Effects of Androgen on Corpus Cavernosum: Role of the Plasma Membrane Calcium Pumps

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ABSTRACT

This study was designed to investigate the relative importance of the intracellular and extracellular calcium stores in mediating the contraction and relaxation of corporal cavernosal smooth muscle (CCSM) tissues. This study also investigated the effects of androgens on the calcium pump mechanism(s) responsible for the relaxation of this tissue.

To determine the relative importance of the intracellular and extracellular calcium stores in CCSM relaxation, isolated CCSM tissues were treated with cyclopanazoic acid (CPA) or DMSO (control) before being contracted by norepinepherine (NE). Sodium nitroprusside (SNP) was then added to the tissues to induce relaxation. Finally, percent relaxations were recorded for each group.

The effects of androgens on the calcium pump mechanism(s) responsible for CCSM relaxation were studied by dividing rats into intact, castrate, and testosterone replacement treatment groups. Isolated CCSM tissues were placed in calcium free media and treated with CPA. Next, CCSM contractions were induced by the addition of NE and $CaCl_2$ to the media. SNP was then added to the media to induce relaxation. Again, percent relaxations were recorded for each group.

Results from this study indicate that the most important mechanism responsible for the removal of cytosolic calcium from CCSM is located on the plasma membrane. This study also shows that castration significantly reduces the relaxation of CCSM, presumably by affecting the plasma membrane mechanism(s) responsible for the removal of calcium from the cytosol. Testosterone replacement was able to restore CCSM relaxation to normal levels. These results indicate that the calcium regulating mechanism utilized by CCSM is, to some extent, androgen regulated.

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TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Impotence is a serious problem that currently affects approximately ten million men worldwide and statistics show that the incidence of this disease increases with age. By the age of 65, 25% of all men are afflicted with impotence. By the age of 80, the incidence increases to 80% (DePalma, 1996). The general cause of impotence has been attributed to three major factors. These factors are a decrease in arterial blood flow into the corpora cavernosal tissue, a lack of veno-occlusion from the corpora cavernosal tissue, and a lack of corporal smooth muscle relaxation (Lue and Tanagho, 1987). The underlying cause of impotence is most commonly attributed to vascular abnormalities in which a decreased amount of blood is supplied to the erectile portion of the penis. This lack of blood flow is the cause of 45% of impotence cases. Less common causes of erectile dysfuction have been identified as endocrine, metabolic, neurologic, and psychogenic factors (DePalma, 1996).

The treatment for impotence has changed dramatically over the past two decades. In the late 1970's and early 1980's, treatments of impotence consisted of corpus cavernosal revascularization (DePalma, 1996). However, once it had been established that intracorporal administration of vasoactive agents, such as papaverine and phentolamine, could stimulate tumescence (Virag, 1982), therapy for impotence began to change. Testing and therapy for impotence became less concerned with increasing arterial blood flow and more concerned with the functioning of corporal smooth muscle (DePalma, 1996). Currently, a major source of treatment comes in the source of oral drugs. Isoxsuprine hydrochoride, which acts as an α_1-adrenergic blocker, yohimbe hydochloride, a pre-synaptic adrenergic-receptor agonist, and trazadone hydrochloride are among the most common

oral treatments currently used. However, the effectiveness of these drugs is still questionable (DePalma, 1996).

Another form of treatment currently used today is the use of a penile prosthesis. A penile prosthesis is a self-contained built-in cylinder pump surgically placed in the penis. During times of intercourse, the pump is activated and the cylinder begins to fill, causing an erection. An estimated 250,000 men have received this form of therapy, recommended mainly for those with diabetes or neurovascular dysfunction. While approximately 90% of patients reported proper functioning from the prosthesis pump, the overall satisfaction rate of this device is only 71% (DePalma, 1996).

Among the latest surgical forms of therapy for erectile dysfunction is the soft Subrini implant (Subrini, 1994). The soft Subrini implant is a device that fills the corporal cavernosal body. However, it is not inflatable nor does it provide much rigidity. This device does, however, allow the maintenance of normal erection during sexual intercourse (DePalma, 1996).

One of the latest and most popular treatments to male impotence is the drug sildenafil (Viagra). Sildenafil is an orally administered drug that acts to inhibit c-GMP specific phosphodiesterase (PDE5), an enzyme known to catalyze the hydrolysis of c-GMP (Illarion *et al.,* 1999; Carter *et al.,* 1998), the second messenger in the relaxation pathway of the corporal cavernosal smooth muscle of the penis. While sildenafil has been shown to weakly bind other isozymes of c-GMP phopshodiesterase (PDE), specifically PDE 2 and PDE6 (Chuang *et al.,* 1998), it has the strongest binding affinity for PDE5, the most abundant PDE found in corporal cavernosal smooth muscle (Boolell, *et al.,* 1996). Inhibition of PDE5 by sildenafil allows a build up of c-GMP to occur in corporal cavernosal smooth muscle cells. This build up

of c-GMP allows a greater relaxation of these smooth muscle cells and, therefore, a better result of tumescence.

The use of sildenafil appears to be an effective treatment for erectile dysfunction in response to sexual stimulation with very few side effects (Hawton, 1998). Studies have shown that 93% of users reported improved erections when taking the drug within one hour of sexual relations (Christianen *et al.,* 1996) and 82% reported improved erections after a single dosage of the drug (Boolell *et al.,* 1996).

In order to treat the occurrence of impotence, it is very important to understand the mechanism of tumescence. Researchers have studied the pathway of events leading to the development of erection for many years. The general mechanism involves an increased inflow of blood into the penile tissue, as well as a decreased outflow of blood from this tissue (Newman and Northup, 1981). However, to completely comprehend the specifics involved in the process of erection, one must first completely understand the anatomical structures of the penis.

The portion of the penis involved in erection consists of the corpus spongiosum and the paired corpora cavernosa, all surrounded by a compressing fibrous connective tissue called the tunica albuginea. The corpus spongiosum, acting as a channel for the passage of urine and semen, lies in the medial portion of the penis and contains the urethra. The two corpora cavernosa can be found lateral to the corpus spongiosum and act solely for the purpose of erection. The corpus spongiosum also has erectile functioning, but only for the purpose of maintaining the urethra during tumescence (Newman and Northup, 1981). Each of these erectile bodies is composed of a smooth muscle matrix containing large amounts of sinusoidal spaces. The smooth muscle portions of the corpora cavernosa and corpus

spongiosum are attached to the pubic bone via striated muscles, the ischiocavernosus and bulbocavernosus respectively (Sachs, 1995). All of the structures described above play an active role in the development of tumescence.

Since a major factor in the development of tumescence is an increase in arterial flow, a thorough comprehension of the blood vessels supplying the erectile bodies of the penis is necessary. A pair of internal pudendal arteries provides the entire blood supply to the penis. After giving off two branches, the bulbar artery and the urethral artery, to supply the corpus spongiosum (Lue and Tanagho, 1987), the pudendal arteries continue as the artery of the penis (Newsome and Northup, 1981). The artery of the penis then branches into the dorsal artery, also supplying the corpus spongiosum (Lue and Tanagho, 1987) and the deep penile artery (Newsome and Northup, 1981). Finally, the deep penile artery branches into two cavernous arteries, supplying the corpora cavernosa (DePalma, 1996). In humans, blood flow out of the corporal tissues occurs through several emissary veins that pass through the tunica albuginea, a layer of connective tissue surrounding both the corpora cavernosa and the corporal spongiosum, (Mills *et al.*, 1996). After passing through the tunica albuginea, these emissary veins merge together, eventually forming the central penile vein (Newsome and Northup, 1981). During tumescence, increased arterial blood flow leads to the filling of corporal sinuses. This filling causes the corporal tissue to expand and press against the tunica albuginea. The pressure of the tunica causes veno-occlusion to occur on the emissary veins, slowing the flow of blood out of the corporal sinuses and permitting the maintenance of erection (Mills, *et al.*, 1996). In the rat, instead of passing through the tunica albuginea, these vessels form channels that run parallel to the tunica albuginea (Mills *et al.*,

1996). It is believed that the compression of these channels is the cause for veno-occlusion in rats (Hernandez, *et al.* 1991).

The corporal tissue of all species having a vascular penis is innervated by neurons that originate from one of two sources. Parasympathetic neurons that promote erection originate in either the lower lumbar, as is the case in rodents, or the sacral, as is the case for primates, region of the spinal cord. These neurons travel through the major pelvic ganglion before reaching the corporal tissue via the penile nerve. Sympathetic fibers that are thought to play a role in the erectile process originate in the lower thoracic and upper lumbar portion of the spinal cord. These neurons pass through the inferior mesenteric ganglion, the hypogastric nerves, and the major pelvic ganglion before innervating the corporal tissue, also via the penile nerve (Sachs, 1995).

This nerve innervation is believed to play a key role in the onset of erection. While the penis is in a flaccid state, sympathetic innervation, primarily through the hypogastric nerve, predominates. The sympathetic release of noradrenaline causes a pronounced contraction of the corporal smooth muscle cells, limiting the amount of arterial blood flow through the penile arteries and into the caversosal spaces. While noradrenaline appears to play a major role in maintaining smooth muscle tone in the flaccid state, other chemicals such as prostanoids, Neuropeptide Y, endothelin polypeptide, and vasoactive polypeptide have also been identified in the penile smooth muscle tissue. However, the function of these chemicals has not yet been identified (Andersson and Holmquist, 1994).

Neurotransmission from inhibitory cholinergic and non-adrenergic non-cholinergic (NANC) nerve fibers, neurons that release neurotransmitters other than noradrenaline or acetylcholine, are responsible for the onset of

erection (Suh *et al.,* 1995; Trigo-Rocha, *et al.,* 1993; Bush, *et al.,* 1992). The signals sent from these fibers lead to a relaxation of the corporal cavernosal smooth muscle cells, allowing a dramatic increase in the arterial flow into the cavernosal spaces (Lue *et al.,* 1983). The parasympathetic actions induced by these fibers facilitate the relaxation of corporal smooth muscle in three possible ways. First, the sympathetic release of noradrenaline may be inhibited by the stimulation of prejuctional muscarinic receptors. Second, endothelium-derived relaxant factors may be released through stimulation of postjunctional muscarinic receptors. Finally, NANC relaxant factors may be released directly from the parasympathetic nerves (Andersson and Holquist, 1994).

The major neurotransmitter believed to mediate the relaxation of corpora smooth muscle cells is nitric oxide (NO). NO was originally termed Endothelium Derived Relaxing Factor (EDRF), however, it was discovered that the two molecules were actually the same (Ignarro *et al.,* 1987). This has been shown to be the case in canine (Trigo-Rocha *et al.,* 1993), rabbit (Seftel *et al.,* 1996), rat (Penson *et al.,* 1996), and man (Rajfer *et al.,* 1992). NO is formed as a result of the conversion of L-arginine into L-citrulline by nitric oxide synthase (NOS), an enzyme that has been localized to the pelvic plexus, cavernosal nerves, and the nerve plexus of the penile arteries (Burnett, 1995). Production of NO from these nerves, and possibly the corporal smooth muscle and endothelium, then leads to smooth muscle relaxation via the c-GMP pathway, a critical second messenger pathway for the relaxation of corpora cavernosal smooth muscle cells (Burnett, 1995).

Nitric oxide initiates the relaxation of corporal smooth muscle cells by crossing the plasma membrane via simple diffusion. Upon entering the cytosol, NO binds to the enzyme soluble guanylate cyclase (sGC), a

heterodimeric hemoprotein composed of α and β subunits. Binding of NO to the heme portion of sGC activates the enzyme. Once activated, sGC catalyzes the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3', 5'-monophosphate (cGMP). This nucleotide then interacts in a variety of intracellular pathways, including smooth muscle relaxation. Also present in smooth muscle cells is the enzyme particulate guanylate cyclase (pGC). pGC is a membrane bound homodimeric protein, which closely resembles sGC. While this enzyme has been useful in better understanding the pathways of sGC, it is not believed that pGC has a significant role in any smooth muscle relaxation (Denninger and Marletta, 1999).

Cyclic GMP is a second messenger with a variety of target proteins, allowing a wide range of effects, depending on cell types. However, for the purpose of smooth muscle relaxation, c-GMP acts primarily to decrease the cytosolic Ca^{+2} concentration. Cytosolic Ca^{+2} concentrations are decreased either by increasing the efflux of Ca^{+2} out of the cytosol, decreasing the influx of Ca^{+2} into the cytosol, or by modifying interactions with intracellular organelles responsible for the internal sequestering of calcium (Vrolix *et al.,* 1988). To accomplish this, cGMP interacts with three known target proteins (Denninger and Marletta, 1999). The first target for cGMP is cGMP-regulated phosphodiesterase (Degerman *et al.,* 1997; Houslay and Milligan, 1997). Cyclic GMP-regulated phosphodiesterase induces the breakdown of cGMP by hydrolyzing the 3'-phosphodiester bond of cGMP, resulting in the formation of guanylate monophosphate (GMP) and lower levels of cGMP (Denninger and Marletta, 1999).

The second target for cGMP is the cGMP-gated ion channels (Zagotta and Siegelbaum, 1996). These channels are non-specific cation channels

found in a different tissues throughout the body (Denninger and Marletta, 1999), however, the exact purpose of these channels in smooth muscle relaxation has not yet been defined.

The third, and possibly most important, target protein associated with cGMP is cGMP-dependent protein kinase (Lohmann *et al.,* 1997). This protein, in the presence of high cGMP concentrations, phosphorylates its own target proteins throughout the cell. A major target protein associated with protein kinase G in smooth muscle cells is the inositol 1,4,5-triphosphate (IP_3) receptor (Denninger and Marletta, 1999). Inositol 1,4,5-triphosphate mobilizes calcium from non-mitochondrial stores by interacting with the IP_3 receptor located on the sarcoplasmic reticulum. Binding of these receptors by IP_3 allows the influx of calcium into the cytosol, causing contraction (Missiaen, *et al.,* 1992). The presence of c-GMP, however, has been shown to inhibit IP_3 dependent calcium release (Murthy and Makhlouf, 1995). This inhibiton of calcium release leads to lower cytosolic concentrations of calcium and ultimately to smooth muscle cell relaxation (Denninger and Marletta, 1999).

Cytosolic calcium levels are often lowered through the use of intracellular organelles that function in the internal sequestering of calcium. While some researchers feel that the mitochondria may play a major role in the intracellular uptake of calcium from the cytosol (Drummond and Fay, 1996), little evidence has been given to prove this claim. Most researchers feel that the mitochondria play a minor part in the internal removal of calcium from the cytosol.

The major organelle responsible for intracellular sequestering of cytosolic calcium appears to be the sarcoplasmic reticulum. The transport mechanism for the movement of calcium from the cytosol into the lumen of

the sarcoplasmic reticulum is a protein with a high affinity for binding calcium, Ca^{+2} ATPase (Martonosi, 1996). This protein can be phosphorylated by cAMP-dependent protein kinase, Ca^{+2}-calmodulin-dependent protein kinase, or cGMP protein kinase (Raeymaekers and Jones, 1986). Experimental evidence shows that phosphorylation of the Ca^{+2} ATPase on the sarcoplasmic reticulum by cGMP, stimulated the uptake of cytosolic calcium into the organelle (Raeymaekers *et al.,* 1988).

The decrease in cytosolic calcium concentrations indicative of vascular smooth muscle relaxation is not necessarily always accomplished by the internal sequestering of calcium by the sarcoplasmic reticulum. Calcium can also be removed from the cytosol by passing across the plasma membrane and into the extracellular space. The first mechanism for this type of extrusion has been identified as a calmodulin-dependent Ca^{+2} transport ATPase located in the plasma membrane of smooth muscle cells (Eggermont *et al.,* 1988). While this protein has a high affinity for calcium, it also has a low total transport capacity. Fortunately, the high affinity for calcium allows the Ca^{+2} ATPase to continuously remove calcium from the cytosol, making this protein a major factor in the removal of cytosolic calcium (Carafoli, 1988).

Although it is widely accepted that the calmodulin-dependent Ca^{+2} ATPase plays a significant role in the regulation of cytosolic calcium concentrations, at least in some smooth muscle tissues, the functioning of this mechanism is still not understood. Originally, it was believed that protein kinase G activated this plasmalemmal ATPase via direct phosphorylation of the Ca^{+2} transport protein (Furukawa and Nakamura, 1987). However, later studies have shown this not to be the case. Experiments performed in 1988 by Vrolix *et al.,* have shown that while

protein kinase G was necessary for the activation of this membrane pump, it was not sufficient. The presence of phosphotidylinositol (PI) was also required for the plasma membrane Ca^{+2} pump to be activated. Further experiments showing phosphotidylinositol-4,5-bisphosphate (PIP) as the most effective stimulator of the ATPase, even in the absence of protein kinase G and PI, allowed a better understanding of the activation of this Ca^{+2} transport (Vrolix *et al.,* 1988). These experiments led to the belief that protein kinase G activates a phosphotidylinositol phosphate kinase (PI kinase), most likely a variant of a type 1 inositol 1,4,5-trisphosphate receptor, via phosphorylation (Vrolix *et al.,* 1988; Yoshida *et al.,* 1992; Koga *et al.,* 1994). However, more recent studies on the functioning of the plasma membrane Ca^{+2}-pump ATPase have yielded results that cannot substantiate this hypothesis. The latest results obtained from research of this Ca^{+2}-pump ATPase indicate that the protein exists in a monomeric form at low concentrations and an oligomeric form at high concentrations (Kosk-Kosicka *et al.,* 1988). In the oligomeric form, the Ca^{+2}-pump ATPase is completely activated in the absence of cGMP kinase and appears to be insensitive to the presence of calmodulin. The monomeric form of the Ca^{+2}-pump ATPase can be stimulated by type Iα cGMP kinase without any evidence of enzyme phosphorylation and is apparently sensitive to the presence of calmodulin (Yoshida *et al.,* 1999).

Regardless of the exact functioning of the plasma membrane Ca^{+2}-pump ATPase, it is still believed that the enzyme is critical in allowing the movement of calcium across the plasma membrane and out of the cytosol, leading to a decrease in the cytosolic Ca^{+2} concentrations (Vrolix, *et al.,* 1988; Yoshida *et al.,* 1992; Koga *et al.,* 1994; Yoshida *et al.,* 1999).

A second mechanism in which calcium can be passed into the extracellular space is the sodium/calcium (Na^{+}/Ca^{+2}) exchanger. The role of the Na^{+}/Ca^{+2} exchanger in cardiac tissue has long been established (Carafoli *et al.,* 1980). However, strong experimental evidence for the existence of this exchanger in smooth muscle had not been confirmed until the early 1990's (McCarron *et al.,* 1994).

The Na^{+}/Ca^{+2} exchanger is a membrane bound protein that functions in the expelling of calcium from the cytosol, into the extracellular space. This exchanger has a low affinity for calcium binding (Carafoli, 1988). Despite this apparent low affinity for calcium, experimental evidence shows that the Na^{+}/Ca^{+2} exchanger is still able to act as a major mechanism for the removal of intracellular calcium. There are currently two possible explanations to answer this discrepancy. The first explanation is that the Na^{+}/Ca^{+2} exchanger is somehow modified, probably through phosphorylation, in vivo (McCarron *et al.,* 1994). The second explanation for the effectiveness of Na^{+}/Ca^{+2} exchanger is the possibility that calcium concentrations are higher just inside the plasma membrane (Moore *et al,* 1991). It is still unclear whether or not either c-GMP or protein kinase G has any affect on the Na^{+}/Ca^{+2} exchanger, since no evidence exists demonstrating the phophorylation of a specific substrate protein in this mechanism (Schmidt *et al.,* 1993; Cornwell *et al.,* 1991 as reviewed in Lohmann *et al.,* 1997).

Regardless of the manner in which it is mediated, the cytosolic calcium concentration is the primary factor in determining the contraction or relaxation of smooth muscle (Eggermont, 1988). This is true of all smooth muscle including the corporal cavernosal smooth muscle (CCSM) tissue responsible for tumescence. It is widely accepted that removal of calcium

from the cytosol induces the relaxation of corporal cavernosal smooth muscle cells. This smooth muscle relaxation allows an increase in arterial flow into the corporal sinuses, filling the cavernosal tissue causing its expansion. As the corporal cavernosal tissue expands against the tunica albuginea, veno-occlusion occurs. This occlusion effectively limits the outflow of blood from the corporeal sinuses, trapping the blood and maintaining the erectile state of the penis (Mills *et al.,* 1996).

Evidence has been presented showing that the erectile process is androgen sensitive in rats (Mills *et al.,* 1996; Leipheimer and Sachs, 1993); Penson *et al.,* 1996). Each of these laboratories has shown that after castration, there is a significant decline in erectile functioning. These laboratories have also shown that exogenous replacement of testosterone resulted in an increase in corporal cavernosal smooth muscle relaxations of castrated animals to near or above those of intact animals. However, despite the evidence supporting the hypothesis that erectile functioning is androgen dependent, the mechanisms by which testosterone acts to regulate the process remains undetermined.

Testosterone is the principle androgen responsible for the regulation of penile erection. Testosterone is produced and released by the Leydig cells of testes upon stimulation from lutenizing hormone, released by the anterior pituitary gland. Once released, testosterone enters the general systemic circulation and travels to the erectile tissues. Upon reaching the erectile tissues, testosterone must be converted intracellularly to dihydrotestosterone (DHT) by the 5-α-reductase enzyme (Anderson, K.M. and Liao, S., 1968; Bruchovsky, N. and Wilson, J.D., 1968). DHT then binds to a receptor protein located either in the cytosol (Fang, S., *et al.,* 1969; Mainwaring, W., 1969) or on the nuclear chromatid (Fang, S., *et al.,* 1969). If the androgen

binds to a receptor protein in the cytosol, the DHT-receptor complex must undergo a temperature dependent change and migrate into the cell nucleus to interact with acceptor sites on the nuclear chromatid (Mainwaring, W.I.P. and Peterkin B.M., 1971; Liao, S., *et al.,* 1972). It is in this form that testosterone is able to exert its effects on the erectile tissues (Rajfer, *et al.,* 1980).

The penile tissue, as well as the striated muscles and smooth muscles associated with the penile tissue, has been shown to be androgen sensitive (Leipheimer and Sachs, 1993). In these experiments, it was demonstrated that, over a time period of a couple of weeks, castration led to a decline in reflexive erections. However, treatment with either testosterone or DHT led to a return of erectile functioning to those levels expressed by intact rats (Leipheimer and Sachs, 1993; Greg *et al.,* 1998). In fact, both testosterone and DHT were equally effective in restoring penile erection without affecting the vasculature normally responsible for increasing blood flow into the penile tissue. Also, since no changes occurred in the mean arterial blood pressure of treated and untreated animals, it is reasonable to assume that all stimulation presented from these androgens was confined to corpora cavernosa smooth muscle (Garban *et al.,* 1995).

While the use of DHT was just as effective as testosterone in restoring erectile functioning, use of the 5α-reductase inhibitor, finastride, along with testosterone prevented any return of reflexive erections. These results support the theory that testosterone must be converted to DHT before any androgenic effects will occur in this tissue (Lugg *et al.,* 1995).

Even as the form of testosterone that is used to regulate erectile functioning becomes better understood, it is just as important to understand the mechanism in which these androgens exert their effects. One belief is

that the androgen receptors present in androgen dependent tissues are affected by the presence of testosterone (Rajfer *et al.,* 1980; Gonzalez-Cadavid *et al.,* 1991; Takane *et al.,* 1990; Mills, *et al.,* 1996). Experimental evidence shows that androgen receptor activity in the corporal cavernosal smooth muscle tissue of rats reaches a maximum level during puberty. This receptor activity then drops significantly, as do testosterone concentrations, as the rats get older (Gonzalez-Cadavid *et al.,* 1991). These results are consistent with those of the DHT receptor activity of penile tissue, in which receptor activity was at a maximum in the youngest age group studied (16 days). The activity of these receptors gradually decreased until, by day 70, almost no activity was perceivable (Rajfer *et al.,* 1980). Other experiments indicate that not only is the activity of the androgen receptors mediated by the presence of androgens, but the number of receptors present in androgen sensitive tissues is also mediated by the presence of androgens. Experiments by Takane *et al.,* have shown that castration of rats prior to puberty prevents the disappearance of androgen receptors that normally occurs. However, when castration occurs after puberty, androgen receptor levels are not restored. This indicates that the reduction of androgen receptors that normally occurs with age is both age mediated and irreversible (Takane *et al.,* 1990; Mills *et al.,* 1996).

Many researchers have also proposed the idea that testosterone regulates erectile activity by up-regulating NOS activity in penile tissue (Mills *et al.,* 1996; Lugg *et al.,* 1995; Zvara *et al.,* 1995). This theory is supported by the observation that addition of testosterone to castrated animals increases NOS activity to normal levels in vivo (Lugg *et al.,* 1995; Zvara *et al.,* 1995).

While androgens may very well regulate NOS activity, it has previously been observed in this laboratory that testosterone also regulates corpus cavernosum smooth muscle relaxation in vitro (Leipheimer and Toepfer, 1996). Later studies in this laboratory have established that by adding a NO donator, such as sodium nitro prusside (SNP), and bypassing the NOS activity, relaxations of corporal cavernosal smooth muscle tissues are less effective in castrated animals than in those that were left intact. Tissues treated with testosterone regained the ability to relax to near normal intact levels. These results show that testosterone must have some regulatory ability at an intracellular step in the NO-guanylate cyclase-cGMP pathway of corporal cavernosal smooth muscle cells. Also, this regulation must take place downstream of NO release (Alcorn *et al.,* 1999). These studies also demonstrated that the direct addition of 8-Br-cGMP to the corporal cavernosal smooth muscle tissue, in effect bypassing the actions of guanylate cyclase, also resulted in a significant decrease in the relaxation of this tissue in castrated animals. Again, corporal tissues that had been treated with testosterone replacement regained the ability to relax to the levels expressed by intact animals. These results show that testosterone not only effects the corporal tissue downstream of the NOS release, but also downstream of the guanylate cyclase activation (Alcorn *et al.,* 1999).

The present study was designed to investigate the relative importance of intracellular and extracellular Ca^{+2} stores in mediating the contraction and relaxation of corporal cavernosal smooth muscle tissue. The second portion of this study also determined the effects of castration and testosterone replacement on the calcium pump mechanism(s) required for the relaxation of this tissue.

Materials and Methods

Animals

Animals were housed in plastic cages in groups of three prior to any surgical procedures. After surgeries, the animals were housed individually in the same type of cages. All animals were given a continuous supply of LabDiet Rat Chow and water. The animals were kept on a reverse light-dark schedule, with lights off from 11 a.m. to 11 p.m., at a room temperature of 22°C. For experiment I, ten intact mature male Long/Evans rats were used. Sixteen mature male Long/Evans rats were used, for experiment II

Surgeries

For all surgical procedures, animals were anesthetized with intramuscular injections of rompun, 8 mg/ kg, and ketamine, 50 mg/ kg. The castrate group consisted of five rats that had undergone castration surgery consisting of a midline scrotal incision under aseptic conditions. The tunica albuginea surrounding the testes was also cut. The spermatic cord was tied off with 3-O surgical silk and the testes and epididymides were removed. All incisions were sutured with 3-O silk suture. The intact (control) group included five rats that had undergone a sham castration surgery similar to the surgery described above, however, the testes were not removed. The testosterone replacement group consisted of six castrated rats that had been subcutaneously implanted on the right flank with testosterone containing capsules, Silastic medical grade tubing, Dow Corning, Midland, Michigan. These capsules, 45 mm in active length, contained an internal diameter of 1.6 mm, and external diameter of 3.2 mm. All capsules were sealed at each end with a 5 mm wooden applicator stick and Silastic medical adhesive silicon type A, Dow Corning, Midland, Michigan. The capsules were similar

to those used by (Smith *et al.,* 1977) and to those used in a study to maintain reflexive erections ex copula (Leipheimer and Sachs, 1993). All animals were given 3 weeks recovery time prior to being used for any data collection.

Tissue Specimens

On the day of experimentation, rats were quickly euthanized with CO_2. Segments of the penis, approximately 2.5-3.0 cm in length, were removed from the animal and placed in a modified Kreb's solution on ice, see compositions below. The surrounding connective tissue, urethra, and dorsal penile vein were quickly removed. The corporal cavernosal tissue was bisected along the central channel into two portions. The proximal end of each portion was tied to a glass anchoring rod while the distal end of each portion was tied to a thin connecting wire via 3-O silk suture. The tissues were placed into a 40 ml chamber of modified Kreb's solution and allowed to equilibrate at 37^0C. The Kreb's solution was continuously bubbled with a 95% O_2, 5% CO_2 gas mixture to maintain proper pH and oxygen supply. At the end of each experiment, wet tissue weights of all specimens were taken.

Drugs and Solutions

The modified Kreb's solution used in these experiments consisted of the following concentrations: NaCl (119 mM); KCl (4.6 mM); NaH_2PO_4 (1.2 mM); $CaCl_2$ (1.5 mM); NaHCO3 (15 mM); MgCl (1.2 mM); and glucose (11 mM). This solution was then adjusted to pH 7.2 to 7.4.

The calcium free media used in some of these experiments substituted EGTA (1.5 mM) for $CaCl_2$.

Norepinephrine, Ascorbic Acid, Sodium Nitroprusside dihydrate (SNP), and Cyclopanazoic Acid (CPA) were obtained from Sigma, St. Louis, Missouri. Solutions of NE and SNP for each experiment were made up in the appropriate media. Solutions of CPA were made up in a 1:10 dilution of DMSO to a final concentration of 10^{-4} M.

Experimental Protocol

Experiment I: SR-Pump Assay

Corporal cavernosal tissues were obtained from 10 intact animals (20 tissues) as described above and tissue assemblies were placed in a 40 ml modified Kreb's bath. The connecting wire of the tissue assembly was attached to a metal hook on a Grass Instruments Force Transducer (Quincy, Massachusetts). Grass Instruments model P122 strain gauge amplifier amplified output from the force transducer and a Grass Instruments Polyview, version 2.1, 4-channel recorder software program then received the signal. The transducer was calibrated to measure all forces in mgs. Resting tensions averaged 174.48 +/- 5.90 mg for the corporal cavernosal smooth muscle (CCSM) tissues that were used in the experimental group, while resting tension averaged 177.45 +/- 4.27 mg for the control tissues (Fig. 1). The tissues were allowed to equilibrate at 37^{0} for 45-60 minutes. After equilibration, the media was flushed, resting tensions were reset and either CPA (final concentration of 10^{-4} M), n=9 tissue halves, or DMSO (control), n=9 tissue halves, was added to the chamber of all experimental tissues. Preliminary studies tested several concentrations of CPA, ranging from 10^{-6} M to 10^{-3} M. A concentration of 10^{-4} M was the lowest concentration to show phasic contractions upon the addition of NE, indicating that CPA had entered the cells and exerted an effect on the

tissues. These results confirm those achieved in previous experiments in which rhythmic contractions occurred upon the application of phenylephrine to CPA treated smooth muscle tissues (Omete and Mizusawa, 1994; Huang and Cheung, 1997). Based on these preliminary experiments, a final CPA concentration of 10^{-4} M was used in this study. Again, the tissues were given 45 minutes to equilibrate. After this equilibration period, resting tensions were reset and norepinephrine (NE) (final concentration equal to 10^{-4} M) was added to the bath to induce contractions as described by Alcorn *et al.*, 1999. The corporal tissue began contracting within 1 minute of the addition of NE; it was then allowed approximately 20 minutes to maintain a higher stable baseline. In order to prevent any influence caused by transducer drift, tissues contracting less than 75 mg were not used for any further experimental data. Upon stabilization of a contracted baseline, SNP (a final concentration of 10^{-3} M) was added to the tissue bath in order to relax the tissue. The corporal cavernosal tissues were given approximately 25 minutes to achieve complete relaxation as described by Alcorn *et al.*, 1999. Following this period, tissues were removed from the force transducer and weighed. Data analysis was performed and changes in tension in mg were calculated. Results were then converted to a percent relaxation of norepinepherine-induced contraction by dividing the mg of SNP-induced relaxation into the difference in mg of tension due to norpeinepherine-induced contraction, once the contraction baseline had stabilized.

Experiment II: Assay of Plasma Membrane Calcium Pumps

Corporal cavernosal tissues were obtained from sham castrate (n=8 tissue halves), castrate (n=8 tissue halves), and testosterone replacement rats (n=10 tissue halves) as described above and tissue assemblies were placed in

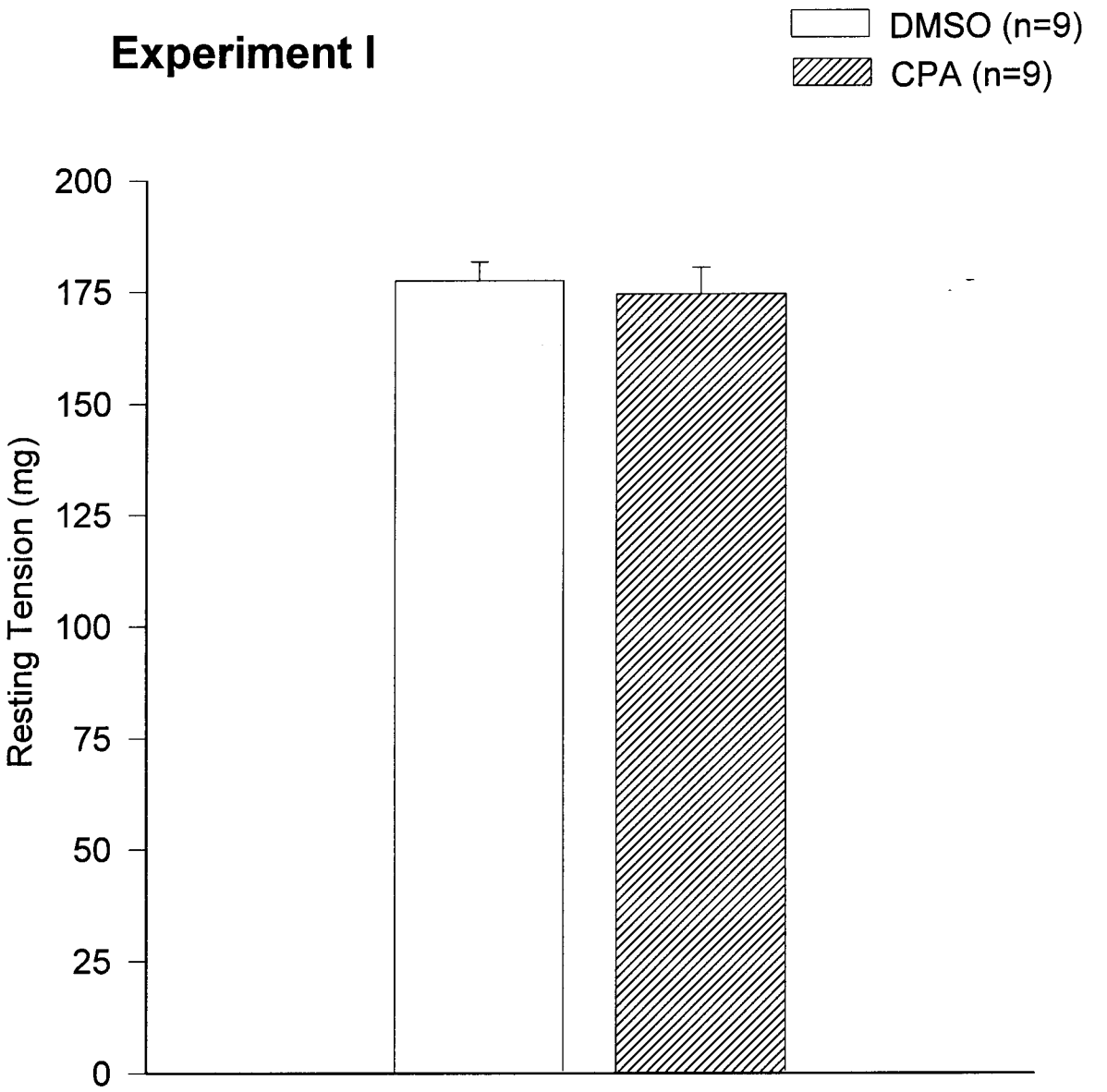
a calcium free media. The tissue assembly was attached to the force transducer and allowed to equilibrate as described in Experiment I. After equilibration, the media was flushed from the chamber and CPA (final concentration equal to 10^{-4} M) was added to the bath. Resting tensions were set and averaged 175.50 +/- 3.29 mg for those tissues removed from the sham castrate animals, 183.90 +/- 7.62 mg for those tissues removed from the castrated animals, and 157.30 +/- 3.60 mg for those tissues removed from the animals treated with testosterone replacement (Fig. 2). Again, the corporal cavernosal tissues were given 45 minutes to allow equilibration. After this period, resting tensions were reset and NE (final concentration equal to 10^{-4} M) was added to the bath. Tensions were closely observed for 5 minutes to assure no contraction had occurred. Because the tension did not increase after the addition of NE, this suggests that the intracellular stores of calcium do not contribute to smooth muscle contraction in the corporal cavernosal tissue. Therefore, $CaCl_2$ (7.5 mM) was added to the bath to induce contraction. The tissue was given between 20 and 25 minutes to attain complete contraction. As in experiment I, to prevent any influence from transducer drift, tissues contracting less than 75 mg were not used for further experimental data collection. Once the contraction had stabilized, SNP (final concentration equal to 10^{-3} M) was added to the bath to induce relaxation. Corporal cavernosal tissues were given approximately 25 minutes to achieve maximal relaxation. After complete relaxation had been achieved, the tissues were removed from the force transducer and weighed. Data analysis was performed and changes in tension in mg were calculated. Results were then converted into percent relaxations of norepinepherine-induced contractions, as described for experiment I.

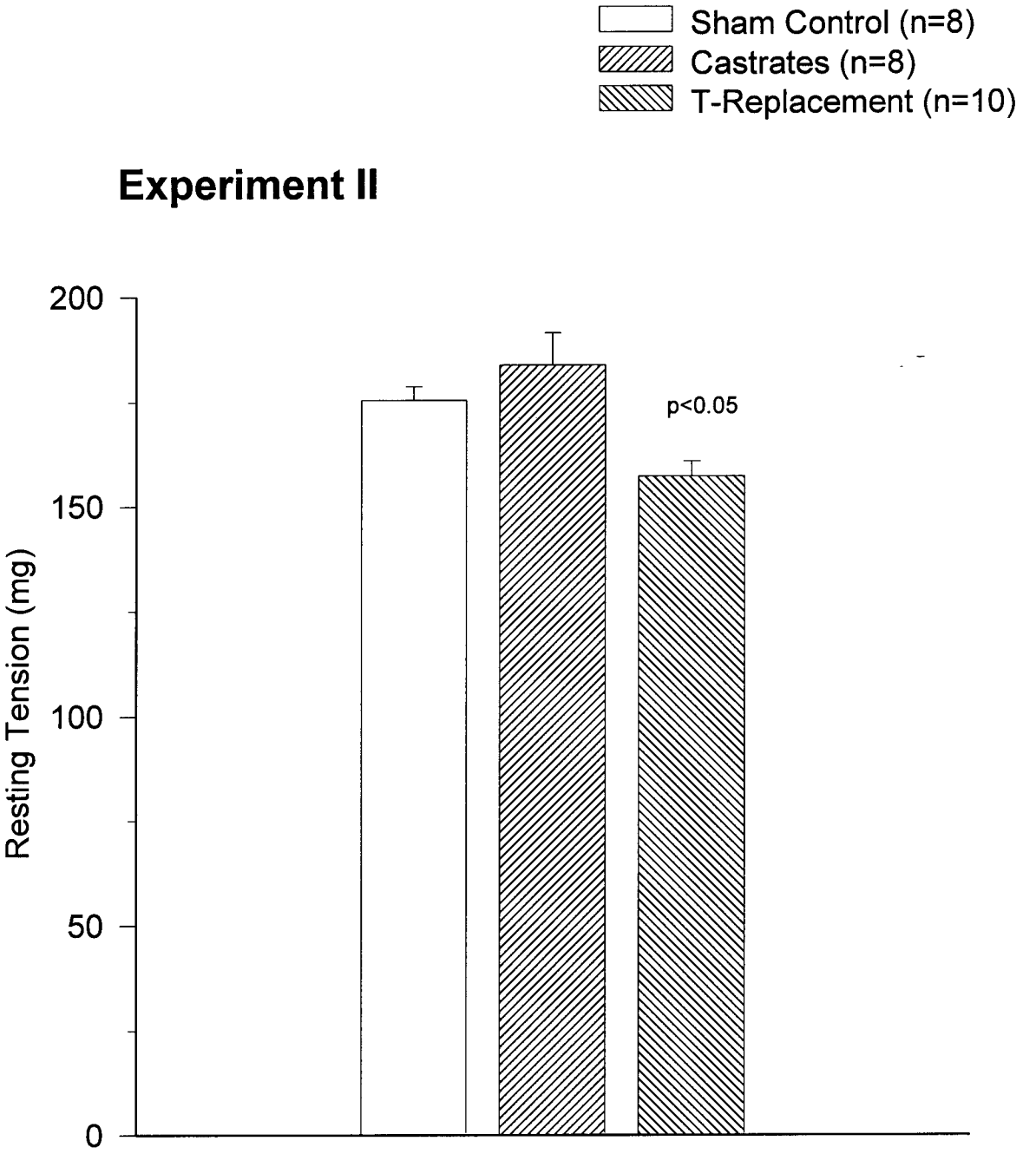
Data Analysis

Percent relaxation data acquired in this study was converted to arcsin transformation and analyzed using the SigmaStats program, version 1.0, Jandel Scientific, Corte Madera, California. Results were subjected to either paired t-tests or one-way ANOVA, with $p < 0.05$ as the minimum for significant differences. Significant differences between treatment groups were further tested using the Student-Newman-Keuls multiple comparison test, again using $p < 0.05$ as the minimum for significant difference. All graphs were constructed using SigmaPlot, version 1.02, Jandel Scientific, Corte Madera, California.

Animal Usage

All animal procedures, as described in Protocol #05-99, were approved by The Institutional Animal Care and Use Committee of Youngstown State University.


Experiment I
DMSO (n=9)
CPA (n=9)
Resting Tension (mg)
200
175
150
125
100
75
50
25
0


Sham Control (n=8)
Castrates (n=8)
T-Replacement (n=10)
Experiment II
200
150
100
50
0
Resting Tension (mg)
p<0.05

Results

Experiment I

The average tissue weights of the corporal cavernosal tissues that were used in this study were 80.78 +/- 4.99 mg for the tissues treated with CPA, and 81.03 +/- 3.25 mg for the tissues treated with DMSO control. These weights showed no significant difference between groups (Fig. 3). The average peak contractions induced by application of norepineprine (final concentration equal to 10^{-4} M) in this experiment were 213.97 +/- 29.0 mg for those tissues treated with CPA. DMSO control treated tissues showed an average peak contraction of 207.02 +/- 29.3 mg. There was no significant difference between groupings (Fig. 4). To ensure that peak contractions were not influenced by tissue weight, contractions were calculated in the form of mg contractions per mg tissue mass. The results of these calculations are as follows; 2.76 +/- 0.42 mg for CPA treated tissues and 2.57 +/- 0.36 mg for DMSO control treated tissues, with no significant difference between groups (Fig. 5).

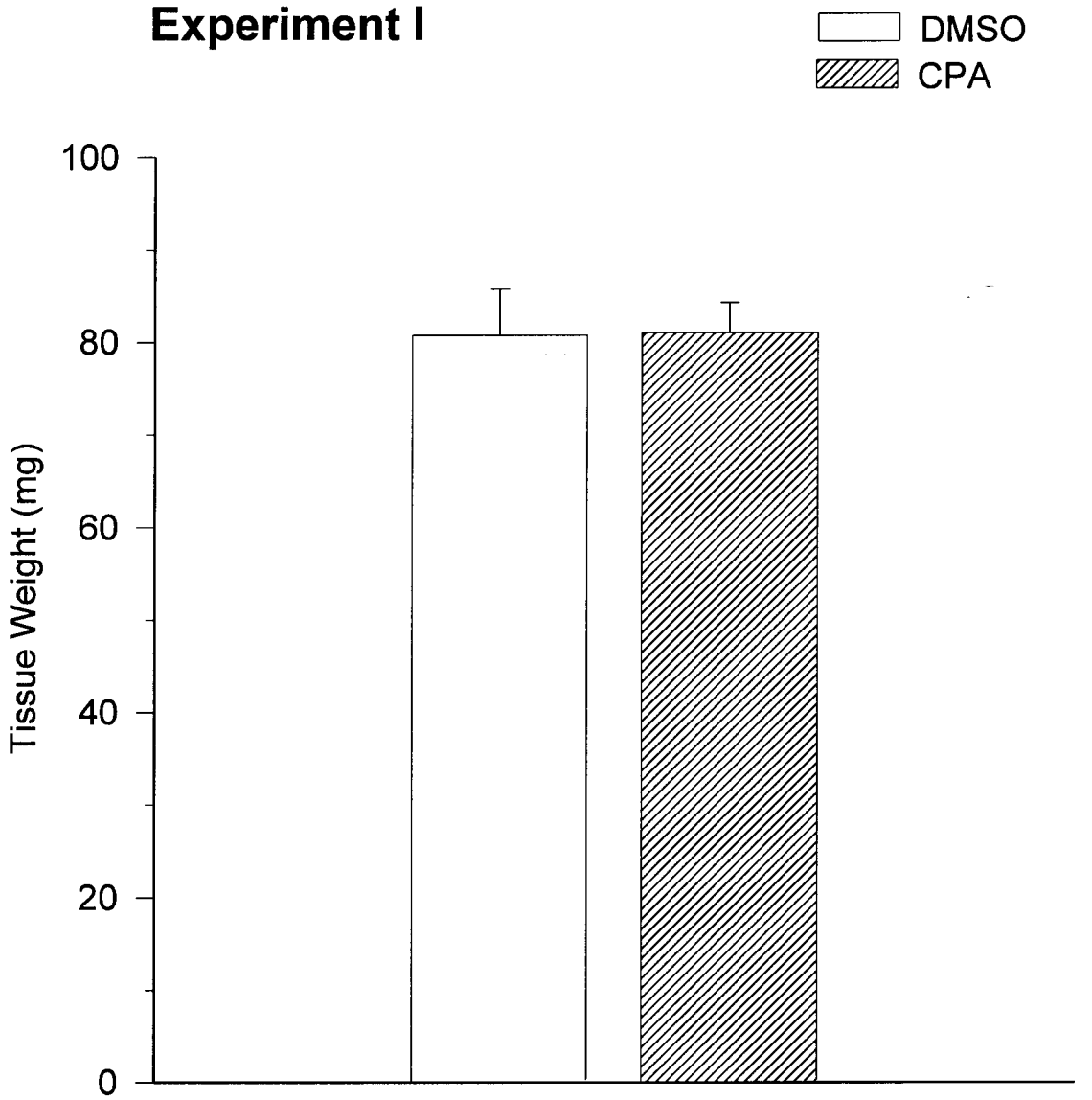
The average percent relaxations after treatment with SNP (final concentration equal to 10^{-3} M) were calculated to be: 89.9 +/- 4.39% for those tissues treated with CPA and 71.6 +/- 1.63% for those tissues treated with DMSO control. Paired t-test, under arcsine transformation, showed a significant difference between groupings, $p = 0.01$ (Fig. 6).

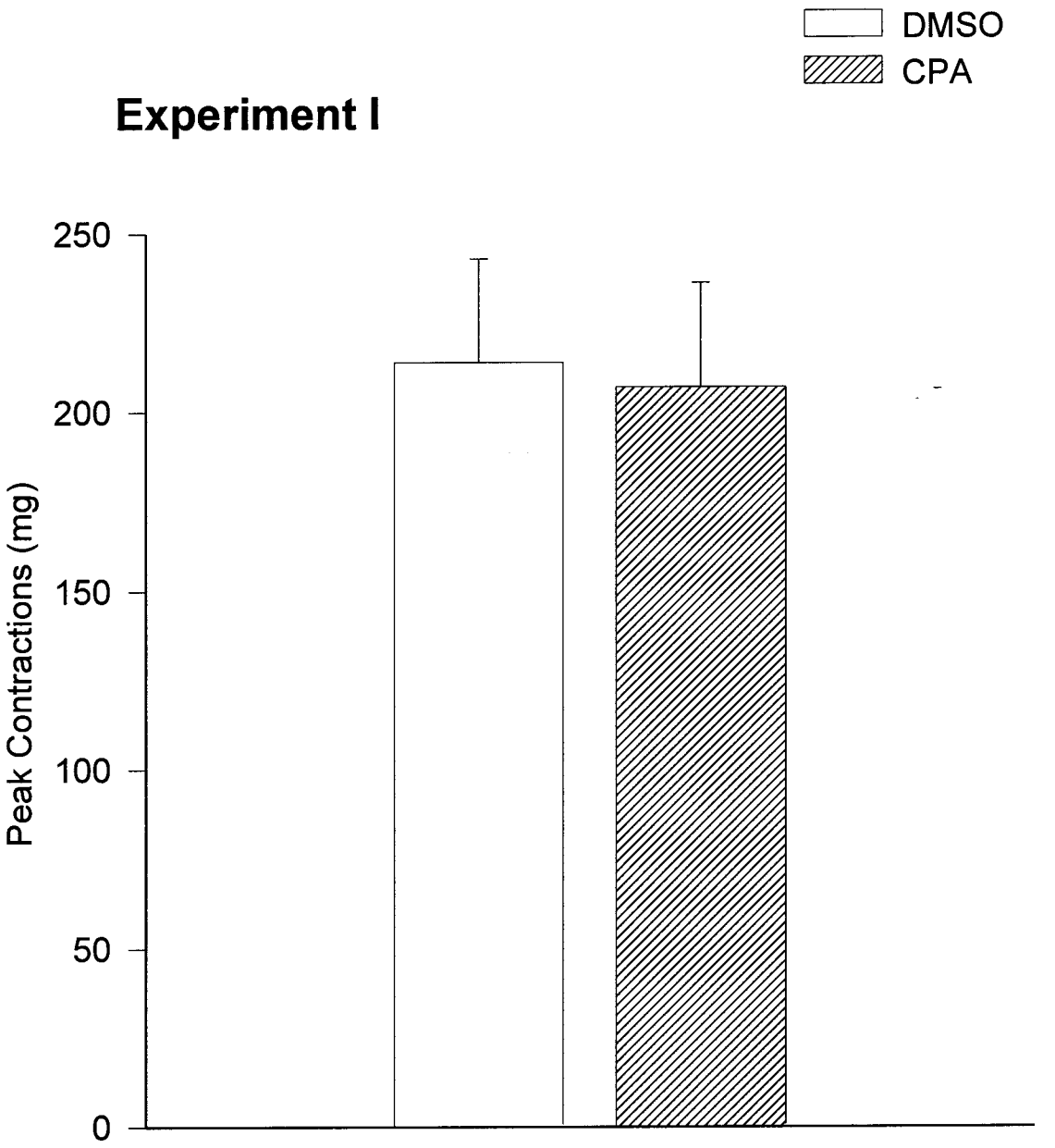
Experiment II

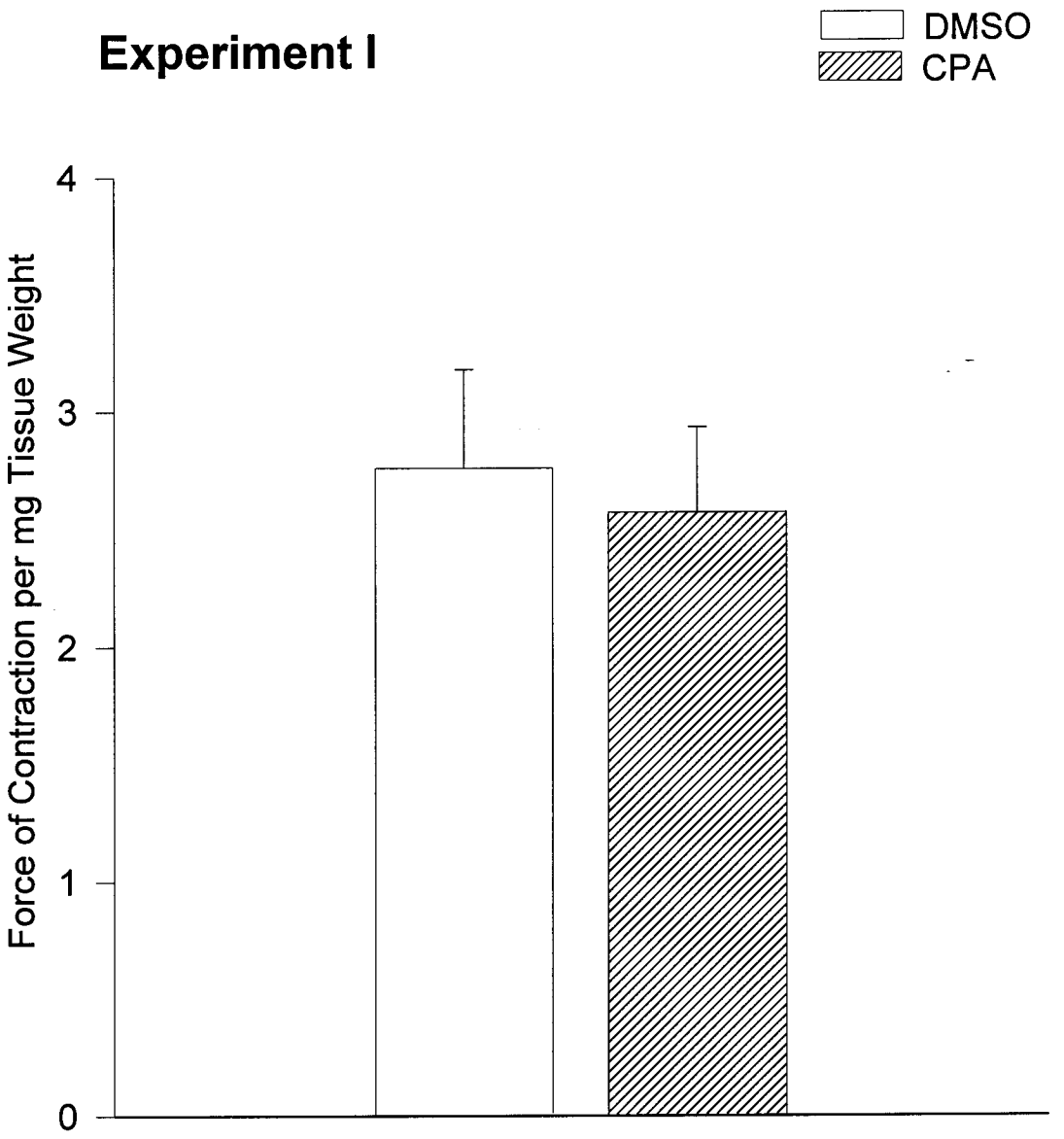
The average tissue weights of the coporal cavernosal tissues used in this experiment were 81.6 +/- 2.66 mg for the intact (control) group, 74.9 +/- 6.55 mg for the castrate group, and 100.7 +/- 3.19 for the testosterone

replacement group. While there were no significant differences between the intact and the castrated groups, the testosterone replacement group displayed a significant difference compared to both the intact and the castrate groups, p = 0.005 one-way ANOVA (Fig. 7). Peak contractions established upon the introduction of norepinephrine (final concentration equal to 10^{-4} M) and calcium (final concentration equal to 7.5 mM) to the calcium free media were 131.2 +/- 18.4 mg for the intact group, 146.4 +/- 14.0 mg for the castrate group, and 137.3 +/- 10.7 mg for the testosterone replacement group. One-way ANOVA testing displayed no significant differences between testing groups (Fig. 8). To further ensure that peak contractions were not significantly different due to increased tissue mass of the testosterone replacement group (Fig. 7), they were calculated in terms of mg contraction per mg tissue mass. The results are as follows; 1.65 +/- 0.26 for the intact group, 2.06 +/- 0.26 for the castrate group, and 1.37 +/- 0.11 for the testosterone replacement group. One-way ANOVA showed no significant differences between groups (Fig. 9).

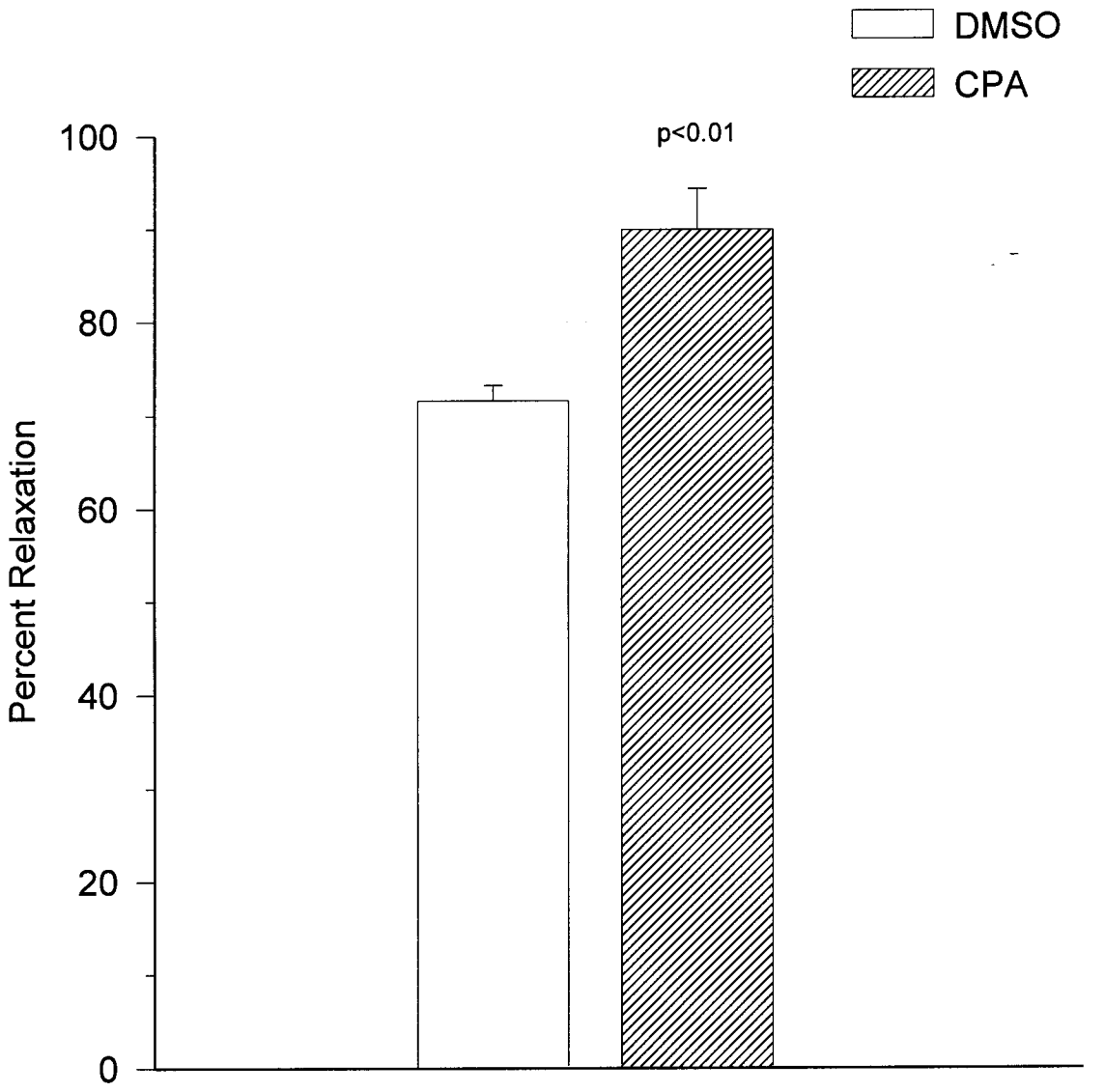
The percent relaxations, upon introduction of SNP (final concentration equal to 10^{-3} M), were determined to be 81.0 +/- 3.16% for the intact group, 56.0 +/- 3.03% for the castrate group, and 74.8 +/- 3.00% for the testosterone replacement group. One-way ANOVA, after arcsine transformation, indicated that there were no significant differences between the intact and the testosterone replacement groups. However, significant differences were observed between the castrate group and both the intact, $p < .001$, and the testosterone replacement groups, $p < .001$ (Fig. 10).

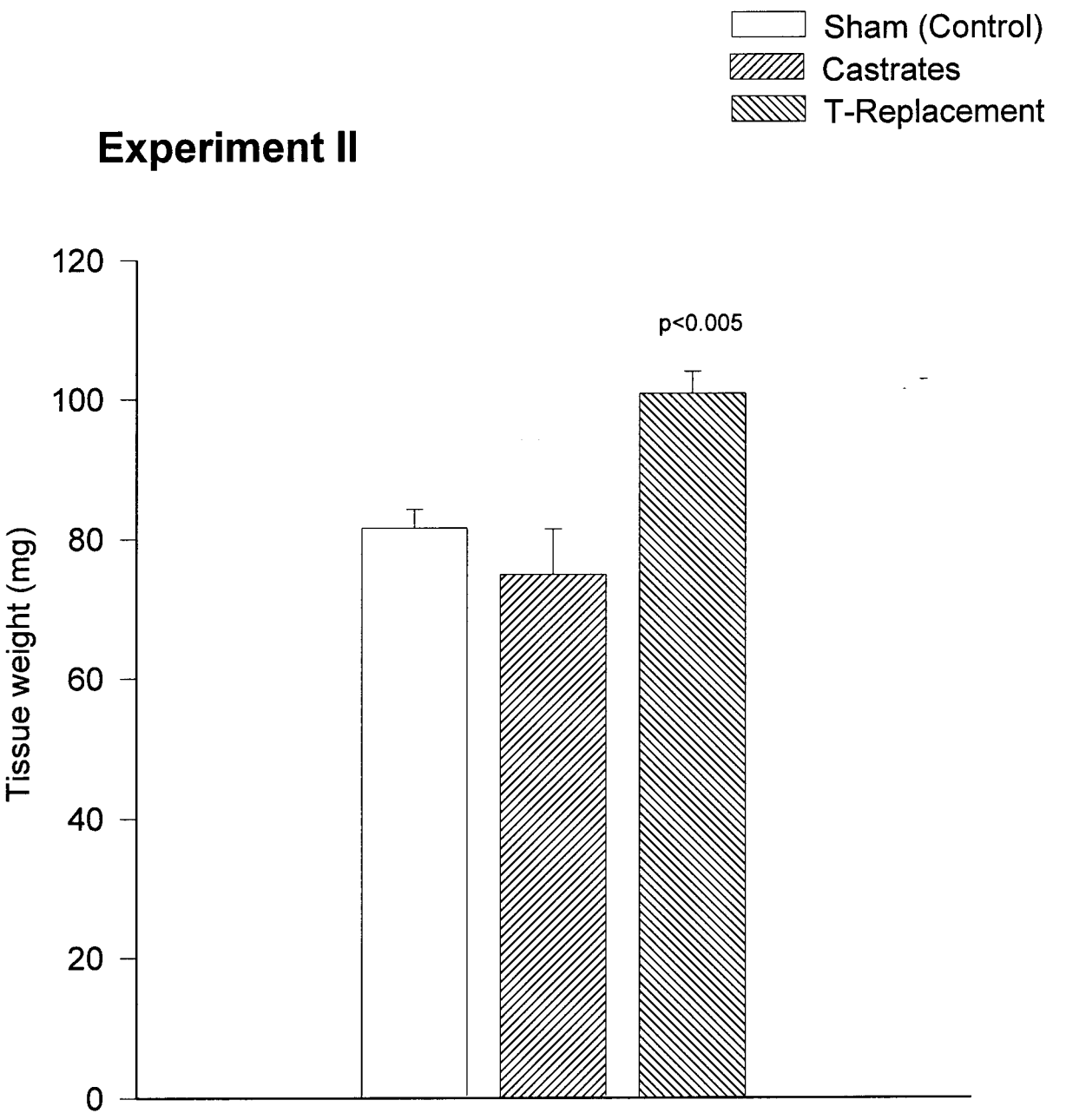

Experiment I
DMSO
CPA
Tissue Weight (mg)
100
80
60
40
20
0

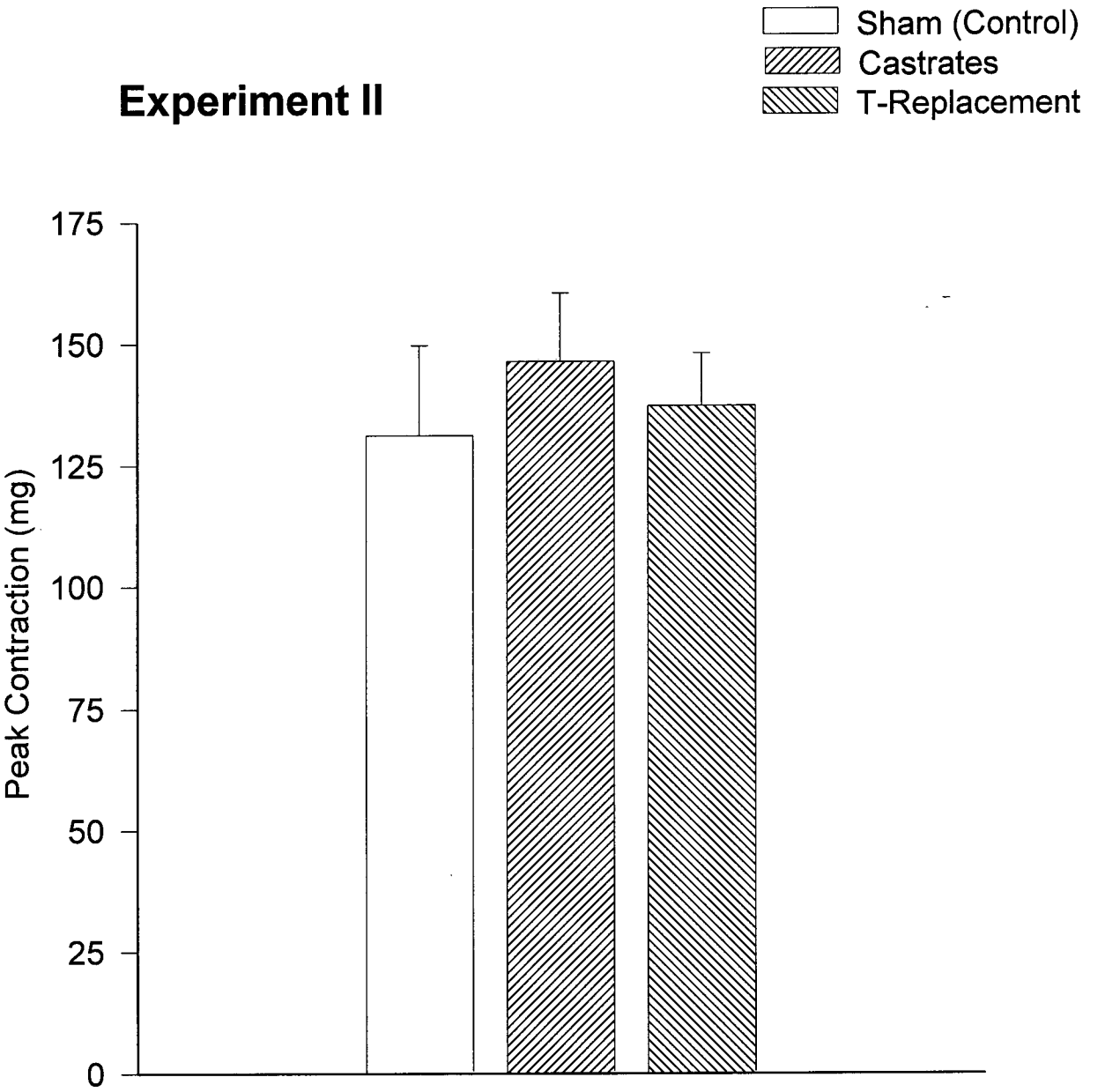

DMSO
CPA
Experiment I
250
200
150
100
50
0
Peak Contractions (mg)

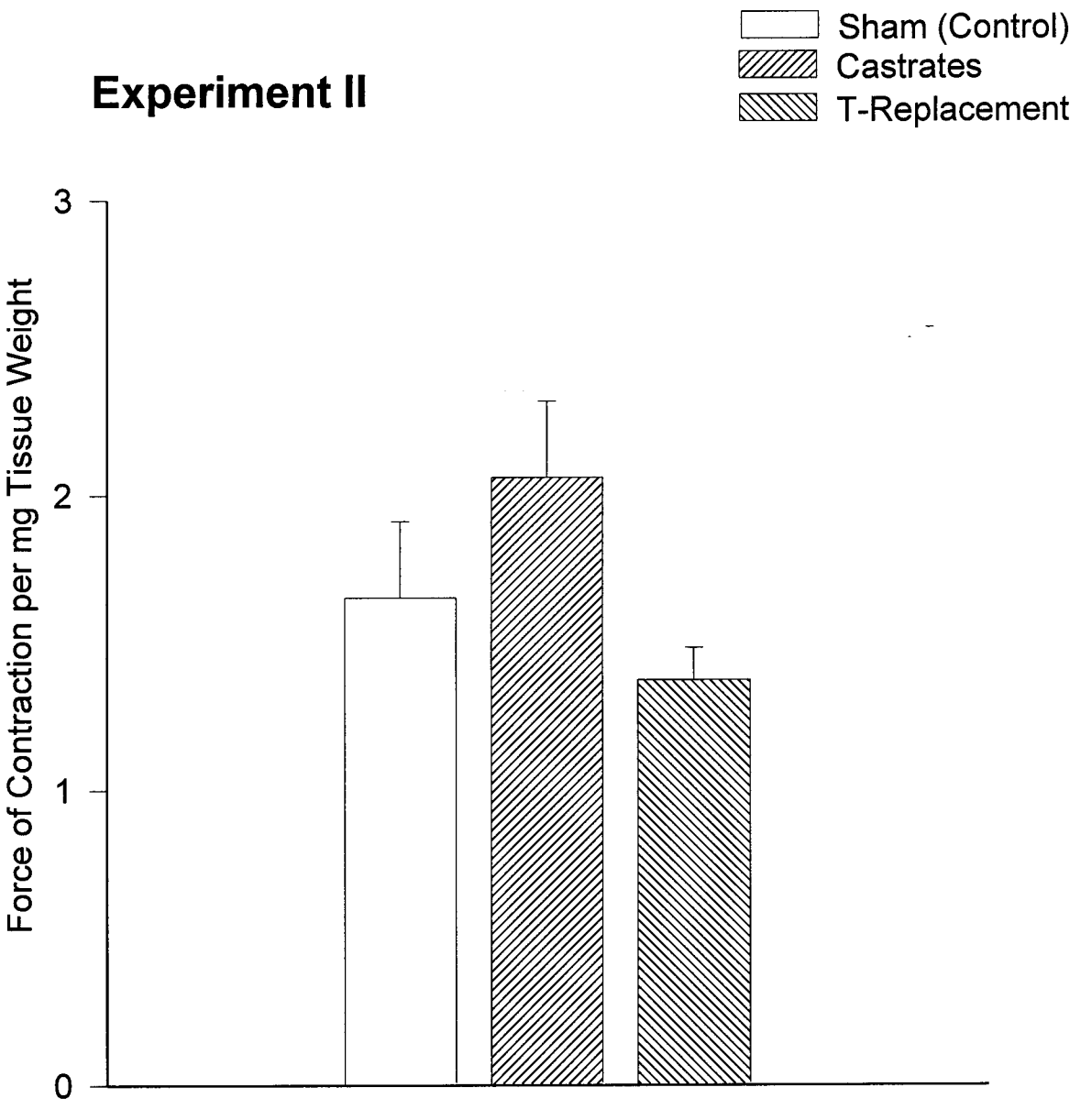

Experiment I
DMSO
CPA
Force of Contraction per mg Tissue Weight
4
3
2
1
0

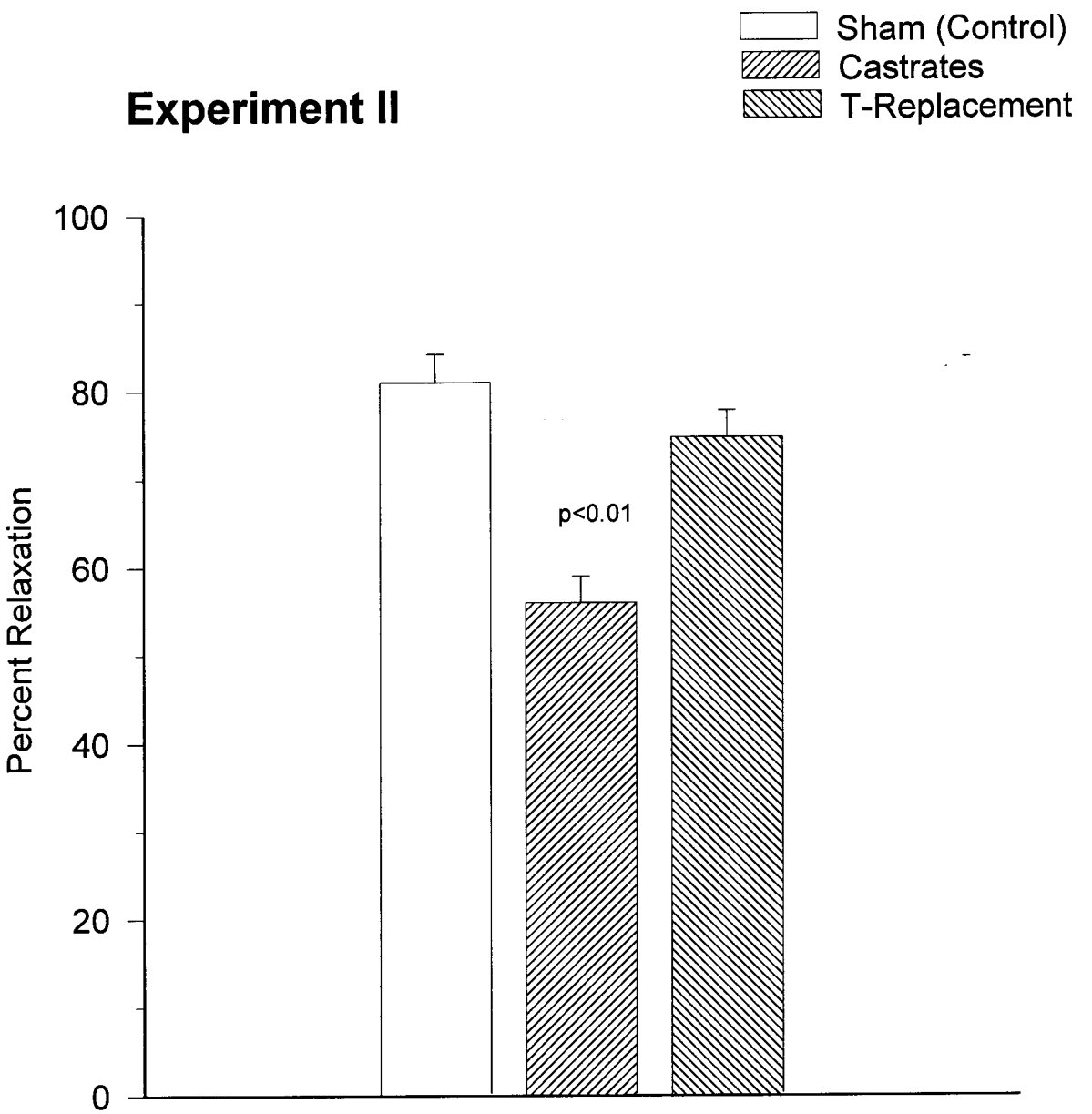
Experiment I




Sham (Control)
Castrates
T-Replacement
Experiment II
120
100
80
60
40
20
0
Tissue weight (mg)
p<0.005


Sham (Control)
Castrates
T-Replacement
Experiment II
Peak Contraction (mg)
175
150
125
100
75
50
25
0


Experiment II
Sham (Control)
Castrates
T-Replacement
Force of Contraction per mg Tissue Weight
3
2
1
0


Sham (Control)
Castrates
T-Replacement
Experiment II
Percent Relaxation
100
80
60
40
20
0
p<0.01

Discussion

This study shows that by blocking the sarcoplasmic reticulum (SR) ATPase with cyclopanazoic acid (CPA), no inhibition occurs to the SNP-induced relaxation of the corporal cavernosal smooth muscle cells (Fig. 6). In fact, tissues treated with CPA actually relaxed more than the control tissues that had been treated with only DMSO. These results suggest that the most important mechanism for the removal of calcium from the cells is external, either through the calmodulin-dependent ATPase or the Na^{+}/Ca^{+2} exchanger. These findings were further supported by the results of the second portion of this study in which the CCSM tissue equilibrated in Ca^{+2} free media failed to contract in the presence of norepinephrine. The CCSM tissue did not contract until the addition of $CaCl_2$ (final concentration equal to 7.5mM) to the media, further suggesting that the most important source of Ca^{+2} for the regulation of contraction in this tissue is from the extracellular fluid.

These experiments also demonstrated that castration significantly reduces the relaxation of CCSM. By blocking the SR ATPase, along with the use of calcium free media, it is evident that castration leads to a significant reduction in the relaxation of CCSM, presumably by affecting the plasma membrane mechanisms responsible for the removal of Ca^{+2} out of the cytosol. This study also shows that CCSM relaxation could be restored through the use of testosterone replacement, supporting the results previously attained in this laboratory (Leipheimer and Toepfer, 1996; Alcorn *et al.,* 1999). Since the differences in contraction were not due to differences in tissue mass, it must be assumed that any changes in relaxation are due to the presence or absence of androgens. Other calculations

associated with this experiment show that the different tissue groups were not affected in their ability to contract in the presence of norepinepherine (NE), the neurotransmitter responsible for the maintenance of CCSM tone (Andersson and Holmquist, 1994). These results show that androgens exert little or no control on the contraction of CCSM, supporting the results of previous experiments in this laboratory (Alcorn *et al.,* 1999).

The cytosolic calcium concentration is the primary factor in determining the contraction or relaxation of smooth muscle, including the corporal cavernosal smooth muscle cells (Eggermont, 1988). This concentration can be regulated through either internal storage or extracellular removal of calcium from the cytosol. Intracellular storage of calcium in smooth muscle cells is accomplished by ATPases located on the sarcoplasmic reticulum (Raeymaekers *et al.,* 1988). Extracellular removal of calcium from the cytosol occurs through one of two mechanisms. The first possibility is the calmodulin-dependent ATPase located on the plasmalemmal membrane (Eggermont *et al.,* 1988). This mechanism, despite a low transport capacity, uses a high affinity for calcium to continuously pump calcium across the plasma membrane out of the cytosol (Carafoli, 1988). The second possible means of exporting calcium across the plasma membrane is through the Na^{+}/Ca^{+2} exchanger; which, despite a low binding affinity, is able to move large amounts of calcium across the plasma membrane and out of the cytosol (McCarron *et al.,* 1994). Our results provide evidence that the plasma membrane mechanisms for Ca^{+2} removal are more effective than the intracellular storage offered by the sarcoplasmic reticulum in regulating relaxation in the corpus cavernosum. However, these experiments did not address the relative contributions that

may be offered by each of the plasma membrane mechanisms in the regulation of relaxation in this tissue.

It is widely believed that androgen regulation is a key component in the mechanism of tumescence (Gray *et al.,* 1980; Leipheimer and Sachs, 1993; Mills *et al.,* 1996; Penson *et al.,* 1996). However, the exact manner in which androgens exert their effects remains unclear. Some experimental evidence suggests that androgens regulate the activity of nitric oxide synthase (NOS), the enzyme responsible for the production of nitric oxide and the initiator of the NO/c-GMP pathway responsible for the relaxation of CCSM (Lugg *et al.,* 1995). While this regulation may indeed occur, previous studies in this laboratory provide evidence that androgen regulation also occurs at intracellular steps of the NO/c-GMP pathway downstream of NOS activity. In fact, these studies also demonstrate androgen regulation past the activity of guanylate cyclase, an enzyme responsible for the formation of c-GMP, the second messenger of the CCSM relaxation pathway (Alcorn *et al.,* 1999). Results of the present study support these results previously obtained in this laboratory. Our results suggest that the plasma membrane Ca^{+2} pump mechanisms are regulated by testosterone. In this study, corporal cavernosal tissues removed from castrated animals relaxed significantly less than those tissues removed from either sham controls or castrated animals given testosterone replacement when treated with the NO donor, SNP. Therefore, androgens must be exerting some regulatory function on the mechanisms that regulate the activity of the Ca^{+2} pumps in the CCSM plasma membrane.

In conclusion, the current study extends the previous experiments performed in this laboratory by demonstrating that the calcium removal processes regulating CCSM relaxation are due to the plasma membrane Ca^{+2}

pump mechanisms. Furthermore, our results indicate that these plasma membrane Ca^{+2} pump mechanisms are regulated, at least to some extent, by the presence of androgens.

Future studies in this laboratory will determine specifically whether the calmodulin-dependent ATPase or the Na^{+}/Ca^{+2} exchanger is primarily responsible for the removal of calcium from CCSM during the relaxation of this tissue. These studies will also examine whether androgens exert any effects on this mechanism and, if so, how these effects are exerted.

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Appendix A

Approval from The Institutional Animal Care and Use Committee

YSU

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August 6, 1999

Dr. Robert Leipheimer
Department of Biological Sciences
UNIVERSITY

Dear Dr. Leipheimer:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed and accepted the modifications you provided to Protocol #05-99 entitled "Androgen Regulation of Isolated Corpus Cavernosum Activity in the Rat." Your protocol is now fully approved.

You must adhere to the procedures described in your approved request. The Institutional Animal Care and Use Committee must first authorize any modification to the project.

Sincerely,

Dr. Peter J. Kasvinsky
Dean of Graduate Studies

PJK:cc

c: Dr. Paul Peterson, Chair
Department of Biological Sciences