The physiological mechanisms of scrotal temperature regulation and the effects of its dysregulation on endocrine function of the testes

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Abstract

The aims of the present project were to extend understanding of the contractile physiology of the tunica dartos muscle, especially the cellular mechanisms and the substances involved in activating contraction and relaxation of the dartos during electrical, pharmacological, and thermal stimulation and also study the effects of dysregulation of intra-scrotal temperature on the endocrine function of the testes.

In scrotal mammals, testicular thermoregulation is achieved by local and central mechanisms that are independent of the temperature regulatory mechanisms of the body core. The scrotum plays this important role in thermoregulation via several structural and functional adaptations, including the contraction of the cremaster and the dartos muscles, the presence of a large number of apocrine sweat glands, the absence of subcutaneous fat and the activity of the counter-current heat exchange system in the pampiniform plexus.

The tunica dartos is a smooth muscle found in the subdermal layer of the scrotal skin. Contraction of the dartos reduces the surface area of the scrotum and blood flow to the scrotal skin, preventing heat loss. Dartos relaxation causes excess heat to be removed. Therefore, the dartos plays an important role in the thermoregulation of the testes and congenital absence of the dartos leads to infertility.

It was known that the isolated dartos muscle contracts in response to electrical field stimulation (EFS) and that it is temperature sensitive.

The relative contractile forces produced by explants of tunica dartos of the Wistar rat were 100%, 134% and 137% respectively to EFS, noradrenaline and cooling (15^{o}C). Extension of these findings suggested that the dartos receives sympathetic innervation and contracted in response to noradrenaline via α-1 receptor stimulation. However, the

cooling induced contractile response which is greater than the maximal response to noradrenaline, is independent of nerves or noradrenaline stimulation.

The cooling induced contraction was inhibited up to 70% using the IP_3 blocker (2-APB) and 85% using the Rho kinase inhibitor (Y-27632) separately and almost 90% when both were used in combination. This forms the basis of the hypothesis that, like some airway smooth muscles, the dartos contracts by producing intracellular Ca^{2+} oscillations and Ca^{2+} sensitization. According to this hypothesis, the cooling induced contraction is activated initially by a mediator that brings about Ca^{2+} oscillations via activation of IP_3 receptor and the release of Ca^{2+} from the sarcoplasmic reticulum. These Ca^{2+} oscillations then activate the Rho-kinase pathway inhibiting myosin phosphatase leading to Ca^{2+} sensitization. The same mediator may be activating the Rho/Rho kinase pathway separately further enhancing the Ca^{2+} sensitization. Extracellular Ca^{2+} also contributes for a small percentage of the Ca^{2+} required for cooling-induced contraction. The identity of the mediator that causes the onset of Ca^{2+} release through IP_3 formation still remains a mystery. However, my experiments facilitate the ruling out of histamine, serotonin, ATP, adenosine, nitric oxide and prostaglandins.

Measurement of Ca^{2+} transients was undertaken using the fluorescent Ca^{2+} indicator Fura-2. Lack of literature in this area made it necessary for all steps of the experiment to be conducted on the basis of trial and error. Both freshly obtained strips of tunica dartos muscle and enzyme dispersed cells of the muscle strips obtained failed to produce Ca^{2+} transients. The cultured cells produced small Ca^{2+} changes in response to agonist stimulation in 11% of the cells tested, and Ca^{2+} oscillations in 4% of the cells tested. However, the low rate of success and the inability to be stimulated repeatedly made the cultured dartos cells inappropriate for the present experiments.

The final experiment of the investigation addressed the effect of scrotal temperature on steroid production in the testes in an animal model - the Merino ram (*Ovis aries*). The attempt at cooling the scrotum using cold gel packs was successful as I was able to achieve a reduction of scrotal temperature by an average of 5^{o}C. The attempt at warming the scrotum using insulation and inhibition of sweat evaporation did not succeed in significantly elevating the intra-scrotal temperature.

The reduction in scrotal temperature did not affect testosterone production from the testicular Leydig cells. Long term stimulation of the pituitary with the GnRH agonist Leuprorelin acetate lead to a gradual reduction in the circulating LH level, and an increase in the testosterone secreted for a given concentration of LH.

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Acknowledgements

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Statement of Candidate Contribution

This thesis contains published work and/or work prepared for publication, some of which has been co-authored. The bibliographical details of the work are included in the appendix.

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Co-ordinating supervisor signature.....

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CHAPTER 1

General Introduction

1.1 Overview

Infertility is a major clinical problem affecting approximately 1 in 7 couples in most countries in the world (Dunson *et al.*, 2004). In 30% of cases the problem is with the male partner (Poland *et al.*, 1985). For many men the only treatment option available is assisted reproductive technology with the use of techniques such as *in vitro* fertilization and intra-cytoplasmic sperm injection (Brugh and Lipshultz, 2004). Compared to female infertility, relatively little research has been presented on the factors that contribute to male fertility. What we do know is that male fertility depends on a normally functioning hypothalamo-pituitary-gonadal hormonal axis and normal sperm production. Spermatogenesis (sperm production) in turn requires structurally and functionally normal testes and the maintenance of the testicles cooler than the body core. The smooth muscle in the scrotal skin, the tunica dartos, is an important contributor to this temperature maintenance, but its physiology remains relatively unknown.

The research presented in this thesis will extend the understanding of the physiological role played by the tunica dartos muscle in regulating scrotal temperature. Specifically the cellular mechanisms and the substances involved in contraction and relaxation of the dartos are investigated, as well as the importance of maintaining scrotal temperature for testicular hormone production. In the research presented in chapter 2 of this thesis, I studied the contractile physiology of the isolated tunica dartos muscle in response to electrical and pharmacological stimulation. In the research presented in chapter 3, the cooling induced contraction of the isolated dartos muscle was studied in an attempt to identify the chemical mediators responsible for the contraction. In the research presented in chapter 4, intracellular calcium imaging was

carried out in order to study the cooling induced calcium availability and the calcium sensitization in the tunica dartos muscle. Primary culturing of tunica dartos muscle was carried out in order to do this. The Wistar rat was used as the animal model for all of the experiments in chapters two, three and four. In the research presented in chapter 5, an attempt was made to quantify the effects of cooling and warming of the scrotal sac and the testis, on the hormones related to spermatogenesis, namely luteinizing hormone and testosterone, using the Merino ram as the animal model.

1.2 Regulation of testicular temperature

For effective sperm production , the male gonads in most mammals require an environment that is 2 to 6°C cooler than the body core (Maloney and Mitchell, 1996; Janowski *et al.*, 1971). The cytological changes occurring after heat exposure remain controversial. The most vulnerable cell type to heat also seems species specific; Young *et al*. (1927) reported that in the guinea pig, spermatocytes degenerated first, but in the mouse the earliest heat induced changes occurred in the spermatozoa (Payne, 1956). In humans, spermatids and spermatozoa are reported to be fragile at high temperatures compared to spermatogonia and spermatocytes (Nakamura *et al.*, 1987). Cellular studies conducted more recently have suggested that heat induced damage to cells are brought about as a part of cellular stress response by inducing expression of heat shock proteins. The temperature at which heat shock protein expression is induced varies with the tissue concerned even in the same species (Sarge and Cullen, 1997) with germ cells having a lower threshold of expression compared to liver cells in the mouse model. Although it is not yet possible to explain fully why spermatogenesis is heat sensitive, there is evidence to support the case that heat causes infertility in humans (Thonneau *et al.*, 1997; Parazzini *et al.*, 1995;

Mieusset and Bujan, 1995; Kandeel and Swerdloff, 1988) as well as other mammals (Setchell, 1978; Setchell, 2006; Setchell, 1998).

Many mammals have a skin-covered pouch that holds the testes outside the body cavity, the scrotum, that helps to maintain the testes at a temperature lower than the body core. The temperature of the scrotum appears to be controlled independently of core temperature (Maloney and Mitchell, 1996). Some mammals that retain their testes internally have either alternate mechanisms to circumvent the adverse effect of high temperature on their gonads (e.g. seals and sea lions) or have developed more heat resistant gametes (e.g. elephants, rhinoceros) (Setchell, 1978). There is limited knowledge about the exact mechanisms of spermatogenesis in these animals with internal testes.

Having the testes suspended from the perineum in a scrotum, makes them vulnerable to external insults, at times causing torsion or rupture. Hence, from an evolutionary perspective there is bound to be a benefit to having the gonads housed this way. The scrotum is not simply a pouch that houses the testis, it plays an active role in providing the necessary thermal environment for the testis. There are five main anatomical features that contribute to the maintenance of the testicular temperature lower than that of the body core. They are,

a. the tunica dartos smooth muscle

b. the striated cremaster muscle

c. a counter-current heat exchange system

d. the absence of a subcutaneous fat layer

e. an abundance of sweat glands

The dartos muscle is the smooth muscle that lines the scrotum and contributes to the wrinkled appearance of the skin overlying it (Shafik, 1973a; Holstein *et al.*, 1974; Eroschenko, 1993). The dartos muscle relaxes when the ambient or core temperature increases (Maloney and Mitchell, 1996). The relaxation of the dartos contributes to cooling of the testes partly by holding them away from the body (Setchell, 1978) which is the local heat source; and partly by causing the blood vessels that run through the muscle to dilate, improving blood flow and facilitating heat loss from the skin (Shafik, 1973a). Cooler ambient temperatures, on the other hand, stimulate contraction of the muscle, reducing the skin surface area and the blood flow to the skin, while holding the testes closer to the core body heat source (Fig.1.1).

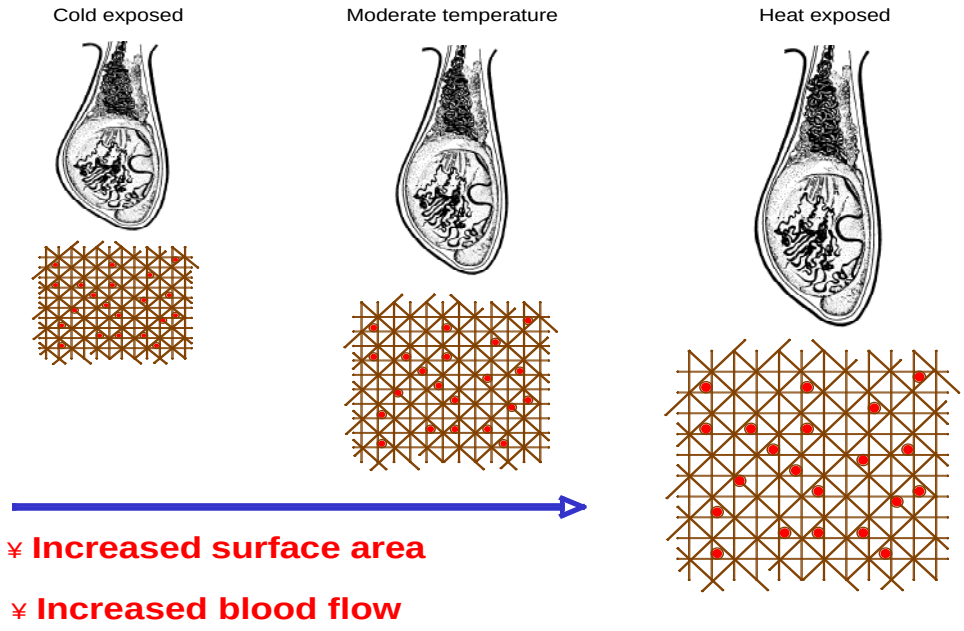

Figure 1.1: Diagramatic representation of how the contraction of the dartos muscle layer (brown lines) controls the skin and muscle blood flow (red circles) thereby influencing heat loss from the skin surface of the scrotum [diagram based on Shafik (1973a) and Waites (1970) for the longitudinal section of the scrotum]

The cremaster muscle fibres, which are skeletal muscle fibres in the spermatic cord, are more directly involved in controlling the distance at which the testes are held from the body (Shafik, 1973b). Contraction of the cremaster causes elevation of the testes and relaxation of cremaster causes descension of the testes. Thus the dartos controls the scrotal skin while the cremaster raises and lowers the testes (Pineda and Dooley, 2003).

The testicular artery that takes blood to the testis from the abdominal aorta descends through the pampiniform plexus of veins that return blood from the testis. Heat exchange between the artery and the surrounding veins via this arrangement also helps in maintaining scrotal temperature (Gunn and Gould, 1975; Cook *et al.*, 1994; Waites and Moule, 1961). Because of the counter-current system, the testes receive blood that is cooler compared to other organs in the body. Blood returning from the testis has lost heat to the environment and is thus cooler than arterial blood. When arterial blood in the testicular artery enters the pampiniform plexus it loses heat to the cooler blood in the pampiniform plexus, and is gradually cooled as it travels towards the testis (Johnson *et al.*, 1970). This mechanism is effective however, only if the scrotum loses heat by radiation (Coulter, 1988), convection or evaporation (Waites and Voglmayr, 1963; Waites and Voglmayr, 1962a) to the environment (Fig.1.2).

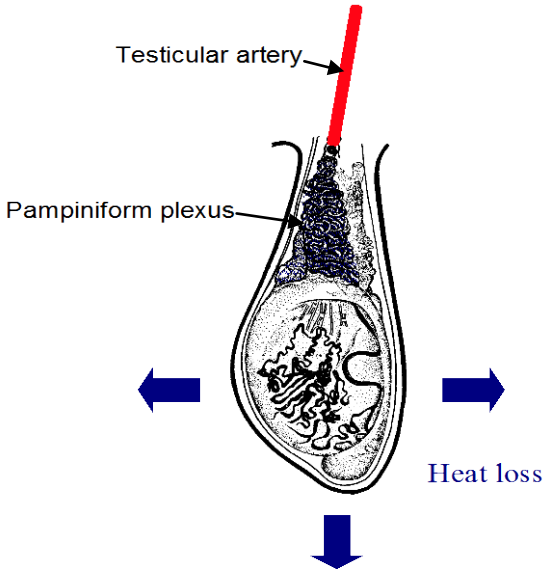

Figure 1.2: Diagrammatic representation of the arrangement of the testicular artery and pampiniform plexus that facilitates the maintenance of the testes at a lower temperature with the aid of heat loss from the surface of the scrotum [diagram based on Waites (1970)].

A subcutaneous fat layer generally has an insulating effect, reducing conductive heat loss to the environment from the body core. The absence of the subcutaneous fat in the scrotal skin (Holstein *et al.*, 1974), therefore, facilitates the loss of heat from the testes.

Finally, mechanism of sweating on the scrotal skin helps to maintain the testicular temperature below that of the body core. There is a high density of sweat glands on the skin of the scrotum (Blazquez *et al.*, 1988) which are stimulated by adrenergic sympathetic nerves (Waites and Voglmayr, 1963). It has been shown that cutaneous warmth receptors in the scrotum exert a strong control over sweating as they do over panting. Experiments performed by Waites and others in the 1960s on rams showed that there is maintenance of testicular temperature even at the expense of body core temperature in certain situations. Merino rams responded to scrotal heating by

either panting (when not shorn) or by peripheral vasodilatation (when shorn) which resulted in a decrease in core temperature (Waites and Voglmayr, 1962a). This evidence highlights the importance that is placed on lower testicular temperature by the thermoregulatory system (Morgentaler *et al.*, 1999).

1.3 Structural overview of the testes, scrotum and the tunica dartos

In humans, the testes are small ovoid organs that are about 4 to 5 cm in length, flattened from side to side that sit inside the scrotal sac (Setchell, 1978; Thibodeau and Patton, 2007). Each testis is encased in a tough fibrous capsule, known as the *tunica albuginia.* This capsule comprises three layers (Figure 1.3).

The innermost *tunica vasculosa*, the middle *tunica albuginia* proper and the outermost *tunica vaginalis* (Setchell, 1978). Internally, a septum divides two sacs that house each testis, epididymis, and lower part of the spermatic cord. The wall of the scrotal sac consists of the skin, the tunica dartos muscle, cremasteric fascia, internal spermatic fascia and parietal *tunica vaginalis* (Knobil and Neill, 1994) in order from outside to inside. The subcutaneous tissue does not contain any fat (Shafik, 1973a).

In the embryo, the testes develop from the gonadal ridges located in the posterior abdominal wall. In mammals that have a scrotum, the testes descend from this point of origin to the scrotum during development. However, there is considerable variation between mammalian orders in the degree of descent of the testis. Structurally, mammalian testes have been grouped into 6 types. In type 1 the testes remain just caudal to the kidneys, as in elephants. Type 2 testes migrate to lie near the bladder, but remain close to the dorsal abdominal wall, like in dolphins. Type 3 testes migrate to or just through the abdominal wall, as in armadillos. Type 4 testes pass caudally into the cremasteric sac near the base of the tail but do not produce an external swelling, as in

moles. Type 5 testes descend into a non-pendulous scrotum with no definite neck as in the rat. In rats, outside of the breeding season the testes assume an abdominal position and move to the scrotum during the mating season (Johnson *et al.*, 1970). Type 6 testes descend into an obvious, pendulous scrotum with a definite neck, as in ruminants and primates. The descent of the testes was believed to be induced by fetal testosterone (Setchell, 1978).

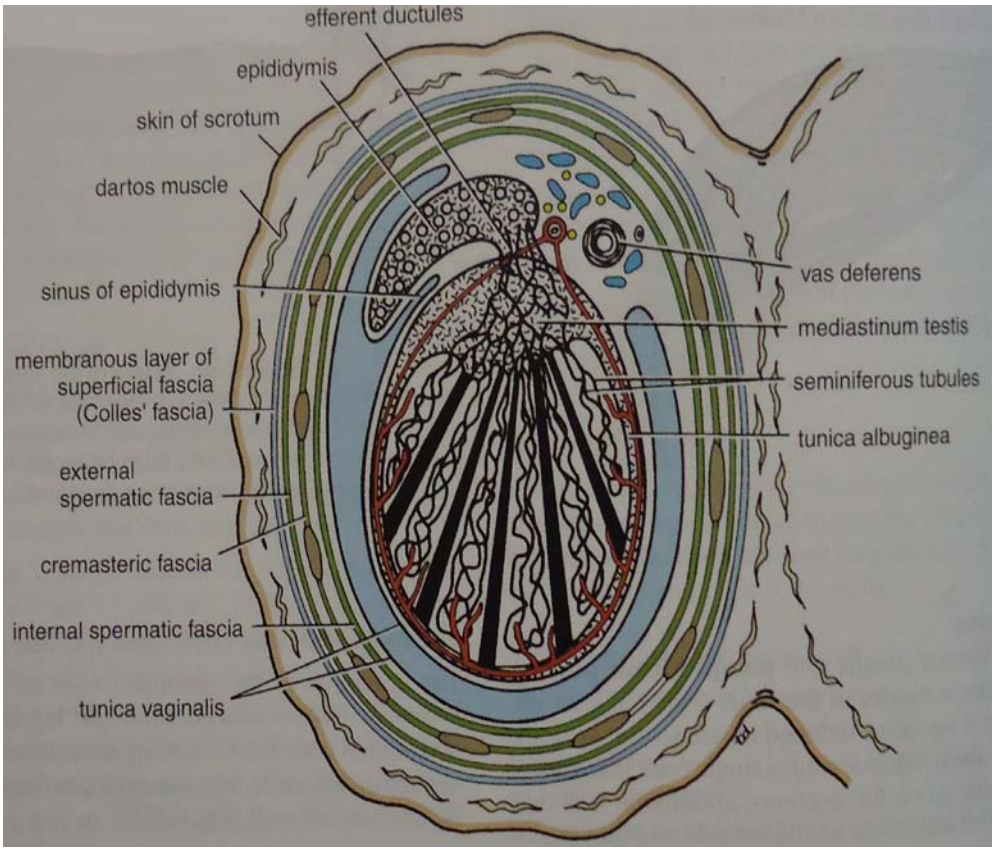

Figure 1.3: Longitudinal section of the scrotum showing the layers of the wall of the scrotum and the relationship of the tunica dartos to other structures [diagram from Grant's atlas of Anatomy].

More recently, testicular descent from the abdomen to scrotum has been thought to occur in 2 phases: namely the trans-abdominal phase and the inguino-scrotal phase. Leydig insulin-like hormone also known as *Insl3* is a member of the insulin hormone

super family which is expressed in the developing testes (Zimmermann *et al.*, 1997). It has been shown experimentally that lack of Insl3 causes cryptorchidism in mice confirming its role in promoting descent of the testes in the abdominal cavity (Nef and Parada, 1999).

Connecting the scrotum to the viscera, the spermatic cord runs from the level of the inguinal canal to the scrotum. It contains the spermatic artery, pampiniform plexus of veins, cremaster muscle, scrotal ligament and the *ductus deferens* (Knobil and Neill, 1994). The spermatic artery is highly tortuous within the spermatic cord, and the testicular veins divide into an interconnected network at the upper pole of the testis and surround the coiled spermatic artery until it enters the inguinal canal. Upon entering the inguinal canal the veins form several venous trunks. This vascular arrangement is called the pampiniform plexus, and as mentioned in the previous section, it facilitates counter-current heat exchange between arteries and veins in the spermatic cord.

The cremaster muscle, which is attached to the *tunica vaginalis,* carries the weight of the testes under normal temperatures. In the cold, via an undescribed mechanism, the cremaster is thought to contract, and at the same time exert a sphincter like action on the cord arteries, reducing blood flow and thereby preventing heat loss. In warm temperatures, the opposite is thought to occur with relaxation of the cremaster lowering the testes and also increasing blood flow, promoting heat loss (Shafik, 1973b). Contraction of the dartos muscle, which lines the skin of the scrotum, will cause slight elevation of the testes with wrinkling of the skin of the scrotum.

The testes are anchored to the skin at the bottom of the scrotum via the scrotal ligament. Histologically, the scrotal ligament consists of collagen and elastin (Shafik, 1977). It penetrates the fibromuscular tube around the testes and is attached to the tunica vaginalis at the lower pole of the testes. The ligament keeps testicular movement synchronous with the movement of the dartos. Relaxation of the dartos muscle pulls

down the ligaments and the testicles, and potentiates relaxation of the cremaster muscle. Exposure to cold causes contraction of the cremaster, which pulls up the ligament and the dartos, enhancing its contraction.

1.3.1 Microscopic structure of *tunica dartos*

The dartos muscle is found beneath the scrotal skin and contains muscle bundles arranged in a crisscross pattern intermingled with fibroelastic tissue (Shafik, 1973a). The overlying scrotal skin, similar to any thin skin, has an epidermis, a dermis (corium) and a subcutaneous (subcutis) layer (Eroschenko, 1993). Ultrastructural features of the dartos have been studied in detail using light, fluorescence, and electron microscopy (Holstein *et al.*, 1974). Some of the features that are relevant to its contractile physiology will be highlighted here. The unique feature of the tunica dartos is the presence of two types of smooth muscle cells. Firstly, there are the typical smooth muscle bundles mainly in the subcutis and sparingly in the corium (Holstein *et al.*, 1974). The thick bundles of smooth muscle cells found in the subcutis are reported to be well delimited from the surrounding connective tissue and show a plexus like arrangement. The muscle bundles are arranged in longitudinal, circular and oblique layers. Wide spaces between the muscle bundles in each of these layers are occupied by blood vessels (Shafik, 1973a).

Secondly, there are the single smooth muscle cells in the corium, which show characteristics of modified fibrocytes. These single muscles in the corium appear to be surrounded by intervening microfilaments, which penetrate into neighbouring collagen fibres and elastin. Parts of the microfilament extend into the specialized sub epithelial connective tissue. These intermediary cells – also referred to as ‘myofibroblasts’ in the smooth muscle literature- are thought to be smooth muscle cells differentiated from

fibroblasts (Ross and Klebanoff, 1971; Guldner *et al.*, 1972). Moreover, throughout the dartos, the smooth muscle cells have extensive structural integration into connective tissue elements, with a cover of microfilaments binding them together and extending beyond the ends. The cell poles are glued together by elastin material.

Apart from the differences in the smooth muscle component, there are also differences in the connective tissue fibres in the corium and the subcutis. In the corium, thin collagen fibrils are prevalent with microfilaments containing elastin being interspersed. In contrast in the subcutis, electron dense large collagen fibrils are prevalent with a small amount of microfilament bundles.

Adrenergic nerves innervating bundles of smooth muscle in both the subcutis and corium have been demonstrated by fluorescent microscopy (Holstein *et al.*, 1974). The single myofibroblasts do not have adrenergic innervation.

1.4 The dartos as a smooth muscle

Muscle is the tissue that is involved with motion in the body. Out of the three types of muscles in the body – skeletal, cardiac and smooth - smooth muscle is found in the walls of visceral organs and blood vessels and is under involuntary control. Generally smooth muscle occurs in large sheets or layers, as in the wall of hollow viscera like the blood vessels, respiratory tract, digestive tract, uterus, bladder, ureters, bile duct, and other visceral tubes. The function of smooth muscle in hollow viscera is to mix and propel the contents by contracting and relaxing rhythmically as a unit (Webb, 2003). In blood vessels and in the bronchial tree, smooth muscle is arranged in a circular pattern allowing it to modify the diameter of the lumen and thereby alter the blood pressure and airway resistance, respectively. There are small bundles of smooth

muscle cells attached to the iris and the lens of the eye, which help to control the amount of light passing into the eye and helps keep images in focus. Such small bundles are also seen in the hairs of the skin which, when activated, cause the hairs to become erect. The tunica dartos, in contrast, is unique in that it is a muscle closely attached to the skin. Its function therefore, is performed together with the skin as a unit. Contraction of the dartos causes wrinkling of the scrotal skin together with a reduction in the surface area. Relaxation of the dartos causes a stretching and an increase of the scrotal skin surface area (Setchell, 1978).

Smooth muscles are categorized as either phasic or tonic, but the demarcation between them is ill defined. In general, it is the vascular and the bronchial smooth muscles that are considered to be tonic and smooth muscles in the other visceral organs that are considered phasic. Phasic muscles have high shortening velocities and can display regenerative action potentials. Tonic smooth muscles do not display action potentials or regenerative electrical activity. Tonic smooth muscles have slower shortening velocities and are more effective in maintaining tone (Sherwood, 2001). The dartos is a tonic smooth muscle (Holstein *et al.*, 1974), its tonic contraction giving the characteristic wrinkled appearance to the scrotal skin.

Smooth muscles receive innervation from the autonomic nervous system usually via both sympathetic and parasympathetic efferents. Contractile activity is modified by hormones, autocrine and paracrine agents and other local chemical signals (Webb, 2003). In addition, mechanical stretch of the contractile proteins actin and myosin can induce contraction (Zeiden *et al.*, 2003). Similarly, a change in membrane potential brought about by an action potential or by activation of stretch dependent ion channels can also trigger contraction (Rapoport and Bevan, 1981).

The two components of the autonomic nervous system, the sympathetic and the parasympathetic, often have opposing effects on a particular group of smooth muscles

in an organ (Sherwood, 2001). For example, in the bronchial tree, sympathetic stimulation dilates, while the parasympathetic stimulation constricts, the bronchi (van der Velden and Hulsmann, 1999). In addition, different organs have variable responses to sympathetic and parasympathetic stimulation (Guyton and Hall, 2000). For example, sympathetic activation predominantly constricts systemic arterioles, but relaxes respiratory smooth muscle, and smooth muscle of the gut. This may be due to the variability of the neurotransmitter released at the nerve ending and / or the type of receptor found in the end organ. In addition, the effect of neurotransmitter binding to a receptor on a cell is variable. In some instances, the smooth muscles respond only to one component of the autonomic nervous system and do not have receptors for neurotransmitters secreted by the other component. An example of such is systemic arteriolar smooth muscle which does not respond to parasympathetic nervous stimulation (Hirst and Edwards, 1989).

In the case of innervation of the dartos, the scrotal nerves originate from L_5, L_6, S_1 roots in the Wistar rat (Garcia *et al.*, 2007) and then travel in the pudendal, inguinal and ilioinguinal nerves carrying somatic and sympathetic fibres (Setchell and Breed, 2006). These nerves supply the dartos muscle, the skin of the scrotum, cremaster muscle and the spermatic cord. Fluorescent microscopy performed on tissue from the human dartos has revealed a very characteristic pattern of adrenergic nerves confined to the tunica dartos (Holstein *et al.*, 1974). These nerves appear as bundles in the connective tissue spaces between smooth muscle fascicles or single axons between smooth muscle fibres in the subcutis. Single smooth muscles in the corium are not associated with accompanying adrenergic nerves. There is no reported parasympathetic innervation of the dartos muscle in the ram, rat, or human.

1.4.1 Ultra-structure of smooth muscles

Structurally, smooth muscles are single nucleated, spindle shaped cells, 2 to 10µm in diameter and 50 to 400 µm in length. The special contractile apparatus is made of thin actin filaments and thick stationary myosin filaments (Somlyo, 1985). However, smooth muscles do not show visible cross striations because the contractile fibres are not arranged in a regular pattern forming myofibrils, as they are in skeletal and cardiac muscle (Somlyo, 1980).

In addition to actin and myosin there is a third type of filament, intermediate in size and made of desmin. Desmin fibres form the cytoskeleton by attaching to dense bodies, which are positioned throughout the smooth muscle (Fig.1.4). The actin filaments are anchored to the dense bodies (Bond and Somlyo, 1982) and the myosin filaments are arranged between the actin filaments.

The contractile elements are arranged slightly diagonally in the smooth muscle cell in an elongated, diamond shaped meshwork rather than running parallel to the long axis, as in the skeletal muscle (Fig.1.4). Sliding of thin filaments over the thick filaments during contraction causes the filament meshwork to shorten and bulge from side to side, producing a shortening of the muscle cell with bulging out between the dense bodies. Smooth muscles are capable of generating more force per cross sectional area than striated muscles, probably due to the geometric arrangement of filaments, or due to the molecules themselves (Somlyo, 1985).

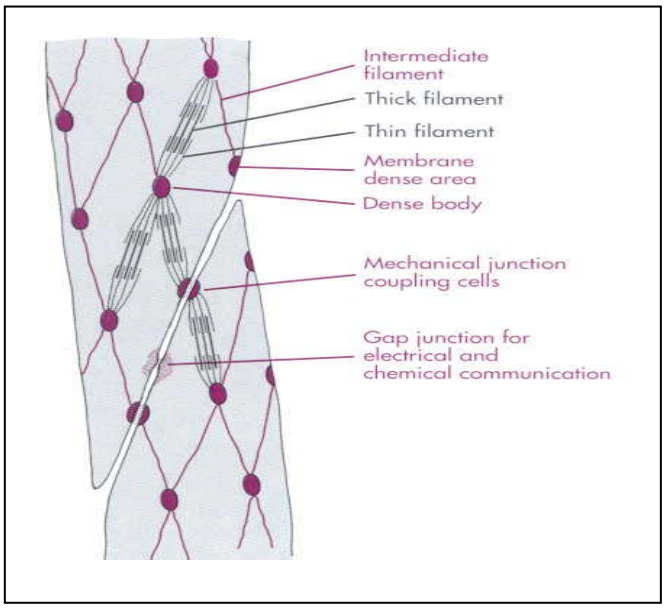

Figure 1.4: Schematic representation of the arrangement of thick and thin filaments in smooth muscle *(source: www.mona.uwi.edu/..../muscles/smooth muscle.htm)*

In some ways the smooth muscle contractile filaments (actin & myosin) are similar to those found in skeletal muscle. However, there are a few differences (Koeppen and Stanton, 2008). Smooth muscle thin filaments are not associated with the protein troponin or nebulin as they are in skeletal muscle. However, smooth muscles have two proteins that are not found in striated muscle; caldesmon and calponin. The exact function of these proteins is not fully understood however, caldesmon has replaced troponin as the calcium dependent regulator of tropomyosin located on the thin filaments. Another difference between the skeletal muscle contractile proteins and the smooth muscle proteins is that smooth muscle contains a unique myosin light-chain subunit which is phosphorylated by the enzyme myosin light-chain kinase (MLCK).

Each thick filament is composed of several hundred myosin molecules in a specific arrangement. A single molecule of myosin comprises two identical protein subunits, each with a head and a tail somewhat like a golf club (Fig. 1.5). The tail ends are intertwined with the heads projecting out at one end (Somlyo, 1980). Each myosin

head contains two important sites for the contractile process, the actin binding site and the myosin ATPase, which hydrolyses ATP.

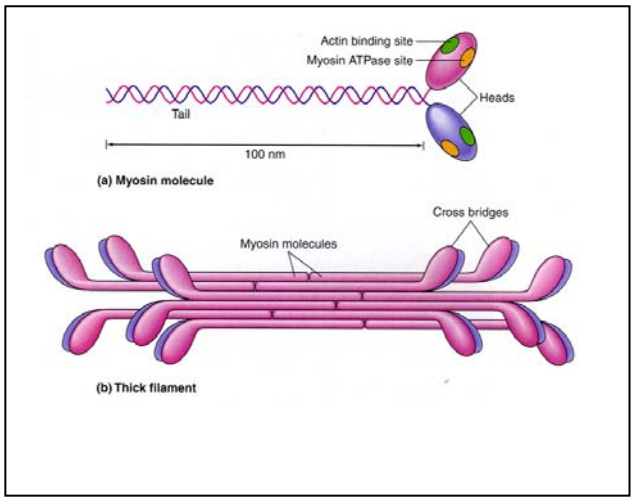

Figure 1.5: The molecular structure of myosin filaments of smooth muscle. The myosin molecule containing a head and a tail is arranged in a stack to form thick filaments with the heads projecting outwards *(Sherwood, 2001).*

The thin filaments are composed of two proteins, actin and tropomyosin. The two chains of actin and tropomyosin are twisted together like a string of pearls and contain binding sites for the formation of cross bridges with myosin (Fig. 1.6).

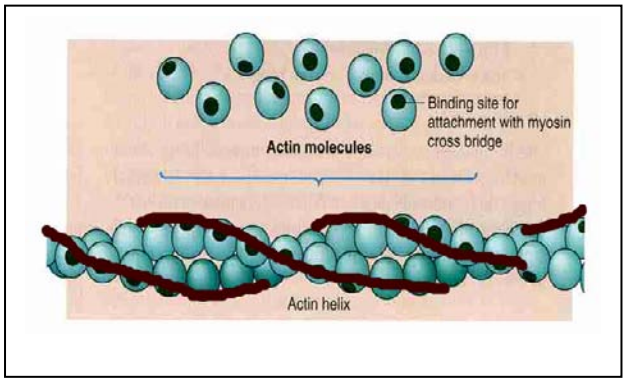

Figure 1.6: The molecular structure of actin filaments of smooth muscle. The actin molecule and tropomyosin are twisted around each other in a chain *(Sherwood, 2001).*

It is now generally agreed that the sarcoplasmic reticulum is the important sub cellular structure in the control of the contraction of smooth muscle (Somlyo, 1985).

Ultrastructurally, it is an intracellular membrane system of tubules, which has no direct ionic communication with the extracellular space. It occupies no more than 2% of the cell volume but contains enough Ca^{2+} to activate contraction (Bond *et al.,* 1984).

1.4.2 The mechanism of smooth muscle contraction

Regardless of the stimulus that initiates muscle contraction, the contraction is usually associated with a rise in intracellular Ca^{2+} (Kamm and Stull, 1985; Somlyo and Somlyo, 1994). The increased intracellular Ca^{2+} mediates changes in the thick myosin filaments. To activate the contractile apparatus, Ca^{2+} must increase globally throughout the cytoplasm. The Ca^{2+} necessary for activation of the contractile apparatus either enters from the extracellular fluid or is released from intracellular store the sarcoplasmic reticulum (Sanders, 2001; McFadzean and Gibson, 2002).

1.4.2.1 Extracellular Ca^{2+} entry mechanisms

Best characterized Ca^{2+} entry pathway is the voltage operated calcium channels (VOCC), the most researched VOCC being the *dihydropyridine sensitive L-type Ca^{2+} channels (DHP)*(McFadzean and Gibson, 2002). They carry voltage sensors to be activated by change in voltage (McDonald *et al.*, 1994b; McDonald *et al.*, 1994a). In some smooth muscles however, chemicals such as nitric oxide activate DHP-sensitive (Clapp and Gurney, 1991). Other *voltage dependent Ca^{2+} channels* have been found in some smooth muscles. These are insensitive to DHP and have been reported in mesenteric arteries (Morita *et al.,* 1999).

Endogenous agonists activate *non-selective cation channels* and Ca^{2+} dependent Cl^- currents in smooth muscles. These are also referred to as *receptor operated*

cationic channels (ROCC) (McFadzean and Gibson, 2002). This in turn contributes to Ca^{2+} entry via voltage dependent Ca^{2+} channels by depolarization. Acetylcholine and adrenergic agents activate this type of non-selective cation channels (Sanders, 2001).

P_2X receptors which are activated by ATP are also known to cause Ca^{2+} entry through the *P_2X channels* (Loirand and Pacaud, 1995).

Mechanical stretch is also known to cause Ca^{2+} entry through non-selective Ca^{2+} channels in the urinary bladder muscles (Wellner and Isenberg, 1994). The channels concerned are referred to as *stretch-sensitive non-selective cation channels.*

In many cells depletion of internal stores is coupled to activation of Ca^{2+} entry from the extracellular space which is referred to as *store-operated Ca^{2+} channels* (SOCC) *or capacitative Ca^{2+} channels* (Putney and Ribeiro, 2000).

1.4.2.2 Intracellular Ca^{2+} release mechanisms

Ryanodine receptors (RyR) are one of the channels that release Ca^{2+} from the sarcoplasmic reticulum. Cytoplasmic Ca^{2+} activates RyR receptors and thus they are called Calcium induced calcium release (CICR) channels (Sanders, 2001). These channels are activated by caffeine and locked into a subconductance state by ryanodine (Hymel *et al.*, 1988). Recent studies have shown that Ca^{2+} entry through DHP-sensitive Ca^{2+} channels can activate CICR in smooth muscle cells of the bladder and that it is coupled to the occurrence of Ca^{2+} waves and sparks (Collier *et al.*, 2000).

IP_3 receptors are the other important calcium release channels. A variety of agonists binding to G protein coupled receptors in smooth muscle results in

activation of phospholipase C which produces IP_3 (Mikoshiba *et al.*, 1994). IP_3 and diacylglycerol (DAG) are soluble products formed when the enzyme phospholipase C acts on the membrane phospholipid phosphatidylinositol 4,5 biphosphate (PIP_2) (Berridge, 1993; Somlyo and Somlyo, 1994). After formation, IP_3 diffuses to the sarcoplasmic reticulum where it binds to and opens a Ca^{2+} channel which causes release of stored Ca^{2+} to the cytoplasm. The activation of the enzyme phospholipase C occurs via G protein coupled receptor stimulation (Somlyo *et al.*, 1988a; Abdel-Latif, 1986; Berridge, 1988).

The Ca^{2+} in the cytosol (either from extracellular or intracellular sources) in turn combines with the acidic protein calmodulin and together the Ca^{2+}/calmodulin complex activates the enzyme myosin light chain kinase (MLCK) (Hai and Murphy, 1989; Kamm and Stull, 1985) (Fig. 1.7). MLCK is responsible for phosphorylation of the 20-kDa myosin light chain (de Lanerolle and Paul, 1991) which enables myosin to interact with actin. Myosin light chain phosphorylation serves two major functions. Firstly, it facilitates the ability of monomeric myosin to assemble into filaments, and secondly, it increases the ATPase activity of myosin by about 100 fold. Energy released from ATP by myosin ATPase activity results in the cycling of myosin cross-bridges with actin and thus smooth muscle contraction (Sommerville and Hartshorne, 1986; Kamm and Stull, 1985; Hai and Murphy, 1989).

Although it has been established earlier that smooth muscle contraction involves an elevation of intracellular Ca^{2+}, understanding of the nature of this elevation has changed over time. Earlier it was thought that increase of intracellular Ca^{2+} took place as an initial spike followed by a sustained plateau which was lower. However, this is not always the case. Recent experiments on mouse, pig and human airway smooth muscles using high resolution microscopy have shown that the Ca^{2+} signal involves a

series of oscillations (Pabelick *et al.*, 2001; Lee *et al.*, 2005; Perez and Sanderson, 2005; Prakash *et al.*, 1997). The peak amplitude of the oscillations generally remains the same and is not in itself vitally important. The important feature of the Ca^{2+} signal is the frequency of oscillations which correlate with the increase in agonist concentration and force of muscle contraction produced (Sanderson *et al.*, 2008). Despite there being differences in the contractile process of different smooth muscles in the body, there are also many similarities. Since the tunica dartos, the muscle under study, is a tonic smooth muscle and airway smooth muscles too are tonic smooth muscles, it may be not entirely wrong to extrapolate similar mechanisms to be operating in the two locations.

The cross bridge cycling that ensues following the elevation of Ca^{2+} is similar to the process that occurs in skeletal muscle (Goldman, 1987; Webb and Trentham, 1983) (Fig. 1.7). Force generation occurs with the formation of cross-bridges between the actin filament and myosin head. In the relaxed state, unattached cross-bridges are present as high energy myosin-ADP-P*i* complexes in the presence of ATP. When myosin light chains become phosphorylated, in the presence of Ca^{2+}, rapid cross-bridge cycling is initiated. The free myosin head with its ATP hydrolysis products (ADP + P*i*) binds the active site of actin and displaces the ADP and P*i*. The arrangement of the actin and myosin filaments is such that when cross-bridges form, the actin filaments are pulled towards the centre of the thick filaments. A new ATP molecule then occupies the myosin head, decreasing the affinity of myosin to actin. This causes release of actin from myosin. Energy from the newly bound ATP is then used to produce a conformational change in the myosin head so that the cross-bridge is ready for another contraction cycle with the hydrolysis of an ATP. The cross-bridge cycling continues while ever the cytoplasmic Ca^{2+} remains elevated. One molecule of ATP is hydrolysed per cycle. With a decrease in cytoplasmic Ca^{2+}, MLCK becomes inactive and myosin is dephosphorylated by myosin phosphatase (MP) (Fig. 1.7 & 1.8).

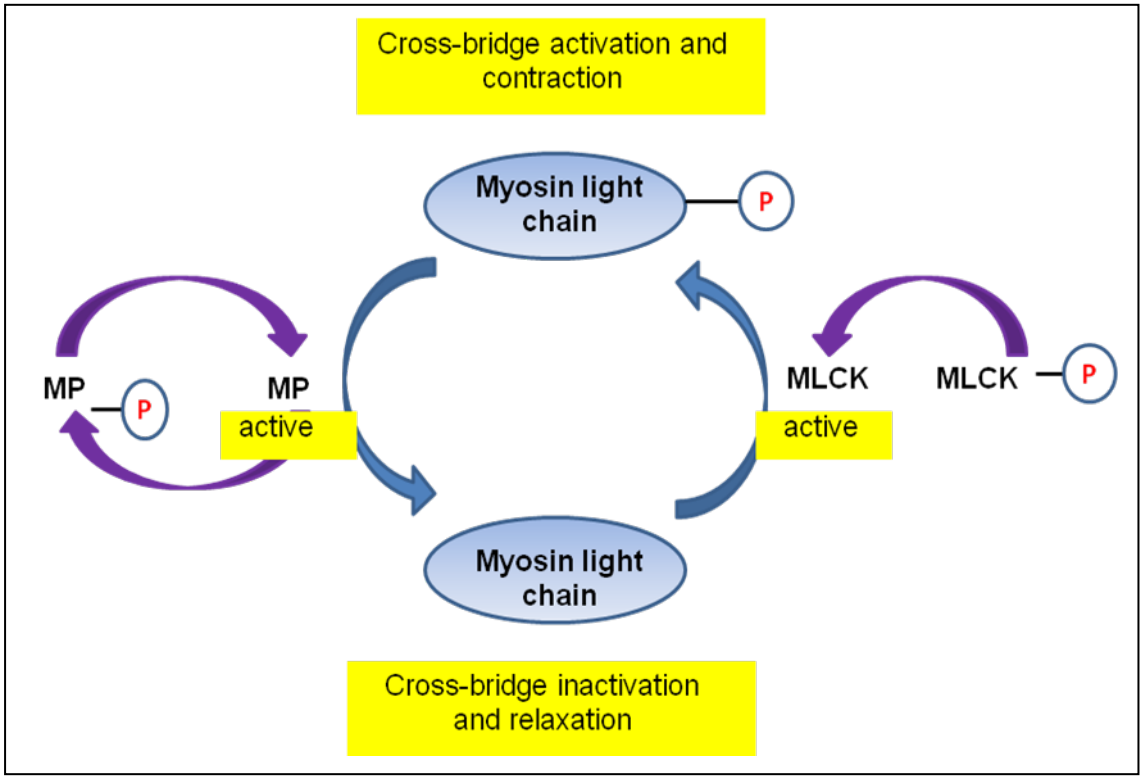

Figure 1.7: The degree of contraction of a smooth muscle depends on the balance between the activity of myosin light chain kinase and myosin phosphatase. These two enzymes are inactive when phosphorylated. The active form acts on myosin light chains to control smooth muscle contraction.

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Although intracellular Ca^{2+} plays an important role in contraction of smooth muscle, early observations using Ca^{2+} indicators have revealed that the degree of contraction is not always proportional to the intracellular Ca^{2+} concentration (Bradley and Morgan, 1987). The force of contraction and the degree of phosphorylation of the myosin light chains induced by agonist stimulation is higher than that of K^{+} depolarization at an equal concentration of intracellular Ca^{2+}. This is referred to as Ca^{2+} sensitization (Somlyo and Somlyo, 1994). The monomeric G-protein binding molecule Rho has been identified as a molecule involved in the Ca^{2+} sensitization by agonists in smooth muscle (Gong *et al.*, 1996). Furthermore, it was found that, increment in phosphorylation of myosin light chains at a constant Ca^{2+} concentration of is due to a reduction in the dephosphorylation rate and not an increase in the phosphorylation rate

(Noda *et al.*, 1995). Thus Rho is supposed to regulate Ca^{2+} sensitivity via MP which would be discussed in more detail later in this chapter.

Smooth muscle relaxation occurs in response to either a decrease in intracellular calcium and/or an increase in myosin phosphatase (MP) activity. MP removes the high energy phosphate from the myosin light chain. MP is tightly bound to myosin under physiological conditions (Horowitz *et al.*, 1996). Several mechanisms contribute to the removal of Ca^{2+} from the cytoplasm, and primarily involve Ca^{2+} pumps on the sarcoplasmic reticulum and the plasma membrane (Horowitz *et al.*, 1996). Ca^{2+} uptake into the sarcoplasmic reticulum depends on the activity of the Ca^{2+}-Mg^{2+}-ATPase on the sarcoplasmic membrane. This binds two Ca^{2+} ions which are then translocated to the lumen of the sarcoplasmic reticulum and released. Sarcoplasmic reticular calcium binding proteins such as Calsequestrin and Calreticulin also contribute to sarcoplasmic eticular Ca^{2+} sequestration by decreasing sarcoplasmic reticular free Ca^{2+} levels (Horowitz *et al.*, 1996). Calcium is also removed from the cytoplasm via Na^{+}/Ca^{2+} exchanger and Ca^{2+} ATPase (Martinez-Serrano *et al.*, 1992).

1.4.3. Regulation of smooth muscle contraction and relaxation

The balance of activity of MLCK and MP determines the degree of phosphorylation of myosin light chains (Pfitzer, 2001), and both MLCK and MP are targets for intracellular signaling pathways which modulate their activities independent of changes in Ca^{2+} (Fig.1.7). It is the covalent modification of myosin by phosphorylation of the light chain that initiates smooth muscle contraction. This mechanism of control is important mainly for generating phasic contractions and the initial development of tonic contractions (Fig. 1.8). The primary regulator of MLCK is

the Ca^{2+}- calmodulin complex which binds to and activates MLCK. MLCK in turn phosphorylates the light chain of myosin and initiates the contractile process.

Phosphorylation of the enzyme MLCK reduces its affinity to calmodulin. This phosphorylation can occur at either of two sites, A or B. Only phosphorylation at site A reduces the affinity to calmodulin (Horowitz *et al.*, 1996)

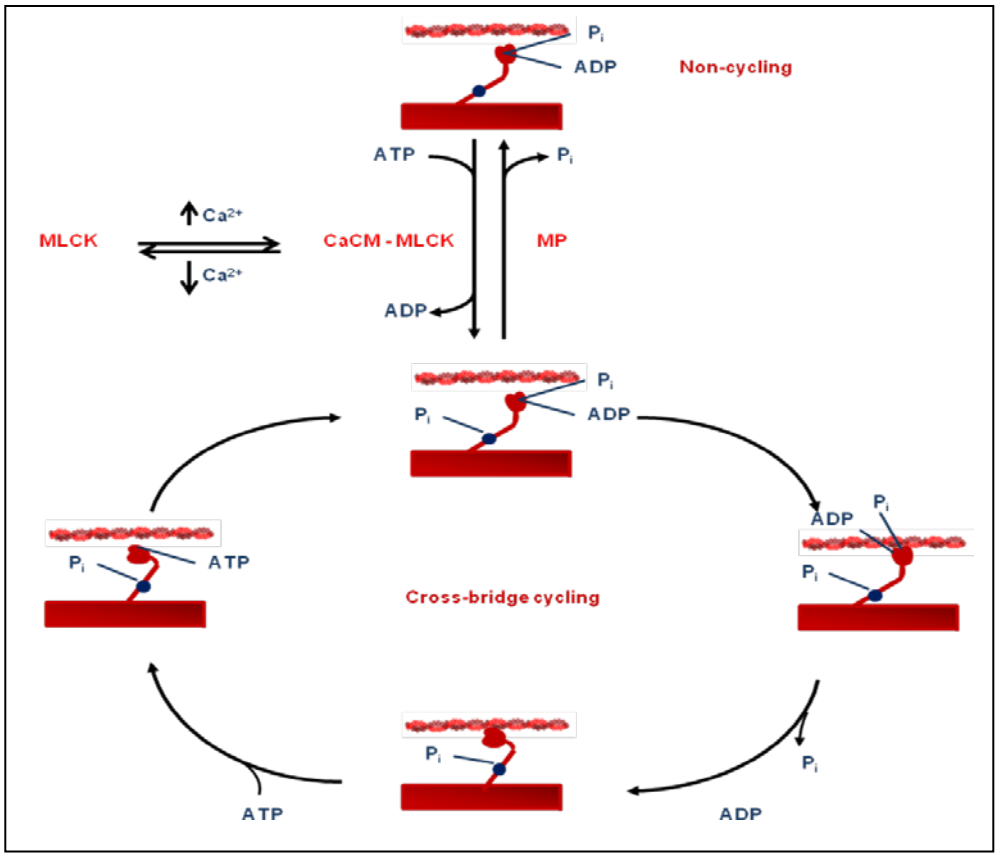


Figure 1.8: Cross-bridge cycling by phosphorylation of the light chain of myosin with the calcium-calmodulin complex. [Diagram based on (Koeppen and Stanton, 2008)].

During phasic contraction in smooth muscle, cytoplasmic Ca^{2+} increases producing cross-bridge phosphorylation. The force reaches a peak and then returns to baseline levels. In contrast, in tonic contraction of smooth muscle in blood vessels, bronchi and tunica dartos, cytoplasmic Ca^{2+} concentration and cross-bridge phosphorylation decline after an initial spike but do not return to the basal level. During

this latter phase, force decreases slowly and is maintained at above basal level. This happens with only 20% - 30% of cross-bridges phosphorylated, reducing the total ATP utilization. The term ‘latch state’ is used for this state of tonic contraction with low energy expenditure. The ‘latch state’ occurs when a cross-bridge is formed and the myosin light chain is dephosphorylated but still bound to actin (Hai and Murphy, 1989). When the myosin light chain is phosphorylated, the cross-bridges cycle as long as intra cytoplasmic [Ca^{2+}] is elevated. However, if an attached cross-bridge is dephosphorylated by MP, the rate of cross-bridge cycling is decreased because the detachment of cross-bridges is a slower process than attachment. The myosin light chain has to be phosphorylated again before the next cycle can start, and if intracellular Ca^{2+} is high (MLCK:MP ratio is high) cross-bridges are phosphorylated and the cycle continues. But when intracellular Ca^{2+} decreases during tonic contraction, it is more likely that the myosin light chains will be in the dephosphorylated but attached cross-bridge state rather than the phosphorylated state. A low rate of Ca^{2+} dependent myosin light chain phosphorylation is essential for tonic contraction. If Ca^{2+} concentration falls below that required for activation of MLCK, the muscle will relax.

In summary, covalent regulation of contraction allows the formation of eight cross-bridge states in smooth muscle. Phosphorylation of MLCK in the presence of high Ca^{2+} is necessary for cross-bridge attachment. Phosphorylated cross bridges cycle rapidly. Dephosphorylation of cross-bridges by MP slows down the cycling rate and produces the latch state. ATP is needed for both the regulation

and cycling of cross bridges.

The small G-protein rho regulates myosin light chain phosphorylation through myosin binding subunit (MBS) of MP and rho kinase (Gong *et al.*, 1996; Fukata *et al.*, 2001). Agonists of smooth muscle contraction, activates rho through their interaction with the G-protein couple receptor. Activated rho in turn, activates rho-kinase.

Activated rho-kinase subsequently phosphorylates MBS of MP resulting in a reduction in the dephosphorylation of myosin light chains (Kimura *et al.*, 1996). Concomitantly, rho kinase directly phosphorylates myosin light chains at the same site that is phosphorylated by MLCK (Amano *et al.*, 1996). Thus rho-kinase is able regulate smooth muscle contraction via two processes namely, inactivation of myosin phosphatase and direct phosphorylation of myosin light chains (Fig. 1.9) (Fukata *et al.*, 2001; Kimura *et al.*, 1996; Amano *et al.*, 1996). The activity of rho-kinase and calcium sensitization process has become an important area in smooth muscle contraction, especially in relation to vascular smooth muscles and certain diseases such as hypertension and coronary vasospasm.

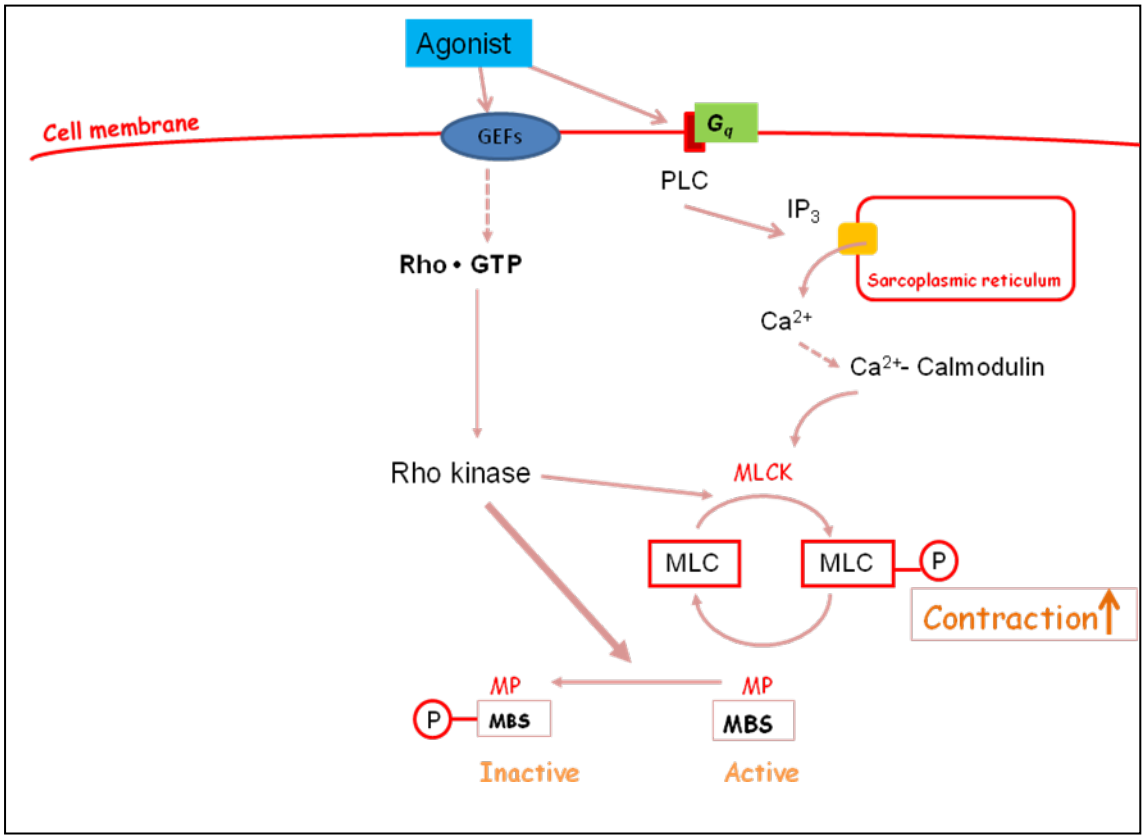


Figure 1.9: Regulation of smooth muscle contraction by Rho-kinase and myosin phosphatase downstream of Rho. Activated Rho activates Rho-kinase, which then phosphorylates MBS of MP and inhibits the MP activity. Rho-kinase also phosphorylates myosin light chains directly thus resulting in greater contraction.

As mentioned at the outset, smooth muscles are broadly categorized as those that exhibit phasic contraction and those that exhibit tonic contraction. Although the demarcation between the two is quite hazy, the excitation-contraction coupling in these two categories differs. Electromechanical coupling is more predominant in phasic smooth muscles such as those found in the gut (Milanov *et al.*, 1984) where the depolarization of the membrane cause entry of ionized calcium through voltage operated channels (VOCCs). Pharmacomechanical coupling is predominant in tonic smooth muscles such as those found in blood vessels and bronchi (McFadzean and Gibson, 2002) where the intracellular calcium rise is due to either store release of calcium from the sarcoplasmic reticulum or entry of calcium from the extracellular space by opening of receptor operated calcium channels (ROCCs) or store operated calcium channels (SOCCs). Dartos being a muscle that exhibit tonic contraction, the assumption to make is that it would exhibit predominantly a pharmacomechanical coupling.

The mechanism of smooth muscle contraction and its control has been highlighted at this point because the bulk of the experiments which form this thesis revolve around the contraction of tunica dartos which is the smooth muscle attached to the scrotal skin. The final experiment which forms this thesis deals with how a change in temperature of the testes affects its endocrine function.

Therefore, it is necessary to understand the basic endocrine function of the testes and the multitude of factors that affect the endocrine secretions from the testes in the live animal model.

1.5 Endocrine function of the Testes

In addition to being the site of spermatogenesis, the testes produce androgens which are important for the development of secondary sexual characteristics and libido in the male. Although the effect of temperature on spermatogenesis has been studied in various mammals, including humans (Meistrich *et al.*, 1973; Freidman *et al.*, 1991; Casady *et al.*, 1953), there are very few conclusive studies probing the effect of temperature on the endocrine function of the testes.

1.5.1 Testosterone secretion

Testosterone is the main steroid hormone secreted by the testes (Darbeida and Brudieux, 1980; Schanbacher, 1976). It is secreted by the Leydig cells when luteinizing hormone (LH) binds the specific receptors on the Leydig cell surface (Barenton and Pelletier, 1983; Cooke *et al.*, 1992). LH in turn is secreted from the anterior pituitary gonadotrophs in response to stimulation by hypothalamic gonadotrophin releasing hormone (GnRH) (Fig. 1.10). Each pulse of LH evokes a pulse of testosterone (Sanford *et al.*, 1974b; Lincoln, 1978). There is an average delay of 35-160 minutes from the peak of LH to the peak of testosterone (Sanford *et al.*, 1974b; Schanbacher and Ford, 1976; Lincoln, 1978; Terqui *et al.*, 1980; Martin and White, 1992; Blache *et al.*, 1997a). Testosterone also has a delay in returning to basal levels, after LH stimulation has ceased.

GnRH is secreted as a stream of pulses from the median eminence of the hypothalamus into the hypothalamo-hypophyseal portal system (Clarke and Cummins, 1982). The frequency of GnRH pulses is controlled by the GnRH pulse generator, a neural network distributed over several regions of the brain (Lincoln *et al.*, 1985). Generally, the preoptic-hypothalamic continuum is considered the most

important population of cells in this system (Blache and Martin, 1995). From the pre-optic continuum neurons project to the median eminence where GnRH is secreted.

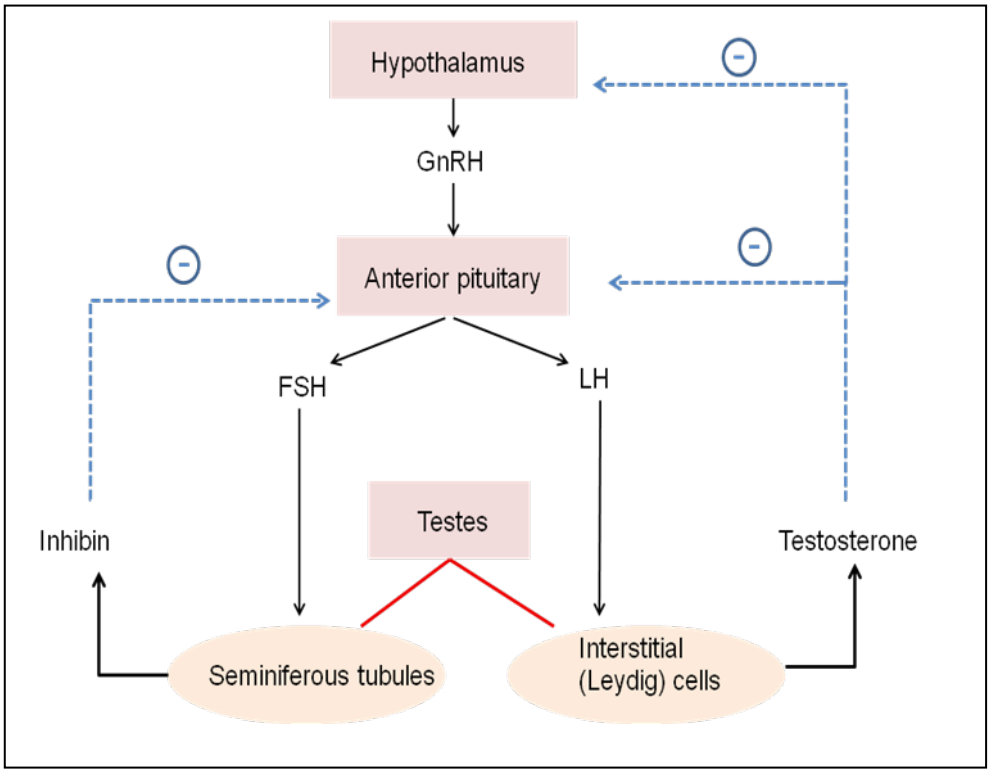


Figure 1.10: Hypothalamo-pituitary-gonadal axis showing the interactions between various hormones [diagram based on (Sherwood, 2001)].

GnRH neurons receive inputs via a variety of neurotransmitters and neuromodulators. Some of these inputs have a direct effect on the GnRH pulsatility (GABA, glutamate) while others provide facilitatory signals (norepinephrine, neuropeptide Y) to the GnRH neural network (Herbison, 2006). These transmitters act on receptors located on GnRH neurone cell bodies, dendrites, or terminals. A newly discovered neuropeptide, kisspeptin, has been implicated as an important stimulator of GnRH secretion. Kisspeptin is a product of the *Kiss1* gene. It binds the G-protein coupled receptor GPR54 and stimulates GnRH secretion directly (Messager *et al.*, 2005). It is now postulated that the onset of puberty may be

triggered by an increase in either *Kiss1* expression, kisspeptin input to GnRH neurons, and/or GnRH neuron sensitivity to kisspeptins (Shahab *et al.*, 2005; Ojeda and Skinner, 2006; Smith and Clarke, 2007). Kisspeptin expression in the brain is correlated positively with conditions known to stimulate the reproductive axis. Some of the other factors known to influence GnRH secretion and the HPG axis are nutritional state, photoperiod and the feedback activity from other hormones.

1.5.2 Testosterone feedback

Testosterone's role in negative feedback inhibition of GnRH neuronal function is probably the dominant influence on reproductive axis function (Jackson *et al.*, 1991; Tilbrook *et al.*, 1991). The inhibition of GnRH pulse frequency by testosterone is accompanied by a concurrent reduction in the pulse frequency of LH. The inhibition of the axis occurs both at the level of the hypothalamus and the pituitary (Steiner *et al.*, 1982; D'Occhio *et al.*, 1982).

The identity of the neurons in the hypothalamus on which testosterone acts to regulate GnRH secretion remain unclear. Recent evidence suggests that kisspeptin neurons may account for at least part of the population of cells linking sex steroids to GnRH (Navarro *et al.*, 2004; Smith *et al.*, 2005; Smith and Clarke, 2007). The kisspeptin cells of the hypothalamus seem to be the missing link between gonadal steroids and the GnRH secreting cells (Smith and Clarke, 2007).

The frequency of GnRH pulses is influenced by factors other than the blood concentration of testosterone. The frequency changes quickly with other stimuli that can be both internal and external to the animal such as the level of nutrition, photoperiod, and socio-sexual cues (Lincoln, 1978; Sanford and Howland, 1984; Rhim *et al.*, 1993; Hotzel *et al.*, 2003).

Luteinizing hormone is a glycoprotein (Pierce and Parsons, 1981) secreted by the gonadotrophs in the anterior pituitary, in a pulsatile manner in response to GnRH stimulation (Clarke and Cummins, 1982). Therefore, LH performs the transduction and amplification of a neural signal that originates in the brain (Martin *et al.*, 1984). Conversely, continuous infusion of GnRH leads to a rapid suppression of LH (Bremner *et al.*, 1976; Nett *et al.*, 1981; Clarke *et al.*, 1986).

After an exogenous dose of GnRH there is a rapid and abrupt rise in LH secretion after about 10 minutes that lasts for 2-6 minutes (Kanchev *et al.*, 1984; Kanchev *et al.*, 1987). After this increase there is an exponential decrease in LH beginning about 30 minutes after the injection (Rasmussen and Malven, 1982; Caldani *et al.*, 1993).

The frequency of GnRH pulses and therefore, LH pulses, is a key factor in the control of reproductive status (Knobil, 1980; Lincoln and Short, 1980; Martin *et al.*, 1984; Caldani *et al.*, 1993). An increased frequency of LH pulses induces ovulation in ewes (Thomas and Oldham, 1984) and an increase in sperm production in rams (Lincoln, 1976), while a decrease of LH pulses causes a decline of gonadal activity in both rams and ewes (Lincoln, 1976).

LH secretion occurs when GnRH occupies GnRH receptors on the plasma membrane of pituitary gonadotrophs. While the secretion of LH is not related to GnRH receptor density, the down-regulation of receptors can lead to the complete suppression of LH secretion (Nett *et al.*, 1981). A profound increase in pulsatile GnRH has been observed in ewes during the pre-ovulatory period, which continues for several hours after the LH surge is completed (Moenter *et al.*, 1990; Moenter *et al.*, 1991). The decrease in LH levels despite the presence of high GnRH levels, and the termination of the surge, may be a result of down-regulation of the GnRH receptors (Crowder and Nett, 1984; Hamenik *et al.*, 1995).

Hence, according to the available literature, the down-regulation of GnRH receptors occurs when the pituitary is stimulated with a continuous infusion of GnRH or with high frequency GnRH pulses.

With the forgoing literature survey forming the background for this thesis it is necessary to state the main aims of the project at this point.

1.6 Aims and objectives of the present project

This project revolves around smooth muscle physiology and the manipulation of the hypothalamo-pituitary-gonadal axis to determine the effect of scrotal temperature on the endocrine function of the testes. The particular smooth muscle under study was the tunica dartos, which is the smooth muscle of the skin of the scrotum. The study started at the cellular level with a comprehensive investigation of the contractile physiology in relation to electrical and pharmacological stimulation. A special focus was made on the response of the tunica dartos to thermal stimulation, with an attempt to find the substance responsible for the marked cooling induced contraction and the intracellular processes involved. Therefore, this was aimed to extend the understanding of the physiological role played by the tunica dartos muscle in regulating testicular temperature and specifically the cellular mechanisms and the substances involved in contraction and relaxation of the dartos. It was also aimed to shed light on the effect of dysregulation of testicular temperature on the endocrine function of the testes.

The foregoing literature review was an attempt to summarize the published literature available on this area of study.

The objectives of the present study stated broadly were;

i. To study the contractile physiology of the tunica dartos as a smooth muscle.

ii. To characterize the cooling induced contractile response of the tunica dartos and to determine the substance/s involved in its activation.

iii. To determine whether the cooling induced contractile response of the tunica dartos is due to an increase in intracellular calcium availability or calcium sensitivity.

iv. To determine the effect of altered testicular temperature on the testosterone output from the testes.

CHAPTER 2

Contractile physiology of the isolated tunica dartos muscle in response to electrical and pharmacological stimulation

2.1. Introduction:

Male fertility is decreasing in the western world (Bledsoe *et al.*, 2000; Carlsen *et al.*, 1992). Many environmental (Jensen *et al.*, 1995), behavioural (Sharpe, 2000) and genetic (Douglas, 2007) factors are thought to affect male fertility. According to a metanalysis by Carlsen et al. (1992), the sperm count has fallen from 113 x 10^6 per ml^3 in 1940 to 66 x 10^6 per ml^3 in 1990. In addition to this quantitative decrease there is also evidence for decreasing quality of semen during the past 50 years (Carlsen *et al.*, 1992). To investigate the underlying physiological processes responsible for this fall in fertility, it is important that all physiological mechanisms responsible for maintaining male fertility are well understood.

In male mammals, the tunica dartos is a smooth muscle that lines the scrotal skin, giving the skin its characteristic wrinkled appearance. Functionally, it is reported to play an important role in testicular thermoregulation (Eroschenko, 1993; Maloney *et al.*, 2005; Holstein *et al.*, 1974; Shafik, 1973a; Setchell, 1978; Morgentaler *et al.*, 1999).

In most mammals testicular temperature is maintained 2-6°C below the body core temperature (Janowski *et al.*, 1971; Maloney and Mitchell, 1996). Sperm production in the testes is a temperature sensitive process and apparently requires testicular temperature to be controlled within a lower range compared to the body core. Elevated testicular temperature has been reported to be detrimental for effective sperm production. Cryptorchidism is a well known cause of infertility, and the infertility is

known to occur because the non-descent of the testes exposes the testes to abdominal temperature (Fukui, 1923; Moore, 1924; Moore and Quick, 1924). Varicoceles (abnormal dilatation of veins in the scrotum draining the testicles) results in elevations in testicular temperature and are also associated with infertility (Goldstein and Eid, 1989). Further, the repair of the varicocele improves fertility (Su *et al.*, 1995) unless permanent damage has occurred to the germinal epithelium.

The available literature suggests that the dartos contributes to testicular thermoregulation in several ways. Firstly, it changes the surface area of the scrotal skin by contracting or relaxing depending on whether the ambient temperature is low or high. Experimental evidence have shown that an elevation of scrotal skin temperature increases its blood flow (Fowler and Setchell, 1971; Waites *et al.*, 1973). Since blood vessels supplying the scrotal skin are arranged between the meshwork of smooth muscle fibres of the dartos, contraction of this muscle may have a constricting effect on the blood vessels thereby reducing the skin blood flow (Shafik, 1973a). Secondly, it contracts or relaxes the scrotal sac, thereby helping to determine the distance at which the testes are held from the body. This process is aided by the cremaster muscle, which has a direct functional role of lengthening and shortening the spermatic cord and thereby controlling the distance at which the testes are held from the body. Thirdly, the lengthening and shortening of the scrotal sac causes lengthening or shortening of the pampiniform plexus (Gunn and Gould, 1975; Cook *et al.*, 1994; Johnson *et al.*, 1970), controlling the counter-current heat exchange between the testicular artery and the veins.

Thus, when the dartos contract, the surface area of the scrotal skin is reduced and blood flow to the skin becomes minimal, limiting heat loss from the scrotal sac. Because the scrotal sac is drawn closer to the body and the pampiniform plexus becomes shorter, warmer arterial blood is delivered to the testes and helps maintain scrotal temperature in colder environmental conditions. The opposite happens when the

dartos relaxes, with increased skin surface area and vasodilatation of the skin promoting heat loss. The lengthening of the plexus may also facilitate counter-current exchange in the pampiniform plexus, by increasing the cooling of the warmer testicular arterial blood. This clear inverse relationship between the level of contraction of the dartos and the external environmental temperature has been shown in several animal studies (Maloney & Mitchell, 1996: El-Darawany, 1999).

The dartos muscle, being a smooth muscle, is innervated by the autonomic nervous system. The nerve supply comes through the genital branch of the genitofemoral nerve that carries sympathetic fibres from the lumber segments of the spinal cord (Garcia *et al.*, 2007; Dean and Pegington, 1996).

To understand the contractile physiology of the dartos, it is necessary to examine the ultrastructural features of the dartos (see chapter 1). As mentioned previously, the wide spaces between the muscle bundles in the subcutis and corium are occupied by connective tissue and are rich in blood vessels (Shafik, 1973a). Thus the contraction of the smooth muscle causes constriction of the vessels running between the muscle fibres in the connective tissue. Innervating the smooth muscle bundles in both the subcutis and corium are adrenergic nerves that have been identified by fluorescence and electron microscopy (Holstein *et al.*, 1974). Accordingly, they have reported a lack of these adrenergic nerves accompanying the myofibroblasts or the single smooth muscle elements of the corium.

The dartos itself appears to be essential for temperature regulation of the testes. Shafik (1978) reported a case of absent dartos muscle in an infertile male underscoring the importance of dartos in male fertility. The available literature highlights the importance of maintaining a scrotal temperature below core temperature for optimal reproductive function, and the possible role played by the dartos for proper scrotal thermoregulation.

However, there is very limited knowledge about the basic contractile physiology of the dartos. Following is a summary of the knowledge available. The contraction of the dartos *in vitro* in response to EFS was found to be abolished by exposure to tetrodotoxin (sodium channel blocker in nerves), phentolamine (alpha adrenergic agonist) or guanethidine (neurotransmitter release blocker) but was unaffected by atropine (cholinergic blocker) or nitroarginine (nitric oxide synthase blocker). The contraction in response to exogenous noradrenaline was shown to be abolished by phentolamine and prazosin. These findings suggest that the dartos contracts responds to noradrenaline via alpha-receptors (Gibson *et al.*, 2002; Maloney *et al.*, 2005) and that there are no cholinergic receptors in this tissue.

The main aim of this chapter therefore was to investigate the contractile physiology of the dartos at its normal resting temperature of 33^{o}C.

2.2 Materials and Methods

2.2.1 Tissue preparation

Male Wistar rats weighing at least 400g (10 weeks or older) were sacrificed using intra-peritoneal injections of Lethabarb (sodium phentobarbitone), 160 mg/kg body weight. The glabrous section of the scrotal sac was removed along with the underlying dartos muscle immediately after death. The tissue was transferred to a dissecting dish and cut longitudinally into 2-3 mm wide strips of 10-15 mm length, while in Krebs-Ringer solution (NaCl 121 mM, KCl 5.4 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, $NaHCO_3$ 25 mM, HEPES 5 mM, glucose 11.5 mM, $CaCl_2$ 2.5 mM) and bubbled with carbogen (5% CO_2 :95% O_2). Krebs-Ringer solution was previously used in this laboratory (Maloney *et al.*, 2005) and HEPES was included to maintain pH after additionof drugs and other substancesto the bath solution. Four to six muscle strips were cut from a single scrotum. Experiments were performed on intact strips (muscle with overlying skin) and isolated muscle preparations (skin removed). When the muscle alone was used, the skin was separated from the muscle under a dissecting microscope.

2.2.2 Equipment and chemicals

The muscle strips (with or without the skin attached) were suspended in an organ chamber of 35 ml volume. The muscle was attached at one end to a tissue attachment post and the other end was attached to a force displacement transducer using size 3/0 black surgical silk (Figure 2.1). The organ chamber was filled with Krebs-Ringer solution and aerated with carbogen gas (5% CO_2:95% O_2). The chamber had a double walled water jacket connected to a re-circulating heated water bath (Haake DC 20) and the chamber temperature was monitored using a thermocouple probe (Analogue devices, AD592) calibrated against a certified mercury in glass thermometer.

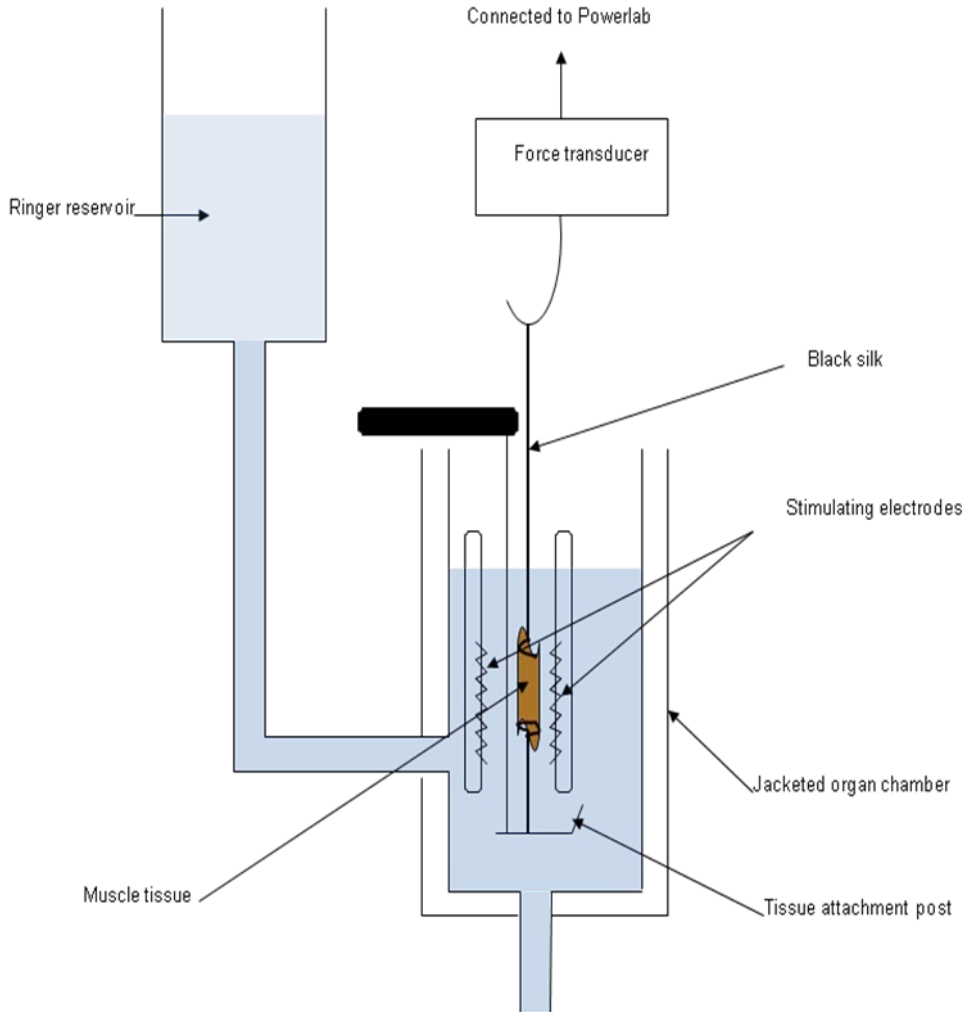


Figure 2.1: Schematic of the organ bath used for force measurements with the connections to the Powerlab and computer

The force transducer output was acquired using a PowerLab 4/20 data acquisition system (ADInstruments), connected to a personal computer. PowerLab Chart software was used for data display and analysis.

2.2.2.1 Calibration of instruments and equipment

The force displacement transducer was calibrated by recording the voltage response to known weights. The voltage output was then converted to tension in grams using a calibration formula.

The thermocouple probe was calibrated against a NATA certified mercury-in-glass thermometer (AS2831, AMA) by placing both thermometers in a circulating water bath and increasing temperature by 5°C increments from 10°C to 45°C.

2.2.2.2 Solutions and drugs

Krebs-Ringer solution was used as the bathing solution for the tissues and maintained at a pH range of 7.35 -7.45. All solutions had the pH correction done prior to experimentation. Calcium free Ringer solution was normally free of Ca^{2+} (i.e. no Ca^{2+} added to the solution) and EGTA was added for chelating Ca^{2+} ions.

Pharmacological agents such as noradrenaline, adrenaline, propranolol and ATP were dissolved in the Krebs-Ringer solution immediately prior to experiments. Ascorbic acid was added to the noradrenaline stock solution (50 μmol/l) to minimize oxidation. Non- water soluble chemicals were dissolved in DMSO (dimethyl sulphoxide). The pharmacological agents, when used, were added to the 35 ml organ bath (stated concentrations are concentrations in the 35 ml bath) and left there for 40 minutes for uniform diffusion before any further experiments were carried out. A detailed list of the drugs used, their suppliers and the solvents used are given in table 2.1.

Table 2.1: The drugs used for organ bath experiments with their suppliers and the solvents used

Name of drug	Solvent used	Supplier
Noradrenaline	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Adrenaline	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Propranolol	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
ATP	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Phentolamine hydrochloride	DMSO	Sigma Chemical Co., St Louis, MO, USA
Prazosin hydrochloride	DMSO	Sigma Chemical Co., St Louis, MO, USA
Guanethidine	Krebs-Ringer	Tokyo Chemical Industry Co. Ltd, Tokyo, Japan
2-APB	DMSO	Sigma Chemical Co., St Louis, MO, USA
Y-27632	DMSO	Sigma Chemical Co., St Louis, MO, USA
Suramin	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Chlorpheniramine maleate	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Cimetidine	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Serotonin	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Caffeine	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Tetrodotoxin	Krebs-Ringer	Sigma Chemical Co., St Louis, MO, USA
Menthol	DMSO	Sigma Chemical Co., St Louis, MO, USA

2.2.3 Experimental procedure

All experiments were carried out at a bath temperature of 33°C unless the effect of temperature was being investigated.

2.2.3.1 Electrical field stimulation (EFS)

The isolated dartos tissue preparations were stimulated using an isolated pulse stimulator (model 2100, A-M systems) in series with a 250 Watt power amplifier (EP 500B, Ebony) connected to a pair of platinum stimulating electrodes. The stimulation consisted of a sequence of supra-maximal 0.5 ms pulses delivered as a 50 second train with a frequency of 50 Hz. The optimum frequency of stimulation was initially investigated by using a fixed pulse duration of 0.25 ms and gradually increasing the frequency of stimulation to obtain the frequency that produced peak force. Similarly, thereafter the frequency thus obtained (50 Hz) was used and the pulse duration was gradually increased from 0.1 ms to 1 ms, to obtain the pulse duration that produced peak force.

2.2.3.2 Pharmacological stimulation

The response of the muscle preparation (with and without the skin) to noradrenaline (NA) was determined by measuring the contractile response to bath concentrations of NA ranging from 10^{-8} M to 10^{-3} M (Sigma Chemical Co., St Louis, MO, USA), at bath temperature of 33°C.

2.2.4 Data analysis and statistics

All muscle contractions were measured as change in tension in grams. Data were normalized by expressing tension as a percentage of the response to maximum EFS measured at 33°C at the optimal length, unless stated otherwise. Normalization of the raw data was performed to eliminate differences in force that could be produced by differences in size of the individual muscle strips.

In dose response measurements, contraction-response curves were performed by sequentially increasing the concentration in the organ bath and not by cumulative increase.

Sigmoidal dose response curves were fitted to data from each tissue using Instat (Graphpad), and the slope and EC_{50} values found in each group were compared statistically. When the comparisons of values were between two groups, Student t tests were performed. In the case of comparisons of values between 3 groups; a two-way ANOVA was used with main effects of tissue type and repeated measures of temperature. When the ANOVA indicated significant main effects and interactions, individual means were compared with a Students Newman Keuls post hoc test.

For display, sigmoidal dose response curves were fitted to the mean data to obtain a single slope and EC_{50} value using Prism software (GraphPad). All results involving continuous variables are expressed as mean ± standard error of the mean.

2.3 Results:

2.3.1 Basic contractile physiology of the tunica dartos at 33°C

To optimize the conditions for electrical field stimulation of the tunica dartos, the relationship between both stimulus frequency and stimulus pulse duration on the muscle force output were examined. The optimal stimulus frequency for maximum force output in response to EFS in the tunica dartos muscle was found to be approximately 50Hz (Fig. 2.2 A). There was no difference between the lowest and the highest stimulus pulse durations tested (ANOVA p=0.4777), and so a duration of 0.5 ms (highest mean) was selected for the experiments in this study (Fig. 2.2 B).

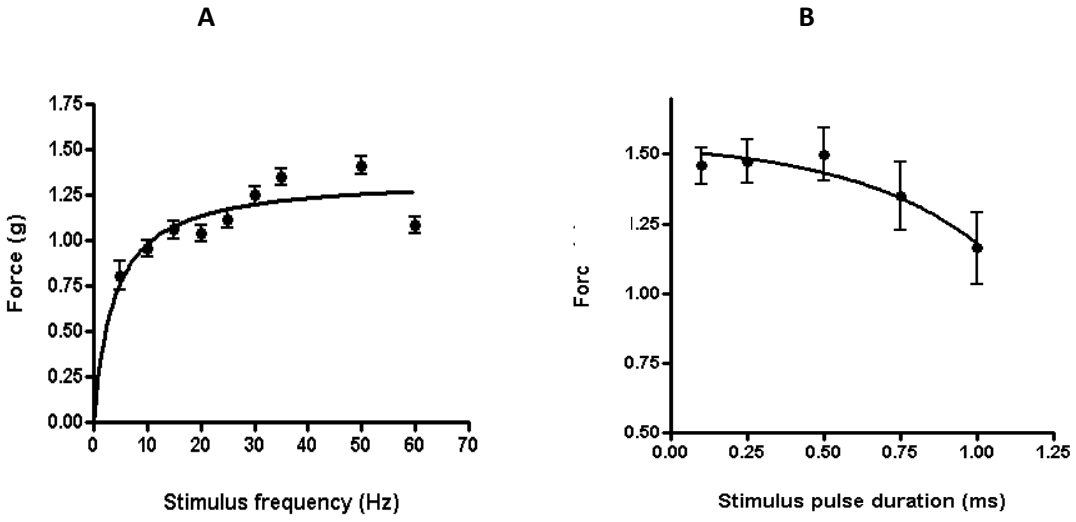


Figure 2.2: (A) The relationship between force output and stimulus frequency in isolated tunica dartos strips at a pulse duration of 0.5 ms. The optimum frequency was found to be 50 Hz. It should be noted that 10 Hz stimulation produced 50% of maximum force. (B) The force generation and the stimulus pulse duration in isolated tunica dartos strips. Points show mean ± SEM. Data obtained from at least 6 muscle strips from 3 different rats.

Typical force responses obtained following EFS and NA (10^{-4}M) are shown in Fig. 2.3. The response to the maximum stimulation with NA was 134% of the EFS response at 33°C (Fig.2.4).

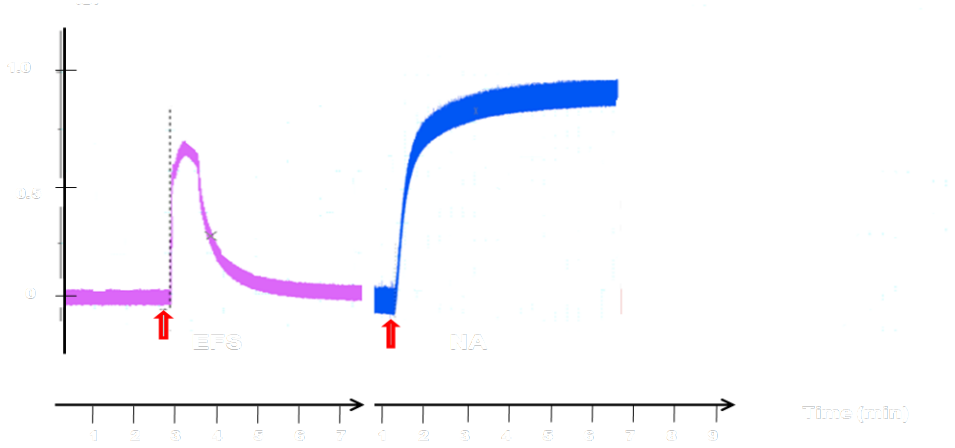


Figure 2.3: Representative force responses obtained from the same strip of muscle (skin attached) exposed to EFS and NA (10^{-4}M). The maximum response to NA stimulation was 133 ± 5.7 % of the maximum EFS response. The responses shown were performed on the same strip of dartos muscle 15 minutes apart. Red arrow indicates the application of the stimulus.

2.3.2 The contractile response to noradrenaline stimulation

Isolated dartos muscle strips maintained at 33°C responded with contraction to exogenous noradrenaline (NA) application. The dose response curve to noradrenaline was a typical sigmoid shape with a maximum response at a concentration of 10^{-4}M, and an EC_{50} of 5 ± 0.03 x 10^{-6}M. The slope was 1.02 ± 0.06. There was no significant difference between the response curves for the muscle alone and the muscle with skin attached (Fig.2.4).

The response to a maximum concentration of NA was significantly larger compared to the response to maximal electrical field stimulation (EFS) in the dartos muscle both with and without the overlying skin attached ($p<0.01$) (Fig.2.5).

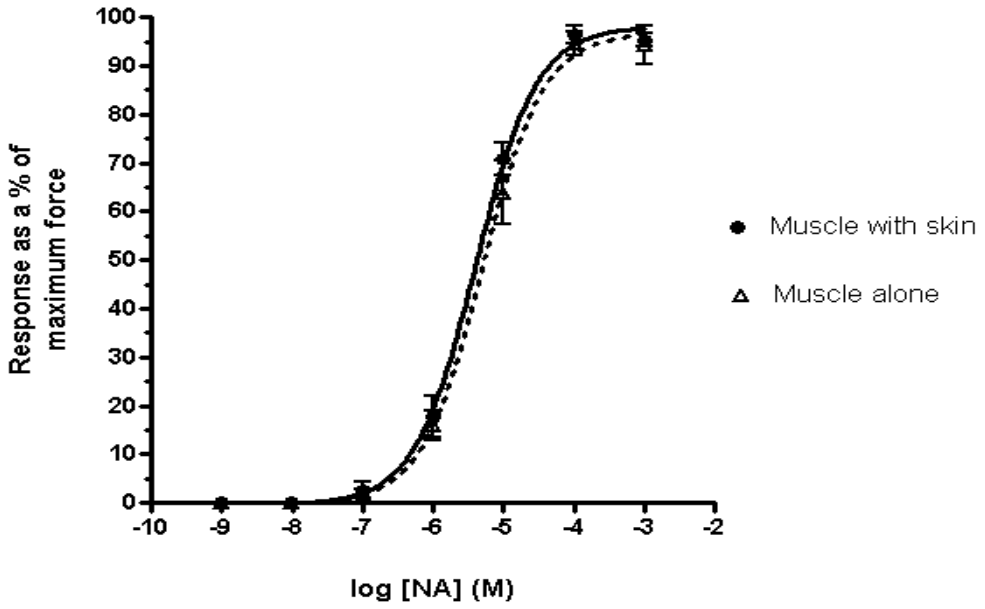


Figure 2.4: Dose-response curves for NA obtained in preparations with and without overlying skin attached. The hill slope was not significantly different for muscle with and without skin (p=0.952). Data obtained for at least 8 muscle strips from 4 rats. Points show mean ± SEM

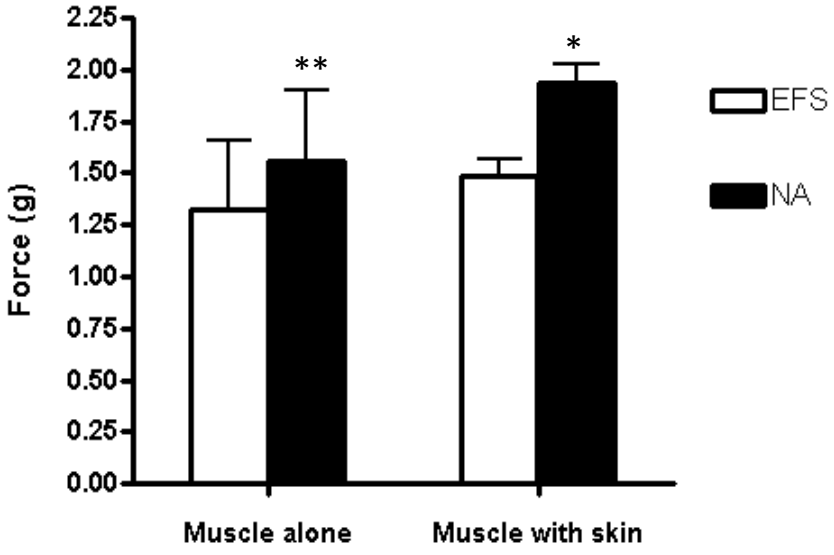


Figure 2.5: Contractile forces generated in response to maximal EFS and a maximal dose of NA (10^{-3} M) respectively in muscle alone (p=0.004) and muscle with skin attached (p=0.0001). There was no significant difference in the response between muscle alone and muscle with skin either with EFS or with NA. 25 strips from 8 rats for muscle alone and 77 strips from 27 rats for muscle with skin. Each bar shows mean ± SEM.

To confirm the type of adrenergic receptor involved in the NA induced force response of the tunica dartos, a non-specific alpha adrenergic receptor blocker was used at first with exogenous NA stimulation. At a temperature of 33^{o}C the contractile response to a maximum concentration of NA (10^{-4}M) was abolished by up to 80% by a concentration of 10^{-3}M phentolamine. Similarly, the alpha-1 specific adrenergic receptor blocker prazosin, abolished more than 90% of the contractile response to NA at a concentration of 10^{-4}M (Fig. 2.6 A and B).

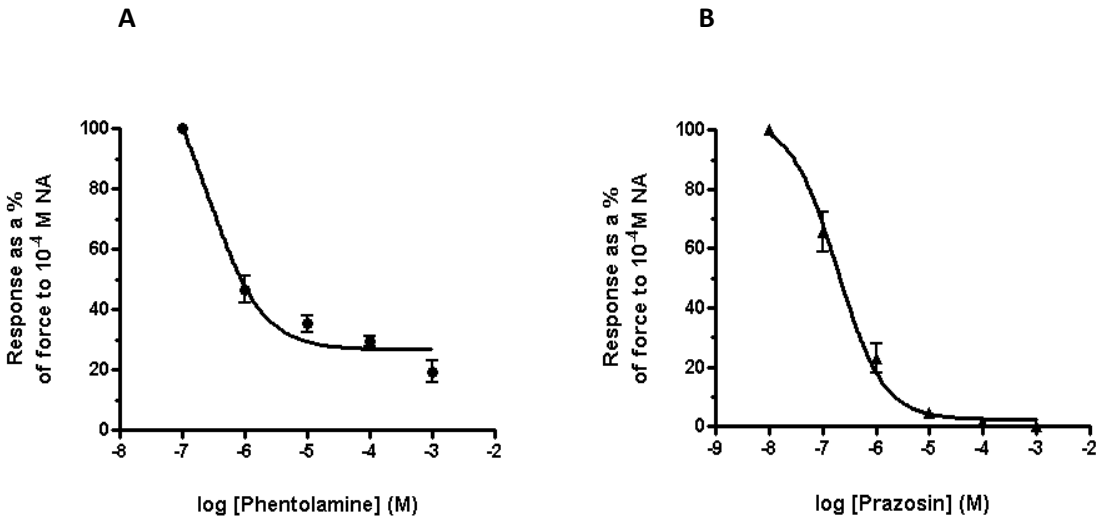


Figure 2.6: The dose-response curves for phentolamine (A) and prazosin (B) showing attenuation of the contractile response of the tunica dartos to NA (10^{4}M) with a series of concentrations. Response expressed as a % of response to the maximum dose of NA in the absence of any antagonist. From the fitted curve, the calculated EC_{50} of phentolamine block was 4.2 x 10^{-7} M and prazosin block was 1.8 x 10^{-7} M. Eight muscle strips from at least 3 rats used. Points indicate mean ± SEM.

These findings indicate that the NA acted predominantly on alpha-1 receptors on the tunica dartos muscle to produce contraction.

The catecholamine release blocker, guanethidine, reduced the EFS induced force response by 95% at a bath concentration of 100 µM. However, guanethidine did not affect the force response elicited in response to the application of exogenous NA (Fig. 2.7).

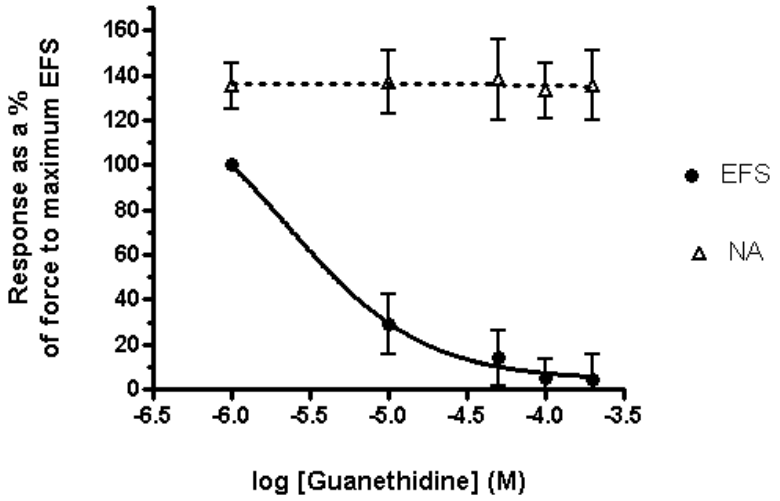


Figure 2.7: Force response to EFS and exogenous NA after adding Guanethidine to the organ bath. Nine muscle strips from 3 rats used. Each bar represents mean ± SEM.

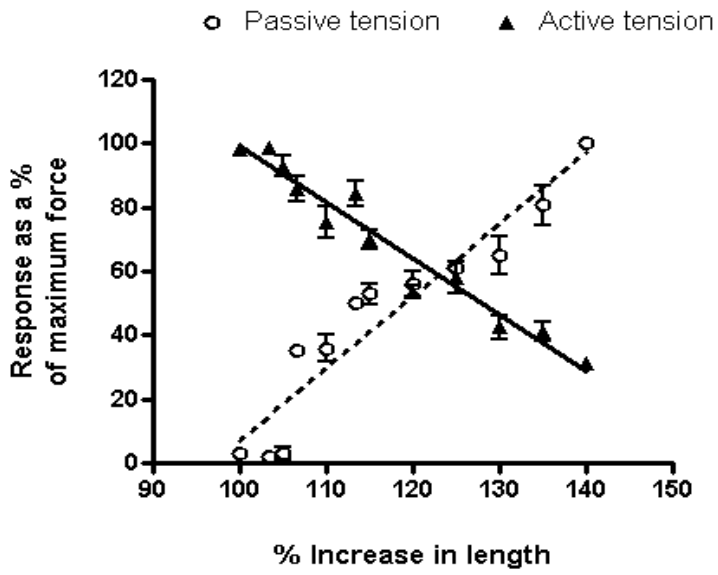
Therefore, it can be concluded that the tunica dartos responds to EFS mainly via release of catecholamines (NA) from sympathetic nerves.

2.3.3 The tunica dartos length tension relationship

In these experiments, the tunica dartos muscle strips were initially held at slack length with enough tension to straighten the muscle strip, but not stretch it (This tension was approximately 0.25 g). The relationship between muscle strip length and the tension generation was then studied by gradually increasing the length in defined increments and recording both the passive tension and the active tension generated in response to electrical field stimulation (EFS). The length of the muscle was converted to a percentage of its initial length; in order to pool data acquired using muscles with slightly different lengths. Tension generated was expressed as a percentage of the maximum tension for that particular muscle strip.

It was found that active tension steadily decreased as length increased, indicating that maximum force generation occurred when the muscle length was very close to its resting or slack length. Similar results were found in muscle strips with and without the skin attached (Fig. 2.8). Passive tension increased with increasing stretch, as expected.

A



B

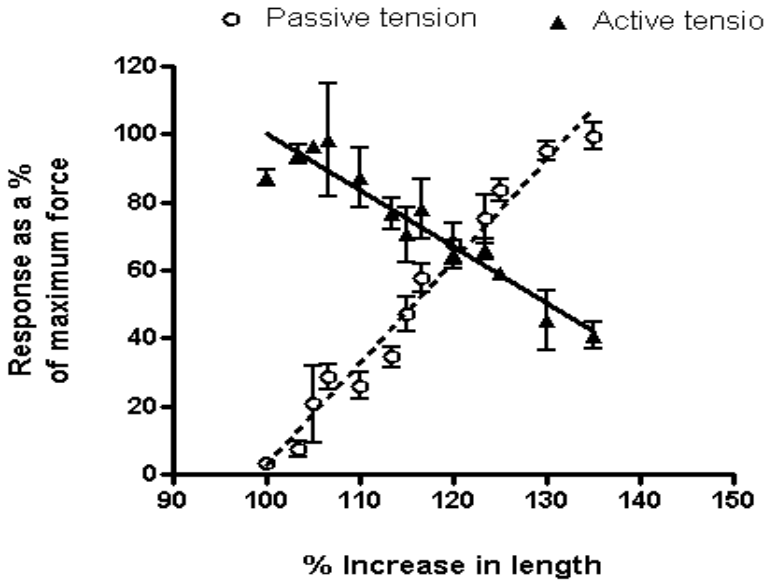


Figure 2.8: The relationship between the percentage change in length and passive and active tension of tunica dartos muscle alone (A) and muscle with the overlying skin attached (B) and the percentage increase in length of the muscle. Each point is the mean obtained from at least 6 muscle strips from 3 different rats. Points show mean ± SEM.

2.3.4 The response of the dartos to purinoceptor stimulation

The dartos responded to exogenous ATP application with a small and transient increase in tension (Fig. 2.9A). The response was approximately 25% and 28% of the response to EFS and a maximum concentration of NA respectively (Fig. 2.9B). There was a hyperbolic relationship between the force and the bath concentration of ATP. The maximum response occurred at a concentration of 1mM (10^{-3}M) , and a concentration of 0.1 mM (10^{-4}M) produced half of the maximal response (Fig.2.10).

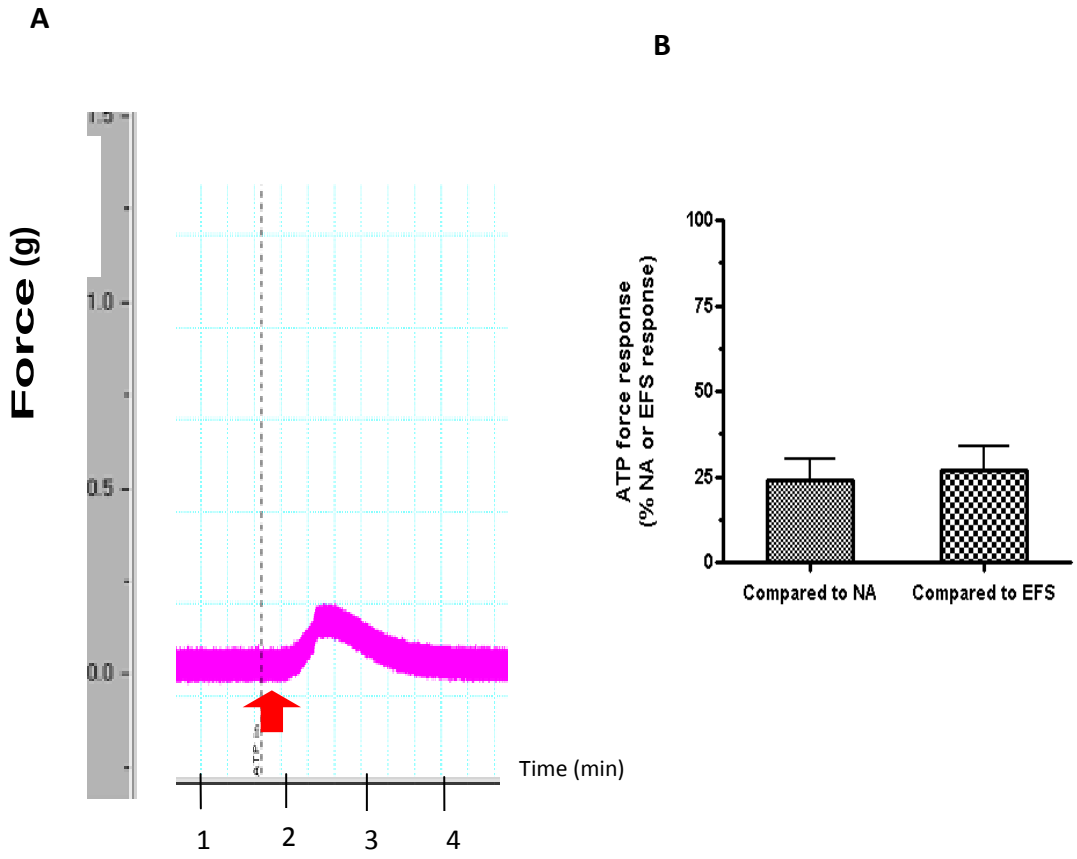


Figure 2.9: (A) An example of a force response in a tunica dartos muscle strip (skin attached) after exposure to ATP. Note the transient nature of the response in comparison to NA (Fig. 2.3). (B) The effect of ATP exposure on force as a percentage of the maximal NA and EFS-induced force responses in the same muscle strip. Each bar represents mean ± SEM. Data from 12 muscle strips from 3 rats.

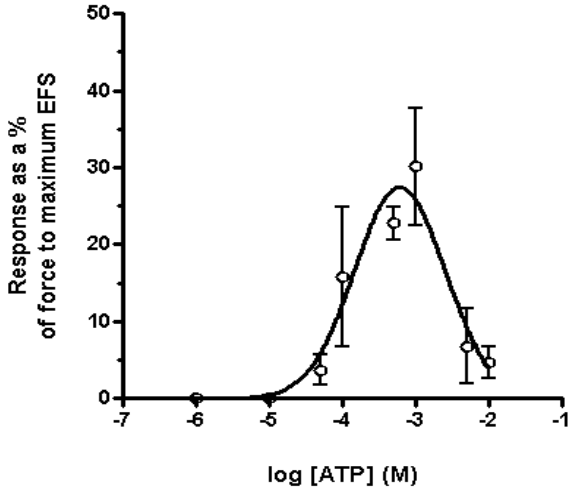


Figure 2.10: The dose response relationship of tunica dartos muscle (skin attached) to ATP. Points show mean ± SEM. Data obtained from 12 muscle strips from at least 3 rats.

2.3.5. Stimulus-contraction coupling of the tunica dartos

2.3.5.1 Extracellular calcium

Since a number of different pathways can link an extracellular stimulus to contraction in smooth muscle, experiments were conducted with EFS and NA stimulation in the absence of extra-cellular calcium to provide information about the relative importance of intra-cellular and extra-cellular sources of calcium for the contraction of the tunica dartos. As it is very difficult decrease the extracellular Ca^{2+} concentrationto very low levels in tissue preparations such as the dartos,due to extracellular Ca^{2+} binding and other effects. In this study, the EGTA solution was expected to significantly reduce the free calcium in the dartos preparation, rather than eliminate it altogether.

Stimulation of the dartos with EFS in Ca^{2+} free Ringers solution produced a contraction that was 83% ($p = 0.007$) of the force response in normal Ringers solution. Stimulation with 10^{-4} M NA, in Ca^{2+} free Ringer led to a contraction that was 68% of the NA induced contraction elicited in normal Ringer ($p < 0.0001$). Removing Ca^{2+} from the extracellular fluid, therefore, had a larger effect on the response to NA than to EFS (* $p = 0.004$) (Fig. 2.11).

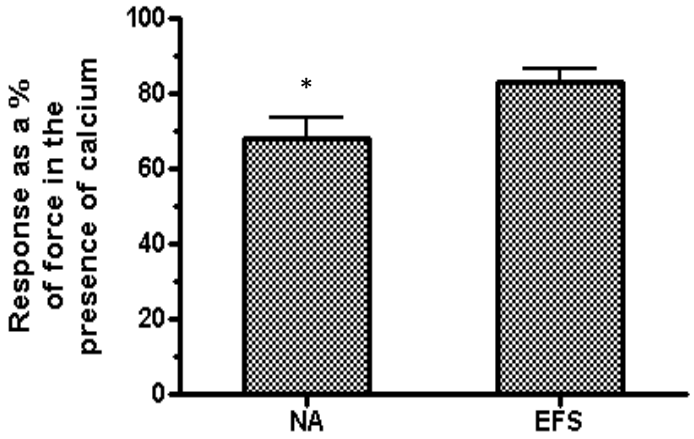


Figure 2.11: The force generated by tunica dartos in response to a maximum dose of NA and EFS in the absence of extracellular calcium in the medium, expressed as a % of the force generated in normal Ringer. The difference between the NA and EFS response was significant with a p=0.0038 and the reduction of force in the absence of calcium was also significant in the case of EFS (p=0.007) and NA (p<0.0001). 12 muscle strips from at least 3 rats used. Each bar represents mean ± SEM.

2.3.5.2 Intracellular store release of calcium

Since the tunica dartos response to NA was found to be by stimulation of alpha-1 adrenergic receptors, it was assumed that the main source of intracellular Ca^{2+} for contraction of the dartos muscle was from the intracellular stores. NA is known to cause Ca^{2+} release via the phospholipase C/IP_3 pathway. In this pathway, inositol

triphosphate (IP_3) diffuses to the sarcoplasmic reticulum and induces the release of calcium from the sarcoplasmic reticulum via stimulation of IP_3 receptors. 2-APB (2-Aminoethoxydiphenyl borate) is a widely used IP_3 receptor blocker that inhibits the IP_3-induced release of Ca^{2+} from the sarcoplasmic reticulum. Therefore, 2-APB was used in the bath at varying concentrations during EFS and NA stimulation of the tunica dartos to determine whether the main source of intracellular Ca^{2+} release was via sarcoplasmic IP_3 receptors.

The tunica dartos responded with a reduction in the force response in a concentration dependent nature after addition of 2-APB into the bath. At a bath concentration of 50µM, there was no response to either EFS or NA stimulation (NA results shown in Fig. 2.12).

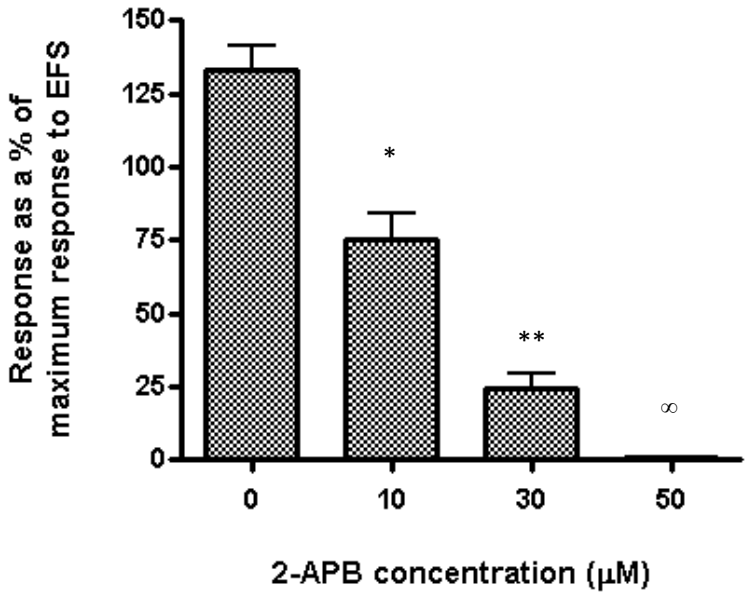


Figure 2.12: The response of the tunica dartos muscle to NA (10^{-4}M) in the presence of varying concentrations of the blocker 2-APB (expressed as a % of EFS response). There was significant inhibition of the NA induced contractile response at 10 µM ($p = 0.03$) and 30 µM ($p = 0.021$) . The NA induced contractile response was totally abolished by a 2-APB concentration of 50 µM ($p = 0.0045$). Bars represent mean ± SEM. 13 muscle strips from 5 rats used.

This experiment confirmed that the tunica dartos responds to NA stimulation (released by sympathetic nerve terminals or added exogenously) mainly by inducing Ca^{2+} release from the sarcoplasmic reticulum via IP_3 receptors.

2.3.5.3 Calcium sensitization in the dartos

In many smooth muscle types, Rho kinase activates pathways resulting in the inhibition of myosin phosphatase which can lead to Ca^{2+} independent enhancement in force production. The specific Rho kinase inhibitor, Y-27632, was used to determine whether this pathway was operating in the dartos.

The Y-27632 compound alone at a bath concentration of 10μM did not cause any change in the tension of the tunica dartos when used at slack length at a temperature of 33^{o}C. This was probably because the tunica dartos muscle did not have a resting tension at this temperature in the absence of an agonist.

The effect of Y-27632 on NA induced tension was examined and the force response of tunica dartos to exogenous NA (10^{-4}M) in the presence of varying concentrations of Y-27632 in the bath is shown in Fig. 2.13.

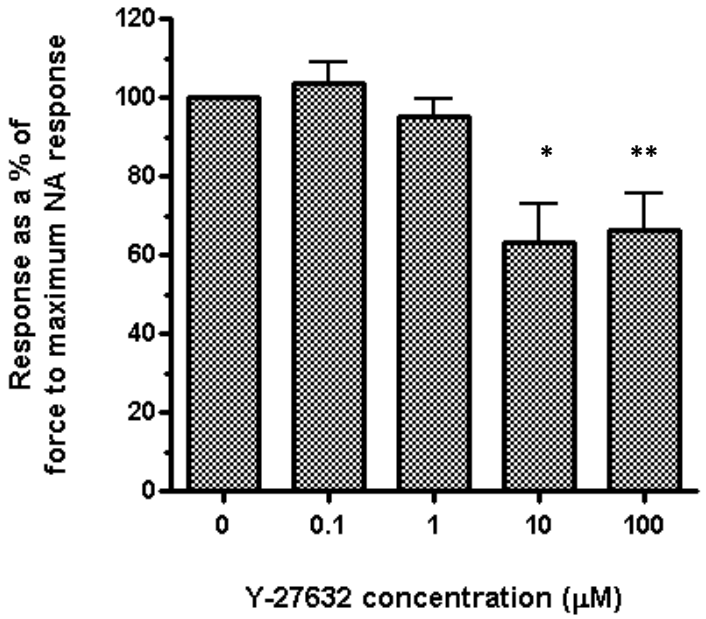


Figure 2.13: Contractile response of the tunica dartos muscle in the presence of varying concentrations of Y-27632 to NA 10^{-4}M. The contractile response to NA was significantly inhibited at Y-27632 concentrations of 10 µM ($p = 0.02$) and 100 µM ($p = 0.042$). Response in the absence of the inhibitor is also given for comparison on the left extreme. Each bar represents mean ± SEM. 6 muscle strips from 3 rats used.

At bath concentration of 0.1 µM, and 1 µM the Y-27632 compound the tunica dartos did not produce a significant change in the force response to NA. At a concentration of 10 µM and above of Y-27632, the NA induced force production was reduced to approximately 63% of the force generated in the absence of Y-27632 ($p=0.043$).

Overall, these results suggested that NA induced force production also had a component of calcium sensitization in addition to the main force being produced by IP_3 induced calcium release from the sarcoplasmic reticulum.

2.3.5.4 The presence of ryanodine receptors

Although the previous experiments have suggested that the NA induced contraction of the tunica dartos occurs via activation of the phospholipase C/IP_3 pathway, it was of interest to rule out the possibility that the dartos also had ryanodine receptors on its sarcoplasmic reticulum, and these could play a small role in dartos activation. Although rare in smooth muscle, ryanodine receptors can allow calcium release when stimulated.

These receptors can be activated by exposure of the tissue to caffeine at high concentrations. Caffeine (Sigma Chemical Co., St Louis, MO, USA) was applied at a bath concentration of 50 mM at a temperature of 33^{o}C and did not significantly affect the resting tension of the dartos strips (Fig. 2.14).

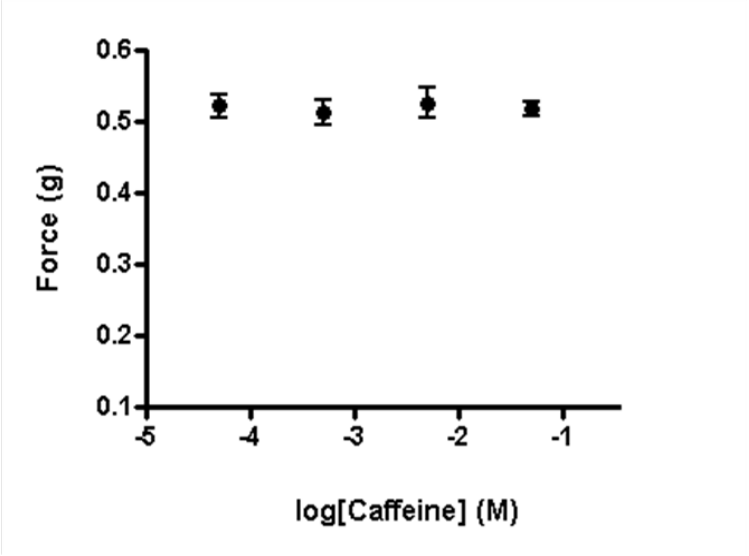


Figure 2.14: Force produced by tunica dartos muscle strips with the skin attached in the presence of varying concentrations of caffeine. There was no change in the tension generated with addition of caffeine to the organ bath. Each point indicates mean ± SEM.

The caffeine dose which opens ryanodine receptors would have non-specific effects on the cells. Therefore, the result of this experiment does not necessarily rule out the possibility of having ryanodine receptors.

2.4 Discussion:

The contractile physiology of the tunica dartos was studied at 33^{o}C using tissues from the Wistar rat in an organ bath set up at physiological conditions. Isolated tunica dartos muscle contracted in response to electrical field stimulation (EFS), exogenous noradrenaline (NA) and ATP. The response to EFS and NA were blocked by adrenergic blockers phentolamine and prazosin indicating that the response was mediated through alpha receptors. Guanethidine inhibited the response to EFS suggesting that the contractile response was mediated through release of catecholamines from the sympathetic nerve endings. This sympathetically mediated contractile response was almost totally abolished by 2-APB which is an indication that the NA acted through synthesis of IP_3 via breakdown of phosphatidylinositol bisphosphate through the phospholipase C / IP_3 pathway. Therefore, it can be assumed that the NA stimulation caused intracellular store release of Ca^{2+}. However, as there was a thirty five percent reduction in this contractile response by the use of Y-27632 (the Rho kinase inhibitor) it can be assumed that there is a component of Ca^{2+} sensitization in the contractile response.

The experiment designed to reduce the extracellular a^{2+} free and thereby stop extracellular Ca^{2+} entry, did not succeed entirely as it is very difficult to make a Ca^{2+} free environment. The attempt at removal of extracellular Ca^{2+} decreased the contractile response to both EFS and NA by about 20%. However, when this result is considered in the light of the fact that 80% of the EFS response remained, it is suggestive that the medium is not entirely Ca^{2+} free. This is because the contractile response to EFS is activated by neurotransmitter release from the motor nerves and extracellular calcium is required for neurotransmitter release. If a total absence of extracellular calcium was achieved, the response to EFS should have been heavily attenuated. Therefore, the findings of calcium free experiments fail to provide firm conclusions regarding the

degree of calcium entry from the extracellular space that occurs during activation in the dartos..

Excitation-contraction coupling in smooth muscle is said to occur by two overlapping mechanisms; electromechanical coupling and pharmacomechanical coupling. Electromechanical coupling occurs when voltage operated calcium channels (VOCC) open following membrane depolarization. Agents which block Ca^{2+} entry though VOCC such as nifedipine will block the process of electromechanical coupling. In smooth muscles that exhibit pharmacomechanical coupling, the rise in intracellular Ca^{2+} is achieved by a combination of store release of Ca^{2+} and Ca^{2+} entry through non-voltage operated channels such as receptor operated calcium channels (ROCC) or store operated calcium channels (SOCC). Generally, the calcium signal that is observed in pharmacomechanical coupling is an initial rapid rise, followed by a smaller, but sustained increase of Ca^{2+}. The latter is related to the Ca^{2+} sensitization process which is an energy saving adaptation in smooth muscle where a greater force is developed at the same low concentration of Ca^{2+}. Electromechanical and pharmacomechanical coupling co-exists in smooth muscles and the relative importance of each mechanism varies depending on what smooth muscle it is. Electromechanical coupling is more predominant in phasic smooth muscles such as those found in the gut (Milanov *et al.*, 1984) where as pharmacomechanical coupling is predominant in tonic smooth muscles such as those found in blood vessels and bronchi (McFadzean and Gibson, 2002).

Investigation into the mechanism of Ca^{2+} entry into dartos cells has not been studied in the present experiments. Further experimentation is needed to differentiate whether it is VOCC, ROCC, SOCC or a combination of those three mechanisms which occur in the dartos muscle. The use of VOCC blockers such as nifedipine would eliminate the calcium entry through voltage operated channels. This has been utilized by other investigators in the mouse anococcygeous (Gibson *et al.*, 1994), gastric fundus of

several species (Petkov and Boev, 1996a; Petkov and Boev, 1996b). Store-operated Ca^{2+} channels open due to depletion of sarcoplasmic reticular calcium store. These can be activated by the use of sarco-endoplasmic reticular Ca^{2+}-ATPase (SERCA) pump blockers like thapsigargin and cyclopiazonic acid. These drugs have been proved very valuable in tissues such as rat aorta (Cohen *et al.*, 1999), mouse anococcygeus (Gibson *et al.*, 1994), rat carotid (Sekiguchi *et al.*, 1996).

It can be concluded from the experimental findings that the dartos exhibits pharmacomechanical coupling typical of tonic smooth muscle as well as some form of electromechanical coupling. Its contractions are induced by noradrenaline via alpha-1 receptors, with intracellular Ca^{2+} release from sarcoplasmic reticulum by stimulation of IP_3 receptors as well as calcium entry by some unknown mechanism from the extracellular space (e.g. through ROCCs or SOCCs). Pharmacomechanical coupling is to be expected in a tonic smooth muscle which maintains contraction for extended periods of time (Somlyo *et al.*, 1999) as would be the case in the dartos in cold environments. The response to exogenous noradrenaline was found to be 134% of the response to EFS in the dartos. The contractile response of the dartos to EFS is brought about by release of NA (with or without other catecholamines) from sympathetic nerve endings. This was confirmed by the finding that guanethidine (a catecholamine release blocker) abolished the response to EFS. The response to NA was blocked by both the non-specific alpha blocker, phentolamine, and specific alpha-1 blocker, prazosin, confirming that NA acted on alpha-1 receptors on the dartos muscle. The maximum response to NA was significantly higher than the maximum response to EFS probably because the concentration of NA released from sympathetic vesicles was lower than the maximum concentration of NA used exogenously (10^{-4}M) in these experiments. Note that at high concentrations, prazosin may havee non-selectively actions on other receptors in addition to alpha-1 receptors.

2-APB at concentrations above 10 µm abolished the contraction induced by a maximum concentration of NA. 2-APB is a reliable and widely used IP_3 receptor blocker that inhibits Ca^{2+} release from the sarcoplasmic reticulum. Inhibition of contractile response with the use of 2-APB suggests that NA induced contraction is mostly by release of intracellular Ca^{2+} from the sarcoplasmic reticulum.

However, extracellular Ca^{2+} also seems to play a role because the removal of ECF Ca^{2+} using Ringer without Ca^{2+} reduced the contractile force to both EFS and exogenous NA. Since 50 µM of 2-APB totally abolished the force produced by NA, all Ca^{2+} release could be taken as being through via IP_3 receptor stimulation. If that is the case, then why should the removal of extracellular Ca^{2+} reduce the EFS and NA response significantly? The answer may be that there is a Ca^{2+} influx through SOCC. If this hypothesis is correct, then blocking IP_3 receptors with 2-APB will not only inhibit contraction, but because there is no decrease in Ca^{2+} concentration in the sarcoplasmic reticulum the SOCC will also not occur. It seems logical that normally when SOCC is stimulated, that some Ca^{2+} entering the cell will contribute directly to contractility. Therefore, the removal of extracellular Ca^{2+} would still reduce contractility.

Moreover, SOCC is reported to be enhanced at low concentrations (1 – 5 µM) of 2-APB and inhibited at high concentrations (≥ 10 µM) (Diver *et al.*, 2001; Prakriya and Lewis, 2001; Peppiatt *et al.*, 2003). Since the concentration used in these experiments were above 10 µM it is likely to have blocked the store mediated calcium entry in addition to IP_3 receptors. However, the presence of ryanodine receptors could not be entirely ruled out. Since the concentration of caffeine that activates ryanodine receptors is very high, and at this concentration there are non-specific effects on smooth muscle contraction the non-response to caffeine does not entirely rule out the absence of ryanodine receptors. Therefore, it is not definite whether there is concomitant activation

of ryanodine recpetors together with IP_3 receptors in the contraction of tunica dartos muscle.

Rho kinase activation in smooth muscle causes an inhibition of myosin phosphatase, reducing the dephosphorylation of myosin light chains and therefore, Ca^{2+} sensitization. Use of the Rho kinase inhibitor (Y-27632) while inducing contraction with NA, caused a reduction of force up to 35% indicating that the NA induced contractile response has a Ca^{2+} sensitization component of 35 %.

The length-tension experiments of the tunica dartos muscle strips with and without the overlying skin proved very difficult to perform as the muscle produced maximum tension at slack length (with a tension of 0.25g). It was a tedious task to have the muscle at slack length to record a maximum force without making the muscle so slack that the force transducer would not detect small changes in force generated. However, the length tension curves may not tell a complete story as there is some evidence that the contractile apparatus of smooth muscles undergoes structural reorganization in response to stretch. For example if a smooth muscle is stretched for a long period of time, it reconstructs its contractile machinery so that the new length is its optimal length for the generation of tension, similar to that reported in airway smooth muscle (Gunst and Wu, 2001). This may be occurring in the tunica dartos muscle, but was not a focus of the present studies.

It is possible that ATP is released from the sympathetic nerve endings as a co-transmitter with NA as has been reported for some other tissues, such as the rodent vas deferens (Westfall *et al.*, 2002).The weak response to ATP could indicate low receptor numbers or insensitive intracellular signalling pathways. The complexity of the stimulus frequency verses force graph may also be due to co-transmission occurring (with ATP and noradrenaline) at the nerve endings. Therefore, it could be further speculated that ATP is actually a co-transmitter at the sympathetic nerves of the dartos.

The experiments outlined in this chapter were undertaken to broaden the understanding of the contractile physiology of the tunica dartos as a smooth muscle. The dartos was found to be a muscle with mainlypharmaco-mechanical coupling with IP_3 activated release of Ca^{2+} from the sarcoplasmic reticulum and store mediated Ca^{2+} entry from the ECF. The dartos has a sympathetic innervation with NA being the neurotransmitter, possibly with ATP as a co-transmitter. The NA acted by binding alpha-1 adrenoceptors, producing release of Ca^{2+} from the intracellular stores via IP_3 receptors. In addition some amount of electromechanical coupling may also be taking place.

CHAPTER 3

Cooling induced contraction of the tunica dartos and an investigation into the mechanism responsible

3.1 Introduction:

It is known that the tunica dartos muscle receives sympathetic innervation from the lumbo-sacral spinal region (Pacheco *et al.*, 1997). From the findings in the previous chapter and from published literature (Maloney *et al.*, 2005; Gibson *et al.*, 2002) it is also known that the dartos muscle responds to EFS and NA through stimulation of alpha-1 receptors.

It has also been reported that isolated strips of the tunica dartos muscle from the rat scrotum contract in response to cooling independently of any neural connections (Maloney *et al.*, 2005) and that the contractile force was greater in the presence of the overlying skin. At this point a question arises whether there is some factor released from the skin that acts on the muscle in a paracrine fashion to potentiate the contractile response of the dartos muscle to a lowering of temperature.

Where epithelia lie close to smooth muscles in other areas of the body, there are paracrine substances released from certain cells in the epithelial layer that can influence the function of the underlying smooth muscle. In the walls of the airways for example, histamine released from the bronchial epithelial cells acts on the smooth muscles of the airway to cause bronchoconstriction (Chand and Deroth, 1978). Interleukins, also produced by airway epithelial cells, cause contraction of bronchial smooth muscles and are known to contribute to bronchial hyperresponsiveness in asthma (Amrani and Panettieri, 2002; Rizzo *et al.*, 2002). Similarly, the gut epithelial layer secretes substances like 5-HT (serotonin), neuropeptide-Y and adenosine, which act on the

underlying smooth muscles of the gut wall causing changes in their contractile status (Murthy, 2006). In blood vessels, endothelial cells release endothelin which causes blood vessel constriction (Lovenberg and Miller, 1990) and nitric oxide, which causes blood vessel relaxation (Bredt and Snyder, 1994) due to effects on the underlying smooth muscle. Hence it is possible that substances such as histamine, 5-HT (serotonin), prostaglandins, interleukins, or nitric oxide secreted by the skin may cause contraction of the underlying dartos muscle. Finding the substance/s responsible for inducing the contraction of the tunica dartos during cooling can only be done by using agonists and antagonists of the possible candidate paracrine agents. A comprehensive list of candidate paracrine substances is extensive and testing them all is beyond the scope of this project. Therefore, this study focused on the most likely candidate substances as described to investigate the mechanism involved in cooling induced contraction of the dartos.

Whatever the extracellular mediator ultimately responsible for the cooling induced contraction in dartos, the intracellular mechanisms that bring about cooling induced contraction would likely involve one or several of the mechanisms that commonly elevate intracellular Ca^{2+}. One of the mechanisms of elevation of Ca^{2+} is by activation of IP_3 receptors via phospholipase C pathway (shown to be the main mechanism in the case of NA stimulation; chapter 2). The other possible mechanisms of elevation of intracellular are influx of extracellular calcium through voltage operated, store operated or receptor operated channels and release of intracellular calcium via activation of ryanodine receptors.

An increase in calcium sensitivity could also contribute to cooling induced contraction, with or without an increase in the available calcium. A potential mechanism for a change in calcium sensitivity is the inhibition of myosin phosphatase, because there is evidence that the activity of this enzyme is temperature sensitive (Mitsui *et al.*,

1994). Gibson *et al*. (2002) showed that the contractile response of the isolated dartos to both field stimulation and noradrenaline was lower at 40^{o}C than it was at the more physiological temperature of 30^{o}C. The heating induced relaxation was attenuated by the addition of Calyculin-A (a myosin phosphatase inhibitor), suggesting that myosin phosphatase plays a role in temperature induced relaxation of the dartos muscle. Gibson *et al.* (2002) concluded therefore, that the relaxation of the tunica dartos in response to elevated temperature was due to increased activation of myosin phosphatase at elevated temperatures. This hypothesis could also explain the cooling induced contraction of the dartos, as the inhibition of myosin phosphatase at lower temperatures would produce a contraction by increasing the proportion of phosphorylated myosin light chains. This could be tested by using a myosin phosphatase inhibitor while exposing the muscle to a reduction in temperature.

Therefore, the main aim of the experiments outlined in this chapter was to characterize the response to cooling of the tunica dartos muscle and to investigate the mechanisms responsible.

3.2 Materials and Method

3.2.1 Tissue preparation and equipment

The preparation of the rat dartos muscle for the experimental procedures and the experimental set up used in these experiments are similar to those outlined in sections 2.2.1 and 2.2.2.

3.2.2 Experimental procedure

The protocols for the experiments described in this chapter were standardized as follows: The bath was initially set at 33^{o}C (the normal skin temperature of the rat scrotum) and an EFS was performed for standardization and, the compound of interest was added to the organ bath and left to equilibrate at 33°C for 40 minutes. Only one compound was tested on each muscle strip in order to prevent the effects of any tightly bound, non-washable remains of the compound on subsequent experiments. All responses were normalized to the peak of the initial EFS responses of that particular muscle strip, unless mentioned otherwise. The bath was then equilibrated at temperatures of 15°C and 40°C, respectively, and the tension generated was compared to the tension at 33°C. A comparison was made between the cooling response of the muscle with and without the skin using different sample strips for each preparation. The process of decreasing the organ bath temperature to 15^{o}C took approximately 5-7 minutes. Similarly, re-warming the bath to 33^{o}C took another 5-7 minutes. Experiments performed at 15^{o}C or any other temperature lower than 33^{o}C, took approximately 15-20 minutes.

To remove the influence of temperature variation on expansion/contraction of the muscle attachment post, and other such metal equipment, dead pieces of muscle were used as controls. Black silk alone was also suspended between the muscle

attachment post and the force transducer to test for an effect of tension changes in the black silk. The basic characterization of the temperature response of the tunica dartos was first determined. Thereafter, a possible mechanism for cooling induced contraction was studied.

3.3 Results:

3.3.1 Characterization of the temperature response of the dartos

The isolated dartos muscle responded to cooling. To compare the tensions generated by cooling with other ways of exciting the muscle (EFS, NA) the same muscle was subjected in random order to EFS, NA or cooling. The magnitude of the responses to each of these stimuli by a single muscle strip with the overlying skin is shown in Fig. 3.1. The maximum response to NA stimulation was 133 ± 5.7 % of the maximum EFS response and the response to cooling was 136 ± 8.4 % of the maximum EFS response.

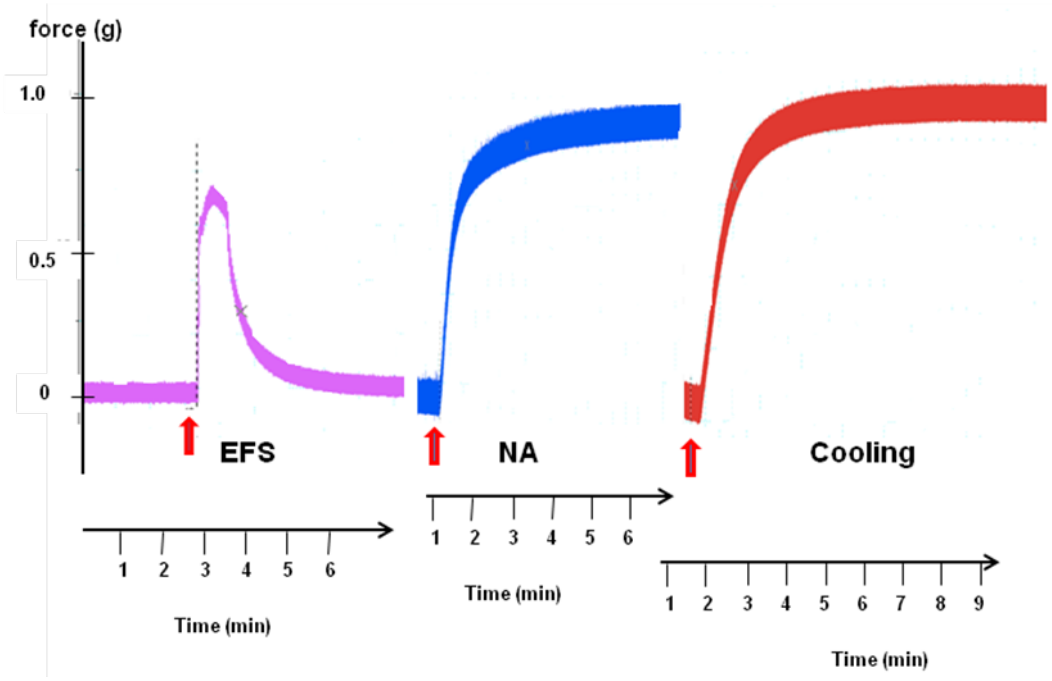


Figure 3.1: Representative force responses obtained from the same strip of muscle (skin attached) exposed to EFS, NA stimulation and cold stimulation. The onset of the stimulus in each case is marked with the red arrow.

Previous investigations have reported that the cooling induced contraction of the dartos is about four times higher in magnitude when the overlying skin remains connected to the muscle, than if the skin is removed (Maloney *et al.*, 2005). This was repeated to confirm these findings. Tunica dartos muscle preparations consisting of muscle with overlying skin attached or muscle alone were cooled from 33^{o}C to 15^{o}C and the resulting force response measured. When the skin was present, cooling to 15^{o}C induced a contraction that was 136 % of the response to EFS at 33^{o}C (Fig. 3.2A). When the muscle alone was cooled the contractile response was only 35 % of the EFS response at 33°C (Fig. 3.2A). Exposure to temperatures above 33°C produced a relaxation which again was greater when the skin was attached (Fig. 3.2B). In summary, the response to both cooling and warming was larger when the overlying skin was present compared to that of muscle alone.

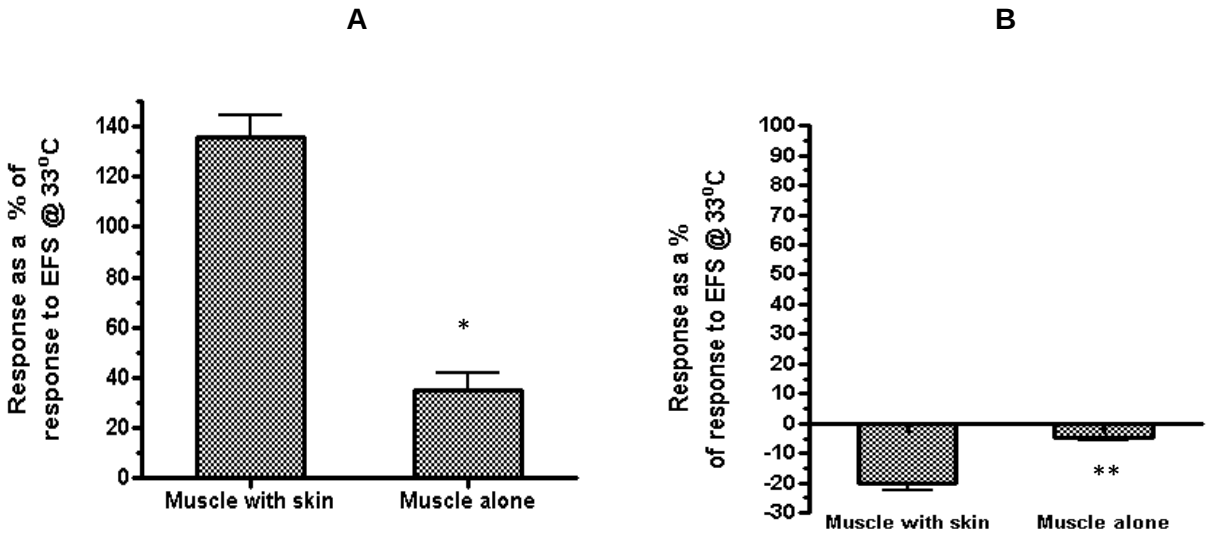


Figure 3.2: Comparison of force generated by cooling from 33^{o}C to 15^{o}C (A) and warming from 33^{o}C to 40^{o}C (B) of the muscle with the skin and the muscle alone, expressed as a percentage of response to EFS. The differences were statistically significant (*p = 0.0001 & **p = 0. 0081). The bars indicate the mean ± SEM of 24 strips from 18 rats for cooling and 18 strip from 6 rats for warming.

Since the response to cooling was more pronounced than the response to warming, and the heating effect has already been examined in some detail (Gibson *et al.*, 2002), the cooling response was examined in greater detail in the present experiments.

To determine the importance of extracellular Ca^{2+} for the cooling induced contractile response of tunica dartos, the muscle preparations were cooled in the absence of extracellular Ca^{2+} (in Krebs Ringer solution containing no Ca^{2+} with added EGTA). Removing Ca^{2+} from the bathing medium reduced the contractile response to cooling by 22% ($p = 0.038$) compared to the cooling response in normal Ringers solution in a preparation with the overlying skin. However, it is notoriously difficult to make the medium entirely calcium free, especially when it is a preparation like the dartos muscle with the overlying skin. Therefore, it can only be said that Ca^{2+} influx from the extracellular space contributes to a proportion of the cooling induced force response in the dartos (Fig. 3.3) using these findings. What exact proportion it is, cannot be derived by this experiment.

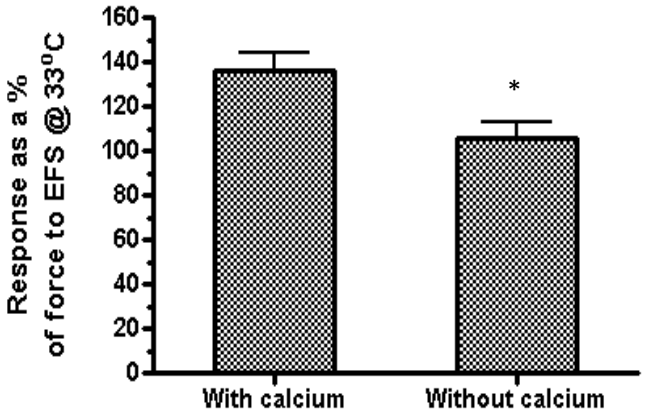


Figure 3.3: The response of the tunica dartos to cooling in calcium free medium and cooling in normal Ringer medium. Force is expressed as a % of force developed at 33°C to EFS. Each bar represents mean ± SEM for at least 9 muscle strips from each of 4 rats. The difference was statistically significant with the response in calcium free Ringer being 78% of the response in normal Ringer (* $p = 0.038$).

The temperature induced changes in dartos contraction that have been reported to date (Gibson *et al.*, 2002; Maloney *et al.*, 2005) have utilized acute temperature exposures. I investigated the effect of persistent and prolonged cooling on the tunica dartos by cooling the tissue from a temperature of 33^{o}C to 15^{o}C and maintaining it at this temperature.

When the tissue was cooled from 33°C to 15^{o}C and maintained at 15^{o}C for 60 to 90 minutes, the tension gradually declined to a similar tension to that observed at 33°C (Fig.3.4). The mean time taken to reach the baseline was 68.6 ± 3.6 min for muscle with skin, and 80.4 ± 8 min for muscle alone (difference not significant p = 0.135).

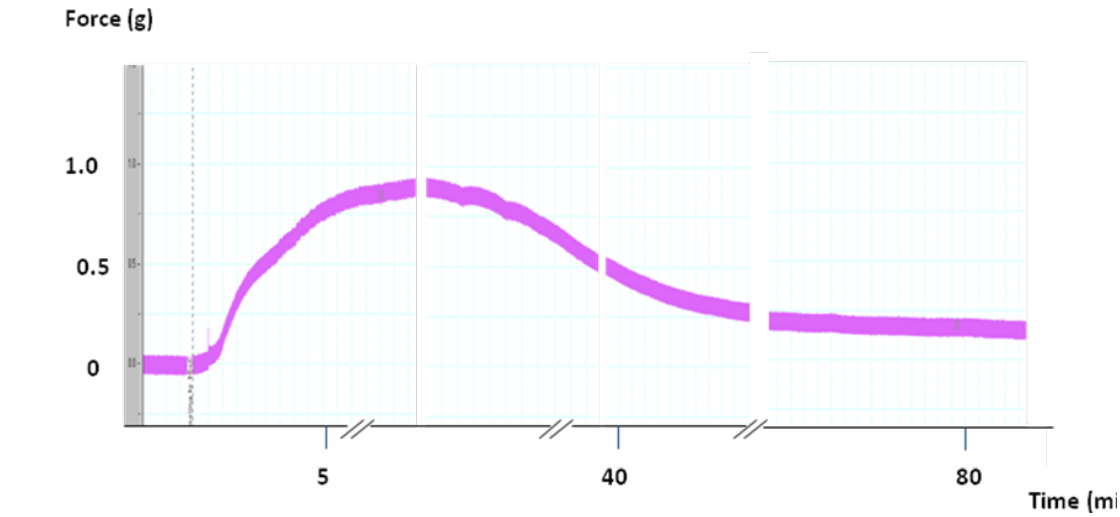


Figure 3.4: The change in tension of the dartos with cooling to 15°C and maintenance at that temperature. There was a gradual loss of tension over time both with and without the skin (no difference in the time taken to come back to the basal tension).

The effect of repeated cold stimulation was studied using tunica dartos preparations consisting of muscle alone and the muscle with the overlying skin. The procedure of decreasing the temperature to 15^{o}C took a minimum of 5-7 minutes after

which the preparation was rewarmed to 33^{o}C (which took another 5-7 minutes). The 2nd and the 3rd cooling took place immediately after this and used similar time durations. Repeated cooling of the tunica dartos muscle to 15^{o}C followed by re-warming to 33°C, led to a reduction in the contractile response to cooling (p = 0.0002) (Fig.3.5). The 3rd peak tension response to cooling was 28.5 ± 11.7 % of the 1st cooling peak response (n=9). This run down in the contractile response to repeated cooling was not observed after repeated exposure to EFS or exogenous noradrenaline stimulation at 33^{o}C (Fig.3.5).

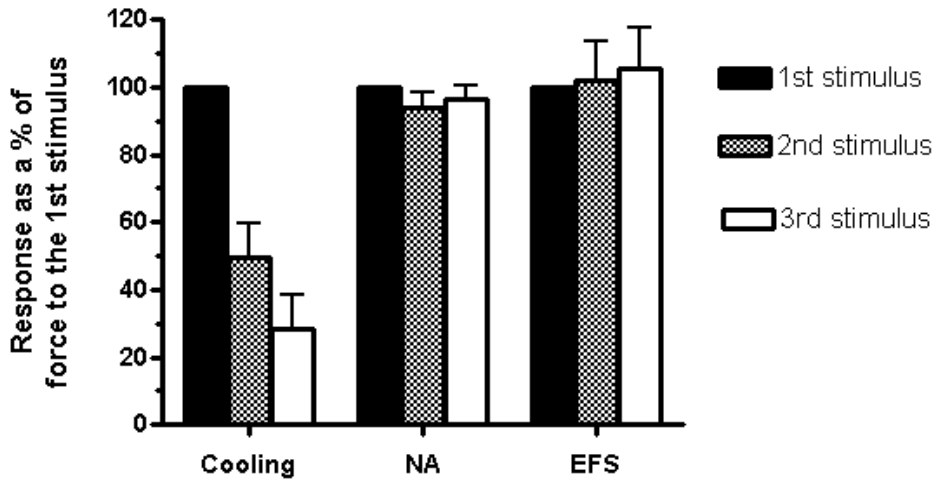


Figure 3.5: The effect of three consecutive stimulations on the same muscle strips with the skin attached, using EFS (effect of repeated stimulation with EFS p=0.92) and NA (effect of repeated stimulation with NA p=0.49) at 33^{o}C and cooling from 33^{o}C to 15^{o}C. The response to repeated cooling stimulation was significantly lower when comparing the three responses (ANOVA p = 0.0002). Twelve muscle strips from 3 rats were used. Each bar represents mean ± SEM.

The time intervals between the three NA stimulations (all at 33^{o}C) were slightly longer than that used for the cooling experiments as the washing off of NA took a minimum of 40 minutes. EFS stimulation (all at 33^{o}C) was undertaken using 15 minute intervals similar to the cooling responses. The three described stimuli were applied to three different sets of muscle strips.

The run down in the contractile response to repeated cold stimulation shown in this study but with undiminished response to other contractile stimuli suggests that the mechanism which brings about the contractile response to cooling diminished after repeated activation. These findings support the hypothesis that a paracrine agent secreted from the skin in response to cold stimulation mediates the cooling induced contraction of the tunica dartos and that the stores of that agent in the skin are limited. Similarly, the run down with time and the rundown with repeated stimulation may also mean that the receptors are getting desensitized. However, the theory of 'a paracrine agent coming from the skin' was the line of reasoning that formed the basis for the experiments outlined in section 3.3.5.

3.3.2 Effect of temperature on the EFS and noradrenaline response

As shown in the previous section, prolonged maintenance of the tissue at 15^{o}C caused the cooling induced tension to gradually decrease to baseline (in 60 to 90 minutes) (Fig. 3.4). In the present experiment to determine the response of the muscle with skin attached to NA at different temperatures, the response to noradrenaline at lower temperatures was examined after the cooling-induced force had returned to baseline. It was found that the contractile response to NA at lower temperatures was significantly larger in magnitude than the response at higher temperatures (Fig. 3.6).

If the contractile force generated by 10^{-5}M of NA (a sub-maximal dose) at 33°C is considered to be 100%, the force generated by the same concentration of noradrenaline at 15°C was 180% (one way ANOVA: $p<0.0001$). Conversely, when the same concentration of NA was used at 40°C, the force was 75% of the force generated at 33°C (one way ANOVA: $p<0.001$). The EC_{50} values at 15^{o}C, 33^{o}C and 40^{o}C were 7.3 ± 0.2 x 10^{-6} M, 5 ± 0.03 x 10^{-6} M, and 3.1 ± 0.01 x 10^{-6} M respectively and they were

not significantly different. Similarly, the hill slope values (at 15°C, 33°C and 40°C were 1.12 ± 0.08, 1.02 ± 0.06 and 1.01 ± 0.01 respectively) were also not significantly different.

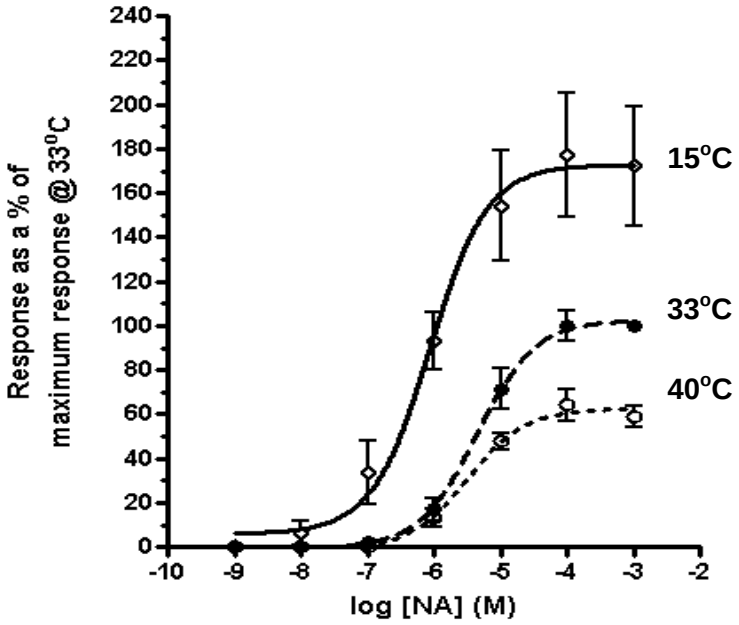


Figure 3.6: The dose response curves for NA at three different temperatures in the tunica dartos muscle (skin attached). Maximum response obtained at the three temperatures (which were 180% and 75% at 15°C and 40°C respectively compared to the response at 33°C taken as 100%) were significantly different (one way ANOVA: p = 0.0001). The Hill slope & EC_{50} were not significantly different at the three temperatures. Each curve consisted of data from at least 12 muscle strips from 3 rats. Points show mean ± SEM.

The contractile response of muscle alone to exposure to 10^{-5}M NA was not different to the response exhibited by muscle with skin attached (p=0.76) (Fig. 3.7). This result indicates that the enhancement of the contractile response to NA at 15°C is unaffected by the presence of the skin.

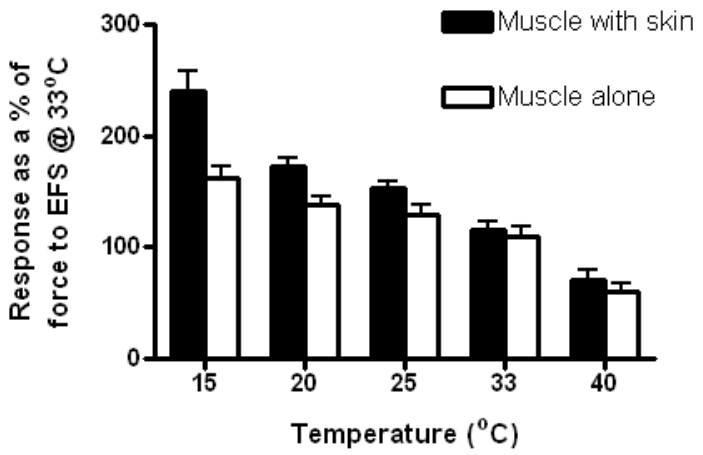


Figure 3.7: Contractile response of the dartos with and without the overlying skin to a sub maximal dose of 10^{-5}M NA at different temperatures. At least 10 muscle strips from 3 rats. Magnitude of response was not significantly different between the strips with the skin on and off. Points show mean ± SEM.

The EFS-induced force responses were also temperature sensitive. The contractile response to EFS increased at 15°C and decreased at 40°C relative to that observed at 33°C (Fig. 3.8). Note that at 15°C the measurements were made after the cooling induced force response had returned to baseline (see Fig. 3.4).

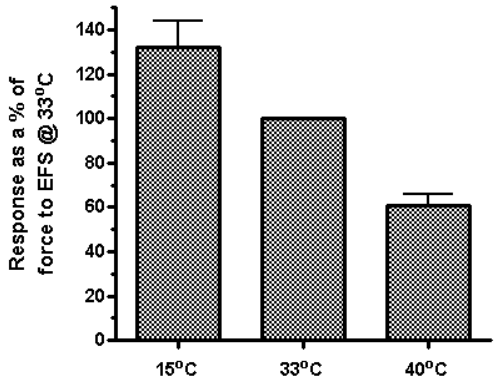


Figure 3.8: Contractile response of the tunica dartos muscle strips with skin attached to EFS at different temperatures (ANOVA: p=0.0011). At least 5 muscle strips from 3 rats used. Bars represent mean ± SEM.

To compare the forces generated by the cooling induced and NA stimulated contractions, dartos preparations were exposed to NA (10^{-4}M) during the plateau of the cooling induced force response, or the preparations were exposed to cooling during the plateau of the NA-induced force response.

In these experiments, cooling to 15°C and then exposing the muscle to NA (10^{-4}M) induced a contractile response which was markedly greater (154%) than the response to NA at 33°C (Fig. 3.9A). Similarly, cooling to 15°C in the presence of NA (10^{-4}M), produced a contractile response that was significantly larger (186%) than the response to cooling alone (Fig. 3.9B).

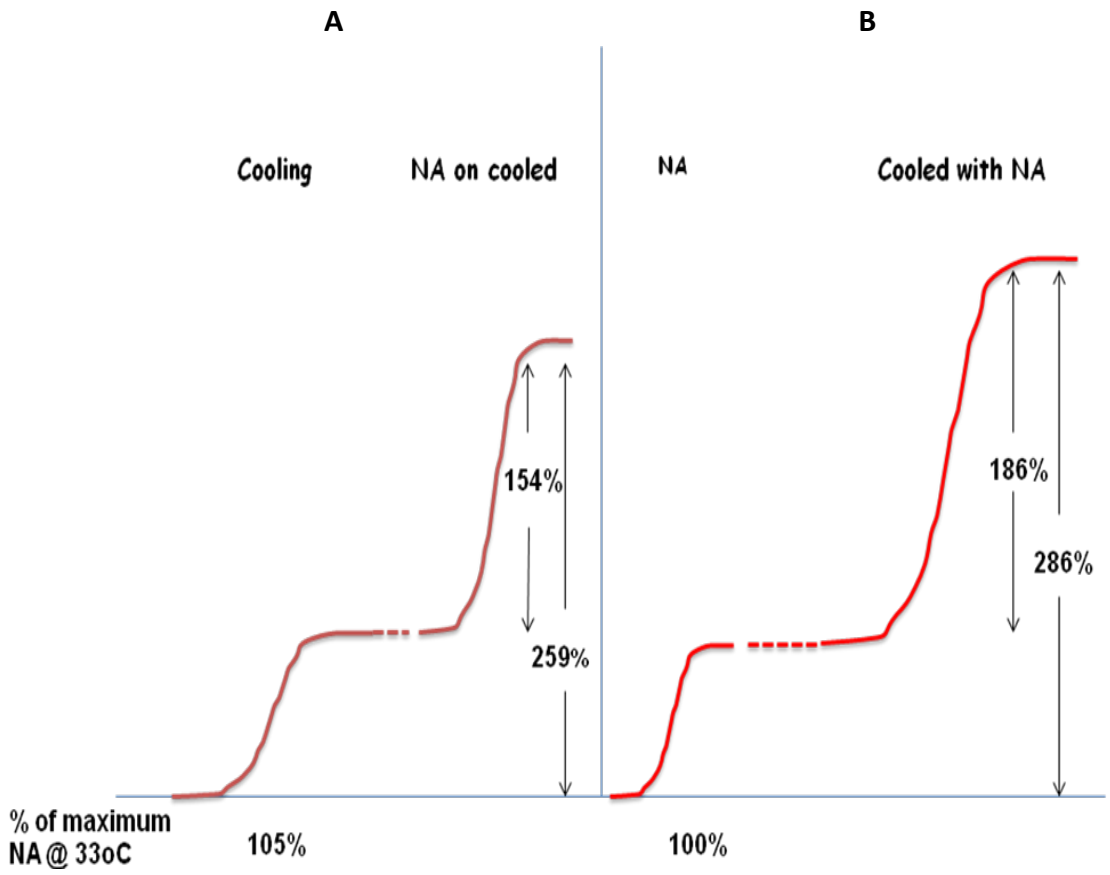


Figure 3.9: Diagrammatic representation of the responses to cooling to 15°C and then NA stimulation (A) compared to NA stimulation and then cooling to 15°C (B) of the tunica dartos. Percentages are expressed as % of response to maximum NA stimulation

These results indicate that the effects of cooling and NA are additive, and are therefore, likely to involve different mechanisms that are independent of each other (Fig. 3.10).

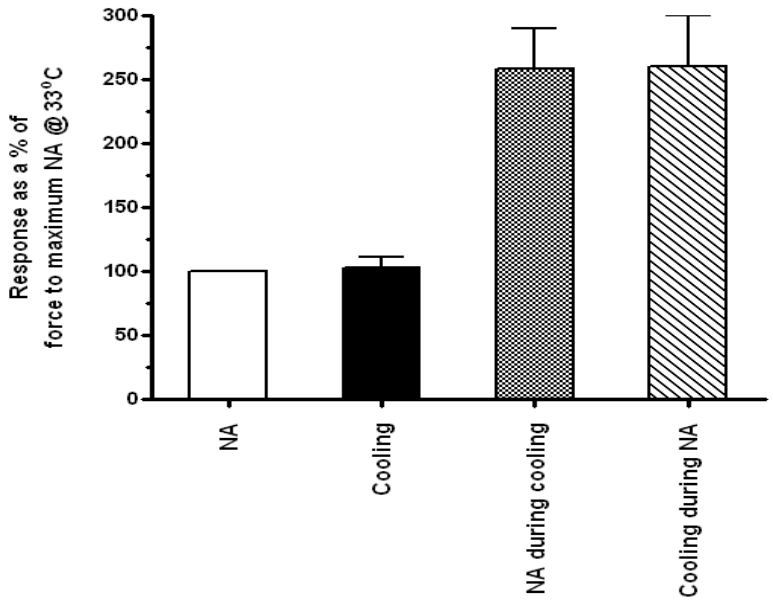


Figure 3.10: The contractile response of tunica dartos to cooling during the plateau phase of the response to (10^{-4}M) NA or exposure to NA during the plateau of the cooling response. All values are expressed as a percentage of response to NA 10^{-4}M at 33°C. Data obtained from at least 20 strips from 6 rats.

Having obtained this result, it was of interest to work out the intracellular mechanisms involved in the contractile response of the cooling induced contraction in the tunica dartos.

3.3.3 The effect of receptor blockers on cooling induced contraction

The concentration of exogenous noradrenaline (NA) that produced the maximal response with or without the overlying skin was 10^{-4} M (chapter 2). To test if cooling induced contraction was mediated by noradrenaline acting on alpha-receptors, the non-selective alpha-receptor antagonist phentolamine hydrochloride (Sigma Chemical Co., St Louis, MO, USA) and specific alpha-1 antagonist prazosin hydrochloride (Sigma Chemical Co., St Louis, MO, USA) were used separately. The dose of the antagonist required to block the effect of a maximum concentration of NA (10^{-4}M) were first found. These concentrations were then used to see if NA antagonism attenuated the response to cooling. Since there was no significant difference in the response to NA of muscle alone and muscle with the overlying skin, the latter was used.

As shown in Table 3.1, the cooling response was only inhibited by 15% with a concentration of 10^{-4}M phentolamine. Yet this concentration of phentolamine completely blocked the effect of 10^{-5}M NA. A concentration of Phentolamine of 10^{-3}M inhibited the cooling response by 85%. However, this is a very high concentration which is likely to also inhibit some other mechanisms in addition to receptor antagonism. The concentration of prazosin that inhibited the response to NA (10^{-4} M) had no effect on the cooling response.

To rule out the possibility that beta-adrenergic receptors were involved in the cooling-induced contraction, muscles were cooled in the presence of the non-specific beta-adrenergic antagonist, propranolol hydrochloride (Sigma Chemical Co., St Louis, MO, USA). Propranolol did not have any significant effect on the cooling induced contractile response at a concentration of 10^{-5}M or less.

Cooling of the tunica dartos was then undertaken in the presence of the adrenergic neurotransmitter release blocker, guanethidine hemisulfate (Tokyo Chemical

Industry Co. Ltd., Toshima, Kita-Ku, Tokyo, Japan), to ascertain whether the substance responsible for cooling induced contraction was released from adrenergic nerves endings, possibly located in the muscle-skin margin. However, guanethidine failed to inhibit the cooling response at the range of concentrations used (20 µM to 200 µM) (Table 3.1). A concentration of 200 µM of guanethidine was found to be necessary to block 90-95% of the EFS response, as outline in chapter 2.

Table 3.1: The contractile response of the tunica dartos during cooling with some adrenergic blocking agents (ns = not statistically significant) Phentolamine and prazosin were dissolved in dimethyl sulphoxide (DMSO). Control solution had a similar concentration of DMSO. The final concentration of DMSO in the bath did not exceed 1:1000.

Contractile response as a % of the response in the absence of the inhibitors				
	Phentolamine 10^{-4}M	**Prazosin 10^{-4}M**	**Guanethidine 2×10^{-4}M**	**Propranolol 10^{-5}M**
Mean ± SEM	114.7 ± 1.4	124.6 ± 15.9	110.3 ± 5.7	98.4 ± 5.8
n	11	12	8	10
Significance when compared to normal cooling response	ns (p=0.093)	ns (p=0.137)	ns (p=0.097)	ns (p=0.432)

However, one argument that could be made is that the drugs like phentolamine, prazosin and guanethidine may be losing its activity at the lower temperatures. One control experiment that could be performed is to test the effect of phentolamine and prazosin on the NA induced contractile force at 15^{o}C. Guanethidine blocked the response to EFS response even at the low temperature of 15^{o}C. The effect of adrenergic receptor blockers on the NA response at 15^{o}C was not performed during this study.

Therefore, the cooling induced contractile response of the tunica dartos is unlikely to be mediated by NA or by substances released from sympathetic nerves assuming the antagonists are not totally ineffective at low temperatures.

3.3.4 Intracellular mechanism responsible for cooling induced contraction

The results presented to this point have shown that the tunica dartos contracts to NA stimulation mainly as a result of Ca^{2+} released from the sarcoplasmic reticulum presumably via the phospholipase C/IP_3 pathway (see chapter 1). In this pathway, IP_3 formed at the cell membrane diffuses to the sarcoplasmic reticulum and induces the release of Ca^{2+} from the sarcoplasmic reticulum via stimulation of IP_3 receptors. To examine whether cooling- induced contraction involves IP_3-mediated Ca^{2+} release, the effect of the IP_3 receptor antagonist, 2-APB (dissolved in DMSO) on the cooling induced contraction of dartos muscle strips (skin attached) was examined.

2-APB at a bath concentration of 50 μM caused the cooling response to decrease to 32% of the cooling response observed in the absence of the inhibitor ($p = 0.0001$) (Fig. 3.11). The reduction in the cooling response was further decreased to 16% of control levels, when cooling occurred in the presence of 2-APB (50μM) in calcium free Ringer ($p=0.0057$) (Fig. 3.11).

These results indicate that the cooling-induced contractile response is mainly taking place through activation of the IP_3 receptors in the sarcoplasmic reticular membrane with some contribution from extracellular calcium.

The findings up to this point indicate that the cooling-induced contractile response is mainly mediated by Ca^{2+} release from the sarcoplasmic reticulum through activation of the phospholipase C/IP_3 pathway combined with a smaller proportion of extracellular Ca^{2+} influx.

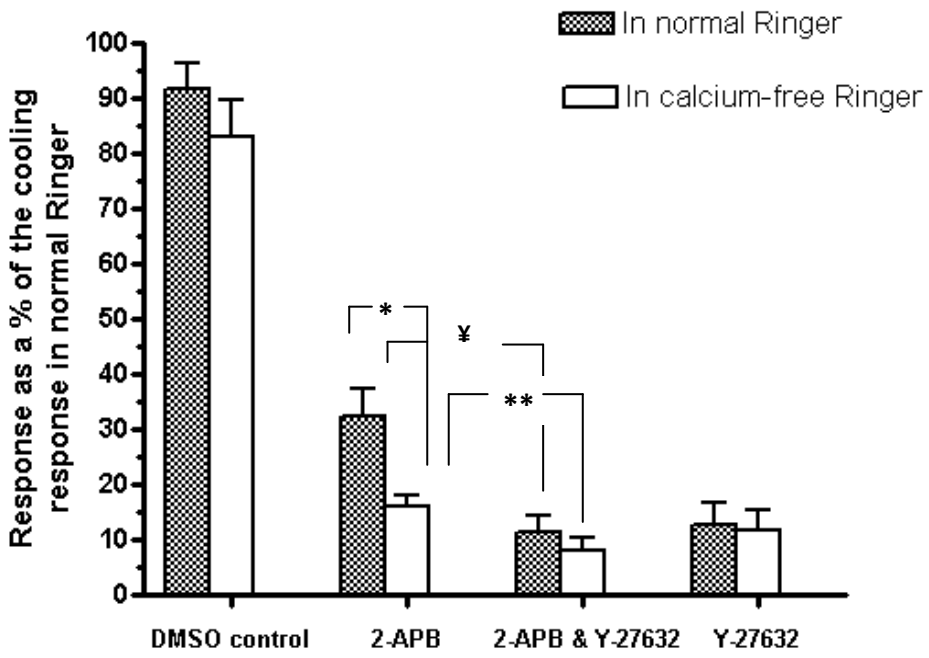


Figure 3.11: Inhibition of the cooling induced contraction by 2-APB (50µM), the IP_3 receptor blocker expressed as a percentage of the cooling induced response when used in the normal Krebs-Ringer solution (NR) and the calcium-free Ringer (CFR) solution (difference significant at * p = 0.0057). Since 2-APB was dissolved in DMSO, the DMSO control is given alongside which showed no significant difference in the cooling response (p = 0.1048). The graph also shows the contractile response for cooling when the Rho-kinase inhibitor Y-27632 was used with 2-APB (IP_3 blocker). There was a significant reduction in the response compared to that obtained with 2-APB alone (p = 0.0065 [¥] in normal Ringer and p = 0.0273 in calcium free Ringer [**]). Removing the calcium in the extracellular medium did not alter the contractile response significantly (p=0.72). Each bar represents mean ± SEM and data from at least 8 muscle strips from 3 rats.

Because 2-APB in the presence of Ca^{2+} free Ringer failed to completely abolish the cooling-induced contraction in the dartos, it is possible that the cooling-induced contractile process in the tunica dartos also has a Ca^{2+} sensitization component. This was examined using the specific Rho kinase inhibitor, Y-27632 (Sigma Chemical Co., St Louis, MO, USA) at a concentration 10µm while cooling the dartos to 15^{o}C. Activation of the Rho kinase pathway inhibits myosin phosphatase and is a common Ca^{2+} sensitization pathway in smooth muscle (Kimura *et al.*, 1996; Amano *et al.*, 1996). If the Rho-kinase pathway is present in tunica dartos smooth muscle, then cooling

induced inhibition of the myosin phosphatase may be responsible for promoting contraction during cooling.

Cooling the dartos in the presence of Y-27632 alone caused almost a complete inhibition of the cooling response to 11 % ($p=0.0001$) of control values (Fig. 3.11).

Cooling in the presence of both 2-APB and Y-27632 decreased the response significantly from cooling response to 2-APB alone in normal Ringer ($p = 0.0065$). Similarly, there was a significantly greater inhibitory effect when both these inhibitors were used together in calcium free Ringer than when 2-APB alone was used ($p = 0.0273$).

3.3.5 Evidence for a soluble mediator released from the skin of the scrotum during cooling induced contraction

The cooling-induced contractile response was several times greater in the presence of the skin compared to that of the muscle alone (section 3.3.1). The following experiment was designed to discover whether a soluble mediator released from the skin acts on the neighbouring muscle to augment the contractile response to cooling in a paracrine fashion.

In these experiments, tunica dartos preparations where the muscle and skin had been physically separated from each other and then tied together at the ends to bring the skin and muscle back into reasonably close proximity were cooled. The preparations responded well to cooling and the effects were compared between preparations consisting of the muscle with the skin, muscle alone, skin and muscle separated from each other but kept in close proximity during cooling as given in Figure 3.12.

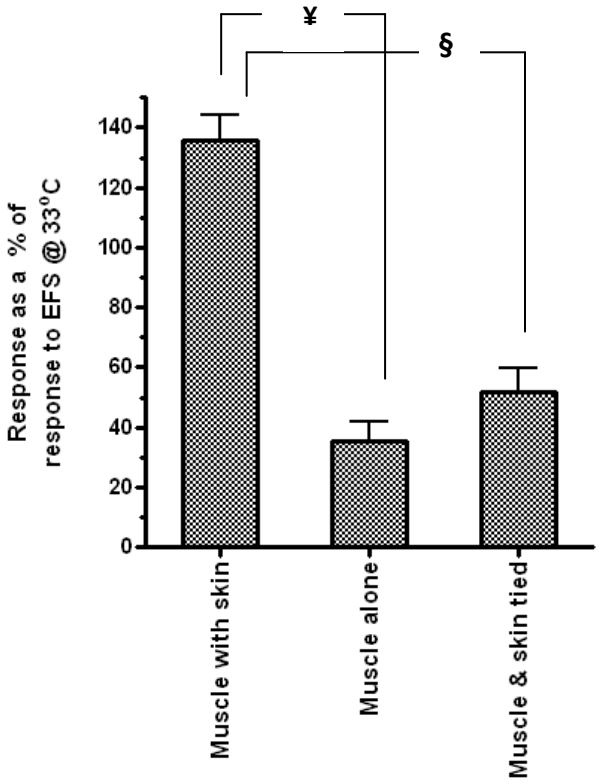


Figure 3.12: Contractile response for cooling of the dartos comparing the responses of muscle with overlying skin, muscle alone and muscle separate from the skin but tied together. Statistical significance obtained between cooling the muscle and muscle with the overlying skin **(¥)** and cooling the muscle skin separated preparation and muscle with the overlying skin **(§)** ($p=<0.001$). Each bar represents mean ± SEM and the data are obtained from at least 8 muscle strip of 3 different rats.

The cooling induced contractile force elicited in rejoined skin and muscle preparation was 51.6 ± 7.9 % of the force generated by EFS and not significantly different to the force generated by the muscle alone, which was 35.1 ± 6.7 % of EFS ($p>0.05$). The contractile forces of both groups were significantly lower than the force generated when the muscle with the overlying skin was cooled, which was 135.8 ± 8.5 % of the initial EFS response.

In the second experiment, a large area of scrotal skin of the Wistar rat, denuded of the muscle, was placed in Ringer solution and cooled to 15^{o}C for 15 minutes and then rapidly rewarmed to 33^{o}C. The cooled skin perfusate was then poured into the organ bath containing strips of dartos muscle alone (no skin) and the changes in tension were monitored. Exposure of the muscle strips to the skin perfusate at 33^{o}C did not produce any change in tension.

3.3.6 The effect of ATP and adenosine on cooling induced contraction

Since exogenous ATP produced a contractile response in the tunica dartos muscle at low concentrations (10^{-6}M -10^{-3}M), it was of interest to examine whether it played a role in the cooling induced contractile process. The cooling induced contractile response of the dartos was inhibited to a minimum level of 47.16% of the EFS response by a high concentration of 10^{-2}M ATP (Fig.3.13). However, at low concentrations of ATP (10^{-6}M and 10^{-5}M), the cooling-induced contractile response of the tunica dartos was enhanced. This enhancement of the cooling induced contractile response by ATP was seen with the skin attached to the muscle.

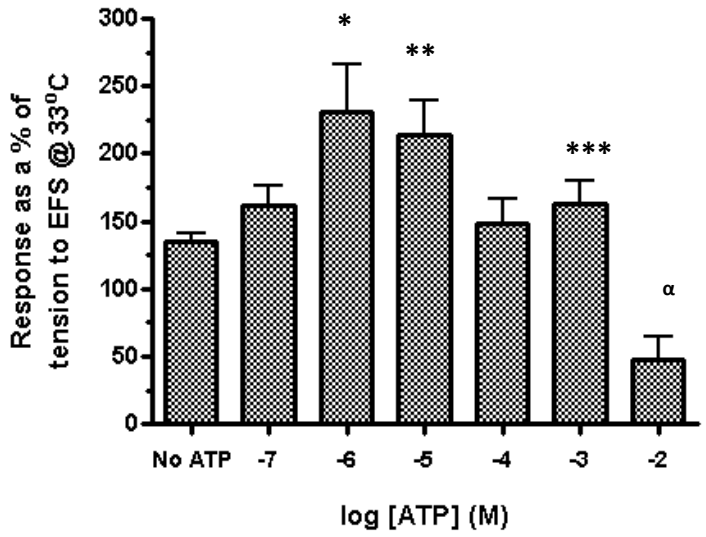


Figure 3.13: The maximum inhibition of the cooling induced contractile response of the dartos was by a concentration of 10 mM (10^{-2}M) of ATP (α). At lower concentration of 0.001 mM (*), 0.01 mM (**), and 0.1 mM (***), there was a significant enhancement of the cooling response. Each point indicates mean ± SEM for 4-5 muscle strips from 3-5 rats.

A similar enhancement of the cooling induced contractile response was also observed with the addition of adenosine (a purine nucleoside that acts at cellular level by stimulating [via A_2 receptors] or inhibiting [via A_1 receptors] cAMP synthesis) to the organ bath during cooling of the tunica dartos (Fig.3.14). It was found to be statistically

significant only at two concentrations. It may be due to low temperatures causing some change in the affinity of A_1 receptors, augmenting the cold induced contraction.

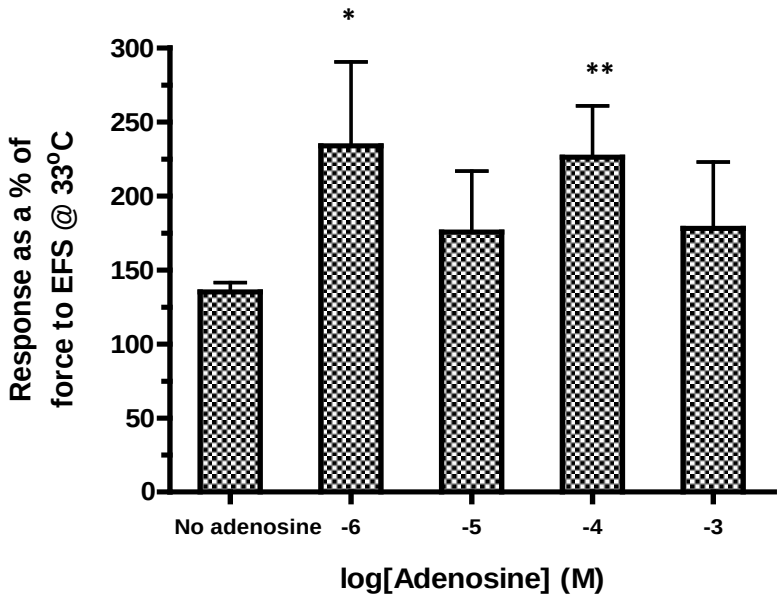


Figure 3.14: Comparison of the response to cooling of the muscle (skin attached) with varying bath concentrations of exogenous adenosine. The response to cooling without adenosine is also given at the left corner for comparison. There was a significant enhancement of the response to cooling at the lower concentrations (* $p = 0.0002$ and ** $p = 2.8 \times 10^{-5}$). Each bar indicates mean ± SEM of 3 muscle strips for each concentration.

Since the cooling contraction of the dartos was enhanced by exposure to exogenous ATP (see above), the effect of blocking P_2 purinergic receptors on the cooling induced contraction was examined. Blockade of purinergic receptors using the non-specific P_2 receptor antagonist suramin (5×10^{-4}M) (Sigma Chemical Co., St Louis, MO, USA) had no significant effect on the cooling response (Table 3.2). Therefore, it was concluded that ATP was unlikely to play a significant role in the cooling induced contractile response of the tunica dartos.

3.3.7 Investigation of other possible paracrine mediators of the cooling induced contraction

An investigation into potential compounds responsible for cooling induced contraction of the dartos was performed by a process of elimination, using the common substances responsible for contraction in other smooth muscles of the body. Since histamine is a known contractile mediator in smooth muscle, I used a varying concentration of Histamine to see whether there was a contractile response to it at any concentration. No such response was found, however, I blocked histamine receptors during cooling of the tunica dartos. Histamine has 7 known receptor subtypes. However, it was not possible to find a substance which would block all receptor subtypes. Blockade of histamine receptors was performed using two common antihistamines, chlorpheniramine maleate 10 µM and cimetidine 10 µM (both from Sigma Chemical Co., St Louis, MO, USA) both separately and in combination, during cooling induced contraction. Chlorpheniramine (10^{-5}M) and cimetidine (2×10^{-5}M) did not have an effect on the cooling induced contractile response of the dartos (Table 3.2).

Table 3.2: Cooling induced contractile response tested with the use of various agents to block possible receptors that may be involved. (ns = not statistically significant)

Contractile response as a % of the response to cooling in the absence of the substance			
	Suramin 5×10^{-4} M	**Chlorpheniramine 10^{-5} M**	**Cimetidine 2×10^{-5} M**
Mean ± SEM	107.7 ± 17.4	94.3 ± 18.3	101.6 ± 20.2
n	10	12	12
Significance when compared to normal cooling response	ns (p=0.55)	ns (p=0.49)	ns (p=0.71)

Serotonin, commonly known as 5-HT, is another contractile mediator found in smooth muscle tissues. Since propranolol is also a 5-HT_1 and 5-HT_2 antagonist in addition to being a beta-adrenoceptor antagonist. Our previous results showing that propranolol did not alter the cooling induced contraction of the dartos (Table 3.1) indicate that the 5-HT_1 and 5-HT_2 serotonin receptor subtypes, are not involved in the cooling effect.

The results are summarised in Table 3.2 with comparison to the response obtained in cooling without the blockers.

The TRPM8 are cold sensitive channels (McKemy *et al.*, 2002) that are opened by stimulation with compounds like menthol (Daniels *et al.*, 2009). The $TRPM_8$ receptor agonist menthol was used to determine whether the receptor for cooling induced contraction was of this type. There was no change in tension in tunica dartos muscle strips when menthol (10 μM, 100 μM and 500 μM) was added to it at 33^{o}C. Therefore, the tunica dartos or skin is unlikely to express these receptors.

3.4 Discussion:

The scrotum is temperature responsive, contracting in cold environments and relaxing in hot environments. The importance of this response for normal function of the testes was outlined in the introduction and the main role played by the dartos was also established. The main objective of the experiments outlined in this chapter was to characterize the cooling induced contractile response of the tunica dartos and to determine the intracellular and extracellular mechanisms involved in this response.

3.4.1 Sympathetic induced contraction and cooling induced contraction

The cooling induced contractile response of the tunica dartos was found to be 136 ± 8.4 % of the response to EFS (100 %) while the response to NA was 133 ± 5.7 % of the response to EFS. Findings outlined in the previous chapter confirmed that the tunica dartos was sympathetically innervated and responded to exogenous NA via alpha-1 receptors, as also outlined in the literature (Gibson *et al.*, 2002; Maloney *et al.*, 2005). However, the present research has increased the understanding of the autonomic control of the scrotal function in thermoregulation; the enhancement of the NA effect at low temperatures. What this means is that the same sympathetic output will have a much greater contractile effect when the scrotum is cool. This will make the tissues increasingly sensitive to sympathetic output as the temperature decreases below 33^{o}C. This would be a useful mechanism for thermoregulation of the testes at lower temperature environments. The Norwegian rat (*Rattus norvegicus*) a close relative of the Wistar rat, swims in water close to zero celsius and will make use of this mechanism to conserve the loss of heat from the scrotum exposed to cold water. On the other hand, at higher temperatures, sympathetic activation will be less effective at stimulating dartos contraction. Perhaps this is important for thermoregulation of the testes during exertion.

During increased physical exertion, skin temperature would increase with sweating and vasodilatation. But adrenal catecholamines could oppose the relaxation of the dartos and the maintenance of temperature. The insensitivity to NA at higher temperatures will therefore, help to maintain testicular temperature.

The possible mechanism for this variation in the response to NA at different ambient temperatures may be due to an alteration in the contractile machinery of the cells or a change in receptor affinity to NA. Conversely, it could also be a reduction in the uptake of NA into the nerve terminals at low temperatures. Uptake of NA into sympathetic nerve endings is a process that can be temperature sensitive (Iversen, 1971). One way of testing whether it is an increase in the activity of the cellular contractile machinery or whether it is the reduction of uptake of NA that is causing the greater response at 15^{o}C is to use a substance such as cocaine (Banerjee *et al.*, 1987). Cocaine being a compound that inhibit reuptake of NA altogether would cause the same magnitude of response that is produced at 15^{o}C to NA even at 33^{o}C. If that happens it could be concluded that the increased force obtained to NA at 15^{o}C is due to lack of reuptake of NA.

In cooling induced contraction of bladder smooth muscle and bronchial smooth muscle the alteration in contractility was due to the attenuation of the sarcolemmal Ca^{2+} pump or the leak of Ca^{2+} from the intracellular stores, resulting in a raised Ca^{2+} in the cytoplasm (Mustafa *et al.*, 1999b; Mustafa and Thulesius, 1999c). In some skin vessels the alpha-2 receptor affinity for NA increases with a reduction in temperature (Flavahan, 1991). In the case of the tunica dartos the reason may be any one or a combination of the above mentioned reasons or a differential effect of temperature on the MLCK or MP that regulates the degree of phosphorylation of the myosin light chains. It is known also that MP is influenced by Rho and that its activation changes with temperature.

There is evidence to suggest that the mechanism of cooling induced contraction of the dartos is different to the mechanism of contraction induced by sympathetic stimulation. The contractile effects of cooling and NA were additive. Performing consecutive cooling induced contraction resulted in a run-down which was not observed with consecutive EFS stimulation or NA stimulation. It is of course a possibility that the cooling causes a desensitization of the receptors which produced a gradual run down of the response.

Phentolamine and prazosin totally abolished the response to NA but failed to have an effect on the cooling induced contractile response. This last finding was in contrast to Maloney *et al*. (2005), who found that phentolamine reduced the cooling response to 78 ± 5 % of control. The difference may be due to the low numbers used by them. In addition, 50 µM of 2-APB (the IP_3 receptor blocker) completely abolished the NA response at 33^{o}C but only partially (by 70 %) abolished the cooling response. However, Y-27632 (Rho-kinase inhibitor) only decreased the NA effect by 40% but decreased the cooling response by 85%. This favours a theory of a larger component of calcium sensitization involved in cooling induced contraction of the dartos muscle.

3.4.2 Intracellular mechanism responsible for cooling

From the observations made during the experiments on intracellular mechanism of cooling induced contractile response, the key findings were that inhibition of Rho kinase and IP_3 receptors caused large reductions in the force response. When the inhibitors were used together, the response was reduced by more than 90%. This suggests that IP_3 receptor stimulation and Rho kinase inhibition happen consecutively in the same pathway. The attempt at removal of extracellular Ca^{2+} reduced the response by a further 15-20 % although the medium was not made totally calcium free. Therefore,

extracellular Ca^{2+} probably played a role through one of the calcium entry mechanisms mentioned previously such as SOCCs, ROCCs or VOCCs.

Some smooth muscles demonstrate intracellular Ca^{2+} oscillations during contraction. In airway smooth muscle, it has been found that the important phenomenon during contraction is not the peak Ca^{2+} increase, but the frequency of Ca^{2+} oscillations (Sanderson *et al.*, 2008); An increased frequency of oscillation causes a higher force of contraction of the muscle. There is evidence that these Ca^{2+} oscillations can activate the Rho kinase system (Sanderson *et al.*, 2008) and there by inhibit myosin phosphatase, promoting contraction. If we make the assumption that cooling induced contraction of tunica dartos brings about Ca^{2+} oscillations by activating the phospholipase C/IP_3 pathway and then these oscillations in turn, activate the Rho kinase pathway, both these processes together will promote the phosphorylated state of myosin light chains promoting contraction of the dartos (Fig.3.15). It should be noted that Rho kinase could also be activated independent of IP_3 pathway. If we assume that there is a mediator which acts as an agonist to activate both PLC and the Rho kinase pathway during cold stimulation of the tunica dartos. The resultant Ca^{2+} release from the sarcoplasmic reticulum would then lead to Ca^{2+} oscillations and further activation of Rho kinase. If this hypothesis is true, if the Rho kinase is inhibited, myosin phosphatase is unaffected and the only process that elevates the intracellular Ca^{2+} would be the activation of the IP_3 receptors which is probably of relatively little importance to cooling induced contraction. This is probably the reason for the reduction in contractile force by approximately 85% by the use of Rho kinase inhibitor. Alternatively, if Ca^{2+} oscillations are prevented through the use of 2-APB by blocking the IP_3 receptors, downstream Rho kinase is partly inhibited and the force is reduced by only 70%. This model assumes that MLCK activation is relatively unimportant during cooling (Fig. 315). Therefore, the bulk of the cooling response comes through activation of the Rho kinase/MP system and

most of the EFS and NA stimulation acts via MLCK activation as mentioned in chapter 2. This would explain also how the two systems are independent but additive, as observed during the consecutive cooling and NA stimulation experiments.

In the present investigation Ca^{2+} sensitization was indirectly inhibited with the use of the Rho kinase inhibitor Y-27632, which would have prevented the inactivation of MP via Rho kinase. The MP therefore dephosphorylated myosin light chains, promoting relaxation. Gibson *et al*. (2002) used Calyculin-A (a type I and II phosphatase inhibitor) to directly inhibit myosin phosphatase to abolish the heat induced relaxation of the muscle which was already contracted by an agonist. Since Calyculin-A is able to inhibit other phosphatases in smooth muscle cells it was decided to use a specific Rho-kinase inhibitor in the present study. Thus the Y-27632 compound would have prevented the inhibition of myosin phosphatase indirectly thereby stopping the cooling induced contraction due to a Ca^{2+} sensitization.

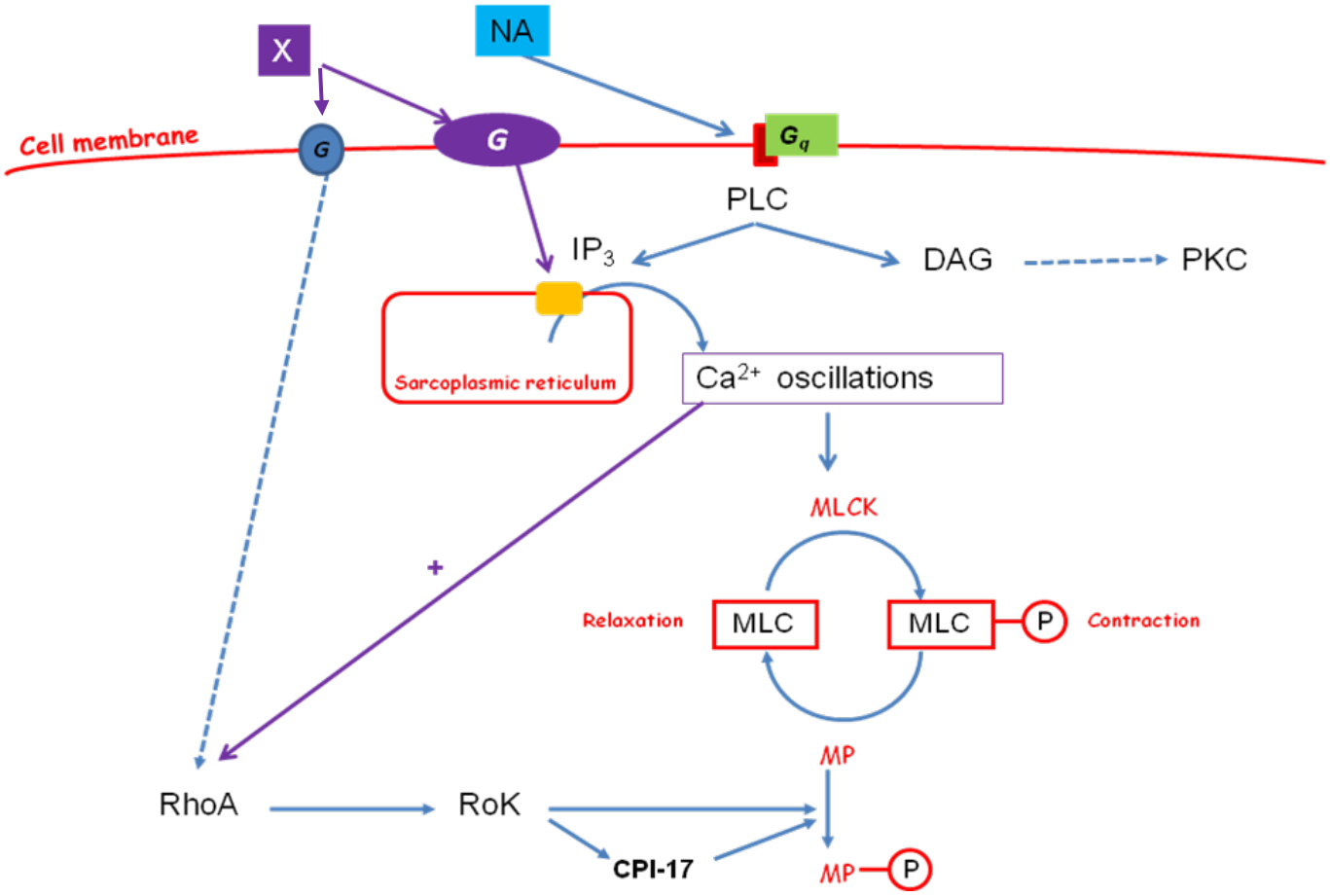


Figure 3.15: Schematic diagram representing the possible intracellular processes taking place in a typical tunica dartos cell during the cooling induced contractile process.

3.4.3 Cooling induced contraction and the role of the skin

According to my findings the temperature response of the tunica dartos was enhanced in the presence of the skin. This is in agreement with the findings by Maloney *et al*. (2005) who postulated the possibility of the presence of a paracrine agent secreted from the skin or skin/muscle margin which acts on the underlying smooth muscle to promote contraction. This is true of some other tissues of the body such as blood vessel endothelia (Lovenberg and Miller, 1990) and bronchial epithelia (Amrani and Panettieri, 2002; Rizzo *et al.*, 2002) secreting paracrine agents which have a contractile effect on the underlying smooth muscle.

The difference in contractile force with and without the skin observed with cold stimulation was not seen in the case of stimulation with EFS or NA (Fig. 2.6). Additionally, the skin produced no force on its own. Therefore, it is unlikely that removal of the skin caused any damage to the muscle, because if that was the case the response to EFS and NA would also have been affected.

However, experiments performed with the skin removed from the muscle, then put back together with severed physical connections failed to produce the same force, generated initially before separation, in contrast to the observations of Maloney *et al.* (2005) where the force generation after physically approximating the separated skin and muscle was not significantly different to that of the force generated with the skin and muscle intact. In the present experiment the force generation by the combined skin + muscle preparation was significantly less than the muscle with the skin attached. Ideally, in this experiment the same muscle strip should have acted as its own control. But this was not possible because of the run down phenomenon of the force response observed with repeated cooling.

The skin perfusate experiment performed by putting the perfusate obtained from cooled skin over the muscle at 33^{o}C also did not provide any evidence of the skin producing some mediator that acted on the muscle. It is, however, possible that there is still a compound being released from the skin, but is too short lived or gets too diluted in the bath during the process of obtaining the perfusate. It is also possible that the compound was degraded during the time lapse that occurs during cooling of the skin and obtaining the perfusate.

It is important to recapitulate at this point that when the Rho kinase inhibitor together with the IP_3 blocker was used in Ca^{2+} free medium there was still approximately 10% of the contractile force left in the muscle with skin preparation. Therefore, it must be some other Y-27632 insensitive sensitization pathway in the muscle which causes this small percentage of contraction. Since the experiments investigating the IP_3 and Rho kinase pathways were performed with the muscle with the skin attached, the activation of Rho kinase or IP_3 could still be due to an unknown mediator coming from the skin.

3.4.4 The mediator responsible for cooling induced contraction

From the experimental findings it is also apparent that the major contributor to the cooling induced contraction of the dartos muscle is Ca^{2+} sensitization. The substance responsible for bringing about activation of the Rho-kinase and Ca^{2+} oscillations could not be identified during the course of the experiments performed. Having excluded noradrenaline and a sympathetic involvement, substances such as ATP, histamine, serotonin and menthol were also excluded as the mediator of the cooling induced contractile response. Previous investigators (Maloney *et al.*, 2005) have excluded any possibility of involvement of prostaglandins and nitric oxide by using indomethacin and

N-L-nitroarginine. Therefore, this study has taken a step further in excluding certain substances as being responsible for bringing about the cooling induced contractile response. Other possible candidates which could be tested in a future study would include substances like GTP (Ayman *et al.*, 2003) or diguanosine pentaphosphate (Tolle *et al.*, 2006).

The present study, therefore, has extended the understanding of the contractile physiology of the tunica dartos by identifying a possible intracellular mechanism that takes place during cooling induced contraction of the dartos and by proving that this mechanism is independent from the sympathetic contractile mechanism. The presence of an independent contractile mechanism in addition to the autonomics in cooling induced contraction of the dartos, aids testicular thermoregulation further with enhancement of speed and magnitude of response. This will ensure contraction of the dartos even when the sympathetic nerves are compromised. The tunica dartos should be able to facilitate testicular thermoregulation even when the sympathetic nervous system is not in an active state.

CHAPTER 4

Calcium indicator measurements in tunic dartos

4.1 Introduction:

4.1.1 Mechanism of smooth muscle contraction

Contraction of the tunica dartos requires myosin light chain phosphorylation as outlined in Chapter 1 (Hartshorne, 1987; Somlyo and Somlyo, 1994). Usually, this phosphorylation occurs following a rise in the intracellular ionic calcium (Ca^{2+}) concentration either after the arrival of an action potential or an agonist binding to a G-protein linked cell surface receptor. When Ca^{2+} is elevated in the cytoplasm it binds to the protein calmodulin (Sommerville and Hartshorne, 1986). Thereafter, the calcium-calmodulin complex activates myosin light chain kinase (MLCK) which, in turn, phosphorylates the regulatory myosin light chains (Hai and Murphy, 1989; Kamm and Stull, 1985). The primary regulator of MLCK is the Ca^{2+}/Calmodulin complex (Horowitz *et al*., 1996). The Ca^{2+}/Calmodulin complex formation is a critical step for the interaction between smooth muscle myosin and actin and the muscle contraction. The detailed mechanism of contraction of smooth muscle is described in detail in Chapter 1. When the intracellular Ca^{2+} levels fall, MLCK becomes inactive. The myosin light chains are dephosphorylated by myosin phosphatase (MP) (Hartshorne *et al*., 1998) and relaxation ensues. Therefore, the relative activity of MLCK and MP determines the degree of myosin phosphorylation (Pfitzer, 2001) which, in turn, determines the state of contraction of the smooth muscle.

The understanding of the nature of intracellular Ca^{2+} signalling during smooth muscle contraction has improved over time. In many smooth muscle types, Ca^{2+} signalling consists of a spike of Ca^{2+} after agonist stimulation, followed by a lower sustained plateau of Ca^{2+}(Somlyo and Himpens, 1989). However, more recently, it has been shown in some smooth muscle types that the Ca^{2+} signal consists of a series of oscillations, the frequency of which determines the force of contraction (Sanderson et al., 2008). Ca^{2+} oscillations have been recorded from smooth muscles of the airways, systemic vessels, colon, uterus and pulmonary vessels in many species including mice, pigs and humans (Kasai *et al*., 1994; Pabelick *et al*., 2001; Lee *et al.*, 2005; Perez and Sanderson, 2005; Prakash *et al*., 1997; Mayer *et al*., 1992). The Ca^{2+} oscillations usually originate in one end of a smooth muscle cell and are propagated along the full length. These oscillations could be due to repetitive Ca^{2+} release from the intracellular Ca^{2+} stores. The Ca^{2+} stores would then be replenished by store-mediated Ca^{2+} influx (Prakash *et al.*, 1997; Perez and Sanderson, 2005; Pabelick *et al*., 2001; Dai *et al*., 2006). Alternatively, the oscillations may arise due to oscillations in the membrane potential, which could periodically activate voltage gated cation channels.

The mechanism responsible for Ca^{2+} release from the internal stores varies with smooth muscle type and location, and usually involves either IP_3 receptors or ryanodine receptors. However, in this study, it was not possible to demonstrate definitely whether the dartos muscle carried ryanodine receptors. They may have been few in number and their effects unmeasurable. Definitive evidence from experiments using 2APB indicate that the dartos exhibited significant intracellular Ca^{2+} release through IP_3 receptors.

Calcium sensitization is also an important modulator of smooth muscle contraction (Somlyo and Somlyo, 1994; Pfitzer, 2001). The mechanism of Ca^{2+} sensitization most often

reflects inhibition of MP, which leads to an increase in net myosin light chain phosphorylation for a given concentration of Ca^{2+}.

A common pathway leading to inhibition of MP is via activation of the monomeric G protein RhoA signal cascade (Hartshorne *et al*., 1998). RhoA activates Rho kinase which, in turn, inhibits MP by two mechanisms (Pfitzer, 2001; Solaro, 2000). First, it directly inhibits by phosphorylating the myosin binding subunit of MP. Indirectly, Rho kinase also phosphorylates CPI-17, an endogenous protein which, in turn, inhibits MP (Somlyo and Somlyo, 2003; Somlyo and Somlyo, 2000; Webb, 2003). Many substances including catecholamines, vasopressin, endothelin, angiotensin and muscarinic agonists are known to increase the sensitivity of smooth muscle contraction to Ca^{2+} through activation of the Rho signalling cascade. Rho kinase can also be activated by the products of arachidonic (Somlyo and Somlyo, 2000; Gong *et al*., 1992).

4.1.2 Tunica dartos muscle and calcium sensitivity

In 2002, Gibson and others published a study on the pharmacology and thermosensitivity of the tunica dartos muscle. The most striking observation this group made was the presence of a marked thermosensitivity of contraction of the dartos to temperatures above 33^{o}C. Contractility was reduced by approximately 80% for a 10°C increase in temperature above 33^{o}C, giving a Q_{10}value of around 5. Although temperature affects several processes involved in smooth muscle excitation-contraction coupling, the Q_{10} of most of these is between 1 and 3. An exception is myosin phosphatase (MP), which has a Q_{10} value of 5.3 (Mitsui *et al*., 1994). This led (Gibson *et al.*, 2002) to investigate the role of MP in the temperature response to NA of the dartos using Calyculin-A, a permeable

type I and II phosphatase inhibitor. This drug has been used extensively to investigate the role of myosin phosphatase in smooth muscle excitation-contraction (Ishihara *et al.*, 1989; Shiozaki *et al*., 2000; Burdyga and Wray, 2002). In the presence of Calycullin-A, Gibson *et al.*, (2002) found that the dartos lost its temperature sensitivity and concluded that the temperature-induced relaxation was due to increased activity of myosin phosphatase. If the decrease in contractility of the dartos at elevated temperatures is due to an increased activity of myosin phosphatase, then the cooling induced contraction observed by Maloney *et al*., (2005) and characterized in the present thesis in Chapter 3, may occur, at least partly, from inhibition of myosin phosphatase, resulting in a raised Ca^{2+} sensitivity at lower temperatures.

In other smooth muscle types, where cooling induced contraction independent of neural stimulation is present, other mechanisms have been implicated. For example, in cooling induced contraction of airway smooth muscle, an increase in intracellular Ca^{2+} has been demonstrated upon cooling resulting from increased Ca^{2+} release from intracellular stores. There was also a concurrent inhibition of the cytosolic Ca^{2+} removal mechanisms during these contractions, including attenuation of sarcolemmal calcium pumps and increased Ca^{2+} leak from the Ca^{2+} store (Mustafa *et al*., 1999b). A similar increase in intracellular Ca^{2+} has been reported in cooling induced contraction of bladder smooth muscle (Mustafa and Thulesius, 1999c). Turkey gizzard smooth muscle, in contrast, shows a differential effect of temperature on MLCK and MP, altering the calcium sensitivity of the contractile process (Mitsui *et al*., 1994) leading to contraction with cooling.

Therefore, in the cooling induced contraction of the tunica dartos, the intracellular signaling pathway responsible may involve increased intracellular calcium level or an increase in calcium sensitivity, or both.

In this series of experiments an attempt was made to measure intracellular calcium transients in tunica dartos preparations using the fluorescent indicator

(fura-2), in response to the known contractile agonist noradrenaline, electrical field stimulation and decreased temperature. In this section of the study, my aim was to determine primarily whether the cooling induced contraction of the tunica dartos involved changes in intracellular calcium and the nature of the calcium signal responsible.

4.2 Materials and Method:

Changes in intracellular Ca^{2+} concentration are associated with a wide variety of cellular processes. This has led to the development of techniques to measure intracellular Ca^{2+} concentrations in living cells. A family of fluorescent calcium indicators used commonly at present to measure intracellular Ca^{2+} include fura-2 and Indo-1 (Grynkiewicz *et al.*, 1985). These ratiometric indicators offer important advantages over previous single wavelength indicators in that they allow quantitative measurement of Ca^{2+} and the measurement is independent of the concentration of the dye and minimise the problem of high signal/noise ratio. They can be loaded as a membrane-permeant Ca^{2+} insensitive ester derivative which readily enters the cell. Once inside the cell, intracellular esterases remove the carboxyl groups from the dye, leaving the membrane impermeable Ca^{2+} sensitive form in the cell. Several reports have described the application of these indicators for studying the free calcium in smooth muscle cells (Williams and Fay, 1985; Ashley and Pritchard, 1985; Sumimoto and Kuriyama, 1986; Himpens and Somlyo, 1988). The most suitable indicator presently available is fura-2, which offers improvements over its predecessors due to increased brightness and the possibility of ratiometric determination of absolute free calcium levels by comparison of the fluorescence emitted at different excitation wavelengths (Grynkiewicz *et al.*, 1985; Williams *et al.*, 1985; Poenie *et al.*, 1985).

Once inside the cell, fura-2 changes its excitation wavelength from 340 nm to 380 nm in response to Ca^{2+} binding. Cells loaded with fura-2 are alternatively illuminated with light of wavelength 340 nm and 380 nm, and the resulting fluorescence emission is measured at a wavelength of 510 nm. The fluorescence emission ratio (340/380) provides an estimation of the intracellular Ca^{2+} concentration.

4.2.1 Calcium indicator measurement using whole muscle bundles

Cytosolic calcium measurements have not been performed previously in the tunica dartos muscle. Therefore, methodology had to be developed to obtain either very small muscle bundles of the dartos or individual muscle cells. Small bundles were employed in order to minimize the total movement that occurs during muscle activation.

The glabrous part of the scrotum of Wistar rats was removed and placed in Krebs-Ringer solution (method described in Chapter 2 – section 2.2.1). Thereafter, the dartos muscle bundles were gently dissected away from the skin to obtain small muscle strips of approximately 1 mm in diameter and 5 mm in length.

The muscle strips were then loaded with fura-2 as follows: The muscle strips were incubated for 45 minutes at room temperature in the dark in a fura-2 'load' solution consisting of physiological rodent saline solution (PRS : Composition in mM: 138 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1.06 $MgCl_2$, 12.4 HEPES and 5.6 glucose, pH 7.3) containing 3 µM fura-2/AM (TefLabs Inc., Austin, Tx, USA) and 0.0125% (w/v) Pluronic F-127 (a detergent that aids dye dispersion) (Molecular Probes, Eugene, OR). The fura-2/AM (50 µg) was dissolved in 50 µl of dimethyl sulphoxide (DMSO) prior to use. The fura-2 loaded muscle strips were then washed three times in fresh PRS before use.

A single muscle strip was placed in a chamber (with a volume of 5ml) and attached at one end to a tissue attachment post and the other end to a force transducer with black surgical silk. The chamber was then mounted on the stage of a Nikon TE 200 microscope. (Fig. 4.1 demonstrates the connections between the different apparatus used for this imaging technique). With light provided by a xenon lamp and Cairn dual monochromator system (Cairn, UK), the muscle strips were illuminated alternatively at excitation wavelengths of 340 nm and 380 nm (bandwidth = 10 nm), and the fura-2 fluorescence

emission at 510 nm (20nm band pass) was acquired with a photomultiplier tube. The photomultiplier tube current output signal was transformed into a voltage, before being monitored and stored on a personal computer. The sampling frequency of the system was 50 Hz (one ratiometric measurement every 20 ms), and the post-PMT gain was set to 10.

Attempts were made to record calcium transients following electrical field stimulation (EFS) and exogenous noradrenaline (NA). The fluorescence ratio was used as an indicator of changes in cytoplasmic Ca^{2+} concentration inside the dartos muscle cell. A total of 21 muscle bundles from 5 rats were used for calcium measurement using this technique.

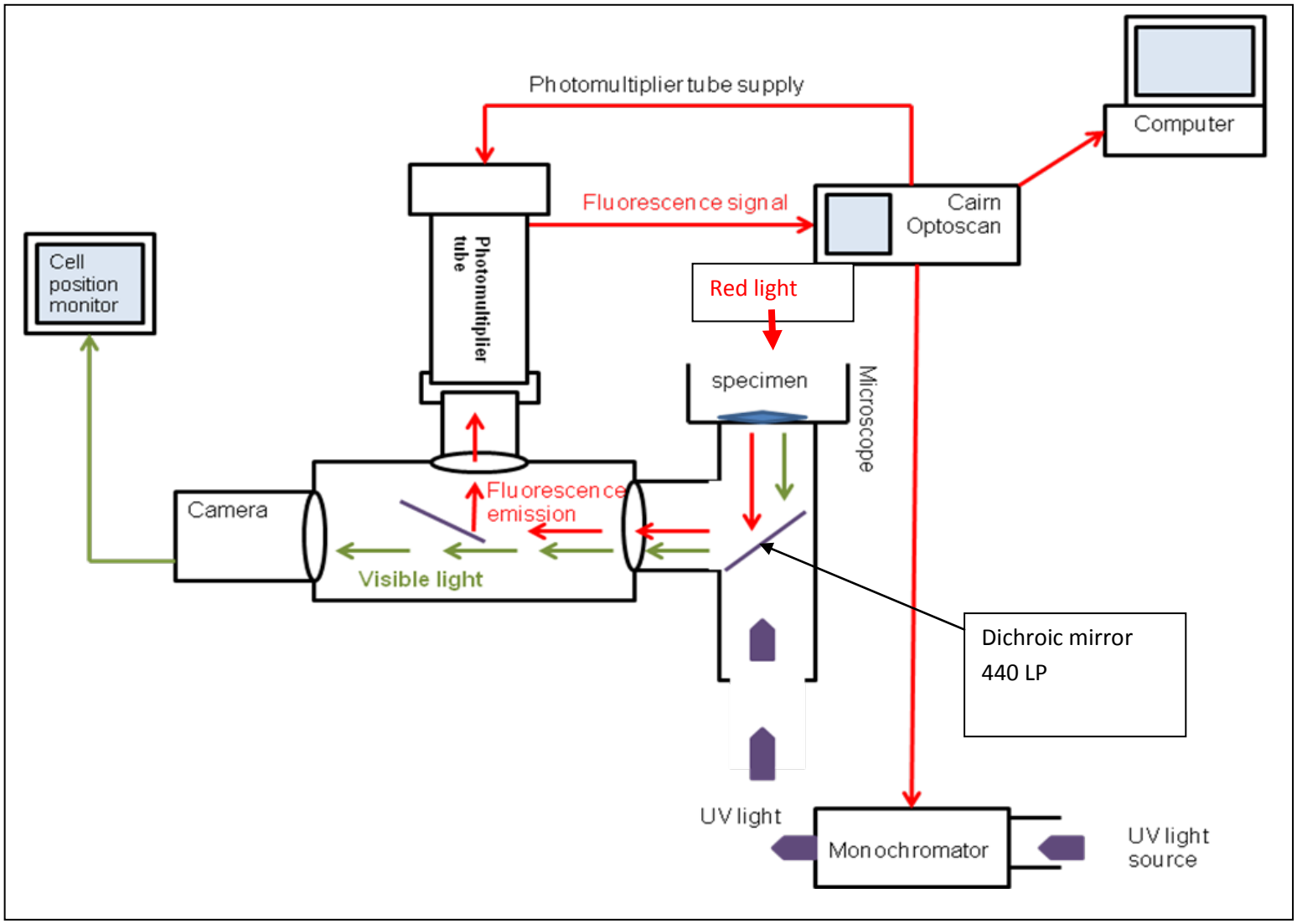


Figure 4.1: Schematic diagram of the set up used for intracellular calcium imaging with the fluorescent calcium indicator Fura-2.

4.2.2 Calcium indicator measurement using enzyme dispersed tunica dartos cells

In order to obtain single muscle cells for Ca^{2+} measurements, it was necessary to develop a method to either disperse cells effectively from the freshly obtained rat dartos or to perform primary culture from the dispersed cells obtained.

To obtain these cells thin strips of tunica dartos muscle were obtained from Wistar rats as described previously (Chapter 2 – Section 2.2.1). The muscle was then cut into small pieces, placed in PRS and transferred to a 15 ml Falcon tube containing an enzymatic digestion mixture 3 (shown in Table 4.1). Thereafter, the Falcon tube was sealed and incubated at 37°C in a shaking water bath for 60 minutes following brief aeration with carbogen (95% O_2; 5% CO_2) to maintain pH. The resulting digested muscle tissue was then exposed to gentle trituration with a Pasteur pipette with a heat-smoothed tip until a suspension of single smooth muscle cells was generated. The mixture was then centrifuged at 300 G for 5 minutes to separate the cells from the digestion solution. The pellet was dispersed in fresh PRS, placed on collagen-coated glass cover slips (200 µl in each) and left to adhere for 30 minutes. The cells readily attached to the treated glass cover slips for the duration of the experiment.

Table 4.1: Compositions of the digestion mixtures

Constituent	Digestion medium 1	Digestion medium 2	Digestion medium 3
5% FCS in BSS (ml)	2	2	2
Collagenase I (units)	600	1200	1200
Elastase IV (units)	10	20	10
Dithiotreitol (mM)	-	-	5

The cells were then loaded with fura-2 by incubating for 20 minutes at room temperature in the fura-2 loading solution described previously. The fura-2 loaded cells were then washed with PRS three times before the cell coated cover slips (containing approximately 10 cells) were placed in a cell bath and mounted on the stage of the Nikon TE 200 inverted microscope. The cells were illuminated with the excitation wavelengths outlined above (in Section 4.2.1) and the fluorescence emission recorded while the cells were stimulated with noradrenaline or exposed to cooling from 33^{o}C to 15°C. The magnification of the objective lens was x40. Two to four cells were imaged at one time. The cells obtained were utilized within 6 hours of preparation. Enzyme dispersions were carried out using tissues from 4 rats. A total of 40 cells were imaged.

4.2.3 Calcium indicator measurement using cultured tunica dartos cells

Enzymatically dispersed tunica dartos muscle cells were also grown in primary culture for intracellular calcium measurement experiments. The freshly obtained tissues were first subjected to an enzyme process digestion (as described above) and then maintained in culture as outlined below.

The media used for digestion and dispersion of smooth muscle from the freshly obtained tunica dartos tissues is given in the first two columns of Table 4.1. The digestion enzymes were dissolved in fetal calf serum (FCS) in buffered salt solution (BSS).

The BSS was made using the compositions given in Table 4.2. The enzymes Collagenase I and Elastase IV were pre-dissolved in small volumes of BSS and stored at a temperature of -20^{o}C in aliquots adequate for single digestion. The medium was prepared fresh on the day of the culture.

Table 4.2: Composition of buffered salt solution (BSS).

Constituent salt	Concentration (g/l)
$NaH_2PO_4.H_2O$	0.021
Na_2HPO_4	0.1905
$NaHCO_3$	0.5
Glucose	1
NaCl	8
KCl	0.22
$CaCl_2$. $2H_2O$	0.1617
$MgCl_2.6H_2O$	0.2233
Phenol red (ml)	1

Following preparation of the desired volume of BSS, the pH was adjusted to 7.3 with a pH meter and the osmolarity was adjusted to295 mOsm/l using an osmometer (located in the culture lab). The solution was sterilized by filtering with a millipore 0.22 µm filter before being stored in the refrigerator for further use.

4.2.3.1 Cell culture medium

Since smooth muscle cells in culture can became non-contractile in the continued presence of serum, a serum deprived medium (SDM) similar to what has been used in airway smooth muscle cultures (Halayko *et al.*, 1999) was used in these culture studies. The compositions of the media used for cell culture in this study are given in Table 4.3.

Table 4.3: Composition of media used for primary culture of dartos

A – Growth medium, B – Serum deprived medium. **DMEM/F12 – Dulbecco's Modified Eagle Medium : Nutrient Mixture Ham's F-12 All chemicals were obtained from Invitrogen, Australia except ITS which was from Sigma Chemical Co., St Louis, MO, USA.

Growth medium (100 ml)	
DMEM/F12 (**) [ml]	95
Fetal calf serum [ml]	5
Streptomycin [mg]	10
Amphoteracin [µg]	25
Penicillin [units]	10000
Serum deprived medium (60 ml)	
DMEM/F12 (**) [ml]	59
ITS [µl]	600
Streptomycin [mg]	6
Penicillin [units]	6000

4.2.3.2 Procedure of primary culture of tunica dartos

Tunica dartos muscle strips were obtained as described previously (Chapter 2 – Section 2.2.1), placed in PRS and dissected under the microscope to obtain very small sections (1 mm x 1 mm) of smooth muscle. These were then placed in 15 ml Falcon tubes containing the digestion medium-1 (Table 4.1) in a shaking water bath at 37°C for 60 minutes after brief carbogenation. Digested smooth muscle bands were then washed and centrifuged twice (500 G for 5 minutes) before being suspended in digestion medium-2 (Table 4.1). The preparation was also subjected to brief carbogenation again prior to being incubated at 37°C for 60 minutes. After the second digestion, the cell suspension was centrifuged (500 G for 5 minutes) and washed in PRS after which it was

placed in the growth medium containing DMEM/F12 with 5% FCS (Table 4.3). At this stage the cells were round and the majority of cells were loosely associated. The cell suspension was then centrifuged again (500 G for 5 minutes) and the medium was aspirated and replaced with fresh growth medium (in order to remove all traces of the enzyme in the digestion mixture). Gentle trituration using a Pasteur pipette was used to dissociate the loosely associated cells into single cells. The cell suspension was then seeded on small Petri dishes containing collagen coated glass cover slips in a 2 ml volume of the growth medium (estimated final seeding density of 5×10^4 cells.cm^{-2}). The cells were maintained in a sterile humidified atmosphere of 95% O_2: 5% CO_2 at a temperature of 37°C until the cells achieved approximately 70% confluence (72 hours).

At this point the medium was aspirated completely and replaced with the SDM (Table 4.3). The cells were then maintained in SDM for 10-12 days until they were used for recording of calcium transients (4.2.2). The loading procedure for fura-2 was similar to that described in previous sections. Seventy five percent of the medium was replaced with fresh serum-deprived medium every 48 hours (Halayko *et al.*, 1999). A total of 84 cells were loaded and imaged from 7 cultures.

4.3 Results:

4.3.1 Calcium indicator measurements using whole muscle bundles from tunica dartos

The tunica dartos strips readily loaded with fura-2. The mean resting ratio of the dartos strips was 0.6234 ± 0.0507 (±SEM). Large force responses were elicited upon electrical field stimulation indicating that the dartos strips were fully functional (mean ± SEM of the 11 strip where force was measured 0.2194 ± 0.04 g) (Fig.4.3). (It was possible to measure force only in the case of muscle strips and not in the case of cells). However, an accurate transient change in intracellular Ca^{2+} was not observed following electrical field stimulation or exposure to noradrenaline. This was most likely due to large movement artefacts associated with the force response altering the florescence output and obscuring the true change in Ca^{2+}. The fluorescence ratio (340 nm / 380 nm) of fura-2 rose as would be expected during contraction. However, this increase was unlikely to be accurate. The fluorescence emission at 340 nm excitation should increase with increasing Ca^{2+} while the 380 nm emission should fall with increasing $Ca^{2+.}$ However, as can be seen in Fig. 4.2.A, both the 340 nm and 380 nm emissions decreased after stimulation, indicating the change in the ratio shown (Fig. 4.2.B) is not exclusively due to a true elevation of Ca^{2+}. Repeated attempts were made to measure calcium transients in the dartos muscle strips while concurrently recording force output (number of attempts: 21 muscle strips from 5 rats) but no believable changes in the ratio were observed.

Several attempts were made to inhibit the movement of the muscle strip using the myosin II inhibitor Blebbistatin, which is reported to inhibit force production without altering intracellular Ca^{2+} signalling (Kovacs *et al.*, 2004). In the presence of Blebbistatin (0.2 mg dissolved in 10 µl of DMSO), no recordable force response occurred in response to EFS or NA (Fig. 4.4).

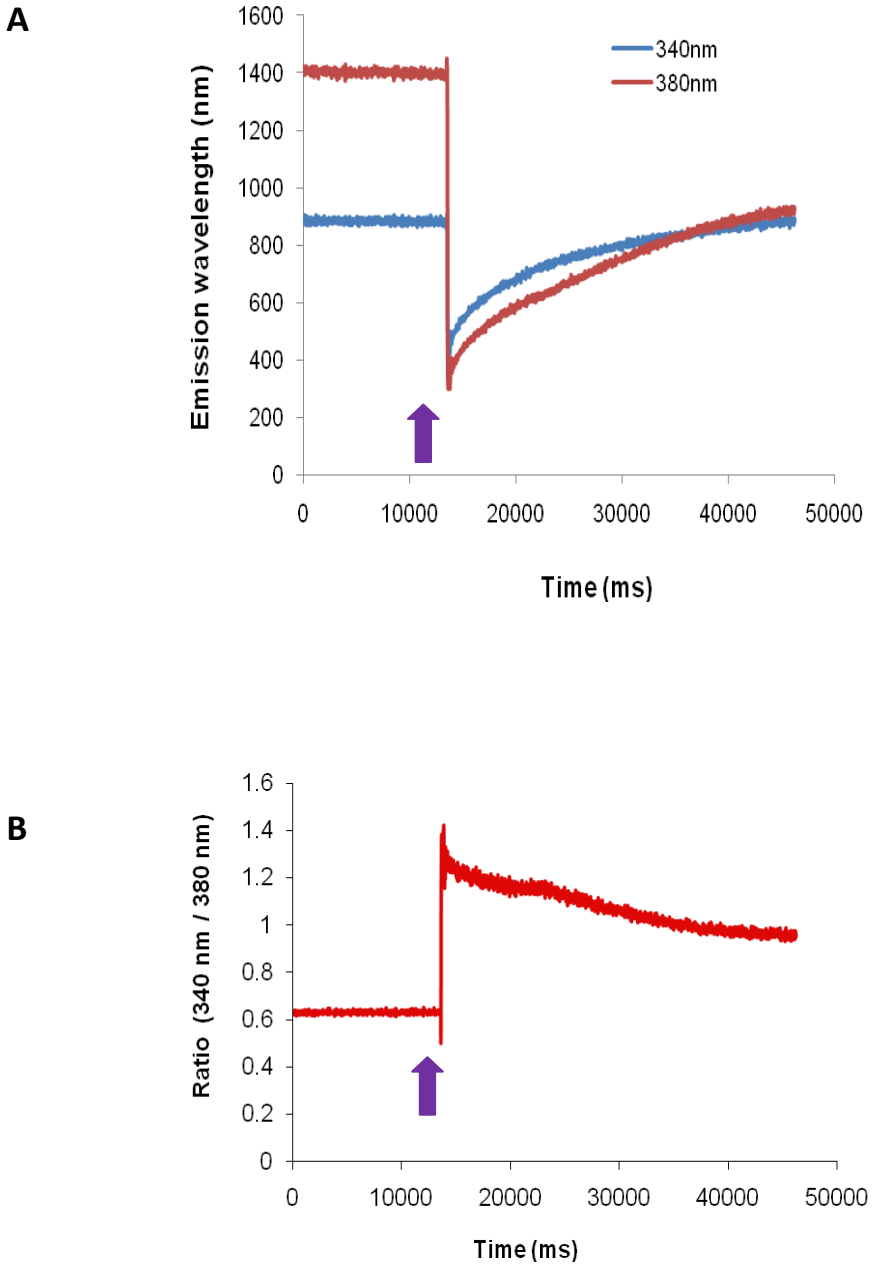


Figure 4.2: Ca^{2+} transients recorded from a tunica dartos muscle bundle stimulated with NA (A) shows emission waves of 340 nm and 380 nm and (B) shows the ratio (340 nm/380 nm).

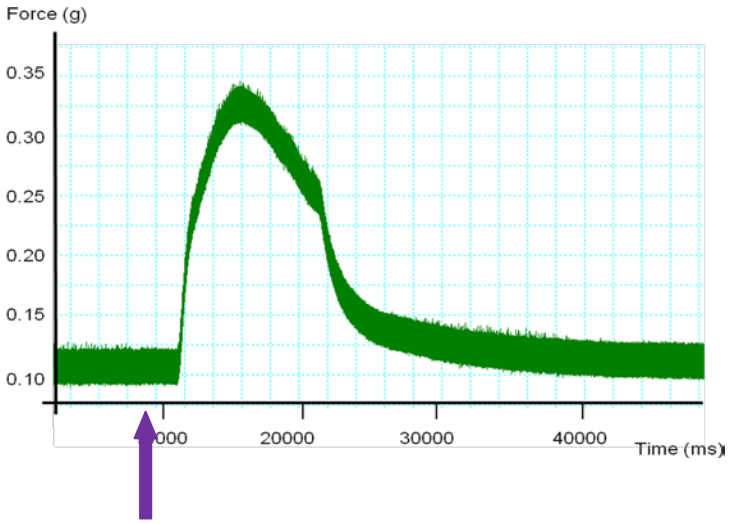


Figure 4.3: Force response recorded during stimulation of the tunica dartos muscle bundles with NA while concurrently recording Ca2+ transients shown in Figure 4.2.

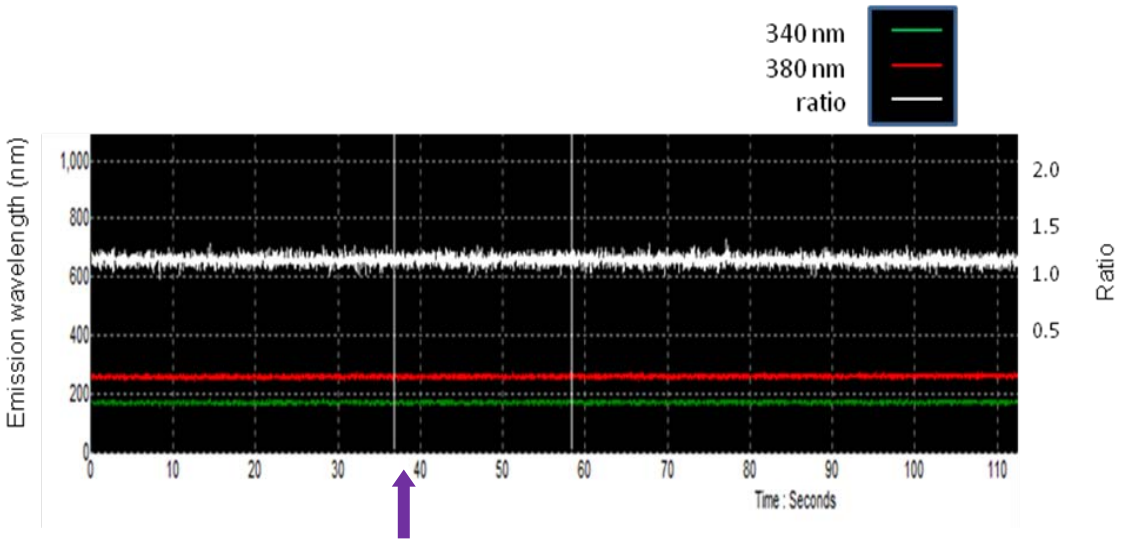


Figure 4.4: Ca^{2+} transients recorded from a tunica dartos muscle bundle stimulated with NA (at arrow) after treatment with Blebbistatin to remove movement artefact. The recordings indicate no change in the emission wavelengths of 340 nm and 380 nm or in the ratio.

The results referred to above prompted an investigation of other potential tunica dartos preparations for the measurement of cytosolic calcium during tunica dartos activation.

4.3.2 Calcium indicator measurements using enzyme dispersed cells from the tunica dartos

Experiments were undertaken attempting to measure Ca^{2+} transients in enzymatically dispersed single tunica dartos muscle cells. Ca^{2+} transients have been successfully recorded in dispersed cells from other smooth muscle types (Frangez *et al.*, 2008; Maruyama *et al.*, 1988). Within the samples of enzyme-dispersed cells, there appeared to be two types of cells present. First group was the larger and the most abundant and the dye distribution within the cell appeared somewhat less homogeneous. These exhibited a medium amount of resting fluorescence. The other group consisted of smaller cells that appeared to take up the dye less well, and the dye distribution was more homogeneous.

The larger granular cells were used for imaging because the mean resting ratio was 0.682 which was closer to what was observed in the cells of the intact dartos muscle strips studied previously. The Ca^{2+} transients that were observed were very small, and the success rate was approximately 10%. Four cells from 40 cells examined (taken from 4 cell dispersions) exhibited ambiguous changes in Ca^{2+} (Fig. 4.5). This was obtained using a maximum concentration of noradrenaline (10^{-4}M). Neither ATP nor cooling produced any change in intracellular Ca^{2+}in the cells used in this study. The smaller cells had a resting ratio of 0.832 on average and no cells produced a change in cytosolic Ca^{2+} in response to maximal noradrenaline exposure.

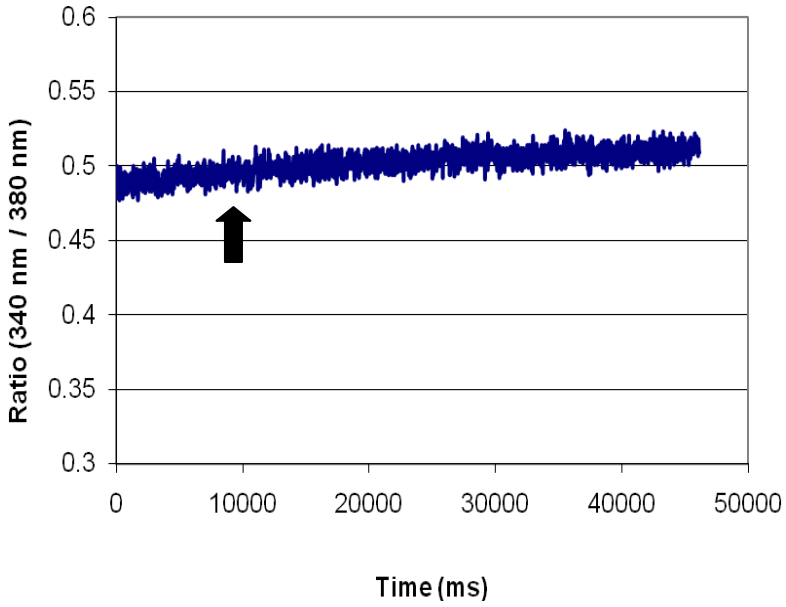


Figure 4.5: An example of a Ca^{2+} measurement obtained from the enzyme-dispersed cells. The cells were probably not in their optimum health due to the time lag between harvesting them and imaging. Black arrow indicates stimulation with NA.

4.3.3 Calcium indicator measurements using cultured tunica dartos cells

Ca^{2+} transients have also been measured in cultured smooth muscle types (Goldman *et al.*, 1990; Reynolds and Dubyak, 1986), so an attempt was made to determine whether tunica dartos muscle cells could be cultured and changes in cytosolic Ca^{2+} measured in response to NA activation. Since no published data or protocols on performing primary culture of tunica dartos existed, a protocol had to be established to obtain viable cells.

4.3.3.1 Obtaining a viable culture of tunica dartos cells

The procedure outlined in the methodology section 4.2.3.3 is the one that was found to be successful in producing viable cells in this study. The viability was tested using the 0.4% trypan blue stain exclusion method. Only non-viable cells took up the dye. Viable cells were able to exclude the dye, and these cells were counted using a haemocytometer (Freshney, 1987) at 48 hours after culture. Approximately 90% of the cells were found to be viable.

At 48 hours, the culture plates exhibited 60% confluence, with approximately 70% of the cells being elongated and 30% of the cells small and circular. If left in the serum containing medium for longer, the percentage of elongated cells decreased and the small rounded cells become the predominant type. From the available literature (Holstein *et al.*, 1974), it was assumed that the elongated cells most closely resembled functional smooth muscle cells. Serum deprivation is reported to maintain smooth muscle cells in the elongated, contractile state (Somlyo, 1985), and therefore, all cultured cells used in this study were grown under serum-free conditions.

4.3.3.2 Recording Ca^{2+} transients in cultured tunica dartos cells

The proportion of the cultured cells that responded with measurable calcium change following exposure to a maximum concentration of noradrenaline was 11% (Fig. 4.6). Further attempts at refining the protocol by addition of testosterone (4 $ng.ml^{-1}$), nutrients like glutamine, did not help improve the number of cells which responded to NA stimulation.

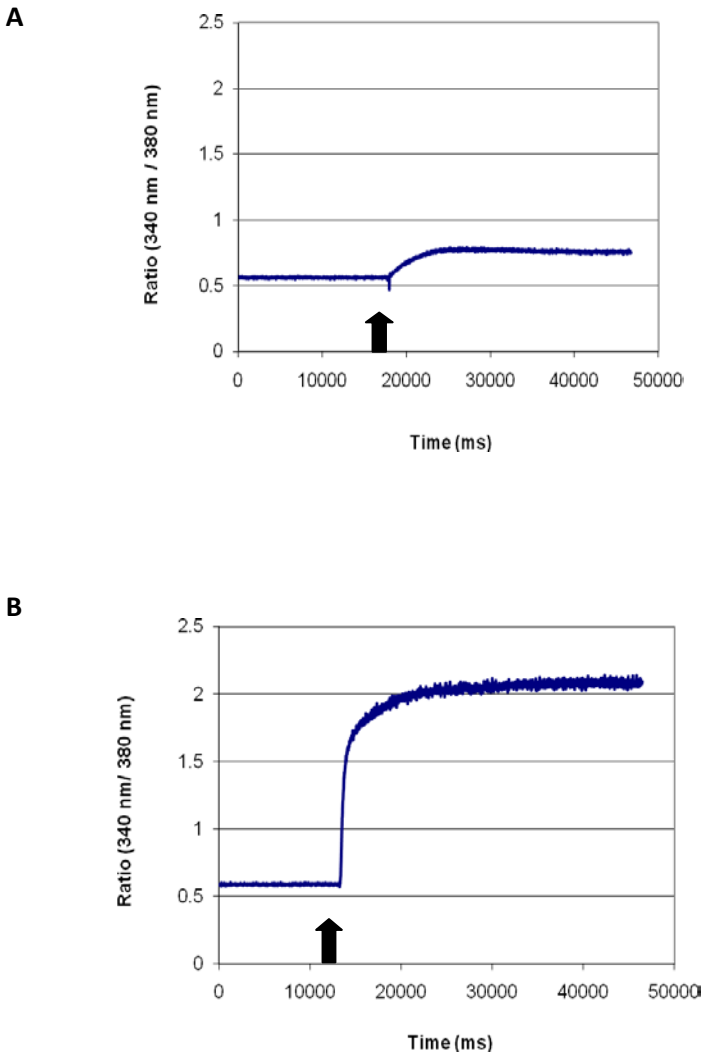


Figure 4.6: A typical calcium response elicited in cultured elongated smooth muscle cells (A) An example of a Ca^{2+} change observed in 11% of the cultured cells examined after exposure to a maximum concentration of the agonist NA (B) An example of the change in fluorescence ratio observed in response to ionomycin. The ratio remained elevated for the rest of the time. Arrow indicates the stimulus.

Table 4.4 shows the peak amplitudes (Amp) of the ratios of Ca^{2+} transients observed during stimulation with NA and ionomycin using the cultured cells. The mean amplitude of the Ca^{2+} transients measured after exposure to NA was 18.85% of the maximum ratio amplitude possible using the ionophore, ionomycin (a compound that creates Ca^{2+} permeable pores in the plasmalemma) (Table 4.4). There was no difference in the average time to peak from that obtained with using ionomycin ($p = 0.7332$, $n=9$).

Table 4.4: The peak amplitudes (Amp) of the calcium changes observed during stimulation with NA and Ionomycin (n = 9)

Peak amplitudes of ratios for NA stimulation (340nm/380 nm) [Amp]	Peak amplitudes of ratios for Ionomycin [Amp]
0.327	1.216
0.078	1.156
0.562	1.326
0.199	1.187
0.175	1.047
0.154	1.132
0.223	1.228
0.158	1.476
0.213	1.203
0.232 ± 0.047	1.219 ± 0.04

In addition to producing a sustained rise in cytosolic Ca^{2+}, a few of the cells (4 out of the 84 cells used) also showed small oscillations in the fluorescence after stimulation with NA (Fig. 4.7).

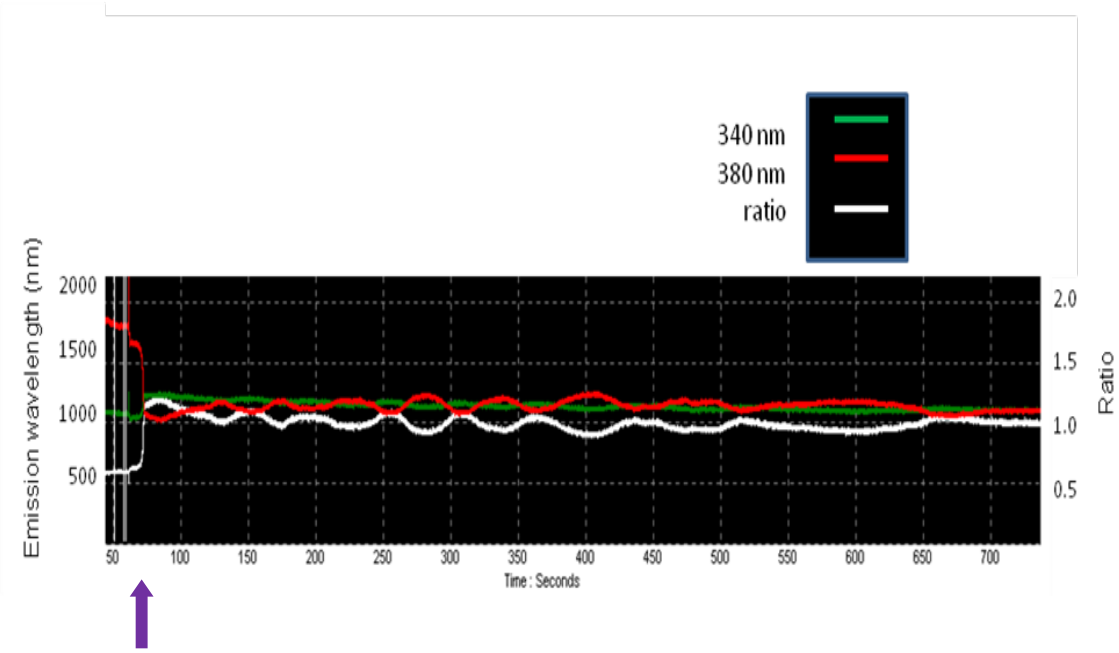


Figure 4.7: The oscillations in the ratio observed after stimulation with NA in a few cultured smooth muscle cells. Arrow indicates the stimulation with NA.

The main problem faced in using the cultured cells was the inability to stimulate again once NA was used and removed by washing in order to study the cooling response of the same cells. No cells responded to cooling (33^{o}C to 15^{o}C) with a change in intracellular Ca^{2+}. Therefore, cultured tunica dartos cells did not provide reliable preparations for measurement of the effect of cooling to 15°C in intracellular Ca^{2+} in this study.

4.4 Discussion:

Measurement of Ca^{2+} transients was undertaken using the fluorescent Ca^{2+} indicator Fura-2. Since this was the first time such measurements were undertaken, the exact form of the tissue/cells that should be used was uncertain. Lack of literature in this area made it necessary for all steps of the experiment to be conducted on the basis of trial and error. Both freshly obtained strips of tunica dartos muscle and enzyme dispersed cells of the muscle strips obtained failed to produce Ca^{2+} transients. The cultured cells produced small Ca^{2+} transients in response to agonist stimulation in 11% of the cells tested, and Ca^{2+} oscillations in 4% of the cells tested.

The first approach was to attempt to measure cytosolic Ca^{2+} changes in intact muscle strips, similar to that used for force measurements (Chapters 2 & 3). Force was measured concurrently in the muscle strips in order to compare the Ca^{2+} and force changes during cooling. However, the apparent change in Ca^{2+} measured (signified by the change in the 340/380 nm fluorescence ratio, Fig. 4.2 B) could not be relied upon because the fluorescence emission at 340 nm decreased during activation, when it is known to increase with increasing Ca^{2+} concentration (Sato *et al.*, 1988).

The working hypothesis to explain this result was that the movement produced by the contracting muscle bundle was producing artifactual changes in the fura-2 fluorescence that was masking the actual change in Ca^{2+}. Experiments using the myosin-II inhibitor, blebbistatin (Kovacs *et al.*, 2004) were then performed in order to test this hypothesis. In the presence of blebbistatin, the force responses elicited in the dartos strips were reduced to almost zero, indicating that blebbistatin effectively inhibited contraction in this smooth muscle. However, in the absence of any movement, no change in cytosolic Ca^{2+} occurred in response to NA application. Poor dye loading was not a contributing factor, as the muscle tissue successfully took up the Ca^{2+} indicator, as shown by the ample resting fluorescence in the samples (e.g. note the basal

fluorescence levels in for the 340 nm and 380 nm traces in Fig 4.2A). From the findings outlined in Chapter 2, removal of external Ca^{2+} or inhibition of sarcoplasmic reticulum Ca^{2+} release with 2-APB had marked inhibitory effects on contractile input in the dartos muscle preparations. These results show that changes in intracellular Ca^{2+} are essential for contraction in the tunica dartos muscle. Therefore, it would appear that the intracellular Ca^{2+} must be changing in some way, but the present experimental procedures were unable to detect this change.

One possible explanation is that the change in intracellular Ca^{2+} may be occurring in the form of oscillations rather than a large sustained rise. Intracellular Ca^{2+} signaling in the form of Ca^{2+} oscillations has been reported in smooth muscle from the airways of various species (Sanderson *et al.*, 2008). These Ca^{2+} oscillations were found to be mediated by the repetitive release of Ca^{2+} from the sarcoplasmic reticulum, with store- mediated Ca^{2+} influx required to maintain sufficient sarcoplasmic reticular Ca^{2+} levels (Prakash et al, 1997). Ca^{2+} oscillations have been shown to be both synchronous and asynchronous in multicellular intact smooth muscle preparations (Ruehlmann et al, 2000). The failure to demonstrate the presence of Ca^{2+} signalling in the whole muscle bundles of tunica dartos muscle used in this study may have been due to the existence of Ca^{2+} oscillations, which occurred asynchronously in individual smooth muscle cells, and were therefore out of phase with each other and hence unidentifiable. However, if increased asynchronous Ca^{2+} oscillations did occur after NA application, one might expect that there would be an increase in the noise levels of the Ca^{2+} signal, but such an increase was not observed in this study (see Fig.4.4).

Past studies have demonstrated the existence of Ca^{2+} oscillations in smooth muscle using confocal microscopy (e.g. Ruehlmann et al, 2000), which can be used to detect Ca^{2+} changes within individual smooth muscle cells in intact tissue. Unfortunately, a confocal microscope was not available for use in the present study.

However, future studies using confocal microscopy could be used to determine the effect of temperature and contractile agonists on Ca^{2+} signalling in the tunica dartos muscle.

An attempt was made to identify Ca^{2+} oscillations in individual tunica dartos cells by examining Ca^{2+} signalling in single enzymatically isolated cells. However, no Ca^{2+} signalling was observed in the enzymatically isolated cells. This is most likely because the cells did not appear to remain fully functional after the digestion/dispersion process and the fura-2 loading procedure. The cells failed to respond to any known agonists with a change in cytosolic Ca^{2+}. Even the Ca^{2+} ionophore, ionomycin, failed to alter intracellular Ca^{2+} suggesting the cells were non-viable.

This study described the first attempts to grow tunica dartos cells in culture in order to provide cells for use in experiments to identify Ca^{2+} oscillations in individual tunica dartos cells. Smooth muscles are known to become non-contractile in culture in the presence of serum (Chamley-Campbell *et al.*, 1979). In airway smooth muscle this has been overcome by depriving the cells of serum (Halayko *et al.*, 1999) after they reach a certain confluence in the culture. The serum-free medium prompts the muscle cells to differentiate into the contractile phenotype from the proliferative, secretary phenotype (Halayko *et al.*, 1999). Although the cultured cells were grown under serum-deprived conditions in the present study, only 11% of the cells responded to the contractile agent NA by producing a change in intracellular Ca^{2+}.

This raised the possibility that many of the cells were not smooth muscle cells. Of all smooth muscles, primary culturing of smooth muscles of blood vessels has been the most commonly reported method (Chamley *et al.*, 1977a; Bodin *et al.*, 1987; Berta *et al.*, 1986). The media of mammalian blood vessels consists entirely of smooth muscle cells (Karrer, 1961). Therefore, by removing the adventitia and intima, pure cultures of vascular smooth muscle could be obtained (Ross and Kariya, 1980). As opposed to this,

visceral smooth muscle tissue (such as those of vas deferens, taenia coli, ureter, and uterus) usually has fibroblasts distributed throughout the muscle and, therefore, cultures of these tissues are known to contain a mixture of smooth muscle and fibroblasts (Chamley and Campbell, 1975; Chamley *et al.*, 1973). This may also be the case in the tunica dartos. Firstly, it is very closely associated with the skin of the scrotum (Holstein *et al.*, 1974) so much so that smooth muscle cells are reported to be found in the corium of the skin of the scrotum. Secondly, in the subcutis, where the smooth muscles are arranged into longitudinal and transverse bundles, the smooth muscle cells have a large amount of connective tissue and blood vessels running amongst them. Therefore, a primary culture of this tissue would contain a variety of cells like fibroblasts, cells from blood vessels and even epithelial cells from the skin. Smooth muscle cells of primary and subcultures are reported to have a morphological similarity to fibroblasts (Chamley-Campbell *et al.*, 1979). The presence of a large proportion of non-smooth muscle types would explain why such a large percentage of the cultured cells did not respond to the known smooth muscle agonist, NA with a change in intracellular Ca^{2+}. All cells studied appeared viable, as they took up, and metabolized the fura-2AM into the Ca^{2+} sensitive fluorescent form of the indicator and they all responded to the Ca^{2+} ionophore, ionomycin, with a large sustained increase in Ca^{2+}.

One way of differentiating is to stain with fluoresceinated antibodies against smooth muscle actin and tropomyosin, which does not stain the fibroblasts (Chamley *et al.*, 1977). This was beyond the scope of the present project. However, this would be the logical next step to follow in further studies that require culturing the dartos. Performing a sorting technique to separate smooth muscle cells from the other cells such as performing flow cytometry following fluorescent labeling of smooth muscles may be a possible next step.

Even though only a small proportion of the cultured cells responded to stimulation in this study, Ca^{2+} oscillations were successfully measured in response to NA in a small proportion of the cultured cells examined (4 out of 84 cells). Small but prolonged elevations in Ca^{2+} were also observed in the cultured cells (10 out of 84 cells). These results suggest that some of the cultured cells were of smooth muscle origin. However, the low success rate for obtaining Ca^{2+} signals the cultured cells and the fact that the responding cultured tunica dartos cells were incapable of being repeatedly stimulated, made it impractical to use these cells for experimentation. If these obstacles could be overcome through further experimentation, the cultured tunica dartos cells could be used to provide information about the effect of temperature on Ca^{2+} signalling in the tunica dartos. While it has been well established that cultured smooth muscle may not accurately represent the behaviour of intact in vivo smooth muscle (Chamley-Campbell *et al.*, 1979), these results suggest that tunica dartos cells may have the capacity to generate oscillatory Ca^{2+} signals in vivo.

CHAPTER 5

The effects of dysregulation of scrotal temperature on the testicular endocrine function in rams

5.1 Introduction:

5.1.1 The importance of regulating testicular temperature

The factors that contribute to the maintenance of testicular temperature have been discussed in detail in the introductory chapter of this thesis. Therefore, the physiological mechanisms that contribute to the maintenance of testicular temperature and the anatomical features that play an important role need not be reiterated here.

In clinical medicine there are at least three situations that affect scrotal temperature and thereby affect fertility. These are cryptorchidism, varicocele, and fever. Cryptorchidism, or non-descent of the testes, has been recognized for many centuries as a cause of infertility (Fukui, 1923; Moore, 1924; Moore and Quick, 1924). Varicocele is the abnormal enlargement of the scrotal veins and the degree of enlargement is positively correlated with elevated testicular temperature (Goldstein and Eid, 1989). Moreover, surgical correction of the varicocele reduces the testicular temperature (Wright *et al.*, 1997) and improves fertility (Agger, 1971). Fever has not been studied much as a cause of infertility in humans. In a study using rams, fever did not cause a significant change in the scrotal temperature (Maloney and Mitchell, 1996). However, fever is recognized among clinicians to reduce sperm count and quality (Carlsen *et al.*, 2003; Levine *et al.*, 1990).

Primary spermatocytes and early spermatids are the stages of sperm development most vulnerable to heat exposure. There is also an accompanying

generalized thinning of the germinal epithelium (Gomes and Jain, 1976; Yin *et al.*, 1997). p53 is a tumour suppressor gene that produces a protein which arrests the cell cycle in damaged cells. This causes the cell to undergo apoptosis or programmed cell death. Apoptosis in cells are known to occur through both p53 dependent and p53 independent pathways which are interrelated (Lowe *et al.*, 1993; Fisher, 1994). Apoptosis or programmed cell death is now recognized as the cellular process that leads to germ cell loss and it is thought to occur through several pathways, both p53-dependent and p53- independent (Lowe *et al.*, 1993; Fisher, 1994).

In almost all of the large number of published studies on testicular temperature, the effect on sperm quality and quantity seems to be the main focus. There are few studies addressing the effects of temperature on the other function of the testes, steroidogenesis. Su *et al.* (1995) reported an improvement in the serum testosterone levels after varicocele repair in humans. Because spermatogenesis requires testosterone, this observation raises the possibility that the effect of heat on spermatogenesis might be mediated by lower intra-scrotal testosterone. Further, there is some evidence to support the notion that altered hormones might cause apoptosis of the germ cells. Withdrawal of testosterone is reported to induce germ cell apoptosis (Tapanainen *et al.*, 1993; Troiano *et al.*, 1994) and in bilaterally cryptorchid mice, apoptosis is preceded by a reduction in testicular testosterone (Ohta *et al.*, 1996). Testicular testosterone is also reduced in the cryptorchid testes (Keel and Abney, 1981; Sharpe *et al.*, 1986). On the other hand, in vitro studies suggest that heat causes germ cell damage even in the presence of adequate hormone levels (Yin *et al.*, 1998).

In patients with varicocele there is a decreased responsiveness of the Leydig cells to intramuscular injections of human chorionic gonadotrophin (Ando *et al.*, 1983; Castro-Magana *et al.*, 1990) while varicocelectomy increases serum

testosterone levels (Su *et al.*, 1995). Taken together these results suggest that altered endocrine function of the testes may occur with changes in scrotal temperature, and that the altered endocrine function might contribute to infertility.

In this part of my study, I attempt to fill the gap in knowledge that exists regarding how changing scrotal temperature affects the endocrine function of the testes. Since the endocrine function of the testes is under the control of the hypothalamus and the pituitary, and since the basic control mechanisms of the hypothalamo-pituitary-gonadal (HPG axis) were dealt with in detail in the first chapter, here I will only outline the literature available on the reproductive axis of the ram.

5.1.2 Reproductive endocrine axis of the ram

The basic interactions between the components of the reproductive endocrine axis are illustrated in Fig. 5.1.

Endocrine activity of the reproductive processes in rams, like in all mammals, is controlled by the hypothalamo-hypophyseal-gonadal (HPG) axis. Gonadotrophin-releasing hormone (GnRH), a neuro-hormone secreted by the hypothalamus, is the substance that determines the activity of the hypothalamo-pituitary-gonadal axis (Caldani *et al.*, 1988; Jansen *et al.*, 1997). Kisspeptins, a family of neuropeptides secreted from the hypothalamus, have recently been implicated as upstream regulators of GnRH secretion (Popa *et al.*, 2008; Colledge, 2008). The discovery of kisspeptins and its relevance to the reproductive system has been the highlight in reproductive endocrinology in the past decade.

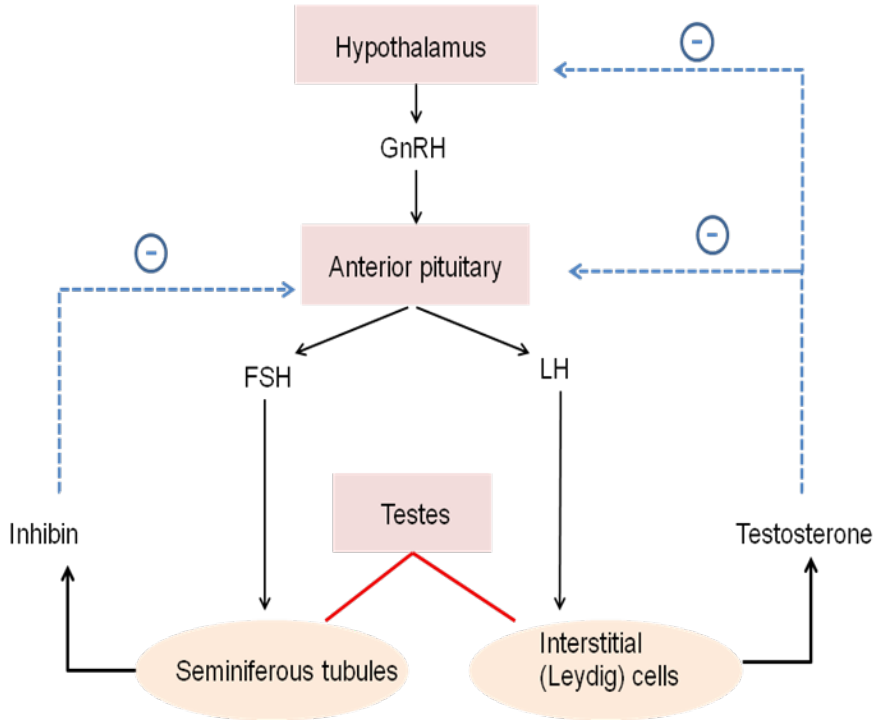


Figure 5.1: Schematic representation of the hypothalamo-pituitary-gonadal axis which indicates the positive and negative controls of the various components [Diagram based on (Sherwood, 2001)].

GnRH is secreted in a pulsatile manner from the hypothalamus and acts on the pituitary gonadotrophs to promote the secretion of luteinizing hormone (LH). LH is secreted in a pulsatile manner in a 1:1 relationship with GnRH (Clarke and Cummins, 1982). Pituitary derived LH acting on the Leydig cells of the testes stimulates the secretion of testosterone (Sanford *et al.*, 1974b). Testosterone thus secreted, has a negative feedback inhibitory effect on hypothalamic GnRH secretion, reducing its pulse frequency (Tilbrook *et al.*, 1991; Jackson *et al.*, 1991) a process now known to involve changes in kisspeptin (Popa *et al.*, 2008).

In sheep, GnRH pulse frequency is influenced by many internal and external factors in addition to the testosterone level in blood. Some of these are

metabolic inputs from hormones such as insulin, the duration of the photoperiod, nutritional status, and socio-sexual cues. The relative responsiveness to these factors depends on the breed of ram, and in some breeds factors like nutrition and photoperiod (Blache *et al.*, 2000; Miller *et al.*, 1998; Zhang *et al.*, 2004) can be so strong that they override the powerful inhibitory signal of testosterone (Blache *et al.*, 2003). Whether core or scrotal temperature influences the HPG axis is unknown.

5.1.3 Experimentation on the reproductive endocrine axis of animal models

Experimentation on hormone profiles in live animals is complicated by the fact that it is influenced by the hormones from the hypothalamus and pituitary gland. There are many ways in which the hypothalamic and pituitary influences could be minimized.

5.1.3.1 Blockade of action of GnRH

The action of GnRH on the pituitary gonadotrophs to cause LH secretion can be blocked using an antagonist of GnRH receptors (Clayton *et al.*, 1982). It can also be achieved by using a super-agonist of GnRH that generally causes hyper-stimulation of the pituitary followed by suppression.

5.1.3.2 GnRH super-agonist Lucrin

Lucrin, or Leuprorelin acetate, is a synthetic nonapeptide analogue of the naturally occurring gonadotrophin-releasing hormone (GnRH). The analogue possesses greater potency than the natural hormone. While leuprorelin acetate is chemically unrelated to steroids, it acts as an inhibitor of gonadotrophin production when given

continuously. Chronic administration of a GnRH super agonist causes an initial hyper-stimulation of LH followed by a marked decrease in both LH and FSH (Fraser and Lincoln, 1980; Haynes *et al.*, 1977).

Investigating the effect of scrotal temperature on the endocrine function of the testes has not been undertaken in large mammals. It is with this background and knowledge that the present study was undertaken to investigate the effect of local testicular temperature on the endocrine activity of the testes. The hypothesis in this experiment was that dysregulation of scrotal temperature would reduce the levels of testosterone in the blood and cause derangement of the hypothalamo-pituitary-gonadal axis.

The specific aims of the present experiment were therefore, to:

- investigate whether scrotal temperature could be effectively manipulated both above and below the normal level
- study the effect of local scrotal temperature manipulation on steroid output from the testes

5.2 Materials and Method:

5.3.1 Experiment design

All procedures were conducted with the prior approval of the University of Western Australia Animal Ethics Committee (AEC RA/3/100/827).

Nine mature Merino rams (weighing 67.1 ± 4.8 kg, mean ± SD and 2-3 years old) were transported to, and housed in individual pens in, the large animal facility (LAF) at the University of Western Australia within a temperature-controlled room. They were provided with 14 hours light and 10 hours dark, and fed with standard ration once a day (before experiments began) and were allowed free access to water. They were acclimated to captivity for two weeks after which surgical implantation of temperature loggers for the measurement and recording of scrotal and core temperature took place with the rams under general anaesthesia. Rams were allowed a minimum period of 14 days for post-operative recovery before the experimentation began.

Each ram was exposed once to each of three treatments; i.e. (i) scrotal warming with insulation packs made with three layers of air filled plastic (bubble wrap), (ii) scrotal cooling with specially designed cold packs made using freezer bags and bubble wrap and (iii) a control intervention involving no manipulation of scrotal temperature. The cooling and insulating interventions were carried out for a period of 8 hours on two consecutive days, with 3 rams receiving the same treatment at a given time (Fig.5.2). Between treatments there was a 5 day gap of an intervention-free period after which each ram group received a different treatment.

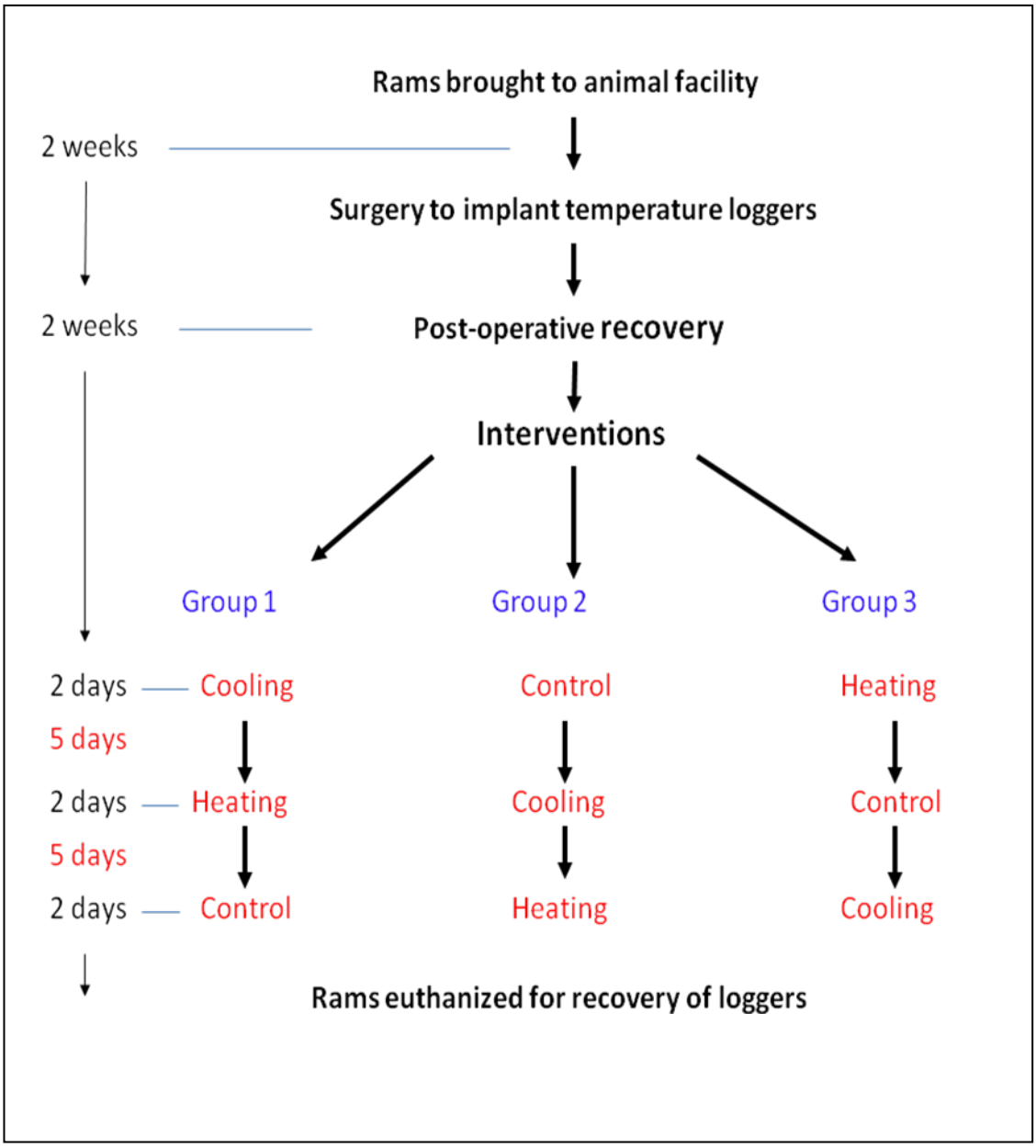


Figure. 5.2: Flow chart summarizing the design of the experiment.

This design allowed each animal to be its own control. The rams were allocated initially into the three groups randomly. During the first day of the intervention, the scrotum was insulated, cooled or left without manipulation. Blood samples of 3 ml were withdrawn through a jugular venous catheter (see below) every 20 minutes for 8 hours. The samples were centrifuged immediately and the plasma frozen for later

analysis of luteinizing hormone (LH) and testosterone levels. On the second day, similar pulse bleeds were performed following a bolus injection of the GnRH super-agonist leuprorelin. At the end of the experimental period, the thermo loggers were recovered after euthanizing the animals with an overdose of sodium pentobarbitone (Lethabarb).

5.3.2 Calibration of temperature loggers (i-buttons)

Temperature recording was performed using i-buttons (i-button, Maxore USA) (17mm diameter and 4g weight) implanted into the abdominal cavity and the scrotum. They recorded temperatures every 10 minutes for a period of 28 days. The i-buttons were calibrated twice against a certified mercury in glass thermometer, at three temperatures of 30°C, 35°C and 40°C, once prior to launching and once after recovery at the end of the experiment. Calibration equations were constructed.

5.3.3 Surgical procedure for implanting i-buttons

Each ram was implanted with two temperature loggers one in the peritoneal cavity and the other in the scrotal sac. Surgery was performed while the rams were under general anaesthesia induced with intravenous sodium thiopentone (20mg/kg) and maintained with inhalation of 3% isoflurane in oxygen following endotracheal intubation. Arterial oxygen saturation, respiratory rate, heart rate and tidal volume were monitored throughout surgery.

The ram was positioned in the right lateral position. The sites of surgical incision were clipped free of wool, shaved, and scrubbed with chlorhexidine and povidone iodine. The peritoneal cavity was accessed via a paralumbar incision and blunt dissection through the muscle and the fascial layers. The wax coated i-button (17mm in diameter and 4g in weight) was inserted into the peritoneal cavity and sutured in place with non-absorbable Vetafil suture. The i-buttons were sutured to the fatty connective

tissue of the paralumbar region so that they would lie in the peritoneal cavity in the flank area of the rams. The overlying muscle and fascia were sutured with 2.0 Vicryl and the skin was sutured with 1.0 Vicryl.

Similarly, a mid-scrotal incision was made in the skin after which blunt dissection was performed through the superficial scrotal fascia to reach the space between the testes. The wax-coated i-button buttons (17mm in diameter and 4g in weight) was placed between the testes and sutured in place using Vetafil suture. Strict aseptic procedures were followed at all times and a dose of a broad-spectrum antibiotic was administered intra-operatively (1 ml.25 kg^{-1} Norocillin i.m.). The entire surgical procedure lasted approximately 25 minutes.

The rams were allowed to recover with postoperative analgesia, using Carprofen 5mg/kg s.c. administered once prior to general anaesthesia and again 12 hours later. Analgesia was maintained with Carprofen 5mg/kg s.c. daily for the next 2 days, following the guidelines recommended by the Canadian Council for Animal Care.

Postoperative monitoring was carried out twice daily in the immediate postoperative period of 72 hours, for changes in physiological parameters and behaviour such as eating and drinking patterns, general alertness and responsiveness, passage of urine and faeces, presence of startle reflex, presence of guarded appearance and a change in body temperature. Thereafter, examination was done once daily for the rest of the two-week period.

5.3.4 Jugular venous cannulation

Two weeks after the surgical procedure when the wounds were healed and the rams were in good health, jugular cannulation was performed to facilitate sequential

blood sampling. Sedation was achieved with a dose of Ilium Xylazil 100 (Rompun) 0.1 ml i.m. injected 10 minutes before the procedure. The neck wool was shorn and the skin was cleaned with surgical spirit before inserting the catheter into the internal jugular vein under aseptic conditions. All catheters were flushed and kept filled with heparinised saline (40 $IU.ml^{-1}$). Three-way taps were fixed to catheters on the day of the pulse bleeds. The catheters were also flushed with heparinised saline (5 $IU.ml^{-1}$) after each bleed.

5.3.5 Scrotal temperature manipulation

Warming of the scrotum was attempted using a pack made with three layers of bubble wrap and duct tape. Cooling was done by a specially designed pack made of gel filled ice packs, bubble wrap, and tape. There was a layer of bubble wrap between the scrotal skin and ice packs to prevent cold injury to the skin. The cold packs were changed every 90 minutes, a time determined during trials prior to the actual experiments with skin thermocouples applied to the scrotal skin to confirm that the interventions were actually manipulating scrotal skin temperature.

5.3.6 The pulse bleeds

Pulse bleeds were carried out for an 8 hour period over two consecutive days starting at 9.00 a.m., during which time scrotal temperature manipulation took place. A volume of 3ml of blood was drawn from each ram every 20 minutes. The catheter was kept filled with heparinised (5 $IU.ml^{-1}$) isotonic saline between blood samples. During a single pulse bleed, a volume of 3ml, which contained a mixture of saline and blood, was drawn using a syringe fixed to one end of the three way tap, after which the actual blood sample was drawn using a syringe fixed to another spigot of the 3-way tap. The blood that was withdrawn initially (mixed with heparinised saline) was then

injected back after which the catheter was flushed with 3 ml of 5 $IU.ml^{-1}$ heparinised saline, equivalent to the volume of the blood withdrawn. The blood sample was immediately transferred into a plastic tube containing EDTA, and centrifuged. Plasma thus obtained was stored in plastic tubes at -20°C until analysed.

5.3.7 GnRH challenge

The synthetic GnRH superagonist leuprorelin acetate (Lucrin) was injected subcutaneously (0.2 mg) to all rams on day two just before the beginning of the second hour of pulse bleeds. By the end of the experiment, each ram had received 3 doses of the GnRH analogue, one week after the previous dose.

5.3.8 Recovery of i-buttons

At the end of the experimental period, the i-buttons were recovered from the scrotum and the peritoneum after the rams were euthanized with an overdose of Lethabarb (sodium pentobarbitone) 150 $mg.kg^{-1}$ i.v. Any signs of infection around the loggers were noted. Temperature data were downloaded from the i-buttons using the 'eTemperature' software package (*version 5.10)* and calibrations applied as determined for each logger during the calibration procedures.

5.3.9 Hormone Assays: LH

LH was measured by a double antibody RIA technique (Scaramuzzi *et al.* 1970) that was described and validated by Martin *et al.* (1980), with further modifications that were described by Martin *et al.* (1994). Each assay had 3 standard curves which were located at the beginning, middle and end of the assay, and each standard curve included 4 tubes of total counts (TC), 4 tubes of non specific binding

(NSB), 9 replicates of zeros, 3 replicates of each standard, and 6 replicates each of three quality control samples.

5.3.9.1 Buffers

The phosphate buffer solutions were prepared according to Table 5.1.

Table 5.1: Constituents and their concentrations of the different phosphate buffers used for LH assay

	Buffer # 1 (g/l)	**Buffer # 2** (g/l)	**Buffer # 3** (g/l)	**Buffer # 4** (g/l)
Na_2HPO_4	12.26	12.26	12.26	12.26
$NaH_2PO_4.2H_2O$	2.22	2.22	2.22	2.22
NaN_3	1	1	1	1
NaCl	8.9	8.9	8.9	8.9
Other constituents		% BSA	% BSA 0.05M EDTA NRS	1% BSA 0.001M EDTA
pH	7.5	7.5	7.5	7.5

BSA= bovine serum albumin (BSA, Fraction V, Sigma) NRS = Normal rabbit serum

5.3.9.2 LH standards

Ovine LH for standard and iodination (NIDDK-oLH-I-4 (AFP-8614B)) was obtained from Dr Parlow (National Hormone & Peptide Program). The stock solution (32 $ng.ml^{-1}$) was stored in 4 ml aliquots in Buffer #2 at -20˚C. One aliquot was thawed and diluted serially to the following concentrations: 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 $ng.ml^{-1}$ in Buffer #2 on the day of assay.

5.3.9.3 Antiserum to LH

The antiserum R1 was raised in our laboratory. It was diluted to 1:125,000 in Buffer #3 with 1:500 normal rabbit serum (NRS), to give a final titre of 1:1,250,000 (dilution in the assay tube). Cross reactions of this antibody were 100% with NIH-LH-S1, 97% with NIH-LH-S20, 100%, 18% with NIAMDD-oFSH-RP1, 0.93% with NIH-FSH-S12, 8.2% with oGH and 5% with NIH-TSH-S8.

5.3.9.4 Second antibody

Donkey anti-rabbit serum was diluted to 1:60 in Buffer #1 (Sapphire Bioscience, Australia G4004).

5.3.9.5 Iodination of LH

The hormone (stored in 5 µg aliquots in 10 µl 0.05M phosphate buffer not containing BSA or azide) was iodinated by the lactoperoxidase method and purified using a Sephadex G100 column (Pharmacia, Sweden). Sephadex G100 was soaked in buffer #1 for 3 days at room temperature (25˚C), and subsequently poured into a K9 column (30 cm x 0.9 cm i.d.; Pharmacia, Sweden). The column was then equilibrated with 30 ml Buffer #1 with 2% BSA. Fifteen micrograms of Lactoperoxidase (120 $U.mg^{-1}$ protein, Sigma, Bovine milk, lyophilised powder) was dissolved in 50 µl of 0.05M phosphate buffer prior to iodination, and 10µl of this was added to 5 µg of the hormone in a reaction vessel. Ten microlitres of NaI^{125} (1 mCi) (Perkin Elmer, NEZ033A) was added to the mixture, followed by 5 µl of H_2O_2 solution (0.006% in double deionised water) and vortexed for 20 seconds. The reaction was stopped by adding 200 µl KI/Albumin/Azide (made as 1:1 mixture of KI (20 mg/ml) in PBS) and albumin/azide (10% BSA in Buffer #1). The mixture was then transferred onto a Sephadex G100 column, and the reaction vessel was washed with 200 µl of Buffer #4, and then run onto

the column. Finally the column was eluted with 30 ml of the same buffer, and fractions were collected in 13 x 100 mm glass culture tubes (Borex, Australia) using a fraction collector. The activity of the fractions was determined on a gamma counter, and the fractions around the peak were tested for percent binding and non-specific binding (NSB) and then diluted to a stock solution of 250 000 cpm/50µl in Buffer #3 and stored at -20˚C for up to 4 weeks.

5.3.9.6 Assay procedure for LH

All unknown plasma samples were assayed in duplicate. Each assay included three standard curves, which were located at the beginning, middle and end of the assay.

On Day 1, standard or unknown plasma (100 µl) were diluted to 400 µl with Buffer #1 in 11 x 75 mm polystyrene assay tubes. The first antibody (50 µl) was then added to each tube except the TCs and NSBs. The assay was incubated at 4˚C overnight before adding 50 µl of labelled tracer (approximately 10,000 cpm) on Day 2 to all tubes. After further incubation at 4˚C, on Day 5, 100 µl of the second antibody, diluted in Buffer # 1, was added. The tubes were vortexed for 5 seconds after the addition of each reagent. On Day 6, 1.0 ml of Buffer #1 was added to each tube except the TCs. The assay was centrifuged at 1500 G for 25 minutes (Beckman, J-6M/E, USA), and the supernatant was decanted. The tubes containing the precipitate were counted for two minutes each in a gamma counter (Packard Cobra-II, Auto Gamma) after drying at room temperature.

5.3.10 Hormone Assay - Testosterone

The plasma testosterone levels were measured using a modification of the method described by Puri *et al.* (1981). Each assay had two standard curves which consisted of triplicate tubes of total count (TC) and non specific binding (NSB), 10 replicates of

zero standard and triplicates of each standard duplicates of unknown samples, 4 replicates of ether blanks and 6 replicates of two known quality control (QC) samples.

5.3.10.1 Extraction of steroids from plasma samples

Plasma from duplicates of all unknown samples and six replicates of two known QC samples were extracted with 2 ml of diethyl ether. The tubes were vortexed for 2 minutes and the ether extracts were separated by freezing the aqueous layer in a dry-ice acetone bath. The ether extracts were dried under a gentle stream of compressed air and were reconstituted in 200 µl of gelatinized phosphate buffer saline (0.1% Gelatine; GPB; viz Buffer # 5).

5.3.10.2 Testosterone standards

A stock solution of testosterone (Sigma Chemical Co. Batch 108T-0777) was prepared in AR grade ethanol. A sub-stock of 12.2 $ng.ml^{-1}$ was made in GPB and stored at -20°C. Standards of 0.01, 0.02, 0.09, 0.185, 0.375, 0.75, 1.5, 3, 6.1, 12.2 $ng.ml^{-1}$ were made by serial dilution of sub-stock in GPB.

5.3.10.3 Antiserum to testosterone

The antiserum (Fitzgerald Industries International, Cat # 20C-CR214OR) was raised in a rabbit against Testosterone-3 BSA. This antiserum cross-reacted, 100% with testosterone, 0.5% with Androstenedione and 0.01% with Dihydrotestosterone (DHT).

5.3.10.4 Assay Procedure for Testosterone

To all tubes containing 25 µl of the standards, 175 µl of GPB was also added. Thereafter, one hundred microliters of antibody (1 : 60,000 in GPB) was added to standards and the ether extracted samples except the 3 (total counts) TCs and 3 (non

specific binding) NSBs. Thereafter, 100 µl of testosterone tracer (2,4,6,7 ^{3}H-testosterone; Amersham Life Science, Buckinghamshire, England) counting 13000 dpm was added to all tubes. Individual tubes were vortexed and left at 4°C overnight. Following overnight incubation, 200 µl of 0.5% dextran coated charcoal was added to all tubes except the TCs while being stirred at 4°C, and left for 20 minutes at that temperature. At the end of 20 minutes tubes were centrifuged at 3000 rpm in a refrigerated centrifuge for 10 minutes. (Total volumes of TCs were made up to 600 µl by adding 500 µl of GPB). After centrifugation, 500 µl of the supernatant was aspirated from each tube into counting vials and 2 ml scintillation fluid (Ultima Gold, Packard Instrument Company, Meriden, CT, USA) was added to all tubes (including TCs). All tubes were thoroughly mixed and then counted for 3 minutes in a liquid scintillation counter (Packard Tri Carb 1500).

5.3.11 Statistical analysis

Mean ± SEM or SD (as stated) were calculated for temperature and hormone levels. Temperature and hormone measurements from the 9 rams were analysed by repeated measures ANOVA and post hoc tests.

5.4 Results:

5.4.1 Pre-treatment temperatures

The mean (± SD) core temperature of the nine Merino rams used for this experiment as recorded prior to administration of treatments was 38.6°C (± 0.34). This was calculated by averaging temperatures from 11.00 hrs to 17.00 hrs for 5 days before the interventions started. The scrotal temperature of all rams was lower than the core temperature with the mean value being 4.5°C lower (Fig. 5.3). Scrotal temperature was more variable compared to the core temperature as shown in Fig. 5.4 and confirmed by the significant difference between the standard deviations of core temperature and scrotal temperature ($p = 0.0085$).

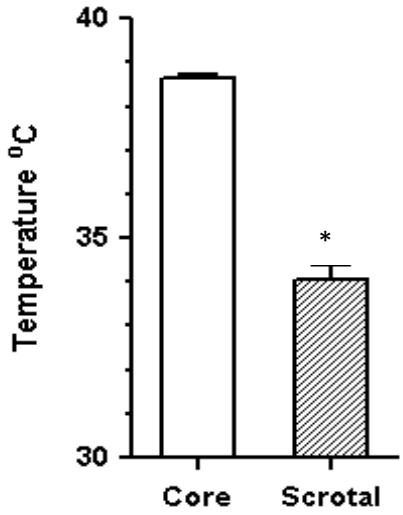


Figure 5.3: Pre-treatment temperatures recorded prior to the intervention in the 9 rams. The bars indicate the mean ± SD. The mean core temperature (38.6 ± 0.34°C) was significantly higher than the mean scrotal temperature (34.1 ± 0.91°C) [$p = 1.45 \times 10^{-7}$].

Since the loggers recorded every 10 minutes for over 4 weeks it was possible to look at the 24-hour variation in temperature in all rams. An interesting observation was a fall in scrotal temperature every morning when 24-hour temperatures were charted in all 9 rams (Fig.5.4). This reduction was probably due to the redistribution of blood as it occurred for about 3 to 4 hours after the time they were fed each morning.

Furthermore, there was a corresponding increase in the core temperature as recorded by the abdominal i-buttons. The likely reason for this may be post-prandial thermogenesis (Henry *et al.*, 2010) together with increased gut perfusion concurrent with feeding due probably to the close proximity of the bowel to the recording loggers. Since there was a depression of the scrotal temperature in all rams until midday, the temperature data for the purpose of analysis were considered from observations after midday.

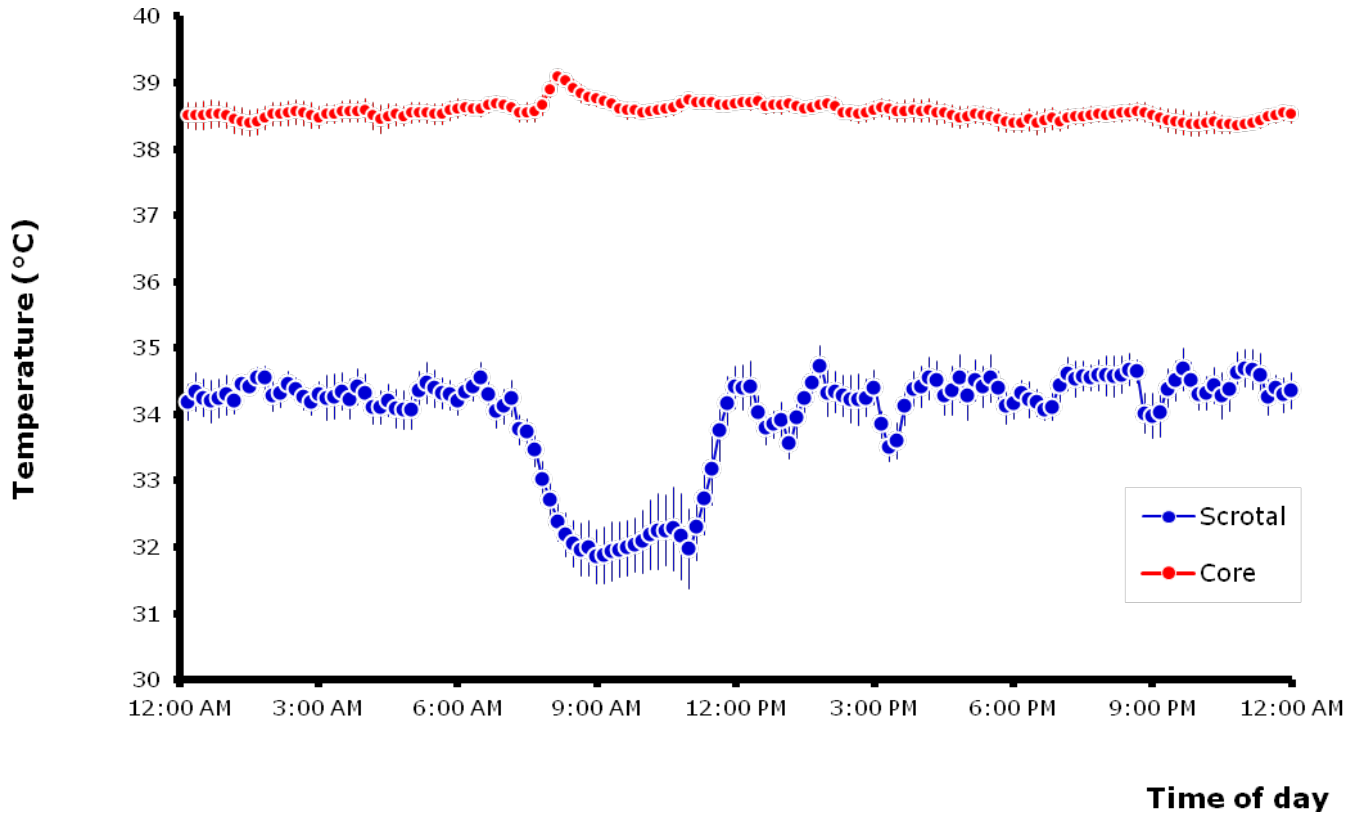


Figure 5.4: Mean ± SEM for core temperature and scrotal temperature is graphed for the nine rams for a period of two weeks before the onset of interventions. Greater variability was observed with scrotal temperature compared to core temperature [significant difference between SD of each animal ($p = 0.0085$)]

During each of the three interventions, one group was referred to as the control group and did not have scrotal temperature manipulations while the others had either scrotal cooling by the application of cold packs or scrotal warming by insulation. A comparison of temperatures of the control group was performed with pre-treatment temperatures. Figure 5.5 shows that there was no significant difference in the temperatures recorded either in the scrotum or the core during the pulse bleeds, without scrotal temperature manipulations, compared to an average pre-treatment day. Stated in another way, the pulse bleeds performed every 20 minutes did not have a significant effect on the scrotal or core temperatures.

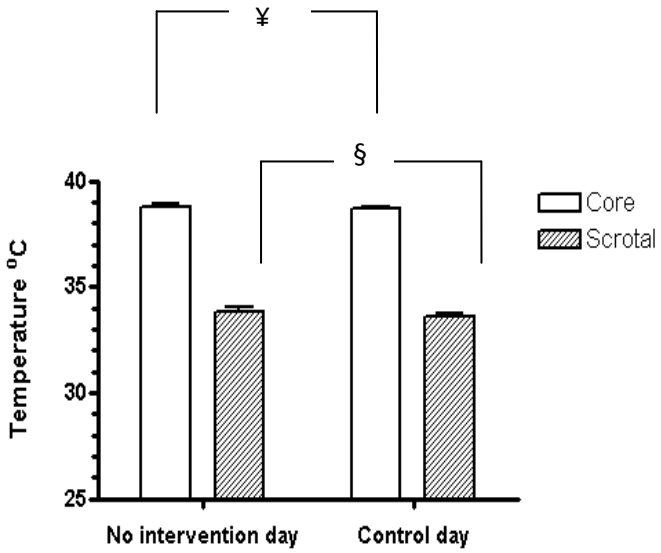


Figure 5.5: Comparison of core and scrotal temperature on a pretreatment day (before the interventions began) and on a day with intervention (pulse bleeds) but no scrotal temperature manipulation (control day). The differences in temperature were not statistically significant in the case of core (¥, $p = 0.45$) or scrotal (§ $p = 0.17$) temperature. Each bar represents mean ± SEM for the 9 rams.

5.4.2 Temperatures during scrotal temperature manipulations

Effective scrotal temperature lowering was achieved by the use of cold gel packs in all rams (Fig. 5.6). With the exception of ram # 1, who was later found to have a scrotal infection, all scrotal temperatures were below 30^{o}C on cooling days. The mean scrotal temperature achieved by application of the cold packs was 28.3°C, which was significantly lower than the temperatures in the control group [33.6°C] and the scrotal warming group [34.8°C] (Fig. 5.7).

Insulation of the scrotum (scrotal warming) with bubble wrap did not significantly elevate the scrotal temperature from that of the normal day, though it was significantly higher than that of a day with the application of cold packs [Fig. 5.7].

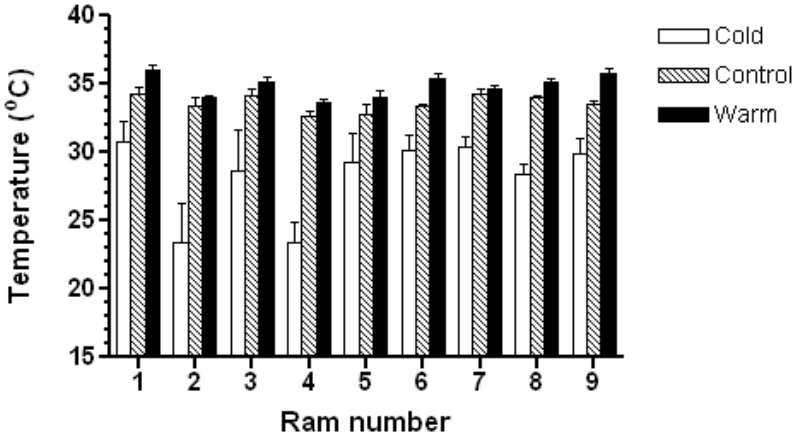


Figure 5.6: Mean ± SD of the scrotal temperatures of the 9 Merino rams during scrotal temperature manipulation with cold packs, scrotal insulation and no scrotal temperature manipulation. All 9 rams showed a reduction in scrotal temperature during cooling and an elevation of temperature during warming. However, the elevation of temperature was not significantly higher than the temperature during no temperature manipulation (see Fig. 5.7).

When observing the core temperatures on the days that scrotal cooling was applied (Fig. 5.7B), the general trend was a slight rise in core temperature. Similarly, the core temperature tended to be lower on the days that scrotal warming was applied. However, these differences were not statistically significant.

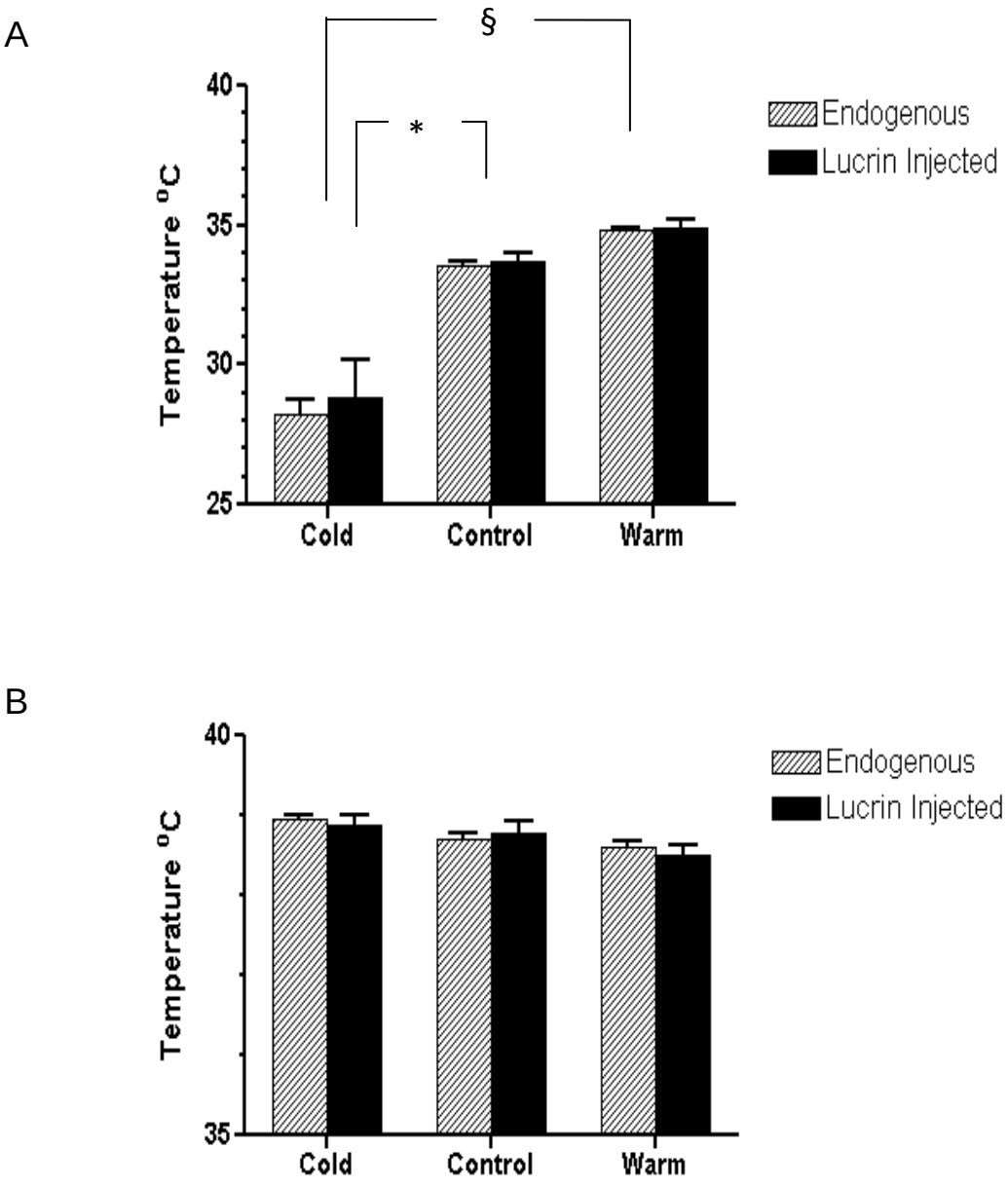


Figure 5.7: The mean ± SEM scrotal (A) and core (B) temperatures achieved during scrotal temperature manipulation. Temperature achieved with cold packs was significantly lower than the normal (* p = 0.00017) and the insulated (§ p = 0.00015) days. There were no significant differences in the core temperatures. The temperatures were not affected by Lucrin injections.

On the days when scrotal warming was applied, the respiratory rates were significantly higher than on the days of scrotal cooling or no scrotal temperature manipulation (Fig. 5.8).

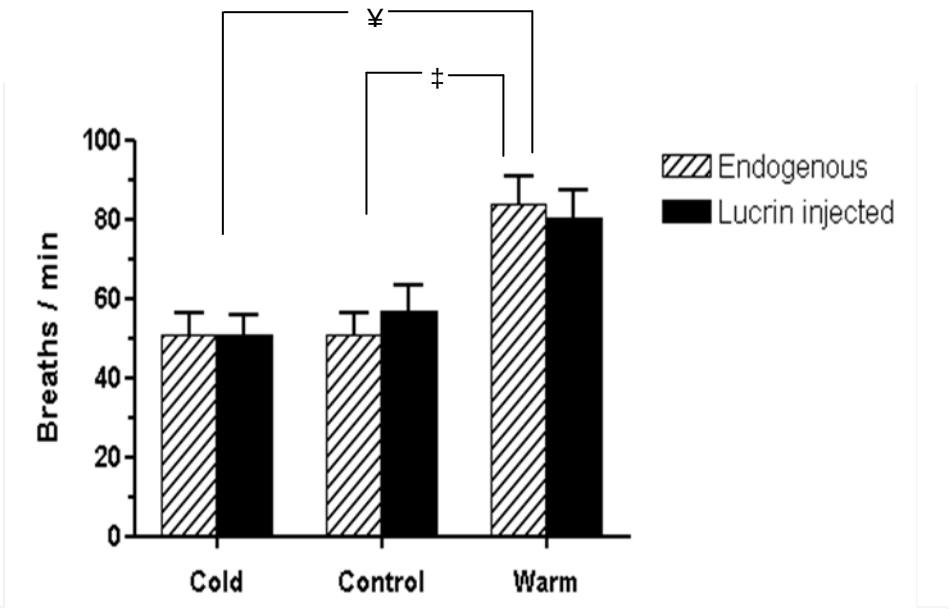


Figure 5.8: Respiratory rates during scrotal temperature manipulation on the days with and without Lucrin injections. The respiratory rate was significantly higher when the scrotum was warmed compared to when it was cooled (¥ p = 0.002) or no manipulation of temperature (‡ p = 0.001) was done both with either endogenous hormones or injection of Lucrin.

There was profuse sweating on the scrotal skin of all rams during scrotal warming. Therefore, it can be assumed that the warming of the scrotum promoted the initiation of cooling mechanisms in the body, by activation of panting and sweating.

5.4.3. Hormonal changes during manipulation of scrotal temperatures

Most rams had very few or no endogenous LH pulses during the interventions prior to the injection of exogenous Lucrin. Scrotal cooling or warming did not change the circulating level of either LH or testosterone (Fig. 5.9). The mean basal level of LH was 0.25 ng.ml^{-1} and the peak observed was 5.26 ng.ml^{-1}.

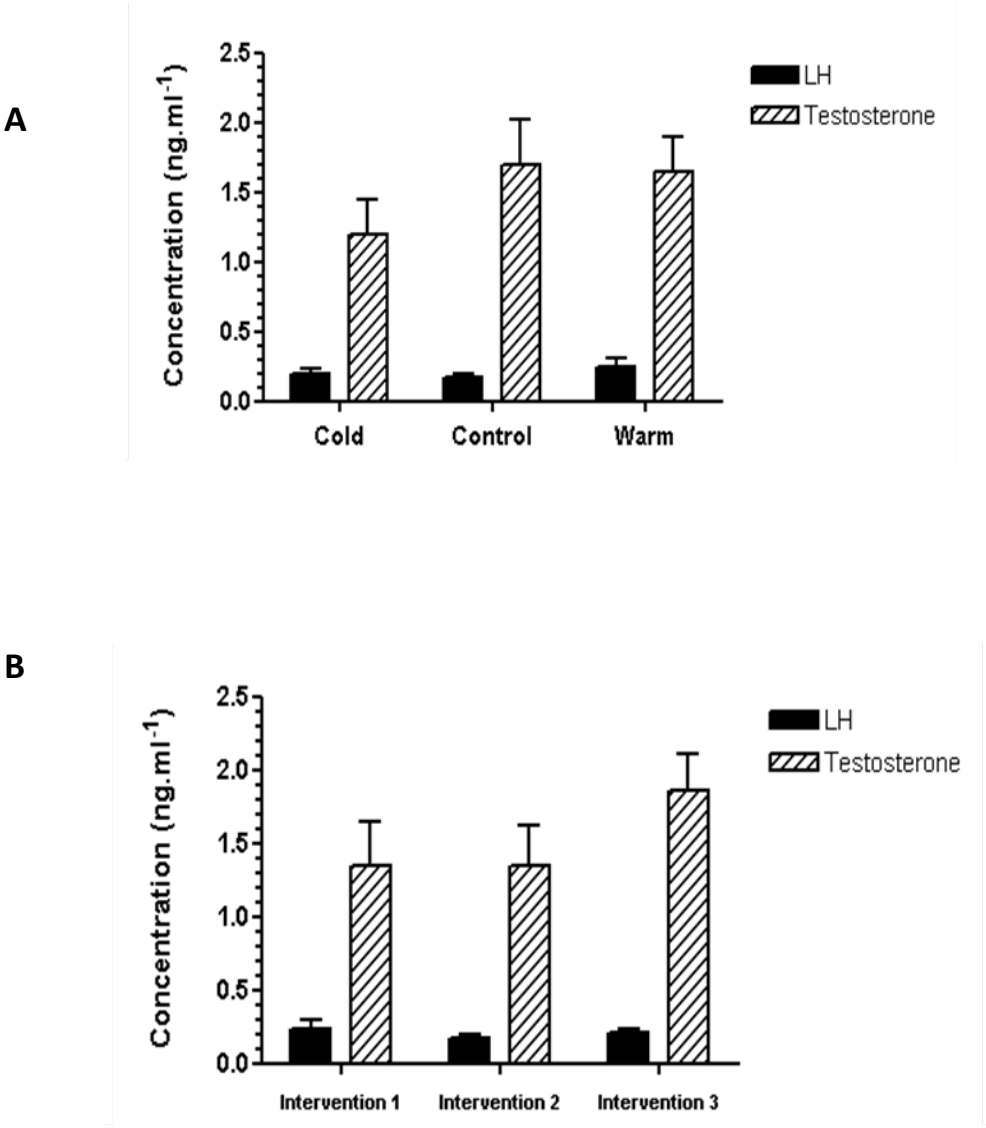


Figure 5.9: Mean ± SEM serum concentrations of endogenous LH and testosterone presented according to the treatment given (A) and the order of intervention (B) on the days when no Lucrin was administered. There was no significant effect on the hormone levels produced by the different treatments or the order in which they were given according to repeated measures ANOVA.

Following an injection of 0.2 mg of Lucrin into the rams there was a rapid and prolonged increase in LH to more than 50 times the baseline level (Figure 5.10). The testosterone level also increased by 6 to 8 times the baseline level.

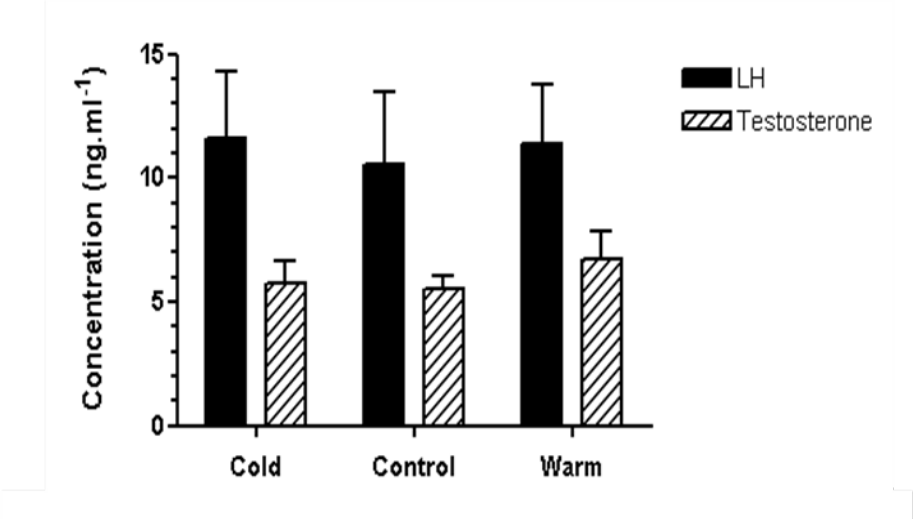


Figure 5.10: The effect of scrotal temperature manipulation on the plasma level of LH and testosterone. Each bar indicates the mean ± SEM of 9 rams. Repeated measures ANOVA indicated there was no significant effect of temperature on the hormone levels.

The manipulation of scrotal temperature did not affect the pattern of LH or testosterone following an injection of Lucrin. This is well demonstrated in Fig.5.11 where the average hormone levels of rams in each group are graphed.

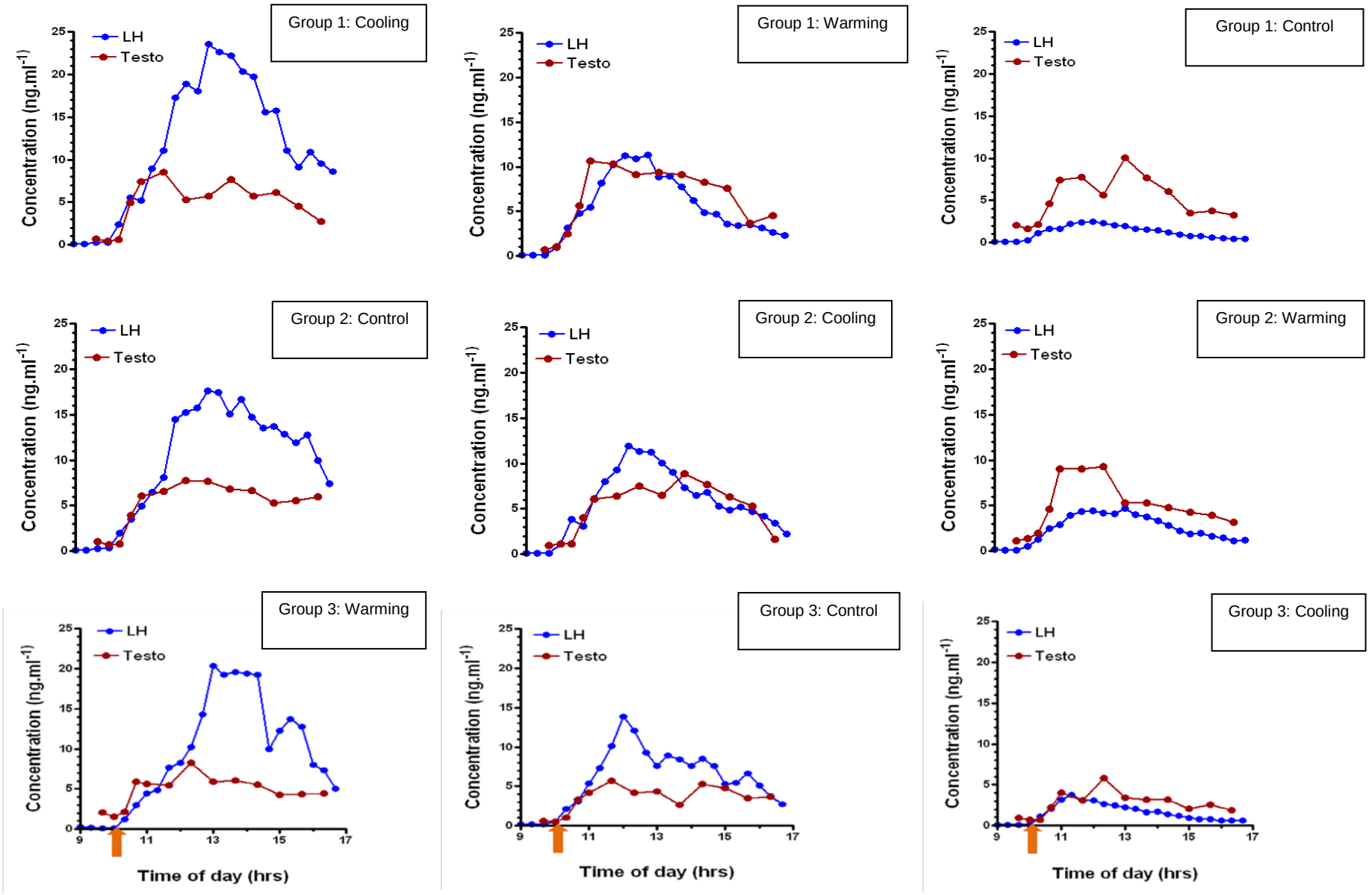


Figure 5.11: Average hormone levels of LH and testosterone in the three groups (1,2 & 3) of rams following injection of Lucrin. The treatment each group received is given in the box above the graph. The graphs indicate an overall pattern of reduction in the LH output in response to the same dose of Lucrin irrespective of the scrotal temperature manipulation the rams received. The arrows indicate the point in time at which Lucrin was injected.

While there was no effect of scrotal temperature manipulation, there was a significant decrease in the level of LH secreted in response to Lucrin over the three interventions (Fig. 5.12).

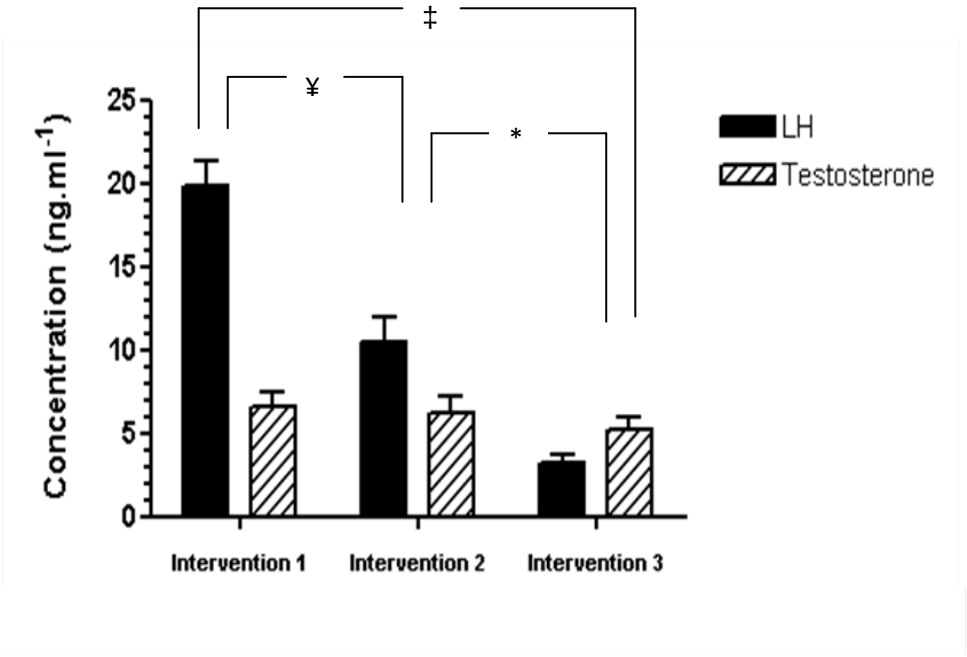


Figure 5.12: The levels of LH and testosterone on the three days of intervention following subcutaneous administration of Lucrin. Repeated measures ANOVA on LH levels: $p = 5x10^{-7}$ (post hoc ‡p = 0.0001, ¥p = 0.0003, *p = 0.0016)

The concentration of testosterone resulting from Lucrin injection was the same on each of the three repeats. Thus the concentration of testosterone secreted in response to a given concentration of LH increased over the three Lucrin injections during scrotal temperature manipulation. The testosterone to LH ratio increased from 0.348 during the first intervention to 2.635 on the third intervention (Fig. 5.13).

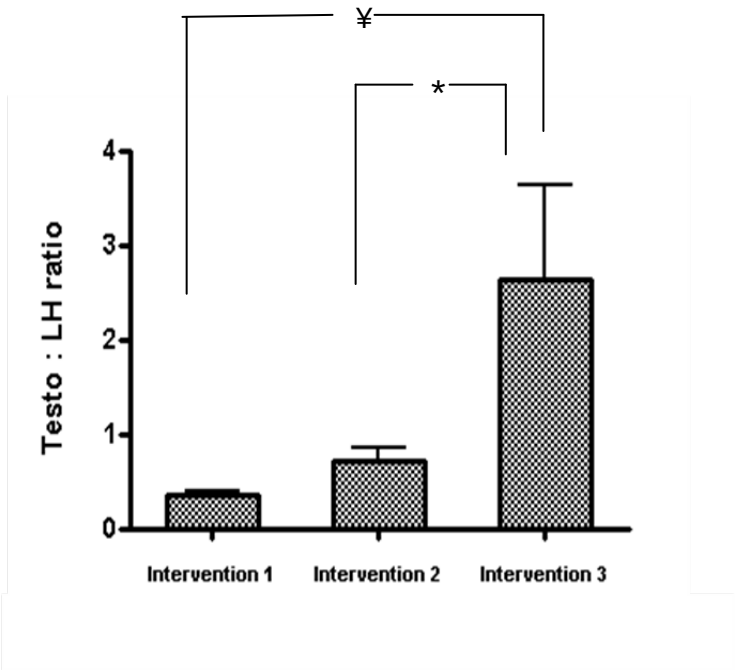


Figure 5.13: The testosterone : LH ratio on the three days of intervention after Lucrin injection showed a progressive rise. The Testosterone: LH of the 3rd intervention was statistically different from the ratio measured at interventions 1 and 2. (¥ p = 0.025, * p = 0.026)

5.5 Discussion:

Testicular temperature is known to be regulated 2 to 6 ^{o}C lower than the body core temperature in most mammals, because the lower temperature is facilitatory for spermatogenesis. The effect that temperature has on the other function of the testes, androgen production, is not clearly identified specially in humans (O'Shaughnessy and Sheffield, 1991; Pearson *et al.,* 1976; Manning and Kime, 1984). Therefore, this experiment was designed first to investigate whether scrotal temperature could be effectively manipulated both above and below the normal levels in an *in vivo* model, and second to study the effect of local scrotal temperature manipulation on androgen output from the testes. In addition, the effect that temperature has on testosterone secretion for a given concentration of LH was studied with the use of an exogenous GnRH super-agonist.

The experiments were performed on an *in vivo* animal model using 9 Merino rams. Surgery was performed prior to experimentation to implant temperature loggers in the abdomen and the scrotum of each animal. Each ram was subjected to scrotal cooling, warming, and no scrotal temperature manipulation in a cross-over design so that each animal acted as its own control. Pulse bleeds were performed every 20 minutes to assay testosterone and LH levels during scrotal temperature manipulation to study the possible effects on these hormone levels. A GnRH super-agonist was used to stimulate LH secretion from the pituitary and to study whether temperature had an effect on the secretion of testosterone in response to a give concentration of LH.

Scrotal temperature was manipulated effectively with cold packs containing cold gel. Using these packs I was able to reduce the scrotal temperature from the mean of 33.6°C to 28.3°C. However, the packs needed to be changed every 90 minutes to maintain the necessary cold temperature.

The reason for the large variability of scrotal temperature (SD of 1.6 with cold packs as oppose to 0.32 with hot packs and 0.42 when no temperature manipulation was performed) during cooling was due to the gradual warming process that took place as a result of conductive heat loss from the ram scrotum to the cold packs. It is also well demonstrated by data from one ram during the three interventions (Fig. 5. 14). Since this type of scrotal temperature manipulation had not been used previously, it was the best approach I could take for the purpose of reducing the testicular temperature.

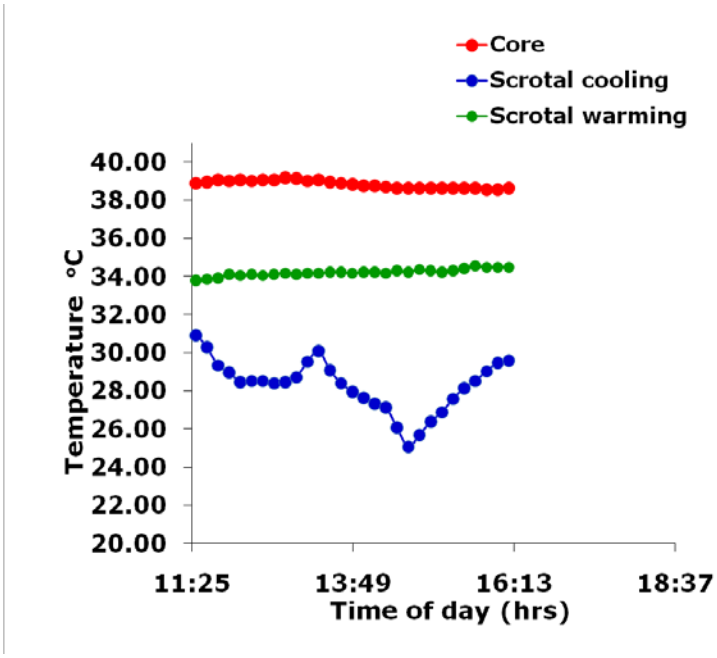


Figure 5.14: The variation in scrotal and core temperature during scrotal warming and cooling in a single ram. This indicates the wide variability that occurred to the scrotal temperature during cooling.

Effective elevation of the temperature of the scrotum proved to be a more difficult task. I expected that adding layers of insulation and inhibiting evaporation from the skin surface would elevate scrotal temperature to close to core temperature. But this turned out to be wrong. The mean temperature elevation achieved was only by 1.2°C,

as recorded by the i-buttons and the change was not significant. But, a similar insulation had achieved a temperature elevation of 1.4 to 2.2 ^{o}C previously (Mieusset *et al.*, 1992). However, the difference between this study and the present study was the duration of application of insulation.

Despite the intra-scrotal temperature not being elevated significantly, the respiratory data indicated that the insulation effectively stimulated the thermal afferents of the skin producing panting in the rams. There was also profuse sweat production apparent on examination of the skin of the scrotum. Evaporation of sweat could not take place because of the bubble wrap placed next to the skin, and sweat remained on the skin. Panting in the animal probably caused heat loss through the respiratory tract, and produced a decrease in core temperature. This finding is consistent with what has been reported previously in the literature which is a lowering of core body temperature in response to scrotal warming via panting and vasomotor reactions (Waites, 1962b; Hales and Hutchinson, 1971) in addition to local scrotal reactions including sweating from the scrotal skin (Waites and Voglmayr, 1963; Maloney and Mitchell, 1996). However, despite these changes, the intra-scrotal temperature was not affected by the intervention in the present experiment.

There has been a large amount of research into the central projections and signal processing of thermal afferents from the scrotum. There are neurons in many parts of the brain that increase the firing rate in response to scrotal heating (Waites, 1991; Li and Thornhill, 1996; Li and Thornhill, 1998; Hellon and Mitchell, 1975; Ishikawa *et al.*, 1984; Kanosue *et al.*, 1984; Nakayama *et al.*, 1983). A good correlation has been established between the firing rates of the central neurons and thermoregulatory effector function (Ishikawa *et al.*, 1984; Li and Thornhill, 1997). The observations in these studies suggest that an increase in scrotal temperature would tend to lower the core body temperature and a decrease would tend to increase the core temperature. Scrotal

heating is also observed to stimulates panting and reduce body temperature similarly in febrile and non-febrile rams (Ovis aries) (Maloney *et al.*, 2003). Findings from the present study confirm the existence of very efficient mechanisms that operate to reduce scrotal temperatures, despite insulation with the bubble-wrap packs which prevented cooling by evaporative heat loss and provided insulation against dry heat loss. Despite the significant increase in panting on the days on which scrotal insulation took place, the core temperature was only marginally lower (38.6°C) as oppose to the other two treatments (cold: 39.0°C and control: 38.7°C). Similarly, on studying the core temperatures on the days that scrotal cooling was carried out, the general trend was a slight rise in core temperature. However, none of these changes in temperature were statistically significant. One explanation for the above findings is that stimulation of respiration and activation of sweating are set off by a reflex initiated from the temperature change sensed by the skin thermal afferents and that the temperature manipulations are not registered by a logger implanted in the intra-scrotal region because the thermoregulatory mechanisms of the testes (i. e. the relaxation/contraction of the dartos and cremaster and the counter-current heat exchange in the pampiniform plexus) buffers the temperature rise before it reached the testes and the recording temperature logger.

In answer to the second research question of this experiment; whether changing scrotal temperature would affect the endocrine function of the testis, the answer is negative. Temperature alteration, or the degree to which it was done, failed to significantly affect the amount of testosterone produced by the Leydig cells in response to LH stimulation. However, there were only very few LH pulses in the 9 rams despite it being the early part of the breeding season.

In vitro studies performed on cultured cells have shown that an increase in temperature accelerates the proliferation of Leydig cells but decreases the testosterone

production when co-cultured with Sertoli cells (Wu and Murono, 1994). The decrease in testosterone was attributed to mitogenic factor which is produced by Sertoli cells at elevated temperatures when cells are maintained in cultures. This *in vitro* model has been used in studying pathological changes that takes place in the cryptorchid testis. My results differ from what would be predicted from these *in vivo* studies for two reasons. Firstly, my experiment was performed on an *in vivo* model. Secondly, the temperature in my *in vivo* model was not as high as in the cultured cells which were maintained 4^{o}C above the core temperature. Since my experimental cooling managed to reduce the intra scrotal temperature from the normal, if an elevated temperature caused a reduction in testosterone output a decrease of temperature could be hypothesised to increase the testosterone production. However, that is not what was observed. Therefore, it can be argued that changes observed in cell culture situations may not be applicable to a living model and that a reduction of 5^{o}C in temperature does not cause a significant change in testosterone secretion.

The GnRH super-agonist leuprorelin acetate (Lucrin) was used to stimulate GnRH receptors on pituitary gonadotrophs and thereby make the LH secretion supra-physiological. That would aid our experimental aims by preventing the hypothalamo-pituitary complex responding to changes in testosterone levels by negative feedback inhibition. If it did, that itself would set in motion a chain of reactions to cause secondary alterations in the testosterone levels. It is now well established that testosterone exerts its feedback action mainly on the GnRH secreting neurons via kisspeptins and has little or no effect directly on the pituitary in the ram (Tilbrook and Clarke, 2001). The use of Leuprorelin (Lucrin) in my experiment successfully stimulated the GnRH receptors and therefore LH secretion from the pituitary. However, the effect of 0.2mg given subcutaneously may have lasted longer than expected in the body of the rams producing a down regulation of receptors for GnRH in the pituitary to

produce the observed progressive decline in LH levels over the three consecutive interventions. Similarly, the amount of testosterone secreted for a given concentration of LH increased from the 1st intervention day to the 3rd, suggesting an up regulation of LH receptors on the testicular tissue presumably because of the persistently lowered endogenous LH levels in blood. This was an unexpected observation because one would assume a reduction of testosterone level to occur with a reduction in the LH levels, as LH is the immediate regulator of testosterone secretion in the body.

Experimentation on the hormone profiles in live animal models is always complicated by multiple confounding factors. Therefore, in making conclusions, we need to consider the multifactorial interactions between the many internal and external factors I have discussed in chapter 1. It is very difficult to control the effects of multiple environmental factors and the genotype. It is further complicated by having to study tissues that are not easily accessible or anatomically identifiable such as the hypothalamus, pituitary and the testes. All these factors were taken into consideration when this experiment was designed.

The unilateral cryptorchid mouse model has been the widely used animal model utilized for investigating the effect of temperature on the testes. Serum testosterone and gonadotrophin levels have been shown to undergo minimal change if any in an experiment performed on this model, which also demonstrated concurrent changes to the germ cell layers (Gomes and Jain, 1976). However, this model was not used in the present study because of the potential unknown adverse effects of artificially inducing cryptorchidism and also because I wanted to study temperature alterations in a natural system.

From this *in vivo* study conducted on 9 rams in the reproductive age the main conclusions drawn are as follows. The scrotal temperature could be effectively lowered

using cold gel packs and this lowering of scrotal temperature had no significant effect on the androgens levels in blood. The exogenous injection of a GnRH analogue also failed to alter the secretion of testosterone regardless of the scrotal temperature. However, the repeated administration of the GnRH analogue caused a reduction in LH secretion presumably via a down regulation of the receptors in the anterior pituitary, and an increase in the testosterone secreted for a given LH stimulus presumably via a changed sensitivity of the testes to LH.

CHAPTER 6

General Discussion: Retrospect

The basic theme behind this investigation was scrotal temperature regulation with special focus on the tunica dartos muscle – the smooth muscle that is found in the subdermal layer of the scrotal skin. Contraction of the dartos in cold environmental conditions causes a reduction in the surface area of the skin and lowers the cutaneous blood flow (Waites *et al.*, 1973). This probably occurs by the direct effect of temperature on the vascular smooth muscles to cause constriction in low temperatures plus an effect of constriction between the contracting muscles as suggested by previous researches (Shafik, 1973a). Relaxation of the dartos in warm surroundings increases the blood flow and the surface area of the skin. This is how the dartos contributes to thermoregulation of the testes which are maintained 2-6 ^{o}C lower than the body core. The process of maintaining a suitable environment for effective spermatogenesis is a complex one which involves local and central control mechanisms. The dartos is an important contributor to these local regulatory mechanisms. Some of the other adaptation processes include contraction of the cremaster, the presence of a large number of apocrine sweat glands in the scrotal skin, the absence of a layer of subcutaneous fat and the activity of the counter-current heat exchange system in the pampiniform plexus between the testicular artery and the veins.

The present study explored in detail the intracellular mechanisms of cold induced contraction of the tunica dartos. Some aspects of its temperature responsiveness have been investigated previously. Gibson *et al*. (2002) reported that the contractile responses of the isolated tunica dartos muscle to field stimulation and noradrenaline were larger at 30^{o}C than at 40^{o}C. They demonstrated this by measuring the contraction at 30^{o}C for a known stimulus and then heating the muscle by 10^{o}C to cause relaxation.

They presented evidence that this heating induced relaxation was due to inhibition of myosin phosphatase because Calyculin-A (phosphatase inhibitor) inhibited the relaxation at 40^{o}C.

Maloney *et al.* (2005), reported that explants of tunica dartos muscle from the rat scrotum contracted in response to cooling and that the cooling induced contractile response was potentiated by the presence of the overlying skin. Here the authors questioned whether the contractile response to cooling was brought about by a soluble mediator released by the skin which acted in a paracrine fashion on the muscle to cause this cold induced contractile response.

It was on the backdrop of these findings that the first part of the present study was planned to extend the understanding of the general contractile physiology of the tunica dartos muscle, to investigate the cooling induced contractile response and to characterize it in detail, to probe the intracellular mechanisms involved, and to image intracellular calcium transients using fluorescent calcium indicators. The experimental findings of this part of the present study (outlined in chapters 2, 3, and 4) revealed that the cooling induced contractile response was larger than the response to electrical field stimulation (EFS), and noradrenaline (NA) stimulation, but operated in an independent fashion to these responses. The contractile responses to both EFS and NA were confirmed to be mediated via adrenergic mechanisms acting through alpha-1 receptors in the dartos muscle. The cooling induced contractile response was found to be independent of nervous mechanisms, being activated and maintained by a process which became exhausted by prolonged cold stimulation (more than 1 hour) and with repeated cold stimulation (with rewarming to 33^{o}C between cooling). These findings taken together were suggestive of the presence of a soluble mediator which may have undergone degradation over time. This is also supported by the hypothesis which was put forward by Maloney *et al.* (2005), of a possible mediator being released from the

overlying skin causing contraction of the muscle during cooling. According to the present experiments, having the overlying skin present accentuated both the cooling induced contractile response and the heating induced relaxation. However, the theory of a mediator being secreted by the skin was not supported because the experiment involving the physical separation of skin muscle connections, but having them in close proximity did not produce similar findings to those of Maloney *et al*. (2005). The contractile response when the muscle was separated from the skin, but positioned close to the skin while cooled, was significantly lower than when muscle with the overlying skin attached was cooled, contrary to that reported in the literature (Maloney *et al.*, 2005). Moreover, the experiment where the cooled perfusate obtained from the overlying skin was used for stimulation of the muscle at 33^{o}C did not produce a contractile response in the muscle, suggesting that there was no such mediator released from the skin or that if a mediator was released it was too short lived or was over diluted to be effective in the small organ bath. A possible reason for the isolated muscle to have a smaller response to cooling than muscle with the skin attached might be damage to the muscle during dissection of the skin from the muscle.

Despite being unable to identify the mediator or the causative compound responsible for the cooling induced contractile response, a possible intracellular mechanism that cause contraction with cooling has been identified as being through activation of the Rho kinase / MP pathway.

To recapitulate at this point the main findings regarding the contractile physiology of the tunica dartos, it is a muscle with 100% pharmaco-mechanical coupling. Cooling causes activation of phospholipase C which in turn releases Ca^{2+} via stimulation of IP_3 receptors. The Ca^{2+} thus released promotes the extrusion of more Ca^{2+} from the neighbouring sarcoplasmic reticulum. Binding of released Ca^{2+} to another site on the IP_3 receptor causes a reduction in the opening probability of IP_3 receptors,

which now closes and results in a decrease in the intracellular Ca^{2+}. The depletion of store Ca^{2+}also contributes to the decrease in intracellular Ca^{2+}. This is the phenomenon that occurs repeatedly to produce Ca^{2+} oscillations in smooth muscle cells, first reported in porcine tracheal smooth muscle (Sanderson *et al.*, 2008). These oscillations in intracellular Ca^{2+} are likely to cause stimulation of the Rho kinase pathway which, when activated, causes Ca^{2+} sensitization via the inhibition of myosin phosphatase. As outlined in chapter 3, myosin phosphatase inhibition and the resulting Ca^{2+} sensitization seems to play a greater role in cooling induced contraction of the dartos according to the results obtained using Rho kinase inhibitor and IP_3 receptor antagonist in combination and separately. Since Gibson *et al*. (2002) reported that heating induced relaxation of the dartos was caused by activation of myosin phosphatase, the phenomenon of cooling induced contraction could be due to inhibition of myosin phosphatase. I present experimental evidence in support of such an hypothesis in chapter 3.

Ca^{2+} imaging using fluorescent Ca^{2+} indicators in the dartos muscle cells in the experiments outlined in chapter 4 was attempted to clarify whether the cooling induced contraction was due to a rise in intracellular Ca^{2+}, or to Ca^{2+} sensitization. Unfortunately, the cultured dartos cells used for calcium imaging did not produce significant and reliable transients. The cultured cells were heterogeneous and not consistent in producing Ca^{2+} changes to the known agonist NA, without which it was not possible to test cooling induced contraction (as it was not possible to confirm that the cells were smooth muscle and not fibroblasts, epithelial cells, or blood vessel cells). The same cell could not be used first for NA stimulation and then for cooling, because once a cell was stimulated with NA it was not possible to wash the NA off and use the same cell for cooling due to permanent changes in the emission ratio once stimulated with NA. The positive outcome of the attempt at Ca^{2+} imaging in the dartos was the development of a protocol for culture of the tunica dartos for the first time. However, similar to any other

smooth muscle tissue which contains a large amount of intermingled connective tissue, a pure muscle cell sample could not be harvested. This is a documented problem in culturing of smooth muscle tissue (Chamley-Campbell *et al.*, 1979). In future if this culturing could be refined further and the smooth muscle cells could be isolated from the fibroblasts and epithelial cells in the culture, it would be possible to reliably record intracellular Ca^{2+} transients, which would enable conclusions to be drawn in relation to cold induced contraction and Ca^{2+} sensitization. Performing a sorting technique to separate smooth muscle cells from the fibroblasts and other cells such as flow cytometry following fluorescent labelling of smooth muscles actin or myosin may be a possible next step.

Possible alternatives for studying Ca^{2+} sensitization in the dartos cells could be looked into in the future. One such possibility is β-escin permeabilisation (skinning) for example. This has been used in other smooth muscles of the body (Chiba *et al.*, 1999; Karaki *et al.*, 1997).

The importance of the tunica dartos muscle for testicular thermoregulation was outlined previously. The importance of having the testes cooler than body core temperature in scrotal mammals was also highlighted in the general introduction of this thesis. Male fertility contributes about one third of the problems of infertility in human couples, but is almost ignored in the scientific literature. I therefore, probed further into aspects of temperature and the function of the testes. Elevation of testicular temperature is known to affect fertility as evidenced by a decrease in fertility with conditions that elevate testicular temperature such as varicocele and cryptorchidism (Goldstein and Eid, 1989; Moore and Quick, 1924; Carlsen *et al.*, 2003). Exactly which cells in the germ cell layer are affected by elevated temperatures and the specific mechanism of injury to these cells is still debatable, although the association of elevated levels of heat shock

protein with conditions that increase testicular temperature and the onset of cellular stress response seem to be a popular explanation.

The evidence on the effect of endocrine function of the testes with elevation of testicular temperature is not definite. Therefore, as outlined in chapter 5, the final experiment of this investigation was performed to ascertain whether dysregulation of scrotal temperature affected the output of testosterone from the testes. I was able to achieve effective reduction of testicular temperature (by 5^{o}C) with the aid of cold gel packs, but failed to significantly elevate scrotal temperature despite adding insulation and preventing evaporation. Therefore, it was not possible to draw conclusions about elevated testicular temperature and hormone production. There was no change in the testosterone output with a 5^{o}C reduction in intra-scrotal temperature.

Mieusset *et al.* (1992) have reported a reduction in fertility with insulation of the scrotum for 16 hours a day for 21 consecutive days. The mean temperature elevation achieved during this experiment was 1.4 to 2.2 ^{o}C, which was higher than achieved by my experiment. Therefore, to further improve the experiment it could be suggested that greater degree of insulation for a longer period of time should be undertaken to obtain a significant elevation of temperature. This would enable making firm conclusions regarding dysregulation of scrotal temperature and the endocrine function of the testes.

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Appendix

The following presentations were done during the PhD candidature:

1. Nanayakkara SDI, Maloney SK, Bakker AJ (2008). The response of isolated tunica dartos muscle acute and prolonged cold stimulation and noradrenaline. *Proceedings of the Australian Physiological Society*. 39: 98.

2. Nanayakkara SDI, Bakker AJ, Maloney SK (2009). Contractile physiology of isolated tunica dartos muscle in response to electrical thermal and pharmacological stimulation. *Proceeding of the 3rd International Symposium on Physiology and Pharmacology of Temperature Regulation.* 130.