LOCAL FEEDBACK REGULATION OF SALT & WATER TRANSPORT ACROSS PUMPING EPITHELIA.
EXPERIMENTAL & MATHEMATICAL INVESTIGATIONS IN THE ISOLATED ABDOMINAL SKIN OF Bufo marinus.

Susmita Thomson
B.Sc. (Hons.), B.E. (Hons.)

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

Department of Physiology
2002
The Cane Toad

Four to nine inches long and up to four pounds in weight, cane toads are big and fat, greenish-yellow and able to put rabbits to shame with their ability to reproduce. A pair of cane toads would produce 60 000, or more toadies every year if all their eggs became mature adults. Hopping rampant in Australia, feeding at night, these amphibians were imported once upon a time from South America so the toads would eat beetles attacking sugar cane fields. Taking a liking to Down Under, the toads proliferated to the point where they’re thicker than fleas on a dingo’s back. In addition to insects, cane toads will eat meat, including small birds and each other. Crushed, the toads stink vilely, but that’s only a small part of the problem

Two glands on the sides of their wart-strewn bodies constantly secrete a venom called bufotenine. When the cane toad is pressured the secretion rate will increase and, when the cane toad is really upset, the venom will shoot up to 40 inches, causing temporary blindness if it lands in your eye. Anything attempting to eat a cane toad, a really upsetting experience for the toad, dies from the poison. A growing number of humans with questionable intelligence remove and dry the toad’s skin and smoke it for what is reportedly an acceptable high. A growing number of the growing number are dying. On the ragged edge of humanity, a few have actually licked the toad for its hallucinogenic effect. Now there’s an interesting way to croak.

-Buck Tilton
Preface

This study describes the results of a four and a half year investigation examining local regulation of ion transport through pumping epithelial cells. The study focussed on the standard isolated toad skin preparation, made famous by Hans Ussing.

Originally, the objective was to perform some simple manipulations on the isolated toad skin, a standard and well-tested epithelial layer, which, according to the literature, was a well-behaved and stable preparation. The purpose of doing these toad skin experiments was to gain familiarity with the experimental techniques, such as measuring the open-circuit voltage (Voc) and the short-circuit current (Isc) across an epithelium. In the process, the experimental information that was obtained was to assist in the development and refinement of a mathematical model of a single pumping epithelial cell. The technical skills that were learnt, along with the insights from the mathematical model, would then be applied in the investigation of a more complex pumping epithelial layer, *stria vascularis* in the mammalian cochlea.

As it turned out, stable Voc and Isc recordings could not be measured across toad skins exposed symmetrically to standard 250mOsm/kgH2O NaCl Ringers solutions in many of the experiments conducted in this study, contrary to the findings of other experimenters (Hillyard, personal communication). Instead, Voc and Isc often decayed over the first five hours of experimentation, which encompassed the experimental times reported by most other investigators. Moreover, the responses were variable between different skins, and even between experiments conducted separately on tissue taken from the same animal. However, the general ‘rundown’ of Voc and Isc over time was quite reproducible, despite experiments being conducted at different times of the day (anywhere between 8.30a.m and 11p.m.), in different seasons, using different batches of toads, different recording equipment and different clamp protocols. Therefore, it appeared that the Voc and Isc responses were physiological and were not artefacts.

The obvious question was ‘Why weren’t these preparations stable?’ Without an adequate answer to this question, there seemed little point in investigating salt and water movements in more complicated epithelial layers. Therefore, to address this issue, attempts were made to stabilise the preparations, as described below.

First, the effect of the voltage-dependent Cl⁻ channels on the stability of the electrophysiological responses recorded across the skin was investigated. These voltage-dependent Cl⁻ channels were originally discovered by Larsen & Kristensen (1978), and subsequently localised to the apical membranes of the mitochondria-rich cells (MRCs) in the toad skin (Larsen et al., 1987; Larsen & Harvey, 1994). It was thought in the
present study that if, for some reason, the apical membrane potential of these cells was changing, then the voltage-dependent Cl\(^-\) conductance would also be changing, which might affect the stability of Voc.

However, before testing this hypothesis it was necessary to gain familiarity with the technique of voltage clamping. This was done by replicating the experiments of Larsen et al. (1987). Thus, a passive, ‘amiloride-treated’ skin (where the active Na\(^+\) pumping had been abolished) was instantaneously changed from a negative holding potential of –40mV (with respect to the apical bathing solution) to a positive potential. Under these conditions the Cl\(^-\) conductance slowly activated over 30-90 seconds, following a delay of around 10 seconds. That is, the Cl\(^-\) channels opened slowly upon depolarisation of the apical membrane. A return to the negative holding potential caused a subsequent inactivation of the Cl\(^-\) conductance over a similar time course, again, after a delay of about 10 seconds. These observations were consistent with the findings of Larsen et al. In the present study, the delay in the activation and inactivation of the Cl\(^-\) conductance appeared to be important in the generation of a damped oscillatory behaviour with a period of 60-68 seconds, which was seen in some skins when suddenly voltage-clamped away from the –40mV holding potential to +100mV. This previously unreported oscillatory behaviour was important as it clearly indicated that local feedback was occurring in the isolated skins. It was also of interest as it seemed consistent with changes in the kinetics of the Cl\(^-\) channels. That is, it was hypothesised that a depolarisation of the apical membrane potential from a negative to a positive potential (with respect to the apical bathing solution) caused gating charges associated with the channel proteins, to migrate from a closed to an open state. Conversely, an hyperpolarisation from a positive to a negative membrane potential caused the gating charges to migrate from an open, to a closed state. But because of the slow activation and inactivation, it seemed that there was more than one open, and one closed state – indeed, there was evidence to suggest here that there may be 6 open and 6 closed states. This led to a detailed mathematical analysis of a 12-state Cl\(^-\) channel model which showed how kinetic arguments could possibly explain the delays observed in the activation and inactivation of the voltage-dependent Cl\(^-\) conductance.

Of course, the conditions under which these voltage-clamp experiments were performed were somewhat artificial, as toads in their natural habitat would not be subjected to instantaneous voltage-clamp conditions, nor would they be exposed to amiloride. But the experiments did highlight the voltage-dependent nature of the Cl\(^-\) channels, and that these channels would close as the apical membrane potential became
more negative. Presumably then, if the apical membranes hyperpolarised slowly, due to the closure of apical $\mathrm{Na}^+$ channels for example, or the excessive uptake of $\mathrm{Cl}^-$, the $\mathrm{Cl}^-$ channels would also close slowly. It was considered possible that the migration of cells from a $\mathrm{Cl}^-$ permeable state to a $\mathrm{Cl}^-$ impermeable one could cause changes in $\mathrm{Voc}$, which might cause an instability in this parameter. Testing this hypothesis in actively pumping skins, it was shown that after voltage-clamping the skins briefly at $-40\,$mV for a period of 2 minutes to turn the $\mathrm{Cl}^-$ channels off, the subsequent release of the skins back to their open circuit conditions, caused changes in $\mathrm{Voc}$ that resembled those recorded from skins under open-circuit conditions over periods of up to 20 hours. This suggested that in many skins, the $\mathrm{Cl}^-$ conductance was turning off during the course of the experiments and was consequently an inherent source of variability between skin samples.

During the course of experimentation a second method that was investigated to stabilise the preparations was to bathe the skins in different solutions. It became clear that more stable Isc responses could be obtained when the skins were bathed symmetrically in 100mOsm/kgH$_2$O NaCl and not the standard 250mOsm/kgH$_2$O NaCl solution used by Ussing and other experimenters. But it was unclear whether the improvement in stability in the 100mOsm/kgH$_2$O NaCl solution was due to a reduction in the ionic concentration, or due to an increase in the water concentration of the solution. This issue was resolved, however, by a series of experiments where the ionic concentrations were held constant, but the osmolality of the solution was successively increased in 50mOsm/kgH$_2$O steps by the addition of sucrose. It was shown from these experiments that increasing the extracellular osmolality, which presumably caused the cells to shrink, resulted in a rundown of Isc. (This supported Ussing’s findings in 1965 that there was a close correlation between the epithelial volume and Isc). Therefore, it was concluded in the present study that the pumping epithelial cells were sensitive to changes in volume, which also agreed well with direct volume measurements made by Spring & Ussing (1986) in individual mitochondria-rich cells of the toad skin. As a result of the findings of the present study, and because Isc is largely a measure of active $\mathrm{Na}^+$ pumping across the epithelial layer (as demonstrated by the rapid reduction in Isc upon apical application of amiloride), it was proposed that the apical $\mathrm{Na}^+$ channels were volume-sensitive, opening as the cells swelled, and closing as the cells shrunk. Although, it was not possible to test this hypothesis directly because cell volume was not measured in this study, the experimental and mathematical modelling results presented in this manuscript were certainly consistent with this proposal. The main point to note here is that if the cells were shrinking in the high-osmolar
250mOsm/kgH₂O NaCl solutions, then the putative Na⁺ channels would be closing during the course of experimentation. With the strong possibility of dynamic changes in both the apical Na⁺ and Cl⁻ permeabilities experimentally it seemed unlikely that under these conditions Isc and Voc would be stable over time.

To demonstrate that apical Na⁺ and Cl⁻ channel closure could produce the results observed experimentally in this study, thus limiting the amount of salt (and water) uptake under these conditions, a single-cell spreadsheet model of a mitochondria-rich cell was developed in Microsoft Excel. The volume-sensitive Na⁺ channels and the voltage-dependent Cl⁻ channels were modelled using simple first-order Boltzman activation curves. Other passive permeabilities, which were fixed, were described using the standard Goldman-Hodgkin-Katz equation, while ionic movements via pumps and symports were described using (saturating) arctan functions. Solver, an optimisation routine in Excel, was used to determine either voltage steady-state or ionic steady-state solutions for particular cell topologies. In addition, a time-domain solution was developed which showed the dynamic changes in a number of cell parameters over time.

While the shutdown of Isc and Voc could be simulated in the model by the closure of the apical volume-sensitive Na⁺ channels and the voltage-dependent Cl⁻ channels respectively, a satisfactory explanation for the differences between the results presented here, and Hillyard’s stable Voc and Isc observations, remained elusive. It was not until the findings of experiments conducted with the K⁺ channel blocker, tetraethylammonium (TEA) were incorporated into the model that the discrepancy seemed to be resolved. Earlier, during the experimental phase, it had been shown that when TEA was applied to the apical bathing solution, increases of between 50-100% in Isc and Voc could be observed. This was consistent with blockade of apical K⁺ channels. It was found that the incorporation of apical K⁺ channels into the model greatly assisted in manipulating the start conditions (and the subsequent responses). The greater the apical K⁺ permeability, the smaller the cell volume and the more negative the apical membrane potential. When these high K⁺ permeable cells were exposed symmetrically to high salt solutions (i.e. 250mOsm/kgH₂O NaCl) the Na⁺ and Cl⁻ channels tended to close off. However, the modelling results suggested that cells with lower potassium permeabilities were more swollen to begin with, and possessed more positive membrane potentials. Consequently, comparatively large changes in the cell volume and apical membrane potentials had to occur before the apical Na⁺ and Cl⁻ channels began to close. In those cases where these changes were not sufficiently large,
channel closure would not result and Voc and Isc would remain stable. This seemed to be consistent with Hillyard’s observations. Therefore, it is suggested that the rundown observed in the results presented in this study, compared with those of other investigators, may have been due to a difference in cellular apical K⁺ permeabilities.

Finally, it should be emphasised the toad skin was a convenient tissue model for exploring more general issues such as:

(i) how pumping epithelial cells may adjust to changes in the extracellular environment by locally regulating their membrane conductances;

(2) how the topology of a cell can influence its function (i.e. the topology can determine whether a cell is optimised for salt transport or water transport).

(3) how different cells, with different functions, may be positioned in apposition in a pumping epithelial tissue so that gradients generated by one cell type can be utilised by another.

From a broader perspective, it is likely that such issues are also applicable to other pumping epithelia, and ultimately, may assist in understanding how these epithelia function.
I would like to thank my supervisor, Dr. Robert Patuzzi for his guidance and assistance during this project. His tremendous enthusiasm for science is contagious and his lateral approach, which splices engineering and biological principles together, has been the source of many interesting, lively, (and sometimes heated!) discussions. I would like to thank him for his many insights, helpful suggestions, valuable criticisms, and his tireless reading of this manuscript. I hope that I have gained some of his remarkable qualities for analytical thinking and critical assessment.

I would like to thank Mr. Greg Nancarrow for his technical assistance which was much appreciated. And both he, and Dr. Simon Marcon should be thanked for their tremendous humour – wherever these two went in the laboratory there was always laughter.

I extend my thanks to Mr. Ross Oxwell who is a fount of knowledge in practical and technical matters. It is great to discuss ideas with Ross, who always gives so willingly and cheerfully of his time. Other technical assistance was also gratefully received from Mr. Charles Biddle and the staff in the Preclinical Workshop.

I would also like to acknowledge Dr. Graeme Yates, whose quiet and courteous ways were greatly admired and his helpful advice always appreciated. His unfortunate passing was a sad loss for the laboratory.

I also extend my gratitude to Professor Don Robertson for his helpful insights and his encouragement, particularly in attending conferences.

While the people acknowledged above were directly involved in my development as a PhD student, there are a number of other people I wish to thank for their encouragement throughout my studies:

I would like to thank the following people associated with the department for their help and friendship during my studies there: Ms. Kirsty Spalding, Mr. Greg O’Beirne, Ms. Catherine McMahon, Ms. Helen Berg, Dr. Helmy Mulders, and Mrs. Georgie Bennet.

I would also like to thank Mr. David Miller, Ms. Kristy Panton, Mrs. Nicole Hedley, Mr. & Mrs. Sam & Alina McAdam and Mrs. & Dr. Naomi & Vaughan Langford for their terrific friendship and support during my undergraduate and postgraduate work.

Finally, I would like to express my sincere appreciation to my family for their continual love and support. In particular, I would like to pay special tribute to my mother, Dr. Judith Thomson, whose incredible qualities never cease to amaze!

*   *   *
The Labview software written in this study to control the electronic circuits, was solely the work of the author. The circuits themselves were designed by Dr. Patuzzi, while the construction of the circuits was carried out by the author. Technical assistance in debugging the circuits was gratefully received from Mr. Greg Nancarrow and Dr. Patuzzi.

The author designed the majority of experiments. The implementation of all experiments, and the subsequent data reduction was also performed by the author.

The development of the single-cell mathematical model, and the mathematical analysis of the 12-state kinetic model for the Cl\(^-\) channels was the combined work of Dr. Patuzzi and the author. The majority of the Visual Basic programming used in this study was the work of the author.

The author was a recipient of an Australian Postgraduate Award. Travel assistance to attend the IUPS 2001 conference in Christchurch, New Zealand was gratefully received from The University of Western Australia.
Abbreviations

Many of the abbreviations are associated with the mathematical modelling.

A−  negatively charged intracellular proteins
ADP  adenosine diphosphate
Al3+  aluminium
ATP  adenosine triphosphate
AVT  arginine vasotocin
(b)  belly (basolateral) side
B  voltage-dependent rate constant, k12
BCs  basal cells
°C  degrees Celcius
Ca2+  calcium
cAMP  cyclic adenosine monophosphate
Cl−  chloride
[Cl−]c  intracellular Na+ concentration
[Cl−]L  Cl− concentration on the left (apical) side
[Cl−]R  Cl− concentration on the right (basolateral) side
Clsats  Michaelis constant for symports: half saturation concentration for Cl−
Clsf  number of Cl− ions translocated per symport cycle
Cu2+  copper
dd  displacement away from d12 required to increase the open probability
     from 1/2 to 10/11 or to decrease it from 1/2 to 1/11.
d12  displacement for which the open probability is 1/2 (i.e. 50%)
DAC  digital to analogue conversion
dC  rate of change of concentration
dL  decilitre
dM2  rate of change of second messenger 2
dM3  rate of change of second messenger 3
dM4  rate of change of second messenger 4
dp  rate of change of the probability of occupancy of a kinetic state
DpR  Density of the Na+/K+-ATPase pumps on the right side
DsL  Density of the symports on the left side
dt  rate of change of time
dV  rate of change of volume
E10  scaling factor (E10 = 2.303*RT/zF ≈ 60mV)
e  charge on an electron (1.6 x 10−19 C)
Ei  energy level of the i-th state
Ea  activation energy
ENaCs  epithelial Na+ channels
EP  endocochlear potential
F  Faraday’s constant (9.659 x 104 C/mol)
FU  furosemide (Na+/K+/2Cl− symport blocker)
φH2OL  water flux across the left membrane
φH2OR  water flux across the right membrane
φi  flux through a channel for some ionic species, i
Γ  mass action ratio (for cytoplasmic ATP, Γ = [ADP][Pi]/[ATP])
Ga  apical conductance
Gb  basolateral conductance
go  unitary channel conductance
Gs\textsubscript{L} Gibbs free energy for the symports on the left side
h Planck’s constant \((6.62 \times 10^{-34} \text{ Js} \text{ or } 1.06 \times 10^{-54} \text{ eVs})\)
Hg\textsuperscript{2+} mercury
Hz Hertz
I\text{Cl}\text{c} total Cl\textsuperscript{-} current into the cell
I\text{Cl}\text{L} Cl\textsuperscript{-} current across the left membrane via passive Cl\textsuperscript{-} channels
I\text{Cl}\text{para} paracellular Cl\textsuperscript{-} current
I\text{Cl}\text{R} Cl\textsuperscript{-} current across the right membrane via passive Cl\textsuperscript{-} channels
I\text{Cl}\text{sL} Cl\textsuperscript{-} component of symport current across the left membrane
ICs intermediate cells
I\text{K}\text{L} K\textsuperscript{+} current across the left membrane via passive K\textsuperscript{+} channels
I\text{K}\text{c} total K\textsuperscript{+} current into the cell
I\text{K}\text{para} Paracellular K\textsuperscript{+} current
I\text{K}\text{pR} K\textsuperscript{+} component of pump current across the right membrane
I\text{K}\text{R} K\textsuperscript{+} current across the right membrane via passive K\textsuperscript{+} channels
I\text{K}\text{sL} K\textsuperscript{+} component of sympot current across the left membrane
I\text{L} total current across the left membrane
I\text{Na}\text{c} total Na\textsuperscript{+} current into the cell
I\text{Na}\text{L} Na\textsuperscript{+} current across the left membrane via passive Na\textsuperscript{+} channels
I\text{Na}\text{para} Paracellular Na\textsuperscript{+} current
I\text{Na}\text{pR} Na\textsuperscript{+} component of pump current across the right membrane
I\text{Na}\text{R} Na\textsuperscript{+} current across the right membrane via passive Na\textsuperscript{+} channels
I\text{Na}\text{sL} Na\textsuperscript{+} component of symport current across the left membrane
I/O input / output
I\text{R} total current across the right membrane
I\text{sc} short-circuit current
ISS ionic steady-state
I-V current-voltage
\(\kappa\) transmission co-efficient (~1)
k Boltzmann constant \((1.38 \times 10^{-13} \text{J/K or 8.62 x 10^{-5} eV/K})\)
K\textsuperscript{+} potassium
[K\textsuperscript{+}]\text{c} intracellular K\textsuperscript{+} concentration
[K\textsuperscript{+}]\text{L} K\textsuperscript{+} concentration on the left (apical) side
[K\textsuperscript{+}]\text{R} K\textsuperscript{+} concentration on the right (basolateral) side
K\text{ATP} equilibrium constant for the reduction of ATP to ADP+P\textsubscript{i}
K\text{f} number of K\textsuperscript{+} ions translocated per pump cycle
k\textsubscript{ij} transition rate constant between the i\textsuperscript{th} and j\textsuperscript{th} state
k\text{\Omega} kilohm
K\text{Sat} Michaelis constant for pumps: half saturation concentration for K\textsuperscript{+}
K\text{Sats} Michaelis constant for sympots: half saturation concentration for K\textsuperscript{+}
K\text{sf} number of K\textsuperscript{+} ions translocated per symport cycle
\(\lambda\) root (zero) of an equation
L subscript ‘L’ – left (apical) side
La\textsuperscript{3+} lanthanum
LPF low pass filter
LH\textsubscript{2}O\textsubscript{L} hydraulic conductivity on the left side
LH\textsubscript{2}O\textsubscript{R} hydraulic conductivity on the right side
M\textsubscript{1} (primary) messenger concentration 1
M\textsubscript{2} second messenger concentration 2
M\textsubscript{3} second messenger concentration 3
M\textsubscript{4} second messenger concentration 4
MCs marginal cells
min minutes
mL millilitre
mM millimolar
MRC mitochondria-rich cell
mV millivolts
µA microamps
µg micrograms
µM micromolar
n the average charge per mole of non-diffusible intracellular protein
N Avogadro’s number \( (6.02 \times 10^{23}) \)
\( \text{Na}^+ \) sodium
\([\text{Na}^+]_i\) intracellular \( \text{Na}^+ \) concentration
\([\text{Na}^+]_L\) \( \text{Na}^+ \) concentration on the left (apical) side
\([\text{Na}^+]_R\) \( \text{Na}^+ \) concentration on the right (basolateral) side
\( \text{Na}_f \) number of \( \text{Na}^+ \) ions translocated per pump cycle
\( \text{Na}_{\text{sat}} \) Michaelis constant for pumps: half saturation concentration for \( \text{Na}^+ \)
\( \text{Na}_{\text{sat}} \) Michaelis constant for symports: half saturation concentration for \( \text{Na}^+ \)
\( \text{Na}_f \) number of \( \text{Na}^+ \) ions translocated per symport cycle
OSS osmotic steady-state
(p) pond (apical) side
\( \Pi \) osmotic pressure
\( \text{Pb}^{2+} \) lead
PC principal cell
PCI \( \text{Cl}^- \) permeability
PCI\(_L\) \( \text{Cl}^- \) permeability on the left side
PCI\(_{\text{max}}\) maximum \( \text{Cl}^- \) permeability on the left side
PCI\(_{\text{para}}\) paracellular \( \text{Cl}^- \) permeability
PCI\(_R\) \( \text{Cl}^- \) permeability on the right side
\( P(d)_{\text{max}} \) maximal density of the volume-sensitive \( \text{Na}^+ \) channels
\( p_i \) probability of a gating particle being in the starting state
\( P_i \) intracellular inorganic phosphate concentration
PK \( \text{K}^+ \) permeability
PKA protein kinase A
PK\(_L\) \( \text{K}^+ \) permeability on the left side
PK\(_{\text{para}}\) paracellular \( \text{K}^+ \) permeability
PK\(_R\) \( \text{K}^+ \) permeability on the right side
PNa \( \text{Na}^+ \) permeability
PNa\(_L\) \( \text{Na}^+ \) permeability on the left side
PNa\(_{\text{max}}\) maximum \( \text{Na}^+ \) permeability on the left side
PNa\(_{\text{para}}\) paracellular \( \text{Na}^+ \) permeability
\( p_o \) open probability of a channel
\( p_{o\text{d}} \) delayed open probability of a channel
\([\text{Prot}]_L\) protein concentration on the left side
\([\text{Prot}]_R\) protein concentration on the right side
\( P_s \) electrochemical sensitivity of the symports
\( P_v \) voltage sensitivity of the pumps
\( P(V)_{\text{max}} \) maximal density of the voltage-dependent \( \text{Cl}^- \) channels
Q\(_c\) total charge in the cell
\( R \) subscript ‘R’ – right (basolateral) side
\( R \) universal gas constant \( (8.314 \times 10^4 \text{J/kg mol.K}) \)
r\(_{ij}\) rate at which a transition between the \( i^{\text{th}} \) and \( j^{\text{th}} \) state can occur
RVD  regulatory volume decrease
RVU  regulatory volume increase
s    seconds
[S]c  intracellular sugar concentration
[S]L  sugar concentration on the left side
[S]R  sugar concentration on the right side
ΣICl  total Cl⁻ current into the cell
ΣIK  total K⁺ current into the cell
ΣINa  total Na⁺ current into the cell
τ    time constant
t    time
T    absolute temperature (K)
TEA  tetraethylammonium (K⁺ channel blocker)
V₁₁  the voltage away from V₁₂ required to increase the open probability from 1/2 to 10/11 or to decrease it from 1/2 to 1/11.
V₁₂  the membrane potential for which the open probability is 1/2 (i.e. 50%)
Vcell/left  voltage across the apical membrane
Vₘ    membrane potential
Vright  voltage across the right membrane
Vskin  voltage across the skin
VI    virtual instrument
V-I   (open-circuit) voltage, (short-circuit) current
Voc   open-circuit voltage
Vol    volume
Volnow  instantaneous cell volume
VSS    voltage steady-state
VSS (OC) voltage steady-state under open-circuit conditions
VSS (SC) voltage steady-state under short-circuit conditions
x₁₁   value of the generic Boltzmann parameter away from x₁₂ necessary to increase the open probability of the channels from 1/2 to 10/11, or to decrease it from 1/2 to 1/11
x₁₂   generic Boltzmann parameter for which the channel open probability is 50%
zᵢ    valence of the ionic species
CONTENTS

PREFACE.........................................................................................................................i
ACKNOWLEDGMENTS ........................................................................................................vi
ABBREVIATIONS ..................................................................................................................viii

INTRODUCTION ..................................................................................................................2

1.1 GENERAL INTRODUCTION .........................................................................................2

1.2 PART 1: STRUCTURE OF THE ANURAN EPIDERMIS ..................................................7

1.2.1 Morphology of the anuran skin ...............................................................................7

1.2.1.1 Principal Cells (PCs) .........................................................................................9

1.2.1.2 Mitochondria-Rich Cells (MRCs) .....................................................................9

1.2.1.3 Merkel Cells ...................................................................................................12

1.2.2 Blood supply .........................................................................................................12

1.2.3 Effect of temperature ............................................................................................12

1.2.4 Moulting cycle .......................................................................................................13

1.3 PART 2: FUNCTION OF THE ANURAN EPIDERMIS ................................................14

1.3.1 Transport mechanisms in the PCs and MRCs ........................................................15

1.3.2 MRCs as the main Cl⁻ pathway ............................................................................18

1.3.3 PCs as the main Na⁺ pathway .................................................................................20

1.3.4 Overall toad skin function .....................................................................................24

1.4 PART 3: TRANSPORT MECHANISMS IN THE TOAD SKIN ........................................26

1.4.1 Principal Cells .......................................................................................................27

1.4.1.1 Evidence for apical Na⁺ channels in PCs ..........................................................27

1.4.1.2 Evidence for basolateral Na⁺/K⁺-ATPase pumps in PCs ..................................28

1.4.1.3 Evidence for basolateral K⁺ channels in PCs ...................................................29

1.4.1.4 Evidence for apical K⁺ channels ......................................................................35

1.4.1.5 Evidence against apical Cl⁻ channels in PCs .....................................................36

1.4.1.6 Conflicting evidence for basolateral Cl⁻ channels in PCs ................................37

1.4.1.7 Evidence for Cl⁻/HCO₃⁻ antiports in PCs .........................................................38

1.4.1.8 Evidence for Na⁺/H⁺ exchangers in PCs ...........................................................43

1.4.1.9 Evidence for basolateral Na⁺/Ca²⁺ transporters in PCs ...................................47

1.4.1.10 Evidence for basolateral Na⁺/K⁺/2Cl⁻ symports in PCs ..................................48

1.4.2 Mitochondria-Rich Cells .......................................................................................49

1.4.2.1 Evidence for apical Cl⁻ channels in MRCs ........................................................51

1.4.2.2 Types of apical Cl⁻ channels in MRCs ...............................................................60

1.4.2.3 Evidence for apical Na⁺ channels in MRCs .......................................................60

1.4.2.4 Evidence for apical H⁺ pumps & Cl⁻/HCO₃⁻ exchangers in MRCs ..................64

1.4.2.5 Evidence for basolateral Na⁺/K⁺-ATPase pumps in MRCs ...............................66

1.4.2.6 Evidence for basolateral K⁺ channels in MRCs ...............................................68

1.4.2.7 Evidence for basolateral Cl⁻ channels in MRCs ...............................................68

1.4.2.8 Evidence for Na⁺/K⁺/2Cl⁻ symports in MRCs ..................................................69

1.4.3 Paracellular pathways ............................................................................................70

1.4.4 Cell volume and hydraulic conductivity of the toad skin ....................................72

1.5 ORGANISATION OF THE THESIS ..........................................................................77

2.METHODS ....................................................................................................................80

2.1 INTRODUCTION .........................................................................................................80

2.2 BACKGROUND PREPARATION ................................................................................80

2.2.1 Animals ................................................................................................................80

2.2.2 In Vitro tissue preparation ......................................................................................83

2.2.3 Solutions .................................................................................................................90
2.3 EXPERIMENTAL EQUIPMENT .................................................................91
  2.3.1 Ussing chambers and calomel electrodes .......................................91
2.4 ELECTRONICS .....................................................................................92
  2.4.1 Standard electrophysiological techniques & electronic measurements ....92
  2.4.2 Requirements and constraints ..........................................................93
  2.4.3 Circuit designs ..................................................................................97
  2.4.4 Specifications and limitations of the apparatus .................................102
2.5 SOFTWARE .........................................................................................103
  2.5.1 Software features .................................................................106

3. VOLTAGE DEPENDENCE & OSCILLATIONS IN THE CL- CONDUCTANCE OF THE TOAD SKIN .................................................................110
  3.1 INTRODUCTION ..............................................................................110
  3.2 METHODS .......................................................................................112
    3.2.1 Tissue preparation .................................................................112
    3.2.2 Monitoring equipment ..............................................................113
    3.2.3 Voc & Isc experiments .............................................................114
    3.2.4 Voltage step-sequence experiments .........................................114
    3.2.5 Single voltage-clamps followed by Voc and Isc recordings ..........114
  3.3 RESULTS .........................................................................................116
    3.3.1 Variability within and between skins ...........................................116
    3.3.2 Step sequence voltage-clamp experiments ...............................118
    3.3.3 Voltage-clamps & oscillations in actively pumping skins ..........124
  3.4 DISCUSSION ....................................................................................126
    3.4.1 Possible cause of the slow decrease in the skin conductance .........126
    3.4.2 Voltage clamping of the skin and voltage divider effects ............127
    3.4.3 What is the mechanism of the delayed Cl- channel gating? .........128
  3.5 THEORETICAL MODELLING .............................................................130
    3.5.1 Kinetics of the voltage-dependent Cl- channel: An analytical approach 130
    3.5.2 General filter method .............................................................141
  3.6 CONCLUSIONS ..............................................................................144

4. VOLUME REGULATION .......................................................................148
  4.1 INTRODUCTION ..............................................................................148
  4.2 METHODS .......................................................................................154
  4.3 RESULTS .........................................................................................154
    4.3.1 Archetypal response .................................................................154
    4.3.2 ‘Independent’ and ‘interleaved’ controls ......................................155
    4.3.3 Metabolic shutdown due to lack of ATP .....................................158
    4.3.4 Effect of the osmolality of the bathing solution on Isc ................161
    4.3.5 Effect of osmolality & ionic concentration of the bathing solution on Isc & Voc .................................................................163
  4.4 DISCUSSION ....................................................................................172
    4.4.1 Control data ...............................................................................172
    4.4.2 Volumetrically sensitive Isc .......................................................174
    4.4.3 Mechanism regulating Isc: volume-sensitive PNa? .................175
    4.4.4 Are the putative volume-sensitive Na+ channels in the toad skin ENaCs? .................................................................177
    4.4.5 Phase I: The order of simple- and solute-induced water movements 178
    4.4.6 Phase II: RVI- and RVD-like behaviour in toad skins ..............181
    4.4.7 ATP effect ..................................................................................183
  4.5 CONCLUSIONS ..............................................................................184
5. HEAVY METALS: EFFECTS OF Hg^{2+} & Pb^{2+} ......................................................188
5.1 INTRODUCTION .................................................................188
5.2 METHODS ..................................................................189
5.3 RESULTS ...................................................................190
  5.3.1 Apical Hg^{2+} dose response curve .................................190
  5.3.2 Effect of Hg^{2+} on the basolateral side of the toad skin ........192
  5.3.3 Effect of Pb^{2+} .........................................................192
  5.3.4 Effect of Al^{3+}, La^{3+} and Cu^{2+} .................................196
  5.3.5 Interaction of Pb^{2+} and Hg^{2+} ...................................198
  5.3.6 Interactions of Pb^{2+} and TEA ...................................200
  5.3.7 Interactions of Pb^{2+} and Furosemide .........................203
5.4 DISCUSSION ..................................................................205
  5.4.1 Heavy metal effects are not due to charge screening .........205
  5.4.2 The action of Hg^{2+} ..................................................206
  5.4.3 The action of Pb^{2+} ..................................................209
5.5 CONCLUSIONS ..............................................................212

6. STEADY-STATE SPREADSHEET MODELLING OF TRANSPORTING EPITHELIAL CELLS ......................................................216
6.1 INTRODUCTION ..............................................................216
  6.1.1 Layout of the general cell model ..................................217
6.2 MATHEMATICAL DETAILS OF THE MODEL .................219
  6.2.1 The state vector .......................................................219
  6.2.2 Charge and fluxes .....................................................221
  6.2.3 Constraint equations ...............................................225
  6.2.4 Steady states ...........................................................225
6.3 MODELLING ISSUES ......................................................227
  6.3.1 Validation of the numerical model using the GHK equation 227
  6.3.2 Specifying starting conditions ....................................228
  6.3.3 Uniqueness of solutions ............................................230
  6.3.4 Error terms and numerical stability .........................234
6.4 APPLICATIONS OF THE MODEL .........................................234
  6.4.1 Investigating different cell topologies and different environments .... 235
  6.4.2 IV plots: An application of ISS under short-circuit conditions .....235
  6.4.3 VI Loci: Application of ISS (or VSS) under open- & short-circuit conditions ..........................................................237
  6.4.4 Reverse engineering ...............................................238
  6.4.5 Limitations .............................................................238

7. TIME-DOMAIN MODELLLING SIMULATING IONIC TRANSPORT ACROSS EPITHELIAL CELLS ......................................................242
7.1 INTRODUCTION ..............................................................242
7.2 OVERVIEW OF TIME-DOMAIN MODELLING ..................243
7.3 SIMULATING STRUCTURAL CHANGES IN THE GENERAL CELL MODEL ..............................................................243
  7.3.1 Modelling variable passive permeabilities: The Boltzmann equation .......244
  7.3.2 Modelling delays in sensitivity to a parameter ................245
7.4 ADDITIONS TO THE STATE VECTOR .................................247
7.5 IMPLEMENTING THE SOLUTION PROCEDURE ..................248
  7.5.1 For which conditions were solutions found? .................248
  7.5.2 Incrementing cell volume, intracellular concentrations and permeabilities ..........................................................249
  7.5.3 Accuracy and numerical stability ................................251
1. Introduction
1. Introduction

1.1 General Introduction

The initial purpose of this doctoral study was to investigate the function of *stria vascularis*, an epithelial layer located on the bony otic capsule of the mammalian inner ear or cochlea (Figure 1.1A,B). The cochlea consists of three-fluid filled compartments: *scala vestibuli* and *scala tympani* which are filled with a Na\(^+\)-rich fluid called perilymph and *scala media* which is filled with a K\(^+\)-rich fluid called endolymph. *Stria vascularis* actively secretes K\(^+\) into endolymph and the accumulation of this ion results in the generation of a positive potential in scala media (relative to scala tympani), known as the endocochlear potential (EP) (Tasaki & Spyropoulos, 1959). This EP is fundamental for hearing. However, the K\(^+\) ions that generate this potential are constantly leaving scala media through the organ of Corti. Interestingly, the +90mV EP seems to remain remarkably steady as indicated by stable hearing thresholds in the absence of any pathology. It would seem that to maintain such a stable potential the K\(^+\) supply through *stria vascularis* must be tightly regulated. Yet it does not appear to be regulated by external factors. Unlike some pumping epithelial layers such as in the kidney where ionic transport is regulated largely by neural or hormonal control, *stria vascularis* is not innervated and there is little evidence in the literature to suggest that it is hormonally sensitive. An alternative possibility is that *stria vascularis* is regulated locally via feedback mechanisms within the strial cells which sense changes in the intra- and extracellular environments.

However, testing the hypothesis that *stria vascularis* is locally regulated is problematical. First, *stria vascularis* is a complex structure consisting of three cell types in series (Figure 1.2A). There are the marginal cells (MCs), which form a single cell layer adjacent to the fluid in scala media. Opposite these cells on the other side of the epithelium next to the spiral ligament (which is attached to the bony cochlear wall and is in contact with perilymph) there are the basal cells (BCs) which form a layer of one to two cells. Sandwiched between the MCs and the BCs are the melanin-containing intermediate cells (ICs) which interdigitate with the MCs and BCs as well as capillaries (Hilding & Ginzberg, 1977; Kikuchi & Hilding, 1966; Kimura & Schuknecht, 1970). ICs are also intimately associated with one another, linked by gap junctions (Bagger-Sjoback et al., 1987). To understand how *stria vascularis* functions as an actively pumping epithelial layer, it is necessary to understand ionic transport across the individual cell layers and the interactions between the different cell types.
The second problem with investigating *stria vascularis* is that it is difficult to isolate and monitor only strial responses *in vivo*. One way to assess strial function is to monitor the EP, but as described above, the electrical loading effects of the organ of Corti affect this measurement. A second method used to monitor strial function is by microelectrode impalement of individual strial cells. However, this is technically challenging as *stria vascularis* is small (<1mm across) and relatively inaccessible (Figure 1.2B). For example, in the guinea-pig (a widely used experimental model in hearing research), a difficult ventrolateral approach is necessary to ensure maximal exposure of the cochlea and optimal approachability to *stria vascularis*. Once the cochlea is accessible, the bone overlying the stria can be removed. However, care must be taken during this process as the cochlea is fragile and it is easy to damage strial cells during the removal process. In addition, bone fragments frequently need to be left in place to avoid macroscopic structural damage to *stria vascularis*. This often makes microelectrode impalements into strial cells difficult. Even once strial cells have been well-exposed problems still exist. First, cell identification is often difficult unless cells are subsequently dye-marked. Second, due to geometric constraints, only two microelectrodes can generally be positioned in the vicinity of the cochlea enabling either two strial cell measurements or one cell measurement and one EP recording. Thus, only limited data can be collected during any cochlear perturbation.

Although technically difficult, if strial function is to be understood it is desirable to investigate it *in vivo*. This is because *stria vascularis* operates optimally when perfused with blood, is kept at 38°C, and is exposed to perilymph on the basolateral face and endolymph on the apical face. These conditions cannot be met *in vitro* with the result that strial function is compromised. This can be illustrated by the rapid degeneration of cell potentials over the 30-40 minutes following the removal of the cochlea from the animal (Thomson, 1995).

Obtaining reliable experimental data from *stria vascularis* was clearly going to be arduous and problematical. The usefulness of *in vitro* data was limited as the tissue only remained viable for short periods and changes in environmental factors introduced a number of variables that could not be controlled. On the other hand *in vivo* data was going to be difficult to obtain. Reassessing the situation, it became obvious that the fundamental reason for obtaining the experimental data in the first place was to develop a mathematical model which could be used to understand strial behaviour. Given the iterative nature of the project, where modelling results were to simulate experimental results, and experimental results were to aid in refinement of the model, it became
Figure 1.1: (A) Three dimensional cross section of the mammalian cochlea showing scala vestibuli and scala tympani filled with perilymph (PL), and scala media filled with endolymph (EL). The pumping epithelial layer, stria vascularis required for the generation of the positive endocochlear potential in scala media is located on the bony cochlea wall (see box); (B) An enlarged version of stria vascularis showing the 3 cell types: the marginal cells (MCs), the intermediate cells (ICs) and the basal cells (BCs). (Taken from Sterkers et al., 1987)
Figure 1.2: (A) Model of *stria vascularis* showing the transport mechanisms in a marginal cell and two basal cells. (B) Looking down on the (grey) strial segments from the scala media side. The stria is attached to the otic capsule via the spiral ligament. (Taken from Thomson, 1995).
apparent that one efficient and systematic way to investigate ionic transport across *stria vascularis* was to begin with the mathematical modelling. The basic framework of an open-loop, single-cell mathematical model already existed from previous work that we had conducted (Thomson, 1995). However, if *stria vascularis* was indeed regulated locally then this model had to be modified extensively to include feedback mechanisms in one or more of the three cell types. Examining the issue of feedback in a multiple cell model first required an understanding of feedback in a single cell model. Even then, the model would only be as good as its predictions, which had to be checked against experimental data. Since strial data was going to prove a problem to collect and the main aim was now to verify modelling procedures, it was decided that data should be collected from a ‘less complex’ pumping epithelial layer so that a number of mathematical modelling issues pertinent to any epithelial layer could be investigated.

The isolated abdominal skin of the toad, *Bufo marinus*, was chosen because the primary passage of ions was across two cell layers and not three. In addition, *Bufo marinus* were inexpensive and were readily available in Australia, and the abdominal tissue was large and easily accessible, had been well-studied, was known to function at room temperature without a blood supply, and was thought to be well-behaved and stable.

As it turned out, the experiments conducted on toad skins mounted in Ussing chambers were more complex to analyse and subsequently model than first anticipated. Standard electrophysiological parameters were not stable and displayed significant variability between skins and from tissue taken from the same skin. Consequently, the focus of the doctoral study soon changed. It became evident that, if it were not possible to understand ionic transport across the isolated toad skin and explain the interactions that were occurring there, it was certainly not going to be possible to do this in a more complicated *in vivo* system where electrical loading effects also had to be considered.

Thus, this thesis concentrates on ionic transport across the abdominal skin of the toad, using this as an experimental model to investigate the broader issues of ion interactions, local feedback and volume regulation in epithelial layers.

* * * * *

This introductory chapter is divided into three main sections. The first section describes the structure of the toad skin and factors affecting the growth and development of the skin cells. The second section gives an overview of how the toad skin is believed to function based on the literature and results from this dissertation. The third section is a more detailed review of the literature outlining the experimental evidence for the different transporting mechanisms in the amphibian skin.
1.2 Part 1: Structure of the anuran epidermis

Frogs and toads belong to the order ‘Anura,’ (meaning ‘without a tail’). Anura are grouped together with two other orders (Caudata and Gymnophiona) to form the generic class ‘Amphibia.’

The order ‘Anura’ is subdivided into four families consisting of: two families of frogs (namely Hylidae [tree frogs] and Ranidae [true frogs]), and two families of toads (namely Bufonidae [toads] and Leiopelmatidae [bell toads]). Scientifically, there are few distinctions between toads and frogs, although some broad generalizations can be used to differentiate between them, as described below:

First, frogs have teeth in both their upper and lower jaws, but toads do not possess teeth. Second, frogs generally lay their eggs in frothy clumps found at the water’s surface, but toad spawn is characterised by long tangled strings of eggs. Third, frogs generally have relatively longer hind legs compared with toads, and tend to jump, instead of hopping like toads. Fourth, frogs are distinguishable from toads by their dorsal skins: frogs typically have smooth, wet dorsal skins, while toads have dry, warty dorsal skins. (When threatened, toads unlike frogs, will secrete a toxic substance through their paratoid glands, which are located on the dorsal surface near the neck region). In both cases however, the abdominal skins are smooth and have similar cellular structures as described in Section 1.2.1. Given that there is scant discussion in the literature suggesting otherwise, it is assumed that the transport properties in the abdominal skin of frogs and toads are similar. Certainly, both are $\text{Na}^+$ absorbing epithelia.

Finally, it should be noted here that there is no clear distinction between the habitats of frogs and toads. Both have been found in hot/dry, moist/tropical or cold environments and have developed specific behaviours to assist them in adapting to their habitats. For example, toads and frogs exposed to extreme cold may reduce their activity, or enter a state of torpor. In contrast, toads and frogs living in extremely hot and arid conditions may regulate their body water content by burrowing underground, or covering themselves in a thick mucous to reduce water loss. Unlike both these groups, toads and frogs living in temperate zones may maintain a moist skin to assist in evaporative cooling.

1.2.1 Morphology of the anuran skin

The anuran skin is a multilayered squamous epithelium consisting of 7 distinct regions (Figure 1.3). The outermost layer, *stratum corneum*, is in direct contact with the
external environment and consists of keratinized, flat, squamous cells, one to two cells thick (Farquhar & Palade, 1965). The sub-corneal space separates stratum corneum from stratum granulosum and stratum spinosum which, together, consist of 3-4 layers of cuboidal or polyhedral cells. Beneath stratum spinosum is stratum germinativum characterised by cylindrical cells 30-40µm thick (Whittembury, 1964), and underneath this layer is the basal lamina. Adjacent to this, and forming the inner face of the anuran epidermis, is a layer of connective tissue known as the corium, which is 40-150µm thick. Capillary networks embedded within the corium service the metabolically active cells of the anuran skin, supplying nutrients and removing waste products. As described earlier, there are two main types of cells which are involved in the transport of electrolytes and water across the anuran skin: the principal cells (PCs) and the mitochondria-rich cells (MRCs).

Figure 1.3: The anuran skin is a multilayered epithelium consisting of (1) stratum corneum; (2) the sub-corneal space; (3) stratum granulosum; (4) stratum spinosum; (5) stratum germinativum; (6) the basal lamina and (7) the corium (adapted from Larsen, 1991).
1.2.1.1 Principal Cells (PCs)

The principal cells, also known as keratinocytes, are the granular, spiny and germinal cells found in the granulosum, spinosum and germinativum layers (Rick et al., 1978). As the name suggests, they constitute the principal component of the cells in the anuran epithelium. The principal cells are interconnected with one another via tight junctions and form a functional syncytium. As discussed in the following section, it is widely accepted that these cells are involved in the active absorption of Na\(^+\) from the external environment.

1.2.1.2 Mitochondria-Rich Cells (MRCs)

The flask shaped mitochondria-rich cells, first identified in the anuran skin back in 1865 (Rudneff, 1865), are located in all layers of the anuran epithelium between the basal layer and the stratum corneum (Lavker, 1971; Whitear, 1975). They make up between 2-5% of the total epithelial cell pool (Budtz, 1995; Fox, 1986) or 40% of the living cells beneath the keratinized layer (Ehrenfeld et al., 1976). In the toad skin it has been estimated that there are about 10\(^5\) MRCs/cm\(^2\), and each cell has an approximate volume of 500µm\(^3\). Overall, the MRCs have a total volume of around 45nL/cm\(^2\) of skin, which is less that 2% of the epidermal volume of 3500nL/cm\(^2\). Discounting the area of the microvilli, the apical surface area of the MRCs is approximately 10µm\(^2\), which is less than 1% of the total epidermal surface area (Larsen, 1991).\(^1\)

It is believed that MRCs are originally differentiated from the epidermal cells of the basal layer (Whitear, 1975). Although they are not often observed, the juvenile MRCs are found just above the basal layer and are rounded in appearance. They are characterised by numerous mitochondria located around the nucleus, and a cytoplasm that is largely free of fibrils (Whitear, 1975). As a MRC matures, the apical region elongates and grows toward the stratum corneum until the apex lies close to or in the junction between two cornified cells. The apical region of the MRCs is often characterised by microvillar ridges, which increase the apical surface area. While MRCs have a longer lifespan than surrounding epithelial cells, there is evidence to suggest that old MRCs are pulled from the surrounding cells and shed with the slough material during a moult cycle (Whitear, 1975).

\(^1\) This contrasts greatly to estimations made by Ehrenfeld et al., (1989) who calculated that the ‘total apical surface area of the MRCs can represent between 13-60% of the exposed apical area of the first transporting cell layer.’
MRCs can display considerable variation in their shape; some MRCs project into the apical layers on an angle while others may have a squat appearance, be elongated or have narrow or broad neck regions (Whitear, 1975). The exact reason for these differences is not known although the maturity of the cell may be an important factor. Despite these differences in shape, MRCs can be distinguished from surrounding cells by their neck regions, which contain many mitochondria and possess a high density of vesicles including lucent vesicles, electron dense vesicles and other multivesicular bodies. In addition, MRCs can sometimes be identified by the presence of nerve fibres which synapse onto these cells around the neck region (Whitear, 1974).

Unlike the principal cells, MRCs do not form a functional syncytium. Cell-to-cell contact is mainly confined to the apical region of the MRCs where contact is made with the overlying cornified layer via bipartite desmosomes. Contact between MRCs and principal cells is limited to a few tight junctions generally located in the upper portions of the neck region (Larsen, 1991). However, sometimes microvilli in the lateral and basal regions of the MRCs may interdigitate with similar processes of neighbouring cells (Whitear, 1975).

Various studies have shown that it is possible to regulate the densities of MRCs by altering certain environmental factors such as the salinity of the bathing solutions. In general, toads that were acclimatised to low salt solutions such as distilled water displayed an increased density of MRCs (and an associated increase in the chloride flux) compared with salt-adapted toads. (Budtz et al., 1995; Devuyst et al., 1991; Ilic & Brown, 1980). The rate of change depended on the mode of acclimatisation: small incremental changes in salt concentrations of the bathing solutions were associated with small changes in the density of the MRCs (Katz et al., 1995). For more rapid concentration changes, it has been shown in *Bufo bufo*, pre-treated with tap water, that a doubling in the density of MRCs can occur within 2 days of exposure to distilled water (Budtz et al., 1995). While these cells were morphologically classified as MRCs using the standard technique of silver staining (Willumsen & Larsen, 1986), they were not fully functional as they displayed a reduced chloride current and a lower conductive hydrogen flux compared with measurements recorded from more mature cells. By day 7 the cells displayed a chloride transporting capacity similar to controls, and by day 14 the

---

2 Katz and Gabbay (1993) also observed an increase in mitochondria-rich cells density in *Bufo viridis* upon exposure to low salt environments. However, this increase was over a much longer time period (60 days). Budtz et al., (1995) suggested that this discrepancy was due to differences in species or methodology.
proton secretion was also comparable to control responses, suggesting that the proteins for chloride channels were inserted into the membranes earlier than the transport proteins for proton secretion. Budtz at al. (1995) concluded that MRCs could be recruited within 2 days or so of being exposed to a low salt environment, but these cells did not attain full functionality until up to 2 weeks later. Kinetic studies performed by the same group also showed that the increased density of the MRCs was not due to an increase in cell birth rate, but was instead due to a reduction in cell apoptosis and an increased rate of cell differentiation. With the exception of high KCl bathing solutions, other studies where toads were adapted to increasingly salty environments were consistent with the above findings, showing a reduction in the density of MRCs (Katz & Gabbay, 1995). The anomalous results where KCl caused an increase in MRC density are so far unclear.

Since MRCs are functionally distinct from the principal cells, it is not surprising that the intracellular concentrations of the two types of cells are different. When the anuran skin is bathed in normal chloride amphibian Ringer on both sides the intracellular Cl⁻ concentration in MRCs is around 15-25mM, while in principal cells it is between approximately 40-50mM. However, both types of cells have similar Na⁺ and K⁺ concentrations of about 10mM and 110-140mM, respectively (Larsen et al., 1987; MacRobbie & Ussing, 1961; Rick et al., 1980; Rick et al., 1978, Rick et al., 1984).

In the past, the role of the MRCs was not entirely clear. It was believed that they could be involved in sensory reception because of their innervation (even if somewhat sparse). Alternatively, it was proposed that they might have provided some form of mechanical stability, anchoring down the overlying stratum corneum, or they might have had a secretory function aiding in the release of the cornified material during the moultting period (Whitear, 1975). As discussed later in this chapter, and in Chapter 3, it is now well established that one of the primary functions of MRCs is the uptake of Cl⁻ from the external environment (Larsen, 1991).

---

3 In solutions other than Normal Amphibian Ringer, it is likely that the intracellular concentrations would be considerably different to the values stated and that the Na⁺ and K⁺ concentrations for both cell types would not be similar.

4 It is assumed that negatively charged proteins are present within the cells in order that electrical neutrality within the cells is maintained.
1.2.1.3 Merckel Cells

Another type of cell that is present in the anuran epithelium is the Merckel cell which constitutes only 0.3% of the anuran epidermal cell population (Budtz & Larsen, 1975). Merckel cells lie at the same level as juvenile MRCs, just above the basal layer. Generally, they are difficult to identify, except in the differentiation phase where they can be distinguished from juvenile MRCs by large projections which protrude from an otherwise smooth cell surface (Whitear, 1975). The function of Merckel cells is still unknown, although it has been proposed that they have some role in co-ordinating epithelial differentiation (Budtz & Larsen, 1975).

1.2.2 Blood supply

The anuran skin consists of 2 capillary networks: the subepidermal network located in the upper portion of the corium close to the basal layer, and a deeper capillary network located in the lower regions of the corium. The two networks are not directly connected, but they are supplied and drained by common arteries and veins located between them (Jasinski & Miodonski, 1978). The capillaries of both networks have a mesh-like appearance such that the blood flows in a circular fashion around a small area of skin, the centre of which is defined by a draining vessel.

The meshes of the subepidermal network are much smaller than the deeper network and vary considerably in shape. The subepidermal meshes are not entirely uniplanar, as the capillaries invaginate into the epidermal layers assisting in gaseous exchange and the absorption of water and electrolytes across the ventral skin (Czopek, 1967; Goniakowska-Witalinska & Kubiczek, 1998). Since the deep capillary network is less dense than the subepidermal one, and is located further from the skin surface, it is unlikely that it has any function in gaseous exchange with the external environment. Instead, the deep capillary network is more likely to have a trophic role (Jasinski & Miodonski, 1978).

1.2.3 Effect of temperature

Toads are poikilothermic vertebrates and, in the wild, species such as *Bufo marinus* can survive temperatures between 7°C to in excess of 42°C (Tyler, 1975). However, there is a remarkable dearth of information in the literature regarding the effects of temperature on the function of the toad skin. From a histological perspective, it would appear that few, if any, studies have been done to ascertain whether the ambient temperature affects tissue kinetics in the skin (i.e. cell birth rate and differentiation rate). From an electrophysiological viewpoint, there have only been a few studies controlling
for the effects of temperature. Some of the major findings showed that the short-circuit current across the skin was temperature-dependent (Snell & Leeman, 1957; Dalton & Snart, 1968; Park & Hong, 1976). Skins taken from *Rana temporaria* acclimatised to an ambient temperature of 6°C displayed a higher short-circuit current compared with those from frogs acclimatised to higher temperatures of 23°C (Lagerspetz & Skyttä, 1975). Interestingly, Lagerspetz & Skyttä also found that the short-circuit current measured at the particular temperature to which the frogs had been acclimatised was regulated at 13µA/cm². This is surprising because chemical reactions typically speed up as the temperature increases, so it might be expected that Na⁺ pumping (and hence Isc) would increase with temperature. However, if the Na⁺/K⁺-ATPase began to denature as the temperature increased, the efficiency of Na⁺ pumping would decrease and an increase in Isc might not be seen. This idea is consistent with the findings of Geering & Rossier (1979) who showed that the Na⁺/K⁺-ATPase activity of the toad kidney versus temperature deviated from a standard linear Arrhenius plot at 15°C. Thus, it is suggested that toad skins may have evolved an autoregulatory mechanism for current: as the temperature increases, the current is maintained at a constant level, possibly because of denaturation of the Na⁺/K⁺-ATPase.

1.2.4 Moulting cycle

The moulting cycle of toads kept at room temperature takes approximately 1 week. During this period various morphological changes occur which Budtz and Larsen (1975) have divided into 4 main stages consisting of: the intermoult phase, the preparation and early shedding phase, the late shedding phase and the differentiation phase. For a detailed description of the events that characterise each phase the interested reader is referred to Barker-Jorgensen (1949), Budtz & Larsen (1975) and Fox, (1984). It has been shown that during the shedding phase, the water permeability is increased 3- to 4-fold and the permeability to salts is increased by up to 20-fold, compared with the intermoult phase (Barker-Jorgensen, 1949). It was suggested that this passive and transient leakiness, located in the vicinity of the MRCs, might be due to the disintegration of zonula occludentes (Whitear, 1974b). However, within 24 hours of detachment of the slough material, a new stratum corneum reforms from the underlying replacement cells, and zonula occludentes on the lateral sides of cells in both the stratum corneum and stratum germinativum layers can be identified (Budtz & Larsen, 1975). This feature is characteristic of the intermoult phase. These morphological observations are important in terms of the present study because in response to a
particular solution, similar trends were recorded over a 10-20 hour period from randomly selected toads at various stages in their moult cycles. Therefore, it cannot be argued that the responses observed were simply due to morphological changes in the skin related to the moult cycle; instead a more rigorous electrophysiological analysis is necessary.

1.3 Part 2: Function of the anuran epidermis

In 1904, Overton noted that when a live frog was placed in a watery solution it would not drink water through its mouth, but instead would absorb water through its skin by osmosis. Only in salty solutions greater than 140mM NaCl (i.e. 280mOsm/kgH₂O) would the frog drink water though its mouth. These findings were some of the earliest to suggest that the anuran skin was involved in water uptake over a large range of salt concentrations.

Just before World War II, the Nobel laureate, August Krogh, became interested in water and electrolyte homeostasis in frogs and other aquatic animals. In 1937 he showed that the frog could absorb salt from fresh water and dilute salt solutions. While recognising that this mechanism was useful in replenishing ions lost in the urine or across the skin through respiration, of greater interest was the realisation that the salts were actively taken up from a dilute (external) solution to a more concentrated blood plasma of just over 100mM (Krogh, 1939). He also demonstrated in vivo that Cl⁻ could be absorbed independently of Na⁺ or an accompanying cation, and likewise that Na⁺ could be absorbed independently of Cl⁻ or an accompanying anion (Krogh, 1937; Krogh, 1938). These findings demonstrated conclusively that the anuran skin had the capacity for active transport and could absorb ions selectively, although active transport was not understood until much later.

---

5 August Krogh (1874-1949) published his thesis on respiration through the skin and lungs in frogs in 1915 entitled Respiratory Exchange of Animals. In 1916, he became a professor of zoophysiology at the University of Copenhagen, and in 1920 he was awarded the Nobel Prize in Physiology and Medicine for his discovery of the mechanism of regulation of the capillaries in skeletal muscle. Krogh was not only respected for his research, but was also widely known for his construction of scientific apparatus (e.g. the spirometer) and for developing techniques for the extraction of insulin from the pancreatic glands of pigs using ethanol, giving the Dutch rapid access to insulin soon after its discovery in 1922 by Banting and Best in Toronto (Schmidt-Nielsen, 1995).

6 Anurans can absorb salt from solutions with NaCl concentrations below 1mM, and under extreme conditions NaCl can be taken up from solutions with concentrations of 0.01mM or less (Krogh, 1939).
With little knowledge of the different cell types in the anuran skin, and the transporting mechanisms within each cell type (many of which had not been discovered at the time), Krogh (1939) wrote at the conclusion of his book (without any specific reference to the anuran skin): ‘*A priori*, one would expect the mechanism for cation and anion absorption respectively to be located in separate cells and these to show somewhat different reactions to stains and precipitants.’ This statement has influenced the course of epithelial physiology over the last 60 years, raising many contentious issues regarding the uptake mechanisms for Na\(^+\) and Cl\(^-\) in the anuran skin over a range of salt concentrations. Even now, there is still debate and some confusion over the function and regulation of the PCs and the MRCs, the two main types of cells that are involved in the transport of water and electrolytes across the anuran skin. This section aims to: (1) review the evidence for Na\(^+\) and Cl\(^-\) uptake in the PCs and the MRCs, focussing specifically on whether these ions are taken up through the same cells or separate cell types; (2) examine the conditions under which the PCs and MRCs operate and (3) discuss the possibility that the function of the PCs is for salt uptake from watery solutions while the function of the MRCs is for water uptake from salty solutions.

1.3.1 *Transport mechanisms in the PCs and MRCs.*

A detailed description of the structure of the toad skin has already been presented in section 1.2, while evidence for the different transporting mechanisms is discussed extensively in section 1.4. For now, a summary of the transport mechanisms present in the main transporting cells of the anuran skin, namely the PCs and the MRCs, is shown diagrammatically in Figure 1.4.

Briefly, in the apical membrane of the PCs there is evidence to suggest that there are passive Na\(^+\) channels (Rick et al, 1978), Na\(^+\)/H\(^+\) antiports (Drewnowska & Biber, 1985; Drewnowska et al., 1988) and Cl\(^-\)/HCO\(_3\)\(^-\) antiports (Drewnowska & Biber, 1985). In the basolateral membrane of the PCs there are passive K\(^+\) channels (Harvey et al., 1991; Harvey & Urbach, 1992; Urbach & Harvey, 1993; Urbach et al., 1994; Urbach et al., 1996), Na\(^+\)/K\(^+\)-ATPase pumps (Mills & DiBona, 1977; Rick et al., 1978), Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporters (Ussing, 1986) and passive Cl\(^-\) channels (Ussing, 1982; Ussing, 1986; Ussing, 1988; Dorge et al., 1989; Larsen, 1991).

The apical membrane of the MRCs contain voltage-activated Cl\(^-\) channels (Larsen & Kristensen, 1978; Voûte & Meier, 1978; Foskett & Ussing, 1986; Willumsen & Larsen, 1986; Larsen et al., 1987; Larsen & Harvey, 1994; Larsen et al., 1996; Sorensen & Larsen, 1996), passive Na\(^+\) channels (Larsen et al., 1987; Rick, 1992; Harvey & Larsen,
Figure 1.4: A two cell model of a principal cell (PC) and a mitochondria-rich cell (MRC) in apposition. (Adapted from Harvey et al., 1991; Larsen, 1991; Larsen et al., 1996 & Acher et al., 1997).
1993), H⁺ pumps (Brown, 1989; Harvey, 1992; Jensen et al., 1997) and Cl⁻/HCO₃⁻ antiports (Willumsen & Larsen, 1995). Although there is little direct evidence for the presence of Na⁺/K⁺/2Cl⁻ cotransporters in the MRCs, it is likely that these cells possess these cotransporters for the purposes of volume regulation. They have therefore been included in the apical membrane, although further experiments are required to determine their existence and exact location. In the basolateral membrane of the MRCs there are passive Cl⁻ channels (Larsen et al., 1987; Nagel & Dorge, 1990; Willumsen & Larsen, 1995), Na⁺/K⁺-ATPase pumps (Mills et al., 1977; Larsen et al., 1987) and passive K⁺ channels (Larsen et al., 1987; Urbach et al., 1994).

In addition to the ion transport mechanisms described above, both the PCs and MRCs can rapidly change in volume, suggesting the presence of aquaporins. Much of the experimental work that has been conducted however, has focussed on the PCs which contain hormonally- and β-agonist-sensitive aquaporins in the apical membranes, which are quite different from the spontaneously occurring aquaporins in the basolateral membranes (Harvey et al., 1991). In contrast, little has been published about the types of aquaporins that exist in the MRCs. One reason for this is that morphological changes, which correlate well with induced volume changes, have been easier to identify in the PCs than the MRCs. For example, the stimulation of transepithelial water flow by the neurohypophoseal hormone, vasopressin, or the β–agonist, isoproterenol, has been associated with the appearance of intramembrane particle aggregates in the apical membrane of PCs (Brown et al., 1983; Handler, 1988; Grosso et al., 1994). It is now widely held that these aggregates are released from intracytoplasmic structures called aggrephores, lying just below the apical membrane (Berman et al., 1989). Although the exact chain of intracellular events is not known, a proposed model of a vasopressin- and isoproterenol-sensitive hydromotic epithelial cell such as in the PCs in the anuran is included in Figure 1.4. It is believed that neurohypophoseal hormones bind to vasopressin (V2) receptors located in the basolateral membrane of the cell, and this triggers a conformational change in a Gαs protein complex that then activates adenylate cyclase, resulting in the formation of cAMP, and the activation of protein kinase A (PKA) (Acher et al., 1997). Likewise, in the presence of cellular ATP, the activation of basolateral β–adrenergic receptors also leads to the formation of cAMP and PKA. The elevated cAMP level stimulates the movement of aggrephores towards...

---

7 It is known that the MRCs are insensitive to arginine vasotocin (AVT) and oxytocin (Ehrenfeld et al., 1989).
the apical membrane along microfilaments, which are regulated by a Ca^{2+}-calmodulin complex (Berman et al., 1989). Each aggrephore fuses with the apical membrane and a quantum of intramembrane particle aggregates is inserted into the membrane (Berman et al., 1989; Verkman, 1992). These aggregates may each represent a functional water channel or they may simply be subunits of one. Alternatively, it is possible that they are deactivated water channels, which are mobilised through phosphorylation by PKA (Berman et al., 1989). The remnants of the aggrephores are subsequently endocytosed and move back into the cell along a microtubule network, which like the microfilaments, is regulated by a Ca^{2+}-calmodulin complex.

1.3.2 MRCs as the main Cl⁻ pathway

A fundamental requirement for a cell type to be regarded as a transepithelial transport pathway for a particular ion is that it possesses both apical and basolateral membrane conductances for that ion. Both the MRCs and the PCs satisfy this requirement, although the transport mechanisms for Cl⁻ across the two cells are different. As outlined in the previous section, the MRCs possess apical Cl⁻/HCO₃⁻ cotransporters (Willumsen & Larsen, 1995), and apical and basolateral Cl⁻ channels, of which the apical channels display a voltage-dependence, opening upon depolarisation (Larsen & Kristensen, 1978; Larsen et al., 1987). The findings of Chapter 3 suggest that these channels are maximally open for transepithelial voltage clamps of +20mV (basolateral side positive with respect to the apical side) and maximally closed for voltage clamps of -20mV.

In contrast, the apical membrane of the PCs does not appear to contain Cl⁻ channels. Evidence for this includes: (1) electrophysiological and tracer flux measurements which have shown that there is little Cl⁻ exchange between the apical solution and the PCs (Nagel, 1977; Helman et al., 1979; Hudson, 1980; Stoddard and Helman, 1985; Ferreira & Ferreira, 1981); (2) apical Cl⁻ substitution experiments, transepithelial voltage clamp experiments and hyperpolarisation experiments (induced by amiloride in short-circuited skins) which have shown that there is no change in cellular Cl⁻ concentration of the PCs (Biber et al., 1985; Willumsen & Larsen, 1986, Biber et al., 1985; Nagel et al., 1981); and (3) volume measurements which have shown that only the MRCs swell when Cl⁻ is added to the apical bathing solution (Voûte & Meier, 1978). However, like the MRCs, the PCs possess basolateral Cl⁻ channels (Ussing, 1986; Ussing, 1988; Dorge et al., 1989; Larsen, 1991) and apical Cl⁻/HCO₃⁻ cotransporters (Drewnowska & Biber, 1985).

One of the features of the Cl⁻/HCO₃⁻ cotransporters found in both the PCs and the MRCs is that they exhibit saturation kinetics in the frog skin with a $V_{\text{max}}$ obtained for an
external Cl⁻ concentration of approximately 2mM (Alvarado, Dietz & Mullen, 1975; Ehrenfeld & Garcia-Romeu, 1978; Ehrenfeld & Garcia-Romeu, 1980). This is important because it brings into perspective the relative Cl⁻ transporting capacities of the PCs and the MRCs. Under conditions where the voltage-dependent Cl⁻ channels of the MRCs are closed, both the MRCs and the PCs are limited in their Cl⁻ uptake by the saturation of the Cl⁻/HCO₃⁻ cotransporters. In these circumstances, the main Cl⁻ pathway may be through either the PCs or the MRCs, and is largely determined by four factors: (1) the number of Cl⁻/HCO₃⁻ cotransporters per MRC or PC; (2) the ratio of the MRCs to PCs, which is partially determined by the salinity of the solution to which the anuran is exposed in the days leading up to the experiment (Budtz et al., 1995; Devuyst et al., 1991; Ilic & Brown, 1980); (3) the relative basolateral Cl⁻ conductances of the MRCs and the PCs and (4) the pH of the extracellular and intracellular environments.

However, if the apical Cl⁻ channels of the MRCs are open, the Cl⁻ flux is the sum of the secondary active uptake through the Cl⁻/HCO₃⁻ cotransporters and the flux through the Cl⁻ channels. The larger the inward driving electrochemical gradient for Cl⁻ across the apical membrane of the MRCs, the larger the contribution of the MRCs to the overall transepithelial Cl⁻ uptake. Of course, if the electrochemical gradient were outwardly directed so that Cl⁻ left the cell across the apical membrane (as might occur in a low NaCl solution), then this would diminish the amount of Cl⁻ taken up by the skin via the MRCs. However, it is likely that the MRCs have evolved such that the apical membrane potential for “physiological” conditions (i.e. low apical salt concentrations and open-circuit) is sufficiently negative that the (rectifying) Cl⁻ channels remain closed thereby preventing significant Cl⁻ loss. For higher NaCl solutions it is likely that the Cl⁻ permeability is determined by the influx of Na⁺, which results in a slight depolarisation of the apical membrane of the MRCs. This is consistent with the experimental findings that the passive component of the Cl⁻ uptake is directly linked with Na⁺ uptake (Garcia-Romeu & Ehrenfeld, 1975).

It may be concluded that the question of whether the MRCs are the main Cl⁻ transporting pathway depends largely upon the experimental conditions, namely the salt concentration of the apical bathing solution and the clamp condition (open-circuit,

---

8 This appears to be the case when the apical side of the isolated short-circuited skin is bathed in a low NaCl solution of around 2mM. Under these circumstances transepithelial Cl⁻ uptake is predominantly active (Erlrij, 1971; Kristensen, 1972; Larsen et al., 1996; Martin & Curran, 1966). In contrast, when the apical solution has a high extracellular NaCl concentration of around 110mM, Cl⁻ uptake is predominantly passive (Koefoed-Johnsen et al., 1952; Ussing & Zerahn, 1951).
short-circuit or +/- voltage clamp). In (salty) Ringer solutions Cl⁻ transport is mainly via the MRCs under both short-circuit and open-circuit conditions, primarily because the PCs possess no apical Cl⁻ permeability. In low salt solutions, where the active Cl⁻ component predominates under open-circuit conditions, Cl⁻ uptake is presumably via both the PCs and the MRCs.⁹

1.3.3 PCs as the main Na⁺ pathway

In 1958 Koefoed-Johnsen and Ussing suggested tentatively that the stratum germinativum cells were responsible for the active uptake of Na⁺ ions across the anuran skin (Koefoed-Johnsen & Ussing, 1958). They proposed that this occurred via the apical influx of Na⁺ through passive channels and the active extrusion of Na⁺ through basolateral Na⁺/K⁺-ATPases. As more experimental data were generated, it became apparent that many of the cells throughout the granulosum, spinosum and germinativum layers were connected by intercellular bridges of low electrical resistance forming a syncytial structure (Rick et al., 1978). The Koefoed-Johnsen/Ussing model was modified to reflect this syncytium of PCs by moving the site of passive Na⁺ uptake to the apical membrane of the outermost granulosum cells. This was consistent with experimental findings which showed that (1) Na⁺ entered the PCs through amiloride-blockable Na⁺ channels in the apical membrane of the granulosum cells; (2) the Na⁺ then diffused throughout the PCs reaching the deeper germinativum layer and (3) all Na⁺ in the germinativum layer was derived from the apical bathing solution (Rick et al., 1978). Further support for the model came with the discovery that the basolateral membranes of all living cells in the frog skin epithelium showed evidence of Na⁺/K⁺-ATPases (Mills & Di Bona, 1977; Mills et al., 1977). Thus, the PCs possessed the basic mechanisms for active Na⁺ uptake.

There are several arguments that are used in the literature to support the view that the PCs are the site of most Na⁺ uptake. First, Na⁺ and Cl⁻ can each be transported independently of the other ion in low salt concentrations (Ehrenfeld et al., 1989). Second, based on the delayed voltage responses to fast changes in the Na⁺ concentration

⁹ Of course, the passive component of the Cl⁻ uptake can be artificially manipulated by biasing the transepithelial voltage. For example, in Ringer solutions the passive component can be negated by applying a large negative voltage clamp (basolateral side negative with respect to apical side) such that the voltage-activated Cl⁻ channels remain closed. Alternatively, in low salt concentrations the passive Cl⁻ component could be introduced by applying a large positive clamp, which, presumably opens the Cl⁻ channels and alters the electrochemical gradient so that Cl⁻ is directed inwards. The positive clamp would also alter the electrochemical driving potential for Na⁺.
of the apical solution, it has been estimated that the distance of Na$^+$ diffusion to the Na$^+$-selective membrane is not much greater than the thickness of the cornified layer suggesting that Na$^+$ uptake occurs across the first “reactive layer.” (Fuchs et al., 1972). Third, according to Brown et al. (1981), but in contrast to Ehrenfeld et al. (1989), the density of the MRCs show no correlation with the active Na$^+$ flux. Fourth, whole-cell Na$^+$ currents and channel density are five times greater in the PCs than the MRCs (Harvey and Larsen, 1992). Fifth, there is a reduction in cell Na$^+$ throughout the syncytium when the apical solution is Na$^+$-free or contains amiloride, but cell Na$^+$ levels approach that of the outside bathing solution when the basolateral pumps are blocked with ouabain (Rick et al., 1980).

On the other hand, the MRCs may be a possible site of Na$^+$ transport. On the other hand, the MRCs may be a possible site of Na$^+$ transport. First, according to Harvey (1992), (but in contrast to Larsen et al., 1987), the MRCs can theoretically account for all of the transepithelial Na$^+$ flux. Second, as their name suggests, the MRCs contain high concentrations of mitochondria (Whitear, 1975). This suggests that the MRCs are more metabolically active than the PCs. [Interestingly, while the presence of mitochondria is not direct evidence for Na$^+$/K$^+$-ATPase pumps, it has been found that 60% of the oxygen consumption of tight epithelia is used to supply these pumps (Harvey & Urbach, 1992)]. Third, there is a strong positive correlation between MRC numbers and Na$^+$ absorption rates when the apical side is bathed in a low NaCl solution (Ehrenfeld et al., 1989).

So far only the reasons favouring either the PCs or the MRCs as the site for Na$^+$ transport have been presented. It is therefore appropriate to discuss the validity of these observations and conclusions with reference to other experimental findings to discern where discrepancies arise. Where possible, data are discussed in the context of experimental conditions (i.e. extracellular salt concentrations and clamp conditions) so that comparisons can be made between different conditions.

*Forgotten Data: Volume-activated Na$^+$ channels*

In the early 1960’s, Ussing and his co-workers conducted a series of experiment which investigated changes in the epithelial volume and the short-circuit current that occurred when the osmolarities or concentrations of the bathing solutions were changed (MacRobbie & Ussing, 1961, Ussing, 1963; Ussing, 1965; Ussing, 1966). It was found that epithelial swelling occurred when the basolateral bathing solution (but not the apical solution) was made hypotonic (with respect to standard Ringer solution) and that epithelial shrinkage occurred when either the apical or basolateral bathing solution was
made hypertonic (Ussing, 1965). In addition, epithelial swelling was associated with an increase in Isc (and thus active Na$^+$ transport) while shrinkage almost always resulted in a reduction in Isc. Thus, there appeared to be a strong positive correlation between volume and Isc (Ussing, 1977). These findings were further substantiated and extended by Costa et al., (1987) who showed that there was a reduction in the apical Na$^+$ permeability ($P_{Na_a}$) of 30-40% upon increasing the tonicity of the basolateral bathing solution or removing basolateral Cl$, and a reduction of 10-20% in $P_{Na_a}$ when Na$^+$ was removed from the basolateral solution.

Of interest, however, is the repeated claim that “changes in the osmolarity or composition of the outside [apical] solution had virtually no effect on the epithelial volume” (Ussing, 1982; Ussing 1977; Spring & Ussing, 1986). Yet Zeiske & Van Driessche (1984) showed a clear reduction in Isc when the osmolarity of the apical bathing solution was increased, and based on kinetic studies they suggested that $P_{Na_a}$ was reduced by ‘hypertonic shocks.’ Based on these conflicting reports, Isc did not appear to be closely correlated with volume. These anomalous results suggested that either epithelial volume changes did indeed occur as the apical bathing solution was changed, or that syncytial (or PC) volume was not always a good indicator of Isc.

Ussing’s experimental data (1965) certainly did suggest that changes in the osmolarity and salt concentration of the apical solution had little effect on epithelial volume. However, it should be noted that the hypotonic solutions (standard Ringer solutions diluted by 50%) still had quite a high osmolality (~115mOsm/kgH$_2$O), which is much higher than the fresh water solutions to which anurans are typically exposed in their natural habitats. Therefore, it is possible that even in 50% dilute Ringer solution the PCs were sufficiently shrunken that the putative stretch-activated Na$^+$ channels were predominantly closed. $^{10}$

Such a postulate would also be consistent with the very low apical Cl$^-$ permeability in the PCs (see sections 1.3.2 & 1.4.1.5). With only apical Cl$^-$/HCO$_3^-$ exchangers present, which saturate at low concentrations and limit apical Cl$^-$ uptake, volume regulation of the PCs by NaCl uptake from the apical solution at high salt concentrations would be restricted. Consequently, a large apical PNa (and hence large Na$^+$ flux) in high salt concentrations would seem unnecessary for volume regulation. At low salt

$^{10}$ A reduction in osmolarity of the apical solution necessitates a reduction in salt (NaCl) concentration. But as the apical Na$^+$ concentration is reduced, so too is Isc. If the NaCl concentration is reduced too far, measurement difficulties often result owing to the (small) magnitude of Isc. This is possibly why Ussing limited reductions in the osmolarity of the apical solution to 1/2 Ringer.
concentrations, however, Na\(^+\) uptake in the PCs would either be through the Na\(^+\)/H\(^+\) exchangers [as seen by the inhibition of the Na\(^+\) absorption and H\(^+\) excretion upon apical application of 1\(\mu\)M acetazolamide (Garcia-Romeu & Ehrenfeld, 1975)], or by uptake through the (partially) open Na\(^+\) channels (electrochemical gradient permitting).

The alternative explanation that the volume of the PCs is not a good indicator of Isc might suggest that Na\(^+\) is transported across the MRCs. With slightly different transport mechanisms, it is likely that MRCs display similar, but sometimes different, volumetric responses to the PCs. Therefore, volume-sensitive Na\(^+\) channels may be present in the anuran skin in MRCs, not in PCs. Extensive work on the volume of the MRCs performed by Ussing and his coworkers in the mid-1980’s showed that: (1) removal of Cl\(^-\) from the apical solution while maintaining the same osmolality caused MRC shrinkage, even though Cl\(^-\) was present in the basolateral solution (Spring & Ussing, 1986); (2) most MRCs swelled when the basolateral solution was clamped positive with respect to the apical solution. This effect could be prevented by removal of Cl\(^-\) from the apical solution (Foskett & Ussing, 1986; Larsen et al., 1987); (3) changes in the volume of the MRCs had little effect on the Cl\(^-\) conductance under voltage clamp conditions. (Larsen et al., 1987); (4) the MRCs acted as ideal osmometers with constant, osmotically inactive volumes of 21% (Larsen et al., 1987). More specifically, the MRCs, unlike the PCs, were sensitive to changes in the osmolality of the apical bathing solution, shrinking and swelling respectively, in solutions that were hyper- and hypotonic to standard Ringer (Larsen et al., 1987; Spring & Ussing, 1986). However, volume changes were more pronounced when the osmolality of the basolateral solution was altered. Spring and Ussing calculated the hydraulic conductivity of the apical membrane to be approximately 20% that of the basolateral membrane. They also found that the MRCs were swollen when the apical side was bathed in pond water, and calculated the osmolality under these conditions to be \(\approx 165\text{mOsm/kgH}_{2}\text{O}\); (5) Under short-circuit conditions the MRCs shrank upon apical exposure to 50\(\mu\)M amiloride or Na\(^+\)-free solutions, but swelled significantly when 3mM ouabain was added to the basolateral solution. This effect could be abolished by the simultaneous application of 50\(\mu\)M (apical) amiloride or by bilateral removal of Cl\(^-\) (Larsen et al., 1987). Clearly, the MRCs are volumetrically responsive, adapting to concentration and osmotic changes in both the apical and basolateral solutions. Therefore, stretch-activated channels (and in particular Na\(^+\) stretch-activated channels) may play an important role in ion transport across these cells.
1.3.4 Overall Toad Skin Function

Although there has been some discussion that Na\(^+\) and Cl\(^-\) ions pass through the same cell compartment (Candia, 1978; Kristensen, 1981, Larsen et al., 1987, Ehrenfeld et al., 1989), there has been a view that the main route for Na\(^+\) uptake is through the PCs, while Cl\(^-\) uptake is through the MRCs. One of the major flaws with this view, however, is that it does not address the issue of volume regulation in each cell type, which must occur if an ionic steady-state is to be reached.

A reassessment of past experimental observations in terms of environmental conditions (i.e. extracellular osmolality, salt concentrations and voltage clamp conditions) suggests that Na\(^+\) and Cl\(^-\) ions do indeed pass through the same cells, but the extracellular environment determines which cell type dominates salt uptake (i.e. PCs or MRCs). More specifically, under in vivo-like conditions, where the basolateral solution resembles blood plasma and does not change significantly in ionic concentration or osmolality, it is hypothesised here that the dominant pathway is through the PCs in apical solutions of low salt concentration (and low osmolality), but in solutions of higher salt concentrations salt uptake is through the MRCs. That is, it is postulated that the function of the PCs is for salt uptake from watery solutions while the function of the MRCs is for water uptake from salty solutions.

Such mechanisms would presumably allow the anuran to regulate water uptake over a wide range of salt concentrations, as found by Overton (1904). In watery solutions, given a sufficient transcellular hydraulic conductivity, water uptake would occur by simple osmosis. In these conditions it is likely that salt uptake is of secondary importance, necessary only to replenish the salts lost through the (dilute) urine. The PCs are likely candidates for this role given their low salt uptake capacity (restricted by the passive Cl\(^-\) impermeability and the saturation of the Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)\(^-\) exchangers), and their low mitochondrial density (which suggests a low pumping capacity). It is likely that most regulation of the PCs is through the hormonal control of the ADH-sensitive apical aquaporins.\(^{11,12}\) Of course, salt uptake by the PCs is quite possibly

---

\(^{11}\) Ussing’s findings that the PCs are unresponsive to relatively salty solutions suggests that the apical Na\(^+\) channels may be volume-sensitive, closing as the anuran moves into increasingly salty solutions whereupon water uptake is controlled by the MRCs.

\(^{12}\) From an experimental perspective it should be pointed out that removal of the skin from the toad isolates it from neurohypophoseal hormones, such as AVT, which are circulating in the blood. Therefore, aquaporin insertion ceases and it is reasonable to assume that immediately after skin removal there is a finite number of aquaporins present in the PCs. But if these AVT-sensitive aquaporins behave similarly to
supplemented by the MRCs, particularly when the anuran is exposed to very low salt solutions for long periods of time. This is consistent with the finding that anurans, acclimated in distilled water for a month, displayed an increase in the number of MRCs compared with those acclimated in tap-water (Ilic & Brown, 1980; Devuyst et al., 1991).

As the apical solution becomes saltier, regulatory control of salt uptake becomes increasingly important. There is the risk that if the apical solution became saltier than blood plasma, water could be lost by the anuran and unnecessary salts could be gained, compromising its survival. Thus, the amount of salt uptake and the rate of absorption must be regulated within certain physiological limits. Outside these limits it is possible that the skin becomes impermeable to salts to prevent excessive uptake. Presumably, the upper limit of apical salt concentration is around 140mM in the light of Overton’s observation that anurans consume water orally rather than absorb it across their skins at this concentration.

It would appear that the MRCs may have evolved with the necessary mechanisms to regulate salt uptake by the elegant combination of voltage-dependent Cl⁻ channels and volume-sensitive Na⁺ channels. First, satisfying the requirement that the skin becomes impermeable to salt in excessive external salt concentrations: it is known that the MRCs shrink and Isc decreases when the tonicity of the apical solution is increased (Zeiske & Van Driessche, 1984; Larsen et al., 1987; this study). It is also likely that under these conditions the apical voltage-dependent Cl⁻ channels are closed. This is based on the observation that, when the apical solution is bathed in Ringer’s solution under short-circuit conditions, Na⁺ channel closure (induced by amiloride) results in the apical membrane potential of the MRCs dropping from approximately –40 to –120mV (Nagel

their ADH-sensitive analogues in the mammalian kidney then they disintegrate over approximately 15 minutes making the membrane water impermeable.

In this study, approximately 20 minutes elapsed between toad pithing and the commencement of measurements on 2 tissue specimens, and approximately 60 minutes elapsed when 8 specimens were used. During this time, it is possible that the apical membranes of the PCs had become water impermeable. No change in PC cell volume would therefore be expected when the osmolality or salt concentration of the apical solution was changed which is consistent with Ussing’s observations.

However, a water-flux across the isolated skin (that is Hg²⁺-inhibitable) can still be observed after the 20 minute period following pithing (Grosso & De Sousa, 1993). This suggests that water absorption is occurring via the MRCs, which possess AVT-insensitive aquaporins (that presumably do not disintegrate in the absence of this hormone).
& Dorge, 1996). Consequently, salt loss across the apical membrane is presumably minimised by closure of both the apical Na\(^+\) and Cl\(^-\) channels.

Second, with increasing hypotonicity of the apical solution the MRCs swell and Isc increases (due to an increase in PNa) until Isc becomes limited by the reduction in the apical (and therefore cellular) Na\(^+\) concentration (see footnote 12). This suggests a graded opening of the Na\(^+\) channels. These findings are consistent with the MRCs appearing swollen when exposed to pond water (Larsen et al., 1987). Under these circumstances, Cl\(^-\) channel opening is presumably largely dependent on the opening of the Na\(^+\) channels. It would be expected that as the Na\(^+\) channels open, a rapid charge reshuffling across the membranes occur with (positively charged) Na\(^+\) ions accumulating at the inner face of the apical membrane, thus depolarising it. Assessing the degree to which the Cl\(^-\) channels are open is therefore difficult, particularly in a non-steady-state where the apical membrane potential is constantly changing. However, it can be said that in 2mM apical NaCl solutions under steady-state open-circuit conditions, the apical membrane potential is approximately −40mV, corresponding to a transepithelial potential across the MRCs of approximately +40mV (Nagel & Dorge, 1996). A voltage clamp of +40mV is sufficient to achieve a maximal Cl\(^-\) conductance (Chapter 3). Therefore, it is reasonable to assume that the Cl\(^-\) channels are at least partially open when the MRCs are bathed in low salt solutions, but tend to close as PNa closes in higher salt solutions. This link between Na\(^+\) flux and membrane potential, resulting in Cl\(^-\) channel opening or closure, is consistent with the idea that Na\(^+\) and Cl\(^-\) uptake appear to be inter-dependent as the apical salt concentrations increases above 2mM (Watlington, 1972; Garcia-Romeu & Ehrenfeld, 1975a,b). This inter-dependence is supported by the experimental observations that (1) ouabain and amiloride, both specific inhibitors of Na\(^+\) transport, also inhibit Cl\(^-\) transport (Garcia-Romeu & Ehrenfeld, 1975a,b) and (2) that application of the Na\(^+\) channel activator, benzimidazolylguanidin (BIG) to the apical solution stimulates the Cl\(^-\) flux through the short-circuit ed skin (Kristensen, 1981).

1.4 Part 3: Transport mechanisms in the toad skin

With an overview of how the toad skin could function in salty and watery conditions, it is now appropriate to review in detail the experimental data presented in the literature that was used to determine the different transporting mechanisms in the PCs and MRCs, which has led to our present understanding of the toad skin.

* * * * *
1.4.1 Principal Cells

1.4.1.1 Evidence for apical Na\(^+\) channels in PCs

Koeffoed-Johnsen and Ussing (1958) hypothesised that Na\(^+\) entry across the apical face of the isolated anuran skin was passive because it provided a simple, yet sufficient solution to explain their experimental data. Although it has been challenged on occasions (Cereijido and Rotunno, 1968), it has stood the test of time, and is now widely accepted. This section reviews the evidence leading to this conclusion and focuses, in particular, on the data advocating the existence of apical Na\(^+\) channels in the PCs. For a comprehensive treatment addressing other properties of amiloride-blockable Na\(^+\)-channels in the apical membranes of high-resistance epithelia, the reader is referred to Abramcheck et al. (1985), Schultz (1985), Garty & Benos (1985), Turnheim (1991) and Schultz (1992).

There are two compelling arguments favouring the existence of apical Na\(^+\) channels in the anuran skin. First, modifications to the apical bathing solution, such as the removal of Na\(^+\) ions, or the addition of the Na\(^+\) channel blocker, amiloride, cause a marked reduction of the short-circuit current, which is a measure of Na\(^+\) movement across the epithelium. These results suggest that Na\(^+\) entry is by diffusion through Na\(^+\) selective ion channels. Second, if apical Na\(^+\) uptake were via these channels, then the current-voltage characteristics of Na\(^+\) movement should be described by the Goldman-Hodgkin-Katz (GHK) equation, a relationship widely used to model electrodiffusion across ion channels. Fuchs et al., (1977) showed that this was indeed the case for a variety of extracellular Na\(^+\) concentrations, thereby supporting existing evidence for apical Na\(^+\) channels (Leblanc & Morel, 1975; Morel & Leblanc, 1975).

The existence of these channels in the PCs is based upon measurements of the intracellular concentration using electron microprobe techniques (Rick et al., 1978). In this elegant experiment it was demonstrated that the PCs derive all Na\(^+\) from the apical bathing solution, and that this Na\(^+\) enters through amiloride-blockable channels. First, Rick et al. reduced the extracellular Na\(^+\) concentration on the apical side and showed a concomitant lowering of the Na\(^+\) concentration of the PCs, but this effect was not elicited if the basolateral Na\(^+\) concentration was lowered. This suggested that the bulk of Na\(^+\) uptake occurred apically. Then they applied the Na\(^+\)/K\(^+\)-ATPase blocker, ouabain, to the basolateral bathing solution inhibiting the Na\(^+\) exit pathway. As a result they saw
an increase in the intracellular Na$^+$ concentration.\textsuperscript{13} Having measured this ouabain-induced effect, Rick et al. then showed that the intracellular Na$^+$ levels in the PCs could be restored to control values by removing Na$^+$ from the apical solution and generating an efflux of Na$^+$ down its concentration gradient. However, if amiloride were added to the apical solution, then the Na$^+$ concentration of the PCs remained elevated, indicating that apical Na$^+$ movement in the PCs is via amiloride-blockable channels.

It is worth noting that apical Na$^+$ uptake is limited mainly to stratum granulosum, the outer layer of living cells, just beneath stratum corneum (Fuchs et al., 1972; Voute & Ussing, 1968; Voute et al., 1975). The PCs in this layer are often referred to as the ‘first reactive principal cell layer’ because these cells possess polarised apical and basolateral cell membranes (Urbach et al., 1994). PCs in the deeper layers are regarded as non-polar due to the passive redistribution of Na$^+$ ions via the low-resistance gap junctions. In essence then, PCs located towards the corium, behave electrically as extended basolateral membranes of the first reactive cell layer.

1.4.1.2 Evidence for basolateral Na$^+/K^+$-ATPase pumps in PCs

Na$^+$ uptake across the anuran skin is a two-step process involving passive entry at the outward facing membrane and active extrusion across the inner barrier (Ussing, 1949). This active pumping of Na$^+$ involves the expenditure of energy provided by oxidative metabolism (Leaf & Renshaw, 1957; Zerahn, 1956) and was originally thought to occur at the innermost cell layers in stratum germinativum (Koefoed-Johnsen & Ussing, 1958). However, multi-step potential profile measurements across the anuran skin (Ussing and Windhager, 1964) challenged this view. It was argued that to obtain such measurements, pumps must also be located in more apical cell layers.

Using staining techniques, and light and electron microscopy, Farquhar and Palade (1966) attempted to determine the exact location of Na$^+/K^+$-ATPase activity in the anuran skin, because this enzyme was thought to be closely linked to the Na$^+$ pump (Skou, 1965). However, while they found evidence of ATPase activity in all living cell layers, the staining techniques that were adopted were later questioned by Mills et al (1977), because Na$^+/K^+$-ATPase was not stained directly. Rather Mg$^{2+}$-ATPase was stained and evidence of this enzyme was assumed to be proportional to Na$^+/K^+$-ATPase activity. Farquhar and Palade had reasoned that, since membrane ATPases in the toad

\textsuperscript{13} This effect could be abolished in the presence of amiloride or Na$^+$-free solution on the apical side further supporting the idea that Na$^+$ uptake occurs predominantly across the apical membrane of the PCs.
skin could be activated by Mg$^{2+}$, and further stimulated by Na$^{+}$ and K$^{-}$, it was possible that the enzymes were simply convertible isoforms, a view that had also been advanced by other researchers of the time (Askari & Fratantoni, 1964; Skou & Hilberg, 1965). Therefore, although the results did not directly show Na$^{+}$/K$^{+}$-ATPase activity, they were thought to be a qualitative measure of Na$^{+}$ pump location.

A quantitative determination of Na$^{+}$/K$^{+}$-ATPase location in the anuran skin was undertaken by Mills et al. (1977), using freeze-dry radioautographic techniques. Skins were poisoned with radiolabelled [$^{3}$H]ouabain, which specifically binds to Na$^{+}$/K$^{+}$-ATPase, inhibiting active Na$^{+}$ transport. The advantage of this technique was that Na$^{+}$ transport could be monitored using standard electrophysiological measurements in vitro, before and after ouabain application. The amount of ouabain required to produce a particular effect could be measured subsequently by counting the number of disintegrations per unit time using a liquid scintillation counter. Alternatively, the location of ouabain binding sites could be determined by radioautographic imaging. Using these procedures, it was shown that ouabain-sensitive Na$^{+}$/K$^{+}$-ATPase pumps were located in all non-cornified cells of the anuran epidermis, which was consistent with the preliminary findings of Farquhar and Palade (1966). However, it was found that the pump density was greatest in cells of stratum spinosum and stratum granulosum (Mills & DiBona., 1977; Mills et al., 1977) suggesting that cells located deeper in the epidermis are involved in the extrusion of Na$^{+}$ across the basolateral membranes.

While Na$^{+}$/K$^{+}$-ATPase pumps are present in all the living cells of the anuran skin, it has been shown that some of these are specifically located in the PCs. This was demonstrated convincingly by Rick et al. (1978), when they measured the intracellular Na$^{+}$ concentration of the PCs using electron microbe analysis. As described earlier, the addition of 10$^{-4}$M ouabain to the basolateral bathing solution resulted in an increase in Na$^{+}$ levels in the PCs. These results are consistent with blocking active Na$^{+}$ extrusion via Na$^{+}$/K$^{+}$-ATPase pumps.

1.4.1.3 Evidence for basolateral K$^{+}$ channels in PCs

As early as 1958 it was hypothesised that the basolateral membrane conductance of the anuran skin was dominated by potassium (Koeffoed-Johnsen & Ussing, 1958). Although many experiments have been performed using the whole skin preparation to show the presence of a basolateral K$^{+}$ conductance (Nielsen, 1985; Ussing et al, 1965; Ussing, 1987), until recently, little was known about the potassium transport mechanisms in the individual cell types, because of difficulties in isolating viable cells
from layers deep within an epithelium. However, recent experiments have demonstrated that it is possible to expose the basolateral membranes of cells in the first reactive layer of the anuran skin by employing a combination of enzymatic and mechanical separation techniques (Urbach et al., 1994). Basically, the corial side of the isolated ventral skin is treated with collagenase under 10 cm of hydrostatic pressure at 30°C. After about an hour of this treatment, blisters formed between the corium and the epithelium. By injecting Ringer solution into these blisters using a catheter, it was possible to get epithelial detachment. The epithelium was then exposed to Ca^{2+}-free Ringer to remove the basement membrane, before being mounted in a miniature Ussing chamber in preparation for whole-cell or single channel, patch-clamp experiments. Under such conditions, both PCs and MRCs can be identified. However, the following discussion pertains only to PCs.

One of the advantages of exposing the basolateral membrane of the PCs was that the macroscopic, current-voltage (I-V) relationship of these membranes could be determined directly using standard cell impalement techniques. Due to the inaccessibility of these membranes, this had previously not been possible (Urbach et al., 1994). The I-V measurements showed that there was steep inward current rectification, either favouring the entry of cations or the efflux of anions. Several lines of evidence indicated that this rectification was K^+ dependent. First, increasing the K^+ concentration of the basolateral bathing solution shifted the reversal potential in a positive direction and increased the degree of rectification. Second, basolateral application of the broad-spectrum K^+ channel blocker, Ba^{2+}, abolished the current rectification and rendered the basolateral membrane insensitive to changes in K^+ concentration (Urbach et al., 1994). Overall, these findings suggested the existence of a passive K^+ conductance in the basolateral membranes of the PCs.

Single channel studies of many epithelia have shown that epithelial cells often contain more than one type of K^+ channel (Dawson et al, 1990). This appears to be true for the PCs of the anuran skin. Using both cell-attached and inside-out configurations of the patch clamp technique (Hamill et al., 1981), it has been shown that at least two types of K^+ channels exist in the basolateral membrane of the PCs. These channels are the 15-25pS spontaneously active, inward-rectifying, ATP-sensitive K^+ channel (K_{ATP}) and the 35pS Ca^{2+}-activated outward-rectifying K^+ channel (K_{Ca}) (Harvey et al., 1991; 14 Early papers refer to the K_{ATP} channel as the inward-rectifying potassium channel (K_i). According to Urbach et al., (1996) these channels are the same. Therefore, to avoid confusion, only the notation K_{ATP} is used in this dissertation.)
Harvey & Urbach 1992; Urbach & Harvey, 1993; Urbach et al., 1994; Urbach et al., 1996).

Of the two channels, it would appear that the $K_{ATP}$ channel is the main $K^+$ flux pathway in the basolateral membrane. Two arguments support this notion. First, if the $K_{Ca}$ channels were a major $K^+$ pathway, then inhibiting them would significantly affect the basolateral membrane conductance. However, pharmacological agents such as TEA, tetrápentylammonium, choline, the honeybee venom, apamin, and charybdotoxin, normally used to block $Ca^{2+}$-activated $K^+$ channels, had no effect on the membrane conductance (Urbach et al., 1994). Second, out of 500 cell-attached patch-clamp recordings made in sodium Ringer solution, only one type of $K^+$ channel, namely the spontaneously active $K_{ATP}$ channel, was identified (Urbach et al., 1994). These results strongly suggest that the role of the $K_{Ca}$ channel is small.

The $K_{ATP}$ channel

The $K_{ATP}$ channel, first reported in heart muscle in 1983 (Noma, 1983), has several interesting properties. First, its open probability, $p_o$, is dependent on a number of metabolic factors such as cytosolic ATP, $H^+$ and $Ca^{2+}$. Urbach & Harvey (1993) showed that, in excised inside-out patches, $p_o$ was reduced by cytosolic ATP, with a half-maximal inhibition ($K_i$) between 30-80$\mu$M. However, in the presence of 100$\mu$M ADP, which is within physiologically acceptable limits, the half-maximal inhibition for ATP was shifted into the millimolar range (Harvey & Urbach, 1992). This is of interest because when the PCs were bathed in sodium Ringer solution, which is the solution used routinely in anuran experiments, the intracellular ATP concentration was about 1.5mM (Harvey & Urbach, 1992). If ATP were the sole regulator of the $K_{ATP}$ channels then one would expect them to be closed! However, physiological levels of ADP open the $K_{ATP}$ channel following inactivation by high ATP concentrations. Therefore, the ratio of ATP:ADP may be one of the important factors in determining $p_o$ (Miki et al., 1990). Another metabolic factor influencing $p_o$ is the cytosolic $H^+$ concentration. Urbach et al., (1991) reported that for a cytosolic pH=6.9 the $K_{ATP}$ channels were maximally closed, but at pH=7.4 the channels were fully open. In other words, any factor causing intracellular acidification closes $K_{ATP}$ channels. Finally, an increase in cytosolic $Ca^{2+}$ ($K_i$=180$\mu$M) also causes $K_{ATP}$ channel inhibition (Harvey & Urbach, 1992). This may play an important role in maintaining the intracellular ionic milieu when PCs are exposed to hypo-osmotic solutions. Under such circumstances stretch-sensitive $Ca^{2+}$ channels in the basolateral membrane are assumed to be activated (Harvey & Urbach, 1992). In addition to inactivation by metabolic factors, the $K_{ATP}$
channel is blocked by Ba\(^{2+}\). However, unlike other types of K\(^+\) channels, the K\(_{ATP}\) channel is insensitive to TEA and quinidine (Harvey & Urbach, 1992).\(^{15}\)

Just as some substances inactivate the K\(_{ATP}\) channel, others activate it. As discussed above, at physiological levels, ADP will reactivate the channels.\(^{16}\) Other substances resulting in channel reactivation include 10µM dibutyl cGMP, which has an effect in less than 5 minutes, 10µM cytosolic GTP\(_{\gamma}\)S (guanosine 5'-O-(3-thiotriphosphate)) and 10nM aldosterone. Aldosterone-induced activation is associated with an increase in pH and a decrease in ATP concentration, both of which would tend to open channels. The exact site of aldosterone action is not known, although there is evidence to suggest that this hormone activates the basolateral Na\(^+\)/H\(^+\)-exchanger in the PCs, as the effect is abolished in the presence of the Na\(^+\)/H\(^+\)-exchange blocker, amiloride (Urbach & Harvey, 1993).

Single channel recordings made in the ‘cell-attached’ patch-clamp mode (Figure 1.5A) indicate several interesting properties of the K\(_{ATP}\) channel (Urbach et al., 1994). First, when the bath and pipette solutions contained standard NaCl Ringer the probability of the channel being open increased as the membrane was depolarised and was close to maximal (p\(_o\)=0.8) at the resting cell potential (approx. –70 to –40mV). As the membrane was hyperpolarised beyond the K\(^+\) reversal potential (E\(_K\) ~ -95mV), p\(_o\) decreased linearly (Figure 1.5B). However, despite this reduction in p\(_o\), the channels were more conductive for inward flowing K\(^+\) ions (Figure 1.5C).\(^{17}\) Similar results were observed in conductivity when the pipette contained a 125mM KCl solution and the bath contained NaCl Ringer. In this situation, the channels showed almost complete inward rectification with a negligible outward current (Figure 1.6A). However, this time the channels closed upon depolarisation (Figure 1.6B).

\(^{15}\) At high quinidine concentrations (100µM), inward rectification of the K\(^+\) current can be abolished (Urbach et al., 1994).

\(^{16}\) It was reported that in high concentrations (i.e. K\(_i\)>5mM), cytosolic ADP will block the K\(_{ATP}\) channel. However, this is well outside the physiological range for ADP (and is at least 5 times greater than the ATP concentration that will cause channel inactivation) (Urbach & Harvey, 1993; Urbach et al., 1996).

\(^{17}\) Unlike other passive channels, the K\(_{ATP}\) channel cannot be described by the standard GHK equation because of the inward current rectification.
Figure 1.5: (A) Single channel recordings of basolateral $K_{\text{ATP}}$ channels made in the ‘cell-attached’ patch clamp mode with standard NaCl Ringer in both the bath and the patch pipette. The dashed line represents the closed state. Inward currents are represented in the downward direction while outward currents are represented in the upward excursions; (B) the opening probability ($p_o$) of $K_{\text{ATP}}$ as a function of membrane potential (represented by the pipette voltage, $V_p$); (C) the voltage dependence of the single-channel $K_{\text{ATP}}$ current (●) and the macroscopic basolateral membrane current (○). (Taken from Urbach et al., 1994).
Finally, in the last part of the study, it was demonstrated that inward current rectification was strongly dependent on cytosolic Mg\(^{2+}\) ions. Removal of these ions abolished rectification such that the single channel I-V curve became ohmic (Figure 1.7).

Two main points are worth noting from this data. First, inward rectification of the K\(_{\text{ATP}}\) channel is not voltage dependent. If it were, then the channels would open upon hyperpolarisation. This is contrary to the finding where the channels were bathed in extracellular NaCl Ringer. Second, when the basolateral membrane is depolarised in NaCl Ringer as in Figure 1.5, the probability of the channels being open is very high and K\(^+\) can be conducted out of the cell. Under such circumstances, the channels approach a constant current source.

The K\(_{\text{Ca}}\) channel

As its name suggests, the 35pS Ca\(^{2+}\)-activated, outward rectifying, K\(^+\)-channel (K\(_{\text{Ca}}\)) is activated by increasing cytosolic Ca\(^{2+}\), with a half-maximal activation, K\(_a\) = 380µM (Harvey & Urbach, 1992). It is also activated by hypotonic shock, dibutryl-cAMP
Unlike the KATP channels it is sensitive to TEA.

It would appear that the main role of the K\textsubscript{Ca} channels is in volume regulation. When the PCs are exposed to hypo-osmotic solutions, a 26pS inward rectifying, stretch-activated Ca\textsuperscript{2+}-channel (SACa) is activated in the basolateral membrane of the PCs, permitting the influx of Ca\textsuperscript{2+} and an increase in intracellular Ca\textsuperscript{2+} concentration (Harvey & Urbach, 1992). This results in the basolateral insertion of the outward-rectifying K\textsubscript{Ca} channels via a Ca\textsuperscript{2+} dependent pathway (Harvey & Urbach, 1992). But as with the K\textsubscript{ATP} channels, at first glance, the rectification appears to be in the wrong direction. Upon exposure to hypo-osmotic solutions, the cells should swell and gain KCl. In such circumstances an outward rectifying channel would not appear to provide an optimal solution.

1.4.1.4 Evidence for apical K\textsuperscript{+} channels

Noise analysis and microelectrode techniques have indicated the existence of apical K\textsuperscript{+} channels in the anuran skin (Van Driessche, 1984; Zeiske, 1990). These channels have not yet been localised to a particular type of cell such as the PCs or the MRCs, because investigations were made on whole skin preparations. However, mention of the existence of these channels, which is consistent with findings in this study supporting an apical K\textsuperscript{+} conductance, seems appropriate in developing the overall picture of transporting mechanisms in the toad skin.

Figure 1.7: When excised inside-out basolateral membrane patches of PCs are exposed to 125mM KCl solutions on both sides, the single-channel K\textsubscript{ATP} current-voltage relationship, which (A) normally displays inward-rectification, becomes (B) ohmic in the absence of cytosolic magnesium. (Taken from Urbach et al., 1994).
Previous investigations showed that there was some variability in apical K\(^+\) permeability (P\(_K\)), of anuran skins. Skins from the same species displayed high or low K\(^+\) permeabilities (Nagel & Hirschmann, 1980; Zeiske & Van Driessche 1979) but this variation was not seasonally dependent. In a series of ion substitution experiments, used in conjunction with the Ca\(^{2+}\) channel blocker, quinidine, it was demonstrated that the apical P\(_K\) could be increased, by either a reduction in cytosolic Ca\(^{2+}\) levels, or a rise in intracellular K\(^+\). This effect could be inhibited by the application of Ba\(^{2+}\) or Cs\(^+\) to the apical bathing solution, suggesting the presence of a specific K\(^+\) pathway (Nielsen, 1984; Van Driessche, 1984).

1.4.1.5 Evidence against apical Cl\(^-\) channels in PCs

There is strong experimental evidence to suggest that the apical membrane of the PCs is not passively permeable to chloride. One argument favouring this conclusion is the lack of change in cellular Cl\(^-\) when the concentration gradient across the membrane is altered. For example, if there were a passive apical Cl\(^-\) permeability, then a change from a high chloride concentration of 115mM in sodium chloride Ringer, to a solution containing low chloride (i.e. 0.1mM) would cause a (Nerstian) membrane potential change of about 180mV and a loss of cellular chloride. However, Biber et al. (1985) showed that removal of Cl\(^-\) from the apical bathing solution under short-circuit conditions (when the skin voltage is clamped at 0mV) caused no change in cellular chloride over a 40 minute period. This result strongly suggests the absence of an apical Cl\(^-\) permeability in the PCs.

An alternative way to determine the conductivity of a membrane to a particular ion is to bias the electrical gradient across it. If the membrane is permeable, then the ions will redistribute according to the Nernst equation and a change in concentration will occur. Using this logic, Willumsen & Larsen (1986) tested the Cl\(^-\) permeability of the apical membrane of the PCs. They clamped the voltage across the skin at \(-40mV\)\(^{18}\) (known to inactivate the transepithelial Cl\(^-\) conductance, (Larsen, 1982)) and impaled double-barrelled microelectrodes into the PCs to monitor the apical membrane potential and the cellular chloride activity. With the microelectrodes in place, the voltage clamp across the skin was reversed to \(+100mV\), activating the transepithelial Cl\(^-\) conductance. Although there was a marked change in the transepithelial current during the

\(^{18}\) In this dissertation all skin potentials were measured with respect to the apical bathing solution. When referring to the work of other researchers who have used the basolateral bathing solution as the reference, it should be noted that the signs of their clamp voltages have been reversed to avoid confusion.
experiment, there was no change in the Cl− concentration in the PCs. Similar results were observed in short-circuited skins (V_{skin}=0mV), where the apical membrane of the PCs was hyperpolarised by blocking Na+ channels with amiloride (Biber et al., 1985; Nagel et al., 1981; Willumsen & Larsen, 1986). Although hyperpolarisation resulted in almost a doubling of the driving force, and was therefore expected to cause loss of cellular Cl−, little change was observed over a 30-minute period. These results suggest that, irrespective of the skin voltage, there is no passive apical Cl− permeability in the PCs.

Other evidence supporting the above findings include radioactive tracer studies and vibrating probe experiments. It was demonstrated that, in skins loaded with radiolabelled^{36}Cl−, there was little Cl− exchange between the apical bathing solution and the PCs (Ferreira & Ferreira, 1981; Stoddard et al., 1985). This would be expected if there were no major apical chloride pathway. In addition, when the skin was depolarised (measurements made with respect to the apical bath), it was shown, using a vibrating probe, that chloride-dependent current density peaks only occurred over MRCs. None were evident over PCs (Katz, 1984). These findings are consistent with the idea that PCs are impermeable to apical chloride.

1.4.1.6 Conflicting evidence for basolateral Cl− channels in PCs

There is conflicting evidence regarding the existence of basolateral Cl− channels in the PCs. Some researchers have reported that the change in membrane potential after Cl− removal from the basolateral solution is too slow to be consistent with Cl− channels (Giraldez & Ferreira, 1984), and that manipulating the transepithelial potential has little effect on the cellular Cl− activity (Willumsen & Larsen, 1986). Others have reported that there is no detectable Cl− diffusion potential (Biber et al., 1985; Helman et al., 1979). In contrast to these findings, some groups have described Cl− channels that are volume-sensitive, opening upon cellular swelling (Ussing 1986), or that are blockable with diphenylamine-2-carboxylate (Dorge et al., 1989) or MK-196 (Ussing, 1986), or that display voltage-dependent properties similar to the Cl− channels located in the apical membranes of MRCs (Larsen, 1991).

The reason for the apparent discrepancy is not entirely clear. However, two points are worth commenting upon. First, if the Cl− channels were voltage-dependent, then it is

---

19 These voltage-dependent, poorly-selective anion channels in the PCs were activated upon membrane depolarisation, by blocking basolateral K+ channels, or by adding K+ to the basolateral solution (Larsen, 1991).
surprising that Willumsen and Larsen (1986) did not see rapid changes in chloride activity upon transepithelial depolarisation. They concluded that the PCs did 'not contribute significantly to the voltage-dependent Cl' conductance,' which is in direct contrast to Larsen's 1991 statements, described above. Second, as discussed in section 1.4.2.1, apical voltage-dependent Cl' channels in MRCs open upon depolarisation. Assuming that the potential profiles across the MRCs and the PCs are similar, then presumably, every time the skin is depolarised to open the Cl' channels in the MRCs, the Cl' conductance in the PCs drops, as the basolateral membrane is hyperpolarised. In such circumstances a volume increase in one type of cells would be accompanied by a volume decrease in the other. However, such a simple relationship does not appear to have been reported in the literature. Alternatively, the Cl' channels in the PCs open upon hyperpolarisation, in which case, they do not have the same properties as the Cl' channels in the MRCs.

1.4.1.7 Evidence for Cl'/HCO₃⁻ antiports in PCs

Although the question of basolateral Cl' channels in the PCs has not been resolved, isotopic tracer studies have shown that Cl' enters and leaves the cell. One component of this flux may be passive, but because the cellular Cl' concentration is well above electrochemical equilibrium (Nagel et al., 1981; Giraldez & Ferreira, 1984), it would appear that Cl' is also actively transported into these cells against is electrochemical gradient. One form of secondary active Cl' transport in many epithelia is via the Cl'/HCO₃⁻ antiport (Drewnowska & Biber, 1988). There is significant evidence to suggest that this exchanger may also be present in the PCs of the anuran skin. However, there is still some debate as to the location of the antiports. As discussed below, experimental findings suggest that it may be located in either the apical or basolateral membranes.

Apical Cl'/HCO₃⁻ antiports in PCs

There are two main arguments favouring the existence of apical Cl'/HCO₃⁻ antiports, as found in *Rana pipiens*. First, the addition of DIDS (4,4'-diisothiocyanostilbene-2,2'-disulphonic acid) to the apical bathing solution caused a 65% reduction in the transepithelial isotopic ³⁶Cl' influx (Drewnowska & Biber, 1985). Both DIDS and SITS (4-acetomido-4'isothiocyanostilbene-2,2'-disulphonic acid) are potent Cl'/HCO₃⁻ exchange inhibitors (Knauf, 1979).

Second, Drewnowska & Biber (1985), used double-barrelled microelectrodes to measure cellular Cl' activity and showed that when skins, initially bathed in NaCl Ringer, were subsequently bathed in Cl'-free Ringer, the rate of cellular Cl' loss was
HCO$_3^-$-dependent. To summarise, the experiments were based upon a step process and where an inhibitory effect was desired, DIDS was always applied to the apical side whereas DIDS + bumetanide was applied to the basolateral solution.\textsuperscript{20} The results showed that, upon removal of Cl$^-$ from the bathing solution, skins treated with DIDS on both sides had a lower rate of Cl$^-$ loss (0.036mM/s) compared with untreated skins (0.227mM/s).\textsuperscript{21} These findings suggest that (a) most Cl$^-$ loss is via a DIDS-inhibitable process and (b) there is little passive Cl$^-$ movement, supporting the idea that there are no apical Cl$^-$ channels in the PCs (section 1.4.1.5). Next, it was shown that the rate of Cl$^-$ loss was not affected significantly in DIDS treated skins bathed in Cl$^-$-free solutions with, or without HCO$_3^-$- This indicates that once the antiports are inhibited, the presence of HCO$_3^-$ has little effect. However, if only the basolateral side of the skin were treated with DIDS, then the addition of 10mM HCO$_3^-$ to the apical Cl$^-$-free solution increased the rate of Cl$^-$ loss from 0.026mM/s to 0.087mM/s. This is consistent with the existence of Cl$^-$/HCO$_3^-$ antiports located in the apical membranes of PCs. Finally, if only the apical side of the skin were treated with DIDS then the addition of 10mM HCO$_3^-$ to the apical Cl$^-$-free solution had little effect, as would be expected if the Cl$^-$/HCO$_3^-$ were already blocked.

Two points are worthy of comment. First, under the experimental conditions described above, the antiports appear to be transporting Cl$^-$ out of the cells towards the apical solution, which is not consistent with a transepithelial Cl$^-$ flux from the apical to basolateral side. However, these measurements were made following a large change in Cl$^-$ concentration and only represent rapid initial, and transient changes. In such cases, it is possible that reversal of the Cl$^-$/HCO$_3^-$ exchanger could occur. Second, it should be noted that secondary active Cl$^-$ influx across the apical membranes of the PCs would result in intracellular acidification as HCO$_3^-$ leaves. It is possible that the accumulation of intracellular H$^+$ results in the slowing of the Cl$^-$/HCO$_3^-$ antiports. Alternatively, intracellular pH may be regulated by some other mechanism such as Na$^+$/H$^+$ exchangers, so slowing of the antiports does not occur (see section 1.4.1.8).

\textsuperscript{20} Presumably, the addition of bumetanide to the basolateral solution inhibited Cl$^-$ loss via Na$^+$/K$^+$/2Cl$^-$ symports.

\textsuperscript{21} Using the reverse argument, it was found that when Cl$^-$ was restored to the apical bathing solution, the initial rate of uptake by Cl$^-$-depleted cells was 93% inhibited when 10$^{-3}$M DIDS was added to the apical bathing solution (Drewnowska & Biber, 1985).
Basolateral Cl-/HCO₃⁻ antiports in PCs

Several experiments provide convincing arguments for the existence of basolateral Cl-/HCO₃⁻ antiports in the anuran skin. One such experiment was conducted in the PCs of *Rana pipiens* using double-barreled microelectrodes to record the intracellular Cl⁻ activity. Prior to the experiment the cells were depleted of Cl⁻ by bathing the skins in Cl⁻-free Ringer solutions. DIDS was then added to the apical solution to inhibit the loss of cellular chloride across this surface and cellular impalements were made. Cl⁻ was then restored to the basolateral bathing solution. and the rate of Cl⁻ uptake was measured. In high-conductance skins (G>0.5mS/cm²) the basolateral Cl⁻ uptake rate was 5.03mM/s. The uptake rate was 98% inhibited by the addition of 10⁻³M SITS + 10⁻³M bumetanide to the basolateral solution, strongly suggesting the presence of a Cl⁻/HCO₃⁻ exchange mechanism (Drewnowska & Biber, 1988). This observation is consistent with the earlier findings of Stoddard et al., (1985) who showed that Cl⁻ efflux from cells loaded with the radioisotope ³⁶Cl was inhibited by basolateral application of 5*10⁻⁴M SITS + 1mM furosemide.²²

In another study, the intracellular Cl⁻ activity and pH regulation of PCs in *Rana esculenta* were investigated, following the exposure of the basolateral solution to an alkaline load (Lacoste et al., 1991). Again, double-barreled microelectrodes were used to measure either the intracellular chloride activity, or the pH. In the first part of the experiment investigating chloride activity, skins were pre-incubated in Cl⁻-free Ringer on both sides and then exposed to Cl⁻ Ringer on the basolateral side resulting in an increase in intracellular chloride activity from ≈7.7nequiv/L to 13.5nequiv/L (Figure 1.8). In each case the bathing solution had been gassed with air ('air Ringer') and had a pH=7.34. The basolateral solution was then changed to 'CO₂/HCO₃⁻' Ringer which had been gassed with 5%CO₂ and 95%O₂ and also had a pH=7.34. This produced a small change in the basolateral membrane potential, and an additional increase in the chloride activity of 3.8nequiv/L, which was attributed to the presence of HCO₃⁻ ions in the bathing solution. It was concluded that intracellular Cl⁻ activity increased as the basolateral HCO₃⁻ concentration increased. This was consistent with the idea of basolateral Cl⁻/HCO₃⁻ antiports that transported Cl⁻ into the cells. In the second part of

---

²² At low concentrations (10⁻⁶ to 10⁻⁴M) furosemide and bumetanide are specific cotransport inhibitors. At higher concentrations they are less specific and are known to block Cl⁻/HCO₃⁻ antiports in erythrocytes (Lacoste et al., 1991).
the experiment, changes in the intracellular pH after an alkaline load, and its dependence on basolateral Cl⁻ was investigated. In this case, the basolateral solution was changed from 'CO₂/HCO₃⁻' Ringer to 'Air' Cl⁻-Ringer which resulted in a transitory increase in pH from 7.3 to 7.6. In addition, a transient hyperpolarisation of the basolateral membrane and an increase in the short-circuit current were observed, both of which were attributed to the combined stimulation of the apical Na⁺ and basolateral K⁺ conductances in alkaline conditions. Upon restoration of ‘CO₂/HCO₃⁻’ Ringer, control conditions were again attained (Figure 1.9). When the experiment was repeated in 'Air' Cl⁻-free Ringer the parameters plateaued, and recovery was only observed when Cl⁻ was restored to the bathing solution. This indicated that the regulation of intracellular pH in response to an alkaline load was dependent on Cl⁻ in the basolateral solution. Finally, in an experiment that complemented the findings of Lacoste et al., it was shown, using pH-sensitive microelectrodes, that removal of Cl⁻ from the basolateral solution alkalised the cells of Rana pipiens (Duffey et al., 1986). These findings are consistent with either an increase in HCO₃⁻ influx, or a reduction in HCO₃⁻ efflux, depending on the direction of operation of the putative Cl⁻/HCO₃⁻ antiport.

**Figure 1.8:** Following the perfusion of a frog skin with Cl⁻-free Ringer on both sides, an increase in the intracellular Cl⁻ activity occurred upon exposure to Cl⁻ Ringer on the basolateral side. An additional increase was elicited when the basolateral solution, initially gassed with air, was changed to a CO₂/HCO₃⁻-buffered Ringer solution (bars). Little change in the intracellular potential (Vₜₜ) was observed with these manipulations. (Taken from Lacoste et al., 1991).
Finally, it has been suggested that the Cl-/HCO₃⁻ exchanger may exist in both the apical and basolateral membranes of the PCs as opposed to just one membrane (Biber et al., 1985). Certainly, this would provide a possible transcellular Cl⁻ pathway across the PCs if the apical Cl⁻/HCO₃⁻ exchanger transported Cl⁻ into the cell while the basolateral exchanger transported Cl⁻ out. Although this is conceivable, the experimental evidence presented in the preceding section suggests that this is probably not the case, as Cl⁻

**Figure 1.9:** A transient alkalinisation, a hyperpolarisation of the intracellular potential ($V_b$), and an increase in the Isc occurs when a CO₂/HCO₃⁻-buffered solution is replaced by an ‘Air Ringer’ solution on the basolateral side of the frog skin. Control conditions are restored upon return of the CO₂/HCO₃⁻-buffered solution. When the experiment is repeated in the absence of Cl⁻ in the ‘Air Ringer’ solution, the response is not transient until Cl⁻ is then added, indicating that the regulation of the intracellular pH, following the alkaline load, is dependent on basolateral Cl⁻. (Taken from Lacoste et al., 1991).
appears to be transported across the basolateral membrane into the PCs. This is reinforced further by the finding that the intracellular Cl⁻ concentration appears to be more dependent on basolateral Cl⁻ uptake than apical uptake (Drewnowska et al., 1988). The difference in uptake rates is probably because the basolateral Cl⁻ permeability is up to 6.5 times greater than the apical Cl⁻ permeability (Lacoste et al., 1991; Stoddard et al., 1985).

If Cl⁻ is transported into the cell across the basolateral membrane via the Cl⁻/HCO₃⁻ exchanger, then presumably there must be an additional electroneutral mechanism that transports Cl⁻ out of the cell to the basolateral side. Otherwise, there would be no net Cl⁻ transfer through the PCs, which would be contrary to the findings of isotopic tracer studies. However, such a configuration appears to be somewhat inefficient. Whatever Cl⁻ concentration gradient is generated by the transport of Cl⁻ out of the PCs into the basolateral solution, is immediately dissipated by the Cl⁻/HCO₃⁻ exchanger transferring Cl⁻ back into the PCs. In fact, the Cl⁻/HCO₃⁻ exchanger almost appears redundant in the presence of another electroneutral Cl⁻ mechanism. However, it may be necessary for intracellular pH regulation or it may provide a stabilising action, preventing cellular parameters from moving outside physiologically acceptable levels. What is apparent, is that there are still some uncertainties about transcellular Cl⁻ transport across the PCs, despite the vast quantities of experimental data.

1.4.1.8 Evidence for Na⁺/H⁺ exchangers in PCs

There is some evidence to suggest the presence of Na⁺/H⁺ exchangers in the PCs of the anuran skin. The exact location of the Na⁺/H⁺ exchangers are still the subject of some debate: some investigators have reported that they are located in the apical membrane while others suggest that they are in the basolateral membrane. The discrepancies may simply be due to a difference in the species used in the various experiments. This section reviews the data supporting each view.

Apical Na⁺/H⁺ exchangers in PCs

The main argument favouring the existence of apical Na⁺/H⁺ exchangers was based on radioisotope studies in Rana pipiens, where ³⁶Cl or ²²Na was added to one bathing solutions and the rate of appearance of the isotope in the other bathing solution was measured (Drewnowska & Biber; 1985; Drewnowska et al., 1988). It was found that

---

23 The mechanism would have to be electroneutral because of the absence of a basolateral Cl⁻ diffusion potential. (Biber et al., 1985; Drewnowska et al., 1985).
when \(10^{-4}\text{M}\) amiloride was added to the apical bathing solution, the active transcellular Cl\(^{-}\) flux was inhibited. Although amiloride results in blockage of Na\(^{+}\) channels and hyperpolarisation of the cell, which could cause a decrease in cellular Cl\(^{-}\), Nagel et al. (1981) showed experimentally that there was no change in cellular Cl\(^{-}\) activity after amiloride. Instead, Drewnowska & Biber (1985) proposed that amiloride blocked apical Na\(^{+}\)/H\(^{+}\) exchangers (as well as the apical Na\(^{+}\) channels), resulting in intracellular acidification. They suggested that this would slow Cl\(^{-}\) uptake via the apical (pH-sensitive) Cl\(^{-}\)/HCO\(_3\)- exchangers, inhibiting transcellular Cl\(^{-}\) flux.\(^{24}\)

**Basolateral Na\(^{+}\)/H\(^{+}\) exchangers in PCs**

In three elegant experiments using double-barrelled Na\(^{+}\)- and H\(^{+}\)-sensitive microelectrodes to impale cells (presumably PCs), Harvey & Ehrenfeld (1988) presented evidence of basolateral Na\(^{+}\)/H\(^{+}\) exchangers in *Rana esculenta*. First, they applied amiloride to the basolateral bathing solution and measured a reversible decrease in both intracellular pH and Na\(^{+}\) activity under steady state, short-circuit conditions. They concluded this finding was consistent with basolateral Na\(^{+}\)/H\(^{+}\) exchangers operating to transport Na\(^{+}\) into, and H\(^{+}\) out of, the cell. In addition, it indicated that the putative exchangers are operating in the steady-state condition.

In the second experiment, the effects of acidifying the intracellular environment were investigated, on the premise that the intracellular pH is regulated by the basolateral Na\(^{+}\)/H\(^{+}\) exchangers. The pH was measured under control conditions in NaCl (CO\(_2\)-free) Ringer, and then the apical side was exposed to a Ringer solution buffered in 5\% CO\(_2\) and 24mM NaHCO\(_3\) which produced a prolonged intracellular acidosis. When the control Ringer was restored to the apical solution, the pH recovered. However, this effect was significantly slowed when either Na\(^{+}\) was absent, or amiloride was present, in the basolateral bathing solution. This finding strongly suggests that the pH is “changed” partially by the Na\(^{+}\)/H\(^{+}\) exchangers.

The third experiment investigated the possibility of reversal of the Na\(^{+}\)/H\(^{+}\) exchangers. It was hypothesised that H\(^{+}\) transport out of the cell depended on an inwardly directed Na\(^{+}\) chemical gradient across the basolateral membrane. If this gradient were disrupted, say by lowering basolateral Na\(^{+}\), then this could reduce the activity of the exchangers or, in extreme circumstances, even cause a reversal in the flux.

\(^{24}\) Presumably, for there to be no change in cellular Cl\(^{-}\) activity, Cl\(^{-}\} entry across the basolateral membrane would have to increase to compensate for the reduction in apical Cl\(^{-}\). However, this would not show up in transcellular isotope measurements.
directions. This would result in Na\(^+\) extrusion and H\(^+\) uptake. When this experiment was performed and basolateral Na\(^+\) levels were lowered, these effects were indeed observed. Cellular acidification and a reduction in Na\(^+\) activity occurred. (In fact, Harvey & Ehrenfeld (1988), estimated that in a low basolateral Na\(^+\) solution, 60% of Na\(^+\) extrusion was via an amiloride-blockable pathway, which was presumably the Na\(^+\)/H\(^+\) exchangers operating in reverse, while only 40% occurred via the Na\(^+\)/K\(^+\)-ATPase pumps).\(^{25}\) Acidification of the PCs by the reversal of the Na\(^+\)/H\(^+\) exchangers could be slowed by addition of basolateral amiloride to the low Na\(^+\) solution. Overall, these results strongly suggest the presence of basolateral Na\(^+\)/H\(^+\) exchangers in the PCs.

The presence of basolateral Na\(^+\)/H\(^+\) exchangers accounts for several experimental findings that would otherwise be difficult to explain. For example, the addition of ouabain to the basolateral solution causes both the apical and basolateral membrane conductances to drop (Harvey & Ehrenfeld, 1988). In contrast, the natriiferic hormones, aldosterone and insulin, have the opposite effect, increasing both the membrane conductances (Harvey & Ehrenfeld, 1988; Moore, 1986). These effects may be explained in terms of intracellular pH. As the pH drops and the cells become more acidic, the apical Na\(^+\) and basolateral K\(^+\) channels close, and the basolateral Na\(^+\)/K\(^+\)-ATPase activity slows (Drewnowska et al., 1988; Eaton et al., 1984; Harvey et al., 1988; Urbach et al., 1991). In other words, the Na\(^+\) and K\(^+\) channels and Na\(^+\)/K\(^+\)-ATPase pumps display a partial dependence on pH. This makes the intracellular pH a possible candidate for the transcellular coupling of ion movements. Presumably, when ouabain is added to the basolateral solution, there is a rapid initial increase in cellular Na\(^+\) as the pumps are blocked (Harvey & Kernan, 1984), which in turn slows the Na\(^+\)/H\(^+\) exchangers. H\(^+\) begins to accumulate in the PCs, causing an acidic shift in pH, which then results in a further slowing of the pumps and closure of the apical Na\(^+\) and basolateral K\(^+\) channels (Harvey et al., 1988). Conversely, aldosterone and insulin appear to stimulate the basolateral Na\(^+\)/H\(^+\) exchangers, resulting in an alkaline shift as H\(^+\) is transported out of the cells. This in turn is believed to cause the apical Na\(^+\) and basolateral K\(^+\) channels to open (Harvey & Ehrenfeld, 1988) and to stimulate the Na\(^+\)/K\(^+\)-ATPase pumps.

In the above studies, irrespective of the particular perturbation (i.e. ouabain, aldosterone or some other substance), the overall effect of altering the membrane conductances was a change in the transepithelial short-circuit current (Isc). That is, an

\(^{25}\) The low pump activity is presumably because of the reduction in the Na\(^+\) electrochemical gradient.
increase in conductances was associated with an increase in Isc. Extending the logic, if intracellular pH alters the conductance, and changes in the conductance affects Isc, then it is possible that a correlation exists between pH and Isc. Harvey & Ehrenfeld (1988) clearly demonstrated this when they showed that there was a monotonically increasing relationship between Isc and physiological pH levels (Figure 1.10).

![Figure 1.10: A monotonically increasing relationship exists between Isc and physiological levels of intracellular pH. Thus, to a first approximation, Isc can be used as a measure of changes in pHi. (Taken from Harvey & Ehrenfeld, 1988).](image)

However, there are 2 examples in the literature that challenge the notion that the intracellular pH regulates transcellular ion movements. First, because both the Na⁺/H⁺- and Cl⁻/HCO₃⁻ exchangers contribute to the pH, acute removal of Cl⁻ from the basolateral bathing solution should slow Cl⁻/HCO₃⁻ exchange activity, causing an alkaline shift in intracellular pH, an increase in membrane conductance, and an increase in Isc. However, it has been reported that basolateral Cl⁻ removal has little effect on Isc or other electrophysiological parameters (Stoddard & Helman, 1981). This strongly suggests that intracellular pH is not dependent on the Cl⁻/HCO₃⁻ exchangers, which conflicts with some of the evidence presented earlier. Second, a change in intracellular pH should, presumably, affect both Na⁺/H⁺- and Cl⁻/HCO₃⁻ exchangers. This appears not to be the case. The induction of intracellular acidification appears to change the
driving potential for the basolateral Na⁺/H⁺ exchangers, as measured by ²²Na uptake across this membrane. However, it does not affect Cl⁻ uptake significantly (Lacoste et al., 1991). This result suggests that the Na⁺/H⁺- and Cl⁻/HCO₃⁻ exchangers are independent mechanisms that are not closely coupled. Overall, the two examples described suggest that changes in pH may alter Na⁺ and K⁺ transport without affecting Cl⁻ transport. This is puzzling since it is Cl⁻ that is coupled with the transport of a pH-determining ion. The reason for this apparent anomaly is still unclear.

1.4.1.9 Evidence for basolateral Na⁺/Ca²⁺ transporters in PCs

Grinstein and Erlij (1978) found that when the basolateral membrane was exposed to a Na⁺-free solution there was a reduction in transepithelial Na⁺ transport and a threefold stimulation of ⁴⁵Ca uptake across the basolateral surface of the anuran skin. When Ca²⁺ was absent from the basolateral solution, the reduction in Na⁺ transport was not observed. Based on these findings they concluded that elevated intracellular Ca²⁺ inhibited transepithelial Na⁺ transport. These findings led to the proposal of a basolateral Na⁺/Ca²⁺ exchanger operating to transport Na⁺ into, and Ca²⁺ out of the skin (Taylor & Windhager, 1979).

Since then, there have been a number of investigations examining whether intracellular Ca²⁺ mediates the transcellular 'cross-talk' of ions by altering particular apical and basolateral channel conductances in the PCs (Grinstein et al., 1978; Chase & Al-Awqati, 1981; Harvey & Urbach, 1992; Windhager, 1983). Despite these investigations, a direct correlation between a rise in intracellular Ca²⁺ and a decrease in Na⁺ transport rate still remains to be shown. This is because of the difficulty in measuring intracellular Ca²⁺ levels, as Ca²⁺ is taken up by many cytoplasmic organelles such as the sarcoplasmic reticulum, or packaged into cytoplasmic vesicles. In these circumstances, ion selective microelectrodes are not useful in determining intracellular Ca²⁺ levels. Thus, because of experimental setbacks, it has been difficult to determine the exact influence of intracellular Ca²⁺ on various transport mechanisms. Therefore, there is still much debate surrounding the effects of Ca²⁺. However, it is worth pointing out that both the apical Na⁺- and basolateral K⁺ conductances, believed to be sensitive to Ca²⁺, are also sensitive to changes in pH. Therefore, it is possible that one regulatory mechanism is dominant over the other. For example, cross-talk may be mediated predominantly by pH, but in the case of severe ionic perturbations, Ca²⁺ may provide a backup regulatory mechanism (Chase, 1984).
1.4.1.10 Evidence for basolateral Na⁺/K⁺/2Cl⁻ symports in PCs

There is some discussion regarding the existence of basolateral Na⁺/K⁺/2Cl⁻ symports in the PCs of the anuran skin. Some investigators have found little evidence for the symports, while others regard them as an integral part of cell volume regulation. As an example of the discrepancies in findings, Lacoste, (1991) reported that 'the possibility of a Cl⁻ uptake mediated by an electroneutral Na:Cl or 2Cl/Na/K cotransport system [was] excluded…, since the basolateral membrane Cl⁻ transport rate was unchanged in a sodium-free Ringer solution and Na⁺ uptake through the basolateral membranes was insensitive to furosemide.' This is inconsistent with the earlier observations of Giraldez and Ferreira (1984), who showed that the intracellular chloride activity decreased when either Na⁺ or Cl⁻ was removed from the basolateral bathing solution. This effect was presumably due to an electroneutral mechanism as there was no concurrent change in the basolateral membrane potential.26

The apparent disparity may be reconciled if such a transport mechanism were dormant under some circumstances but not others. This idea was demonstrated clearly by Cox & Helman, (1983) who investigated the alteration of ²²Na flux when the Na⁺/K⁺/2Cl⁻ symport blocker, furosemide, was added to the basolateral bathing solution. In control skins, furosemide had little effect on the ²²Na flux. However, when ouabain was added to the basolateral solution to block the Na⁺/K⁺-ATPase pumps, furosemide caused a significant inhibition of a neutral, Cl⁻-dependent, ouabain-insensitive Na⁺ efflux. It would appear that prior to ouabain, the Na⁺/K⁺/2Cl⁻ symports were dormant, yet after the cells were pharmacologically perturbed, the symports began to function, possibly due to a change in the driving potentials for the Na⁺, K⁺ or Cl⁻ ions.

A change in cell volume appears to be the trigger for Na⁺/K⁺/2Cl⁻ symport activation. This was first illustrated in an elegant experiment by MacRobbie and Ussing (1961) who showed that a loss of cellular KCl caused a reduction in epithelial volume, which they attributed mainly to the PCs (Ussing, 1986). Cell volume could only be restored if Na⁺, K⁺ and Cl⁻ were all present in the basolateral solution and recovery could be

---

26 As mentioned in section 1.4.1.5, it is unlikely that this effect was due to an outward Cl⁻ flux across the apical membranes of the PCs because these membranes have a low Cl⁻ permeabilities compared with the basolateral membranes.
inhibited by basolateral furosemide or bumetanide.\textsuperscript{27} This suggests that the symports are responsible for restoration of cell volume.

As a final comment, a symport that is normally dormant, but activates when cells have shrunk, certainly complements a stretch-activated Cl\textsuperscript{−}-channel that is normally closed, but opens upon cell expansion (section 1.4.1.6). This may well be the way that PCs regulate their volume and intracellular ionic concentrations. For example, if too much Cl\textsuperscript{−} is dumped from the cells when the Cl\textsuperscript{−} channels are open and the cells shrink, then although the channels have closed, there is still a mechanism by which to restore intracellular K\textsuperscript{+} and Cl\textsuperscript{−} concentrations.

* * * * *

1.4.2 Mitochondria-Rich Cells

There are at least 3 subpopulations of MRCs that exist in an anuran skin all of which exhibit significant carbonic anhydrase activity (Rosen & Friedley, 1973; Larsen, 1991). These subpopulations include: the $\alpha$-MRCs which are associated with acid (H\textsuperscript{+}) transport, the $\beta$-MRCs which are associated with base (HCO\textsubscript{3}\textsuperscript{−}) transport, and the $\gamma$-MRCs which are associated with the rheogenic uptake of Cl\textsuperscript{−} (Figure 1.11). The relative proportion of each subpopulation depends largely on MRC differentiation and proliferation, which can be influenced by the external environment (Illic & Brown 1980; Devust et al., 1991). However, the Cl\textsuperscript{−}-transporting $\gamma$-MRCs (which are the only MRCs that display a significant apical Na\textsuperscript{+} conductance) are believed to be the predominant subpopulation in the toad skin (Larsen, 1991).\textsuperscript{28} It is these $\gamma$-MRCs that are the focus of the following sections.

---

\textsuperscript{27} Ussing found that potassium (and hence cell volume), could not be restored in frogs that had been starved in the laboratory or had been hibernating over the winter season. But in well-nourished animals, cell shrinkage was quite reversible (Ussing, 1978).

\textsuperscript{28} Using \textit{Rana pipiens}, Rick, (1992) used a different classification of MRCs again dividing them into 3 main groups. Of the MRCs that were investigated: (1) 50\% were ouabain insensitive (2) 25\% were ouabain sensitive but amiloride insensitive and (3) 25\% were ouabain sensitive and amiloride sensitive (which were described as equivalent to Larsen's $\gamma$-MRCs). This would suggest that in this investigation, the $\gamma$-MRCs were not the predominant subpopulation. However, Rick points out that the frequency of each type of MRC subpopulation varied considerably between frogs without any apparent reason. Presumably, the same is true across species. Variation may also depend, to some extent, on the season (Voute & Meier, 1978).
Figure 1.11: Three subpopulations of mitochondria-rich cells exist in the anuran skin: (A) the $\alpha$-MRCs associated with acid ($H^+$) transport; (B) the $\beta$-MRCs associated with base ($HCO_3^-$) transport, and (C) the $\gamma$-MRCs associated with the rheogenic uptake of $Cl^-$. The $\gamma$-MRCs are believed to be the predominant subpopulation of MRCs. (Taken from Larsen, 1991).
1.4.2.1 Evidence for apical Cl⁻ channels in MRCs

As discussed in section 1.4.1.5 the passive transepithelial uptake of Cl⁻ across the anuran skin does not appear to occur via the PCs. Instead, there are two other possible Cl⁻ routes: transcellular uptake across the MRCs or paracellular movement through tight junctions (see section 1.4.3). This section reviews the evidence that has led to the belief that the MRCs are the site of Cl⁻ uptake. In particular, it focuses on the findings from three main types of experiments including morphological studies, cell volume experiments, and voltage clamp procedures. It also presents some of the experimental findings from vibrating probe techniques that are currently not explicable in terms of the present model.

As early as 1935, Huf demonstrated that the isolated anuran skin was capable of transepithelial uptake of Cl⁻ from the apical (pond) solution (cited in Krogh, 1939). However, it was only in the late 1970's that considerable interest was shown in the MRCs as the possible site for this Cl⁻ uptake. Until then, techniques for examining the individual cell populations were rudimentary and, consequently, the majority of the early experiments were performed macroscopically on the isolated anuran skin, investigating changes in the transepithelial electrical parameters.

By 1978, Voute and Meier were using light micrographs to examine the changes in MRCs when exposed to different bathing solutions. Briefly, they attempted to create a Cl⁻ concentration gradient across short-circuited skins of Rana esculenta by exposing the apical side of the skin to KCl Ringer and the basolateral side to K⁺-gluconate Ringer. In doing so, any current that was measured was presumably due to the transcellular movement of Cl⁻, and not Na⁺ (which was absent from the bathing solutions), or K⁺ (unless it was actively transported across the skin in the absence of a transepithelial electrochemical gradient). Following exposure to the Cl⁻ gradient, the skins were fixed and sectioned in preparation for examination by light microscopy whereupon the MRCs were counted and morphological changes were noted. Several conclusions were drawn from their study. First, the MRCs that were in contact with the apical bathing solution swelled when the skin was exposed to the Cl⁻ gradient described above, suggesting that the Cl⁻ conductance of the skin was localised to the apical membrane of the MRCs. Second, it was found that the (Cl⁻-dependent) current through
the skin increased linearly, in proportion to the number of MRCs (Figure 1.12). Both these findings suggested that the MRCs were the site of Cl⁻ uptake.

However, because these findings are used extensively in the literature to argue that the MRCs are the main transcellular Cl⁻ pathway, it is important to investigate two questions that may challenge these results. First, were the sections made when the skins had reached an osmotic steady-state? The answer to this is not readily apparent from the methods section of the paper. However, it is an important question, because presumably changes in the larger volume of the PCs syncytium occur more slowly than changes in the smaller, individual MRCs. In addition, volume changes may be transient, so at the instant that the skins were fixed, the MRCs may have been undergoing a transient regulatory volume increase thereby affecting the validity of the results.

Figure 1.12: A linear correlation was shown to exist between the transepithelial Cl⁻-dependent current and the number of mitochondria-rich cells (Taken from Voûte & Meier, 1978). It should be noted that other researchers have not observed this correlation (see text for details).

29 Nagel & Dorge (1990) did not observe this linear relationship between MRC density and chloride conductance in either *Rana esculenta* or *Rana pipiens*.
Second, was there any quantitative correlation between the PCs and the Cl\(^-\) current? The morphological studies indicated that the PCs remained unchanged or shrank with the applied Cl\(^-\) gradient in K\(^+\) solutions but no relationship (or lack thereof) was presented between the number of PCs and the current. Given that there is quite strong evidence to suggest that Cl\(^-\) is taken up by secondary active transport mechanisms in the PCs (section 1.4.1.7), it would be surprising if there were no correlation. But if a correlation were indeed observed, this might suggest that the MRCs were not necessarily the main Cl\(^-\) transport pathway. However, based on the data presented in the paper, it is not possible to resolve this issue at the present time.

In 1986, Foskett and Ussing presented new evidence suggesting that the chloride conductance was localised to the MRCs. They used two methods: video imaging to measure the volume of the MRCs, and a vibrating probe technique to electrically localise the Cl\(^-\) conductance to these cells (discussed later).\(^{30}\) The cell volume experiments were based on the premise that, if the MRCs had a significant apical Cl\(^-\) conductance (and a basolateral K\(^+\) conductance), then when a positive voltage clamp of \(V_{\text{skin}}=150-200\text{mV}\) was applied across the epithelium (apical side grounded), both K\(^+\) and Cl\(^-\) would enter the MRCs resulting in cell swelling. This hypothesis was tested in both Cl\(^-\) and Cl\(^-\)-free Ringer. As shown in Figure 1.13, which depicts the volume changes in one mitochondria-rich cell when skins were exposed to Cl\(^-\) Ringer on both sides, and a positive voltage clamp was applied, the cell swelled. When the positive voltage clamp was removed, and the skin returned to the (control) short-circuit condition where \(V_{\text{skin}}=0\text{mV}\), the mitochondria-rich cell shrank. (Out of the 25 cells where this procedure was repeated with exposure to Cl\(^-\) Ringer on the apical and basolateral sides, cell swelling was observed in 64\% of MRCs while 24\% shrank and 12\% remained unchanged). When the skin was then exposed to Cl\(^-\)-free solution (Figure 1.13), as expected, no change was observed in the mitochondria-rich cell volume with- or without, positive voltage clamping. (Out of the 41 cells investigated, 10\% swelled, 12\% shrank and 78\% remained unchanged). But with reintroduction of Cl\(^-\) on both sides of the epithelium during a positive voltage clamp (Figure 1.13), the mitochondria-rich cell again swelled. Subsequent removal of the clamp caused cell shrinkage.\(^{31}\)

\(^{30}\) Katz & Scheffey, (1986) also used the vibrating probe technique to localise the Cl\(^-\) conductance to the MRCs.

\(^{31}\) Although, Foskett and Ussing’s study (1986) was conducted on MRCs they did comment that no change in the volume of the PCs was observed upon application of a positive voltage clamp. This
Larsen et al. (1987) investigated the possibility that the Cl\textsuperscript{−} channels were volume-sensitive and that voltage-clamping somehow initiated a volume change. However, this idea was dismissed when they showed that in amiloride-treated skins, the transepithelial current was largely unaffected by changes in the osmolarity of the bathing solution, which caused volume changes during a voltage clamp (Figure 1.14). Had the Cl\textsuperscript{−} channels been volume-sensitive, then altering the osmolarity of the bathing solution would have presumably initiated a change in the current as the Cl\textsuperscript{−} channels opened or closed. Instead, it was concluded in both studies that the majority of the MRCs possess a voltage-sensitive Cl\textsuperscript{−} conductance that is activated when the basolateral solution is positive with respect to the apical solution. (In Figure 1.13 the Cl\textsuperscript{−} channels appear to inactivate under control conditions when $V_{\text{skin}}=0\text{mV}$).\textsuperscript{32}

In terms of the localisation of the voltage-sensitive Cl\textsuperscript{−} channels, Foskett and Ussing (1986) showed that the channels appeared to be positioned in the apical membrane, and

\[\text{Figure 1.13: The effect of voltage clamping on MRC volume when the skin was exposed to normal Cl\textsuperscript{−} Ringer or 1mm Cl\textsuperscript{−} Ringer (Cl\textsuperscript{−}-free) on both sides. Arrows denote periods of voltage clamping (basolateral side $\approx+150\text{mV}$ with respect to the apical side). During other periods the transepithelial voltage was 0mV. (Taken from Foskett & Ussing, 1986).}\]

\[\text{suggests that either there are no voltage-sensitive tansporting mechanisms in the PCs, or that a positive voltage clamp does not activate them.}\]

\textsuperscript{32} 100% inactivation was shown by Larsen et al., (1987) to occur for a transepithelial voltage of around $–30$ to $–40\text{mV}$, but in this skin the channels appear to have closed off at 0mV.
not the basolateral membrane, of the MRCs. Summarising briefly, a control was performed by exposing both sides of the epithelium to a Cl⁻-free solution and applying a positive voltage clamp. No volume change occurred (Figure 1.15). The same procedure was repeated, but with Cl⁻ Ringer’s present on the basolateral side and again no volume change was observed (Figure 1.15A). But when Cl⁻ was introduced into the apical bath (with Cl⁻-free in the basolateral solution) and a positive voltage applied across the skin, there was a significant increase in the volume (Figure 1.15B). These findings indicated that the MRCs were more sensitive to the effects of voltage clamping when Cl⁻ was present in the apical solution. By extension then, it would appear that the voltage sensitive Cl⁻ channels are located in the apical membrane of the MRCs.

Figure 1.14: Effects on MRC volume when the osmolality of the bathing solution was changed with sucrose during a transepithelial voltage clamp going from −30mV to +100mV (basolateral side positive). (A) Skin was bathed bilaterally in 219mOsm NaCl Ringer’s with 50µM amiloride on apical side; (B) Skin was bathed in half strength NaCl Ringer’s with 50µM amiloride on apical side and the osmolarity increased to 235mOsm with sucrose. Note how the transepithelial (Cl⁻) current changes with voltage but is largely unaffected by osmotic perturbations. For consistency with the results presented in Chapter 3, the sign of the currents has been reversed. (Adapted from Larsen et al., 1987).
As mentioned earlier, the work on apical Cl⁻ channels in the MRCs by Foskett and Ussing (1986) was furthered by Larsen et al. (1987). Using NaCl Ringer's to bathe both sides of the preparation with 50μM amiloride in the apical bathing solution, they showed that, when a positive voltage clamp was applied across the skin, the rate of swelling of the MRCs had a similar time course to the activation of the transepithelial

**Figure 1.15:** The effect of voltage clamping on MRC cell volume when the skin was exposed to 1mm Cl⁻ Ringer (Cl⁻-free) on both sides and then to (A) normal Cl⁻ Ringer in the basolateral solution (with Cl⁻- free in the apical solution), or (B) normal Cl⁻ Ringer in the apical solution (with Cl⁻-free in the basolateral solution). The volume does not appear to be dependent on basolateral Cl⁻ but is affected by apical Cl⁻. As before, arrows denote periods of voltage clamping (basolateral side ≈+150mV with respect to the apical side). During other periods the transepithelial voltage was 0mV. (Taken from Foskett & Ussing, 1986).

As mentioned earlier, the work on apical Cl⁻ channels in the MRCs by Foskett and Ussing (1986) was furthered by Larsen et al. (1987). Using NaCl Ringer's to bathe both sides of the preparation with 50μM amiloride in the apical bathing solution, they showed that, when a positive voltage clamp was applied across the skin, the rate of swelling of the MRCs had a similar time course to the activation of the transepithelial
current. (Figure 1.16).\textsuperscript{33} When this was repeated in Cl\textsuperscript{−}-free solution the MRCs did not swell and the magnitude of the current was significantly reduced. It was concluded that transepithelial depolarisation increased the chloride conductance and cell swelling was probably due to the rapid accumulation of apical Cl\textsuperscript{−}, and basolateral K\textsuperscript{+} ions (since the Na\textsuperscript{+} entry had been eliminated by amiloride).

\textbf{Figure 1.16:} The effect of voltage clamping on transepithelial current and MRC volume. Voltage clamps were performed at −30mV and +80mV (with respect to the apical solution, which contained 50µM amiloride). The left-hand side shows the effects in normal Cl\textsuperscript{−} Ringer. The right-hand side shows the effects when Cl\textsuperscript{−} is removed from the apical solution and replaced with gluconate, suggesting that the cell volume and a component of the transepithelial current are dependent on apical Cl\textsuperscript{−}. For consistency with the results presented in Chapter 3, the sign of the currents has been reversed. (Taken from Larsen et al., 1987).

\textsuperscript{33} In this study the transepithelial potentials were referenced to the basolateral bathing solution. However, throughout this dissertation the apical bathing solution is the reference. Therefore, a negative transepithelial voltage clamp cited in Larsen's paper is positive using the sign convention adopted in this review. Diagrams have been adjusted accordingly.
It was therefore hypothesised that if swelling was dependent on K⁺ entry, then reducing the basolateral K⁺ concentration should slow the rate of volume increase. This indeed appeared to be the case. When the basolateral K⁺ concentration was reduced from 5mM to 0.5mM, the time taken to reach a new steady state increased significantly (Figure 1.17). But when the K⁺ concentration was increased to 10mM there was not much change in the rate of swelling. The main conclusion from this experiment was that the cation involved in swelling of the MRCs appears to be K⁺. One other interesting point arising from Figure 1.17 is that increasing the basolateral K⁺ concentration seems to cause the transepithelial current to oscillate. This suggests that the time delays between certain cellular events that assist in returning a cell to a steady-state following a perturbation, have been modified in some way.

![Figure 1.17: The rate of change of the transepithelial current and MRC volume in response to voltage clamping, which activates and deactivates the Cl⁻ conductance, is dependent on the basolateral K⁺ concentration. In each case, the apical solution contained 50µM amiloride. For consistency with the results presented in Chapter 3, the sign of the currents has been reversed. (Taken from Larsen et al., 1987).](image)

As yet, the sensitivity of the apical voltage gated Cl⁻ channels of the MRCs has not been discussed. Larsen et al. (1987) addressed this issue by eliminating the Na⁺ concentration above 2.4mM.

---

34 In fact, Larsen reported that the rate of volume change was not affected greatly for basolateral K⁺ concentrations above 2.4mM.
transport pathway with amiloride and using the transepithelial current as a measure of the Cl⁻ conductance. The voltage sensitivity of these channels was demonstrated by applying different voltages across the skin and measuring the current (Figure 1.18). It is clear that as the voltage clamp becomes more positive the magnitude of the transepithelial current increases which is consistent with more Cl⁻ channels opening upon depolarisation. In addition, it is evident that there is a brief delay before the Cl⁻ channels have opened maximally, and the current has reached a steady-state. Upon removal of the clamp there is also a delay before the channels close, as shown by the finite period of time taken before the current reaches its pre-clamp value. As mentioned above, these time delays may play an important role in the feedback processes of the cell, and are discussed later.

**Figure 1.18:** A superimposed family of currents in response to a series of transepithelial voltage clamps between a holding potential of –30mV and a maximum of +60mV (referenced to the apical solution, which contained 50µM amiloride). The left-hand side shows the effects in normal Cl⁻ Ringer on both sides. The right-hand side shows the effects when Cl⁻ is removed from the apical solution and replaced with gluconate. (Taken from Larsen et al., 1987). For consistency with the results presented in Chapter 3, the sign of the currents has been reversed. That is, a positive voltage-clamp corresponds to the movement of negative ions from the apical side to the basolateral side.
Although there is much evidence to suggest the presence of voltage-activated Cl⁻ channels in the apical membranes of the MRCs, recent evidence using vibrating probe techniques challenges this notion (Somieski & Nagel, 1998). Developed in the early 1970's (Jaffe & Nuccitelli, 1974), the vibrating probe can be used to measure the local electrical current density above a current source. That is, current peaks in the extracellular fluid above particular cells can be detected. This technique was used by Foskett & Ussing (1986), and Katz & Scheffey, (1986) to demonstrate that such current peaks were identifiable above the MRCs. While it appeared that these regions of high current density were probably due to Cl⁻ fluxes, this could not be concluded definitely, as the transepithelial current density was not reported. In addition, it was only possible to locate this elevation in current density above a few MRCs because of variable conductances within the subpopulations of the MRCs and the poor resolution of the technique. However, with several technological improvements it became possible to measure the current density profile of large areas of tissue under transepithelial current clamp conditions, while simultaneously recording the tissue morphology. Interestingly, it was found that only 10% of MRCs were associated with current density peaks (Nagel et al., 1998). In addition, only 20% of the transepithelial current clamp was due to current density peaks; the other 80% was homogeneously distributed over the tissue and not closely associated with any particular morphological structure. These results, in conjunction with the theoretical simulations performed by Somieski & Nagel (1998), led these investigators to the conclusion that the MRCs were not the site of the voltage-activated Cl⁻ conductance in the anuran skin. Instead, they suggested that the voltage-activated Cl⁻ conductance may be localised to the paracellular pathways (section 1.4.3). At present, the apparent disparity between the vibrating probe experiments and the evidence favouring a voltage-activated Cl⁻ conductance in the MRCs has not been resolved.

1.4.2.2 Types of apical Cl⁻ channels in MRCs

Although there appears to be some confusion regarding the transepithelial Cl⁻ pathway, it is not possible to rule out MRCs as a feasible route based on morphological arguments, because these cells do possess Cl⁻ channels. At least 3 different types of Cl⁻ channels exist in the MRCs: channels with a small conductance, an intermediate conductance or a giant conductance (Sorensen & Larsen, 1996). By isolating MRCs from the whole skin using an enzymatic treatment of collagenase and trypsin, it is
possible to use patch clamp techniques to distinguish between these channels. The properties of each of these types of channels are discussed below.

**Small conductance Cl⁻ channels**

The most frequently encountered ion channels in the apical membrane of the MRCs are the small conductance Cl⁻ channels, being found in approximately 26% of patches (Sorensen & Larsen, 1996). These channels have a small unitary conductance between 7-10pS (Sorensen & Larsen, 1995(a) & (b); Sorensen & Larsen, 1996) and display a linear current-voltage (I-V) relationship when they are exposed bilaterally to a 125mM Cl⁻ solution. When the channels are exposed to asymmetrical Cl⁻ solutions, the channel I-V characteristic can be described by the GHK equation. The channels do not exhibit voltage dependence and they appear to be unaffected by cytoplasmic ATP or Ca²⁺. However, they appear to be activated by cAMP as was found when the MRCs were preincubated in forskolin (Larsen et al., 1996). Forskolin, which is believed to activate a membrane-bound adenyl cyclase, which in turn elevates cytoplasmic ATP, led to an almost two-fold increase in the number of patches containing the small conductance Cl⁻ channels. Finally, in terms of channel kinetics, where recordings were possible, the channels appeared to have 1 open state and 1- or 2- closed states. More states may exist but have not been identified because of deterioration of the patch-clamp seals (Sorensen & Larsen, 1996).

**Intermediate conductance Cl⁻ channels**

Sorensen & Larsen, (1996) found Cl⁻ channels with a mean slope conductance of around 10–30pS in approximately 14% of patches. This group of 'intermediate conductance' channels could be subdivided further into 3 subpopulations consisting of: (i) channels with a linear or slightly outwardly rectifying I-V relationship, which were identifiable in about 5% of patches. These channels could be activated by ATP and, like the small conductance channels, appeared to have 1 open state and 1- or 2- closed states, (ii) channels that exhibited a voltage-dependent open probability, again, identifiable in about 5% of patches. and, (iii) channels with unresolvable kinetics seen in approximately 4% of patches.

**Giant conductance Cl⁻ channels**

Observed in approximately 3% of patches, the giant conductance Cl⁻ channels have a high unitary conductance of 120pS when bilaterally exposed to 125mM Cl⁻. These
channels exhibit GHK rectification but only open upon depolarisation, with a half-maximal activation in the range of 0mV<V_{cell}<50mV where V_{cell} is the voltage across the isolated cell (Larsen and Harvey, 1994). When open, the channels have a conductance ranging between 150-550pS (Sorensen & Larsen, 1996). With regards to the kinetics of these channels, there is still some discussion. Sorensen and Larsen, (1996) reported that the openings and closings of the giant conductance Cl\(^{-}\) channels were characterised by complicated kinetics generally involving multiple steps. This is in contrast to the earlier findings of Larsen and Harvey (1994) who observed random channel fluctuations between one open and one closed state.

But perhaps of greater importance are the discrepancies in the literature regarding the magnitude of the fully voltage-activated component of the Cl\(^{-}\) conductance in the MRCs. Willumsen & Larsen (1986) estimated the transcellular Cl\(^{-}\) conductance to be around 260mS cm\(^{-2}\) of apical MRC surface area, whereas Foskett & Ussing (1987) predicted it to be around 100mS cm\(^{-2}\) and Larsen and Harvey (1994) calculated it to be about 20mS cm\(^{-2}\). One possible reason for the low value reported by Larsen and Harvey (1994) may have been because trypsin, used in the enzymatic process to isolate the MRCs, has recently been found to inhibit the voltage-activated Cl\(^{-}\) conductance in the anuran skin epithelium (Nagel & Katz, 1999). This could also explain why these giant conductance Cl\(^{-}\) channels were only observed in a small percentage of patches.

Overall, it is clear that different types of Cl\(^{-}\) channels contribute to the apical Cl\(^{-}\) conductance of the MRCs, and at least one type appears to be voltage-sensitive.

1.4.2.3 Evidence for apical Na\(^{+}\) channels in MRCs

There are at least 3 compelling arguments that support the idea of Na\(^{+}\) channels in the apical membranes of MRCs. This section reviews these arguments, focussing on the experimental findings from patch-clamp studies, volume experiments and electron microprobe analyses.

Perhaps the most direct evidence for the existence of apical Na\(^{+}\) channels in the MRCs comes from patch-clamp studies. Using the excised 'inside-out' patch configuration, Harvey and Larsen (1993) made recordings of single-channel currents in the apical membranes of MRCs that exhibited a high Na\(^{+}\) selectivity and could be fitted using the GHK equation. When the channel was bilaterally exposed to 120mM Na\(^{+}\) solutions, the I-V relationship was linear with a slope conductance of 3.5pS. In asymmetrical solution ([Na\(^{+}\)]_{pipette}=120mM, [Na\(^{+}\)]_{bath}=12mM), a chord conductance of 9pS was observed. Whole-cell patch techniques yielded similar results (Harvey, 1992).
By subtracting the whole cell I-V characteristics recorded in the presence of 5μM amiloride from the whole-cell I-V characteristics in the absence of amiloride, it was possible to obtain a measure of the amiloride-sensitive I-V relationship of the MRCs. This data, representing the apical Na⁺ flux, could be described well by the GHK equation, further supporting the idea of apical Na⁺ channels in the MRCs.

Larsen et al., (1987) also presented convincing evidence for apical Na⁺ channels. Using video-imaging, the volume of the MRCs were recorded upon apical exposure of these cells to Na⁺-free Ringer or to NaCl Ringer containing amiloride (Figure 1.19). In both cases approximately a 10-15% reduction in volume was observed compared with the (NaCl Ringer) control. These effects were presumably due to a reduction in cellular Na⁺ either as the Na⁺ entry pathway was blocked, as in the case of amiloride, or due to Na⁺ leaving the cell passively down its concentration gradient, as in the case of low extracellular Na⁺. In both experiments the effects were reversible. Again, these results are consistent with the idea of apical Na⁺ channels in the MRCs.

![Figure 1.19: The volume of a single MRC under short-circuit conditions (Vskin=0mV) in response to Cl⁻ Ringer in both the apical and basolateral solutions (control) followed by: (A) a Na⁺-free solution on the apical side and then recovery under control conditions and (B) the addition of 50μM amiloride to the apical solution and then recovery under control conditions. (Taken from Larsen et al., 1987).](image)
Some electron microprobe experiments have also shown changes in cellular Na⁺ levels that are consistent with apical Na⁺ channels (Rick, 1992). In ouabain-sensitive MRCs, approximately half were sensitive to amiloride when it was added to the apical bathing solution, as shown by a reduction in intracellular Na⁺ concentration. However, these findings were not consistent with those of Dorge et al. (1990), who observed no amiloride effect using similar techniques. One possibility for the discrepancy might be that subpopulations of MRCs were expressed in different proportions in each experiment. This is entirely plausible, because factors such as environmental conditions prior to experimentation can influence the type of MRCs that are expressed. For example, the skins of frogs adapted to Na⁺-free solutions display an increase in the number of MRCs and an associated increase in transepithelial Na⁺ transport compared with skins where the frogs have not been exposed to low Na⁺ environments (Ehrenfeld et al., 1989). Under these circumstances, it would appear that 'Na⁺-transporting' MRCs are expressed preferentially to other subpopulations. In addition, it has also been suggested that the experimental conditions play a role in Na⁺ reabsorption across the MRCs (Dorge et al., 1990). When the skins are subjected to in vivo-like conditions (i.e. open-circuit), most of the Na⁺ appears to pass through the MRCs, but under experimental condition (i.e. short-circuit conditions with NaCl Ringer in both bathing solutions), the Na⁺ flux through the MRCs only contributes a few percent of the total short-circuit current (Larsen et al., 1987).

Finally, it has been estimated that MRCs contain up to 450 Na⁺ channels per cell (or 45*10⁶ channels per cm² assuming 10⁵ MRCs per cm²) (Harvey, 1992). Theoretically then, the MRCs could account for all transepithelial Na⁺ uptake. However, while the MRCs have the capacity to absorb Na⁺ passively through amiloride-sensitive channels in the apical membrane, the contribution that these cells make to the total transepithelial Na⁺ uptake appears to be somewhat variable. In fact, there is some speculation that when skins are exposed to low extracellular Na⁺ levels, absorption occurs primarily via the MRCs, but in the presence of high extracellular Na⁺, the main absorption pathway is through the PCs (Ehrenfeld et al., 1989).

1.4.2.4 Evidence for apical H⁺ pumps & Cl⁻/HCO₃⁻ exchangers in MRCs

In γ-MRCs, the passive uptake of Cl⁻ across the apical membrane is supplemented by an active Cl⁻ flux via a Cl⁻/HCO₃⁻ exchange mechanism, that operates in parallel with a rheogenic proton (H⁺) pump. This section reviews the experimental evidence demonstrating the existence of the proton pumps and the Cl⁻/HCO₃⁻ exchangers.
It has been known for some time that the anuran skin has the capacity to acidify the apical bathing solution, and that this response can be attributed directly to the MRCs (Page & Frazier, 1987). Several experimental observations suggest this is due to the presence of apical proton pumps. First, it has been shown repeatedly that there exists a steep H⁺ gradient above the MRCs (Harvey, 1992; Larsen et al., 1992; Larsen et al., 1996; Jensen et al., 1997). This was shown using pH-sensitive double-barrelled microelectrodes in Cl⁻ free solutions. It was found that the H⁺ concentration was high when the electrodes were close to the MRCs (i.e.~10µM) but this decreased as the electrodes were moved vertically away from the cells into the bulk solution. No gradient was observed when the electrodes were moved sideways across the skin and away from the MRCs, indicating that the adjacent PCs do not generate a significant proton gradient. When Cl⁻ was restored to the bathing solution, the proton gradient was abolished above the MRCs. This is consistent with the idea that a neutralisation reaction was occurring between H⁺ transported out of the cell via the proton pumps and HCO₃⁻ exchanged with Cl⁻ as it was taken up via the Cl⁻/HCO₃⁻ exchanger. These observations strongly suggest both the presence of apical proton pumps and Cl⁻/HCO₃⁻ exchangers in the MRCs.

A second line of evidence supporting the existence of proton pumps is the effect of removing CO₂ from the basolateral bathing solution. According to the Stirling Cycle, CO₂ (which can diffuse into the cells from the bathing solutions, or be generated by metabolic processes) combines with cellular H₂O to form H₂CO₃, a reaction that is catalysed by carbonic anhydrase (CA). The H₂CO₃ subsequently dissociates into H⁺ and HCO₃⁻ ions, which are transported out of the MRCs into the apical bathing solution. Jensen et al., (1997) predicted that if CO₂ were removed from the basolateral bathing solution, then a reduction in cellular H⁺ and HCO₃⁻ would occur. Under these conditions, and in the absence of Cl⁻ in the bathing solutions to eliminate the effects of the Cl⁻/HCO₃⁻ exchanger, the pH gradient above the MRCs should be reduced, as less H⁺ was pumped out of the MRCs. This indeed was shown to be the case and was fully reversible upon restoration of CO₂. This data is therefore consistent with apical proton pumps in the MRCs.

Another experimental manipulation to test for proton pumps was the inhibition of the CA enzyme using acetozolamide or ethoxzolamide applied to the apical bathing solution. (Ehrenfeld & Garcia-Romeu 1977; Ehrenfeld et al., 1985; Harvey, 1992). The MRCs are known to contain particularly high levels of CA (Rosen & Friedley, 1973), so it was predicted that the inhibitors would cause a marked reduction in H⁺ and HCO₃⁻
formation. As with CO₂ depletion, in the absence of Cl⁻ in the bathing solutions, there was a marked decrease in the pH gradient above the MRCs.

Acidification of the apical bathing solution was also reduced using the proton pump inhibitors, dicyclohexyl-carbodiimide (DCCD) and oligomycin (Ehrenfeld et al., 1985). Furthermore, preventing oxidative metabolism by starving the putative proton pumps of ATP (Jensen et al., 1997), deoxygenating the bathing solutions (Ehrenfeld et al., 1985) or using metabolic inhibitors such as dinitrophenol and Antimycin A (Machen & Erlij, 1975) also caused a reduction in MRC proton secretion, which is consistent with the existence of apical proton pumps. Finally, these results are in agreement with rod-shaped particles found in freeze-fracture replicas of the apical membrane of the MRCs. These particles are believed to be part of the H⁺-ATPase (Brown, 1989).

In 1995, Willumsen & Larsen stated that "transport of Cl⁻ across the apical membranes of MRCs [had] been shown to occur either passively through voltage-gated anion channel in the case of high external Cl⁻ concentrations, or by secondary active transport via a Cl⁻/HCO₃⁻ exchanger energised by an apical proton ATPase in the case of low (<1mM) external Cl⁻ concentration, typical for freshwater." The latter part of this statement had its genesis in several experimental findings. First, it was shown that there was a net inward flux of Cl⁻ that affected the short-circuit current (Zadunaisky et al., 1963; Bruus et al., 1976). This Cl⁻ influx could be described by a saturating function, with a half maximal saturation of around [Cl⁻]₀=0.2mM (Larsen, et al., 1996). These observations strongly suggested that the mechanism responsible was a cotransporter. The process was also inhibitable by acetazolamide (Bruus et al., 1976). Second, the net Cl⁻ flux was stoichiometrically related to the HCO₃⁻ secreted (Garcia-Romeu et al., 1969). And finally, as discussed above, in the presence of Cl⁻ in the bathing solution, the pH gradient above MRCs could be abolished, consistent with the idea of a neutralisation reaction occurring between H⁺ and HCO₃⁻ secretion. When viewed overall, these findings strongly suggest the presence of Cl⁻/HCO₃⁻ exchangers in the apical membranes of the MRCs.

1.4.2.5 Evidence for basolateral Na⁺/K⁺-ATPase pumps in MRCs

Using freeze-dry radioautographic techniques, Na⁺/K⁺-ATPase pumps have been localised to the basolateral membranes of all living cells of the anuran skin (Mills et al., 1977). Discussed below is the evidence supporting the existence of these pumps in the MRCs.
Cell volume studies performed by Larsen et al. (1987) on the MRCs have shown that when the potent Na⁺/K⁺-ATPase inhibitor, ouabain, is applied to the basolateral bathing solution, the MRCs swell (Figure 1.20). As the pumps are blocked, there is a net increase in the intracellular Na⁺ concentration, resulting in water entry and volume expansion. Thus, this finding strongly suggests that Na⁺/K⁺-ATPase pumps are located in the basolateral membrane of the MRCs. The presence of these pumps is consistent with estimations that the MRCs have the capacity to generate large active Na⁺ currents of 80-90µA/cm² which is approximately 4-5 times larger than that estimated for the PCs (20-25µA/cm²) (Larsen et al., 1987). To generate such large currents, it is likely that the MRCs have large energy requirements. Morphological studies support this premise, indicating that the MRCs contain high concentrations of mitochondria (Whitear, 1975). While the presence of mitochondria is not direct evidence of Na⁺/K⁺-ATPase pumps, it is in agreement with the observation that greater than 60% of the oxygen consumption of tight epithelia is used to supply energy to this active transporting mechanism (Harvey & Urbach, 1992).

Figure 1.20: The volume response of a single MRC to the basolateral application of 3mM ouabain. (Taken from Larsen et al., 1991).
1.4.2.6 Evidence for basolateral $K^+$ channels in MRCs

A perusal of the literature shows that few experiments have been conducted directly on the MRCs to determine the presence of passive $K^+$ channels in the basolateral membrane. However, two lines of evidence suggest that these channels do exist. First, a passing comment in a paper on $K^+$ channels in PCs by Urbach et al. (1994) stated that inward rectifier $K^+$ channels had been identified in the basolateral membrane of MRCs using standard patch-clamp techniques. Second, as shown in Figure 1.17, lowering the basolateral $K^+$ concentration to 0.5mM slowed the MRC volume response to voltage activation/deactivation quite considerably (Larsen et al., 1987). It is unlikely that this response is attributable to basolateral Na$^+$/K$^+$-ATPase pumps as they had been starved of Na$^+$ by the apical application of 50µM amiloride. Therefore, with a negligible apical $K^+$ conductance, these results suggest that $K^+$ entry into the MRCs was via passive channels located in the basolateral membrane, and that low basolateral $K^+$ concentrations can become the rate-limiting step in MRC volume regulation.

1.4.2.7 Evidence for basolateral $Cl^-$ channels in MRCs

There are at least three lines of evidence that suggest the presence of $Cl^-$ channels in the basolateral membranes of MRCs. This section reviews this evidence, focusing on the experimental findings from cell volume studies, ion substitution experiments and patch-clamp techniques.

Already, it has been shown that ouabain causes the MRCs to swell (Figure 1.20). This swelling is presumably dependent on Na$^+$ and $Cl^-$ entry (where Na$^+$ gain is in excess of $K^+$ loss). In a series of experiments conducted by Larsen et al., (1987) it was demonstrated that $Cl^-$ could enter across either the apical or basolateral membrane. The underlying logic was that if a cell volume increase was observed after replacement of $Cl^-$ with gluconate in one of the bathing solutions, then $Cl^-$ was entering the cell from the opposite bathing solution. This protocol was carried out in both the apical and basolateral solutions, and in each case cell swelling was observed. Consequently, it was concluded that the MRCs possessed a $Cl^-$ permeability in both membranes.

This finding was further substantiated by electron microprobe analysis where the intracellular concentrations were determined following ionic substitutions of the bathing solutions (Nagel and Dorge, 1990). It was found that intracellular $Cl^-$ could be exchanged with $Br^-$ entering from either the apical or basolateral bathing solutions. However, the rate of exchange across both membranes was quite variable in different
MRCs from the same skin. One possibility for this variability may be due to the heterogeneous population of Cl\(^-\) channels in both membranes.

The different types of Cl\(^-\) channels in the apical membrane of MRCs have already been discussed (section 1.4.2.2). Using patch-clamp techniques, three types of chloride channels have been localised to the basolateral membrane of the MRCs (Willumsen and Larsen, 1995). These include (1) medium sized channels with a single channel conductance of 31-37pS, (2) smaller channels with a single channel conductance of 9-12pS, and (3) channels that exhibit noisy patch currents but do not display distinct single channel events. Thus, these findings indicate conclusively that basolateral Cl\(^-\) channels exist in the MRCs.

1.4.2.8 Evidence for Na\(^+\)/K\(^+\)/2Cl\(^-\) symports in MRCs

Many of the studies that have investigated the existence of symports in the anuran skin have been carried out on whole skins without investigating the PCs or MRCs individually. Therefore, it is not possible to say conclusively that either the PCs or the MRCs contain symports. However, many experiments have suggested that Na\(^+\)/K\(^+\)/2Cl\(^-\) symports are indeed present in the anuran skin (Cox & Helman, 1983; Ferreira & Ferreira, 1981; Ussing, 1985). But invariably this transport mechanism has been ascribed to the PCs. One of the reasons for this is that the PCs make up a considerable component of the epithelial volume. Therefore, to a first approximation, changes in the volume of the whole skin probably reflected changes in the volume of the PCs. Another reason is that the maintenance of a high cellular Cl\(^-\) concentration above equilibrium in the PCs required the (secondary) active uptake of Cl\(^-\). Whatever the mechanism, it also had to be consistent with findings that restoration of cellular Cl\(^-\) following cell shrinkage was dependent on the presence of Na\(^+\), K\(^+\) and Cl\(^-\) in the basolateral bathing solutions (Ussing, 1985). A Na\(^+\)/K\(^+\)/2Cl\(^-\) symport in the basolateral membrane of the PCs fitted the requirements, and was in agreement with many of the experimental findings. However, the presence of Na\(^+\)/K\(^+\)/2Cl\(^-\) symports in the PCs is not a convincing argument for the preclusion of symports in the MRCs, particularly as (1) experiments have not been conducted independently on either the PCs or the MRCs and (2) a percentage of MRCs also has a high Cl\(^-\) concentration well above equilibrium (Dorge et al., 1990). Thus, some arguments favouring the presence of symports in PCs are equally applicable to MRCs. Indeed, it may be argued that in particular circumstances, because the MRCs have such a significant role in transepithelial Cl\(^-\) transport, these cells may be more likely than PCs to lose cellular Cl\(^-\) and shrink. If this were the case, then the
MRCs may be even more likely candidates for symports than the PCs, to ensure rapid restoration of cellular ionic concentrations. Therefore, while there is little direct evidence for symports in the MRCs, it is proposed tentatively that these transporting mechanisms do exist in either the apical or basolateral membranes. Based on experimental results from this study on whole skins, if the MRCs indeed possess Na$^+$/K$^+$/2Cl$^-$ symports, it is more likely that these transport proteins are located in the apical membrane of these cells, at least in Bufo marinus, because addition of the Na$^+$/K$^+$/2Cl$^-$ symport blocker, furosemide, had little effect when added to the basolateral solution, but caused an increase in both Isc and Voc when added to the apical solution (see Chapter 5).

1.4.3 Paracellular Pathways

There is strong morphological evidence showing the existence of paracellular pathways in the anuran skin (Farquhar & Palade, 1965). However, the cell-to-cell contacts that make up the paracellular pathways in this epithelial layer are slightly different from those found in many other epithelia. This section looks at these differences, and also examines the ionic selectivity of these pathways, and how the conductances can be altered.

In most epithelia the paracellular pathways consist of a tripartite junctional complex comprised of the tight junctions (or zonula occludentes), adherens junctions and desmosomes (Figure 1.21). The tight junctions (TJs) are the most apical element of the complex and form the major paracellular barrier. The adherens junctions link adjacent cells together via Ca$^{2+}$-dependent cell-cell adhesion molecules that are linked to actin and myosin filaments within the cells, while the desmosomes form point contacts between cells and provide anchoring sites for the intermediate filaments (Denker & Nigam, 1998). In the anuran skin a tripartite junctional complex is only apparent in the early larval stage; in larvae approaching metamorphosis and in the adult stage the junctional complex only consists of TJs and various types of desmosomes. More specifically, in stratum corneum the TJs lie on the lateral surface of the cells, directly above a series of 'modified' desmosomes which have a denser plate of intercellular material and occupy a significantly larger surface area than ordinary desmosomes (Farquhar & Palade, 1965). The basolateral membranes of the deepest cornified cells are connected with the underlying cells via bipartite desmosomes. The desmosomal half that is associated with the cornified cells is characterised by a modified desmosome, while the half associated with the cells of stratum granulosum are characterised by the
smaller, ordinary desmosome. Similar to *stratum corneum*, the lateral junctional complex of *stratum granulosum* consists of TJs but these lie apical to ordinary desmosomes. Again basolateral cell-to-cell contacts are via bipartite desmosomes. In the deeper strata, no TJs are evident; contact is via ordinary desmosomes and *maculae occludentes*.

It has only been in the last two decades or so that it has become apparent that the permeability of the TJs can vary over several orders of magnitudes under different conditions (Anderson & Van Itallie, 1995; Madara, 1988). Before that, the TJs were regarded as fixed barriers. However, now they are seen as sieve-like structures, consisting of a number of distinct trans junctional 'pores,' rather like a series of channels in close apposition (Madara, 1988). It is now well accepted that the TJs are highly regulated, but there are a number of different factors, such as intracellular second messengers, involved in this regulation.\(^{35}\) These factors may vary among cell types and the dominant regulatory mechanism may be different for different tissues (Anderson &

\(^{35}\)It is beyond the scope of this review to address some of the known 2nd messenger pathways that control the permeabilities of the tight junctions. The interested reader is referred to the recent reviews by Anderson & Van Itallie (1995) and Denker & Nigam, (1998).
Van Itallie, 1995). Some of the factors that have been implicated in increasing the permeability of the TJs in the anuran skin include apical hypertonicity (Erlij & Martinez-Palomo, 1972; Gonzales et al., 1978; Mills et al., 1977; Ussing & Windhager, 1964), acidification of the apical bathing solution (Benedictis & Lacaz-Viera, 1982; Fischbarg & Whittembury, 1978; Gonzales et al., 1978), and the accumulation of Li⁺ within the cells, which may induce an increase in the intracellular Ca²⁺ concentration (Aboulafia et al., 1983). In other epithelia and cultured monolayers, TJ permeability is increased by a reduction in the extracellular Ca²⁺ concentration (Castro et al., 1993; Cereijido et al., 1978), an increase in intracellular Ca²⁺ (Cereijido et al., 1981), a reduction in intracellular ATP (Denker & Nigam, 1998) and lateral mechanical stress (Madara, 1988). Factors known to reduce the permeability of the TJs in various epithelia include 2,4,6-triaminopyramidine (Moreno, 1974), protamine (Bentzel et al., 1987) and plant cytokinins (Bentzel et al., 1980). In the anuran skin, BaSO₄ is known to block the paracellular pathways (Castro et al., 1993).

An interesting feature of the paracellular pathways in the anuran skin is that they exhibit a degree of ionic selectivity, favouring the permeation of cations over anions. Estimates of the paracellular Na⁺ and Cl⁻ permeabilities in non-moulting anuran skins by Larsen (1991) indicated that the Na⁺ permeability was approximately double that of Cl⁻ (PNa ~ 2x 10⁻⁸cm/s cf. PCl ~ 1x10⁻⁸cm/s). Indeed, some researchers have found that there is a close correlation between the permeability of the paracellular paths and the rate of Na⁺ transport, such that the lower the transport rate, the lower the paracellular permeability (Voute & Ussing, 1970). However, it is not known if this effect can be attributed directly to Na⁺, or if Na⁺ somehow triggers an intracellular event, which in turn causes a reduction in paracellular permeability. The main point is that in non-moulting anuran skins, the paracellular pathways do contribute to vectorial transport and can assist in creating and maintaining the composition of the extracellular fluids in the intercellular spaces.³⁶

³⁶ During the moult phase, the paracellular permeability increases significantly such that it has the "property of a free diffusion leak for Na⁺ and Cl⁻." (Larsen, 1991).

1.4.4 Cell volume and hydraulic conductivity of the toad skin

The cell volume of polarised epithelial cells (which are typically exposed to different extracellular osmolarities on their apical and basolateral sides) is largely determined by two factors: (1) the permeability of the apical and basolateral cell membranes to water (i.e. hydraulic conductivity) and (2) the difference in osmolarity between the
intracellular and extracellular fluids (where any imbalance due to osmosis is corrected by an hydrostatic pressure relative to the surrounding fluid). Therefore, assuming a water flow across these polarised epithelial cells (i.e. both the apical and basolateral membranes possess hydraulic conductivities), regulation of cell volume can be achieved either by altering the relative hydraulic conductivity of the cell by upregulating or downregulating the number of aquaporins (water pores), or by changing the osmotic gradients across the cell membrane by increasing or decreasing the osmolarity of the internal or external environments.37

The water permeability of many epithelia is highly variable. In many cases, such as in the cells of the late distal tubules and collecting ducts of the kidney, the permeability differs greatly between the apical surface, which is almost impermeable to water under most circumstances, and the basolateral surface, which is highly water permeable. The same differences between apical and basolateral water permeabilities occurs in the anuran skin. In the absence of neurohypophyseal hormones such as arginine vasotocin (AVT), corticosteroids such as aldosterone, or β-agonists such as isoprenaline, the apical surface of the anuran skin is tight to water. In contrast, the basolateral water permeability is generally quite high (MacRobbie & Ussing, 1961).

It should be emphasised however, that the macroscopic properties of an epithelium are not necessarily representative of what is occurring at a cellular level. For example, it has been demonstrated that when an hypotonic solution bathes the apical surface of the anuran skin, the PC volume remains constant, which is consistent with the idea that the epithelium is tight to water on the apical side. However, Larsen et al. (1987) demonstrated that the MRCs swell (which is consistent with the earlier suggestion in Part 2 that volume-sensitive Na⁺ channels may exist in the apical membrane of these cells). This observation are important for two reasons. First, they demonstrate that different cell types can have different volume responses to the same perturbation. This may be due to different water (and ionic) permeabilities of each cell type, either at the apical or basolateral membranes. Alternatively, the variation in response may occur due to each cell type having a different intracellular osmolarity, which may be determined by differences in intracellular ionic concentrations, or the presence of organic

37 It should be noted that in non-polarised cells which are surrounded by the same extracellular fluid, changing the hydraulic conductivity will simply alter the rate at which a steady-state volume is attained but not the steady-state volume per se. This is in contrast with polarised epithelial cells which can generate different apical and basolateral extracellular osmolarities, with the result that a change in the relative membrane hydraulic conductivities can result in a change in the steady-state cell volume.
osmolytes. Second, and on a broader philosophical note, two cell types in apposition in an epithelium having disparate responses will each alter the shared environment of the intercellular spaces. This means that although there may be no physical contact between the MRCs and the PCs, one cell type could affect the other in principle, simply by the alteration of ionic and electrical gradients. This has important implications when mathematically modelling epithelial layers consisting of two or more cells positioned in series or in parallel. The main point here, however, is that the water permeability of an epithelium is determined by the water permeabilities of different cell types, which may differ considerably.

When considering the water permeability of the anuran skin, it is worth noting that it was only in 1991 that the first aquaporins were isolated in any tissue (Preston & Agre, 1991; Preston et al., 1992). These aquaporins, now known as AQP1 (or CHIP28) were found in human erythrocytes. Since then at least four other homologous mammalian aquaporins have been identified: AQ2, which is found in the rat collecting duct and is vasopressin-sensitive and Hg$^{2+}$-inhibitable; AQP3, which is found in the basolateral membranes of rat collecting duct and is permeable to urea and is Hg$^{2+}$-inhibitable; AQP4, which is found in several rat tissues and is Hg$^{2+}$-insensitive; and AQP5, which is found in exocrine glands and respiratory parenchyma and is Hg$^{2+}$-inhibitable (Lazowski et al., 1995). The point is that there are different aquaporins, all possessing slightly different properties. Recently, interest in aquaporins has been extended to anurans with the finding of a frog aquaporin (FA-CHIP) in frog urinary bladder that was 77.4% identical to human AQP1 (Abrami et al., 1994) and a toad aquaporin (AQP-t1) that was 76% identical to human AQP1 and 88% identical to the FA-CHIP (Ma et al., 1996). This vasopressin-insensitive AQP-t1 was first cloned from toad urinary bladder, but Northern blot analysis also showed AQP-t1 mRNA expression in toad skin (Ma et al., 1996). Presumably, it is simply a matter of time before other anuran aquaporins are isolated.

In terms of this review, the interest in anuran aquaporins is two-fold. First, it important to know what stimuli activate aquaporins in a particular cell type because this gives some indication of (a) the presence of different types of aquaporins and/or (b) the presence of different types of receptors. In PCs there are hormonally- and β-agonist-sensitive aquaporins in the apical membrane, which are different from the spontaneously occurring aquaporins in the basolateral membrane which display no such sensitivity (Berman et al., 1989; Verkman, 1992; Acher et al., 1997). In contrast to the
PCs, there is little documented about aquaporins in the MRCs at the present time. However it is possible that the MRCs possess:

1. **the same types of aquaporins and receptors as the PCs.** Under these circumstances, the different ionic concentrations and/or the different aquaporin densities of each cell type could explain the different volume responses of the PCs and the MRCs to the same osmotic perturbation, as described earlier.

2. **the same types of aquaporins but a different activating system.** This scenario is consistent with Ehrenfeld et al.’s postulate (1989) that the MRCs lack the receptors for some neurohypophoseal hormones such as AVT and oxytocin, which would explain why these cells are insensitive to such stimuli.

3. **different types of aquaporins from the PCs.** This scenario might also partly explain why the MRCs display different volume responses from the PCs.

Irrespective of the exact details, the main point is that the regulatory mechanisms for water transport across each cell type may not necessarily be the same.

The second reason for the interest in the water pores is that, if the PCs and the MRCs express different types of aquaporins, there may be particular conditions where one cell type becomes the dominant route for water (and ionic) transport across the skin. This idea was elegantly developed in terms of ionic transport by Ehrenfeld et al. (1989), but the same ideas are applicable in terms of water transport, particularly where different types of cells display different types, and numbers, of aquaporins. Ehrenfeld et al. showed that both the MRCs and the PCs could transport Na\(^+\), but the relative contribution made by each cell type depended heavily upon the extracellular NaCl concentration and the degree of coupling between the net Na\(^+\) and H\(^+\) fluxes. When the apical NaCl concentration was high, most Na\(^+\) was transported through the PCs because of the favourable Na\(^+\) electrochemical gradient. However, acute cell acidification resulted in the closure of Na\(^+\) channels in the PCs, and under such conditions Na\(^+\) uptake was predominantly via the MRCs (Ehrenfeld & Klein, 1997). Thus, the relative ionic (and water) fluxes carried by each cell type appears to be variable.

The idea that different cell types can alter their contribution to the transepithelial flux depending on their intracellular and ionic environments may seem trivial at one level, but there has been a prevailing view in the literature that the PCs are primarily responsible for Na\(^+\) transport, while the MRCs are primarily responsible for Cl\(^-\) transport. To an extent, this idea originated from the early studies of Krogh (1939) who showed that in Cl\(^-\)-free solutions, Na\(^+\) was absorbed across the skin, and likewise, in Na\(^+\)-free solutions, Cl\(^-\) was transported across the skin, even from dilute solutions. He
concluded that both Na$^+$ and Cl$^-$ could be actively absorbed across the skin independently of one another. However, he did not show that these events occurred independently through different cell types. But he is often thought to have said this largely because in the final chapter of his book, he wrote: "A priori, one would expect the mechanism for cation and anion absorption respectively to be located in separate cells…." Taken out of context as an isolated statement it would appear that Krogh was stating that cations were only absorbed by one cell type in the toad skin, while another absorbed only anions. This is an incorrect misconception that has been perpetuated throughout the literature because of the unfortunate phrasing of a concluding statement, where general principles of active transport in epithelia were being discussed with no specific reference to the anuran skin. (In fact in the paragraph in which this statement appears, Krogh talks only of fish, crustacea and arthropoda, with no mention of anura). When taken in context, it is apparent that Krogh is merely speculating about a general mechanism. Indeed, he was the first to acknowledge that the study of ion transport at that time was in its formative stages and was not well understood.\textsuperscript{38} Besides, if this interpretation were the case, then there would be little volume regulation in either cell type! But of course some form of volume regulation occurs in each cell type, as exemplified at the beginning of this section, with regard to the effects of bathing the skin in a hypotonic solution. In addition, separate cells for the transport of cations and anions is not at all consistent with the information presented throughout this review which has demonstrated that, both the PCs and MRCs have the mechanisms required to transport cations \textit{and} anions.

\textsuperscript{38} Krogh’s recognition that ion transport across pumping epithelia at the time was not well understood is borne out by three comments, which appear in the same concluding remarks as his ‘A priori’ statement. He writes: (1) ‘The demonstration of active and selective ion-absorbing mechanisms in organs which are comparatively easy of access opens up interesting possibilities for further study, possibilities which must be followed up before we can hope to obtain any rational conception of what is going on;’ (2) ‘When definitely located, suitable cells should be studied cytologically in the living state, during rest and under exposure to various stimulants and depressants;’ (3) ‘Possible mechanisms for the ion transport have been proposed in special cases… and models constructed, but I do not think that the time is at all ripe for this final step, and I submit that the most specialised mechanisms should be studied preferentially, because they are more likely to furnish clues.’ When read in the context of these statements, the speculative nature of his ‘A priori’ statement becomes apparent.
1.5 Organisation of the thesis

As described at the beginning of this chapter, the objective of this study was to obtain a better understanding of salt and water movements across epithelial layers and how these were regulated.

Using isolated toad skins mounted in Ussing chambers as an epithelial model, one of the first requirements from an experimental perspective was to develop a reliable monitoring system which could control and record the currents and voltages across the tissue preparation under different circumstances. Although manual procedures were considered briefly, an automatic monitoring system was a more attractive option having the advantages that: (1) an investigator was not dedicated to a single experiment; (2) the history of the skin, once recordings had commenced, was not lost if the investigator was interrupted during the course of experimentation and (3) fast transients occurring over seconds, or slow transients occurring over periods of up to 20 hours could be recorded continuously without any compromise in data resolution. The details of the electronic circuits and accompanying software are outlined in Chapter 2, along with a description of the methods used in tissue preparation.

Once the automated monitoring system had been developed, it was necessary to compare recordings made with this system, with those of other investigators to establish the validity of the automated recordings. The findings of Larsen and Kristensen (1978), which demonstrated the presence of voltage activated Cl\(^-\) channels were chosen arbitrarily as a benchmark. Their voltage clamp experiments were replicated, as described in Chapter 3, and voltage dependent Cl\(^-\) channels were shown to exist. In addition, the activation and inactivation of the Cl\(^-\) conductance was investigated. It was found that delays in the activation of about 10 seconds occurred when the transepithelial voltage was positively clamped away from the –40mV holding potential (basolateral side positive). Similar delays were observed for inactivation of the Cl\(^-\) conductance when the voltages were returned from positive voltage clamps to the holding potential. It is suggested that these delays in both the activation and inactivation of the Cl\(^-\) conductance were important in initiating the damped oscillatory behaviour observed in some skins when positive voltage biases of up to +100mV were applied.

One of difficulties encountered early in the study was how to obtain stable control traces. Each time a ‘control’ experiment was performed in standard 250mOsm/kgH\(_2\)O NaCl amphibian Ringer, transients in Isc and Voc were observed. This posed a problem because without stable control traces it was difficult to interpret changes in electrophysiological parameters following a perturbation. However, after performing a
series of experiments comparing responses in different solutions it was argued that the instabilities, which occurred over hours, were not due to Cl for two reasons. First, the instabilities occurred when skins were bathed in Cl- and Cl'-free solutions suggesting that Cl- was not the main determinant. Second, the results from Chapter 3 showed that Cl- transients occurred rapidly over seconds. While the Cl- conductance may have been altering slowly due to slow changes in the membrane potentials over time, this was a secondary effect and was not the main cause of the instabilities. Instead, some of the experimental data from this study suggested that the osmolality of the bathing solution influenced the electrophysiological responses. This information, along with comments made by Ussing (1965) about the possible volume sensitivity of the apical Na+ channels in the frog skin resulted in an extensive investigation of epithelial volume, the results of which are presented in Chapter 4. Briefly, the results of this investigation suggested that the apical Na+ permeability was indeed volume-sensitive (although it was not determined whether this volume-sensitive Na+ conductance was located in the PCs or the MRCs, or both cell types). It was also shown that volume changes had a time-constant of about 20-30 minutes which was too slow to explain the slow transients observed over many hours. The possible cause of these transients is discussed in Chapter 5.

Once it had been established that the toad skins possessed dynamic Na+ and Cl- conductances, and that stable controls could not be obtained, the effects of various pharmacological perturbations could be better understood without over-interpretation of the data. In Chapter 5, toad skins were perturbed with a variety of agents with the main focus on the effects of two heavy metals, namely Hg2+ and Pb2+ although references were made to Al3+, La3+, Cu2+, ATP, the K+ channel blocker TEA, and the Na+/K+/Cl- symport blocker furosemide. Although many agents could have been used to perturb the toad skins to investigate how salt and water fluxes were affected, heavy metals were investigated because of the increasing pollution problem caused by them and their detrimental effects on the environment and the health of many species including humans and toads. While the systemic effects of heavy metals have been well documented in humans, Chapter 5 shows that at least some heavy metals can also have a local effect on pumping epithelia by altering the ion transporting capacities of the cells.

Finally, having presented detailed experimental responses of the toad skins over hours under different conditions, salt and water movements were examined from a theoretical perspective. Chapter 6 describes the development, and applications of a general steady-
state mathematical model of a single cell. The ideas are then expanded further in Chapter 7 where a general method for simulating ion transport across single cells as a function of time is described. Chapter 8 then focuses on how the steady-state and time domain models can be used to investigate ion transport across the toad skin.
2. Methods
2. Methods

2.1 Introduction

Many research groups obtained current and voltage responses of the anuran skin by manually altering the transepithelial voltage or current clamp conditions and then recording the response. There are several problems with a manual technique. First, because each manual operation is of finite duration, it is often only possible to conduct one experiment at a time. Thus, an investigator becomes dedicated to a single experimental setup, performing mundane and repetitive tasks which become somewhat frustrating if the experiment continues for an extended period. Second, data resolution is frequently compromised using a manual technique, often being collected after several minutes have elapsed such that fast, transient responses are not recorded. Third, if data recording is interrupted during the experiment then the history of the skin is lost, which becomes an issue when the response of the skin to a perturbation depends on the state prior to the manipulation. Clearly, there are limitations with the manual approach, many of which can be overcome by automating the clamp procedures and the subsequent acquisition of data. This chapter looks at how procedures were automated in the present study. However, to put this automation procedure in context, some familiarity with the available equipment and background tissue preparation is important. Therefore, the chapter has been divided into 4 main sections describing (1) the background preparation necessary before an experiment was conducted, (2) the experimental apparatus (3) the electronics and (4) the software used to control procedures. Block diagrams of the two design solutions discussed in this chapter are presented in Figures 2.1 & 2.2.

2.2 Background Preparation

2.2.1 Animals

In the present study, approximately three hundred in vitro experiments were conducted on one hundred and thirty-nine cane toads of both sexes. The cane toads were imported from Queensland and held in the animal housing unit at the University of Western Australia prior to experimentation. During the holding period, which ranged from several days to a year, the animals were unfed and kept in a dark environment with constant access to tap water. The room temperature was maintained between 22°C and 24°C.
Figure 2.1: Block diagram of the apparatus used for 2 setups (reed relay configuration)
Figure 2.2: (A) Block diagram of the apparatus used for 8 setups (analogue multiplexer configuration); (B) PCB layout.
2.2.2 *In Vitro Tissue Preparation*

Toads were double pithed (at time t=0 minutes) and the right forepaw removed in accordance with the vivisection protocol outlined by the Animal Experimentation Ethics Committee at the University of Western Australia.\(^1\) Where necessary, the abdomen of each toad was rinsed in tap water to remove any blood. A paper towel was then gently pressed against the skin to absorb excess water. The pithing of 3 toads took approximately 5 minutes.

Following pithing, the abdominal skin was removed by making a small pelvic incision, just ventral to the anus, and then cutting towards either leg. The skin flap was then lifted, either by hand or with a pair of forceps and, using blunt dissection techniques, the entire ventral skin was separated from the underlying connective tissue and fascia. When experiments were performed on several toads, skins were removed sequentially from each toad and laid across the associated animal so that the basolateral (belly) side of the skin remained in contact with the moist, underlying organs. No solution was placed on the outward facing (pond) surface. Once all skins had been removed, each one was taken in turn, and divided into 2 or 3 smaller pieces. Where 2 pieces of skin were required, the tissue between the fore- and hind-legs was divided down the centre-line of the toad; where 3 pieces were required, an extra section was removed from beneath the animal’s throat (Figure 2.3). The skins were divided into multiple pieces for 2 reasons: first, it meant that internal controls could be performed and second, it reduced the number of animals that were killed. In terms of identification, each toad was designated by a number (e.g. Toad 86), and each skin sample was described by the letters a, b or c following the toad number. An abbreviated form of this information (e.g. ‘86a’) is shown on each of the traces presented in the experimental section of this thesis.

Once each skin had been subdivided, the pieces were mounted in an Ussing chamber. Calomel electrodes were inserted into the chambers bathing either side of the skin and clamps were placed on the drainage tubes beneath each chamber (Figures 2.4 & 2.5). The procedure was then repeated for the other toad skins. When all the Ussing chambers were set up, complete with skin, electrodes and drainage clamps, the appropriate solutions were added to the bathing chambers before the software was started.

---

\(^1\) Animals were killed by double pithing and not anaesthesia because the standard amphibian anaesthetic, MS 222 (also known as tricaine methane sulphonate (TMS) or 3-aminobenzoic acid ethyl ester) is absorbed dermally, affecting the epithelial layer of interest.
Figure 2.3: (a) The initial incision for the removal of the abdominal skin of the toad was made just ventral to the anus. (1), (2) & (3) When the skin had been dissected it was sub-divided into 2 or 3 pieces according to the sections shown.
Figure 2.4: The experimental apparatus used for 2 setups (reed relay configuration). The perspex Ussing chambers were mounted on retort stands above a splash tray. Behind the Ussing chambers were the electronic circuits housed in a grey box (see section 2.4).
Figure 2.5: The experimental apparatus for 8 setups (analogue multiplexer configuration). (A) Individual components used for one setup including the perspex Ussing chamber, rubber O-ring, 2 Mohr clips, 2 calomel electrodes and the electronic clamp circuit; (B) Ussing chambers mounted on retort stands; (C) the entire experimental apparatus showing the 8 setups surrounded by a Faraday cage, the hard drive and power supply below the desk and the monitor and keyboard on the desk; (D) the Lab PC terminal block (left) and PCI-6704 terminal block (right) providing inputs and outputs to the computer.
Table 2.1: Ionic concentrations of the solutions used in the present study. All concentrations are in mM and calculated osmolarities are in mOsm/kg H2O.
Normal Cl + Hg

Normal Cl + Cu

Normal Cl + Ba
High K, Cl-free (sulphate)
Zero K, Cl-free (sulphate)
Sucrose -loaded Na-free (magnesium)
Low-osmolar Na-free (magnesium)
Iso-osmotic 50% dilute sulphate
Hypo-osmotic 50% dilute Na sulphate
sucrose-loaded Normal Cl
50% dilute Normal Cl- (hypo-osmotic)
50% dilute Normal Cl- with sucrose (iso-osmotic)
1/8 Normal Cl, 1/8 sulphate
1/8 Normal Cl, 1/8 sulphate +sucrose
1/8 Normal Cl, 1/8 sulphate + sucrose
1/8 Normal Cl, 1/8 sulphate + sucrose
1/8 Normal Cl, 1/8 sulphate + sucrose
1/8 Normal Cl, 1/8 sulphate + sucrose
1/8 Normal Cl, normal hepes
1/8 Normal Cl, normal hepes +sucrose
1/8 Normal Cl, normal hepes +sucrose
Modified 50% Normal Cl
Modified 50% Normal Cl +sucrose
Modified 50% Normal Cl +sucrose
Modified 50% Normal Cl +sucrose
Modified 50% Normal Cl +sucrose
Modified 50% Normal Cl +sucrose
Modified 50% Normal Cl +sucrose
Modified 50% Cl-free (sulphate)

Modified 50% Normal Cl +Hg

Modified 50% Normal Cl +Hg

Modified 50% Normal Cl +Hg

Modified 50% Normal Cl +Hg

Modified 50% Normal Cl +Hg

Modified 50% Normal Cl +Hg

Modified 50% Normal Cl +Hg

Modified 50% Normal Cl +Hg

Modified 50% Cl-free (sulphate) + sucrose
Modified 50% Cl-free (sulphate) + sucrose
Modified 50% Cl-free (sulphate) + sucrose
Modified 50% Cl-free (sulphate) + sucrose
Modified Cl-free (sulphate) (Soln 2)
Modified 50% Cl-free (sulphate)
Modified 50% Cl-free (sulphate) + sucrose
Modified 50% Cl-free (sulphate) + sucrose
Modified 50% Cl-free (sulphate) + sucrose
High KCl, Low NaCl
High K sulphate, Low Na sulphate
High K sulphate, Low NaCl
Modified Normal Cl- (to cf Soln 40)

17

18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44

44.5

45

46

47

48

49

50

51

52
53
54
55
56
57
58
59
60
61
62
63
64

Description
Normal Cl
Cl-free (sulphate)
Na-free Cl (magnesium)
Na-free Cl (choline)
Cl-free (gluconate)
High Ca, Na-free
Zero Ca, Normal Cl
High K, Low Na
Zero K, Low Na
Sucrose -loaded Normal Cl
Na-free, Cl-free
Zero K. High Na
Na & Cl-free, High K
Cl-free sucrose-loaded Na
Cl + Hg

16

Solution #
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

45
111

45

45

45

45

45

45

45

45

45

111
55.5
55.5
13.88
13.88
13.88
13.88
13.88
13.88
13.88
13.88
13.88
45
45
45
45
45
45
45

91

101

101

111

111

115

NaCl
1M
111

dissolve 15.5g in 500mL

Choline Chloride
(wt)

10
10
10
10
20
10
10
10
10
10
10
10
10

10

10

10

10

10

10

10

10

20
20
10
10
20
10
10
20
20
20
20
20
20
20
20
20
10
10
10
10
10
10
10
10

20

20

20

20
20
20

20
20

K Hepes
0.5M (wrt Hepes)
20
20
20
20
20
20
20

20
20

20

20
20

Na Hepes
0.5M (wrt Hepes)

20.5

20
20
20
20
50
20.5
20.5
20.5
20.5

20

6.25
6.25

6.25
6.25
6.25
6.25
6.25
6.25

25
50

50

50

Na2SO4
1.11M

89
123

139

K2SO4
0.4M

127

198

1.35
1.35

2.7

1.35

1.35

1.35

1.35

1.35

1.35

1.35

1.35

2.7
1.35
1.35
0.34
0.34
0.34
0.34
0.34
0.34
0.34
0.34
0.34
1.35
1.35
1.35
1.35
1.35
1.35
1.35

2.7
2.7

2.7

2.7

2.7

2.7

2.7

2.7
2.7
2.7

58.2

2.7
2.7

KCl CaCl2
0.56M
1M
2.7

40

20
20
20
20
40
20
20
20
20

20

5
5

5
5
5
5
5
5

20
20

40
40

10
10
10
10

10

3.5
3.5

3.5
3.5
3.5
3.5
3.5
3.5

14.0
14.0

55.5

2.5

2.5

111.0
111.0

2.5
2.5
2.5
2.5
5
2.5
2.5
2.5
2.5
2.5
2.5
2.5
2.5

2.5

2.5

2.5

2.5

2.5

2.5

2.5

2.5

5
5
5
5
5
2.5
2.5
5
2.5
2.5
5
5
5
5
5
5
5
5
5
2.5
2.5
2.5
2.5
2.5
2.5
2.5
2.5

5

5

14

57

49.35
99.35
149.35
199.35
56
18
68
118
168

45.95
95.95
145.95
195.95
245.95
295.95

222.72
422.72

122.72
322.72
522.72
722.72
922.72

125.1

125.1

141.1

10
56
111.5
56

CaGluconate NaGluconate KGluconate MgSO4 MgCl2 Glucose
Sucrose
67.5mM
0.5M
0.5M
1M
0.5M
1M
1M
5
40
28
5
28
111.0
5
5
40
222
5
5
56
5
5
28
111.0
5
5
dissolve 342.3g in soln
40
83
5
56
5
40
222
5
40
5
dissolve 342.3g in solution
28
5

Table 2.2: Volumes (mL) of the stock solutions used in the present study.

941
941
941
941
941
941
940

0.5M HgCl2 = 0.02mL
0.5M HgCl2 = 0.05mL
0.5M HgCl2 = 0.1mL
0.5M HgCl2 = 0.2mL
0.5M HgCl2 = 0.4mL
0.5M HgCl2 = 0.6mL
0.5M HgCl2 = 0.8mL
0.5M HgCl2 = 1mL

888
838
788
738
829
929
879
829
779
813
781
818
861

940

819
771
740
768
805
861
787
904
736
931
806
946
823
623
423
223
23
961
723
523
941
895
845
795
745
695
645
938

819
BaCl2.2H2O= 2.443g in 100mL

HgCl2= 15.1g in 500mL
CuCl2.2H2O= 0.8525g in 50mL

HgCl2= 1.3575g in 50mL

dH2O
861
857
833
472
713
861
860
774
833
861
796
861
713
885
444

HgCl / CuCl2.2H2O /
BaCl2.2H20


2.2.3 Solutions

A number of different bathing solutions were used in the present study. Each bathing solution was prepared by mixing aliquots of various stock solutions. The ionic concentration of each bathing solution is shown in Table 2.1, while the volumes of the stock solutions used to obtain these concentrations are outlined in Table 2.2. While there was often some error in the higher (250mOsm/kgH₂O) solutions due to the hygroscopic nature of the solutes used (generally of about 20mOsm/L), the lower 100mOsm/kgH₂O solution could be reproduced consistently to within a few mOsm/L. Unless otherwise stated, bathing solutions were maintained at room temperature throughout the experiment and were not oxygenated. In addition, all solutions were buffered to a pH of 7.5 using either Na⁺ Hepes or K⁺ Hepes. For ease of reference throughout this dissertation, each solution has been named and numbered.

As stated above, solutions were not bubbled with oxygen. From an experimental perspective, internal controls showed that Isc was stable over periods of 10 hours in 100mOsm/kgH₂O NaCl solutions (see Figure 4.9). These findings strongly suggested that the cells could maintain their metabolism, in the absence of oxygenation of the bathing solution, to sustain active pumping at a constant rate for at least a 10-hour period. Given these internal controls, it is argued here that cell metabolism is relatively constant, at least for the first 10 hours of experimentation. Of course, cell metabolism may have been sub-maximal, but this was not a parameter that could be monitored experimentally, nor would oxygenation of the bathing solution have necessarily guaranteed this condition anyway.

The delivery of solutions to the bathing chambers was via a 60mL syringe. The syringe was filled and the contents injected into the appropriate chambers at the desired time. Each time a new solution was used in an experiment the chamber was emptied and the new solution was flushed through 2-3 times to wash out the previous solution and to remove residual solution from the skin. (This washing procedure was also used when skins were initially mounted in the chambers at the beginning of each experiment).²

² Prior to every experiment, each Ussing chamber was washed and scrubbed in distilled water and then dried by hand.
2.3 Experimental Equipment

2.3.1 Ussing Chambers and Calomel Electrodes.

The Ussing chambers used in the present study each consisted of 2 half-cells machined from perspex blocks (Figure 2.5A). The inner surface of one half-cell had a raised circular area (A= 1.41cm²) upon which the toad skin was laid. (All currents in this study were measured as absolute currents across this skin area and were not normalised to an area of 1cm²). A rubber O-ring was then placed over the raised area, clamping the tissue in place. The second half-cell had a circular depression machined into the inner surface such that the first half-cell formed a press fit with the second when the toad skin and O-ring were in place. When joined, the half-cells could be mounted on a retort stand.

The inner surface of each half-cell was directly connected to a conical bath, machined into the body of the perspex blocks. When a toad skin was mounted in the Ussing chamber, the apical side of the tissue was exposed to one bath and the basolateral side to the other. Each bath had the capacity to hold approximately 9mL of solution. Bathing solutions were introduced into the conical baths through a ‘solution injection hole’ located at the top of each half-cell. Fluid was subsequently released through silastic tubing attached to drainage outlets positioned below each conical bath. Mohr clips clamped the silastic tubing in between drainage procedures, preventing the loss of bathing fluid.

Voltage measurements across the toad skin were measured using 2 calomel electrodes, one of which was inserted into each bath through ‘electrode holes’ which were positioned as close to the skins as possible to reduce ohmic losses in the bathing fluid. Each electrode was supported by a silastic sleeve which slipped over the electrode and rested on top of the electrode hole (Figure 2.5A). The sleeves were necessary to ensure that the electrodes were positioned at the same place relative to the skin during the experiment, as there sometimes appeared to be non-uniform regions of current density in the skin. Had the electrodes not been constrained, whenever the electrodes were moved, an artefact would have been recorded. The electrodes themselves were generally stable, often only drifting about 5mV over the course of 15 hours.

The last noteworthy feature of the Ussing chamber was the current injecting electrodes protruding from small side baths attached to the outer surface of each half-cell. The injecting electrodes were made from sterling silver and, as their name suggests, they were used for injecting current into the system when it was desirable to
clamp the transepithelial current to a particular value. The side baths were generally filled with either 50mM KCl or normal NaCl Ringers solution and a porous wooden wick, made from the wooden ends of cotton wool buds, acted as a salt bridge connecting the small side chambers with the conical baths, allowing current to flow.

2.4 Electronics

Designing, costing, manufacturing, prototyping and testing the electronic circuits was an ongoing part of the present study. As requirements and constraints changed, different circuits evolved and the software was rewritten. Although a number of prototypes were tried, the majority of the experiments were performed using either a 'reed relay circuit' or an 'analogue multiplexer circuit'. Therefore, this section focuses on these 2 configurations and is divided into 3 main sub-sections looking at (1) the general tasks that had to be performed by the electronics, (2) the requirements and constraints that had to be satisfied during the development process and (3) the final design and operation of the circuits.

2.4.1 Standard electrophysiological techniques & electronic measurements

Current clamping and voltage clamping are two techniques that are routinely used in the study of ionic transport across any pumping epithelium. Current clamping is where the current passing through the epithelium is fixed at a particular value and the transepithelial voltage that is generated by the current is recorded. Conversely, voltage clamping is where the voltage across the epithelium is fixed and the resulting current is measured (Figure 2.6).

Two measurements that are commonly recorded in epithelial physiology are the open circuit voltage (Voc) and the short-circuit current (Isc). These measurements are simply special cases of current clamping and voltage clamping, respectively. Voc is defined as the voltage across the epithelium that occurs when the current is clamped to 0µA whereas Isc is the current across the epithelium when the transepithelial voltage is clamped to 0mV (Figure 2.6)³.

In automating the measurement procedure, it was necessary to develop a feedback circuit that could both voltage clamp and current clamp. That way, not only could Voc

³ In this dissertation, Voc and Isc are arbitrarily defined to be independent of the transepithelial concentration gradient. For example, the term Voc is used when Iclamp=0µA and either ΔCpond/ΔC_belly = 1 or ΔCpond/ΔC_belly ≠ 1.
and Isc be measured over time, but other clamp cases could also be investigated.

In addition to these tasks, it was desirable that the circuit had to provide verification of the clamp conditions. This meant, for example, that whenever the circuit was voltage clamping it was not sufficient to collect only the associated current information, it was also necessary to record the actual voltage across the skin to demonstrate that the circuit was indeed clamping.

2.4.2 Requirements and Constraints

A number of requirements and constraints provided a framework for the possible design solution. This sub-section presents some of the major considerations that had to be taken into account, focusing in particular on (1) experimental requirements, (2) computer constraints, (3) electronic requirements and (4) geometric limitations.
(1) Experimental Requirements

There were 4 main experimental requirements that had to be satisfied. First, as described in section 2.4.1, the fundamental experimental requirement was the capacity to automatically clamp the transepithelial voltage and current, and to measure Voc and Isc over an extended time period. As a precaution, it was also necessary to have a verification procedure in place to ensure that the clamp value that was specified was the same as the clamp that was actually applied across the skin.

Second, there was often a DC offset between the calomel electrodes. To ensure that this offset did not contribute to the potential developed across the skin due to the transepithelial movement of ions, it was necessary to have an offset control that enabled the calomel electrodes to be 'zeroed.' The 'zeroing' procedure involved placing the calomel electrodes in the same bathing solution under open circuit conditions. Theoretically, in this situation, no potential difference should have existed between the electrodes. Whatever potential difference was apparent was not physiological and could be ascribed to the electrodes. This DC offset was nulled using the offset control so that there was no potential difference between the electrodes.4

The third requirement was that the investigator had ultimate control of the experiment and could override the sequence of tasks that was being performed repetitively by the electronics. This enabled the investigator to rezero the electrodes, for example, or to acquire a current-voltage (I-V) plot, or to impose a particular clamp condition for an indefinite period of time, whenever it was deemed appropriate. To accomplish this requirement, it was simply a matter of writing the software to check routinely for external inputs from the investigator. If no input were registered, the software would continue to run through a specified set of tasks. If an input were registered, the software would interrupt the sequence of events it was performing and execute the new tasks specified by the investigator.

Finally, for the purposes of efficiency, it was desirable to maximise the number of experiments that were running concurrently. It should be noted, however, that at the

4 Technically, the output (Pin 6) of the high-speed FET-input instrumentation amplifier (INA 111 chip) is referred to the output reference terminal (Pin5), which is normally grounded. However, if the output reference terminal is adjustable (or 'floating') then the amplifier output can be set to zero, as desired (see Fig. 2.7 & 2.8). As described in the text, one condition where the amplifier output should be zero is when the calomel electrodes are in the same bathing solution under open circuit conditions. If it isn't, then there is a DC offset between the electrodes which can be eliminated by changing the voltage on the output reference terminal (or 'offset control').
beginning of the study, this was not a high priority. Experiments were short (lasting only 1-2 hours) and multiple experiments could be performed in a day. Under these circumstances, two setups were sufficient. However, as the experimental technique was refined and different solutions were used, useful Voc and Isc measurements could be recorded for up to 20 hours. Although the process was automated, certain events such as solution changes still required the presence of the investigator throughout much of the experiment. Adding in the preparation time (such as planning the experiment, making solutions and dissecting the tissue) left little time in any 24-hour period. It rapidly became apparent that performing these time-consuming experiments using only 2 setups was not efficient. Therefore, to rectify the problem, 2 avenues were investigated. First, attempts were made to speed up the skin responses by heating the bathing solutions. This complicated matters somewhat, as various physiological parameters were modified by this manipulation. At that stage of the study, the aim was to simplify responses as much as possible to gain a better understanding of the interactions across the epithelium. Consequently, it was decided not to pursue the effects of heating. A second option that was investigated was improving the overall efficiency by increasing the number of experimental setups. Instead of acquiring data from only 2 setups during an experiment, data from 8 setups was attained. However, because this 4-fold increase in the number of setups had not been anticipated in the initial circuit design using reed relays, it meant that the electronics had to be redesigned because of a limited number of terminals on the data acquisition card. This, in addition to the requirement that each setup operated independently having the capacity to execute tasks as specified by the investigator, necessitated the rewriting of the software.

(2) Hardware (computer) Constraints

The design criteria for the 2 circuit configurations used in this study were somewhat different. At the beginning of the study, the emphasis was on developing one functional automated system. There was little regard for optimising the circuit design to maximise the number of setups that could be operated by the existing hardware. This was primarily because the study was at its formative stages where attempts were still being made to show that automation was indeed a feasible idea. As it turned out, the reed relay circuit used only half the available number of computer input/output lines so it was

5 The number of setups in operation at any one time was determined by 2 factors: (1) the circuit design and (2) the number of analogue input/output terminals on the data acquisition card.
possible to run 2 independent setups concomitantly. Later, when the system worked automatically and experiments were running over a long time period, the design criteria changed and maximising efficiency, by increasing the number of setups, became a priority. Thus, the number of available computer input/output lines became an important constraint factor: the fewer lines utilised by each setup, the more setups that could operate.

Initially, the electronics were designed around the Lab PC+ data acquisition card because it was available in the laboratory at the time. The card is versatile with 2 digital-to-analogue conversion (DAC) channels, 8 analogue input channels, and 3 ports of 8 (i.e. 24) digital input/output (I/O) channels. This card was sufficient to operate an electronic circuit where (1) an analogue input voltage (Vcontrol) was required to enable the circuit to clamp to a particular value and (2) the transepithelial current and voltage data from the skin was acquired and sent back to the computer. However, as more setups were required it was necessary to rethink the reed relay circuit and how channels on the PC+ card were utilised. Many of the inefficiencies of the relay design were overcome with the analogue multiplexer circuit. Some of the major changes involved moving away from bipolar inputs to single ended inputs and interleaving current and voltage measurements so they could be acquired by the computer as a single stream of information rather than collecting this data simultaneously on separate computer lines. It was also necessary to supplement the PC+ card with a PCI-6704 card to supply independent analogue control voltages to the 8 setups.

(3) Electronic Constraints and Requirements

There were 3 main requirements that had to be satisfied in terms of the electronics. First, the feedback circuit had to be stable for all experimental conditions that were likely to be encountered (i.e. the circuits were designed to be stable for skin resistances ranging from approximately 1kΩ to 10kΩ). Second, the electronics could not be powered by the Labview PC+ card. Although the card could supply sufficient voltage, the external voltage lines were contaminated by high frequency computer noise. Therefore, all circuits were supplied by an independent, mains-operated, ±9V power supply. The third requirement was that sufficient time was left between the initiation of a clamp condition and subsequent data collection. This was necessary to allow transient responses in the electronic components to settle before the data was sampled. Empirically, such responses were complete within approximately 10-20ms of a command voltage. Data was therefore collected after the elapse of this time.
The main design constraint in this study was cost. Significant emphasis was placed on achieving a solution that was inexpensive to produce. All components were therefore priced, and where possible, the cheapest components were used.

(4) Geometric Considerations

Each circuit was positioned as close as possible to the Ussing chamber in order to reduce high-frequency capacitive pickup along the wires leading from the calomel electrodes to the instrumentation amplifier. However, having a circuit in close proximity to the experimental apparatus meant that it was prone to being splashed by the salty solutions used to bathe the tissue. Therefore a compromise had to be reached where the circuits were close to the apparatus so that the wires were short, but were positioned in such a way that they were unlikely to be splashed.

In terms of the reed relay circuit configuration, the two circuits were housed in a single box (30x16x6cm) that sat on a platform, above the level of the silastic tubing leading from the drainage outlets in the Ussing chambers (Figure 2.4). That way, the circuits were close to the Ussing chambers, but when the fluid was drained from the baths, it was unlikely that any would splash up and enter the box containing the electronics. With the analogue multiplexer circuits, each was accommodated in an individual plastic box (7.5x5.5x2.5cm) which was clamped above, and off to the side of, the associated Ussing chamber (Figure 2.5A,C). The input and output wires and offset control were all positioned below the box to prevent the accumulation of fluid near possible sites of entry. Again, each circuit was close to the experimental apparatus, but because the boxes were above the drainage outlets, inadvertent splashing of the boxes was minimised. Nonetheless, care was taken when injecting and draining fluid from the Ussing chambers.

2.4.3 Circuit designs

The schematics for 2 possible design solutions that satisfy the constraints and requirements discussed in section 2.4.2 are presented in Figure 2.7 (reed relay configuration) and Figure 2.8 (analogue multiplexer configuration). This section gives a brief description of how each circuit works.

(1) Reed Relay Configuration

A reed relay is basically an electromagnetic switch. By applying a voltage to the coils inside the relay it induces a switch to be thrown from its default contact to a second
One such type of relay, where there is only one switch, is known as a ‘single pole change over’ (SPCO) relay. When there are 2 independent switches that can be thrown simultaneously by application of the same voltage, the relay is called a ‘double pole change over’ (DPCO) relay. Three of these DPCO relays were used in the present study. The first relay was used to change between a clamp condition (default), and the open circuit condition. The second relay determined whether the skin was subjected to a current clamp (default) or a voltage clamp, while the third relay passed the appropriate clamp voltage, arising from the current clamp (default) or voltage clamp, back into the LF356 chip to be compared with the control voltage.

Under voltage clamp conditions (Figure 2.7A), a control voltage was generated by the computer and passed out via a DAC line to the circuit whereupon it entered the feedback circuit at the LF356 chip. The output of this chip was then passed through reed relay 1 operating in its closed circuit mode, then into reed Relay 2 where the switches had been thrown and it was functioning in its Vclamp mode. Here, both the switches were incorporated into the circuit. The first switch carried the output from reed relay 1, which subsequently passed out of reed relay 2 via Pin 7, crossing a 5.1kΩ resistor before being attached to silver electrode #1 on one side of the experimental apparatus. The other switch was grounded and its output (Pin 6) was attached to silver electrode #2 on the other side of the Ussing chamber such that a potential difference was generated across the toad skin. This was monitored by the calomel electrodes which acted as inputs to the INA 111 amplifier. The output, which had been amplified by 20 times, was then passed (a) out to the computer which displayed whether the circuit was clamping properly and (b) into reed relay 3, operating in its voltage clamp mode. The output from reed relay 3 was passed back into the LF356 chip. While this voltage clamping procedure was occurring, the current was also being monitored by recording the voltage drop across the 5.1kΩ resistor. The software was then used to apply Ohm’s law thereby obtaining a value for the current flowing through the skin.

Much the same process as described above, occurred under current clamp conditions (Figure 2.7B). A control voltage entered the feedback circuit at the LF356 chip and passed through reed relay 1, again operating in its clamp mode. The output of this relay

---

6 Reed relay 2 and 3 were synchronous, being operated by the same +5V signal from the Labview card. To ensure that sufficient current was passed through each coil in the 2 Reed relays to facilitate switching, it was necessary to use a transistor, as shown in the circuit diagram.
Figure 2.7: Schematic diagram of the reed relay circuit operating in (A) voltage clamp mode and (B) current clamp mode.
Figure 2.8: Schematic diagram of the analogue multiplexer circuit.
was passed into reed relay 2 (functioning in its current clamp mode) and it exited at Pin 8 before being connected to silver electrode #2 on the Ussing chamber. The output from the second switch in reed relay 2 (pin 5) was grounded and was connected to one end of the 5.1kΩ resistor. The other end of the resistor entered a node point where connections were made with silver electrode #1 and also pin 8 of reed relay 3, which subsequently fed back into the LF356 chip. As before, the voltage drop across the resistor was acquired by the computer and converted into a current, this time giving an indication of how well the circuit was current clamping. In addition, the calomel electrodes monitored the voltage across the skin, which was amplified 20 times and then logged by the computer.

Finally, it is worth mentioning that the special case of open circuit voltage was not generated by clamping the current to 0µA. Instead, it was obtained by opening the switch at reed relay 1 so that there was no possibility of current flowing in the circuit.

(2) Analogue multiplexer configuration

The design criteria for the analogue multiplexer circuit were slightly different from the reed relay circuit. As described in section 4.2.2 the main differences were that the outputs should be single-ended to minimise the number of computer lines required, and that the current and voltage data were to be collected sequentially and not simultaneously. While the basic clamping ideas remain the same between the 2 configurations, the introduction of these criteria meant some modifications to the circuit design, the main one of which, was the introduction of a single (MC14053B) analogue multiplexer chip, which replaced the 3 reed relays. This chip contained 3 switches that were all independently controlled by digital I/O lines enabling the current or voltage to be measured under either current or voltage clamp conditions. The first switch was used to decide between open circuit (default) and clamp conditions, the second switch swapped between voltage clamp (default) and current clamp conditions, while the third switch moved between measuring the voltage (default) and measuring the current (Figure 2.8).

When in voltage or current clamp mode a control voltage was introduced into the feedback system at the positive terminal of one of the four LM348 opamps. (This LM348 opamp replaced the LF356 chip in the reed relay configuration). The output passed across switch A (in the clamp position) before being connected to silver electrode #1 on one side of the Ussing chamber. Silver electrode #2 was part of a node which was connected to (a) the positive terminal of a second LM348 opamp (used for
current measurements), and (b) a 1kΩ resistor (that was grounded at the other end). Calomel electrodes were used to measure the voltage across the toad skin which, in this case, was ‘a floating voltage,’ as neither silver electrode was grounded (as in the reed relay configuration). The calomel electrode measurements became the inputs to the INA 111 instrumentation amplifier.

Under voltage clamp conditions, the INA 111 amplifier output was returned to the negative terminal of the first LM348 opamp via switch B, which was in the voltage clamp position. In addition, this amplifier output was sampled periodically by the computer when switch C was in the voltage measure position, to demonstrate that the circuit was indeed voltage clamping. However, because the transepithelial current was the main point of interest when the skin was voltage clamped, switch C existed mainly in the current measure position.

In current clamp mode the output from the second LM348 opamp mentioned above, was fed back into the first LM348 opamp via switch B, this time working in the current clamp position. The output was also sampled by the computer when switch C was in the current measure position, providing an internal check to show that the circuit was current clamping correctly. However, the transepithelial voltage was the main point of interest under current clamp conditions so switch C was mostly in the voltage measure position.

Finally, as with the reed relay configuration, recordings made under open circuit condition were not made by clamping the current across the skin to 0µA. Rather, switch A was held in its default position, thereby breaking the circuit so no current flowed.

2.4.4 Specifications and Limitations of the Apparatus

The circuits could operate over a voltage clamp range of ±250mV and current clamp range of ±500µA for skin resistances between 1kΩ and 10kΩ and wick resistances of 50kΩ or less. If the resistances exceeded these ranges then sometimes it was not possible to get the circuits to clamp to the appropriate value or, in extreme cases the electronics became unstable and oscillated. However, under most experimental conditions these limits were not violated so after the initial design phase these problems were not significant.

---

7 One simple solution that was sometimes used to improve the voltage clamping capacity of the circuit involved placing tissue wicks between the side chamber and the main chamber, effectively reducing the resistance of the wooden wicks and enabling more current to flow through the system.
Some care was taken to ensure that the dominant source of noise in the raw trace was physiological and not due to the electronics. Noise from the electronic components contributed less than 0.5mV peak to peak to the overall noise which was often several millivolts. In addition, the data acquisition process meant that 0.12mV quantisation noise was introduced via the computer. This was deemed to be insignificant compared with the biological noise from the toad skin.

Finally, during the design phase some stability problems were encountered due to the presence of earth loops and capacitive and radiative noise. Appropriate measures (such as removal of the earth loops, shielding the circuits from external noise sources using a Faraday cage and ensuring that wires entering and leaving the circuits were as short as possible) were used to eliminate these stability problems.

2.5 Software

Labview was the software used in the present study to control the electronics and acquire data from each of the skins. Labview is a versatile, general-purpose iconographic programming system. It is based on the concept of a virtual instrument (VI) which imitates an actual instrument both in appearance and operation. A VI consists of 2 panels: (1) a user interface panel, also known as the front panel, where the user interacts with the instrument, entering variables and viewing the data acquired by the computer (Figure 2.9) and (2) a block diagram where the programmer develops the code to perform specific functions executed in a particular order (Figure 2.10). Unlike text based code though, Labview utilises a graphical language such that the programmer wires together objects enabling data to be sent from one object to the next.

Some of the attractive features of Labview are that the data can be: (1) modified by functions such as division and subtraction, (2) controlled by standard programming structures such as WHILE or FOR loops and (3) sent out of the computer or acquired from an external source via a Labview compatible data acquisition card (i.e. the Labview PC+ card). Labview also promotes the idea of modular programming such that tasks are broken down into a series of simpler tasks (or subVIs), which are analogous to subroutines or procedures in text based programming.

This section presents some of the main features of the software that was written in the present study using Labview. Individual programmes for the reed relay and analogue multiplexer configurations will not be discussed because, although the programmes were quite different, the main features available to the user were similar.
Figure 2.9: Front panel of the virtual instrument Toad1.VI used to control the 8 analogue multiplexer setups.
Figure 2.10: Block diagram of the virtual instrument Toad1.VI used to control the 8 analogue multiplexer setups
2.5.1 Software Features

The software was designed around a sequence of events (for each setup) that was repeated indefinitely until the user specified otherwise. Some of the available sequences included, measuring Voc followed by Isc, current clamping the skin and then measuring Voc, or voltage clamping over a series of different values and measuring the current (Figure 2.11A).

The way in which the sequences were specified is illustrated in Figure 2.11B which shows a series of ‘sequence combination boxes’ with ID numbers entered in a particular order. These ID numbers represented a particular clamp condition as described by the list located to the left of the diagram. (If the user wanted to specify their own sequence of events and it was not presented on the front panel) it was simply a matter of entering the ID numbers that represented particular events, such as Isc, into a particular sequence combination box). The main advantage of designing the software in this way was that each setup could be subjected to a different sequence of events at any one time. This meant, for example, that one skin could be voltage clamped at a specified value for a particular period while routine Voc/Isc measurements were performed on the other skins.

Another feature of the software was that the duration time for each of the clamp events in the sequence could be specified. For example, the third event could be maintained for twice the length of time of the first event. However, although this feature was available, the default case was usually used where the duration of each event was the same (and set to 1 second). Under these conditions, and using the information in Figure 2.11B, it can be seen that in a 20 second epoch for a Voc/Isc sequence, the open circuit voltage was measured for 9 out of every 10 seconds. During the tenth second Isc was recorded. The cycle was then repeated. In a similar manner the time spent recording the different clamp conditions for the other sequences can be determined. However, it should be noted that the most frequently used sequences were the ‘Voc/Isc test sequence’; the ‘Isc/Voc test sequence’ and the ‘Voc/Inc Vclamp/Imeasure under Inc Vclamp/Isc test sequence’.

Interestingly, the long-term

---

8 It should be noted that the timing was the same for all circuits. So if the third event was twice the time of the first event, all setups, irrespective of the sequence being used, would be subjected to this condition.

9 The ‘Voc/Inc Vclamp/Imeasure under Inc Vclamp/Isc test sequence’ was a sequence developed to measure the skin current when the tissue was voltage clamped 5 or 10mV away from the open circuit voltage. This information was used during experiments as a rough indicator of the instantaneous skin resistance.
Figure 2.11: (A) Schematic depiction of events in a cycle showing measurements of (i) mainly Voc with a brief recording of Isc; (ii) mainly Isc with a brief recording of Voc. (iii) The duration of an event in the sequence could be altered. (B) The sequence of events to which a skin was subjected could be described by a series of ID numbers, each representing a particular event.
behaviour of the skins did not appear to be greatly affected by the different protocols as shown in Chapter 4.

Finally, one of the most useful properties of the software was that the current and voltage data was displayed on the front panel in real time, as it was collected and written to disk (Figure 2.9). The capacity to display the recorded data rapidly meant that the user had an immediate overview of the experiment and could plan further manipulations based on this information.
3. Voltage dependence & oscillations in the Cl⁻ conductance of the toad skin
3. Voltage dependence & oscillations in the Cl\textsuperscript{−} conductance of the toad skin

3.1 Introduction

In the late 1970's, voltage-clamp experiments conducted on the isolated abdominal skin of anurans showed that the current-voltage (I-V) relationship measured across skins was influenced by the duration of the clamp. When a fast (50ms) staircase-shaped voltage pulse was applied across the skin, the I-V relationship was essentially linear but when each voltage-clamp condition was presented for 3-5 minutes, the steady-state I-V curve became non-linear (Larsen & Kristensen, 1978). This phenomenon was demonstrated in skins bilaterally exposed to NaCl Ringer solution, and was not affected by the addition of 60µM amiloride to the pond side (referred to elsewhere as the apical or mucosal side), 1mM ouabain to the belly side (also known as the basolateral or serosal side), or the substitution of Na\textsuperscript{+} by K\textsuperscript{+} in the apical bathing solution. However, the voltage-induced current activation could not be demonstrated in skins where Cl\textsuperscript{−} had been removed from the bathing solutions and replaced with gluconate. These findings suggest that it was the Cl\textsuperscript{−} component of the current that was voltage-activated, and not the Na\textsuperscript{+} or K\textsuperscript{+} currents.

Subsequent experiments showed that the main transepithelial Cl\textsuperscript{−} conductive pathway was localised to the mitochondria-rich cells (MRCs), and not the principal cells (PCs) of the anuran skin. Experimental data supporting this view include: (1) the apical membrane of PCs is impermeable to Cl\textsuperscript{−}, as demonstrated by tracer studies which showed little exchange between radiolabelled Cl\textsuperscript{−} in the apical bathing solution and Cl\textsuperscript{−} in the PCs (Ferreira & Ferreira, 1981); (2) the Cl\textsuperscript{−}-dependent current through the skin increases linearly in proportion to the number of MRCs (Voûte & Meier, 1978; Willumsen & Larsen, 1985); (3) in voltage-clamped skins where the Cl\textsuperscript{−}-dependent conductance was activated, regions of peak conductance were shown to exist above MRCs and not PCs when a vibrating probe was moved across the apical surface of the skin (Foskett & Ussing, 1986); and (4) the MRCs but not the PCs undergo a reversible volume increase upon activation of the voltage-dependent current, consistent with the movement of Cl\textsuperscript{−} (and K\textsuperscript{+}) into the cells. This response could only be initiated when Cl\textsuperscript{−} was present in the apical bathing solution and no change in cell volume occurred under similar voltage-clamp conditions, either in the presence or absence of Cl\textsuperscript{−} in the basolateral solution. These findings strongly suggest that it is the apical and not the basolateral membrane of the MRCs that is voltage-sensitive (Foskett & Ussing, 1986). Nevertheless, the basolateral membranes of MRCs are Cl\textsuperscript{−} permeable. The evidence for
this includes: (1) Cl⁻ channels have been located in the basolateral membrane of the MRCs using single cell patch-clamp techniques (Willumsen & Larsen, 1995); (2) using electron microprobe analysis the intracellular ionic concentrations were determined following ionic substitutions of the bathing solutions. It was found that Cl⁻ could be exchanged with Br⁻ entering from either the apical or basolateral bathing solutions (Nagel & Dorge, 1990) and (3) given that the Na⁺/K⁺-ATPase blocker, ouabain, causes MRCs to swell, presumably due to the entry of Na⁺ and Cl⁻ into the cells (where Na⁺ gain is in excess of K⁺ loss), then if a cell volume increase was observed after replacement of Cl⁻ with gluconate in one of the bathing solutions, then Cl⁻ must have been entering the cell from the opposite bathing solution. This protocol was performed in both the apical and basolateral solutions, and in each case cell swelling was observed. Consequently, it was concluded that the MRCs possessed a Cl⁻ permeability in both membranes (Larsen et al., 1987).

In addition to the Cl⁻ channels, the other main transport mechanisms present in the MRCs include apical Na⁺ channels (Dorge et al., 1990; Harvey, 1992; Harvey & Larsen, 1993; Larsen et al., 1987; Rick 1992), basolateral Na⁺/K⁺-ATPase pumps (Mills et al., 1977; Larsen et al., 1987; Larsen 1991) and basolateral K⁺ channels (Urbach et al., 1994) (Figure 3.1). When ions move through these transporters, they contribute to the total conductance of the membrane in which they are located. If these conductances were invariant, then the voltage drop across each membrane could be determined easily assuming a simple voltage divider. However, the presence of a variable apical conductance (due to the voltage-dependent Cl⁻ conductance (Figure 3.1A arrow) complicates the situation. The voltage divider approach is still applicable, but the membrane potentials change as channels open, potentially providing local voltage-dependent feedback (positive or negative) in the skin.

The purpose of this chapter was to examine some of the features of the voltage-dependent Cl⁻ conductance, and how the relative apical and basolateral conductances influence the behaviour of the skin under voltage-clamp and open-circuit conditions (Figure 3.1B). More specifically, this chapter focuses on: (1) replicating the findings of Larsen and Kristensen (1978) who showed the activation of a Cl⁻-dependent current, in the absence of any Na⁺ effect, when a positive voltage-clamp (with respect to the apical bathing solution) is applied across the skin; (2) investigating the activation and inactivation time-courses of the Cl⁻ conductance when the skin voltage is changed; (3) presenting examples of the Cl⁻-dependent oscillatory behaviour observed in skins under both voltage-clamp and open-circuit conditions; (4) presenting the findings of a
A mathematical model which simulates the slow Cl\(^-\) activation and inactivation, and the oscillatory behaviour under voltage-clamp conditions and (5) discussing how the conductance ratio of the apical and basolateral membranes may affect the stability of the skin.

3.2 METHODS

3.2.1 Tissue Preparation

Toads were double pithed (at time t=0 minutes) and the right forepaw was removed for animal counting purposes. (Cane toads are vermin in Australia and containment of their spread requires careful monitoring). The abdominal skin was removed by making a small pelvic incision, just ventral to the anus, and cutting towards either hind leg. The

---

1 Pithing and removal of the forepaw was conducted in accordance with the vivisection protocol outlined by the Animal Experimentation Ethics Committee at the University of Western Australia. Animals were killed by pithing and not anaesthesia because the standard amphibian anaesthetic, MS 222 (also known as tricaine methane sulphonate (TMS), or 3-aminobenzoic acid ethyl ester) is absorbed dermally, affecting the epithelial layer of interest.
skin flap was then lifted, either by hand or with a pair of forceps and, using blunt
dissection techniques, the entire ventral skin was separated from the underlying
connective tissue and fascia. When experiments were performed on several toads, skins
were removed sequentially from each toad and laid across the associated animal so that
the basolateral (belly) side of the skin remained in contact with the moist, underlying
organs. No solution was placed on the outward facing (pond) surface. Once all skins
had been removed, each one was taken in turn, and divided into 2 or 3 smaller pieces.
Where 2 pieces of skin were required, the tissue between the fore- and hind-legs was
divided down the centre-line of the toad. Where 3 pieces were required, an extra section
was removed from beneath the animal’s throat (see Figure 2.3). In this study, each toad
was designated by a number, and each skin sample was described by the letters a,b or c
following the toad number. For example, the alphanumeric text ‘101a’ seen at the
bottom left hand corner of Figure 3.2A indicates that the experiment was conducted on
sample ‘a’ taken from Toad 101. All experimental data presented throughout this thesis
uses this naming convention.

In this series, all experiments were conducted at room temperature. The isolated skins
were mounted in Ussing chambers and were bilaterally exposed to either
250mOsm/kgH₂O NaCl Ringer (Soln. 1) or 250mOsm/kgH₂O sulphate Ringer (Soln. 2),
with the following composition in mmol/L: NaCl Ringer: Na⁺=111, K⁺=5, Ca²⁺=2.7, Cl⁻
=116.4, Hepes⁻=10, Glucose=5; Sulphate Ringer: Na⁺=111, K⁺=5, Ca²⁺=2.7, Mg²⁺=28,
Hepes⁻=10, Gluconate⁻=5.4, SO₄²⁻=83.5, Glucose=5).

3.2.2 Monitoring Equipment

An automated control system consisting of electronic analogue feedback circuits and
associated software was custom-designed and built to monitor skin responses under
voltage-clamp or current-clamp conditions. In many cases this involved monitoring
either the open-circuit voltage (V_{oc}) with calomel electrodes (where the current flow
across the skin was 0µA) or the short-circuit current (I_{sc}) with current electrodes (where
the voltage across the skin was 0mV). However, it was also possible to clamp the skin
to specified voltages or currents and record the resulting transepithelial currents or
voltages with a 2 second time resolution. In this study, V_{oc} and I_{sc} were monitored in a
series of skins, and the ratio of these was used to calculate the nominal skin resistance
(R=V_{oc}/I_{sc}). In a second series of experiments, voltages across the skins were clamped
in a step-sequence, and the currents during these clamps monitored. Finally, the effect
on $V_{oc}$ and $I_{sc}$ when skins were released from a voltage-clamp condition was investigated.

All skin potentials were measured with respect to the apical bathing solution, which was defined as ground (0mV). Thus, a voltage-clamp of $+100\text{mV}$ in this study corresponded to a voltage-clamp of $-100\text{mV}$ as defined by Larsen and Kristensen (1978). Finally, unless otherwise stated, positive current flowing into the skin was defined as positive.

3.2.3 $V_{oc}$ & $I_{sc}$ Experiments

Skins were bathed on both sides in 250mOsm/kgH$_2$O NaCl Ringer solution and $V_{oc}$ and $I_{sc}$ recordings were made for up to 20 hours. Calomel electrode drift was generally of the order of 3-5mV during this period. Recordings in actively pumping skins were discarded when the current dropped below 0.25$\mu$A.

3.2.4 Voltage step-sequence experiments

Voltage step experiments were conducted after 50µM of amiloride (an epithelial Na$^+$-channel blocker), had been added to the apical bathing solution, 110-225 minutes after pithing. This was done following the protocol of Larsen and Kristensen (1978), ensuring removal of active Na$^+$ pumping (by blocking apical Na$^+$ entry), and a predominantly Cl$^-$-permeable skin. The short-circuit current ($I_{sc}$) was abolished within approximately 30 seconds of amiloride application, and voltage-clamp experiments were commenced within 12-32 minutes (Figure 3.2).

At the beginning of the voltage-clamp protocol, skins were clamped at a holding potential of $-40\text{mV}$ until a steady-state current was obtained. The skins were then voltage-clamped for a period of either 5 or 10 minutes, at 20mV increments, up to a maximum of 100mV. In between each voltage-clamp, the skin was returned to the $-40\text{mV}$ holding potential for 5 minutes to mimic Larsen’s experiments. This was sufficient time to allow complete closure of the Cl$^-$ conductance. The rise time of the transepithelial voltage steps was between 10-20ms.

3.2.5 Single Voltage-clamps followed by $V_{oc}$ and $I_{sc}$ Recordings

In two actively pumping skins the transepithelial voltage was clamped to $-100\text{mV}$ for two minutes to close the apical Cl$^-$ channels. The clamp was then released and $V_{oc}$ and $I_{sc}$ were measured as the channels reopened. This protocol was performed in both 250mOsm/kgH$_2$O NaCl and Cl$^-$-free Ringer solutions. The presence of oscillatory
Figure 3.2: Application of 50µM amiloride to the apical/pond (p) solution in (A-B) 250mOsm/kgH₂O NaCl Ringer and (C-D) 250mOsm/kgH₂O Na₂SO₄ Ringer results in a rapid decrease in Isc (grey lines) and Voc (black lines) within 2.8 minutes.
behaviour in NaCl but not Cl\textsuperscript{-}-free solutions suggest that the responses were physiological and were not due to the monitoring equipment.

### 3.3 RESULTS

#### 3.3.1 Variability within and between skins

While not reported previously, one of the significant problems in investigating a toad skin is that $V_{oc}$ and $I_{sc}$ can change over the 10-20 hour experimental period and not settle to a steady-state. In addition, these changes are not the same in different skins, despite all skins being bathed in 250mOsm/kgH\textsubscript{2}O NaCl Ringer (Figure 3.3) This is manifested clearly in the nominal resistance (shown below each $V_{oc}/I_{sc}$ trace), which shows the resistance rising monotonically in some skins (C & D), but falling in others (B), or displaying multiphasic changes (E & F). Plotting $V_{oc}$ against $I_{sc}$, in what we have termed the V-I locus (insert), highlights the differences between skins. The data are superimposed on iso-resistance lines (for 0.2, 0.4, 0.6, 0.8, 1, 1.25, 1.67, 2.5, and 5kΩ in ascending order), and show a variety of responses, with some skins following the iso-resistance lines closely (A & B), while others show a migration across iso-resistance lines (D & E).

The variability in the responses of the skin to the same extracellular solution emphasises that the skins are different. This is not to say that the fundamental mechanisms governing the movement of ions are different, simply that the skins start with different properties (such as different intracellular ionic concentrations, different relative permeabilities of the apical and basolateral membranes, or different densities of transport mechanisms in the same membrane). Therefore, we have not found it possible to obtain standard control conditions before perturbing a skin. However, an awareness of the slow transient changes in $V_{oc}$ and $I_{sc}$, most often resulting in a movement towards higher iso-resistance lines can prevent incorrect or over-interpretation of results. It is certainly true that no reports have appeared in the literature which present detailed data over periods greater than 300 minutes, suggesting that others may have had similar problems, although they do not mention them. Part of this study therefore focussed on the role of the voltage-dependent Cl\textsuperscript{-} channels in this skin instability.
Figure 3.3: Upper Panels: Changes in Voc (black) and Isc (grey) as a function of time for skins bathed in 250mOsm/kgH₂O NaCl Ringer. The V-I locus (insert) shows Voc plotted against Isc, superimposed on iso-resistance lines for R=0.2kΩ to R=5kΩ (see text). Lower Panels: Time-dependent resistance determined using the ratio of Voc to Isc. Notice the variability between different skins.
3.3.2 Step sequence voltage-clamp experiments

One way to eliminate the variability in $I_{sc}$ and $V_{oc}$ is to remove the effect of Na$^+$, either by removing Na$^+$ from the bathing solutions or by blocking Na$^+$ transport using pharmacological agents such as the Na$^+$ channel blocker, amiloride. This results in the abolition of $I_{sc}$ within 30 seconds. Any current that is then observed is presumably dominated by the passive movement of KCl.\(^2\) Under open circuit conditions, this current is typically less than 5µA.

By applying a positive voltage across the skin with respect to the apical side (Figure 3.4A), the passive KCl flux can be greatly increased when the skin is bathed in

\[2\text{ If uncoupling of ion transport occurred within the cells, then ion movement through the Na}^+/K^+/2Cl^- symports could account for a small proportion of the current.\]
250mOsm/kg H2O NaCl Ringer (Figure 3.4C). A sequence of voltage-clamps stepping from a –40mV holding potential up to +80mV in 20mV steps shows that as the voltage-clamp becomes more positive, the time-dependent current increases in magnitude. When the skin is returned to the holding potential before stepping up to the next clamp value, the current reverses and slowly decreases back to its original steady-state value. Repeating the experiment in 250mOsm/kg H2O Cl– free (sulphate) Ringer shows the abolition of the time-dependent turn-on and a reduction in the absolute current by one order of magnitude (Figure 3.4B).

When the steady-state current obtained during the voltage-clamp, is plotted as a function of the bias voltage to produce an I-V curve, there is strong rectification in amiloride-treated skins bathed in NaCl Ringer that is not present in skins bathed in Cl– free Ringer (Figure 3.5A). The non-linearity of the Cl– data is even clearer when the data is normalised and the mean and standard deviations are presented (Figure 3.5B). (In this case the data were normalised to the steady-state currents at 80mV in Figure 3.5A because the data at 100mV was not available in all examples). For comparative purposes, a normalised steady-state I-V plot derived from the mathematical modelling described later, is included in Figure 3.5B. This illustrates the strong similarities between the experimental and the theoretical results. The main point of the data shown in Figures 3.4 & 3.5 is that the time-variant component of the voltage-induced current depends on extracellular Cl–, as suggested by Larsen & Kristensen (1978). The results presented here are presumably representative of behaviour present in previous experiments by others, but not reported in detail.

In some amiloride-treated skins bathed in NaCl Ringer, voltage-clamping initiated oscillatory behaviour in the current. This is important because the presence of oscillations suggests a closed-loop feedback system, and that the isolated epithelial layer has the capacity for local regulation. Figure 3.6 presents three skins showing varying degrees of damped oscillations, which are most obvious for large excursions away from the holding potential (i.e. +60, +80 & +100mV). The oscillation is least damped in Skin 101a, where the oscillation frequency is 0.015-0.017 Hz for +80 & +100mV (1 cycle every 60-68 seconds). An example of the oscillatory behaviour generated by the mathematical modelling, discussed later in this chapter, is presented in the last column. The skin conductance, derived from the current measurements and presented directly below, illustrates how the skins become more permeable to Cl– with a positive clamp, but almost impermeable when the voltage is returned to the holding
Figure 3.5: (A) Steady-state I-V curves for amiloride-treated skins bathed in 250mOsm/kgH₂O NaCl (filled circles) or 250mOsm/kgH₂O Cl⁻-free Ringer (open circles). Notice the non-linearities in the Cl⁻ data and the linearity of the Cl⁻-free data. (B) The NaCl data in (A), normalised to the 'adjusted' Cl⁻ currents at 80mV (arrow) and then averaged. The adjusted currents were the measured Cl⁻ currents shown in (A) minus the Cl⁻-free currents, estimated from the line of best fit given by: ICl-free=0.156V-0.963. The normalised results from the mathematical modelling are superimposed for comparison (dashed line). (C) Cl⁻ conductance activation curve derived from the normalised data presented in (B) (dark line). The data is fitted using the bell shaped curve given by: gCl= 1.168*exp(-1.69*10^-4*(V-100)^2).
Figure 3.6: Voltage-clamp experiments (columns 1-3) and theoretical modelling (column 4) showing variation in oscillatory behaviour for non-pumping (amiloride-treated) skins bathed in 250mOsm/kgH₂O NaCl Ringer on both sides. (A) Voltage step sequences were performed from -40mV (holding potential) to +100mV in 20mV steps. (B) The transepithelial current recorded under voltage-clamp conditions. A positive current corresponds to the movement of negative ions from the pond side to the belly side. (C) The transepithelial conductance, derived by dividing the current by the clamp potential. (D) The normalised transepithelial conductance, derived by converting the conductance into a percentage of the conductance range (i.e. max - min conductance) for each clamp. For clarity, normalised conductances in (D) are only shown for voltage-clamps greater than or equal to +20mV.
potential. This is consistent with the idea that Cl⁻ channels open with a positive transepithelial voltage-clamp but close with a negative voltage-clamp. The small conductance observed at the holding potential or in a Cl⁻-free medium is presumably due to cellular leakage pathways of Na⁺, K⁺ or Cl⁻. The leakage conductances in these toads ranged from 0.2-0.66mS, corresponding to skin resistances between 5 and 1.5kΩ, respectively.

The oscillations suggest the presence of delays in the system that determines the Cl⁻ conductance. Figure 3.7A presents the detailed time course of the Cl⁻ conductance as skins are clamped stepwise from −40mV to +100mV, while Figure 3.7B shows the conductance normalised as a percentage of the conductance range.³ It is clear from this data that the sharp change in skin potential is not reflected by a rapid change in conductance. Instead, there is a delay of up to 10 seconds before there is a rise in the conductance, similar to the observations of Larsen & Kristensen (1978). It is also clear that the more positive the voltage-clamp, the faster the conductance activation. At first glance, this may suggest that the activation time-constants are voltage-dependent. However, it should be emphasised that clamping the voltage across the skin does not mean that the individual apical and basolateral membrane potentials are clamped due to the voltage divider effect and the change in apical conductance. Therefore, the interactions between the apical and basolateral membranes must be taken into account when considering the skin current in estimating channel-gating kinetics.

Delays can also be observed in the inactivation of the Cl⁻ conductance. Figure 3.8 shows the detailed time-course of the conductance and the normalised conductance when the voltage across the skin is returned to the holding potential after 5 or 10 minutes of a positive voltage-clamp. These data show that delays of up to 13 seconds can occur before the conductance begins to inactivate, a process which is typically complete within 1-1.5 minutes of returning to the holding potential. It is also apparent that the more positive the clamp potential, the longer it takes for inactivation. The data in Figure 3.8B are described well by equations of the form $Ae^{-bt^2}$ where $A$ is a scaling constant, $b$ changes the steepness of the curve and $t$ is the time in minutes. An example of this curve fit is shown in Figure 3.8B (Skin 101b#1) using the constants $A=100$ and

³ The conductance range was defined as the difference between the maximal and the minimal conductances during the voltage-clamp. It should be noted however, that there were some variations in the time at which the maximal conductance occurred due to transients in the currents.
Figure 3.7: (A) Delayed activation of Cl⁻ conductance when the voltage-clamp is changed from -40mV (holding potential) in 20mV steps up to a maximum clamp of +80mV (left 2 columns) or +100mV (right 2 columns). The arrows show the region of nominal maximal conductance typically used to calculate the conductance range. (B) Conductance as a percentage of conductance range. Notice that the faster conductance activations occur for larger clamp potentials.

Figure 3.8: (A) Turn-off of conductance when the voltage-clamp is returned to -40mV (holding potential) in 20mV steps from a maximum clamp of +80mV (left 2 columns) or +100mV (right 2 columns). The initial transient on returning to the holding potential is assumed to be a switching transient in the monitoring system, and so the arrows show the nominal maximal conductance used to calculate the conductance range. (B) Conductance as a percentage of conductance range. Notice the larger the clamp potential, the slower the conductance inactivation. The data in Skin 101b#1 have been fitted using the equation $100 \times \exp(-2.6 \times t^2)$ (dashed line), which has been offset to the right of the experimental data by 0.2 minutes.
b=−2.6. So that the experimental data can be seen, the theoretical curve has been offset to the right by 0.2 minutes.

3.3.3 Voltage-clamps & oscillations in actively pumping skins.

In the preceding section, some of the properties of the Cl− conductance were presented without the influence of any Na+ interactions. It was shown that the Cl− conductance fully activates within 0.5-1 minute of application of a positive voltage-clamp, and inactivates within 1-1.5 minutes when returned to the negative holding potential. It was also shown that there was a delay before the onset of activation and inactivation, which may be responsible for the oscillatory behaviour sometimes seen. But what happens in the presence of Na+? Figure 3.9 shows the initial (control) response of a skin when it was placed in 250mOsm/kg H₂O NaCl, 19 minutes after pithing. Following this, two – 100mV voltage-clamps were applied for two minutes. In between the voltage-clamps, V₋ and Iₛc were monitored and the nominal resistance was calculated (Figure 3.9B). Before focussing on the effects of the negative voltage clamps, it is important to point out that there was a gradual increase in the skin resistance throughout the experiment, comparable to the resistance changes in other skins where no voltage clamp experiments had been conducted (Figure 3.9B & Figure 3.3C, D). The effects of the voltage-clamp experiments are merely superimposed upon this slow resistance increase.

Nevertheless, it is clear that the voltage clamps have a pronounced short-term effect on V₋ and a smaller, but noteworthy effect on Iₛc. Immediately after the release of both negative clamps, V₋ was significantly elevated, but within about 6-6.5 minutes V₋ had fallen to a local minimum, and had begun to rise again, the peak of which was reached 5-6 minutes later (i.e. see arrow Figure 3.9A). Iₛc also displayed evidence of a bounce or damped oscillation, rising transiently to a local maximum and then decreasing. This was clearest after the release of the second voltage clamp.

The V-I-locus is useful in summarising the changes in V₋ and Iₛc and shows both the effects of short-term extrinsic factors (such as voltage clamping), and long-term intrinsic factors. When the V-I locus of Figure 3.9 is compared with the V-I loci presented in Figure 3.3, it is clear that the skin shows the beginnings of a typical shut-down resembling the V-I locus of Skin 60b in particular (Figure 3.3D). The negative voltage-clamps clearly divert the skin from its shut-down trajectory, causing a transient increase in resistance.
Figure 3.9: (A) Voc (black) and Isc (grey) recordings and (B) resistance changes made in 250mOsm NaCl Ringer, 19 minutes after toad pithing. Note (i) the large change in Voc compared with the small change in Isc (circle) immediately after release from a -100mV voltage-clamp (vertical lines) and (ii) the large oscillatory change in Voc, not evident in Isc, (arrow) which is emphasised in the variation of skin resistance compared with the initial resistance of another skin (grey); (C) V-I locus for data presented in (A) showing the migration from a low resistance skin (R~0.7kΩ initially) to a higher resistance skin (R~3kΩ at the end of the measurements). Points (a), (b) and (c) indicate changes in Voc and Isc at the beginning of the experiment, and after the first, and second -100mV voltage-clamps, respectively.
As an extension of the findings presented in Figure 3.4, the oscillatory behaviour in $V_{oc}$ after a negative voltage-clamp appears to be dependent on extracellular Cl$. Figure 3.10 shows the absence of oscillatory behaviour for one skin bathed in Cl$^{-}$-free Ringer, and a 50% drop in the percentage change in resistance after a voltage-clamp of $-100$mV. In contrast, the oscillatory behaviour was present in the NaCl Ringer following a $-100$mV clamp, and this was associated with a 100% increase in the change in resistance, similar to the findings of Figure 3.8. Interestingly, there was a doubling in $I_{sc}$ immediately after each voltage clamp, in both Cl$^{-}$ and Cl$^{-}$-free solutions. $I_{sc}$ then decreased slowly returning to control values over the next 10-15 minutes. Since $I_{sc}$ is dominated by the active pumping of Na$^{+}$ ions, and there was a similar increase in $I_{sc}$ in both Cl$^{-}$ and Cl$^{-}$-free solutions, it can be concluded that the observed oscillatory behaviour was not due to Na$^{+}$.

![Graph of Skin 26b](image)

**Figure 3.10:** Short-circuit current (grey) and open-circuit voltage (black) before and after a 2 minute voltage-clamp at -100mV (dashed lines) for a skin bathed successively in Cl$^{-}$-free Ringer then NaCl Ringer. The percentage change in resistance for each perturbation is shown below. (Note that the vertical range is between 50% and 200%).

### 3.4 DISCUSSION

#### 3.4.1 Possible cause of the slow decrease in skin conductance

One of the problems highlighted in this study was the slow change in $V_{oc}$ and $I_{sc}$ (and therefore the nominal resistance) in actively pumping skins over a 10-20 hour period.
(Figure 3.3). When considering these changes as a function of time it was often difficult to determine the similarities between the skins. For example, $V_{oc}$ and $I_{sc}$ in A, C and F of Figure 3.3 appear to be quite different. Yet when the V-I loci for the six skins are examined closely, a ‘zig-zagging’ trend can be observed. The skin resistances initially tend to migrate along (or across) lines of low iso-resistance before a sharp upward inflection, followed by a downward migration along a higher iso-resistance line (‘the line of death’), towards zero. This general trend is most simply seen in Figure 3.3B. The other skins show more complex patterns following variations on this same theme.

One possible cause for this complex behaviour is that the apical $Na^+$ channels slowly close due to shrinkage of the cells in the $250mOsm/kgH_2O$ bathing solutions, as discussed in Chapter 4. With $Na^+$ channel closure, $I_{sc}$ would decrease, resulting in a drop in the apical membrane potential and closure of the voltage-dependent Cl$^-$ channels. With $Na^+$ and Cl$^-$ channel closure, the apical membrane would become largely impermeable to salts.

3.4.2 Voltage clamping of the skin & voltage divider effects.

It has already been pointed out in the introduction that under voltage-clamp conditions, the voltage applied across the entire skin is clamped, but the voltages across the individual membranes are not. Thus, even though the basolateral membrane resistance may be fixed, the presence of the voltage-dependent Cl$^-$ channels in the apical membrane causes a change in the ratio of the membrane resistances. Consequently, both the apical and basolateral membrane potentials change due to the voltage-divider effect as the apical Cl$^-$ channels open and close.

One of the effects of two membranes in series is that both membranes contribute to the total skin conductance. Consequently, the skin conductance is often more complex than the conductance of a single membrane. This is highlighted in Figure 3.5C which shows the Cl$^-$ conductance activation curve across the skin. The data are not well-fitted by a simple Boltzmann function, describing the probability of opening of voltage-

---

4 The upward inflection is characterised by a significant increase in $V_{oc}$ compared with a small change in $I_{sc}$ ($I_{sc}$ either increases or decreases slightly which changes the gradient of the inflection from positive to negative).

5 A decrease in $I_{sc}$ could occur if the basolateral $Na^+/K^+$-ATPases ceased to function (which might occur for example, if cellular ATP reserves were slowly being depleted), or if the apical $Na^+$ channels closed.

6 The Boltzmann function takes the form: $g_{Cl^\text{-}}/(1+10^{(g_{m}-g_{12})/g_{11}})$, where $g_{Cl^\text{-}}$ is a conductance scaling factor, $g_{m}$ is the membrane conductance, $g_{12}$ is the conductance for which the opening probability is 1/2.
dependent channels in a single membrane. Instead, the conductance curve was better approximated by a 2nd order bell-shaped function. The main point here is that as the epithelial layer becomes more complex due to more cells in series (or parallel), so too do the transepithelial responses because of the increasingly complicated contributions made by the individual membranes.

3.4.3 What is the mechanism of the delayed Cl⁻ channel gating?

From the results it is clear that there is a delay in the activation and inactivation of the Cl⁻ conductance after a voltage perturbation. It is suggested that this delay may be due to one of two mechanisms: a direct, kinetic effect in channel gating or an indirect effect, as described below.

First, it is possible that the voltage acts directly on the Cl⁻ channels causing kinetic changes in the channel proteins. In certain conformations (or states) ions can pass through the channels, but in other states the channels are relatively non-conducting. If the movement from one state to another takes a finite amount of time, then a delay between the voltage stimulus and a change in the channel conductivity will result (Armstrong & Matteson, 1984). Of course, if the proteins undergo multiple conformational changes due to the existence of multiple states, the delay will lengthen and, for a particular voltage stimulus, the activation and inactivation times of the Cl⁻ conductance will increase. Thus, channel kinetics is one mechanism which could explain the delayed changes in Cl⁻ conductance after a voltage perturbation. This issue is discussed in more detail in the following section on theoretical modelling where the mathematics describing the probability of a channel existing in one of many states in a sequence is addressed.

A second mechanism that could explain the delayed activation and inactivation of the Cl⁻ conductance is an indirect effect where the voltage modulates some intracellular parameter which in turn determines the conductivity of the Cl⁻ channels. For example, it is possible that a positive transepithelial voltage clamp (basolateral side positive) could modulate some process such as an intracellular second messenger system where the concentration of the second messenger determines the conductivity of the Cl⁻ channels. If the levels of several precursors determine the concentration of the second messenger, then a delay would be expected between a change in voltage and a change in Cl⁻ conductivity, because of the time taken before the precursors reached sufficiently high

and \( g_{11} \) is the conductance away from \( g_{12} \) necessary to either increase the opening probability from 1/2 to 10/11, or to decrease it from 1/2 to 1/11.
or low levels to initiate a change in the second messenger concentration, and the integration time inherent in a change in the second messenger concentration. This finite period, combined with the time required for the second messenger to bind and take effect, is an example of a delay that could occur as a result of an indirect voltage effect.

Regardless of whether the mechanism is a direct or an indirect one, the time delay that results in the delayed activation and inactivation is significant because it is this which causes the observed oscillatory behaviour. For example, assuming a fixed basolateral Cl\(^-\) conductance, a positive transepithelial voltage clamp (basolateral side positive) will cause the apical Cl\(^-\) channels to open. This changes the total skin conductance \(G_{TOT}\), given by \(G_{TOT} = G_a G_b/(G_a+G_b)\), where the subscripts ‘a’ and ‘b’ stand for apical and basolateral, respectively. As the apical Cl\(^-\) channels open, Cl\(^-\) ions enter or leave the cell across the apical membrane, as determined by the electrochemical gradient. The resultant reshuffling of charges changes the apical membrane potential, and the Cl\(^-\) channels begin to open or close in response. But because of the delayed activation and inactivation, by the time the Cl\(^-\) channels have responded, the membrane potential has changed again due to the further reshuffling of charge. In some cases, this can lead to the overshooting or undershooting in the transepithelial Cl\(^-\) current and damped oscillatory behaviour, until a steady-state Cl\(^-\) conductance is reached.

It is of interest to note that there has been little discussion in the literature about the oscillatory behaviour of the Cl\(^-\) conductance in the abdominal skin of the toad under either voltage clamp, or open-circuit conditions. The most likely reason for this is that the oscillations have not been observed, presumably because only a limited number of voltage-step experiments have been conducted. Even so, it is surprising that the oscillatory behaviour does not occur more often because delays, such as those seen in the activation and inactivation of the Cl\(^-\) conductance, are one of the main causes of instability in a system.

That oscillations do occur in an isolated, actively-pumping epithelial layer is important because it demonstrates that delayed feedback is occurring. This in turn, raises the possibility that some epithelia can be regulated locally (without the influence of ‘external’ factors such as neural or hormonal regulation), simply by applying biophysical principles to different transport mechanisms, arranged with a particular topology in the cell membranes.
3.5 Theoretical Modelling

Because of the oscillations observed experimentally, one of the aims of the mathematical modelling was to ensure that the model had the capacity to oscillate under voltage clamp conditions when the permeability of the cell was dominated by Cl⁻. As discussed below, two approaches were investigated. First, attempts were made to model the kinetics of the Cl⁻ channel. Second, a more general approach was examined which introduced delays in the system using a series of filters. This general approach was useful because it was applicable, irrespective of whether a direct or indirect mechanism was responsible for the delayed activation and inactivation of the Cl⁻ conductance (section 3.4). While both approaches are described, the final model utilised the more general filter description.

3.5.1 Kinetics of the voltage-dependent Cl⁻ channel: An analytical approach

One of the most distinctive features of the data presented here, and in previous studies (Larsen et al., 1987), is that there is a delay of 0.5-1 minutes before the current begins to turn on during a voltage clamp away from the holding potential. When the skin is returned to the holding potential, there is another delay of 1-1.5 minutes before the current turns off. Therefore, this data suggests that it takes a finite time for the channels to open and close. This is in distinct contrast to the activation kinetics of most voltage-activated Cl⁻ channels, having opening and closing times of the order of milliseconds (Procopio, 1997; Weiss, 1994).

In 1952 Hodgkin and Huxley observed a similar delay in the onset of the K⁺ conductance upon depolarisation of the squid giant axon, but in this case they saw no significant delay in the decline of the K⁺ conductance upon repolarization (Hodgkin & Huxley, 1952). It was found that the repolarization results could be described using a simple exponential equation derived from a first-order kinetic model, consisting of a conductive (open) and non-conductive (closed) state for the K⁺ conductance where the transition rates between the two states were described by two voltage-dependent rate coefficients (Figure 3.11A). However, at least a third- or fourth-order equation was necessary to describe the depolarisation results to account for the delayed increase in the K⁺ conductance. This was achieved by modelling the K⁺ conductance as being 'proportional to the fourth power of a variable which obeyed a first-order equation' (Hodgkin & Huxley, 1952) (Figure 3.11B). Although not strictly accurate in terms of representing an actual gating mechanism, this approach ensured that the mathematical
equations remained simple, while describing the K$^+$ conductance with reasonable accuracy.

In a similar fashion to Hodgkin and Huxley, Larsen has used a first-order kinetic model to describe the voltage-controlled Cl$^-$ conductance in MRCs (Larsen, 1982). However, in calculating the time course of activation of the Cl$^-$ current, the probability of entering the conductive state was raised to the power of 6 to best match the shape of the current activation. But two main problems arise when this analysis is used to describe the Cl$^-$ conductance. First, Larsen noted in 1982 that in some skins a transient reduction in the current could be observed before the current activation began. Although not seen in the present study, this phenomenon cannot be explained using first-order kinetics regardless of how many first-order particles are used. Second, the slow current inactivation cannot be described by a simple exponential equation. If this were the case then the current would drop away rapidly (Figure 3.12). Instead, the real current inactivates slowly at first and then more rapidly with time. Again, first-order kinetics

---

**Figure 3.11:** (A) A simple two state model where the two states i and j, at energy levels $E_i$ and $E_j$ respectively, are separated by an activation barrier. The activation energy for a gating particle to move from state i to state j is defined as $E_{aij}$ and the rate ($r_{ij}$) at which a transition from state i to state j can occur is dependent on the transition rate constant $k_{ij}$, and the probability of a channel being in that state, $p_i$; (B) Hodgkin & Huxley modelled the delayed activation of the voltage-dependent K$^+$ conductance in the squid giant axon by taking a simple two state model and raising the probability of the channel being open to the power of 4.
cannot explain this behaviour. It would appear then, that a 2 state model simply consisting of a single closed and open state has its limitations in describing the Cl− conductance.

An alternative method to describe the macroscopic, time-dependent behaviour of channels is a multiple-state mechanism, representing the kinetic folding of channel proteins into a number of open, closed or inactive states. When subjected to a particular perturbation, such as a change in membrane voltage, the mobile gating charges associated with the channel proteins are influenced by the electrostatic forces across the membrane and relocate into more energetically favourable positions in the membrane. This relocation process takes a finite period and a time-dependent shuffling of the probability of being in a particular state occurs. This shuffling could be manifested as delays in the activation and inactivation of the Cl− conductance.7

Figure 3.12: Qualitative representation of the activation and inactivation of the apical Cl− conductance based on Larsen's 1991 theoretical model and what is seen experimentally. The approach used by Larsen where the Cl− conductance was modelled as being proportional to the sixth power of a variable which obeys a first-order equation gives a delayed activation in the Cl− conductance, consistent with experimental results. However, using first-order mathematics no delay in the inactivation of the Cl− conductance can occur, which is inconsistent with what is observed experimentally (black line).

7 This is because the more closed (or open) states there are the greater the lag in the activation (or inactivation) of the channels since, in a sequential model, more states must be traversed before reaching the open (or closed) states (Armstrong & Matteson, 1984).
The major disadvantage in adopting a multiple-state kinetic model is that the mathematics becomes increasingly complex the greater the number of states (or conformations) that the protein can adopt. In developing this kinetic model it appeared that a minimum of 6 closed states existed because this description was consistent with the delayed current activation seen by Larsen and his first-order kinetic model raised to the power of 6. Because no Cl⁻ inactivation models were available in the literature, 6 open states were assumed because this yielded a delayed current inactivation. Thus, the kinetics of a 12 state model, with 24 rate coefficients separating the 12 states were investigated.

In addition, a simple sequential model, where the states are adjacent to each other was adopted because (1) sequential models are necessary to produce a time lag or phase delay (Armstrong & Matteson, 1984); and (2) there appeared to be no data in the literature to suggest that the states were topologically interconnected in a more complex fashion. For the sake of simplicity, the rate constants were all assumed to be equal in magnitude, except for the forward and backward rate constants associated with the one voltage-dependent state (which was defined to be the closed state located at the end of the sequence (see Figure 3.13)). These two rate constants were described as a function of membrane voltage.

But before considering the complexities of a 12-state model solution, the rate kinetics of a simpler 2-state model are presented. As shown in Figure 3.11A, the 2-state model consists of 2 energy levels at energies Eᵢ and Eᵣ (corresponding to state i and state j) and separated by an energy barrier. The activation energy for a gating particle to move from state i to state j is defined as Eaᵢj, and the rate (rᵢj) at which a transition from state i to state j can occur is dependent on the transition rate constant (kᵢj), and the probability of a gating particle being in the starting state (pᵢ) according to equation (3.1) (Adamson, 1973; Armstrong & Matteson, 1984; Patuzzi, 1998; Weiss, 1994):

\[ r_{ij} = k_{ij} \cdot p_i \]  

(3.1)

where

\[ k_{ij} = \kappa \cdot (kT/h) \cdot e^{-E_{ij}/kT} \]  

(3.2)

The constant terms in (3.2) are defined as \( \kappa \), the transmission coefficient, which is approximately 1; \( k \), the Boltzmann constant, \( (1.38 \times 10^{-13} \text{ J/K or } 8.62 \times 10^{-5} \text{ eV/K}) \); \( h \) which is Planck’s constant \( (6.62 \times 10^{-34} \text{ Js or } 1.06 \times 10^{-54} \text{ eVs}) \) and \( T \), the absolute
temperature. Assuming $\kappa=1$, then taking the natural logarithm of (3.2) and rearranging gives an explicit expression for the activation energy level $E_{aij}$:

$$E_{aij} = kT \cdot \ln\left(\frac{kt}{h}/k_{ij}\right) \quad (3.3)$$

By substituting the appropriate form of (3.3) into (3.2) the ratio of the forward and backward rate constants is defined as:

$$k_{ij}/k_{ji} = e^{-(E_{aij}-E_{aji})/kT} = e^{(E_i-E_j)/kT} \quad (3.4)$$

The main point to notice before complicating the system is that for a constant temperature, the rate constant $k_{ij}$ can only change if the activation energy $E_{aij}$ is adjusted, which effectively means that the energy level $E_i$ must alter.

The 12-state model used in this chapter is simply the repeated juxtaposition of the 2-state model described above. For simplicity, all the energy states, except for the leftmost closed state (state 1) in Figure 3.13, were defined as equal and were referenced to zero.\(^8\,9\) However, the energy level, $E_1$, was allowed to vary in order to bestow voltage dependence upon the channel. Clearly, the lowering of $E_1$ below the energy level of the other states, biases the distribution of gating particles towards the closed states. Experimentally, this is analogous to applying a negative potential across the skin, as is done when the skin is voltage clamped at the holding potential of –40mV. Conversely, raising $E_1$ above the energy level of the other states (analogous to a positive voltage clamp, experimentally) biases the distribution of gating particles towards the open state.

As a mobile gating charge associated with a channel protein begins to ‘shuffle’ from one conformation to another, the probability of it existing in any given state changes as a function of time. More specifically, the ‘rate of change of the occupancy of a particular state is defined as the difference between the movement into and out of that state’ (Patuzzi, 1998). With this in mind, it is possible to describe the dynamic behaviour of a gating charge using a series of differential equations, where all that needs

---

8 Although any state(s) could have been chosen, the experimental data was insufficient to allow determination of the relative rate constants or energy levels. Therefore, it was appropriate to choose a model that simplified the mathematical analysis and presented the conceptual ideas clearly.

9 Since the energy levels for states 2-12 were all fixed this meant that all the associated rate constants were also fixed. The only variable rate constant was $k_{12}$, associated with the energy state, $E_1$. 
to be known is the probability of occupancy at any instant and the 24 rate constants (23 of which are constant in this case). The differential equations for a 12-state sequential system, are given by:

\[
\frac{dp_1}{dt} = k_{21} p_2 - k_{12} p_1 \quad (3.5)
\]

\[
\frac{dp_2}{dt} = k_{12} p_1 + k_{32} p_3 - (k_{21} + k_{23}) p_2 \quad (3.6)
\]

\[
\frac{dp_3}{dt} = k_{23} p_2 + k_{43} p_4 - (k_{32} + k_{34}) p_3 \quad (3.7)
\]

\[
\frac{dp_4}{dt} = k_{34} p_3 + k_{54} p_5 - (k_{43} + k_{45}) p_4 \quad (3.8)
\]

\[
\frac{dp_5}{dt} = k_{45} p_4 + k_{65} p_6 - (k_{54} + k_{56}) p_5 \quad (3.9)
\]

\[
\frac{dp_6}{dt} = k_{56} p_5 + k_{76} p_7 - (k_{65} + k_{67}) p_6 \quad (3.10)
\]

\[
\frac{dp_7}{dt} = k_{67} p_6 + k_{87} p_8 - (k_{76} + k_{78}) p_7 \quad (3.11)
\]

**Figure 3.13:** 12-state sequential model depicting the possible kinetics of the apical voltage-dependent Cl– channels. Six open and six closed states are represented. All states are separated by equal activation energies with equal rate constants k, except for the leftmost one which has been arbitrarily ascribed voltage dependence. Depolarisation of the membrane (referenced to the extracellular juice) increases the energy level of the first state such that there is an increased probability that gating particles migrate sequentially from state 1 to state 2 to state 3…to state ‘n’ such that there is a delayed activation of the Cl– conductance.
\[
\begin{align*}
\frac{dp_8}{dt} &= k_{78}p_7 + k_{98}p_9 - (k_{87} + k_{89})p_8 \\
\frac{dp_9}{dt} &= k_{98}p_8 + k_{10_9}p_{10} - (k_{89} + k_{9_10})p_9 \\
\frac{dp_{10}}{dt} &= k_{9_10}p_9 + k_{11_10}p_{11} - (k_{10_9} + k_{10_11})p_{10} \\
\frac{dp_{11}}{dt} &= k_{10_11}p_{10} + k_{12_11}p_{12} - (k_{11_10} + k_{11_12})p_{11} \\
\frac{dp_{12}}{dt} &= k_{11_12}p_{11} - k_{12_11}p_{12}
\end{align*}
\]

The analytical solution of these equations (yielding an explicit description of the probability of occupancy, \( p_i \), for each state) can be found using the method of Colquhoun and Hawkes (1981). The general form of \( p_i \) is given by:

\[
p_i(t) = p_i(\infty) + a_2e^{-t/\tau_2} + a_3e^{-t/\tau_3} + a_4e^{-t/\tau_4} \ldots + a_n e^{-t/\tau_n} \tag{3.17}
\]

where \( p_i(\infty) \) = the probability of occupancy of the \( i^{th} \) state at equilibrium, or in the quiescent state that exists just prior to a perturbation and \( a_n e^{-t/\tau_n} \) = the contribution of gating particles shuffling from the \( n^{th} \) state into the \( i^{th} \) state where the magnitude \( a_n \) is determined from the initial conditions, and the time constant \( \tau_n \) is the reciprocal of the root (\( \lambda_n \)) found from the characteristic equation. (The subscript ‘n’ in (3.17) denotes the maximum number of states in the system).

Although it is relatively simple to find the characteristic equation from the 12 differential equations using the proprietary software package, Mathematica (see equation 3.18), the solutions to this equation cannot be expressed in closed form. That is, there is no explicit algebraic expression for each of the 12 roots (eigenvalues).

\[
0 = (11Bk^{10} + k^{11})x + (220Bk^9 + 66k^{10})x^2 + (1287Bk^8 + 715k^9)x^3 + \\
(3432Bk^7 + 3003k^8)x^4 + (5005Bk^6 + 6435k^7)x^5 + (4368Bk^5 + 8008k^6)x^6 + \\
(2380Bk^4 + 6188k^5)x^7 + (816Bk^3 + 3060k^4)x^8 + \\
(171Bk^2 + 969k^3)x^9 + (20Bk + 190k^2)x^{10} + (B + 2k)x^{11} + x^{12} \tag{3.18}
\]

where \( B = \) the voltage-dependent rate constant, \( k_{12} \).
Instead, the roots (and therefore the time constants) must be determined numerically. But there is a further complication: due to the voltage sensitivity of the channel, the rate constant \( k_{12} (=B) \) is variable and therefore many of the co-efficients of the characteristic equation change. As the characteristic equation changes, so too do the roots. For example, Figure 3.14A shows the characteristic equation for \( B/k=1 \) and for \( B/k=5 \). Clearly, as \( B/k \) increases the roots (or the intercept points along the x-axis) migrate outwards, becoming larger in magnitude. This is important because it affects the time constants in (17), altering the contribution of gating particles shuffling from the \( n^{th} \) state into the \( i^{th} \) state.

The question then arises: ‘by how much do the roots and time constants change as a function of the \( B/k \) ratio?’ Figure 3.14B illustrates how each of the 12 roots increase in magnitude as \( B/k \) increases (corresponding to a depolarisation of the membrane potential), while Figure 3.14C shows the associated reduction in time constants.\(^{10,11}\) The main point to note is that for a 1000-fold change in \( B/k \), there is remarkably little variation in the magnitude of most of the roots. However, for a \( B/k \) ratio greater than 2, the outermost zero in the characteristic equation migrates dramatically away from its starting position (at \( \lambda \approx 4 \)) becoming increasingly more positive. This is associated with a reduction in the corresponding time constant.

---

10 Throughout this section voltage dependence was expressed in terms of rate constants (i.e. the \( B/k \) ratio) and not as a function of the more conventional membrane potential. This was for 3 reasons. First, it was desirable to develop the analytical solution using the most general parameters. Second, it was inappropriate to specify particular membrane potentials since experimentally voltage clamp trials were conducted across the whole epithelium and not a particular membrane. As discussed in section 3.4.2 only a fraction of the clamp voltage appears across the apical membrane. Therefore, to replicate experimental conditions, it would be necessary to scale the apical voltages accordingly, requiring knowledge of the basolateral conductance. Third, even if appropriate membrane potentials were available, it would still have been necessary to convert these values into the corresponding \( B/k \) ratios in order to determine the roots of the characteristic equation. Therefore, it was more efficient to use a \( B/k \) ratio throughout the development of the analytical solution.

11 The characteristic equation is a 12\(^{th} \) order polynomial and therefore is regarded as an ‘even’ function. Consequently, one root will always pass through the origin resulting in an undefined rate constant. Thus, there are only 11 rate constants.
Figure 3.14: (A) Outward migration of the roots (zeroes) away from the origin as the B/k ratio increases in magnitude; (B) Changes in the magnitude of each of the 12 roots as the B/k ratio increases upon membrane depolarisation (referenced to the outside of the cell) and (C) associated reduction in time constants, $\tau$. 
Once the roots have been determined for a particular B/k ratio, the co-efficients in (17) can be found by applying Colquhoun and Hawkes’ method (1981). From this, the dynamic behaviour of each state can be expressed explicitly. When the B/k ratio is unity, the system settles down to equilibrium with gating charges having an equal probability of being in any one of the 12 states. When the B/k ratio is less than one (corresponding to a negative voltage clamp), there is a greater probability that the gating charges exist in state 1 and the channels are closed. If this B/k ratio is suddenly increased in magnitude (corresponding to a more positive voltage clamp being applied across the skin) it is likely that the gating charges move under the influence of the new electric field, such that the probability of them existing in the open states increases. However, as the gating charges migrate through the membrane they must pass through the adjacent closed states before reaching the open states. Consequently, there is a ‘sloshing’ effect where there is a rapid migration of gating charges from state 1 to state 2 and then from state 2 to state 3 onwards.13

An example of these migrations through the different states is illustrated in Figure 3.15A which shows the dynamic changes in the probability of a gating charge existing in a particular state when the B/K ratio is (i) changed from 0.25 to 1.2 and then (ii) changed back to 0.25. As described above, the application of the positive voltage biases at (i) increases the probability of a gating charge leaving state one and successively entering states 2 to 12. When the clamp is subsequently removed at (ii),14 there is a rapid reduction in the probability of a gating charge existing in states 2 to 12, and it becomes increasingly likely that it will exist in state 1. The important point to note here is that it takes a finite period for a gating charge to move between the different states.

---

12 One slight modification that was necessary because of the use of the B/k ratio was that the W_{ij} matrix used by Colquhoun and Hawkes (1981) had to be multiplied by a factor of B to ensure that the sum of all the probabilities at any instant was 1.

13 The analogy is a series of 12 troughs filled with water. As the water form trough 1 is poured into trough 2 it fills until it begins to overflow, emptying into trough 3 which in turn fills and overflows into trough 4. This process continues until the water redistributes and a new steady-state is reached.

14 From an experimental perspective, the time at which a voltage clamp is removed can affect the recovery pattern.
Figure 3.15: (A) The probability of gating charges existing in any 1 of 12 states. Point (i) shows the effects of the membrane voltage moving from a negative to a positive value such that gating charges migrate from the closed states towards the open states and the Cl⁻ conductance increases. Point (ii) is the reverse case where the membrane voltage moves from a positive to a negative value such that gating charges migrate from the open states towards the closed states and the Cl⁻ conductance decreases. The dotted line represents equilibrium where the energy levels of all states are equal. (B) Total open probability, defined as \( p_\text{open} = p_7 + p_8 + p_9 + p_{10} + p_{11} + p_{12} \), for different voltage clamp values (i.e. B/K ratios). (i) Steady-state \( p_\text{open} \) for B/k=0.25; (ii) Delayed increase in the open probability for B/k>0.25 representing a change in the transepithelial voltage from a negative holding potential to a positive voltage clamp and (iii) the delayed decrease in the open probability upon subsequent return to the negative holding potential.
An alternative way to assess the effects of different B/k ratios is to sum the probabilities of a gating charge being in any one of the six open states ($p_{\text{open}}$), and consider the changes in the overall channel conductance with time. Figure 3.15B illustrates the theoretical changes that occur in $p_{\text{open}}$ for a range of B/k ratios. The main points that are apparent as the B/k ratio is first increased, and then decreased are: (1) there is a delay in both the activation and inactivation of the conductance; (2) the channels begin to open faster as indicated by a leftwards shift in $p_{\text{open}}$ and (3) the proportion of channels adopting an open conformation increases as demonstrated by a successive rise in the magnitude of steady-state values. These findings are consistent with the experimental observations of Figure 3.6 suggesting that a multi-state model for the apical voltage-dependent Cl⁻ channels in the toad skin is reasonable.

However, it should be emphasised that the analytical solution presented in this section is only a model for voltage-dependent channels in a single membrane. It does not include the voltage divider effect that occurs when two membranes are placed in apposition. Consequently, there was no means for local feedback to occur so the system was perfectly stable, with no indication of an oscillatory response.

Attempts were made to model a 12-state voltage dependent apical Cl⁻ conductance in apposition with a fixed basolateral Cl⁻ conductance using a version of the single-cell Excel spreadsheet model described in Chapters 6 & 7. However, Nyquist’s criteria for oscillatory behaviour (namely a phase delay of 180 degrees or more, and a loop gain of 1 or more) were clearly not satisfied as oscillations could not be elicited theoretically using this method. This was almost certainly due to a poor choice of parameters, because we had demonstrated earlier, using a simple voltage divider approach, that oscillatory behaviour could be elicited with a 12-state channel in series with a fixed basolateral conductance. However, although many different permutations and combinations were investigated, time limitations did not permit an exhaustive study to be conducted to find the optimal parameters to elicit oscillations using the 12-state representation of the Cl⁻ channel in the single-cell model. Therefore, although it is most likely that the system would have oscillated given the correct parameters, a simpler mathematical model was sought.

3.5.2 General filter method:

While the ideal approach was to develop a mathematical model that gave insight into the mechanism causing the delay in the activation and inactivation of the Cl⁻ conductance, the overall purpose of the mathematical model was not to explain
mechanisms, but to replicate experimental results as closely as possible. With this in mind, the objective then became the development of a general method which gave a delay in the opening and closing of the Cl\(^-\) channels which could be used in whole cell modelling, without consideration of the actual mechanisms. This section describes such a method based on a Boltzmann activation function and the use of low-pass filters.

It is possible to model the apical Cl\(^-\) permeability using a simple first-order voltage-dependent Boltzmann activation function where the probability of the channels being open \(p_o\), is given by the equation:

\[
p_o = \frac{1}{1 + 10^{(V_m - V_{12})/V_{11}}} \quad (3.19)
\]

where: \(V_m\) = the membrane potential across the apical membrane
\(V_{12}\) = is the membrane potential for which the opening probability is a half.
\(V_{11}\) = is the voltage away from \(V_{12}\) required to increase the opening probability from 1/2 to 10/11 or to decrease it from 1/2 to 1/11.

However, this only yields a sigmoidally shaped curve. It does not produce the delay seen in the activation and inactivation of the Cl\(^-\) channels. One way to model the delays is to pass the voltage-dependent open probability \(p_o\), generated by the Boltzmann function, through ‘n’ low-pass filters (LPFs) arranged in series, to obtain a series of delayed open probability outputs denoted respectively by \(p_{o1}\), \(p_{o2}\), \(p_{o3}\),… \(p_{on}\) (Figure 3.16A). By multiplying the final delayed open probability \(p_{on}\), by the maximal Cl\(^-\) permeability \(P_{Cl_{sat}}\), a delayed activation and inactivation of the Cl\(^-\) permeability can be generated to a step-like voltage stimuli applied across the membrane (Figure 3.16B). Thus, a voltage dependent Cl\(^-\) permeability can be represented using a Boltzmann activation function followed by LPFs. (More specifically, it was found in this study that to match the experimental results presented, a minimum of three identical LPFs were required to obtain the phase rotation and loop gain necessary to satisfy Nyquist’s criteria).

To calculate the delayed open probability outputs \(p_{o1}\), \(p_{o2}\) and \(p_{o3}\) in the time-domain, it was necessary to calculate the incremental changes in these parameters denoted respectively by \(dp_{o1}\), \(dp_{o2}\) and \(dp_{o3}\) as described explicitly in equation 20.

\[
\begin{align*}
dp_{o1} &= \frac{dt(p_o - p_{o1})}{\tau_1} \\
dp_{o2} &= \frac{dt(p_{o1} - p_{o2})}{\tau_2} \\
bp_{o3} &= \frac{dt(p_{o2} - p_{o3})}{\tau_3}
\end{align*}
\quad (3.20)
\]
where: $\text{dt} = \text{the incremental time step}$

$\tau = \text{the time constant for each filter (in this case because the filters were assumed to be identical, } \tau_1 = \tau_2 = \tau_3)$. 

The incremental values were then added to their associated existing instantaneous probability values $p_{od1}$, $p_{od2}$ and $p_{od3}$ to obtain the next probability values according to equation 21.

$$p_{od1\text{next}} = p_{od1} + dp_{od1} \quad p_{od2\text{next}} = p_{od2} + dp_{od2} \quad p_{od3\text{next}} = p_{od3} + dp_{od3} \quad (3.21)$$

Figure 3.16: (A) The apical Cl$^-$ conductance can be modelled by passing the open probability $p_o$, determined from a first order Boltzmann activation function, through a series of low pass filters; (B) By multiplying the final delayed open probability $p_{od3}$, by the maximal Cl$^-$ permeability a delayed activation and inactivation of the Cl$^-$ permeability can be generated to a step-like voltage stimuli applied across the membrane.
Using the expression for the voltage-dependent apical Cl⁻ permeability described above in the single-cell time domain model described in Chapter 7, it was possible to investigate the delayed opening and closing of the Cl⁻ channels under transepithelial voltage clamp conditions. As described earlier (see Figure 3.6) it was found that under certain circumstances, oscillatory behaviour was possible due to the voltage divider effect across the apical and basolateral membranes which permitted local feedback to occur.

The main parameters used in achieving the oscillatory result are presented in Tables 3.1–3.4. Table 3.1 describes the relative permeabilities of the apical, basolateral and paracellular transport pathways. (It should be noted that to replicate experimental conditions where the skins had been amiloride-treated, the apical Na⁺ permeability was small). Table 3.2 presents the relevant bath and cell parameters, where the theoretical ‘bathing solutions’ resembled the composition of the 250mOsm/kgH₂O NaCl Ringer used experimentally. Table 3.3 presents salient pump and symport parameters. Table 3.4 presents the parameters used in developing the voltage-dependent expression for the apical Cl⁻ channels.

Finally, it should be noted that during the course of the study, it was necessary to alter slightly the $V_{12LPF}$ and $V_{11LPF}$ parameters of the Cl⁻ channels as new information became available. In the final model that is appended in the form of a compact disk, the values that were used were $V_{12LPF} = -18\,\text{mV}$ and $V_{11LPF} = -5\,\text{mV}$ and not $-35\,\text{mV}$ and $-25\,\text{mV}$ respectively, as described in Table 3.4. Since these parameters are inherent properties of the channel, ideally the variables in the models (such as cell permeabilities, cell compliances and intracellular sugar and protein concentrations) should have been optimised so that the $V_{12LPF}$ and $V_{11LPF}$ values were the same for both the oscillatory responses modelled in this chapter and the final time-domain model of the toad discussed in Chapter 8. Despite this discrepancy, the main point to note is that it is possible to model a Cl⁻-dependent oscillatory behaviour by delaying the opening and closing of apical Cl⁻ channels in a single-cell, two-membrane model with feedback.

3.6 Conclusions

The purpose of this chapter was to investigate the voltage-dependence of the Cl⁻ conductance and the oscillations that sometimes result. The main conclusions of this study are as follows:
### Table 3.1: Relative permeabilities of the apical, basolateral & paracellular pathways.

<table>
<thead>
<tr>
<th>Transport Mechanism</th>
<th>Apical</th>
<th>Basolateral</th>
<th>Paracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquaporins</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase pump</td>
<td>0</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Cl⁻ channels</td>
<td>variable</td>
<td>15</td>
<td>0.1</td>
</tr>
<tr>
<td>K⁺ channels</td>
<td>0</td>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td>Na⁺ channels</td>
<td>0.0001</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Na⁺/K⁺/2Cl⁻ symport</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3.2: Extracellular and intracellular parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Apical</th>
<th>Intracellular</th>
<th>Basolateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Na⁺] mM</td>
<td>111</td>
<td>variable</td>
<td>111</td>
</tr>
<tr>
<td>[K⁺] mM</td>
<td>5</td>
<td>variable</td>
<td>5</td>
</tr>
<tr>
<td>[Cl⁻] mM</td>
<td>116</td>
<td>variable</td>
<td>116</td>
</tr>
<tr>
<td>[sugar] mM</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>[prot-] mM</td>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>A⁻ (relative)</td>
<td>0</td>
<td>74</td>
<td>0</td>
</tr>
<tr>
<td>Hydrostatic pressure (relative)</td>
<td>0</td>
<td>variable</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 3.3: Na⁺/K⁺-ATPase pump and Na⁺/K⁺/2Cl⁻ symport parameters

<table>
<thead>
<tr>
<th>Pump Parameter</th>
<th>Value</th>
<th>Symport Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_{ATP}</td>
<td>$1 \times 10^4$</td>
<td>Na_{sat}</td>
<td>1</td>
</tr>
<tr>
<td>gamma</td>
<td>$5 \times 10^{-4}$</td>
<td>K_{Sat}</td>
<td>1</td>
</tr>
<tr>
<td>Na_{sat}</td>
<td>30</td>
<td>Cl_{Sat}</td>
<td>2</td>
</tr>
<tr>
<td>K_{sat}</td>
<td>1</td>
<td>P_s</td>
<td>0.1</td>
</tr>
<tr>
<td>P_V</td>
<td>0.1</td>
<td>Na_{sats}</td>
<td>$1 \times 10^{-8}$</td>
</tr>
<tr>
<td>[ATP] mM</td>
<td>20</td>
<td>K_{sats}</td>
<td>$1 \times 10^{-8}$</td>
</tr>
<tr>
<td>[ADP] mM</td>
<td>0.1</td>
<td>Cl_{sats}</td>
<td>$1 \times 10^{-8}$</td>
</tr>
<tr>
<td>[P_i] mM</td>
<td>0.1</td>
<td>Na/K</td>
<td>1</td>
</tr>
<tr>
<td>K_f</td>
<td>2</td>
<td>Na/Cl</td>
<td>0.5</td>
</tr>
<tr>
<td>Na_f</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K/Na</td>
<td>2/3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.4: Voltage dependent Cl⁻ channel parameters.

<table>
<thead>
<tr>
<th>Transport Mechanism</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{Cl}_{sat}</td>
<td>25</td>
</tr>
<tr>
<td>V_{12_{LPF}} (mV)</td>
<td>-35</td>
</tr>
<tr>
<td>V_{11_{LPF}} (mV)</td>
<td>-15</td>
</tr>
<tr>
<td>τ₂ (relative)</td>
<td>0.06</td>
</tr>
<tr>
<td>τ₃ (relative)</td>
<td>0.06</td>
</tr>
<tr>
<td>τ₄ (relative)</td>
<td>0.06</td>
</tr>
</tbody>
</table>
(1) A gradual increase in the skin resistance \( (R=V_{oc}/I_{sc}) \) was observed in skins bathed in 250mOsm/kgH₂O NaCl Ringer over 10-20 hours, consistent with a slow closure of apical Cl⁻ channels.

(2) Voltage-dependent currents could be elicited from skins clamped at positive potentials (with reference to the apical solution) in Cl⁻ Ringer, but not in Cl⁻-free Ringer.

(3) When stepping the skin voltage from a –40mV holding potential to a positive voltage of up to +100mV, delays of up to 10s occurred before the voltage-dependent Cl⁻-current began to activate.

(4) The activation process was typically complete within 0.5-1min of application of the positive clamp. When skins were returned to the holding potential, delays of up to 13s were observed before the current began to inactivate. Inactivation was typically complete within 1-1.5min.

(5) Damped oscillatory behaviour was also observed under voltage-clamp conditions in some skins when a positive bias of up to +100mV was applied. In one skin where the oscillations were least damped, the frequency of oscillation was between 0.015-0.017Hz (i.e. a period of 60-68 seconds).

(6) Damped oscillations were also present in the open-circuit voltage in actively pumping skins following release from a 2-minute voltage-clamp at -100mV. The oscillations were superimposed on the slower increase in skin resistance.

While it is possible that channel kinetics are directly responsible for the slow activation and inactivation of the Cl⁻ conductance, further information is required about the voltage sensitivity of the channel proteins before this issue can be resolved completely. However, using a simple low-pass filter model it was shown that delays in the opening and closing of the Cl⁻ conductance in a whole cell model could create the oscillations observed experimentally.
4. Volume Regulation
4. Volume Regulation

4.1 Introduction

In 1992 Spring and Hoffman wrote in their review on cellular volume control that “when cells are experimentally subjected to a sudden change in medium osmolality, the first event which occurs is the rapid flux of water across the membranes in the direction determined by the chemical gradient for water. This swelling or shrinkage alters intracellular ionic and solute concentrations, leading to alterations in the flux of these substances across the cell membranes as well as to the activation of otherwise quiescent mechanisms.” Due to the ordering of events, these statements may be misleading because they suggest that for water- and ion permeable cells, water transients always occur before ion transients. While this may be true in some cases, it is also possible that ion transients (which may create net salt movements) occur before water movements, or that both water and ions transients occur simultaneously.

As discussed below, the importance of distinguishing between the order of the transients becomes apparent when considering water movements and cell volume. In many texts and review articles, cell volume changes are often described in terms of a net water movement occurring as a result of a difference in osmolarity between the cells and the surrounding fluid. An alternative (conceptual) way of looking at water movement is to separate it into two component parts and to consider (1) ‘simple water movements’ and (2) ‘solute-induced water movements.’

Simple water movements are defined here as those which are independent of changes in cell solute content. For example, a simple water movement can occur when a membrane, which is permeable to water but not to ions (or other osmolytes), separates two fluid compartments of different osmolarities. Under these conditions water will move across the membrane until the osmotic pressure on both sides equilibrates.

Solute-induced water movements are those that occur following changes in cell solute content. Such water movements balance the small dilution (or concentration) effects that occur when the intracellular solute concentration changes.

Of course, because the water movements occur through the same pathway via the aquaporins only one net water movement can be measured. However, because of the different forces initiating the water movements the function describing the net water movement may not follow just a simple exponential.

One of the advantages of specifying the two components of the net water movement is that conditions where simple- and solute-induced water movements oppose or reinforce
each other can be considered. For example, Figure 4.1 shows five examples of how volume can be affected by different bathing solutions (all referenced to the intracellular environment). Under isotonic conditions (Figure 4.1A) there are no net simple- and solute-induced water movements, so the cell volume remains constant. When cells are placed in a solution with a low ionic concentration and high osmolality (such as a dilute salt solution where an osmotically inactive substance like sucrose has been added) simple- and solute-induced water movements reinforce so that there is a total water movement out of the cell, and the cell shrinks. Conversely, if simple- and solute-

![Figure 4.1](image)

**Figure 4.1:** (i) Schematic diagram showing the directions of simple- and solute-induced water movements when placed in different bathing solutions (where the osmolarities and ionic concentrations are relative to the intracellular condition); (ii) The effects of simple water movements (due to water moving down its concentration gradient) and solute-induced water movements (due to net salt (or other solute) movement) on total cell volume; (iii) Theoretical changes in Isc as the intracellular Na\(^+\) concentration rises or falls for a fixed Na\(^+\) permeability; (iv) Theoretical changes in Isc as the intracellular Na\(^+\) concentration rises or falls for a variable Na\(^+\) permeability which is volume-sensitive. A short delay between volume changes and Isc changes has been included.
induced water movements are both inwardly directed the cell would swell (Figure 4.1E).\(^1\) In a high ionic, high osmolarity solution (such as the standard 250mOsm/kgH\(_2\)O NaCl Ringer solution used in this study), simple water movements may be directed out of the cell while solute-induced water movements may oppose this movement by entering the cell (Figure 4.1C). Depending on the relative magnitudes of these water movements and their time-courses (determined by the water and ionic permeabilities of the cell), the cell may shrink (due to rapid simple water movements) then swell (due to slower solute-induced water movements), undergoing a regulatory volume increase (RVI). Alternatively, the cell may swell (due to rapid solute-induced water movements) then shrink (due to slower simple water movements), undergoing a regulatory volume decrease (RVD). In both cases the same mechanisms are operative, yet different responses in the total volume are observed. Similar problems arise when the cell is bathed in a low ionic, low osmolality solution such as distilled water or a very dilute salt solution (Figure 4.1D). Here the simple water gradient is directed into the cell, but salts will tend to move out of the cell. Again, depending on the gradients and the permeabilities of the cell, it may shrink then swell undergoing RVI, or it may swell then shrink as RVD occurs. In both high ionic, high osmolality and low ionic, low osmolality solutions differentiating between the opposing simple- and solute-induced water movements experimentally is not easy, unless the water and ion permeabilities of the cells are significantly different. It has been noted that many animal cells are highly permeable to water (Hoffman & Simonsen, 1989). In such cells it is possible that water transients are faster than ionic transients. Certainly, this has been demonstrated in human peripheral blood mononuclear lymphocytes (Grinstein et al., 1983), and in Ehrlich ascites tumour cells (Hoffman et al., 1983) (Figure 4.2). But it is noteworthy that, under physiological conditions, these cells, like most mammalian cells (with the exception of some cells such as those in the kidneys), are not exposed to anisotonicity, with the osmolality of body fluids being closely regulated within ±3% of 285mOsm/kgH\(_2\)O (Hoffman & Simonsen, 1989). In cells that are exposed to rapidly changing extracellular environments (such as dermal cells) the apical water permeability is possibly much lower, or can be varied by the animal, depending on the environment.

\(^1\) For inwardly directed simple- and solute-induced water movements the bathing solution would have to be a high ionic, low osmolality solution. Generally speaking, unless the cell is shrunken so the osmolality is higher than the external medium, it is not possible to achieve this because a high ionic concentration necessarily means a high osmolality.
Under such circumstances the time courses of simple- and solute-induced water movements may be difficult to separate. In addition, the situation may be further complicated if the ion permeabilities are also variable.

The cells of the toad skin are possibly a good example where it is difficult to separate the simple- and solute-induced water movements. In the first place, it is likely that the water permeability of the apical side of the skin is hormonally controlled (Acher et al., 1997). Second, as shown in Chapter 3, and described in the literature, there is evidence to suggest that at least the apical Cl⁻ permeability of the MRCs is variable, depending on

Figure 4.2: (A) Overall reduction in relative cell volume after human peripheral blood mononuclear lymphocytes were exposed to a solution made hypertonic by addition of 300mOsm/L NaCl or sucrose. Notice how the cells act as perfect osmometers with sucrose but there is a small regulatory volume increase with NaCl. Points are representative of four experiments; (B) Transient volume increase and regulatory volume decrease of cells after exposure to hypotonic solutions followed by a rapid reduction in volume and a regulatory volume increase upon exposure to an isotonic solution. Cell volumes were measured using a Coulter counter. (Taken from Grinstein et al., 1983). (C) KCl uptake in Ehrlich ascites tumour cells following an increase in external osmolarity from a hypotonic solution of 225mOsm (in which the cells had been bathed for 20-40mins solution) to a solution with a tonicity of 300mOsm at t = 0 minutes. Notice that in this case cell water and cell ion content were measured and not volume (Taken from Hoffman et al., 1983).
the membrane voltage. Studies where the MRC volumes have been recorded show that when the osmolality of the bathing solution is increased by around 50 mOsm/kgH₂O during a transepithelial voltage clamp at 100 mV (serosa positive), where the Cl⁻ channels are presumably open, the MRCs undergo a volume reduction of approximately 20% within about 1 minute, and then partially recover this volume (Figure 4.3A, Larsen et al., 1987). Because there was no change in the ionic concentration it can be surmised that the rapid shrinkage in this case was due to simple water movements, and the slower regulatory volume increase was due to solute-induced water movements. However, this is not always the case. Figure 4.3B, taken from Foskett & Ussing’s work in 1986, shows that when the solution is kept constant, a 40-50% volume increase can occur within approximately 1-2 minutes (so long as apical Cl⁻ is present), when the skin is clamped at 150 mV (serosa positive).² With no change in the osmolality or ionic composition of the bathing solution, the rapid volume change may be attributed to solute-induced water movements as the apical Cl⁻ permeability increases. But in the absence of a voltage clamp, it is not always possible to identify whether the contribution to the overall movement of water is dominated by simple water movements or by solute-induced water movements. One method that has been adopted is to separate simple- and solute-induced water movements in a two-step process where the salt concentration, and then the osmolality, is successively changed. Figure 4.3C shows such an example where the NaCl concentration of the mucosal solution was reduced from 108 to 5 mM while maintaining the osmolality with mannitol. This resulted in a 10% (solute-induced) volume reduction over 19 minutes. Subsequent reduction of the osmolality of the mucosal solution from around 200 to 10 mOsm/kgH₂O resulted in about a 30% volume increase over 24 minutes, presumably due to simple water movements (Spring & Ussing, 1986). Clearly, the MRCs have the capacity to respond volumetrically. Yet, from these results, it is not clear whether water is catching up with solute movements, or whether solutes are catching up to water movements under pseudo steady-state conditions. Resolution of this issue is important if ion transport in cells of the toad skin (and of other epithelia) is to be accurately modelled.

In addition to the variable Cl⁻ permeability, it should be noted that in 1965 Ussing commented that “no matter how the volume changes were brought about, there usually

² If Cl⁻ has been previously removed and then restored to the bathing solution it may take longer for the volume change to occur as shown by the second voltage clamp in Figure 4.3B.
was for each individual skin a close correlation between the epithelial volume and the rate of active sodium transport as measured by the short-circuit current.” Although he does not explicitly say it, his results suggest that the apical Na⁺ permeability (PNa), either in the PCs or MRCs, may be volume-sensitive. (Interestingly, Ussing later writes in 1982, in reference to swelling of frog skin cells that: “The selective sodium permeability of the apical membrane is probably increased (Ussing, 1965) but in most cases that has only a minor effect on the volume regulation”). One of the aims of the present study was to test the hypothesis that PNa was indeed volume-sensitive. If this were true, then presumably Isc would decrease in hyperosmotic solutions and increase

Figure 4.3: (A) Increasing the osmolality of the bathing solution during a transepithelial voltage clamp (serosa positive) caused a rapid shrinkage in the MRCs presumably due to simple water movements The slower regulatory volume increase that followed was probably due to solute-induced water movements (Taken from Larsen et al., 1987); (B) The rapid volume change upon clamping the skin (serosa positive) was probably due to solute-induced water movements since there was no change in osmolality or ionic composition of the bathing solution (Taken from Foskett & Ussing, 1986); (C) Solute-induced water movements followed by simple water movements as the solutions are changed from the control condition of normal Ringer containing 108mM NaCl with an osmolality of ~200mOsm/kgH₂O to a low (5mM) NaCl isotonic solution and then to a hypotonic (5mM NaCl, 10mOsm/kgH₂O) solution (Taken from Spring & Ussing, 1986).
in hyposmotic solutions, as well as showing evidence of volume regulatory increases or decreases (see row (iv) Figure 4.1). If PNa were not volume-sensitive, then for a constant Na$^+$ concentration, Isc should remain the same when the bathing solution was changed between hyper- and hyposmotic solutions, assuming of course, minimal dilution effects (i.e. the change in volume is small compared with the change in cell Na$^+$ concentration). In addition no form of regulatory volume increases or decreases should be observed in the Isc parameter (see row (iii) Figure 4.1).$^3$

Thus, the main aims of this volume study were twofold: (1) to determine whether simple water transients occurred before solute-induced transients, and (2) to investigate the possible volume-sensitivity of PNa.

### 4.2 Methods

As described in Chapter 2, toad skins were dissected and mounted in Ussing chambers and the open-circuit voltage (Voc) and short-circuit current (Isc) were monitored for up to 20 hours, using custom-built software and electronics.

The composition of the solutions used in this series of experiments to bathe the skins on both sides is listed in Table 4.1. In addition to these solutions several pharmacological agents were used, including adenosine triphosphate (ATP), the Na$^+$ channel blocker, amiloride, and the Na$^+$/K$^+$-ATPase inhibitor, ouabain. These agents were applied to the apical/pond (p) side or the basolateral/belly (b) side, as shown in the results section and, where indicated, were washed out by flushing the appropriate bath three times with fresh aliquots of the desired bathing solution.

### 4.3 Results

#### 4.3.1 Archetypal Response

For the purposes of discussion later, and to draw the reader’s attention to some of the main features present in the following responses, an archetypal response and its associated V-I locus are presented in Figure 4.4. The response is based on data collected from a skin bathed in 250mOsm/kgH$_2$O, reduced NaCl Ringer (solution 40), but it has been modified manually to depict features seen in some, but not all other skins. When

---

$^3$ The Na$^+$ concentration in the bathing solution must be maintained at a sufficient level for Isc to be measurable (personal observations suggest a lower limit of 20mM Na$^+$ is necessary). This precluded the use of low ionic, low osmolality solutions such as distilled water to determine the volume-sensitivity of PNa using Isc as an indicator.
viewed in this way, it would appear that a slow, damped oscillation is occurring. However, because different skins display different features, the archetypal response has been divided into two phases. Phase 1 refers to the initial oscillation consisting of an increase in Isc and Voc followed by a decrease. As shown in the ensuing sections the initial increase is not always present. Phase 2 encompasses the second and third peaks of the oscillatory behaviour. Typically, the time taken to reach the peaks gets progressively longer, and the magnitude of the peaks gets successively smaller. In most cases the system is so damped that the third peak does not occur. The reasons for the variations in the archetypal response are examined later in the discussion.

4.3.2 ‘Independent’ and ‘Interleaved’ Controls

‘Independent’ control traces where only the open-circuit voltage (Voc), or the short-circuit current (Isc) were recorded in 250mOsm/kgH2O NaCl Ringer (Solution 1) and 250mOsm/kgH2O Na2SO4 (Solution 2) are shown in Figure 4.5A-D. It was found in both the Cl− and SO4^{2−} solutions that independent Voc and Isc recordings were not stable over periods of up to 20 hours. A small but fast upward transient lasting approximately

Table 4.1: Ionic concentrations of the solutions used in the present study. All concentrations are in mmol/L.

<table>
<thead>
<tr>
<th>Soln</th>
<th>Description</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Cl⁻</th>
<th>Hapes⁻</th>
<th>Gluconate</th>
<th>SO₄^{2−}</th>
<th>Glucose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>500mOsm/kgH2O NaCl</td>
<td>238</td>
<td>5</td>
<td>2.7</td>
<td>240.7</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>250mOsm/kgH2O NaCl</td>
<td>111</td>
<td>5</td>
<td>2.7</td>
<td>116.4</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>100mOsm/kgH2O NaCl</td>
<td>45</td>
<td>2.5</td>
<td>1.35</td>
<td>47.7</td>
<td>5</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>150mOsm/kgH2O NaCl</td>
<td>45</td>
<td>2.5</td>
<td>1.35</td>
<td>47.7</td>
<td>5</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>200mOsm/kgH2O NaCl</td>
<td>45</td>
<td>2.5</td>
<td>1.35</td>
<td>47.7</td>
<td>5</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>250mOsm/kgH2O NaCl (low K⁺ cf. Soln 63)</td>
<td>45</td>
<td>2.5</td>
<td>1.35</td>
<td>47.7</td>
<td>5</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>300mOsm/kgH2O NaCl</td>
<td>45</td>
<td>2.5</td>
<td>1.35</td>
<td>47.7</td>
<td>5</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>350mOsm/kgH2O NaCl</td>
<td>45</td>
<td>2.5</td>
<td>1.35</td>
<td>47.7</td>
<td>5</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>500mOsm/kgH2O Na2SO4</td>
<td>237.5</td>
<td>5</td>
<td>2.7</td>
<td>58</td>
<td>10</td>
<td>5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>250mOsm/kgH2O Na2SO4</td>
<td>111</td>
<td>5</td>
<td>2.7</td>
<td>28</td>
<td>10</td>
<td>5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>100mOsm/kgH2O Na2SO4</td>
<td>45.51</td>
<td>2.5</td>
<td>1.35</td>
<td>5</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>150mOsm/kgH2O Na2SO4</td>
<td>45.51</td>
<td>2.5</td>
<td>1.35</td>
<td>5</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>200mOsm/kgH2O Na2SO4</td>
<td>45.51</td>
<td>2.5</td>
<td>1.35</td>
<td>5</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>250mOsm/kgH2O medium NaCl, high K⁺</td>
<td>45</td>
<td>100.9</td>
<td>1.35</td>
<td>47.7</td>
<td>5</td>
<td>49.2</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2-3 minutes was sometimes observed in both Isc and Voc at the commencement of experiments, examples of which are shown in the Isc traces of Figure 4.5C & D. Furthermore, because this transient was present in Cl\(^-\) and SO\(_4^{2-}\) solutions it was concluded that this was a Cl\(^-\) independent event. Following this transient, a slower rundown in both the independent Voc and Isc recordings was generally observed over the ensuing 30-80 minutes, although the time course was variable. This was most often followed by an increase in both Voc and Isc until a local maximum was reached. After this, Voc and Isc would typically decrease again, sometimes in a two-step process, where the gradient of the Voc or Isc decline would suddenly change. This change in gradient did not appear to be linked to a particular time after toad pithing. In some cases, such as in Figure 4.5D which shows changes in Isc, a second local maximum could be observed as if the electrophysiological parameters were oscillating slowly over hours.

‘Interleaved’ control traces where Voc and Isc measurements were recorded alternately in the same skin displayed similar transients to independent control traces.
Figure 4.5: Toad skin responses measured (A & B) under only open-circuit voltage (Voc) conditions; (C & D) under only short-circuit current (Isc) conditions; (E & F) under mainly Voc conditions but with interleaving of Isc (G & H) under mainly Isc conditions but with interleaving of Voc in either standard 250mOsm/kgH₂O NaCl amphibian Ringer (column 1) or 250mOsm/kgH₂O Na₂SO₄ (Cl⁻-free) Ringer (column 2). Notice that in all cases both Isc and Voc are not stable over hours.
where only Voc or Isc were recorded. That is, under conditions where Voc was mainly recorded with intermittent measurements of Isc (Figure 4.5E & F), or where Isc was mainly recorded with intermittent measurements of Voc (Figure 4.5G & H), an initial rundown, followed by an increase to a local maximum and then a slow decrease could be observed in the Voc and Isc traces in both Cl− and SO4²⁻ solutions.

From these control traces, three observations can be made: (1) interleaving the Voc and Isc recordings is not the main cause of the transient behaviours because the instabilities are evident in independent recordings of Voc and Isc; (2) whatever is causing the transients appears to be independent of the time spent under either Voc or Isc conditions and (3) although slightly muted in the SO4²⁻ solution, the transients can be observed in both the Cl− and SO4²⁻ solutions.

4.3.3 Metabolic shutdown due to lack of ATP

One issue that was considered was whether the initial rundown of Isc and Voc was due to cytoplasmic depletion of ATP stores. It was hypothesised that when the isolated skins were bathed in high NaCl solutions, apical Na⁺ entry would cause a transient increase in the intracellular Na⁺ concentration. The additional Na⁺ ions could bind to the basolateral Na⁺/K⁺-ATPase pumps, assuming that these were not already kinetically limited by Na⁺, resulting in an increase in the number of ions translocated per unit time. In the process energy would be expended and cytoplasmic ATP stores would diminish. As the ATP stores were reduced, the pumps would slow due to an ATP kinetic limit, causing a reduction in Isc (and Voc). If this were the case, then supplementing cytoplasmic ATP would presumably slow the rundown, or even cause an increase in Isc and Voc. Attempts were made to do this by adding ATP to the apical bathing solution. (Addition of ATP to the basolateral bathing solution had no effect).

Following the typical rundown of Isc and Voc in 250mOsm/kgH₂O Na₂SO₄ solution, the addition of 10mM apical ATP caused a rapid increase in both parameters (Figure 4.6A). Isc increased from 8.8 to 19.0 µA with a time constant of $\tau=5.3$ minutes. This was followed by a slow decline with a time constant of $\tau=422.3$ minutes back to 5.9 µA recorded at t=1200 minutes. Voc behaved in a similar manner upon application of ATP, increasing from 29.9 to 53.6mV with a time constant of $\tau=3.1$ minutes, and then

---

4 Presumably, ATP production is part of a feedback loop. It would therefore be expected that following a drop in ATP levels there would be a subsequent upregulation of ATP production. In the isolated toad skin, this feedback loop may or may not be operational.
Figure 4.6: Changes in Isc (grey lines) and Voc (black lines) after ATP in (A) 250mOsm/kg H2O Na2SO4; (B) 100mOsm/kg H2O NaCl; (C) 250mOsm/kg H2O NaCl. The effects of the P2x, P2y purinoceptor inhibitor, suramin is also shown in (B) & (C). Note: (p) = pond and (b) = belly.
declining to 19.9mV at t=1200 minutes with a time constant of $\tau=564.1$ minutes. The trends observed in Isc and Voc initially seemed consistent with the diffusion of ATP into the cells supplementing ATP stores that were slowly being depleted.

Only at the relatively high concentrations of 10mM ATP could an effect be elicited, as shown by the cumulative dose response curve of Figure 4.6B. Following the initial rundown in 100mOsm/kgH2O NaCl solution, apical application of 0.1mM and 1mM ATP had little effect. But application of 10mM ATP caused an increase in Isc from 8.3 to 13.9$\mu$A which subsequently decayed to 9.3$\mu$A at t= 318.0 minutes (although this was not a minimum). A large increase in Voc was also observed from 9.8 to 39.4mV with a time constant of $\tau=1.8$ minutes. This was followed by a decrease in Voc which exhibited initial trends similar to the sulphate example of Figure 4.6A. Interestingly, increasing the ATP concentration further with two successive doses of 30mM ATP had negligible effects, suggesting that an ATP saturation limit had been attained. It was concluded therefore that 10mM ATP was a suitable ‘test’ dose from which a rapid and definable response from the toad skin could be elicited.

Although the ATP effect appeared to be saturable, it was also reversible. When ATP was washed from the apical bathing solution a rapid reduction in Isc and Voc was observed. In the 100mOsm/kgH2O NaCl solution, washing out the accumulated 71.1mM ATP (Figure 4.6B at ‘g’) caused a rapid increase in Isc from 6.8 to 7.8$\mu$A which then dropped to 2.7$\mu$A and remained quite stable over the ensuing 37 minutes. Voc went through a rapid transient, first increasing from 15.6 to 19.9mV then dropping to 8.4mV and then increasing to 17.6 mV over the following 61 minutes, at which point 10mM ATP was again added to the apical bathing solution. Isc and Voc increased to a peak as before, and then slowly decreased until the ATP was washed out of the bathing solution, whereupon Isc and Voc dropped from 6.6 to 2.4$\mu$A and from 38.2 to 17.0mV, respectively. This reversible and reproducible ATP effect was also demonstrated in 250mOsm/kgH2O NaCl solution (Figure 4.6C). Apical application of 10mM ATP caused an increase in Isc from 5.1 $\mu$A to a peak of 8.2 $\mu$A at t=121.0 minutes and an increase in Voc from 5.3mV to a peak of 21.6mV at t=137.1 minutes. Washing out the ATP resulted in a drop in Isc from 5.1 to 2.5$\mu$A while Voc dropped from 17.9 to 4.4mV and then slowly increased to 5.9mV at t=235.0 minutes. At this point 10mM ATP was again added to the apical solution showing that the response could be elicited repeatedly. Isc increased from 2.9 to 6.3 $\mu$A while Voc increased from 5.9 to 23.4mV.
As before, washing out the ATP caused a drop in Isc from 4.0 µA to a local minimum of 2.7 µA, and a drop in Voc from 22.8mV to a minimum of 6.5mV over 9.3 minutes.

Because the ATP effect was rapidly reversible, saturable and could be re-initiated, the possibility that ATP was binding to extracellular receptors instead of diffusing into the cells was considered. In many tissues ATP has an affinity for P2x and P2Y purinoceptors (Abbracchio & Burnstock, 1994; Ralevic & Burnstock, 1998). Therefore, the broad-spectrum P2x and P2Y inhibitor, suramin, was applied to the apical solution at a concentration of 100µM to see whether this would reduce any subsequent response to ATP. As shown in Figure 4.6 the ATP effect was not diminished after suramin application with Isc increasing from 2.7µA to a peak of 9.0µA (Figure 4.6B) and from 3.4µA to a peak of 8.3µA (Figure 4.6C). Moreover, the rise in Voc in both examples was larger after suramin than before it, with increases in Voc from 21.9mV to a peak of 48.6mV (Figure 4.6B), and from 11.0mV to a peak of 36.2mV (Figure 4.6C). However, although the magnitudes of the responses were not reduced, the ATP effects before suramin had a more rounded peak than those recorded after suramin, which rose sharply and then decayed more rapidly with signs of an oscillation.

4.3.4 Effect of the osmolality of the bathing solution on Isc

Another possible cause for the initial rundown seen in the control experiments was that the cells were shrinking in the 250mOsm/kgH2O solutions. Early experiments conducted in the study had shown that the skins were osmotically sensitive. In general, it had been found that when the osmolality of the bathing solution was increased in steps using sucrose, without changing the ionic concentrations, there was a corresponding decrease in Isc.5 But when the osmolality was reduced, a transient increase in Isc was observed.

Examples of the osmotic sensitivity of Isc are presented in Figure 4.7, showing the reduction in Isc in Cl--free Ringers (column 1) and Cl– Ringers (column 2) as the osmolality of the bathing solution was increased in 50mOsm/kgH2O steps. The grey exponentially decreasing curves overlayed upon the data are a representative Isc response recorded from one toad bathed in 250mOsm/kgH2O Na2SO4 (Cl--free) Ringer (see Figure 4.8F). The time constant of this response was τ=23.3 minutes. (The grey

---

5 Voc traces are not shown in this chapter because, as has already been discussed in Chapter 3 (see Section 3.3.3), Voc is largely determined by the open probability of the apical voltage-dependent Cl channels. It is not a ‘first-order’ indicator of what is occurring volumetrically.
**Figure 4.7:** Effect on Isc (black) when the osmolality of the bathing solution was changed using sucrose (as shown by the numbers on each trace in mOsm/kgH2O). Experiments were conducted in Na2SO4 (Cl\(^-\)-free) Ringer (column 1) and NaCl Ringer (column 2). Superimposed on each trace (grey) is the Isc trace recorded from Toad 107c in 250mOsm/kgH2O Na2SO4 Ringer (see Figure 4.7F). This trace was fixed in the horizontal direction so that the time constant was not affected but it was scaled by eye in the vertical direction to best fit the Phase I 'rundown' of the data in each trace. Note: In (F) r.e.=rezeroed electrodes.
exponentially *increasing* curves were constructed using the same time constant). The overlays shared the same abscissa so comparisons within and between skins could be made but the individual graphs were scaled in the vertical direction by eye to obtain a fit to the data. The overlays were used to show that the rundown responses were similar in the Cl⁻ and Cl⁻-free solutions, and that the same mechanism was probably responsible for the initial rundown in the different solutions. In terms of the mechanism, it should be emphasised that only the sucrose concentrations were altered and not the ionic concentrations. Therefore, the observed rundown with increasing osmolality was probably due to a simple shrinkage as water moved out of the cells. This is also consistent with the observed increase when the osmolality was subsequently reduced and water presumably entered the cells. Using the time constant from the overlayed Cl⁻-free data, it would appear that simple water movements have a time constant of around $\tau_{\text{simple}} = 23$ minutes.

Following the initial rundown it is evident that in some cases when the osmolality was increased, a secondary effect occurred with Isc increasing transiently until a local maximum was reached, after which a decline was observed (see cross-hatched regions in A, E, F, G). This was possibly due to an RVI response, which incidentally appears to be more pronounced in the Cl⁻ solutions than the Cl⁻-free solutions. In contrast, when the external osmolality was reduced, a decrease in Isc was observed following the initial increase (see hatched regions in A, B, C, F, H). This may have been due to an RVD response, which again appears to be stronger in the Cl⁻ solutions.

Finally, the changes in Isc as the osmolality is varied suggest that Isc is osmotically sensitive. This is important because, since Isc is dominated by the Na⁺ current (Ussing & Zerahn, 1950), it suggests that Na⁺ is possibly volumetrically sensitive. This issue is addressed in more detail in the discussion.

### 4.3.5 Effect of osmolality & ionic concentration of the bathing solution on Isc and Voc

As described in the introduction, it is possible to divide the total water movement into simple- and solute-induced water movements. So far, the issue of simple water movement has been considered, and it was found that, irrespective of the osmolality of the bathing solution, $\tau_{\text{simple}} = 23$ minutes. In this section, the effect on Isc and Voc of skins bathed in solutions with different ionic concentrations (and osmolalities) is examined over periods of up to twenty hours.⁶ Because of the variability between skins, measurements were made mainly under open-circuit conditions with intermittent samplings of Isc.

---

⁶ Measurements were made mainly under open-circuit conditions with intermittent samplings of Isc.
Figure 4.8: Open-circuit voltage, Voc (dark lines) and short-circuit current, Isc (grey lines) measured over periods of up to 20 hours in 500mOsm/kgH₂O Na₂SO₄ Ringer (column 1), 250mOsm/kgH₂O Na₂SO₄ Ringer (column 2) and 100mOsm/kgH₂O Na₂SO₄ Ringer (column 3). The associated V-I loci are shown as inserts. Note the different vertical scales.
Figure 4.9: Open-circuit voltage, \( \text{Voc} \) (dark lines) and short-circuit current, \( \text{Isc} \) (grey lines) measured over periods of up to 20 hours in 500mOsm/kgH\(_2\)O NaCl Ringer (column 1), 250mOsm/kgH\(_2\)O NaCl Ringer (column 2) and 100mOsm/kgH\(_2\)O NaCl Ringer (column 3). The associated V-I loci are shown as inserts. Note the different vertical scales.

IONIC CHANGES: Cl\(^{-}\) PRESENT

[Na\(^{+}\)]=238mM, [K\(^{+}\)]=5mM, [Cl\(^{-}\)]=241mM

[Na\(^{+}\)]=111mM, [K\(^{+}\)]=5mM, [Cl\(^{-}\)]=116mM

[Na\(^{+}\)]=45mM, [K\(^{+}\)]=2.5mM, [Cl\(^{-}\)]=48mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=238mM, [Cl\(^{-}\)]=45mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=2.5mM, [Cl\(^{-}\)]=241mM

[Na\(^{+}\)]=238mM, [K\(^{+}\)]=5mM, [Cl\(^{-}\)]=116mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=2.5mM, [Cl\(^{-}\)]=45mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=238mM, [Cl\(^{-}\)]=241mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=2.5mM, [Cl\(^{-}\)]=116mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=238mM, [Cl\(^{-}\)]=45mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=2.5mM, [Cl\(^{-}\)]=241mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=238mM, [Cl\(^{-}\)]=116mM

[Na\(^{+}\)]=5mM, [K\(^{+}\)]=2.5mM, [Cl\(^{-}\)]=45mM
five representative examples recorded in each solution have been presented so that responses within and between groups may be observed. The data are presented in three main groups, showing the effects of (i) 500, 250 & 100mOsm/kgH₂O Na₂SO₄ (Cl⁻-free) solutions (Figure 4.8); (ii) 500, 250 & 100mOsm/kgH₂O NaCl solutions (Figure 4.9), and (iii) 250mOsm/kgH₂O low and high K⁺ solutions (Figure 4.10). The five Isc results recorded in each solution have then been normalised and overlayed giving an overview of the major trends and differences (Figure 4.11) as summarised below.

(1) Na₂SO₄ data

It can be seen in the 500mOsm/kgH₂O solution (Figure 4.8, column 1) that Isc typically decreased, plateaued and then increased. Initial Isc values ranged between 15.5µA (C) and 47.9µA (E) while the final values ranged between 0µA (E) and 46.8µA (C). Voc followed much the same trend as Isc although the final increase was much less pronounced. Initial Voc values ranged between 14.8mV (B) and 61.6mV (E) and final values ranged between 0mV (E) and 5.5mV (C). Variations on the ‘decrease, plateau, increase’ theme are seen in D, where Isc and Voc increased rapidly at the very beginning before decreasing, and in E, where in addition to the same initial transient as seen in D, Isc displayed oscillatory behaviour with local minima occurring at 302.5 minutes and 801.7 minutes.

Isc and Voc responses recorded in the 250mOsm/kgH₂O Na₂SO₄ solution (Figure 4.8, column 2) were somewhat different from those measured in the 500mOsm/kgH₂O Na₂SO₄ solution. As in the 500mOsm/kgH₂O Na₂SO₄ traces, the initial rundown was evident in all examples (with (H) preceded by a brief increasing transient similar to (D) and (E)). Initial Isc values ranged between 8.2µA (H) and 60.6µA (F) while initial Voc values ranged between 44.7mV (H) and 107.7mV (G). Unlike the 500mOsm/kgH₂O Na₂SO₄ traces a ‘hump’ region occurred in Isc and Voc after the initial rundown in all 250mOsm/kgH₂O Na₂SO₄ traces. This region looked similar in shape to what appeared.

7 It is possible that the increases in Isc observed in the 500mOsm/kgH₂O Na₂SO₄ and NaCl experiments after about 10 hours were artefactual in accordance with Ohm’s Law (V=IR). That is, because the resistance of these skins had become so low (i.e. 100-200 ohms), small electrode drifts of around 5mV meant that relatively large currents were observed (i.e. 50µA). Certainly, this is consistent with the observation that bathing the apical membranes of anuran skins in hypertonic solutions can cause the tight junctions to open, and hence the skin resistance to decrease (see Section 1.3.4). However, in higher resistance skins (i.e. skins in the early stages of the 500mOsm/kgH₂O experiments or skins bathed in 100- or 250mOsm/kgH₂O solutions), this was not an issue.
to be RVD responses in Figure 4.7. It is therefore possible that the ‘hump’ region was due to RVD. The local Isc maxima in this region ranged between 4.4\(\mu\)A (H) and 15.7\(\mu\)A (I) occurring anywhere between 317.7 minutes (H) and 419.7 minutes (J), while the local Voc maxima ranged between 41.0 (F) and 73.9mV (G), occurring between 308.8 minutes (H) and 426.2 minutes (I). After reaching the local maxima, Voc and Isc tended to decline. In some cases (G-J), there was evidence of a subsequent increase in Isc, and in particular Voc, as if these parameters were tending toward a second, but smaller local maximum, and perhaps oscillating (H, J).

Recordings from skins bathed in the 100mOsm/kgH\(_2\)O Na\(_2\)SO\(_4\) solution (Figure 4.8, column 3) appeared to be much more stable over time when compared with those made in the 500- or 250mOsm/kgH\(_2\)O Na\(_2\)SO\(_4\) solutions. Initial Isc values ranged between 16.5\(\mu\)A (O) and 59.1\(\mu\)A (L) while initial Voc values ranged between 60.2mV (M) and 89.6mV (L). Following an initial transient period generally occurring within the first 45 minutes, Isc in three out of the five examples shown (M-O) remained quite stable until around 600 minutes (10 hours), after which a slow decrease was observed. Of the other two examples, Isc remained stable until approximately 300 minutes in (L) but decreased monotonically in (K). In all cases Voc exhibited overall trends similar to Isc.

(2) NaCl data

All skins bathed in the 500mOsm/kgH\(_2\)O NaCl solution (Figure 4.9, column 1) displayed an initial transient where Isc and Voc started off low and then peaked over the next 15-34 minutes with Isc consistently peaking 3-6 minutes earlier than Voc. Initial Isc values ranged between 0.4\(\mu\)A (A) and 15.4\(\mu\)A (E) and initial Voc values ranged between 0.3mV (A) and 3.1mV (E). Peak Isc values were 2-3 times larger than peak Voc values and ranged between 9.3\(\mu\)A (C) and 40.7\(\mu\)A (E), and 3.9mV (C) and 14.2mV (D), respectively. Following the peaks, Isc and Voc generally decreased, plateaued and then most often increased (see Footnote 7), with most change observed in Isc. This ‘rundown, plateau, increase’ behaviour was similar to responses observed in the 500mOsm/kgH\(_2\)O Na\(_2\)SO\(_4\) solution.

The initial Isc and Voc transients so clear in the 500mOsm/kgH\(_2\)O NaCl solution were notably absent in three out of the five examples (F-H) shown for skins bathed in the 250mOsm/kgH\(_2\)O NaCl solution (Figure 4.9, column 2). The Isc transient was observed in (I) and (J), but the Voc transient was only seen in (I). Because the transients appeared in some but not other examples, the ranges in the initial Isc and Voc values were larger compared with other solutions and differed between 1.2\(\mu\)A (J) and 71.7\(\mu\)A (G), and
11.9mV (I) and 50.1mV (F), respectively. Similar to the 250mOsm/kgH2O Na2SO4 solution, three traces (G, H, J) displayed evidence of a ‘hump’ or local maximum in Isc and Voc occurring between approximately 460 minutes and 730 minutes and ranging respectively from 7.8µA (J) to 12.7µA (G), and 19.9mV (J) to 24.0mV (G,H). Figure 4.9(I) displayed evidence of two local maxima after the initial transient, whereas (F) did not exhibit this behaviour.

Traces recorded from skins bathed in the 100mOsm/kgH2O NaCl solution (Figure 4.9, column 3) appeared to be much more stable than recordings made from skins bathed in the 500- and 250mOsm/kgH2O NaCl solutions. Initial Isc values ranged from 14.8µA (L) to 53.7µA (O) while initial Voc values ranged from 17.8mV (L) to 76.4mV (K). Following an initial transient period lasting approximately 35-80 minutes, Isc remained reasonably constant in all examples except (N), until around 600 minutes, after which Isc decreased. Having said that, a muted oscillation could be seen in some of these examples. However, the magnitudes were small and therefore Isc values appeared relatively constant. These oscillatory effects are shown more clearly in Figure 4.11F where Isc has been normalised. While Voc displayed similar trends to Isc in some cases such as (K) & (N), in other examples such as (L) & (O), Voc increased slowly during the relatively constant Isc phase prior to t=600 minutes. But perhaps of most interest was the pronounced increase in Voc shown in (M) which possibly represented the spontaneous inactivation of the apical voltage-dependent Cl− permeability from a fully open state to a fully closed state (see Chapter 3).

(3) KCl data

To investigate whether part of the rundown observed in the Na2SO4 and NaCl solutions was due to the efflux of KCl from cells, the effect of varying the K+ concentration of the bathing solution was examined. Figure 4.10 shows Voc and Isc traces recorded in a low K+ solution (column 1) and in a high K+ solution (column 2), both with an osmolality of 250mOsm/kgH2O so that simple water movements were similar.

In the low K+ solution initial Isc values ranged from 13.3µA (B) to 42.2µA (A), while the initial Voc values ranged from 26.5mV (C) to 66.8mV (A). An increasing Isc transient in (E) and a Voc transient in (B) preceded a rundown in Isc and Voc that was present in all examples. Following the rundown, local maxima in Isc and Voc were seen in traces (B) & (D), but were absent in (E). The local Isc maxima ranged from 3.6µA
Figure 4.10: Open-circuit voltage, Voc (dark lines) and short-circuit current, Isc (grey lines) measured over periods of up to 20 hours in 250mOsm/kgH2O medium NaCl, Low K⁺ Ringer (column 1) and 250mOsm/kgH2O medium NaCl, High K⁺ Ringer (column 2) The associated V-I loci are shown as inserts. Note the different vertical scales.
(B) to 9.3µA (D), occurring at 249.7 and 263.6 minutes, respectively. The local Voc maxima ranged from 9.5 (B) to 18.8mV (D) occurring at 248.0 and 325.1 minutes, respectively. The plateau regions seen in (A) and (C) at around t=200-230 minutes were probably due to the same mechanism responsible for the local maxima in (B) & (D). Evidence of a second, but smaller peak between t=512-596 minutes in (A) and (C) could also be observed.

In the high K⁺ solution the initial Isc values ranged from 14.2µA (G) to 22.8µA (H) while the initial Voc values ranged from 6.7mV (F) to 27.3mV (J). Although the ranges were slightly smaller than for the low K⁺ solutions, the high extracellular K⁺ concentration appeared to mute the rundown typically observed in the low K⁺ solution. This may have been due to a slowing of whatever was causing the rundown, or because the mechanism responsible for the local maxima seen in the low K⁺ solution was having an increasingly dominant effect.⁸ (Certainly, the local peak values in the high K⁺ solutions occurred over a similar time period to the local maxima in the low K⁺ solutions, ranging between 132.5 minutes (J) to 209.4 minutes (G) for Isc, and from 144.7 minutes (H) to 316.0 minutes (G) for Voc). Whatever the case, the Voc and Isc traces in the high K⁺ solution remained elevated during a period typically characterised by a decline in magnitude of these parameters in the low K⁺ solution.

(4) Normalised Isc Overlays

For comparative purposes the Isc data presented in Figures 4.8, 4.9 & 4.10 were each graphed separately with each abscissa the same length over a period of 0-1200 minutes. The five graphs in each group described earlier were then shifted in the horizontal direction (but not resized), and then scaled by eye in the vertical direction until the initial transient, in most cases, overlay. (The initial transient in the 250mOsm/kgH₂O high K⁺ data was so muted that the data were normalised to the final rundown). Because the abscissas were all the same, a direct comparison of the time constants within and between the different groups could be made. In addition, other nuances were easily observed. Figure 4.11 shows the Isc overlays for each of the Na₂SO₄, NaCl and K⁺ groups. Inserts of the unadjusted Isc data over 600 minutes have been included for convenience. As summarised below, some interesting points arise when the data are presented in this way.

---

⁸ It should be noted that from this data it is not possible to tell whether the same mechanism, or separate mechanisms are responsible for the differences seen in the high and low KCl solutions.
NORMALISED COMPARISONS

A. 500mOsm/kg H₂O Na₂SO₄

B. 500mOsm/kg H₂O NaCl

C. 250mOsm/kg H₂O Na₂SO₄

D. 250mOsm/kg H₂O NaCl

E. 100mOsm/kg H₂O Na₂SO₄

F. 100mOsm/kg H₂O NaCl

G. 250mOsm/kg H₂O medium NaCl, low K⁺

H. 250mOsm/kg H₂O medium NaCl, high K⁺

**Figure 4.11:** Overlays of the short-circuit currents (Isc) presented in Figures 4.8, 4.9 & 4.10 normalised by eye in the vertical direction but not in the horizontal direction so that the time constants remained fixed. The grey curve is an exponential with a time constant of 23 minutes. This time constant was the time constant of the curve used to fit the sucrose data of Figure 4.7 where only first-order water movements occurred.
First, the local ‘hump’ region, possibly due to a regulatory volume increase, can be seen quite clearly in the 250mOsm/kgH$_2$O Na$_2$SO$_4$ solution (C) and the 100mOsm/kgH$_2$O NaCl solution (F). A muted version of this, shifted slightly to the left, is evident in the low K$^+$ solution (G) and appears to dominate the high K$^+$ solution (H). There is also some evidence of the local maxima appearing in the 250mOsm/kgH$_2$O NaCl solution (D). Furthermore, it is possible that the slow increases in Isc observed towards the end of the experiments in both the 500mOsm/kgH$_2$O Na$_2$SO$_4$ and NaCl solutions (A, B) may be local maxima that have just been delayed.

Second, the grey overlays, representing the exponential for simple water movements with a time constant of 23 minutes, fit the Phase I rundown reasonably well in most cases, despite up to a five-fold increase in osmolalities and a five-fold increase in the extracellular Na$^+$ and Cl$^-$ concentrations. The obvious exceptions are the skins bathed in the 250mOsm/kgH$_2$O NaCl and high K$^+$ solutions, where the Phase I rundown are markedly slower than expected for simple water movements. Other less pronounced deviations from the simple water movement rundown include the slightly faster decrease in Isc in the 100mOsm/kgH$_2$O Na$_2$SO$_4$ examples, and the departure of the traces in the 500mOsm/kgH$_2$O solutions just prior to the simple water exponential settling to a constant value. The reasons for this behaviour are addressed overleaf.

4.4 Discussion

4.4.1 Control Data

Based on a lack of evidence to the contrary in the literature, one of our main reasons for investigating ionic transport in vitro in the abdominal skin of the toad was the belief that the standard electrophysiological parameters, Isc and Voc were relatively stable over several hours. Originally, this seemed important because some of our previous work conducted in vitro on the mammalian pumping epithelia, stria vascularis of the inner ear, had shown that cell potentials were not stable, degenerating over the first 30-60 minutes following removal of the tissue from the animal (Figure 4.12). Without a stable control situation, it was difficult to perform meaningful experimental manipulations to show how ions were transported. Wanting to avoid such a situation again, and not realising at the time that the slow change of cell potentials may have been
Figure 4.12: Similar rundown of electrophysiological parameters recorded *in vitro* from (A) & (B) marginal cells of *stria vascularis*, a mammalian pumping epithelial layer of the inner ear, and (C) across the (amphibian) abdominal skin of *B. marinus*. For comparative purposes recordings in (C) were shifted horizontally so that the start times of the experiments corresponded. In (A) and (B) the intracellular potentials were measured and averaged from different tissues bathed in artificial endolymph, a K⁺-rich solution, and artificial perilymph, a Na⁺ solution. (Endolymph and perilymph are solutions found in the inner ear). The toad skin in (C) was bathed in a 250mOsm/kg H₂O NaCl Ringer solution with [Na⁺]=45mM, [Cl⁻]=48mM, [K⁺]=2.5mM and [sucrose]=146mM (see Figure 4.9E). (Strial data taken from Thomson, 1995).
an intrinsic property of how the epithelia worked, the ‘simpler’ and ‘more stable’ toad skin preparation was investigated.

Although some investigators have reportedly measured stable Isc currents (at $V_{clamp}=0$ mV) over several hours (Hillyard, personal communication), the findings of Figure 4.5 are interesting because they show clear examples where Isc and Voc are not stable over periods of up to 20 hours when bathed in 250mOsm/kgH$_2$O NaCl Ringer, a standard solution used by Ussing and subsequent investigators, and 250mOsm/kgH$_2$O Cl$^-$ free solutions. It is also noteworthy that the parameters often declined significantly during the first few hours of experimentation, a period during which other investigators have evidently measured Isc, and presumably Voc. Just why this unstable behaviour has not been reported previously is not entirely clear. But the overall trends presented in this study are quite reproducible despite experiments commencing at different times of the day (anywhere between 8.30 a.m and 11 p.m.), in different seasons, using different batches of toads, different recording equipment and different clamp protocols. Even when the skins were bathed in different solutions, points of commonality can be identified. For these reasons, it would appear that the responses are physiological in origin and are not artifactual.

4.4.2 Volumetrically sensitive Isc

One of the interesting findings of this study was the apparent volumetric sensitivity of Isc. This was demonstrated by the step-wise reduction in Isc when a water gradient was created by successively increasing the osmolality of the bathing solution using sucrose whilst maintaining the ionic concentration (Figure 4.7). Conversely, a reduction of the bathing solution osmolality resulted in an increase in Isc. Thus, there appeared to be a correlation between osmolality (presumably affecting cell volume) and Isc: as the bathing osmolality increased and the epithelial cells presumably shrank, Isc decreased; as the epithelial cells presumably swelled Isc increased.

These findings are consistent with those of Spring and Ussing (1986) who showed a direct increase in cell volume when the apical solution was made hypotonic (Figure 4.3C). In their experiments, the time course of this change appeared to be about 25-30 minutes (although the entire response was not presented). This is of the same order of

\footnote{It remains to be verified that the rundown of the cell potentials was indeed due to an intrinsic change in \textit{stria vascularis} such as permeability changes. This is only one of many possible causes of the observed rundown and it should be emphasised that in moving from an \textit{in vivo} to and \textit{in vitro} situation, it was not possible to control for many variables which may have contributed to the decline.}
magnitude as the 23-minute time constant used to fit the Isc data in this study when the osmolality was altered. Also, it is worth mentioning that Ussing and Spring’s results show that the 200mOsm/kgH₂O ‘isotonic’ solution, applied just prior to the hypotonic solution, caused a reduction in the mitochondria-rich cell (MRC) volume. But if the solution were truly isotonic with the cells, then no volume change would have occurred. Therefore, the results suggest that the intracellular osmolality of this cell at least, was lower than 200mOsm/kgH₂O. The exact osmolality will of course change from cell to cell, but it is certainly noteworthy that in the present study, the volumetrically sensitive Isc remained more stable in the 100mOsm/kgH₂O solutions than in the 250- or 500 mOsm/kgH₂O solutions (Figures 4.8, 4.9). Combining the information from Spring and Ussing’s paper and this study, it would appear that overall, the pumping epithelial cells of the toad skin have an osmolality somewhere between 100 and 200mOsm/kgH₂O, with values probably biased towards the lower limit.

4.4.3 Mechanism regulating Isc: volume-sensitive PNa?

The relative ease with which Isc can be measured makes it a useful experimental tool. However, it should be remembered that Isc is not a simple function. For example, theoretically determining Isc using a single cell model as the simplest approximation of a toad skin requires that there is no net charge build-up within the cell, the sum of the transcellular and paracellular currents across the skin is zero, and the sum of the individual ionic currents into and out of the cell is also zero (see Chapter 6). Based on this model it is easy to see that Isc is influenced by a number of parameters.

Despite this, one of the simplest statements that can be made about the mechanism responsible for the changes in Isc is that it is not Cl⁻ dependent, because similar effects are observed in Cl⁻ and Cl⁻ free bathing solutions when the osmolality is increased or decreased (Figure 4.7). It can therefore be concluded that Isc is not largely dependent on Cl⁻ ion transport via the apical or basolateral Cl⁻ channels or the Na⁺/K⁺/2Cl⁻ symports.

Using the steady-state mathematical model presented in Chapter 6 it can be shown that reducing the apical Na⁺ permeability or basolateral K⁺ permeability, or increasing the putative apical K⁺ permeability, produces a reduction in Isc and Voc, consistent with the experimental results of Figure 4.7. While there are a variety of ways in which the ion permeability of cells can be altered,¹⁰ this study suggests that changes in cell volume

¹⁰ Broadly speaking, two common methods for altering the ion permeability of a cell are: (1) changing the channel pool size by the incorporation or disintegration of channels into a membrane; or (2) altering the opening probability of channels. In the case of the toad skin, the rapid initiation of changes in Isc and
may cause changes in cell permeability of the toad skin. Thus, from a theoretical perspective, ascribing any one of the three permeabilities described above with a volume-sensitivity, such that the apical Na⁺ permeability or basolateral K⁺ permeability decreased, or the apical K⁺ permeability increased as the cells shrank, would be feasible. However, from an experimental perspective, it has been shown that Isc is dependent on apical Na⁺ and not K⁺ (Ussing & Zerahn, 1950), and a rapid reduction in Isc occurs when the apical side is bathed in a Na⁺-free solution, or the apical Na⁺ permeability is blocked with amiloride (see Chapter 3). Based on this information, the simplest explanation for the reduction in Isc with increasing osmolality is that the apical Na⁺ permeability shuts down as the cells shrink.

From a ‘design’ perspective, it certainly makes good ‘sense’ to regulate the apical Na⁺ permeability because the osmolality of most solutions which the toad would encounter in the wild would be determined by NaCl and not KCl. Of course, in freshwater it is unlikely that there is any need for Na⁺ regulation: the toad absorbs water through aquaporins until it is sufficiently hydrated, at which point arginine vasotocin levels decrease, mRNA expression of the aquaporins is reduced, aquaporin insertion into the apical membranes ceases and any residual apical aquaporins presumably disintegrate. (Alternatively, after hydration, the toad jumps out of the water so no Na⁺ regulation is required). But in brackish water the cane toad relies upon the uptake of salt to create a sufficient osmotic gradient for water absorption across its skin. However, the rate of salt uptake and the amount of salt absorbed are both critical factors in determining the toad’s survival. The magnitude and rate of salt uptake must be sufficient that the toad can rehydrate reasonably rapidly, but not so great that cellular water is lost or the toad’s kidneys are overloaded. Regulating the apical Na⁺ permeability according to the volume of the cells would provide an elegant way of locally controlling the amount of Na⁺ entry. The issue of PNa regulation as part of a local, self-adjusting feedback system

Voc, commencing within 10-30 seconds of an increase or decrease in the extracellular osmolality, make it unlikely that the pool size was altering. (A slower time course would be expected if this were the case. Furthermore, an asymmetry in the inactivation and activation times of the ion permeability of the cell would be expected to account for differences in the time for channel disintegration, and the time required for manufacturing the channel proteins and subsequent incorporation into the membranes). A more likely explanation is that the open probability of channels was altered due to the channels being sensitive to some cell parameter such as volume.

11 Rehydration times appear to be of the order of several hours as found by Hillyard and Larsen (2001) who showed that toads dehydrated by 10-15% of their standard weight rehydrated to almost the same extent when bathed in deionized water for 120 minutes or in 120mmolL⁻¹ NaCl for 180 minutes.
which can alter dynamically as a cell interacts with, and adjusts to its environment, is discussed in detail in Chapter 8.

4.4.4 Are the putative volume-sensitive Na⁺ channels in the toad skin ENaCs?

In 1993 a new superfamily of ion channels were defined when three homologous channel subunits called α-, β-, and γ-epithelial Na⁺ channels (ENaCs) were cloned from rat colon (Canessa et al., 1993; Lingueglia et al., 1993). These amiloride-sensitive, Na⁺-conducting channels have since been identified in a variety of absorbing and secreting epithelia (Garty & Palmer, 1997). Members of the superfamily display a wide range of biophysical properties and are characterised by a single channel conductance of 4-7pS, possess a high PNa/PK selectivity (>10), and typically have relatively long open and closed times (Benos et al., 1996). They are also genetically similar to proteins involved in mechanosensory transduction in nematodes, prompting the question of whether ENaCs are mechano-, volume- or stretch-sensitive (Garty & Palmer, 1997). Given that the putative volume-sensitive Na⁺ channel described above shares some of the characteristics of the ENaC (i.e. it is a major Na⁺ conducting pathway and it is amiloride- and volume-sensitive), it is plausible that it belongs to the ENaC superfamily, although as yet its presence has not been determined in the toad skin (Garty & Palmer, 1997).

Having said that, the Na⁺ permeability of the toad skin displays some differences with known ENaCs. For example, in Xenopus oocytes osmotic shrinkage appears to activate ENaCs (Bohmer et al., 2000; Bohmer & Wehner 2001, Ji et al., 1998). This is opposite to the findings of this, and other studies (Zeiske & Van Driessche, 1984) which suggest that Isc (and hence PNa) is activated by cell swelling.

There is also evidence to suggest that at least some ENaCs are regulated by the intracellular Na⁺ concentration. In Xenopus oocytes, increasing the intracellular Na⁺ concentration caused an inhibition of ENaC currents, but increasing the K⁺ concentration or the tonicity had no effect (Awayda, 1999). Although there has been some discussion that Na⁺ channels in the toad skin are regulated in this way (Turnheim, 1991), or are regulated by the extracellular Na⁺ concentration (Garty & Palmer, 1997), the findings of Figure 4.7 do not support the idea of a Na⁺-induced, Na⁺-regulatory mechanism, because changes in Isc were observed in the absence of changes in the Na⁺ concentration gradient.

While the Na⁺ permeability of the toad skin is certainly different from the ENaCs of Xenopus oocytes, it has been pointed out that the ENaC may simply be a ‘core
conduction element’ around which other polypeptides arrange themselves, forming distinctive Na⁺ transporting pathways with different kinetic and regulatory properties (Benos et al., 1996). It may be only a matter of time before the apical Na⁺ channels in the toad skin are found to belong to the ENaC superfamily.

4.4.5 Phase I: The order of simple- and solute-induced water movements

Regardless of volume sensitivity, if the toad skin were Na⁺ permeable on the apical side, then it would be expected that when the skins were first bathed in a solution with a high extracellular Na⁺ concentration, the Na⁺ flux into the cell would increase as Na⁺ flowed down its electrochemical gradient (from the apical solution into the cell), causing a rise in Isc. Experimentally, there is evidence of this initial rise in Phase I in both SO₄²⁻ (Figure 4.8D, H) and Cl⁻ Ringer (Figure 4.9A-E, H, J, K, N). The response is generally strongest in the 500mOsm/kgH₂O solutions where the Na⁺ concentrations are highest, becoming less apparent in solutions with lower Na⁺ concentrations.

However, this rising transient was not present in all examples where skins were bathed in high extracellular Na⁺, being notably absent in four out of the five SO₄²⁻ examples (although present in all five of the 500mOsm/kgH₂O Cl⁻ examples). One possible reason for this could have been that the recordings were not begun soon enough after the skins were bathed in the solutions and consequently the transients were missed. However, only five minutes or so elapsed between the time of filling the first of the eight Ussing chambers and commencing data acquisition. During this time all baths were filled and then emptied sequentially, a process that was repeated three times. Thus, the skins were not actually bathed in the solutions for the entire five minutes. But even if they had been, the tail end of the transient before it peaked should still have been apparent in most cases, given that in those examples where the rising transient was present, it took 10-30 minutes before the Isc peak of Phase I was reached. Therefore, the absence of the rising transient was probably not because the data was not recorded.

The time taken to reach the Isc peak is an important clue in determining the mechanism responsible for the initial rise in Isc. The 10-30 minute period is sufficiently long for it to be argued that volume changes were probably occurring. Certainly, Spring and Ussing (1986) had demonstrated that the MRCs at least, had the capacity for considerable volume changes, of up to 40-50%, over 20-30 minutes in an unclamped skin (Figure 4.3C). Of course, if volume changes were responsible for the initial rise in Isc, then the changes would be due to rapid, inwardly directed solute-induced water
Figure 4.13: (A) The total volume can rise and fall when the simple- and salt-induced water movements are in opposite directions; (B) the time constant of the total volume is the same as the time constants for the simple- and salt-induced water movements (C) the time constant of the total volume is faster than the simple water movement because of salt-induced water movements; (D) the time constant of the total volume is slower than the simple water movement because of salt-induced water movements; (E) the time constant of the total volume is biphasic because the simple water movements are much faster than the salt-induced water movements. NB: The dotted line represents the total volume when the time constants for the bulk- and salt-induced water movements are equal.
movements, and not simple water movements, which would necessarily be outwardly
directed in the high osmolar solutions (Figure 4.13A). The inwardly directed solute-
induced water movements would only occur if salts were moving into the cells down
their electrochemical gradient. In the case of high extracellular NaCl solutions it is
likely that both Na\(^+\) and Cl\(^-\) move into the cells creating an inward salt movements,
provided of course, that the cells are Na\(^+\) and Cl\(^-\) permeable. But in the case of high
extracellular Na\(_2\)SO\(_4\) solutions, Na\(^+\) might move into the cells, but unless the cells were
highly positive with respect to the extracellular solution, Cl\(^-\) would leave the cells,
preumably followed by K\(^+\), resulting in a net loss of salts, and cell shrinkage.\(^{12}\) That is,
under these circumstances, a net outward and not inward movement of salts could
actually occur (unless, of course, the cells were permeable to some other anion).
Consequently, it would be unlikely to observe cell swelling due to solute-induced water
movements, or to observe the associated increase in the volumetrically sensitive Isc.
This is consistent with the observation that Isc does not generally display the Phase I
increase in high osmolar (i.e. 500mOsm/kgH\(_2\)O) Na\(_2\)SO\(_4\) solutions which is seen in the
high osmolar NaCl solutions.

After the initial increase in Isc, a Phase I decrease of varying degrees can be observed
in all the experimental bathing solutions. In the 500mOsm/kgH\(_2\)O NaCl solution, this
decrease would appear to be due to simple water movements, as described above.
Certainly, the exponential with a time constant of 23 minutes, used to fit the simple
water movements of Figure 4.7, also fits the Phase I rundown of the 500mOsm/kgH\(_2\)O
NaCl solution (Figure 4.11B). With the exception of the 250mOsm/kgH\(_2\)O NaCl, and
250mOsm/kgH\(_2\)O high K\(^+\) solutions (Figure 4.11D,H), the ‘simple water exponential’ is
also a reasonably good fit for the Phase I rundowns observed in the other solutions. This
suggests that in most cases: (1) simple water movements are directed out of the cells
and (2) the intracellular osmolality is generally low relative to the bathing solution.

From a theoretical perspective, it would be expected that the time constant of the
volumetrically sensitive Isc Phase I rundown would be the same as the time constant for
the total volume changes. If the time constant of the total volume changes is dominated
by the simple water time constant, either because there is no solute-induced water
movement, or the time constant of the solute-induced water movement is the same as
the simple water movement, then the Phase I rundown in Isc should be well fitted by the

\(^{12}\) Furthermore, if the loss of Cl\(^-\) occurred predominantly across the basolateral membrane, Voc and Isc
would also decrease because this would effectively depolarise the basolateral membrane.
simple water exponential (Figure 4.13B). Experimentally, this appears to describe the rundown for the 250mOsm/kgH\(_2\)O Na\(_2\)SO\(_4\) and low K\(^+\) solutions, and the 100mOsm/kgH\(_2\)O NaCl solutions (Figure 4.11C, F, G).

Finally, it should be noted that the individual rundowns in the 500mOsm/kgH\(_2\)O Na\(_2\)SO\(_4\) and NaCl solutions (Figures 4.8, 4.9) are generally not smooth, but are often characterised by a rapid drop followed by a slower exponential decline. Theoretically, this could occur if two exponentials were summed and the time constant of one was significantly larger than the other (Figure 4.13E). Experimentally, because the initial part of the biphasic rundown is well fitted by the simple water exponential with the 23-minute time constant, it is suggested that the second part of the rundown is due to an outwardly directed salt movement with a time constant slower than 23 minutes. In light of the high extracellular Na\(^+\) concentrations in both 500mOsm/kgH\(_2\)O solutions, it is also suggested that this salt loss is due to the efflux of K\(^+\), and either Cl\(^-\) (in the case of the Na\(_2\)SO\(_4\) solution) or some other anion (in the case of the NaCl solution).

To summarise, the main changes in Phase I of the volume-sensitive Isc response can be explained by the addition of two exponentials with the same or different time constants.

4.4.6 Phase II: RVI- and RVD-like behaviour in toad skins

If Isc is an indicator of cell volume, as suggested by Figure 4.7, then the transients seen in Isc following the initial simple exponential response to a change in bathing osmolality are possibly due to RVI’s or RVDs. Several examples of ‘RVI-like’ and ‘RVD-like’ behaviour in Cl\(^-\) and Cl\(^-\)free Ringer are evident (Figure 4.7). From these examples two main points are worthy of note.

First, Isc does not necessarily stabilise at a particular value. For example, after the osmolality was increased in Figure 4.7, Isc typically dropped, and was often followed by an upward rebound (or RVI) which subsequently fell. This is different from the theoretical behaviour of Figure 4.1 where the final volume, generated by the addition of two exponentials for simple- and solute-induced water movements, approached a constant value following an initial transient. The disparity between the experimental and theoretical results suggests that the toad skin behaviour is not entirely explicable in
terms of a simple regulatory volume response. Clearly, some other mechanism is occurring which prevents Isc from settling to a stable value.\(^\text{13}\)

One clue to the problem is found in the skin responses recorded over 20 hours. Over this time course it is sometimes possible to observe a slow oscillation in Isc (see Figure 4.8 E, H, J & Figure 4.9 I, J, L, O).\(^\text{14}\) The presence of this oscillation is important because it indicates that there is a negative feedback loop regulating Isc (and probably the volume). Although the details of the loop are not entirely clear from the data presented in this study, the mechanism is probably more complex than: a change in the bathing solution causing a change in volume, and hence a change in the apical Na\(^+\) permeability, Na\(^+\) flux, apical membrane potential, apical Cl\(^-\) permeability and net salt flux, which in turn causes a net water movement and a change in cell volume. While such a system would be capable of oscillating, it has already been shown that volume changes have a time constant around 20-30 minutes, which is too fast to explain the slow oscillations observed over many hours. Clearly, a slower mechanism (such as the upregulation and downregulation of intracellular protein expression) is influencing the overall cellular responses.

The second point regarding the RVI and RVD-like behaviour that requires comment is that this behaviour is present in Cl\(^-\) and Cl\(^-\)-free solutions. It was argued by Zeiske and Van Driessche (1984) that any secondary rise in Isc following the initial decrease when the osmolality of the sulphate bathing solution was raised was not due to a volume effect because 'cell volume [could] not be restored by regaining salt and water'. While it is accepted that restoration of cell volume due to Cl\(^-\) re-uptake in the sulphate solutions is not possible, it is plausible that the volume was restored by the re-uptake of other anions such as sulphate, which Larsen and Simonsen, (1987) suggested may move across the apical membrane of the MRCs via the Cl\(^-\) pathways.\(^\text{15}\) Alternatively, (and consistent with the slow oscillations described above), it is possible that the cells were upregulating mRNA and manufacturing organic osmolytes, similar to renal medullary

\(^{13}\) It should be noted that whatever the mechanism, it is not trivial given that a regulatory increase of around 50% (i.e. 25mOsm/kgH\(_2\)O) can occur in some cases (Figure 4.7E), following a 50mOsm/kgH\(_2\)O change in osmolality.

\(^{14}\) If the regulatory volume increase is due to salt movement and the salts cannot flow into the cells, say because PCI is closed, then no oscillations will be observed.

\(^{15}\) It is possible that salt movements supplement other volume regulatory processes. But any net salt movement in Cl\(^-\)-free Ringer, due to other anions such as sulphate, is probably less than in Cl\(^-\)-Ringer given that the RVI-like responses in Cl\(^-\) Ringer are generally larger than in Cl\(^-\)-free Ringer.
cells exposed to hypertonic stress (Beck et al., 1998; Beck et al., 1992; García-Perez & Burg, 1991). The event initiating the proposed upregulation is unknown, but appears to be somewhat variable since the time at which the RVI-like behaviour is observed is not fixed. For example, in Figure 4.7 where only the osmolality was changed and not the ionic concentrations, the RVI-like behaviour was initiated relatively rapidly, sometimes within 30-40 minutes. However, in the examples where the ionic concentrations were changed (Figures 4.8, 4.9, 4.10), the initiation of RVI-like behaviour was quite variable, occurring within about 30-60 minutes after commencement of recordings in the 100mOsm/kgH₂O solutions, but not until up to 8½ hours after some skins were first bathed in 500mOsm/kgH₂O solutions. The main point is that not all volume regulatory processes are dependent on extracellular Cl⁻. Therefore, Zeiske and Van Driessche's rejection of the proposal that Isc is volumetrically sensitive, based simply on the responses observed in Cl⁻-free solutions, would appear unfounded. Of course, to resolve this issue completely and to establish whether or not RVI's and RVD's occur due to some mechanisms other than Cl⁻ movement in the toad skin, direct and detailed measurements of cell volume from skins bathed in Cl⁻ and Cl⁻-free solutions over a period of hours is required. Unfortunately, this information is not available at the present time.

4.4.7 ATP Effect

Originally, before the relationship between cell volume and Isc was established, it was hypothesised that the rundown effect in Phase I was due to a depletion of intracellular ATP stores. In view of subsequent findings (i.e. stable Isc over 20 hours), this now does not appear to be the case. However, several points are worth noting about the ATP effect.

First, the rise in Isc and Voc is rapid with a time constant of around 5-6 minutes. This is probably too fast to be a volume effect. More likely, ATP is binding to (apical) receptor-operated channels and altering the skin resistance. Such an action is consistent with the resistance change observed in the V-I loci after ATP application. It also agrees with the observations that the ATP effect is saturable and can be washed out and then re-instated. In terms of the type of channel affected, if ATP opens channels, then

16 It is probably not the case that ATP was entering the cells and then leaving the cells upon washout of the apical bathing solution, as first thought. If this were the case, then any time the apical bath were flushed with a solution not enriched with ATP, a decrease in Isc would be expected as ATP was washed out of the cells. This was not observed experimentally.
presumably it is acting on the apical Na⁺ pathway. But if it causes channel closure, then it could be acting via either the Cl⁻ or K⁺ channels. Any one of these options would result in the observed increase in Isc and Voc.

Whatever the site of ATP action, its long-term effect is also of interest because it appears to damp the RVI-like behaviour discussed in previous sections. This is clearly demonstrated when the Isc responses recorded in 250mOsm/kgH₂O Na₂SO₄ Ringer (Figure 4.11C), are compared with the Isc response recorded after treatment with ATP, where the RVI-like behaviour is notably absent (Figure 4.6A). This may be because the RVI-like behaviour is not initiated, or because the ATP effect is so large that it dominates the changes in Isc so that no RVI-like effect is observed. Interestingly, the damping effect of ATP seemed to be nulled however, when the skins were pre-treated with suramin before ATP was added. Under these circumstances there was evidence of a slow oscillation (possibly volumetrically related) in both Isc and Voc. (In Figure 4.6C the oscillation in Isc is probably lost in the noise floor). While further investigation is required, it is possible that suramin is somehow altering the loop gain or phase rotation of the negative feedback loop which appears to be regulating ion and water movement across the toad skin.

4.5 Conclusions

The primary goal of this chapter was to investigate volume regulation in the toad skin. The main conclusions of this study are as follows:

(1) Isc is volume sensitive. This volume sensitivity may be due to the possible existence of apical volume-sensitive Na⁺ channels.

(2) Isc is not stable when skins are bathed on both sides in the standard 250mOsm/kgH₂O NaCl solution used by Ussing. This is probably because the cells shrink in such a salty solution. More stable Isc measurements can be obtained however, when skins are bathed in a 100mOsm/kgH₂O NaCl solution. This solution has sufficient Na⁺ in it to permit measurement of Isc, but appears to be less osmotically challenging to toad skin cells than the 250mOsm/kgH₂O NaCl solution.

(3) In the 100mOsm/kgH₂O NaCl solutions, Isc values remained relatively stable over periods of 10 hours or more, suggesting that metabolic decline was not occurring over this period, and that cellular ATP levels were not limiting the operation of basolateral Na⁺/K⁺-ATPase pumps (see Figure 4.8).
(4) Total water movement can be considered in two parts: simple water movements and solute-induced water movements. In the toad skin, simple water movements follow an exponential with a time course of about 23 minutes. Solute-induced water movements follow an exponential with a variable time constant (largely dependent on the permeability of the skin and the electrochemical gradients of the participating ions). The time constant of the solute-induced exponential determines the time constant of the total volume movements, which in turn determines the rate of change of the volume-sensitive Isc.

(5) There is evidence of a slow oscillation in Isc. This oscillation, occurring over hours, is distinct from the much faster Cl⁻ oscillation observed over minutes when the toad skins were voltage-clamped (see Chapter 3).

(6) The Isc oscillations may be due to oscillations in cell volume, particularly given the RVI- and RVD-like behaviour that was observed. Moreover, the presence of the oscillatory behaviour suggests that Isc (and volume) are part of a negative feedback loop, which may be regulating ion and water transport across the toad skin.

(7) The rapid and saturable increase in Isc and Voc observed with 10mM apical ATP, which could be washed out and repeated, suggested that extracellular ATP was acting via receptor operated channels, either opening apical Na⁺ channels, or closing K⁺ or Cl⁻ channels.
5. Heavy Metals: Effects of Hg$^{2+}$ & Pb$^{2+}$
5. Heavy Metals: Effects of Hg$^{2+}$ & Pb$^{2+}$

5.1 Introduction

Present exposure levels in industrialised countries to heavy metals are significantly higher than preindustrial levels, with pollutants such as Pb$^{2+}$ often more than 10 times (and sometimes up to 10,000 times) higher than naturally occurring levels in the environment (Flegal & Smith, 1995). If absorbed at high enough concentrations, heavy metals such as Hg$^{2+}$ (which was one of the first known diuretics) and Pb$^{2+}$, can have serious effects on humans and other species. For example, in humans, acute poisoning by Pb$^{2+}$ is characterised by vomiting, diarrhoea, and severe internal injury, while chronic effects include loss of appetite, anaemia, constipation, pallor and colic (Muir, 1977). Acute poisoning by inorganic Hg$^{2+}$, is characterised by abdominal pain and vomiting, pharyngitis, bloody diarrhoea (containing necrotic parts of the intestinal mucosa), oliguria, anuria and, in extreme cases, death (HMSO, 1976; Friberg & Vostal, 1972). Symptoms of chronic Hg$^{3+}$ poisoning include weakness, fatigue, either dry mouths or excessive salivation, mercurial tremors, micromercurialism (producing psychological changes such as memory loss and depressive mood changes), renal symptoms including proteinuria which can lead to development of nephrotic syndrome, and ocular symptoms, including the deposition of mercury on the lens of the eye, known as mercurialentis (Friberg & Vostal, 1972). Of course, such symptoms are only manifested for relatively high heavy metal blood concentrations. In terms of Pb$^{2+}$, early toxic effects, at least in children, are manifested at blood Pb$^{2+}$ levels of 10µg/dL or 480nM (Flegal & Smith, 1995). This is about 3-4 times higher than the average blood Pb$^{2+}$ level in citizens of modern USA who are not occupationally exposed to Pb$^{2+}$, and about 600 times higher than estimated preindustrial blood Pb$^{2+}$ levels (Flegal & Smith, 1995). In contrast, humans appear to be more Hg$^{2+}$- than Pb$^{2+}$ tolerant, with Hg$^{2+}$ poisoning in humans occurring at micromolar, and not fractions of micromolar, blood

---

1 In extreme cases, several litres of saliva can be collected per day (Friberg & Vostal, 1972).

2 Many of the symptoms of chronic Hg$^{2+}$ poisoning were first observed in hat makers between 1550 and 1850 when beaver felt hats were fashionable in Europe. As part of the curing process, the beaver pelts were treated with mercurious nitrate. This produced a yellow-red colour on the fur tips and roughened the individual hair fibres (which improved the matting ability of the fur at a later stage). However, subsequent drying of the pelts caused the release of mercury vapours, which were inhaled by the hat makers, and resulted in Hg$^{2+}$ accumulation in these craftsmen over time. The mercurial tremors and mental degeneration associated with hat makers because of this Hg$^{2+}$ poisoning lead to the coining of the term ‘mad as a hatter.’
concentrations. More specifically, symptoms of Hg\(^{2+}\) poisoning have been recorded for blood Hg\(^{2+}\) levels as low as 0.2 ppm or 1\(\mu\)M, although typically, symptoms are not observed until levels reach 1-4 ppm or 5-20\(\mu\)M. Fatal poisoning has been recorded for a blood Hg\(^{2+}\) level of 4 ppm or 20\(\mu\)M (HMSO, 1976). For comparative purposes, a blood Hg\(^{2+}\) level of 20\(\mu\)M is about 400-800 times greater than the average blood Hg\(^{2+}\) level of people not occupationally exposed to Hg\(^{2+}\), and eating a ‘normal’ western diet.

One of the noteworthy points about heavy metal toxicity is that symptoms are often presented in terms of systemic failure, with little consideration of what is occurring at a cellular and sub-cellular level. Yet at least one heavy metal Hg\(^{2+}\), has an effect at a cellular level, blocking aquaporins and acting as a diuretic (Snigirevskaia & Komissarchik, 1999). Despite this, with the exception of Hillyard & Gonick’s work with Cd\(^{2+}\)(1976), there have been few studies monitoring the changes in the electrophysiological parameters of ion-transporting epithelia due to the local action of heavy metals. But if heavy metals were acting locally to alter salt and water transport, then presumably standard electrophysiological measurements would be affected. To investigate this premise, the open-circuit voltage (Voc), and short-circuit current (Isc), in the toad skin were recorded in this study, in response to the local application of three heavy metals, namely Hg\(^{2+}\), Pb\(^{2+}\), and Cu\(^{2+}\), with particular emphasis on the first two of these.

5.2 Methods

As described previously in Chapter 2, toad skins were dissected and mounted in Ussing chambers and the open-circuit voltage (Voc) and short-circuit current (Isc) were monitored for up to 20 hours, using custom-built software and electronics. All voltages were referenced to the apical (pond) bathing solution.

Skins were bathed on both sides in 100mOsm/kgH\(_2\)O NaCl (Soln 37), with the following composition in mmol/L: Na\(^+\) = 45, K\(^+\) = 2.5, Cl\(^-\) = 47.7, Ca\(^{2+}\) = 1.35, Hepes\(^-\) = 5 and Glucose = 2.5. Mercuric chloride and lead chloride were applied to the apical and basolateral bathing solutions, most often at a concentration of 100\(\mu\)M, although other concentrations were used, as described in the results section. (To put the Hg\(^{2+}\) concentrations used in this study in perspective, the World Health Organisation specifies that the acceptable upper limit for Hg\(^{2+}\) concentrations in drinkable water is 0.001 ppm by weight (equivalent to 0.001mg/L or 5nM) (HMSO, 1976). This level has been adopted in the Australian Drinking Water Guidelines (1996) developed by the National Health and Medical Research Committee. In terms of Pb\(^{2+}\), an acceptable Pb\(^{2+}\)
concentration in drinking water is 0.01mg/L or 50nM, as specified by the Australian Drinking Water Guidelines).

Other agents used to investigate possible causes of the changes observed included the heavy metal Cu^{2+}, the transition metals La^{2+} and Al^{3+}, the potassium channel blocker tetraethylammonium (TEA), the sodium channel blocker amiloride, and the Na^+/K^+/2Cl^- symport blocker furosemide (generically known as Lasix, with an active ingredient of 20mg/mL furosemide). Where indicated in the results, drugs were washed out of the bathing solution by flushing the appropriate bath three times with fresh aliquots of the 100mOsm/kgH_2O NaCl solution.

5.3 Results

5.3.1 Apical Hg^{2+} dose response curve

A cumulative dose response curve for Hg^{2+} applied to the apical or pond (p) side of the toad skin indicated that 0.5µM Hg^{2+} had little effect on Isc or Voc (Figure 5.1A). Increasing the concentration 10-fold to 5µM Hg^{2+} initiated an exponential decrease in Isc from 15.8 to 6.7µA with a time constant of \( \tau = 60.9 \) minutes, after a delay of approximately 10 minutes in the example shown. Unlike Isc, Voc did not drop with 5µM Hg^{2+}, but continued to increase in a 2-step manner, first following an exponential increase from 23.7 to 33.2mV, with a time constant of \( \tau = 54.5 \) minutes, and then increasing linearly, probably following its pre-Hg^{2+} trend line. (The maximum was not attained before the next dose of Hg^{2+} was administered). On the V-I locus, the application of 5µM Hg^{2+} (‘b’) occurred at what we have termed a ‘breakpoint,’ where there is a change in the sign of the resistance. For example, before point ‘b’ the skin resistance traversed positive iso-resistance lines but then, due to the change in direction of Isc and not Voc, the skin resistance entered a negative resistance region between ‘b’ and ‘c’. Interestingly, several examples suggest that 5µM Hg^{2+} on the pond side acts to initiate such a breakpoint, irrespective of prior perturbations such as Pb^{2+} (cf. Fig. 5.1A, Fig. 5.5D & Fig. 5.3B).

Upon application of 50µM Hg^{2+} at point ‘c,’ a second breakpoint occurred on the V-I locus as the skin resistance moved from a negative to a positive resistance. As seen on the adjacent time trace this was due to Voc and Isc moving in the same direction once again. The higher Hg^{2+} dose caused a biphasic drop in Voc from 35.6mV (point ‘c’) to 8.7mV (point ‘d’), and a rapid exponential drop in Isc from 6.7 to 1.5µA with a time constant of \( \tau = 6.6 \) minutes. (Attempts to reverse the Hg^{2+} effect with 100µM Pb^{2+} on the
Figure 5.1: Effects of the diuretic and heavy metal, mercury (Hg$^{2+}$) on Voc and Isc when applied to (A), and (B) the belly (b) side, and (C) the pond (p) side. The arrow in (B) denotes a spontaneous change in the resistance of the skin. Notice in (C) that 5mM Hg$^{2+}$ (p) initiates a reduction in Isc. All experiments were started in 100mOsm/kgH$_2$O NaCl Ringer (Soln. 37). The time trace for (C) does not show the initial transients in 100mOsm/kgH$_2$O NaCl Ringer but this has been included on the V-I locus (a*) on the right to illustrate that behaviour at the beginning of the experiments was not extraordinary.
pond side (point ‘d’) were unsuccessful. The effects of Pb\(^{2+}\) are described in more detail later in this section). From this data, and similar data in other skins, it was concluded that 50\(\mu\)M Hg\(^{2+}\) was sufficient to elicit a rapid and definable response from the toad skin. This dose was therefore used as a standard to test for Hg\(^{2+}\)-sensitivity.

5.3.2 Effect of Hg\(^{2+}\) on the basolateral side of the toad skin

In Figure 5.1A it was demonstrated that application of 50\(\mu\)M Hg\(^{2+}\) to the pond side of the toad skin caused a reduction in Isc and Voc. When this same dose was applied to the basolateral or belly (b) side however, the main effect (albeit smaller than the change on the apical side) was an increase in Isc and Voc. This is illustrated in Figure 5.1B, which shows an exponential increase in Isc from 12.8 to 15.8\(\mu\)A with a time constant of \(\tau=4.8\) minutes and, after a very brief downward transient (seen as a breakpoint on the V-I locus), a transient rise in Voc from 17.0 to 19.4mV which peaked 22.5 minutes after Hg\(^{2+}\) application. Little effect was observed when Hg\(^{2+}\) was washed out of the basolateral bathing solution, suggesting that once bound, Hg\(^{2+}\) did not unbind readily and its effects were not reversible. Figure 5.1C is a second example of the effects of 50\(\mu\)M Hg\(^{2+}\) on the belly side which shows an increase in Isc from 13.4 to 17.1\(\mu\)A, peaking 15 minutes after application, and an increase in Voc from 24.9 to 30.3mV, peaking 17.7 minutes after application. In this particular example, the 50\(\mu\)M Hg\(^{2+}\) application appears to initiate a slow oscillatory response in Isc and Voc with a cycle time of approximately 127 to 150 minutes (assuming that Hg\(^{2+}\) washout had little effect).

5.3.3 Effect of Pb\(^{2+}\)

In the light of the Hg\(^{2+}\) experiments and the pronounced effect that this heavy metal had, other heavy metals were applied to see if they produced similar effects. One such series of experiments examined the effects of Pb\(^{2+}\). It was found that application of 100\(\mu\)M Pb\(^{2+}\) to the pond side of the toad skin typically caused a rapid and significant increase in Isc and Voc. An example of this is shown in Figure 5.2A, where Isc increased from 10.7 to 31.2\(\mu\)A with an initial time constant of \(\tau=18.8\) minutes, and Voc increased from 17.9 to 36.0mV with a time constant of \(\tau=26.9\) minutes. In this particular example the (maximal) Pb\(^{2+}\) effect on Voc was sustained for 163 minutes before Voc increased by approximately 2.5mV over 221 minutes (resulting in a breakpoint on the V-I locus), and then decreased monotonically over the final 543 minutes. The (maximal) Pb\(^{2+}\) effect on Isc was sustained over a period of 134 minutes.
Figure 5.2: Effect of lead (Pb$^{2+}$) on Voc and Isc when applied to the the pond (p) and belly (b) sides of the toad skin. A significant rise in Voc and Isc was often seen when Pb$^{2+}$ was applied to the pond side as shown in examples (A) to (D), but there was little effect when it was applied to the belly side (see (C) to (D)). Notice also that Pb$^{2+}$ effects do not wash out readily on the pond side (C) & (D). All experiments were conducted in 100mOsm/kgH$_2$O NaCl Ringer (Soln. 37). (NB: r.e.=rezeroed electrodes in (B)).
before Isc decreased monotonically until the end of the trace some 11.5 hours later. It should be noted though that the sustained behaviour of Isc after Pb$^{2+}$ was not always evident, with some skins exhibiting a transient rise and fall in Isc, often resulting in a breakpoint, as shown on the associated V-I loci (see Figure 5.2B & D). In these examples, it was difficult to determine the effect of washing Pb$^{2+}$ out of the apical bathing solution because the Pb$^{2+}$ transients were superimposed on transients which occurred routinely when placed in 100mOsm/kgH$_2$O NaCl solutions (see Chapters 3 & 4). Nevertheless, in Figure 5.2C where the application of 100µM Pb$^{2+}$ to the pond side resulted in the sustained increase of Voc from 58.4 to 77.1mV, and Isc from 19.5 to 31.4µA (with time constants of $\tau$=22.0 minutes and $\tau$=19.8 minutes, respectively), there were no significant changes in Voc and Isc following the washout of Pb$^{2+}$ from the apical solution. In fact, these parameters remained remarkably stable at approximately 73mV and 30µA over the 10 hours after Pb$^{2+}$ washout, suggesting that Pb$^{2+}$ is not easily removed from the skin, and its effects are not readily reversed.

Since 100µM Pb$^{2+}$ gave such a large response on the pond side, it was of interest to determine whether this was a maximal or sub-maximal response, and whether the effect saturated. Three cumulative dose response curves in different skins were determined to examine the effects of 1, 10, 100 and 300µM Pb$^{2+}$ on the pond side (Figure 5.3). Interestingly, only small changes in Voc and Isc were observed in each case (i.e. around 5mV or 5µA), even at concentrations of 100µM, already known to elicit significant responses in other skins. (Pb$^{2+}$ responses were elicited from other skins at a concentration of 100µM on the same day, using the same solution, invalidating the argument that the Pb$^{2+}$ solution had in some way been compromised). Thus, these findings suggest that while some skins were sensitive to the effects of Pb$^{2+}$ others were not, and were ‘Pb$^{2+}$-insensitive’ or ‘Pb$^{2+}$-inactivated’ and showed no rapid changes in Voc and Isc with Pb$^{2+}$.

Finally, when 100µM Pb$^{2+}$ was applied to the basolateral side of skins (which were clearly sensitive to Pb$^{2+}$ on the pond side), little effect was observed (Figure 5.1C & D). When the Pb$^{2+}$ was subsequently washed out of the basolateral solution again, no effect

---

3 Interestingly, the curved region on the V-I locus of Figure 5.2A (shown as the rundown of the skin at the end of the experiment in the adjacent time trace) is remarkably similar to the V-I loci of skins bathed in sulphate Ringer, as shown in Chapter 4.
Figure 5.3: Three examples of skins that were not sensitive to Pb$^{2+}$ on the pond (p) side. All experiments were conducted in 100mOsm/kgH$_2$O NaCl Ringer (Soln. 37).
was apparent. Therefore, it was concluded that skins were not sensitive to Pb\(^{2+}\) on the basolateral side.

### 5.3.4 Effect of Al\(^{3+}\), La\(^{3+}\) and Cu\(^{2+}\)

In addition to Hg\(^{2+}\) and Pb\(^{2+}\), several other metals were investigated to determine whether the effects were due to simple charge-screening of the exposed cell membranes (Hille, 1991). It was found that Al\(^{3+}\) had little effect on Isc and Voc when applied to the pond and belly side at concentrations of 100 and 200µM, and the effects of washing the metal ion out of the solutions was negligible\(^5\) (Figure 5.4A & B). When a further 400µM was added to the pond solution, which already contained 200 µM Al\(^{3+}\) (Figure 5.4B), a small transient increase of 4.2µA and 2.2mV was observed respectively in Isc and Voc. These responses were negligible, and it was concluded that at these concentrations Al\(^{3+}\) had little effect on the pond or belly side. Experiments conducted using La\(^{3+}\) also showed little effect at 100µM on the pond and belly side, and no washout effect was evident. A small increase of approximately 4.9µA and 4.1mV was observed for a concentration of 200µM on the pond side, but no effect at this concentration was evident on the belly side (Figure 5.4C & D). Application of an additional 400µM to the pond side, which already contained 200µM La\(^{3+}\), did not have an additive effect (Figure 5.4D). Again, it was concluded that La\(^{3+}\) had little effect on the pond or belly sides at the concentrations shown. Finally, it was found that, unlike the effects of the other heavy metals described in the previous sections, application of 50µM Cu\(^{2+}\) to the pond and belly side had little effect on Isc or Voc, and no significant washout effect could be observed (Figure 5.4E). These results suggested that the charge per se of the metal ions were not important and that charge-screening effects were minimal.

---

\(^4\) It is not possible to attribute the changes in Voc and Isc seen at points ‘d’ and ‘e’ in Figure 5.2C to the effects of 100µM Pb\(^{2+}\) and its respective washout because of the normally occurring transients in 100mOsm/kgH\(_2\)O NaCl solutions (see Chapter 3). In fact, given the lack of response to basolateral Pb\(^{2+}\) seen in Figure 5.2C & D it is likely that there is no Pb\(^{2+}\) effect and that what is observed is simply a standard 100mOsm/kgH\(_2\)O NaCl response.

\(^5\) If no effect were observed upon application of a drug, then it might reasonably be assumed that no effect would be observed upon washout. However, experiments conducted in our laboratory have shown that the local anaesthetic Nembutal, has little effect when applied to the apical side of the skin, but initiates a response when washed out of the bathing solution.
Figure 5.4: (A) & (B) Effect of aluminium, (C) & (D) effect of lanthanum, and (E) effect of copper on the pond (p) and belly (b) sides of the toad skin. All experiments were conducted in 100mOsm/kgH2O NaCl Ringer (Soln. 37).
5.3.5 Interaction of Pb$^{2+}$ and Hg$^{2+}$

Given the opposite effects of Pb$^{2+}$ and Hg$^{2+}$ it was of interest to determine whether it was possible to reverse, or at least slow the effects of Hg$^{2+}$ by pre-treating the skins with Pb$^{2+}$. This information was important as it gave some information about the mechanism of action of Pb$^{2+}$.

Figure 5.5 show attempts to abolish the Hg$^{2+}$ effects in three skins that were Pb$^{2+}$ sensitive (A-C), and in one skin that was not Pb$^{2+}$ sensitive (D), all of which were bathed in 100mOsm/kgH$_2$O NaCl solutions. Traces ‘A’ and ‘B’ clearly illustrate the lack of Pb$^{2+}$ effect on the belly side, as described earlier in this paper. Upon application of 100µM Pb$^{2+}$ to the pond side increases in Isc (from (A) 16.8 to 34.4µA and from (B) 13.9 to 21.5µA) and increases in Voc (from (A) 29.4 to 46.8mV and from (B) 36.7 to 46.3mV) were observed. Application of 50µM Hg$^{2+}$ to the pond side led to an almost complete abolition of Isc in both cases, dropping from (A) 25.1 to 2µA and from (B) 15.6 to 1.9µA with time constants of $\tau$=7.5 minutes and $\tau$=3.2 minutes, respectively. Rapid decreases in Voc were also observed falling from (A) 46.4 to 12.8mV and from (B) 44.6 to 7.1mV. Similar Hg$^{2+}$ responses were observed in trace ‘c’ where 100µM Pb$^{2+}$ was first applied to the pond side causing an increase in Isc from 10.7 to 38.7µA and an increase in Voc from 17.4 to 39.0mV. Removal of Pb$^{2+}$ from the pond side resulted in little change in Voc, but a small reduction in Isc of 4µA over 123 minutes. Both trends continued when 100µM Pb$^{2+}$ was applied to the belly side. When 50µM Hg$^{2+}$ was then applied to the pond side, a significant reduction in Voc from 37.9 to 2.7mV with a time constant of $\tau$=36.8 minutes was observed, and Isc dropped from 28.0 to 0.7µA with a time constant of $\tau$=7.9 minutes. Again, it was evident that Hg$^{2+}$ effects were not annulled by pre-treatment with Pb$^{2+}$. Figure 5.5D illustrates that Hg$^{2+}$ effects were also elicited in Pb$^{2+}$-insensitive skins. In this example, 10µM Pb$^{2+}$ on the pond side had no significant effect on Isc or Voc which could be distinguished from transients that normally occurred in 100mOsm/kgH$_2$O NaCl solutions (see Chapters 3 & 4). Apical application of 5µM Hg$^{2+}$ however caused an increase in Voc from 19.4mV to a maximum of 31.5mV, 147 minutes later and a transient increase in Isc from 16.4 to 18.6µA which peaked 30 minutes after addition of Hg$^{2+}$. This was followed by a

---

6 Toad 132a was deemed to be Pb$^{2+}$-insensitive based on (1) its lack of response to Pb$^{2+}$ and (2) the lack of response of tissue taken from the same toad where a Pb$^{2+}$ dose response curve had failed to elicit significant increases in Voc and Isc (Figure 5.3B).
Figure 5.5: Attempts to abolish the effects of Hg$^{2+}$ on Isc and Voc using Pb$^{2+}$. All experiments were conducted in 100mOsm/kgH$_2$O NaCl Ringer (Soln. 37). (Note the vertical axis in (D)). (p=pond, b=belly).
reduction in Isc to 9.3µA at t=481.3 minutes, although a minimum was not attained. This Hg\(^{2+}\)-induced change in the direction of Isc, with no concomitant change in Voc, initiated a breakpoint on the associated V-I locus and the skin resistance moved into a negative resistance region. Subsequent application of 100µM Pb\(^{2+}\) stabilised the decline in Isc at 9.5µA, and caused an increase of Voc from 31.7 to 36.8mV, but these responses were clearly not the same as the Pb\(^{2+}\) responses observed in Pb\(^{2+}\)-sensitive skins. Before Voc had reached a maximal value, however, 50µM Hg\(^{2+}\) was applied to the pond side which caused an exponential drop in Isc from 9.3 to 0.73µA with a time constant of $\tau$=10.1 minutes and a biphasic drop in Voc from 36.8mV to an intermediate value of 5.5mV, and then to 1.5mV at the end of the experiment. These results indicate that significant Hg\(^{2+}\) effects were observable even when Pb\(^{2+}\) effects were negligible.

5.3.6 Interaction of Pb\(^{2+}\) and TEA

To gather further information about the action of Pb\(^{2+}\), a series of experiments was conducted with Pb\(^{2+}\) and the potassium channel blocker, tetraethylammonium (TEA). From earlier experiments, TEA was known to have similar effects to Pb\(^{2+}\) when applied to the pond side (i.e. a rapid rise in Isc and Voc). Summarising the effects of TEA briefly, the dose response curve of Figure 5.6A shows that 10mM TEA was sufficient to elicit an observable (albeit sub-maximal) effect on the pond side.\(^7\) Other examples at this concentration (Figure 5.6B & C) showed that TEA, caused increases in Isc from (b) 20.9 to 36.3µA and from (c) 13.2 to 29.2µA within 11.3 minutes and 41.6 minutes respectively, and increases in Voc from (b) 45.9 to 58.5mV and from (c) 23.1 to 33.2mV. Responses to 10mM TEA on the belly side were in the opposite direction and were small compared with those on the pond side, with Isc dropping from (b) 22.9 to 18.9µA and from (c) 17.3 to 15µA within 12.0 minutes and 11.3 minutes respectively, and Voc dropping from (b) 50.3 to 43.9mV and from (c) 27.2 to 24.4mV. Finally, in all examples TEA effects were reversible upon washout of the drug.

It was postulated that if Pb\(^{2+}\) and TEA were acting on the pond side via the same mechanism to increase Voc and Isc, then application of one agent followed by the other would either have no effect (due to the first causing a maximal change in Voc and Isc), or have an additive effect (due to the first agent causing a sub-maximal change in these parameters). As shown in Figure 5.7A, the application of 100µM Pb\(^{2+}\) to the pond side

\(^7\) Larger changes in Isc and Voc were observed at 30mM as shown in Figure 5.6A. Above this concentration TEA began to precipitate in the bathing solution.
Figure 5.6: Effect of the potassium channel blocker, tetraethylammonium (TEA) on Voc and Isc when applied to the pond (p) and belly (b) sides of the toad skin. All experiments were conducted in 100mOsm/kgH₂O NaCl Ringer (Soln. 37). (Note the differences in time scales).
Figure 5.7: The effects of lead (Pb^{2+}) and the potassium channel blocker, tetraethylammonium (TEA) on Voc and Isc when applied to the pond (p) and belly (b) of the toad skin. (A) & (B) Voc and Isc decrease upon TEA application when in the presence of Pb^{2+}. (C) & (D) The Pb^{2+} response is abolished after washout of TEA and Pb^{2+} on the pond side. All experiments were conducted in 100mOsm/kgH_2O NaCl Ringer (Soln. 37). (Note the differences in time scales).
caused an exponential increase in Isc from 15.0 to 25.5\,\mu A with a time constant $\tau=11.3$ minutes and an increase in Voc from 36.7 to 54.0mV (at $t=271.1$ minutes although a maximum was not attained). Subsequent addition of 10mM TEA to the apical solution caused little change in Isc and Voc. In Figure 5.7B the protocol was reversed and a significant increase was observed in Isc from 5.0 to 16.8\,\mu A and in Voc from 10.3 to 22.7mV upon apical application of 10mM TEA. Further increases were observed when 100\,\mu M Pb$^{2+}$ was added with Isc rising from 16.9\,\mu A to a maximum of 26.6\,\mu A over 35.7 minutes and Voc rising from 22.7mV to a maximum of 30.5mV over 34.2 minutes. A decline in magnitudes of both parameters was observed after each parameter had peaked. The protocol of Figure 5.7B was repeated in the skin of Figure 5.7C. Again, an exponential increase in Isc from 60.0 to 80.0\,\mu A with a time constant of $\tau=2.4$ minutes was observed, along with a smaller change in Voc, which increased from 92.0mV to a peak of 100.5mV and then declined. Addition of 100\,\mu M Pb$^{2+}$ had little effect on Voc but caused a transient increase in Isc from 81.2\,\mu A to a maximum of 92.4\,\mu A 78.6 minutes later. Overall, the results of Figure 5.7A-C were consistent with the proposed hypothesis that Pb$^{2+}$ and TEA were acting via the same mechanism. However, the results of Figure 5.7D were not consistent with this proposal. In this case, a standard Pb$^{2+}$ effect on the pond side was elicited causing an increase in Isc from 6.9 to 20.8\,\mu A with a time constant of $\tau=37.6$ minutes and an increase in Voc from 13.7 to 32.3mV (at $t=396.4$ minutes). However, subsequent application of 10mM TEA resulted in a small and very rapid increase in Isc of 2.1\,\mu A followed by a decline to a local minimum of 17.2\,\mu A over 111.3 minutes. Voc also declined from 32.3mV to a local minimum of 20.4mV, 84.1 minutes later. Finally, it was unclear whether the transient increase in Voc over the following 8 hours (peaking at 960.3 minutes) and then declining, and the slow drop in Isc over the same interval, were a direct effect of TEA or whether the responses were affected by the additional spontaneous changes that occurred when the skins were bathed in 100mOsm/kgH$_2$O NaCl solutions.

5.3.7 Interactions of Pb$^{2+}$ and Furosemide

Another response that was similar to the effect of Pb$^{2+}$ was that of the diuretic and Na$^+$/K$^+$/2Cl$^-$ symport blocker furosemide (FU). Therefore, as part of the Pb$^{2+}$ investigation it was of interest to examine whether FU effects (at a bath concentration of 20mg/L) could be elicited in the presence of Pb$^{2+}$. Based on similar arguments used for TEA, it was hypothesised that if Pb$^{2+}$ and FU were acting on the same mechanism, then
**Figure 5.8:** The effects of lead (Pb$^{2+}$) and the Na$^+$/K$^+$/2Cl$^-$ symport blocker, furosemide (FU) on Voc and Isc when applied to the pond (p) and belly (b) of the toad skin. (A) Voc and Isc increase upon FU application on the pond side. (B) Isc decreases when FU is applied on the pond side. All experiments were conducted in 100mOsm/kgH$_2$O NaCl Ringer (Soln. 37). (Note the differences in time scales).
either no effect or an additive effect would be observed when one agent was applied directly after the other on the pond side.

Figure 5.8A shows a typical FU response for a skin bathed in a 100mOsm/kgH₂O NaCl solution with a fast initial increase in Voc from 9.2 to 15.6mV, 5.8 minutes after application, and an exponential increase in Isc from 12.0 to 19.8µA with a time constant of $\tau=7.7$ minutes. The addition of 100µM Pb²⁺ resulted in a further increase in Isc to a maximum of 28.3µA over 33.3 minutes. It also caused an increase in Voc from 19.6mV to 29.9mV at $t=464.5$ minutes, although a maximum was not reached due to a subsequent perturbation (not shown). Thus, this result indicated that an additive effect could be elicited.

However, a reversal of the drug protocol did not demonstrate this additive effect. Figure 5.8B showed an increase in Voc from 52.8 to 60.3mV (at $t=205.9$ minutes) with a time constant of $\tau=7.6$ minutes upon application of 100µM Pb²⁺ to the pond side (although this response did not reach a maximum). A concomitant increase in Isc was also observed from 46.4µA to 64.2µA (at $t=218.4$ minutes) with a time constant of $\tau=13.1$ minutes. The subsequent application of FU resulted in an exponential increase of Voc from 65.1 to 75.6mV (at $t=364.0$ minutes) with a time constant of $\tau=48.2$ minutes. However, an atypical change in Isc occurred, as depicted by a rapid increase in Isc over 4.7 minutes from 66.6 to 69.4µA followed by a slow decline to 61.1µA at $t=398.0$ minutes (at which point a third perturbation was carried out (not shown)).

5.4 Discussion

5.4.1 Heavy metal effects are not due to charge screening

Before discussing the Hg²⁺ and Pb²⁺ results it is worthwhile noting that the effects of these metals do not appear to be due to the simple ionic charge of the ions. This issue was considered because it was possible that metal cations were accumulating on the membrane surface having a ‘charge-screening’ effect and altering the potential profile across the membrane (Hille, 1991). Therefore, it was necessary to investigate whether application of differently charged ions elicited different responses in the skins and whether increasing or reducing the concentration of these ions in the bathing solution caused graded changes in the potential profiles, which in turn affected ion transport (and Isc and Voc).

As shown in Figure 5.4, studies conducted using the transition metals Al³⁺, La³⁺ and Cu²⁺ showed that there were no significant changes in Isc and Voc when metal ions of
different charges and different concentrations were applied to the apical or basolateral sides of the skin. This finding suggested that charge screening effects were minimal, having at most an effect of approximately 5 µA on Isc and 5 mV on Voc. Therefore, it was concluded that simple charge screening effects were not responsible for the observed Hg\(^{2+}\) and Pb\(^{2+}\) effects. This conclusion was also consistent with the fact that the Hg\(^{2+}\) and Pb\(^{2+}\) effects were in opposite directions, with 100 µM Hg\(^{2+}\) typically causing a reduction in Voc and Isc, but 100 µM Pb\(^{2+}\) generally resulting in an increase in Voc and Isc. Had these effects been due to charge screening, changes in Voc and Isc would have been in the same direction.

5.4.2 The action of Hg\(^{2+}\)

Of the ten aquaporins described in mammalian epithelial tissue and the two described in amphibian epithelial tissue, all aquaporins except AQP4 are inhibited by mercurial reagents which specifically bind to cysteine residues on the aquaporins and block the aqueous pores (Brooks et al., 2000; Kowarhara et al., 1997; Snigiirevskaia & Komissarchik, 1999, Verkman, 1992). Consequently mercuric compounds such as HgCl\(_2\) have been used in other studies as an experimental tool to investigate the water permeability of Hg\(^{2+}\)-sensitive epithelia. Grosso and De Sousa (1993) monitored the net water flow across the abdominal skin of the toad and showed that 1 mM Hg\(^{2+}\) had little effect on the osmotic water permeability (and if anything, slightly increased it) when it was applied to the basolateral (belly) solution, while it ‘reversed the state of high water permeability induced by vasopressin’ when applied to the apical solution, causing a ‘rapid’ reduction in the net water flow during the first twenty minutes. (Using an automated technique net water flow measurements were made continuously, and the results were presented as the average water flow over time epochs of two minutes). They concluded that this suggested the presence of a Hg\(^{2+}\)-sensitive apical water pathway in the vasopressin-stimulated skins.

At first glance, the experimental data presented in this chapter appears consistent with the findings of Grosso and De Sousa. Assuming a net water flux across the skin from the pond side to the belly side, as is the case for live toads when they absorb water across their abdominal skins, blockage of the apical water channels was expected to cause cellular shrinkage as water ceased to enter the cells but continued to leave across the basolateral side. As the cells shrank the putative apical volume-sensitive Na\(^+\) channels described in the previous chapter were expected to close, resulting in a reduction in Isc as shown in Figure 5.1A. Reversing the argument, application of Hg\(^{2+}\)
to the basolateral side was expected to prevent basolateral cellular water loss but not inhibit apical water entry, resulting in a net volume increase that opened apical Na\(^+\) channels and increased Isc. Again, the findings of Figure 5.1B & C support this view, showing a small but distinctive transient increase in Isc.

While it is probable that Hg\(^{2+}\) is blocking aquaporins in the toad skin, there are several arguments suggesting that the Hg\(^{2+}\) effects seen in Figure 5.1 are not entirely attributable to this action. First, the experiments on isolated skins in this study were performed with no osmotic gradient across the skin, and hence no net water movement from one side to the other. Assuming initially the simplest model possible, consisting of a single cell layer with aquaporins in the apical and basolateral membranes, it is evident that when the skin is bathed in the same solution on both sides water will either enter the cell, or leave the cell from both sides. That is, the direction of water flow across each membrane relative to the cell is the same due to the symmetry of the bathing solutions. As a result, blocking either the apical or basolateral aquaporins should not alter whether the cells shrink or swell: it may slow the rate of cell volume change, but it will not change the direction of the water flow. Based on these arguments, no effect should be observed with Hg\(^{2+}\) due to blockage of aquaporins.

Clearly, apical Hg\(^{2+}\) had a large effect on skins suggesting that either (1) an osmotic gradient did exist across the pumping cells or (2) Hg\(^{2+}\) was not acting simply via aquaporins blockade in the isolated skins. In considering the first option, it is necessary to expand the single cell layer approximation, and consider multiple cells in the epithelium, separated by interstitial fluid. If the osmolarity of the interstitial fluid were sufficiently different from the bathing solution, then net local water fluxes could develop. If these net water fluxes occurred through pumping epithelial cells, such as the MRCs in the first reactive layer of the skin, it is possible that local volume changes could be produced by aquaporin blockade, and affect the putative volume-sensitive Na\(^+\) channels and hence Isc. (Since the MRCs are located close to the apical bathing solution it is likely that a significant percentage of the Hg\(^{2+}\) applied to the apical solution reaches the apical membrane of the MRCs. When Hg\(^{2+}\) is applied to the basolateral solution a smaller percentage probably reaches the basolateral membrane of the MRCs due to the cells lying beneath the first reactive layer. This may account for the muted Hg\(^{2+}\) response on the basolateral side).

Presumably, if cell volume changes were responsible for the Hg\(^{2+}\) effects reported here, then they would be rapid, because application of 100\(\mu\)M Hg\(^{2+}\) to the apical side of the toad skin could cause a reduction in Isc with a time constant of \(\tau=6.6\) minutes.
This raises the important question of how fast volume changes occur. As mentioned earlier, Grosso and De Sousa (1993) found that 1mM Hg\(^{2+}\) applied apically caused a reduction in net water flow in the first twenty minutes after application. This is consistent with experiments conducted in this study that examined the effects of water movement on Isc. It was found that Isc dropped with a time constant of \(\tau=15-35\) minutes when the osmolality of the bathing solution was increased from 100 to 250mOsm/kg\(\text{H}_2\text{O}\) by the addition of sucrose (without changing the ionic concentrations). Since there was only a change in water gradients across the skin in these experiments, the drop in Isc was attributed to water movements and changes in cell volume as described in Chapter 4. The main point here is that both Grosso and De Sousa’s results and those described here indicate that volume change occurs relatively slowly over 15–35 minutes. This suggests that the rapid Hg\(^{2+}\) effects seen at higher concentrations on the pond side, which were completed within minutes, were not primarily due to aquaporin blockage.

Assuming fixed channel densities, one possible explanation for the observed effects on the pond side is that Hg\(^{2+}\) is blocking apical transport proteins causing a reduction in Isc. By a process of elimination experimental results suggest that Hg\(^{2+}\) is not acting via \(K^+\) channels or Na\(^+\)/K\(^+\)/2Cl\(^-\) symports because apical application of TEA or furosemide results in an increase, and not a decrease in Isc and Voc (Figures 5.6 & 5.8). Hg\(^{2+}\) is also probably not blocking apical voltage-activated Cl\(^-\) channels. If this were the case then a rapid (transient) increase in Voc (and Isc) would be expected, due to an overall increase in the resistance of the skin. This was not observed experimentally (Figure 5.1A). Out of all the apical transport proteins it is suggested that Hg\(^{2+}\) is blocking the volume-sensitive Na\(^+\) channels. It has already been reported earlier in Chapter 3 that blockage of these Na\(^+\) channels with 50\(\mu\)M amiloride causes a rapid drop in Isc and Voc within 30 seconds to 2 minutes. The Hg\(^{2+}\) effects at 100\(\mu\)M are consistent with these findings, although the time course is slower which may simply be a function of a lower Hg\(^{2+}\) toxicity compared with amiloride.\(^8\)

Finally, if volume changes are relatively slow, then the small but rapid increase in Isc after basolateral Hg\(^{2+}\) application (\(\tau=4.8\) minutes as shown in Figure 5.1B) suggests that the dominant action of Hg\(^{2+}\) is not via aquaporins. Since there is little evidence of

\(^8\) The existence of Hg\(^{2+}\)-sensitive, amiloride–blockable Na\(^+\) channels has been suggested previously by Jungwirth et al., (1991) who showed evidence for such channels in the principal cells of distal convoluted tubules of the rat.
basolateral Na\(^+\) channels in the toad skin, it is not entirely clear how Hg\(^{2+}\) is acting on this side. However, it is clear that this effect is small and is of secondary importance to the much larger response that occurs when Hg\(^{2+}\) is applied to the apical solution.

### 5.4.3 The action of Pb\(^{2+}\)

Although the systemic toxicity of Pb\(^{2+}\) is widely documented (Friberg & Vostal, 1972; HMSO, 1976), there is surprisingly little in the literature describing the local electrophysiological effects of Pb\(^{2+}\) on ion-transporting epithelia. Clearly though, the results from this study show that Pb\(^{2+}\) can have a significant effect on the pumping epithelia of the toad skin, causing a marked increase in Voc and Isc. This effect appears to be localised to the apical side, with little effect observed when Pb\(^{2+}\) is applied to the basolateral bathing solution. It is therefore suggested that Pb\(^{2+}\) is acting on a particular apical transport protein or is entering cells via the apical membrane and altering the intracellular environment. The following discussion, based on the MRC model presented in Chapter 8, examines five possible sites of action of Pb\(^{2+}\) on the apical side, namely its action on aquaporins, Cl\(^-\) channels, K\(^+\) channels, Na\(^+\) channels and Na\(^+\)/K\(^+\)/2Cl\(^-\) symports. It also examines the possibility of an intracellular event being initiated. However before doing so, the issue of why some skins appear to be sensitive to Pb\(^{2+}\) while others are not is addressed.

As shown in the results section, there is evidence that not all skins respond to apical Pb\(^{2+}\) application. There are at least two possible explanations for this behaviour. First, a Pb\(^{2+}\) effect may not have been present due to a physical limitation. For example, the disintegration of particular (Pb\(^{2+}\)-sensitive) transport proteins during the course of the experiment, or the failure of such proteins to insert into the apical membranes would result in no observable effect upon apical application of Pb\(^{2+}\). A second possibility is that the response had saturated as some limit had been reached (such as clearance of a metabolite or the production rate of an intracellular macromolecule). However, it should be noted that the proposed limit does not appear to affect the magnitudes of Isc and Voc. That is, it is not the case that skins with low Isc or Voc magnitudes are Pb\(^{2+}\)-sensitive while those displaying higher currents and voltages are Pb\(^{2+}\)-insensitive (compare Figures 5.7D, 5.2A and 5.8B with 5.3A, 5.3B and 5.7C).

Considering, each of the apical transport properties in turn now, Pb\(^{2+}\) does not appear to act directly on the voltage-activated Cl\(^-\) channels. If Pb\(^{2+}\) were opening (or closing) Cl\(^-\) channels, then a change in the resistance of the skin would be expected, with a rapid migration away from the iso-resistance trajectory being traversed immediately prior to
Pb\textsuperscript{2+}, towards a lower (or higher) resistance state on the V-I locus. Several examples show that this was not necessarily the case, with little change in the resistance of the skin just before and after Pb\textsuperscript{2+} application (Figures 5.2A,B,D & 5.5C). This suggests that either (1) the action of Pb\textsuperscript{2+} was not via Cl\textsuperscript{−} channels because there was no migration from the iso-resistance line with Pb\textsuperscript{2+}, or (2) the lack of change in the resistance was because the Cl\textsuperscript{−} channels were already maximally activated. (Had they been maximally closed the skin resistance would have followed the ‘line of death’). But if the channels were maximally activated prior to Pb\textsuperscript{2+}, then presumably no effect would be observed when Pb\textsuperscript{2+} was added to the bathing solution in these cases. However, this argument is inconsistent with experimental findings, which showed a significant change in Voc and Isc. Therefore it is concluded that the first option is correct, and that Pb\textsuperscript{2+} action is independent of the Cl\textsuperscript{−} channels.

A second possibility is that Pb\textsuperscript{2+} was operating via apical K\textsuperscript{+} channels.\textsuperscript{9} Assuming a simple shunt resistance and a fixed basolateral resistance, any agent that blocked K\textsuperscript{+} channels would depolarise the apical membrane (and increase the net active current (Isc) in the apical to basolateral direction), while any agent that increased the K\textsuperscript{+} permeability would hyperpolarise the membrane and decrease Isc. Because Pb\textsuperscript{2+} caused an increase in Isc and Voc (which, for a fixed basolateral conductance, meant a depolarisation of the apical membrane), it was proposed that Pb\textsuperscript{2+} was blocking K\textsuperscript{+} channels. With TEA, a known K\textsuperscript{+} channel blocker, having the same effect, it was argued that application of one agent followed by the other would either have no effect, due to a maximal block being elicited by the first agent, or an additive effect, as blockade by the second agent supplemented the first response. However, the experimental findings of Figure 5.7D showed that Pb\textsuperscript{2+} and TEA had opposite effects, and were presumably not both blocking K\textsuperscript{+} channels. Therefore, on the basis that TEA was indeed blocking these channels, it is suggested that Pb\textsuperscript{2+} was not acting via this mechanism.\textsuperscript{10}

In regards to Pb\textsuperscript{2+} acting on aquaporins, it was pointed out earlier in the discussion of Hg\textsuperscript{2+} that for any effect to be observed an osmotic gradient must exist across the

\textsuperscript{9} The presence of apical K\textsuperscript{+} channels, based on TEA results, was an interesting finding in itself because it suggested that the skins were not operating with maximal efficiency. That is, the voltage gradient across the skin generated by the net Na\textsuperscript{+} current from the apical to the basolateral side was partially dissipated by the net K\textsuperscript{+} current in the opposite direction.

\textsuperscript{10} If Pb\textsuperscript{2+} had somehow altered the topology of the cell or altered the basolateral conductance then blocking K\textsuperscript{+} channels with TEA might not produce a depolarisation of the apical membrane and/or manifest itself as an increase in Voc.
pumping cells. This may, or may not exist, in isolated skins bathed in the same solution on both sides. But if an osmotic gradient did exist, and Pb\(^{2+}\) were acting via the aquaporins, to observe an increase Isc (and Voc) it would presumably have to initiate cellular swelling to open the putative volume-sensitive Na\(^{+}\) channels. This would require either: (1) a reduction in the basolateral hydraulic conductivity, or (2) an increase in the apical hydraulic conductivity (i.e. by aquaporin insertion). With respect to the first premise, it is unlikely that Pb\(^{2+}\) altered the basolateral hydraulic conductivity because of its minimal effect when applied to the basolateral solution. In terms of the second proposition, assuming that the aquaporins were Hg\(^{2+}\)-blockable and that the apical membranes were water permeable, it may be argued from the Hg\(^{2+}\) data that because the volumetric effect due to Hg\(^{2+}\) was small, altering the aquaporin density had little influence on the overall response of the cell. Thus, even if Pb\(^{2+}\) were acting on the apical aquaporins, this alone would not explain the sizeable effect observed with apical Pb\(^{2+}\) application. Therefore, it is suggested that the main of action of Pb\(^{2+}\) is not via the apical aquaporins.

Another alternative was that Pb\(^{2+}\) acted to increase the Na\(^{+}\) permeability, either by opening existing channels or by channel insertion, thereby increasing Isc and Voc. However, as pointed out earlier, Pb\(^{2+}\) does not alter the resistance of the skin as demonstrated by a move along the same diagonal on the V-I locus immediately before and after its application (Figures 5.2A,B,D & 5.5C). This poses a problem, because both opening Na\(^{+}\) channels and Na\(^{+}\) channel insertion would change the resistance of the skin and result in a movement off a given ‘iso-resistance diagonal’ which is not consistent with the experimental results. Consequently, it appears that Pb\(^{2+}\) action is not via apical Na\(^{+}\) channels.

With regards to the symports, it was proposed that if Pb\(^{2+}\) were blocking these transport proteins, then preceding or following it with FU (a known Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\) symport blocker) would either have no effect, due to a maximal block being elicited by the first agent, or an additive effect as the second agent supplemented the first. Figure 5.8A shows that Pb\(^{2+}\) following FU could elicit an additive affect.\(^{11}\) On the other hand, FU following Pb\(^{2+}\) (Figure 5.8B) caused a slow reduction in Isc, not consistent with the idea that Pb\(^{2+}\) and FU both acted via Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\) symports. However, it should be noted that the current and voltage scales for the two examples are quite different and

\(^{11}\) The action of FU is interesting as it suggests the presence of apical Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\) symports in the abdominal skin of *Bufo marinus*. (Basolateral FU has little effect (data not shown)). This is in contrast to evidence of basolateral Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\) symports in *Rana temporaria* described by Ussing (1985).
consequently, it could be argued that current saturation had occurred in Figure 5.8B, but not in Figure 5.8A, which was why no further increases in Isc were observed with FU. (Moreover, with a high active current, the ATP utilisation was presumably higher which could explain the subsequent decline in Isc). Thus, it remains a possibility that Pb$^{2+}$ could be acting via apical symports.

Finally, it is plausible that Pb$^{2+}$ was diffusing across the apical face into the cytoplasm of cells where it altered the intracellular environment or attached to the cytosolic side of transport proteins in the basolateral membrane of cells. Eliminating at least one possible site of action though, the literature shows that Pb$^{2+}$ has a direct inhibitory, and not a stimulatory effect, on the Na$^+$/K$^+$-ATPase (Swarts et al., 1987). This suggests that the large, and relatively rapid increases in Isc and Voc were not due to Pb$^{2+}$ stimulating the flux via direct action on the basolateral Na$^+$/K$^+$-ATPase pumps. Furthermore, in a separate series of experiments in tissue slices of the rat kidney cortex, it was shown that ‘O$_2$ consumption and the ATP content were 25-30% lower in slices incubated with 200 microM Pb$^{2+}$ than in control slices’ although ‘the effect on ATP content was not observed until incubation had continued for 30 min’ (van Rossum et al., 1985). These findings suggest that Pb$^{2+}$ was not causing the release of ATP from intracellular stores unless of course, a bolus of ATP was released rapidly and subsequently converted to ADP+Pi (thereby lowering ATP levels) during the first 30 minutes before the ATP levels were measured. But if this were the case, then the overall O$_2$ consumption would presumably have been elevated due to the increased levels of oxidative metabolism. However, this was not observed experimentally. Therefore, it would appear that Pb$^{2+}$ was not causing the release of ATP from intracellular stores. Thus, while it is clear that Pb$^{2+}$ has a significant local action on the abdominal skin of the toad causing a quadrupling in Isc in some cases (Figure 5.5C), the exact mechanism of its action remains unclear.

5.5 Conclusions

In this chapter, previously unreported local effects of the heavy metals Hg$^{2+}$ and Pb$^{2+}$ on Isc and Voc in the toad skin were demonstrated. The main findings were that: (1) the effects were not due to charge screening; (2) Hg$^{2+}$ and Pb$^{2+}$ had opposite and significant local effects; (3) the reduction in Isc was too fast just to be a volume change and was probably due to some other mechanism such as blockade of apical Na$^+$ channels and (4) Pb$^{2+}$ was not acting via apical aquaporins or Cl$^-$, K$^+$ or Na$^+$ channels. Of these findings, possibly the most important, from a general perspective, is that some heavy metals can
have a local effect on pumping epithelia by altering the ion transporting capacities of cells.
6. Steady-state spreadsheet modelling of transporting epithelial cells
6. Steady-state spreadsheet modelling of transporting epithelial cells

6.1 Introduction

Ionic transport across cells is complicated and depends on a number of factors, such as the type of transport mechanisms in the different membranes, the fluxes through these mechanisms and the transmembrane voltage and concentration gradients. Until now the complex interactions within and between membranes, governed by non-linear equations, have typically been modelled using programming languages, such as C++ and Fortran, which require specialist programming knowledge. But with the advent of sophisticated software packages such as Microsoft Excel, and the ever-increasing speed of computer processors, it is feasible to solve the non-linear ion transport problems rapidly using spreadsheet mathematics.

This chapter, the first of three chapters on mathematical modelling, is about the development of a general single-cell model using spreadsheets, which can be used to find voltage or ionic steady-state solutions for cells with different topologies. The main purpose of the steady-state model is to demonstrate the validity of the modelling techniques and to show that the equations can converge to a steady-state solution. The techniques developed for the steady-state model are then extended in Chapters 7 and 8 which focus on osmoregulation in the time-domain.

The model contains two membranes representing the apical and basolateral membranes of a cell, each containing aquaporins, passive Na⁺, K⁺ and Cl⁻ channels, Na⁺/K⁺-ATPase pumps and Na⁺/K⁺/2Cl⁻ symports which can be activated or deactivated according to the configuration of the cell under investigation. Paracellular Na⁺, K⁺ and Cl⁻ pathways are also included. The steady-state model does not contain volume-sensitive permeabilities (such as the putative volume-sensitive Na⁺ channels) because it is assumed that this cell model is isometric, and therefore no volume changes occur, and there are no dilution / concentrating effects of the intracellular solutes. The steady-state model also does not contain voltage-sensitive permeabilities (such as the voltage-sensitive Cl⁻ channels). However, variable permeabilities are incorporated into the time domain solution discussed in the following two chapters.

This chapter is divided broadly into three parts which describe:

1. the mathematical details of the model. This section presents the flux equations for both passive and active transporting mechanisms in a general cell and how they are used to formulate six constraint equations that are necessary to solve for voltage and ionic steady-states.
(2) **important issues that were addressed in developing the model.** This section examines the validity of the Goldman equation, how to specify the initial conditions under different conditions and the issues of uniqueness, and error handling.

(3) **applications of the steady-state model.** This section describes some of the uses of the steady-state model including I-V plots and V-I loci.

To orient the reader first, a brief description of the layout of the cell model is presented.

### 6.1.1 Layout of the general cell model

As described above, many different cell topologies exist depending on the type of transport mechanisms that are present in a cell, and in which membrane these transport proteins are found. Figure 6.1 is a screenshot of the cell model developed in Excel. At the top of the figure, a box showing the different transport mechanisms in the right and left membranes and the paracellular pathways beneath, represents the general cell (Fig. 6.1A). Adjacent to each transport mechanism is a spreadsheet cell containing a number. These numbers define the density of the associated transport mechanism relative to the other transport mechanisms.\(^1\) (If a transport mechanism is not part of the system, then placing a zero in the appropriate cell prevents a flux through the transport mechanism).\(^2\)

Below the topological representation of the cell are bar graphs which show the intracellular and extracellular ionic concentrations, the osmolarities and the potential profile across the cell membranes (Fig. 6.1B). The numerical values of these parameters are shown in the ‘cell environment’ block located under the graphs (Fig. 6.1C). Numbers on the left and right side of the block represent parameters associated with the left and right bathing solutions respectively (and are specified by the user), while numbers in the central column are associated with the intracellular environment.

---

\(^1\) The relative densities of the transporting processes and not the exact physiological densities are important in determining the steady-state ionic concentrations and transmembrane potentials. This is easily demonstrated by considering two separate but identical cells where the densities of the transporting mechanisms are equal and the cells are bathed in the same solutions. In a steady-state the respective intracellular ionic concentrations and membrane potentials are the same. If these cells were then joined together to form one large cell, there would be no change in the intracellular ionic concentrations or membrane potentials. Yet the total number of transporting mechanisms has doubled and the total flux for a particular ionic species has scaled by a factor of two. Thus, scaling all the densities by a constant factor simply scales the magnitude of the fluxes through the cell.

\(^2\) The permeability of channels was obtained by scaling the transport densities by a fixed unitary conductance, \(g_o\).
Figure 6.1: Layout of the single cell model developed in Microsoft Excel. (A) Topological representation of the cell showing paracellular pathways and transport mechanisms in the left and right membranes and their associated densities; (B) bar graphs showing the intracellular and extracellular ionic concentrations, the osmolarities in the different compartments and the potential profile across the cell membranes; (C) ‘cell environment’ block showing the extracellular ionic concentrations in the left and right compartments, and the intracellular concentrations and membrane potentials; (D) fluxes through the individual transport mechanisms; (E) constants describing the current-voltage (I-V) characteristics of the Na⁺/K⁺-ATPase pumps and the Na⁺/K⁺/2Cl⁻ symports; (F) ‘Error’ block describing the errors in the constraint equations; (G) voltage or ionic steady-states equations under open-circuit or short-circuit conditions. The grey buttons beside each spreadsheet cell initiate the macros that solve for the steady-state solutions.
Fluxes through the individual transport mechanisms are calculated based on the intra- and extracellular environments, membrane potentials and transport densities. The numerical values are shown in a ribbon located below the cell environment block (Fig. 6.1D) while the magnitudes are depicted graphically in the cell diagram. The equations describing these fluxes are presented in section 6.2.2.

Constants necessary to describe the current-voltage (I-V) characteristics of the Na\(^+/\)K\(^+\)-ATPase pumps and the Na\(^+/\)K\(^+\)/2Cl\(^-\) symports are listed in blocks located in the middle of the diagram (Fig. 6.1E). Beneath these constants is a set of constraint equations (Fig. 6.1F) used to find voltage or ionic steady-states under open-circuit or short-circuit conditions (Fig. 6.1G) (see section 6.2.4). Two main sign conventions were adopted in the model. First, in terms of ionic fluxes, a positive flux was always defined as a positive ion entering the cell from either the right or left side (or a negative ion leaving the cell). Second, with regard to the membrane voltages, the voltage inside the cell (V\(_{\text{left}}\)) and the voltage across the epithelial layer (V\(_{\text{skin}}\)) were always measured with respect to the left hand compartment. The difference between V\(_{\text{skin}}\) and V\(_{\text{left}}\) was therefore V\(_{\text{right}}\), the voltage across the membrane on the right hand of the cell.

6.2 Mathematical details of the model

6.2.1 The state vector

Mathematically, the single-cell model was formulated as a steady-state problem. The user specified the topology and environment of the cell and then the model searched for a steady-state solution (the state to which the system naturally settled) by allowing the intracellular concentrations, membrane potentials, fluxes and certain ionic conductances to change.\(^3\) The state of the cell at any instant in time was described by approximately 50 parameters collectively referred to as the ‘state vector’. These parameters included: (1) structural attributes of the cell describing the densities of the transport mechanisms; (2) environmental parameters describing the intracellular and extracellular environments; and (3) values describing the I-V characteristics of the Na\(^+/\)K\(^+\)-ATPase pumps, and Na\(^+/\)K\(^+\)/2Cl\(^-\) symports. Explicit expressions for the parameters in the state vector are listed below, all of which had to be specified initially by the user.\(^4\)

---

\(^3\) This situation can be likened to plucking a violin string. The initial condition refers to the displaced state of the string just prior to release (i.e. position, taughtness, mass, velocity) while the steady-state refers to the state of the string when it comes to rest. The transient vibration of the string in between displacement and rest is a function of the system.

\(^4\) The initial conditions specify the state vector at time, t=0.
addition, it was also necessary to specify some scaling constants which are also listed below. Values that were used consistently throughout the modelling have been listed next to the appropriate parameters and where available, experimental values from the literature have been cited in the description.

(1) Structural attributes: Densities of the transport mechanisms

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNa_L</td>
<td>left sodium density</td>
</tr>
<tr>
<td>PK_L</td>
<td>left potassium density</td>
</tr>
<tr>
<td>PCl_L</td>
<td>left chloride density</td>
</tr>
<tr>
<td>DP_L</td>
<td>left Na⁺/K⁺-ATPase pump density</td>
</tr>
<tr>
<td>DS_L</td>
<td>left Na⁺/K⁺/2Cl⁻ symport density</td>
</tr>
<tr>
<td>PNa_R</td>
<td>right sodium density</td>
</tr>
<tr>
<td>PK_R</td>
<td>right potassium density</td>
</tr>
<tr>
<td>PCl_R</td>
<td>right chloride density</td>
</tr>
<tr>
<td>DP_R</td>
<td>right Na⁺/K⁺-ATPase pump density</td>
</tr>
<tr>
<td>DS_R</td>
<td>right Na⁺/K⁺/2Cl⁻ symport density</td>
</tr>
<tr>
<td>PCl_para</td>
<td>paracellular Cl⁻ density</td>
</tr>
<tr>
<td>PK_para</td>
<td>paracellular K⁺ density</td>
</tr>
<tr>
<td>PNapara</td>
<td>paracellular Na⁺ density</td>
</tr>
</tbody>
</table>

(2) Intracellular and extracellular environments

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Na]_L</td>
<td>left Na⁺ concentration</td>
</tr>
<tr>
<td>[K]_L</td>
<td>left K⁺ concentration</td>
</tr>
<tr>
<td>[Cl]_L</td>
<td>left Cl⁻ concentration</td>
</tr>
<tr>
<td>[S]_L</td>
<td>left sugar concentration</td>
</tr>
<tr>
<td>[Prot]_L</td>
<td>left protein concentration</td>
</tr>
<tr>
<td>P_L</td>
<td>left hydrostatic pressure</td>
</tr>
<tr>
<td>[Na]_c</td>
<td>intracellular Na⁺ concentration</td>
</tr>
<tr>
<td>[K]_c</td>
<td>intracellular K⁺ concentration</td>
</tr>
<tr>
<td>[Cl]_c</td>
<td>intracellular Cl⁻ concentration</td>
</tr>
<tr>
<td>[S]_c</td>
<td>intracellular sugar concentration</td>
</tr>
<tr>
<td>[A-]_c</td>
<td>intracellular protein concentration</td>
</tr>
<tr>
<td>[Na]_R</td>
<td>right Na⁺ concentration</td>
</tr>
<tr>
<td>[K]_R</td>
<td>right K⁺ concentration</td>
</tr>
<tr>
<td>[Cl]_R</td>
<td>right Cl⁻ concentration</td>
</tr>
<tr>
<td>[S]_R</td>
<td>right sugar concentration</td>
</tr>
<tr>
<td>[Prot]_R</td>
<td>right protein concentration</td>
</tr>
<tr>
<td>P_R</td>
<td>right hydrostatic pressure</td>
</tr>
<tr>
<td>Volnow</td>
<td>cell volume</td>
</tr>
<tr>
<td>V_L</td>
<td>left/apical membrane potential</td>
</tr>
<tr>
<td>V_skin</td>
<td>voltage across the cell layer</td>
</tr>
<tr>
<td>(NB: the right potential is V_R=V_L-V_skin)</td>
<td></td>
</tr>
</tbody>
</table>

(3) values for the I-V curves of the Na⁺/K⁺-ATPase pumps, & Na⁺/K⁺/2Cl⁻ symports

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_ATP</td>
<td>equilib. const. for reduction of ATP to ADP+P_i (Nicholls, 1982: 1E-8)</td>
</tr>
<tr>
<td>Na_sat</td>
<td>half sat. conc. for Na⁺ (Michaelis const.) (Beague 1984: 20mM)</td>
</tr>
<tr>
<td>K_sat</td>
<td>half sat. conc. for K⁺ (Michaelis const.) (Garrahan &amp; Glynn, 1967a: 1-2mM)</td>
</tr>
<tr>
<td>P_i</td>
<td>voltage sensitivity of the pump</td>
</tr>
<tr>
<td>ATP</td>
<td>intracellular ATP concentration (Stein, 1990: 5mM)</td>
</tr>
<tr>
<td>ADP</td>
<td>intracellular ADP concentration (Stein, 1990: 0.05mM)</td>
</tr>
<tr>
<td>P_i</td>
<td>intracellular inorganic phosphate concentration (Stein, 1990; 5mM)</td>
</tr>
<tr>
<td>K_f</td>
<td># of K⁺ ions translocated per pump cycle (Sen &amp; Post, 1964: 2)</td>
</tr>
<tr>
<td>N_R</td>
<td># of Na⁺ ions translocated per pump cycle (Sen &amp; Post, 1964: 3)</td>
</tr>
<tr>
<td>P_e</td>
<td>electrochemical sensitivity of the symport</td>
</tr>
<tr>
<td>N_sat</td>
<td>(symport) half saturation concentration for Na⁺ (Michaelis constant)</td>
</tr>
</tbody>
</table>
In specifying the parameters described above, several assumptions were made. First, the extracellular solutions were assumed to be infinitely buffered with uniform compositions and infinite volume. Therefore, the movement of ions into or out of the extracellular solutions did not affect the ionic concentrations. (The intracellular environment was assumed to be uniform but in this case the volume was sufficiently small that the concentrations did vary). Second, the hydrostatic pressure in the extracellular solutions was zero. Third, only three diffusible ions (Na\(^+\), K\(^+\) and Cl\(^-\)) significantly influenced the response of the system.

6.2.2 Charge and Fluxes

Once the state vector had been specified for an instant in time, it was possible to calculate the charge within the cell and the fluxes through each transport mechanism. This section summarises the equations used to calculate (1) the charge, and the fluxes for (2) passive transport, (3) primary active transport and (4) secondary active transport.

(1) Total charge in cell

The total charge in the cell, \(Q_c\) was defined as the sum of the charges on the Na\(^+\), K\(^+\) and Cl\(^-\) ions and negatively charged proteins, A\(^-\) (eq. 6.1). This was calculated to ensure that electrical charge neutrality was maintained within the cell, under steady-state conditions.

\[
Q_c = e \times N(z[Na^+]_c + z[K^+]_c - z[Cl^-]_c - n[A^-]_c)
\]

where \(e\) is charge on an electron (1.6*10\(^{-19}\)C), \(N\) is Avogadro’s number (6.02*10\(^{23}\)), \(z\) is the valence of the ion and \(n\) is the average charge per mole of non-diffusible intracellular protein.
(2) Passive Fluxes

All passive ion fluxes (including paracellular fluxes) were modelled using the Goldman-Hodgkin-Katz (GHK) flux equation (eq. 6.2) of the form:

\[
\Phi_i = zV_m P_i [C_{in}] - [C_{out}] \frac{10^{-zV_m}}{10^{E_{10}}} - \frac{10^{E_{10}}}{10^{-zV_m}}
\]

where \(\Phi_i\) is the flux through the channel for some ionic species \(i\), \(z\) is the valence of the ion, \(V_m\) is the voltage across the membrane (or skin in the case of paracellular fluxes), \(P_i\) is the permeability of the ion (see below), \(C_{in}\) & \(C_{out}\) are the intracellular and extracellular ionic concentrations respectively and \(E_{10}\) is the activation energy required to cause a 10-fold change in concentration. It is approximately 60mV at 37°C. \((E_{10} = 2.303RT/zF\) where \(R\) is the universal gas constant, \(T\) is the temperature in Kelvin, \(F\) is the Faraday constant and the value 2.303 is a factor used to convert natural logarithms into base 10 logarithms).

(3) Primary Active Fluxes

The \(\Phi-V\) characteristic of the Na/K-ATPase pump was modelled using an arctan function. This saturating function was chosen for two reasons. First, it was in agreement with the observation that, when operating to actively transport K\(^+\) into the cell and Na\(^+\) out of the cell, the Na\(^+\)/K\(^+\)-ATPase \(\Phi-V\) characteristic was hyperbolic (Gadsby et al., 1985; Gradmann et al., 1978). Second, it was consistent with the observation that, in the absence of cytoplasmic ATP, the Na\(^+\)/K\(^+\)-ATPase pumps reversed resulting in a net K\(^+\) efflux and Na\(^+\) influx (Garrahan and Glynn, 1967b; De Weer and Rokowski, 1984 cited in Lauger and Apell, 1986). Assuming that the I-V characteristic for the ATPase operating in one direction was similar to it operating in the opposite direction, then the total I-V characteristic for the pump would be sigmoidally shaped (i.e. two hyperbolas at 180° to each other, which may be approximated by an arctan function).\(^5\)

The net current through the Na\(^+\)/K\(^+\)-ATPase molecule at a particular membrane voltage was determined by the Gibbs free energy of the pump \((G_p)\), which was the energy available to translocate the ions across the membrane. \(G_p\) (the argument of the arctan function) was defined as the sum of the free energy available from moving ions through their electrical and concentration gradients and the free energy released by the hydrolysis of ATP to ADP+Pi. Equation 6.3 gives the mathematical formula for \(G_p\).

\(^{5}\) It should be noted that experimentally, it is difficult to characterise the I-V characteristic for Na\(^+\)/K\(^+\)-ATPases operating in both directions in animal cells because the application of transmembrane potentials greater in magnitude than the pump reversal potential (which is generally of the order of –250mV), usually results in irreversible damage to the cell membrane (De Weer, 1984).
Explicitly, in order of the terms presented, it states that $G_p$ is the work done in moving $K^+$ through its electrical gradient, minus the work done in moving $Na^+$ through its electrical gradient, plus the Gibbs free energy from the ATP reaction at equilibrium, minus the Gibbs free energy from the ATP reaction not at equilibrium, plus the work done in moving $K^+$ through its concentration gradient, minus the work done in moving $Na^+$ through its concentration gradient. Graphically speaking, altering the magnitude of $G_p$ translates the pump $\Phi-V$ curve in the horizontal direction.

\[
(6.3a) \quad G_p = [K_r V_m - Na_r V_m] + [E_{10} \log_{10} \Gamma - E_{10} \log_{10} K_{ATP}] + \left( K_r E_{10} \log_{10} \frac{[K^+]_c}{[K^+]_o} - Na_r E_{10} \log_{10} \frac{[Na^+]_c}{[Na^+]_o} \right)
\]

\[
(6.3b) \quad G_p = V_m [K_r - Na_r] + E_{10} \log_{10} \left[ \Gamma \left( \frac{[K^+]_c}{[K^+]_o} \right)^{K_r} \left( \frac{[Na^+]_c}{[Na^+]_o} \right)^{Na_r} \right]
\]

where $T = [ADP][Pi]/[ATP]$ and is the mass action ratio for cytoplasmic ATP in a steady-state and $K_{ATP}$ is the equilibrium constant for the reduction of cytoplasmic ATP to ADP+Pi.

Although thermodynamic or energy constraints were incorporated into the $\Phi-V$ characteristic of the pump using $G_p$, to approximate experimental observations well, it was also necessary to include a kinetic limit which scaled the current according to the availability of the limiting substrates (i.e. $[Na^+]_{in}$, $[K^+]_{out}$ and $[ATP]_{in}$). These concentrations were compared with their respective half saturation concentrations (Michaelis constants) to give a fractional value between 0 and 1. Thus, as the concentration of a substrate decreased and approached its half saturation value, the $Na^+$ and $K^+$ ion fluxes were reduced and the net pump current decreased. Of course, if all substrates were present in sufficient concentrations and the pump was operating optimally, the current saturated since all binding sites were filled and no further ions could be translocated in that cycle.\(^1\)

Finally, in developing the $\Phi-V$ characteristic of the $Na^+/K^+$-ATPase pump it was assumed that: (1) the $Na^+/K^+$-ATPase molecule itself was insensitive to voltage and denaturation of the protein did not occur. That is, as the membrane voltage changed, there was no statistical biasing causing the protein to remain in one particular conformation; (2) all ions bound to the transporting enzyme simultaneously and the

\(^1\) A second kinetic issue that can affect the rate of ion translocation (and therefore current) is protein folding. Irrespective of how full the binding sites are, if the protein conformational changes are slow, the transfer of ions across the membrane will be slow. It was assumed in the current model that ‘conformational kinetic limits’ were negligible.
order of binding was not important and (3) ions were tightly coupled\(^7\) thereby side-stepping the issue of variations in the ratio of Na\(^+\) to K\(^+\) due to uncoupled ionic transport.

In light of these assumptions, the Φ-V relationship for the Na\(^+\) flux through the pump is given by:

\[
(6.4) \quad \Phi_{Na_p} = -D_p \left( \frac{[ATP]}{[ATP]^0 + [ATP]_{ext}} \right) \left( \frac{[K^+]_{out}}{[K^+]_{out} + [Na^+]_{sat}} \right)^{K_s} \left( \frac{[Na^+]_{in}}{[Na^+]_{in} + [Na^+]_{sat}} \right)^{N_a} \arctan(P^*G_p)
\]

where \(D_p\) is the density of the pump, \([x]_{sat}\) was the half-saturation concentration and \(P_v\) was an empirical term introduced to alter the voltage sensitivity of the pump.

That is, Na\(^+\) was transported out of the across the basolateral membrane (hence the negative value). The K\(^+\) flux through the pump was simply \(-2/3\Phi_{Na_p}\).

**4) Modelling secondary active fluxes through the electroneutral Na/K/2Cl symport**

Technically, the I-V relationship of an electroneutral symport is meaningless since the transport of ions via the symport is unaffected by the voltage across the membrane and there is no net current through the transporting mechanism. Of greater significance is the flux-electrochemical potential (Φ-Ψ) dependence of the symport, which saturates, and was assumed to be similar to the Φ-V characteristic for the pump.\(^8\) In the present study the Φ-Ψ was modelled using an arctan function where the net flux through the molecule at a particular electrochemical potential was determined by the Gibbs free energy of the symport (\(G_s\)). \(G_s\) was similar to \(G_p\) except that no energy was injected into the system by the hydrolysis of ATP. Broadly speaking, \(G_s\) was defined as sum of the energy required to move ions through an electrical gradient, and through their respective concentration gradients.

\[
(6.5) \quad G_s = \frac{E_0}{m} \left[ [Na_s + K_s - Cl_s] - E_1 \log_{10} \left( \frac{[Na^+]_{out}}{[Na^+]_{cell}} \right) \left( \frac{[K^+]_{out}}{[K^+]_{cell}} \right) \left( \frac{[Cl^-]_{out}}{[Cl^-]_{cell}} \right) \right]
\]

For an **electroneutral symport**, the sum of \(Na_s\), \(K_s\) and \(Cl_s\) is zero so the first term in equation 6.5, representing the work done in moving ions through an electrical gradient is zero.

\(^7\) The issue of ionic coupling in active transport mechanisms is addressed in detail by Stein (1986).

\(^8\) The subject of mathematically modelling co-transporting mechanisms has been extensively analysed by numerous researchers. For a detailed review of this topic the reader is referred to Stein (1986).
As with the pump, the symport was assumed to be kinetically constrained by the availability of the limiting substrates (i.e. \([K^+]_{\text{out}}\), \([\text{Na}^+]_{\text{in}}\), and \([\text{Cl}^-]_{\text{in}}\)). These concentrations were compared with their respective half saturation concentrations, which were maintained at the low value of \(1E-8\). Consequently, this meant that the symport fluxes were generally not kinetically limited.

The \(\Phi-\Psi\) relationship for the \(\text{Na}^+\) (and therefore \(\text{K}^+\)) flux through the symport is given by equation 66. The \(\text{Cl}^-\) flux was found by multiplying this flux by \(-2\).

\[
\Phi_{\text{Na}} = -D_s \left( \frac{[K^+]_{\text{out}}}{[K^+]_{\text{in}} + [K^+]_{\text{out}}} \right)^{K_{\text{Na}}} \left( \frac{[\text{Na}^+]_{\text{in}}}{[\text{Na}^+]_{\text{in}} + [\text{Na}^+]_{\text{out}}} \right)^{N_{\text{Na}}} \left( \frac{[\text{Cl}^-]_{\text{in}}}{[\text{Cl}^-]_{\text{in}} + [\text{Cl}^-]_{\text{out}}} \right)^{C_{\text{Cl}}} \arctan(P_s \ast G_s)
\]

where \(D_s\) is the density of the symport, \([x]_{\text{sat}}\) was the half-saturation concentration and \(P_s\) was an empirical term introduced to alter the sensitivity of the symport to the electrochemical gradient across the membrane.

### 6.2.3 Constraint Equations

Six constraint equations (or error terms) were formulated based on the fluxes and charges that were calculated from the state vector (eq. 6.7-6.12). Combinations of these constraint equations were used to solve for the different steady-states (section 6.2.4). Listed in the order presented below, the constraint equations described the charge build up in the cell, the current through the cell, the current across the cell layer, the \(\text{Na}^+\) current into the cell, the \(\text{K}^+\) current into the cell and the \(\text{Cl}^-\) current into the cell. The superscripts \(\text{Na}, \text{K}\) and \(\text{Cl}\) refer to the species of ion while the subscripts are denoted by \(c = \text{channel}\), \(p = \text{pump}\), \(s = \text{symport}\), \(\text{para} = \text{paracellular pathway}\), and \(L\) \& \(R\) refer to the left and right membranes, respectively.

\[
\begin{align}
\text{6.7) } Q_{\text{cell}} & = e^* N(z[A_{\text{cell}}]^+ + z[C_{\text{cell}}] - n[A_{\text{cell}}]) \quad \text{(from eq. 6.1)} \\
\text{6.8) } I_{\text{cell}} & = zF \left( \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} \right) \\
\text{6.9) } I_{\text{skin}} & = zF \left( \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} + \Phi_{\text{Na}}^{\text{K}} \right) \\
\text{6.10) } I_{\text{Na}}^{\text{cell}} & = zF \left( \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} + \Phi_{\text{Na}}^{\text{Na}} \right) \\
\text{6.11) } I_{\text{K}}^{\text{cell}} & = zF \left( \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} + \Phi_{\text{K}}^{\text{K}} \right) \\
\text{6.12) } I_{\text{Cl}}^{\text{cell}} & = zF \left( \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} + \Phi_{\text{Cl}}^{\text{Cl}} \right)
\end{align}
\]

### 6.2.4 Steady States

There are three main types of steady-states in which a cell (or epithelial layer) can exist: voltage steady state (VSS), osmotic steady-state (OSS) or ionic steady-state (ISS). These states can be found under either open-circuit conditions, where there is no voltage...
applied across the cell layer, or short circuit conditions, where an external voltage is applied across the cell to artificially bias the distribution of ions. As discussed below the constraints for steady-states under open-circuit or short-circuit are slightly different.

VSS occurs within milliseconds, and involves the rapid shuffling of ions across a membrane until the voltage stabilises at a particular value which gives zero net current into a cell, or across a cell layer. The time to reach a VSS is limited by the capacitance and conductance of the cell membranes. As the number of ions that re-distribute across the membrane is small compared with the total number of ions in the cell, the intracellular ionic concentration remains essentially unchanged. In terms of the model, two conditions had to be satisfied to reach a VSS. First, a state of bulk charge neutrality had to exist. That is, there could be no charge accumulation in the cell (i.e. \( Q_c = 0 \)). The second requirement was the cessation of charge reshuffling. Under short-circuit conditions, for this to be true there could be no net current into the cell (\( I_c = 0 \)). For open-circuit conditions, this, and the condition that there was no net current across the cell layer (\( I_{\text{skin}} \)) had to be satisfied. Equations 6.13 & 6.14 respectively give the explicit expressions that were minimised by Solver to obtain a VSS under short-circuit and open-circuit conditions.

\[
(6.13) \quad \text{VSS}_{\text{SC}} \text{ occurs when: } (Q_c)^2 + (I_c)^2 = 0
\]

\[
(6.14) \quad \text{VSS}_{\text{OC}} \text{ occurs when: } (Q_c)^2 + (I_c)^2 + (I_{\text{skin}})^2 = 0
\]

An OSS is where the water into the cell is balanced by the water leaving the cell. OSS was not solved for explicitly because it occurred between VSS and ISS, the two states of interest. However, it could be observed as a matter of course in the time domain modelling, where it was necessary to include the effects of water fluxes (and therefore cell volume) to model permeability changes in volume-sensitive channels. Equations for the incremental changes in volume that produced an OSS are presented in the following chapter.

ISS happens after VSS typically occurring over minutes or hours. It is defined as the condition where there is no net ionic movement of any species. To satisfy this, a state of bulk charge neutrality must exist, there can be no charge reshuffling and the inward movement of a particular species of ions must equal the outward movement. As with VSS, ISS can be solved under either short-circuit or open-circuit conditions, the difference being the additional constraint under open circuit conditions that there be no current across the cell layer. The explicit equations (6.15 & 6.16) defining ISS under short- and open-circuit conditions respectively, are presented overleaf.
All constraint equation terms were squared to avoid errors negating one another and the prediction of a ‘steady-state’ where appropriate constraints were not satisfied.

6.3 Modelling Issues

So far, a spreadsheet model has been presented that simulates ionic transport across a general cell and that can be configured in different ways with a variety of passive and active transport mechanisms in the apical and basolateral membranes. Having presented the mathematical framework in the previous section, attention is now focussed on some of the issues that were pertinent in developing cell modelling.

6.3.1 Validation of the numerical model using the GHK equation

As an internal verification of the spreadsheet calculations it was good practice to check the numerical solutions for the transmembrane voltages against the analytical Goldman-Hodgkin-Katz (GHK) equation. It was found that the GHK equation produced the same estimate for membrane potentials as the numerical spreadsheet model when certain assumptions, applicable to the GHK equation, were satisfied.

The GHK equation which describes the ‘potential reached when two or more ions with unequal equilibrium potentials are free to move across [a] membrane’ is based on several assumptions (Matthews, 1991). According to Goldman (1943), it was assumed that: (1) ‘ions mov[e] under the combined influence of diffusion and electrical forces’; (2) ‘the membrane is assumed to be uniform system.’ That is, the electrical field across the membrane is assumed to be constant and (3) ‘the ion mobility and and activity co-efficients are constant.’ Although the GHK equation accurately represents experimental data under passive conditions (Goldman 1943), the equation does not adequately describe the transmembrane voltage when electrogenic transporting mechanisms are present within the cell membrane (Mullins & Noda; 1963; Sjodin, 1983; Thomas, 1972). Indeed, it is worth remembering that the GHK equation was first formulated (from constant field equations) in 1943, well before the discovery of electrogenic pumps such as the Na⁺/K⁺-ATPases in the 1950’s (Skou, 1957).

The simplest application of the Goldman equation occurs when ions move passively down their electrochemical gradients through a single membrane. As would be expected for VSS conditions, the calculated transmembrane voltage and the Goldman potential are equivalent. Interestingly, one point that arose from the computer modelling was that...
intracellular negatively charged proteins were required for the Goldman equation to apply across a single, passive cell membrane. This issue was originally pointed out by Goldman (1943) in his discussion of ‘fixed ions’ and later addressed by Lew et al., (1979) but otherwise seldom raised. Without these proteins, VSS could not be reached because the constraint equations were not satisfied. That is, at the very least the conditions for VSS under open-circuit (i.e. no charge build-up on either side of the membrane \(Q_c=0\) and no net current across the membrane \(I_c=0\), could not be satisfied simultaneously. In the past, this issue has been avoided in many conceptual models not underpinned by any mathematical analysis by implicitly assuming that the relevant cellular constraint equations were satisfied when the Goldman equation was applied.

Some points that may be worth noting are that, as a general rule, the Goldman equation does not apply across cell membranes where electrogenic transporting mechanisms are present and operational. (Of course, if the active transport mechanisms are non-electrogenic such that there is no net charge transfer across the membrane, the Goldman equation will apply). In addition, the Goldman potentials across both the apical and basolateral membranes of a cell do not approximate the values calculated by the numerical steady-state model when paracellular pathways are present, regardless of whether the concentrations for individual ionic species in the compartments on either side of the cell are identical. This is because a net current can flow through the cells, driven by a concentration gradient, while a neutralising current flows between the cells. This violates Goldman’s basic premise that all ions were transported through membrane channels only.

6.3.2 Specifying Starting Conditions

One of the basic requirements in generating the initial conditions is that the pumping epithelium is bathed on either side in fluids. Without this, the voltage across the membrane that is not in contact with the fluid, and the voltage across the entire epithelial layer cannot be specified as there is no reference point. Consequently, the state vector is indeterminate.

Since many pumping epithelial layers are located within a living entity and are therefore in constant contact with extracellular fluid, indeterminate starting conditions are often not an issue. But they become a problem in cases where the epithelial layer is located on the outside of the entity and is exposed routinely to dry conditions. Such an example occurs in the toad where its abdominal skin, a well known pumping epithelial
layer, is exposed alternately to dry conditions when the toad is on land and watery solutions when the toad moves into a pond environment.

The question becomes how to model the transition between the dry (indeterminate) conditions and the wet conditions (or for that matter, move in the opposite direction from a determinate to an indeterminate situation). The answer is evident from a consideration of epithelial function in the live toad. When the toad skin is exposed to air on its external face it is quite evident that salt and water is not being taken up by the skin. Therefore, it is reasonable to assume that there are no paracellular fluxes, or fluxes (either passive or active) across the outermost epithelial membranes. In terms of the mathematical model, it is appropriate then to set these fluxes arbitrarily to 0\(\mu A\). (Under these circumstances the basolateral membrane voltages and fluxes will adjust accordingly to reach a VSS). But it should be emphasised that although the fluxes may be zero, this in no way suggests that the membrane permeabilities are zero (or that the densities of the transporting mechanisms have decreased). In fact, in vitro results from the toad skin indicate that the apical Cl\(^-\) permeability is actually quite high at the instant when the skin is first bathed in 250mOsm/kgH\(_2\)O NaCl Ringer solution on both sides. Only after exposure to the solution does the Cl\(^-\) permeability begin to decrease. Assuming permeability continuity (and not current continuity) in the instant before and immediately after wetting of the apical membranes, it is suggested that in dry conditions the toad skin may be in a state of suspended animation, ready for operation in the event that the external environment suddenly changes to a wet one. Under these circumstances it is acceptable to initialise the state vector in the model with non-zero (positive) values for the permeabilities and densities of transporting mechanisms located in the apical membranes. Likewise, paracellular pathways may be attributed with a finite permeability. (The exact values to be used must be determined by trial and error until the simulation replicates experimental findings).

Finally, indeterminate variables in the state vector (such as voltage) should be assigned arbitrary values. These do not mean anything in the ‘air-bathed’ case (and have no influence on membrane fluxes because these have been set to 0\(\mu A\)), but they do

---

9 This issue may also be of interest from a clinical perspective as it is possible that some pathologies associated with pumping epithelia may be a result of the epithelium entering an indeterminate state. Given the correct perturbation may enable a resetting or redetermining of membrane voltages, permeabilities and intracellular conditions necessary to restore function.

10 Setting the paracellular and apical membrane fluxes to 0\(\mu A\) can be done quite simply using a ‘dummy’ flux multiplier which is set to 0 to simulate dry conditions and 1 to simulate wet conditions.
provide a start point at the instant when a wet environment is encountered. It is worth pointing out here that where such indeterminate variables influence feedback elements located in the air-exposed membrane (such as voltage-sensitive channels), the initial values for the membrane voltage and the voltage dependent permeability do not have to be consistent. For example, say a voltage-sensitive channels, opens upon depolarisation and has a 50% open probability at +50mV and is maximally closed at +40mV. If half the channels were specified to be open in the state vector, this does not mean that the membrane voltage must initialised with a value of 50. It could just as well be -10 or 80 because in the air-bathed scenario the voltage is not definable. However, as soon as the air-bathed membrane comes into contact with a solution, the model will use the start value of −10(mV) or 80(mV) and iterate to find an appropriate steady-state solution, changing the membrane potential and permeability so that that they are consistent.

6.3.3 Uniqueness of solutions

One important issue that requires consideration in developing a non-linear mathematical model is that of uniqueness of solution. That is, if a cell with a known topology is perturbed in different ways will it converge to the same solution?

The answer to this appears to depend on the topology of the cell and the relative densities of the transporting mechanisms. In some cases, cells with a particular topology can start with quite different membrane potentials and intracellular concentrations and consistently converge to the same solution. An example of this is shown in Figure 6.2 based on Greger’s colonic carcinoma cell model (Greger, 1994) These cells are Cl\(^-\) secreting cells and contain Cl\(^-\) channels in the left (luminal) membrane and K\(^+\) channels, Na\(^+\)/K\(^+\)/2Cl\(^-\) symports and Na\(^+\)/K\(^+\)-ATPase pumps in the right (basolateral) membrane. Condition 1 shows the cell bathed on both sides in ~150mM NaCl solution (a standard experimental solution \textit{in vitro}) and started with intracellular Na\(^+\), K\(^+\) and Cl\(^-\) concentrations of 20mM, 100mM and 46mM respectively, and a voltage across the cell layer of −20mV and a luminal membrane potential of +20mV. Condition 2 is the same cell in the same solution but started with a different intracellular environment with Na\(^+\), K\(^+\) and Cl\(^-\) concentrations of 2.5mM, 170mM and 98.5mM respectively and a voltage across the cell layer of +50mV and a luminal membrane potential of +70mV. In both cases, when an ISS under open-circuit conditions is sought, the cell converges to the same solution with intracellular Na\(^+\), K\(^+\) and Cl\(^-\) concentrations of 12mM, 153.3mM and 91.2mM., respectively. The left membrane potential is −68.6mV and the right
Figure 6.2: An example of Greger's colonic carcinoma cell with different initial intracellular conditions (yellow arrows) converging to the same solution.
Figure 6.3: An example of a cell topology based on a mitochondrial-rich cell of the toad skin. With the transporting densities shown, starting the cell with different intracellular conditions (yellow arrows) resulted in the convergence to different steady-state solutions.
membrane potential is –78.6mV, consistent with Greger’s values of –64mV and -75mV, respectively (i.e. cell negative with respect to the extracellular solution). Other perturbations to the cell (not shown) yield the same result. Thus, although the perturbations are different, the cell appears quite robust repeatedly converging to the same ISS solution, suggesting the existence of a (local) unique solution.

The qualifier is important however, because it is not possible to state unequivocally without a mathematical theorem, that this is the only solution. In a separate region of state space, it is possible that there exists another solution, which is equally valid, but has not been encountered simply because the perturbations were not sufficiently different to cause the cell to converge toward one solution in one case and the other, in the other case. Such an example is illustrated in Figure 6.3 based on a mitochondria-rich cell of the toad skin. (A lower salt concentration on the left side compared to the right side was modelled to simulate brackish pond water and blood plasma, respectively). For the transporting densities shown, at least two ISS solutions exist when the intracellular conditions are started from different values.\(^\text{11}\) In the first case, the intracellular Na\(^+\), K\(^+\) and Cl\(^-\) concentrations are 15mM, 80mM and 21mM respectively with a voltage of +80mV across the cell layer and –40mV across the left membrane. An ISS solution is attained for Na\(^+\), K\(^+\) and Cl\(^-\) concentrations of 6.5mM, 88.6mM and 21.0mM respectively, and a voltage of 2.2mV across the cell layer and –24.2mV across the left hand membrane. In contrast, when the intracellular conditions are started with Na\(^+\), K\(^+\) and Cl\(^-\) concentrations of 15mM, 115mM and 56mM respectively, and a voltage across the cell layer of 10mV and a membrane potential of –10mV across the left membrane, an ISS is reached when the intracellular concentrations are 1.4mM, 112.2mM and 39.6mM respectively, for Na\(^+\), K\(^+\) and Cl\(^-\) and the voltage across the cell layer is 2.2mV and the potential across the left membrane is –12.4mV. This solution is somewhat different from the first solution showing a doubling in Cl\(^-\), a 25% increase in K\(^+\) and a halving of the voltage across the left membrane. Clearly, at least two solutions exist, which may be local unique solutions, which are located ‘close’ together in state space or not unique solutions at all.

The existence of two or more solution states occurs frequently in non-linear biological systems. One example in physiology where different solutions may be attained, depending on the value of a stimulus parameter, occurs in the alveoli of the lung Here, \(^\text{11}\) A cell with the same topology but a different ratio of transporting mechanisms may be more robust, converging toward the same solution over a wider range of intracellular conditions.
the alveoli may be open for different inward pressures and there may appear to be only one solution: the alveoli are always open. But at some point, an incremental increase in the inward pressure suddenly causes the alveoli to collapse in a process called atelectasis. A different state (i.e. closed) is attained just by altering the magnitude of the perturbation. So the fact that a unique solution may not exist in the model is not indicative of a deficiency in the model but rather that one of the numerous parameters has changed sufficiently to cause the cell to move into a different solution state.

The idea that there may exist different steady-state solutions towards which a cell may converge raises many philosophical questions. How many steady-state solutions exist? Are they physiologically attainable? What parameter causes the change to a different state? Can reversing this parameter return the cell to the original steady-state or is there hysteresis in the system and the cell is effectively ‘locked-out’ of the original state? Does the new state affect the stability or robustness of the cell and does this alter function?\textsuperscript{12} Suffice it to say, many questions remain unanswered in the study of non-linear systems such as epithelial cells.

6.3.4 Error terms and numerical stability

One question that was considered was whether the magnitude of individual error terms was biasing the optimisation process and therefore influencing the final solution. If this were the case, then it was expected that multiplying any one of the error terms by a dummy variable, to scale its magnitude, would result in a different steady-state solution. Testing this idea suggested that this was not the case and that scaling the error terms (by a factor of 100) did not affect the final solution for the steady-state model presented in this chapter with fixed transporting densities. Under these conditions the solutions were considered to be accurate.

6.4 Applications of the model

So far, the mathematical equations defining the model have been presented and some of the issues arising from the development of the simulation have been explored. It is now appropriate to discuss some of the applications of the model and how the different steady-state solutions can be used.

\textsuperscript{12} One example where cells appear to move from a stable to a less stable (chaotic) state occurs in non-pacemaker cardiac cells. This behaviour is associated with cardiac dysrhythmias and may be stabilised by pharmacological treatment (Chialvo et al., 1990).
6.4.1 Investigating different cell topologies and different environments

The most significant applications of the model have arisen through reaching the design objectives: the mathematical simulation can be used to investigate cells with different configurations exposed to different environments. While an exhaustive examination of different epithelial cells is well beyond the scope of this research, the model has been used in the detailed analysis of marginal and basal cells of *stria vascularis* in the mammalian cochlea and the investigation of the mitochondrial-rich cells in the toad skin. In each case, not only has the feasibility of proposed configurations been assessed but the responses of the cells to different extracellular environments have been examined. (Details of the toad skin modelling are discussed in Chapter 8).

As a small subset of the broad uses outlined above, it should also be pointed out that the model can be used to analyse a particular cell migrating from one topology to another. Such a situation occurs for example, where environmental factors or pharmacological intervention result in the activation or inactivation of certain permeabilities, an issue is of particular interest in the current study.

In addition, it should be emphasised that the model is not only useful where the structure of the epithelial cells are known. It can also be used as a predictive tool to test whether certain topologies are feasible. This is particularly useful in analysing epithelial layers that have not been well studied and where the transport mechanisms are still to be identified. The model’s predictive capacities are also useful for testing hypothesis prior to experimentation, which can prevent valuable loss of time and resources.

6.4.2 IV plots: An application of ISS under short-circuit conditions

While solving for a steady-state solution in its own right is useful, it is often beneficial to apply protocols which assist in categorising cells in a systematic way. One such protocol utilises the short-circuit ISS (eq. 6.15) to simulate voltage clamp conditions over a range of voltages. By recording the simulated current across the cell layer (eq. 6.9) at each voltage, it is possible to construct the I-V characteristic of the cell. This is useful as it gives insight into how a cell in ionic-steady state, with a certain topology, is affected by voltage. As that topology changes, (either as transporting densities alter or

---

13 A compact disk (under copyright) with the steady-state model is included at the back of the dissertation so that the reader may investigate cells in which they are interested.
as other cells are studied) the I-V characteristic will change and comparisons can be made within or between cells, and verified experimentally where desirable.\textsuperscript{14}

One attractive feature of the simulation is that the I-V characteristics of the transporting mechanisms in each of the cell membranes can also be constructed, if the simulated ionic currents and membrane voltages are recorded for each transcellular clamp voltage. An example of this is illustrated in Figure 6.4 which shows a cell containing Na\textsuperscript{+} and Cl\textsuperscript{-} channels in the left membrane and K\textsuperscript{+} and Cl\textsuperscript{-} channels and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase pumps in the right hand membrane.

\textbf{Figure 6.4:} Simulated steady-state I-V relationships showing the total current through the skin for a given skin voltage clamp (top) and the currents through the individual transport mechanisms as functions of the resulting transmembrane voltages (left and right). Paracellular contributions are shown beneath. Note the change in scale.

\textsuperscript{14} If the cell is the main ion-transporting route in an epithelial layer, then its I-V characteristic will approximate that of the epithelium.
Small paracellular leakages for Na⁺, K⁺ and Cl⁻ are also included. The voltage across the cell layer was ‘clamped’ at 100mV intervals between ±300mV and the relevant data recorded to give the steady-state I-V characteristics for each mechanism. This is useful as it shows which ionic species is dominating the ionic current across a membrane and through which mechanism.15

6.4.3 VI Loci: Application of ISS (or VSS) under open- & short-circuit conditions.

Another useful way to analyse and categorise cells is to use, what we have termed in our laboratory, the V-I locus. The V-I locus can be determined under either ISS or VSS and combines information from the open–circuit and short-circuit solutions. In both steady-states cases, the open-circuit voltage (Voc) is plotted as a function of the short-circuit current (Isc) to give an x,y (Isc, Voc) co-ordinate pair. Depending on the steady-state, this pair summarises the instantaneous (VSS), or long-term (ISS) response of a cell with a given topology and density of transporting mechanisms.

One way that the V-I locus concept has been explored under ISS conditions is through the use of the ‘fingerprinting’ method. Fingerprinting is simply a term that has been coined in our laboratory to describe the V-I locus of a cell that has been subjected to a series of different perturbations. Essentially, a cell with a fixed topology is set up in the model, and Isc and Voc are found. The resulting co-ordinate is regarded as a control point on the V-I locus. Then the cell is subjected to a number of perturbations, and for each perturbation the corresponding Isc, Voc co-ordinate is found and mapped on the V-I locus. (The sorts of perturbations that have been simulated so far include changing the extracellular bathing solutions and pharmacologically blocking different transporting mechanisms by reducing the appropriate densities by a certain percentage). By comparing the perturbation co-ordinates with the control case it is possible to get an overview of how that cell responds to different circumstances. This ‘fingerprint’ can then be compared with other cell topologies that were subjected to the same perturbations enabling cells to be categorised based on responses.

The use of the V-I locus under VSS conditions is slightly different. Because VSS is attained rapidly without the movement of ions, it only represents a transient point in the overall behaviour of the cell as it moves towards an ISS solution. By incrementing the

---

15 The I-V characteristic for a particular membrane can be generated by summing the net current through each transporting mechanism in the membrane at a given voltage (see Patuzzi, 1998). However, the overall I-V characteristic for the cell is not the sum of the I-V characteristics of the individual membranes as paracellular influences must also be included. These influences are negligible in the example shown.
ionic concentrations and membrane voltages and solving for a new VSS, a point close to, but different from the original VSS can be obtained. Repeating this process a number of times it is possible to summarise how the cell migrates from an initial start condition to a final steady-state position. However, the incrementing process is a time-dependent one. This issue is addressed in greater detail in the following chapters.

6.4.4 Reverse engineering

One attractive feature of the numerical model is the ease with which the user can specify the parameters for which they seek a solution. Since the model used simple spreadsheet mathematics, as opposed to program code, it was possible to optimise different parameters by altering the conditions given to the Solver optimisation routine. For example, given the densities of the transporting mechanisms in the cell, the intracellular ionic concentrations and transmembrane voltages can be calculated. Reversing this logic, if the concentrations and potentials are specified (as might be the case after experimentation) then it is possible to reverse engineer the situation to determine the relative densities of the transporting mechanisms required to reach a steady-state using Solver. Both approaches are equally valid for determining a steady-state solution for a particular fixed cell topology.16

6.4.5 Limitations

As with any model, the steady-state model presented in this paper has limitations. One of its main limitations is that it is a single cell model. Only with the addition of more constraint equations can it be combined with other cells to model multicellular systems. (The additional equations are determined by the organization of the cells with different constraints necessary to model cells in parallel and in series). A second limitation is that the extracellular concentrations were fixed. This is not a problem where the extracellular volumes are much larger than the intracellular volumes, as assumed with the toad skin. However, in modelling multicellular systems where the interstitial volumes are not large, this constraint should be relaxed. Third, the steady-state model cannot be used to simulate delays in the system. This poses a problem if there exist channels, for example, that have time-dependent activations and inactivations, an issue that is addressed in greater detail in the chapter on modelling in the time-domain. Lastly, the steady-state model is limited insofar as only the initial and final states are known. The manner in which state variables change over time to bring about an ionic)

16 It is not possible to reverse engineer a cell where the topology changes dynamically over time.
steady-state solution is not assessed. Again, this issue is examined more extensively in the following chapter on time-domain solutions.
7. Time-domain modelling simulating ionic transport across epithelial cells
7. Time-domain modelling simulating ionic transport across epithelial cells

7.1 Introduction

In the previous chapter, the mathematical equations required to solve for a voltage steady-state (VSS) or ionic steady-state (ISS) were presented for a cell with fixed transporting densities. This model was designed to show whether a steady-state solution could be found for a particular cell topology. It was not designed to show how parameters in the state vector changed to reach this solution. While this was perhaps not such an important issue when solving for a single VSS (where only voltages changed in the state-vector) it was of greater importance when solving for ISS. Under these conditions many parameters in the state vector were affected. Since one of the main objectives of this research, was to investigate the dynamic interactions occurring as ions were transported across epithelial layers, it was necessary to incorporate time-dependence into the model, so that changes in the parameters over time could be investigated, as an ionic steady-state solution was approached. This gave rise to the ‘time-domain model,’ an extension of the steady-state model.

Before describing the process of modelling in the time-domain it should be emphasised that, in the last chapter, only cells with fixed topologies were considered. However, this represents only a small subset of epithelial cells. Many epithelial cells undergo ‘structural changes’, a term used here to refer to changes in the cell membranes as transport proteins are inserted, degenerate or change in conformation. These changes often occur in response to changes in the cell’s environment. But because the structural changes affect the efficiency of ion (or water) transport across the cell, the environment is affected, which in turn causes further incremental changes in cell structure, and so on. That is, the structure of the cell evolves in tandem with the cell’s environment: the two are time-interdependent and cannot be separated. In the general time-domain model presented in this chapter, it was assumed that the densities for active transporting mechanisms and aquaporins were fixed. However, the permeabilities of passive transport mechanisms could be fixed, volume-sensitive, voltage-dependent or sensitive to intracellular second messenger levels, enabling complex inter-relationships between cell structure and environment to be modelled.

This chapter is divided into 5 main sections including: (1) an overview of how the time-domain model works; (2) the equations used to model structural (passive permeability) changes; (3) a description of the additional parameters required in the
state vector; (4) the method used to update parameters and (5) the utility of the time-domain model.

7.2 Overview of time-domain modelling

The time-domain model was based on the idea that a series of steps existed between the start point (specified by the initial conditions) and the final ionic steady-state. In the process of stepping between the two points three criteria had to be satisfied. First, at any point examined, conditions of bulk charge neutrality had to exist. To ensure this, there could be no net current into the cell (under short-circuit conditions) and across the cell (open-circuit conditions). Essentially, this meant a VSS had to exist at each step. Second, at some point before reaching the ISS, the water into the cell had to balance the water leaving the cell satisfying the condition of osmotic steady-state. Third, and by definition, by the time an ionic steady-state was attained there could be no net fluxes of any ionic species into the cell (short-circuit conditions) and across the cell layer (open-circuit conditions).

By solving for a sequence of VSS, each incrementally different from the preceding one in terms of concentrations, cell volume and variable permeabilities, the three criteria were satisfied. That is, during the process of solving for successive VSS, the net water fluxes settled to zero so an OSS was reached, and the net currents for the individual ions gradually tended to zero until an ISS steady-state was reached. This process is illustrated in Figure 7.1 which shows a cell with different transporting mechanism in each membrane, representing a general cell. The first VSS (or ‘snapshot’) was found from the initial conditions. Then the voltages, permeabilities and cell volume were updated to give the next state vector from which the next VSS was found. This process was repeated until no further changes occurred and ISS was reached.

7.3 Simulating structural changes in the general cell model

To develop a time-domain solution where the cell adapts to its environment it was necessary to install adaptive mechanisms in the cell. Therefore, this section present a general method, and a variation on this theme, which can be used to model three adaptive mechanisms, namely volume-sensitive, voltage-dependent and intracellular 2nd messenger-sensitive passive permeabilities.
7.3.1 Modelling variable passive permeabilities: The Boltzmann equation

For a population of channels that is sensitive to a particular parameter, at any given value of that parameter, some channels will be closed while some will be open. The probability of channels being open ($p_o$) for a specified parameter value can be described by a first-order Boltzmann activation function (eq. 7.1).

$$p_o = \frac{1}{1 + 10^{\frac{x - x_{12}}{x_{11}}}}$$

where $x$ represents the instantaneous value of the parameter (i.e. cell volume, voltage or 2nd messenger level), $x_{12}$ represents the value of the parameter for which the open probability of the channels is 1/2 and $x_{11}$ is the value of the parameter away from $x_{12}$ necessary to either increase the open probability of the channels from 1/2 to 10/11, or decrease it from 1/2 to 1/11.

Graphically, the Boltzmann function saturates indicating that there comes a point where the probability of the channels being open approaches 1 (and no more channels can open) or the probability approaches 0 (and all channels are closed) (Figure 7.1). Assuming no delays between a change in stimulus parameter and permeability changes,
the number of channels open at any instant \((n_{\text{ch inst}})\) is the product of the open probability fraction of channels and the total number of channels in the population.

![Boltzmann activation curve](image)

**Figure 7.2:** Boltzmann activation curve showing 0,50 and 100% channel activation for a parameter, \(P\) where \(P_{12}\) is the value of the parameter that gives 50% activation and \(P_{11}\) is the distance that must be moved away from \(P_{12}\) to increase channel activation from 1/2 to 10/11 or to decrease it from 1/2 to 1/11. \(P_{11}\) determines the steepness of the curve.

\[(n_{\text{max}}) \text{ (eq. 7.2). Scaling } n_{\text{ch inst}} \text{ by the unitary conductance of the channels (}g_o\text{) gives the instantaneous, parameter-sensitive permeability, } P_{\text{parameter}} \text{ (eq. 7.3).}\]

\[
(7.2) \quad n_{\text{ch inst}} = p_o \cdot n_{\text{max}}
\]

\[
(7.3) \quad P_{\text{parameter}} = g_o \cdot n_{\text{ch inst}}
\]

Thus, equations 7.1-7.3 are the general equations used to model variable channel permeabilities, where the channels are instantaneously sensitive to parameters such as volume, voltage, or an intracellular 2\textsuperscript{nd} messenger event.

### 7.3.2 Modelling delays in sensitivity to a parameter

In living cells, it is often the case that, while the channels may be sensitive to a parameter, there is a delay between changes in the parameter, and changes in channel permeability. For example, conformational changes in the channel protein itself, or the increase/decrease in different intracellular messenger levels, may both result in delays between stimulus and response. Whatever the cause, delays affect ion transport.
Therefore, incorporating such delays into a time-domain model was important to understand transient changes in cellular volume, concentrations and voltages.

One general method that could be used to model delays, without regard for the cause of the delay, was to introduce a series of delay elements. These elements were essentially low-pass filters (LPFs), each having a capacity to filter, and delay the stimulus (which was determined by a time constant, $\tau$). From equation 7.1 it is known that a change in stimulus is manifested as a change in $p_o$. Mathematically then, delaying $p_o$ was equivalent to delaying the stimulus and causing a permeability change. Thus, passing $p_o$ through a delay element resulted in a delayed (and filtered) version of $p_o$ given by $p_{od1}$. Passing $p_{od1}$ through a second delay element resulted in an output of $p_{od2}$ which could then be passed through a third delay element to give an output of $p_{od3}$ and so on as shown in Figure 7.3. (The number of delay elements and the value of the time constants were determined experimentally from channel activation/inactivation curves). The final delayed probability output, $p_{odn}$ was then substituted into eq. 7.2 in place of $p_o$ to give the number of channels open at an instant in time. The permeability of the channel was described as before using eq. 7.3. However, now there existed a delay between when the stimulus parameter changed and when the channels responded to this change.

![Diagram](image)

**Figure 7.3:** Delaying the probability of channels opening using a series of low-pass filters delays the activation and inactivation of channels.

Implementing the delay in channel activation and inactivation over time meant that for each instant, not only did $p_o$ have to be calculated as the stimulus parameter changed, but so did the delayed probability outputs, $p_{od1}$, $p_{od2}$, $p_{od3}$, ......, $p_{odn}$. The incremental changes in $p_{od1}$, $p_{od2}$, $p_{od3}$, ......, $p_{odn}$ as $p_o$ altered were defined as $dp_{od1}$, $dp_{od2}$, $dp_{od3}$, ......, $dp_{odn}$ and were calculated based on instantaneous values of the existing probability values as shown in equation 7.4.

$$
\begin{align*}
\tau_1 \frac{dt}{p_o - p_{od1}} & = \frac{dp_{od1}}{\tau_1} \\
\tau_2 \frac{dt}{p_{od1} - p_{od2}} & = \frac{dp_{od2}}{\tau_2} \\
\tau_3 \frac{dt}{p_{od2} - p_{od3}} & = \frac{dp_{od3}}{\tau_3} \\
& \vdots \\
\tau_n \frac{dt}{p_{od(n-1)} - p_{odn}} & = \frac{dp_{odn}}{\tau_n}
\end{align*}
$$

where $dt$ was the incremental time step
These incremental values were then added to their associated existing instantaneous probability values $p_{od1}$, $p_{od2}$, $p_{od3}$, ….., $p_{odn}$ to obtain the next probability values. The existing value of $p_{odn}$ was then replaced by the new value of $p_{odn}$ in eq.7.2, in preparation for the VSS calculations at the next instant in time.

To summarise, the main advantage of applying the general delay technique described above was its independence of the delay mechanism: any channel that did not respond instantaneously to a stimulus could be modelled using it, and different activation/inactivation times for channels sensitive to different parameters could be modelled by altering the time constants. This means, for example, that second messenger-sensitive channels which activated slowly, and voltage-dependent channels which activated more rapidly, but still with a delay, could both be modelled in the same epithelial cell using the same general principles.1

7.4 Additions to the state vector

As discussed in the previous chapter, the state of the single-cell model at any time was described by a set of parameters known as the state-vector. For the state vector to completely describe the state of the cell in the time-domain required the specification of additional parameters. These parameters included (1) Boltzmann activation parameters for any channel sensitive to a given stimulus and (2) delay parameters for any channel where a delay in the activation or inactivation occurred. Explicit expressions for the parameters required in the state vector are listed below. (If a cell topology only contained channels that were sensitive to a particular stimulus, then only the Boltzmann parameters associated with that stimulus required specification).

1. Boltzmann activation parameters

- $P(d)_{\text{max}}$: maximal density of the volume-sensitive channels
- $d_{1/2}$: displacement for which the open probability is 1/2
- $d_{11}$: displacement away from $d_{12}$ necessary to either increase the open probability of the channels from 1/2 to 10/11, or decrease it from 1/2 to 1/11.

- $P(V)_{\text{max}}$: maximal density of the voltage-sensitive channels
- $V_{1/2}$: voltage for which the open probability is 1/2
- $V_{11}$: voltage away from $V_{12}$ necessary to either increase the open probability of the channels from 1/2 to 10/11, or decrease it from 1/2 to 1/11.

1 A compact disk version of the time-domain model (under copyright) is included at the back of the dissertation. This model includes an apical voltage-dependent Cl\(^{-}\) permeability which displays a delayed activation and inactivation, and an apical volume-sensitive Na\(^{+}\) permeability.
\( P(M)_{\text{max}} \) maximal density of the 2\textsuperscript{nd} messenger-sensitive channels

\( M_{1/2} \) 2\textsuperscript{nd} messenger conc. for which the open probability is 1/2

\( M_{11} \) 2\textsuperscript{nd} messenger conc. away from \( M_{12} \) necessary to either increase the open probability of the channels from 1/2 to 10/11, or decrease it from 1/2 to 1/11.

**2) Delay parameters**

For each type of channel with delayed activations and inactivations, it was necessary to specify the following:

\[ \tau_1, \tau_2, \ldots, \tau_n \] time constants of the \( n \) low-pass filters that delayed the activation and inactivation of the channel conductance

\[ p_{\text{od1}}, p_{\text{od2}}, \ldots, p_{\text{odn}} \] output values of the \( n \) low-pass filters in series

### 7.5 Implementing the solution procedure

Having presented the equations used to model variable permeabilities, it is now pertinent to discuss some of the procedural issues that arose when modelling in the time-domain. This section is divided into three parts which focus on (1) the conditions for which a solution was found; (2) the parameters that had to be updated for each time-increment and how the updating process occurred and (3) issues regarding accuracy and numerical stability.

#### 7.5.1 For which conditions were solutions found?

Solutions to a variety of situations, such as open-circuit, voltage-clamp or current clamp conditions, could be found depending on how the macros were written. While these features were all useful, from a practical perspective, the aim was to model experimental results. Most of the experiments in this project were conducted under open-circuit conditions where the voltage (Voc) was measured, with intermittent recordings of the short-circuit current, Isc which did not affect the state of the cell. (In most experiments where Voc and Isc were recorded, 9 out of every 10 seconds were spent recording Voc and the remaining second was spent recording Isc).

To replicate this scenario, the macros for the time-domain solution were written such that Voc and Isc were found for each snapshot in time. However, since most time was spent under open-circuit conditions experimentally, the intracellular concentrations, cell volume and permeabilities in the model were updated under open-circuit conditions and not short-circuit conditions.\(^2\) This is important to note because under Voc or Isc

\(^2\) Had most time been spent under Isc conditions then theoretical updates would have occurred under this condition and not Voc. If significant time had been spent measuring both Isc and Voc interchangeably,
conditions quite different fluxes through each transporting mechanism can arise, resulting in different changes in concentrations, volume and permeabilities.

7.5.2 Incrementing cell volume, intracellular concentrations and permeabilities

In the steady-state model, when an ionic steady-state solution were sought and it was not important to know how the cell parameters changed over time, it was sufficient to allow the Solver optimisation routine to solve for the cell volume and concentrations. However, when analysing cells in the time-domain it was necessary to develop a procedure for incrementing the cell volume, intracellular concentrations and permeabilities, enabling VSS solutions to be found for a series of snapshots, each slightly different from the last. This section describes how the incremental changes were implemented.

(1) Incrementing cell volume

Water fluxes across the left and right membranes ($\phi_{H2OL}$ and $\phi_{H2OR}$) determined the incremental change in cell volume, $dV$ that occurred at every snapshot in time. Each water flux was described as the osmotic pressure difference minus the hydrostatic pressure difference across the membrane of interest and scaled by the hydraulic conductivity ($L_{H2O}$) according to equation 7.5.

$$\Phi_{H2O} = L_{H2O} [(\Pi_{cell} - \Pi_{out}) - (HydroP_{cell} - HydroP_{out})]$$

where the hydraulic conductivity was the product of the number of aquaporins and the unitary conductance (i.e. $L_{H2O} = n_{H2O} \cdot g_{oH2O}$)

The osmotic pressures $\Pi_{out}$ and $\Pi_{cell}$ were proportional to the extracellular and intracellular solute concentrations, respectively and the hydrostatic pressures acting from the extracellular sides were arbitrarily defined as zero ($HydroP_{out} = 0$). As described by equation 7.6 the intracellular hydrostatic pressure $HydroP_{cell}$ was a function of the stiffness of the cell and the instantaneous volume ($Vol_{now}$) referenced to an arbitrary volume (refvolume).

$$HydroP_{cell} = (Vol_{now} - refvolume) \cdot \text{stiffness}$$

then theoretical updates of the volume, concentrations and permeabilities would be required in both situations.
It was assumed that the stiffness of the apical and basolateral membranes was the same, and was sufficiently high that the volume changes were negligible (i.e. the cell was isometric). (The different relative areas of the membranes could be calculated back from the stiffness, if need be, but these parameters were not expressed explicitly in the model). In order to determine the magnitude of the stiffness, a process of trial and error was used until the cell behaved isometrically.

The sum of the water fluxes into the cell during a time increment, dt, scaled by a factor, delta gave the incremental change in volume, dV as described by equation 7.7.

\[
(7.7) \quad dV = \delta \cdot dt \cdot (\Phi_{H2O_a} + \Phi_{H2O_b})
\]

The instantaneous cell volume (Vol\textsubscript{now}) was then updated to Vol\textsubscript{next} by the addition of the incremental volume change according to equation 7.8.

\[
(7.8) \quad Vol_{next} = Vol_{now} + dV
\]

Thus, at every instant in time, the osmotic and hydrostatic pressures were calculated based on the instantaneous cellular conditions and were used to calculate the water fluxes. The water fluxes were used to calculate the incremental change in volume, which was then added to the instantaneous volume to determine the volume at the next instant in time.

(2) Incrementing ionic concentrations

Ionic concentrations were incremented when a fractional change in concentration (created by the sum of the flux into the cell for a particular ionic species during the time increment, dt) was added to the instantaneous concentration. This fractional concentration was divided by the instantaneous volume to ensure dimensional correctness. The concentrations were constrained to positive values by considering only the absolute values. Finally, to ensure charge balance, the intracellular Cl\textsuperscript{−} concentration was defined as the sum of the Na\textsuperscript{+}, K\textsuperscript{+} and negatively charged protein (A\textsuperscript{−}) concentrations as described by Lew et al., (1979). The explicit expressions for the ionic concentrations that were used in the time-domain modelling were:

\[
\text{Vol}_{next} = \text{Vol}_{now} + dV
\]

3 The ‘scaleit’ factor used in the model was a scaling factor that effectively altered all the flux magnitudes by the same amount.
Incrementing intracellular second messenger levels proceeds along similar lines to eq. 7.9 except that the flux term is replaced by a term describing the difference in second messenger synthesis and consumption within the cell. (In addition if the second messenger is localized to a particular compartment within the cell, the volume of the compartment should be substituted in place of the instantaneous cell volume).

(3) Incrementing permeabilities

At the end of each snapshot when a VSS had been found and the next predicted values of any delayed permeabilities had been determined, the volume and intracellular concentrations were updated. This process was accomplished with a macro that utilised the ‘cut’ and ‘paste’ functions in Excel and it resulted in the instantaneous change of any variable permeabilities where delays were not important. The new predicted permeabilities (both delayed and otherwise), along with the new cellular concentrations and volume were then used in the calculation of the next VSS snapshot.

7.5.3 Accuracy and numerical stability

Two problems that were encountered in both steady-state and time-domain models were those of accuracy and numerical stability. In terms of accuracy, it was important to set the precision, tolerance and convergence parameters in the Solver optimisation routine to sufficiently low values that error terms for the constraint equations, described in the previous chapter, were reduced to zero.4 If this were not done, the solution did not converge and an optimised steady-state solution could not be found. The following values were satisfactory for obtaining steady-state solutions for the cell topologies tested: Precision = 0.0000000001, Tolerance 5% and Convergence 0.001.

Numerical stability was also an issue of interest and depended on the size of the time step, dt. If the time step were too large then the system became numerically unstable.

4 These values are found by going to the Tools menu in the standard toolbar of Excel, activating the Solver menu which brings up the Solver Parameter display box, and clicking on the ‘options’ button.
and constraints for the VSS snapshots were not satisfied. For the topologies that were investigated in this project a time step of $dt = 0.016$ consistently produced numerically stable solutions in the time-domain. Reducing this time step produced the same solution, but it took longer to generate it. If the value of $dt$ was increased above 0.016, it was possible that an ionic steady-state was found more quickly, but the likelihood of the system becoming numerically unstable increased.

7.6 Utility of time-domain modelling

For each snapshot, a row or ‘ribbon’ of values describing the state of the cell was generated in the spreadsheet containing the cell model and all the parameters describing it. This ribbon included the ionic fluxes and membrane voltages under short-circuit conditions, and the ionic fluxes, membrane voltages, intracellular concentrations, water fluxes, volume and permeabilities under open-circuit conditions. At the end of each snapshot, the ribbon (which corresponded to a particular instant in time), was copied and pasted into a second spreadsheet. Values for the volume and the intracellular concentrations were then updated in preparation for the next snapshot. This procedure was repeated at each instant and the new ribbons pasted beneath the old ones in sequential order so that an array of numbers was generated. Each row in the array was the ribbon and each column in the array was an individual parameter changing as a function of time.

Plotting the individual parameters against time showed the dynamic interactions between the membrane potentials, fluxes, ionic concentrations and time-variant permeabilities for a particular cell topology, under short-circuit and open-circuit conditions. This was extremely useful because it meant that experimental results, where it was only possible to measure $V_{oc}$ and $I_{sc}$, could be investigated in terms of the simulated ion movements across the epithelial layer.
8. Mathematical modelling of MRCs in the toad skin
8. Mathematical modelling of MRCs in the toad skin

8.1 Introduction

In Chapter 6 a general mathematical model was developed for ion transport across a pumping epithelium consisting of a single layer of one type of cell. In that chapter mathematical equations were presented which described the different ion transporting mechanisms. Equations for determining the VSS and ISS under open- and short-circuit conditions were also given. Chapter 7 then extended the ideas developed in Chapter 6, describing how transient changes in the time-domain could be modelled. However, the analysis so far has focussed on the general method used in developing the model. In Chapter 8 the emphasis changes to consider the practical applications of the model, with reference to the MRCs of the toad skin. The chapter is divided into 3 main sections describing: (1) the archetypal MRC model; (2) how parameters were optimised to obtain realistic time-domain solutions and (3) a comparison of selected modelling results with experimental data. The main aim of this chapter is to demonstrate how different ion transporting elements can be combined in a single cell in such a way that salt and water uptake are regulated locally, without neural or hormonal control.

8.2 Archetypal Cell Configuration

Consistent with the single MRC model discussed throughout this dissertation, the archetypal MRC used in the mathematical model had the following ion transporting mechanisms: located in the apical membranes of the cell were aquaporins, voltage-dependent Cl⁻ channels, volume-sensitive Na⁺ channels and Na⁺/K⁺/2Cl⁻ symports, while aquaporins, K⁺ and Cl⁻ channels and Na⁺/K⁺-ATPase pumps were positioned in the basolateral membrane. Paracellular Na⁺, K⁺ and Cl⁻ permeabilities separated the apical and basolateral sides.

Ionic steady-state solutions were determined for the archetypal MRC bathed on both sides in a 100mOsm/kgH₂O ‘NaCl-like’ solution, consisting of 45mM Na⁺, 2mM K⁺ and 47mM Cl⁻. As will be discussed in section 8.6, the initial steady-state solution that was obtained for the cell prior to the ‘experimental’ perturbation was dependent on the relative ion conductances of the cell. Thus, although the ionic perturbation is the same in both cases, and the type of transporting mechanisms that are present are the same, the differences in ion conductances mean that the system or cell itself is different.

While on the topic of the bathing solutions, it is also worth commenting on one of the techniques that was employed in the modelling to ensure a directional water movement. In Chapter 5 it was argued that for a single cell possessing both an apical and
basolateral water permeability, water would either be lost or gained by the cell when placed in the same solution on both sides. Under these circumstances no directional water movement would occur where water moved from the apical side to the basolateral side or vice versa. This is obviously not how the toad skin functions since this pumping epithelial layer is the primary layer in which the toad absorbs water from the pond environment. This suggests a directional water movement from the pond to the belly side. In a similar manner, to ensure a directional movement in the mathematical model, a small osmotic gradient was introduced across the cell in the form of a 25mM sugar bias added to the basolateral side. This ensured a directional movement consistent with in situ observations.

8.3 Finding a solution

The most common method for recording experimental data in this study was to measure the open-circuit voltage most of the time, with brief interludes where Isc was measured. In an attempt to most accurately model the experimental results a ‘Voc/Isc’ macro was developed in Visual Basic for the spreadsheet model, as described in Chapter 7 (section 7.5). Briefly, a VSS was found (and temporarily stored) under open-circuit conditions. Isc was then found using the intracellular ion concentrations and membrane permeabilities determined under Voc. Restoring appropriate membrane voltages for the Voc conditions, the intracellular concentrations and permeabilities were updated and the next VSS was determined.

8.4 Block Diagram

Before looking at the details of the mathematical modelling in the time-domain, a block diagram has been presented to show the possible feedback pathways that were considered in the archetypal model of the MRC. The block diagram (Figure 8.1B), which is positioned below the schematic of the MRC (Figure 8.1A) is laid out in a similar manner to the schematic of the MRC: parameters associated with the apical membrane are located on the left and parameters associated with the basolateral membrane are located on the right. Intracellular and paracellular parameters are positioned towards the middle of the diagram.

As can be seen, the feedback pathways are complicated. Starting at the top of the diagram, it is apparent that changes in the apical and/or basolateral membrane potentials (and hence the voltage across the entire cell layer) affect the fluxes across the respective membranes. Changes in the fluxes in turn feedback and affect the membrane potentials.
Figure 8.1: (A) Schematic representation of ion-transport mechanisms and aquaporins in the mitochondria-rich cells (MRCs) of the toad skin. (B) Feedback loop showing the interactions between membrane voltages, fluxes, the relative densities of the transporting mechanisms, the extra- and intracellular ionic concentrations, and cell volume.
They also affect the intracellular ionic concentrations. Altering the ionic concentrations results in changes in the osmotic pressure of the cell, which leads to water movements across the apical and basolateral membranes, and changes in the volume of the cell. These volume changes are important as they result in the opening/closure of the volume-sensitive Na\(^+\) channels as the cells swell/shrink. This dynamic change in the apical Na\(^+\) permeability, along with the change in the voltage-dependent Cl\(^-\) permeability (caused by fluctuations in the apical membrane potential) can lead to changes in the ion fluxes across the apical membrane. As described above, changes in the fluxes then feed back onto the membrane voltage and intracellular concentrations.

Having identified the main feedback pathways in the single cell model, the aim was to determine whether the various model parameters could be optimised in such a way as to replicate some of the experimental results. Confirmation of this issue would support the idea that ion transport across an epithelial layer could be locally regulated. Methods for optimising the parameters so that the model responded reasonably to environmental perturbations are discussed in the following section.

8.5 Feedback and Optimising the Parameters

In order to fine tune the feedback circuits presented in Figure 8.1, three basic questions were asked: (1) Is there a relationship between membrane voltage and the volume of a single cell? (2) If so, can this relationship be adjusted/optimised to explain the observed experimental results, and (3) What is the predicted relationship between the membrane voltage and cell volume? Unfortunately, while the questions are relatively simple, the answers, which are largely interdependent, are not. But some clues can be obtained from the experimental data presented previously in this study, as summarised below.

It was shown in Chapter 3 that the Cl\(^-\) channels could open and close maximally over 10-15 seconds under an imposed voltage clamp. This was demonstrated in Figure 3.6 where the skin voltage was clamped at successively more positive values (belly side positive), separated each time by a return to a holding potential of –40mV. From this data it was clear that, for quite significant electrical perturbations, the Cl\(^-\) conductance

---

1 Changes in the basolateral Na\(^+\) and K\(^+\) pump fluxes presumably also influences the intracellular ATP concentration. The effect of this was considered during the course of developing the model. However, it was found that many of the experimental results could be modelled quite closely without the addition of a fluctuating ATP concentration. Consequently, the ATP concentration was fixed at a constant value for the modelling results that are presented in this chapter.
could change rapidly. However, in circumstances where an external voltage was not applied, it appeared that the Cl⁻ conductance changed relatively slowly over several hours. Examples of this were frequently observed upon exposure of a skin to a ‘NaCl containing’ bathing solution, which often yielded the familiar ‘N’ shaped V-I locus. As shown in Figure 8.2, the experimentally derived V-I locus is consistent with the idea that Cl⁻ channels are open initially (point ‘a’), as depicted by a low resistance skin. But as the apical membrane potential presumably hyperpolarises, the Cl⁻ channels begin to close, until all of the Cl⁻ channels are fully closed and the skin enters a higher resistance state.

![Figure 8.2: V-I locus showing a typical 'N-shaped' change in the resistance of the skin during the experiment. Notice the migration from a low-resistance at the beginning of the trace ('a'), where the apical Cl- channels are mostly open, to a higher resistance at the end, where the Cl- channels are mostly closed. The trace is a modified version of experimental data collected from Toad 86a bathed in 250mOsm/kgH₂O reduced NaCl Ringer (Solution 40). The insert depicts a Boltzman function. As the apical membrane potential hyperpolarises (i.e. moving towards the left), the voltage-dependent Cl⁻ channels begin to close off.](image_url)

If the ‘N’ shaped curve is due to the closure of the Cl⁻ channels, it suggests several points: First, it suggests that a significant change occurs in the apical membrane potential. Second, given that the Cl⁻ channels have the capacity to open and close rapidly (over 15 seconds), and yet the Cl⁻ conductance changes slowly, over a matter of
hours, it would appear that the voltage changes are not the dominant variable in the feedback circuit. Instead, it is suggested that changes in some other parameter (possibly PNa) are causing the membrane potential to change, which in turn affects the Cl⁻ conductance. So, effectively, the Cl⁻ conductance is in a pseudo-equilibrium, following the changes in other cell parameters. As a corollary of this, the third point is that transients in the Cl⁻ permeability are finished well before the completion of changes in other cell parameters. More specifically, it was hypothesised that PNa changes were still occurring after PCl was closed. Certainly, the experimental data supported this proposal, with Voc and Isc continuing to drop in Figure 8.2, after the skin had entered the high resistance region (where the Cl⁻ channels were closed). As a result, feedback in the single cell model was examined on the basis that the voltage range determining the upper and lower limits of the Cl⁻ conductance occurred somewhere within the volume range which determined the upper and lower Na⁺ conductance limits. As discussed in the following section, the Na⁺ and Cl⁻ Boltzmann parameters were therefore adjusted accordingly, so that the Cl⁻ Boltzmann curve was traversed within the period in which the Na⁺ Boltzmann was traversed.

8.6 Optimising the Boltzmann Parameters

Determining the relative parameters for the Na⁺ and Cl⁻ Boltzmann curves required an iterative process which involved a significant amount of trial and error due to the number of variables that changed with even the simplest perturbation of the system. Because of these changes it was often difficult to separate the important first-order ‘driving’ events from the less important, second-order ‘driven’ events. The problem was compounded further by a lack of direct experimental data. Only the transepithelial open-circuit voltages and short-circuit currents had been measured whereas, ideally, temporal changes in the apical MRC membrane potential and cell volume should also have been recorded to determine the half activations and sensitivities of the Na⁺ and Cl⁻ channels. Since this experimental information was not available from this, or other studies, it was necessary to adjust the Na⁺ and Cl⁻ Boltzmann parameters to see how the cell model responded to a standard perturbation (in this case changing from a 100mOsm/kgH₂O ‘medium salt’ NaCl solution to a 250mOsm/kgH₂O ‘high salt’ NaCl solution. Thus, as shown in Figure 8.3, the objective was to set up a cell model where a simple change in the bathing solution (from a 100mOsm/kgH₂O NaCl solution to a 250mOsm/kgH₂O NaCl solution) caused sufficient changes in the apical MRC membrane potential that the probability of the Cl⁻ channels being open dropped from a relatively high percentage...
Figure 8.3: Ideally, the aim was to optimise the cell parameters such that when moving from a steady-state condition in a 100mOsm/kgH2O NaCl solution to a 250mOsm/kgH2O NaCl solution the Cl⁻ Boltzman would be traversed within the period during which the Na⁺ Boltzman was traversed. Thus, during the period in which the Na⁺ channels were closing due to cell shrinkage, the Cl⁻ channels would have closed off.
to a low percentage. At the same time, the solution changes also had to initiate a sufficient reduction in cell volume such that the probability of the Na\(^+\) channels being open dropped from a high percentage to a low percentage. However, the rates of change (i.e. steepness) of the Boltzmann curves could be different, as could be the values of the initial and final open probabilities for the Na\(^+\) and Cl\(^-\) channels.

8.6.1 PCl starting Values

It was found that, when moving from a medium salt solution to a high salt solution, the Cl\(^-\) Boltzmann parameters should be adjusted such that the open probability of the Cl\(^-\) channels should be 50% or less in the medium salt solution. That way, the Cl\(^-\) channels could open transiently in response to the rapid depolarisation that appears to occur upon placement of the cells in the high salt solution (compare \(V_L\) in Figure 8.6M and Appendix 11M at \(t = 64\) minutes). Had the Cl\(^-\) channels been fully open, or close to it in the medium salt solution, placement in the high salt solution would have had little effect on the open probability of the Cl\(^-\) channels. However, the operating point (defined as the point where the instantaneous value of the parameter defining the x-axis of the Boltzmann curve intersects with the Boltzmann) is driven further to the right, into the (100%) saturated region of the Boltzmann curve. The problem with this is that subsequent placement in a medium salt solution, may not sufficiently hyperpolarise the apical membrane to cause the operating point to enter the linear region of the Cl\(^-\) Boltzmann curve. Consequently, the Cl\(^-\) channels may remain open for a perturbation that would typically be expected to cause channel closure.

8.6.2 PNa Starting Values

In contrast, it was found that the Na\(^+\) Boltzmann parameters should be adjusted so that the probability of the Na\(^+\) channels being open was close to 100% in the medium salt solution. (That is, the Na\(^+\) operating point should be set near to the edge of the upper saturated region). Manipulating the Boltzmann parameters during the initial development of the model to ensure that PNa was almost a maximum in the 100mOsm/kgH\(_2\)O medium salt solution was important for three main reasons.

First, it meant that certain cell configurations existed where Isc remained stable over time. This helped establish the credibility of the model because examples where Voc and Isc remained stable over hours (following an initial transient period) had been recorded experimentally across skins bathed in 100mOsm/kgH\(_2\)O NaCl solutions (see Figure 4.9K).
Second, it meant that as the volume was increased (shifting the operating point to the right) PNa increased slightly until 100% of the channels were open. This meant that when placed in moderately dilute NaCl solutions (say, 50mOsm/kgH\textsubscript{2}O NaCl), slightly higher values of Isc could be modelled consistent with experimental observations. However, as the NaCl concentration was further reduced on the apical side Isc began to drop, although all of the Na\textsuperscript{+} channels were open due to the swollen state of the cell. This reduction in Isc was due to the reduced, inwardly directed electrochemical gradient for Na\textsuperscript{+} across the apical face. With less Na\textsuperscript{+} entering the cell, less Na\textsuperscript{+} was actively pumped out of the cell across the basolateral membranes.\footnote{Laboratory results (not shown) indicated that the Na\textsuperscript{+}/K\textsuperscript{-}-ATPase pumps became kinetically limited by Na\textsuperscript{+} when the apical extracellular Na\textsuperscript{+} concentration dropped below approximately 5mM. Above this, Isc remained relatively stable (until the osmolality of the bathing solution caused the cells to shrink and PNa to close).} Again, this behaviour (modelled in Appendix 42), was consistent with experimental observations.

The third reason for ensuring that PNa was almost maximally open in a 100mOsm/kgH\textsubscript{2}O NaCl solution was to maximise the cells capacity to recover from a high salt (shrinking solution). Setting the cell volume so that the cells were swollen and the Na\textsuperscript{+} operating point was located at the upper end of the linear region of the Boltzmann for example, gave the greatest range for cell shrinkage during which PNa could alter. Had the operating point been located midway along the linear part of the Boltzmann for example, the PNa regulation range would have been diminished by 50%. That is the volume changes required to cause complete Na\textsuperscript{+} channel closure would be half that of the volume change required to cause channel closure when the Na\textsuperscript{+} operating point was located at the upper end of the linear region of the Boltzmann curve. Since the experimental results (see Figure 8.2) suggested that the Cl\textsuperscript{-} channels close during the period in which the Na\textsuperscript{+} channels are closing, maximising the PNa range was also advantageous as it yielded a more detailed PCl response. (i.e. the larger the volume range the smaller the change in the apical membrane potential per unit change in volume). This meant improved feedback control of the Cl\textsuperscript{-} conductance over a wider volume range. The overall effect was greater dynamic control (i.e. better regulation) of NaCl movement across the apical membrane.

\subsection{Effect of Apical PK}

During the course of optimising the Boltzmann parameters the importance of including an apical K\textsuperscript{+} permeability (PK) in the model became apparent. Originally, an
apical PK had not been included in the model, primarily because it had not been included in the models developed by other investigators (Koeffoed-Johnsen & Ussing, 1958; Larsen, 1991). But the experimental findings of this study showed that the application of TEA to the apical solution caused an increase in Isc and Voc which was consistent with apical K⁺ channel blockade (Figure 5.6). These findings appeared somewhat counter-intuitive initially, as the presence of K⁺ channels in the apical membrane of MRCs would presumably (1) cause uncoupling between the Na⁺ and K⁺ transport across the basolateral membrane, thereby reducing the efficiency of Na⁺ uptake across the epithelial layer and (2) result in KCl fluxes across the apical membrane, again reducing the efficiency of Na⁺ uptake. However, because the TEA experimental results could not be explained in the absence of an apical K⁺ channels, the effects of including these K⁺ channels in the model were investigated.

The results from two theoretical simulations provided some interesting results. In the first case, cells were modelled in the same 100mOsm/kgH₂O NaCl solution and the effects of altering only the apical PK on the steady-state apical membrane potential and cell volume were investigated (Figure 8.4). It was found that, as PK was increased, the steady-state apical membrane potential became increasingly more negative, and the cell volume decreased. Consequently, the initial open probabilities for the Na⁺ and Cl⁻ channels were respectively higher for the cells with lower apical PKs.

The second important finding was that in different solutions, the steady-state cell volume and membrane potentials (and hence Na⁺ and Cl⁻ open probabilities) could be quite different for different apical K⁺ permeabilities. Figure 8.5 depicts this schematically. The main point to note is that for low apical K⁺ values, the steady-state apical membrane potential becomes more positive, and the cells swell when the bathing solution is changed from a 100mOsm/kgH₂O NaCl solution to a more concentrated 250mOsm/kgH₂O NaCl solution on both sides. (Under such circumstances, if the channels were already open, little effect in the overall resistance would be observed). In contrast, when the bathing solution was changed from a 100- to a 250mOsm/kgH₂O NaCl solution on both sides for cells with comparatively high apical PK values, a hyperpolarisation of the apical membrane potential was observed and the cells shrank, resulting in the respective closure of the Cl⁻ and Na⁺ channels.

These findings were extremely promising for two reasons. First, from a modelling perspective the existence of an apical PK was important because it provided a single variable for manipulating the initial apical membrane permeabilities without interfering with the feedback mechanism between volume and voltage. Perhaps cells are
Figure 8.4: Different operating points on (A) the Cl⁻ Boltzmann activation curve and (B) the Na⁺ Boltzmann activation curve for MRCs bathed in the same 100mOsm/kgH₂O NaCl solution under steady-state conditions, but with different apical K⁺ permeabilities. For (i) low values of PK, the Na⁺ and Cl⁻ channels have a greater probability of being open than for (ii) higher apical K⁺ permeabilities.
Figure 8.5: The (steady-state) start conditions in a 100mOsm/kgH₂O NaCl solution and the end conditions in a 250mOsm/kgH₂O NaCl solution depend largely on the apical K⁺ permeability (PK). For low values of PK, increasing the osmolality of the bathing solution does not cause the cells to enter into either the Na⁺ or the Cl⁻ Boltzman regulatory regions, and hence no shutdown of the Na⁺ and Cl⁻ channels occur. For higher values of PK, the steady-state starting permeabilities are lower and salt uptake is more likely to be regulated by the closure of apical Na⁺ and Cl⁻ channels.
genetically equipped with this mechanism for setting/altering the ‘initial’ conditions by the regulation of mRNA for K⁺ channel expression.

The second reason why these steady-state results were promising was that they began to explain why simple perturbations of cells with slightly different transporting densities gave what had initially appeared to be quite different, and sometimes contradictory, results. However when it was realised that for low K⁺ permeabilities, increasing the osmolality of the bathing solution did not cause the cells to enter the Na⁺ or Cl⁻ regulatory regions (i.e. the linear sections of the respective Boltzmann curves), it was not surprising that neither Na⁺, nor the Cl⁻ channels closed. For higher K⁺ permeabilities however, the lower steady-state open probabilities made it more likely for the Na⁺ or Cl⁻ channels to either enter, or already exist within the regulatory ranges of the Boltzmann curves. Hence, with a high apical PK it was more likely for the Na⁺ and Cl⁻ channels to close upon exposure to the high salt, high osmolar solutions. Further implications of an apical PK in the MRCs are discussed in more detail in section 8.7.2, which examines the transient changes that occur between the initial steady-state and the final steady-states considered above.

Before looking at the time-domain solutions, one final issue requires discussion. During the course of developing the model and optimising the Na⁺ and Cl⁻ Boltzmann curves to most accurately explain the experimental results in this study, it was found that the apical PK had to be the same order of magnitude as the apical Na⁺ permeability. Two pieces of experimental evidence suggested this. First, amiloride experiments (Figure 3.2) showed that neither Voc nor Isc became highly negative after Na⁺ channel blockade. Had the PK been much larger than the Na⁺ permeability in these toad skins, such behaviour would have been expected. Second, placement of the skins in high 250mOsm/kgH₂O NaCl solutions did not result in Voc or Isc becoming significantly negative. Therefore, the value of the apical PK in the model could not be set so high that Voc and Isc became very negative when in a 250mOsm/kgH₂O shrinking solution. If anything, there was evidence to suggest that, in some cases, Voc and Isc appeared to stabilise at slightly positive values. In these examples, it is suggested that the apical Na⁺ permeability was marginally larger than the PK. In any case, for the purposes of the modelling, K⁺ permeabilities of about the same value as the Na⁺ permeabilities were investigated, and the Boltzmann curves were adjusted so that for different K⁺ permeabilities there was a range of steady-state open probabilities for the apical Na⁺ and Cl⁻ channels.
Time-Domain Modelling

8.7 Presentation of results

The theoretical results of this chapter are presented in both a ‘distilled’ and ‘expanded’ form. The distilled form is shown in the main body of the chapter, and summarises changes in the apical membrane potential, cell volume, PCl, PNa, Voc and Isc over time for a particular perturbation. Generally, several variations of a perturbation are shown per page so that the overall trends are clear. However, for each perturbation there are changes in many variables, all of which contribute to the overall behaviour of the cell, as clear from the feedback diagram of Figure 8.1. Due to the large number of variables, it was not feasible to present these detailed changes in the main body of the text and still maintain a focus on the important issues. Consequently, the particulars of each perturbation are presented in ‘expanded’ form in the Appendices at the end of this dissertation. For ease of reference, the appropriate appendix number is located above each perturbation in the diagrams as ‘App.#’.

One example showing a template of the Appendices is presented in Figure 8.6. Briefly, the cell topology, the relative ion transporting densities, and the bathing solutions are shown at the top of each page for the initial steady-state condition (left), and the final steady-state conditions (right) to which the cell settles after the perturbation. Parameters, which are highlighted in bright yellow, indicate the main, or initial perturbation that was applied to the cell, while parameters shaded in light yellow indicate other changes in conditions.

Beneath each cell configuration is a series of graphs showing how the various parameters changed over time to reach the final steady-state solution. It should be emphasised that each modelling run was started under (open-circuit) ionic steady-state conditions for the cell as depicted in the ‘initial state.’ Therefore, points shown at t=0 minutes represent the initial steady-state and, in figures which are not a function of time (such as the V-I locus in Figure 8.6D), this steady-state is denoted by a large square. Any subsequent migrations away from this steady-state represent the effects due to the ‘experimental’ perturbation.

Each parameter in the graphs is labelled in a colour-coded manner. Where necessary, descriptions of each can be found in the Abbreviations List at the beginning of this thesis. Generally, the meaning of each parameter can be determined from the vertical axis and the cell topologies at the top of the page. The main abbreviations that were used were: ‘s’ and ‘p’ referring to ‘symports’ and ‘pumps’; ‘P’ referring to the permeability of the cell to a particular ion; ‘OC’ and ‘SC’ denoting ‘open-circuit and
Figure 8.6: Archetypal MRC (with an apical PK=2) under steady-state conditions in a 100mOsm/kgH₂
‘short-circuit’ conditions and the subscripts ‘L’ and ‘R’ referring to the ‘left’ and ‘right’ membranes, respectively. In addition, a summation sign was used to indicate the total ionic current into the cell (ΣI), square brackets ([ ] ) were used to denote ionic concentrations and φ was used to represent hydraulic fluxes. (A negative water flux symbolises water movement out of the cell). Finally, unless otherwise indicated, all graphs were presented as a function of time (in minutes).

8.7.1 Time Step and Numerical Stability

Before looking at the results generated by the model it is worth commenting on the size of the time step (dt) used in the time domain modelling. In order to minimise the amount of computing time it was desirable to make the time increment between successive voltage steady-state solutions as large as possible. However, if the time steps were made too large, then the system could become unstable due to the size of the numerical errors.

To determine an incremental time step for which the simulations were stable for most perturbations, the model was run with different values of dt. As shown in the representative examples of Appendices 1-5, it was found that similar trends were observed for dt=0.004, 0.008, 0.016 and 0.032 indicating only small numerical errors in the modelling. However, as the time step was increased further to 0.064, the numerical errors were sufficiently large that instabilities could be observed in the results (App. 5). Clearly, a time step of 0.064 was too large to produce stable traces. Therefore, a smaller time step was used. As a compromise between computing time and numerical stability a time step of 0.016 was chosen.

Finally, since this value of dt was only a relative term, the time scale was scaled to convert it into one that was comparable with the experimental results. Thus, the modelling results are presented as a function of time over ‘1200 minutes.’ It should also be noted that similar to the time step, the cell volume, the conductances and the fluxes/currents were all relative terms which were scaled within the model to obtain ‘user friendly values’ such as 1 or 2, or were scaled to resemble experimental values, as in the case of the currents. The only absolute units in the model were the voltages (upon which the GHK equation and Boltzmann voltage activation relied). The voltages depended on kT (i.e. the product of the Boltzmann constant k, and the absolute temperature, T), both of which are absolute, and not relative terms.
8.7.2 Modelling Changes in apical PK in the Time-Domain

With the steady-state results indicating the importance of the apical K⁺ channels, a natural progression was to model the changes that occurred over time as the fixed K⁺ channel density was increased. Using the archetypal cell configurations, the steady-state conditions were found for cells bathed in a 100mOsm/kgH₂O NaCl solution, with relative apical K⁺ permeabilities of 0, 0.1, 0.25, 0.5, 0.75 and 1, respectively. Having determined the start conditions, the cells were then perturbed at t=64 minutes by simulating a change in the bathing solution to a 250mOsm/kgH₂O NaCl solution. This mimicked the standard *in vitro* protocol of exposing a skin to ‘Normal’ Amphibian Ringers.

The results of these simulations are presented in Figure 8.7 (and Appendices 6-11). The top line of graphs show the time-course of the apical membrane potential (V_{apex}) for each cell configuration and the position of this parameter relative to the Cl⁻ Boltzmann activation curve, indicated by the horizontal dotted lines at V_{12} ± V_{11}. To the right of each voltage time-course is a diagram showing the changes in PCl superimposed on the associated (voltage) Boltzmann activation curve.

Beneath the row of membrane potential graphs is a set of cell volume graphs showing the relative volume of the cell with respect to the Na⁺ Boltzmann activation curve, as shown by the horizontal dotted lines at d_{12} ± d_{11}. Shown to the right of each volume time-course is the change in the Na⁺ permeability overlaid on its associated (volume) Boltzmann activation curve.

One of the important points that is apparent from this progression in the apical PK is that the steady-state starting values for cell volume and apical membrane voltage are different, and become more negative as PK increases. As was stated earlier in section 8.6, the starting PCl and PNa values appear to affect the subsequent behaviour of the cell. If these parameters start in the upper saturated regions of the Boltzmann activation curves (as is the case for low PK values - i.e. PK=0, 0.1), no feedback ‘regulation’ occurs, and the channels remain open in the salty solutions. In fact, the modelling seems to suggest that cell swelling occurs. Yet, if these parameters start sub-maximally, with PCl set at less than 50% (i.e. PK=0.5, 0.75 and 1), PCl and PNa both follow their respective Boltzmann activation curves as the channels close.

These results are also interesting because they provide an explanation for the ‘unstable’ results observed in this study, and perhaps why such instability has not been described previously in the literature. Given that most of the results published in this area have been derived from toads living in the northern hemisphere, whereas these
Figure 8.7: Simulated effects of different apical K⁺ permeabilities of MRCs after changing the apical and basolateral bathing solutions from a 100mOsm/kgH₂O NaCl solution (for which steady-state conditions were found) to a 250mOsm/kgH₂O NaCl solution (at t=64 min). (A & B) Notice that for low K⁺ permeabilities, changing to the higher osmolality solution does not bring the apical membrane potential or the cell volume into the Boltzman range and therefore, under these circumstances, the apical voltage-activated Cl⁻ permeability and the volume-sensitive Na⁺ permeability remain maximally on. (C & D) As the apical K⁺ permeability is increased, changing to the higher osmolality solution results in the cell volume and apical membrane voltage entering the respective Boltzman ranges such that regulation of the apical Na⁺ and Cl⁻ permeabilities occurs. (E & F) For high apical K⁺ permeabilities cell volume is lost more readily and the apical membranes hyperpolarise more upon changing to the 250mOsm/kgH₂O NaCl solution such that the lower limits of the Boltzman curves are reached or exceeded. This results in closure of the apical Na⁺ and Cl⁻ channels. (It is interesting to note that because of the difference in time courses of Na⁺ and Cl⁻ shutdown, the faster Cl⁻ shutdown partially limits the loss of cell volume (by KCl loss). Presumably, this mechanism has evolved to slow cell shrinkage outside the lower (volume) Boltzman limit so that subsequent recovery is possible. If the cells shrink too far then irreparable damage to the cells may occur and subsequent reinflation may not be possible). Note: In these examples $V_{12}=-18$ mV and $d_{12}=12$ (relative units).
experiments were conducted on toads in the southern hemisphere (originally from Queensland, Australia), it is plausible that the apical PKs were different for environmental or climatic reasons. More specifically, the simulations of skins bathed symmetrically on both sides in the same solution suggest that the northern hemisphere toads possess a much lower apical PK compared with the southern hemisphere toads used in this study. Such a proposal is consistent with the fact that Voc and Isc measurements can be recorded from northern hemisphere toads over hours (Hillyard, personal communication), but not from these southern hemisphere toads. In the southern hemisphere toads, the simulations suggest that to initiate the cell shrinkage necessary for the experimentally observed shutdown in Voc and Isc, a relatively high apical PK is required.

To summarise, it is suggested that:

(1) when bathed in the same solution on both sides, toad skins which possess a low apical PK remain stable whereas toad skins which have a higher apical PK become unstable (i.e. Voc and Isc drop markedly over time);

(2) MRCs probably operate somewhere along a continuum between these two extremes, where a low apical PK occurs in cells optimised for water uptake from watery (i.e. low salinity) conditions while high apical PKs are characteristic of cells optimised for salt uptake in salty conditions. This is in slight contrast to the original proposal in Chapter 1 (section 1.3) where it was suggested that the MRCs regulated salt uptake in salty conditions while the PCs regulated salt uptake under watery conditions. With the benefit of the

It is interesting to note that the idea of the MRCs being optimised for the regulation of water uptake, or salt uptake, is consistent with morphological observations made by Katz & Gabbay (1995), as described in section 1.2.1.2. They found that the density of the MRCs could be regulated by altering the salinity of the bathing solutions, with toads acclimated to low salt (distilled water) solutions displaying an increased density of MRCs compared with toads adapted to 65-100mmol/L NaCl for 6-10 days. Katz and Gabbay then went on to say that the increased density of MRCs was not due to an increased rate of cell birth, but rather a reduction in cell apoptosis and an increased rate of cell differentiation. The interpretation of these results is simplified by assuming that acclimation in low salt solutions is the norm (consistent with what toads would typically experience in a pond environment), while acclimation in salty solutions is the exception, resulting in an increase in MRC apoptosis and a reduction in MRCs. It is suggested here that, in the saltier environments, up-regulation of the K⁺ permeability may occur, resulting in the shrinkage of the MRCs (as demonstrated by the mathematical modelling). If excessive shrinkage occurs, then it is plausible that the recovery of cell volume is not possible when the osmolality of the bathing solution is later lowered, and the cells die. Interestingly, Katz and Gabbay observed no reduction in MRC density in salty KCl environments, which was presumably due to an increase in cell volume.
mathematical modelling and the apparent importance of the apical PK, it now seems that the MRCs could adapt to either watery or salty solutions given sufficient time to up-regulate or down-regulate the apical PK.

8.7.3 Modelling the Amiloride Effect

Amiloride is a well-known, fast-acting inhibitor of Na\(^+\) channels in tight epithelia (Irish & Stitzel; 1986). When applied to the apical side of toad skins it causes a rapid reduction in Isc and Voc within approximately 30 seconds (Figure 8.8D). Because of its rapid action, amiloride effects have been simulated in the mathematical modelling by an instantaneous reduction in PNa to near-zero values. Figure 8.8 shows three examples of amiloride effects, laid out in a similar format to Figure 8.7. Shown in the first two examples is the effect of amiloride when applied to cells in an ionic-steady-state, bathed in 100mOsm/kgH\(_2\)O NaCl, and differing only in the apical PK values. In the third example, the amiloride effect is superimposed on a non-steady-state cell, undergoing a slow volume reduction due to exposure to a 250mOsm/kgH\(_2\)O NaCl solution at t=64 minutes. (Initial steady-state conditions were determined in a 100mOsm/kgH\(_2\)O NaCl solution allowing a direct comparison of Figure 8.8C with Figure 8.7D).

Two main results are clear from the amiloride simulations. First, a rapid reduction in PNa causes an hyperpolarisation of the apical membrane potential, consistent with experimental findings (Helman & Fischer, 1976; Nagel, 1976) and Lew et al’s., (1979) theoretical modelling. Second, closure of the sodium channels does not necessarily cause a drop in cell volume (which can remain stable while PNa is perturbed even for relatively high PK values). However, reductions in both Isc and Voc occur due to a reduction in the net Na\(^+\) uptake across the cell from the apical solution. That is, with minimal Na\(^+\) being supplied from the apical side, the simulations suggest that the intracellular Na\(^+\) concentration drops (App. 12R-14R), resulting in a reduction in the Na\(^+\) and K\(^+\) pump fluxes in the basolateral membrane (App. 12I-14I) and a fall in Isc. Due to the hyperpolarisation of the apical membrane potential (which of course results in Cl\(^-\) channel closure), the amount of K\(^+\) leaving the cell across the apical membrane is reduced (App. 12H-14H), due to a weakening in the outwardly directed electrochemical K\(^+\) gradient. Consequently, the intracellular K\(^+\) concentration begins to increase (App. 12S-14S), so that Na\(^+\)/K\(^+\) exchange effectively occurs and the cell volume is maintained.
Figure 8.8: (A)-(C) Theoretical and (D) experimental effect of the Na⁺ channel blocker amiloride, on the apical/pond (p) side of the toad skin. The mathematical modelling predicts that amiloride application (denoted by arrows) causes a rapid hyperpolarisation of the apical membrane which results in apical voltage-sensitive Cl⁻ channels closing. However, as shown in (A) and (B) for simulations of skins in a steady-state condition in 100mOsm/kgH₂O NaCl, the (steady-state) volume of the cells does not alter significantly as a result of Na⁺ channel blockade. But if the cell is already undergoing a volume decrease such as in (C), which simulates the rundown of MRCs from a steady-state in 100mOsm/kgH₂O NaCl upon exposure to 250mOsm/kgH₂O NaCl on both sides, then Na⁺ channel blockade accelerates the rate of volume reduction. (D) Experimental time course and associated V-I locus showing the rapid reduction in Voc and Isc when amiloride is applied to the pond side of a skin bathed bilaterally in 250mOsm/kgH₂O NaCl. Note: In these examples V_{12} = -18mV and d_{12} = 12 (relative units).
The effect of artificially manipulating PNa is, in many respects, similar to manipulating the apical PK. Essentially, it enables the external adjustment of PNa and PCl. That is, PNa is reset by Na$^+$ blockade and PCl is reset by the resulting membrane hyperpolarisation. Therefore, used in moderation to create a partial block, amiloride could possibly be used as an experimental tool to manipulate the degree of feedback control in MRCs.

Another point that is worthy of comment is the absence of the ‘N’-shaped V-I locus, both experimentally and theoretically, when amiloride is added prior to Cl$^-$ channel closure. This is because PNa shuts off more quickly than PCl (App. 12K-14K), whereas experimentally it would appear that the Cl$^-$ channels in the MRCs close off before PNa, as discussed in section 8.6. The result of PNa closing before PCl is that, although the Cl$^-$ Boltzmann activation curve is traversed (as demonstrated by the sigmoidal drop in PCl (App. 12K-14K), the Boltzmann range in which Cl$^-$ channel closure would normally have occurred have already been covered during the almost instantaneous drop in Voc and Isc (App. 12E-14E). That is, the perturbation is over before a change in PCl can be observed. Therefore, no ‘kick-up’ corresponding to Cl$^-$ channel closure is apparent in the V-I locus.

While discussing the rapid drop in Voc and Isc, it is worth noting that these theoretical results are consistent with amiloride simulations (Figure 8.9) performed by Lew et al., (1979) using a single-cell, time-domain model of a toad skin which neither incorporated feedback, nor possessed dynamically changing Na$^+$ and Cl$^-$ permeabilities. The point here is that even without feedback, a rapid decrease in the Na$^+$ permeability causes an almost instantaneous hyperpolarisation of the apical membrane potential (similar to increasing the apical PK). The rapid voltage changes that are both simulated and observed experimentally upon altering the apical permeability are presumably due to the rapid reshuffling of a small number of ions across the membranes of the cells. Both these simulations and those of Lew et al. suggest that it is only after these rapid changes that the ionic concentrations begin to alter.

---

4 The difference between artificially altering PK and PNa (or PCl), whether it be theoretically or experimentally with the assistance of pharmacological drugs, is that PK does not appear to alter the degree of feedback between volume and voltage but simply affects the starting values. However, altering PNa or PCl results in an inherent adjustment of the level of feedback.
8.7.4 Modelling the Ouabain Effect

The well known Na\(^+\)/K\(^+\)-ATPase pump inhibitor ouabain, causes a slow decline in \(V_{oc}\) and \(I_{sc}\) over several hours when applied basolaterally, as shown by the experimental results of Figure 8.10. This is in contrast to the experimental observations of Lew et al., (1979) ‘of a small, sharp, initial drop in \(I_{sc}\) or intracellular potential following ouabain addition’. The ouabain effect presented here appears to be sped up by prior application of the K\(^+\) channel blocker TEA, to the apical bathing solution (Figure 8.10C), which may reduce the amount of KCl flowing across the apical face. The effect appears to be partially reversible, as demonstrated by a partial recovery in \(V_{oc}\) and \(I_{sc}\) following the removal of 100\(\mu\)M ouabain from the basolateral solution (Figure 8.10B). However, full reversibility does not occur, presumably because of the superposition of the ouabain effect upon the shrinkage effect brought about by exposure to a 250mOsm/kgH\(_2\)O NaCl solution.
Figure 8.10: Experimental effect 100µM ouabain (a Na⁺/K⁺-ATPase blocker) on Voc and Isc when applied to the pond (p) and belly (b) sides of a toad skin bathed in a 100mOsm/kg H₂O NaCl solution. Results are shown as a function of time in the first column, and summarised using the associated VI-locus in the second column.
In terms of the modelling, the ouabain effect was simulated by an instantaneous reduction in the Na\(^+\)/K\(^-\)-ATPase pump density from a relative value of 1 to 0.1 (Figure 8.11). This caused an instantaneous drop in Isc and Voc in all cases modelled. Following this instantaneous change, a slower reduction in Isc and Voc occurred in most cases (except Figure 8.11F), consistent with the dissipation of ion gradients as described by Lew et al. This dissipation involved: (1) the reduction in intracellular K\(^+\) concentration (App. 15-20S) due the outwardly directed but diminishing K\(^+\) currents across the apical and basolateral membranes (App. 15-20A,B,H,I); (2) the increase in intracellular Na\(^+\) concentration (App. 15-20R) due to the inwardly directed, but again diminishing, Na\(^+\) currents across the apical membrane (App. 15-20A,B,H,I); and (3) the increase in intracellular Cl\(^-\) concentration (App. 15-20R), due largely to the inwardly directed, but decreasing apical Cl\(^-\) current (App. 15-20A,H). Thus, due to the removal of electrogenic pumping, intracellular K\(^+\) was effectively replaced by Na\(^+\). However, in the process, a net NaCl accumulation occurred resulting in a water influx (App. 15-20F), which resulted in a volume increase (App. 15-20L). This is consistent with the observation that in some cell types (e.g. cardiac cells) oedema occurs upon the cessation of Na\(^+\)/K\(^-\) pumping via the Na\(^+\)/K\(^-\)-ATPase (Sokolow et al., 1990).

The modelling results presented here are similar to the ouabain simulations published in 1979 by Lew et al. (Figure 8.12). These investigators showed a rapid decrease in Isc and Voc followed by a slower reduction in these parameters. They also presented intracellular concentration changes showing an increase in intracellular Na\(^+\) and Cl\(^-\), but a decrease in K\(^+\). Although the results are similar, the interpretation is slightly different. In Lew et al.’s paper it was suggested that the initial reduction in Isc and Voc ‘resulted from the sudden arrest of electrogenic pumping [i.e. rapid binding of ouabain] and correspond[ed] to the full magnitude of the electrogenic effect on the basic model.’ The slower rundown in Isc and Voc that followed resulted from the dissipation of ionic gradients.

Finally, one other feature arising from the ouabain modelling conducted in the present study is that the time of application of the pharmacological agent can result in different transient behaviours. For example, when ouabain is ‘applied’ at t=768 minutes in Figure 8.11F, there is some evidence of a recovery of Isc and Voc above the pre-ouabain state. This behaviour is not seen when ouabain is ‘applied’ at t=268 minutes (Figure 8.11E).

\footnote{As a point of reference, it has been found that in rat vas deferens the association rate constant of ouabain is 2.87-3.6\(\mu\)M/min (Noel et al., 1998).}
Figure 8.11: Simulated effects of the Na⁺/K⁺-ATPase pump blocker, ouabain on the basolateral / belly side applied at times t=108 min, 268 min or 768 min (see arrows) for skins changed from a steady-state in a 100mOsm/kgH₂O NaCl solution to a non-steady-state in a 250mOsm/kgH₂O NaCl solution at t=64 min. Examples were simulated for MRCs with either an apical K⁺ permeability of (A-C) PK=0.25, or (D-E) PK=0.5. The relative density of the basolateral pumps was reduced from 1 to 0.1 in each case. Note: In these examples V₁₂ = -18mV and d₁₂=12 (relative units).
The point here is that even though the perturbation of the cell is the same in both cases, the state of the cell is not, because the cell is in the process of adapting to a change in the bathing solution from a 100- to a 250mOsm/kgH₂O NaCl solution.

### 8.7.5 Modelling the TEA Effect

The K⁺ channel blocker tetraethylammonium chloride (TEA) causes a rapid increase in Isc and Voc over 30 seconds to several minutes when applied to the apical side of the isolated toad skin. This effect is illustrated in Figure 8.13 which shows a TEA dose-response curve (A) and the effects of applying TEA to the pond and belly sides (B & C). (The dose-response curve indicates that a strong, but sub-maximal response can be elicited with 10mM TEA). The TEA effect is mostly reversible, but similar to other pharmacological agents in this study the effect appears to be superimposed on a slow volume regulatory effect brought about by the exposure of the skin to a relatively salty solution (i.e. 100mOsm/kgH₂O NaCl compared with tap water). The TEA effect is generally quite stable over an hour or so, suggesting that TEA does not unbind during
Figure 8.13: As shown earlier in Chapter 5, the experimentally derived effect of the potassium channel blocker, tetraethylammonium (TEA) on Voc and Isc when applied to the pond (p) and belly (b) sides of the toad skin. All experiments were conducted in 100mOsm/kgH₂O NaCl Ringer (Soln. 37). (Note the differences in time scales).
Figure 8.14: Simulated effects of the K⁺ channel blocker, tetraethylammonium (TEA) on the apical / pond side applied at times t=108 mins, 268 mins or 768 mins (see arrows) for skins changed from a steady-state in a 100mOsm/kg H₂O NaCl solution to a non-steady-state in a 250mOsm/kg H₂O NaCl solution at t=64 mins. Examples were simulated for MRCs with a 10-fold reduction in the apical K⁺ permeability starting at (A-C) PK=0.1, or (D-E) PK=0.25.
Figure 8.15: Simulated effects of the K⁺ channel blocker, tetraethylammonium (TEA) on the apical / pond side applied at times $t=108\text{mins}$, $268\text{mins}$ or $768\text{mins}$ (see arrows). Simulations A-E show changes from a steady-state in a 100mOsm/kg$\text{H}_2\text{O}$ NaCl solution to a non-steady-state in a 250mOsm/kg$\text{H}_2\text{O}$ NaCl solution at $t=64\text{mins}$ for different PK values. Example E shows the simulated effect of TEA for MRCs already in a steady-state in a 100mOsm/kg$\text{H}_2\text{O}$ NaCl solution.
this time period. However, in some cases where TEA is applied around t=500-600 minutes, there is some loss in the stability of Isc and Voc (Figure 8.13A). It is suggested that this is probably due to the effects of Cl⁻ channel closure, which typically occurs around this time in 100mOsm/kgH₂O NaCl solutions, if it is going to occur (Figure 8.13A(h)).

The modelling indicates that the effect of instantaneously decreasing the K⁺ channel permeability causes (1) a depolarisation of the apical membrane potential (and hence an opening of Cl⁻ channels), and (2) an increase in cell volume (and an associated opening of Na⁺ channels). This is shown in Figures 8.14 & 8.15, which illustrate up to ten-fold reductions in different starting PK values, at t=108, 268 and 768 minutes. However, as discussed previously in section 8.7.1, the effect of manipulating the apical PK is not necessarily observed (or is muted) in the Isc and Voc traces if the Na⁺ and Cl⁻ operating points are located in the saturated regions of their respective Boltzmann activation curves, and the channels are fully open. This concept is presented in Figure 8.14 where only minimal effects on Isc and Voc are observed upon K⁺ channel blockade. However, when operating in the linear region of the Na⁺ and Cl⁻ Boltzmann curves, which is more likely with higher apical PK values (Figure 8.15), the effects on Isc and Voc are more pronounced. As would be expected, one of the main changes that occurs with K⁺ channel blockade is an increase in intracellular K⁺ (App. 21-32S) due to a reduction in the outwardly directed apical K⁺ current (App. 21-32A,H). As the K⁺ concentration increases, Na⁺/K⁺ exchange occurs and the intracellular Na⁺ concentration decreases (App. 21-32R), due to a reduced influx of apical Na⁺ (App. 21-32A,H). Because the Na⁺ and K⁺ currents are not equally matched there is a net inward Cl⁻ current across the apical face (App. 21-32A,H), which results in an increase in intracellular Cl⁻ (App. 21-32R). With an overall accumulation of KCl, there is a net entry of water into the cell (App. 21-32F) and the volume increases (App. 21-32L). As a result, the sodium channels begin to open (App. 21-32K).

As with other pharmacological agents, the time of TEA application is important, because different responses can be induced in cells that are already undergoing volume changes. It has already been mentioned in this section that the modelling suggests that little or no effect is observed when TEA is applied to swollen cells. From an experimental perspective, this often occurs early on in the experiment when skins have just been removed from toads exposed to tap water. In some cases, however, TEA may initiate oscillations in the apical membrane potential (App. 29M) and in PCl (App. 29K), which may then manifest as an oscillatory behaviour in Voc (App. 29E).
Theoretically, it would appear that to initiate these oscillations, the cell must be operating close to the lower saturation point on the Na⁺ Boltzmann (App. 29L), and outside the lower region of the Cl⁻ Boltzmann envelope (App. 29M) (i.e. the Cl⁻ channels are closed and the Na⁺ channels are almost closed). In practice, for cells with relatively high PK values bathed in shrinking solutions, these conditions often appear to exist towards the later stages of an experiment, after t=600 minutes.

To summarise, if the objective is to develop and maintain a stable in vitro toad skin preparation, one way to achieve this is to apply TEA apically, early on in an experiment (or while the Na⁺ and Cl⁻ operating points are in, or on the edge of the upper saturated regions of their respective Boltzmann activation curves). This will minimise the loss of apical KCl and prevent cell shrinkage. The stability will presumably be enhanced by ensuring that: (1) prior to experimentation, the live toad is exposed to water and (2) once experimentation has commenced the isolated skin is bathed on both sides in as low a salt solution as possible, such that Isc is still measurable, but that cell shrinkage is minimised.

8.7.6 Modelling the Furosemide Effect

Experimentally, the Na⁺/K⁺/2Cl⁻ symport blocker and loop diuretic furosemide, causes a rapid increase in Isc and Voc within 30 seconds or so of application to the apical solution bathing an isolated toad skin. This response is presumably due to an improvement in the Na⁺/K⁺ coupling across the basolateral face as less Na⁺ and K⁺ are lost across the apical membrane. Within minutes Voc and Isc peak, as presented in Figure 8.16 which shows the responses of 3 tissue samples to furosemide after a series of -100mV voltage clamps (with respect to the apical solution). In the case of B & C, furosemide effects are demonstrated in a 250mOsm/kgH₂O NaCl solution after bilateral exposure to a Na⁺-free solution. These findings, which could not be replicated upon application of furosemide to the belly side, suggested the presence of Na⁺/K⁺/2Cl⁻ symports in the apical membrane of the toad skin. Interestingly, this is in contrast to other pumping epithelial layers such as the shark rectal gland, parotid gland and mammalian kidneys (Palfrey, 1994) where the Na⁺/K⁺/2Cl⁻ symports are reportedly located in the basolateral cell membranes. Because of the experimental results presented in this study, the Na⁺/K⁺/2Cl⁻ symports were modelled as existing in the apical membrane.⁶ although it was demonstrated during the course of the modelling that, for a

⁶ Interestingly, the modelling produced similar results for single cells bathed symmetrically in the same solution, upon blockade of symports in the apical or basolateral membranes.
single cell bathed symmetrically in the same solution, the theoretical results were identical regardless of which membrane the (electroneutral) symports were positioned.

In modelling the furosemide response, the density of the symports (Ds) was instantaneously reduced to simulate a reduction in ion transport via this mechanism. The effect of this reduction in Ds was investigated in MRCs which (1) possessed different PK values (i.e. PK=0.25 & 1) and (2) were undergoing a volumetric change due to an alteration in the baths from a 100- to a 250mOsm/kgH2O NaCl solution. The effects of applying furosemide at different times during the (non steady-state) volumetric changes were also investigated. The modelling results are summarised in Figure 8.17 and presented in detail in Appendices 33-38.

There are several important results that are important from the modelling. First, a furosemide effect was not observed in the Voc and Isc traces in all model examples, as demonstrated in Figure 8.17 A-C. However, it should be emphasised that just because an effect is not observed using the standard electrophysiological measurements, it should not be assumed that the pharmacological agent is having no effect. To the contrary, it can be seen in Appendices 34 & 35 that upon application of furosemide, there is a depolarisation of the apical membrane potential, a marked increase in the

---

As a technical point it should be noted that, in operating the model, if the relative magnitude of Ds is set too high, the system may become numerically unstable.

Figure 8.16: Experimental results showing the effect of the Na⁺/K⁺/2Cl⁻ symport blocker furosemide (FU), when applied to the pond (p) and belly (b) sides of skins bathed in 250mOsm/kgH₂O NaCl. The arrows highlight the effect of applying furosemide to (A) both sides and (B & C) just the pond side. The absolute time in minutes is also shown. Note the difference in horizontal scales. (Active ingredient: 10mg/mL furosemide).
Figure 8.17: Simulated effects of the Na⁺/K⁺/2Cl⁻ symport blocker, furosemide on the apical / pond side applied at times t=108 min, 268 min or 768 min (see arrows) for skins changed from a steady-state in a 100mOsm/kgH₂O NaCl solution to a non-steady-state in a 250mOsm/kgH₂O NaCl solution at t=0 min. Examples were simulated for MRCs with either an apical K⁺ permeability of (A-C) PK=0.25, or (D-E) PK=1. The relative density of the apical symports was reduced from 0.1 to 0.05 for examples (A-C) and from 0.1 to 0.03 for examples (D-F).
apical Na\(^+\) and K\(^+\) concentrations and cell volume, and an opening of the apical Na\(^+\) channels. However, these changes are either small, or Voc and Isc are already saturated, such that the responses are not manifested in the experimentally observable parameters.

The second point to note is that, as with other pharmacological blockers, the furosemide effect appears to be dependent on the state of the cell, as demonstrated by the application of the drug at different times to a non steady-state cell. For example, in Figure 8.17A, application of furosemide soon after a change in the bathing solution has little observable effect (presumably because it is dominated by other effects), but when applied at t=268 & 768 minutes an increase in Voc and Isc is seen. It might be argued that furosemide caused a reversal in the direction of ion transport via the symports. However, the modelling demonstrates that the symport blockade does not result in a change in the sign of the driving potential for the symport (Gs), and hence there is no reversal in the salt transport across this mechanism (see Appendices 34T, 35T, 37T & 38T).

Having said that, it is interesting to note that the furosemide effect is only apparent in Voc and Isc when Gs is positive (and the net transport of Na\(^+\), K\(^+\) and Cl\(^-\) ions by the co-transporter is out of the cells). Thus, from an experimental perspective care should be taken when interpreting a null result where, after the application of furosemide, no effect is observed. This is because symports may exist in the membranes of interest, but may simply be transporting ions into and not out of the cells.

The question then arises ‘Under what conditions are the Na\(^+\)/K\(^+\)/2Cl\(^-\) symports operating to transport ions out of the MRCs?’ From Figure 8.17 it can be seen that furosemide responses are only elicited in examples E&F. In all cases no significant response is observed. The main difference between E & F compared with the other examples is that at the point of furosemide ‘application,’ the apical membrane potential is outside the lower limits of the Cl\(^-\) Boltzmann activation curve. That is, most of the Cl\(^-\) channels are closed. But interestingly, the furosemide effect can be elicited while a percentage of the Na\(^+\) channels are still open, as demonstrated by Figure 8.17E, which shows that, at the time of furosemide application, the cell volume is still within the limits of the Na\(^+\) Boltzmann activation curve. Thus, it is suggested that the effect of furosemide is most evident when the Cl\(^-\) channels are closed, but a small percentage of the Na\(^+\) channels are open.
8.7.7 Modelling the Sulphate Effect

One of the standard perturbations used experimentally in this, and other studies is the placement of isolated toad skins in Na$_2$SO$_4$ (Cl$^-$-free solutions). Attempts were made to model the SO$_4^{2-}$ effect by changing the bathing solution from a 100mOsm/kgH$_2$O NaCl solution to a 250mOsm/kgH$_2$O low Cl$^-$ solution (Figure 8.18). It should be noted that for numerical purposes it was necessary to maintain the extracellular Cl$^-$ concentration at a nominal level, in this case 10mM. The main point to note is that upon changing to the 250mOsm/kgH$_2$O low Cl$^-$ solution, there is a rapid depolarisation of the membrane potential, which opens the apical Cl$^-$ channels. This appears consistent with the initial rapid increase seen experimentally in Voc when the isolated skin is placed in a Cl$^-$-free solution (see Figure 4.8H). With the apical Cl$^-$ channels opening, and the low Cl$^-$ concentration in the baths, the modelling suggests that Cl$^-$ leaches out of the cells and is not restored. At the same time there is a water movement out of the cells due to the high tonicity of the bathing solution. This loss of cell volume results in a reduction in PNa. In turn, Na$^+$ channel closure results in the hyperpolarisation of the apical membrane (as demonstrated in section 8.7.3 upon Na$^+$ channel blockade). As a result, the Cl$^-$ channels begin to close. Because there is no net restorative salt flux into the cell, the modelling suggests that the cell volume decreases more rapidly compared with cases of comparable osmolality where extracellular Cl$^-$ is present (App. 10). Consequently, the apical PNa shuts down faster than the apical PCI.

8.7.8 The Toad in the Pond: Serial Dilution Effect on the Pond Side

One of the issues that was raised in Chapter 1 was how Na$^+$ (and Cl$^-$) ions could be transported across toad skins from very low concentration (<1mM) in a pond environment. So far, the mathematical model developed here has been used to describe a number of features that were observed experimentally in this study when the isolated toad skins were bathed in the same solutions on both the pond and belly sides. However, this experimental protocol is not representative of the natural habitat of a toad in a pond. A live toad is typically exposed to a watery solution containing only low salt concentrations on the pond side. On the basolateral side the cells are assumed to be in contact with blood plasma, which in this study was mimicked by the high osmolar, high ionic 250mOsm/kgH$_2$O NaCl Ringers solution.

The main reasons for bathing the isolated skins in the same solution on both sides in the experiments presented here were to minimise the effects of ionic diffusion, and to simplify the complex Isc and Voc responses. Now, with a better understanding of the
Figure 8.18: Simulated and experimental results in 250mOsm/kgH$_2$O Na$_2$SO$_4$ Ringer on both the apical and basolateral sides. (A) Simulations were conducted on the 'archetypal' MRC, initially in a steady-state condition in 100mOsm/kgH$_2$O NaCl solution, with a relative apical K$^+$ permeability of PK=0.5. At t=64 min the concentrations of the apical and basolateral solutions were changed to simulate a Na$_2$SO$_4$ solution. Notice how in Na$_2$SO$_4$, the apical membrane potential starts off positive (due to the rapid influx of Na$^+$ ions upon changing from an apical [Na$^+$]=45mM in 100mOsm/kgH$_2$O NaCl to a [Na$^+$]=111mM in 250mOsm/kgH$_2$O Na$_2$SO$_4$) such that the Cl$^-$ channels, which are mainly closed at t=0 min, undergo a delayed opening. This change in the voltage-activated Cl$^-$ permeability occurs even in the absence of Cl$^-$ ions. Over time however, the apical volume-sensitive Na$^+$ channels begin to close due to a loss of cell volume to the high-osmolar external environment. This reduction in PNa results in the hyperpolarisation of the apical membrane and Cl$^-$ channel closure; (B) Simulated changes in Voc and Isc; (C) Experimental recordings showing the changes in Voc and Isc of a toad skin bathed in 250mOsm/kgH$_2$O Na$_2$SO$_4$ Ringer (Soln 2).
interactions between membrane potentials, concentrations, cell volume and ionic permeabilities, it is of interest to investigate how a toad skin is predicted to respond when only the pond solution is changed, thus emulating how a toad adapts to different external environments.

Figure 8.19 and Appendix 40 show the simulated effects of a serial concentration of the apical solution, followed by a serial dilution for MRCs with an apical PK=0.01. The ionic concentrations of the pond solution were described as fractions of the basolateral concentrations which were fixed at \([Na^+]=111\text{mM}, [K^+]=2\text{mM} \text{ and } [Cl^-]=113\text{mM}\). These basolateral concentrations represented the ‘undiluted’ case. Thus, using the basolateral solution as a reference, a ‘1%’ pond solution contained \([Na^+]=1.11\text{mM}, [K^+]=0.02\text{mM} \text{ and } [Cl^-]=1.13\text{mM}\) and a ‘10%’ pond solution contained \([Na^+]=11.1\text{mM}, [K^+]=0.2\text{mM} \text{ and } [Cl^-]=11.3\text{mM}\). Concentrations for the ‘20%’ and ‘50%’ solutions were determined in a similar manner. As described below, several important points are evident from Figure 8.19.

First, as the pond solution becomes increasingly more concentrated, the apical membrane potential successively depolarises (Figure 8.19A). However, the same trend is not observed in \(V_{oc}\) (Figure 8.19C), where a drop in \(V_{oc}\) occurs upon exposure to the 50% and 100% pond solutions. This drop is presumably due to the opening of the apical voltage-controlled Cl\(^-\) channels, causing a reduction in the overall skin resistance.

Second, while focusing on \(V_{oc}\), it is also interesting to note the relative changes in \(V_{oc}\) and \(I_{sc}\). Initially, as the pond solution is concentrated, \(I_{sc}\) and \(V_{oc}\) increase together. However, for the 50% and 100% pond solution, \(V_{oc}\) decreases, as mentioned above, while \(I_{sc}\) increase, suggesting that a change in phase between the two parameters has occurred. This phenomenon, where \(V_{oc}\) and \(I_{sc}\) move together in phase, but later move in different directions is known to occur experimentally, as demonstrated in Figures 3.9A, 5.2B&D, 5.5D, 5.7D & 5.8B. It is an important phenomenon because it suggests that the loop gain of the feedback system alters dynamically. This is an extremely important point because it effectively illustrates a local self-adjusting system which can speed up/slow down as a cell (with a particular topology) interacts with, and adapts to, its environment.

The third point to note is that at first, as the pond solution is successively concentrated, only small changes in the intracellular volume are observed, despite up to 10-fold increments in the water concentration. It is only when a significant accumulation of intracellular salts begins to occur in the 50% pond solution that the volume begins to undergo an observable increase. That is, salt accumulation, and not a
Figure 8.19: Serial concentration of the apical solution followed by a serial dilution for cells with an apical PK=0.01. The basolateral solution was kept constant throughout with [Na⁺]=111mM, [K⁺]=2mM, [Cl⁻]=113mM. This represented the undiluted case (100%). The apical solution was described as a fraction of this undiluted case. For example, a 100-fold dilution (1%) was described by [Na⁺] = 1.11mM, [K⁺]=0.02mM, [Cl⁻]=1.13mM. and a 10-fold dilution (10%) contained [Na⁺]=11.1mM, [K⁺]=0.2mM, [Cl⁻]=11.3mM.
simple water gradient, appears to be the important factor in initiating a change in cell volume, in this case. It is also interesting to note that the accumulation of salts in the cell seems to be initiated by the apical membrane potential depolarising and moving into the lower range of the Cl⁻ Boltzmann activation curve, which causes a small percentage of Cl⁻ channels to open. This appears to cause a small influx of salts, resulting in a small increase in volume. In turn, the volume change causes a small percentage of volume-sensitive Na⁺ channels to open, and the further accumulation of salts until a steady-state is reached. Interestingly, in the 100% solution, a steady-state is not reached until all the Na⁺ and Cl⁻ channels have ‘snapped’ open, in response to the cell volume and apical membrane potential moving respectively through the full range of the Na⁺ and Cl⁻ Boltzmann activation curves.⁸

For higher PK values, the cells do not appear to ‘snap’ open. This can be seen from Figure 8.20, which shows the final steady-state values for different parameters after successively increasing the pond solution from a (dilute) 1% solution to a more concentrated solution, as specified by the values along the horizontal axis. Each graph contains four traces representing cells with different PK values. For the relatively high PK values of 0.1 and 0.5, little change in volume (M), V_{apex} (B), P_{Na} (G), P_{Cl} (H), ionic currents (P-U) and intracellular ionic concentrations (D-F) occur as the pond solution is changed from a 1% to a 50% (or more concentrated) solution. Essentially, the cells remain quiescent in terms of ion transport, although they still have the capacity for water uptake, as indicated by Figures 8.19J&K. In contrast, a ‘snapping’ effect or ‘atelectasis’ occurs for cells with lower PK values. Such cells appear to have the capacity to absorb both ions and water. Thus, it is suggested, that by simply altering the K⁺ permeability, cells may be involved in both salt and water uptake or just water uptake.

One of the shortcomings of this model however, which is clear from Figure 8.19E, is that there is no net water uptake in the steady-state. That is, although the transient water fluxes are in the correct directions (such that, as the pond concentration is increased, the magnitude of the water fluxes entering the cell across the apical face, and leaving the cell across the basolateral face, both decrease), the total steady-state water flux always settles to zero. Given that the skin is the primary means for water uptake in the toad

---

⁸ Interestingly, due to the non-linearity of the system some hysteresis is evident, particularly in the volume and concentration traces (Figure 8.19B, F&G). That is, depending on whether the pond solution is successively concentrated or diluted, the cell volume and concentrations will tend to different values for the same pond solution.
Figure 8.20: Steady-state values for different concentrations of the pond solution relative to the basolateral solution (which had a fixed ionic concentration of [Na$^+$]=111mM, [K$^+$]=2mM and [Cl$^-$]=113mM. The different lines represent different apical PK values. (open circles: PK=0.001; filled triangles: PK=0.01; open squares: PK=0.1; filled diamonds: PK=0.5).
(Overton, 1904) the total water flux across the skin in Figure 8.19E, would be expected to asymptote to successively more negative values as the pond solution was diluted. The reason that this does not occur is because the basolateral solution is maintained with a constant ionic concentration. If this constraint were removed, and the ionic concentrations on the belly side were allowed to change, then presumably a net water flux would be observed which increased and decreased in magnitude as the toad moved into more dilute and concentrated pond environments, respectively. However, releasing this constraint is beyond the scope of the present study.

8.7.9 Overview: Two types of cells

So far, two different cases have been considered:

1. the symmetrical case where skins were bathed in the same (salty) solution on both sides. In this case, both experimental and simulated results were presented;

2. the asymmetrical case where the toad skins were exposed to a low salt solution on the pond side and a high salt solution on the belly side to simulate the toad in the pond. In this case, only simulated results were presented.

As summarised in Figure 8.21, the steady-state start conditions for the symmetrical and asymmetrical cases were different, which led to quite different conclusions in terms of the relative magnitude of the apical PK. That is, it was found from the simulations that to get the apical \( \text{Na}^+ \) and \( \text{Cl}^- \) channels in the MRCs to close in the high salt solution for the symmetrical case a high PK was required, but to get these channels to open for high apical salt concentrations in the asymmetrical case, a low PK was required. The reasons for these differences are summarised below.

In the symmetrical case, it had been shown experimentally that Voc and Isc could be relatively stable over hours in a 100mOsm/kgH\(_2\)O NaCl solution and yet, in many cases, these parameters decreased in magnitude (or ‘shut down’) in a 250mOsm/kgH\(_2\)O NaCl solution (see Figure 4.9). To mimic this in the simulations a stable steady-state was found for the cells in a 100mOsm/kgH\(_2\)O NaCl (medium salt) solution (Figure 8.21A(ii)). As described in section 8.6, to simulate the subsequent shutdown in a 250mOsm/kgH\(_2\)O NaCl solution it was necessary that, in the medium salt solution, the apical PK was relatively high, almost all the apical \( \text{Na}^+ \) channels were open, and that 50% (or less) of the apical \( \text{Cl}^- \) channels were open. Only under these circumstances would the simulations produce a sufficient volume change that \( \text{PNa} \) and \( \text{PCl} \) would close in the 250mOsm/kgH\(_2\)O NaCl solution and Isc and Voc would fall in magnitude.
Figure 8.21: Cells bathed (A) symmetrically, and (B) asymmetrically in watery, medium salt, and high salt solutions. (A) Experimentally, toad skins were bathed symmetrically in the same solutions on both sides. Cells behaved as if they had a relatively high apical PK and were initially adapted to (ii) medium salt conditions (boxed case) which, (iii) then closed down and became impermeable to salt and water uptake when exposed to high salt solutions, such as 250mOsm/kgH₂O NaCl, on both sides. Presumably, some water could be absorbed in (i) the low salt solutions, although this was minimal due to the absence of a significant osmotic gradient. This case was difficult to test experimentally because of the small magnitudes of Voc and Isc in the low Na⁺ environment. (B) The toad in the pond was most closely simulated by a low salt concentration on the pond side and a high salt concentration on the belly side as shown in the boxed case in (i). Using this as a reference, the ionic concentration of the pond solution was successively increased in the simulations. For cells with a low PK, initially adapted to the low salt environments, it was shown that the apical membrane became increasingly permeable to Na⁺ and Cl⁻ as the saltiness on the pond side was increased.
In terms of water uptake for the symmetrical case, it is suggested that cells with a high PK which are adapted to a medium salt environment absorb water ‘indirectly’ by first absorbing salt, which creates a hyperosmotic environment on the belly side. Water would then follow down its osmotic gradient. Clearly, however, in high salt environments water is not taken up by this mechanism because the Na⁺ and Cl⁻ channels have closed and there is no net salt uptake. This is obviously not conducive to long-term survival in high salt environments. But for a toad adapted to medium salt solutions, the high apical PK may become a useful protective mechanism when the toad temporarily enters a high salt environment. That is, Na⁺ and Cl⁻ channel closure presumably (1) protects the toad from excessive NaCl uptake (and cellular NaCl toxicity) and (2) means that the apical membrane is effectively transformed into a Nernstian membrane, permeable only to K⁺. Consequently, excessive K⁺ loss to the environment is prevented.

At the other extreme, placement of the ‘medium salt-adapted’ cells in a low salt (watery) environment on both sides (Figure 8.21A(i)) is presumably not detrimental to the toad because, although Na⁺ and Cl⁻ uptake from the pond side will be minimal, and thus, water will not follow salt, water will be absorbed directly from the pond down its own concentration gradient. This was difficult to demonstrate experimentally because in low salt environments Isc and Voc tend to 0µA and 0mV, respectively). It was also not possible to effectively demonstrate a net water uptake in the steady-state using the simulations because the ionic concentrations of the belly solutions were not allowed to change in this model. However, the point here is that for cells with high apical PKs which are adapted to medium salt solutions, water probably follows NaCl down its osmotic gradient from the pond to the belly side. In high salt solutions, water uptake via this mechanisms ceases as the apical Na⁺ and Cl⁻ permeability is decreased.

For asymmetrically bathed cells adapted to low salt (watery) conditions on the pond side, water uptake appears to be through a direct mechanism (i.e. no salt uptake is required). However, as the pond solution becomes increasingly salty, less water appears to be taken up by this mechanism. Instead, water absorption probably follows down the osmotic gradient created by the absorption of NaCl, at least for cells with a low apical PK.

---

9 Because Na⁺ uptake is minimal, Na⁺/K⁺-ATPase activity will be limited. Therefore, for cells with a high apical PK, intracellular K⁺ may be lost to the environment but presumably this can be restored from the basolateral side when the toad subsequently moves into more brackish water and ATPase activity recommences.
PK. These low PK cells appear to undergo a process of ‘bootstrapping’ whereby salts begin to accumulate inside the cells causing water to enter and the cells to swell, as the ionic concentration of the pond solution is increased. As the cells swell, the modelling suggests that the Na\(^+\) channels, many of which are closed in the watery solutions, begin to open and Na\(^+\) enters, depolarising the apical membrane. This in turn, results in the apical Cl\(^-\) channels beginning to open. The net NaCl gain that results causes further cell swelling more Na\(^+\) channels to open and subsequently, more Cl\(^-\) channels to open. As the intracellular Na\(^+\) concentration builds up the Na\(^+\)/K\(^+\)-ATPase activity increases and Na\(^+\) is pumped out of the cell Cl\(^-\) then follows, diffusing down its electrical gradient into the belly solution. Finally, the net NaCl accumulation on the belly side causes water to be absorbed across the cell layer. Thus, in high salt solutions, low PK cells which are initially adapted to low salt solutions appear to open (Figure 8.21B) in contrast to high PK cells adapted to medium salt solutions, which close (Figure 8.21A).

To summarise, it is suggested that there may be (at least) two different types of MRCs, one with a high apical PK that is adapted to medium salt environments, which ‘snaps’ closed as the extracellular NaCl is increased, and one with a low PK, that is adapted to low salt environments, and which ‘snaps’ open as the pond solution becomes more salty.

In both cell types the mechanism for water uptake in low salt solutions is probably via the direct absorption of water as it flows down its concentration gradient from a high water concentration to a low water concentration. In higher salt solutions water is probably absorbed in both cell types following NaCl absorption.

8.8 Conclusions

The main objective of this chapter was to demonstrate how the toad skin could be modelled in the time-domain, and how a single-cell approximation, with inbuilt feedback, could regulate itself locally to produce changes which, in many cases, were similar to those observed experimentally. The main conclusions of this chapter are summarised below.

With particular reference to the symmetrically bathed cells:

10 The simulations suggest that cells with a high apical PK which are adapted to low salt solutions remain impermeable to Na\(^+\) and Cl\(^-\) over a range of pond concentrations because the apical membrane potential is sufficiently negative due to the high PK that it never enters the lower regions of the Cl\(^-\) Boltzmann activation curve. Therefore, no net salt accumulation can occur and the cells do not swell. Thus, in increasingly salty solutions fewer Na\(^+\) channels open, as with the low PK case.

11 This appears to be consistent with Larsen’s remarks (1991) that there are several types of MRCs, namely α-, β-, & γ-MRCs.
(1) Experimentally, there is evidence for an apical PK in the toad skin. The inclusion of this apical PK in the model assists in ‘manipulating’ the apical membrane potential and setting the relative PCl and PNa values initially. (That is, increasing PK hyperpolarises the apical membrane potential and increases the likelihood of the cell operating within the linear/regulatory regions of the Na\(^+\) and Cl\(^-\) Boltzmann curves). It is possible that in real life the apical PK is up-regulated and down-regulated by the toad depending on its environment. This may explain why some toad skins, presumably with a low PK, remain stable over hours, while others, such as in this study, do not.

(2) The starting conditions of cells are extremely important in determining how the cell will respond to a perturbation.

(3) The response of the cells to pharmacological agents depends largely on the state of the cells at the time of application, which in turn is determined by the topology of the cell and the cell’s environment.

(4) Simulating Na\(^+\) channel blockade with amiloride resulted in an abrupt reduction in the Na\(^+\) permeability and the movement to another Boltzmann activation curve associated with a lower Na\(^+\) channel density. This did not cause any significant change in the cell volume but did cause a hyperpolarisation of the apical membrane potential and a closure of the apical Cl\(^-\) channels.

(5) Simulating a ouabain effect typically caused a reduction in Isc and Voc, from which the cells did not recover in most cases. Depending on the state of the cell Na\(^+\)/K\(^+\)-ATPase blockade caused either an increase or decrease in the apical membrane potential (increases were associated with high PK values). In addition, and consistent with the data of Lew et al., (1979) an increase in cell volume was generally observed for cells with high PK values, but little change in volume was observed for cells with low PK values.

(6) The modelling demonstrated that blockade of the apical K\(^+\) channels with TEA could cause a transient increase in Isc and Voc. The simulations also suggested that application of TEA early on in the experiment, just after exposure to a 250mOsm/kgH\(_2\)O solution, helped stabilise the cells, causing the Na\(^+\) and Cl\(^-\) permeabilities to open and move into the saturated regions of their respective Boltzmann curves.

(7) The furosemide simulations (conducted in NaCl solutions) suggested that the effect of furosemide when applied to the pond solution was most evident when
the Cl\textsuperscript{−} channels were closed but a small percentage of the Na\textsuperscript{+} channels were open.

(8) Bathing the cells in a high osmolar, low Cl\textsuperscript{−} solution to mimic a 250mOsm/kgH\textsubscript{2}O Na\textsubscript{2}SO\textsubscript{4} solution resulted in a rapid depolarisation of the apical membrane potential such that the Cl\textsuperscript{−} channels were initially opening while the volume-sensitive Na\textsuperscript{+} channels were closing. This effect, where the Cl\textsuperscript{−} channels close more slowly than the Na\textsuperscript{+} channels, appears to be characterised by a V-I locus where the ‘N’-shaped curve is either muted or absent.

Finally, on a more general note:

(1) The analysis in this study suggests that two different types of MRCs may exist: (i) those possessing a medium to high apical PK that have adapted to medium salt solutions and which close when exposed to high (i.e. 250mOsm/kgH\textsubscript{2}O) NaCl solutions and (ii) those possessing a low apical PK which have adapted to watery solutions on the pond side, which open when exposed to high salt solutions.

(2) Water is probably absorbed in one of two ways depending on the concentration of the bathing solution and the permeability of the cells: (i) in watery pond solutions, water would appear to flow down its osmotic gradient from a high water concentration on the pond side to a low water concentration on the belly side and (ii) in medium/high salt solutions, salt uptake from the pond solution probably creates a hyperosmotic environment on the belly side of the cells and water then follows.
9. Complexity Theory
9. Complexity Theory

9.1 Introduction

In the previous theoretical chapters the dynamic behaviour of a complex single-cell system was modelled using feedback and a set of non-linear equations. Supplied with the initial conditions, the system was then allowed to adapt to its environment until an ionic steady-state solution was found.

The modelling of such ‘complex adaptive systems’ is a relatively new area in science and only burgeoned in the early 1990’s. It has required a radical shift in thinking from the deterministic idea that predicting the outcome is of primary importance, towards a broader philosophy where predicting the outcome is secondary to how the system evolves towards a particular solution. The purpose of this chapter is to give a brief overview of how non-linear, complex, adaptive systems fit into modern science, and to discuss some of the issues associated with them, and how these may be relevant to epithelial transport.

9.2 Historical Overview

The meteorologist Edward Lorenz, showed in (1963) that it was not possible to numerically predict the weather because the equations were sensitive to infinitesimal differences in the initial conditions. Thus, small changes in the initial conditions resulted in unpredictable and chaotic behaviour of the system. From this study, the relatively new field of chaos originated where there are no specific solutions but only a certain probability of a result occurring. Since then it has been determined that for a system to be chaotic the system must have ‘independent dynamical variables and the equations [must]…contain a nonlinear term that couples several of the variables’ (Baker and Golub, 1996).

That is not to say that systems fitting this description are necessarily chaotic. In fact, depending on the choice of parameters it is possible that the same system behaves in a constant, periodic or chaotic manner. This can be shown using the ‘Logistic Function’ which is commonly used to describe population growth. It is given by equation 9.1.

\[ P_{n+1} = r * P_n * (1 - P_n) \]  

where \( P \) is the population, \( r \) is the reproduction rate and \( n \) is the number of generations. (In this example if \( P=1 \) the population becomes extinct).
The effect of changing the reproduction rate for an initial population of 0.5 is shown in Figure 9.1. For rates less than 2.8 the population settles to a constant value over a number of generations.1 Plotting the population as a function of the reproduction rate yields a monotonically increasing curve which, depending on the initial population, may approach a straight line. For reproduction rates of 2.8-3.4 the population oscillates cyclically between two values (Figure 9.1E) As the rate gets closer to 3.4, the amplitude of the oscillations increase. Under these circumstances plotting the population against the reproduction rate shows a splitting or bifurcation in the value of the population. Increasing the reproduction rate to between 3.4 –3.5 results in the population cycling repeatedly between four values in a process called period doubling. The population/rate graph shows further bifurcations in the value of the population occurring at each of the last branches. Increasing the reproduction rate still further results in the population fluctuating between 8, 16 and 32 levels and so on with period doublings at each level until finally, at a reproduction rate approaching four, the population fluctuates in an unpredictable and chaotic manner.2,3

The population dynamics model is important because it illustrates clearly the move between stability and instability when the value of just one parameter is altered and the transition between a system that can be described by (i) fixed (Newtonian) equations where linear approximations work well, (ii) non-linear equations which are not well approximated using linear equations and (iii) chaos, which due to a statistical approach based on averages and distributions, can be well approximated using linear equations (Figure 9.2). Of interest in terms of epithelial modelling are systems described by non-linear equations which come under the auspices of ‘complexity theory’ and are stable yet can be unpredictable.

Complexity theory encompasses a number of issues including positive and negative feedback, non-linear interactions, non-equilibrium and non-steady-state systems, and systems that adapt to changing environments. In dealing with these issues, there is the

---

1 More specifically, for reproduction rates less than 1 the population gradually becomes extinct, for rates between 1 and 2 the population settles monotonically to a constant value and for rates between 2 and 3 a damped oscillation is seen in the population before it settles to a constant value.


3 A Feigenbaum fractal is the name of the fractal that shows successive bifurcations which finally break into the chaotic behaviour when a parameter, such as the reproduction rate in this example, is increased progressively.
Figure 9.1: The effect of changing the reproduction rate on the population. For rates less than 2.8 the population settles to a constant value (A) & (C) and the population vs. rate graphs are monotonically increasing curves (B) and (D). For rates between 2.8 and 3.4 the population oscillates between two values (E) and the population vs. rate graphs bifurcates (F). For rates between 3.4 and 3.5 the population oscillates between four values (known as period doubling) (G) and the population vs. rate graphs bifurcate again (H). Further period doublings and bifurcations occur until at a rate very close to 4, the population breaks into a chaotic oscillation (I & J). (Adapted from http://www.saltspring.com/brochmann/math/).
common theme of interactions between multiple parameters and how order can result. In the following section the types of order that can occur are examined in the context of attractors.

9.3 Attractors

When simulating a dynamical system, it is often the case that a system will settle in such a way that it traverses a well-defined (but sometimes complicated) set of points known as an attractor (Stewart & Dewar, 2000). That is, attractors are a set of points to which nearby trajectories converge thus ‘attracting’ a system to behave in a particular region of state space.\(^4\) When the system is behaving within that state space, the attractor defines the (local) solution space. There are four main types of attractors: static point attractors and time-varying cyclic (periodic) attractors, torus attractors and chaotic (strange) attractors (Figure 9.3).

One of the problems in dealing with attractors is that they are somewhat abstract. A system first needs to be defined and then the attractors can be developed as the system responds to different conditions and settles to a point or a series of points. In other words, it should be emphasised that *attractors are a result of the system and cannot be*

\(^4\) See http://www.wfu.edu/~petrejh4/Attractor.htm
defined before the system. But, so long as the system’s structure does not change, the attractors do not change and it is possible to develop a static ‘attractor map’ in state space defining areas of stability and convergence. However, the epithelial cells modelled in this study presumably ‘self-organise,’ co-evolving with the environment by opening and closing channels. In such dynamic systems it is not possible to construct static ‘attractor map’ because as the system changes so too do the attractors. Therefore, to convey some of the important ideas in complexity theory, the following discussion assumes a simple fixed system which does not evolve over time. This limits discussion to point attractors.

Having said that, it is possible to imagine that the same, or different attractors lie next to each other in state space. For example, cyclic attractors may be located next to strange attractors or, as a more concrete example where an attractor map can be generated for a fixed system, point attractors may be separated from each other by their respective ‘basins of attraction’ forming a mountainous surface (Figure 9.4). Whatever the case, if a non-linear system with feedback is perturbed in some way and multiple
Figure 9.4: Changing parameters for a fixed system can result in multiple attractors located adjacent to each other in state space. In this case the attractors are point attractors separated by bowls of attraction which create a mountainous surface. The initial conditions define where the system is first positioned (i.e. on a peak such as ‘A’ or on the side of a ‘mountain’). Small changes in parameters then determine into what basin the system enters (if is not already located in one) and the pathway taken to reach the local steady-state condition at the bottom of each valley. A large perturbation may result in the system leaving one basin of attraction and entering another. (Image from http://www.calresco.org).

If these attractors are different types then the system can display quite different behaviour, moving for example, from a stable cyclical pattern to a chaotic one.

The movement of a system within an attractor or between different attractors influences the behaviour of non-linear systems, raising at least three interesting points worth discussion. These include the issues of (1) multiple solutions, (2) lockout and irreversibility, and (3) system stability.

9.3.1 Multiple Solutions

One of the implications of different attractors is the existence of many steady-state solutions for a given set of initial conditions. This can be illustrated clearly using Figure 9.4. In this example, if a system were started at a peak ‘A’ changing different parameters could cause the system to migrate towards one of five or so point attractors. (The attractor towards which the system moves depends largely on the parameters that
are changed [which determines the initial direction of migration of the system], the magnitude of these changes, and the strength of each attractor [in this case the strength of the attractor is determined by the gradient from the peak into the attractor basin]). Here, it is relatively easy to visualise the different steady-state solutions. But in epithelial physiology the attractors and their properties are not known. Therefore, it is not possible to assess a priori the solution to which the system will converge for different initial conditions or how many steady-state solutions exist. This was illustrated in Chapter 6 with Greger’s carcinoma cell and a cell based on the MRCs in the toad skin. With Greger’s cell it appeared that the model consistently converged to the same steady-state solution. This may have been because there existed a unique solution. Alternatively, the initial conditions were sufficiently similar that the system started off in the same basin of attraction each time such that there appeared to be a unique solution. Had other initial conditions been used then the system may have started off in, or migrated towards a different basin of attraction. This appears to be the case for the MRC example. Of course, initial conditions should be specified within physiologically reasonable limits for biological systems. This restriction may prevent certain attractors from ever being realised and ensure that the system functions in a particular way which may be advantageous for long-term survival. Moving outside physiological limits may result in death (the ultimate attractor for living systems).

9.3.2 Lockout and Irreversibility

At a bifurcation point where the system can follow one of two (or more) pathways, the issue of lockout arises. Lockout occurs when commitment to one pathway closes off access to other pathways at the bifurcation point. Under these circumstances the system is ‘locked out’ of particular modes of operation. Lockout is an important phenomenon because it minimises the number of ways in which a system can function, thus promoting self-selection and diversification.

If the system and environment were reversed and returned to the exact conditions at the point of bifurcation, then the system could, in theory, traverse a different pathway. However, the process would require a reversal in entropy (which would have increased due to changes in the environment and, for a variable system, changes in the system as it adapted to its changing environment). In most cases this is not possible, so the process is regarded as irreversible.

The phenomenon of irreversibility arose in this study during the early stages of experimentation when the local feedback mechanisms in the toad skin were not fully
appreciated. In a series of volume regulation experiments where skins were exposed to NaCl or Na2SO4 solutions of increasing osmolality, attempts were made at the end of the experiment to replicate Isc conditions observed at the beginning of the experiment. These attempts were not successful, with Isc typically rising to only a fraction of the original value (Figure 9.5). However, when it was realised that the toad skin was constantly adapting to its environment such that the number of voltage-dependent Cl\textsuperscript{−} channels and volume-activated Na\textsuperscript{+} channels that were open at the end of the experiment was generally lower than that at the beginning, the reduced Isc results were not surprising. That is, attempting to demonstrate reversibility by exposing the skin to the same solution at the beginning and end of the experiment was flawed because the system was different due to the dynamically changing ion permeabilities.

9.3.3 System stability

It was shown previously in Chapter 3 that some toad skins became unstable and oscillated due to a voltage divider effect between the apical and basolateral membranes. This Cl\textsuperscript{−} dependent oscillatory behaviour was a ‘forced response’ because it was observed under an imposed transepithelial voltage clamp when the Na\textsuperscript{+} channels were blocked. However, some toad skins break into spontaneous oscillations, sometimes within the first couple of hours of experimentation (Figure 9.6), but more often during the final stages of experimentation (Figure 9.7), when the skins have entered a high resistance state (‘line of death’) and the Cl\textsuperscript{−} channels have presumably closed (see Chapter 3).\textsuperscript{5} The cause of these oscillations is currently unknown.

Whatever the mechanism, the main point is that some toad skins oscillate spontaneously. Under conditions where these oscillations appear to be superimposed upon the ‘line of death’ it is suggested that these toad skins have entered the basin of attraction of the ‘death attractor’ but within this basin there exist sub-states between which the toad skins are oscillating. This abstract idea may be visualised using the ball and bowl analogy described earlier in this chapter, where the system, represented by the ball, is assumed to be fixed for the purposes of illustration and the basin of attraction is represented by the bowl. If the surface of the bowl is not smooth, then as the ball rolls around the bowl towards its final resting state at the bottom (i.e. death), it is possible that it becomes trapped in particular sub-states, oscillating between them.

\textsuperscript{5} Interestingly, spontaneous oscillations have also been observed in a case where only Voc, and not Isc, was measured strongly suggesting that the oscillatory behaviour is not determined by Isc.
Figure 9.5: Reproducibility of initial conditions conducted at the end of volume experiments, where cells had been progressively shrunk by exposure to solutions of increasing osmolalities, were unsuccessful. This was presumably due to channels closing during the experiment and changes in the intracellular environment such that the skin was intrinsically different at the end compared with its initial conditions. (A) and (B) show lack of reproducibility in NaCl solutions while (C) and (D) illustrate similar behaviour in Cl⁻ free (sulphate) solutions. All osmolalities are measured in mOsm/kgH₂O but have been abbreviated on each trace to mOsm. Details of ionic concentrations are given in Chapter 2. (NB: r.e = rezeroed electrodes.)
Figure 9.6: Spontaneous oscillations in Voc in three different skins bathed in 250mOsm/kgH₂O NaCl Ringer (Soln 1). (A) & (B) Recordings made mainly under Isc conditions with intermittent checks of Voc; (C) Recordings made mainly under Voc conditions with intermittent checks of Isc. V-I loci for each example are shown on the right.
Figure 9.7: Spontaneous oscillations in skins during the final stages of the experimentation after the skins had entered the high resistance region, as shown on the V-I loci, where the Cl⁻ channels have presumably closed. All experiments were conducted in 250mOsm/kgH₂O NaCl Ringer (Soln 1) under mainly Voc conditions with intermittent checks of Isc.
While the above description is somewhat esoteric, attempts were made using small signal analysis, to investigate the theoretical conditions for which a system became unstable and to identify which parameters caused this. (Small signal analysis is a standard engineering technique used to obtain a linear approximation of a simple non-linear system). However, after some investigation it was concluded that this approach was unsuitable, primarily because it was attempting to squeeze a complex and variable non-linear system into the confines of a linear (Newtonian–like) system. Unfortunately, there exist no standard analytical techniques in the physical sciences at the moment to investigate the stability of variable complex adaptive systems, illustrating the present limits of complexity theory.

9.4 Conclusion

This short chapter has attempted to show how non-linear systems, such as epithelial systems, can be thought about using attractors. However, at the present time complexity theory is still in its developmental phase and it is not at the stage where broad generalisations can be made. Instead, much like the work conducted in this study, many groups are still in the process of developing models of complex adaptive systems as a first step towards understanding how they work.
10. Summary
10. Summary

10.1 Introduction

As stated in the introductory chapter, although there has been some discussion that Na\(^+\) and Cl\(^-\) ions pass though the same cell compartment in the isolated toad skin (Candia, 1978; Kristensen, 1981; Larsen et al., 1987; Ehrenfeld et al., 1989), there has been a prevailing view in the literature that the main route for Na\(^+\) uptake is through the PCs while Cl\(^-\) uptake is through the MRCs. One of the major flaws with this view however, is that it does not adequately address how an ionic steady-state within each cell type is reached. To investigate this issue of Na\(^+\) and Cl\(^-\) transport through the same cell type in the toad skin, a single-cell mathematical model was developed, with the objective of mimicking experimental findings, and demonstrating that an ionic steady-state could be reached.

Since the MRCs possessed both apical Na\(^+\) and Cl\(^-\) permeabilities, much of this study focussed on these cells and not the PCs. In future studies the valuable insights gained from modelling the MRCs can be applied to PCs and other cells. The following section gives an overview of the main directions during this study, the reasons for pursuing them and the conclusions arising from them. The final section looks at possible future investigations.

10.2 Overview

In order to develop a comprehensive mathematical model of the toad skin it was necessary to benchmark it against experimental data. It was soon found however, that to collect the detailed Voc and Isc data over many hours it was necessary to develop an automated data acquisition system.

As described in Chapter 2, a number of electronic circuits were built, and software was written to control them, which enabled measurements to be recorded automatically across the isolated toad skins under voltage-clamp, current-clamp and open-circuit conditions. Having developed an automatic monitoring system, it was then possible to collect detailed Voc and Isc data from many skins simultaneously, over many hours.

One important series of experiments that was conducted with the automated system investigated the presence of voltage-dependent Cl\(^-\) channels in the toad skin, in an attempt to replicate Larsen and Kristensen’s findings (1978). Consistent with their published observations, it was found that voltage-dependent transepithelial currents could be elicited from skins clamped at positive potentials (with reference to the apical bathing solution) in Cl\(^-\), but not in Cl\(^-\)-free Ringer’s solutions and, when stepping the
voltage from a –40mV holding potential to a positive voltage of up to +100mV, a delay of up to 10 seconds occurred before the voltage-dependent Cl⁻ current was activated (Chapter 3). The activation process was complete within 1 minute of application of the positive voltage clamp. In addition, when the skins were returned to the –40mV holding potential, delays of up to 13 seconds were observed before the current began to inactivate, with inactivation typically being complete within 1.5 minutes. These delays in the activation and inactivation of the Cl⁻ currents were necessary to initiate the damped oscillatory behaviour observed in some skins under voltage clamp conditions. The oscillatory behaviour had a period of approximately 1 minute, and was of interest because it demonstrated that feedback could occur in the isolated toad skin. In the same series of experiments it was also shown that a gradual increase over 10-20 hours occurred in the resistance of (unclamped) skins bathed symmetrically in 250mOsm/kgH₂O NaCl. This appeared consistent with the slow closure of voltage-activated Cl⁻ channels, as the skins adapted to the high-salt, high-osmolar solutions. It was also found that when actively pumping skins (mainly under open-circuit conditions) were voltage-clamped briefly for 2 minute at –100mV (to bias the Cl⁻ channels into a closed state), and then suddenly released back to the open-circuit condition, damped oscillations were present in Voc. This was presumably due to a transient adaptation in the number of open Cl⁻ channels immediately after release from the voltage-clamp. Finally, while it is possible that channel kinetics are directly responsible for this slow activation and inactivation of the Cl⁻ currents, the voltage-dependent Cl⁻ channels were ultimately modelled using a simple low-pass filter implementation.

One of the problems that was experienced early on during the experimental phase was that stable Isc measurements could not be obtained when the isolated skins were bathed symmetrically in 250mOsm/kgH₂O NaCl (the standard solution used by Ussing and many other investigators of toad skin function). In this study, Isc typically dropped in magnitude over 1-2 hours in this solution, making it difficult to interpret the effects of experimental perturbations. This drop in Isc did not appear to be due to cellular ATP limiting the operation of the basolateral Na⁺/K⁺-ATPase pumps, because Isc values of up to 45µA could be measured for 20 hours after toad pithing in some cases. In an attempt to obtain stable Isc traces, skin responses to solutions with different ionic concentrations and osmolalities were investigated. As described in Chapter 4, it was found that, in many cases, Isc remained stable over hours when bathed in a 100mOsm/kgH₂O NaCl solution. A series of sucrose experiments where only the osmolality (and not the ionic concentration) of the bathing solution was altered
indicated that Isc was more stable in solutions with lower osmolalities. This led to the suggestion that Isc was volume-sensitive, possibly due to apical volume-sensitive Na⁺ channels. These channels appeared not to be ‘Na⁺-activated Na⁺ channels,’ because a shutdown in Isc could be observed in the absence of a change in Na⁺ concentration, just by increasing sucrose. These sucrose experiments were also important because they showed that simple water movements across isolated toad skins bathed in the same solutions on both sides followed an exponential time course with a time-constant of about 23 minutes.

To further investigate the effects of volume on Isc, a series of Hg²⁺ experiments were conducted in an attempt to block aquaporins. However, it became evident that the reduction in Isc upon apical application of Hg²⁺ was too fast to be explained simply by aquaporin blockade, and was possibly due to some other mechanism, such as blockade of the apical Na⁺ channels. Nonetheless, the experimental results were important because they indicated that this heavy metal was having a local (non-neural) effect on pumping epithelia. Extending this, it was found that other heavy metals such as Pb²⁺ also had local effects on pumping epithelia. However, unlike Hg²⁺ Pb²⁺ had the opposite effect, causing significant increases in Isc and Voc. As discussed in Chapter 5, this effect appeared not to be due to Pb²⁺ acting directly on aquaporins, Cl⁻, K⁺ or Na⁺ channels. Nor did the effect appear to be due to charge screening, because the two heavy metals (with the same charge) had opposite effects and other multivalent metals had little effect. Instead, it is possible that Pb²⁺ was intrinsically altering the cell in some way, such as by causing a shift in the activation curve of the volume-sensitive Na⁺ channels. The main point to note here is that the effects of some heavy metals such as Hg²⁺ and Pb²⁺ are not only systemic (via neural and hormonal modulation), but can be local, effecting pumping epithelia directly by altering the ion transporting capacities of these cells.

With respect to the mathematical simulations, the single-cell model developed in this study was based largely on the experimental evidence, which supported the existence of voltage-activated Cl⁻ channels and volume-sensitive Na⁺ channels. The model was then ‘fine-tuned’ using other experimental data collected in this study, with particular reference to the effects of various pharmacological blockers. The two most significant of these findings were that:

(i) a TEA effect could be elicited on the apical side only of isolated skins, suggesting the presence of apical TEA-blockable K⁺ channels, and
(ii) a furosemide effect was observed on the apical side only, suggesting the presence of apical Na\(^{+}/K^{+}/2\text{Cl}^{-}\) symports.

Without the inclusion of these apical transporting elements the modelling results did not closely approximate the experimental results.

While the details of many different perturbations are presented in the main body of the text, there are several important points arising from the modelling. First, the starting conditions of the cells are extremely important in determining how the cells respond to a perturbation. For example, it was shown using the model that altering the apical PK (and thus ‘manipulating’ the apical membrane potential) was fundamental in determining the initial PCl and PNa values. Based on this, it was also demonstrated that when simulated cells with low PKs were bathed symmetrically in 250mOsm/kgH\(_2\)O NaCl, Voc and Isc remained stable over hours. However, for simulated cells with high PKs (which seemed to be representative of the experimental results presented here), Voc and Isc decreased in magnitude as the apical Na\(^{+}\) and Cl\(^{-}\) channels closed over time.

Second, it was shown that the response of the cells to pharmacological agents depended largely on the state of the cells at the time of drug application, which in turn was determined by the topology of the cell and the cell’s environment. Third, a comparison of simulated cells bathed symmetrically in the same solution, with simulated cells bathed asymmetrically (in a watery solution on the pond side and 250mOsm/kgH\(_2\)O NaCl on the basolateral side) suggested that two different types of MRCs may exist:

(i) those possessing a relatively high apical PK which have adapted to medium salt solutions and which close when exposed to high salt solutions (i.e. 250mOsm/kgH\(_2\)O NaCl) and

(ii) those possessing a low apical PK which have adapted to watery solutions on the pond side which open when exposed to high salt solutions.

Finally, water uptake for toads adapted to, and exposed to a salty environment would appear to be dominated by ‘salt-induced’ water movements, where salt uptake from the apical to the basolateral solution creates an hyperosmotic basolateral environment, causing water to follow. Water uptake for toads adapted to, and exposed to a watery pond environment is probably via simple water movements, where water moves directly down its osmotic gradient.

10.3 Future Directions

Currently, we are in the very strong position of being able to explain many of the experimental results observed in the isolated toad skin using a simple single-cell model.
containing local feedback. Looking forwards, however, there are several valuable experiments which would complement the results presented here. These include:

(i) successively concentrating and diluting the apical bathing solution (while the basolateral solution is maintained throughout with 250mOsm/kgH₂O NaCl) to investigate whether the changes in Voc and Isc predicted in this study compare well with those observed experimentally.

(ii) investigating whether Voc and Isc can be restored experimentally following ‘shutdown,’ by bathing the skins in moderately high K⁺ solutions in an attempt to ‘reinflate’ the cells.

(iii) using assays to determine conclusively whether K⁺ channels are expressed in the apical membrane of toads, as the results presented here seem to suggest, and if so, what factors are involved in the upregulation / downregulation of these channels.

(iv) developing a system for monitoring volume changes over time.

From a broader perspective, with a much better understanding of ion and water movements in the toad skin, it is of interest to investigate other epithelial layers, such as lung epithelia and stria vascularis in the mammalian cochlea. No doubt the insights gained from working with amphibian pumping epithelia can be applied to mammalian epithelia in the near future, hopefully improving our understanding of such diseases as cystic fibrosis.

With regard to the mathematical modelling, the next theoretical step is to release the constraint of a fixed ionic concentration in the basolateral solution. Allowing these concentrations to vary will enable a net water uptake to occur across the skin in the steady-state. Currently, transient water changes can be observed but the steady-state water flux settles to zero. Finally, in the more distant future the objective is to model two (or more) cells in apposition, where cells share (and can alter) common extracellular environments, but may have different intracellular environments.
11. References
11. References


Garrahan PJ, & Glynn IM. (1967a). Factors affecting the relative magnitude of the sodium : potassium and sodium:sodium exchanges catalysed by the sodium pump. J. Physiol. 192, 189-216.

Garrahan PJ, & Glynn IM. (1967b). The incorporation of inorganic phosphate into adenosine triphosphate by reversal of the sodium pump. J. Physiol. 192, 237-256.


12. Appendices
Appendix 1: Effect of changing from an initial steady-state condition in 100mOsm/kgH2O NaCl to a 250mOsm/kgH2O NaCl solution for an MRC with an apical PK=0.5. The incremental time step used in modelling this example was dt=0.004.
Appendix 2: Effect of changing from an initial steady-state condition in 100mOsm/kg H2O NaCl to a 250mOsm/kg H2O NaCl solution for an MRC with an apical $PK=0.5$. The incremental time step used in modelling this example was $dt=0.008$. 
Appendix 3: Effect of changing from an initial steady-state condition in 100mOsm/kgH2O NaCl to a 250mOsm/kgH2O NaCl solution for an MRC with an apical PK=0.5. The incremental time step used in modelling this example was dt=0.016.
Appendix 4: Effect of changing from an initial steady-state condition in 100mOsm/kgH₂O NaCl to a 250mOsm/kgH₂O NaCl solution for an MRC with an apical PK=0.5. The incremental time step used in modelling this example was dt=0.032.
Appendix 5: Effect of changing from an initial steady-state condition in 100mOsm/kgH2O NaCl to a 250mOsm/kgH2O NaCl solution for an MRC with an apical PK=0.5. The incremental time step used in modelling this example was dt=0.064. Notice that the numerical errors introduced with this time step are quite apparent and cause instabilities upon perturbation of the system.
Appendix 6: Effect of changing from an initial steady-state condition in 100mOsm/kgH2O NaCl to a 250mOsm/kgH2O NaCl solution for an MRC with an apical PK=0.
Appendix 7: Effect of changing from an initial steady-state condition in 100mOsm/kgH₂O NaCl to a 250mOsm/kgH₂O NaCl solution for an MRC with an apical PK=0.1.
Appendix 8: Effect of changing from an initial steady-state condition in 100mOsm/kgH2O NaCl to a 250mOsm/kgH2O NaCl solution for an MRC with an apical PK=0.25.
Appendix 9: Effect of changing from an initial steady-state condition in 100mOsm/kg H2O NaCl to a 250mOsm/kg H2O NaCl solution for an MRC with an apical PK=0.5.
Appendix 10: Effect of changing from an initial steady-state condition in 100mOsm/kgH2O NaCl to a 250mOsm/kgH2O NaCl solution for an MRC with an apical PK=0.75.
Appendix 11: Effect of changing from an initial steady-state condition in 100mOsm/kgH2O NaCl to a 250mOsm/kgH2O NaCl solution for an MRC with an apical PK=1.
Appendix 12: Effects of the Na⁺ channel blocker amiloride, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl (t=0 mins). The perturbation was simulated by a 10-fold drop in the apical PNa from 1 to 0.1 at t=188 mins for an MRC with an apical PK=0.25.
Appendix 13: Effects of the Na$^+$ channel blocker amiloride, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kg H$_2$O NaCl (t=0 mins). The perturbation was simulated by a 10-fold drop in the apical PNa from 1 to 0.1 at t=188 mins for an MRC with an apical PK=0.5.
Appendix 14: Effects of the Na⁺ channel blocker amiloride, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl (t=0 mins) and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in the apical PNa from 1 to 0.1 at t=188 mins for an MRC with an apical PK=0.5.
Appendix 15: Effect of the Na+/K+-ATPase blocker ouabain, when added to the basolateral solution of a skin initially in a steady-state in a 100mOsm/kg H2O NaCl solution at t=0 mins and then changed to a 250mOsm/kg H2O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in Dp from 1 to 0.1 at t=108 mins, for an MRC with an apical PK=0.25.
Appendix 16: Effect of the Na⁺/K⁺-ATPase blocker ouabain, when added to the basolateral solution of a skin initially in a steady-state in a 100mOsm/kgH₂O NaCl solution at t=0 mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in Dp from 1 to 0.1 at t=268 mins, for an MRC with an apical PK=0.25.
Appendix 17: Effect of the Na⁺/K⁺-ATPase blocker ouabain, when added to the basolateral solution of a skin initially in a steady-state in a 100mOsm/kg H₂O NaCl solution at t=0 mins and then changed to a 250mOsm/kg H₂O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in Dp from 1 to 0.1 at t=768 mins, for an MRC with an apical PK=0.25.
Appendix 18: Effect of the Na⁺/K⁺-ATPase blocker ouabain, when added to the basolateral solution of a skin initially in a steady-state in a 100mOsm/kgH₂O NaCl solution at t=0 mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in Dp from 1 to 0.1 at t=108 mins, for an MRC with an apical PK=0.5.
Appendix 19: Effect of the Na⁺/K⁺-ATPase blocker ouabain, when added to the basolateral solution of a skin initially in a steady-state in a 100mOsm/kgH₂O NaCl solution at t=0 mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in Dp from 1 to 0.1 at t=268 mins, for an MRC with an apical PK=0.5.
Appendix 20: Effect of the Na⁺/K⁺-ATPase blocker ouabain, when added to the basolateral solution of a skin initially in a steady-state in a 100mOsm/kgH₂O NaCl solution at t=0 mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in Dp from 1 to 0.1 at t=768 mins, for an MRC with an apical PK=0.5.
Appendix 21: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0 mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.1 to 0.01 at t=108 mins.
Appendix 22: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.1 to 0.01 at t=268 mins.
Appendix 23: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kg H₂O NaCl at t=0 mins and then changed to a 250mOsm/kg H₂O NaCl solution at t=64 mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.1 to 0.01 at t=768 mins.
Appendix 24: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.25 to 0.025 at t=108 mins.
Appendix 25: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.25 to 0.025 at t=268 mins.
Appendix 26: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.25 to 0.025 at t=768 mins.
Appendix 27: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.5 to 0.05 at t=108 mins.
Appendix 28: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.5 to 0.05 at t=268 mins.
Appendix 29: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins and then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.5 to 0.05 at t=768 mins.
Appendix 30: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kg H₂O NaCl at t=0mins and then changed to a 250mOsm/kg H₂O NaCl solution at t=64mins. The perturbation was simulated by a 4-fold drop in PK from PK=0.5 to 0.125 at t=268 mins.
Appendix 31: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kg H₂O NaCl at t=0mins and then changed to a 250mOsm/kg H₂O NaCl solution at t=64mins. The perturbation was simulated by a 50% drop in PK from PK=0.5 to 0.25 at t=268 mins.
Appendix 32: Effect of the K⁺ channel blocker TEA, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins. The perturbation was simulated by a 10-fold drop in PK from PK=0.5 to 0.05 at t=268 mins.
Appendix 33: Effect of the Na+/K+/2Cl- symport blocker furosemide, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kg H2O NaCl at t=0 mins & then changed to a 250mOsm/kg H2O NaCl solution at t=64 mins. The perturbation was simulated by a 50% drop in the symport density from Ds=0.1 to 0.05 at t=108 mins for an MRC with an apical PK=0.25.
Appendix 34: Effect of the Na⁺/K⁺/2Cl⁻ symport blocker furosemide, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins & then changed to a 250mOsm/kgH₂O NaCl solution at t=64mins. The perturbation was simulated by a 50% drop in the symport density from Dₛ=0.1 to 0.05 at t=286 mins for an MRC with an apical PK=0.25.
Appendix 35: Effect of the Na+/K+/2Cl- symport blocker furosemide, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH2O NaCl at t=0mins & then changed to a 250mOsm/kgH2O NaCl solution at t=64mins. The perturbation was simulated by a 50% drop in the symport density from Ds=0.1 to 0.05 at t=768 mins for an MRC with an apical PK=0.25.
Appendix 36: Effect of the Na⁺/K⁺/2Cl⁻ symport blocker, furosemide, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins & then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a reduction in the symport density from Dₛ=0.1 to 0.03 at t=108mins for an MRC with an apical PK=1.
Appendix 37: Effect of the Na⁺K⁺2Cl⁻ symport blocker, furosemide, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0mins & then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a reduction in the symport density from Ds=0.1 to 0.03 at t=268mins for an MRC with an apical PK=1.
Appendix 38: Effect of the Na⁺K⁺2Cl⁻ symporter blocker, furosemide, when added to the apical solution of a skin initially in a steady-state in 100mOsm/kgH₂O NaCl at t=0 mins & then changed to a 250mOsm/kgH₂O NaCl solution at t=64 mins. The perturbation was simulated by a reduction in the symport density from Dₛ=0.1 to 0.03 at t=768 mins for an MRC with an apical PK=1.
Appendix 39: Effect of changing from an initial steady-state condition in a 100mOsm/kg H2O NaCl solution to one that mimicked a 250mOsm/kg H2O Na2SO4 solution at t=64mins for an MRC with an apical PK=0.5. Note that a minimal extracellular Cl\(^-\) concentration had to be maintained in the simulation to ensure numerical stability and that an increase in the sugar concentration was used to represent the osmotic effect of SO\(_4^{2-}\).
Appendix 40: The simulated effects of a serial concentration followed by a serial dilution of the pond solution for an MRC with an apical PK=0.1 and Ds=0.01. The pond solution was changed successively from 1/100 of the belly solution, to 1/10, 1/5, 1/2 and then finally, at t=4320mins, it was changed to the same ionic concentration as the belly side containing [Na+] = 111mM, [K+] = 2mM and [Cl-] = 113mM. At t=5576mins the pond solution was sequentially diluted in the reverse order (i.e. 1/2, 1/5, 1/10, 1/100). Notice the hysteresis that occurs as the pond solution is diluted.
**STEADY STATE PORTS**

<table>
<thead>
<tr>
<th>Cell</th>
<th>1</th>
<th>-37</th>
<th>2.37137E-11</th>
<th>2.03</th>
<th>48</th>
<th>97</th>
</tr>
</thead>
</table>

- **tau3**: 0.06
- **A**: 0.001
- **Ksf**: 1.0
- **dV**: -1.61
- **T**: 2.60
- **48**: 126
- **OC**:

**LEFT**

- **d11percent**: 0.00
- **ClR**:

**RIGHT**

- **d12vol**: 250
- **M3**: 4.5E-01
- **M3**: 4.5E-01

**Next**

- **K+**: O 1/10 drop of pump density 0.1
- **VOLUME**: 15.151
- **VOLUME**: 15.2
- **indicator lines**:

**VI LOCUS**

<table>
<thead>
<tr>
<th>Σ</th>
<th>HYDROSTAT</th>
<th>P</th>
<th>2.61</th>
<th>48</th>
<th>126</th>
</tr>
</thead>
</table>

**H2OfluxL**:

- **-216.4**: 1315.0
- **50**: 430
- **Ksat**: 1.00

**ARCHY**

- **-104.5**: 1315.0
- **-0.2**: 400.0
- **0.2**: 420.0
- **0.100 PORT PORT**: 0.000

**VSS SC Error**

- **K+**: Cl 1/100 drop in [Cl] on both sides 0.01
- **Naf**: 1.0
- **Clsf**: 2.0
- **F**: 1/100 block of port 0.01

**Ikl**:

- **-216.4**: 950.0
- **-100**: 850.0
- **-100**: 0

**InasR**:

- **216.4**: 570.0
- **100**: 110
- **100**: 0

**216.4 570.0 100**

- **216.4 570.0 100**
- **216.4 800.0 100**
- **216.4 800.0 100**

**Σ time (min)**

- **20**: 0
- **40**: 0
- **60**: 0

**SteadystateVo**

- **20**: 0
- **40**: 0
- **60**: 0

**40 VOLUME**

- **15.151**: 15.2
- **15.2**: 15.1

**24/04/2007, 9:22 AM**
|          | toadwobble6.xls | -6.11 | 9.67E-05 | 4.83E-06 | -0.5861 | 0 | 0.5861 | 06 | 83.90867 | -26.7879 | -20.6904 | 0 | 0 | -4.5E-08 | -2E-09 | -4.7E-08 | -6.11206 | -6.11206 | ... | -5.35758 | -11.5359 | -58.6106 | 39.07371 | 111.9791 | -111.98 | -24.0273 | -24.0273 | -495.6653 | 329.3701 | 6.192477 | 619.5 | 4252.8 |

### Constants

- **INaL**
- **KL**
- **ClL**
- **NapL**
- **KpL**
- **Napara**
- **Kpara**
- **Cl para**
- **NasL**
- **KsL**
- **ClsL**
- **NaR**
- **KR**
- **ClR**
- **NapR**
- **KsR**
- **ClsR**
- **H2OL**
- **H2OR**
- **Vright**
- **Vleft**
- **VfR**

### Salt-induced water movement

- **left Katp**
- **100000000 gamma**
- **0.0005**
- **Nasat**
- **30**
- **Ksat**
- **1**
- **Pv**
- **0.1**
- **ADP**
- **0.1**
- **Pi**
- **0.1**
- **Kf**
- **2**
- **Naf**
- **3**
- **K/Na**
- **0.66666667**

### Additional Values

- **VfR**
- **-495.66529**
- **GsL**
- **7.00715247**
- **GsR**
- **-7.0071525**
- **Naf**
- **1**
- **Ksf**
- **1**
- **Clsf**
- **2**
- **Ps**
- **0.1**
- **Nasats**
- **0.00000001**
- **Ksats**
- **0.00000001**
- **Clsats**
- **0.00000001**
- **Na/K**
- **1**
- **Na/Cl**
- **0.5**
- **E10**
- **57.3**
- **Ea**
- **80**
- **go**
- **0.1**
- **goH2O**
- **0.1**
- **stiffness**
- **10**
- **delta**
- **0.001**
- **PNadLmax**
- **1**
- **PNadRmax**
- **10**
- **d12percentL**
- **85**
- **d11percentL**
- **-8**
- **d12volL**
- **12**
- **d11volL**
- **-6**
- **d12percentR**
- **100**
- **d11percentR**
- **-10**
- **d12volR**
- **250**
- **d11volR**
- **-5**
- **PClLPFsat**
- **2**
- **V12LPF**
- **-18**
- **V11LPF**
- **-5**
- **tau2**
- **0.06**
- **tau3**
- **0.06**
- **tau4**
- **0.06**
- **ATPsat**
- **30**
- **synth_rate**
- **0.05**
- **leak_rate**
- **0**
- **burn_rate**
- **0.01**
- **ATP_reserv...**
- **0.5**
- **Food_start**
- **100**
- **INapump**
- **-58.610565**

### Fixed ATP

- **20**

**Date:** 24/04/2007, 9:22 AM