The Role of Plasma Membrane Calcium Pumps in Regulating Relaxation of Rat Corpus Cavernosum Smooth Muscle *in vitro*

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ABSTRACT

These experiments were designed to investigate the roles of the plasma membrane Ca^{2+} pumps in regulating relaxation of CCSM tissue *in vitro*.

To distinguish the significant importance of these two plasma membrane pumps in determining CCSM relaxation, two different experiments were performed. The first experiment compared the relaxation of isolated CCSM tissues in Kreb's (control) versus Na^{+} -reduced solution. The isolated CCSM tissue was treated with norepinephrine (NE) to stimulate contraction, and sodium nitroprusside (SNP) to induce relaxation. Percent relaxation values were then recorded for each group.
This experiment focused on the Na^{+}/ Ca^{2+} exchanger and its effects on relaxation of the CCSM.

The second experiment compared isolated CCSM tissue in three different groups of media; Kreb's (control) w/ DMSO, carboxyeosin w/ DMSO, and Na^{+} -reduced solution w/ carboxyeosin. The drug carboxyeosin is believed to be an inhibitor of the calmodulin-dependant Ca^{2+} ATPase pump. Again, the isolated CCSM tissue was treated with NE to initiate contraction, carboxyeosin was then added to the media, and finally SNP was given for the CCSM tissue to undergo relaxation. This experiment examined the effects of inhibiting the calmodulin-dependant Ca^{2+} ATPase pump on the relaxation of CCSM.

Our results demonstrated that both the Na^{+}/Ca^{2+} exchange pump and the calmodulin-dependant Ca^{2+} ATPase pump play an important role in mediating relaxation of the CCSM tissue tested with the NO donor, SNP.

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TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Impotence is the inability to sustain an erection for a sufficient amount of time during sexual intercourse. The causes of impotence deal with both physiological and psychological deficiencies within the human body. It is believed that 10 to 15 million American men suffer from this disease. Those incidents of impotence also run parallel to age. Around 5 percent of men at the age of 40 and above develop impotence. Whereas men at the age of 65 or higher, have a 15 to 25 percent chance of developing this condition. Impotence, or Erectile Dysfunction (ED), can be linked to many things. Damage to the arteries, smooth muscle, and fibrous tissue is known to be the most common cause of impotence today. Diabetes also causes 35 to 50 percent of impotence in men. Surgery and injury to the penis and its various structures, such as the corpus cavernosum, can lead to impotence as well. Hypogonadism, 10-20 percent of prevalence, can also be responsible for impotence (Korenman, 1990). Psychological factors including stress, anxiety, guilt, and depression, are known to cause 10 to 20 percent of cases concerning impotence.

There are a variety of diagnostic measures used today to discover if a male patient has ED. Diagnostic classification of ED is based on the disorder (physiological vs. psychological) and the duration and extent of

erectile difficulties (Rosen, 1996). The patient is asked about his sexual and medical history, given various physical examinations, laboratory tests, and extensive psychosocial tests to accurately diagnosis this disorder.

Treatment approaches concerning ED have changed dramatically throughout the years. There are a variety of surgical and medical treatments available to treat this disorder. Such popular treatments include penile implant surgery; intracavernosal injection therapy, vacuum constriction devices, oral drugs, and the administration of topical agents (Rosen, 1996). Surgeries to implant penile prostheses date back to the 1930's, when a piece of human rib was used. It has been estimated that 25,000 surgical implants have been done annually (Rosen, 1996). The inflatable prostheses are now more commonly used but are three times more expensive then the noninflatable ones. Only male individuals with spinal cord injury, severe vascular or neural disease, or pelvic trauma are recommended for this option (Rosen, 1996). The high cost, irreversibility of the surgery, and high-risk of side effects make this the least favorite option to treat ED.

Intracavernosal injection therapy is yet another option that is used to treat ED. Vasoactive drugs injected directly into the corpus cavernosum such as papaverine, phentolamine, and prostaglandin E1, can cause relaxation within the tissue (Rosen, 1996). The overall effective rate was

reported to be 75-80%, although most patients were dissatisfied with this option due to side effects and unwillingness to self inject. Vacuum devices also help cause erections by creating a partial vacuum that draws blood within the penis and causes it to become erect. Side effects are few, but bruising to the penis and painful ejaculations can occur (Rosen, 1996). In addition, the use of oral drugs such as dopamine and trazodone, have been effective in treating ED in men with low levels of natural testosterone. These drugs have limited effectiveness though and cause numerous side effects within the individual (DePalma, 1996). Lastly, topical agents such as minoxidil and nitroglycerin can be used to elicit an erection. However, numerous side effects are often observed with this therapy, such as persistent erection, scarring, allergic reaction, and irritation (Rosen, 1996).

Various psychotherapy techniques, such as those that help reduce anxiety and increase sexual stimulation, have been recorded to work efficiently (Rosen, 1996). The patient's partner can decrease the anxiety level by applying various techniques that develop intimacy and sexual stimulation. Also, fantasy training, a couple's assertiveness, and communication training are factors in treating ED (Rosen, 1996). Using a combination of physiological and psychological treatments to treat ED, has been found to be a more effective way in battling this disorder.

One of the most effective treatments, of ED, is sildenafil citrate (Viagra) a drug approved by the Food and Drug Administration in 1998. Other oral drugs have been used to treat impotence such as dopamine and trazadone, but their effectiveness was questioned (DePalma, 1996). Sildenafil is a selective and potent inhibitor of type-5 cGMP cyclic nucleotide phosphodiesterase (PDE5) isozymes (Chuang *et al*, 1998a). Sildenafil acts to inhibit the hydrolysis of cGMP, which then increases the overall amount of cGMP in the corpus cavernosum smooth muscle (CCSM) tissue (Chuang *et al*, 1998b). Alone sildenafil is an effective drug in treating ED, but sexual arousal is still needed to obtain the desired erectile response. Sildenafil had a 60% efficacy rate, and was chosen the sole treatment by 30% of the 316 patients tested in an experiment (Virag, 1999). Other studies reported very few side effects while using the drug (Hawton, 1998), and 82% of people who have taken the drug only once have had successful erections (Boolell *et al.*, 1996).

The mechanism of erection has to be understood to most effectively treat the disorder of impotence. Recent advances have increased the understanding of the physiology of the erectile process. Blood flow measurements during erections have shown an increase in arterial flow, sinusoidal relaxation and increased venous resistance, resulting in turgidity

(relaxation) of the corpus cavernosum and corpus spongiosum (Lue and Tanagho, 1987). Contraction of the bulbocavernosus and ischiocavernosus muscles compress the proximal corpora, and further fills the CCSM tissue with blood, usually during sexual intercourse (Lue and Tanagho, 1987). Therefore, structural functions of the penis must be understood.

In response to sexual stimulation, a sufficient amount of blood trapped in the corpus cavernosum tissue serves as the main mechanism in maintaining erection. Whereas another important tissue, the corpus spongiosum acts as both a urinary and ejaculatory outlet (Newman and Northup, 1981). The corpus spongiosum surrounds the urethra, and could also develop a slight turgidity similar to that which the corpus cavernosum experiences during erection. The tuninca albuginea is a third important structure that helps to trap the blood in the corpus cavernosum, and is therefore instrumental in causing the penis to become erect. The process by which erection occurs ties in the previous mentioned structures. The CCSM contains two chambers, which are parallel to the length of the penis. The tunica albuginea is an elastic, fibrous connective tissue that covers the corpus cavenosum blood spaces. The corpus spongiosum is the channel that allows urine and semen to pass through, which lies in the medial portion of the penis (Lue and Tanagho, 1987). The CCSM and the corpus spongiosum

are both attached to the crura (pubic bone), by striated muscles. Blood that enters the CCSM spaces will increase the rigidity and diameter of the penis, which is limited by the presence of the tunica albuginea and the crura (Lue and Tanagho, 1987).

Blood flow to the penis is another important aspect that has to be considered in understanding penile erection. Two important internal pudendal arteries are necessary in supplying the entire blood flow to the penis (Newman and Northup, 1981). Each pudendal artery branches off to two arteries, the bulbar and urethral arteries of the kidney (Newman and Northup, 1981). These arteries supply the corpus spongiosum and continue as the artery of the penis. The dorsal artery is formed prior to the artery of the penis (Lue and Tanagho, 1987), which branches off into four or five separate arteries and supplies blood to the corpus spongiosum (Newman and Northup, 1981). The deep penile artery branches into one to two trunks, and supplies blood to the CCSM.

Concerning veins, there are three major penile subdivisions that need to be understood. They are the superficial, intermediate, and deep veins (Newman and Northup, 1981). The superficial veins are deep, extensive veins that lead to the dorsal vein. The intermediate veins are superficial to the tunica albuginea, and take a direct path to the pubis in the sulcus between

the two-corpus cavernosa. The deep veins are large veins near the CCSM, and empty in the pudendal vein or plexus (Newman and Northup, 1981). All the penile veins come from intracavernous venules that drain the sinusoidal spaces (Lue and Tanagho, 1987). During erection, most of the veins are compressed against the tunica albuginea by the blood-filled corpus cavernosum, which then functions to prohibit outflow. Restricted venous outflow, increased arterial inflow into the sinusoidal spaces and CCSM tissue, and shunting of the draining venules all contribute to the erectile response, or tumescence. Interestingly, CCSM relaxation has also been associated with platelet accumulation in causing, and not just involving vascular anatomical changes, in maintaining an erection (Rodrigues *et al.*, 1998). Although further research is needed to determine the underlying mechanism causing this venous restriction and penile erection, due to platelet accumulation.

The nervous system plays an important role in mediating the erectile response. Parasympathetic nervous activity is the most significant system mediating penile erection. The parasympathetic nerve fibers originate from the lower lumbar or sacral portion of the spinal cord and extend to the penis through the major pelvic ganglion (Sachs, 1995). These nerves contribute to penile erection by at least three different mechanisms, which include: 1.

inhibition of neuronal release of noradrenaline by stimulation of the prejunctional muscarinic receptors; 2. endothelium-derived relaxant factors released by postjunctional muscarinic receptors; and 3. nonadrenergic, noncholinergic (NANC) relaxant factors released by the parasympathetic nerves (Anderson and Holmquist, 1994). Most of these nerve interactions are focused on NANC nerve fibers that are responsible for the beginning of erection (Trigo-Rocha, *et al.*, 1993). These nerve fibers release neurotransmitters that do not include the classic neurotransmitters - acetylcholine or noradrenaline. Sympathetic nerve activity involves detumescence, and keeps the penis flaccid until sexual stimulation is activated. Noradrenaline is released and maintains contraction of the CCSM and therefore lack of blood flow in the corpus cavernosum. The sympathetic nerves run from the lower thoracic and upper lumbar segments of the spinal cord and extend to the penis through the inferior mesenteric ganglion, the hypogastric nerves, and major pelvic ganglion (Sachs, 1995).

Nerve interaction helps induce vascular changes that are mediated by the parasympathetic and sympathetic outflow to the peripheral organs (Rampin and Giuliano, 2000). The central nervous system collaborates with the cardiovascular system in response to sexual stimulation that induces an erection. Therefore, as heart rate and blood pressure increase, the

sympathetic functions decrease and the parasympathetic functions increase to the penis. This leads to tumescence, or an erection to occur.

Nitric Oxide (NO) is believed to be the main neurotransmitter involved in erectile stimulation. NO which is a gaseous messenger molecule, is an important mediator of CCSM tissue relaxation (Burnett, 1995). NO was originally known as the endothelium-derived relaxing factor (EDRF), that has been identified as the mediator of smooth muscle relaxation in human and other mammals (Trigo-Rocha *et al.*, 1993). NO is formed during the conversion of L-arginine into L-citrulline, which is catalyzed by the enzyme nitric oxide synthase (NOS), found mostly in the cytosol of cells off the penile tissue (Penson *et al.*, 1996). Stimulation of the NANC neurons causes the release of NO, which causes the relaxation of the CCSM. NO neurotransmitter is not only found in the peripheral nervous system, but in the central nervous system regulating oxytocinergic transmission in the paraventricular nucleus of the hypothalamus and other actions such as yawning responses. (Melis, 1997).

NO is known to initiate smooth muscle relaxation. A variety of second messenger systems continue the relaxation process. It is also known that androgens play an important role in regulating the erectile process. These steroid hormones work together in some way with the

neurotransmitters (NO) and secondary messenger systems (cGMP) in the relaxation of smooth muscles (Mills *et al.*, 1996). The androgen, testosterone, is believed to be the regulating agent. The exact pathway on how testosterone affects smooth muscle tissue involved in erection is still unknown. In addition, little is known concerning how the androgens affect the neurotransmitters response in smooth muscle, or how they regulate the smooth muscle erectile process (Mills *et al.*, 1999).

Testosterone is known to be the main androgen involved in regulating penile erection. It is synthesized by the Leydig cells, and is released when stimulated by luteinizing hormone. To exert its main effect on target tissues, such as the prostate gland, testosterone must be reduced by 5-α-reductase to dihydrotestosterone (DHT) (Rajfer *et al.*, 1980). The DHT then binds to a cytosol receptor protein, where it undergoes a temperature dependant change (Rajfer *et al.*, 1980). Then it enters the cell nucleus where it interacts with nuclear chromatin on its acceptor sites (Mainwaring, W.I.P. and Peterkin B.M., 1971). This form of testosterone is then able to exert its effects on CCSM tissue, modulating the mechanisms responsible for relaxation (Rajfer, *et al.*, 1980).

Many experiments have shown that testosterone levels decline as individuals increase in age. This supports the idea that virility decreases in

old age due to decreased testosterone production (Vermeulen, 1971). An increase in metabolic clearance rate in old age also affects testosterone levels. Free plasma T levels and the apparent free testosterone concentration remain the same during adolescence to the age of 50 (Vermeulen, 1971). However, free testosterone concentration decreases in males aged 60 years and older. Other experiments also show testosterone levels are at their highest and constant levels at a very young age and until the end of puberty. Once puberty is over, the level of free testosterone drops in the cytosol, due to the level of penile androgen receptor decrease (Rajfer *et al.*, 1980).

Although derived from testosterone, DHT levels were not found to be age dependent as are testosterone levels (Gray *et al.*, 1980). DHT found in blood fails to decline with age because most serum DHT is from the reduction of testosterone in peripheral tissue (Gray *et al.*, 1980). Castration experiments also support data previously described. Castrated rats prior to puberty show no disappearance of androgen receptors, while after puberty androgen receptors decline. This shows that age has an irreversible effect on the amount of androgen receptors present in males (Mills *et al.*, 1996). Negative feedback can also be a possibility in effecting DHT formation. When DHT is inhibited the aromatization pathway of testosterone to

estradiol will increase and cause a down regulation of androgen receptor mRNA levels to be produced (Lin *et al.*, 1993).

NOS activity, combined with testosterone, is found to be very important in smooth muscle relaxation. Androgens, such as testosterone, are shown to promote CCSM relaxation *in vitro* (Leipheimer and Toepfer, 1996). In one experiment, testosterone was shown to regulate erectile activity by increasing NOS activity in the penile tissue (Mills *et al.*, 1996). Evidence of this was shown when researchers gave castrated rats testosterone, and NOS activity eventually increased to normal (Lugg *et al.*, 1995). However, other studies have shown that testosterone has the ability to regulate sites downstream from the formation of cGMP in the relaxation pathway (Alcorn, 1999). This research further showed that castrates had a decreased response to relaxing agents, such as the NO donor sodium nitroprusside (SNP) or 8-Br-cGMP, which was restored by androgen replacement. Therefore, testosterone has a direct action on the corporal tissue downstream of the effects of NO to increase cGMP levels. Furthermore, testosterone regulation must be acting further downstream from the guanylate cyclase activation site, since 8-Br-cGMP had a decreased relaxation response in the smooth muscle of the castrated rats (Alcorn, 1999).

Cyclic guanosine 3', 5'-monophosphate (cGMP) is an important second messenger for the penile erectile process that is activated after NO penetrates the cell. Once NO is in the cell cytosol, NO activates soluble guanylate cyclase (sGC), a hemoprotein, which coverts cyclic guanosine 5'-triphosphate (cGTP) into cyclic guanosine 3', 5'-monophosphate. cGMP effects various ion channels, protein kinases, and other effector signals to promote a smooth muscle response (Trigo-Rocha, 1993). To achieve smooth muscle relaxation, cGMP must decrease the amount of cytosolic Ca^{2+} in the cell. This is accomplished by decreasing influx of Ca^{2+} into the cell, modifying internal organelles into taking up more Ca^{2+}, and pumping Ca^{2+} out of the cell (Vrolix *et al.*, 1988).

Cyclic GMP is a second messenger because it requires specific target proteins to have its effect (Denninger and Marletta, 1999). cGMP functions in smooth muscle relaxation to decrease cytosolic Ca^{2+} concentrations. There are three known targets for cGMP to express the NO/cGMP pathway signal. The first is for cGMP-dependant protein kinase, a target protein associated with cGMP, to phosphorylate it's own target proteins in response to an increase of cGMP concentration throughout the cell (Lohmann *et al.*, 1997). Protein kinase G is associated with a major target protein called inositol 1,4,5-triphosphate (IP_3) receptor. The IP_3 receptors, located on the

sarcoplasmic reticulum, are phosphorlyated when cGMP is present causing smooth muscle relaxation by decreasing the cytosol Ca^{2+} concentration inside the cell (Denninger and Marletta, 1999). Consequently, in the absence of cGMP, Ca^{2+} is mobilized from non-mitochondria Ca^{2+} pools, and IP_3 attaches to its receptor cells on the sarcoplasmic reticulum, this allows the influx of calcium into the cell causing smooth cell contraction (Missiaen, 1992).

The second target for cGMP is Cyclic GMP-regulated phosphodiesterase. Cyclic GMP-regulated phosphodiesterase breaks down cGMP using cyclic nucleotide phosphodiesterase to catalyze the hydrolysis of 3'-phosphodiester bond of cAMP and cGMP to yield Amp and guanylate monophoshate (GMP) (Denninger and Marletta, 1999). This yields lower levels of cGMP and cAMP, which helps regulate or inhibit the erectile responses.

The third target for cGMP is a cyclic nucleotide-gated ion channel. These are non-specific cation channels found in many cell membranes, but the exact nature on how this system effects relaxation is still unknown (Denninger and Marletta, 1999).

The plasma membrane has three Ca^{2+} transporting systems within it (Carafoli, 1988). They are the Ca^{2+} channel, the Ca^{2+}-ATPase, and the

Na^+/Ca^{2+} exchanger. The Ca^{2+}-ATPase and Na^+/Ca^{2+} exchangers are considered exporting systems of Ca^{2+}, while the Ca^{2+} channel imports Ca^{2+}. The Na^+/Ca^{2+} exchanger indirectly has been found to function in smooth muscle, or specifically CCSM (McCarron *et al.*, 1994). Although, it's mechanism of action or regulation of Ca^{2+} distribution in smooth muscle is still quite unknown (McCarron *et al.*, 1994).

The Na^+/Ca^{2+} exchanger is a large capacity, low-affinity system, and is active in excitable tissues (Carfoli, 1988). Due to its low affinity system for binding calcium, phosphorlylation of the Na^+/Ca^{2+} exchanger must be executed to transport the Ca^{2+} out of the cell successfully. Although, there is another explanation stating that since possibly the calcium concentrations are usually higher inside of the plasma membrane than outside of the cell, this causes the Na^+/Ca^{2+} exchanger to run (Moore et *al.*, 1991). The Na^+/Ca^{2+} exchanger has been predominately studied in relation to the structure of the heart. The heart operates the exchanger by electrical stimulation, exchanging three Na^+ for one Ca^{2+} (Carfoli, 1988). Therefore, the exchanger has been described as a voltage-dependant system, regulating the amount of calcium allowed into the cytosol of the cell (McCarron, 1994).

The Ca^{2+} transport ATPase is another mechanism that can be used to sequester calcium out of the cytosol to cause relaxation. This Ca^{2+} -ATPase

is calmodulin-dependant (Yoshida *et al.*, 1999). Calmodulin-dependant Ca^{2+} transport ATPase is located in the plasma membrane of a smooth muscle cell. This mechanism has a high affinity for calcium, but also has a low total transport capacity for delivering Ca^{2+}(Eggermont *et al.*, 1988).

However, other research concluded that protein kinase G activated calmodulin-dependant Ca^{2+} transport ATPase through phosphorylation of the Ca^{2+} transport protein (Furukawa and Nakamura, 1987). Although later experiments have shown that the presence of phosphotidylinositol (PI), in addition to protein kinase G was required for the Ca^{2+} pump to work efficiently (Vrolix *et al.*, 1988). Phosphotidylinositol-4, 5-biphosphate (PIP) proved to be the best stimulator of ATPase, which led to the conclusion that phosphotidylinositol phosphate kinase is activated by protein kinase G through phosphorylation (Koga *et al.*, 1994). Although due to previous studies, this hypothesis cannot be maintained (Kosk-Kosicka *et al.*, 1988). The results from the calmodulin-dependant Ca^{2+} transport ATPase pump show that the protein exists in monomeric form at low concentrations and at high concentrations in oligomeric form. The monomeric form of the Ca^{2+} - ATPase pump is stimulated by type Iα cGMP kinase without phosphorylation, and is sensitive to calmodulin. The oligomeric form of the

Ca^{2+} -ATPase pump is fully active in the absence of cGMP kinase, and the presence of calmodulin has no effect on its actions (Yoshida *et al.*, 1999).

Results have shown that the Ca^{2+} transport protein can exist as both a monomeric and oligomeric protein (Kosk-Kosicka *et al.*, 1988). In the monomeric form of the protein, low concentrations of this protein are observed. In the oligomeric form, high concentrations of the protein are seen, and Ca^{2+}-pump ATPase is activated in the absence of cGMP kinase. Whereas the monomeric form can stimulate Ca^{2+}-pump ATPase by type Iα cGMP kinase without phosphorylation (Yoshida *et al.*, 1999). Calmodulin does affect the monomeric, and not the oligomeric protein, in regulating cytosolic concentrations of Ca^{2+} (Yoshida *et al.*, 1999).

The organelle believed to be responsible for the removal of cytosolic calcium in most smooth muscle is the sarcoplasmic reticulum. Ca^{2+}-ATPase, a protein with high affinity for binding calcium, of the sarco/endoplasmic reticulum (SERCA) forms enzymes that catalyze ATP, which pumps Ca^{2+} from the cytosol into the lumen of the reticulum (Martonosi, 1996). Phosphorylation of the Ca^{2+} -ATPase pump can result in the uptake of cytosolic calcium into the sarcoplasmic reticulum by cGMP-dependant protein kinase (Raeymaekers *et al.*, 1986). The mitochondria are

also believed to sequester Ca^{2+} from the cytoplasm of a cell, but only in a limited role (Denninger and Marletta, 1999).

Present studies in our laboratory have shown that the most important mechanism for removal of calcium cells is extracellular, from calmodulin-dependant ATPase, or from the Na^{+}/Ca^{2+} exchanger (Powell, 2000). This study showed that when CCSM was equilibrated in Ca^{2+}-free media, contraction in the CCSM was not observed following the addition of NE. This showed that $Ca^{2+}$is most important factor in regulating contraction of the CCSM tissue from the extracellular fluid source. Also, using cyclopanazoic acid (CPA), a drug used to inhibit the sarcoplasmic reticulum ATPase, showed that intracellular stores of calcium in the sarcoplasmic reticulum did not play a significant role in regulating relaxation in CCSM (Powell, 2000). Therefore, the plasma membrane pumps help regulate the amount of Ca^{2+} coming into and out of the cell, which regulates corpus cavernosum relaxation.

The drugs carboxyeosin and ouabain have been used to inhibit these specific plasma membrane pumps in investigating their specific roles. Carboxyeosin is thought to be a direct inhibitor of the calmodulin-dependant ATPase pump. It is also known to decrease the rate of Ca^{2+} decay or decreased efflux when present (Shmigol et al., 1998). Also, tests

done in Na^+-free media have shown a dramatic decrease in Ca^{2+} decay due to the inactivity of the Na^+/Ca^{2+} exchanger and the carboxyeosin effects. Tests have shown that this drug was effective in specific cardiac and smooth muscle tissues (Shmigol et al., 1998).

Ouabain is another drug that has an indirect inhibitory effect on the Na^+/Ca^{2+} exchanger. It directly inhibits the Na^+, K^+-ATPase, which would increase Na^+ concentration, thereby inhibiting the Na^+/Ca^{2+} exchanger indirectly (Rembold *et al.*, 1992). It is known to increase Na^+ concentration and cause contraction by enhancing norepinephrine release from certain tissue nerve terminals (Rembold *et al.*, 1992). The CCSM tissue response to ouabain was investigated in one study showed an increase in Ca^{2+} in the intracellular fluid and contraction of the tissue resulted (Gupta *et al.*, 1995).

The present study is designed to investigate the relative roles of the plasma membrane Na^+/Ca^{2+} exchanger and the calcium-dependant Ca^{2+} ATPase pump in regulating relaxation of the CCSM tissue *in vitro*.

Materials and Methods

Animals

The animals were housed in groups of three before the experiment was performed. All the animals were given continuous supply of LabDiet Rat Chow and water. The animals were kept on a reverse light-dark schedule. The lights were off from 1100 hours to 2300 hours at a room temperature of 22°C. All experiments performed used mature male Long/Evans rats. All experimental procedures were approved by The Institutional Animal Care and Use Committee (IACUC) at Youngstown State University.

Tissue specimens

At the onset of experimentation, the rats were euthanized with CO_2 within a plastic chamber. Approximately 2.5-3.0 cm length of the penis was removed from the animal and placed in the Kreb's solution on ice. All the remaining connective tissue, dorsal penile vein, and urethra were removed. Once the corporal cavernosal tissue was isolated, it was bisected down it's midsection, or central channel, into two pieces. The proximal end of each portion was tied to a glass rod, while the distal end portions were tied to a thin connecting wire (28 or 30 gauge) using 4-O silk suture. The tissues

were then placed into a 20ml chamber of modified Kreb's solution at 37°C. The Kreb's solution was continuously bubbled with 95% O_2, and 5% CO_2 gas mixtures to maintain pH and oxygen supply. The tissue weights were determined at the end of each experiment.

Drugs and Solutions

The modified Kreb's solution used throughout the experiments had the following concentrations: NaCl (119 mM); KCl (4.6 mM); NaH_2PO_4 (1.2 mM); $CaCl_2$ (1.5 mM); $NaHCO_3$ (15 mM); MgCl (1.2 mM); and glucose (11mM). The solution was then adjusted to pH 7.2 to 7.4.

Norepinephrine (NE), Ascorbic Acid, Carboxyeosin, and Sodium Nitroprusside Dihydrate (SNP) were obtained from Sigma, St. Louis, Missouri. The NE and SNP solutions were made in the appropriate media. Stock carboxyeosin solution was made up in a 1:10 dilution using DMSO to a final concentration of 10^{-4} M.

The reduced sodium media was used in some of these experiments substituting choline chloride (119 mM) for NaCl (119 mM) (Smith, 1989).

Experimental Protocol

Experiment I: Na^+/Ca^{2+} Plasma Membrane Pump

A corpus cavernosal tissues were obtained from 10 intact animals (20 tissue halves; although 3 tissue halves were discarded due to error) as described above and half of the total tissue specimens were placed in either a 20 ml modified Kreb's solution bath or reduced sodium media. The tissue was attached to a connecting wire, which was also attached to a metal hook on a Grass Instruments Force Transducer (Quincy, Massachusetts). The amplifier, Grass Instruments model P122 strain gauge amplifier, modified the signal output received from the force transducer and the data was analyzed on the Grass Instruments Polyview Program, version 2.1. The transducer was calibrated to convert all signals from volts to mg. The tissues were allowed to equilibrate for 45 minutes at 37°C. The solution was then flushed, the resting tensions were then reset, and the baseline was stabilized. Five ml of norepinephrine was then added to the bath to induce contractions. The final concentration used was 10^{-4} M (Alcorn *et al.,* 1999). In order to avoid any occurrences of transducer drift, tissues contracting less than 75 mg were not used for experimental data (Alcorn *et al.* 1999; Powell, 2000). Once contraction baseline was stabilized, 20 minutes after the

addition of NE, 1 ml of SNP (final concentration of 10^{-3} M) was then added to relax the tissue. The corpus cavernosum tissue was allowed 20 minutes after SNP treatment to relax. After this period, data analysis was performed and changes in tension were obtained and calculated in mg.

Preliminary Experiment: Carboxyeosin Dose Response Trials

Dilutions of 10^{-3}M, 10^{-4}M, and 10^{-5}M carboxyeosin doses were made to observe their effects on the CCSM tissue. The same experimental procedure was performed as in Experiment II, substituting only the carboxyeosin dilution being tested with the carboxyeosin (10^{-4}M) step.

Experiment II: Effects of Carboxyeosin on Corpus Cavernosum Relaxation

Corpus cavernosum tissues were obtained from 12 intact animals (n= 24 tissue halves), although additional rats were later needed to validate certain data obtained. The CCSM tissues were assigned to three different group experiments; Kreb's control solution with DMSO vehicle (n=11), Kreb's control solution with carboxyeosin (n=8), and reduced Na^+ solution with carboxyeosin (n=14). For each experiment, tissue was placed in 20 ml of one of the solutions listed above. The tissue again was placed on a force transducer as described in Experiment I above and was allowed 45 minutes

to equilibrate. After equilibration, the solution was flushed, and resting tensions were reset. Five ml of NE (final concentration equal to 10^{-4} M) was then added to each of the four chambers. An additional 20 minutes was given to observe contraction. Once contraction was stabilized, either DMSO (control) or 200 μl of carboxyeosin (final concentration equal to 10^{-4} M) was added to each chamber. Twenty minutes was allowed effects of the drug to take place. Then 1ml of SNP (final concentration equal to 10^{-3} M) was added to the bath to produce relaxation. Another 20 minutes was given to achieve relaxation. Data analysis was again performed and changes in tension in mg were calculated. Results were then converted into of percent relaxations of norepinepherine-induced contractions, as in Experiment 1.

Data Analysis

Percent relaxation data acquired from the experiment was converted to arcsin transformation and was analyzed using the SigmaStats program, version 1.0, Jandel Scientific, Corte Madera, California. Paired t-tests or one-way ANOVA with $p< 0.05$ were used as the minimum for significant differences. Student-Newman-Keuls multiple comparison tests were then used for significant differences in treatment groups with $p< 0.05$.

Results

Experiment I: Effects of Na^+ -Reduced Media on Na^+/Ca^{2+} Plasma Membrane Pump Function/CCSM Relaxation

The average resting tension placed on the corporal cavernosal tissues prior to contraction with norepinephrine (10^{-4}M) were 186 +/- 4.2 mg for the tissues treated with Kreb's (control) solution, and 187.2 +/- 6.03 mg for the tissues treated with Na^+ -reduced (experimental) solution. There were no significant differences between groups (Fig. 1). The average tissue weights of the corporal cavernosal tissues that were used in this study were 75.9 +/- 2.539 mg for the tissues treated with Kreb's (control) solution, and 78.2 +/- .953 mg for the tissues treated with Na^+ -reduced (experimental) solution. These weights showed no significant difference between groups (Fig. 2). The average peak contractions obtained by the addition of norepineprine (final concentration equal to 10^{-4} M) in this experiment were 124.0 +/- 10.8 mg for those tissues treated with Kreb's solution, while Na^+ -reduced treated tissues showed an average peak contraction of 151.0 +/- 15.3 mg. There were no significant differences between groups (Fig. 3). Peak contractions were also expressed as mg contractions per mg tissue mass. Calculations are as follows; 1.63 +/- 0.133 mg for Kreb's solution and 1.92 +/- 0.183 mg for Na^+ -reduced treated tissues, with no significant difference between groups

(Fig. 4). The average percent relaxations after treatment with SNP (final concentration equal to 10^{-3} M) were determined to be: 79.9 +/- 4.91% for those tissues treated with Kreb's solution and 55.0 +/- 8.18% for those tissues treated with Na^+ -reduced solution ($p<0.0295$, Figure 5).

Experiment II: Effects of Carboxyeosin on Corpus Cavernosum Relaxation

The average resting tension placed on the corporal cavernosal tissues prior to contraction with norepinephrine (10^{-4}M) were 197.4 +/- 7.41 mg for the tissues treated with Kreb's (control) solution with DMSO, 211.8 +/- 4.58 mg for the Kreb's solution with carboxyeosin, and 212.8 +/- 2.83 for the Na^+ -reduced solution with carboxyeosin (Fig. 6). There were no significant differences between groups. The average tissue weights of the coporal cavernosal tissues used in this experiment were 63.7 +/- 3.21 mg for the Kreb's (control) solution with DMSO, 61.0 +/- 3.71 mg for the Kreb's solution with carboxyeosin, and 60.3 +/- 1.45 for the Na^+ -reduced solution with carboxyeosin. There were no significant differences between the control and the treatment groups (Fig. 7). Peak contractions obtained upon the introduction of norepinephrine (final concentration equal to 10^{-4} M) in the Kreb's solution with DMSO were 143.6 +/- 17.6 mg, 152.3 +/- 15.0 mg for the Kreb's solution with carboxyeosin, and 147.6 +/- 23.8 mg for the Na^+

-reduced solution with carboxyeosin. One-way ANOVA testing displayed no significant differences between groups (Fig. 8). Again, peak contractions were expressed in the form of mg contractions per mg tissue mass. The results were as follows; 2.36 +/- .33 for the Kreb's solution with DMSO, 2.51 +/- .229 for the Kreb's solution with carboxyeosin, and 2.47 +/- .4 for the Na^+ -reduced solution with carboxyeosin. One-way ANOVA showed no significant differences between groups (Fig. 9).

Carboxyeosin (final concentration equal to 10^{-4}M) treatment was shown to significantly inhibit the relaxation of the CCSM tissue difference. The percent relaxations, upon introduction of SNP (final concentration equal to 10^{-3} M), were determined to be 74.0 +/- 4.67% for the Kreb's solution with DMSO, 51.9 +/- 5.65% for the Kreb's solution with carboxyeosin, and 89.4 +/- 3.85% for the Na^+ -reduced solution with carboxyeosin. Carboxyeosin significantly inhibited the relaxation of CCSM tissue (Figure 10).

Fig. 1. Resting tension placed on CCSM tissue prior to contraction with Norepinephrine (10^{-4}M). Tissues were treated with either Na^{+}-reduced (experimental) or Kreb's (control) solutions. There were no significant differences between groups. All values in this and other figures are the mean +/- S.E.M.

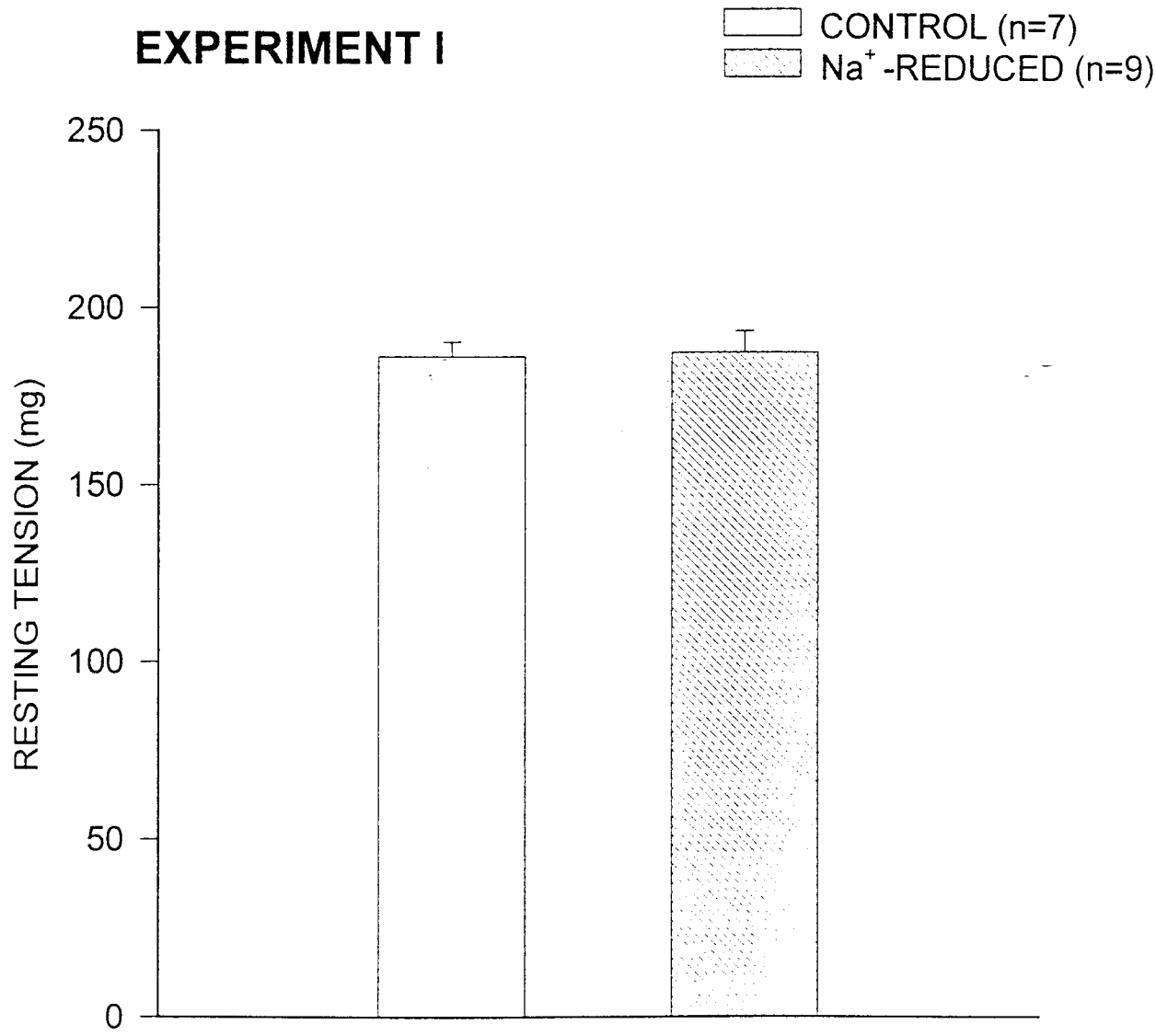

EXPERIMENT I
CONTROL (n=7)
Na+ -REDUCED (n=9)
RESTING TENSION (mg)
250
200
150
100
50
0

Fig. 2. Tissue weights taken from CCSM tissue. Tissues were treated with either Kreb's (control) or Na^+-reduced (experimental) solutions. No significant differences were found between groups.

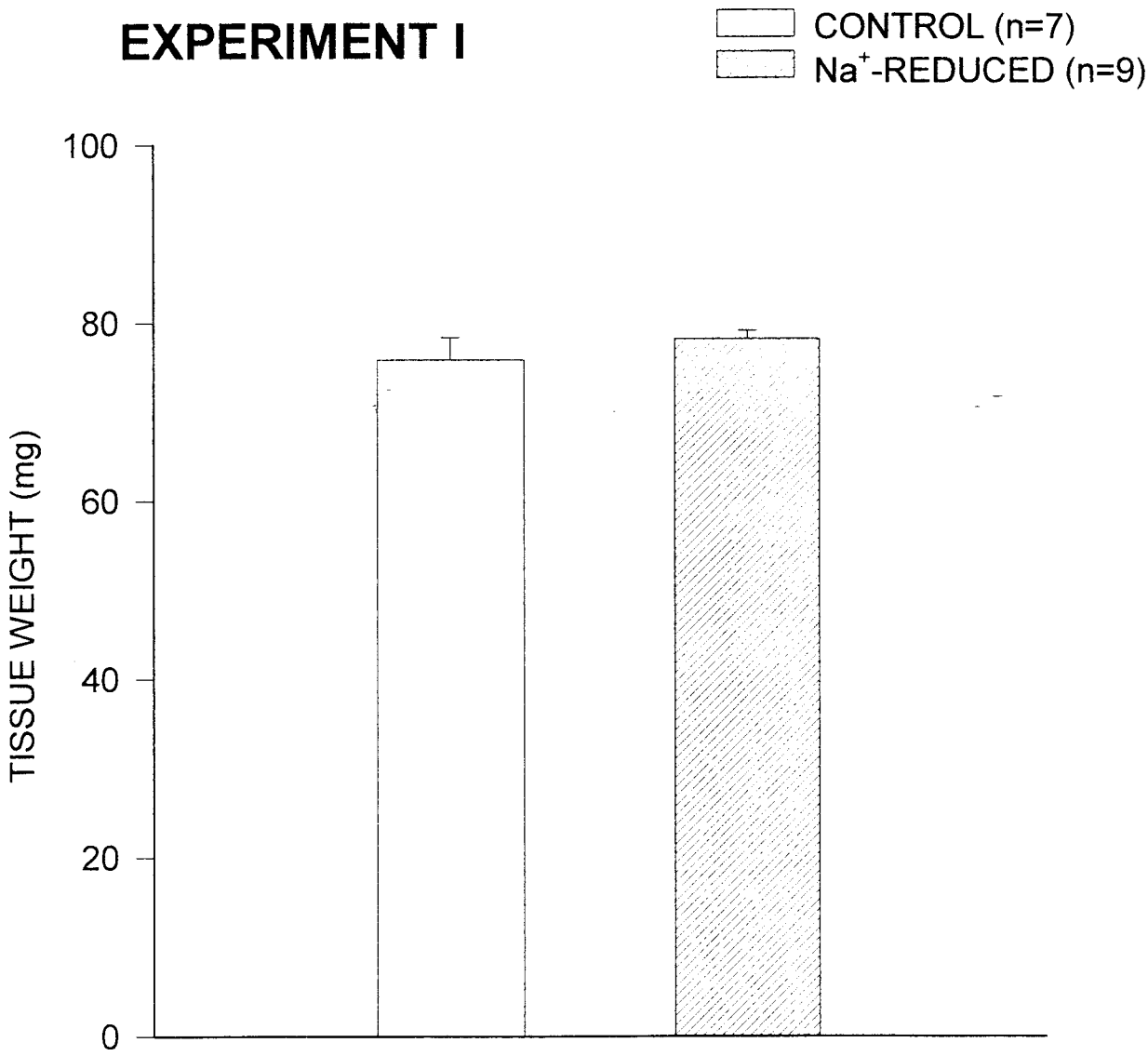

EXPERIMENT I
CONTROL (n=7)
Na+-REDUCED (n=9)
TISSUE WEIGHT (mg)
100
80
60
40
20
0

Fig. 3. Peak contractions of CCSM tissue treated with NE (final concentration equal to 10^{-4} M). Tissues were treated previously with either Na^{+}-reduced (experimental) or Kreb's (control) solutions. There were no significant differences between groups.

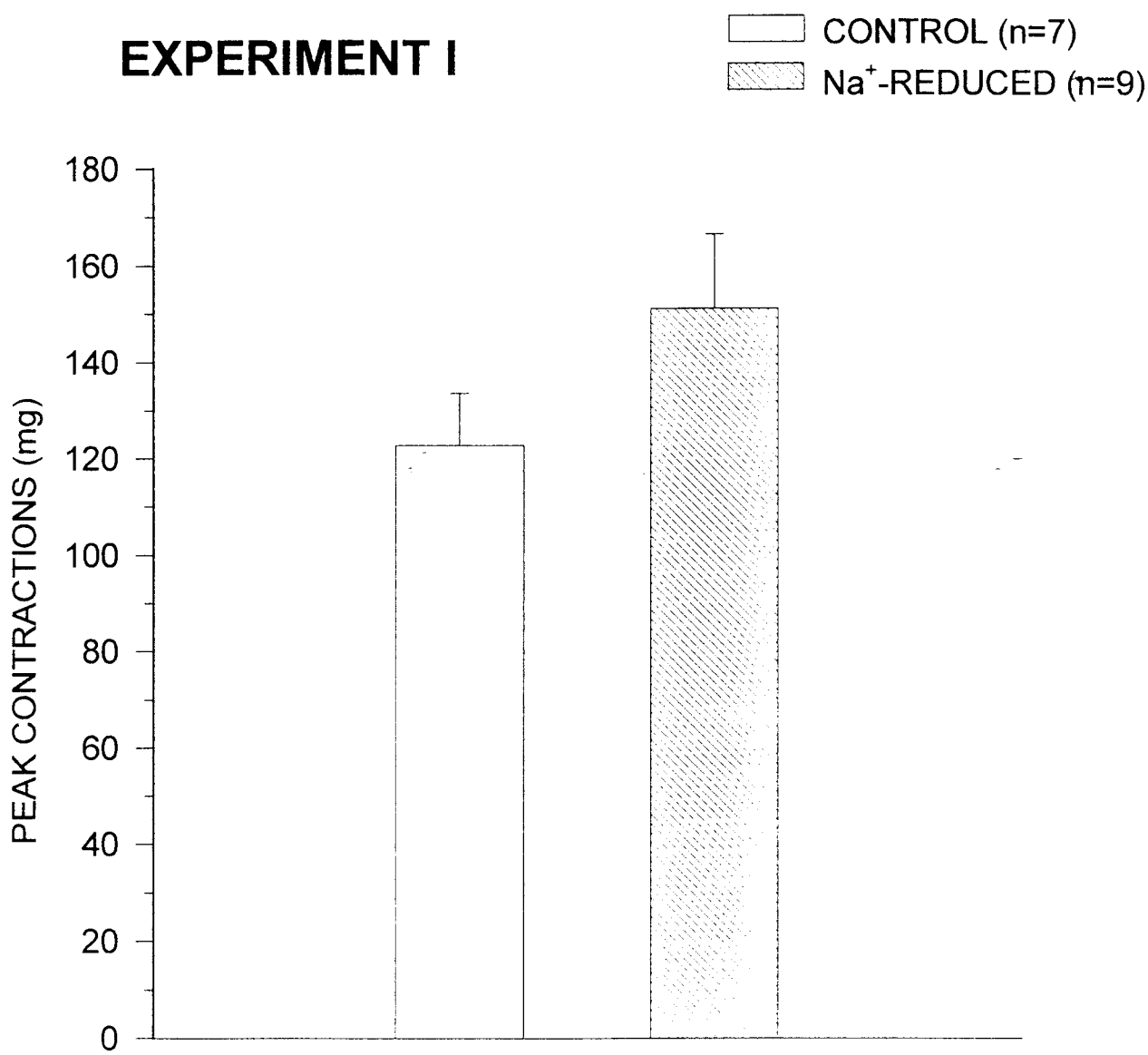

EXPERIMENT I
CONTROL (n=7)
Na+-REDUCED (n=9)
PEAK CONTRACTIONS (mg)
180
160
140
120
100
80
60
40
20
0

Fig. 4. Peak contractions of CCSM tissue as measured in mg contraction per mg tissue weight. Tissues were treated with either Na^+-reduced (experimental) or Kreb's (control) solutions. There were no significant differences between groups.

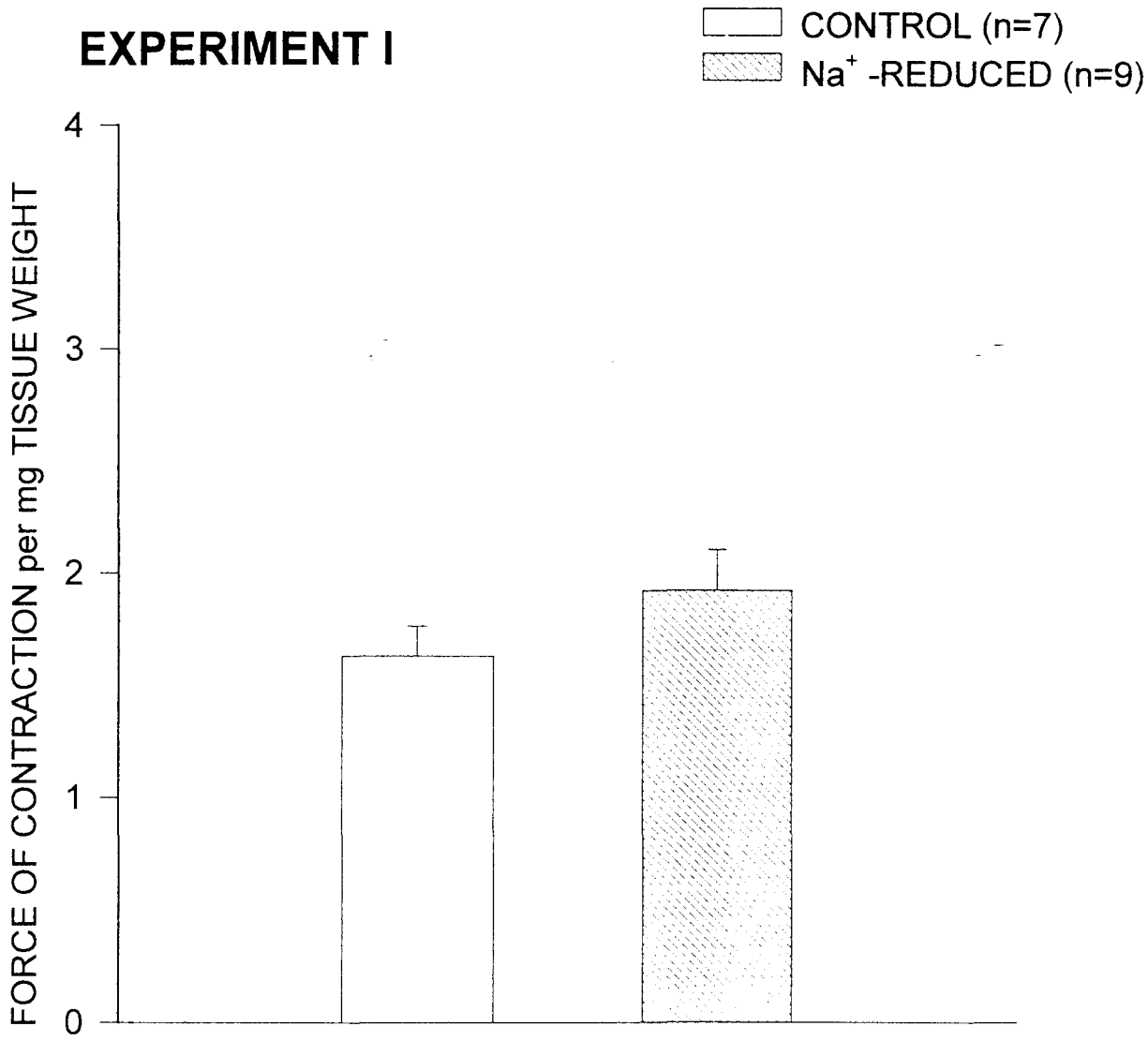

EXPERIMENT I
CONTROL (n=7)
Na+ -REDUCED (n=9)
FORCE OF CONTRACTION per mg TISSUE WEIGHT
4
3
2
1
0

Fig. 5. Average percent relaxations of CCSM tissues treated with SNP (final concentrations equal to 10^{-3} M). Tissues were previously treated with either Na^+-reduced (experimental) or Kreb's (control) solutions. The Na^+-reduced treated tissues relaxed significantly more than the control tissues, $p<0.0295$.

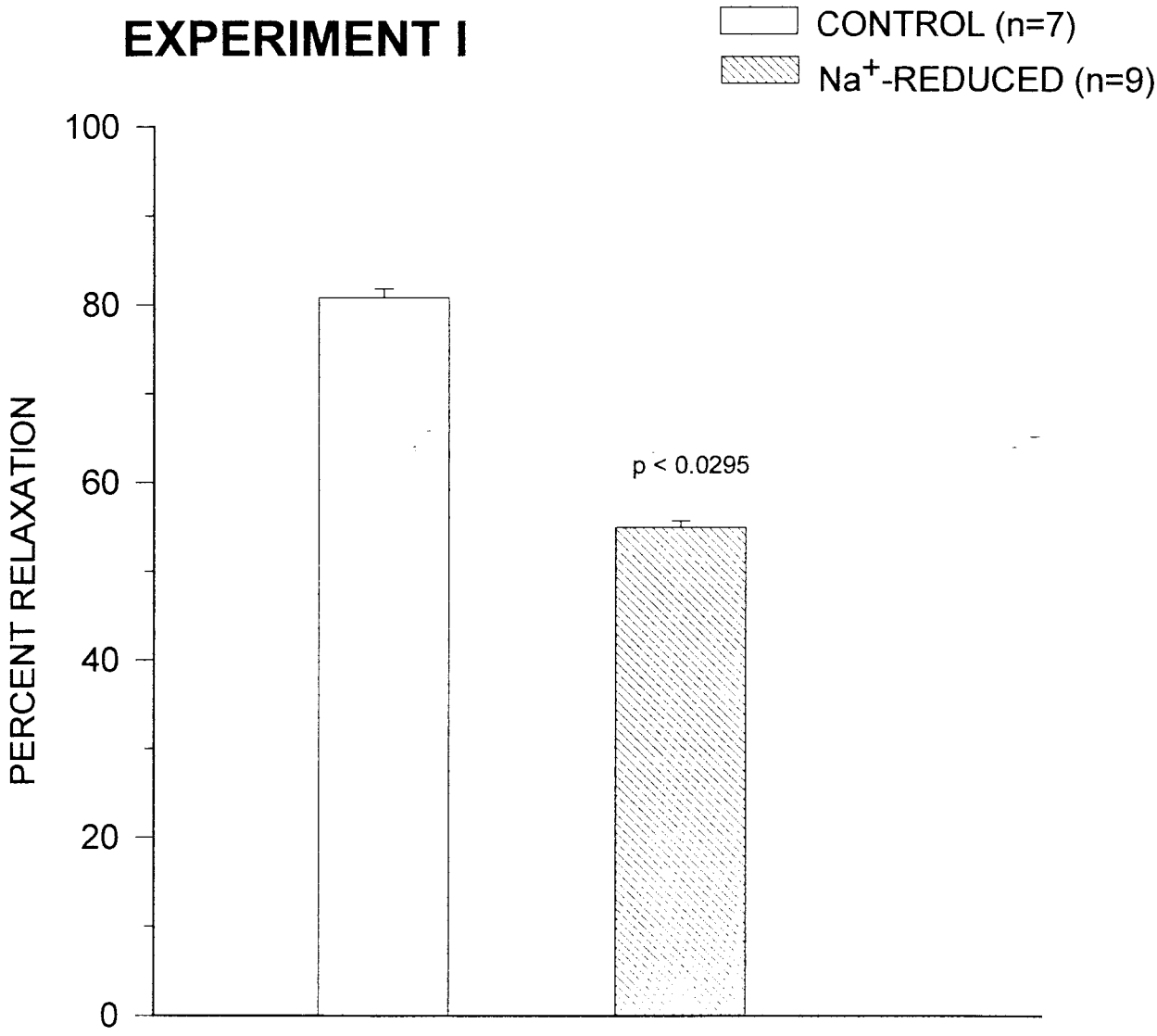

EXPERIMENT I
CONTROL (n=7)
Na+-REDUCED (n=9)
PERCENT RELAXATION
100
80
60
40
20
0
p < 0.0295

Fig. 6. Resting tensions placed on CCSM tissue prior to contraction with Norepinephrine (10^{-4}). Tissues were taken from Kreb's (control), Kreb's w/Carboxyeosin (experimental), or Na^{+}-reduced w/Carboxyeosin (experimental) animals. There is no significant differences between groups. N values are consistent for all the graphs in experiment II.

EXPERIMENT II

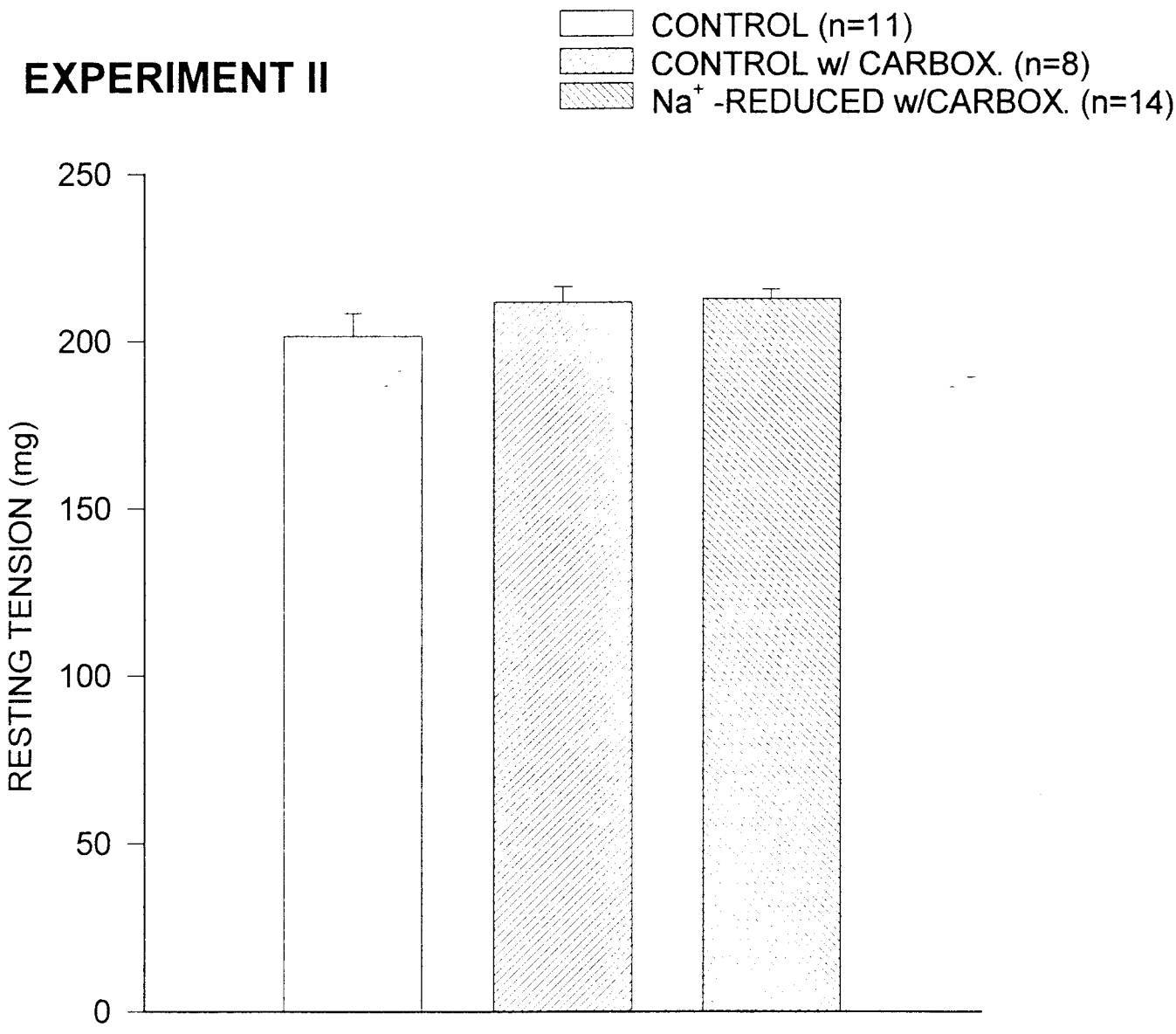


Fig. 7. Tissue weights of CCSM tissue removed from Kreb's (control), Kreb's w/Carboxyeosin (experimental), or Na^+-reduced w/Carboxyeosin (experimental) animals. There were no significant differences between groups.

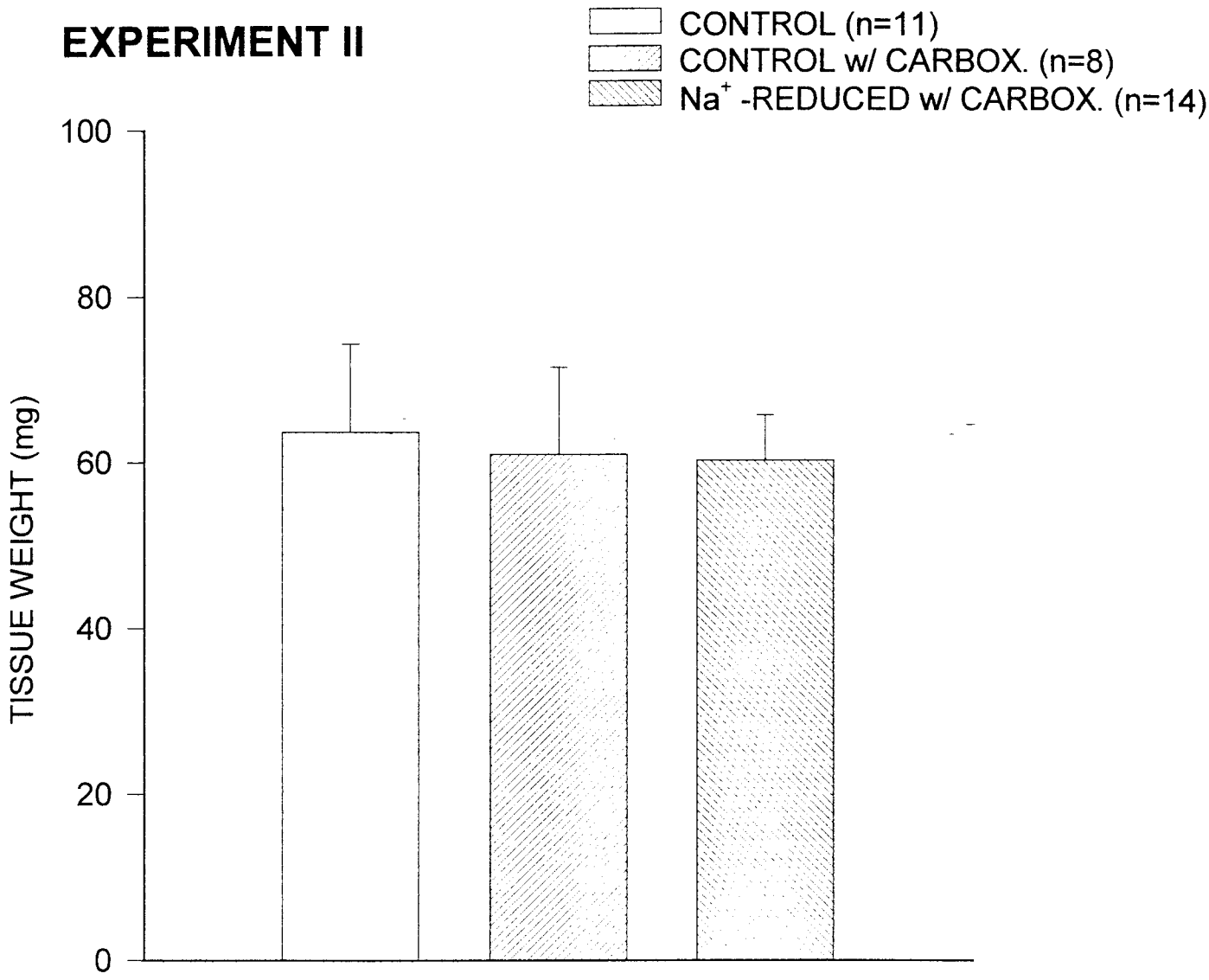

EXPERIMENT II
CONTROL (n=11)
CONTROL w/ CARBOX. (n=8)
Na+ -REDUCED w/ CARBOX. (n=14)
TISSUE WEIGHT (mg)
100
80
60
40
20
0

Fig. 8. Peak contractions of CCSM tissues. Tissues were taken from Kreb's (control), Kreb's w/Carboxyeosin (experimental), or Na^+-reduced w/Carboxyeosin (experimental) rats. There were no significant differences between groups.

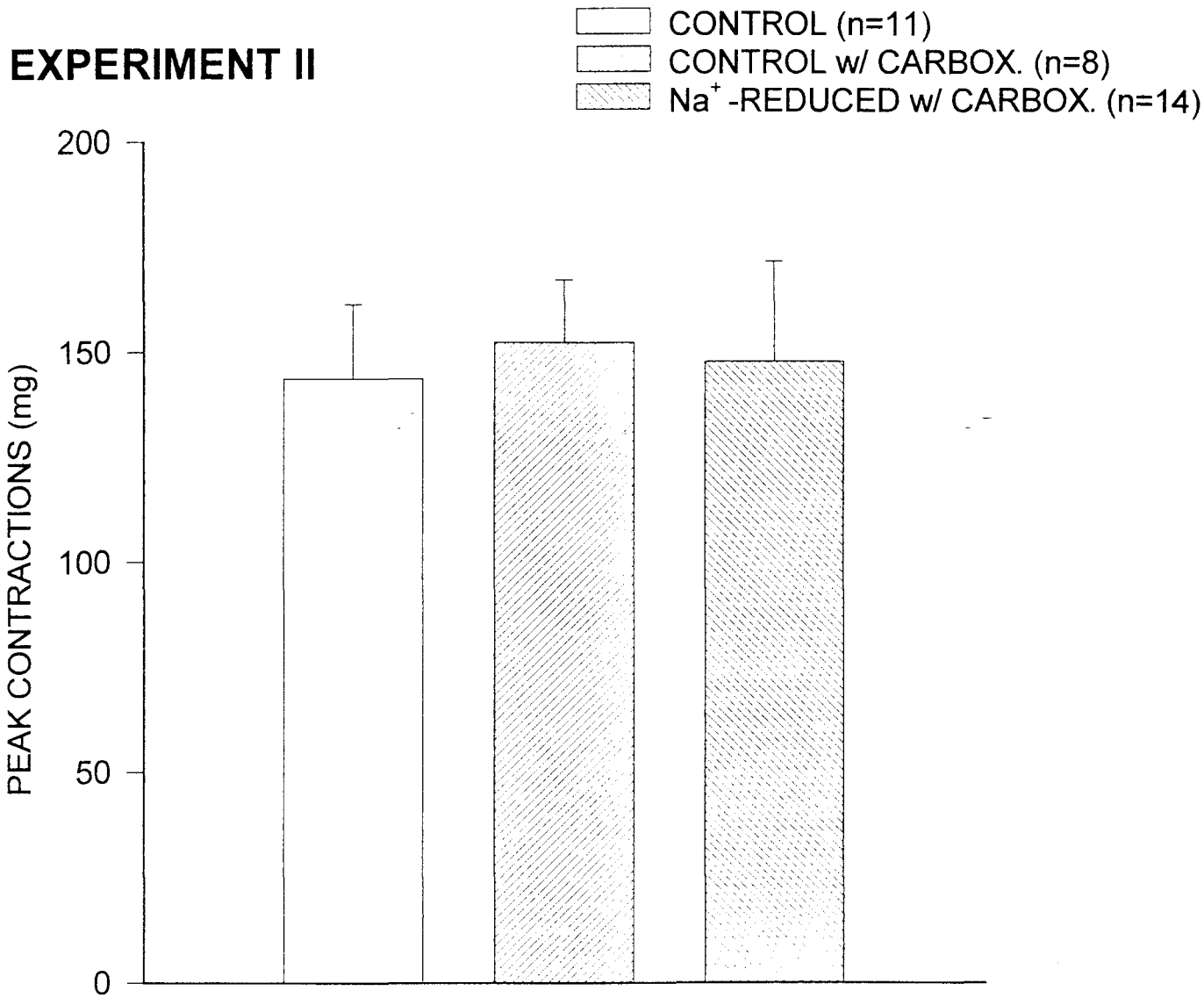

EXPERIMENT II
CONTROL (n=11)
CONTROL w/ CARBOX. (n=8)
Na+ -REDUCED w/ CARBOX. (n=14)
PEAK CONTRACTIONS (mg)
200
150
100
50
0

Fig. 9. Peak contractions of CCSM tissue as measured in mg contraction per tissue weight. All CCSM tissues were contracted with NE (final concentration equal to 10^{-4} M). There were no significant differences between groups.

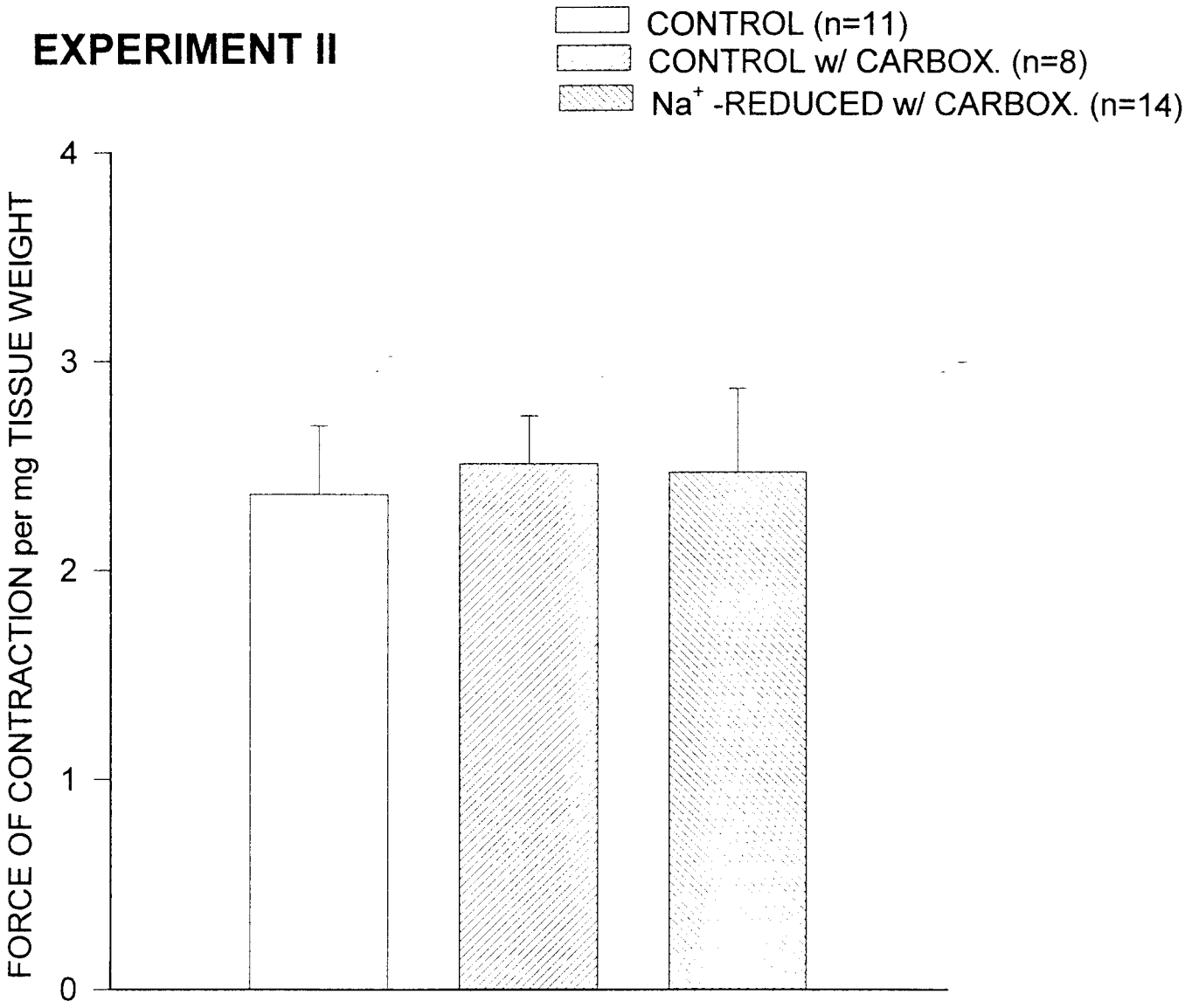
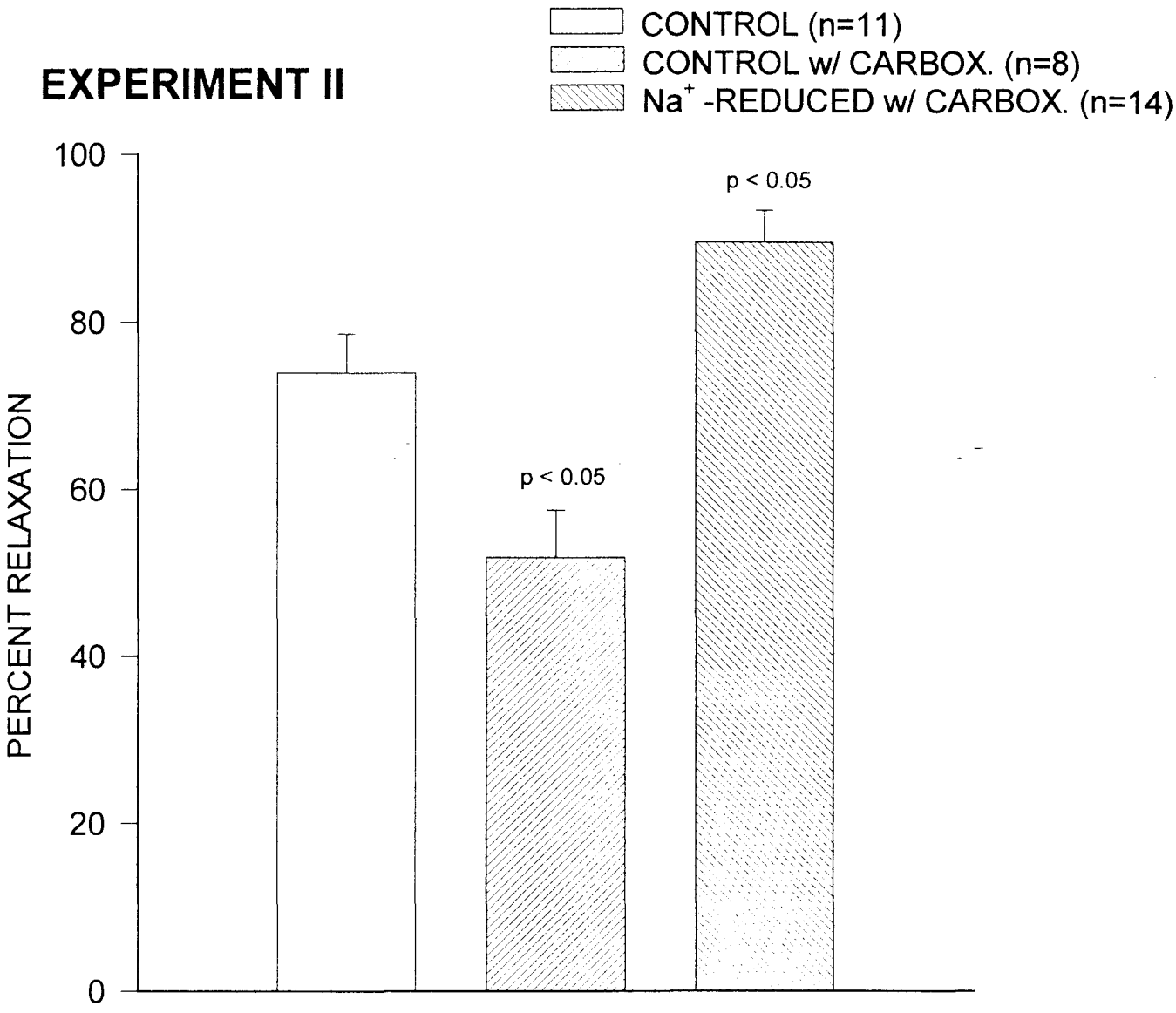

EXPERIMENT II
CONTROL (n=11)
CONTROL w/ CARBOX. (n=8)
Na+ -REDUCED w/ CARBOX. (n=14)
FORCE OF CONTRACTION per mg TISSUE WEIGHT
4
3
2
1
0

Fig. 10. Average percent relaxations of CCSM tissues treated with SNP (final concentrations equal to 10^{-3} M). Carboxyeosin significantly reduced the relaxation of CCSM tissue, $p<0.05$. The control w/ carboxyeosin (n=8) and Na^+ -reduced w/ carboxyeosin (n=14) both were significantly different, $p<0.05$, than control (n=11) treated tissues.


EXPERIMENT II
CONTROL (n=11)
CONTROL w/ CARBOX. (n=8)
Na+ -REDUCED w/ CARBOX. (n=14)
PERCENT RELAXATION
100
80
60
40
20
0
p < 0.05
p < 0.05

Discussion

These experiments were designed to investigate the roles of the plasma membrane Ca^{2+} pumps in regulating relaxation of CCSM tissue *in vitro*. Our results demonstrated that both the Na^{+}/Ca^{2+} exchange pump and the calmodulin-dependant Ca^{2+} ATPase pump play an important role in mediating relaxation of the CCSM tissue tested with the NO donor, SNP.

The first study showed that when sodium (Na^{+}) is excluded from the external fluid, a significant decrease in the percent relaxation of the CCSM tissue results (Figure 5). Therefore, inhibition of the Na^{+}/Ca^{2+} exchanger is affected, which is located in the plasma membrane of the cell. This suggests that Na^{+} is needed for the exchanger to work effectively in the relaxation process of the CCSM tissue. Past studies have indicated that the Na^{+}/Ca^{2+} exchanger is a large capacity, low-affinity system for binding calcium, needing phosphorylation to transport Ca^{2+} out of the cell successfully (Carfoli, 1988). Taking Na^{+} out of the extracellular media, displays may disrupt the low-affinity Ca^{2+} binding system, which may explain the significant decrease in CCSM tissue relaxation observed in this study. Although the exact mechanism of how the Na^{+}/Ca^{2+} exchanger works in

distribution of Ca^{2+} within smooth muscle cells is still quite unknown (McCarron et al., 1994).

The second portion of the study focused on the other plasma membrane pump in question, the calmodulin-dependant Ca^{2+} ATPase pump. The calmodulin-dependant Ca^{2+} ATPase pump is an exporting Ca^{2+} system in the plasma membrane, similar to the Na^{+}/Ca^{2+} exchanger in regulating relaxation (Powell 2000). This mechanism is known to have a high affinity for transporting calcium, but has a low total transport capacity for delivering Ca^{2+} (Eggermont *et al.*, 1988). The calmodulin-dependant Ca^{2+} ATPase pump uses a Ca^{2+} transport protein that can exist as both a monomeric and oligomeric protein (Kosk-Kosicka *et al.*, 1988). Calmodulin itself affects the monomeric, and not the oligomeric protein in regulating Ca^{2+} in the cytosol (Yoshida *et al.*, 1999). It has been reported that the chemical carboxyeosin (final concentration equal to 10^{-4} M) inhibits this pump (Shmigol *et al.* 1998). Therefore, when carboxyeosin was placed in the Kreb's media, a significant inhibition of relaxation occurred within the CCSM tissue (Figure 10). To further assses this, we analyzed the tension levels in the Kreb's (control) and carboxyeosin treated tissues prior to the addition of SNP. No significant differences were observed, which indicated

that carboxyeosin inhibited the calmodulin-dependant Ca^{2+} ATPase pump and significantly reduced relaxation of the CCSM.

Unexpected results were also present primarily in the second portion of the experiment. When carboxyeosin (10^{-4}M) was placed with the Na^{+}-reduced media, an increase of percent relaxation occurred in the CCSM tissue (Figure 10). This result was not expected because both the Na^{+}-reduced media and carboxyeosin alone resulted in a decrease in relaxation of the CCSM. Unknown side effects due to the use of carboxyeosin on the CCSM tissue could have been obtained when using the Na^{+}-reduced media. However previous studies did not report any side effects when using this dose of carboxyeosin in aortic smooth muscle. Comparative studies using carboxyeosin in the CCSM are also lacking.

In conclusion, our results support the hypothesis that both the plasma membrane Na^{+}/Ca^{2+} exchanger and calmodulin-dependant Ca^{2+} ATPase-pump have instrumental roles in mediating the NO-cGMP induced relaxation the CCSM tissue. However, the relative importance of one mechanism over the other in regulating relaxation of CCSM remains unknown. Future studies in this laboratory will continue to focus on these plasma membrane pumps using quite different approaches. The drug ouabain, which is known to inhibit the Na^{+}/Ca^{2+} exchanger, will be used in

place of carboxyeosin to examine its effects on relaxation of the CCSM tissue. Also, radioactive labeled Ca^{2+} isotopes will be used to determine the role of extracellular Ca^{2+} during the contraction and relaxation periods during these experiments.

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Appendix A

Approval from The Institutional Animal Care and Use Committee

YSU

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Dean of Graduate Studies
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FAX (330) 742-1580
E-Mail: amgradØ3@ysub.ysu.edu

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