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Androgen-induced norepinephrine release in male accessory sex organ smooth muscle growth and differentiation

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I. Abstract

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The goal of this research was to determine the role of norepinephrine (NE) in androgen-dependent pubertal proliferation and adult terminal differentiation in normal male accessory sex organ smooth muscle. The guinea pig seminal vesicle served as a model as its unique anatomy allowed clean harvest of smooth muscle without epithelium.

Androgen significantly increased *in vivo* DNA synthesis, total DNA, and NE release and down-regulated protein kinase C (PKC) in prepubertal seminal vesicle smooth muscle (SVSM) relative to prepubertal orchiectomized controls. High performance liquid chromotography quantified NE release as an increased concentration of 1) endogenous NE-metabolite, 3,4-dihydroxyphenolglycol per NE, and 2) NE *in vitro* SVSM minces. Binding studies using [^{3}H]RX821002, an α_2-adrenoceptor antagonist, showed a concurrent four-fold decrease in α_2-adrenoceptor concentration, indicating reduced feedback as a potential mechanism for the increase in norepinephrine release. *In vivo* NE-depletion of $\geq$ 99% by reserpine selectively antagonized the androgen-induced increases in SVSM-DNA

(measured spectrofluorophotometrically) and protein kinase C (PKC) down-regulation (measured by $\gamma[^{32}P]$ATP transfer). Gel electrophoresis detected no apoptosis, supporting other evidence that cell number is proliferation-dependent. Cell culture experiments demonstrated that SVSM α_1-adrenoceptors mediated the proliferative response to NE. Functional α_1-adrenoceptor expression, exhibited by electrical field stimulation of SVSM neurons, correlated with the onset of androgen-induced proliferative response.

In terminally differentiated adult SVSM, elevated NE release and decreased α_2-adrenoceptor concentration were maintained from puberty and developed androgen-resistance. PKC, however, returned to prepubertal castrate control levels (Mariotti *et al.*, 1992). Androgen withdrawal combined with NE depletion caused no reduction in adult SVSM-DNA relative to adult castrates. These findings suggest that while increased NE release plays an obligatory role in pubertal SVSM proliferation, NE release becomes uncoupled from the mitogenic response at or before PKC stable activation in adulthood.

Human benign prostatic hypertrophy (BPH) is a fibromuscular neoplasia. The data presented here suggest BPH may be due to defective uncoupling between sustained,

androgen-induced NE release and PKC activation. New pharmacological therapies for BPH can be envisioned based on either antagonizing the reduction in presynaptic α_2-adrenoceptors to prevent increased NE release or inducing PKC resistance to generate the normal amitotic state.

II. Literature Review

A. Benign Prostatic Hypertrophy: General Background

Benign prostatic hypertrophy (BPH) is primarily a stromal neoplasia of the innermost aspect of the prostate. The origin of the neoplasia is confined primarily to periurethral tissue around the verumontanum, in proximity to the cylindrical urinary sphincter that arises from the bladder neck (Reischauer, 1925; Deming and Wolf, 1939; Pradhan and Chandra, 1975; McNeal, 1978). Glandular epithelial tissue comprises approximately 20-30% of BPH mass, whereas 70-80% is fibromuscular (Rohr and Bartsch, 1980). Although the cause of BPH is not understood, the adenoma is androgen and age dependent (Huggins and Stevens, 1940; Wu and Gu, 1987).

By examining random specimens at autopsy, benign prostatic hypertrophy (BPH) has been found to begin as a microscopic, non-symptomatic nodule as early as age 25-30 years of age when it has a prevalence of 10%. With advancing age, histological prevalence of BPH rises such that by age 60, 70% of men are effected and by age 80, 90% of men have histologically identifiable BPH (Arrighi *et al.*, 1991). There is a lag period of approximately 5 years in which 50% of microscopic BPH develops into macroscopic

BPH that can be palpated through digital rectal examination (Isaacs and Coffey, 1989). Of these macroscopic nodules, half will become clinically significant, meaning that only 25% of men with histologically identifiable BPH eventually develop clinically relevant pathology (Lytton *et al.*, 1969). However, on the basis that 25% of all men with histologically identifiable BPH will need therapeutic intervention, it appears that the probability that a 50-year-old man will undergo medical treatment for BPH in his lifetime is estimated to be 35% (Glynn *et al.*, 1985). The high incidence of this neoplasia makes the development of future pharmacological prophylactics for BPH prevention a practical concept.

At clinical presentation of sympomatic disease, the enlarged adenoma displaces the normal organ and weighs between 60 and 100 grams, although weights of up to 200 grams have been seen. These nodular enlargements encroach upon the walls of the urethra, contributing to a set of symptoms collectively termed clinical BPH. Clinical BPH symptoms include: 1) urethral compression causing difficulty in urination and 2) urinary retention causing subsequent bladder distension and hypertrophy. These secondary effects can then lead to tertiary complications such as urinary tract infections, cystitis, renal

infections, and decreased bladder continence due to thinning of the bladder musculature. The severity of clinical, symptomatic BPH does not correlate with the size of the adenomatous mass. Additional pathologies may be required for the presentation of clincally significant disease. For example, environment and genetic predisposition (Ekman, 1989), lymphocytic infiltration (Kohnen and Drach, 1979), infarcts (Franks, 1975), nonandrogenic testicular factors (Sutkowski *et al.*, 1993), the stromal-epithelial interaction (Cunha, 1973), and a variety of hormones and neurotransmitters have been suggested to contribute to the development of BPH. None of the above, however, have been shown to be directly responsible for the development of adenomatous, smooth muscle cell BPH as will be the focus of the research presented here.

By 1989, the large number of men with clinical BPH made procedures such as the transurethral resection of the prostate (TURP) the second most common surgical procedure, following cataract removal, for men 65 years of age and older, costing the health care system more than five billion dollars (Graversen *et al.*, 1989). This dollar amount includes only surgical treatment of BPH and overlooks widely prescribed medical treatments such as 5α-

reductase inhibitors (finasteride), α_1-adrenoceptor antagonists (terazosin and doxazosin) and the newer, less well-evaluated therapies such as thermoablation and stents.

Unfortunately, no treatment regimen has been highly satisfactory to the patient. The surgical treatments, TURP and open prostatectomy, represent the most effective form of treatment with 75-96% of patients reporting symptom improvement and only a 2.2% reoperation rate (Oesterling, 1995). Surgery also has the highest rate of severe complications such as impotence in 5-10% and urinary incontinence in up to 4% (Geller *et al.*, 1995).

None of the various pharmacological therapies cure the disease, but rather help to alleviate obstruction by different mechanisms. While the 5-α-reductase inhibitor, finasteride, inhibits epithelial growth, the peak onset of effect is 6-12 months after treatment. Thirty percent of men received some benefit as measured by symptom scoring (Geller *et al.*, 1995). However, the change in symptom score is only marginally different from placebo (2.6 vs 1 for placebo) (Lepor *et al.*, 1996). Side effects include impotence and decreased libido. α_1-adrenoceptor antagonists reduce sympathetic tone around the bladder neck to improve urinary outflow in 60-80% of patients within 4-6 weeks of

treatment initiation. However, 10% of patients suffer adverse effects such as hypotension and headache (Jonler *et al.*, 1994). All of the current pharmacological therapies must be maintained chronically, since they only relieve secondary symptoms in reversible fashion.

By the year 2000, forty million men will begin to reach middle age (Bouvier and DeVita, 1991). They represent the largest population in history that will face the problems of BPH. (They also represent an enormous market for pharmaceutical companies.) There is a definite need to develop more effective treatments for clinical BPH, and even to ultimately develop a therapy for prevention of the adenomatous growth.

B. Overview of Androgen Effects on Male Accessory Sex Organs

Androgens effect a wide variety of target tissues such as external and internal reproductive structures, sebaceous glands, muscle and even the peripheral and central nervous systems. Androgenic effects range from the morphogenesis of the male phenotype (Jost, 1953) to excitatory or

maintenance actions on cellular growth and secretion via androgen receptor-mediated induction of gene transciption (Price and Williams-Ashman, 1961; Liao and Fang, 1970; Roy and Chatterjee, 1995).

While much of the recent research of androgen action has focused on genomic effects, nongenomic effects of androgens at physiological concentrations have also been identified within reproductive tissues. These nongenomic effects occur too rapidly to be compatible with changes in mRNA and protein synthesis. In general these non-genomic effects have been implicated in signal transduction events such as changing intracellular calcium concentrations and effecting chloride flux in rat Sertoli cells (Revelli *et al.*, 1998). Androgens may also function to modulate biochemical reactions within some cells by serving as coenzymes for the transfer of hydrogen between NADPH and NAD (Talalay and Williams-Ashman, 1958).

Testosterone can diffuse passively into cells and can either bind directly to the androgen receptor or can be reduced to 5α-dihydrotestosterone (DHT) by reduction with 5α-reductase (Farnsworth and Brown, 1963; Bruchovsky and Wilson, 1968). DHT can also bind the androgen receptor and will do so with greater affinity and stability than testosterone itself (Krieg *et al.*, 1976). The gene coding

for the androgen receptor is located on the X chromosome and has been cloned (Lubahn *et al*., 1988). 5α-dihydrotestosterone (DHT) binds the steroid binding domain of the receptor. Chaperone proteins such as heat shock proteins then dissociate from the receptor, enabling the activated receptor to dimerically bind DNA within the nucleus at a specific DNA enhancer sequence called the androgen-responsive element (Lindzey *et al*., 1994). Testosterone also binds the receptor but has a diminished effect on the tissue compared to DHT (Wenderoth *et al*., 1983; Geller, 1990). Upon binding to the androgen-responsive element, the androgen receptor then can modulate transcriptional activity ultimately effecting morphogenic and other growth-promoting and stimulatory processes in target tissues.

Most research on male accessory sex organs have focused on androgen regulation of the epithelium. However, the clinical importance of the fibromuscular stroma in BPH and the role of the mesenchyme in embryonic development of male accessory sex organ (Cunha, 1973) has begun to focus attention on the hormonal regulation of smooth muscle. After castration, the rat ventral prostate epithelial cells showed a 92% reduction in number and an 85% decrease in cell size. The fibromuscular stroma was also effected but

to a lesser degree, showing a 39% decrease in cell number and a 23% decrease in cell size. Exogenous testosterone replacement reversed these changes (DeKlerk and Coffey, 1978). In this same organ, intra-acinar stromal cells which may be smooth muscle cells, were unlike other prostatic cells in that castration and androgen replacement did not affect cell number (English *et al*., 1985).

Relevant to the focus of the research presented here, were prior studies of androgen action on male accessory sex organ fibromuscular stroma. Mariotti *et al*. (1992) examined the effects of castration and androgen replacement on DNA in prepubertal and adult guinea pig seminal vesicle smooth muscle. Castration of prepubertal guinea pigs prevented the postnatal development of the seminal vesicle smooth muscle number as measured by DNA. This development was shown to be androgen dependent as the treatment of these prepubertal castrates with dihydrotestosterone would restore smooth muscle development. Adult smooth muscle, however, appeared to be terminally differentiated. Neither androgen depletion (castration) nor repletion had any effect on adult smooth muscle cell number which were similar to observations recorded earlier by English *et al*. (1985). Dihydrotestosterone's actions on the seminal

vesicle smooth muscle have been shown to be androgen receptor mediated (Neubauer and Mawhinney, 1981).

Male accessory sex organs also contain estrogen receptors which are concentrated in the fibromuscular stroma (Belis *et al*, 1976; Neubauer and Mawhinney, 1981). Estrogen treatment of castrate male guinea pigs induced growth in smooth muscle tissue via an estrogen receptor dependent mechanism (Mariotti and Mawhinney, 1981; Neubauer *et al*.,1989). However, several lines of evidence have indicated that estrogen is not of physiological significance in this growth (Neubauer *et al*., 1981). However, intraneuronal aromatization of testosterone to estradiol in the medial preoptic area of male rats is required for normal androgen effects on male sexual behavior, and both dihydrotestosterone and estradiol are required to generate normal virilization of this neural site (Christensen and Clemens, 1975). The extent to which aromatization is involved in androgen action in peripheral neurons is questionable. However, neurosteroids, formed *de novo* in neurons, have been identified in both central and peripheral neurons (Jung-Testas *et al*., 1989; Melcangi *et al*., 1990) and may modulate neurotransmission either directly or through gene transcription. The degree of

estrogen receptor involvement in male accessory sex organ neurotransmission remains to be defined.

C. Autonomic Innervation of the Male Accessory Sex Organs

In the human, the preganglionic sympathetic innervation of the prostate is from the last 3 thoracic (T10, T11 and T12) and first 2 lumbar (L1 and L2) segments of the spinal cord. These form 2 pathways, the superior hypogastric plexus and the continuation of the pelvic sympathetic trunks. The preganglionic parasympathetic innervation comes from the second through fourth sacral spinal nerves to form the pelvic splanchnic nerves which join other sympathetic hypogastric nerves to form the inferior hypogastric plexus. The sympathetic and parasympathetic nerves form what is called the pelvic plexus which innervates the pelvic organs as well as the external genitalia.

The smooth muscle of the guinea pig seminal vesicle is largely comprised of circularly oriented smooth muscle cells with an additional longitudinal layer toward the proximal, urethral end. Based on light microscopy, both smooth muscle layers are abundantly innervated with

catecholamine-containing nerves, which are particularly concentrated at the base of the organs. The sympathetic adrenergic innervation originates from long preganglionic hypogastric neurons which synapse with short postganglionic neurons whose cell bodies are in the adventitia of the seminal vesicle (Al-Zuhair *et al*., 1977). Cholinesterase-containing nerves are rarely encountered in the outer layer, but can be identified in modest amounts in the circular inner layer. The submucosa and epithelia are rich in cholinesterase-containing nerves, and catecholamine-containing nerves are rare (Al-Zuhair *et al*., 1975). Because the epithelium is cholinergically innervated and has minimal to no adrenergic fibers, this tissue can therefore serve as an indicator of androgenic responses that have no direct link to cellular adrenergic innervation.

By using agonists and antagonists in vas deferens, a biphasic contractile response to electrical field stimulation has been defined. This initial contraction has been described as being ATP-mediated in the guinea pig vas deferens (Burnstock *et al*., 1972; Westfall and Stitzel, 1978). The latter component of the contractile response is mediated by the post-junctional α_1-adrenoceptor which was abolished by prazosin in the rat vas deferens. Rauwolscine

enhanced the contractile response, indicating the existance of the pre-junctional α_2-adrenoceptor which mediates norepinephrine feedback (Brown *et al.*, 1983).

Prostatic α_1-adrenoceptors are found in the smooth muscle. At this time, evidence supports the existence of three α_1-adrenoceptor subtypes, A, B and D as well as subtypes with high affinity (α_{1H}) and low affinity (α_{1L}) to prazosin, which appear to be variants of the α_{1A}-adrenoceptors (Andersson *et al.*, 1997). Norepinephrine-feedback-mediating α_2-adrenoceptors also exist in several subtypes, A, B, C and D. The A and D subtypes are considered to be species homologues (Bylund, 1995).

D. Androgen Effects on Neurotransmission in Male Accessory Sex Organs

Several studies have examined the effect of puberty on the development of neurotransmission in the rat vas deferens. The post-natal development of α_1-adrenoceptor mediated contraction in the rat vas deferens coincided with the large increase in endogenous androgen production at

puberty (Swedin, 1972). Rauwolscine-induced enhancement of electrically field stimulated rat vas deferens also did not occur until the rat was pubertal (MacDonald and McGrath, 1984). However, because of the lack of a prepubertal α_1-mediated response, it was not possible to determine whether the function of the α_2-adrenoceptor was also androgen dependent.

More studies have linked androgens to the modulation of adult adrenergic neurotransmission in target tissue smooth muscle. Twelve weeks after the castration of adult Wistar rats, the adrenergic component of the contractile response to electrical field stimulation was lost. Spontaneous contractions that were not suppressed by adrenergic antagonists, also developed post-castration. Exogenous testosterone replacement daily for ten days partially reversed this castration-induced loss of the adrenergic contractile response and abolished the spontaneous contractions (MacDonald and McGrath, 1980). Total activities of tyrosine hydroxylase, the rate-limiting enzyme of norepinephrine biosynthesis, as well as dopamine-beta-hydroxylase, which catalyzes the last step of norepinephrine synthesis were also shown to decrease per pair of rat vasa deferentia after castration. Neither castration nor androgen repletion effected changes in

enzyme concentrations. Norepinephrine content per pair of rat vasa deferentia also fell but as was observed with the contractile response, testosterone replacement reversed these decreases such that norepinephrine returned to control levels (Bustamante *et al.*, 1989).

E. Norepinephrine as a Component of Cell Growth in Male Accessory Sex Organ Smooth Muscle

It is clinically established that α_1-adrenergic antagonists reduce smooth muscle tone and thus relieve some of the obstructive symptoms of BPH, but any impact on smooth muscle cell growth in human prostatic smooth muscle has not been formally evaluated. However, laboratory research has demonstrated that catecholamines may play a significant role in the proliferation of a variety of cells including smooth muscle.

In 1989, Marino *et al.* established that norepinephrine enhanced cellular proliferation in dividing fetal rat cardiocytes. The next year, it was reported that low passages of subconfluent primary rat aorta cultures exhibited an increase in DNA synthesis in the presence of low concentrations of norepinephrine (1 nM) which was

mediated through α_1-adrenoceptors (Nakaki *et al.*, 1990). On the other hand, a high concentration of norepinephrine (10 μM) inhibited DNA synthesis via β_2-adrenoceptors, as confirmed with β_2-adrenoceptor agonist, isoproterenol (Nakaki *et al.*, 1990). Later studies on primary cultures of rat aortic smooth muscle showed that epinephrine-induced increases in proliferation rate could be inhibited by α_1-adrenoceptor antagonist, prazosin, but not by α_2- nor β-adrenoceptor antagonists, yohimbine or propranolol (Mimura *et al*, 1995). Thus it was confirmed that catecholamine-induced proliferation was an α_1-adrenoceptor effect.

This enhanced proliferation is not limited to cardiocytes and the vasculature. Hepatocyte cultures also showed proliferative effects due to catecholamines. Epinephrine and norepinephrine were found to accelerate the entry into S phase but inhibit cells if added in late G_1. Phenylephrine accelerated proliferation, but at a lower rate (Refsnes *et al.*, 1992). It has also been shown that α_1-receptor blockade by prazosin abolished the twenty-four hour peak of DNA synthesis in regenerating rat liver post partial hepatectomy (Cruise *et al.*, 1987).

The relationship between catecholamines and cell proliferation has also been studied in atherosclerosis.

Plasma adrenaline and noradrenaline levels in patients with or without arteriosclerosis significantly and positively correlated with the severity of vascular smooth muscle thickening (Hauss *et al*., 1990). It has also been shown that prazosin and urapidil, α_1-adrenoreceptor antagonists, orally administered to rats whose carotid arteries were experimentally injured with a balloon catheter reduced the formation of neointima. The formation of neointima, as measured by DNA analysis, was significantly reduced in both groups of rats, while the urapidil rats also showed significant reductions in growth as morphometrically measured in terms of area on histological cross-section. These reductions in DNA and in cross-sectional area were dose-dependent (Fingerle *et al*., 1991).

The role of catecholamines has been studied not only in cellular proliferation, but also in apoptosis. This has resulted in some potentially conflicting hypotheses about the role of norepinephrine. Twenty-four hour norepinephrine (10 μM) exposure in rat ventricular myocytes in vitro was shown to decrease the number of viable myocytes by 35% and to increase the percentage of apoptotic cells two-fold (Communal *et al*., 1998). Mouse spleen cells and macrophages were also shown to undergo norepinephrine-induced apoptosis in a dose-dependent manner (Josefsson *et*

al., 1996). Another laboratory found that it was 3,4-dihydroxyphenylglycolaldehyde (DOPGAL), a monoamine oxidase A metabolite of norepinephrine, that induced apoptosis in PC-12 (rat pheochromocytoma) cells, a model for adrenergic neurons (Burke *et al*., 1997 and 1998). The metabolite DOPGAL, but not norepinephrine itself, was able to induce apoptosis. Communal *et al*. (1998) and Josefsson *et al*. (1996), cited above, did not examine norepinephrine metabolites in their studies. On the opposing side is research showing that norepinephrine reverses the apoptotic effect of activin A in rat hepatocyte cultures (Zhang *et al*., 1996), showing that more work needs to be done to determine what is truly occurring.

While evidence has been accrued on catecholamine-enhanced proliferation in organ systems such as the vascular smooth muscle and hepatocytes, almost no work has been done in the field of accessory sex organs such as the prostate. Determining what role catecholamines play in accessory sex organ smooth muscle growth could provide important clues in the understanding and treatment of associated diseases such as BPH.

F. Norepinephrine and Transmembrane Signaling via α_1-Adrenoceptors of Protein Kinase C

Activation of α_1-adrenoreceptors by norepinephrine is known to activate a G-protein which then stimulates phospholipase C (PLC) (Das, 1993). PLC cleaves phosphoinositol-bisphosphate (PIP_2) which is a phosphorylated derivative of phosphatidylinositol, a phospholipid in cell membranes. PIP_2 cleavage results in inositol 1,4,5-triphosphate which releases calcium from the endoplasmic reticulum and activates calcium channels (Larsson *et al.*, 1984). The other product of PIP_2 cleavage is diacylglycerol (DAG) which in turn temporarily activates protein kinase C (PKC). The temporary activation of PKC results in translocation of the enzyme from the cytoplasm to the plasma membrane (Kishimoto, *et al.*, 1980; Kraft and Anderson, 1983).

Phorbol esters are often called tumor promoters because of their ability to enhance cellular proliferation leading to tumor formation. Phorbol esters such as PMA structurally mimic diacylglycerol (DAG). DAG produces only temporary association of PKC with the membrane due to activation of sphingomyelinase (Kolesnick, 1987). Sphingomyelinase activation can result in increased

concentrations of sphingosine which compete for the regulatory unit of PKC and thus inhibit PKC activity (Hannun *et al.*, 1986). However, relative to DAG, PMA can stablely activate PKC which may be due in part to its inability to activate sphingomyelinase (Kolesnick, 1987). Stable activation of PKC was demonstrated in 1988 by the addition of PMA to cultured neonatal rat cardiomyocytes. A five minute exposure to PMA doubled total PKC activity as compared to the control. By forty-eight hours, PMA treatment of these neonatal cardiomyocytes resulted in a down-regulation of total PKC activity to just 3% of control (Henrich and Simpson, 1988). It is thought that this down-regulation is not a decrease in synthesis, but a result of increased degradation by calpain, a calcium-dependent protease (Young *et al.*, 1987). To summarize, unlike temporary activation by diacylglycerol, phorbol esters lead to stable activation of PKC, followed by cellular PKC depletion and subsequently to cellular proliferation (Hansen *et al.*, 1990; Brooks *et al.*, 1991).

Similarly to PMA down-regulation of PKC, activation of α_1-adrenoreceptors with 10 μM norepinephrine for 4 hours in vascular smooth muscle cell culture down-regulated PKC (Hu *et al.*, 1992). Although the decrease in PKC with prolonged α_1-adrenoceptor activation has not been directly related to

smooth muscle growth, there are many studies linking α_1-adrenergic effects to proliferation. With this in mind, it becomes a possibility that prolonged α_1-adrenoreceptor activation and down-regulated PKC are dependent upon one another in association with proliferation. This would fit well with the hypothesis of this research linking prepubertal, androgen-induced increased norepinephrine release with, PKC down-regulation and male accessory sex organ smooth muscle proliferation.

It is necessary to recognize the complexity within the PKC superfamily. Molecular cloning has identified and characterized eleven PKC isoforms and has established that PKC is a multigene family. However, which PKC isozyme is responsible for smooth muscle cell proliferation is still being examined. PKC isozymes α, β, δ, ε, γ, and ζ were studied in cultured porcine aortic vascular smooth muscle cells. PKCα and PKCε were implicated as possible mediators of G_1/S cell cycle inhibition with PKCα predominating. PKCα down-regulated completely with PMA. PKCε also down-regulated in response to PMA, although not completely (Sasaguri *et al.*, 1993). A later study using cultured rat aortic vascular smooth muscle cells, quantified PKC isoforms α, β and γ, and found PKCα was the only significant

isoform associated with proliferation, also by down-regulation (Haller *et al*., 1995).

Reduced amounts of total PKC activity (Mariotti *et al*., 1992) and enhanced calpain activation (Gerbrosky *et al*., 1997) also occur in response to androgen-induced accessory sex organ smooth muscle proliferation (Mariotti *et al*., 1992). Prepubertal guinea pigs showed a doubling of DNA and a drop in PKC in their seminal vesicle smooth muscles in contrast to their adult counterparts. Adult guinea pigs showed no changes in PKC, calpain, or in DNA in response to androgen. However, normal adult seminal vesicle smooth muscle which failed to proliferate with androgen treatment, still continued to hypertrophy in response to androgen, reflecting a selective loss in proliferative ability in which PKC is resistant to changes produced by androgen. What remains to be explored is the mechanism and potential link of PKC activation and subsequent depletion to androgen regulation of neurotransmission in male accessory sex organ smooth muscle.

G. Rationale for the Use of Guinea Pig Seminal Vesicle Smooth Muscle

Benign prostatic hypertrophy is a smooth muscle neoplasia. The goal of the research presented here is to understand the normal mitogenic and terminal differentiation mechanisms of androgen-dependent male accessory sex organ smooth muscle cells. Understanding this could ultimately be used to develop improved treatment and preventative therapies to pharmacologically interfere with the growth of the adenoma and the clinical presentation of BPH.

Unfortunately, a good animal model for the study of BPH does not exist and it has not been possible to establish androgen-sensitive culture systems for normal or neoplastic smooth muscle cells. It should be recognized that the true human prostate gland (the anterior, posterior and lateral lobes) do not develop BPH. It is the periurethral, fibromuscular stroma that is the origin the adenoma (Reischauer, 1925; Pradhan and Chandra, 1975). Rodent male accessory sex organs do not exhibit spontaneous prostatic hyperplasia. The hormonal induction of hypertrophy has never been achieved in the rat prostate, and most mice strains show only marginal diethylstilbestrol

induction of prostatic hyperplasia (Fingerhut and Veenema, 1966). While aging dogs develop BPH, the neoplasia is primarily a diffuse parenchymal hyperplasia. Dog prostate also does not show nodular growth that compresses surrounding normal tissue, as seen in man (Moore, 1944).

Implants and xenografts of various prostate tissues into hosts have also been tried in attempts to induce abnormal growth. Implantation of intact fetal urogenital sinus into the ventral prostate gland of athymic mice caused a 10-20 fold overgrowth within 4-9 weeks (Chung *et al.*, 1984), but it has been difficult to quantify changes due to growth of the embryonic specimen versus the host response. Human BPH tissue xenografts in nude mice have been explored. But these xenografts are difficult to maintain, show gradual atrophy and increasing squamous metaplasia and have not been successfully maintained for longer than 16 weeks (Debiec-Rychter *et al.*, 1994).

Given the absence of a definitive animal model for human BPH, androgen-dependent proliferation and differentiation of normal accessory sex organ smooth muscle was studied. The long term goal was to develop a hypothesis for the potential etiology and treatment of the human neoplasia of the periurethral fibromuscular stroma.

The guinea pig seminal vesicle was employed in this investigation because of its unique anatomy. Architecturally, the seminal vesicle is a cylindrical tube, with a distinct inner epithelial layer and an outer smooth muscular compartment. The smooth muscle can be quickly separated from the epithelium, leaving pure smooth muscle specimens which can be employed in a variety of experimental analyses. This preparation was initially employed by Levey and Szego (1955).

The normal guinea pig seminal vesicle smooth muscle shows a prepubertal, androgen-sensitive state in which DNA in smooth muscle cells increase, and an adult terminally differentiated state that is selectively resistant in this response to androgen (Mariotti *et al.*, 1992). Comparing the prepubertal state in guinea pig seminal vesicle in which DNA increases in response to androgen against the adult which is resistant to androgen-induced proliferation could uncover the mechanisms for normal accessory sex organ smooth muscle growth and terminal differentiation. Defects in the normal terminal differentiation process for smooth muscle tissue may prove to be an essential component in the proliferation of accessory sex organ fibromuscular stroma leading to human BPH.

III. Specific Aims

The long term goal of this research is to understand norepinephrine's role in the androgen-mediated proliferation of male accessory sex organ smooth muscle and to ultimately use this knowledge to develop possible treatments and preventative therapies of benign prostatic hypertrophy (BPH). The working hypothesis of this research is that androgen-dependent prepubertal proliferation of male accessory sex organ smooth muscle is due to an androgen-dependent increase in norepinephrine release and down-regulation of PKC. With this in mind, studies were done to determine physiological relevance of norepinephrine in the development of seminal vesicle smooth muscle, then the mechanism by which norepinephrine release occurred, using both *ex vivo* and *in vitro* experimental designs.

A. Specific Aim 1: Determine if androgen *in vivo* induces the release of norepinephrine in seminal vesicle smooth muscle in association with proliferation and is resistant in terminal differentiation.

B. Specific Aim 2: Determine the role of norepinephrine as a mediator of growth in androgen-induced proliferation of seminal vesicle smooth muscle.

1. Determine whether reserpine at maximal norepinephrine-depleting doses *in vivo* selectively antagonizes seminal vesicle smooth muscle proliferation and PKC down-regulation in androgen-treated animals.
2. Determine if norepinephrine *in vitro* stimulates increases in cell number of seminal vesicle-derived smooth muscle cells via α_1-adrenoceptors.
3. Determine if norepinephrine may function anti-apoptotically in seminal vesicle smooth muscle cells to effect changes in cell number.

C. Specific Aim 3: Determine whether the androgen-induced increase in norepinephrine release is due to a significant reduction in the pre-synaptic feedback inhibition of norepinphrine release.

IV. Materials and Methods

A. *In Vivo* Orchiectomy and Hypogastric Nerve Decentralization

Adult male Hartley guinea pigs (Hilltop) were inhalationally anesthetized with metafane and underwent scrotal route castration. The skin incisions were closed using stainless steel surgical staples. Prepubertal, 5-7 day old, male Hartley guinea pigs (Hilltop) were inhalationally anesthetized with ether and underwent castration by midline abdominal incision. In some animals, hypogastric nerve decentralization was done simultaneously with the castration by severing the hypogastric nerve within the intestinal mesentery. The hypogastric nerve was positively identified prior to surgery with preliminary *in situ* electrical stimulation of the hypogastric nerve resulting in seminal vesicle contraction. To close, abdominal walls were sutured with silk thread, then the skin was stapled together.

B. Dihydrotestosterone, Estradiol, Testosterone, and Reserpine Administration

After a ten day recovery period, 10 mg/kg of 5α-androstan-17β-ol-3-one, aka. dihydrotestosterone (DHT) (Sigma), 0.01 mg/kg of estradiol benzoate (E_2B) (Sigma), or 10 mg/kg testosterone (Sigma) dissolved in peanut oil (Planters), was administered subcutaneously.

These doses were chosen since it was previously established that this dose was the maximally effective dose to observe seminal vesicle smooth muscle growth in the guinea pig (Neubauer and Mawhinney, 1981). Steroids were administered for a minimum of one week since one week was the time period established previously to show a significant increase in DNA levels and DNA synthesis (Mariotti *et al*., 1992).

Control groups received subcutaneous peanut oil only.

Reserpine (Sigma) was freshly prepared on each day of use. The varying concentratrations of reserpine were first dissolved in 100 µl benzyl alcohol (Fisher) and 13.67 mg citric acid monohydrate (Sigma) with slight heat. 500 µl propylene glycol (Sigma) was then added and mixed. Water

was used to q.s. to 5 ml. Reserpine was then administered intraperitoneally.

Control groups received intraperitoneally administered vehicle only.

C. Harvesting of Seminal Vesicle Smooth Muscle Tissue

All guinea pigs were anesthetized by ether inhalation then euthenized via cervical dislocation. Seminal vesicles were immediately removed at the bifurcation and longitudinally opened. Epithelia were carefully separated by manual removal with a blunt scalpel, leaving clean smooth muscle intact. Both epithelia and smooth muscle tissues were weighed.

D. Quantification of Norepinephrine Release

Norepinephrine release was measured both directly in smooth muscle minces and indirectly ex vivo by DHPG/NE ratios in samples from guinea pigs treated *in vivo*. NE and DHPG were quantified by HPLC.

3,4-dihydroxyphenylglycol (DHPG) is the primary norepinephrine metabolite found within the rat vas deferens (Tarlov and Langer, 1971). A previous study to determine the metabolite spectrum in guinea pig seminal vesicle smooth muscle indicated that DHPG was the predominant metabolite and could be clearly detected using the method described here (Johnson, 1996). Previous studies established that DHPG/NE ratio indicated norepinephrine release (Graefe *et al.*, 1972; Cubeddu *et al.*, 1974). Spontaneous release of [^{3}H]norepinephrine was found to yield primarily 3,4-dihydroxyphenylglycol in the rat vas deferens (Graefe *et al.*, 1972). Electrical field stimulation of the rat vas deferens also yielded 3,4-dihydroxyphenylglycol (Cubeddu *et al.*, 1974). By using cocaine, it was determined that the 3,4-dihydroxyphenylglycol formation was the result of presynaptic metabolism by monoamine oxidase of the released norepinephrine which was recaptured by adrenergic nerve endings (Langer and Enero, 1974). Storage-inhibiting agents like reserpine will also increase the amount of 3,4-dihydroxyphenylglycol in tissue as a result of the recapture and rapid presynaptic metabolism of norepinephrine release from the disrupted granule (Cubeddu and Weiner, 1975).

Ultimately, it was determined that using the ratio of the metabolite, 3,4-dihydroxyphenylglycol to norepinephrine (DHPG/NE) did indirectly quantify norepinephrine release (Graefe *et al.*, 1972; Cubeddu *et al.*, 1974; Langer and Enero, 1974).

To quantify endogenous DHPG/NE, samples of seminal vesicle smooth muscle were prepared by making 1.5% homogenates using a McIlwain tissue chopper (0.25 micron^2), Brinkman Polytron (120 seconds at setting 4.5) and a homogenate buffer containing 0.6% $HClO_4$ (Fisher), 0.1% sodium metabisulfite (Fisher), and 1.4 μM 3,4-dihydroxylbenzylamine (Sigma) as an internal standard. After centrifugation at 30,000 x g for 10 minutes, the supernatant was syringe filtered through 0.45 μm Teflon filters (Titan). The filtrate was stored in microcentrifuge tubes and frozen at –80^{o}C until HPLC analysis. Samples, homogenates and filtrates were kept on ice and protected from light.

To quantify NE release *in vitro*, NE was measured in samples of media from seminal vesicle smooth muscle tissue minces. Smooth muscle was minced with a McIlwain Tissue Chopper (0.25 micron^2). Minces were placed in 6 well tissue culture plates in 2 mls Krebs-Henseleit buffer containing 1.13 M NaCl (Fisher), 47 mM KCl (Fisher), 25 mM $CaCl_2$

(Sigma), 12 mM $MgSO_4$ (Fisher), 56 mM dextrose (Sigma), 11.8 mM KH_2PO_4 (Fisher) and 250 mM $NaHCO_3$ (Fisher). 1.4 μM 3,4-dihydroxybenzylamine was also added to the buffer to serve as an internal standard. 100 μM ascorbic acid (Fisher), 1 μM desipramine (RBI) and 1 μM yohimbine (RBI) were added to the Krebs-Henseleit buffer to prevent oxidation, reuptake and feedback inhibition of released norepinephrine. Minces were kept in an atmosphere of 95% oxygen and 5% carbon dioxide.

Three 15-minute washes were initially done to remove any norepinephrine released from the trauma of harvesting and mincing. Following this, 2-12 minute baseline media samples were collected. All samples were kept on ice, protected from light then frozen at –80^{o}C until HPLC analysis. On the day of analysis, samples were thawed, kept on ice and protected from light. Samples were probe sonicated, syringe-filtered through 0.45 μm Teflon filters (Titan), then analyzed by HPLC.

A mobile phase of 0.1 M NaH_2PO_4 (Sigma), 0.325 mM octane sulfonic acid (Acros), 0.1 mM EDTA (Eastman Kodak), and 0.25 mM triethylamine (Fisher), brought to pH 3.35 with H_3PO_4 (Fisher) was pumped by a Waters 6000 pump at a flow rate of 1.0 ml/min. Samples or standards in 50-100 ul

volumes were injected with a Waters U6K injector through a Bioanalytical Systems (BAS) Phase II, 100 mm x 3.2 mm, 3 μm particle size, C_{18} column. Electrochemical detection using a BAS LC-4C detector was done under the following parameters: +0.700 V, 10-50 nAmp range, and 0.10 Hz filter. Results were charted on a Hewlett Packard 3395 Integrator under the following parameters: threshold 5, attenuation 8, chart speed 1, area reject 800,000.

Standard curves were established using 10, 80 and 160 pmols/50 ul of norepinephrine (RBI)and 3,4-dihydroxyphenylglycol (Sigma) and 70 pmol/50 ul of 3,4-dihydroxybenzylamine as an internal standard.

E. DNA Quantification for Seminal Vesicle Smooth Muscle Treated *In Vivo*

DNA quantification followed the methods established by Brunk *et al*. (1979). Samples of smooth muscle were chopped on a McIlwain tissue chopper (0.25 micron^2), then homogenized to a 5% homogenate using a Brinkman Polytron (30 seconds, setting 3). The homogenate buffer consisted of 5 mM MOPS (pH 7.4), 1 mM dithiothreital (DTT), 2 mM EDTA, and 1 mM EGTA. After homogenization samples were

sonicated for 1 minute using a Horn sonicator under the following conditions: power 70, duty cycle 50, continuous. All tubes are kept on ice.

The assay was done using a Shimadzu spectrofluorophotometer at excitation 350 nm, emission 450 nm, band widths 5 nm, and low sensitivity. Fisher Ultra-UV microcuvettes were filled with 1.4 ml of buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris 7.0) and 0.1 ml of 4’,6-diamidino-2-phenylindole (DAPI) working dye (made from 0.06 ml of 1 mg/ml DAPI stock solution in 5 ml buffer). Blank readings were taken. Readings following sequential 10 µl additions of sample homogenates were then taken to establish the linear stoichometric range for each sample. Samples ranged in concentration from 0.1-0.3 µg of DNA/µl.

Standard curves were established using known amounts of DNA (2, 4, 6, 8, and 10 µg). DNA (Calf thymus—Sigma) was prepared as a stock solution of 1 mg/ml in 0.01 N NaOH, and were diluted to 0.2 mg/ml using buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris 7.0) for the assay.

F. Detection of Apoptosis by Gel Electrophoresis

In apoptosis, DNA fragments consisting of 180-200 base pairs can be seen in a “laddering” pattern via agarose gel electrophoresis (Wyllie, 1980). This internucleosomal DNA degradation by endonuclease is characteristically associated with apoptosis and not with necrosis (Wyllie, 1980). Therefore, to detect the presence of apoptosis, agarose gel electrophoresis was used.

Seminal vesicles were harvested and the epithelia removed. Tissues were flash frozen in liquid nitrogen then transferred to –80C until homogenization at a later date. For homogenization, tissues were thawed by placing in ice cold buffer containing 100 mM NaCl, 10 mM EDTA, and 10 mM Tris 7.0. A 2% homogenate was made by first chopping tissues with a McIlwain tissue chopper then homogenizing with a polytron.

DNA was extracted and prepared for agarose gel electrophoresis in conjunction with Dr. Laura Gibson, in the Department of Microbiology and Immunology at West Virginia University. Cells were lysed and protein digested with proteinase K for 1 hour at 37^{o}C. Equal volumes of chloroform and phenol were added. The homogenate mixture was shaken for 30 seconds then spun at 14,000 x g for 5

minutes. The aqueous phase was removed to a new tube and 1/10 volume of 3 M NaOAc and 1.5 volume of 100% ice cold ethanol was added. Precipitation occurred overnight at –20^{o}C. The next day, DNA was pelletted by spinning at 14,000 x g for 15 minutes. The pellet was dried, resuspended in Tris-EDTA, treated with 5 mg/ml RNAse for 1 hour at 37^{o}C, extracted with an equal volume of chloroform, then respun. The aqueous phase was removed and again precipitated using ice cold 100% ethanol.

100 ng of sample DNA and 5 µl dye were loaded into the wells of a 2% agarose gel containing 2% ethidium bromide and covered with 0.04 M tris-acetate, 0.001 M EDTA buffer. The first lane contained a positive control Kb ladder (Gibco BRL) while the last lane contained a negative sample. The power supply was applied for 2 hours at 90 mV. The gel was then exposed to UV light and photographed with a Strategene Eagle Eye II camera.

G. Seminal Vesicle Smooth Muscle Cell Cultures

Male Hartley guinea pigs were sacrificed and submerged in 70% ethanol before the seminal vesicle was harvested and the epithelia removed. The clean smooth muscle tissue was then minced in a petri dish containing 1 mg/ml filter sterilized collagenase (Sigma) in Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro) or Hanks Balanced Salt Solution (Sigma) with no bovine serum, and incubated for one hour at 37^{o}C. The sample was then triturated with decreasing pipet size to 5 ml then transferred to a 50 ml. tube to settle at unit gravity. The pellet was reconstituted in DMEM containing 292 μg/ml L-glutamine (Sigma), 50 μg penicillin/ml (Sigma), 50 μg streptomycin/ml (Sigma), 150 μg neomycin/ml (Sigma) and 10% heat inactivated fetal calf serum (Biowhittaker). The supernatant was harvested and spun on a Sorvall RT 6000B centrifuge (speed 3). The resultant pellet was also reconstituted in culture medium. Two separate cultures were made with the two reconstituted pellets. Cells were then grown in flasks (Costar) in an incubator at 37^{o}C, and 5% CO_2. Previous studies established that cultures appeared to be pure smooth muscle cells based on double staining for nuclei

(DAPI) and immunocytochemical staining for smooth muscle isoactin (Gerbrosky *et al*., 1997).

Cells were passed every 2 weeks and grown in media containing 5% fetal bovine serum (Gibco). Cells were harvested by first removing media and any detached cells, rinsing with HBSS, and trypsinizing (Sigma) for 2 minutes followed by mechanical removal with a rubber policeman to remove attached cells. Media containing 10% fetal bovine serum was used to stop trypsinization.

For *in vitro* proliferation studies, six-well culture plates (Costar) were used. Each well was plated with 6-7 x 10^4 cells/well as determined by hemocytometer, and incubated for 24 hours to allow attachment. Treatment groups were then exposed to various agents such as: 1 µM phenylephrine (Sigma) in 0.1% ethanol, 10 µM phenylephrine in 0.1% ethanol, 1 µM prazosin (Sigma) in 0.1% ethanol, 1 µM isoproterenol (RBI) in water, and 100 µM ascorbic acid (Fisher).

Note: Although phenylephrine is freely soluble in water as well as ethanol, experiments using water vehicle did not show any effect of phenylephrine on cells.

Control cells were exposed to 0.1% ethanol or water and 100 μM ascorbic acid. Media and agents were replaced daily. Cells were counted using a hemocytometer.

H. Organ Bath Electrical Field Stimulation

Seminal vesicles from male Hartley guinea pigs treated *in vivo* were harvested. Adventitia was removed, the lumen opened lengthwise and the epithelia removed. Proximal segments of longitudinal smooth muscle were mounted between two platinum electrodes for electrical field stimulation (Grass S44) and attached to a force displacement transducer (Grass 79D) for isometric contraction recording. The proximal 1.5 cm for adult and 1.0 cm for prepubertal guinea pigs were used in the organ bath due to the inability of the bath to contain an entire longitudinal adult seminal vesicle smooth muscle. Proximal ends were used since longitudinal smooth muscle was more prevalent proximally than elsewhere (Al-Zuhair *et al.*, 1975). Preparations were placed in an organ bath containing Krebs-Henseleit buffer, kept at room temperature and continuously bubbled with 5% CO_2 and 95% O_2. Tissues were given 1 hour to equilibrate at an initial tension of 2 grams.

After equilibration, tissues were electrically field stimulated for 30 seconds at 40 V with train pulses of 0.5 msec. duration, 0.01 msec. delay at varying Hz (2, 5, 10, 20, 30 and 40 Hz). Contractile response was shown to be graded between 2 and 20 Hz, and 30 Hz was previously shown to stimulate supramaximal contraction without causing significant exhaustion (LaPierre *et al.*, 1993). Organ bath contents were drained and replaced 5 minutes after stimulation was initiated. Tissues were not stimulated again until 15 minutes had passed. After all the seminal vesicle segments were stimulated at 2, 5, 10, 20, 30, and 40 Hz, segments 1 μM rauwolscine (RBI) was added to the Krebs-Henseleit buffer and segments were allowed to equilibrate for 1 hour. Tissues were then restimulated at the various Hz. 1 μM prazosin was then added to the buffer and tissues equilibrated again for 30 minutes before receiving a final 40 Hz stimulation. 1 μM prazosin was found in preliminary dose response studies to maximally inhibit the adrenergically-mediated, electrically-field-stimulated contraction.

For the study of post-junctional α_2-adrenoceptors, tissues were prepared as above. However, rather than electrical stimulation, 10 μM BHT-933 [6-Ethyl-5,6,7,8-

tetrahydro-4H-oxazolo[4,5-d]azepine-2-amine dihydrochloride], a selective α_2-adrenoceptor agonist (RBI), or 10 μM methoxamine $\pm$ 0.1 μM prazosin were used to elicit contraction.

I. α_2-Adrenoceptor Characterization and Quantification

Ligand binding studies using [^{3}H]RX821002 [2-methoxy-1,4-[6,7-^{3}H]benzodioxan-2-yl-2-imidazolin HCl] (Amersham) were done to identify, characterize and quantify the α_2-adrenoceptor in prepubertal and adult 7-day-dihydrotestosterone-treated, normal and castrate guinea pigs. The ligand RX821002 is an α_2-adrenoceptor antagonist which exhibits relatively high affinity for all the α_2-adrenoceptor subtypes (O'Rourke *et al.*, 1994). RX821002 is the 2-methoxy analog of idazoxan. In rabbit and human frontal cortex, idazoxan binds with comparable affinity to α_2-adrenoceptors and phentolamine-displacable non-stereoselective sites (Convents *et al.*, 1989). In these same tissues, the 2-methoxy analog of idazoxan, [^{3}H]RX821002, was shown to have a 10-fold higher affinity for the α_2-adrenoceptor (K_d = 2.8 nM) than [^{3}H]idazoxan (K_d =

24 nM) and equal affinity with [^{3}H]rauwolscine (Vauquelin *et al.*, 1990).

Seminal vesicles were freshly harvested, and the adventitia and epithelia were removed. Seminal vesicle smooth muscles were then chopped on a McIlwain tissue chopper to 0.5 micron^2 followed by teflon glass homogenization on ice in a 0.32 M sucrose solution containing 5 mM Tris buffer at pH 7.4. A Brinkman polytron was then used to further homgenize the sample.

Homogenates were centrifuged at 1000 x g for 10 minutes. The supernatants were saved and the pellets were rehomogenized and re-spun as previously described. The supernatants were then pooled, centrifuged at 48,000 x g for 20 minutes, and the resultant supernatant was discarded. The synaptic membrane pellet was then reconstituted in 25 mM sodium phosphate buffer by using the Brinkman polytron (Deupree *et al.*, 1996).

Bradford protein determinations were done on this reconstitution.

Kinetic studies were done to establish protein dependence and equilibration with time of incubation using 0.4 nM [^{3}H]RX821002, the lowest ligand concentration employed in saturation analysis. The analyses for protein dependence and equilibration time were quantified for total

rather that saturable binding to ensure valid [^{3}H]RX821002 binding conditions for all potential components of binding.

For α_2-adrenoceptor saturation binding experiments, 0.40 to 9.0 nM of [^{3}H]RX821002 were added to membrane suspensions and sodium phosphate buffer was added for a final total volume of 200 µl. Reactions were allowed to equilibrate for 20 minutes at room temperature. Saturable binding was determined with a parallel set of reactions that also contained 10 µM rauwolscine. Rauwolscine was initially employed because the non-tritiated RX821002 was commercially unavailable at the time. 10 µM of rauwolscine represented the minimum concentration of rauwolscine necessary to produce greater than 90% inhibition of 5 nM [^{3}H]RX821002 binding. Subsequently, non-radioactive RX821002 became commercially available. When tested, RX821002 at $1x10^{-7}$ M generated the same degree of maximum inhibition as 10 µM rauwolscine at 5 nM [^{3}H]RX821002 binding.

To assess ligand binding specificity, competition experiments were done using 5nM [^{3}H]RX821002 and varying concentrations of prazosin (Sigma), phentolamine(Sigma), clonidine , 5HT, rauwolsince (RBI), and 2-methoxyidazoxan (Sigma). Assays were given 20 minute equilibration time.

Membrane suspensions were then filtered through sodium phosphate presoaked Whatman GF/B glass fiber filter papers with a 10-well minifold (Fisher). Filters were rinsed with sodium phosphate buffer and the trapped radioactivity was counted by liquid scintillation (ScintiSafe by Fisher) photometry.

Scatchard-Rosenthal plots were used to determine K_d and B_{max} values. IC_{50} values were converted to K_i values using the Cheng and Prusoff equation:

$$K_I = \frac{IC_{50}}{1 + \frac{\text{free } {}^3\text{H-ligand}}{K_d \text{ of } {}^3\text{H-ligand}}}$$

J. PKC Analyses

Tissues were homogenized (Brinkman Polytron setting 3, 30 seconds, three times with 30 second intervals) in 20 mM MOPS (pH 7.4) containing, 2 mM DTT, 1 mM EDTA, 1 mM EGTA, 100 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml chymostatin A, 10 µg/ml antipain and 1 mM phenylmethanesulfonyl fluoride (PMSF). Homogenates were then centrifuged at 10,000 x g for 10 minutes to sediment nuclei and unbroken cells. This pellet was then

resuspended, rehomogenized in the above buffer, and recentrifuged. The supernatants of the original spin and of the subsequent spin were combined and centrifuged at 100,000 x g for 60 minutes to generate the EGTA soluble fraction.

To assay the protein kinase, the EGTA-soluble fraction was column fractionated on DEAE-cellulose. Assays were performed at 30^{o}C using a reaction mixture of 100 µl containing 40 mM MOPS (pH 7.2), 5 mM $MgSO_4$, 1 mM DTT, 20 µM $\alpha[^{32}P]$ATP (0.5 µCi), and 600 µg protamine. Cyclic AMP-dependent type I and type II kinases were assayed by reaction with and without 10 µM cAMP, using 200 µg of histone f_2b as a substrate. After a 5 minute incubation at 30^{o}C, duplicate 40 µl aliquots were spotted onto 2.3 cm squares of P-81 phosphocellulose paper and immersed in 1% sodium pyrophosphate, followed by three 15 minutes washes in the same fluid, a 95% ethanol rinse and a 3:1 v/v 95% ethanol:ether rinse. Disks were air-dried then counted in a liquid scintillation spectrophotometer (Durham *et al.*, 1986).

To further isolate particular PKC isozymes, peak enzymatically active fractions from the DEAE column were pooled and loaded in Minifold I (Schleicher & Schuell)

wells. Total proteins from these individual fractions were identical between groups based on Bradford protein analysis of the individual fractions and the pooled sample. The Minifold contained Immobilon P (PVDF) (Millipore) membrane wetted underneath with one sheet of Gel Blot (GB002) (Schleicher & Schuell). 500 μl samples with protein ranging from 1.5 to 25 μg were applied. The membrane was prepared following Tropix, Inc. Western-Light Chemiluminescent Detection System Protocol. Primary mouse monoclonal anti-PKC antibodies (Transduction Labs) were applied to the membrane in a concentration of 2 μg/ml in a 90 minute incubation. Unbound primary antibody was rinsed off before a 90 minute incubation in secondary Donkey anti-mouse IgG, alkaline phosphate-conjugated antibody in a 1 : 5000 dilution with buffer containing 0.2% I-Block (Tropix), 0.1% Tween-20 and $1/10^{th}$ volume PBS. Unbound antibody was again rinsed off and Ultra-Pure CSPD chemiluminescent substrate (Tropix) was applied.

Chemiluminescence was recorded on Kodak X-OMAT film and analyzed and quantified by Optimus densitometer. Antibodies were used to detect PKC isozymes α, β, δ, ε, γ, θ, and ξ. Isozymes α, ε, γ, θ, and ξ were detectable in appreciable quantities. The β and δ isozymes were either

undetected or detected only in trace amount unquantifiable by densitometry. The ε, θ, and ξ isozymes demonstrated wide intra-assay variation and were not subsequesntly pursued. Only the α and γ isozymes exhibited a stoichiometric relationship between protein concentration and chemiluminescence and thus were the 2 isozymes quantified in this study. In preparation for PKCα isozyme quantification by dot blots, a protein dependence study was done using seminal vesicle smooth muscle from prepubertal and adult castrates and PKCα. Although not stoichiometrically linear, relative density did increase with increased protein applied in a linear fashion. For prepubertal castrates, the amount of protein (x) was related to chemiluminescent density (y) such that $y=0.022x+0.671$ with correlation $r^2=0.80$. In adult castrates, $y=0.019x+0.228$, $r^2=0.97$. This showed that chemiluminescent density would be dependent upon the amount of protein applied.

K. Statistical Analyses

Statistical analyses, generally one-way analysis of variance, Students t-test or Tukey-Kramer's test were employed in this work. Statistical significance was defined at $p \leq 0.05$. Graphs were done using JMP Statistical Software, Microsoft Excel 97, or GraphPad.

V. Results

A. Specific Aim 1: Androgen *in vivo* induced increased norepinephrine release in the seminal vesicle smooth muscle of prepubertal castrates.

As shown in previous studies of rat vas deferens (Tarlov and Langer, 1971; Graefe *et al*., 1972; Cubeddu *et al*., 1974), the predominant metabolite of norepinephrine (NE) in the guinea pig seminal vesicle smooth muscle was found to be 3,4-dihydroxyphenylglycol (DHPG) (Johnson, 1996). By using [^{3}H]norepinephrine, it was found that more than 70% of the spontaneous outflow of NE in rat vas

deferens was deaminated to DHPG, with the next highest metabolite via catachol-O-methyltransferase, accounting for 14% (Graefe *et al*., 1972). DHPG was positively identified by comparison of retention times with known DHPG standard on HPLC.

The use of endogenous DHPG/NE ratio has been used as an indicator if *in vivo* NE release. Prepubertal guinea pigs treated *in vivo* for 7 days with 10 mg/kg dihydrotestosterone (DHT) showed a tripling of endogenous DHPG/NE over oil-treated prepubertal controls in seminal vesicle smooth muscle homogenates (fig. 1).

Figure 1: Effects of DHT on DHPG/NE in seminal vesicle smooth muscle

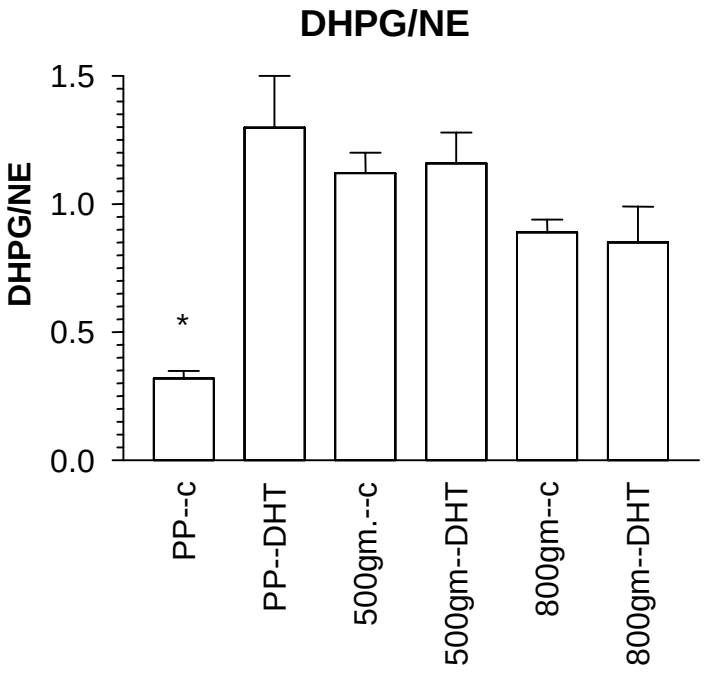


Figure 1: Norepinephrine release in prepubertal (PP) seminal vesicle smooth muscle, as measured by DHPG/NE, increased three-fold with 10 mg/kg DHT replacement as compared to prepubertal castrate (c) control. DHT-treated prepubertal ratios were similar to adult levels which were resistant to androgen-induced change. In prepubertal animals, castration was done prepubertally at 6-7 days of age. Adults were castrated at approximately 500 grams or at 800 grams. Prepubertal groups had n=8-15, weighing approx. 250gms. Adult groups had n=4-8.
*$p \leq 0.05$ compared to all other groups.

Norepinephrine release in adult guinea pigs showed no change with DHT treatment and were similar to levels measured in prepubertal castrates treated with DHT for 7 days. This indicated that once norepinephrine release was

elevated by androgen, it remained elevated into adulthood when it was no longer dependent upon androgens.

The effect of other sex steroids on norepinephrine release was also examined.

Figure 2: The Effect of Various Sex Steroids on Norepinephrine Release in Seminal Vesicle Smooth Muscle

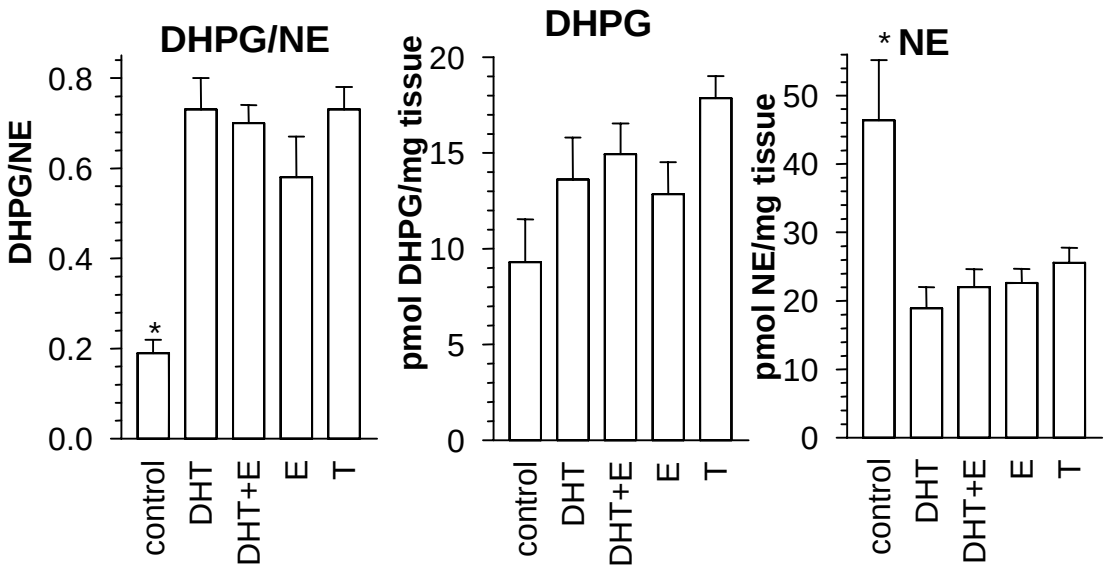


Figure 2: Prepubertal guinea pigs were castrated at 6-7 days of age. 10 mg/kg of dihydrotestosterone (DHT) and testosterone (T), 0.01 mg/kg estradiol benzoate (E), or oil (control) were given daily for 7 days. n=6-15
*$p \leq 0.05$

Previous evidence demonstrated that the actions of testosterone on certain central nervous system neurons

depended upon its aromatization by 5α-reductase and the combined effects of the resultant dihydrotestosterone and estradiol (Christensen and Clemens, 1975). Estradiol was also known to stimulate increased seminal vesicle smoooth muscle DNA (Mariotti and Mawhinney, 1981). Therefore, estradiol was examined with the hypothesis that it may interact with dihydrotestosterone in norepinephrine release. While norepinephrine release was increased in response to estradiol, there was no significant interaction with dihydrotestosterone (fig. 2). The effects of testosterone were duplicated by dihydrotestosterone alone in prepubertal castrate animals. This was consistent with other parameters examined in seminal vesicle smooth muscle (Neubauer and Mawhinney, 1981). Dihydrotestosterone alone was thus the androgen employed as it would eliminate the question of estradiol involvement in any androgenic effect.

Figure 3: Effects of DHT on DHPG and NE concentrations in prepubertal and adult seminal vesicle smooth muscle

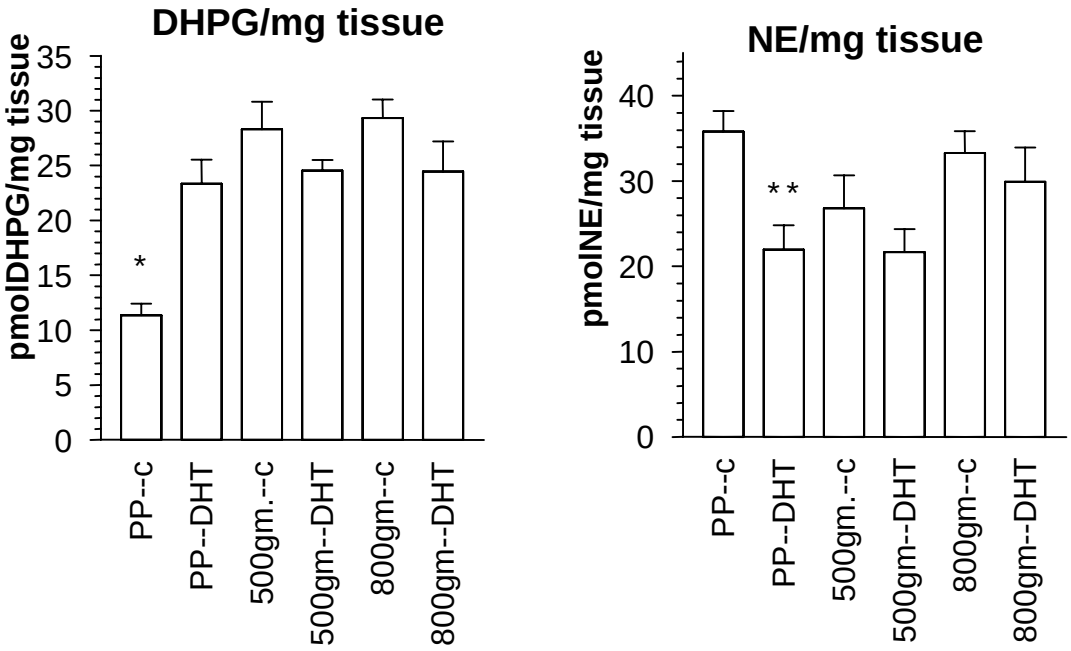


Figure 3: The absolute numbers here were used to calculate the ratios in Fig.1. In prepubertal castrates, norepinephrine (NE) was released and no longer contained within the smooth muscle, resulting in a drop in NE concentration. However, due to the increased NE release, the concentration of metabolite DHPG increased within the tissue.
*$p \leq 0.05$
** $p \leq 0.05$ compared tp PP-c

Evaluating the individual components of the DHPG/NE ratio (fig. 1) revealed that dihydrotestosterone (DHT) had opposite effects on the concentrations of DHPG and norepinephrine (fig. 3). This pattern of change was expected since the release of norepinephrine would cause more deaminated product (DHPG) to be formed while the

stored amount of parent norepinephrine may be diminished. Once again, DHT-treated prepubertal tissues showed similar concentrations of DHPG and NE as measured in adult castrate and adult DHT-treated castrate tissues, indicating NE release was sustained at relatively high levels and became androgen-resistant in adults.

Simultaneous experiments were also done to examine the question of whether severing the preganglionic nerve supply to the guinea pig seminal vesicle antagonized the DHT-induced norepinephrine release during the proliferative phase of growth. Decentralization of the hypogastric nerve was performed and confirmed by the lack of seminal vesicle contraction in response to directly applied electrical stimulation of the hypogastric proximal to the severed nerve *in situ*.

Figure 4: Hypogastric nerve decentralization did not effect DHPG/NE, DHPG or NE

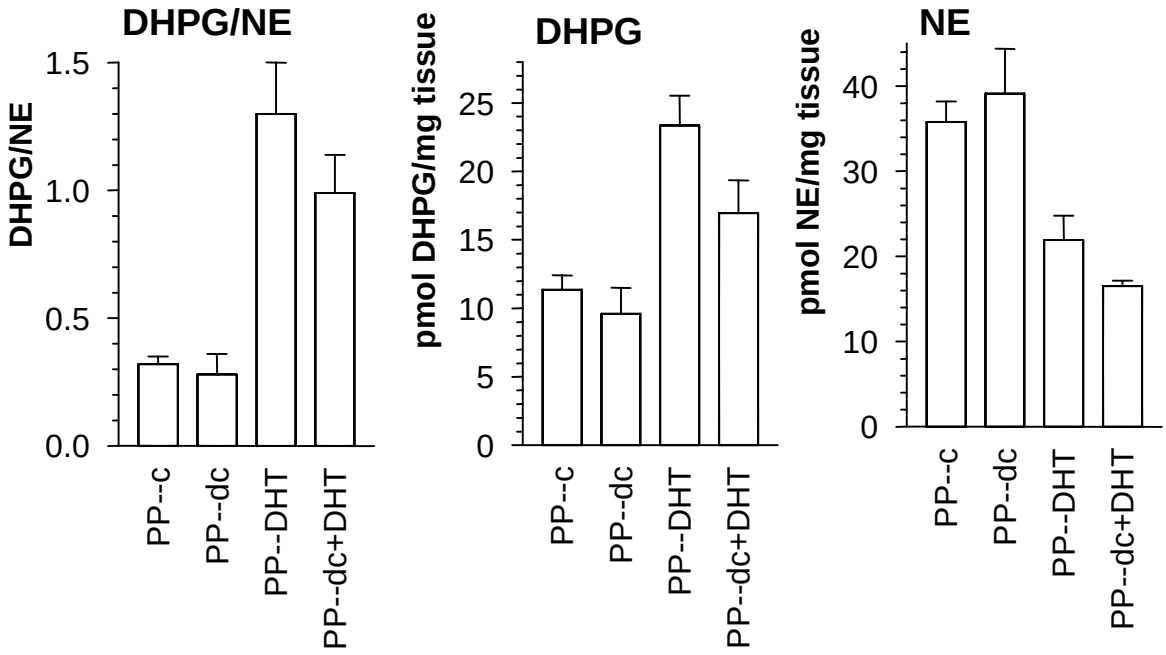


Figure 4: Castration (C) and decentralization (d) were done during the same surgery on days 6-7 of age. n=8-15. There were no significant differences due to decentralization.

Decentralization did not inhibit norepinephrine release as measured by DHPG/NE ratio in prepubertal guinea pigs (fig. 4). This suggested that basal norepinephrine release was also not effected by decentralization and further indicated that androgen-induced neurotransmission was localized to the post-ganglionic fiber.

A time course of the DHPG/NE ratios in prepubertal castrates, completed prior to the research presented here (E. A. Johnson, 1996), showed that significant increases

begin on the third day of DHT treatment and peaks at day 5 of treatment. The ratio continued to be elevated to the seventh and last day of treatment. The increases in these studies showed the same magnitude as recorded in this present investigation (fig. 5).

Figure 5: Time course of measured NE release in seminal vesicle smooth muscle homogenates from prepubertal castrates treated with DHT

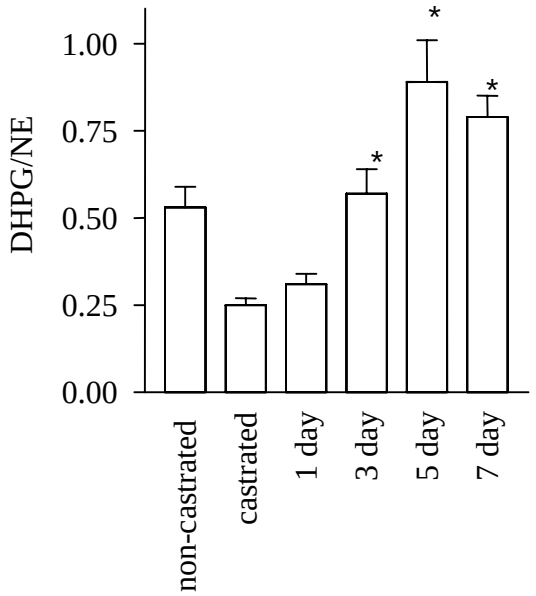


Figure 5: Norepinephrine release (DHPG/NE) increased significantly on day 3, peaking by day 5. (Data by E. A. Johnson, 1996).
*p $\leq$ 0.05 compared to castrate control.

These ex vivo measurements of norepinephrine release from the homogenates were then compared and confirmed with studies in which norepinephrine release was directly

measured from the incubation media from minces of *in vivo* androgen-treated, prepubertal castrate seminal vesicle smooth muscle. Direct measurements of norepinephrine from the minces also eliminated any questions regarding systemic norepinephrine. Ascorbic acid, desipramine and yohimbine were added to the buffer to prevent oxidation, reuptake and feedback inhibition of released norepinephrine respectively.

Basal release, rather than electrically or potassium stimulated release, was of interest in an attempt to relate the findings to proliferation and differentiation. Maximal nerve activity from electrical or potassium stimulation was not pursued since it would more closely resemble the nerve activity seen post-pubertally during ejaculation. To ensure release, rather than leakage due to trauma, was being measured, three 15-minute washes were done prior to collection.

A preliminary study using adult normal seminal vesicle smooth muscle was done to show that norepinephrine released into the 2ml of media was proportionate to the amount of smooth muscle. Using 134 mg and 226 mg of smooth muscle, the NE released into the media were 39.48 and 78.42 pmoles respectively, indicating 0.29 and 0.35 pmole of NE released per mg tissue.

Figure 6: The effect of DHT on baseline norepinephrine release as measured directly in prepubertal castrate minced seminal vesicle smooth muscle

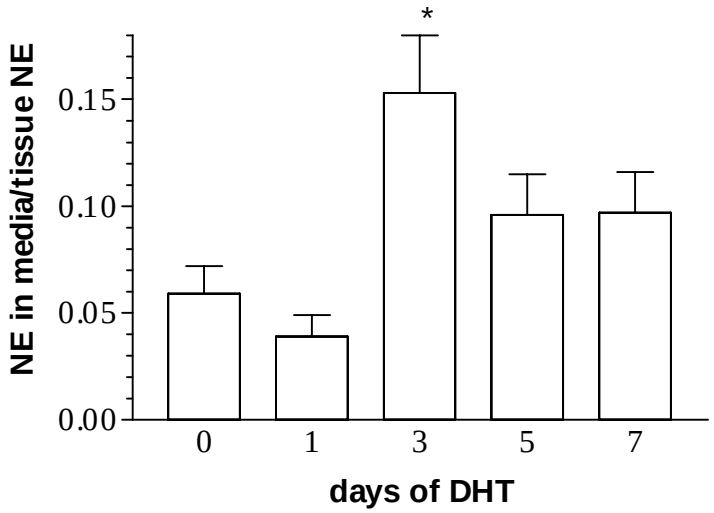


Figure 6: Prepubertal castrate guinea pigs (approx. 250 gms) were treated *in vivo* then seminal vesicle smooth muscle was harvest and minced. Minces underwent 3-15 minute washes, then the Krebs-Henseleit buffer was collected as the baseline after a 12 minute, post-wash interval. Norepinephrine was quantified by HPLC. Release was expressed as norepinephrine released into the media per norpinepinephrine within the tissue. n=3.
*p $\leq$ 0.05 compared to all other groups.

Norepinephrine release in the minces increased with DHT-treatment showing a similar time-course as demonstrated above in figure 5. Androgen treatment caused release to increase significantly by day 3. Release remained high on days 5 and 7 although it was not significantly different from control. The percentage of initial norpepinephrine within the tissue which was released into the media during

the point of collection was as follows: control=5.9%, 1-day DHT=4.0%, 3-day=15.2%, 5-day=11.8%, and 7-day=11.4. Figure 5 showed peak release on day 3 and ex vivo data showed increased release on day 5. This may be due to *ex vivo* and *in vitro* differences in metabolic activity, pathways and uptake.

B. Specific Aim 2: Norepinephrine mediated androgen-induced proliferation of seminal vesicle smooth muscle *in vivo*.

1. Reserpine at the minimum dose to maximally deplete norepinephrine *in vivo* selectively antagonized seminal vesicle smooth muscle proliferation and PKC down-regulation in androgen-treated animals.

Previous studies have shown that 7 day treatment with 10 mg/kg dihydrotestosterone in prepubertal guinea pigs caused proliferation of the seminal vesicle smooth muscle as measured by DNA content (Mariotti *et al*., 1992). The results presented above, indicated that norepinephrine release was increased with androgen treatment in prepubertal guinea pigs concurring with a similar time course of increased DNA synthesis. In adults, NE release

was androgen-resistant under conditions in which cell number was also androgen-resistant (Mariotti *et al*., 1992).

To address the question of whether NE mediated the androgen-induced proliferation of seminal vesicle smooth muscle, *in vivo* experiments were done using the drug reserpine which depletes norepinephrine by disrupting storage and uptake into the granule (Bertler *et al*., 1961). Because norepinephrine is co-transmitted with adenosine triphosphate (ATP) (Burnstock *et al*., 1972), there was some concern that ATP may be effected by reserpine. However, studies showed that reserpine at maximum norepinephrine-depleting dosages did not affect ATP (Kirkpatrick and Burnstock, 1987).

Preliminary studies showed that a single dose of reserpine at 0.30 mg/kg depleted seminal vesicle smooth muscle norepinephrine to levels undetectable by HPLC for 48 hours in 100% of animals. By 72 hours, norepinephrine levels began to rise. However, the crucial study was to determine the regimen of reserpine treatment in androgen-treated and control animals to deplete norepinephrine during an entire 5-day course of treatment.

Reserpine dose-response studies were done in androgen-treated and control groups to determine the minimum dose of reserpine necessary to maximally deplete norepinephrine to

undetectable levels as quantified by HPLC in seminal vesicle smooth muscles of 100% of the animals. Time studies were also done to determine the appropriate reserpine dose necessary to deplete norepinephrine during the entire treatment regimen.

Table 1: 24 hour Reserpine Dose Response in Prepubertal Oil-treated Castrate Controls

Reserpine given	% NE depleted
0.05 mg/kg (n=3)	0
0.15 mg/kg (3)	80
0.25 mg/kg (3)	100
0.50 mg/kg (3)	100

Table 1: HPLC analysis indicated that 0.25 mg/kg reserpine i.p. was necessary to deplete NE to undetectable levels in seminal vesicle smooth muscle in all prepubertal castrate control animals. This 0.25 mg/kg dose was also used to pre-treat DHT-treated animals 24 hours prior to the first DHT administration.

Time course studies were done during which HPLC analysis was done after 48 hours, 72 hours, 4 days and 5 days of daily reserpine treatment. These studies showed that 0.25 mg/kg reserpine, i.p., given daily, depleted norepinephrine to levels undetectable by HPLC in seminal vesicle smooth muscle in 100% of oil-treated castrate

control animals for the entire 5 day oil-treatment regimen (not shown). This regimen had no significant effect on castrate seminal vesicle smooth muscle DNA (SVSM-DNA) (fig. 7).

Figure 7: The Effect of 5-day, 0.25 mg/kg Reserpine Treatment on Seminal Vesicle Smooth Muscle (SVSM)-DNA in prepubertal castrates

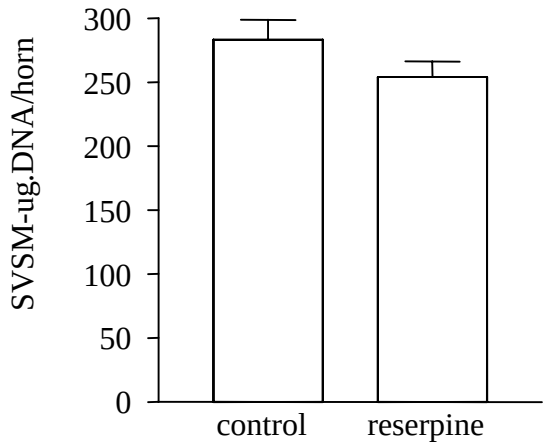


Figure 7: 0.25 mg/kg reserpine, i.p. daily for 5 days did not significantly reduce DNA (µg) content in seminal vesicle smooth muscle in prepubertal castrates.

Animals treated with 10 mg/kg dihydrotestosterone (DHT) were found to require a higher dose of reserpine to deplete norepinephrine for the entire 5 days of DHT treatment regimen. When initially given the same 0.25mg/kg/day dose, it was noticed that DHT-treated animals experienced almost no mortality as well as morbidity such

as lethargy, diarrhea and weight loss, compared to reserpine-only groups. Dose response studies were repeated for guinea pigs treated with androgen for 1, 3 and 5 days. It was determined that after a pretreatment 0.25 mg/kg reserpine dose, 0.75 mg/kg of reserpine given daily intraperitoneally was necessary to deplete norepinephrine in 5 day, DHT-treated guinea pigs. Hence, oil-treated controls were also pretreated with 0.25 mg/kg reserpine.

The reason for the discrepancy in reserpine doses in DHT versus oil-treated controls was not explored. The important issue was to determine the minimum dose of reserpine necessary to deplete seminal vesicle smooth muscle norepinephrine to levels undetectable by HPLC in 100% of animals within the treatment groups.

Figure 8: Effect of Norepinephrine-Depletion on Androgen-Induced Seminal Vesicle Proliferation

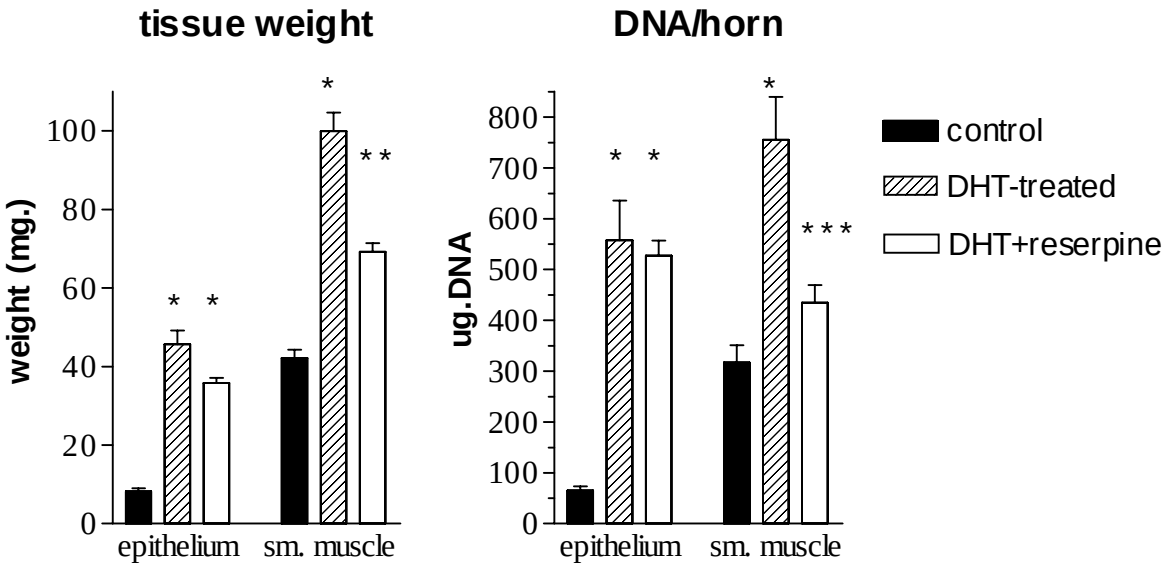


Figure 8: Animals were castrated and decentralized at 6 days of age. After a 10 day post-castration recovery period, animals were pretreated with 0.25 mg/kg reserpine, i.p., or with vehicle. 24 hours after the pretreatment, 10 mg/kg dihydrotestosterone (DHT) or oil treatment, and 0.75 mg/kg reserpine or vehicle was given daily for 5 days. Epithelium, which is cholinergically innervated, was not effected by NE depletion. Control n=5. DHT n=7. DHT+reserpine n=14.
*$p \leq 0.05$ compared to control.
**$p \leq 0.05$ compared to control and DHT-treated.
***$p \leq 0.05$ compared to DHT-treated.

The data show that norpinephrine depletion by reserpine had no significant effect on androgen-induced epithelial growth as measured by wet weight and DNA. The unperturbed epithelium also served as a control for any non-specific and anti-androgenic effects of reserpine.

Because the epithelium is primarily cholinergically innervated, agents acting selectively upon adrenergic innervation would not be expected to affect the epithelium. It has been confirmed in mouse brain that reserpine *in vivo* did not affect acetylcholine levels (Palfai *et al.*, 1986).

Norepinephrine-depletion did affect the adrenergically innervated smooth muscle compartment. Norepinephrine-depletion reduced the androgen-induced increase in wet weight by 31% and in DNA content by 42% without causing a change in cell size as measured by RNA/DNA ratio (0.0014 $\pm$ 0.0003 for DHT versus 0.0018 $\pm$ 0.0003 for DHT+reserpine).

In androgen-treated guinea pigs, reserpine did cause a significantly small decrease in total body weight of the guinea pig when weighed on day of sacrifice. Androgen-treated prepubertal castrates weighed 278.20 $\pm$ 5.65 gms. on day of sacrifice compared to guinea pigs treated additionally with reserpine which weighed 232.12 $\pm$ 10.93 gms. This 16.6% difference in total body weight was not enough to account for the 31% wet weight decrease in seminal vesicle smooth muscle or the 42% decrease in DNA seen in reserpine-treated animals (fig. 8).

An experiment using prepubertal guinea pigs having undergone castration alone was done to determine whether the decentralization, as done in the prepubertal castrates

in the experiment above, had any effect on seminal vesicle smooth muscle response to DHT+reserpine (fig. 9).

Figure 9: Decentralization had no significant effect on castrate, prepubertal guinea pig seminal vesicle smooth muscle

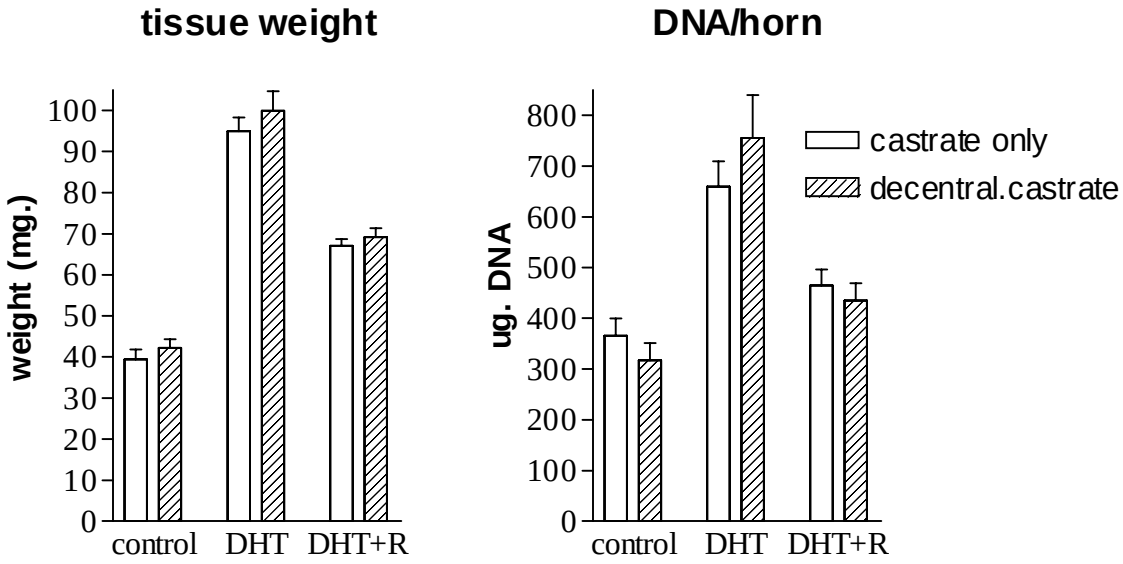


Figure 9: Decentralization had no significant effect on prepubertal castrate, seminal vesicle smooth muscle compared to castration alone. Decentralized data were reproduced from figure 6. For castrate-only animals, n=14 for controls, 10 for DHT-treated groups, and 19 for DHT+Reserpine.

Because decentralization did not effect norepinephrine release (fig. 1), it was anticipated and recorded that decentralization did not effect the smooth muscle response to androgen and reserpine.

The down-regulation of protein kinase C (PKC) and changes in cAMP in response to norepinephrine-depletion by reserpine were also examined. It has been shown that a reduction of total PKC activity occurred in response to androgen-induced proliferation of prepubertal guinea pig seminal vesicle smooth muscle and was androgen-resistant in association with the terminal differentiation seen in adults (Mariotti *et al.*, 1992). Changes in PKC were compared to changes in cAMP-dependent protein kinases which remained androgen-sensitive in both prepubertal and adult animals (fig. 10).

Figure 10: The time course of androgen's effect on PKC and the interaction of reserpine in prepubertal castrate seminal vesicle smooth muscle

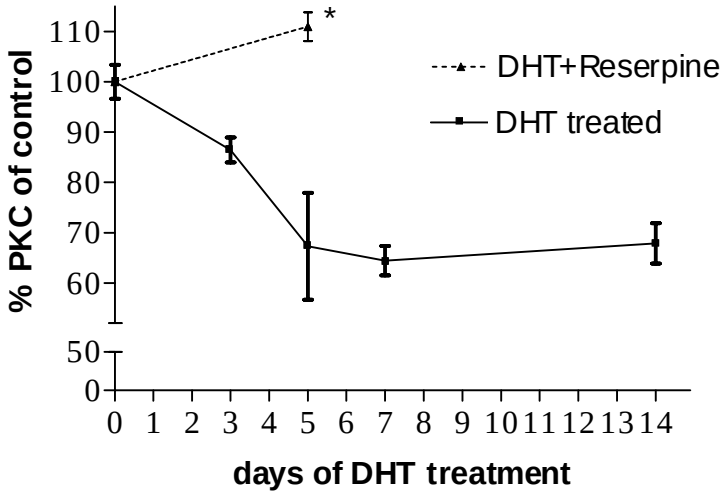


Figure 10: Soluble PKC activity decreased with androgen treatment. Norepinephrine-depletion by reserpine antagonized this effect. Animals treated with reserpine were treated for only 5 days due to the deleterious effects of reserpine. n=3 with each n representing a pool of at least 6 prepubertal animals weighing approx. 250 gms. Control activity=473 $\pm$ 19 pmol/[^{32}P] transferred/fraction/5 mg soluble protein applied to the DEAE column.
*p $\leq$ 0.05 compared to DHT-treated.

Experiments done here confirmed the down-regulation of PKC with androgen-treatment. Norepinephrine-depletion by reserpine did prevent the down-regulation of PKC in androgen-treated animals, such that the level of activity was comparable to control animals that were not exposed to androgen and showed no proliferation. In contrast, cyclic-AMP dependent type II kinase was unchanged by norepinephrine-depletion (239.1 $\pm$ 24.1 for DHT only versus

267.3 ± 32.3 for DHT+ reserpine (units in pmol/[^{32}P] transferred/fraction/mg soluble protein applied to the DEAE column)).

Figure 11: Relative amounts of PKCα as measured by chemiluminesce

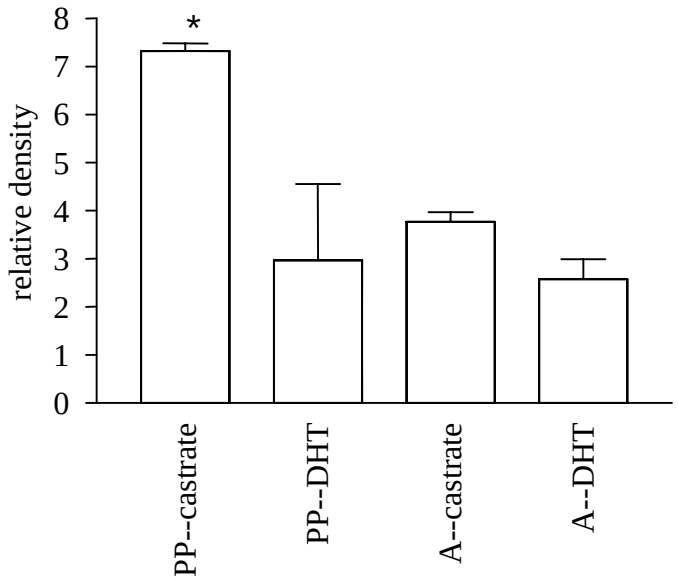


Figure 11: PKCα levels were down-regulated in prepubertal animals with 7-day androgen treatment, yet were unchanged in adults. Chemiluminescence was quantified by densitometer. n=3. 12 μg of protein was used per well and film was exposed for 1hour. PKCγ, which did not change with DHT-treatment, was used as a control. PP=prepubertal (approx. 250 gms). A=Adult (800-900 gms).
*p ≤ 0.05 compared to all other groups.

A brief examination of the immunoreactive PKCα and γ isozymes revealed that PKCα activity in prepubertal castrates directly followed the down-regulation of total

PKC (fig. 10) by 7-day androgen treatment. PKCγ was uneffected by androgen treatment. It was previously shown that PKC enzymatic activity in adult castrate seminal vesicle smooth muscle was unresponsive to androgen treatment (Mariotti *et al.*, 1992), as is shown in figure 11. However, in adulthood, total PKC enzymatic activity returned to prepubertal castrate control levels (Mariotti *et al.*, 1992), which was not represented in the immunoreactive PKCα fraction. A preliminary experiment to determine the effect of 5-day reserpine treatment on androgen-induced changes in PKCα was inconclusive which may be due to differences in length of androgen administration. No further study was conducted on PKC isozymes.

B. Specific Aim 2:

2. Norepinephrine stimulated proliferation *in vitro* in seminal vesicle-derived smooth muscle cells via the α_1-adrenoceptor.

The effect of fetal bovine serum (FBS) concentration on adult guinea pig seminal vesicle smooth muscle cell cultures was studied. Cells were initially plated at 1 x 10^4 cells per flask and cultured for six days with 5%, 7.5%, 10%, or 20% FBS. The concentration of FBS did effect cell number with increasing FBS yielding more cells.

Figure 12: The concentration of fetal bovine serum (FBS) effected cell number in seminal vesicle smooth muscle cultures

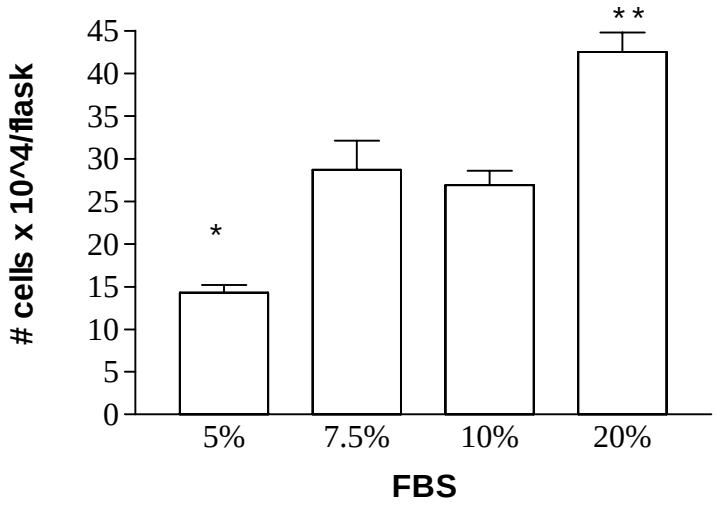


Figure 12: Adult normal guinea pig seminal vesicle smooth muscle cells were plated at 1 x 10^4 cells per flask. Varying concentrations of FBS were added to the media and cells were cultured for 6 days. n=8.
* and **: $p \leq 0.05$ compared to all other groups.

For studying the role of norepinephrine *in vitro*, 5% fetal bovine serum was used. Five percent was chosen since it allowed for increased cell number and was not maximal. A maximal rate of increase in cell number due to serum concentration was to be avoided as it may have masked any changes due to adrenergic agents

In vitro cell culture studies support the role of norepinephrine in male accessory sex organ smooth muscle proliferation. Primary cultures were made from adult

guinea pig seminal vesicle smooth muscles. Cells were then passed 5 times before plating 6-well culture dishes at $1x10^4$ cells per well and 6×10^4 cells per well.

Figure 13: The effect of plating density (1 vs. 6×10^4 cells/well) on 10 μM phenylephrine stimulation of seminal vesicle cell culture

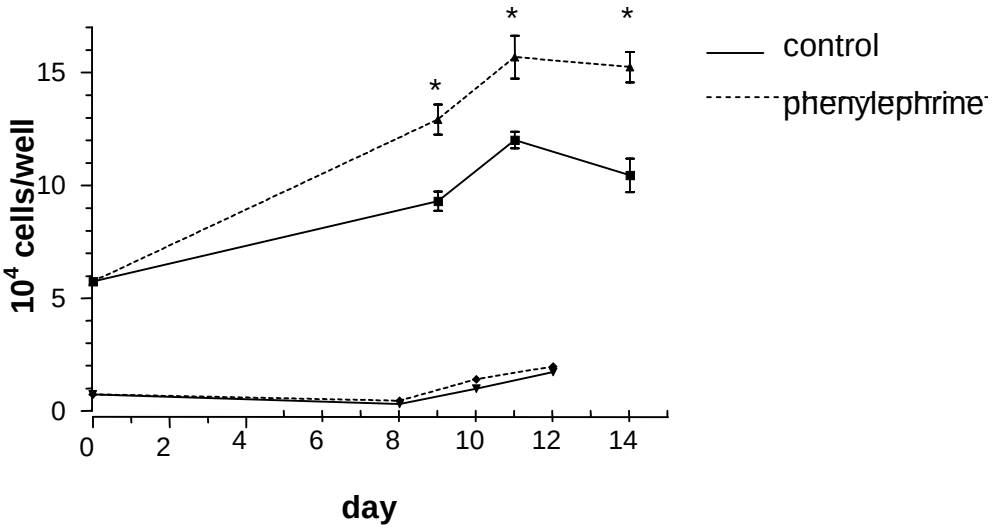


Figure 13: At low density plating, cells number showed no change from control when stimulated by 10 μM phenylephrine (n=6 for all groups and days.) 10 μM phenylephrine significantly enhanced cell number at high plating density. 6-well culture plates were used and media and adrenergic agents were changed daily.
*$p \leq 0.05$ compared to control.

Cells initially plated at the higher density of 6×10^4 were counted on days 9, 11, and day 14 after treatment with 10 μM phenylephrine, an α_1-adrenoceptor agonist, and

compared to controls counted on the same days. Phenylephrine stimulation increased cell number 44.2% on day 9, 31.6% on day 11 and 44.6% on day 14.

Cells plated at the lower density of 1×10^4 cells per well showed no increase in cell number by day 8 and no difference between control and phenylephrine on day 8 as well as subsequent days. Some of the cells initially plated at the lower density were maintained to day 35, with daily media changes as was done in the earlier counts. On day 35, phenylephrine-treated cells showed a significant, almost 2-fold increase over control cells. Control cell number remained constant from day 12 using this low serum concentration. Although not further pursued here, it is possible that growth factors secreted from the cells themselves play a role in the rate of increase of cell number.

Figure 14: Cell number increases in low density (1 x 10^4 cells/well) plated wells

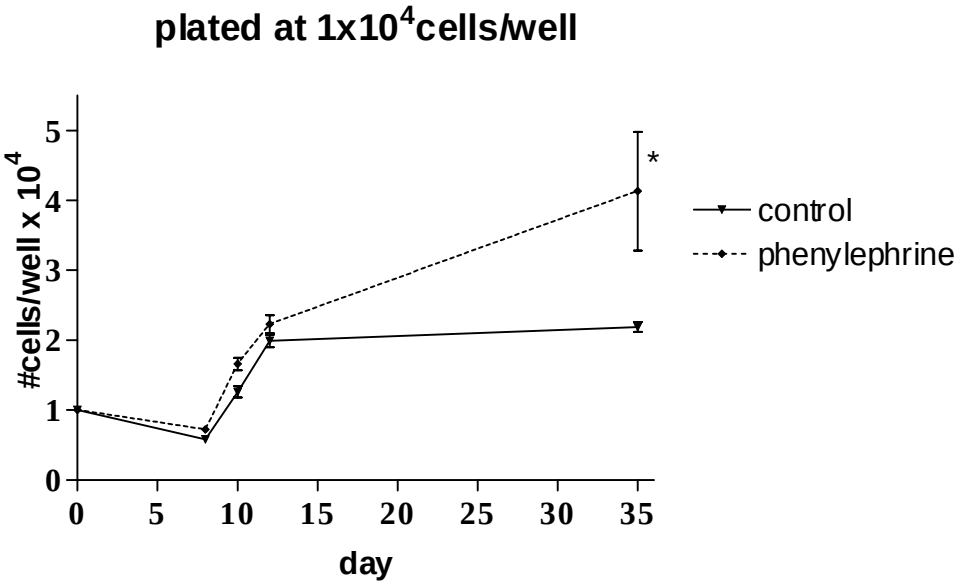


Figure 14: No difference was observed in phenylephrine-treated cells compared to control 8, 10 and 12 days post-plating. With long-term maintenance (35 days), a significant difference was seen. 6-well culture plates were used. n=6.
*p $\leq$ 0.05 compared to control.

Figure 15: The Effect of Various Adrenergic Agents on 14-day Seminal Vesicle Smooth Muscle Cell Culture

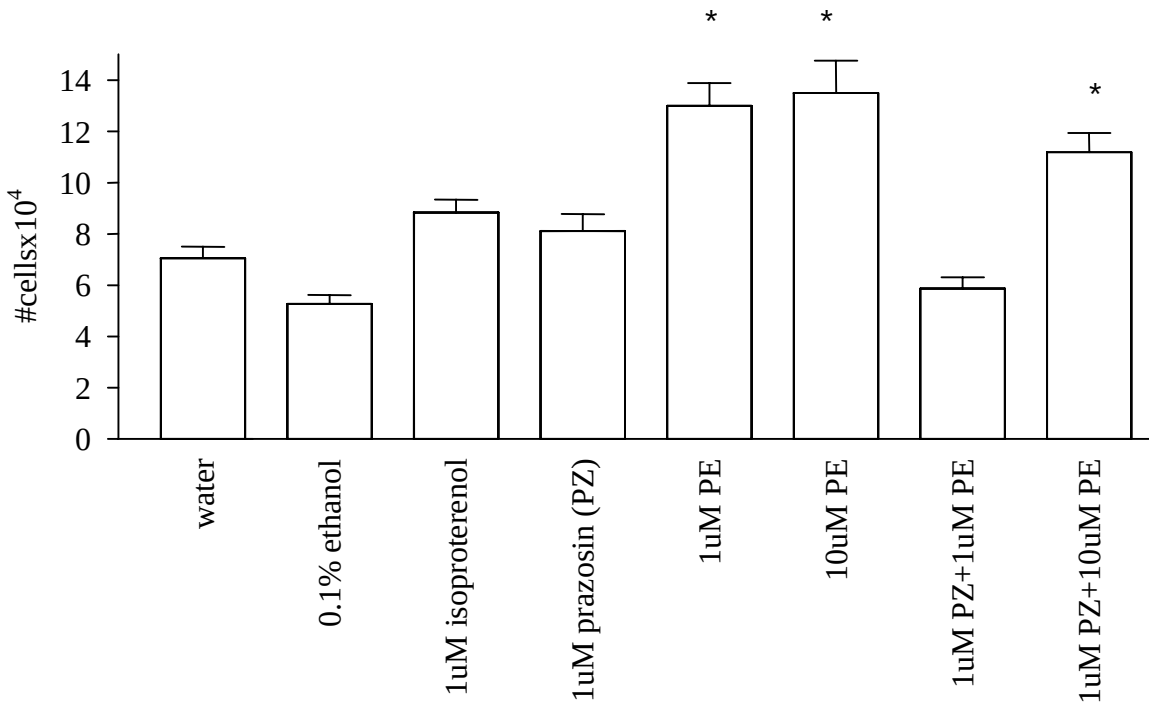


Figure 15: Increased cell number was due to α_1-adrenoceptor stimulation which was blocked by prazosin, but overcome with increased phenylephrine concentration. Water was the vehicle for isoproterenol. 0.1% ethanol was the vehicle for the other agents. Cells were initially plated at 6 x 10^4 cells/well (6 well plates) and maintained for 14 days. Media and adrenergic agents were changed daily. n=3. *$p \leq 0.05$ compared to other groups.

The use of adrenergic antagonists indicated that the increase in cell number was due to an α_1-adrenoceptor-mediated effect. Cells exposed to 1 μM isoproterenol, a β-adrenoceptor agonist, had no effect on cell number compared to its water control. Therefore, it suggested that the β-

adrenoceptor was not involved in the increase in cell number. Furthermore, the effect of 1 μM phenylephrine was blocked by 1 μM prazosin, an α_1-adrenoceptor antagonist. However, the antagonism caused by 1 μM prazosin could be overcome by increasing the phenylephrine concentration to 10 μM. These data indicated that the α_1-adrenoceptor mediated the phenylephrine-induced increase in cell number.

B. Specific Aim 2:

2. Norepinephrine did not function anti-apoptotically in seminal vesicle smooth muscle cells in prepubertal castrates treated with androgen.

Previous data have shown that adult guinea pig seminal vesicle smooth muscle exists in a terminally differentiated state *in vivo* that is resistant to changes in DNA in response to androgen ablation or repletion (Mariotti *et al.*, 1992). Two findings shown above raised the possibility that elevated NE release prevented castration-induced regression of smooth muscle cell number in adults through an anti-apoptotic mechanism. NE release in adult

castrates was 1) as high as in androgen-treated prepubertal castrates (fig. 1) and 2) unchanged by androgen treatment (fig. 1). Therefore, adult guinea pigs were orchiectomized and treated with reserpine to determine whether norepinephrine depletion would effect a net reduction in total DNA and involve an apoptotic mechanism.

Figure 16: Norepinephrine depletion did not effect DNA in adult seminal vesicle smooth muscle

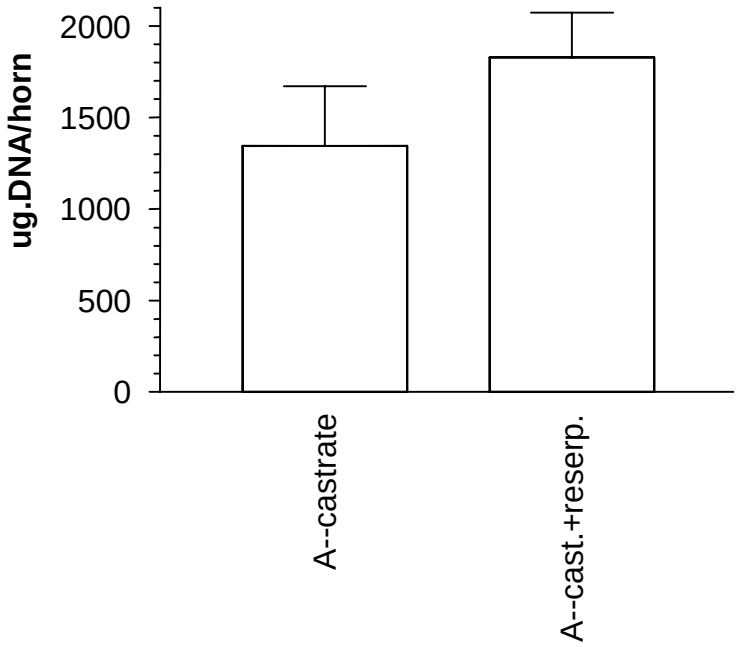


Figure 16: Depletion of norepinephrine by reserpine had no significant effect on µg. DNA compared to weight-matched controls. n=4-10 per group.

In adult castrates, DNA was not effected by reserpine treatment, precluding the proposed subsequent analysis of apoptosis. The potential role of apoptosis was further pursued in androgen and reserpine-treated prepubertal castates which exhibited a 42.4% reduction in cell number relative to their androgen-treated control (fig. 8). However, gel electrophoresis of nuclear extracts revealed that neither group exhibited apoptosis. That is, the characteristic apoptotic laddering from DNA oligonucleosomes was not seen in either group. Generalized heavy smearing indicating larger scale DNA degradation was also not seen, ruling out necrosis.

Figure 17: No apoptosis was detected in norepinephrine depleted prepubertal castrates treated with androgen

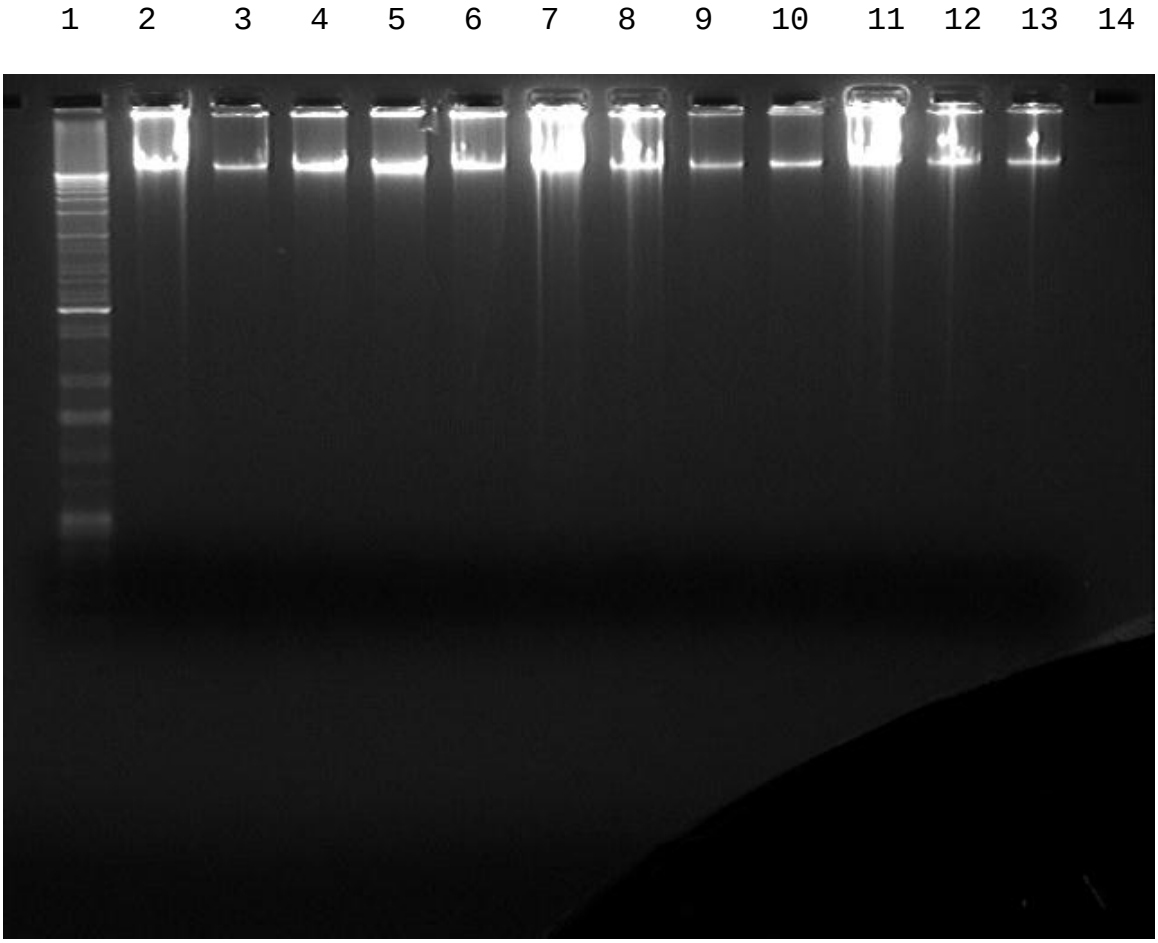


Figure 17: Lane 1 is a positive control (Kb Ladder). Lanes 2-6, and lane 13 are prepubertal castrates treated with 10 mg/kg DHT alone. Lanes 7-12 are prepubertal castrates treated with both DHT and 0.75 mg/kg reserpine. Lane 14 is an empty template, negative control.

It can be concluded that during five days of androgen treatment, concurrent reserpine treatement did not induce apoptosis at that point in time, despite the inhibition of androgen-induced changes in cell number.

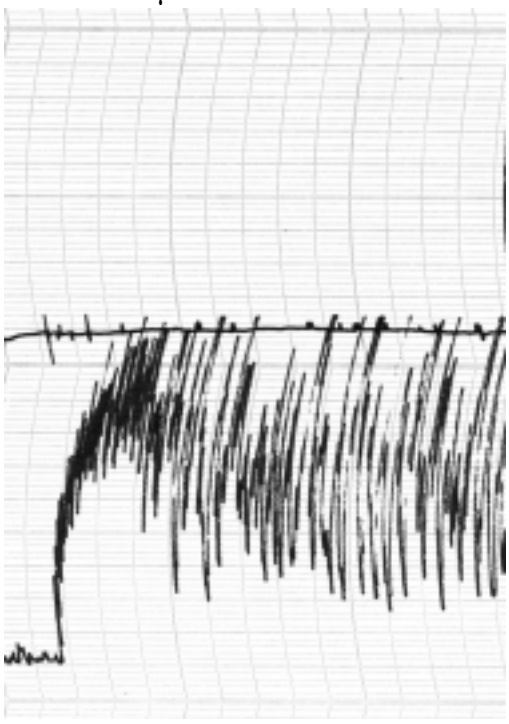
C. Specific Aim 3: Androgen decreased the number of norepinephrine-feedback-mediating, α_2-adrenoceptors in seminal vesicle smooth muscle.

Norepinephrine was shown, in Specific Aim 2 above, to mediate smooth muscle growth in prepubertal castrates treated with DHT. A potential mechanism for increased norepinephrine release involving decreased pre-synaptic feedback by the α_2-adrenoceptor was then examined. Other potential mechanisms were not pursued.

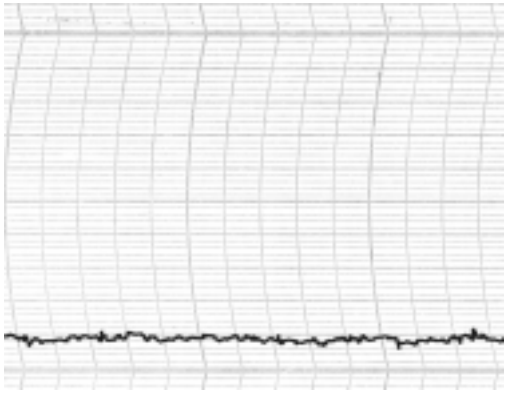
As a prelude to study of the presynaptic α_2-adrenoceptor, experiments were done to determine whether functional post-junctional α_2-adrenoceptors were present. Post-junctional α_2-adrenoceptors can mediate contraction and have been identified in dog vascular smooth muscle post-synaptically (Langer *et al.*, 1980). The presence of such receptors would preclude the use of the proposed electrical field stimulation experiments in isolated organ baths to study the presynaptic feedback of endogenous norepinephrine. By using BHT-933, a post-junctional α_2-adrenoceptor agonist (Timmermans and van Zwieten, 1980), the presence of post-junctional α_2-adrenoceptors was studied, using contractile response as an end-point.

Figure 18: BHT-933 was unable to elicit post-junctional α_2-adrenoceptor-mediated contractions in adult normal guinea pig seminal vesicle smooth muscle

10 μM BHT-933



10 μM methoxamine



10 μM methoxamine after 30 min.incubation with 10^{-7} M prazosin

Figure 18: 10 μM BHT-933 did not elicit a post-junctional α_2-adrenoceptor mediated response. 10 μM methoxamine, an α_1-adrenoceptor agonist, $\pm$ prazosin was used as a positive control in eliciting contraction. n=4.

BHT-933 was unable to elicit a contractile response in seminal vesicle smooth muscle (fig. 18), even at a supramaximal concentrations of 10 μM (Nielson *et al.*, 1989; Timmermans and van Zwieten, 1980), suggesting the absence of functional post-junctional α_2-adrenoceptors for this parameter. The smooth muscle tissues were then tested to confirm viability with 10 μM methoxamine as a positive control, which did elicit contractions. These contractions were completely blocked by prazosin (fig. 18).

Electrical field stimulation (EFS) studies of seminal vesicle smooth muscle contraction in the presence and absence of 1μM rauwolscine, an α_2-adrenoceptor antagonist were performed. It was expected that rauwolscine would block presynaptic α_2-adrenoceptor-mediated norepinephrine feedback, and thus enhance EFS-induced contractions. The working hypothesis was that in prepubertal castrate animals, androgen treatment, which caused an increase in NE release (fig. 1), would result in significant reductions in rauwolscine-enhanced EFS contractions relative to the prepubertal castrate control.

Figure 19: An example of electrically field stimulated contractile tracings from isolated seminal vesicle smooth muscle tissues

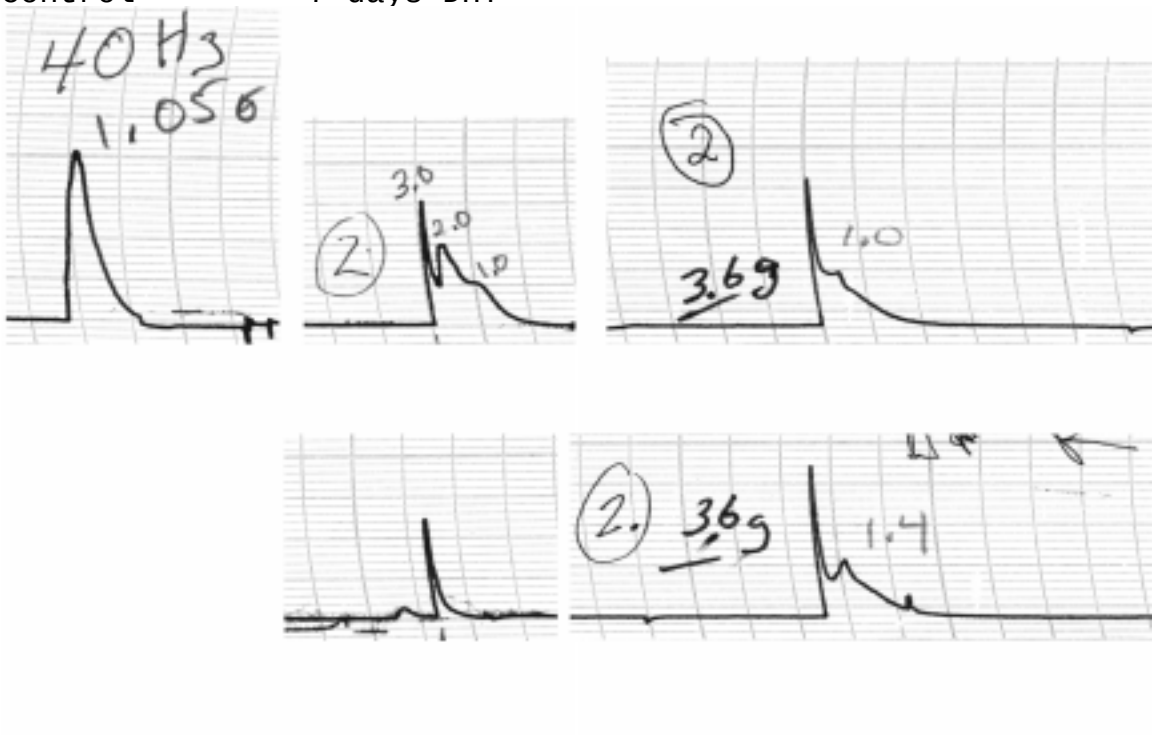


Figure 19: Only qualitative comparisons could be made due to differences in transducer sensitivities and tissue behavior. Tissues were stimulated at 2, 5, 10, 20, 30 and 40 Hz for 30 seconds with each pulse being of 0.5 msec duration. Tracings from a 40 Hz stimulation are shown above. Prepubertal castrates showed only one contractile peak that occurred at the moment of stimulation and was not abolished by prazosin. The other groups had a biphasic or multiphasic response. In these groups, prazosin abolished all peaks except the initial peak. All peaks increased with increasing Hz. Prepubertal guinea pigs weighed approx. 250 gms. Adults weighed 500-550 gms. Each group had an n=6-8.

While all groups showed increasing contractile response to increasing frequency of stimulation, inter-group comparisons could not be made because the contractile responses were qualitatively different. Prepubertal castrate controls exhibited only a single contraction in both the control and rauwolscine-treated tissues that occurred immediately to stimulation. This response was not inhibited by prazosin. Adult normals, castrates, and androgen-treated castrates showed a biphasic response, and prepubertal castrates treated with dihydrotestosterone showed multiple contractile peaks. In all groups, prazosin abolished all contractile responses except the initial, very rapid upsweep of the tracing. This initial contraction has been described as being ATP-mediated in the guinea pig vas deferens (Burnstock *et al.*, 1972; Westfall and Stitzel, 1978), and appears to be present in prepubertal castrates as well as all of the other experimental treatment groups.

In the groups whose tracings did show adrenergic, contractile peaks, 1 μM rauwolscine caused enhancement to varying degrees, indicating that the pre-synaptic α_2-adrenoceptor was functional. The notable absence of the functional α_1-adrenoceptor-mediated contractile response in

prepubertal castrates prevented further use of this particular experimental design to assess changes in pre-synaptic α_2-adrenoceptor mediated effects on NE release. The presence of a functional α_1-adrenoceptor-mediated contractile response in prepubertal castrates after androgen-treatment, however, suggested that androgen may be important in the development of the α_1-adrenoceptor. This follows evidence suggested in earlier studies that the development of α_1-adrenoceptor-mediated contraction in rat vas deferens coincided with the onset of puberty and endogenous androgen production (MacDonald and McGrath, 1984).

The effect of α_2-adrenoceptor antagonism by rauwolscine on EFS overflow of norepinephrine was not studied. Preliminary studies using supramaximal stimulation (40 V, 40 Hz, 30 seconds) in the presence of 1μM rauwolscine in 10 ml organ baths did not yield detectable norepinephrine concentrations without additional extraction techniques.

Ligand binding experiments were then performed using [^{3}H]RX821002 to identify and quantify α_2-adrenoceptors in seminal vesicle from normal adult males (500-550 gms). Studies to determine binding rate and protein dependence

used 0.4 nM of tritiated ligand, the lowest concentration to be employed in subsequent saturation analyses.

Figure 20: [^{3}H]RX821002 binding rate and protein dependence of [^{3}H]RX821002-binding in adult normal guinea pig seminal vesicle smooth muscle

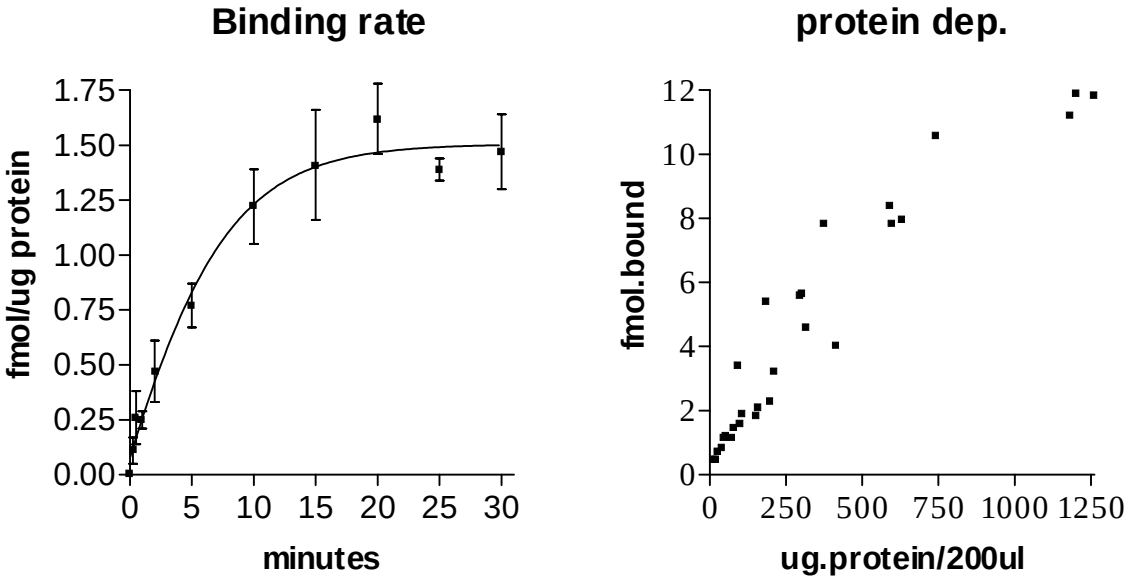


Figure 20: Binding reached equilibrium by 15 minutes, and was linearly protein dependent between 12 and 1260 μg of protein/200 μl when using 0.4 nM [^{3}H]RX821002 in adult normal guinea pigs weighing 500-550 gms. n=4.

Using 0.4 nM [^{3}H]RX821002, data plotted using the GraphPad computer program, showed that a 15 minute incubation provided for binding equilibrium. All subsequent experiments were done using 20 minute incubations. Protein dependence experiments also showed that the femtomoles of α_2-adrenoceptors bound were linearly

dependent upon the amount of protein present between 12 and 1260 μg in the 200 μl reaction mixture.

Binding affinity as well as the number of sites were determined using [^{3}H]RX821002 concentrations ranging from 0.40 to 9.0 nM ± 10^{-4} M non-radioactive rauwolscine. Results were calculated using both saturation binding curves by GraphPad statistical program and its linear transformation to a Scatchard-Rosenthal plot.

Figure 21: Saturation binding curves and Scatchard-Rosenthal plots for the α_2-adrenoceptor using [^{3}H]RX821002 in normal adult guinea pig seminal vesicle smooth muscle

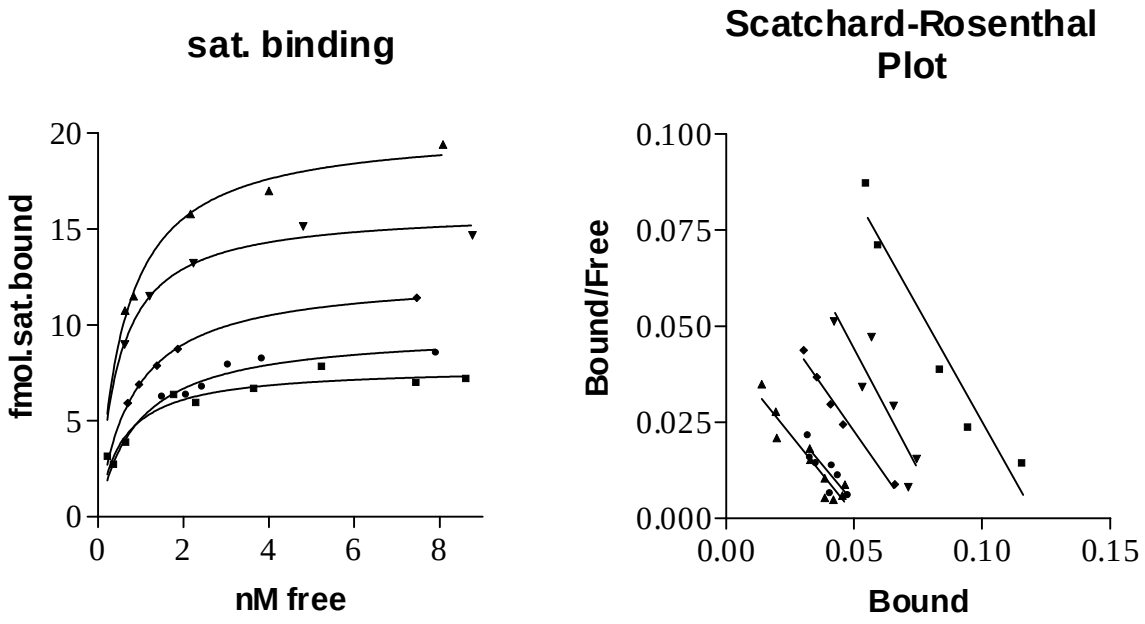


Figure 21: [^{3}H]RX821002 concentrations were used ranging from 0.40 to 9.0 nM ± 10^{-4} M non-radioactive rauwolscine. K_d=0.67 ± 0.08 nM according to the saturation binding curve and 0.65 ± 0.08 nM by Scatchard-Rosenthal linearization. The curves depicted reflect different amounts of protein used on each experimental day. When corrected for protein, B_{max}=11.78 ± 1.62 $^{fmol}/_{mg}$. Adult normal guinea pigs weighing 500-550 gms were used.

The saturation binding curve showed a K_d = 0.67 ± 0.08 nM. The Scatchard-Rosenthal plot gave a K_d = 0.65 ± 0.08 nM. When corrections for the varying protein amounts used on the experimental days were made, the number of α_2-adrenoceptor binding sites (B_{max}) was determined to be 11.78 ± 1.62 $^{fmol}/_{mg}$ membrane protein.

K_d comparisons can only be made to other tissues and species since there were no other known studies using RX821002 in guinea pig seminal vesicle smooth muscle or any other smooth muscle specimen. From research employing rat cortex, spleen and kidney, and guinea pig kidney (Erdbrugger, 1995), the K_d values were 0.6 ± 0.0, 0.4 ± 0.1, 1.5 ± 0.8, and 4.3 nM respectively. The 0.67 nM K_d determined in guinea pig seminal vesicle smooth muscle is within the reported range.

Competition assays using 5 nM [^{3}H]RX821002, a saturating concentration, were done to characterize the receptor subtype present.

Table 2: Comparison of IC50's and affinity (K_i) drugs for the α_2-adrenoceptor, using 5 nM [^{3}H]RX821002 in adult normal guinea pig seminal vesicle smooth muscle

Drug	IC50	K_i
RX821002	0.67 nM(K_d from saturation experiments)	
Phentolamine	220 nM	26.00 nM
Clonidine	310 nM	36.63 nM
Rauwolscine	420 nM	49.63 nM
Prazosin	35000 nM	4136 nM

Table 2: IC50's were determined from hand-drawn, log-logit inhibition plots, and were confirmed by curves drawn by GraphPad computer program. K_i's were calculated using the IC50's derived by the handdrawn curves and the Cheng and Prusoff equation, and were also confirmed by GraphPad. Adult normal guinea pigs weighing 500-550 gms were used. n=4.

The rank order of potency for the drugs employed above is similar to the rank order of drugs quantified in other tissues with α_{2D}-adrenoceptors (Table 3). Qualitatively, the α_{2D}-adrenoceptor differs from subtypes A, B, and C in that phentolamine was a more potent antagonist than rauwolscine (Table 3, below).

Table 3: Ratio of K_i of competetitor to respective K_d of RX821002 in experiments done in guinea pig seminal vesicle smooth muscle compared to other tissues with α_{2D}-adrenoceptors

	seminal vesicle	bovine		rat	human clones[c]		
Drug	smooth muscle	iris[a]	pineal[b]	cloned 2D[b]	2A	2B	2C
phentolamine	38.8	48.3	9.4	9.1	14.1	6.47	35.1
rauwolscine	74.1	85.8	28.8	35.2	1.1	0.35	0.35
prazosin	6173	26241	4151	1648	1041	29.5	29.7

Table 3: a. data from Bylund (1997)
b. data from O'Roarke (1994) and Simmonneaux(1991)
c. data from Bylund (1992) and O'Roarke (1994)

5-hydroxytrypamine (5HT) was used to determine if [^{3}H]RX821002 had any interaction with serotonin receptors. 5HT at concentrations as high as 1 μM had no effect on binding. Since RX821002 has been shown to bind to non-adrenergic imidazoline receptors in rabbit kidney basolateral membranes (Coupry *et al.*, 1987), clonidine was used to rule out the binding of imidazoline sites. The notable finding was that the Ki ratio of phentolamine to clonidine was much lower in α_2-adrenoceptor sites than imidazoline sites in human brain cortex (1.52 versus 17.01) (Miralles *et al.*, 1993). In the seminal vesicle smooth muscle the clonidine to phentolamine K_i ratio was 1.38, indicating that relative to imidazoline sites, [^{3}H]RX821002 preferentially bound to the α_2, subtype D, adrenoceptor.

Using a single 5 nM saturating concentration of [^{3}H]RX821002, experiments utilized seminal vesicle smooth muscle preparations derived from the four treatment groups to assess potential changes in the maximal number of binding sites relative to known changes or lack thereof in NE release.

Figure 22: Number of α_{2D}-adrenoceptor binding sites (B_{max}) in seminal vesicle smooth muscle among various treatment groups.

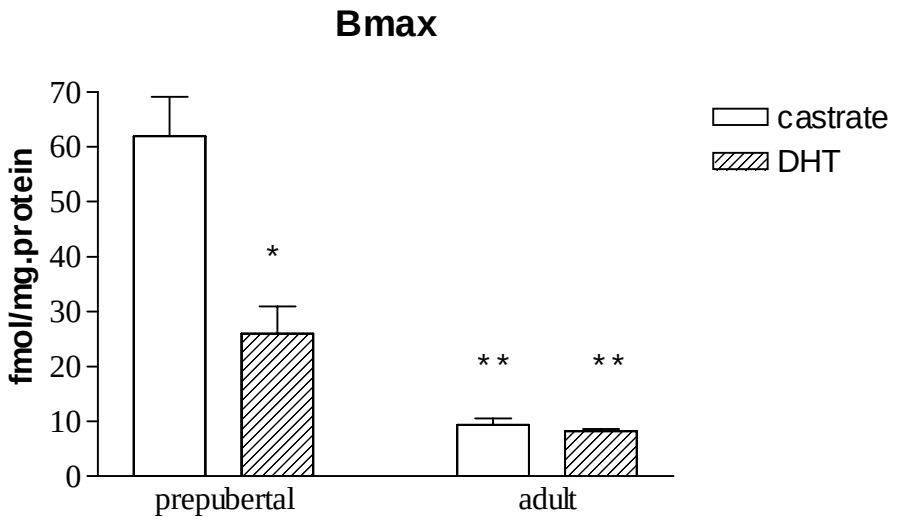


Figure 22: The number of α_{2D}-adrenoceptor binding sites was reduced with androgen exposure in prepubertal seminal vesicle smooth muscle and was unchanged by androgen in adult tissues. The number in parentheses is the ratio of α_2-adrenoceptor B_{max} to norepinephrine released (DHPG/NE) Prepubertal guinea pigs weighed approx. 250 gms. Adults weighed 500-550 gms. Four separate experiments were done using multiple animals in each group.
*$p \leq 0.05$ compared to prepubertal castrate control.
*$p \leq 0.05$ compared to prepubertal castrate control and prepubertal DHT-treated.

The lack of contraction by BHT-933 (fig. 18) could be assumed to be indicative of an absence of post-synaptic, contractile-mediating α_2-adrenoceptors. Therefore, it could be implied that any quantification of α_2-adrenoceptors by RX821002 may be more closely correlated with norepinephrine-feedback-mediating α_2-adrenoceptors. It appeared that androgen down-regulated the number of α_2-adrenoceptors during the proliferative, prepubertal phase in which norepinephrine release is induced by androgen. In adulthood, the number of norepinephrine-feedback-mediating α_2-adrenoceptors remained low and androgen-resistant while norepinephrine release was sustained at high levels equivalent to androgen-treated prepubertal castrates. This androgen-induced decrease in the norepinephrine-feedback-mediating, α_2-adrenoceptor may be mechanistic for the increase in norepinephrine release observed in response to androgens in the prepubertal seminal vesicle (fig. 1).

VI. Discussion

The goal of this research was to understand the role of norepinephrine in the growth of male accessory sex organ smooth muscle. The results showed that androgen-induced post-ganglionic norepinephrine release (fig. 1) in seminal vesicle smooth muscle (fig. 3) and was involved in the regulation of proliferation (fig. 8). This increased norepinephrine release was accompanied by down-regulation of the α_2-adrenoceptor population (fig. 22). This discussion will more closely explore these findings, suggest future directions stemming from this data, and examine the potential therapeutic significance.

In prepubertal castrates treated with androgen, smooth muscle DNA increased 138% over control while epithelial DNA increased 759% (fig. 8). The androgen-induced increase in NE release follows the time course of DNA synthesis (Mariotti *et al.*, 1992) and first significant increase in total DNA. Depletion of norepinephrine by $\leq$ 99% of control by reserpine reduced the smooth muscle increase to 37% over control without disrupting the androgen-induced proliferation of the cholinergically innervated epithelium. This suggests that norepinephrine mediates the androgen-induced proliferative response in smooth muscle. However,

it did not indicate whether this effect was due to enhanced proliferation or decreased apoptosis.

It was hypothesized that the inability of castration to reduce cell number in adult smooth muscle (Mariotti *et al.*, 1992) may have been due to the unexpected finding of the sustained, androgen-resistant elevation in norepinephrine release (fig. 1). If norepinephrine were anti-apoptotic, reserpine-treatment would enhance apoptosis and decrease DNA. However, depletion of norepinephrine in adult castrates by reserpine did not decrease cell number relative to controls (fig. 16). In androgen-treated prepubertal castrates, treated concurrently with reserpine, there was a decrease in DNA compared to androgen-treated controls (fig. 17). However, agarose gel electrophoresis failed to detect any apoptotic laddering. Because apoptotic products are quickly phagocytized (Lockshin and Beaulaton, 1974), it is possible that apoptosis had occurred at an earlier time point and thus was undetected on the fifth day of treatment. Gel electrophoresis is also limited in detecting small numbers of apoptotic nuclei. If apoptosis were to be further examined, double staining to detect both *in situ* end labeling (ISEL) and chromatin condensation should be employed (Tatton *et al.*, 1998). The onset of androgen-induced DNA synthesis is at day 3 of treatment and

increases in graded fashion to peak on day 7. Androgen-induced increases in total DNA were first detected on day 5 (based on analyses from days 3, 5, and 7 of androgen treatment) (Mariotti et. al, 1992). Therefore, the effect of reserpine on androgen-induced increases in cell number probably reflects an anti-proliferative effect. Further pursuit of this could explore cell cycle dependent markers of proliferation such as Ki-67, PCNA or MIB-1 (Schipper *et al.*, 1998) in response to androgen $\pm$ reserpine treatment.

Although there is sustained elevation of norepinephrine release (fig. 1), seminal vesicle smooth muscle from orchiectomized adults failed to exhibit any change in DNA in response to androgen repletion (Mariotti *et al.*, 1992). This androgen-resistant elevation of norepinephrine release in adulthood (fig. 1) combined with the relatively high PKC activity (Mariotti *et al.*, 1992), that is also androgen-resistant, indicate that increased norepinephrine release became uncoupled from the mitogenic response at or before the level of stable activation of PKC. Specifically, with the onset of androgen-exposure in prepubertal castrates, seminal vesicle smooth muscle norepinephrine release was upregulated and maintained into adulthood when it was androgen-resistant (fig. 1). PKC was stablely activated then down regulated in androgen-treated

prepubertal castrates. However, in adulthood, PKC returns to the elevated, prepubertal castrate control levels in association with differentiation to an amitotic state (Mariotti *et al.*, 1992). This uncoupling of increased norepinephrine release from PKC activation and subsequent depletion appears to be a critical event in the resistance to proliferation demonstrated in adult smooth muscle. Future investigation focusing on the mechanism of PKC resistance to α_1-adrenoceptor stimulation should provide pertinent information about the normal terminal differentiation process of this androgen sensitive smooth muscle.

Prepubertally, before uncoupling of norepinephrine and PKC occurs, norepinephrine depletion by reserpine can prevent the androgen-induced down-regulation of PKC (fig. 10). Androgen down-regulated PKCα in prepubertal castrates (fig. 11), but in adulthood, the α-isozyme did not return to prepubertal castrate control levels as has been demonstrated with PKC (Mariotti *et al.*, 1992). However, it is possible that this discrepancy is due to differences in quantification methods. Immunochemilumiscence was employed to determine isozyme quantities while enzymatic activity was employed to quantify PKC.

The androgen-dependent down-regulation of α_2-adrenoceptors (fig.22) may be a potential mechanism for increased norepinephrine release. However, whether these analyses actually represent pre-synaptic α_2-adrenoceptors that mediate the feedback inhibition of norepinephrine release, remains to be determined. Studies of BHT-933 effects failed to identify post-junctional α_2-adrenoceptor mediated smooth muscle contraction (fig. 18), but the failure to detect these post-junctional receptors does not rule out extraneuronal binding sites. Future studies should be pursued to determine whether a cause and effect relationship exists between the increased norepinephrine release and decreased α_2-adrenoceptor population during growth of smooth muscle cells. *Ex vivo* electrical field stimulation studies to measure increased norepinephrine release under conditions in which α_2-adrenoceptor agonist and antagonist effects can be quantified should provide more relevant insight into the role of the pre-synaptic receptor and norepinephrine release. Pursuit of these experiments may require the use of reuptake inhibitors and an organ bath designed with minimal volume to allow for norepinephrine measurement with graded electrical field stimulation rather than supramaximal and prolonged

stimulation commonly employed for these types of experiments (LaPierre *et al*., 1993; Ventura *et al*., 1998).

A major area for future examination is the potential role of the extracellular matrix in proliferation. The cell culture experiments performed here suggest extracellular factors may be involved. *In vivo* adult smooth muscle does not proliferate in response to androgen and the elevated endogenous release of norepinephrine. Yet, adult seminal vesicle smooth muscle cells proliferated in culture and were sensitive to the mitogenic effects of phenylephrine (fig. 13, 14, and 15). Cell number also increased at varying rates that appear to be dependent upon the plating density and confluence (fig. 13 and 14).

This change to a proliferative state in culture may be due to a decrease in anti-proliferative extracellular matrix heparan sulfate proteoglycans. Neonatal or neointimal matrices have been shown to stimulate vascular smooth muscle cell growth more efficiently than matrix from adult cells (Hein *et al*., 1996). This growth was further increased by the enzymatic digestion of extracellular matrix heparan sulfate (Hein *et al*., 1996). Heparan sulfate proteoglycans have the potential to function as growth inhibitors (Castellot *et al*., 1981; Bingley *et al*., 1998) in vascular smooth muscle cell cultures and can

prevent neointimal formation *in vivo* (Bingley *et al*., 1998). An eight-fold greater anti-proliferative potency was measured for cell surface heparan sulfate proteoglycans extracted during confluence as compared to the exponential growth or postconfluent phases (Fritze *et al*., 1985). While the anti-proliferative effect of heparan sulfate has not been clearly elucidated, heparin, a molecule closely related to heparan sulfate, can inhibit PKC activity in intact cells (Feige *et al*., 1989). It has also been shown in mixed micellar assays that heparan sulfate is a weak inhibitor of PKC (Herbert and Maffrand, 1991). These observations raise the possibility that the resistance *in vivo* to proliferation in adult seminal vesicle smooth muscle, which involves PKC resistance to α_1-adrenoceptor stimulation, may be linked to the expression of cell surface anti-proliferative heparan sulfate proteoglycans.

Lastly, the possible therapeutic significance of this work should be recognized. Depletion of norepinephrine by reserpine *in vivo* in prepubertal castrates selectively perturbed the androgen-induced increase in smooth muscle mass and DNA (fig. 8). This, along with other evidence accrued in this work, indicates that norepinephrine does indeed play an obligatory role in androgen-induced proliferation. Importantly, only the smooth muscle was

inhibited. The epithelium was not effected by reserpine. This is the first demonstration of selective antagonism of androgen-induced increases in DNA in accessory sex organ smooth muscle. While systemic reserpine treatment could not be used therapeutically for BPH due to multiple systemic adverse effects, the principle has been established that it is pharmacologically feasible to inhibit androgen-induced growth of seminal vesicle smooth muscle without perturbing the epithelium. Future studies should be performed to determine whether the inhibition of the proliferative response by short-term reserpine treatment in the presence of sustained androgenic stimulation is irreversible. Selective inhibition of the mitogenic response without antagonism of the differentiating effects of androgen may result in irreversible and premature cessation of smooth muscle growth. This experiment could be particularly enlightening regarding the future development of short term therapies in young adults that could prevent symptomatic BPH in men 60 years of age and older.

In addition, if α_2-adrenoceptor agonists decrease norepinephrine release and inhibit androgen-induced smooth muscle proliferation, attempts could be made to pharmacologically upregulate the expression of the pre-

synaptic, norepinephrine-feedback-mediating α_2-adrenoceptor. Understanding the sequencing of the promoter region of the α_2-adrenoceptor gene could lead to the rational development of ligands to possibly maintain irreversible expression at high levels. Theoretically, such compounds may include neurosteroids that could selectively upregulate pre-synaptic α_2-adrenoceptor expression without stimulating androgen receptor mediated male accessory sex organ smooth muscle growth. For example, progesterone has been found to be derived from rat glial cells in culture (Jung-Testas *et al.*, 1989) and functions to modulate GABAergic transmission in the central nervous system (Mahesh *et al.*, 1996). Hammond (1978) has identified progesterone in the human prostate at concentrations (0.39 ± 0.07 ng/g tissue) in the same range as the biologically active androgens, testosterone (0.25 ± 0.04 ng/g tissue) and dihydrotestosterone (1.22 ± 0.14 ng/g tissue). However, no biological role of this steroid in male accessory sex organ has been elucidated to date. The idea that endogenous steroids with neurospecific effects may exist within male accessory sex organ is intriguing and worthy of experimental pursuit.

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Hepatology **23**: 288-293. 1996.

Curriculum Vitae

Education:

Currently in a joint MD/PhD program. West Virginia University School of Medicine.

May 1999*:* PhD in Pharmacology

May 2001: MD expected.

1995: Awarded the Korean-American Medical Association Medical Student Fellowship

Masters in Fine Arts (Creative Writing). Brown University, Providence, RI. May 1993.

Awarded a University Fellowship 1991.

Masters thesis: *"N=1": A Yellow Girl in Appalachia.* A collection of short stories and poetry. Awarded the Rose Lowe Rome Award for best manuscript, 1993.

Wesleyan University, Middletown, CT. BA in English, Magna cum laude, May 1991.

Honors thesis awarded Best Manuscript, 1991.

Phillips Exeter Academy (prep school), Exeter, NH.

Graduated May 1987.

Research:

Studied the role of norepinephrine release in androgen-dependent accessory sex organ smooth muscle proliferation and differentiation using the guinea pig seminal vesicle as a model for human benign prostatic hypertrophy. Worked under the supervision of Michael Mawhinney, Ph.D., Professor of Pharmacology and Toxicology with an appointment in the Dept. of Urology, West Virginia University School of Medicine.

Presentations:

March 1996: Poster at the International Symposium on the Biology of the Prostate, Washington, D.C., sponsored by the NIH

May 1996: Poster at the American Urologic Association, Orlando, FL., sponsored by the AUA.

May 1996: Poster at the Pfizer Pharmaceuticals Research Evening, Orlando, FL, sponsored by Pfizer Pharmaceuticals.

March 1997: Poster at West Virginia University School of Medicine Research Day.

July 1997: Poster at the National Student MD/PhD conference, Aspen, CO.

March 1999: Presentation at West Virginia University School of Medicine Van Liere Convocation.

March 1999: Poster at West Virginia University School of Medicine Research Day.

Teaching Experience:

Assistant Professor of Creative Writing. Brown University. September 1992-May 1993.
Had full responsibility of teaching from planning syllabi, conferences, and grade determination.

Lecturer for Nursing Pharmacology. West Virginia University School of Medicine. Spring 1997 and 1998.
Lectures on Asthma Therapy and Fertility.

Teaching Assistant for Medical Pharmacology. West Virginia University School of Medicine. Spring 1996 and 1998.
Led weekly Problem Based Learning sessions for 10-12 medical students.

Extracurricular Activities:

Mother of 1 and 2 year old daughters. Duties include everything possible 24 hours per day, seven days per week, 52 weeks per year.

Search Committee Member for the Chair of the Dept. of Pharmacology and Toxicology, West Virginia University, School of Medicine. December to July 1999.

Student MD/PhD representative. West Virginia University School of Medicine. July 1996-November 1997.
Scheduled meetings and speakers as well as represented the students in administrative meetings.

Counselor. Rape and Domestic Violence Information Center, Morgantown, WV. July 1995-February 1997.
Duties included meeting victims at emergency rooms and police stations, as well as crisis intervention by telephone.
Volunteered 40 hours per month.

Editor-in-Chief. *The Bedpan: Modern Movements in Medicine*. West Virginia University School of Medicine monthly student newspaper. April 1994-April 1995. Supervised and delegated all tasks necessary to publish a newspaper from assigning articles to lay-out and finance.

Articles Editor. *The Bedpan*. August 1993-March 1994.

Student Academic Committee. West Virginia University School of Medicine. September 1993-May 1995.
Elected to 2 consecutive terms to represent medical students in academic issues ranging from curriculum to exam scheduling.