Regulation of smooth muscle activity in the rat: Effects of castration and iberiotoxin

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Abstract

The mechanism by which smooth muscle contracts and relaxes has been intensely studied; however, many aspects have yet to be explained. The regulation of intracellular calcium concentrations within the smooth muscle cells essentially determines the flaccid/erect state of the penis. One factor identified to influence both contractile and relaxation mechanisms are the presence/absence of testosterone. The present investigated (1) whether castration (androgen-depleted cavernosal tissue) would have a differential effect on contraction and relaxation (Phase 1 or Phase 2) and (2) investigated whether inhibition of BK channels would effect the individual phases (Phase 1 and 2) of relaxation and test whether the presence/absence of androgens influenced BK channels.

Corpus cavernosal tissue was removed from laboratory rats and tested *in vitro* on isometric force transducers. Tissues were contracted with norepinephrine and relaxed by sodium nitroprusside. BK channels were inhibited by the highly-selective antagonist iberiotoxin. Data was collected for contractile tension, percent total relaxation, percent of Phase 1, rate of Phase 1 relaxation, and percent of Phase 2 relaxation.

Castration was found to significantly increase cavernosal tissue response to the norepinephrine; castration also decreased the response of the cavernosal tissue to the sodium nitroprusside. Further analysis of relaxation found the initial phase of relaxation was significantly reduced and the second phase was significantly greater, presumably due to castration. Inhibition of BK channels significantly slowed the rate of relaxation in Phase 1, while it tended to decrease percent total and Phase 1 relaxation. At least part of the supportive role of testosterone in normal erectile physiology is accomplished through the Phase 1 mechanism of relaxation and potentially the BK channels.

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Introduction

The signal transduction pathway of penile tumescence has been an evolving

concept for more than fifty years, while the two most recent decades have identified

specific molecular components essential in normal activity. The presence of androgens

has become a well-accepted stipulation for optimal erectile stimulation and response.

However, specifically where and how androgens exert their effects in the penile tissue

continues to be an area of interest and research. The aim of this experiment focuses on

the relaxation properties of corpus cavernosum tissue from the rat by treatment targeting

calcium-dependent potassium channels in the presence (sham-castrated) and absence

(castrated) of androgens.

Anatomy

The anatomy of the penile tissue directly relates to the erectile function. The

central structure of the erectile response is found in a pair of cylindrical vascular beds, the

corpus cavernosum, which extends the length of the longitudinal axis of the penis and

provides the central structural framework for erectile function (Brock et al., 1997). The

corpus spongiosum also supports erectile function and is found in the ventral-longitudinal

axis. The corpus spongiosum contains the urethra and contracts in such a way so as not

to occlude the lumen so that ejaculation may still occur while the penis is erect

(Aboutseif and Tanagho, 1999). The corpus spongiosum extends distally beyond the

corpus cavernosal columns to form the glans penis. The composition of these vascular

beds includes smooth muscle cells. Though the rat has become a well accepted model for

erectile dysfunction, one group identified the smooth muscle concentration to be much

lower in the rat while the collagen concentration was much higher when compared to

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human penile samples (Pinheiro et al., 2000). Regardless, the smooth muscle found in the cavernosal beds along with strong connective tissue deposits have been recognized as essential components for normal tumescence and detumescence activity (Moreland, 2000).

Skeletal muscles provide a containment and supportive role during the initiation and maintenance of an erection. The ischiocavernosus muscle originates from the ishial ramous and projects to the crus (base) of the penis (Meisel and Sachs, 1994). Contraction of the ischiocavernosus muscle has been identified in rats to produce the "flips", a characteristic flexion movement of the penis, which promotes intromission (Hsu, 2004; Zempoalteca, Lucui, and Eguibar, 2008; Sachs, 1982). The bulbospongiosus muscle surrounds the corpus cavernosum and corpus spongiosum with an origin in the bulbous (Zempoalteca et al, 2008). The bulbospongiosus muscle contracts which results in distension of the cavernosal sinuses by compressing and also slowing venous blood outflow of the corpus cavernosum (Hsu, 2004; Sachs, 1982). Excision of ischiocavernosus muscle in rats resulted in an inability to achieve necessary proximity for intromission; excision of bulbospongiosus muscle resulted in less successful induction of pregnancy of the female rat due to an inability to deliver the seminal plug (Sachs, 1982; OHanlon and Sachs, 1986).

The tunica albuginea is an extension of the ischiocavernosus muscle and also encapsulates the pair of corpus cavernosal tissues and partially that of the corpus spongiosum. The degree to which the tunica can distend under the pressure of the expanding cavernosal and spongiosum tissues contributes to the veno-occlusive mechanism, which will be discussed later. Thick collagen fibers and elastics fibers are

the building blocks of the tunica albuginea and are normally found in two distinct layers (Akkus et al., 1997). The outer portion of the tunica is composed of collagen in a longitudinal fashion while the inner layer is composed with a circumferential orientation (Brock et al., 1997). Thick collagen fibers provide the tensile strength necessary to withstand the pressure forces seen during tumescence, while the elastic fibers enable the penile tissue to return to the flaccid state after tumescence. Underlying pathologies can disrupt the collagen and elastic fiber properties which change the strength and elasticity of the tunica. Peyronie's disease is one example that illustrates a dysfunctional tunica albuginea through one or more underlying pathologies which can lead to erectile dysfunction and impotency (Goldstein et al., 1984, Persson, Deiderichs, and Lue, 1989).

The vascular supply to the penile tissue is particularly important since, via the systemic blood pressure, it provides the force necessary for normal erectile function. The primary artery that supplies the erectile tissue with blood is the internal pudendal which arrives in the urogenital region and passes through the urogenital diaphragm where it becomes the penile artery (Simonsen, Garcia-Sacristan, and Prieto, 2002; Russell and Nehra, 2003). Moving distally from the urogenital diaphragm, the penile artery has several branches, including the bulbourethral and cavernous deep penile arteries, as it extends to the dorsal artery. The dorsal artery continues distally with several lateral circumflex arteries that perfuse the corpus cavernosal tissues to provide the volume and pressure necessary for the erectile process (Simonsen, Garcia-Sacristan, and Prieto, 2002). Within the cavernosal tissue the helicine resistance arteries are connected to cavernosal sinuses which have the potential to create space for receiving relatively large volumes of blood (Moreland, 2000, Montorsi et al., 1998; Russell and Nehra, 2003).

Stimulation for dilation and contraction of the resistance helicine arteries and sinusoid cavities help contribute to the functional status of the penis.

The venous drainage network is composed of three relative levels; superficial, middle, and deep. The superficial network drains areas such as the penile dermis layer and prepuce to the saphenous or external pudendal veins. The middle network is found around the area of the tunica albuginea. The remaining deep tissue receives venous blood from the dorsal and circumflex veins. These vessels lead into the periprostatic venous plexus. The deep network is mainly comprised of the cavernous and crural veins; the cavernosal sinuses drain into the cavernous veins, which travel proximal through the penis to become the crural veins (Fernandez et al., 1991). The venous blood finally exits the penile tissue via the pudendal vein.

Veno-Occlusive Mechanism

Smooth muscle relaxation within the penile tissue under normal conditions results in the erectile response. The mechanical changes that facilitate the erectile response are known as the veno-occlusive mechanism. While the arterial blood pressure is the fundamental driving force for assuring adequate blood volume delivery to the penile tissue it is also the fundamental driving force for the erectile process. As the arteries in the penis and cavernosal sinuses relax and dilate, an increased volume of blood flows into the penis therefore increasing the volume of the sinusoid cavities. The tunica albuginea limits the amount of expansion the dilated tissue may experience by allowing a finite amount of stretch and thus total volume that the penis may accommodate (Akkus et al., 1997). Concurrently the venous vascular structures responsible for the returning the blood supply become compressed between the increasing size and pressure of the corpus

cavernosal sinuses as they expand out, stretching against the tunica albuginea supportive skeletal muscles. This leads to trapping of blood in the cavernosal sinuses.

One final component that supports the veno-occlusive mechanism is the contraction of the ischiocavernosus and perineal muscles. Their contraction also increases the amount of blood retained in the penile tissue by slowing the venous drainage, leading to increased pressure by maintaining increased volume within the erectile tissue. Zempoalteca et al. (2008) showed that dennervation to the ischiocavernous and perineal muscle in rabbits maintained the ability to achieve an erection; however, intromission was failed and resulted in extravaginal ejaculation. This implies a role for the skeletal muscles in the process of reproduction, but not necessarily for tumescence in rabbits. Thus, for the erectile response to be present the following conditions must be satisfied; dilation of the arterial vasculature, dilation of the cavernosal smooth muscle (sinusoid cavities), and adequate impedance of the venous blood exiting the penile tissue.

Central Nervous Regulation

The regulation for the erect and non-erect state of the penis comes from both the central and peripheral nervous systems. Although the spinal cord also represents the mediator between peripheral tissue and the central nervous system, it is also the primary control center for an erectile response. Stimulation from the higher control centers (brain) provide stimulation to the spinal cord that may inhibit or promote an erectile response, however, they are not necessary to initiate the response. The dorsal penile nerve has been identified as a primary affective nerve pathway transmitting information from the sensory tissue of the glans and penile sheath to the central nervous system

(Giuliano and Rampin, 2000; Pickard, Powell, and Schofield, 1994). The efferent tracts representing the sympathetic innervation to the penile tissue has been identified in both the hypogastric nerve and lumbar sympathetic chain (Giuliana and Rampin, 2000; Janig and McLachlan, 1987). Penile parasympathetic innervation, which is pro-erectile, comes from the pelvic and cavernous nerves (Giuliano et al., 1995). Despite similarities in physiology, no universal anatomic pathway exists among species by which parasympathetic innervation exists (Giuliano et al., 1995).

As previously mentioned, erectile function may persist following spinal cord lesion or injury; this is due to the ability of the sacral spinal cord, specifically the sacral parasympathetic nucleus, to receive somatic information from penile tissue (e.g. tactile stimulation) and initiate a response leading to erection (Steers, 2000;Rampin and Giuliano, 2001). The components of the central nervous system that have most closely been associated with an integral role in the erectile process and copulatory behavior are the medial pre-optic area (MPOA) and the paraventricular nucleus (PVN) (Malsbury, 1971; Giuliano et al., 1997;Argiolas and Melis, 2005). Stimulation of the MPOA is proposed to activate the PVN to produce an erectile response, and likewise direct stimulation of the PVN also elicits an erectile response (Giuliano et al., 1997). Therefore the MPOA and PVN are modulatory centers for an erectile response, however, they are not considered primary control centers.

Early investigations on the role of the central nervous system targeted the aspects of central control mechanisms and copulatory behavior influence as the core of erectile physiology (Beach, 1976; McKenna, 2000). However, lesion studies in rats which disconnected the brain from the spinal cord showed that the erectile response remained at

least possible and therefore do not completely depend on the upper level structures of the central nervous system (Meisel and Sachs, 1994; Bors and Comarr, 1960). Despite this, normal erectile function requires the integration of both sensory information as well as neuromuscular control in the urogenital effector tissues (McKenna, 2000).

The neurotransmitters utilized by the central nervous system to regulate the penile tissue have been vastly studied and include the following: 5-Hydroytryptamine (serotonin), dopamine, noradrenaline, γ-Aminobutyric acid (GABA), oxytocin, acetylcholine, and nitric oxide (Andersson, 2001; Martino et al., 2005). While the use of pharmacological agents have been used at receptor sites for these neurotransmitters, the exact mechanism and the role in penile tissue regulation has yet to be clearly established for all. Sato et al. (1995) found that the role of dopamine and its affect on the MPOA are directly related to changes of the behavioral aspect of reproduction so that increases in dopamine increase copulatory activity. Specifically noted, stimulation of dopamine receptors resulted not only in copulatory behavior, but specifically increased the activity of NOS enzymes within the PVN that generated the erectile response suggesting a transduction cascade at the central nervous system level (Melis, Succu, and Argiolas, 1996). Similarly the relationship between dopamine and NOS has been studied while in the presence and absence of testosterone; the supportive role of dopamine in tumescence is facilitated by an up-regulation of NOS enzymes by testosterone via phosphorylation of steroid receptors, whereas hypogonadism resulted in decreased amounts of NOS in the MPOA (Hull et al., 1999).

Smooth Muscle Contraction

Smooth muscle contraction is initiated and regulated by a number of different mechanisms. Mechanical, neural, hormonal, and electrochemical stimuli have all been shown to affect the contractility of smooth muscle. In the penile tissue, smooth muscle tissue that responds to contractile stimuli are found in the helicine arteries that provide blood supply to the tissue and within cavernosal tissue as trabecular smooth muscle.

The cavernosal tissue is maintained in a contracted state under resting conditions. Innervation and stimulation from the sympathetic nervous system determines the tone of the cavernosal tissue. The primary neurotransmitter for the sympathetic nervous system in the cavernosal tissue has been identified as norepinephrine, which is released from adrenergic nerve fibers. The cavernosal tissue is populated with both α-1 and α-2 receptors; α-1 receptors serve as the target receptors while the α-2 receptors serve in an autoregulatory function (Tong and Cheng, 1997). The use of highly specific agonists on alpha-1 receptors in cavernosal tissue has been shown to produce the contraction of smooth muscle (Wingard, Lewis, and Mills, 2001). The use of antagonists targeting the alpha-2 receptors produced the same results because of an increase in alpha-1 stimulation while the autoregulator was inhibited. This has been shown experimentally with administration of α-1 agonist drugs producing cavernosal smooth muscle contraction and α-2 antagonist drugs producing an increased release of norepinephrine. The mechanism for alpha-induced contraction of smooth muscle has been accepted to depend on a second messenger system that results in an increase in intracellular rise in calcium (Sato and Kawatani, 2002).

Endothelin-1(ET-1) has also been identified as a neurotransmitter responsible for contraction of the cavernosal smooth muscle, though not to the extent of norepinephrine (Wingard et al., 2003). Low concentrations of ET-1 demonstrate a synergistic effect when combined with phenylephrine (α-1 specific agonist) to activate downstream modulators that promote smooth muscle contraction while inhibitors of the downstream modulators ceased contraction and promoted relaxation in rat cavernosal tissue (Wingard et al., 2003; Anderson and Stief, 1997). Finally, other chemical messengers that have been associated with the initiation or maintenance of cavernosal smooth muscle contraction include the following, though their mechanisms have yet to be elucidated: Angiotensin II, tromboxane A2, neuropeptide Y, and prostanoids.

Agonists for smooth muscle contraction will bind to their respective receptors found in the plasma membrane. The mechanism that initiates the contraction of the smooth muscle cells first begins by activation of a G-protein linked receptor (Gq protein), followed by activation of phospholipase C (Somlyo and Somlyo, 2002). This molecule is released from a membrane bound state to the intracellular matrix. There phospholipase C is responsible for converting phosphatidylinositol 4,5-biphosphate into inositol-3-phosphate (IP3) and diacylglycerol. The diacylglycerol is then responsible for the activation of protein kinase C.

Protein kinase C in turn is responsible for phosphorylating the calcium channels found in the plasma membrane, opening the channels and allowing calcium to rapidly move into the cytosol following the concentration gradient. The movement of calcium across the plasma membrane illustrates the importance of calcium balance as it relates to the electrochemical stability. Such movement of calcium has been found to occur both

simultaneously throughout the cell as well as isolated local sections within the plasma membrane. These isolated movements of calcium, deemed calcium "sparks", result in the activation of additional ion channels in surrounding areas of the plasma membrane that further regulate the membrane potential (Nelson et al., 1995). One example of these calcium-dependent ion channels, which supports depolarization and muscle contraction, is an inward Cl- ion current that is regulated by voltage-dependent calcium channels; this Cl- current supports the action of stimuli such as phenylephrine to induce contraction of cavernosal myocytes (Karkanis et al., 2003). Experiments in both rat and human cavernosal tissue have demonstrated that blockade of Cl- channels with antagonists resulted in an increase of intracavernosal pressure (Karkanis et al., 2003).

Protein kinase C also regulates the MLC phosphatase through an indirect mechanism by phosphorylating the regulatory molecule CPI-17 (Koyama et al., 2000). CPI-17 then phosphorylates MLC phosphatase, thereby allowing the myosin light chain to remain active in the crossbridge activity. This indirect mechanism contributes to the maintenance of contraction in very small quantities (Somlyo and Somlyo, 2000).

IP3 is also responsible for an increase in the intracellular increase of calcium by directly activating the selective channels found on the sarcoplasmic reticulum, an intracellular storage organelle of calcium (Szado et al., 2001). Though this calcium does contribute, the majority of the calcium that results in the intracellular concentration increase comes from the extracellular space. Patch-clamp and in vitro studies have shown that when extracellular calcium is removed or a blockage of critical plasma membrane channels, such as the voltage-dependent calcium channel, with highly specific agents such as verapamil and nifedipine the ability of smooth muscles cells to increase

intracellular calcium and hence produce contraction was significantly impaired (Christ et al., 1990; Focaeus, Andersson, and Hedlund, 1987).

Calcium increases within the intracellular space result in calcium binding with calmodulin. The calcium-calmodulin union results in conformational changes in calmodulin that allow specific binding sites for myosin light chain kinase to be revealed. The calcium-calmodulin molecule can then phosphorylate the myosin light chain. The phosphorylated myosin light chain then activates the myosin ATPase so that adenosine triphosphate (ATP) can be utilized and crossbridge binding with actin will begin. As more of the MLC become phosphorylated, more contractile force is produced and it becomes more difficult to stimulate the tissue to relax (Chang et al., 2005). The crossbridging, and thus generation of contractile force, will continue so long as there is calcium and ATP available.

A significant increase of intracellular calcium that leads to increased activity of MLC kinase, however, is not the only means by which generation of force is initiated and maintained. The mechanism by which smooth muscle initiates contraction relies upon a second messenger system which results in an increased sensitivity to elevation of intracellular calcium as well as regulation of another important regulatory molecule, RhoA (Somlyo and Somlyo, 2000). Without the substantial increase of intracellular calcium to begin the contractile mechanism, this mechanism has been suggested to operate independent of calcium concentrations (Uehata et al., 1997), however, it has become clearer that the contractile mechanism is becoming more sensitive to small increases of calcium rather than operating independently under the second mechanism.

The G-protein complex is coupled with agonist receptors for contractile agents. When the receptor is activated by an agonist, the G-proteins initiate the conversion of an inactive RhoA molecule bound to a GDP complex to the active form of RhoA which is bound to a GTP complex (Wang et al., 2002). The concentration of this RhoA molecule has been found to be in much higher concentration in the cavernosal tissue as compared to that of other vascular smooth tissue such as in arterial walls. This activated form for RhoA then anchors in the plasma membrane.

From its position in the plasma membrane, RhoA can act upon a number of targets. Of those targets, the Rho kinase molecule has the most profound effect in terms of smooth muscle cell contraction. Rho kinase primarily affects its targets through phosphorylation which results in either activation or deactivation of that target. One target of Rho kinase is myosin light chain (MLC) phosphatase. MLC phosphatase is responsible for the dephosphorylation of the myosin light chain. When the myosin light chain is dephosphorylated crossbridge action between myosin and actin cease and therefore the contraction of the muscle is stopped. As MLC phosphatase is phosphorylated by the Rho kinase molecule it becomes inactive and unable to dephosphorylate the myosin light chain, leaving the crossbridge interaction to continue and sustain contraction.

Rho kinase has also been reported to directly phosphorylate the myosin light chain which leads to contraction (Somlyo and Somlyo, 2002). By directly phosphorylating the myosin light chain, Rho kinase has direct control over the contractile state and therefore the tone of the smooth muscle.

The calcium sensitizing pathway gets its name because the RhoA/Rho kinase system has no direct effect on regulating intracellular calcium levels (Takahashi et al., 2003). The initiation of contraction is brought on by increasing the intracellular calcium as described above. However, these increases in calcium last for only a short period of time in order to maintain a relative homeostatic and electrochemically stable intracellular environment. Therefore, the RhoA/Rho kinase system acts to maintain crossbridging of actin and myosin while intracellular calcium is returned to basal concentrations (Mill et al., 2001). This has been confirmed when the RhoA/Rho kinase systems are disabled, whether genetically or chemically, the initial contractile force was produced but the maintenance of the contraction is lost due to the return of the intracellular calcium level to baseline concentrations (Mills, Chitaley, and Lewis, 2001; Wang et al., 2002; Takahashi et al., 2003).

Smooth Muscle Relaxation

The cavernosal tissue is maintained in the contracted state for the majority of a lifetime. The dominate state of smooth muscle (flaccid) is maintained by crossbridge activity between actin and myosin via calcium-dependent and/or calcium-independent mechanisms; the effort of the erectile mechanism (smooth muscle relaxed) opposes the crossbridge activity by deactivating the calcium-dependent and independent mechanisms. Management of the cavernosal tissue for the erectile process to occur has been identified to revolve around the necessity to decrease the intracellular calcium concentrations, disable the RhoA/Rho kinase mechanism (calcium-independent mechanisms), and increase the electrochemical membrane potential. Developments in the treatment of erectile dysfunction, including identification of specific neurotransmitters and convincing

drug therapies have that resulted in significant insight and clinical outcomes, have only been partially explained the mechanism of tumescence.

Coordination of the signal transduction pathway to transition from the flaccid to erect state of the penis generally begins from the central nervous system. Within the brain, activation of the parasympathetic nervous system begins with increased activity in the PVN and MPOA of the hypothalamus. These sections of the brain are involved in regulating copulatory behavior. The principal neurotransmitter mediating this behavior within these areas of the brain is dopamine. Therefore any disruption in the production, effectiveness, and degradation of dopamine will have downstream effects on tumescence. From these areas, neurotransmission follows the sacral parasympathetic nucleus spinal tract. Exiting the spinal cord, the pudendal and pelvic nerves provide peripheral nerve tracts that both lead to the cavernous nerve. Arriving at the penile tissue, the innervation is supplied to both the helicine arteries as well as the cavernosal trabecular smooth muscle. Electrical field stimulation to these neural structures has commonly been used to induce nitric oxide release and vasodilatation of the cavernosal smooth muscles that lead to an erectile response (Escrig, Gonzalez-Mora, and Mas, 1999).

Nitric oxide has been identified as the primary neurotransmitter in the signal transduction pathway leading to tumescence. Nitric oxide synthase (NOS) is the enzyme responsible for the production of nitric oxide. There have been three isoforms of the NOS enzyme isolated; nNOS, eNOS, and iNOS (Alderton, Cooper, and Knowles, 2001; Maas et al., 2002). Parasympathetic nerve fibers innervating the penile tissue have been shown to contain the nitric oxide synthase enzymes, specifically non-adrenergic non-cholinergic (NANC) nerve fibers, and therefore have since identified the enzyme as

nNOS. Nitric oxide synthase has also been identified in the endothelial tissue and

classified as eNOS. This endothelial tissue is found lining the cavernosal and helicine

arteries. Cholinergic innervation and acetylcholine stimulation on the endothelial cells

results in increased production of eNOS by the endothelial cells (Maas et al., 2002). A

third isoform of the enzyme has been identified as inducible-NOS, this isoform is often a

reactionary byproduct to counteract local mediators of vasoconstriction (Maas et al.,

2002). nNOS is the isoform that is found in the highest relative concentration within the

penile tissue (Burnett *et al*, 1992). NO is produced by the NOS-mediated conversion of

L-arginine to L-citrulline and NO (Burnett, 1997; Ghalayini, 2004). The competitive

inhibitor, N-nitro-L-arginine (LNNA), prevents the substrate L-arginine from binding

with the NOS molecule and therefore prevents the production of NO; erectile responses

are decreased when this competitive antagonist is infused (Mills et al., 1999).

Early investigations in the signal transduction mechanism for smooth muscle

relaxation show that nitric oxide was originally named endothelium-derived relaxing

factor (Furchgott and Zawadzki, 1980; Lincoln, Komalavilas, and Cornwell, 1994). The

observations that lead to understanding the endothelium-derived relaxing factor was

indeed nitric oxide included identification of the similar pharmacological characteristics

as well as the increase in intracellular cyclic guanylyl monophosphate (cGMP) that

resulted from intracellular increase of each compound (Palmer, Ferrige, and Moncada,

1987). When released, nitric oxide travels through the plasma membrane of the smooth

muscle cells and activates the soluble guanylyl cyclase (Lee and Kang, 2001; Behrends et

al., 2000, McDonald and Murad, 1996; Sausbier et al., 2000). This enzyme occurs both

in a membrane bound and soluble form; the target of nitric oxide for relaxation is soluble

guanylyl cyclase (sGC). sGC is an enzyme that is responsible for converting guanylyl

triphosphate (GTP) to cGMP. Evidence has shown that when sGC is activated by YC-1

[3-(5'-hydroxymethyl-2'-furyl)-1-benzlindazole], an increase in the amount of

intracellular cGMP, vasodilatation, and thus relaxation of cavernosal smooth muscle

occurs (Schmidt *et al*, 1993; Wu et al., 1995). When YC-1 treatment is combined with a

nitric oxide donor, tissue relaxation increases synergistically (Nakane et al., 2002). One

proposal for this increased response has been attributed to multiple binding sites found on

the sGC molecule (Hsieh et al., 2003; Nakane et al., 2002). Likewise when the sGC is

inhibited by the compound 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) or

methylene blue, the amount of vasodilatation and relaxation of the cavernosal tissue is

limited when stimulated by NO (Reilly et al., 1997; Hsieh et al., 2003). Multiple

isoforms of the subunits that compose the sGC molecule have been reported in

cavernosal tissue (Behands et al., 2000). However, comparisons among ED patients and

patients with normal erectile function showed no differences in the expression of the

isoforms that form the active sGC (Behrends et al., 2000; Burnett, 1997). The cGMP

produced by sGC is a critical component in the relaxation cascade in both NO-dependent

and –independent mechanisms (Reilly et al., 1997). cGMP has at least three groups of

targets when activated in the relaxation cascade of cavernosal smooth muscle;

phosphodiesterases, protein kinases, and ion channels.

Phosphodiesterases are found throughout the body with varying distribution.

Several isoforms of the enzyme have been identified; however, two specific isoforms

(PDE5, PDE3) have been recognized to play an important role in erectile physiology

(Moreland et al., 2001). PDE5 has further been identified to exist in multiple isoforms as

PDE5A1, PDE5A2, and PDE5A3 (Lin, 2004), with PDE5A3 the most relevant to erectile physiology. Expression of genes and mRNA for PDE5 have been shown to be most concentrated in the cavernosal tissue (Morelli et al., 2004) The phosphodiesterase action occurs by hydrolyzing cGMP into an inactive 5`-guanosine monophosphate (5`-GMP) and therefore inactivating this critical component of the transduction pathway for relaxation (Beavo, 1995). As a target of the cGMP, the phosphodiesterases are deactivated to allow cGMP to remain effective on vasodilatation factors.

The phosphodiesterases have become a significant therapeutic modality in the treatment of erectile dysfunction. The first therapy of this kind to gain significant momentum was sildenafil and its target was PDE5 (Moreland et al., 1998). The PDE5 inhibitor mechanism of action works to competitively bind with the PDE5 molecule in cavernosal tissue and block the receptor site for cGMP, rendering it inactive (Corbin and Francis, 2003). Administration of sildenifil in both in vitro and in vivo studies resulted in normotensive blood pressure, increased cavernosal vasodilatation, increased intracavernosal pressure, and increased erectile responses in previously unresponsive subjects (Carter et al., 1998; Simonsen et al., 2001, Prieto et al., 2006). Though other approaches to the understanding and treatment of erectile dysfunction are being taken, PDE5s (sildenafil, vardenafil, tadalafil) have come to assume the majority of the commercial market of erectile dysfunction (Argiolas, 2005).

Another target of cGMP that contributes significantly to the relaxation and erectile response of cavernosal tissue is protein kinase G (PKG) (Bonnevier et al., 2004). PKG has been found in humans in two different forms; PKG-I and PKG-II (Keilbach, Ruth, and Hofmann, 1992). Of these two subtypes, PKG-I was found to be the most

concentrated. Lin et al. (2002) found that activation of PKG-I could be achieved by cGMP as well as by cross-activation with cAMP. Other cross-activation mechanisms explored include cGMP activating PKA which is was then proposed to activate BK channels, though this occurred at supra-physiological concentrations (Sausbier et al., 2000). In fact, the cross-activation potential between cAMP/PKA transduction systems and cGMP/PKG transduction systems have been proposed as potential therapeutic targets, though the mechanism has been shown to be inadequate for compensating a PKG disruption (Uckert et al., 2004; Stief et al., 2000).

Many potential downstream targets of PKG have been proposed making the precise mechanism of this molecule difficult to pinpoint. Likewise for that of cGMP, the overall goal of PKG is to reduce cytosol calcium concentrations and maintain an intracellular electrochemical balance that sustains the relaxation mechanism. Direct targets of PKG include ion channels found on the sarcoplasmic reticulum, sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps (Szado et al., 2001), that directly sequester calcium, phosphorylation of calcium-dependent potassium channels (BK channels), and inhibition of L-type (voltage-regulated) calcium channels (Lincoln, Dey, and Sellak, 2001). Indirect mechanisms include phosphorylation of IP3 (Rapoport, 1989) and an increase of spontaneous outward transient current (STOCs) that lead to net loss of intracellular calcium concentration (Lincoln, Dey, and Sellak, 2001). PKG has also been reported to inhibit PDE, thus facilitating the erectile response (Corbin and Francis, 2003; Bonnevier et al., 2004).

The calcium-sensitizing pathway based upon the effects of RhoA/Rho kinase is also a key downstream target that leads to tumescence. Cavernosal tissue remains

contracted despite low calcium concentrations with the aid of Rho kinase phosphorylating MLC phosphatase (Mills, Chitaley, and Lewis, 2001). Evidence has indicated that the NO/cGMP cascade specifically targets the RhoA molecule with PKG in order to facilitate the dephosphorylation of MLC, which will in turn lead to tissue relaxation (Sawada et al., 2001; Sauzeau et al., 2000). Additional evidence identified a synergistic relaxation effect when a NO-donor agent was combined with the Rho kinase inhibitor Y-27632 to stimulate cavernosal tissue (Mills et al., 2002). Chitaley and Webb (2002) supported the RhoA/Rho kinase inhibition mechanism to promote relaxation in aortic rings.

Likewise, when calcium was removed from the cytosol and myosin phosphatase was activated, cavernosal tissue relaxed (Hilgers and Webb, 2005). Active removal of calcium from the intracellular space occurs through multiple mechanisms and can be categorized by two basic means: (1) sequestration of calcium into the sarcoplasmic reticulum and (2) export of calcium via plasma membrane cation pumps. Activation of the sarcoplasmic reticulum results in the organelle sequestering more calcium and lowering the intracellular calcium concentration (Szado et al., 2001; Woodrum and Brophy, 2001); similarly inhibition of the calcium pumps on the sarcoplasmic reticulum attenuates the calcium sensitivity when membrane channels release intracellularly (Woodrum and Brophy, 2001).

In the class of plasma membrane ion channel targets that receive upstream signals and promote the decreased tone of cavernosal tissue leading to tumescence, the large-conductance calcium-activated potassium (BK) channels have been extensively reviewed(Ghatta *et al*, 2006; Prieto et al., 2006; Archer 2002). These channels have been

confirmed to be present in cavernosal, spongiosum, and penile arterial vasculature (Werner et al., 2005). The core of the BK channel is formed by alpha-subunits that give shape/structure to form a pore that allows the passage of K+ as well as the molecular structures sensitive to intracellular calcium; beta sub-unit accessory molecules control conformational changes of the channel that allow and inhibit the passage of potassium (Ghatta et al., 2006). Though it should be noted for this project, regulation of the channel occurs not only at the beta sub-units, but can also be controlled by physical blockade of the alpha-pore structure. Examples of such pore blockers include charybdotoxin (ChTX) and iberiotoxin (IbTX). While the BK channel receives much of the attention in terms of smooth muscle physiology, it is one of three different groups of K+ channels characterized by their conductance tendencies: Large-conductance, intermediate-conductance, and small conductance calcium-activated potassium channels (Ghatta *et al*, 2006; Fan *et al*, 1995). A comparative study between rat and human myocytes from cavernosal tissue found no relative functional difference between the two specimens in terms of BK channel morphological and electrochemical properties, making the rat cavernosal tissue an acceptable model for experimentation (Wang et al., 2000).

Throughout the body, BK channels are involved in a number a different tissue functions and are activated independently by calcium influx, cellular depolarization, or direct activation (Schubert and Nelson, 2001; Xia, Zeng, and Lingle, 2002). The source of the calcium as noted above comes from the sarcoplasmic reticulum or the extracellular space, depending on the stimulus. When activated by direct phosphorylation, such as PKG, BK channels open to allow potassium to flow down its concentration gradient, moving from inside the cell to the extracellular space. The loss of this positively charged

ion results in a net loss of positively charged ions and therefore an increase in the membrane potential (Tare et al., 1990). The increase in membrane potential and activity of kinases that phosphorylate L-type calcium channels cause it to close (Christ, 2002). As potassium is leaving and the membrane potential increases, a hyperpolarized state is established and the contractile machinery ceases; much of the tone observed in smooth muscle myocytes is the direct product of the activity of the BK channel to hyperpolarize the membrane potential (Nelson *et al.*, 1990; Bychkov *et al*, 1998, Sausbier *et al*, 2000).

A proposed mechanism by which the BK channel is activated identifies PKG as the molecule responsible for stimulating the channel to open (Archer *et al*, 1994; Robertson *et al*, 1995). Protein kinase G is hypothesized to stimulate the BK channel via direct phosphorylation which results in increased channel activity (Bielefeldt and Jackson, 1994; Schubert and Nelson, 2001). At one time it was thought that nitric oxide was bypassing the cGMP/PKG mechanism and activating the BK channel itself, however, this independent mechanism has been shown to be unlikely (Sausbier *et al*, 2000) and a NO-sGC-cGMP mediated mechanism has been supported experimentally (Lee and Kang, 2001). Agonistic studies targeting the BK channel have confirmed their role in other tissues such as patch-clamp studies (Malysz et al., 2004) of smooth muscle tissue from urinary bladder muscle utilizing the compound NS1608 ((N-3(trifluoromethyl)phenyl)-N`-(2-hydroxy-5-chloropheny-1)urea) (Siemer et al., 2000; Mora and Suarez-Kurtz, 2005; Hu and Kim, 1996). Direct activation of sGC (Paterno et al., 1996) and cGMP (Price and Hellerman, 1997) in rat vascular smooth muscle lead to the BK channel activation which resulted in vasodilatation. Gruhn et al. (2002) compared the responsiveness to nitroglycerin (a NO-donor) while inhibiting BK channels

in human arterial and venous samples, and they found the arterial beds were more responsive to the nitroglycerin as well as an endothelial-dependent response of the arterial tissue. Likewise in coronary arteries, responsiveness to a NO-donor is less sensitive when the BK channels have been inactivated supporting the theory that the BK channel is a critical component of the relaxation cascade (Bychkov et al., 1998). As mentioned previously, the inhibition of PDE5 with compounds such as sildenifil and tadalifil have produced revolutionary treatment success. An increase in BK channel opening from penile arteries was found when sildenifil was administered to inhibit the PDE5; suggesting upstream regulators of each may be shared (Prieto et al., 2006; Lee and Kang, 2001).

The use of genetically mutated, or "knock-out" mice (PKG -/-) has also validated how important of a role BK channels play in the relaxation mechanism. The PKG molecule is genetically eliminated from the organism and both in vivo and in vitro studies have shown that the ability of the cavernosal tissue to relax and produce and erectile response eliminated (Hedlund et al., 2000; Werner et al., 2005). Furthermore, the ability of cAMP to cross-communicate from the same NO stimuli does compensate for the PKG -/- treatment (Waldkrich et al., 2005). Interestingly though, mice that had the NOS gene removed retain the ability to reproduce which implies erectile function remains intact (Huang et al., 1993). Despite differences between species, it was speculated that alternative molecular mechanisms to that of NO exist to support the erectile response.

Iberiotoxin, as mentioned previously, acts antagonistically on BK channels with very high selectivity to prevent efflux of potassium and therefore prevents relaxation and promotes contraction of smooth muscle (Mora and Suarez-Kurtz, 2005). The original

pharmacological agent that was used to target BK channels was charybdotoxin (Miller et al., 1985).   The mechanism by which charybdotoxin works is very similar to that of the iberiotoxin, occupying the external side of the channel to prevent potassium efflux, however charybdotoxin does so with much less specificity to potassium channels (Candia, Garcia, and Latorre, 1992). Iberiotoxin was discovered in the venom from the *Buthmus tamulus* scorpion and comprises less than one percent of the total venom (Galvez et al., 1990). Blockade, or inactivation, of the BK channel has been shown to increase the responsiveness of the smooth muscle tissue to stimulation from alpha-adrenergic innervation making contraction of smooth muscle more likely (Spektor et al., 2002; El-Hajj et al., 2005).   Likewise, inhibition of the BK channel renders tissue less responsive to pro-relaxation stimuli such as nitroglycerin (Satake, Fujimoto, and Shibata, 1996).

Role of Androgens

The relationship between erectile function and androgens has largely been assumed to be based around the effects at the central neurological level. Androgens have long been shown to affect the male's libido and disposition to copulation (Korenman et al., 1990). When present, androgens have been shown to increase interest in sex, and likewise when androgens are absent a decrease in interest is observed. Though strong correlations have been made between erectile function and testosterone availability, much of mechanism by which testosterone supports the erectile response remains to be clarified.

The androgen receptor has been identified in cavernosal tissue across multiple species (Lewis and Mills, 2004). The time in which an organism is transitioning though

puberty appears to be when androgen receptors are in greatest concentrations within the cavernosal tissue and steadily decline after the peak of puberty (Lewis and Mills, 2004). Androgens are transported throughout the body either bound to sex hormone binding globulin (SHBG) or to albumin. Once at the target tissue, the androgen will break free and passively move through the plasma membrane into the intracellular space. Once the androgen binds to the androgen receptor, morphological changes occur to allow interaction with genes for transcription to follow and protein synthesis leading to a cellular response (Lee and Chang, 2003). While an exact relationship of testosterone binding the androgen receptor and the erectile response is still unknown, Penson et al. (1996) found that receptor sites blocked with flutamide in castrate animals produced no erectile response while the sham treatment group displayed a decreased, however present, erectile response. Also in terms of a genetic effect, Bowles et al. (2004) concluded circulating testosterone concentration to have a direct correlation with L-type voltage-gated calcium channels in regards to expression and channel activity, supporting the role of testosterone in the erectile response.

The non-genomic action of testosterone has been explored through a number of tissues and animals. The non-genomic mechanism seems to primarily utilize a G-protein second messenger found in the plasma membrane (Heinlein 2002). The non-genomic changes seen from testosterone occur in a timeframe considered too quickly, 10 seconds to five minutes, to possibly be transcriptional (Tep-areenan et al., 2003). Intramuscular injections of testosterone versus oil-vehicle into castrated rats (four weeks post-surgery) resulted in a demonstration of electromyographic bursts and contractions of the bulbospongiosus muscle, further illustrating the potential of rapid, non-genomic effects of

testosterone in these striated muscles that augment erection (Sachs and Leipheimer, 1988). Alternative approaches to the non-genomic relaxation properties of testosterone blocked the androgen receptor with flutamide (competitive receptor antagonist) and testosterone's ability to produce a relaxation effect on the tissue persisted (Deenadayalu et al., 2001), suggesting the non-genomic effect is created by a mechanism independent of the androgen receptor (Jones et al., 2003). Of active forms of androgens, the 5beta-dihydrotestosterone was found to be the most effective in producing a vasodilatation response via non-genomic, NO-independent mechanism that acted directly on ion channels (Perusquia el al., 1996; Perusquia and Villalon, 1999), most likely calcium regulatory channels. In the review from Jones et al. (2003), the proposal that testosterone is acting both as a calcium-antagonist (preventing calcium-dependent contraction) as well as acting to open BK channels (Tep-areenan et al., 2003) via cGMP elevation and other intracellular messengers (promoting the active relaxation of smooth muscle) suggests that the vasodilatory action of testosterone has many targets to net the overall relaxation effect.

Aside from psychogenic effects, low levels of testosterone have also been correlated with anatomical changes in the body relevant to erectile dysfunction. Terminal axon density at the vas deferens is decreased when circulating testosterone is reduced by chemical/surgical castration or natural aging (Keast et al., 2002). Decrease in circulating androgens could lead to erectile dysfunction by decreasing the amount and efficacy of other efferent pathways: specifically, changes in the pelvic ganglion, dorsal nerve, and cavernosal nerve fibers undergo downregulation and structural degradation (Bialek et al., 2004). Concentration of nerve fibers and dispersal of axon terminals were decreased

along with thickness of the myelin sheaths and restoration of these structures along with erectile function was achieved with supplemental testosterone (Bialek et al., 2004).

Under conditions of hypogonadism the penile tissue has been observed to undergo changes that go beyond cessation of smooth muscle growth and division. The identification of adipocytes, or fat-containing cells, was important not only in terms of identifying potential targets for androgens but also understanding structural changes that occur during erectile dysfunction due to hypogonadism (Traish et al., 2005). Though the exact relationship has yet to established, it was clearly shown that the smooth muscle cells in the trabecular sinuses were being replaced with connective tissue. Again the effects of the hypogonadic state, the adipocytes deposition, were reversible with testosterone supplementation (Traish et al., 2005). Increased venous leaking during the erectile process can at least be partially attributed to the increase adipocytes (Traish and Kim, 2005). Doppler flow rate studies have shown that compared to intact animals, castrate models were observed with higher venous flow rates during erectile stimulation (Palese et al., 2003; Mills et al., 1994). The adipocytes and their inability to dilate leave the cavernosal tissue at the same relative volume thus not expanding outward to tunica albuginea. The veno-occlusive mechanism is therefore compromised because of the continued venous drainage and inability to raise intracavernosal pressure. Another site in which changes occur as result of hypogonadism is in the penile tissue itself. Specifically, androgens have a trophic affect on the trabecular smooth muscle. Promotion of growth and division are some of the primary mechanisms by which androgens contribute to the erectile process: In vivo studies of human cavernosal arteries correlated higher compliancy with increased serum testosterone concentrations (Aversa et al., 2000).

The calcium-sensitizing mechanism of RhoA/Rho kinase has been shown to become up-regulated when rats are castrated and androgens no longer present (Wingard et al., 2003). This results in heightened activity and sensitivity of the smooth muscle contractile mechanism which results in increased tone of the cavernosal tissue. This also provides one explanation as to why hypogonadal subjects are more prone to erectile dysfunction. Initial proposals to the relationship between NO and Rho A suggested NO was directly inhibiting the RhoA-mechanism, however, further research now indicates that the downstream modulator PKG is responsible for the deactivation of RhoA (Wingard et al., 2003, Mills et al., 2002, Sauza et al., 2000.). Supporting research has also found that when the Rho kinase inhibitor Y-27632 is used to treat cavernosal tissue from hypogonadal subjects the tissue response to vasodilators improves (Chitaley et al., 2001), especially when an exogenous NO-donor is present with the inhibitor (Chitaley et al., 2003). Treatment for erectile dysfunction have thus been proposed to target the RhoA/Rho kinase mechanism, especially in hypogonadal subjects (Mills et al., 2001); these in vivo models administered Y-27632 to castrated rats to produce cavernosal relaxation without any significant affect on the blood pressure (Chitaley et al., 2001, Wingard et al., 2003). One downstream target found not to be a crucial target of the androgenic effect is that of sGC (Alcorn, Toepfer, and Leipheimer, 1999). Simulation of its downstream products created no effect in castrate rats and therefore it was speculated androgens target later earlier in the cascade.

The relative sensitivity of the cavernosal tissue to other stimuli also changes depending on the androgen availability. Cavernosal sensitivity to alpha-adrenergic stimulation increases when androgen availability decreases, making the tissue more likely

to maintain a contracted state and less responsive to pro-relaxation stimuli (Riley et al., 1997; Mills et al., 1999). This was further supported by Wingard et al. (2001) using the highly specific alpha-1 agonist methoxamine, which showed NO reduced the effectiveness of methoxamine and provided a dominant stimulus that lead to relaxation of cavernosal tissue. Sensitivity to androgen depletion and restoration has also been demonstrated in the skeletal musculature (bulbospongiosus and ischiocavernosus muscles) that facilitates the erectile response (Leipheimer and Sachs, 1993).

Aging is another factor that can lead to both hypogonadism and erectile dysfunction (Garban et al., 1995). Structurally, young and old human cadaver investigations found that supportive skeletal muscle (bulbospongiousis and ischiocavernosus muscles) content is decreased in the latter group (Hsu et al., 2004). The age-correlated decline circulating levels of testosterone has been suggested as one of the leading causes of erectile dysfunction (Garban et al., 1995; Carrier et al., 1997; Champion et al., 1999). Supplemental testosterone to hypogonadal rats results in full erectile function returning, and specifically restoration of NOS levels (Garbon et al., 1995). One combination therapy that has been investigated is with the Rho kinase inhibitor Y-27632 along with PDE-5 inhibitors and shown aged rats produce an increase in erectile responsiveness by 12-27% compared to the Y-27632 alone (Rajasekaran et al., 2005). Despite strong correlations between aging and erectile dysfunction, one component not affected by aging is PKG-I. No quantitative decrease in the concentration of PKG-I was found in aged rats, however, responsiveness to cGMP was depressed (Lin et al., 2002). Likewise a negligible decrease in the sGC and cGMP concentration of middle-aged rats, used to represent early onset age and hormone related erectile

dysfunction (Hosogai et al., 2003), compared to younger models despite a significant amount of NOS activity. All together, this identified probable targets for androgens as well as non-targets that give rise to potential pathological sources for erectile dysfunction.

Other combination therapies used testosterone and either sildenafil in borderline-hypogonadic males (Aversa et al., 2003) or tadalafil in human subjects that did not respond to the PDE5 therapy alone (Yassin, Saad, and Diede, 2006; Zhang et al., 2005)), and found increases in cavernosal blood flow along with increased vasodilatation that resulted in improved erectile response. Morelli et al. (2004) found a clear decrease in the expression of genes of mRNA for PDE5 in rabbits that were castrated; testosterone supplementation restored PDE5 expression to basal levels which lead to the conclusion androgen availability has a direct relationship to PDE5 expression. Another experiment utilized diabetes-induced erectile dysfunction and found testosterone supplementation along with sildenafil was successful in restoring erectile function (Zhang et al., 2006). With as little as 30-50% of human subjects yielding positive responses to PDE5 inhibitors (Salonia et al., 2003), the success of combination therapies with testosterone emphasizes some of the functional endpoints the hormone maintains in erectile physiology.

The BK channel is one component of the NO-cGMP-PKG pathway that has been proposed to be linked with aging and hypogonadism. In penile tissue from rats, Davies et al. (2007) found increased concentration of the BK channel that lead to low or absent erectile responses. Studies of aged rat coronary artery smooth muscle found a decreased expression in BK channels that lead to increased arterio-constriction (Nishimaru et al.,

2004; Marjic et al., 2001). With no functional BK channels the cavernosal myocytes and penile arterial vasculature are unable to attain a sufficient membrane potential that would promote tissue relaxation and therefore remain chronically contracted.

The NOS enzyme responsible for production of NO has also been speculated as a target for androgens. Some research indicated testosterone was affecting cavernosal tissue by regulating the availability of NO (Mills et al., 1999), however, NOS is identified as a primary target of an androgenic mechanism to support the relaxation of cavernosal tissue (Chamness et al., 1995, Lugg et al., 1995). A decrease in the amount of NOS from castrate rats occurs compared to intact or testosterone-replacement rats (Lewis and Mills, 2004; Carrier et al., 1995). Though this decrease in NOS along with a decreased erectile response is associated with decreased availability of testosterone, some erectile response does remain; the remaining response can be eliminated when androgenic effect is accounted for by surgical castration with concurrent treatment of flutamide (Penson et al., 1996).   This decrease in NOS as also found in diabetic rats, though it was noted that these animals were also found with significantly low plasma testosterone levels (Garbon et al, 1995; Vernet el al., 1995).

Changes in the expression of the nNOS and thus availability of NO, several downstream targets are also affected. The sensitivity and responsiveness of the cavernosal tissue to NO is decreased in castrate rats and therefore thought to be at least semi-dependent on androgen availability (Mills, Wiedmeier, and Stopper, 1992; Alcorn, Toepfer, and Leipheimer, 1999). Cavernosal tissue from middle-aged rats had decreased NOS concentration/activity than compared to younger rats despite only a slight decrease in the concentration of cGMP (Hosogai et al., 2003; Lugg, Rajfer, and Gonzales-Cadavid,

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1995). In addition, the regulation of NOS by androgens has been supported by evidence

illustrating the up-regulation and expression of mRNA for NOS when androgens are

present and complement down-regulation and expression when castrate models were

used, as well as testosterone replacement therapy in castrated animals (Reilly et al., 1997;

Park et al., 1999; Marin et al., 1999).

Summary and Hypothesis

The smooth muscle of the corpus cavernosum tissue is fundamentally identified in

two states; contracted (flaccid) and relaxed (erect). The contracted smooth muscle results

in high amounts of blood returning to systemic circulation, relatively low intracavernosal

pressure, and thus flaccid penis. The mechanism for relaxation serves to decrease the

amount of venous blood return and increase the intracavernosal pressure. Among the

many factors that affect the mechanisms of cavernosal smooth muscle contraction and

relaxation, the presence/absence of androgens has become an undeniable component

directly related to structure and function of the tissue. Androgens have many targets in

penile tissue smooth muscle tissue that support normal physiology and cavernosal tissue

from castrated rats demonstrate decreased relaxation. Additionally, cavernosal smooth

muscle has been found to relax from a NO-donor with a rapid, initial phase (Phase1)

followed by a longer, more sustained phase (Phase 2) (Leipheimer and Toepfer,

unpublished observations). Experiment 1 investigated whether castration (androgen-

depleted cavernosal tissue) would have a differential effect on Phase 1 or Phase 2.

Literature suggests a role for BK channels in mediating relaxation of smooth muscle

tissue. Experiment 2 investigated whether inhibition of BK channels would effect the

specific phases (Phase 1 and 2) of relaxation and test whether the presence or absence of

androgens influenced BK channels.

Materials and Methods

Animals

Long-Evans strain laboratory rats were obtained from Youngstown State University colony from stock originally purchased from Charles Rivers Laboratories (Wilmington, MA). Animals were housed in plastic cages with one to four animals per cage. Food and water were freely available to animals and the light cycle was set for 12 hour light/12 hour dark (lights on at 10:00 p.m.). Temperature was maintained at 22° C. Sexually mature animals were randomly assigned to one of two groups; sham-castrate and surgical castrate. Sham castrate animals were anesthetized with ketamine (50 mg/kg) and xylazine (8 mg/kg). After shaving the animal's hair and under aseptic technique, a midline scrotal incision was made and the underlying subcutaneous and connective tissue was cleared from the area directly over a testis so that the tunica vaginalis could be visualized. A single incision was made in the tunica vaginalis and the testis was visualized but not removed. The tunica vaginalis was then closed with a single 4-0 silk thread suture. This procedure was repeated to the tunica vaginalis superficial to the other testes. The dermal tissue was closed with three to four 4-0 silk thread sutures. Castrate group animals were anesthetized with the same procedure as above. Again a midline scrotal incision was made followed by clearing the subcutaneous and connective tissue to access the tunica vaginalis above one of the testes. After the single incision was made in the tunica vaginalis, the testes and epididymis were extracted through the incision and out of the scrotal cavity. A 3-0 silk thread ligation was applied to the spermatic cord proximal to the testes and epididymis. The testes and epididymis were then excised with surgical scissors by cutting the spermatic cord just distal of the 3-0 silk thread ligation.

The tunica vaginalis was sutured closed with 4-0 silk thread. This procedure was repeated for the remaining testis. Once the second testis was removed and the tunica was sutured, the dermal tissue was closed with three to four 4-0 silk thread sutures. Animals from both groups were allowed at least two weeks of recovery to allow hormone levels to stabilize.

These experiments were approved by the Institutional Animal Care and Use Committee of Youngstown State University.

Tissue Collection

On the day of an experiment, the animal was euthanized with carbon dioxide overdose, a method in compliance with the AVMA Guidelines on Euthanasia 2007 (formerly Report of the AVMA Panel on Euthanasia). The penile tissue was removed from the animal and placed in a modified Kreb's solution (described below). Superficial connective tissue was removed from the penile tissue as well as the urethra and dorsal penile vein. A tissue specimen length of approximately two centimeters was obtained from each animal. The tissue was then bisected longitudinally with a surgical blade to produce two approximately equal strips of cavernosal tissue. Each individual tissue was first anchored to a thin connecting wire at the distal end and the proximal end of the tissue was anchored to the fixed mounting bracket. This tissue assembly was then placed in a water-jacketed tissue chamber containing 10 mL of the modified Kreb's solution, aerated with 95% oxygen / 5% carbon dioxide gas mixture to oxygenate the tissue, and maintained at a constant pH. Heated water was pumped through the jacketed chambers to maintain at a constant temperature of 37° C.

Data Collection

The free end of the thin connecting wire attached to the distal end of the

cavernosal tissue was anchored to a Grass Force-Displacement Transducer FT-03 (Astro-

Med, Inc. West Warwick, RI). The force transducer was connected to a Grass model

P122 strain gauge amplifier (Astro-Med, Inc. West Warwick, RI). The amplified signal

was then received by the PolyView 2.5 computer software (Polybytes, Inc. Cedar Rapids,

Iowa). Data values were recorded in terms of tension (milligrams). Once the tissue

assembly (tissue, fixed mounting bracket, and thin transducer-connecting wire) was set in

the tissue bath with 10 mL of Kreb's solution, a resting tension of approximately 300 mg

was set and the tissue equilibrated for one hour. After equilibration, the Kreb's solution

in the tissue chamber was drained and fresh Kreb's solution was added to the chamber.

The 300 mg tension baseline was re-established and data recording began. Three to five

minutes of baseline tension was recorded and then norepinephrine (NE) was added to the

tissue bath resulting in a final concentration appropriate to the experimental design. After

a 20 minute contractile period the nitric oxide donor, sodium nitroprusside (SNP), was

added to the tissue bath at a final concentration of 10^{-3} M in a 100 μL volume and

recording continued for an additional 30 minutes.

Experimental Design

Experiment 1: Sham versus Castrate

The two treatment groups, sham (n= 6) and castrate (n= 6), followed the

experimental design outlined above. Norepinephrine was added to the chambers

resulting in a final concentration of 10^{-4} M (Tong and Cheng, 1997; Alcorn, Toepfer, and

Leipheimer, 1999). After the 20 minute time period, SNP was delivered to the tissue bath

at a final concentration of 10^{-3} M to relax the tissue. Tissues were allowed 30 minutes to relax.

Experiment 2: Sham-castrate with Vehicle / Iberiotoxin Treatment and. Castrate with Vehicle / Iberiotoxin Treatment

The two treatment groups, sham (n= 19) and castrate (n= 13), followed the general experimental design outlined above. However, norepinephrine was added to the chambers at a final concentration of 10^{-5} M (Tong and Cheng, 1997). This concentration was selected based upon the dose-response curve of norepinephrine in rat cavernosal tissue. Norepinephrine 10^{-5} M stimulated cavernosal tissue at a less-than-maximal response and for the effect of allowing iberiotoxin (described below) to exert its effect (if any). Ten minutes after the addition of the norepinephrine either 100 μL iberiotoxin (highly selective calcium-dependent potassium channel antagonist) for final concentration of 100 nM; or 100 μL of vehicle (modified Kreb's solution) was added to the tissue bath. Ten minutes was allowed for iberiotoxin to equilibrate, and then SNP was added to the tissue bath at a final concentration of 10^{-3} M. The tissue was allowed 30 minutes to relax after the addition of SNP, and then data recording was stopped.

Solutions and Drugs

The modified Kreb's solution used was prepared from the following; NaCl (119 mM), KCl (2.6 mM), NaHCO3 (15 mM), MgCl2*6H2O (1.2 mM), NaH2PO4*H2O (1.2mM), CaCl2 (1.5 mM), and glucose (11 mM) (Alcorn, Toepfer, and Leipheimer, 1999). The pH of the modified Kreb's solution was adjusted to 7.35-7.45 using either HCl or NaOH. Norepinephrine bitartrate salt and SNP were purchased from Sigma-Aldrich, Inc., St. Louis, Missouri. The iberiotoxin used was purchased from Bachem

Bioscience Inc., King of Prussia, Pennsylvania. All drugs used were dissolved in the modified Kreb's solution.

Data Analysis

Raw data was analyzed with the PolyView data analysis software and values measured for contraction and relaxation variables were placed in an Excel spreadsheet and analyzed with SigmaStat software. The total tension developed for the tissue after the addition of NE and the percent relaxation after addition of SNP were analyzed for each experiment. In addition, most groups also demonstrated two distinct phases of relaxation; a rapid relaxation period (phase 1) followed by a slower more sustained period of relaxation (phase 2) as previously observed in this laboratory for cavernosal tissue (unpublished observations).

Contractile tension was determined by measuring the tensile difference between the baseline and the peak tension produced after the addition of NE. The percent total relaxation was calculated by dividing the total relaxation value at 20 minutes after SNP by the contractile tension, and multiplying by 100. Percent phase 1 relaxation was calculated by dividing the amount of phase 1 relaxation by the total relaxation. The percent phase 2 relaxation was calculated by dividing the amount of phase 2 relaxation by the total relaxation. Therefore the percent relaxation of both phases was analyzed as well as the rate of relaxation in phase 1. The rate of relaxation of Phase 1 was measured with the dv/dt function so as to assess the maximum slope of the line. Phase 1 relaxation was measured by the tension difference between that just prior to the addition of the SNP and the point at which the rapid phase of relaxation ceases. Phase 2 relaxation was

measured by the tensile difference between the point at which the slower-relaxing portion of the relaxation phase began and 20 minutes after the addition of SNP.

Percent relaxation data were transformed via arcsine transformation so that between groups analysis could be conducted. Comparisons between control and treatment groups were analyzed with the student's t-test (Experiment 1) and two-way analysis-of-variance (ANOVA) in Experiment 2 (Sigma Stat v3.10). Post-hoc analysis of significant two-way ANOVA was conducted by the Student-Newman-Keuls method. Statistical significance was indicated with p-values less than or equal to 0.05. Graphs were constructed using Sigma Plot v9.0.

Results

Figure 1 is an example of a typical contraction/relaxation profile of cavernosal tissue from a sham-castrated rat. This figure illustrates the response of cavernosal tissue to NE by an increase in tension from the baseline. Additionally, the tension was reduced when SNP was administered to produce the two phases of relaxation in response to the SNP. Phase 1 was shown to relax rapidly, while Phase 2 relaxed more slowly and exhibited a sustained response. Figure 2 demonstrates that the contractile tension produced during the peak effect of norepinephrine during Experiment 1 was significantly increased in tissue isolated from castrated rats (267.02 ± 43.06 mg) compared to that for the sham-castrated animals (163.45 ± 27.27 mg); $p = 0.0348$. Figure 3 illustrates that tissue isolated from castrated animals showed a trend to relax less than cavernosal smooth muscle from sham-castrated rats (sham group: 61.45 ± 5.86 %; castrate group: 49.12 ± 4.26 %), however no significant differences were found between the groups. Figure 4, however, shows that castration significantly inhibited the percent relaxation of Phase 1 (sham group: 39.29 ± 6.34; castrate group: 22.51 ± 3.14 %; $p = 0.0395$). Figure 5 illustrates that cavernosal smooth muscle from castrated animals tended to relax slower than cavernosal smooth muscle from sham-castrated rats. However, these differences were not statistically significant. Figure 6 shows that a greater percentage of the total relaxation shifted to Phase 2 in cavernosal tissue from castrated rats. Cavernosal smooth muscle from castrated animals had significantly greater Phase 2 relaxation than cavernosal tissue from sham-castrated rats (sham group: 51.98 ± 5.27%; castrate group: 72.61 ± 4.56 %; $p = 0.0136$).

Experiment 2 investigated the effects of iberiotoxin (IbTX) treatment in cavernosal tissue from castrated or control (sham-castrate) rats. Peak tension produced after the addition of a sub-maximal dose of norepinephrine (final concentration 10^{-5} M) did not differ among the treatment groups: sham/iberiotoxin group (138.03 ± 21.06 mg), sham/vehicle (162.47 ± 19.26 mg), castrate/vehicle (115.72 ± 12.38 mg), and castrate/iberiotoxin group (121.76 ± 22.09 mg) (Figure 7). Figure 8 demonstrates the percent total relaxation resulting from treatment with sodium nitroprusside. IbTX treatment showed a trend to decrease relaxation in sham-castrated tissue (65.60 ± 1.63 %; sham-vehicle: 73.89 ± 2.12 %), however these differences were not statistically significant. Castration did result in a significant decrease in relaxation compared to cavernosal tissue isolated from sham-castrated animals (castrate-vehicle: 45.12 ± 7.34 %; sham-vehicle: 73.89 ± 2.12 %; $p < 0.01$). The IbTX treatment showed a trend to inhibit the percent phase 1 relaxation in the sham-iberiotoxin group (45.32 ± 1.66 %) compared with the sham/vehicle group (57.48 ± 2.51%), although the differences between the groups were not significant. IbTX treatment had no effect on the Phase 1 relaxation of cavernosal smooth muscle from castrated animals (castrate-vehicle: 61.25 ± 9.12%; castrate-iberiotoxin: 62.00 ± 10.52%; Figure 9). Figure 10 demonstrates that IbTX treatment significantly inhibited the rate of Phase 1 relaxation in sham-castrated rats (sham-IbTX: 0.64 ± 0.16 mg/sec; sham-vehicle: 1.66 ± mg/sec; $p = 0.003$). Figure 11 demonstrates that IbTX treatment tended to increase relaxation during Phase 2 (sham-iberiotoxin: 53.43 ± 2.56 % versus sham-vehicle: 41.75 ± 2.67%). However, there were no significant differences between the groups.
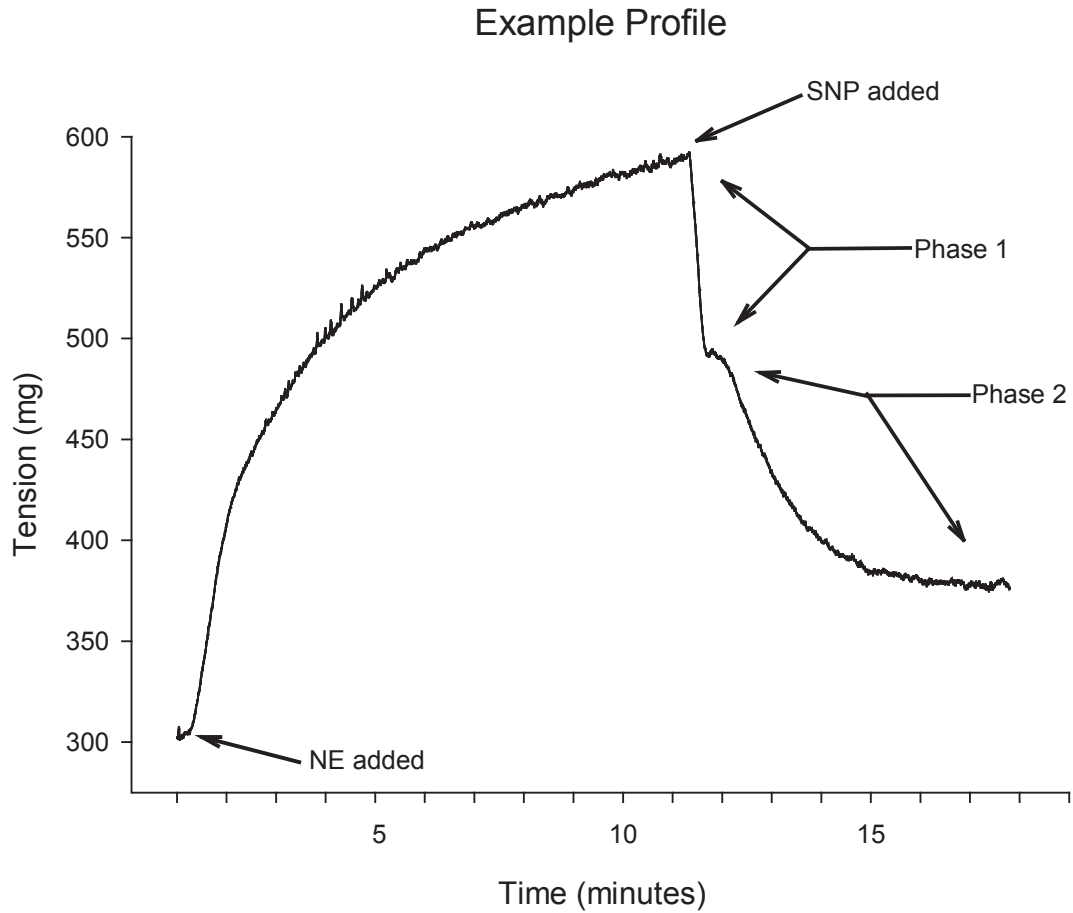
Figure 1. Representative example of a contraction/relaxation response of cavernosal tissue in the experiment. Tension increases following norepinephrine (NE) administration and relaxes following the sodium nitroprusside (SNP) administration. The phases of relaxation are illustrated to the right of the tracing. Time scale of the tracing in terms of the design has been adjusted to illustrate all components of the experiment.
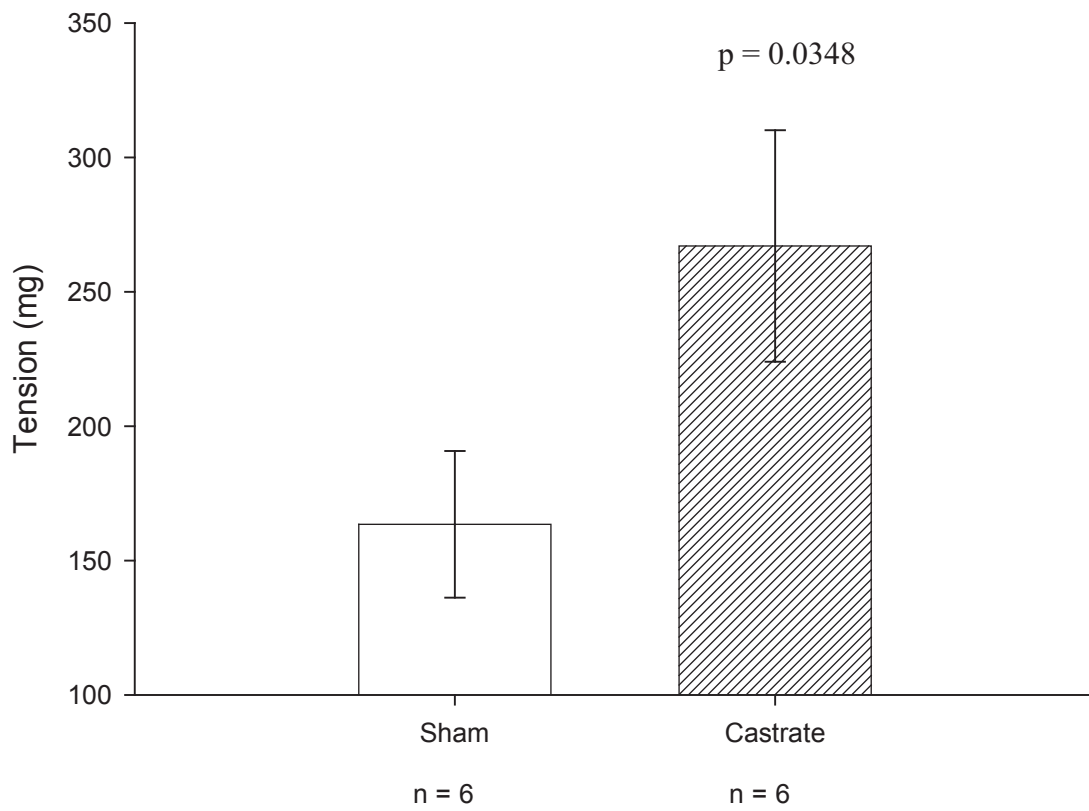
Contractile Tension

Figure 2. Peak contractile tension (mg) reported for Experiment 1 after the addition of NE. Peak tension was significantly greater in corpus cavernosum isolated from castrated rats (p = 0.0348). Values given are the mean ± SEM.
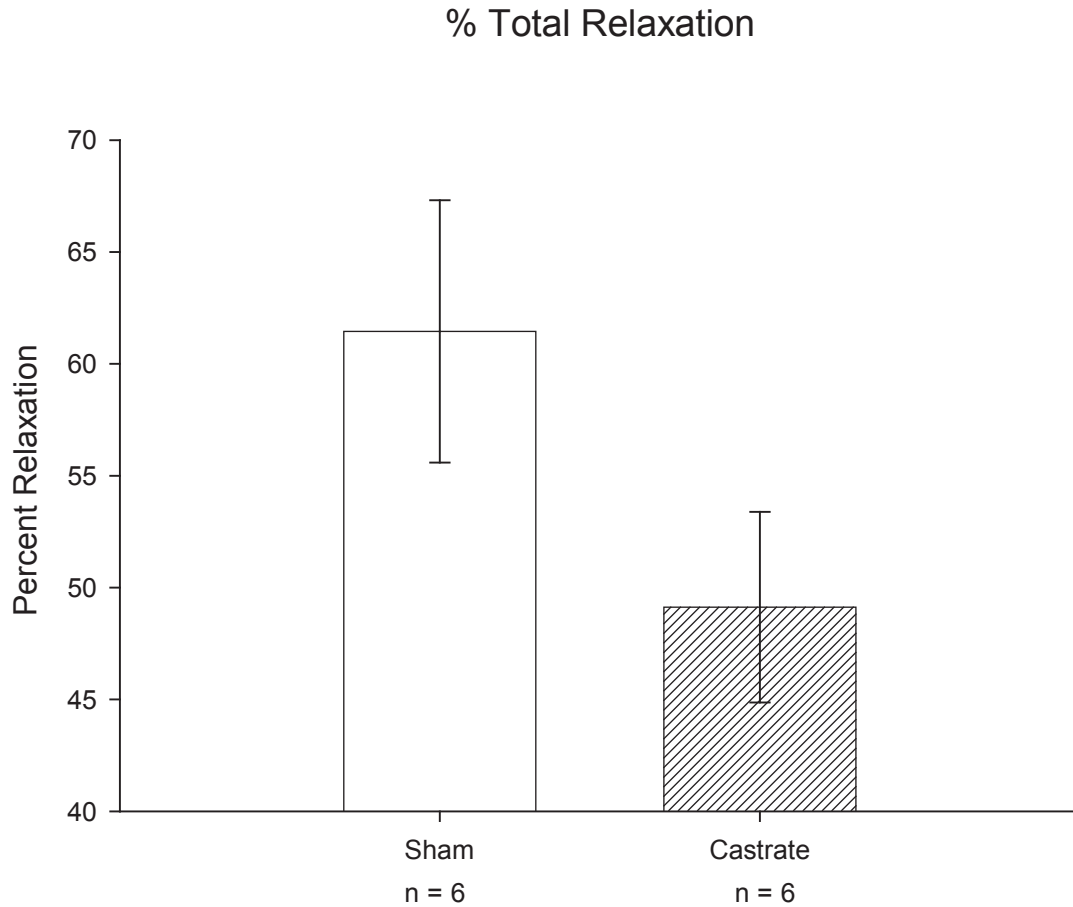
% Total Relaxation

Figure 3. Total percent relaxation of corpus cavernosum isolated from sham and castrated rats after addition of SNP to the chamber in Experiment 1. Although the percent relaxation of tissue from castrated rats tended to be less than that from sham animals, there was no significance difference between the groups. Values given are the mean ± SEM.
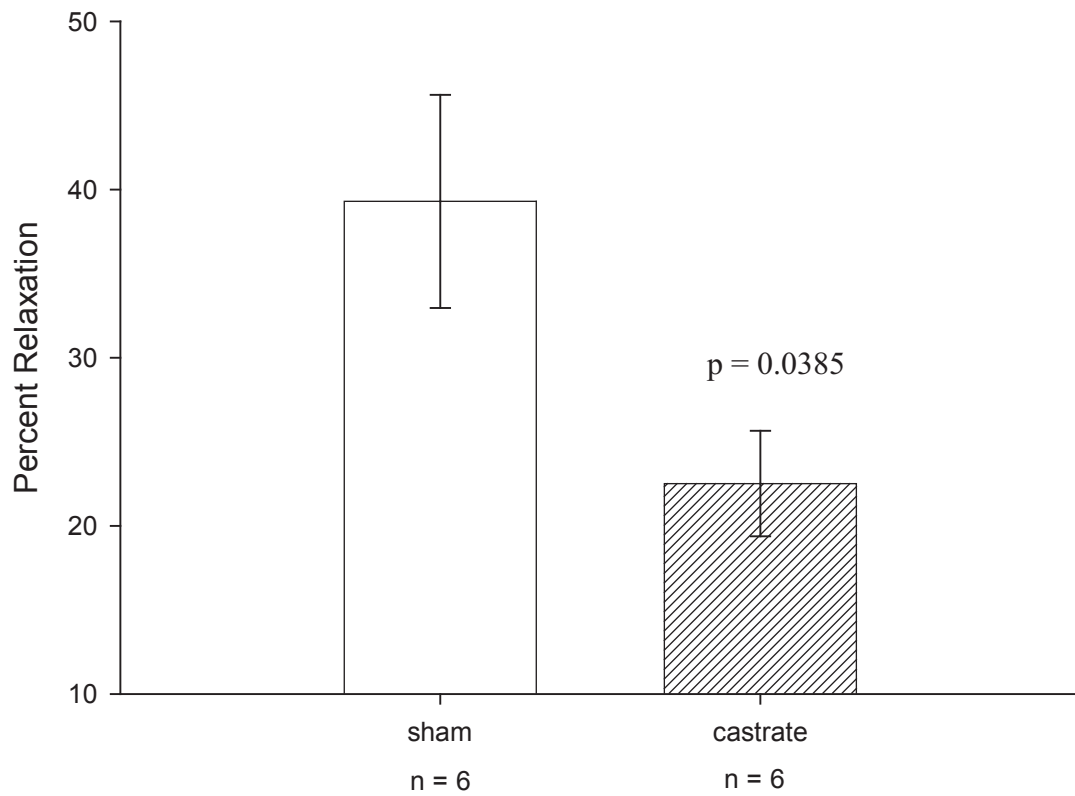
% Phase 1 Relaxation

Figure 4. Percent phase 1 relaxation recorded in Experiment 1. Castration significantly inhibited relaxation during the short, rapid Phase 1 period (p = 0.0385). Values given are the mean ± SEM.
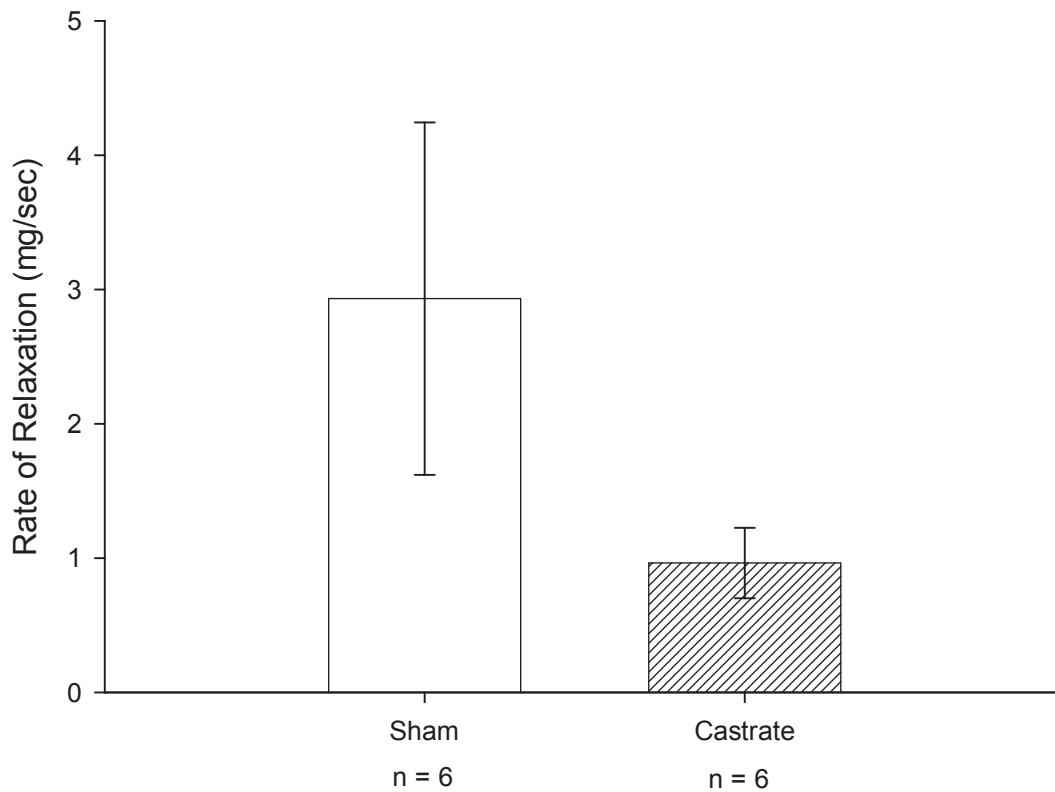
Figure 5. Rate of Phase 1 relaxation of cavernosal tissue in Experiment 1. Cavernosal smooth muscle isolated from castrated rats tended to relax slower than sham-castrate tissue, however there was no statistical difference between groups. Values given are the mean ± SEM.
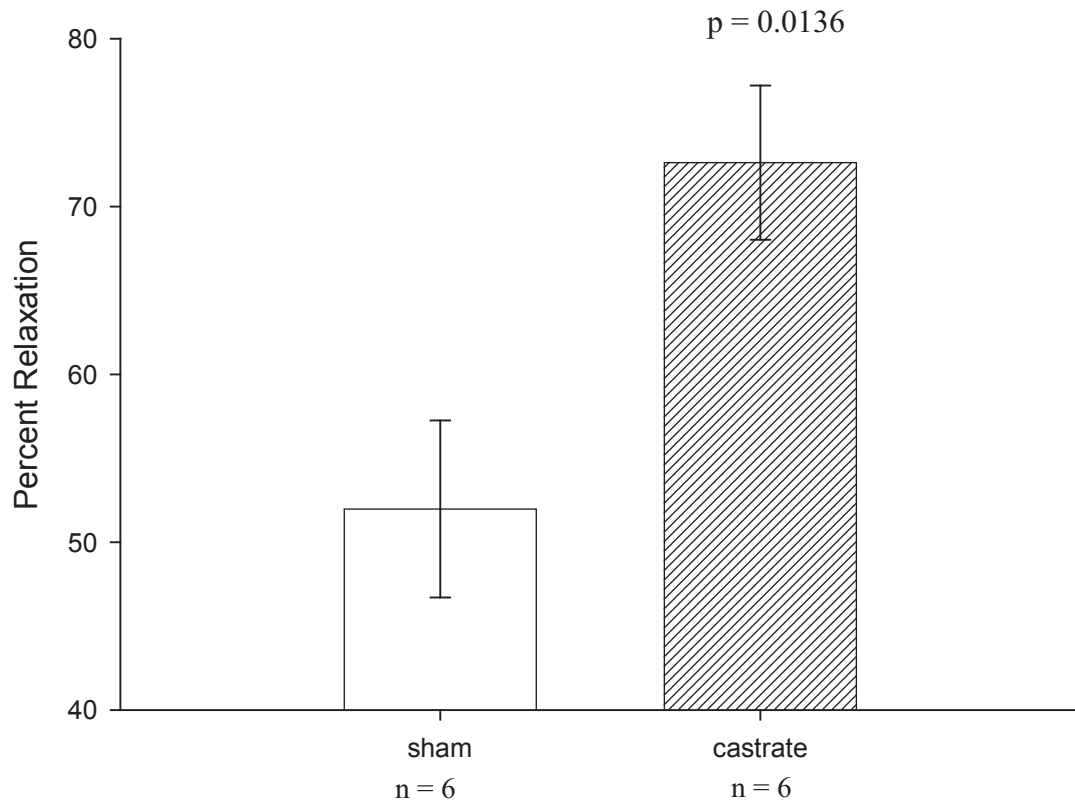
% Phase 2 Relaxation

p = 0.0136

Figure 6. Relaxation of cavernosal tissue in Experiment 1 during phase 2. The castrate tissue clearly relaxed more during phase 2 compared to that of the sham tissue. This difference resulted in a significance level of p = 0.0136. Values given are the mean ± SEM.
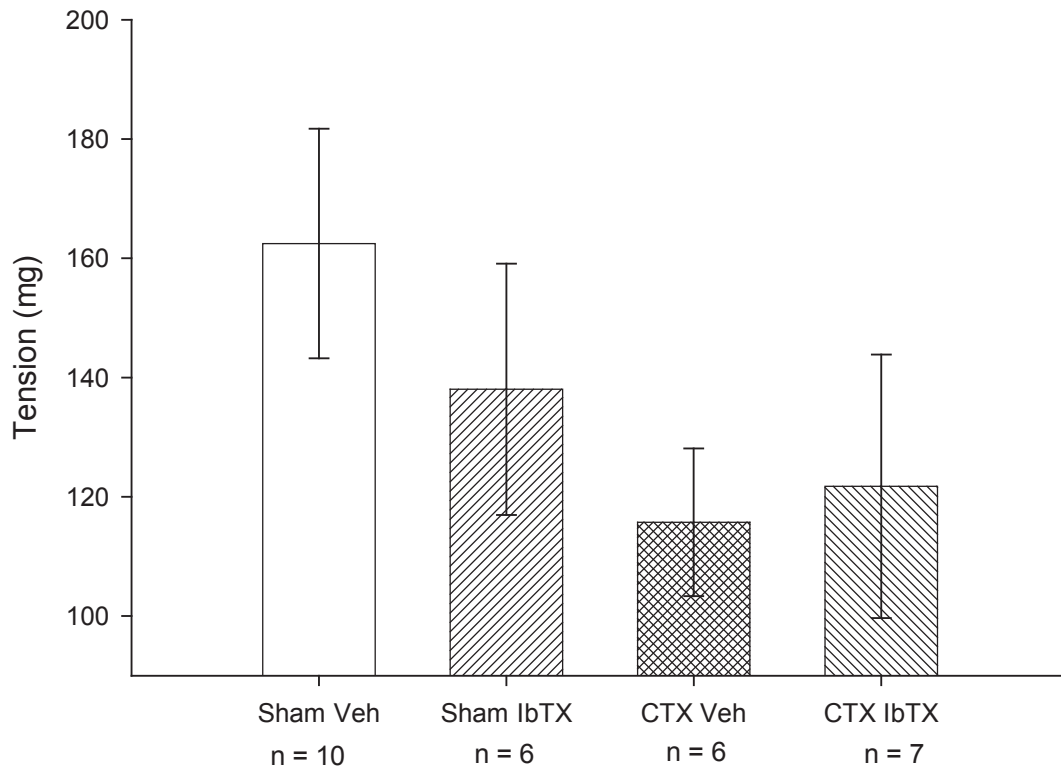
Figure 7. Contractile tension reported for Experiment 2 after the addition of NE. No significant differences were found among the groups. Values given are the mean ± SEM.

% Total Relaxation

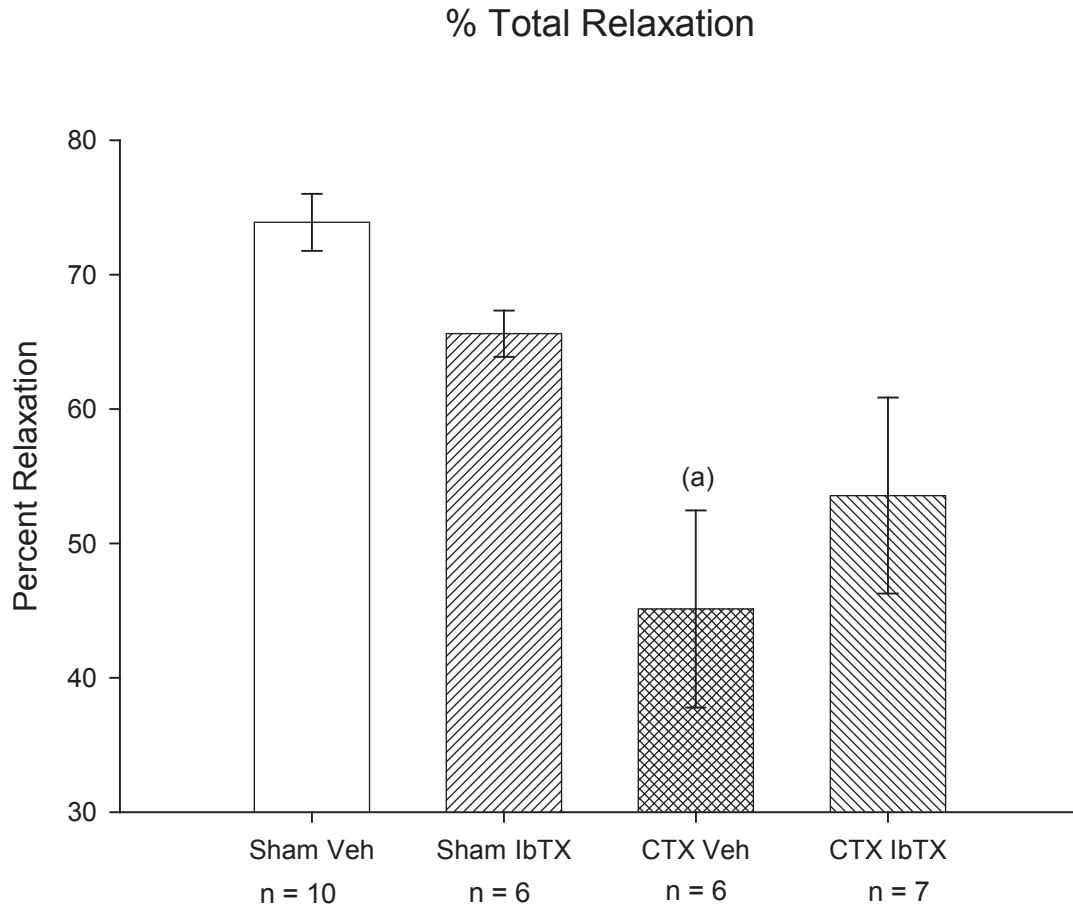**Figure 8.** Total percent relaxation of cavernosal tissue from sham and castrated rats treated with either vehicle or iberiotoxin in Experiment 2. IbTX showed a trend to decrease relaxation compared to the sham-vehicle treated rats, but was not statistically significant. Castration resulted in a significant inhibition of relaxation compared to sham-castrate tissue (a: $p < 0.001$). Values given are the mean $\pm$ SEM.

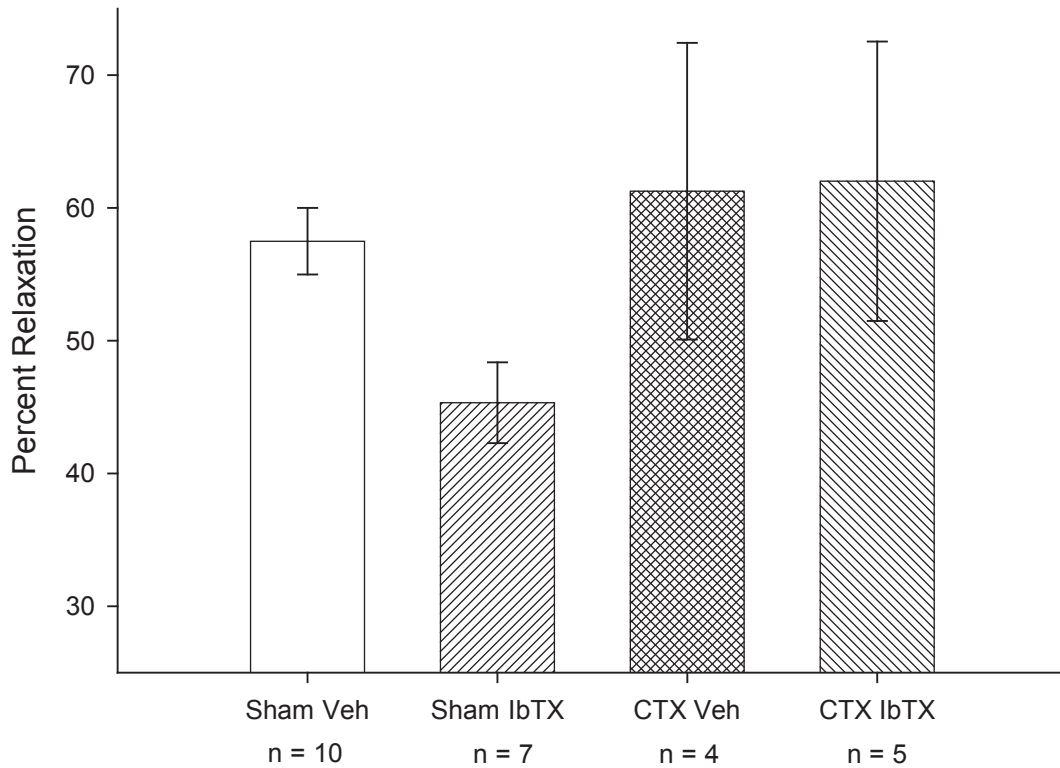## % Phase 1 Relaxation

Figure 9. Percent of the Phase 1 relaxation in Experiment 2. Cavernosal smooth muscle tissue from sham rats treated with IbTX tended to relax less than the cavernosal smooth muscle from the sham-vehicle animals, although the difference was not significant. IbTX had no effect on the relaxation of cavernosal smooth muscle from castrated rats. Values given are the mean ± SEM.

Rate of Phase 1 Relaxation
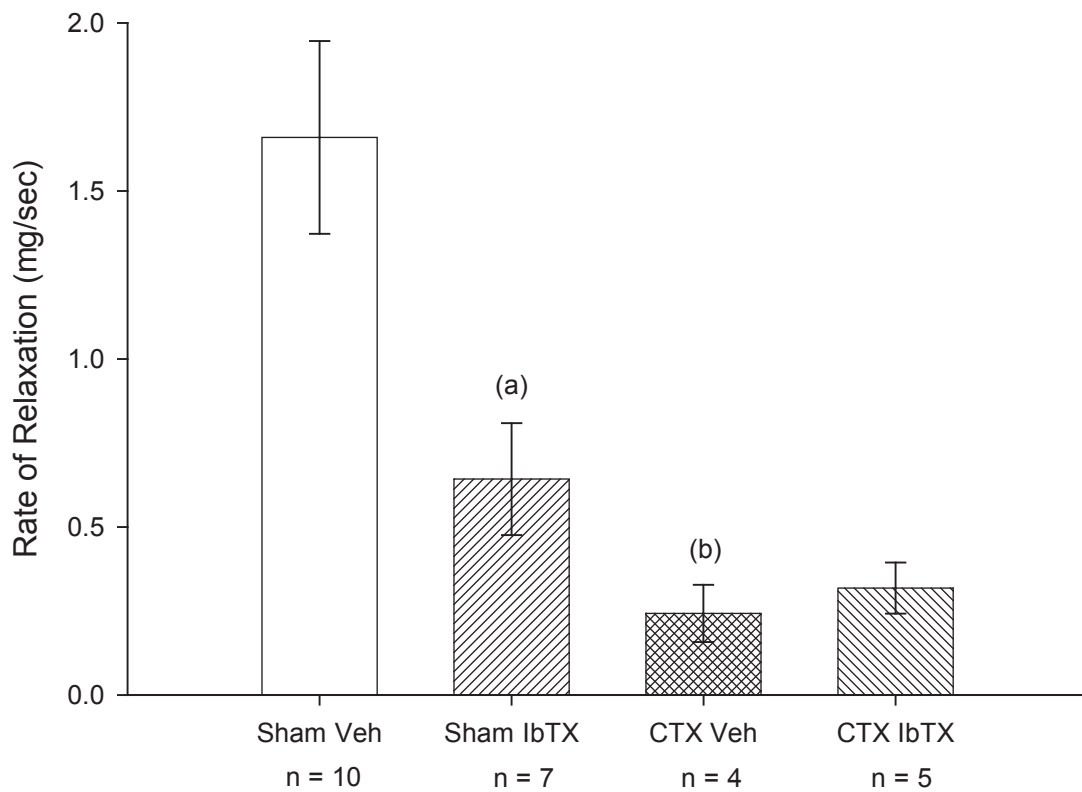
Figure 10. Rate of relaxation during Phase 1 in Experiment 2. (a) IbTX treatment significantly inhibited the relaxation rate in sham-castrated animals compared to the sham-vehicle group; p = 0.003. (b) Castration also significantly inhibited the rate of Phase 1 relaxation compared to sham controls; p = 0.001. Values given are the mean ± SEM.

% Phase 2 Relaxation

Figure 11. Percent Phase 2 relaxation in Experiment 2. IbTX treatment showed a trend to prolong relaxation compared to the sham-vehicle group; however no there were no significant differences between the treatment groups. Values given are the mean ± SEM.

Discussion

Overall, results of these experiments demonstrate that castration significantly affected both the contraction and relaxation of corpus cavernosum smooth muscle. With respect to contraction, Experiment 1 demonstrated 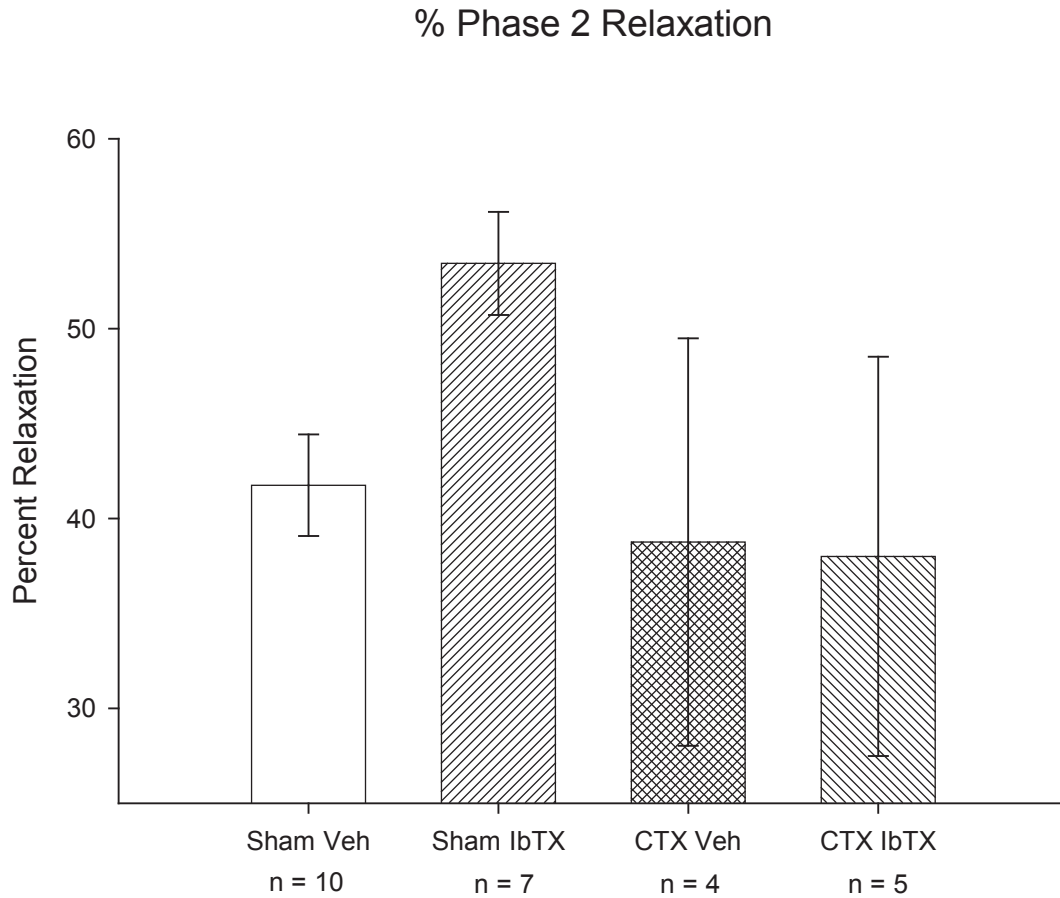an increased tissue sensitivity to the alpha-adrenergic agonist, norepinephrine. Tissue isolated from castrated rats contracted with greater tension compared to the sham tissue (Figure 2). This finding is consistent with other experiments that stimulated cavernosal tissue to contract with an alpha-adrenergic agonist and found an increased responsiveness in castrated tissue (Riley et al., 1997; Mills et al., 1999; Wingard, Lewis, and Mills, 2001). The mechanism for contraction becomes more sensitive to alpha-adrenergic stimulation in castrated animals, presumably due to a decrease in circulating blood androgen concentrations. This decrease in androgen concentration is linked to an increase in tension because components of the contractile mechanism such as RhoA/Rho kinase are more active; when Rho kinase was inhibited by Y-27632 in cavernosal tissue the responsiveness to vasodilators was improved (Chitaley et al., 2001; Takahashi et al., 2003).

In contrast to the effects on contractility, castration significantly inhibited the percent of Phase 1 relaxation of corpus cavernosum smooth muscle. Results depicted in Figure 4 illustrates that tissue from castrated rats relaxed less than tissue isolated from sham animals during the initial period of relaxation. Furthermore, castration tended to inhibit the total relaxation of Experiment 1 as shown in Figure 3; Experiment 2 also support these results with trends of reduction in percent total relaxation (Figure 8), Phase 1 relaxation (Figure 9), and the rate of relaxation of Phase 1 (Figure 10). These results support other experiments that demonstrated castration resulted in an inhibition of

relaxation (Lee and Kang, 2001; Behrends et al., 2000; McDonald and Murad, 1996; Sausbier et al., 2000, Alcorn, Toepfer, and Leipheimer, 1999).

Further analysis of the relaxation properties of the corpus cavernosal tissue can be described as an initial, rapidly relaxing phase (Phase 1) followed by a longer, maintenance-like phase of relaxation (Phase 2), illustrated in Figure 1. It has been hypothesized that the initial phase of relaxation depends on a rapid decrease in intracellular calcium ions by the action of the sarcoplasmic reticulum calcium ion pumps (SERCA), the stimulation of plasma membrane calcium ATPase pumps, the stimulation of plasma Na+-Ca+ exchange pumps, and the inhibition of plasma membrane calcium ion channels (Szado et al., 2001; Woodrum and Brophy, 2001; Lincoln, Dey, and Sellak, 2001). These actions may allow the immediate response following the NO donor stimulus for relaxation. Results from this laboratory have shown inhibition of the SERCA with cyclopiazonic acid (CPA) inhibited the early phase of cavernosal relaxation with no effect on the latter phase (Leipheimer and Toepfer, unpublished observations). Phase 1 appears to be directly dependent on the presence of androgens since Figures 4 and 10 show a significant reduction in the percent of Phase 1 relaxation and in the rate of Phase 1, respectively, from the castrated tissue groups. For that reason, these results suggest that androgens may be involved in regulating the mechanisms that are responsible for the initial, rapid phase of cavernosal smooth muscle relaxation. Experiments have indicated that this initial, rapid phase of relaxation depends on the movement of intracellular ions and membrane potential (Lincoln, Dey, and Sellak, 2001; Rapoport, 1986; Hilgers and Webb, 2005). Figures 5 and 10 illustrate trends that also strengthen this relationship, though not significantly, in cavernosal smooth muscle

isolated from castrated animals. Future experiments are needed to identify specific targets of androgens and how they support the initiation of the erectile process.

While Phase 1 is thought to be the product of cytosolic calcium management, Phase 2 is thought to be due to cellular mechanisms that regulate calcium sensitivity and calcium concentration. Results from our laboratory have shown that treatment of cavernosal smooth muscle tissue with the myosin phosphatase inhibitor, Calyculin A, significantly inhibited the longer, more sustained relaxation associated with Phase 2 with no effect on Phase 1 (Leipheimer and Toepfer, unpublished observations). Because the percent relaxation was decreased in Phase 1 by castration in the present experiment, a significantly greater percentage of total relaxation was found in Phase 2 (Figure 6).

Experiment 2 examined the role of the large conductance, calcium-regulated potassium channels (BK channels), and effects of inhibiting the channel on contraction and relaxation of cavernosal tissue. We also tested whether the actions of the BK channels were influenced by the presence/absence of androgens. With respect to contraction, IbTX had no significant effect on the NE-induced tension in either the sham or castrated rats (Figure 7). The sham tissue contracted with more tension than the remaining treatment groups, however, the wide variability among the groups seemed to hinder any ability to recognize any definitive trends or patterns in the amount of tension generated. Literature support would suggest inhibiting the BK channel would enhance the contractility of the tissue by decreasing the electrochemical potential and increasing the sensitivity to the adrenergic stimuli (Tare et al., 1990, Christ, 2002; Nelson et al., 1990, Bychkov et al., 1998; Sausbier et al., 2000).

Review of the relaxation properties in Experiment 2 began with the percent total relaxation and found that there were no significant differences among the treatment groups (Figure 8), however trends reflects results from Experiment 1 where relaxation was decreased by castration treatment. Castration significantly inhibited percent total relaxation (Figure 8) and the rate of Phase 1 relaxation (Figure 10).

The highly-selective antagonist of the BK channel, iberiotoxin (IbTX), clearly had an effect on relaxation in the sham-castrate (control) animals; the percent total relaxation (Figure 8) and rate of Phase 1 relaxation (Figure 10) had significant reductions in respective groups that received the IbTX compared to vehicle group, while the percent relaxation in phase 1 (Figure 9) demonstrated the same trend. The addition of IbTX to tissue isolated from castrated rats had no effect on any parameter of relaxation. One possible explanation for these results is that the effect of castration may alter the cellular mechanisms that regulate normal erectile physiology such that a significant effect of IbTX treatment could have been masked. Future studies using highly selective BK channel agonists such as NS-1608 (Siemer et al., 2000; Mora and Suarez-Kurtz, 2005; Hu and Kim, 1996) with cavernosal tissue may provide new insights into the mechanisms that regulate BK channels and perhaps a more specific role for androgens in regulating the complex mechanisms in cavernosal smooth muscle physiology. Along with further identification and characterization of BK channels in cavernosal tissue, additional targets may be revealed for the treatment of erectile dysfunction.

We found that both IbTX treatment and castration significantly inhibited the early, rapid phase of relaxation (Figure 4, 9, 10). Therefore, the relationship between androgens and BK channels may, in part at least, be found in the mechanisms that initiate

the rapid period of relaxation, Phase 1. This could be in the form of support by maintenance of molecular mechanisms that facilitate Phase 1, or perhaps the direct effects of androgens that promote relaxation (Alcorn, Toepfer, and Leipheimer, 1999; Heinlein 2002; Deenadayalu et al., 2001; Tep-areenan et al., 2003).

In conclusion, our results support previous studies that have shown androgens play an important regulatory role in the activity of corpus cavernosal smooth muscle necessary for normal erectile function. Our results extend those findings and suggest that androgens are required for the early, initial phase of cavernosal smooth muscle relaxation that is most likely associated with the onset of erection. Inhibition of BK channels also inhibited the early, initial phase of cavernosal relaxation, which supports the hypothesis that the early phase of cavernosal relaxation depends on rapid changes in ion concentrations within the cytosol. The relaxation mechanism that leads to an erectile response by the corpus cavernosum can be thought of as a two-phase mechanism that is initiated by a short, rapid phase followed by the slower, maintenance phase of cavernosal smooth muscle relaxation. The targets by which androgens exert their effects to regulate normal contraction and relaxation physiology in cavernosal smooth muscle require further investigation.   Furthermore, additional experiments investigating the potential interactions between androgens and BK channels could lead to the development of new approaches to the treatment of erectile dysfunction.

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Tuesday, October 23, 2007

Dr. Robert Leipheimer
Department of Biology
UNIVERSITY

Re: IACUC Protocol # 10-07
        Title: Regulation of smooth muscle contraction and relaxation in rats.
        Approval date: October 15, 2007   Expiration date: October 15, 2010

Dear Dr. Leipheimer:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration titled **"Regulation of smooth muscle contraction and relaxation in rats"** and determined it should be unconditionally approved for the period of **October 15, 2007** through its expiration date of **October 15, 2010.**

This protocol is approved for a period of three years; however, it must be updated once a year via the submission of an Annual Review-Request to Use Animals form *prior* to its yearly anniversary date of **October 15, 2008** and **October 15, 2009.** You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Sincerely,

Dr. Peter J. Kásvinsky
Associate Provost for Research
Research Compliance Officer

PJK:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
        Dr. Robert Leipheimer, Chair IACUC, Chair Department of Biological Sciences
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