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Novel *in vitro* models to investigate pharmacological targets in genital resistance vasculature

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Abstract

Introduction

Male and female sexual dysfunctions are prevalent, multifactorial disorders, which significantly impact on the quality of life of sufferers. The development of treatments for male sexual dysfunction has been based on an understanding of the function of the genital tissues. However, much of this knowledge has been gained using techniques to investigate responses of penile and vaginal tissue strips to exogenous agonists and antagonists. In the few studies that have considered the function of isolated male penile arteries it has been demonstrated that vascular responses may differ from those of penile tissues. A fuller understanding of the function of isolated penile arteries will provide a more sophisticated approach to novel pharmacological therapies for male sexual dysfunction.

In addition, very little research has been carried out into the physiology of female genital tissues and no studies have considered the function of isolated female genital arteries. Treatments for sexual dysfunction in the female sex have largely been based on successes in the male field with mixed results. A fuller understanding of the function of female vaginal arteries may provide a more coherent basis for the development of novel pharmacological therapies for female sexual dysfunction.

The present study utilised the method of small vessel wire myography to perform a detailed pharmacological investigation into the vascular function of isolated genital arteries from male and female New Zealand White rabbits. The arteries chosen for study were the male dorsal and cavernous arteries and the female vaginal artery, divided into two preparations, upstream 'extra-vaginal' artery (EVA) and downstream 'intra-vaginal' artery (IVA). Mechanisms of vasoconstriction and vasodilation were examined and related to current published knowledge.

Adrenoceptor-Mediated Responses

Male and female arteries were shown to be innervated by sympathetic adrenergic nerves using immunohistochemistry and/or EFS. During the investigation of adrenoceptor (AR)-mediated pathways, noradrenaline (NA)-induced vasoconstriction and active NA uptake mechanisms were demonstrated in both penile and vaginal arteries. AR-induced vasoconstriction was mediated by a combination of α_{1A} -ARs and α_2 -ARs, potentially the

α_{2A} -AR subtype, in male penile arteries. The α_2 -AR was shown to be of particular importance in the vasoconstriction of dorsal penile arteries; reflected by the potency order of AR agonists, UK 14,304 > Medetomidine > NA = phenylephrine (PE). A clear role for α_{1A} -ARs was demonstrated in the cavernous arteries, while α_2 -ARs could not be excluded.

In female vaginal arteries the presence of functional α_1 -ARs was confirmed. However, α_2 -ARs were shown to make little or no contribution to AR-mediated responses in isolated vaginal arteries. The lack of α_2 -AR-mediated responses highlights a clear gender difference between male and female rabbits that was reflected in the order of agonist potencies in female vaginal arteries, NA = UK 14,304 = PE = Medetomidine.

Basal AR activity, either via tonic adrenergic nerve or constitutive AR activity, was considered to be responsible for the maintenance of endogenous tone in both male and female genital arteries.

Nitric Oxide-Mediated Responses

The contribution of nitric oxide (NO) to endothelium-dependent, direct smooth muscle and nerve mediated relaxation was investigated during the current study. Direct stimulation of smooth muscle by the NO donor drug, sodium nitroprusside (SNP), caused vasodilation of both male and female genital arteries. However, this relaxation was not potentiated by the phosphodiesterase type 5 (PDE-5) inhibitor, UK 343,664; which was unexpected considering the success of Viagra[®], another PDE-5 inhibitor, in the treatment of male erectile dysfunction.

Considering the wealth of data proposing NO as the main non-adrenergic, non-cholinergic (NANC) neurotransmitter in male penile tissues, surprisingly little evidence was found for NO involvement in electrical field stimulation (EFS)-induced relaxation in male penile arteries. In female vaginal arteries, a greater inhibition of EFS-induced relaxation by blockade of nitric oxide synthase (NOS) activity using N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME) was demonstrated than in male penile arteries. In female arteries, L-NAME predominantly inhibited EFS-induced responses at low frequencies while an L-NAME-resistant component persisted at high stimulation frequencies.

Acetylcholine (ACh)-induced, endothelium-dependent relaxation of male and female genital arteries was mediated by a combination of NO and K⁺ channel activity. K⁺ channel activity was demonstrated by inhibition of K⁺ channels using apamin and charybdotoxin

and was, therefore, potentially due to release of endothelial-derived hyperpolarising factor (EDHF). No role was demonstrated for prostacyclin in ACh-induced relaxation of male or female genital arteries. Relative contributions of NO and K⁺ channels (EDHF-like vasodilation) to endothelium-dependent vasodilation demonstrated significant gender differences. Vasodilation was predominantly mediated by NO in male dorsal arteries, K⁺ channels in female EVA and a combination of both in smaller IVA and cavernous arteries. Contrary to previous studies, the involvement of EDHF was demonstrated to be greater in upstream sections of the vaginal artery (EVA) compared to smaller downstream sections (IVA). This study was the first to determine the relative contributions of EDHF and NO to endothelium-dependent relaxation of male and female genital arteries.

Vascular tone was increased in the presence of blockers or inhibitors of NOS or K⁺ channels. Therefore, basal release of NO was found to contribute significantly to the maintenance of endogenous tone in both male and female genital arteries. The unique observation of L-NAME-sensitive, pronounced, spontaneous vasodilations in the EVA demonstrated the importance of NO in the maintenance of vascular tone in this artery. Basal K⁺ channel activity, thought to be due to a basal release of EDHF, contributed to the maintenance of endogenous tone in all genital arteries. In the IVA, NO and EDHF pathways showed some degree of compensation in that blockade of either pathway alone was significantly less effective than blockade of both pathways together.

Peptide- and Purine-Mediated Responses

L-NAME-resistant, EFS-induced vasodilation was shown not to be due to ATP in the female genital arteries. In contrast to published data in vaginal tissues, no direct vasodilator effect of ATP was demonstrated in isolated female vaginal arteries. However, functional P_{2X} receptors (ATP-induced vasoconstrictions) were demonstrated; suggesting that ATP may be involved in sympathetic, excitatory neurotransmission. Until now, no previous studies have investigated this role for ATP in isolated genital arteries.

Functional VIP receptors were demonstrated in female genital arteries and the presence of VIPergic neurons confirmed by immunohistochemical studies. A role for VIP as a neurotransmitter in the EVA could not be excluded. To date no studies have managed to conclusively delineate the physiological function of VIP. However, an additional, unknown vasodilator neurotransmitter remained unidentified in both male and female genital arteries and identification of this compound may solve the characterisation of L-NAME-resistant, non-adrenergic, non-cholinergic neurotransmission.

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Publications

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Abbreviations

α,β,m ATP	Alpha, beta, methylene adenosine 5'-triphosphate
ACh	Acetylcholine
AC	Adenylate cyclase
Apa	Apamin
AR	Adrenoceptor
ATP	Adenosine 5'-triphosphate
BK _{Ca}	Large-conductance calcium-activated potassium channel
cAMP	Cyclic adenosine monophosphate
CEC	Chloroethylclonidine
CI	Confidence interval
cGMP	Cyclic guanosine monophosphate
ChTX	Charybdotoxin
CNS	Central nervous system
CRC	Concentration-response curve
D β H	Dopamine beta hydroxylase
dH ₂ O	Distilled water
ED	Erectile dysfunction
EDHF	Endothelial-derived hyperpolarising factor
EET	Epoxyeicosatrienoic acid
EFS	Electrical field stimulation
eNOS	Endothelial nitric oxide synthase
EVA	Extra-vaginal artery
FSD	Female sexual dysfunction
GTP	Guanosine triphosphate
IK _{Ca}	Intermediate-conductance calcium-activated potassium channel
IP ₃	Inositol trisphosphate
IVA	Intra-vaginal artery

L-NAME	N ^ω -nitro-L-arginine methyl ester hydrochloride
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NANC	Non-adrenergic, non-cholinergic
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
NO	Nitric oxide
PBS	Phosphate buffered saline
PE	Phenylephrine
PECAM	Platelet/endothelial cell adhesion molecule – 1
PDE	Phosphodiesterase
PGE	Prostaglandin
PGP 9.5	Protein gene product 9.5
PKG	cGMP-dependent protein kinase
PSS	Physiological saline solution
sGC	Soluble guanylate cyclase
SK _{Ca}	Small-conductance calcium-activated potassium channel
SNP	Sodium nitroprusside
SR	Sarcoplasmic reticulum
TNP-ATP	2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate monolithium trisodium salt
TTX	Tetrodotoxin
VIP	Vasoactive intestinal polypeptide
VIP (6-28)	Vasoactive intestinal polypeptide fragment 6-28

Chapter 1

General Introduction

Sexual Function and Dysfunction

Sexual dysfunction is an area that has been moved into the public consciousness by the advent of oral medications such as the PDE-5 inhibitors sildenafil citrate (Viagra[®]), tadalafil (Cialis[®]) and vardenafil hydrochloride (Levitra[®]) for the treatment of male erectile dysfunction. These drugs have provided some of the impetus for further scientific research into the underlying aetiology of sexual dysfunction. However, a large volume of basic research has yet to be completed before we can fully understand the mechanisms involved in the normal function of the genital system and, therefore, understand the processes that are responsible for dysfunction.

Male Penile Anatomy

The structure and function of penile arterial, venous and nervous systems have been investigated in many species including rat (Fernandez *et al.*, 1991), rabbit (Fujimoto & Takeshige, 1974), monkey (Fugleholm *et al.*, 1989) and human (Breza *et al.*, 1988; Benoit *et al.*, 1999) and no major anatomical differences were found across mammalian species.

The body of the penis contains three cylindrical masses of erectile tissue, two paired corpora cavernosum and a corpus spongiosum, composed mainly of smooth muscle and connective tissue with endothelium, fibroblasts and nerves (Figure 1-1). The corpora cavernosum are positioned dorsally and divided by a septum which is incomplete in some species e.g. human and rabbit, allowing communication between the two bodies of tissue (Simopoulos *et al.*, 2001) but not in others e.g. dogs (Azadzo *et al.*, 1995). Ventrally the corpus spongiosum tissue surrounds the urethra. Each corpus is surrounded by a layer of tissue called the tunica albuginea composed of collagen and elastin. The tunica albuginea around the corpus spongiosum consists of a single layer while the paired corpora cavernosum are surrounded by three layers, an inner circular, intermediate oblique and outer longitudinal layer. The relative indistensibility provided by the tunica albuginea around the corpus cavernosum is necessary to allow this tissue to act as a blood filled capacitor during erection and to provide a rigid structure to the penis. Proximally in the root of the penis, the two corpora cavernosum separate and taper as penile crura. The crura are attached to the ischial and inferior pubic rami and are covered by the ischiocavernosus muscles. Distally the corpora cavernosum end proximal to the glans penis.

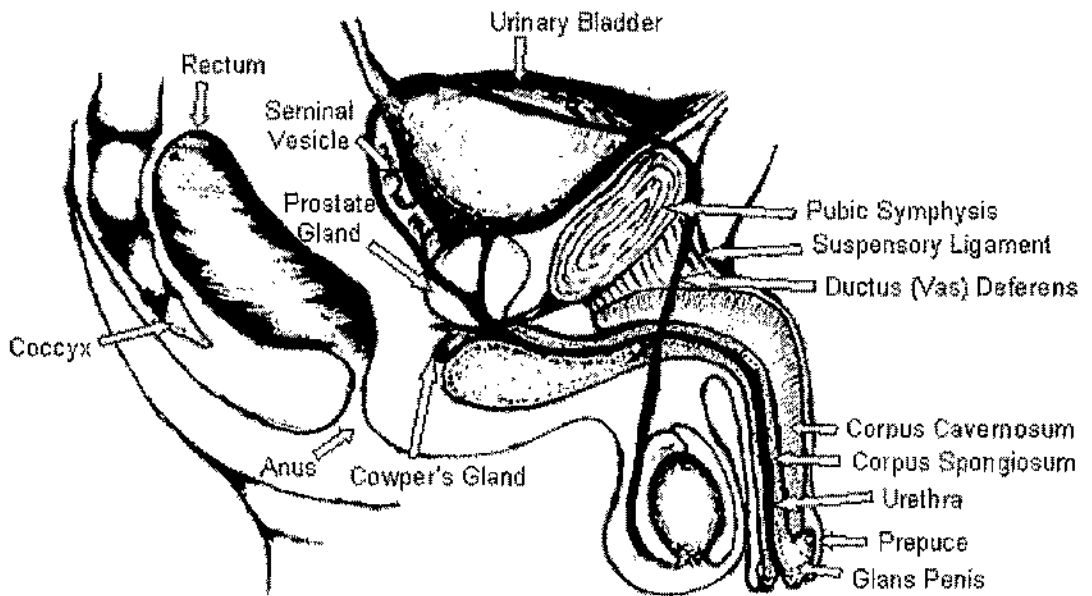


Figure 1-1: The human male genital anatomy. A cross-section of the male reproductive organs showing the tissues of the penis, including the corpus cavernosum, corpus spongiosum, and glans penis, and their positions relative to other organs in the body. (Adapted from Tortora & Grabowski, 2000).

Penile Circulation

The arterial blood supply to the penis originates from the internal iliac (hypogastric) artery, which branches to give rise to the internal pudendal artery. At its terminal end this passes through Alcock's canal where it branches into the perineal and penile arteries. The penile artery further divides into the bulbourethral, cavernous and dorsal arteries.

The cavernous artery enters the junction of the two corpora cavernosum and is the primary blood supply to this tissue. A proximal branch, the crural artery, supplies blood to the penile crura while the cavernous artery continues distally in the centre of the corpus cavernosum tissue (Figure 1-2). Numerous branches, helicine resistance arteries, arise from the cavernous artery, connecting directly into trabecular sinuses in the corpus cavernosum tissue which become filled with blood during the erectile process. Shunt vessels exist, which are open in both flaccid and erect states and bypass the trabecular sinuses; connecting helicine resistance arteries directly to a venous plexus draining the corpus cavernosum tissue (Fugleholm *et al.*, 1989).

The dorsal artery is situated along the dorsal surface of the corpora cavernosum and constitutes the main blood supply to the glans penis and prepuce. Circumflex arteries

branch along the length of the dorsal artery and accompany circumflex veins around the lateral surfaces of the corpora cavernosum. These arteries may provide a secondary blood supply to the corpora cavernosum (Breza *et al.*, 1988).

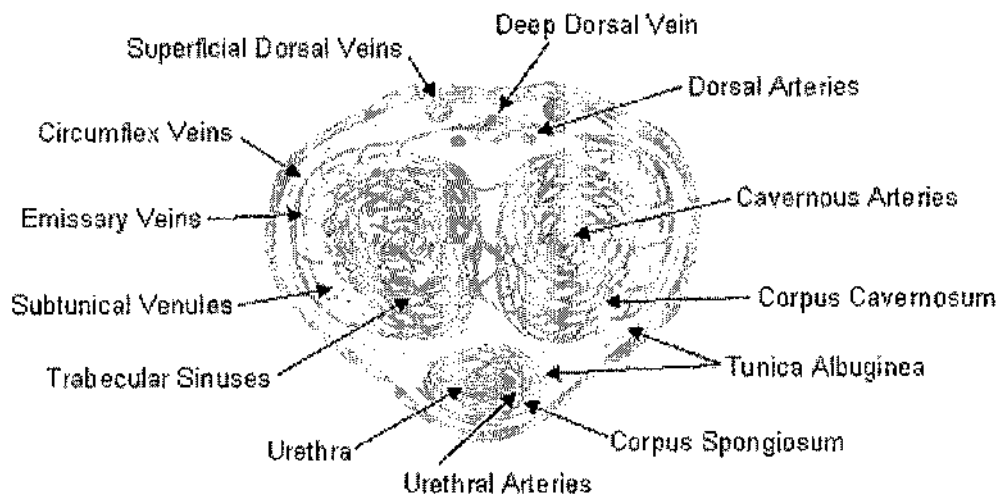


Figure 1-2: The human penile circulation. A cross-section of the penis showing the positions of arteries and veins relative to penile tissues. (Adapted from Tortora & Grabowski, 2000)

The Male Sexual Response

There are three types of erection: psychogenic, reflexogenic and nocturnal (reviewed by Andersson & Wagner, 1995). Auditory, olfactory, visual or imaginative stimuli to the CNS initiate psychogenic erections while reflexogenic erections result from direct tactile stimulation of the genitalia. The three main events that occur during erection are increased penile arterial inflow, relaxation of the trabecular tissue and increased venous resistance. During the flaccid state cavernous arteries and smooth muscle are contracted allowing only a low blood flow through the tissue for nutritional purposes.

Sexual arousal leads to increased parasympathetic activity causing the penile arteries and trabecular smooth muscle to relax. Arterial inflow increases (Lee *et al.*, 1993; Azadzo *et al.*, 1995) and blood fills the corpus cavernosum, corpus spongiosum and glans penis erectile tissues until they become limited by the surrounding tunica albuginea, causing intracavernosal pressure to rise rapidly. Veno-occlusion increases venous resistance and reduces outflow from the erectile tissue resulting in a relatively low flow rate to maintain erection. Intracavernosal pressure rises to just below systolic blood pressure values providing a high pressure system that gives shape and rigidity to the penis. Due to the

relative distensibility of the tunica albuginea surrounding the corpus spongiosum and the glans penis these tissues have a lower pressure system than the corpus cavernosum, which is maintained by a high blood flow rate (Vardi & Siroky, 1990).

It is thought that full rigidity of the penile tissues may require conscious or reflexive stimulation of the ischiocavernosus muscle (Juncmann *et al.*, 1989). Contraction of this muscle causes compression of the crura of the corpora cavernosum resulting in a rise in intracavernosal pressure. Stimulation of the bulbocavernosus muscle is thought to be specifically involved in the process of ejaculation.

A decrease in parasympathetic and an increase in sympathetic activity causes the cavernous arteries and smooth muscle to contract followed by a gradual re-opening of veins draining the corpus cavernosum. Both arterial and venous blood flow return to normal levels and intracavernosal pressure decreases rapidly, returning the penis to the flaccid state.

Female Vaginal Anatomy

In 1998, the first international consensus development conference on female sexual dysfunction (FSD), convened by the American Foundation for Urologic Disease, identified the need for basic research covering the epidemiology, anatomy and biology of FSD concluding that FSD was an “under researched and poorly understood area” (Basson *et al.*, 2000). In particular, investigations of genital arterial, venous and nervous systems and their role in the control of vascular smooth muscle tone, vasodilation and vaginal lubrication were identified as areas of study required to provide a fuller understanding of the physiology of the female sexual response.

The female sexual anatomy is divided into internal and external genitalia (reviewed by Berman & Bassuk, 2002; Munarriz *et al.*, 2002). External genitalia are collectively termed as the vulva and include the labia, interlabial space, vestibular bulbs and the clitoris (Figure 1-3). Embryologically, the clitoris and penis are derived from the same origin and, therefore, are composed of similar parts: a glans and two corpora cavernosum surrounded by a single layer tunica albuginea. Internal genital organs include the uterus, fallopian tubes, ovaries and the vagina. The vagina is a cylindrical organ which lies in the midline of the body. The vaginal wall is composed of three layers, an inner epithelial membrane consisting of mucous-type stratified squamous epithelial cells, a vascular muscularis layer composed of smooth muscle and extensive blood vessels and an outer fibrous layer. The

vaginal wall has numerous folds allowing it to distend and increase in length and luminal diameter during sexual activity.

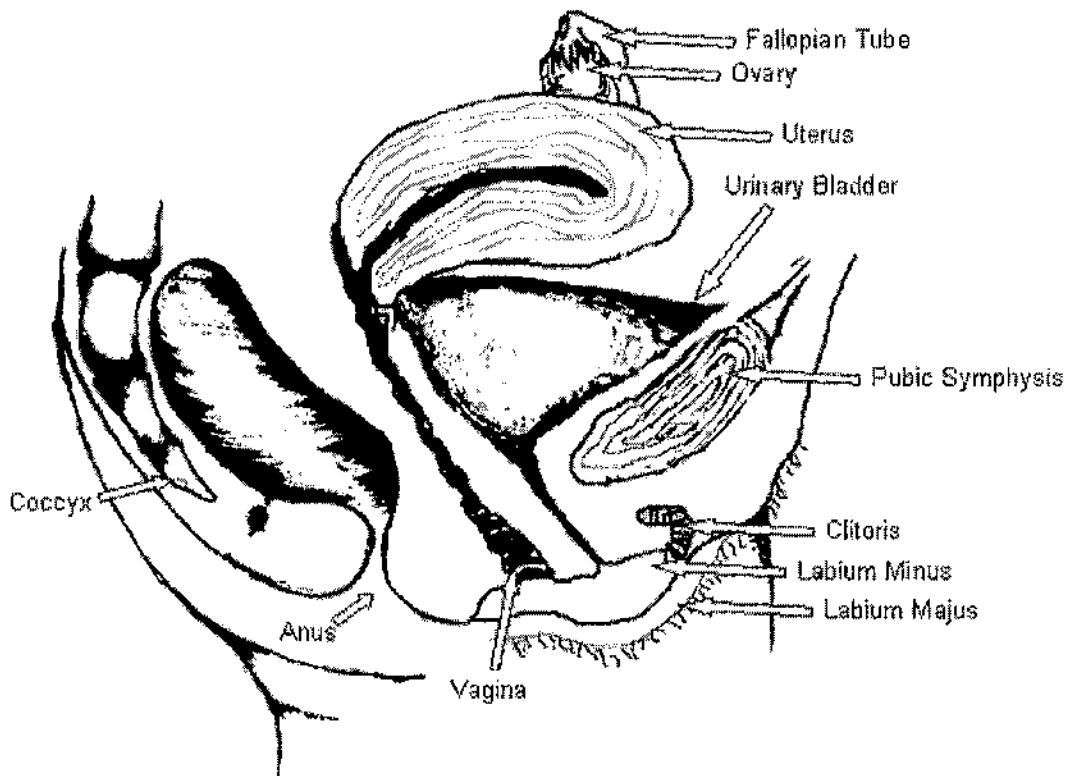


Figure 1-3: The human female genital anatomy. A cross-section of the female reproductive organs showing the vagina and uterus and their positions relative to other organs in the body. (Adapted from Tortora & Grabowski, 2000).

Vaginal Circulation

In males, many investigators have used techniques such as corrosion casts to provide a detailed knowledge of the vascular beds supplying the penile tissues. However, corresponding studies in the female genital tissues are limited to a single study in the rat (Shabsigh *et al.*, 1999). The vascular system in female vaginal tissues is supplied largely by the internal iliac artery and in part by the external iliac artery. The internal iliac artery branches to give the cervical and vaginal arteries. In the rat, multiple collaterals and serpentine vessels connect the two main vaginal arteries on the lateral sides of the vagina. Venous drainage is via a complex network of veins termed the vaginal venous plexus.

The Female Sexual Response

The female sexual response, first defined by Masters & Johnson (1966) and later refined by Kaplan (1974), includes phases of desire, arousal, orgasm and resolution. In the basal state, vaginal and clitoral smooth muscles remain contracted, which is similar to corpus cavernosum smooth muscle in the male. During the desire phase, internal and external stimuli lead to a CNS response involving the hypothalamus; resulting in the release of sex hormones and vasoactive substances. In the female, the brain is considered to be the most important mediator of the sexual response and is affected by biological, e.g. hormones and neurotransmitters, motivational, e.g. relationship factors, and cognitive, e.g. risk and wish, aspects. Female sexual arousal is characterised by vasculogenic changes, including vasodilation, increased vaginal blood flow, increased vaginal lubrication, relaxation of the vaginal wall and engorgement of the clitoris with blood. Orgasm results from a rhythmic contraction of the vagina, uterus and levator ani muscles. Unlike males, sexual satisfaction in females is not reliant on the occurrence of an orgasm response. The final phase involves resolution during which there is a release of muscular tension, a decrease in blood flow to the sexual organs and emotional satisfaction.

Genital Innervation

The innervation of male and female genital tissues is comparable and includes sympathetic, parasympathetic and somatic (sensory and motor) nerves (Lue *et al.*, 1983; Andersson & Wagner, 1995; Giuliano *et al.*, 2002; Munarriz *et al.*, 2002).

In both sexes, sympathetic (thoracolumbar) preganglionic fibres synapse at the paravertebral sympathetic chain ganglia (Figure 1-4). Postganglionic fibres reach the genitalia via three possible routes; in the pudendal, pelvic (nervi erigentes) or hypogastric nerves. Parasympathetic preganglionic fibres travel via the pelvic nerve from the sacral region of the spinal cord to the pelvic plexus. Postganglionic fibres reach the genitalia via the cavernous and dorsal nerves, branches of the pelvic/hypogastric and pudendal nerves respectively. In the male, branches of the cavernous nerve containing sympathetic and parasympathetic fibres innervate the cavernous vein and urethral, dorsal and cavernous arterics. Terminal neurons innervate helicine resistance vessels and trabecular smooth muscle. In females, the dorsal nerve innervates clitoral tissue while the cavernous nerve provides innervation to the vaginal tissue.

Somatic innervation involves afferent sensory fibres from the genitalia that reach the sacral region of the spinal cord through the dorsal nerve in the penis and subsequently the pudendal nerve in both sexes. However, a difference occurs in efferent nerve pathways in males and females. In males, the efferent response occurs either via motor fibres in the pudendal nerve to the ischiocavernosus, bulbocavernosus and other pelvic muscles or via parasympathetic pathways. In females, the efferent pathway is also via the pudendal nerve to the pelvic muscles but in addition the motor response is co-ordinated with sympathetic pathways (Giuliano *et al.*, 2002).

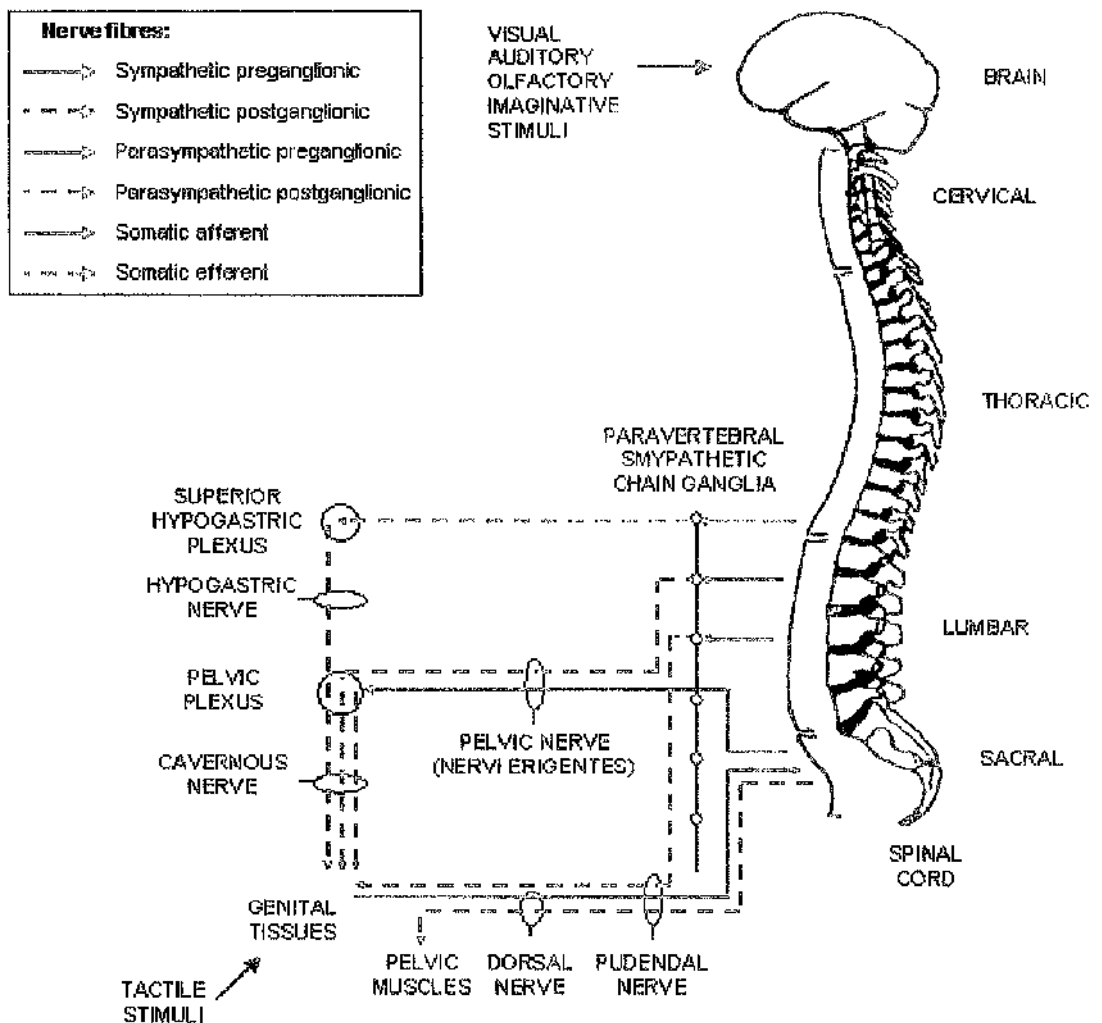


Figure 1-4: Schematic overview of sympathetic, parasympathetic and somatic innervation of the genitalia. Sympathetic innervation originates from the thoracic and lumbar regions of the spinal cord and travels in the hypogastric, pelvic and pudendal nerves to the genitalia. Parasympathetic innervation originates in the sacral region of the spinal cord and travels in the pelvic nerve to the genitalia. Somatic afferent and efferent fibres relay to the sacral region of the spinal cord. (Adapted from Andersson & Wagner, 1995).

Male Sexual Dysfunction

In males, sexual dysfunction can be separated into loss of libido, ejaculatory, orgasmic and erectile dysfunctions. The definition of the latter is an inability to attain or maintain erections sufficiently rigid for vaginal penetration and sexual satisfaction. Various studies have estimated that at any time approximately 18% to 31% of men aged 25 to 80 years suffer from sexual dysfunction (Laumann *et al.*, 1999; Johannes *et al.*, 2000; Martin-Morales *et al.*, 2001; Lewis *et al.*, 2004; Nicolosi *et al.*, 2004). This dysfunction is age related and associated with cardiovascular risk factors, diabetes, psychiatric/psychological disorders and socio-demographics.

Erectile dysfunction (ED) has been classified into five types dependent upon the underlying cause. Of these vasculogenic ED has been estimated to account for approximately 75% of all ED patients (NIH Consensus Conference, 1993) and so is by far the most common class. Other classes include neurogenic, psychogenic, endocrinologic and iatrogenic ED but these were out with the scope of this study. Vasculogenic ED is characterised by an interruption of penile blood flow with arterial insufficiency, venous incompetence or both. Risk factors include diabetes mellitus, hypertension, atherosclerosis, hypercholesterolemia and smoking. The prevalence of ED in diabetic men, 28%, is three times higher than in non-diabetics, 9.6% (Feldman *et al.*, 1994).

Therapeutics

In the last twenty years, since the first reports of penile erections induced by intracavernosal agents (Virag, 1982; Brindley, 1986), pharmacological therapies have developed to become the most effective and reliable treatment of male ED.

Pharmacological therapies generally bypass the normal psycho-neurological stimuli required to initiate an erection by directly activating or inhibiting central (e.g. dopamine) or peripheral receptors (e.g. adrenergic) and signal transduction pathways (e.g. nitric oxide/cGMP).

Intracavernosal injection remains a common route of administration of pharmacological therapies but can prove problematic due to patients' fear of self-injection. The transurethral route can provide a less invasive alternative while oral or topical administrations are non-invasive options. However, transurethral or topical drug administrations present a difficulty in drug delivery because of the anatomy of the tunica albuginea of the corpus cavernosum. More recently developed oral therapies, while providing the benefit of having enhanced

public awareness of sexual dysfunction, are also associated with more common systemic side effects such as flushing, headaches and visual disturbances.

Current therapeutic approaches to erectile dysfunction have been based on knowledge of the physiology and pharmacology of erectile tissues. Pharmacological therapies aim to increase vasodilation (Table 1-1), decrease vasoconstriction (Table 1-2), or act centrally (Table 1-3). Since most drugs target the vasodilator pathways required for erection, they risk causing vasodilation in the systemic circulation leading to hypotension. Systemic vasodilation is responsible for a common side effect of many current therapies, headache. Vasodilator pathways may be over stimulated by the use of drugs leading to the occurrence of prolonged erections (priapism). Improved results have been achieved using combination treatments, including reduced toxicity, priapism and adverse side effects. For example PGE₁, phentolamine, papaverine and vasoactive intestinal polypeptide (VIP) have been used in two or three drug combinations with each other or with additional compounds such as prazosin (an α -AR antagonist) or forskolin (activates adenylate cyclase leading to increased cAMP, Mulhall *et al.*, 1997). Beneficial effects may also be achieved by attaching NO donors to α -AR antagonist or PDE inhibitors (Moreland *et al.*, 2000) to target both vasodilator and vasoconstrictor pathways simultaneously.

Table 1-1: Therapeutic drugs for the treatment of male erectile dysfunction intended to increase smooth muscle relaxation.

Drug	Route of Administration	Mode of Action	References
Sildenafil, vardenafil	oral	selective PDE-5 inhibitor	Boolell <i>et al.</i> , 1996 Goldstein <i>et al.</i> , 1998 Saenz de Tejada <i>et al.</i> , 2001
Linsidomine chlorhydrate (SIN-1)	intracavernous	non-enzymatic liberation of NO	Steif <i>et al.</i> , 1992
Organic nitrates	topical	stimulate soluble GC	Holmquist <i>et al.</i> , 1993
e.g. nitroglycerin		enzymatic liberation of NO	Owen <i>et al.</i> , 1989 Meynoff <i>et al.</i> , 1992
PGE ₁ , alprostadil (synthetic PGE ₁)	intracavernous intraurethral oral topical	↑ cAMP via EP receptors inhibit NA release	Waldhauser & Schramek, 1988; Tsei <i>et al.</i> , 2000 Moreland <i>et al.</i> , 2000
Papaverine	intracavernous topical	multi-level non-selective PDE inhibition ↑ cAMP and cGMP	Delcour <i>et al.</i> , 1987 Kirkeby & Johannesen, 1989
VIP	intracavernous	attenuate α ₁ -AR contraction stimulate AC, ↑ cAMP	Kiely <i>et al.</i> , 1989 McMahon, 1996 Steif <i>et al.</i> , 1991
CGRP	intracavernous	direct action on smooth muscle K ⁺ channel opener stimulate AC, ↑ cAMP	Holmquist <i>et al.</i> , 1990 Vick <i>et al.</i> , 2002
K ⁺ channel openers e.g. minoxidil	topical	K ⁺ channel opener	

Table 1-2: Therapeutic drugs for the treatment of male erectile dysfunction intended to decrease smooth muscle contraction.

Drug	Route of Administration	Mode of Action	References
Phentolamine	intracavernous oral	competitive α_1 -AR antagonist 5-HT receptor antagonist mast cell histamine release NOS activation	Brindley, 1986 Sidi <i>et al.</i> , 1986 Becker <i>et al.</i> , 1998
Thymoxamine, moxisylyate	intracavernous	competitive α_1 -AR antagonist antihistamine	Marquer & Bressolle, 1998
Yohimbine	oral	α_2 -AR antagonist	Kunellus <i>et al.</i> , 1997

Table 1-3: Therapeutic drugs for the treatment of male erectile dysfunction intended to act centrally.

Drug	Route of Administration	Mode of Action	References
Opioid receptor antagonists e.g. naloxone, naltrexone	oral intravenous	opioid receptor antagonist	Brennemann <i>et al.</i> , 1993 Van Ahlen <i>et al.</i> , 1995
Dopamine receptor agonists e.g. apomorphine (D1 and D2), quineforane (D2)	subcutaneous sublingual	central dopamine receptor agonist	Lai <i>et al.</i> , 1987 Dula <i>et al.</i> , 2001
Trazodone	intracavernous oral	antidepressant inhibits central 5-HT uptake \uparrow brain dopamine turnover α -AR antagonist 5-HT _{2c} agonist α -AR antagonist	Costabile & Spevak, 1999 Enzlin <i>et al.</i> , 2000
metabolite, m-CPP			
Melanocortin receptor agonists e.g. melanotan II (α MSH analogue)	subcutaneous	melanocortin receptor agonist mimics ACTH and α MSH activity	Molinoff <i>et al.</i> , 2003 Diamond <i>et al.</i> , 2004

Female Sexual Dysfunction

The first question many people ask when the subject of FSD arises is “does it exist?” In answer to this it may be put forward that no functional biological system is infallible and therefore will be prone to dysfunction. The question, therefore, may not be “does sexual dysfunction exist?” but rather “does it matter?” The primary focus of research in females has generally been in the field of reproduction and less in the area of sexual function, which has been considered passive in comparison to males. Also since sexual dysfunction is not life-threatening it is not always considered a high priority. However, in both males and females, sexual dysfunction has been shown to have a significant impact on an individual’s quality of life and interpersonal relationships and this point alone provides ample reason to strive for a treatment for sexual dysfunction in both sexes. In addition, the prevalence of sexual dysfunction has been shown to be greater in females than in males with 40% to 45% of women experiencing some form of sexual dysfunction (Laumann *et al.*, 1999; Lewis *et al.*, 2004; Nicolosi *et al.*, 2004). Therefore, FSD both exists and is a prevalent disorder of significant consideration for those who are affected by it.

Current definitions of FSD include sexual desire disorder, including hypoactive sexual desire and sexual aversion, sexual arousal disorder, orgasmic disorder and sexual pain disorder, including vaginismus, dyspareunia and non-coital pain (Basson *et al.*, 2000; Basson *et al.*, 2004). Similar to erectile dysfunction in men, FSD can arise from vasculogenic, neurogenic, hormonal/endocrine or psychogenic aetiologies (Berman *et al.*, 1999). FSD also shares common risk factors with male sexual dysfunction, including cardiovascular disease, smoking and hypercholesterolemia and is associated with negative experiences in sexual relationships and general poor health.

Female sexual arousal disorder (FSAD) is defined as the persistent or recurring inability to attain or maintain sufficient sexual excitement, causing personal distress. Arousal disorders may be characterised either by a lack of genital responses, e.g. decreased vaginal lubrication, decreased clitoral and labial sensation or engorgement, and a lack of vaginal smooth muscle relaxation, or by a lack of subjective excitement due to psychological factors, decreased vaginal blood flow, or as a side effect of surgery or medication (Berman *et al.*, 1999).

Therapeutics

Pharmacological therapies for FSD have, to date, largely been based on successes in the male field. This is both because this provides an obvious starting point, but also because little is known about the physiology and pharmacology of female sexual function upon which to base treatment strategies. However, the lack of success of drugs such as PDE-5 inhibitors in treating FSD highlights the inherent problems with trying to infer the function of one system directly from the other.

In addition, a further complication in the treatment of FSD is the multifactorial nature of the disorder and the greater dependence of female sexual arousal upon psychological stimuli. It has been found in many cases that peripheral indicators of genital arousal rarely correlate to subjective feelings of sexual arousal (Laan *et al.*, 1995; Basson, 2002). However, not all FSD is psychological in origin and so a role for pharmacological intervention remains important in the treatment of female sexual arousal disorder. Some drugs that have been considered include oestrogen replacement therapy, methyltestosterone, sildenafil, PGE₁, phentolamine and apomorphine, but most therapies are still in the experimental stages for treatment of FSD. While the use of hormones was initiated largely from a secondary effect of oral contraceptives or hormone replacement therapy (HRT) other options, e.g. sildenafil, PGE₁, phentolamine and apomorphine, have followed on directly from their success in males.

A greater understanding of the physiology and pharmacology of the female sexual response would provide a more coherent basis for the development of novel pharmacological therapies for the treatment of FSD.

Techniques to Study Sexual Function and Dysfunction

Cell Cultures

Human and rabbit genital smooth muscle cells have been successfully cultured to provide a method for the investigation of biochemical and signalling pathways in both male and female genital tissues (Gupta *et al.*, 1998; Traish *et al.*, 1999; Sato & Kawatani, 2002). These cell cultures are considered to retain their metabolic functional integrity allowing the investigation of isolated signalling processes. Using this technique the presence of post-synaptic α_1 -, α_2 - and β -ARs in rabbit corpus cavernosum smooth muscle cells has been shown (Gupta *et al.*, 1998; Sato & Kawatani, 2002). In human and rabbit vaginal smooth

muscle cells the action of sildenafil and zaprinast in inhibiting phosphodiesterase type 5 activity was demonstrated. However, this method is limited in the data which it can provide since it includes only a single cell type.

Organ Baths

The organ bath has been used as an *in vitro* tool to investigate local regulatory mechanisms in the genital tissues. Using this technique, pharmacological responses of corpus cavernosum or vaginal wall strips have been investigated and this has provided the majority of current data on the male and female genital systems. In addition, organ baths can be coupled with EFS to determine responses to endogenously released transmitters, providing more physiologically functional information. While studies have been performed in a variety of species, encompassing horse, human, monkey, dog, chicken, rabbit and rat, most data has been gained from investigations of rabbit or human genital tissues from both males and females.

A few studies have used the organ bath to investigate the function of isolated male genital vessels, but by necessity the vessels studied were from larger species since the technique is only suitable for vessels greater than 1 mm internal diameter. No investigators have used the organ bath technique to study isolated blood vessels from female genital tissues.

The organ bath technique has been used to demonstrate the role of VIP and NO in the vasodilation of human dorsal and cavernous penile arteries (Hedlund & Andersson, 1985c; Segarra *et al.*, 1999). In dorsal penile arteries from the bull, NO was shown to mediate NANC-induced relaxation (Liu *et al.*, 1991) while a combined vasodilator role of NO and K^+ channels was shown in the horse cavernous artery (Simonsen *et al.*, 1995). The importance of K^+ channels relative to NO-mediated vasodilations was found to be greater in human cavernous tissue than isolated cavernous arteries (Hedlund *et al.*, 1994); suggesting that results gained using tissue preparations may not be indicative of the function of isolated genital blood vessels. The same group also highlighted differences in the responses of human cavernous tissues and arteries to various prostanoids (PGI_2 relaxed arteries but not tissues, PGE_2 relaxed tissues but not arteries) and adrenoceptor (AR) agonists (responses were mediated by α_1 -ARs in tissues and α_2 -ARs in arteries) (Hedlund & Andersson, 1985a, 1985b). A single study attempted to delineate the role of α_1 -ARs in the cavernous artery of the rat in an organ bath environment (Mizusawa *et al.*, 2002). ARs, endothelin and NO were also shown to be involved in active regulation of human and horse deep dorsal veins (Holmquist *et al.*, 1992; Segarra *et al.*, 1998a; Recio *et al.*, 2004).

While organ baths have provided a large proportion of the current available knowledge of the functional pathways in genital tissues, a limitation of the method is that responses of isolated vessels have regularly been shown to differ from those in tissue strips. This indicates differences in the signal transduction pathways predominant in vascular and non-vascular smooth muscle and, since both muscle types are present in tissue strips, data gained via the organ bath method may be complicated by the presence of multiple pathways. A detailed study of the function of isolated genital blood vessels using a wire myograph should provide a more sophisticated approach to therapeutic treatments for sexual dysfunction.

In vivo Techniques

In vitro techniques have provided a wealth of information regarding the second messenger pathways functionally active in genital tissues but these pathways ultimately must be also tested in an *in vivo* model where a more physiological environment is provided. In the investigation of sexual function, neurophysiological control of penile erection has been studied by stimulation of the pelvic nerve in anaesthetised animals, including dogs and rabbits (Andersson *et al.*, 1984; Vardi & Siroky, 1990; Trigo-Rocha *et al.*, 1993; Min *et al.*, 2000). However, the pelvic nerve contains both sympathetic and parasympathetic fibres leading to a potentially confusing situation of mixed vasoconstrictor and vasodilator responses. Therefore, stimulation of cavernous and hypogastric nerves has also been used to study erectile responses (Holmquist *et al.*, 1991; Rehman *et al.*, 1998). The end point measurements that can be used in the *in vivo* animal model include measures of blood flow, luminal, wall or intracavernous pressure and dimensional measurements. Primarily, *in vivo* studies have determined the effects of drugs, such as papaverine and prostaglandins, on physiological indicators of sexual function in monkeys and dogs (Aboseif *et al.*, 1993; Trigo-Rocha *et al.*, 1995) due to their larger size. However, techniques have now been refined such that rabbits and rats (Stief *et al.*, 1998; Sazova *et al.*, 2002; Kim *et al.*, 2004) are more commonly used. A conscious animal model has also been developed to test potential compounds for drug development in male rabbits (Bischoff, 2001) and the effect of hormones in female rats (Hale *et al.*, 2003).

Small Vessel Wire Myography as an Experimental Tool

Small vessel wire myography is a technique developed by Mulvany & Halpern (1976) to study responses in isolated third order rat mesenteric arteries. Since then wire myography has become a well developed, reliable *in vitro* technique for the pharmacological and

physiological characterisation of blood vessels. The wire myograph is a miniaturised version of an organ bath which allows investigation of the functional responses of vessels with an internal lumen diameter of 100 to 500 μm . Similar to larger organ baths, wire myography can be coupled with EFS, providing the opportunity to investigate endogenous factors.

In both male and female sexual function an increase in blood flow is central to the sexual response; making the function of blood vessels mediating this increase of great importance. Few investigators have previously used small vessel wire myography in the study of sexual function and none of these studies have involved isolated blood vessels from female genital tissues. Those that have used the technique, used isolated cavernous and helicine resistance arteries from larger species such as human, horse and bull to investigate genital vascular function. To date, studies on genital arteries in the wire myograph have primarily investigated vasodilator pathways. VIP, NO and sildenafil have been shown to cause vasodilation of human and bovine arteries (Hempelmann *et al.*, 1995; Simonsen *et al.*, 2001) while NPY caused both vasoconstriction and vasodilation (Prieto *et al.*, 2004). Endothelium-dependent relaxation was also studied in human and horse arteries (Prieto *et al.*, 1998; Angulo *et al.*, 2003) demonstrating that endothelium-dependent relaxation resulted from NO release in cavernous tissue and both NO and K^+ channels in cavernous arteries. Similarly to studies in larger organ baths, wire myograph studies have demonstrated inconsistencies in the responses of isolated vessels and tissue strips.

In the current thesis, wire myograph and immunohistochemical techniques were used. This will add to data from cell cultures, whole tissues, *in vivo* systems, histological, and mRNA studies to provide an integrated approach to understanding the overall physiological system.

Rabbits as an Experimental Model

Prior to the introduction of the myograph, limitations of vessel size meant that animals such as the rabbit and rat could not be used to study genital blood vessels. From this respect, many studies have been performed in larger species such as horse and dog. However, the development of techniques and experience has led to greater use of smaller species such as the rabbit and rat providing, in addition, a more economic and viable solution. An animal showing potential for future use is the mouse, which is particularly attractive due to the development of genetic knockout strains, but its small size may limit the studies that are achievable. Initial reviews of the field of sexual function have

concluded that both the rabbit and the rat provide systems that will help to guide future research in humans (Bischoff, 2001; Burnett, 2001; Min *et al.*, 2001; Hale *et al.*, 2003; Kim *et al.*, 2004).

The rabbit is particularly useful since pathophysiological models for the study of sexual dysfunction, such as hypercholesterolaemia, diabetes and aging, can be induced. A disadvantage of using female rabbits is their unique endocrine physiology. Female rabbits remain in continuous diestrus until mounting occurs and therefore have low serum oestrogen levels. Therefore, the female rabbit does not provide a good system for the study of hormonal influences on sexual function. In addition, the potency of the adrenergic antagonist prazosin in rabbit tissues has been demonstrated to be lower in rabbit tissues (Docherty, 1988; Muramatsu *et al.*, 1990; Naghadeh, 1996) suggesting that sensitivity of these tissues to adrenergic selective compounds could show species specific differences. While the use of animal tissue is never ideal compared to human samples the lack of available human tissue, in particular normal controls, makes its use inevitable. The relevance of data acquired from animal models must be validated where possible against that obtained in humans.

Male and female New Zealand White rabbits were used in the current study due to their multiple advantages of size, cost and comparability to previous studies. Despite the differences in the hormonal milieu of female rabbits, these animals were used to study female genital vascular function in order to provide comparable data between the sexes. In the current study no investigations were made into hormonal influences on vascular responses. Arteries were investigated in isolation from the *in vivo* environment thereby reducing the involvement of hormones as far as was feasible.

Vascular Responses in Isolated Genital Arteries

Adrenoceptor-Mediated Responses

The involvement of ARs in genital vascular function is one of the few areas that have begun to be investigated in isolated male penile arteries. Using selective agonists and antagonists (noradrenaline, phenylephrine, prazosin and rauwolscine) vasoconstriction of cavernous arteries was shown to be mediated predominantly by α_2 -ARs, while α_1 -ARs were shown to contribute to contraction of cavernous tissues (Hedlund & Andersson, 1985b). In the cavernous artery of the rat, α_{1D} -ARs were considered to be the predominant α_1 -AR subtype mediating noradrenaline-induced vasoconstriction (Mizusawa *et al.*, 2002).

Nitric Oxide-Mediated Responses

The second area that has benefited from investigation in isolated genital arteries is the involvement of NO in vascular vasodilation. NO donor drugs have been shown to cause vasodilation in dorsal and cavernous penile arteries (Hempelmann *et al.*, 1995; Segarra *et al.*, 1999; Simonsen *et al.*, 2001), vasodilation that was increased by the PDE-5 inhibitor, sildenafil. The contribution of NO was investigated using selective inhibitors of NOS. In addition, the contribution of K⁺ channels, considered to be associated with the actions of EDHF, were investigated using blockers of Ca²⁺-activated K⁺ channels, e.g. apamin and charybdotoxin. A combination of NO and K⁺ channels (EDHF-like vasodilation) mediated both NANC-induced relaxation in the dorsal artery (Liu *et al.*, 1991; Simonsen *et al.*, 1995) and endothelium-dependent relaxation in the cavernous artery (Prieto *et al.*, 1998; Angulo *et al.*, 2003), while a predominance of K⁺ channels (EDHF-like vasodilation) was suggested in the cavernous arteries (Hedlund *et al.*, 1994).

Peptide-Mediated Responses

An area that has received recent attention is the involvement of various peptides in the function of genital tissues. The contribution of some peptides, including vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), substance P and vasopressin, to vasoconstriction and vasodilation of isolated vessels has been considered. The role of peptides has been investigated by determining the direct effect of these compounds on basal or pre-contracted tone and their effect on EFS-induced responses. Of the peptides tested only VIP was found to be compatible with the role of a vasodilator neurotransmitter (Hedlund & Andersson, 1985c). VIP-induced vasodilation was increased by sildenafil (Simonsen *et al.*, 2001) but was not found to be synergistic with NO-donor-induced vasodilation (Hempelmann *et al.*, 1995). NPY was shown to have both vasoconstrictor and vasodilator actions (Prieto *et al.*, 2004) while vasopressin also had vasoconstrictor effects (Segarra *et al.*, 1998b).

A comprehensive review of the current literature in the field of adrenoceptors, NO, EDHF, prostaglandins and peptides in the genital tissues are provided in the relevant chapters.

Aims

In this thesis, small vessel wire myography was utilised to perform a detailed pharmacological analysis of isolated male and female New Zealand White rabbit genital

arteries. The vessels chosen were the male dorsal and cavernous penile arteries. In the male, these vessels represented important inflow arteries to the penile tissue and were comparable to previous studies. The female vaginal artery was chosen for study as this represents the main inflow artery to vaginal tissues. No previous studies have been performed on any isolated arteries from female genital tissues presenting a unique opportunity to investigate the function of isolated vaginal arteries.

A focus of the current study was to build on preliminary findings on adrenoceptor pharmacology in isolated genital arteries and to define the predominant ARs. A second focus of the current study was to investigate the involvement of NO in neurogenic, endothelium-dependent and smooth muscle relaxation in both male and female genital arteries. In addition, the relative contributions of NO, endothelium-derived hyperpolarising factor (EDHF) and prostaglandins were investigated. A third focus of the current study was to investigate the role of peptides in neurogenic and direct vascular smooth muscle contraction and relaxation. The aims of the research presented herein were as follows.

- To determine the involvement of adrenoceptors, in particular α_1 - and α_2 -ARs, in vasoconstriction of male and female rabbit genital arteries. Following the establishment of α_1 -AR-mediated contractions in male penile arteries, a second aim was to further subtype this response.
- To investigate NO-induced vasodilator responses in male and female rabbit genital arteries. In addition, to characterise endothelium-dependent, direct smooth muscle and neurogenic NO-mediated vasodilation.
- To investigate endothelium-dependent vasodilation and determine the relative contributions of prostaglandins, NO and K^+ channels/EDHF in male and female rabbit genital arteries.
- To characterise smooth muscle and neurogenic peptide-mediated responses in female rabbit vaginal arteries.
- To highlight similarities or differences in the vasoconstrictor and vasodilator responses of male and female rabbit genital arteries.

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Chapter 2

Materials and Methods

Immunohistochemistry

Tissue Preparation

Paraffin Wax Sections

Isolated vaginal tissue and vaginal artery were embedded in paraffin wax, sectioned at 5 μm thickness and mounted on polysine microscope slides in preparation for immunohistochemistry by J.Owen, Pfizer Global Research and Development. Vaginal tissue was divided distally from the vaginal opening into twelve numbered parts approximately 1 cm in length. Slides were prepared with two consecutive sections mounted on each slide to allow for control and experimental staining. The vaginal artery was divided into four parts and a section from each mounted on a single slide. Paraffin wax sections were stored at room temperature until use.

Frozen Sections

Isolated vaginal tissue was divided distally from the vaginal opening into nine numbered parts approximately 1 cm in length. Each part was snap frozen in liquid nitrogen and sectioned at 6 to 8 μm thickness using a cryostat (Leica CM1900) set at -15 °C chuck temperature and -18 °C cryostat temperature. Two consecutive sections were mounted on each slide to allow for control and experimental staining. Slides were stored at -80 °C until use.

Techniques

Preparation of Slides for Staining

Paraffin wax sections were deparaffinised using one of two methods; either xylene, absolute alcohol and industrial methylated spirit (Pfizer) or tissue clear and a series of graded 100%, 90% and 70% alcohols (University of Glasgow) depending on location. Following deparaffinisation sections were fully re-hydrated in running tap water.

Frozen sections were prepared by air drying slides at room temperature before fixing in acetone (-20 °C). Slides were washed in phosphate buffered saline (PBS) followed by running tap water.

Antigen Retrieval

Antigen retrieval was performed only for paraffin wax sections to improve the accessibility of antigens to antibodies following the wax embedding process. Re-hydrated slides were placed in a plastic carrying rack and dish along with a working dilution of antigen unmasking solution (Vector Laboratories). The solution was brought to the boil and simmered for either six minutes in a microwave (Pfizer) or 20 minutes in a steamer (University of Glasgow) depending on location. Slides were cooled in running tap water.

Experimental Protocols

Sections were delineated using a hydrophobic PAP pen. To quench endogenous peroxidase activity paraffin wax sections were incubated for 10 minutes in peroxidase block (ImmunoCruz staining system - goat, Santa Cruz Biotechnology Inc.). Frozen sections were incubated for 30 minutes in 0.3% H₂O₂ in methanol to achieve an equivalent endogenous peroxidase block. Slides of either paraffin wax or frozen sections were then rinsed in distilled water (dH₂O) followed by PBS. In some cases a step was included at this stage to prevent non-specific binding of secondary antibodies by incubation with protein block (Dako[®] Protein Block, serum free) for 20 minutes. Protein block was tipped off but slides were not rinsed prior to primary antibody incubation.

Slides were incubated with appropriately diluted primary antibodies or negative control for either 60 minutes at room temperature or overnight at 5 °C. Negative controls consisted of incubation with either species specific normal IgG (specific for the primary antibody species) where available or PBS where the correct normal IgG was unavailable. Sections were thoroughly rinsed in PBS prior to secondary antibody incubation. The method of secondary antibody incubation and staining was dependent on the required visualisation technique, i.e. light or fluorescence microscopy.

The majority of sections were incubated with an appropriately diluted biotinylated secondary antibody for 30 minutes followed by a PBS rinse. Subsequent incubation with HRP-streptavidin complex for 30 minutes, followed by a PBS rinse, provided an additional signal amplification step. Thirdly sections were incubated with peroxidase substrate (Vector[®] DAB Substrate) for 5 minutes and rinsed with dH₂O. Slides were counterstained using Vector Haematoxylin (Vector Laboratories), rinsed in tap water, 2% acetic acid solution, tap water a second time, incubated in Shandon Blueing Reagent (Modified Scott's Tap Water) and finally rinsed in tap water for a third time. In preparation for light

microscopy, slides were dehydrated using the appropriate re-hydration method in reverse and mounted using DPX and a coverslip. Sections were viewed using a light microscope. Positive structures were stained brown, while nuclei appeared blue.

Where sections were incubated with fluorescent-labelled secondary antibodies care was taken to ensure the antibody and slides were kept in dark conditions throughout the protocol to prevent bleaching of fluorescent dyes. Sections were incubated for 30 minutes following which they were rinsed thoroughly in PBS followed by dH₂O. Slides were mounted using an aqueous mountant (Dako[®] Fluorescent Mounting Medium) and a coverslip. Sections were viewed using a fluorescence microscope with the appropriate filters for the fluorescent dye used.

A selection of slides was stained using Haematoxylin (blue nuclear stain) and Eosin (pink cytoplasmic stain) by J.Owen, Pfizer Global Research and Development. These slides were used to view the structural properties of the tissue under a light microscope.

Drugs and Solutions

Solutions

The following solutions were used for immunohistochemical studies.

- PBS, composition (M): phosphate buffer 0.01, KCl 0.0027, NaCl 0.137, prepared daily using PBS tablets (Sigma[®]). The pH of this solution was 7.4 at 25 °C.
- Antigen Unmasking Solution (Vector Laboratories, Inc).
- Peroxidase blocking solution and donkey serum block from ImmunoCruz[™] staining system, goat (Santa Cruz Biotechnology).
- Dako[®] Protein Block, serum-free (Dako).
- Dako[®] EnVision[™] system, IIRP (DAB) (Dako).
- Vectastain[®] ABC Kit, mouse (Vector Laboratories, Inc).
- Liquid DAB + Substrate Chromogen System (Vector Laboratories, Inc).

- Haematoxylin nuclear counterstain (Vector Laboratories, Inc).
- Blueing reagent (Modified Scott's Tap Water) (Shandon®).
- DPX (BDH/Merck®).

Primary Antibodies

Primary antibodies, used to identify nerves and endothelial cells in vaginal tissues, were raised in species other than the rabbit to prevent non-specific binding of secondary antibodies. Lists of primary antibodies, their properties and suppliers are given (Table 2-1).

Table 2-1: Primary polyclonal antibodies

	Anti-Protein Gene Product 9.5 (PGP 9.5)	Anti- Platelet/Endothelial Cell Adhesion Molecule-1 (PECAM-1)	Anti-Dopamine- β-Hydroxylase (DβH) (C- Terminal)	Anti-Vasoactive Intestinal Polypeptide (VIP)
Antibody Species	Mouse	Goat	Sheep	Sheep
Supplier	Biogenesis	Santa Cruz Biotechnology Inc.	SigmaAldrich	Chemicon International
Specificity	Neuronal cell bodies and axons in the CNS and PNS, small nerve fibres in PNS	Glycoprotein expressed on cell surfaces of monocytes, neutrophils, platelets and some T cells	Dopamine- β - hydroxylase, abundant in adrenergic neurons	Vasoactive intestinal polypeptide
Species reactivity	Human and rat	Human, mouse and rat	Human, monkey, bovine and rabbit	Guinea-pig
Recommended IHC Dilution	1:50 – 1:200	1:100	-	1:2500 – 1:5000

Secondary Antibodies

Secondary antibodies, selective for the appropriate primary antibody species, i.e. sheep, goat or mouse, were either biotin- or fluorescence-conjugated to allow visualisation of positive structures (Tables 2-2 and 2-3).

Table 2-2: Secondary polyclonal antibodies used for light microscopy

	Anti-Sheep IgG, Biotin Conjugated	Anti-Goat IgG, Biotin Conjugated	Anti-Mouse IgG, Biotin Conjugated
Antibody Species	Donkey	Donkey	Donkey
Supplier	Chemicon International	Santa Cruz Biotechnology Inc.	Santa Cruz Biotechnology Inc.
Specificity	Sheep IgG (H&L)	Goat IgG	Mouse IgG
Species reactivity	Sheep	Goat	Mouse
Recommended IHC Dilution	1:500 – 1:5000	-	-

Table 2-3: Secondary polyclonal antibodies used for fluorescence microscopy

	Anti-Sheep IgG, Cy5 Conjugated	Anti-Sheep IgG (H&L), FITC Conjugated
Antibody Species	Donkey	Donkey
Supplier	Chemicon International	Chemicon International
Specificity	Sheep IgG	Sheep IgG (H&L)
Species reactivity	Sheep	Sheep
Recommended IHC Dilution	1:100 – 1:800	1:50 – 1:200
Wavelength	Absorption peak = 650 nm, Emission peak = 680 nm	Absorption peak = 492 nm, Emission peak = 520 nm

Small Vessel Wire Myography

Tissues

Penile or vaginal tissues were obtained from New Zealand White rabbits (2.5-3.5 Kg) sacrificed by an overdose of sodium pentobarbitone (Euthatal, Rhône Merieux, UK) injected into the ear marginal vein. Animals were supplied by Harlan and were housed in the University animal holding unit. A minimum of one week was allowed between the arrival of animals and their use to allow acclimatisation. Gross dissection of tissue occurred as soon as was possible after time of euthanasia. All further tissue dissections were carried out in ice-cold physiological saline solution (PSS).

Male Penile Resistance Arteries

Identification of Suitable Vessels

An extensive review of the literature was performed to determine the most suitable genital blood vessels for study. Penile erection is brought about by three events; increased arterial inflow, relaxation of trabecular tissue and increased venous resistance. The first two of these processes rely on the state of vasoconstriction of the arteries supplying the penile tissue, making a study of genital resistance arteries worthwhile with respect to their importance in the erectile response. The literature review showed that in male genital tissue three branches of the penile artery, namely the bulbourethral, cavernous and dorsal arteries, compromise the blood supply to the genital tissue. The main blood supply to the penile tissue occurs through the cavernous artery (corpus cavernosum tissue) and the dorsal artery (glans penis and prepuce with a secondary role supplying the corpus cavernosum). In the rabbit, these vessels were of a suitable size to be classified as resistance arteries, less than 400 μm internal lumen diameter, which was to be the focus of this study.

Dissection Techniques

With the male rabbit in a supine position the skin overlying the penis was removed (Figure 2-1). The entire penile tissue was excised from the body, up to and including a section of ischiocavernosus muscle. In removing the penile tissues, the two corpus cavernosum were transected shortly after their separation into penile crura. Further dissection of penile arteries was performed in an agar-filled Petri dish containing ice cold PSS using a Zeiss dissecting microscope (Stemi 2000).

Two dorsal penile arteries, from the left and right dorsal surface of the penis, were isolated from beneath the overlying fascia. Lengths of each artery extending from the ischiocavernosus muscle to a point proximal to the glans penis, approximately 1.5 cm, were dissected free from the penile tissue. Arteries were pinned out and all surrounding tissue dissected away from the artery wall. Each artery was divided into ring segments approximately 2 mm in length with six or seven sections obtainable from each side. A single 2 cm length of 40 μm diameter wire was inserted into the lumen of each artery section in preparation for use on a wire myograph. The passive internal lumen diameter of dorsal arteries was found to be $173.7 \pm 1.1 \mu\text{m}$ ($n(N) = 155(630)$).

Corpus cavernosum tissue from the left and right sides of the dividing septum were isolated from penile tissue by carefully cutting around the inside edge of the tunica albuginea and septum. Cavernous arteries were identified within the corpus cavernosum tissue and all surrounding trabecular and connective tissue carefully removed. While the arteries were pinned out to allow tissue removal, a length of 40 μm diameter wire was inserted down the entire length of each artery. This was necessary due to the convoluted nature and size of the cavernous artery; which meant that insertion of a wire after removal of the pins was more difficult and likely to cause significant damage to the vessel. Once a wire was successfully inserted into the vessel lumen, holding pins were removed and both wire and vessel cut so that each ring segment of vessel, approximately 2 mm in length, remained on a 2 cm length of wire. A similar number of sections as in the dorsal artery, six or seven, were obtainable. Cavernous arteries were generally smaller than dorsal arteries with a passive internal lumen diameter of $138.3 \pm 1.2 \mu\text{m}$ ($n(N) = 152(610)$).

Arteries prepared in this way for use on a wire myograph were stored in PSS at 5 °C and used within 48 hours of dissection. Arteries from the left and right sides of the corpus cavernosum or dorsal surface and from different positions along the length of the penis were randomly assigned to experimental protocols.

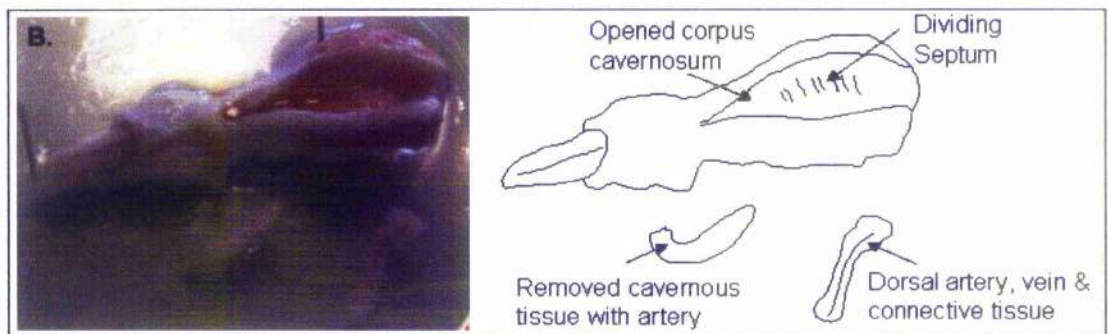
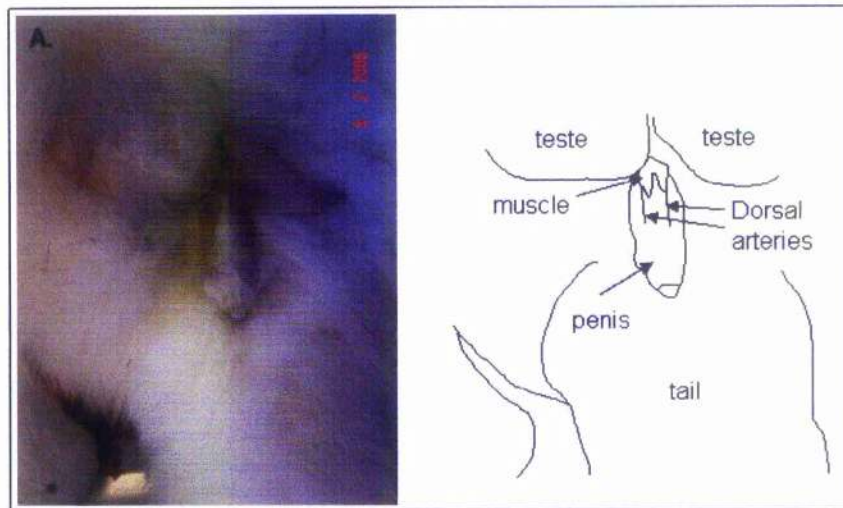


Figure 2-1: Location of the male rabbit penile arteries. A: Genital tissues *in situ* showing position of the penile dorsal arteries. B: Isolated penile tissue showing dissected dorsal and cavernous arteries and opened corpus cavernosum.

Female Vaginal Resistance Arteries

Identification of Suitable Vessels

A review of the current literature in females revealed that although female sexual arousal is known to be a vasocongestive and neuromuscular event (Giuliano *et al.*, 2002) very few studies have been performed to investigate vaginal or clitoral anatomy and vasculature. Sexual stimulation in females is characterised by an increase in vaginal blood flow and smooth muscle relaxation as in male penile tissues. However, studies measuring increased vaginal blood flow have relied on techniques, such as laser Doppler flowmetry, laser oximetry or heated oxygen electrodes, to provide a measure of vaginal tissue perfusion. In an attempt to identify suitable blood vessels for isolation from vaginal tissues investigative dissections were carried out in collaboration with colleagues (Pfizer Global Research and Development) and based on anatomical illustrations (Barone, 1973; Popesko *et al.*, 1990). The main vaginal artery was identified as a branch of the internal iliac artery, which was

traced towards the vaginal tissue and found to form a close association with the vaginal wall. This artery was considered to be the main blood supply for vaginal tissues and hence important in sexual vascular responses. The vaginal artery corresponds well to the male penile arteries chosen for study since both originate as branches of the internal iliac artery.

Dissection Techniques

To remove vaginal tissues, the female rabbit was placed in a supine position. Skin overlying the abdomen was dissected back towards the hindlimbs and the exposed abdominal muscles excised down the midline towards the vaginal opening. Since the vagina lies beneath the symphysis pubis in this position it was necessary to remove a section of pelvic bone to expose the vaginal vestibule. The vaginal artery, left and right branches of the internal iliac artery, was identified and the entire vaginal tissue excised from the animal taking care to preserve a good length of vaginal artery (Figure 2-2). Further dissection of the vaginal artery was performed in ice-cold PSS, under a Zeiss dissecting microscope (Stemi 2000), in an agar-filled Petri dish.

The vaginal artery was identified prior to joining with the vaginal wall. A length of artery approximately 5 cm long, running from out with the vaginal wall to a point proximal to the vaginal opening, was dissected free from both left and right sides of the vaginal tissue. The vaginal artery branches extensively within 2 cm of the vaginal opening and as many as possible of these branches were included in the dissection. Surrounding tissue was removed from the artery walls whilst the vessels were pinned.

In the isolated vaginal artery, the point at which the vessel originally met the vaginal wall *in situ* could be identified by the presence of a single branch point near the beginning of the isolated section, followed by a significant length of vessel devoid of branches. Ring segments of artery approximately 2 mm in length were taken from the vaginal artery proximal to this identifying branch point. These sections were referred to as the 'extra' vaginal artery (EVA) since they originated from a section of artery not closely associated with the vaginal wall. The passive internal lumen diameter was found to be $270.0 \pm 3.5 \mu\text{m}$ ($n(N) = 66(254)$) and as such was the largest artery investigated. In preparation for wire myography, 40 μm diameter wires were inserted into the artery lumens and the vessels were stored in PSS at 5 °C and used within 48 hours of dissection.

Further ring segments of a similar length were taken from the distal end of the vaginal artery; at a point where the vaginal artery branches extensively. Primary branches were

chosen for dissection and all smaller branches removed. Ring segments were taken down to a point where vessel lumens became too narrow for use on a wire myograph. Due to the requirement to insert two 40 μm diameter wires into the artery lumen, see Vessel Mounting Procedure, this equated to around 90 to 100 μm internal lumen diameter. These artery sections were referred to as the 'intra' vaginal artery (IVA) since they were found in close association with the vaginal wall. The mean passive internal lumen diameter was found to be $148.4 \pm 1.5 \mu\text{m}$ ($n(N) = 69(269)$). As with the cavernous artery, the size of these vessels required the insertion of a length of wire into the entire artery before subsequent separation into approximately 2 mm lengths. This helped to prevent undue damage occurring to endothelial cells and vessel walls. Arteries were stored in PSS at 5 $^{\circ}\text{C}$ and were used within 48 hours of dissection.

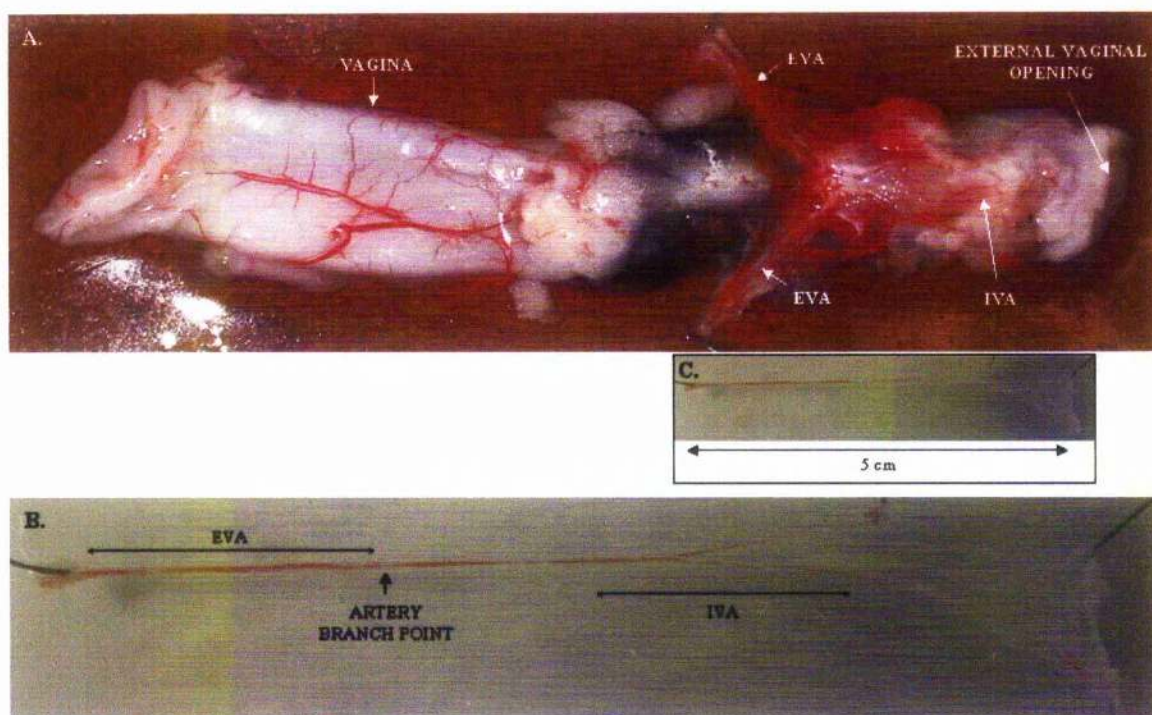


Figure 2-2: Dissection of the female rabbit genital arteries. A: Isolated vaginal tissues showing the position of the vaginal artery (EVA and IVA). B: Isolated vaginal artery showing the branch point from above which the artery was termed 'EVA' and smaller downstream branches termed 'IVA'. C: 'B' shown to scale against the isolated rabbit vagina.

Techniques

The equipment used to carry out pharmacological evaluation of vessel function was the small vessel wire myograph (DMT, Aarhus, Denmark). The particular models used were the multi myograph 600M or 610M, which consist of a set of four baths mounted on a heat

block set to heat the baths to 37 °C. Each bath had provision for a supply of gas and suction fluid removal (Figure 2-3). Within each bath a set of two detachable stainless steel heads were positioned to form a vice-like set of jaws. One head was attached to a micrometer and could be adjusted to allow alignment and application of circumferential tension to the vessel. The second head was attached to a force transducer measuring the tension generated by stretch or vascular activity as force in mN. Output was transmitted via a myograph interface and PowerLab 4/20 data acquisition system to a computer display calibrated in grams force, Chart v4.1.2 (PowerLab ADInstruments Ltd, UK). Myographs were regularly calibrated using a two gram weight and 'T' bar, which exerted a force equivalent to one gram on the transducer head. Little change was observed between calibrations.

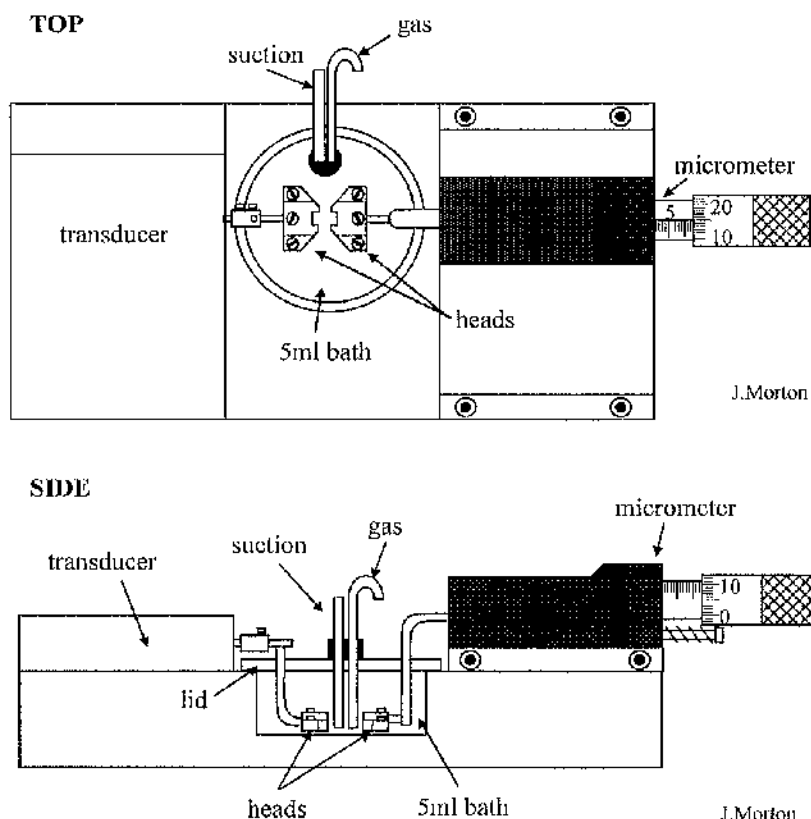


Figure 2-3: Top and side views of a wire myograph bath showing micrometer and transducer heads, gas supply and suction.

Vessel Mounting Procedure

Prior to setting up tissue, the myograph was first rinsed with dH₂O and cold PSS. Each bath could accommodate a single 2 mm section of vessel and was filled with 5 ml of PSS. Prepared arteries were mounted in the baths as follows (Figure 2-4). A vessel with a single

wire through the lumen was placed between the myograph heads and held by closing the jaws together. The wire was then secured to the micrometer head using two small screws. Once in place the heads were separated and a second 40 μm diameter wire inserted through the vessel lumen. In larger vessels this did not present a problem but in smaller vessels the potential for causing endothelial damage was more significant and care was taken to minimise damage. The second wire was secured to the transducer head using two small screws. The heads were positioned with the two wires parallel and adjacent. Excess wire was removed to prevent interference with gas bubbles. A Perspex lid and funnel were fitted to the bath to assist temperature regulation and the regular replacement of PSS. Each bath was bubbled with 95% O_2 , 5% CO_2 to minimise changes in pH and maintain the viability of the blood vessels.

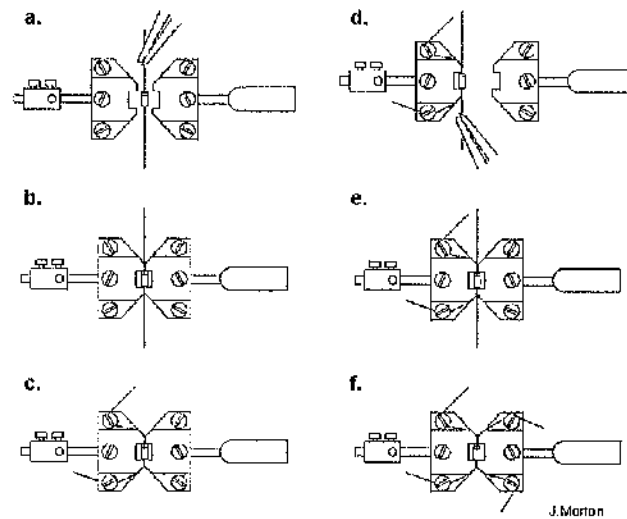


Figure 2-4: Procedure for mounting a blood vessel in a wire myograph bath. a. Positioning of vessel with a single wire through lumen between myograph heads using forceps. b. Closure of heads. c. Attachment to micrometer head. d. Insertion of second wire through lumen. e. Closure of heads. f. Attachment to transducer head.

Protocols

Optimal Isometric Tension

Active-tension experiments were performed to determine the optimal isometric tension for each vessel. Maximal contractions in response to phenylephrine (PE, 10 μM) were determined using a range of resting tensions from zero to one gram force (Figure 2-5). Resting tension was set at 0.25 grams force for all experiments involving cavernous and dorsal penile arteries and at 0.30 grams force for all experiments involving vaginal arteries.

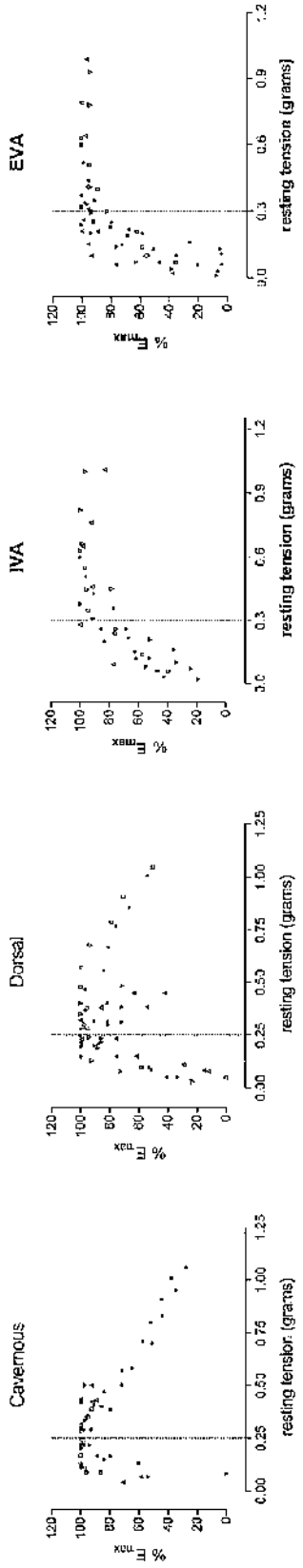


Figure 2-5: Length-tension experiments in the genital arteries to determine optimal isometric tensions. Responses to PE (10 μ M) at different basal resting tensions shown expressed as a percentage of the maximum response in an individual vessel. n(N): cavernous = 5(7), dorsal = 5(8), IVA = 3(7) and EVA = 3(8). Vertical dotted lines show basal tensions selected as being appropriate, i.e. maximal responses were achieved without overstretch of the artery, for each vessel for further experimental protocols.

Wake-Up Protocol

Vessels were allowed to equilibrate under resting tension for 30 minutes, following which a reproducible maximal contraction to noradrenaline (NA, 10 μ M) was determined. Between each addition of NA responses were allowed to plateau, given two washes over a period of eight to ten minutes and allowed to re-equilibrate for a further ten minutes. Each wash, whether during wake-up or experimental protocols, consisted of four total changes of PSS. The mean NA maximal contraction was used as a standard response (sighting response), specific for each vessel, with which to compare experimental responses. Endothelium integrity was confirmed by assessing the ability of acetylcholine (ACh, 3 μ M) to relax NA (10 μ M) pre-contracted vessels. Vessels were allowed to equilibrate for 30 minutes prior to the commencement of experimental protocols.

Experimental Protocols

To assess the properties of vasoconstrictor agonists, concentration response curves (CRCs) were constructed by cumulative addition of agonists in half log increments from 0.1 nM to 300 μ M. During the evaluation of antagonists, first curves to agonists were performed with an absence of antagonist in all preparations. Following a wash out period, vessels were incubated with antagonist (1 nM to 100 μ M) for 30 to 40 minutes prior to second agonist CRCs. This procedure was repeated to establish third or fourth CRCs when necessary. All protocols included a parallel control vessel with an absence of antagonist during each curve. In some cases where a subsequent agonist curve was found to be significantly desensitised pre-incubation of antagonists occurred before single CRCs were constructed. In this situation responses in the presence of antagonist were compared to parallel control vessels.

An alternative protocol was used to determine receptor subtypes involved in responses to the agonist UK 14,304. In this protocol a cumulative CRC to UK 14,304 (either 0.1 nM to 30 nM or 0.1 nM to 30 μ M) was constructed in the absence of antagonists. Following the completion of these curves, and before responses were washed out, CRCs to antagonists were constructed on the background of UK 14,304-induced vasoconstriction.

To investigate agonist-induced vasodilation tone was raised using NA. Following the establishment of a plateau, agonists were added cumulatively in half log increments from 0.1 nM to 30 μ M. As with contractile agonists first curves were performed with an absence of antagonist. After a wash out period preparations were incubated with antagonists (0.001

to 100 μM) for 30 to 40 minutes prior to second agonist CRCs. Where responses to vasoactive intestinal polypeptide (VIP) were investigated a total of two hours was allowed between curves to prevent desensitisation of the response. In a parallel control vessel curves were constructed in the absence of antagonists.

Electrical Field Stimulation

Techniques Additional to Small Vessel Wire Myography

To perform EFS of blood vessels, tissues were dissected and prepared for small vessel wire myography as previously described in this chapter. However, to allow electrical stimulation the stainless steel myograph heads were replaced with Perspex heads, each containing a stimulating electrode. The stimulating electrodes were situated alongside and parallel to the blood vessel. A current stimulator, model CS200 (DMT, Aarhus, Denmark) was used to pass a current through the vessel and cause stimulation of nerves within the vessel walls. The current stimulator could be programmed to generate bipolar pulses of a specified frequency, duration and current (Figure 2-6). The equipment could also generate trains of pulses with a specified duration, delay and mode, i.e. single, continuous or frequency stepped.

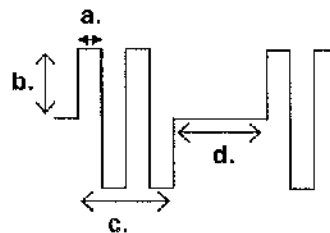


Figure 2-6: Pulses generated by the CS200 electrical field stimulator with a bipolar output, pulse duration ms (a.), current mA (b.), train duration ms (c.) and train delay ms (d.)

Protocols

Dissection and setting up of tissues, application of baseline tension and wake-up protocols proceeded as for general wire myography (see section Small Vessel Wire Myography). Initial experiments were performed using varying currents and frequencies to determine whether stimulation-induced responses could be blocked by tetrodotoxin (TTX), a Na^+ channel blocker that blocks the conduction of nerve impulses. Following initial investigatory experiments stimulation parameters were set at pulse duration 0.3 ms, current 35 mA, train duration 15 s with a delay of five minutes between trains.

Vasoconstrictor responses were investigated from baseline tone. To study vasodilator responses vessels were incubated with 10 μM guanethidine for 1 hour 30 minutes to

prevent adrenergic-induced contraction. Baseline tone was raised using NA (10 μM) and was allowed to plateau before stimulation began. For both vasoconstrictor and vasodilator responses initial frequency-response curves were constructed using frequencies from 0.5 Hz to 64 Hz increasing two-fold with each step. Following a wash out period vessels were incubated with an antagonist (10 nM to 100 μM) for 30 to 40 minutes prior to the construction of a second CRC. This was repeated for third or fourth CRCs where appropriate.

Data Analysis

Data was recorded using Chart v4.1.2 (PowerLab ADInstruments Ltd, UK) and analysed using Prism v3.0 (GraphPad Software Inc, U.S.A.) or Excel (Microsoft[®] Office Excel 2003) software. Data were expressed using mean, standard error (s.e.mean) and 95% confidence intervals (95% CI) where appropriate. Contractions were expressed either as force in grams, a percentage of the sighting response to NA (10 μM) or a percentage of the maximum contraction achieved during a CRC (E_{max}). Vasodilations were expressed as a percentage relaxation from pre-contracted baseline tone. CRCs were fitted with a sigmoidal curve of variable slope using the non-linear regression capabilities of Prism. Agonist responses were expressed using pEC_{50} values; the inverse log of the effective concentration producing 50% of maximum contraction. Where shown n indicates the number of animals used while N indicates the total number of vessel sections. Data were compared using a one-way analysis of variance (ANOVA) with a Bonferroni post-test or a Student's t -test where appropriate. Statistical significance was taken as $P < 0.05$.

In cases where three or more antagonist concentrations caused a parallel shift of an agonist curve in the same tissue a Schild plot of $\log(\text{dose ratio} - 1)$ vs $\log(\text{antagonist concentration})$ was constructed. In circumstances where linear regression of this plot gave a slope that was not significantly different from unity, the antagonist could be considered competitive and the x-intercept of this plot as an estimate of the $\log K_B$. A pA_2 was calculated as the inverse $\log K_B$. The pA_2 is defined as the inverse log of the concentration of antagonist required to shift an agonist response curve by two-fold compared to the control. Where less than three antagonist concentrations were available for analysis pK_B values were calculated for single antagonist concentrations. The pK_B is defined as the inverse log of the concentration of antagonist that occupies 50% of available receptors. Both pA_2 and pK_B values were used to describe receptor/drug interactions and to allow the potency of antagonists to be quantified.

Drugs and Solutions

Solutions

PSS composition (mM): NaCl 119, KCl 4.7, CaCl₂ 2.5, MgSO₄·H₂O 1.2, KH₂PO₄ 1.2, NaHCO₃ 24.9, glucose 11.1, EDTA 0.023 was prepared daily. The pH was 7.3 at room temperature.

Drugs

All drugs used were of analytical grade and purchased from Sigma-Aldrich™ (ACh, apamin, ATP, α,β ,mATP, BMY 7378, corticosterone, 5-MeU, NA, phentolamine, PE, prazosin, SNP, TNP-ATP), Roche Bioscience™, Palo Alto, CA (RS 100329), Calbiochem™ (VIP), Bachem (UK) Ltd™ (VIP, VIP (6-28)), Tocris™ (BRL 44408, rauwolscine, UK 14,304) or manufactured in house (Rec 15/2615, UK 343,664). Stock solutions were made from powder form using de-ionised H₂O (unless otherwise stated) and all further dilutions were made in de-ionised H₂O. Some drugs required sonication to assist the formation of a solution. In the preparation of ATP, solutions were tested for pH and found to be approximately pH 7.2 at the highest dilutions used for small vessel wire myography protocols.

Drugs included:

- ACh (*acetylcholine*): 10 mM
- ATP (*adenosine 5'-triphosphate disodium salt*): 10 mM
- α,β ,mATP (*α,β ,methyleneadenosine 5' triphosphate*): 100 mM
- Apa, Apamin: 1 mM
- BMY 7378 (*8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydro-chloride*): 1 mM
- BRL 44408 (*2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole maleate*): 10mM
- ChTX, Charybdotoxin: 100 μ M
- Cocaine (*cocaine hydrochloride*): 10 mM

- Corticosterone (*4-pregnene-11 β ,21-diol-3,20-dione 21-acetate*): 30 μ M in physiological saline solution
- Guanethidine (*guanethidine sulphate*): 100 mM
- Indomethacin: 10 mM in DMSO (*dimethylsulphoxide*)
- L-NAME (*N ω -nitro-L-arginine methyl ester hydrochloride*): 100 mM
- Medetomidine: 1 mM
- 5-MeU, 5-methylurapidil (*5-methyl-6[[3-[4-(2-methoxy-phenyl)-1-piperazinyl]-propyl]-amino]-1,3-dimethyl-uracil*): 100 nM
- MRS 2179 (*2'-Deoxy-N⁶-methyl adenosine 3',5'-diphosphate Diammonium salt*): 1 mM
- NA, noradrenaline (*(-)-norepinephrine (+)-bitartrate salt hydrate*): 100 mM in 23 μ M EDTA (*diaminoethanetetraacetic acid disodium salt*)
- Phentolamine (*2-(N-[m-hydroxyphenyl]-p-toluidinomethyl) imidazoline*): 10 mM
- PE, phenylephrine (*(R)-(-)-phenylephrine hydrochloride*): 100 mM
- Prazosin (*1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl) piperazine*): 1 mM
- Rauwolscine (*17 α -Hydroxy-20 α -yohimban-16 β -carboxylic acid methyl ester*): 1 mM
- Rec 15/2615 (*1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-[2-[2-methoxy-6-(1-methylethyl)phenoxy]acetyl]piperazine hydrochloride*): 1 mM in DMSO (*dimethylsulphoxide*)
- RS 100329 (*N-[(2-trifluoroethoxy)phenyl],N'-(3-thyminypropyl) piperazine hydrochloride*): 1mM
- SNP, sodium nitroprusside (*sodium nitroferricyanide*): 100 mM
- TNP-ATP (*2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate monolithium trisodium salt*): 10 mM
- UK 14,304 (*5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline*): 10 mM in DMSO (*dimethylsulphoxide*)
- UK 343,664: 10 mM in DMSO (*dimethylsulphoxide*)

- VIP, vasoactive intestinal polypeptide (*vasoactive intestinal peptide acetate salt*):
10 mM
- VIP (6-28), VIP fragment 6-28: 1 mM

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Chapter 3

Adrenergic Mechanisms

Introduction

Adrenoceptors

Adrenoceptors (ARs) belong to the G-protein coupled receptor family. Since their initial conception over a century ago, following the demonstration of an adrenaline-induced pressor effect in 1896 by Oliver & Schafer (Rang *et al.*, 2002), ARs have been through many changing definitions. After an intensive bout of scientific research in the 1980s, the current classification of nine, plus a potential further two, subtypes was reached (Bylund *et al.*, 1994).

The current nine AR subtypes have been characterised both pharmacologically and using molecular techniques. Three major classes exist, namely α_1 , α_2 and β , defined by their drug affinities, second messenger and physiological responses. Post-junctionally, α_1 -ARs are coupled to $G_{q/11}$ -proteins, which cause contraction of vascular smooth muscle by activation of the inositol trisphosphate (IP_3) pathway. Post-junctional α_2 -ARs may inhibit relaxation by coupling to G_i -proteins that in turn inhibit the adenylate cyclase (AC)/cyclic adenosine monophosphate (cAMP) pathway. Pre-junctionally, α_2 -ARs have been shown to inhibit release of neurotransmitters from adrenergic, cholinergic and non-adrenergic, non-cholinergic (NANC) nerve terminals in penile tissues (Saenz de Tejada *et al.*, 1989; Simonsen *et al.*, 1997b). β -ARs are coupled to G_s -proteins and cause stimulation of the AC/cAMP pathway leading to smooth muscle relaxation.

Each class of AR, α_1 , α_2 and β , has been further subdivided. The α_1 -ARs are divided into α_{1A} , α_{1B} and α_{1D} subtypes, the missing 1C reflects the fluxes and discrepancies arising from various methods of characterising and classifying α_1 receptors. Classification of α_1 -AR subtypes originally included only two groups: the α_{1A} - and α_{1B} -ARs (McGrath, 1982). Later definitions accounted for low (α_{1L}) and high (α_{1H}) affinities for prazosin (Flavahan & Vanhoutte, 1986). The current classification into α_{1A} , α_{1B} and α_{1D} subtypes includes three subtypes, which all demonstrate a high affinity for prazosin, leaving the α_{1L} -AR subtype currently unaccounted for. Further research in the late 1990s proposed the α_{1L} -AR as a fourth, distinct subtype (Docherty, 1998). However since the α_{1L} -AR has not been successfully cloned it has been suggested that the α_{1L} -AR may represent an alternative affinity state of the α_{1A} -AR (Ford *et al.*, 1997).

The α_2 -ARs are divided into α_{2A} , α_{2B} and α_{2C} subtypes. While most research to determine α_2 -AR subtypes has shown a good correlation between pharmacological and cloning methods, the only discrepancy was the α_{2D} -AR, which has been proposed as a species variant of the α_{2A} -AR found in rodents.

The β -ARs are divided into β_1 , β_2 and β_3 subtypes (reviewed by Guimaraes & Moura, 2001). β_1 -ARs are found in the heart where they mediate chronotropic and inotropic effects. β_2 -ARs cause relaxation of vascular smooth muscle via stimulation of the AC/cAMP pathway. The third subtype, the β_3 -AR, mediates lipolysis and thermogenesis in skeletal muscle and adipose tissue. A fourth β -AR has been proposed, the β_4 -AR (Bylund *et al.*, 1994; Oriowo, 1995). However, the results of this study may also be explained through an atypical action of a β_3 -AR agonist on the β_1 -AR.

Adrenergic Pathways in Male Genital Tissues

Cavernous trabecular smooth muscle and penile arteries are innervated by adrenergic nerves (Tamura *et al.*, 1995). The neurotransmitter NA is released from these nerves and causes contraction of the smooth muscle of trabecular tissue and vasculature leading to detumescence of the penis. It has also been suggested that tonic sympathetic activity may maintain flaccidity of the penis in the resting state. Support of this theory is based on erections induced by injection of α -AR antagonists and the association of priapism and prolonged erection following treatment with α_1 -AR selective antagonists (Brindley, 1986; Saenz de Tejada *et al.*, 1991; Munoz *et al.*, 1994; Zorogniotti, 1994; Becker *et al.*, 1998; Marquer & Bressolle, 1998).

NA-induced contraction of penile erectile tissue is considered to be predominantly mediated by α_1 -ARs. Sato & Kawatani (2002) have shown that NA-induced increase in cytosolic Ca^{2+} concentration in rabbit corpus cavernosum cells was largely mediated by α_1 -ARs with a small decrease due to β -ARs. Expression of mRNA for α_{1A} , α_{1B} and α_{1D} subtypes has been demonstrated in corpus cavernosum tissue in the human (Dausse *et al.*, 1998; Goepel *et al.*, 1999), rabbit (Peng *et al.*, 1998) and rat (Veronneau-Longueville *et al.*, 1998). However, Goepel *et al.* (1999) showed that only α_{1A} - and α_{1B} -AR mRNA was further translated into protein with no protein expression for the α_{1D} -AR detected.

Functional studies have demonstrated the presence of α_1 -ARs in the corpus cavernosum tissue of the horse (Recio *et al.*, 1997; Simonsen *et al.*, 1997a), rat and dog (Sironi *et al.*,

2000; Mizusawa *et al.*, 2002), human and rabbit (Saenz de Tejada *et al.*, 1989; Traish *et al.*, 1995; Furukawa *et al.*, 1996; Peng *et al.*, 1998; Sato & Kawatani, 2002). In the rabbit corpus cavernosum, despite showing a major mRNA expression of α_{1B} -ARs, Peng *et al.* (1998) attributed the main functional receptor to be the α_{1A} -AR using the selective antagonist 5-methylurapidil. The α_{1A} -AR was also concluded to be the main functional AR in the rat corpus cavernosum by Tong & Cheng (1997) using an alternative α_{1A} -AR selective antagonist, WB4101. Furukawa *et al.* (1996), suggested that functional characteristics in the rabbit corpus cavernosum represented an α_{1B} -AR response based on the sensitivity of PE contractions to chloroethylclonidine (CEC) and a low pA_2 (8.05) to WB4101. However, Hirasawa *et al.* (1997) have shown that CEC is not α_1 -AR subtype-selective and, therefore, cannot be relied on as an α_{1B} -AR selective antagonist as previously believed. Both α_{1A} - (Rec 15/2841) and α_{1B} -AR (Rec 15/2615) antagonists, but not α_{1D} -AR antagonists (Rec 15/3039), have been shown to have pro-erectile properties in rats and dogs (Sironi *et al.*, 2000). In contrast, Mizusawa *et al.* (2002) demonstrated that α_{1B} -ARs had only a minor role in NA-induced contraction of rat corpus cavernosum and cavernous arteries with a major α_{1D} -AR component based on a putative α_{1D} -AR selective compound, A-119637. However, the concentrations of A-119637 used during the study could be acting at all three α_1 -AR subtypes (Carroll *et al.*, 2001).

Functional post-junctional α_2 -ARs have been shown to be present in rabbit trabecular smooth muscle cells (Gupta *et al.*, 1998) and in human corpus cavernosum and cavernous arteries (Hedlund & Andersson, 1985). Pre-junctional α_2 -ARs have been demonstrated in horse cavernosal arteries (Simonsen *et al.*, 1997b). In the penile tissues, pre-junctional α_2 -ARs are thought to modulate both adrenergic neurons, an autoregulatory function inhibiting further NA release (Saenz de Tejada *et al.*, 1989) and NANC neurons, an inhibition of nitric oxide (NO) release (Simonsen *et al.*, 1997b).

Endogenous NA and adrenaline may also act at β -ARs. The presence of functional β -ARs has been demonstrated in horse corpus cavernosum (β_1 - and β_2 -AR subtypes, Recio *et al.*, 1997) and cultured rabbit corpus cavernosum cells (Sato & Kawatani, 2002). It is thought that the β_2 -AR subtype may be important in mediating relaxation of trabecular smooth muscle and penile arteries (Carati *et al.*, 1985; Simonsen *et al.*, 1997a). Evaluation of mRNA expression has shown that β -ARs are outnumbered approximately 10:1 by α -ARs (Levin & Wein, 1980) and, therefore, may not exert a significant vasodilator effect in response to NA (reviewed by Lerner *et al.*, 1993). However, Simonsen *et al.* (2002) have suggested that β -ARs are highly sensitive to adrenaline and that elevated plasma levels of

adrenaline during erection, demonstrated by Becker *et al.* (2000), may contribute to vasodilation of penile arteries.

Adrenergic Pathways in Female Genital Tissues

Human female vaginal tissue has been shown to be densely innervated (Hilliges *et al.*, 1995) and in the pig both adrenergic and cholinergic nerves were present (Lakomy *et al.*, 1987). Similar to the male, sympathetic innervation in females originates from the hypogastric nerve (Liu *et al.*, 2001) and nervous control of sexual function is thought to be comparable in males and females (Giuliano *et al.*, 2001). However unlike males, in females the adrenergic agonist ephedrine, administered *in vivo* to activate the sympathetic nervous system, facilitates the initial stages of sexual arousal, demonstrated by an increase in both subjective and physiological sexual responses (Meston & Heiman, 1998; Meston, 2000).

The presence of α_1 - and α_2 -ARs in female genital tissues has been demonstrated both by expression of mRNA (Munarriz *et al.*, 2003) and by the action of α_1 - and α_2 -AR agonists and antagonists. In contrast to the reported actions of ephedrine in facilitating sexual arousal, the α_2 -AR agonist clonidine was shown to inhibit female sexual arousal (Meston *et al.*, 1997) and a non-selective α -AR antagonist, phentolamine, increased vaginal blood flow and pressure in rabbits *in vivo* (Park *et al.*, 1997). A comprehensive *in vitro* study by Kim *et al.* (2002) demonstrated functional α_1 - and α_2 -ARs in rabbit vaginal and clitoral tissues using the non-selective antagonist phentolamine, the α_1 -AR selective antagonists prazosin, tamsulosin and Rec 15/2615 and the α_2 -AR antagonist dequalamine. In addition, β -ARs have been shown to be present in vaginal and clitoral smooth muscle cell cultures (Traish *et al.*, 1999).

Aims

Significantly more data is available on adrenergic mechanisms in males than in females. All of the major AR classes, α_1 , α_2 and β , have been demonstrated to be present and functionally active within male genital tissues. However, even in this relatively well researched area there exist discrepancies and gaps in the current knowledge. The α_1 -AR has been attributed as the predominant AR mediating smooth muscle contraction in the genital tissues. However, no consensus of opinion has been reached as to the subtype/s that may be responsible for this action; studies exist to support each of the three candidates, α_{1A} -, α_{1B} - and α_{1D} -ARs. In addition, most current research has been performed in genital

tissues with few studies considering the function of isolated genital arteries. Studies in whole tissues may be complicated by the presence of many, potentially conflicting, pathways, while the study of isolated vessels may provide additional information about mechanisms involved in the function of the tissue circulation.

In female genital tissues, considerably more research is required to understand the basic physiology and pharmacology of vascular function. The involvement of the adrenergic system in female sexual function has been suggested but little mechanistic data has been acquired.

The aims of this chapter were as follows.

- To determine the involvement of α_1 - and α_2 -ARs in the function of male and female rabbit genital arteries.
- To subtype the α_1 -AR mediated response in male rabbit genital arteries.
- To compare AR mediated responses in male and female rabbit genital arteries.

Results

Adrenergic Innervation

During this study, immunohistochemistry techniques were used to make preliminary investigations into the innervation of female rabbit vaginal tissues. While it would have been interesting to have also carried out these studies in male penile tissues much of that work has been previously carried out by other investigators and constraints of time did not allow for it in the present study.

Pan-Neuronal Antibody

The initial primary antibody used was a mouse monoclonal antibody specific for protein gene product 9.5 (PGP 9.5, 1:200). PGP 9.5 is present in neuronal cell bodies and axons in the central and peripheral nervous systems and in small nerve fibres in the peripheral nervous system. Therefore, this antibody would be expected to bind to all neurons present in the tissue studied, a pan-neuronal antibody. In negative controls, primary antibody was omitted and replaced with normal mouse IgG.

Following primary antibody incubation, slides were stained for visualisation using the Dako® EnVision™ system (Dako) to label positive structures with horseradish peroxidase (HRP) followed by DAB. Using this system, negative structures were stained blue using a haematoxylin nuclear counterstain, while positive structures stained brown and were visualised under a light microscope. A second series of slides were stained using PGP 9.5 primary antibody (1:200) followed by FITC conjugated secondary antibody (1:50). Positive structures stained fluorescent green and were visualised under a fluorescence microscope using a HQ525/40nm (chroma technology) filter. Secondary antibodies or stains were included in all experimental and control slides.

During the preparation of isolated vaginal artery slides, nearby surrounding tissues and vessels were included in the sections. On staining these sections it could be seen that surrounding tissues included nerve ganglia, veins and arterioles in addition to the main vaginal artery. Arteries were identified by their structure, comprising a circular shape with thick walls and a smaller lumen (Figures 3-1 and 3-2). Veins were identified by a thinner wall structure in addition to a collapsed circular formation and larger lumen (Figure 3-3).

All nerve ganglia in vaginal artery sections were stained positive using PGP 9.5 and were numerous and in close proximity to the vaginal artery (Figure 3-1). In addition, PGP 9.5 staining demonstrated a layer of positive neurons surrounding arteries (Figures 3-1 and 3-2) but not veins (Figure 3-3). This layer, or plexus, of neurons was positioned external to vascular smooth muscle cells; around the media-adventitia border. Positive nerve structures were also demonstrated in close proximity to arterioles (Figure 3-3). Neurons, ganglia and the nerve plexus surrounding blood vessels were additionally visualised by positive staining with the FITC conjugated secondary antibody (Figure 3-4).

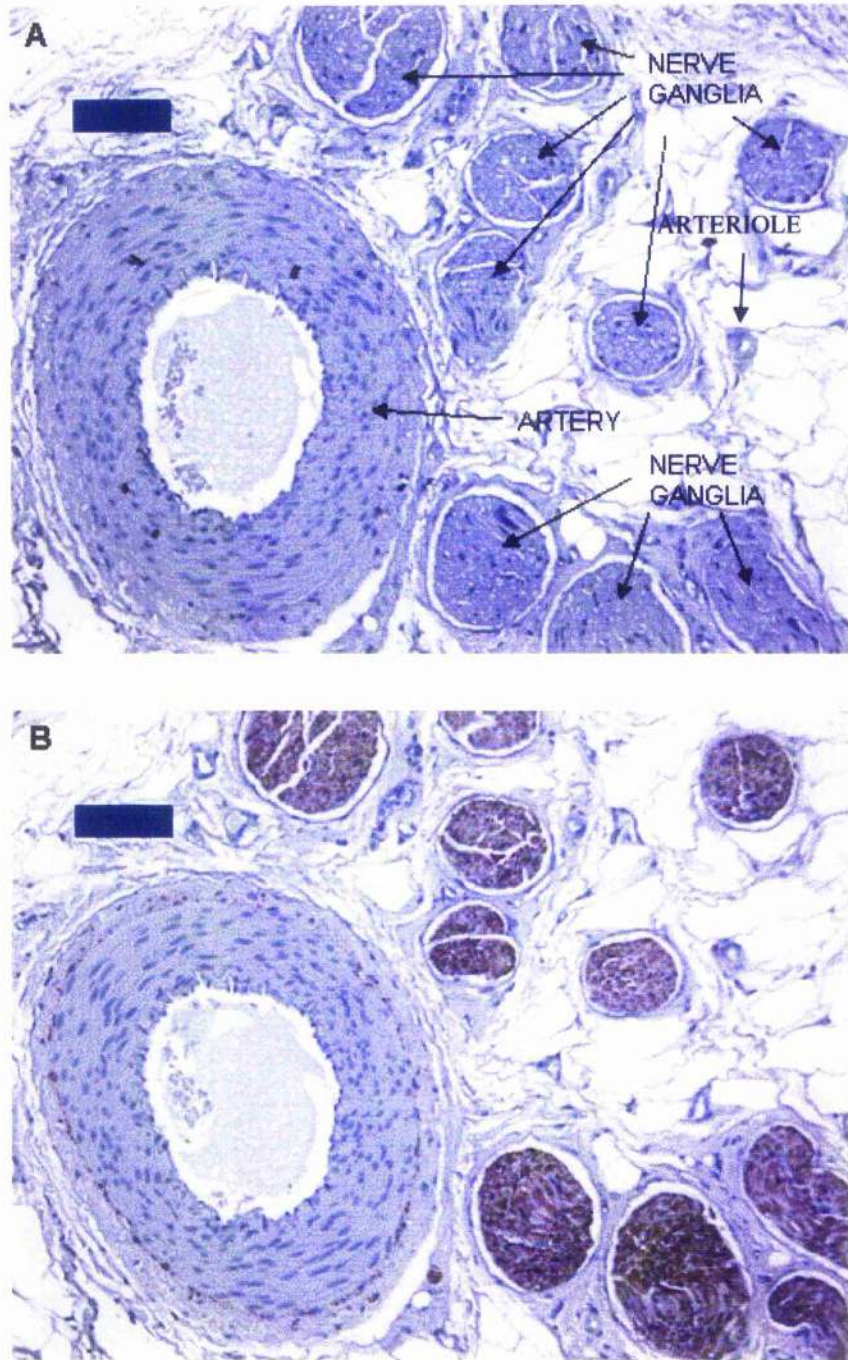


Figure 3-1: PGP 9.5 (1:200 dilution) staining in female vaginal tissue. A: Control, B: Brown staining positive for PGP 9.5 seen in a layer surrounding the arterial smooth muscle, close to an arteriole and in numerous nerve ganglia. Scale bar = 100 μ m.

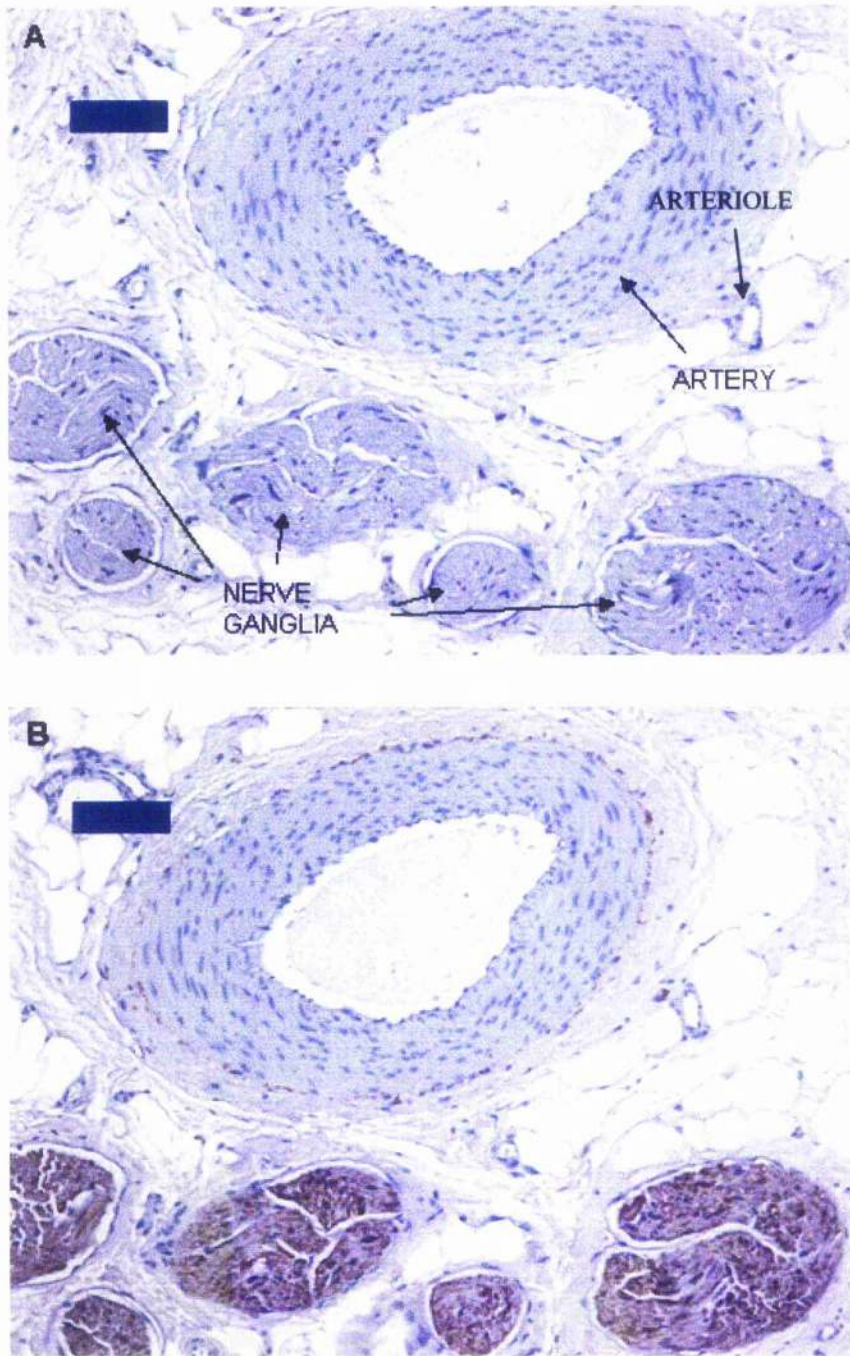


Figure 3-2: PGP 9.5 (1:200 dilution) staining in female vaginal tissue. A: Control, B: Brown staining positive for PGP 9.5 seen in a layer surrounding the arterial smooth muscle, close to an arteriole and in numerous nerve ganglia. Scale bar = 100 μ m.

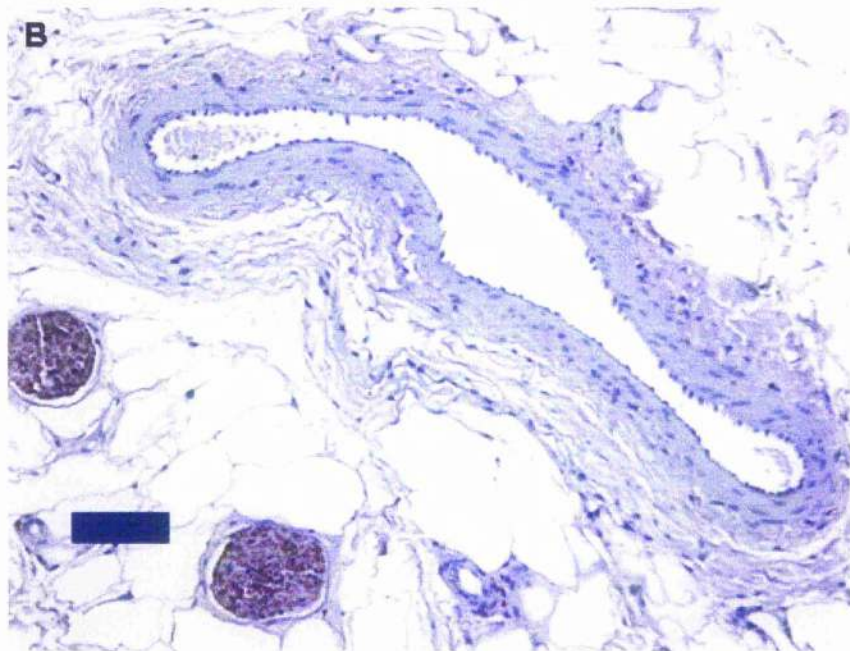
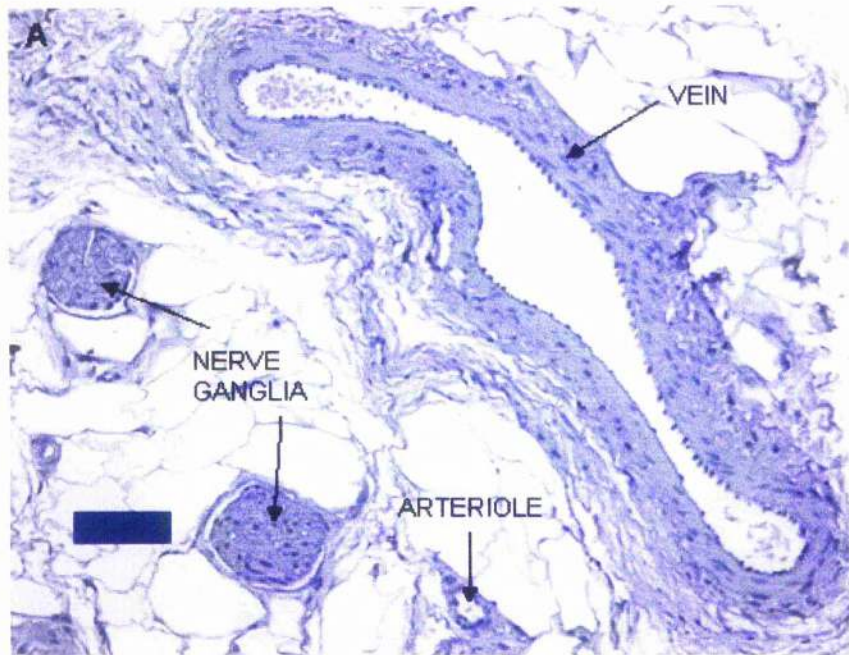


Figure 3-3: PGP 9.5 (1:200 dilution) staining in female vaginal tissue. A: Control, B: Brown staining positive for PGP 9.5 seen close to an arteriole, in nerve ganglia but not in association with a vein. Scale bar = 100 μ m.

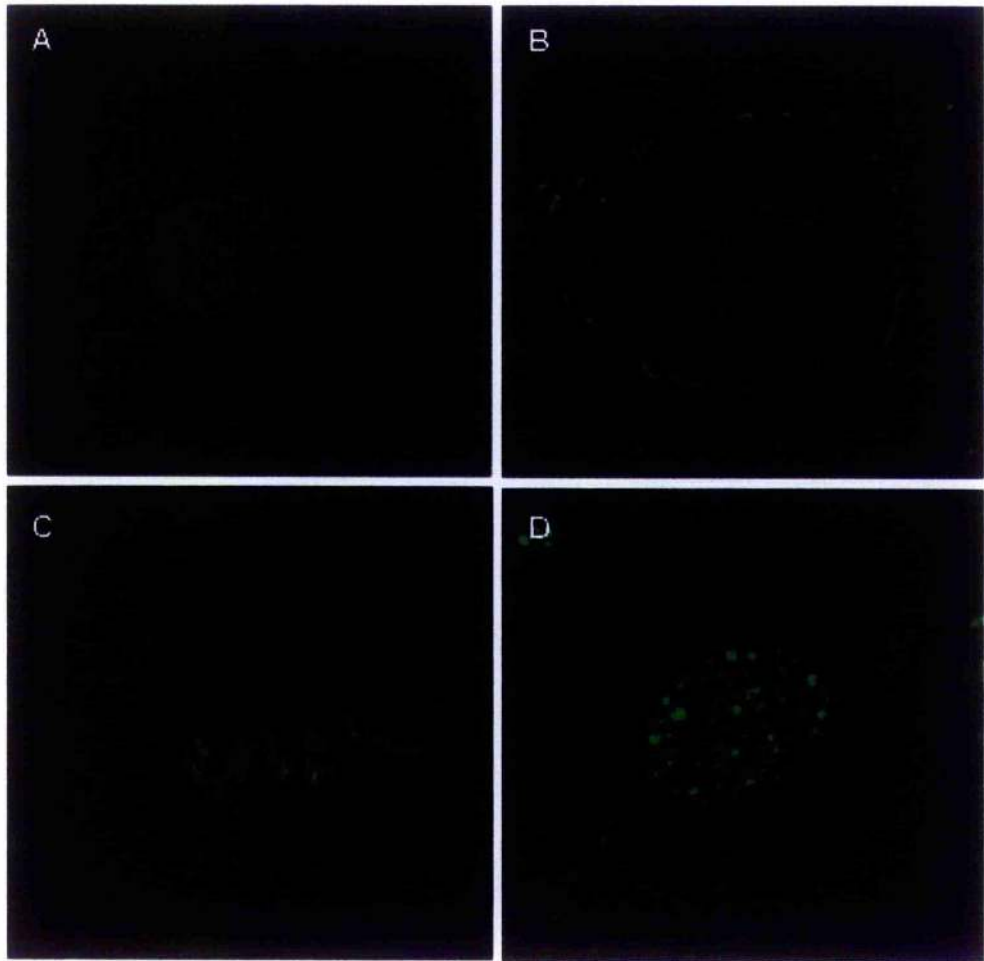


Figure 3-4: PGP 9.5 (1:200 dilution) staining in female vaginal tissue. A: Control demonstrating autofluorescence in red blood cells. Positive green fluorescent staining (FITC 1:50) demonstrating B: A plexus of nerves surrounding a blood vessel, C: A neuron and D: A nerve ganglion.

Adrenergic Neurons

To determine the presence or absence of adrenergic neurons in female genital tissues a primary sheep polyclonal antibody specific for dopamine β -hydroxylase (D β H, 1:1000) was chosen. The secondary antibody used was a donkey anti-sheep antibody conjugated to FITC at a concentration of 1:50, within the manufacturers' guidelines of 1:50 to 1:200. Negative controls consisted of slides where the primary antibody was replaced with PBS since normal sheep IgG was not available. These slides were incubated with secondary antibody as for experimental slides. Using these antibodies, positive structures were stained fluorescent green and were visualised under a fluorescence microscope using a HQ525/40nm (chroma technology) filter.

Positive fluorescent staining for D β H was observed in vaginal tissue sections in an area surrounding arteries that was similar to the plexus of nerves visualised using PGP 9.5 (Figure 3-5). However, no ganglia or veins within the genital tissues were observed as positive for D β H. A large amount of non-specific staining was observed in connective tissues and in the endothelial layer of arteries.

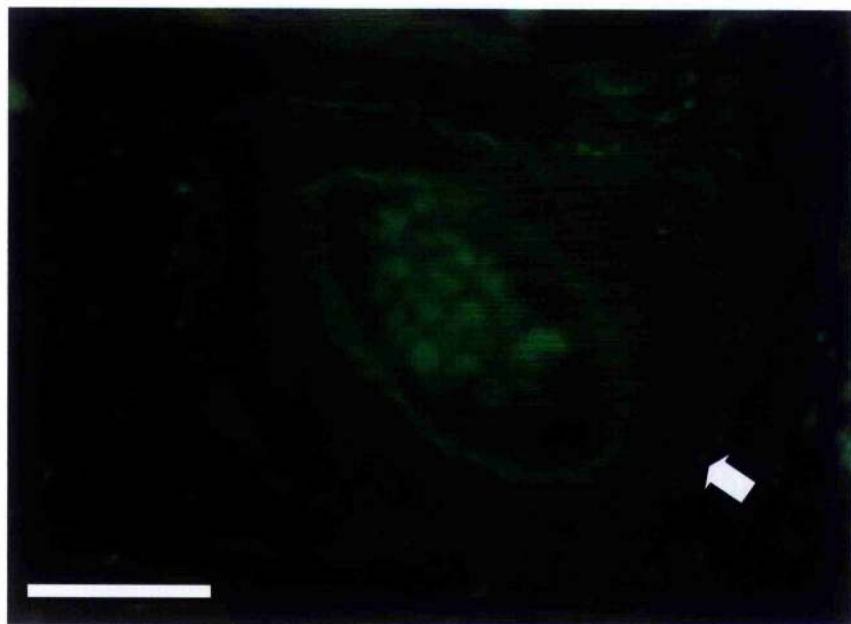
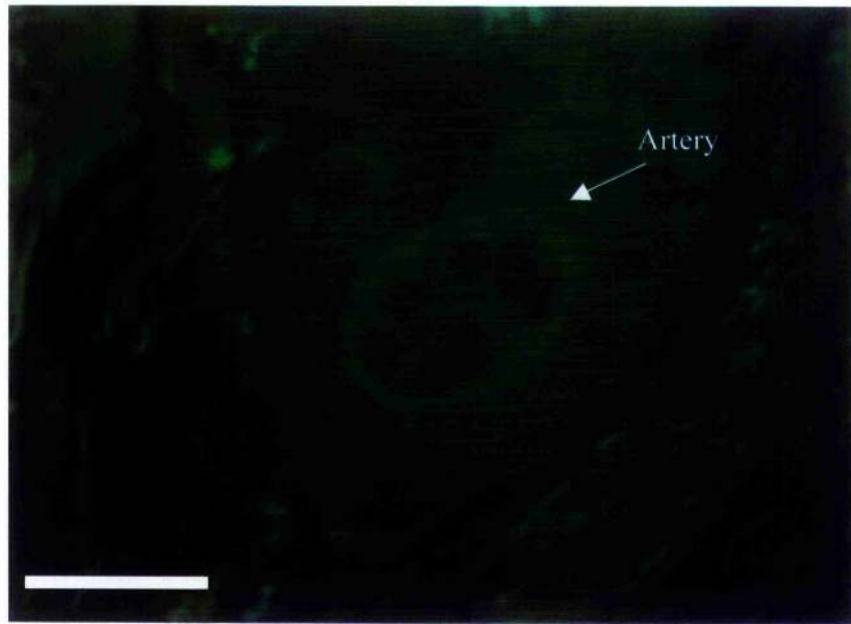


Figure 3-5: D β H (1:1000 dilution) staining the female vaginal tissue. A: Control, B: Green fluorescent staining (FITC 1:50) positive for D β H seen in a layer surrounding the arterial smooth muscle (arrow). Scale bar = 50 μ m.

Adrenoceptor-Mediated Responses

Noradrenaline – Non-Selective Adrenoceptor Agonist

The non-selective AR agonist NA caused vasoconstriction of all four arterial preparations (Figure 3-6, Table 3-1). Maximal contractions were greatest in EVA > dorsal = IVA > cavernous arteries and were of magnitude EVA: 2.88 ± 0.25 g ($n(N) = 12(14)$), dorsal: 1.87 ± 0.12 g ($n(N) = 16(27)$), IVA: 1.81 ± 0.12 g ($n(N) = 11(12)$) and cavernous: 1.08 ± 0.11 g ($n(N) = 18(24)$). In all arterial preparations, NA responses demonstrated similar potencies ($P > 0.05$) and Hill slopes close to unity.

Nitric Oxide Synthase Inhibition

Incubation with the nitric oxide synthase (NOS) inhibitor, N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μ M), caused a significant increase in NA E_{max} in EVA and dorsal arteries ($P < 0.05$, Figure 3-7, Table 3-2). In the dorsal artery, maximal NA contractions were increased approximately 37% from 1.76 ± 0.32 g to 2.78 ± 0.16 g ($n(N) = 6(6)$). The increase in the EVA was of approximately 38% from 3.17 ± 0.25 g to 4.39 ± 0.35 g ($n(N) = 8(10)$). In the female EVA, NA potency was significantly increased from pEC_{50} 5.86 ± 0.06 to 6.33 ± 0.14 ($P < 0.01$). L-NAME had no significant effect on NA-induced vasoconstriction in the IVA and cavernous arteries ($P > 0.05$).

Noradrenaline Uptake Block

Incubation with cocaine (3 μ M) and corticosterone (30 μ M) to block uptake 1 (neuronal reuptake) and uptake 2 (extra-neuronal uptake) mechanisms respectively, caused a significant increase in the sensitivity of all preparations to NA (Figure 3-8, Table 3-3). In male arteries, NA potency increased from pEC_{50} cavernous artery; 6.22 ± 0.14 to 7.20 ± 0.21 , ($n(N) = 5(6)$, $P < 0.01$) and dorsal artery; 5.66 ± 0.13 to 6.86 ± 0.12 , ($n(N) = 5(6)$, $P < 0.001$). In female vessels, NA potency increased from pEC_{50} IVA; 5.90 ± 0.19 to 6.62 ± 0.14 , ($n(N) = 6(6)$, $P < 0.05$) and EVA; 5.85 ± 0.08 to 6.67 ± 0.09 , ($n(N) = 5(6)$, $P < 0.001$). NA uptake blockers had no effect on overall NA E_{max} during a CRC in any artery but decreased responses to the highest concentrations in EVA and dorsal arteries ($P < 0.05$). At 300 μ M, NA-induced vasoconstriction was decreased by $24.1 \pm 9.0\%$ ($n(N) = 5(6)$) and $43.7 \pm 14.5\%$ ($n(N) = 6(7)$) in EVA and dorsal arteries respectively.

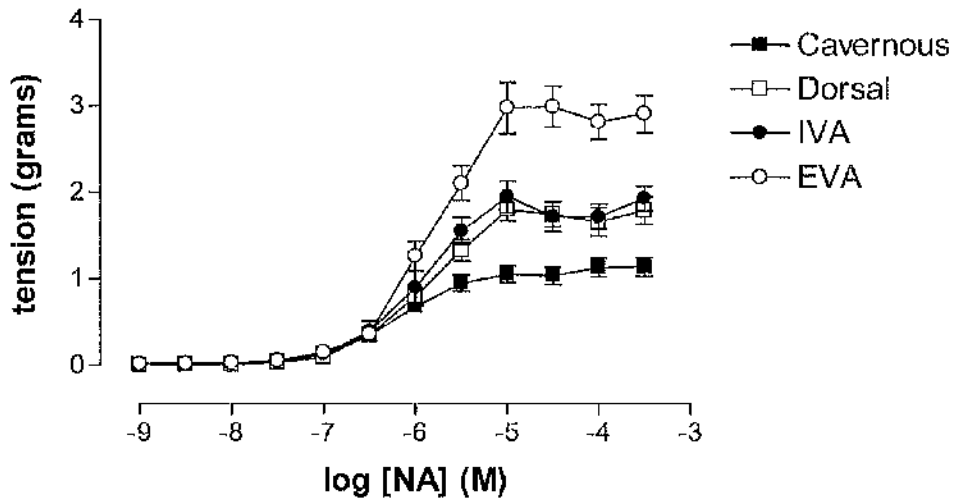


Figure 3-6: NA-induced vasoconstriction in the genital arteries expressed as grams tension \pm s.e.mean.

Table 3-1: Comparison of NA-induced vasoconstriction between vessels.

	n (N)	E_{max} grams (s.e.mean)	Hill Slope (95% CI)	pEC_{50} (s.e.mean)
Cavernous	18 (24)	1.08 (0.11)	1.22 (1.04 to 1.39)	6.27 (0.07)
Dorsal	16 (27)	1.87 (0.12)	1.23 (0.83 to 1.63)	5.92 (0.08)
IVA	11 (12)	1.81 (0.12)	1.36 (0.84 to 1.88)	5.81 (0.19)
EVA	12 (14)	2.88 (0.25)	1.33 (0.99 to 1.67)	5.94 (0.10)

CI: confidence interval

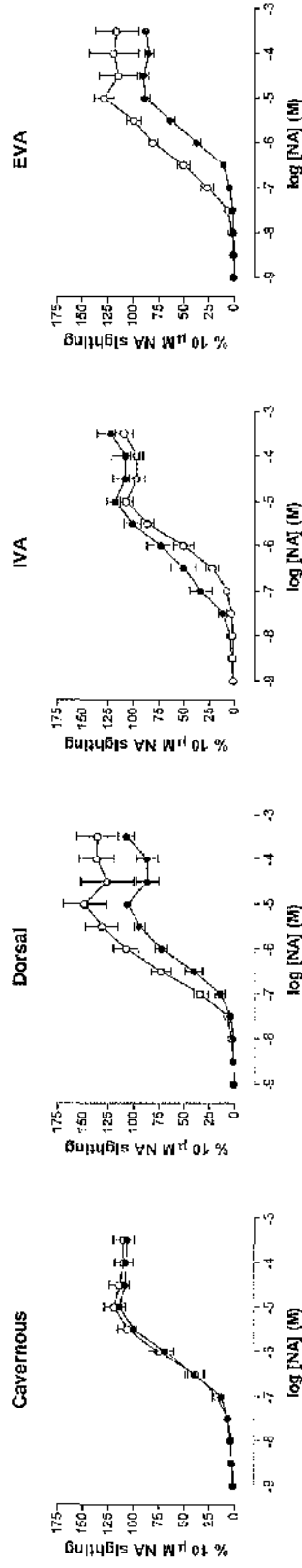


Figure 3-7: NA-induced vasoconstriction in the genital arteries in the absence (●) or presence (○) of L-NAME (100 μM) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-2: NA-induced vasoconstriction in the genital arteries in the absence or presence of L-NAME (100 μM).

	n (N)	E_{max} grams (s.e.mean)	E_{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC_{50} (s.e.mean)	
Cavernous	Control	6 (6)	0.96 (0.16)	115.78 (2.94)	1.21 (0.90 to 1.52)	6.31 (0.15)
	L-NAME	6 (6)	1.10 (0.20)	118.52 (10.57)	1.30 (0.76 to 1.84)	6.33 (0.15)
Dorsal	Control	6 (6)	1.76 (0.32)	110.76 (4.23)	1.37 (0.77 to 1.96)	6.32 (0.09)
	L-NAME	6 (6)	2.78 (0.16)	152.26 (20.63)	1.16 (0.43 to 1.88)	6.49 (0.12)
IVA	Control	8 (8)	2.01 (0.14)	113.31 (6.51)	1.35 (0.86 to 1.84)	5.90 (0.11)
	L-NAME	7 (8)	2.15 (0.20)	133.38 (11.92)	0.82 (0.39 to 1.25)	6.24 (0.16)
EVA	Control	8 (10)	3.17 (0.25)	99.77 (9.07)	1.34 (1.03 to 1.65)	5.86 (0.06)
	L-NAME	8 (10)	4.39 (0.35)	148.17 (19.39)	0.95 (0.38 to 1.53)	6.33 (0.14)

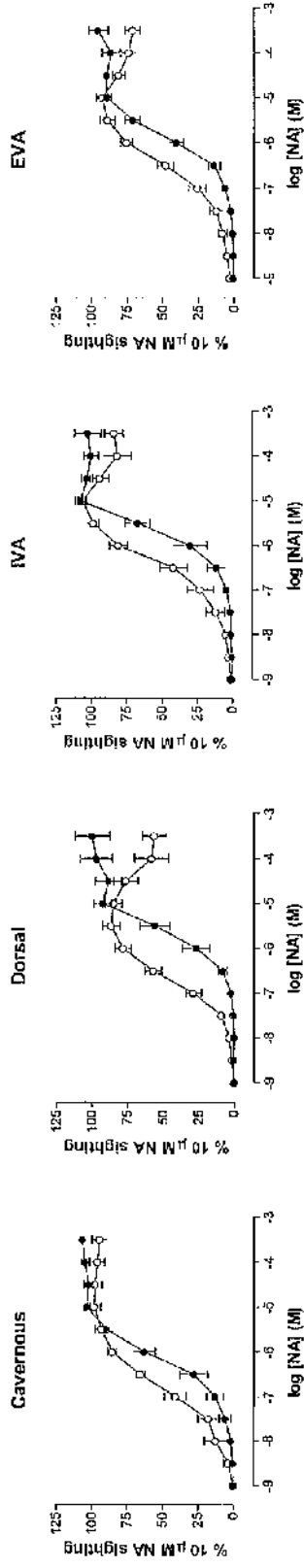


Figure 3-8: NA-induced vasoconstriction in the genital arteries in the absence (●) or presence (○) of NA uptake blockers cocaine (3 μM) plus corticosterone (30 μM) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-3: NA-induced vasoconstriction in the genital arteries in the absence or presence of NA uptake blockers cocaine (3 μM) plus corticosterone (30 μM).

	n (N)	E_{max} grams (s.e.mean)	E_{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC_{50} (s.e.mean)	
Cavernous	Control	5 (6)	1.57 (0.24)	108.61 (0.86)	1.17 (1.01 to 1.32)	6.22 (0.14)
	Uptake Block	6 (7)	1.13 (0.18)	87.69 (14.82)	0.96 (0.77 to 1.16)	7.20 (0.21)
Dorsal	Control	5 (6)	1.67 (0.35)	104.23 (10.46)	1.35 (0.90 to 1.80)	5.66 (0.13)
	Uptake Block	6 (7)	2.19 (0.18)	102.19 (14.42)	1.21 (1.03 to 1.39)	6.86 (0.12)
IVA	Control	6 (6)	1.88 (0.22)	112.72 (5.03)	1.41 (0.85 to 1.97)	5.90 (0.19)
	Uptake Block	6 (7)	2.05 (0.21)	107.19 (3.93)	1.27 (0.70 to 1.84)	6.62 (0.14)
EVA	Control	5 (6)	3.34 (0.37)	99.12 (5.06)	1.34 (1.04 to 1.63)	5.85 (0.08)
	Uptake Block	5 (6)	3.32 (0.64)	97.73 (4.94)	1.01 (0.85 to 1.17)	6.67 (0.09)

Alpha₁-Adrenoceptor-Mediated Responses

Phenylephrine – Alpha₁-Adrenoceptor Selective Agonist

All preparations responded to the α_1 -AR selective agonist, PE, with concentration-dependent vasoconstriction (Figure 3-9, Table 3-4). Maximal vasoconstriction to PE demonstrated the equivalent order of magnitude to NA-induced vasoconstriction, namely EVA > dorsal = IVA > cavernous arteries. The potency of PE-induced vasoconstriction was similar in EVA; 5.84 ± 0.19 (n(N) = 12(12)), IVA; 5.73 ± 0.18 (n(N) = 13(13)) and dorsal; 5.74 ± 0.05 (n(N) = 35(37)) arteries, while cavernous arteries; 6.28 ± 0.04 (n(N) = 38(39)) demonstrated a significantly greater sensitivity to PE than all other preparations ($P < 0.001$).

Vasoconstrictions to PE were reproducible with no change in potency from 1st to 2nd CRCs in any arterial preparation ($P > 0.05$, Figure 3-10, Table 3-5). However, IVA and dorsal artery preparations demonstrated a significant decrease in maximal contraction to PE of approximately 13% and 18% respectively on repetition of the PE CRC ($P < 0.05$). Maximal responses decreased from 2.02 ± 0.07 g to 1.75 ± 0.06 g (n(N) = 7(7)) in IVA and 1.85 ± 0.10 g to 1.52 ± 0.09 g (n(N) = 35(37)) in dorsal arteries. No decrease in E_{\max} was observed from 1st to 2nd CRCs in either the EVA or cavernous arteries ($P > 0.05$).

Nitric Oxide Synthase Inhibition

Incubation with the NOS inhibitor L-NAME significantly increased maximal contractions to PE in IVA, EVA and dorsal arteries ($P < 0.05$), by approximately 24%, 54% and 56% respectively with no effect on potency ($P > 0.05$, Figure 3-11, Table 3-6). PE E_{\max} increased in the IVA from 1.31 ± 0.23 g to 1.63 ± 0.15 g (n(N) = 6(6)), in the EVA from 1.96 ± 0.31 g to 3.02 ± 0.42 g (n(N) = 5(5)) and in the dorsal artery from 1.48 ± 0.25 g to 2.31 ± 0.14 g (n(N) = 5(5)). PE-induced vasoconstrictions in cavernous arteries were not significantly affected by incubation with L-NAME ($P > 0.05$).

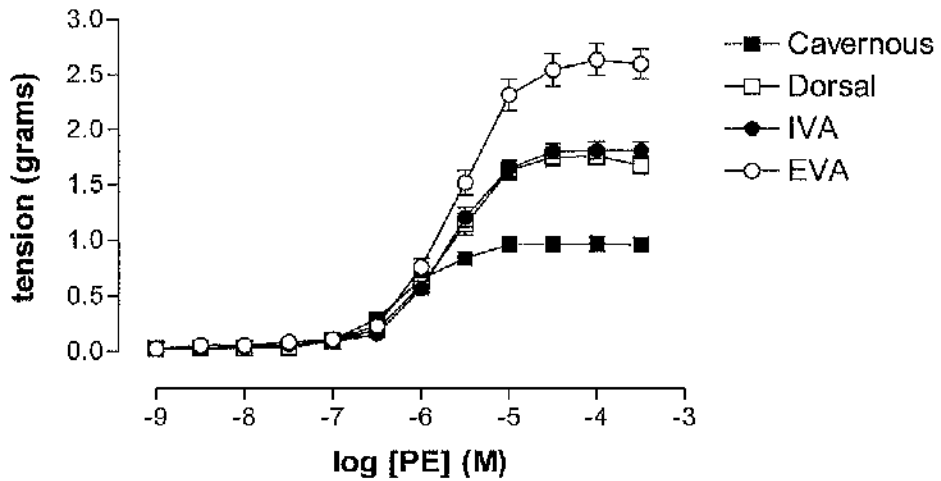


Figure 3-9: PE-induced vasoconstriction in the genital arteries expressed as grams tension ± s.e.mean.

Table 3-4: Comparison of PE-induced vasoconstriction between vessels.

	n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	38 (39)	0.97 (0.08)	102.32 (2.07)	1.28 (1.15 to 1.41)	6.28 (0.04)
Dorsal	35 (37)	1.85 (0.10)	87.64 (2.01)	1.31 (1.08 to 1.53)	5.74 (0.05)
IVA	13 (13)	1.69 (0.15)	101.43 (8.08)	1.36 (1.28 to 1.45)	5.73 (0.18)
EVA	12 (12)	2.55 (0.23)	91.97 (4.77)	1.19 (1.07 to 1.31)	5.84 (0.19)

CI: confidence interval

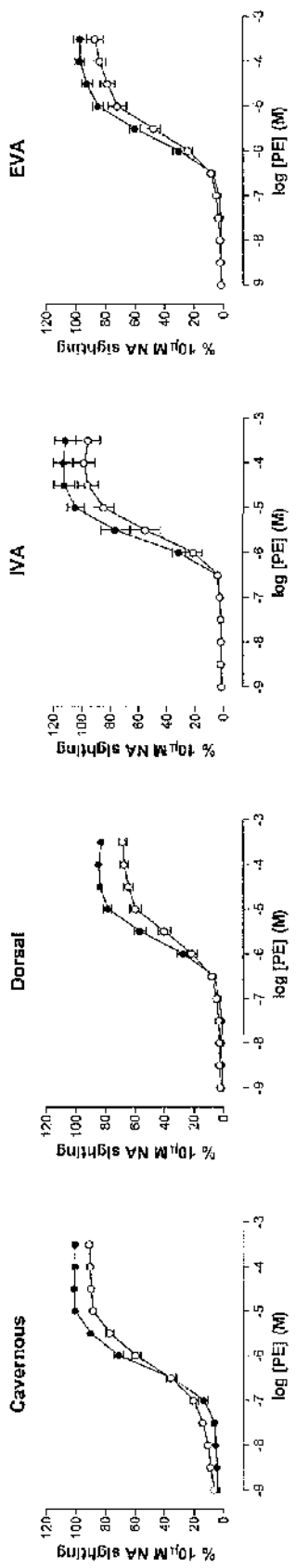


Figure 3-10: PE-induced vasoconstriction in the genital arteries, 1st (●) and 2nd (○) controls, expressed as a percentage of the NA (10 µM) sighting response ± s.e.mean.

Table 3-5: PE-induced vasoconstriction in the genital arteries, 1st and 2nd controls.

	n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)
Cavernous	1 st Control 38 (39)	0.97 (0.08)	102.32 (2.07)	1.28 (1.15 to 1.41)	6.28 (0.04)
	2 nd Control 38 (39)	0.86 (0.08)	89.82 (2.61)	0.98 (0.80 to 1.17)	6.32 (0.07)
Dorsal	1 st Control 35 (37)	1.85 (0.10)	87.64 (2.01)	1.31 (1.08 to 1.53)	5.74 (0.05)
	2 nd Control 35 (37)	1.52 (0.09)	71.82 (2.32)	1.15 (0.87 to 1.43)	5.64 (0.07)
IVA	1 st Control 7 (7)	2.02 (0.07)	115.03 (7.67)	1.36 (1.28 to 1.45)	5.69 (0.08)
	2 nd Control 7 (7)	1.75 (0.06)	100.08 (7.71)	1.44 (0.90 to 1.98)	5.54 (0.08)
EVA	1 st Control 7 (7)	2.79 (0.23)	99.38 (3.91)	1.19 (1.07 to 1.31)	5.71 (0.07)
	2 nd Control 7 (7)	2.58 (0.18)	87.30 (5.47)	1.11 (0.83 to 1.39)	5.59 (0.04)

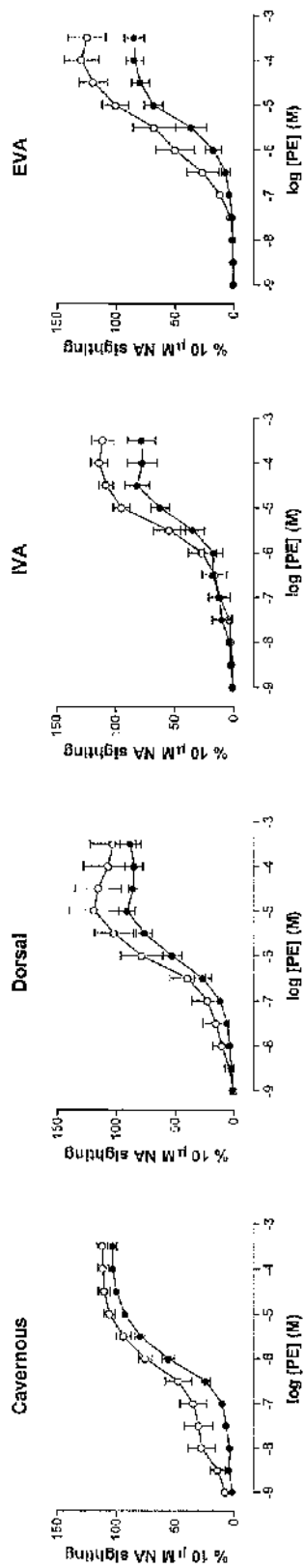


Figure 3-11: PE-induced vasoconstriction in the genital arteries in the absence (●) or presence (○) of L-NAME (100 μM) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-6: PE-induced vasoconstriction in the genital arteries in the absence or presence of L-NAME (100 μM).

	n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)	
Cavernous	Control	6 (6)	0.93 (0.17)	103.91 (3.45)	1.05 (0.86 to 1.25)	6.08 (0.07)
	L-NAME	6 (6)	1.13 (0.18)	108.91 (4.42)	0.73 (0.36 to 1.11)	6.65 (0.33)
Dorsal	Control	6 (6)	1.48 (0.25)	95.02 (6.08)	1.21 (0.73 to 1.70)	6.10 (0.11)
	L-NAME	5 (5)	2.31 (0.14)	128.81 (16.15)	1.25 (0.14 to 2.37)	6.28 (0.14)
IVA	Control	6 (6)	1.31 (0.23)	85.56 (12.80)	1.24 (0.30 to 2.18)	5.79 (0.40)
	L-NAME	6 (6)	1.63 (0.15)	124.62 (10.82)	1.14 (0.62 to 1.65)	5.75 (0.27)
EVA	Control	5 (5)	1.96 (0.37)	81.61 (8.50)	1.20 (0.63 to 1.76)	6.03 (0.45)
	L-NAME	5 (5)	3.02 (0.42)	136.76 (15.34)	0.73 (0.32 to 1.15)	6.02 (0.26)

Phentolamine – Non-Selective Alpha-Adrenoceptor Antagonist

Phentolamine (0.001 to 1 μM), a non-selective α -AR antagonist, was tested against PE-induced vasoconstrictions (Figure 3-12, Table 3-7) in male penile arteries.

In the cavernous artery, vasoconstrictions were significantly antagonised by phentolamine ($P < 0.001$) resulting in a parallel rightward shift of the PE curve. A mean pK_B of 8.33 ± 0.09 ($n(N) = 8(18)$) was calculated and a Schild plot of the data described a linear relationship with a slope not significantly different from unity, 0.81 ± 0.08 , allowing an estimated pA_2 of 8.55, 95% CI 8.09 to 9.12, to be calculated (Figure 3-13).

In the dorsal artery, PE-induced vasoconstrictions were significantly antagonised by phentolamine (0.1 to 1 μM , $P < 0.001$, Figure 3-12, Table 3-7). Analysis of the data gave a mean pK_B of 8.00 ± 0.16 ($n(N) = 8(18)$). The corresponding Schild plot demonstrated a slope not significantly different from unity, 0.72 ± 0.19 and a pA_2 of 8.32, 95% CI 7.34 to 10.31, (Figure 3-13).

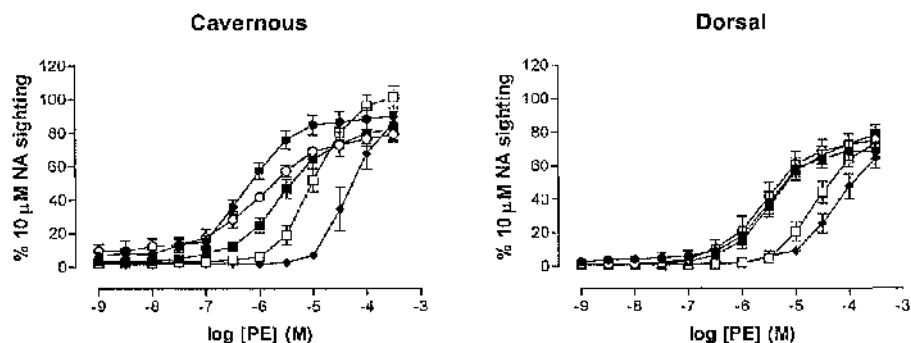


Figure 3-12: PE-induced vasoconstriction in the male genital arteries in the absence (●) or presence of phentolamine, 1 nM (○), 10 nM (■), 100 nM (□) or 1 μM (◆), expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

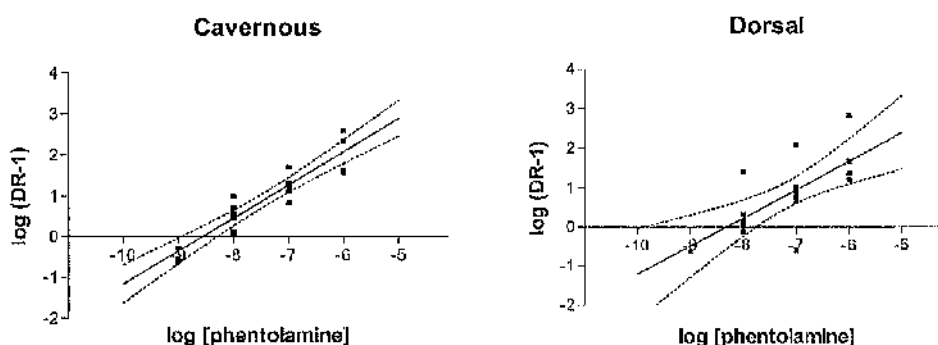


Figure 3-13: Schild plot of PE vs phentolamine in the male genital arteries. Each point shows a single result at a given antagonist concentration. Linear regression ± 95% CI shown.

Table 3-7: PE-induced vasoconstriction in the absence or presence of phentolamine in the male genital arteries.

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	Control	8 (8)	0.90 (0.13)	90.61 (5.82)	1.02 (0.87 to 1.17)	6.34 (0.08)
	1 nM	3 (3)	0.90 (0.05)	78.98 (4.51)	0.79 (0.71 to 0.87)	6.14 (0.09)
	10 nM	5 (5)	0.74 (0.14)	83.03 (7.13)	0.92 (0.83 to 1.00)	5.62 (0.07)
	100 nM	6 (6)	1.27 (0.26)	101.21 (6.78)	1.27 (1.15 to 1.38)	5.02 (0.07)
	1 μM	4 (4)	0.90 (0.14)	85.75 (7.08)	1.55 (1.35 to 1.76)	4.39 (0.12)
Dorsal	Control	8 (8)	1.39 (0.20)	71.33 (4.89)	1.19 (1.05 to 1.33)	5.63 (0.19)
	1 nM	6 (7)	1.46 (0.18)	75.36 (6.06)	1.21 (1.00 to 1.43)	5.59 (0.14)
	10 nM	8 (8)	1.58 (0.16)	79.92 (5.99)	0.99 (0.85 to 1.14)	5.43 (0.16)
	100 nM	8 (8)	1.54 (0.27)	73.27 (4.27)	1.23 (1.12 to 1.35)	4.66 (0.10)
	1 μM	6 (7)	1.58 (0.20)	64.48 (6.17)	1.20 (1.11 to 1.29)	4.33 (0.09)

CI: confidence interval

Prazosin – Non-Subtype-Selective Alpha₁-Adrenoceptor Antagonist

Prazosin (1 to 100 nM), a non-subtype-selective α_1 -AR antagonist, caused a parallel rightward shift of the PE CRC in the cavernous artery ($P < 0.001$, Figure 3-14, Table 3-8). The mean pK_B was calculated to be 8.41 ± 0.15 ($n(N) = 8(16)$). Schild analysis demonstrated a slope equal to unity, 0.99 ± 0.25 , allowing an estimated pA_2 of 8.42, 95% CI 7.66 to 9.85, to be calculated (Figure 3-15).

In the dorsal artery, prazosin (10 to 100 nM) caused a significant rightward shift of the PE CRC ($P < 0.01$, Figure 3-14, Table 3-8). The mean pK_B was 8.60 ± 0.16 ($n(N) = 7(18)$) in these arteries. Plotting a Schild plot of the data revealed a linear regression with a shallow slope, 0.41 ± 0.16 . Re-analysis of calculated pK_B s demonstrated decreasing affinity with increasing concentrations of prazosin: pK_B 9.34 ± 0.29 at 1 nM, 8.64 ± 0.23 at 10 nM and 8.14 ± 0.17 at 100 nM (Figure 3-15).

In the IVA, prazosin (100 nM) significantly inhibited PE-induced vasoconstrictions ($P < 0.001$, Figure 3-14, Table 3-8). The mean affinity of prazosin inhibition in these arteries was pK_B 8.35 ± 0.11 ($n(N) = 7(16)$). A Schild plot of the data showed a linear regression with a shallow slope, 0.61 ± 0.09 . Analysis of the pK_B s demonstrated decreasing affinity with increasing concentrations of prazosin: pK_B 8.85 ± 0.20 at 1 nM, 8.21 ± 0.12 at 10 nM and 8.05 ± 0.05 at 100 nM (Figure 3-15).

Prazosin (10 to 100 nM) significantly inhibited PE responses in the EVA ($P < 0.05$, Figure 3-14, Table 3-8) with a mean pK_B of 8.44 ± 0.09 ($n(N) = 7(19)$). Schild analysis demonstrated a linear regression with a slope close to unity, 0.78 ± 0.10 . The estimated pA_2 from this data was 8.58, 95% CI 8.03 to 9.22 (Figure 3-15).

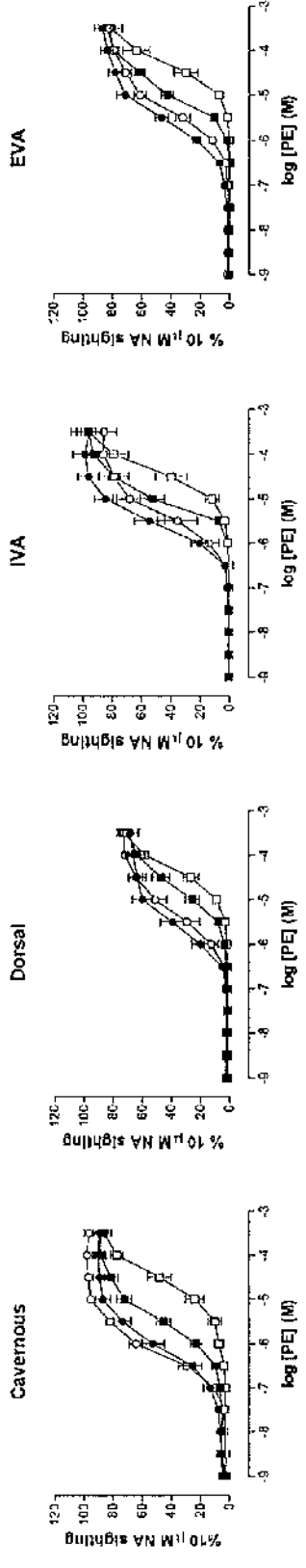


Figure 3-1: PE-induced vasoconstriction in the genital arteries in the absence (●) or presence of prazosin, 1 nM (○), 10 nM (■) or 100 nM (□) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

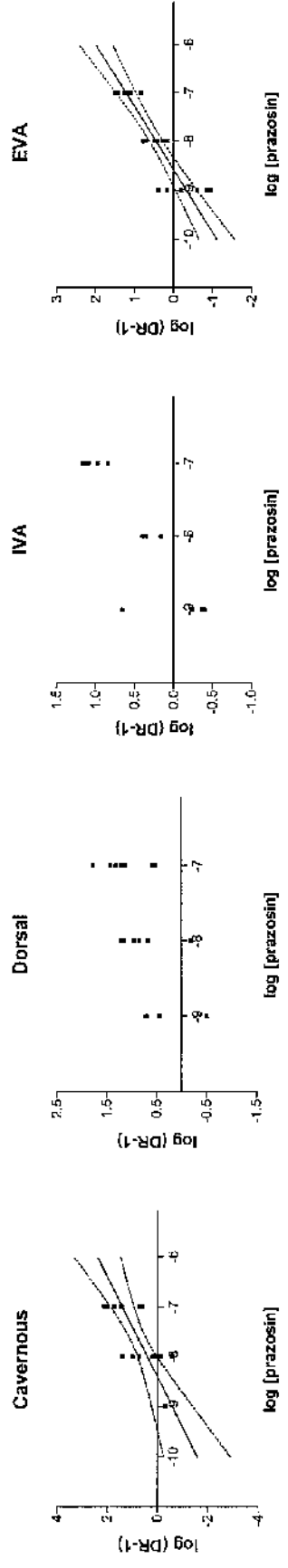


Figure 3-2: Schild plot of PE vs prazosin in the genital arteries. Each point represents a single result at a given concentration. Linear regression ± 95% CI is shown only when slope was not significantly different from unity.

Table 3-8: PE-induced vasoconstriction in the genital arteries in the absence or presence of prazosin.

	<i>n</i> (<i>N</i>)	E_{max} grams (s.e.mean)	E_{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)	
Cavernous	Control	7 (8)	0.98 (0.20)	92.46 (4.04)	1.13 (1.02 to 1.24)	6.13 (0.13)
	1 nM	7 (8)	1.07 (0.08)	99.69 (2.23)	1.26 (1.06 to 1.47)	6.24 (0.08)
	10 nM	8 (9)	0.79 (0.15)	90.36 (4.12)	1.16 (1.05 to 1.28)	5.55 (0.06)
	100 nM	7 (8)	0.93 (0.17)	86.28 (4.84)	1.14 (0.97 to 1.32)	4.67 (0.11)
Dorsal	Control	7 (7)	1.59 (0.28)	71.09 (5.33)	1.23 (1.06 to 1.40)	5.58 (0.14)
	1 nM	7 (7)	1.59 (0.28)	74.06 (2.00)	1.09 (1.02 to 1.16)	5.33 (0.16)
	10 nM	7 (7)	0.91 (0.22)	69.38 (7.01)	1.25 (1.13 to 1.38)	4.79 (0.08)
	100 nM	7 (7)	1.61 (0.16)	72.53 (4.84)	1.49 (1.39 to 1.59)	4.40 (0.08)
IVA	Control	7 (7)	1.75 (0.06)	100.08 (7.71)	1.44 (1.30 to 1.58)	5.54 (0.08)
	1 nM	6 (6)	1.63 (0.28)	87.53 (9.22)	1.30 (1.15 to 1.45)	5.39 (0.16)
	10 nM	6 (6)	1.77 (0.08)	97.59 (5.44)	1.77 (1.27 to 2.27)	5.01 (0.10)
	100 nM	6 (6)	1.76 (0.14)	96.70 (14.25)	1.46 (1.41 to 1.50)	4.42 (0.07)
EVA	Control	7 (7)	2.58 (0.18)	87.30 (5.47)	1.11 (0.98 to 1.24)	5.59 (0.04)
	1 nM	6 (6)	2.24 (0.33)	82.20 (3.58)	1.25 (1.06 to 1.44)	5.37 (0.08)
	10 nM	6 (6)	2.47 (0.21)	83.93 (2.75)	1.27 (0.95 to 1.58)	4.98 (0.05)
	100 nM	6 (6)	2.37 (0.29)	81.40 (7.90)	1.49 (1.45 to 1.53)	4.35 (0.08)

RS 100329 and 5-Methylurapidil – Alpha_{1A}-Adrenoceptor Selective Antagonists

In the cavernous arteries RS 100329 (1 to 100 nM), an α_{1A} -AR selective antagonist, potently inhibited PE-induced vasoconstrictions with a mean pK_B of 8.80 ± 0.09 ($n(N) = 6(13)$), Figure 3-16, Table 3-9). Schild analysis of the data demonstrated a linear relationship with a slope not significantly different from unity, 0.83 ± 0.10 , allowing a pA_2 estimate of 8.98, 95% CI 8.51 to 9.61, to be calculated (Figure 3-17). A second α_{1A} -AR selective antagonist, 5-methylurapidil (10 to 100 nM), inhibited PE vasoconstrictions with variable affinity ($P < 0.05$, Figure 3-18, Table 3-10). The mean pK_B estimate was 8.65 ± 0.21 ($n(N) = 5(16)$). A Schild plot gave a pA_2 of 9.07, 95% CI 7.05 to 17.22, with a slope not significantly different from unity, 0.65 ± 0.26 (Figure 3-19).

PE vasoconstrictions in the dorsal artery were significantly inhibited by RS 100329 at 10 nM, but no further rightward shift was observed at the higher concentration of 100 nM (Figure 3-16, Table 3-9). An estimate of the mean pK_B based on 1 nM and 10 nM data was found to be 9.03 ± 0.07 ($n(N) = 6(12)$). A Schild plot of all data demonstrated a linear relationship with a shallow slope, 0.43 ± 0.11 , which was skewed by data obtained at 100 nM. Excluding this data and re-evaluating the Schild plot resulted in a slope not significantly different from unity, 0.74 ± 0.11 , with an apparent pA_2 of 9.22, 95% CI 8.82 to 9.84, (Figure 3-17). Responses to PE were inhibited by 5-methylurapidil with highly variable affinity (Figure 3-18, Table 3-10). A mean pK_B estimate was 8.45 ± 0.22 ($n(N) = 6(14)$). Schild analysis of the data demonstrated a slope significantly shallower than unity, 0.25 ± 0.17 (Figure 3-19).

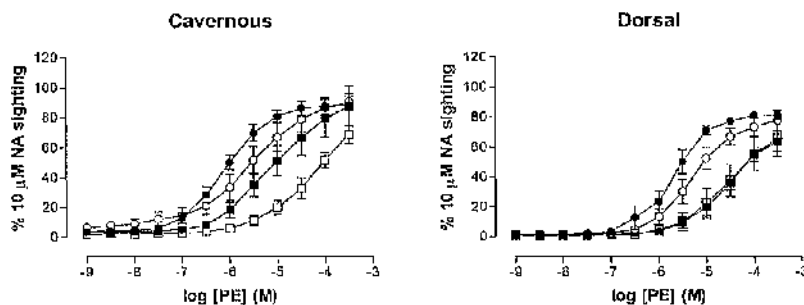


Figure 3-16: PE-induced vasoconstriction in the male genital arteries in the absence (●) or presence of RS 100329, 1 nM (○), 10 nM (■), or 100 nM (□) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

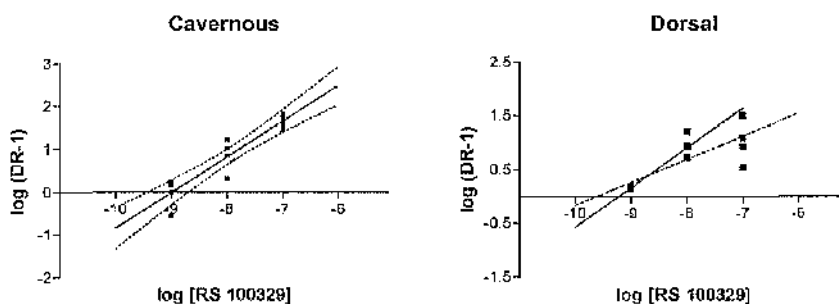


Figure 3-17: Schild plot of PE vs RS 100329 in the male genital arteries. Each point shows a single result at a given antagonist concentration. Linear regression ± 95% CI shown. Dorsal artery plot shows dotted line: linear regression on 1, 10 and 100 nM data, solid line: linear regression on 1 and 10 nM data.

Table 3-9: PE-induced vasoconstriction in the male genital arteries in the absence or presence of RS 100329.

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	Control	6 (6)	0.58 (0.09)	88.99 (5.13)	0.95 (0.92 to 0.99)	6.15 (0.06)
	1 nM	5 (5)	0.44 (0.13)	95.27 (4.28)	0.70 (0.63 to 0.76)	5.66 (0.21)
	10 nM	5 (5)	0.72 (0.28)	87.48 (13.42)	0.73 (0.62 to 0.84)	5.17 (0.17)
	100 nM	5 (5)	0.74 (0.15)	68.70 (6.16)	0.68 (0.62 to 0.73)	4.50 (0.11)
Dorsal	Control	4 (6)	1.54 (0.11)	82.14 (2.62)	1.13 (0.98 to 1.28)	5.70 (0.13)
	1 nM	6 (6)	1.31 (0.18)	77.46 (7.07)	1.03 (0.97 to 1.09)	5.36 (0.14)
	10 nM	6 (6)	0.98 (0.14)	68.40 (9.36)	0.94 (0.85 to 1.04)	4.72 (0.15)
	100 nM	6 (6)	1.32 (0.27)	64.99 (11.96)	0.85 (0.77 to 0.92)	4.63 (0.14)

CI: confidence interval

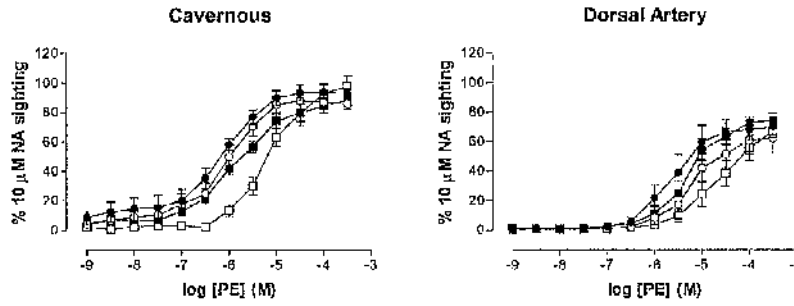


Figure 3-18: PE-induced vasoconstriction in the male genital arteries in the absence (●) or presence of 5-methylurapidil, 1 nM (○), 10 nM (■), or 100 nM (□) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

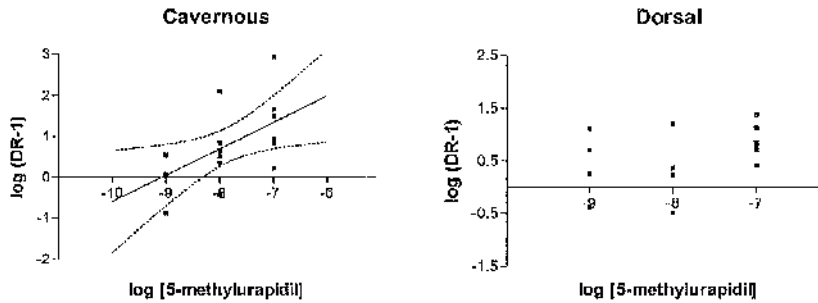


Figure 3-19: Schild plot of PE vs 5-methylurapidil in the male genital arteries. Each point shows a single result at a given antagonist concentration. Linear regression ± 95% CI is shown only when slope was not significantly different from unity.

Table 3-10: PE-induced vasoconstriction in the male genital arteries in the absence or presence of 5-methylurapidil.

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	Control	5 (5)	1.03 (0.25)	95.18 (3.04)	1.00 (0.86 to 1.14)	6.62 (0.26)
	1 nM	5 (5)	1.19 (0.33)	89.76 (5.28)	1.07 (0.84 to 1.30)	6.16 (0.16)
	10 nM	6 (6)	1.20 (0.18)	88.27 (3.97)	0.81 (0.69 to 0.93)	5.90 (0.07)
	100 nM	6 (6)	1.26 (0.18)	97.66 (6.94)	1.08 (0.89 to 1.27)	5.24 (0.06)
Dorsal	Control	6 (6)	1.78 (0.25)	70.22 (6.80)	1.13 (0.98 to 1.29)	5.62 (0.24)
	1 nM	6 (6)	1.26 (0.23)	62.28 (10.16)	1.19 (0.94 to 1.43)	5.29 (0.16)
	10 nM	6 (6)	1.88 (0.12)	75.99 (4.06)	1.19 (0.95 to 1.42)	5.31 (0.15)
	100 nM	6 (6)	1.70 (0.18)	66.92 (5.80)	0.84 (0.73 to 0.95)	4.67 (0.18)

CI: confidence interval

Rec 15/2615 – Alpha_{1B}-Adrenoceptor Selective Antagonist

Rec 15/2615 (1 to 100 nM), an α_{1B} -AR selective antagonist, did not affect PE-induced vasoconstriction in the cavernous arteries ($P > 0.05$, Figure 3-20, Table 3-11).

Concentration ratios at 1 nM, 10 nM and 100 nM were 1.06 ± 0.47 , 2.14 ± 0.72 and 3.00 ± 2.06 ($n/N = 4/4$) respectively; demonstrating that Rec 15/2615 had no effect in these arteries (Figure 3-21).

In the dorsal arteries, Rec 15/2615 (1 to 100 nM) did not significantly affect PE-induced vasoconstriction ($P > 0.05$, Figure 3-20, Table 3-11). Concentration ratios were 1.03 ± 0.28 at 1 nM, 1.50 ± 1.18 at 10 nM and 1.01 ± 0.19 at 100 nM ($n/N = 6/6$, Figure 3-21).

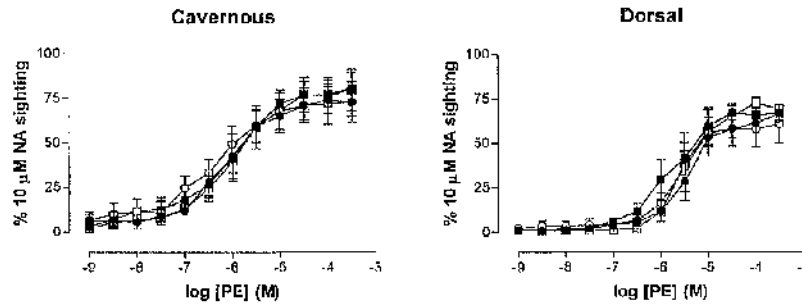


Figure 3-20: PE-induced vasoconstriction in the male genital arteries in the absence (●) or presence of Rec 15/2615, 1 nM (○), 10 nM (■), or 100 nM (□) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

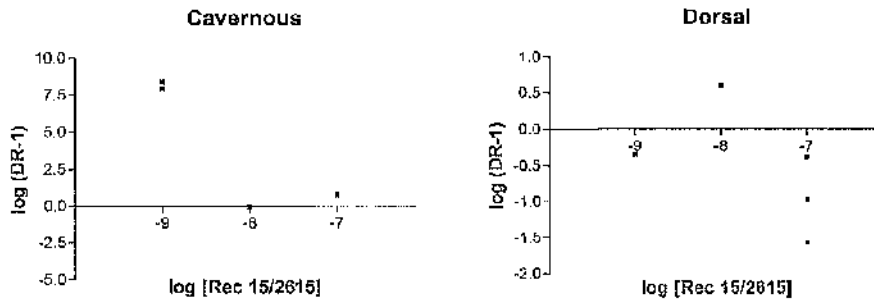


Figure 3-21: Schild plot of PE vs Rec 15/2615 in the male genital arteries. Each point represents a single result at a given concentration. Linear regression not shown since slope was significantly different from unity.

Table 3-11: PE-induced vasoconstriction in the male genital arteries in the absence or presence of Rec 15/2615.

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	Control	3 (3)	0.69 (0.39)	73.92 (7.38)	0.92 (0.76 to 1.08)	6.12 (0.19)
	1 nM	4 (4)	0.78 (0.32)	73.60 (10.78)	0.77 (0.61 to 0.93)	6.36 (0.18)
	10 nM	4 (4)	0.78 (0.14)	80.30 (8.64)	0.79 (0.62 to 0.96)	5.98 (0.14)
	100 nM	4 (4)	0.97 (0.12)	79.75 (11.34)	0.83 (0.71 to 0.95)	5.97 (0.16)
Dorsal	Control	4 (4)	1.20 (0.03)	64.08 (1.45)	1.27 (0.96 to 1.59)	5.49 (0.18)
	1 nM	4 (4)	1.31 (0.02)	59.78 (0.69)	1.48 (1.25 to 1.70)	5.56 (0.18)
	10 nM	4 (4)	1.52 (0.03)	68.31 (1.50)	0.95 (0.74 to 1.16)	5.78 (0.28)
	100 nM	4 (4)	1.43 (0.02)	69.69 (1.31)	1.38 (1.08 to 1.68)	5.51 (0.13)

CI: confidence interval

BMY 7378 – Alpha_{1D}-Adrenoceptor Selective Antagonist

In the cavernous arteries BMY 7378 (1 to 100 nM), an α_{1D} -AR selective antagonist, caused no inhibition of PE-induced vasoconstriction ($P > 0.05$, Figure 3-22, Table 3-12). The concentration ratios of responses at 1 nM, 10 nM and 100 nM were 0.86 ± 0.33 , 1.83 ± 0.69 and 1.30 ± 0.53 respectively ($n(N) = 6(7)$, Figure 3-23).

In the dorsal arteries BMY 7378 (1 to 100 nM) also had no effect on PE-induced vasoconstriction ($P > 0.05$, Figure 3-22, Table 3-12) with concentration ratios at 1 nM, 10 nM and 100 nM of 1.91 ± 0.67 , 1.02 ± 0.38 and 1.30 ± 0.53 respectively ($n(N) = 6(6)$, Figure 3-23).

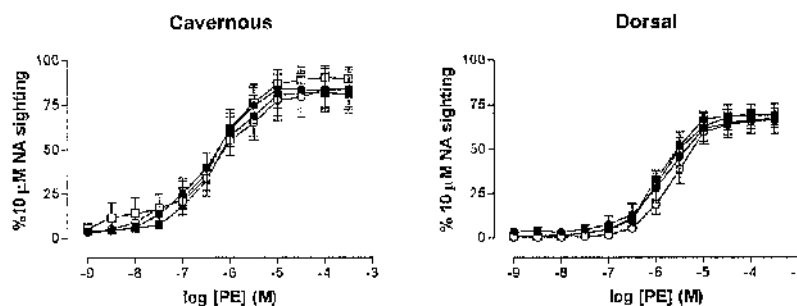


Figure 3-22: PE-induced vasoconstriction in the male genital arteries in the absence (●) or presence of BMY 7378, 1 nM (○), 10 nM (■), or 100 nM (□) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

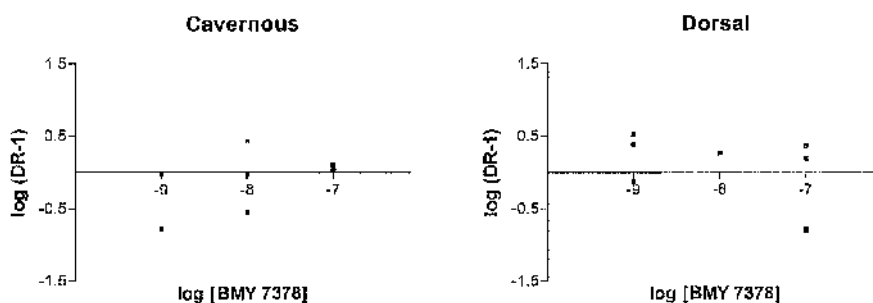


Figure 3-23: Schild plot of PE vs BMY 7378 in the male genital arteries. Each point represents a single result at a given concentration. Linear regression not shown since slope was significantly different from unity.

Table 3-12: PE-induced vasoconstriction in the male genital arteries in the absence or presence of BMY 7378.

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	Control	6 (7)	0.83 (0.15)	88.06 (10.62)	0.87 (0.73 to 1.00)	6.47 (0.16)
	1 nM	6 (7)	1.12 (0.19)	85.84 (11.94)	0.70 (0.54 to 0.85)	6.46 (0.16)
	10 nM	6 (7)	0.93 (0.28)	84.17 (11.17)	1.00 (0.86 to 1.14)	6.25 (0.14)
	100 nM	6 (6)	0.92 (0.23)	92.11 (6.81)	0.95 (0.76 to 1.14)	6.55 (0.36)
Dorsal	Control	6 (6)	1.51 (0.24)	67.79 (8.04)	1.09 (0.97 to 1.21)	5.81 (0.14)
	1 nM	6 (6)	1.52 (0.14)	67.20 (4.58)	1.27 (1.12 to 1.43)	5.69 (0.15)
	10 nM	6 (6)	1.70 (0.06)	70.88 (3.58)	1.24 (1.08 to 1.39)	5.92 (0.08)
	100 nM	6 (6)	1.64 (0.17)	71.21 (6.05)	1.16 (1.07 to 1.26)	5.81 (0.17)

CI: confidence interval

Alpha₂-Adrenoceptor-Mediated Responses

UK 14,304 – Alpha₂-Adrenoceptor Selective Agonist

The α_2 -AR selective agonist UK 14,304 caused concentration-dependent vasoconstrictions in all four genital arterial preparations (Figure 3-24, Table 3-13). Maximal responses to UK 14,304 in male genital arteries were approximately 50% smaller in magnitude than those induced by NA or PE. Maximal vasoconstrictions were cavernous artery 0.43 ± 0.01 g ($n(N) = 24(24)$) and dorsal artery 0.88 ± 0.02 g ($n(N) = 16(16)$). In female genital arteries, contractions to UK 14,304 did not reach a plateau at the highest concentration used during a CRC (30 μ M) precluding the calculation of a pEC₅₀. Maximal responses to UK 14,304 at 30 μ M were IVA, 0.98 ± 0.08 g ($n(N) = 8(22)$) and EVA 0.92 ± 0.13 g ($n(N) = 8(24)$). Responses to UK 14,304 in all preparations demonstrated Hill slopes significantly shallower than unity: cavernous 0.42 ± 0.17 , dorsal 0.59 ± 0.29 , IVA 0.48 ± 0.15 and EVA 0.54 ± 0.22 .

UK 14,304 CRCs in male genital arteries were not reproducible (Figure 3-25, Table 3-14). While the maximal responses did not change from 1st to 2nd CRCs ($P > 0.05$), a significant leftward shift was observed in both cavernous and dorsal arteries demonstrating a decrease in sensitivity to UK 14,304 ($P < 0.05$). In cavernous arteries the pEC₅₀ was changed by approximately a half log step from 7.78 ± 0.15 ($n(N) = 3(4)$) to 7.18 ± 0.11 ($n(N) = 3(4)$), while in dorsal arteries the shift was greater than one log step from 7.09 ± 0.28 ($n(N) = 4(6)$) to 6.25 ± 0.21 ($n(N) = 4(6)$).

In female arteries, although a plateau was not reached, responses were reproducible. No change was observed in maximal vasoconstriction to UK 14,304 at 30 μ M. In the IVA, the pEC calculated at approximately half of the UK 14,304 non-plateaued maximal response, equivalent to 35% NA sighting, was not significantly different from 1st, 6.33 ± 0.72 ($n(N) = 3(3)$), to 2nd, 6.15 ± 0.05 ($n(N) = 3(3)$), CRCs. Similarly in the EVA, the pEC at 20% NA sighting was not significantly different from 1st, 5.39 ± 0.18 ($n(N) = 3(3)$), to 2nd, 5.04 ± 0.68 ($n(N) = 3(3)$), CRCs.

Partial Agonism

In the IVA, NA (10 μ M) induced tone, 1.25 ± 0.15 g ($n(N) = 1(4)$), was decreased by UK 14,304 (Figure 3-26). Relaxation of NA-induced tone amounted to -0.70 ± 0.10 g (-56%) at 30 μ M UK 14,304. However, 5-hydroxytryptamine (3 μ M) induced tone, 0.28 ± 0.08 g

($n(N) = 1(4)$), was not decreased by UK 14,304. In the IVA, UK 14,304 (30 μ M) caused vasoconstriction from baseline, 0.12 ± 0.07 g ($n(N) = 1(4)$), and from 5-hydroxytryptamine-induced, 0.30 ± 0.10 g ($n(N) = 1(4)$), tone.

Similarly, in the EVA, UK 14,304 caused relaxation of NA (10 μ M) but not 5-hydroxytryptamine (3 μ M) pre-contracted vessels (Figure 3-27). Relaxation of NA-induced tone, 1.53 ± 0.05 g ($n(N) = 1(4)$), amounted to -1.00 ± 0.12 g (-65%) at 30 μ M UK 14,304. UK 14,304 caused vasoconstriction from baseline, 0.17 ± 0.03 g ($n(N) = 1(4)$), and from 5-hydroxytryptamine-induced, 0.09 ± 0.06 g ($n(N) = 1(4)$), tone.

Nitric Oxide Synthase Inhibition

The NOS inhibitor L-NAME (100 μ M) caused no change in the magnitude or potency of responses to UK 14,304 in the cavernous artery ($P > 0.05$, Figure 3-28, Table 3-15). In the dorsal artery, L-NAME significantly increased the magnitude of UK 14,304-induced vasoconstrictions ($P < 0.05$) from 0.81 ± 0.18 g to 1.78 ± 0.18 g ($n(N) = 6(6)$) with no change in potency ($P > 0.05$).

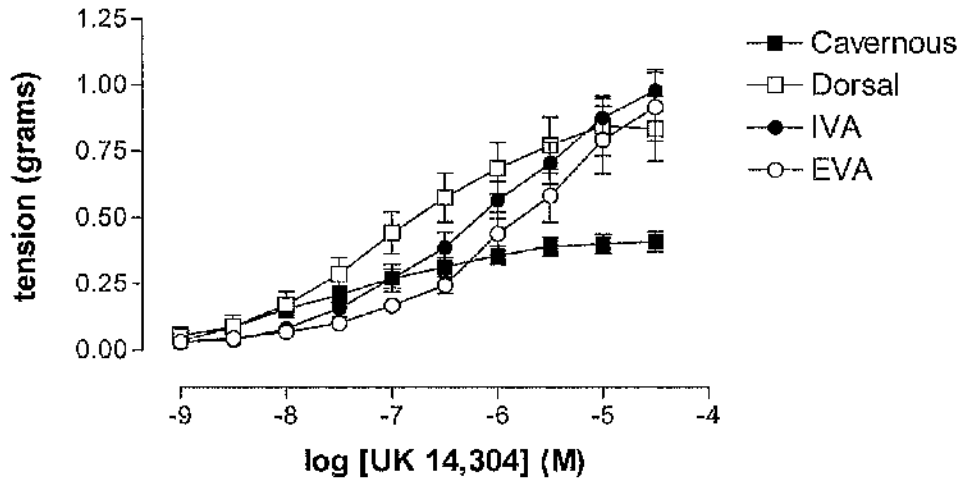


Figure 3-24: UK 14,304-induced vasoconstriction in the genital arteries expressed as grams tension \pm s.e.mean.

Table 3-13: Comparison of UK 14,304-induced vasoconstriction between vessels.

	n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	24 (24)	0.43 (0.01)	60.79 (1.39)	0.42 (0.31 to 0.53)	7.86 (0.16)
Dorsal	16 (16)	0.88 (0.02)	52.26 (1.08)	0.59 (0.45 to 0.73)	7.18 (0.07)
IVA	8 (22)	0.98 (0.08)	53.25 (3.88)	0.48 (0.37 to 0.58)	nd
EVA	8 (24)	0.92 (0.13)	27.97 (3.53)	0.54 (0.41 to 0.67)	nd

CI: confidence interval, nd: not determined

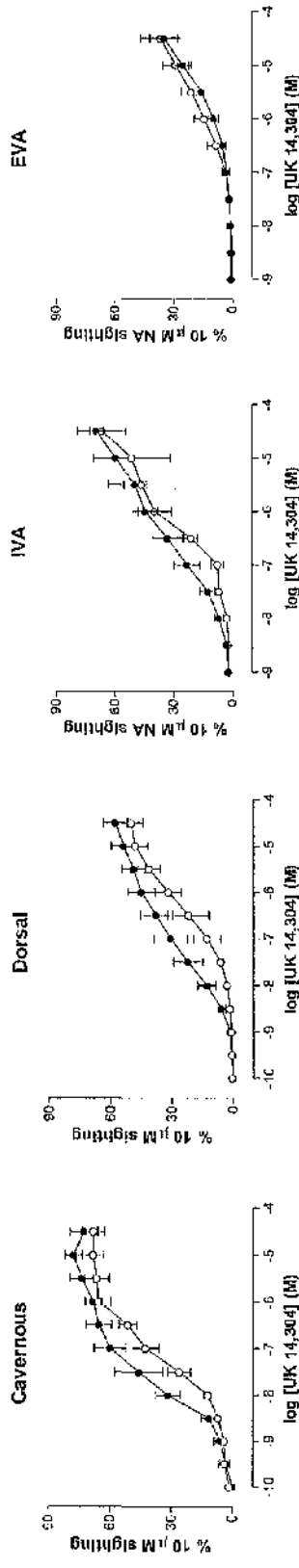


Figure 3-25: UK 14,304-induced vasoconstriction in the genital arteries, 1st (●) and 2nd (○) controls, expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-14: UK 14,304-induced vasoconstriction in the genital arteries, 1st and 2nd controls.

	n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)	
Cavernous	1 st Control	3 (4)	0.58 (0.04)	74.15 (3.25)	0.78 (0.40 to 1.15)	7.78 (0.15)
	2 nd Control	3 (4)	0.55 (0.05)	68.97 (2.65)	0.87 (0.52 to 1.22)	7.18 (0.11)
Dorsal	1 st Control	4 (6)	1.28 (0.20)	59.38 (7.31)	0.49 (0.13 to 0.85)	7.09 (0.28)
	2 nd Control	4 (6)	1.16 (0.18)	53.20 (5.92)	0.71 (0.28 to 1.13)	6.25 (0.21)
IVA	1 st Control	3 (3)	1.11 (0.06)	69.48 (3.55)	0.41 (0.04 to 0.78)	nd
	2 nd Control	3 (3)	1.05 (0.15)	66.72 (12.15)	0.71 (-0.22 to 1.64)	nd
EVA	1 st Control	3 (3)	1.06 (0.14)	35.06 (7.37)	0.61 (0.02 to 1.21)	nd
	2 nd Control	3 (3)	1.09 (0.19)	36.87 (10.15)	0.60 (-0.17 to 1.36)	nd

nd: not determined

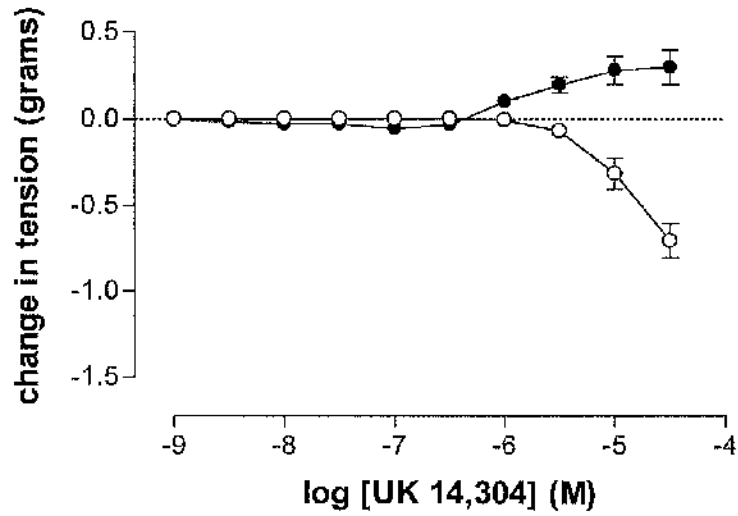


Figure 3-26: Partial agonism of UK 14,304 relative to NA in the IVA expressed as a change in grams tension from NA (10 µM)-induced tone (○) or 5-hydroxytryptamine (3 µM)-induced tone (●).

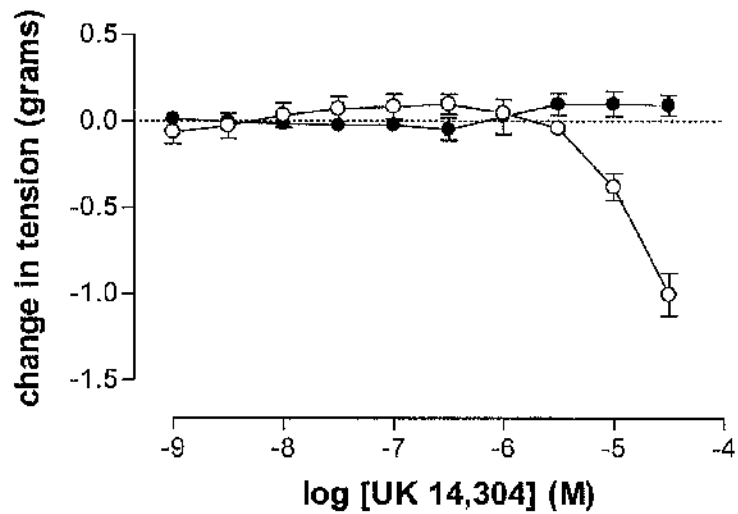


Figure 3-27: Partial agonism of UK 14,304 relative to NA in the EVA expressed as a change in grams tension from NA (10 µM)-induced tone (○) or 5-hydroxytryptamine (3 µM)-induced tone (●).

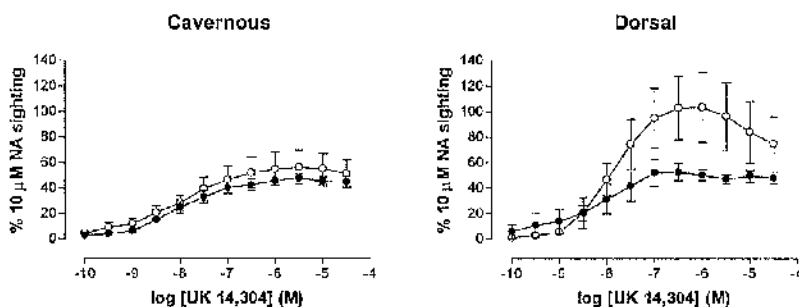


Figure 3-28: UK 14,304-induced vasoconstriction in the male genital arteries in the absence (●) or presence (○) of L-NAME (100 μM) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-15: UK 14,304-induced vasoconstriction in the male genital arteries in the absence or presence of L-NAME (100 μM).

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	Control	7 (7)	0.44 (0.07)	47.89 (3.17)	0.79 (0.61 to 0.96)	8.04 (0.06)
	L-NAME	6 (6)	0.53 (0.13)	56.29 (13.34)	0.72 (0.51 to 0.93)	8.00 (0.09)
Dorsal	Control	6 (6)	0.81 (0.18)	52.58 (6.86)	1.07 (0.55 to 1.60)	8.14 (0.10)
	L-NAME	6 (6)	1.78 (0.18)	103.24 (27.16)	1.37 (0.27 to 2.46)	8.01 (0.13)

CI: confidence interval

Rauwolscine – Non-Subtype-Selective α_2 -Adrenoceptor Antagonist

Due to the desensitisation of control UK 14,304 curves in male arteries, a protocol was used whereby antagonist (0.001 to 1 μM) was added cumulatively in log steps following the construction of a partial (0.1 nM to 30 nM) or full (0.1 nM to 30 μM) UK 14,304 CRC.

In the cavernous artery, rauwolscine (0.001 to 1 μM) reversed UK 14,304-induced tone following a partial CRC (Figure 3-29, Table 3-16). However, this reversal of tone was not significant when compared to the loss of tone with time ($P > 0.05$). Rauwolscine (1 μM) caused $102.1 \pm 3.0\%$ ($n(N) = 5(6)$) relaxation of UK 14,304-induced tone compared to a $76.0 \pm 10.6\%$ ($n(N) = 5(5)$) loss of tone with time. In the cavernous artery, rauwolscine-induced relaxation following a full CRC, $64.5 \pm 4.6\%$ ($n(N) = 5(5)$), was not significantly different to the loss of tone with time, $54.0 \pm 9.5\%$ ($n(N) = 5(6)$), $P > 0.05$, Figure 3-30, Table 3-16).

In the dorsal artery, addition of rauwolscine (0.001 to 1 μM) following the construction of a partial CRC significantly reversed UK 14,304-induced vasoconstriction; $97.3 \pm 6.1\%$ ($n(N) = 5(5)$), compared to a $37.4 \pm 5.3\%$ ($n(N) = 4(6)$) loss of tone with time ($P < 0.001$, Figure 3-29, Table 3-16). Rauwolscine (1 μM) did not significantly decrease UK 14,304-induced tone following a full CRC; $56.1 \pm 9.0\%$ ($n(N) = 7(7)$) compared to $52.8 \pm 8.5\%$ ($n(N) = 6(9)$) loss of tone with time ($P > 0.05$, Figure 3-30, Table 3-16).

In female arteries, control UK 14,304 CRCs were reproducible allowing the traditional protocol of pre-incubating vessels with antagonist prior to an agonist CRC to be used.

In the IVA, rauwolscine (10 nM) did not significantly inhibit UK 14,304-induced vasoconstriction ($P > 0.05$, Figure 3-31, Table 3-17). However, a small parallel rightward shift of the CRC was observed from a pEC, calculated at 35% NA sighting, of 6.15 ± 0.05 ($n(N) = 3(3)$) to 5.70 ± 0.26 ($n(N) = 6(6)$) with a concentration-ratio at 10 nM rauwolscine of 7.79 ± 2.83 . The calculated pK_B was 8.67 ± 0.31 ($n(N) = 3(3)$) suggesting that there was an effect of rauwolscine at this concentration.

Rauwolscine (10 nM) had no effect on UK 14,304-induced vasoconstriction in the EVA ($P > 0.05$, Figure 3-31, Table 3-17). The pEC, calculated at 20% NA sighting, was 5.74 ± 0.10 ($n(N) = 4(4)$), compared to control pEC 5.04 ± 0.68 ($n(N) = 6(6)$), while the concentration-ratio was 0.92 ± 0.10 ($n(N) = 3(3)$).

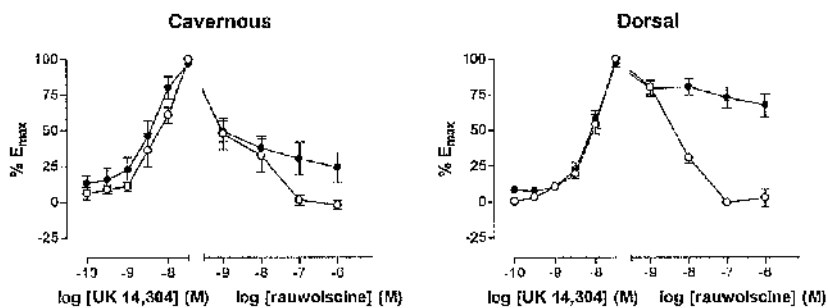


Figure 3-29: Inhibition of a partial UK 14,304 CRC by rauwolscine, 1 nM to 1 μM, (○) compared to a decrease of tone with time (●) in the male genital arteries expressed as a percentage of a vessels own maximum ± s.e.mean.

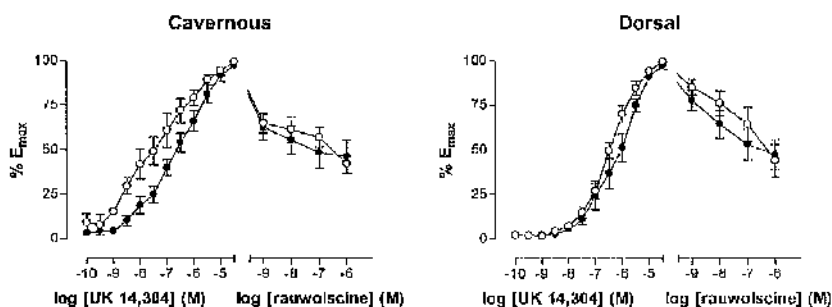


Figure 3-30: Inhibition of a full UK 14,304 CRC by rauwolscine, 1 nM to 1 μM, (○) compared to a decrease of tone with time (●) in the male genital arteries expressed as a percentage of a vessels' own maximum ± s.e.mean.

Table 3-16: Maximum percentage decrease in UK 14,304 tone with time (control) or in response to rauwolscine, 1 nM to 1 μM, following either a full or partial UK 14,304 CRC.

	UK 14,304 CRC	Protocol	n (N)	% Maximum Relaxation (s.e.mean)
Cavernous	Partial	Control	5 (6)	76.02 (10.57)
		Rauwolscine	5 (5)	102.12 (3.04)
	Full	Control	5 (7)	53.98 (9.48)
		Rauwolscine	5 (5)	64.46 (4.64)
Dorsal	Partial	Control	4 (6)	37.37 (5.34)
		Rauwolscine	5 (5)	97.28 (6.11)
	Full	Control	6 (9)	52.76 (8.53)
		Rauwolscine	7 (7)	56.10 (8.97)

CRC: concentration response curve

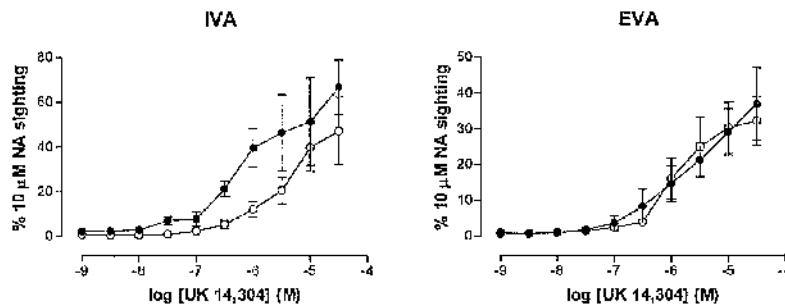


Figure 3-31: UK 14,304-induced vasoconstriction in the female genital arteries in the absence (●) or presence of rauwolscine, 10 nM (○) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-17: UK 14,304-induced vasoconstriction in the female genital arteries in the absence or presence of rauwolscine.

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
IVA	Control	3 (3)	1.05 (0.15)	66.72 (12.15)	0.71 (-0.22 to 1.64)	nd
	10 nM	6 (6)	0.66 (0.17)	47.18 (15.08)	0.97 (-0.13 to 2.07)	nd
EVA	Control	4 (4)	1.09 (0.19)	36.87 (10.15)	0.60 (-0.17 to 1.36)	nd
	10 nM	6 (6)	1.15 (0.35)	32.23 (6.74)	1.38 (-0.03 to 2.79)	nd

CI: confidence interval, nd: not determined

BRL 44408 -- Alpha_{2A}-Adrenoceptor Selective Antagonist

The subtype-selective α_{2A} -AR antagonist, BRL 44408, did not significantly reverse UK 14,304-induced tone in the cavernous artery following the construction of either a partial or full CRC ($P > 0.05$). Following the construction of a partial CRC (Figure 3-32, Table 3-18), a trend was observed whereby BRL 44408 caused reversal of UK 14,304-induced tone; $120.2 \pm 17.8\%$ ($n/N = 7(7)$) compared to a $76.0 \pm 10.6\%$ ($n/N = 5(6)$) loss in the time control. However, due to the variability of responses in cavernous arteries to UK 14,304 this effect was not significant. No significant effect of BRL 44408 was observed following the construction of a full UK 14,304 CRC ($P > 0.05$, Figure 3-33, Table 3-18).

In the dorsal artery, following the construction of a partial UK 14,304 CRC, BRL 44408 significantly reversed UK 14,304-induced tone by $97.4 \pm 7.5\%$ ($n/N = 8(10)$) compared to a $37.4 \pm 5.3\%$ ($n/N = 4(6)$) loss of tone with time ($P < 0.01$, Figure 3-32, Table 3-18). BRL 44408 had no significant effect on UK 14,304-induced tone following a full UK 14,304 CRC, $59.9 \pm 6.8\%$ ($n/N = 9(10)$) compared to $52.8 \pm 8.5\%$ ($n/N = 6(9)$) with time ($P > 0.05$, Figure 3-33, Table 3-18).

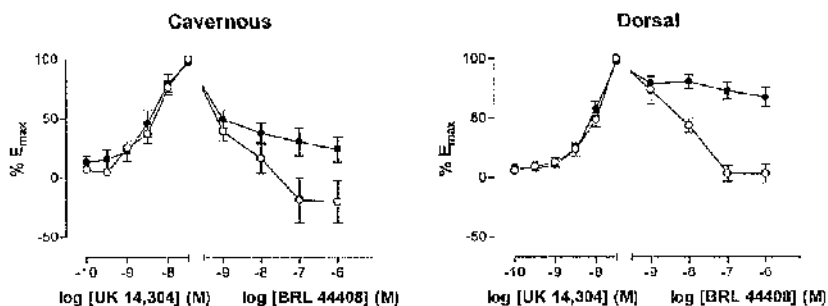


Figure 3-32: Inhibition of a partial UK 14,304 CRC by BRL 44408, 1 nM to 1 μM, (○) compared to a decrease of tone with time (●) in the male genital arteries expressed as a percentage of a vessels own maximum ± s.e.mean.

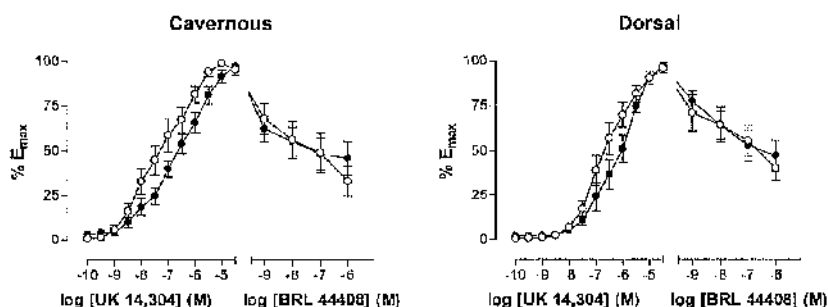


Figure 3-33: Inhibition of a full UK 14,304 CRC by BRL 44408, 1 nM to 1 μM, (○) compared to a decrease of tone with time (●) in the male genital arteries expressed as a percentage of a vessels own maximum ± s.e.mean.

Table 3-18: Maximum percentage decrease in UK 14,304 tone with time (control) or in response to BRL 44408, 1 nM to 1 μM, following either a full or partial UK 14,304 CRC.

	UK 14,304 CRC	Protocol	n (N)	% Maximum Relaxation (s.e.mean)
Cavernous	Partial	Control	5 (6)	76.02 (10.57)
		BRL 44408	7 (7)	120.15 (17.82)
	Full	Control	5 (7)	53.98(9.48)
		BRL 44408	7 (8)	66.93 (8.45)
Dorsal	Partial	Control	4 (6)	37.37 (5.34)
		BRL 44408	8 (10)	97.36 (7.46)
	Full	Control	6 (9)	52.76 (8.53)
		BRL 44408	9 (10)	59.89 (6.82)

CRC: concentration response curve

Prazosin – Non-Subtype-Selective α_1 -Adrenoceptor Antagonist

In the cavernous artery, addition of prazosin following the construction of a partial (Figure 3-34, Table 3-19) or full (Figure 3-35, Table 3-19) CRC was ineffective at causing any significant reversal of UK 14,304-induced tone ($P > 0.05$). A trend was observed following a full UK 14,304 curve whereby prazosin caused $78.5 \pm 9.5\%$ ($n(N) = 5(5)$) reversal of tone compared to $54.0 \pm 9.5\%$ ($n(N) = 5(7)$) with time.

Following the construction of a partial UK 14,304 CRC in the dorsal artery, prazosin demonstrated a non-significant trend towards reversal of UK 14,304 tone; $67.2 \pm 6.3\%$ ($n(N) = 5(6)$) compared to $37.4 \pm 5.3\%$ ($n(N) = 4(6)$) with time ($P > 0.05$, Figure 3-34, Table 3-19). Prazosin ($1 \mu\text{M}$) significantly reversed UK 14,304-induced tone following the construction of a full CRC; $96.1 \pm 3.3\%$ ($n(N) = 7(8)$) compared to $52.8 \pm 8.5\%$ ($n(N) = 6(9)$) with time ($P < 0.01$, Figure 3-35, Table 3-19).

In the IVA, pre-incubation with prazosin (10 nM) significantly ($P < 0.05$) shifted the UK 14,304-induced response from pEC, calculated at 25% NA sighting, of 6.17 ± 0.04 ($n(N) = 3(3)$) to 5.01 ± 0.17 ($n(N) = 6(6)$, Figure 3-36, Table 3-20). The mean pK_B was 8.98 ± 0.56 . A combination of prazosin (10 nM) and rauwolscine (10 nM) produced a similar shift, pEC 4.67 ± 0.27 ($n(N) = 6(7)$, $P < 0.05$), while rauwolscine (10 nM) alone had no significant effect (see previous section, UK 14,340 vs rauwolscine).

Pre-incubation with prazosin in the EVA followed a similar pattern to that observed in the IVA (Figure 3-36, Table 3-20). Prazosin (10 nM) significantly shifted the UK 14,304-induced response from a pEC, calculated at 15% NA sighting, of 5.74 ± 0.07 ($n(N) = 4(4)$) to 4.69 ± 0.29 ($n(N) = 5(5)$, $P < 0.05$). A similar significant shift occurred using a combination of prazosin (10 nM) and rauwolscine (10 nM); pEC, calculated at 15% NA sighting, 4.46 ± 0.29 ($n(N) = 5(5)$, $P < 0.05$), while 10 nM rauwolscine alone had no significant effect (see previous section, UK 14,340 vs rauwolscine). The mean pK_B of prazosin in this tissue was 8.55 ± 0.05 ($n(N) = 2(2)$), while that of the combination was 8.56 ± 0.05 ($n(N) = 3(3)$) and of rauwolscine alone was 6.76 ($n(N) = 1(1)$).

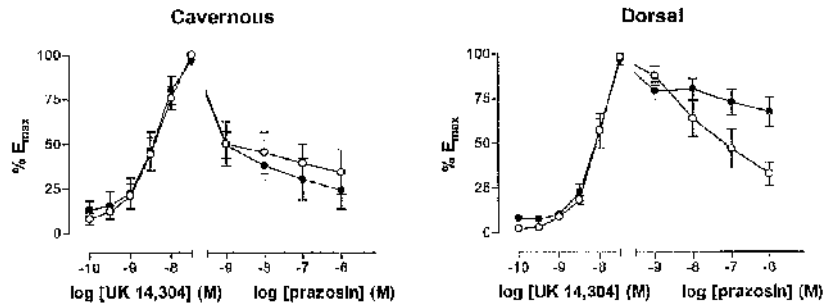


Figure 3-34: Inhibition of a partial UK 14,304 CRC by prazosin, 1 nM to 1 μ M, (○) compared to a decrease of tone with time (●) in the male genital arteries expressed as a percentage of a vessels own maximum \pm s.e.mean.

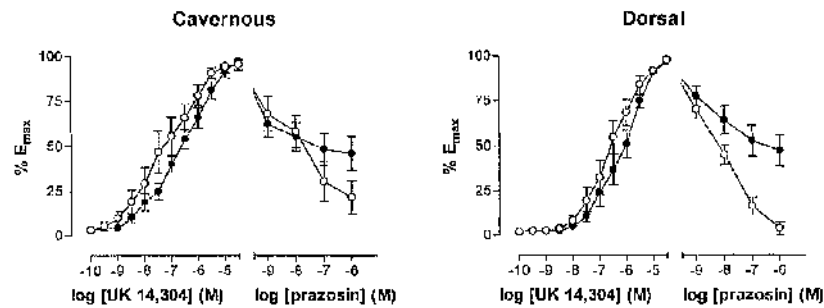


Figure 3-35: Inhibition of a full UK 14,304 CRC by prazosin, 1 nM to 1 μ M, (○) compared to a decrease of tone with time (●) in the male genital arteries expressed as a percentage of a vessels own maximum \pm s.e.mean.

Table 3-19: Maximum percentage decrease in UK 14,304 tone with time (control) or in response to prazosin, 1 nM to 1 μ M, following either a full or partial UK 14,304 CRC.

	UK 14,304 CRC	Protocol	n (N)	% Maximum Relaxation (s.e.mean)
Cavernous	Partial	Control	5 (6)	76.02 (10.57)
		Prazosin	5 (5)	65.92 (12.50)
	Full	Control	5 (7)	53.98(9.48)
		Prazosin	5 (5)	78.46 (9.52)
Dorsal	Partial	Control	4 (6)	37.37 (5.34)
		Prazosin	5 (6)	67.18 (6.25)
	Full	Control	6 (9)	52.76 (8.53)
		Prazosin	7 (8)	96.09 (3.34)

CRC: concentration response curve

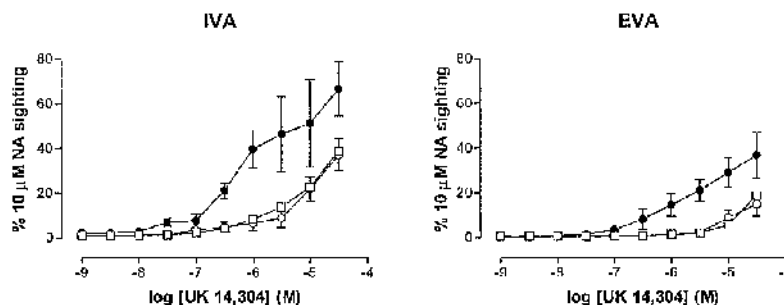


Figure 3-36: UK 14,304-induced vasoconstriction in the female genital arteries in the absence (●) or presence of prazosin 10 nM (○), or a combination of prazosin 10 nM plus rauwolescine 10 nM (□) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-20: UK 14,304-induced vasoconstriction in the female genital arteries in the absence or presence of prazosin or prazosin plus rauwolescine.

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
IVA	Control	3 (3)	1.05 (0.15)	66.72 (12.15)	0.71 (-0.22 to 1.64)	nd
	10 nM	6 (6)	0.86 (0.18)	37.18 (7.15)	0.65 (-0.31 to 1.61)	nd
	Prazosin + Rauwolescine	6 (7)	0.79 (0.18)	38.77 (10.73)	0.53 (-0.83 to 1.90)	nd
EVA	Control	4 (4)	1.09 (0.19)	36.87 (10.15)	0.60 (-0.17 to 1.36)	nd
	10 nM	6 (6)	0.59 (0.28)	14.99 (5.42)	1.77 (-0.75 to 4.29)	nd
	Prazosin + Rauwolescine	5 (6)	0.62 (0.24)	18.87 (7.91)	1.50 (-3.94 to 6.93)	nd

CI: confidence interval, nd: not determined

Medetomidine – Alpha₂-Adrenoceptor Selective Agonist

In addition to UK 14,304, a second non-subtype-selective α_2 -AR agonist was tested in genital arterial preparations to determine if less variable results could be obtained.

Medetomidine caused concentration-dependent vasoconstriction in all genital arterial preparations (Figure 3-37, Table 3-21). Maximal responses to medetomidine in the male genital arteries, similar to UK 14,304, were approximately 50% smaller in magnitude than those induced by NA or PE. Medetomidine demonstrated a similar potency in male cavernous, pEC_{50} 6.43 ± 0.10 ($n/N = 10/34$), and dorsal arteries, pEC_{50} 6.47 ± 0.14 ($n/N = 9/37$) that was significantly less than UK 14,304 ($P < 0.01$). Responses to medetomidine in the female genital arteries did not reach a plateau at the highest concentration used during a CRC (30 μ M), similar to UK 14,304, precluding the calculation of a pEC_{50} .

Medetomidine responses were reproducible in male arteries; no change in either maximal response or potency was observed between 1st and 2nd CRCs ($P > 0.05$, Figure 3-38, Table 3-22). As with UK 14,304, vasoconstrictions were smaller in magnitude than NA-induced vasoconstriction and were approximately 50% to 70% of NA responses. In contrast to UK 14,304, responses to medetomidine demonstrated Hill slopes that were not significantly different from unity: cavernous 0.98 ± 0.21 , dorsal 0.75 ± 0.18 , IVA 0.89 ± 0.19 and EVA 1.02 ± 0.14 .

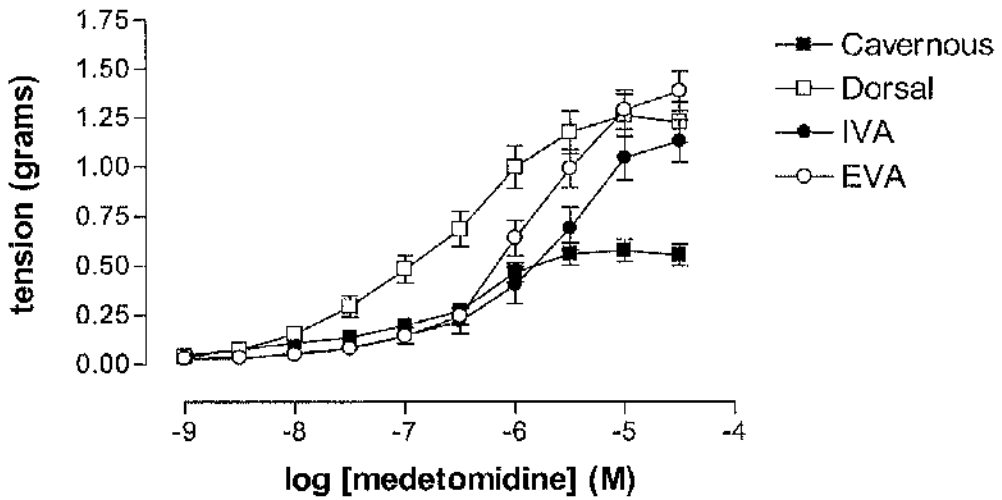


Figure 3-37: Medetomidine-induced vasoconstriction in the genital arteries expressed as grams tension \pm s.e.mean.

Table 3-21: Comparison of medetomidine-induced vasoconstriction between vessels.

	N (N)	E_{max} grams (s.e.mean)	E_{max} % NA sighting (s.e.mean)	Hill Slope (95% CI)	pEC_{50} (s.e.mean)
Cavernous	10 (34)	0.55 (0.06)	60.93 (2.81)	0.98 (0.57 to 1.40)	6.43 (0.10)
Dorsal	9 (37)	1.28 (0.11)	56.41 (3.81)	0.75 (0.40 to 1.10)	6.47 (0.14)
IVA	7 (26)	1.13 (0.11)	71.20 (5.02)	0.89 (0.51 to 1.26)	nd
EVA	7 (27)	1.39 (0.10)	53.05 (2.99)	1.02 (0.74 to 1.30)	nd

CI: confidence interval, nd: not determined

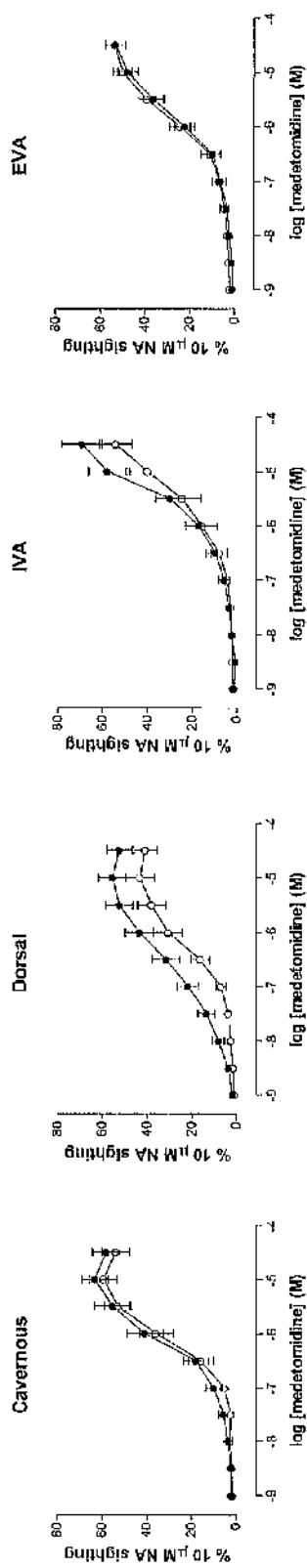


Figure 3-38: Medetomidine-induced vasoconstriction in the genital arteries, 1st (●) and 2nd (○) controls, expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-22: Medetomidine-induced vasoconstriction in the genital arteries, 1st and 2nd controls.

	<i>n</i> (<i>N</i>)	<i>E</i> _{max} grams (s.e.mean)	<i>E</i> _{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)
Cavernous	1 st Control	0.79 (0.14)	63.46 (5.91)	0.78 (0.40 to 1.15)	6.14 (0.14)
	2 nd Control	0.74 (0.14)	59.59 (6.12)	0.87 (0.52 to 1.22)	6.16 (0.11)
Dorsal	1 st Control	1.27 (0.19)	57.69 (5.64)	0.49 (0.13 to 0.85)	6.74 (0.21)
	2 nd Control	1.00 (0.17)	44.96 (6.13)	0.71 (0.28 to 1.13)	6.28 (0.15)
IVA	1 st Control	1.06 (0.15)	70.04 (9.17)	0.41 (0.04 to 0.78)	nd
	2 nd Control	0.73 (0.15)	47.50 (8.14)	0.71 (-0.22 to 1.64)	nd
EVA	1 st Control	1.65 (0.17)	58.72 (3.67)	0.61 (0.02 to 1.21)	nd
	2 nd Control	1.60 (0.20)	56.89 (4.38)	0.60 (-0.17 to 1.36)	nd

nd: not determined

Rauwolscine – Non-Subtype-Selective α_2 -Adrenoceptor Antagonist

Pre-incubation with the non-subtype-selective α_2 -AR antagonist rauwolscine (0.01 to 1 μ M) had no effect on responses to medetomidine in the cavernous artery ($P > 0.05$, Figure 3-39, Table 3-23). Concentration ratios were 0.57 ± 0.27 ($n(N) = 4(4)$), 0.99 ± 0.21 ($n(N) = 3(3)$) and 1.32 ± 0.31 ($n(N) = 3(3)$) at 10 nM, 100 nM and 1 μ M respectively (Figure 3-40).

In the dorsal artery responses to medetomidine were significantly shifted by pre-incubation with rauwolscine ($P < 0.05$, Figure 3-39, Table 3-23). Analysis of the data gave a mean pK_B of 6.96 ± 0.23 ($n(N) = 5(12)$). The corresponding Schild plot demonstrated a slope not significantly different from unity, 0.56 ± 0.24 with an estimated pA_2 of 6.87, 95% CI -3.29 to 11.00 (Figure 3-40).

Rauwolscine (10 nM) did not significantly inhibit vasoconstriction to medetomidine in the IVA ($P > 0.05$, Figure 3-39, Table 3-23). The pEC calculated at 30% NA sighting was 5.16 ± 0.38 ($n(N) = 6(10)$) in controls and 5.48 ± 0.31 ($n(N) = 6(7)$) in the presence of rauwolscine (10 nM) while the concentration-ratio was 0.86 ± 0.19 ($n(N) = 7(7)$).

Vasoconstriction to medetomidine in the EVA was also not affected by rauwolscine (10 nM, $P > 0.05$, Figure 3-39, Table 3-23). The pEC calculated at 30% NA sighting was 5.92 ± 0.13 ($n(N) = 6(10)$) in controls and 5.87 ± 0.12 ($n(N) = 6(6)$) in the presence of rauwolscine (10 nM), while in this artery the concentration-ratio was 2.86 ± 1.15 ($n(N) = 6(6)$).

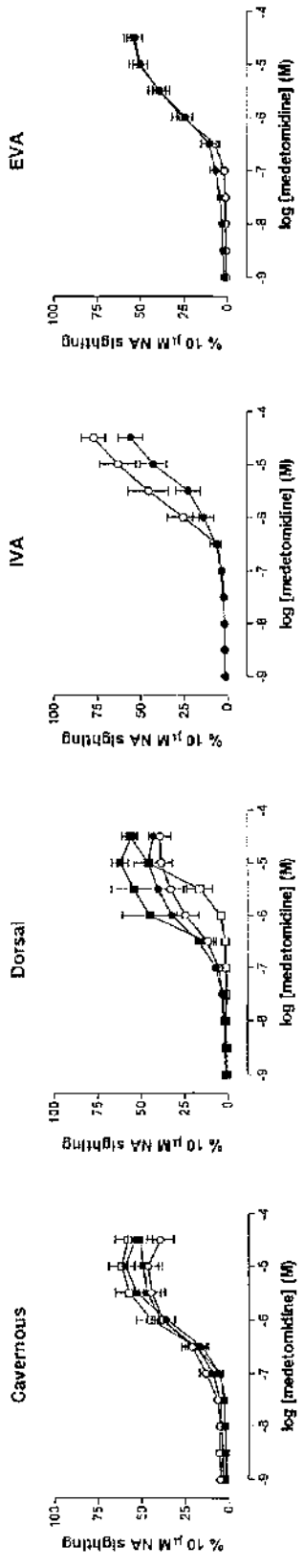


Figure 3-1: Medetomidine-induced vasoconstriction in the genital arteries in the absence (●) or presence of rauwolscine, 10 nM (○), 100 nM (■) or 1 μM (□) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

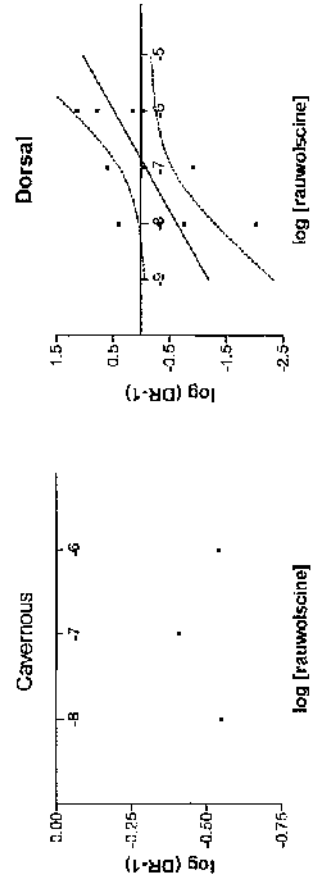


Figure 3-2: Schild plot of medetomidine vs rauwolscine in the male genital arteries. Each point represents a single result at a given concentration. Linear regression ± 95% CI is shown only when slope was not significantly different from unity.

Table 3-23: Medetomidine-induced vasoconstriction in the genital arteries in the absence or presence of rauwolfscine.

	<i>n</i> (<i>N</i>)	E_{max} grams (s.e.mean)	E_{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)
Cavernous	Control	0.74 (0.14)	59.59 (6.12)	1.50 (1.02 to 1.97)	6.16 (0.11)
	10 nM	0.41 (0.12)	47.00 (8.03)	1.49 (0.57 to 2.42)	6.69 (0.17)
	100 nM	0.47 (0.14)	50.81 (8.32)	1.47 (1.21 to 1.73)	6.38 (0.11)
	1 μ M	0.51 (0.11)	61.72 (7.47)	1.66 (1.37 to 1.96)	6.29 (0.11)
Dorsal	Control	1.00 (0.17)	44.96 (6.13)	1.22 (0.99 to 1.44)	6.28 (0.15)
	10 nM	0.82 (0.18)	37.29 (7.27)	1.10 (0.99 to 1.21)	6.11 (0.17)
	100 nM	1.42 (0.38)	53.68 (12.03)	2.19 (1.51 to 2.87)	6.01 (0.15)
	1 μ M	1.06 (0.33)	44.31 (12.77)	1.94 (1.81 to 2.08)	5.45 (0.16)
IVA	Control	0.73 (0.15)	47.50 (8.14)	0.84 (0.67 to 1.01)	nd
	10 nM	1.39 (0.15)	78.71 (8.12)	1.02 (0.79 to 1.26)	nd
EVA	Control	1.60 (0.20)	56.89 (4.38)	1.06 (0.93 to 1.18)	nd
	10 nM	1.49 (0.28)	62.42 (9.57)	1.21 (0.95 to 1.48)	nd

nd: not determined

Prazosin – Non-Subtype-Selective α_1 -Adrenoceptor Antagonist

Prazosin (10 nM), a non-subtype-selective α_1 -AR antagonist, did not significantly inhibit medetomidine-induced vasoconstriction in cavernous arteries ($P > 0.05$, Figure 3-41, Table 3-24). The concentration-ratio was 0.42 ± 0.32 ($n(N) = 2(2)$).

In the dorsal artery, responses to medetomidine were significantly inhibited ($P < 0.01$, Figure 3-41, Table 3-24) by prazosin (10 nM) shifting the CRC from a pEC_{50} of 6.28 ± 0.15 ($n(N) = 8(13)$) to 5.48 ± 0.14 ($n(N) = 4(7)$). The mean pK_B was calculated as 8.24 ± 0.54 ($n(N) = 3(3)$).

Vasoconstriction to medetomidine in the IVA was unaffected by prazosin (10 nM, $P > 0.05$, Figure 3-41, Table 3-24). The pEC , calculated at 25% NA sighting, was 5.17 ± 0.37 ($n(N) = 6(10)$) in control and 4.71 ± 0.32 ($n(N) = 5(5)$) in the presence of prazosin (10 nM) with a concentration-ratio of 1.49 ± 0.17 ($n(N) = 4(4)$).

In the EVA, medetomidine-induced vasoconstriction was significantly inhibited by prazosin (10 nM, $P < 0.001$, Figure 3-41, Table 3-24). The pEC , calculated at 25% NA sighting, decreased from 5.94 ± 0.13 ($n(N) = 6(10)$) to 5.02 ± 0.06 ($n(N) = 6(7)$). The calculated mean pK_B was found to be 9.00 ± 0.23 ($n(N) = 6(7)$).

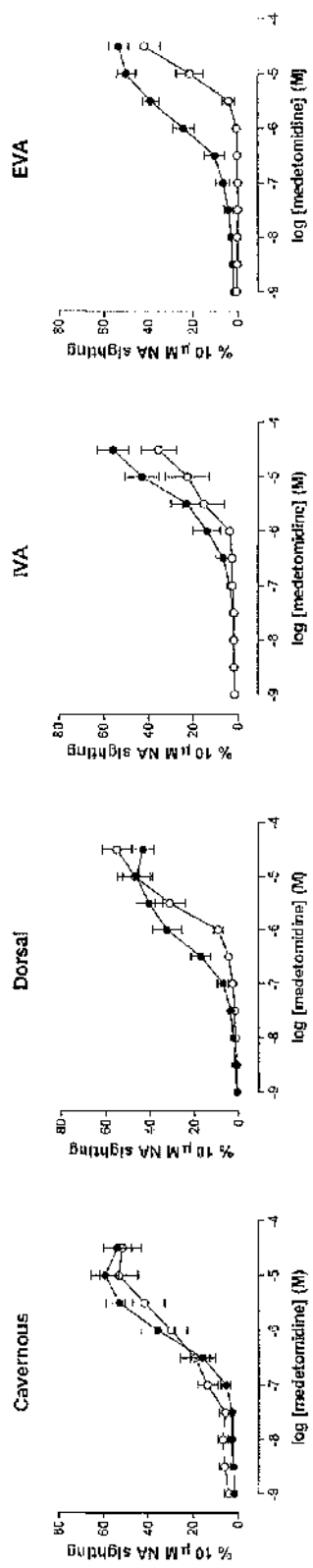


Figure 3-41: Medetomidine-induced vasoconstriction in the genital arteries in the absence (●) or presence of prazosin, 10 nM (○) expressed as a percentage of the NA (10 μM) sighting response ± s.e.mean.

Table 3-24: Medetomidine-induced vasoconstriction in the genital arteries in the absence or presence of prazosin.

	n (N)	E_{max} grams (s.e.mean)	E_{max} % NA Sighting (s.e.mean)	Hill Slope (95% Confidence Interval)	pEC_{50} (s.e.mean)	
Cavernous	Control	6 (7)	0.74 (0.14)	59.59 (6.12)	1.50 (1.02 to 1.97)	6.16 (0.11)
	10 nM	5 (5)	0.63 (0.07)	60.95 (5.44)	0.84 (0.49 to 1.19)	6.17 (0.25)
Dorsal	Control	8 (13)	1.00 (0.17)	44.96 (6.13)	1.22 (0.99 to 1.44)	6.28 (0.15)
	10 nM	4 (7)	1.39 (0.23)	52.38 (7.02)	1.43 (1.16 to 1.70)	5.48 (0.14)
IVA	Control	6 (10)	0.73 (0.15)	47.50 (8.14)	0.84 (0.67 to 1.01)	nd
	10 nM	5 (5)	1.59 (0.21)	38.44 (10.44)	1.02 (0.79 to 1.26)	nd
EVA	Control	6 (10)	1.60 (0.20)	56.89 (4.38)	1.06 (0.93 to 1.18)	nd
	10 nM	6 (7)	1.01 (0.19)	38.49 (7.25)	0.96 (0.51 to 1.41)	nd

nd: not determined

Electrical Field Stimulation

To demonstrate the presence of physiologically functional adrenergic neurons in the genital tissues, small vessel wire myography was coupled with EFS. Adrenoceptor antagonists were tested against responses induced by electrical stimulation of endogenous nerves and the release of endogenous neurotransmitters.

Control Responses

To compare EFS responses in the presence or absence of inhibitors, frequency-response curves were constructed to frequencies of 0.5 to 64 Hz, increasing two-fold with each step. EFS parameters were pulse duration 0.3 ms, current 35 mA, train duration 15 s with a delay of 5 minutes between trains.

TTX, a Na⁺ channel blocker, was tested against various EFS parameters, including different currents, frequencies and pulse widths. However, variable results were observed with no experiments achieving complete abolition of EFS-induced responses by TTX (1 μM). Following a review of the literature, it was noted that experimenters investigating EFS-induced responses in genital tissues from both males and females used a range of parameters. EFS parameters used by these investigators included frequency 0.5 to 64 Hz, current 35 to 75 mA or voltage 4 to 70 V, pulse width 0.1 to 1.0 ms and train duration 1 to 120 s. In many of these experiments EFS-induced responses were abolished by TTX (0.1 to 1 μM). The tissues studied included vaginal wall tissue, corpus cavernosum tissue, dorsal and cavernous arteries from species such as mouse, rat, rabbit, monkey, bull, horse and human (Saenz de Tejada *et al.*, 1988; Simonsen *et al.*, 1997a; Okamura *et al.*, 1998; Recio *et al.*, 1998; Hedlund *et al.*, 1999; Segarra *et al.*, 1999; Hedlund *et al.*, 2000; Simonsen *et al.*, 2001; Ziessen *et al.*, 2002; Prieto *et al.*, 2004). From this literature review it was concluded that a set of parameters at the lower end of the range used to obtain published data would be suitable for investigations in the current study. The chosen parameters were those previously quoted (frequency 0.5 to 64 Hz, current 35 mA, pulse width 0.3 ms, train duration 15 s, train delay 5 minutes) and were similar to those used by Prieto *et al.* (2004) in horse cavernous arteries.

Further experiments were performed to determine the extent to which chosen EFS parameters could be inhibited by TTX in the genital arteries being studied. Responses to EFS during a frequency-response curve were inhibited, but not abolished, in all genital artery preparations (Figure 3-42). The magnitude of this inhibition amounted to 12.3%

($n(N) = 1(1)$), $12.5 \pm 1.2\%$ ($n(N) = 3(4)$), 69.0% ($n(N) = 1(1)$) and $13.0 \pm 2.4\%$ ($n(N) = 2(3)$) in the cavernous artery, dorsal artery, IVA and EVA respectively. Since the same parameters were used for both EFS-induced vasoconstriction and vasodilation and EFS-induced vasodilation was variable, TTX was not further tested against vasodilation responses.

In male and female genital artery preparations, vasoconstrictions in response to frequency-response curves were found to be highly reproducible (Figures 3-43 and 3-44, Table 3-25). No changes were observed in the magnitude of EFS-induced vasoconstriction at any frequency (0.5 to 64 Hz, $P > 0.05$).

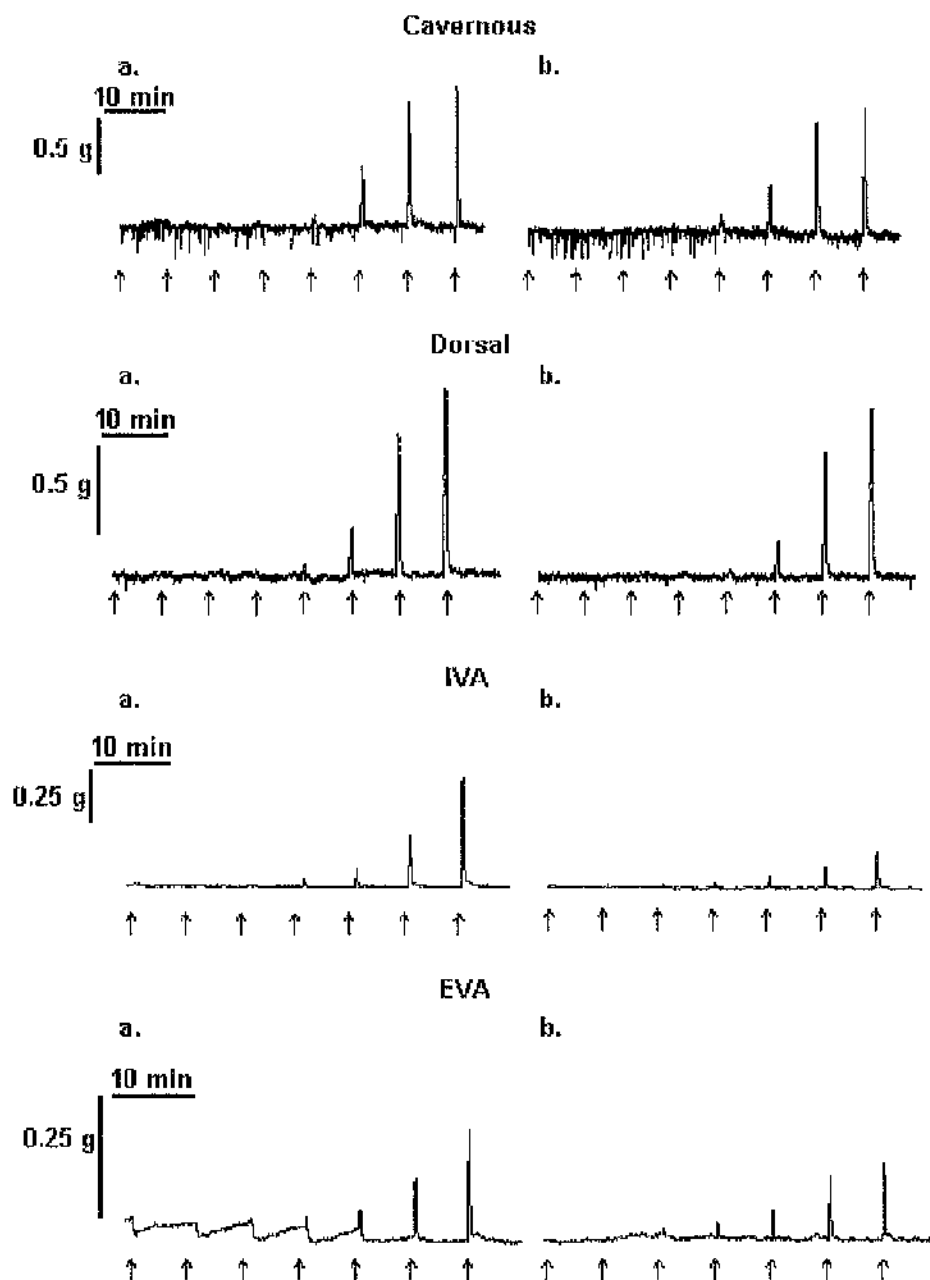


Figure 3-42: EFS-induced responses in the genital arteries in the absence (a.) or presence (b.) of 1 μM TTX. Raw traces obtained with the EFS parameters frequency 0.5 to 64 Hz (arrows), current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

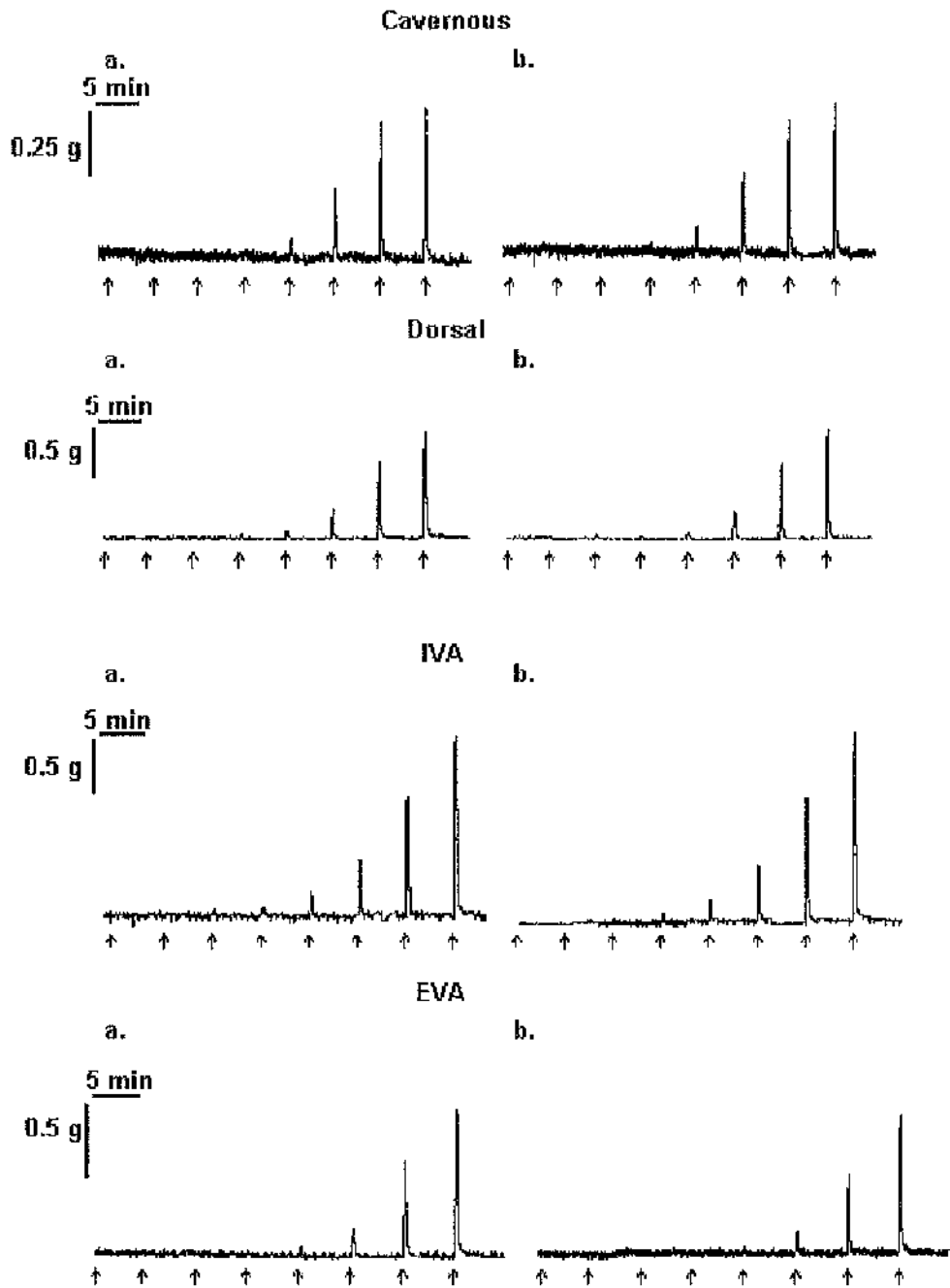


Figure 3-43: Frequency-response curves (arrows, 0.5 to 64 Hz) in the genital arteries, 1st (a.) and 2nd (b.) controls. Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

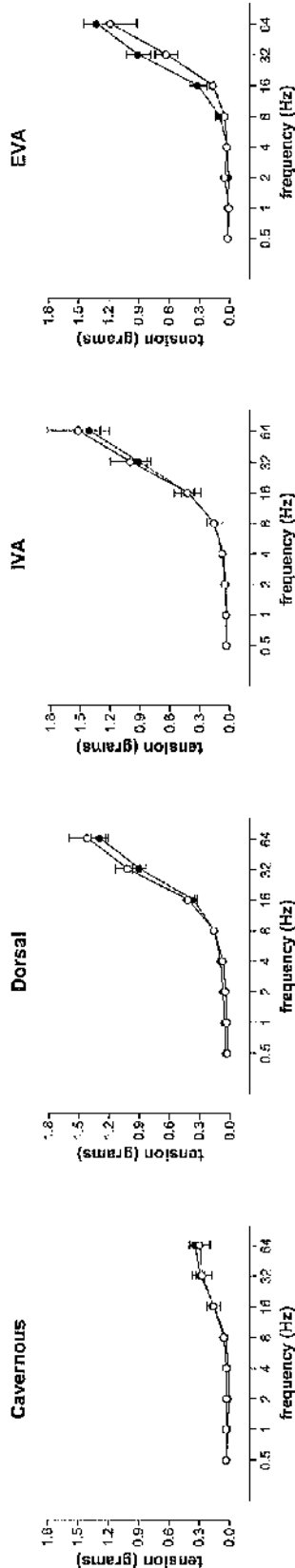


Figure 3-44: EFS-induced vasoconstriction in the genital arteries, 1st (●) and 2nd (○) controls, expressed as grams tension ± s.e.mean.

Table 3-25: EFS-induced vasoconstriction in the genital arteries, 1st and 2nd controls.

	n (N)	0.5 Hz	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz	64 Hz	
Cavernous	1 st Control	6 (17)	0.03 (0.00)	0.02 (0.01)	0.01 (0.01)	0.01 (0.01)	0.04 (0.01)	0.15 (0.03)	0.29 (0.05)	0.34 (0.05)
	2 nd Control	4 (4)	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.06 (0.02)	0.16 (0.07)	0.27 (0.10)	0.30 (0.10)
Dorsal	1 st Control	7 (26)	0.04 (0.01)	0.05 (0.01)	0.07 (0.01)	0.09 (0.01)	0.16 (0.02)	0.36 (0.04)	0.90 (0.07)	1.29 (0.08)
	2 nd Control	5 (5)	0.02 (0.00)	0.03 (0.01)	0.04 (0.02)	0.07 (0.02)	0.15 (0.04)	0.41 (0.06)	1.02 (0.12)	1.41 (0.18)
IVA	1 st Control	5 (13)	0.04 (0.01)	0.03 (0.01)	0.04 (0.01)	0.06 (0.01)	0.16 (0.03)	0.42 (0.06)	0.92 (0.09)	1.41 (0.11)
	2 nd Control	2 (2)	0.03 (0.02)	0.03 (0.00)	0.05 (0.01)	0.07 (0.03)	0.15 (0.08)	0.42 (0.13)	1.00 (0.20)	1.52 (0.31)
EVA	1 st Control	4 (12)	0.02 (0.00)	0.02 (0.00)	0.01 (0.01)	0.02 (0.01)	0.10 (0.04)	0.32 (0.08)	0.91 (0.12)	1.32 (0.13)
	2 nd Control	2 (2)	0.02 (0.00)	0.01 (0.03)	0.05 (0.02)	0.03 (0.01)	0.05 (0.00)	0.16 (0.02)	0.63 (0.11)	1.18 (0.26)

Adrenoceptor Inhibition

A combination of prazosin (10 nM) and rauwolscine (10 nM), inhibitors of α_1 - and α_2 -ARs respectively, were tested against frequency-response curves to determine the involvement of adrenergic neurons in EFS-induced responses.

In cavernous arteries, maximal vasoconstrictions were reduced by AR inhibition (prazosin plus rauwolscine) by $41.8 \pm 3.4\%$ ($n(N) = 4(4)$) at the highest frequency, 64 Hz (Figure 3-45). In dorsal arteries, the reduction in maximum vasoconstriction was less, $16.2 \pm 2.2\%$ ($n(N) = 6(6)$) at the highest frequency. Inhibition of ARs reduced EFS-induced vasoconstriction in IVA by $33.7 \pm 4.9\%$ ($n(N) = 4(4)$), and in EVA by $48.7 \pm 11.4\%$ ($n(N) = 3(3)$). While responses in individual vessels were visibly decreased by adrenergic inhibition, statistical analysis of mean data did not prove any effects to be significant ($P > 0.05$, Figure 3-46, Table 3-26).

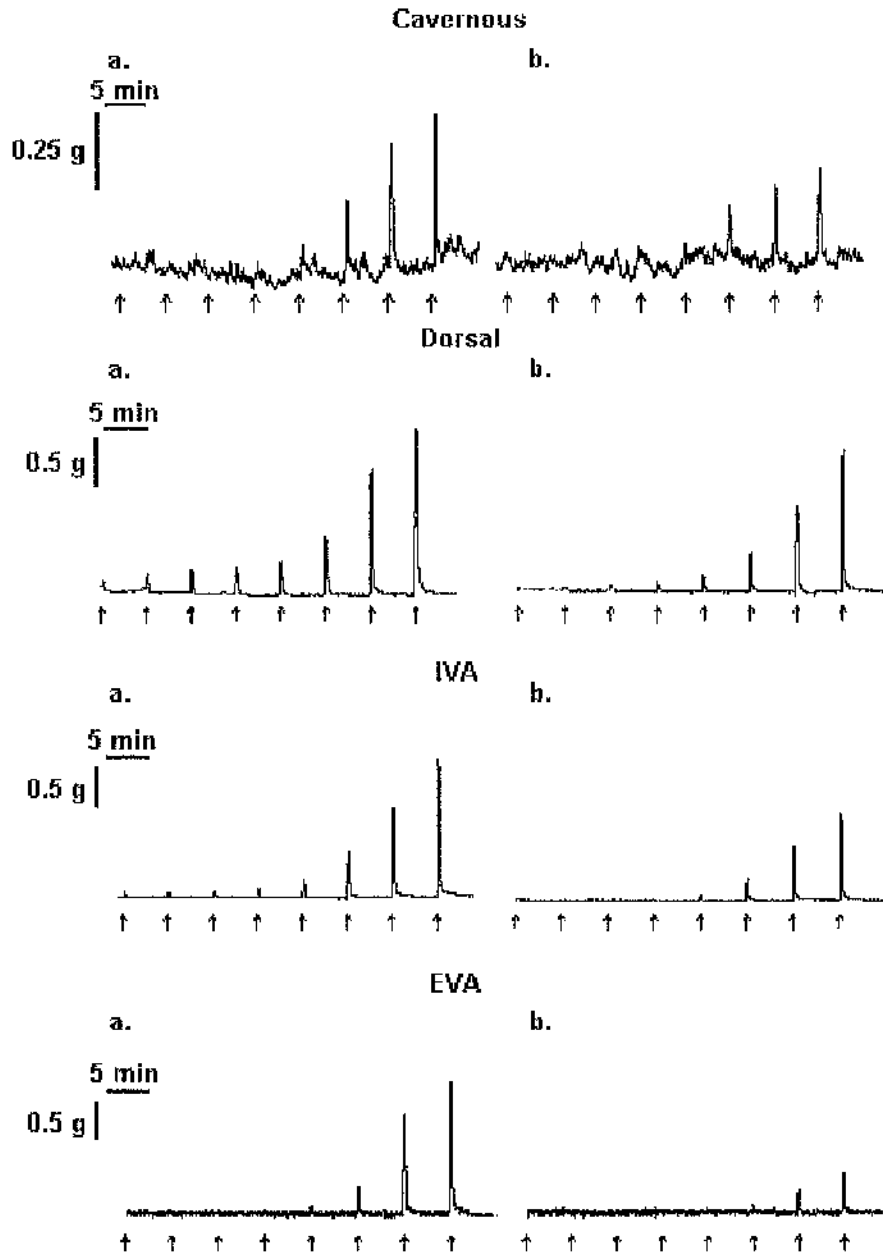


Figure 3-45: Frequency-response curves (arrows, 0.5 to 64 Hz) in the genital arteries in the absence (a.) or presence (b.) of prazosin (10 nM) plus rauwoiscine (10 nM). Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

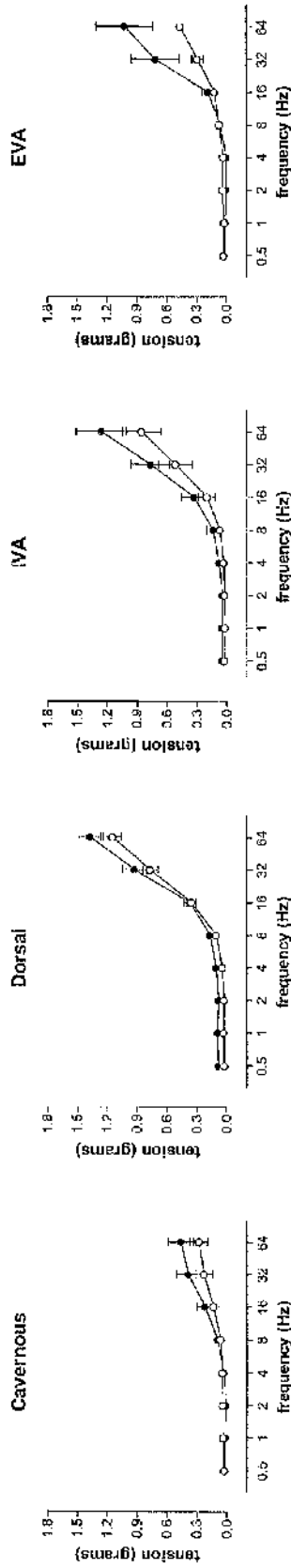


Figure 3-46: EFS-induced vasoconstriction in the genital arteries in the absence (●) or presence (○) of prazosin (10 nM) plus rauwolscine (10 nM) expressed as grams tension ± s.e.mean.

Table 3-26: EFS-induced vasoconstriction in the genital arteries in the absence or presence of prazosin (10 nM) plus rauwolscine (10 nM).

	E_{max} grams (s.e.mean)								
	n (N)	0.5 Hz	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz	64 Hz
Cavernous Control	4 (4)	0.03 (0.01)	0.02 (0.01)	0.01 (0.00)	0.03 (0.01)	0.09 (0.04)	0.22 (0.08)	0.39 (0.11)	0.46 (0.13)
Adrenergic Inhibition	4 (4)	0.03 (0.01)	0.03 (0.02)	0.04 (0.02)	0.05 (0.02)	0.06 (0.03)	0.13 (0.06)	0.23 (0.08)	0.28 (0.09)
Dorsal Control	6 (6)	0.09 (0.03)	0.09 (0.03)	0.08 (0.02)	0.11 (0.02)	0.17 (0.03)	0.37 (0.06)	0.92 (0.12)	1.37 (0.11)
Adrenergic Inhibition	6 (6)	0.03 (0.01)	0.03 (0.01)	0.03 (0.00)	0.04 (0.01)	0.10 (0.02)	0.35 (0.05)	0.77 (0.08)	1.15 (0.09)
IVA Control	4 (4)	0.05 (0.02)	0.05 (0.03)	0.04 (0.02)	0.08 (0.04)	0.14 (0.07)	0.33 (0.13)	0.77 (0.20)	1.26 (0.25)
Adrenergic Inhibition	4 (4)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)	0.03 (0.01)	0.07 (0.04)	0.20 (0.08)	0.51 (0.17)	0.85 (0.20)
EVA Control	3 (3)	0.02 (0.01)	0.01 (0.01)	0.01 (0.01)	0.00 (0.01)	0.06 (0.02)	0.19 (0.06)	0.72 (0.24)	1.03 (0.29)
Adrenergic Inhibition	3 (3)	0.03 (0.01)	0.03 (0.01)	0.04 (0.02)	0.03 (0.02)	0.08 (0.02)	0.12 (0.03)	0.29 (0.06)	0.47 (0.03)

Discussion

Adrenergic Neurons

Male erectile tissues have been extensively studied and as a result both sympathetic and parasympathetic pathways, including adrenergic, cholinergic and NANC neurons, have been demonstrated and localised to the cavernous trabecular smooth muscle and penile arteries (McConnell *et al.*, 1972; Lue *et al.*, 1983; Sathananthan *et al.*, 1991; Tamura *et al.*, 1995; Segarra *et al.*, 1998).

Female genital tissues have been far less extensively investigated and while vaginal tissue has been shown to be densely innervated (Hilliges *et al.*, 1995) and innervation to include adrenergic, cholinergic neurons and NANC neurons (Lakomy *et al.*, 1987; Yucel *et al.*, 2004), few studies have been performed to localise or characterise neurons within female genital tissues. In this study, sections of vaginal tissue and isolated vaginal artery were prepared for immunohistochemical analysis to further investigate innervation of these tissues.

Initial studies were carried out using a pan-neuronal antibody to determine the general density and location of neurons within rabbit vaginal tissues. Many nerve ganglia were observed, which is consistent with Hilliges *et al.* (1995) who reported a dense innervation of human vaginal tissues. These nerve ganglia are likely to be parasympathetic due to their location in the peripheral tissues. In addition, this study showed nerves arranged in a plexus surrounding the vascular smooth muscle of larger arteries, while arterioles also appeared to be closely innervated. Veins, however, were lacking in any innervation indicating that vaginal veins are not directly regulated by neurogenic stimuli. This is an interesting point since it has been postulated that active regulation, in addition to the passive veno-occlusive mechanism, is responsible for decreased venous outflow during the erectile response in males (Kirkeby *et al.*, 1991; Holmquist *et al.*, 1992; Segarra *et al.*, 1998). However, the vaginal tissue does not act as a capacitor; unlike male penile tissues that retain blood to achieve rigidity. Therefore, increased vaginal blood flow may be adequate for sexual arousal in females without controlled venous outflow.

D β H was used in this study to determine the location of adrenergic neurons. D β H is an enzyme involved in catecholamine synthesis (Figure 3-47). It is present in adrenergic neurons and synthesises the conversion of dopamine to the neurotransmitter NA. Tyrosine hydroxylase, another enzyme involved in catecholamine synthesis, has previously been

used to locate adrenergic neurons. However, tyrosine hydroxylase is common to the synthesis of dopamine, NA and adrenaline and will be present in additional locations including dopaminergic neurons. Therefore, D β H has become more commonly used for the identification of noradrenergic neurons.

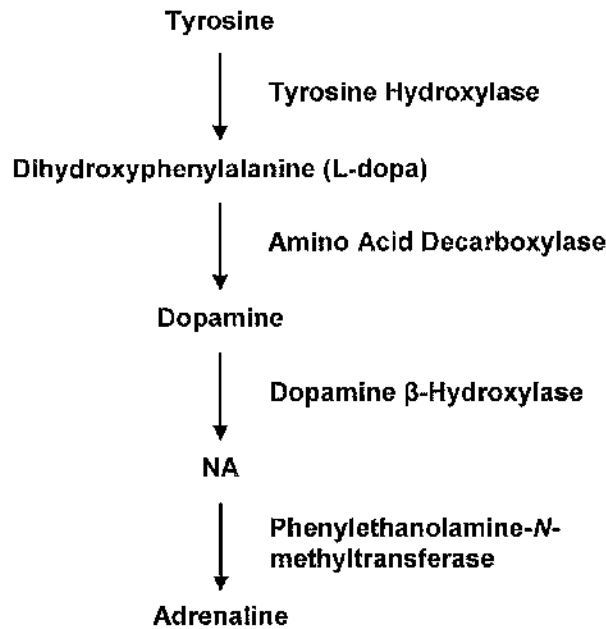


Figure 3-47: Catecholamine biosynthetic pathway.

D β H-positive staining was observed in a similar location to the nerve plexus surrounding vaginal arteries, previously demonstrated by PGP 9.5 staining. This indicates a likely adrenergic involvement in the vasoconstriction of vaginal arteries. D β H was not identified in any nerve ganglia in vaginal tissues, further confirming that these structures were not sympathetic ganglia. The presence of non-specific background staining in the endothelium and connective tissues of vaginal tissue could be removed by using lower concentrations of primary or secondary antibodies. Further experiments are required to refine the technique and further investigate co-localisation of D β H-positive staining with PGP 9.5 to determine a percentage representation of sympathetic innervation.

Dense innervation of both male and female genital tissues includes adrenergic innervation, which in this study was shown to be located in close association with genital arteries. Electrical stimulation of genital arterial preparations caused frequency-dependent vasoconstriction which could be inhibited by a combination of prazosin and rauwolscine in all vessels studied. Therefore, it is likely that sympathetic adrenergic innervation, of both penile and vaginal arteries, has an important physiological role in the vascular function of genital tissues. However, while individual vasoconstriction responses could be inhibited by

up to 69% using prazosin plus rauwolscine, in all arteries a resistant component remained following AR antagonism. This suggested the presence of additional vasoconstrictor mechanisms.

An interesting point was that EFS-induced vasoconstriction could not be abolished by TTX in any arterial preparation. TTX is a powerful neurotoxin from puffer fish which prevents the propagation of nerve impulses along an axon by blocking Na^+ channels and would, therefore, be expected to abolish any nerve-induced responses. EFS parameters are normally chosen such that direct stimulation of the smooth muscle does not occur and it might be implicated that responses observed during the current study were as a result of direct activation of arterial smooth muscle cells. However, if responses were due to direct smooth muscle stimulation no inhibition of EFS-induced vasoconstriction could be achieved by inhibition of receptors, which could only be activated by transmitter release. When compared to TTX inhibition, which amounted to approximately 13% in most arteries, adrenergic inhibition was much greater, approximately 40%. In addition, responses gained via direct activation of smooth muscle would be expected to be longer in duration than the transient, EFS-induced responses observed during the current study.

TTX-resistant, EFS-induced responses were also noted in the rabbit pulmonary artery (Jackson *et al.*, 2002). The authors of this study suggested that electrical stimulation of blood vessels could cause direct depolarisation of nerve varicosities leading to neurotransmitter release, an action which would be TTX-resistant since TTX blocks only the conduction of an action potential. In addition, some neurons have been shown to contain TTX-resistant Na^+ channels (Campbell, 1993; Ikeda *et al.*, 2005).

In summary, inhibition of EFS-induced responses by AR inhibition with prazosin and rauwolscine provides a good indication of the involvement of sympathetic adrenergic neurotransmission in the control of genital arteries. The lack of total inhibition of EFS-induced responses by TTX is a phenomenon that requires further investigation to determine whether it was due to experimental method, depolarisation of nerve varicosities, TTX-resistant Na^+ channels or Na^+ -independent neurotransmission. This would be a novel finding if indeed a TTX-resistant nerve-mediated response could be proven. To investigate this area further, alternative methods could be used to either remove neurons, e.g. sympathetic denervation with 6-hydroxydopamine, or inhibit nerve transmission, e.g. by removal of Na^+ from the bathing media.

Adrenoceptor-Mediated Mechanisms

All four genital arteries responded to the non-selective adrenergic agonist NA with sizable contractions. These responses were relatively insensitive compared to other rabbit blood vessels including aorta, renal vein, saphenous artery and vein, plantaris vein, ear artery and vein, but similar to the rabbit renal artery and vena cava (Dunn *et al.*, 1991; Naghadeh, 1996). Responses to NA were of greatest magnitude in EVA, of equivalent size in IVA and dorsal arteries, and smallest in cavernous arteries. This may reflect either differences in AR density or the relative size and structure of the genital arteries.

In EVA and dorsal arteries, maximal responses to NA were increased by incubation with the NOS inhibitor, L-NAME, suggesting that either a basal release of NO occurs in these arteries, that would normally oppose adrenergic contractile responses, or that NA induces NO release. The involvement of NO and other vasodilator mechanisms are considered in further detail in later chapters.

All vessels studied demonstrated up to a ten-fold increase in NA potency in the presence of NA uptake blockers cocaine and corticosterone, demonstrating active NA uptake mechanisms. Interestingly, in EVA and dorsal arteries at the highest NA concentrations (10 to 300 μM) vasoconstrictions reached a maximum and then decreased instead of maintaining a plateau. In the presence of uptake blockers, NA removal from the vicinity of receptors was prevented, amplifying the effective NA concentration compared to the application of NA in the absence of uptake blockers. The explanation for the observed suppression of responses to supra-maximal NA concentrations may be due to a desensitisation of ARs in the presence of increased concentrations of NA. Alternatively, an increased presence of NA may act at β -ARs, reputed to be present in horse and rabbit penile tissues (Recio *et al.*, 1997; Sato & Kawatani, 2002).

These experiments demonstrated that NA responses could be increased both in sensitivity (all vessels) and size (EVA and dorsal arteries) using uptake blockers or NOS inhibition respectively. However, blockers or inhibitors were not included routinely throughout experimental protocols and so responses obtained to NA could be expected to be sub-maximal. The advantage of not routinely including these inhibitors was to avoid the use of a cocktail of antagonists and blockers during experimental protocols; the potential complex interactions of which would be unknown.

Alpha₁-Adrenoceptors

To characterise adrenergic mechanisms in the genital arteries, both non-subtype- and subtype-selective antagonists were tested against responses to the α_1 -AR-selective agonist PE. Responses to PE were found to be similar in characteristics to NA responses and, as with NA, maximal PE vasoconstrictions in EVA and dorsal arteries were increased by incubation with L-NAME; further confirming evidence of a basal and/or AR-induced release of NO in these vessels. Although a minimal, approximately 13% to 18%, decrease in PE E_{max} was observed between 1st and 2nd control curves in IVA and dorsal arteries, no change in potency was observed. It was therefore considered reasonable to compare 2nd curves to determine the effect of antagonists on PE responses.

In male dorsal and cavernous arteries, vasoconstrictions to PE were inhibited by both phentolamine and prazosin, while responses in female IVA and EVA were inhibited by prazosin (phentolamine not tested). Prazosin would be expected to inhibit responses at all α_1 -AR subtypes with approximately ten-fold greater affinity than phentolamine (Leonardi *et al.*, 1997). However, in the dorsal artery and IVA, prazosin did not demonstrate typical antagonism of PE responses; affinity estimates decreased with increasing concentrations of prazosin and the corresponding Schild plot demonstrated a shallow slope. Both NA and, to a lesser extent, PE can act at α_2 -ARs. Additionally, phentolamine has been shown to inhibit responses at α_2 -ARs; receptors at which prazosin has little effect (Leonardi *et al.*, 1997). Rabbit genital tissues, specifically corpus cavernosum strips (Gupta *et al.*, 1998) and vaginal tissues (Kim *et al.*, 2002), have been demonstrated to possess functional α_2 -ARs in addition to α_1 -ARs. In the present study, antagonism of PE by prazosin in IVA and dorsal arteries may be complicated at higher PE concentrations where responses are due to a mixed receptor population of α_1 - and α_2 -ARs. At these concentrations, both α_1 - and α_2 -AR mediated responses would be inhibited by phentolamine but only α_1 -AR responses would be inhibited by prazosin, reducing the apparent affinity of prazosin in these tissues. Naghadeh (1996) similarly demonstrated that in the rabbit saphenous vein, shown to have a mixed receptor population of α_1 - and α_2 -ARs (Daly *et al.*, 1988a; Naghadeh, 1996), PE responses were inhibited by prazosin with a low pA_2 of 8. The presence of functional α_2 -ARs in the genital arteries is discussed in more detail in later sections.

Prazosin inhibition in EVA and cavernous arteries was, however, more straightforward and demonstrated parallel shifts of CRCs with pK_{BS} of 8.44 and 8.41 respectively. These affinities are relatively low for prazosin, which normally demonstrates subnanomolar

affinities at α_1 -ARs (Hieble *et al.*, 1995). However, low affinity estimates for prazosin have been demonstrated in other rabbit tissues (Daly *et al.*, 1988b; Muramatsu *et al.*, 1990; Van der Graaf *et al.*, 1997; Kava *et al.*, 1998) and this may either represent a mixed receptor population, a species-specific effect or be indicative of a distinct AR subtype with a low affinity for prazosin (Flavahan & Vanhoutte, 1986; Ford *et al.*, 1997).

Alpha₁-Adrenoceptor Subtypes in Male Genital Arteries

To characterise α_1 -AR subtype/s involved in PE-induced vasoconstriction in male genital arteries, four subtype-selective antagonists were tested. RS 100329 and 5-methylurapidil are both selective for the α_{1A} -AR subtype (Hanft & Gross, 1989; Williams *et al.*, 1999). RS 100329 was shown to potently inhibit PE responses in both dorsal and cavernous arteries with pK_B s of 9.03 and 8.80 respectively. Responses in the dorsal artery were atypical in that PE-induced vasoconstriction was not further inhibited by the highest concentration of RS 100329 (100 nM). This may represent a maximum rightward shift of the PE curve; at this point the pEC_{50} was 4.63 ± 0.14 . At high concentrations of agonist, selectivity may be reduced; as previously mentioned PE may act at both α_1 - and α_2 -ARs and it may equally be possible for non-AR mediated responses to occur. Further support for this hypothesis was demonstrated by the failure of any antagonist used during the study to shift the dorsal PE curve significantly further to the right; 1 μ M phentolamine pEC_{50} 4.33 ± 0.09 , 100 nM prazosin pEC_{50} 4.40 ± 0.08 , 100 nM 5-methylurapidil pEC_{50} 4.67 ± 0.18 .

PE-induced vasoconstriction in the presence of 5-methylurapidil was found to be highly variable, particularly in the dorsal artery. Despite this variability, in the cavernous artery PE responses were shown to be significantly inhibited by 5-methylurapidil with a pK_B of 8.65. This further confirmed evidence, indicated by RS 100329 inhibition of PE-induced vasoconstriction, of the presence of functional α_{1A} -ARs in the cavernous arteries. The variability of 5-methylurapidil data in the dorsal artery prevented calculation of a pA_2 but an estimated pK_B of 8.45 ± 0.22 was determined. Taken together, results for both 5-methylurapidil and RS 100329 confirmed the presence of functional α_{1A} -ARs in both dorsal and cavernous male penile arteries. In addition, vessels from various species, including rabbit, rat, dog and human, have demonstrated a relative insensitivity to NA (pA_2 5.7 to 6.8) in tissues possessing the α_{1A} -AR subtype compared to those with either the α_{1B} - or α_{1D} -AR subtypes (pA_2 7.1 to 8.4) (Buckner *et al.*, 1996; Smith *et al.*, 1997; Argyle & McGrath, 2000; Jarajapu *et al.*, 2001; Deighan *et al.*, 2005; Hosoda *et al.*, 2005). The relative insensitivity to NA, previously mentioned, in all arterial preparations would provide further support for the presence of the α_{1A} -AR subtype in these vessels. The

presence of α_{1A} -ARs in erectile tissue is also supported by two studies in rabbit and rat corpus cavernosum tissue (Tong & Cheng, 1997; Peng *et al.*, 1998) and by an *in vivo* investigation of the effects of α_1 -AR antagonists on erections in rats and dogs (Sironi *et al.*, 2000). However, this is the first study to demonstrate the presence of functional α_{1A} -ARs in isolated male genital arteries.

Two antagonists, selective for the α_{1B} -AR subtype (Rec 15/2615) and the α_{1D} -AR subtype (BMY 7378), were ineffective when tested against PE-induced vasoconstriction in both male arteries. Evidence for the α_{1B} -AR subtype in corpus cavernosum tissue has been put forward only by one group (Furukawa *et al.*, 1996) whose results were largely based on the sensitivity of PE responses to CEC. However, a study by Hirasawa *et al.* (1997) has shown that CEC is not subtype-selective for α_1 -ARs and cannot be relied on as an α_{1B} -AR-selective antagonist as previously believed. Rec 15/2615 has been shown to be selective for the α_{1B} -AR subtype (Testa *et al.*, 1997) and has been used in two recent *in vivo* studies of adrenoceptor pharmacology (Sironi *et al.*, 2000; Kim *et al.*, 2002) in rabbits, rats and dogs. The lack of effect of Rec 15/2615 in dorsal and cavernous arteries argues against an involvement of the α_{1B} -AR subtype in these vessels.

Mizusawa *et al.* (2002) previously proposed the α_{1D} -AR as the predominant subtype in rat corpus cavernosum and cavernous artery but based this conclusion on a relatively untested antagonist, A-119637. A-119637 has been shown to possess only a small selectivity for α_{1D} -ARs over α_{1A} -ARs and at the concentrations used during the study, A-119637 could act at all three α_1 -AR subtypes (Carroll *et al.*, 2001). The antagonist used during the present study, BMY 7378, is a widely used α_{1D} -AR antagonist used at concentrations of 1 nM to 100 nM; covering its known affinity at the α_{1D} -AR ($pK_B \sim 8.7$, Kenny *et al.*, 1995; Satoh *et al.*, 1999; Daly *et al.*, 2002; Tanoue *et al.*, 2002; Deighan *et al.*, 2005), while avoiding its affinity at the α_{1A} -AR ($pK_B \sim 6.6$, Lachnit *et al.*, 1997; Zacharia *et al.*, 2004; Deighan *et al.*, 2005). Since no effect on PE-induced vasoconstriction was observed over this concentration range it would be reasonable to conclude that the α_{1D} -AR subtype is not a predominant functional subtype in either dorsal or cavernous penile arteries.

Alpha₂-Adrenoceptors

All four genital arterial preparations studied responded to UK 14,304 with vasoconstrictions that were 28% to 61% of those induced by NA (10 μ M). UK 14,304 was significantly more potent than NA or PE in male, but not female, arteries. Rank order of agonist potency was UK 14,304 > NA > PE in the cavernous and dorsal arteries and NA =

PE = UK 14,304 in the IVA and EVA. In female arteries UK 14,304 was shown to be a partial agonist in comparison to NA. In these arteries, UK 14,304 caused relaxation of NA-induced, but not 5-hydroxytryptamine-induced, tone demonstrating that receptor occupancy by UK 14,304 resulted in a lesser vasoconstriction than that resulting from NA. In the male dorsal artery, responses to UK 14,304 were significantly increased by incubation with L-NAME, which was also shown for responses to both NA and PE in this artery.

Since UK 14,304 is considered to be an α_2 -AR-selective agonist (Turner *et al.*, 1985), UK 14,304-induced vasoconstriction would suggest that functional, post-junctional α_2 -ARs are present and contribute to AR mediated vasoconstriction in the genital arteries. However, responses to UK 14,304 in all preparations demonstrated Hill slopes that were significantly shallower than unity. Potential explanations for shallow Hill slopes include the activation of more than one receptor by the agonist in use. In this case it may be possible that while UK 14,304 is considered to be an α_2 -AR-selective agonist, in the genital arteries being studied it was acting at both α_1 - and α_2 -ARs. To investigate the actions of UK 14,304 further, both α_1 - and α_2 -AR-selective antagonists were tested against agonist responses.

In male genital arteries an alternative protocol was used to test antagonists against UK 14,304 responses during 1st curves. In these protocols, antagonists were added cumulatively following the induction of tone by a partial (0.1 nM to 30 nM) or full (0.1 nM to 30 μ M) UK 14,304 CRC. Responses to UK 14,304 were not significantly reversed by the addition of rauwolscine following the construction of a full CRC in either male penile artery. Interestingly, addition of rauwolscine following a partial CRC did affect UK 14,304-induced tone. In the dorsal and cavernous arteries, rauwolscine completely abolished UK 14,304-induced tone. However, due to the poor maintenance of UK 14,304-induced tone in time controls, the effect of rauwolscine in cavernous arteries was not found to be significant. These results suggest that at low concentrations, UK 14,304 acts at α_2 -ARs, present in both male genital arteries. At high concentrations, UK 14,304-induced responses were due to a mixed population of receptors, which may include both α_2 - and α_1 -ARs.

In female arteries, UK 14,304 CRCs were not significantly desensitised from 1st to 2nd curves allowing pre-incubation with antagonists prior to a 2nd CRC. Rauwolscine did not significantly affect UK 14,304-induced vasoconstriction in the IVA. However, the presence of α_2 -ARs could not be excluded since the corresponding concentration-ratio was greater than one; demonstrating that rauwolscine antagonised UK 14,304-induced

responses in this artery. In the EVA, there was no evidence using pre-incubation with rauwolscine (10 nM) of an α_2 -AR mediated response.

A second α_2 -AR-selective antagonist, BRL 44408, which is claimed to be selective for the α_{2A} -AR subtype (Uhlen *et al.*, 1995), was tested against UK 14,304-induced vasoconstriction in male genital arteries. Addition of BRL 44408 following the construction of a full CRC did not have any effect on UK 14,304 tone. However, the addition of BRL 44408 following a partial UK 14,304 CRC had a striking effect in dorsal arteries; completely abolishing UK 14,304-induced tone as previously observed using the α_2 -AR antagonist rauwolscine. BRL 44408 also completely abolished UK 14,304 tone in cavernous arteries but, again, this effect was not significant due to the considerable loss of tone with time in these vessels. The results gained using the α_{2A} -AR-selective antagonist BRL 44408 confirm those demonstrated by rauwolscine and suggest that UK 14,304 responses in male genital arteries are mediated predominantly by α_2 -ARs at low concentrations of UK 14,304 but by a mixed receptor population at high concentrations of UK 14,304. In addition, since responses to low concentrations of UK 14,304 were completely abolished by BRL 44408, a subtype-selective antagonist, it is likely that the α_2 -AR subtype involved in these responses is the α_{2A} -AR subtype.

UK 14,304 has been previously shown to be a partial agonist at α_{1D} -ARs in the rat carotid artery by Naghadeh (1996). Therefore, to test whether responses to high concentrations of UK 14,304 could be mediated by a mixed receptor population involving both α_2 - and α_1 -ARs, experiments were repeated using the α_1 -AR-selective antagonist prazosin.

Tone induced during a partial UK 14,304 CRC was not significantly reduced by prazosin in the cavernous artery. Following the construction of a full UK 14,304 CRC prazosin reduced UK 14,304-induced tone but, due to the variability of UK 14,304 responses in the cavernous arteries, this effect was not significant. The indication was, however, that prazosin may be inhibiting an α_1 -AR mediated response at high concentrations of UK 14,304; a result which would complement those obtained with the α_2 -AR-selective antagonist rauwolscine.

In the dorsal artery, prazosin completely abolished UK 14,304 tone induced during a full CRC and showed a trend towards reversing UK 14,304 tone resulting from a partial CRC. This indicates that in the dorsal artery, UK 14,304-induced vasoconstrictions are mediated in part by α_1 -ARs at high concentrations of UK 14,304.

In both female genital arteries, pre-incubation with prazosin (10 nM) significantly inhibited the UK 14,304 CRC. In addition, inhibition by a combination of rauwolscine (10 nM) and prazosin (10 nM) had no further effect than that of prazosin alone. Since the effect of pre-incubation with rauwolscine (10 nM) alone was also non-significant, this would suggest that rauwolscine has no effect in either artery. Therefore, UK 14,304 responses in the female arteries are predominantly mediated via α_1 -ARs and, if functional α_2 -AR are present, their contribution is likely to be minimal.

In an attempt to clarify the adrenergic mechanisms functionally active in genital tissues, a second proposed α_2 -AR-selective agonist medetomidine (Bryant *et al.*, 1998) was investigated. This agonist caused concentration-dependent vasoconstriction in all four preparations and was less potent than UK 14,304 in male genital arteries. UK 14,304 responses demonstrated shallow Hill slopes suggesting that vasoconstrictions were mediated by more than one receptor, however, Hill slopes of medetomidine responses were not significantly different from unity.

Pre-incubation with the α_2 -AR-selective antagonist rauwolscine was ineffective at inhibiting medetomidine-induced vasoconstriction in IVA, EVA and cavernous arteries. In the dorsal artery, medetomidine CRCs were significantly shifted to the right by rauwolscine. The mean pK_D/pA_2 of rauwolscine in the dorsal arteries was low, 6.96/6.87. Maximal responses to medetomidine were also significantly increased by 100 nM and 1 μ M rauwolscine ($P < 0.001$, $n(N) = 3(3)$) demonstrating that antagonism of medetomidine responses by rauwolscine was atypical.

Prazosin (10 nM) was also ineffective at inhibiting medetomidine-induced vasoconstriction in IVA and cavernous arteries. However, medetomidine-induced vasoconstriction in EVA and dorsal arteries was significantly inhibited by 10 nM prazosin with a pK_R of 9.00 and 8.24 respectively.

The evidence, therefore, suggests that far from being an α_2 -AR-selective agonist, medetomidine appears to mediate contractile responses in male and female genital arteries predominantly via the α_1 -AR. While responses in IVA and cavernous arteries were inhibited by neither rauwolscine nor prazosin, inhibition of responses may have been achieved using higher antagonist concentrations. It was clear, however, that medetomidine would not prove to be a more selective agonist than UK 14,304 and so this avenue of investigation was not pursued further.

Physiologically, in male erectile tissues, increased sympathetic activity and concomitant release of NA during the flaccid state would lead to activation of α_1 -, and potentially α_2 -ARs, with the predominant functional receptor being the α_{1A} -AR. Vasoconstriction of cavernous arteries, mediated via α_{1A} -ARs, would prevent an increased arterial inflow that is the first stage of an erectile response. During this study it was demonstrated that dorsal arteries had a lower sensitivity to NA than cavernous arteries. This difference in sensitivity may allow dorsal arteries to remain dilated during the flaccid state, allowing a continued blood supply to penile tissues when cavernous arteries are occluded by vasoconstriction.

During this study, dorsal arteries demonstrated increased basal tone and maximal contractions to NA, PE and UK 14,304 in the presence of L-NAME (100 μ M) suggesting a basal or AR-induced level of NO release, an effect which was not observed in cavernous arteries. Dorsal arteries also demonstrated a higher incidence of rhythmic activity that may result from spontaneous or agonist-induced NO release. Decreased sensitivity to adrenergic transmitters and increased levels of NO release would provide a two-pronged approach to maintaining blood flow, via the dorsal arteries, to the penile tissues during the flaccid state. The occurrence of spontaneous activity in all genital artery preparations is considered in more detail in later chapters.

Little is known about the physiology of female genital tissues. However, increased vaginal blood flow, a marker of sexual arousal, has been previously shown to be caused by α -AR-selective antagonists, such as phentolamine (Park *et al.*, 1997). This indicates that the female adrenergic system may act in a similar fashion to the male adrenergic system; by maintaining blood vessels in a contracted state during periods of quiescence. However, unlike male genital tissues, where sympathetic activity has been shown to inhibit the onset of erection, sympathetic activity in the female has been shown to facilitate the first stages of sexual arousal. The vascular function of female genital tissues is, therefore, more complex than first anticipated and cannot be directly inferred from findings in male tissues. In the current study it was demonstrated that α_2 -ARs may be of significantly less importance in female than in male genital arteries, while α_1 -ARs appear to mediate the main functions of the adrenergic system in females.

Summary

In summary, evidence was found supporting the presence of functional adrenergic mechanisms and active NA uptake mechanisms in both male and female genital arteries. In male penile arteries PE responses obtained in the presence of the non-selective antagonists

phentolamine and prazosin suggested that dorsal arteries may have a mixed receptor population composed of α_1 - and α_2 -ARs. Using subtype-selective antagonists, the α_1 -AR mediated response was shown to be due to the α_{1A} -AR subtype in both dorsal and cavernous arteries. No evidence was found for functional α_{1B} - or α_{1D} -AR subtypes in these vessels (Tables 3-27 and 3-28).

Further investigations using the agonist UK 14,304 demonstrated that at low concentrations of UK 14,304, contractions in both dorsal and cavernous arteries were mediated by α_2 -ARs, while contractions in response to high concentrations of UK 14,304 were mediated by a mixed population of α_1 - and α_2 -ARs. In dorsal arteries in particular, α_2 -ARs contributed significantly to adrenergic mediated vasoconstriction. On the basis of BRL 44408 effects, the predominant α_2 -AR subtype was likely to be the α_{2A} -AR. These results were further supported by the rank order of agonist potency. In the cavernous artery this was UK 14,304 > Medetomidine = NA = PE, demonstrating that the α_2 -AR agonists were more potent than the non-selective NA and the α_1 -AR-selective PE. A similar rank order was observed in dorsal arteries, UK 14,304 > Medetomidine > NA = PE, with the notable difference of both α_2 -AR-selective agonists being significantly more potent than NA; confirming the significant contribution of α_2 -ARs in this artery. The presence of functional α_2 -ARs in penile tissues may provide the basis for the use of yohimbine, an α_2 -AR antagonist, for over 100 years in the treatment of erectile dysfunction with variable results (Susset *et al.*, 1989; Vick *et al.*, 2002).

In female vaginal arteries no evidence was found to support the presence of functional α_2 -ARs in the larger EVA while a possible role for the α_2 -AR in the downstream IVA remained. Results supported the presence of functional α_1 -ARs although the predominant subtype was not investigated. The lack of α_2 -AR contribution to vasoconstriction in these vessels was reflected in the rank order of agonist potencies, which was NA = UK 14,304 = PE = Medetomidine in both IVA and EVA. Previously, the presence of functional α_1 - and α_2 -ARs has only been demonstrated by a single study in vaginal tissues (Kim *et al.*, 2002) making this study the first investigation of AR pharmacology in isolated vaginal arteries. The additional finding made during the current study was the lack of significance of an α_2 -AR mediated response in isolated vaginal arteries.

Over the course of experiments, both UK 14,304 and medetomidine were shown to be *non*-selective α -AR agonists, with responses mediated by significant contributions from both α_2 - and α_1 -ARs.

Table 3-1: Summary of adrenergic data in the genital arteries and the effect, increase (↑), decrease (↓) or no change (-), of antagonists and blockers on responses.

Activity	Drugs	Cavernous	Dorsal	IVA	EVA
NA (0.001 to 300 μM)	Control (E_{max})	1.1 g	1.9 g	1.8 g	2.9 g
	L-NAME (100 μM)	-	↑*	-	↑*
	Uptake blockers (Cocaine, 3 μM and Corticosterone, 30 μM)	↑**	↑***	↑*	↑***
	Control (E_{max})	1.0 g	1.9 g	1.7 g	2.6 g
PE (0.001 to 300 μM)	L-NAME (100 μM)	-	↑*	↑*	↑*
	Phentolamine (0.001 to 1 μM)	↓	↓	nd	nd
	Prazosin (1 to 100 nM)	↓	↓	↓	↓
	RS 100329 (1 to 100 nM)	↓	↓	nd	nd
	5-Methylurapidil (1 to 100 nM)	↓	↓	nd	nd
	Rec 15/2615 (1 to 100 nM)	-	-	nd	nd
	BMY 7378 (1 to 100 nM)	-	-	nd	nd

Significance vs controls, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, nd: not determined

Table 3-28: Summary of adrenergic data in the genital arteries and the effect, increase (↑), decrease (↓) or no change (-), of antagonist and blockers on responses.

Activity	Drugs	Cavernous	Dorsal	IVA	EVA
UK 14,304 (0.001 to 30 μM)	Control (E_{max})	0.4 g	0.9 g	1.0 g	0.9 g
	L-NAME (100 μM)	-	↑*	nd	nd
	Rauwolescine (0.001 to 1 μM)	-	↓	-	-
	BRL 44408 (0.001 to 1 μM)	-	(partial CRC)	nd	nd
	Prazosin (0.001 to 1 μM)	-	↓	↓	↓
Medetomidine (0.001 to 30 μM)	Control (E_{max})	0.6 g	1.3 g	1.1 g	1.4 g
	Rauwolescine (0.001 to 1 μM)	-	↓	-	-
	Prazosin (10 nM)	-	↓	-	↓
EFS-induced vasoconstriction	Control (E_{max} at 64 Hz)	0.3 g	1.3 g	1.4 g	1.3 g
	TTX (1 μM)	↓	↓	↓	↓
	Prazosin (10 nM) plus Rauwolescine (10 nM)	↓	↓	↓	↓

Significance vs controls; *, $P < 0.05$, nd: not determined

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Chapter 4

Nitroergic Mechanisms

Introduction

Nitric Oxide as a NANC Neurotransmitter in Genital Tissues

Since the 1970's, when a NANC inhibitory neurotransmission was first described by Gillespie (1972) and Klinge & Sjostrand (1974), a large volume of work has been carried out to uncover the transmitters and pathways responsible. In the following years various candidates, including ACh, VIP, calcitonin gene related peptide (CGRP) and pituitary adenylylate cyclase-activating polypeptide (PACAP), among others, were investigated to determine the identity of the NANC neurotransmitter in male penile erection. Many studies indicated a role for NO: NANC relaxation was inhibited by NOS inhibitors (Gillespie *et al.*, 1989; Holmquist *et al.*, 1991, 1992; Rajfer *et al.*, 1992; Finberg *et al.*, 1993; Trigo-Rocha *et al.*, 1993a; Kim *et al.*, 1994), the NO scavenger oxyhaemoglobin (Bowman *et al.*, 1982; Ignarro *et al.*, 1990; Simonsen *et al.*, 1995) and sulphhydryl inactivating compounds (Liu *et al.*, 1994).

The functional significance of NO release in penile tissue was shown *in vivo* by administration of NO donors which increased intracavernosal pressure and caused erection in male monkey, dog, cat, rat and rabbit (Trigo-Rocha *et al.*, 1993a, 1995; Hellstrom *et al.*, 1994; Wang *et al.*, 1994; Bivalacqua *et al.*, 1999b; Champion *et al.*, 1999; Sazova *et al.*, 2002). *In vitro*, NO, nitrosothiols and NO donors caused relaxation of corpus cavernosum strips (Heaton, 1989; Bush *et al.*, 1992a; Rajfer *et al.*, 1992; Holmquist *et al.*, 1993). In addition, levels of NO (Escrig *et al.*, 1999) and NO breakdown products nitrate and nitrite (Ignarro *et al.*, 1990; Bush *et al.*, 1992b; Leone *et al.*, 1994) were increased following EFS of corpus cavernosum tissue. NO is now widely accepted as the main inhibitory neurotransmitter mediating penile smooth muscle and vascular relaxation.

Female genital tissues have also been shown to be innervated by NOS immunoreactive neurons (Grozdanovic *et al.*, 1994; Majewski *et al.*, 1995; Hoyle *et al.*, 1996; Costagliola *et al.*, 1997; Uckert *et al.*, 2005b). *In vivo*, vaginal blood flow in rats, an indicator of sexual function, was increased by NO donors (Pacher *et al.*, 2003) and decreased by the NOS inhibitor L-NAME (Kim *et al.*, 2004). In female genital tissues *in vitro*, the NO donors nitroglycerin and SNP induced relaxation of human, rabbit and chicken vaginal tissue accompanied by an increase in cyclic guanosine monophosphate (cGMP) (Traish *et al.*, 1999; Costagliola *et al.*, 2004; Uckert *et al.*, 2005a). The PDE-5 inhibitors sildenafil and vardenafil were also shown to cause relaxation of vaginal tissues via an increase in cGMP

(Uckert *et al.*, 2005a). In addition, NANC neurotransmission was inhibited by NOS inhibitors (Al-Hijji *et al.*, 2000; Ziessen *et al.*, 2002; Oh *et al.*, 2003; Costagliola *et al.*, 2004; Kim *et al.*, 2004). However, only a partial inhibition of NANC relaxation was achieved in vaginal tissues. Therefore, while NO has been widely accepted to be the main NANC neurotransmitter in female clitoral tissue, in vaginal tissues NO is thought to have only a partial role (reviewed by Traish *et al.*, 2002 and Munarriz *et al.*, 2003).

Nitric Oxide Pathways

NO and citrulline are produced through the oxidation of L-arginine in a reaction catalysed by NOS and which requires the presence of the prosthetic groups flavin, haem, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin (BH₄). The reaction also requires the presence of calmodulin as a cofactor in promoting electron transfer (reviewed by Moore & Handy, 1997). Both constitutive isoforms of the enzyme, neuronal NOS (nNOS) and endothelial NOS (eNOS), have been demonstrated in penile erectile tissue. In addition, nNOS and eNOS have been demonstrated in female human, rat and rabbit vaginal tissues (Chatterjee *et al.*, 1996; Batra & Al-Hijji, 1998; Yoon *et al.*, 2001; Uckert *et al.*, 2005b).

NO causes smooth muscle relaxation through activation of soluble guanylate cyclase (sGC), which catalyses the conversion of guanosine triphosphate (GTP) to cGMP, leading finally to a decrease in intracellular Ca²⁺ concentration and relaxation. Intracellular concentrations of cGMP, but not cAMP, increase in response to nitrenergic stimulation and NO donors (Bowman & Drummond, 1984; Ignarro *et al.*, 1990; Mirzazadeh *et al.*, 1991; Bush *et al.*, 1992a; Dahiya *et al.*, 1993; Jeremy *et al.*, 1997; Vanhatalo *et al.*, 2000). Inhibition of sGC by methylene blue or 1H-[1,2,4] oxadiazolo [4,3,-a] quinoxalin-1-one (ODQ) decreased both EFS- and NO-induced relaxation in erectile tissues (Simonsen *et al.*, 1995; Recio *et al.*, 1998; Simonsen *et al.*, 2001) and vaginal tissues (Ziessen *et al.*, 2002; Kim *et al.*, 2003).

The mechanisms of signal transduction from cGMP to smooth muscle relaxation are currently uncertain but many pathways have been suggested, which all result in a decreased intracellular concentration of Ca²⁺ (Figure 4-1). cGMP may inhibit voltage-dependent Ca²⁺ channels preventing entry of Ca²⁺ into the cell (Lincoln *et al.*, 1988; Clapp & Gurney, 1991). Alternative pathways involve activation of cGMP-dependent protein kinase (PKG, Cornwell & Lincoln, 1989). Genetic disruption of PKG in mice abolishes relaxation responses to EFS in corpus cavernosum tissues (Hedlund *et al.*, 2000). PKG

may have a number of different actions on smooth muscle cells. Voltage dependent Ca^{2+} channels may be indirectly inhibited by PKG through activation of Ca^{2+} -sensitive K^{+} channels, leading to hyperpolarisation of the plasma membrane (Hampf *et al.*, 1995; Simonsen *et al.*, 1995). PKG may activate Ca^{2+} ATPase (Yoshida *et al.*, 1991) or the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (Furukawa *et al.*, 1991) on the plasma membrane resulting in net Ca^{2+} efflux from the cell. Agonist-induced production of IP_3 by phospholipase C may be inhibited by PKG (Hirata *et al.*, 1990), while the action of IP_3 on the sarcoplasmic reticulum (SR) may also be inhibited by PKG phosphorylation of the SR IP_3 receptor (Komalavilas & Lincoln, 1994). PKG may cause phosphorylation of SR phospholamban resulting in activation of SR Ca^{2+} ATPase and uptake of Ca^{2+} into the internal stores (Cornwell *et al.*, 1991; Cohen *et al.*, 1999). These potential pathways may not be mutually exclusive and may act in either an independent or synergistic manner.

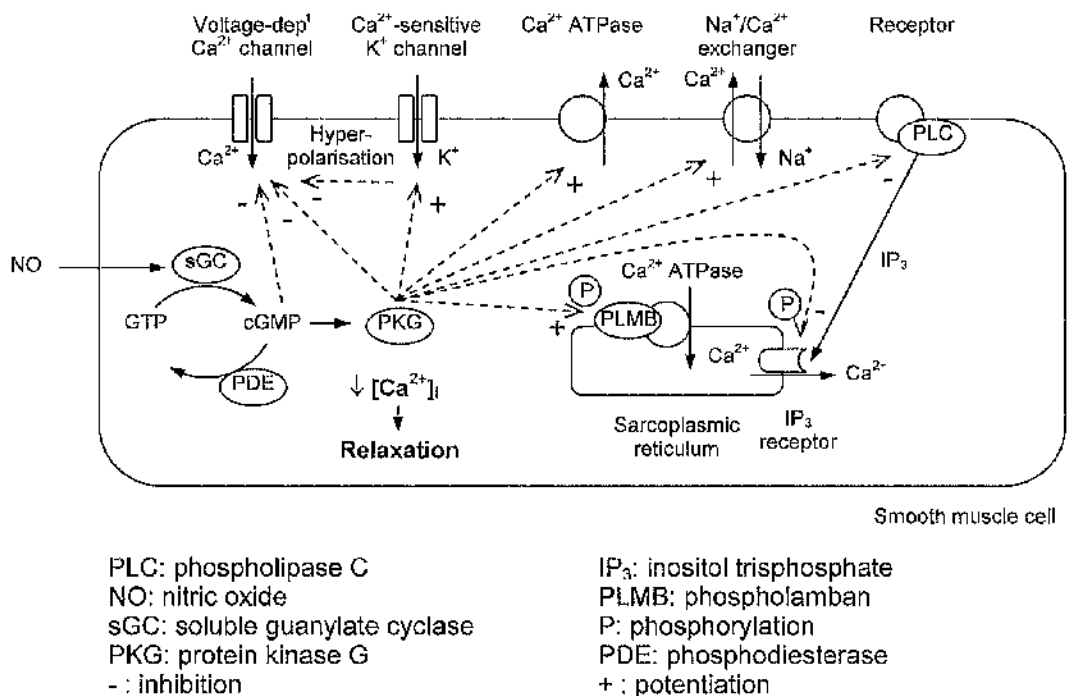


Figure 4-1: Summary of potential NO signal transduction mechanisms in smooth muscle cells.

The actions of cGMP are curtailed by PDE hydrolysis. Currently 11 families of PDE containing greater than 40 isoforms have been characterised (reviewed by Andersson, 2001), of which 13 isoforms have been isolated from human corpus cavernosum tissue (Kuthe *et al.*, 2001, Figure 4-2). Of these, PDE-3A and PDE-5A are thought to be the most functionally relevant isoforms in erectile tissues (Ballard *et al.*, 1998; Stief *et al.*, 1998; Bivalacqua *et al.*, 1999a; Kuthe *et al.*, 2000, 2001). PDE-5 inhibitors have been shown to increase cGMP levels in rabbit, rat and mouse corpus cavernosum (Bowman &

Drummond, 1984; Gibson & Mirzazadeh, 1989; Mirzazadeh *et al.*, 1991; Jeremy *et al.*, 1997), increase nitrenergic-induced relaxation in human corpus cavernosum (Rajfer *et al.*, 1992; Ballard *et al.*, 1998) and enhance EFS-induced intracavernosal pressure increases (Trigo-Rocha *et al.*, 1993b; Boolell *et al.*, 1996; Bivalacqua *et al.*, 1999a). In female vaginal tissues, PDE-4, PDE-5 and PDE-10 have been demonstrated to be present (D'Amati *et al.*, 2002; Uckert *et al.*, 2005b). PDE-5 inhibitors decreased EFS-induced contractions and caused concentration-dependent relaxation of pre-contracted vaginal strips *in vitro* (Gibaldi *et al.*, 2002; Uckert *et al.*, 2005b). *In vivo*, inhibition of PDE-5 increased vaginal blood flow and lubrication in rats and rabbits (Min *et al.*, 2000; Kim *et al.*, 2003, 2004).

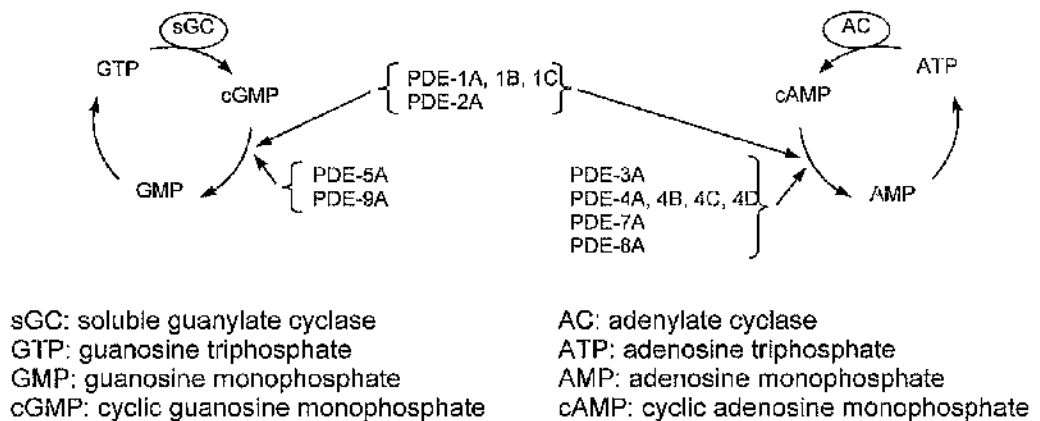


Figure 4-2: Summary of the formation and breakdown by phosphodiesterases of cGMP and cAMP in male erectile tissues. (Adapted from Kuthe *et al.*, 2001).

Aims

Male and female genital tissues have been demonstrated to be abundantly innervated by NOS-IR neurons. Nitrenergic neurons have been accepted as the main inhibitory neurotransmission in penile and clitoral, but not vaginal, tissues. Therefore, the aims of this chapter were as follows.

- To characterise responses of male and female rabbit genital arteries to a NO donor, SNP.
- To confirm the presence of functional nitrenergic neurotransmission in male and female rabbit genital arteries.

The involvement of NO in endothelium-dependent relaxation of genital arteries is considered in detail alongside other endothelial factors in later chapters.

Results

Nitric Oxide Mediated Responses

Noradrenaline-Induced Vasoconstriction

NA (10 μ M) was used to induce tone in all preparations. Sufficient time was allowed for tone to reach a stable plateau before experimental protocols were begun. Responses were found to be most stable in the cavernous artery. In all other preparations NA-induced vasoconstriction demonstrated an initial transient phase followed by a significant loss of tone before a sustained level was reached ($P < 0.001$, Figure 4-3, Table 4-1). Sustained tone was measured and vasodilation responses taken as a decrease from this level of tone.

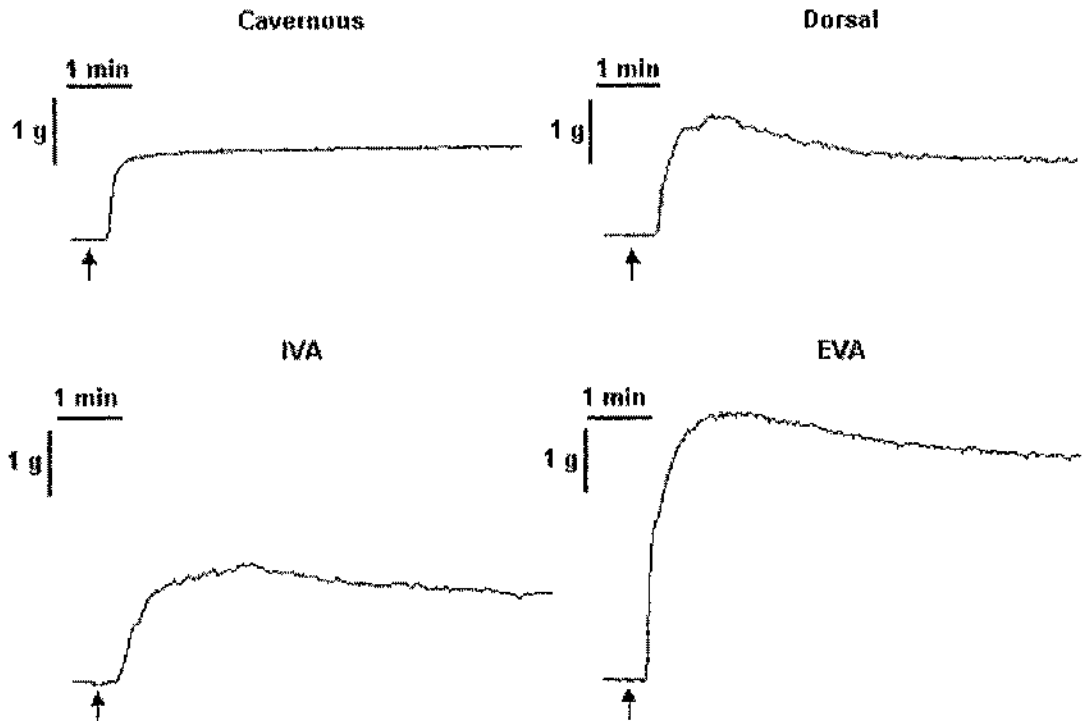


Figure 4-3: NA-induced vasoconstriction in the genital arteries ($10 \mu\text{M}$, arrows). Raw traces from typical experiments demonstrating an initial loss of tone prior to the establishment of a stable plateau in the dorsal artery, EVA and IVA compared to maintained tone in the cavernous artery.

Table 4-1: Comparison of NA-induced ($10 \mu\text{M}$) vasoconstriction between vessels.

	n (N)	E_{max} grams (s.e.mean)	Sustained Tone grams (s.e.mean)
Cavernous	11 (29)	0.92 (0.07)	0.92 (0.08)
Dorsal	13 (34)	2.01 (0.09)	1.51 (0.09)
IVA	15 (30)	1.78 (0.13)	1.03 (0.10)
EVA	16 (30)	2.88 (0.13)	2.15 (0.14)

Sodium Nitroprusside – Nitric Oxide Donor

The nitric oxide donor, SNP, caused a concentration-dependent vasodilation in all genital arterial preparations (Figure 4-4, Table 4-2). Maximal relaxations were greatest in IVA = dorsal = cavernous > EVA and were of magnitude IVA: $97.9 \pm 1.3\%$ ($n(N) = 8(29)$), dorsal: $95.8 \pm 2.6\%$ ($n(N) = 4(16)$), cavernous: $81.8 \pm 3.8\%$ ($n(N) = 3(11)$) and EVA: $65.5 \pm 4.7\%$ ($n(N) = 8(30)$). The potency of SNP was greater in IVA, $pEC_{50} 6.21 \pm 0.12$, and dorsal, $pEC_{50} 6.24 \pm 0.06$, arteries than EVA, $pEC_{50} 5.52 \pm 0.17$, and cavernous, $pEC_{50} 5.46 \pm 0.08$, arteries ($P < 0.01$).

CRCs to SNP were reproducible and showed no significant changes in either potency or maximal responses (Figure 4-5, Table 4-3) from 1st to 2nd curves ($P > 0.05$).

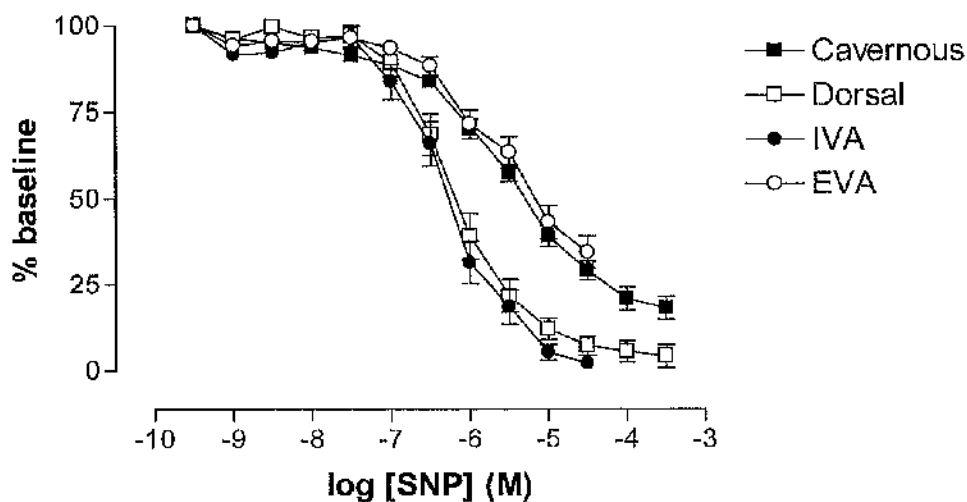


Figure 4-4: SNP-induced vasodilation in the genital arteries expressed as a percentage of NA (10 μ M) induced baseline tone \pm s.e.mean.

Table 4-2: Comparison of SNP-induced vasodilation between vessels.

	<i>n</i> (<i>N</i>)	% Maximum Relaxation (<i>s.e.mean</i>)	-Hill Slope (95% <i>CI</i>)	pEC_{50} (<i>s.e.mean</i>)
Cavernous	3 (11)	81.79 (3.76)	0.66 (0.54 to 0.78)	5.46 (0.08)
Dorsal	4 (16)	95.82 (2.55)	1.09 (0.82 to 1.36)	6.21 (0.12)
IVA	8 (29)	97.88 (1.28)	1.16 (0.84 to 1.48)	6.24 (0.06)
EVA	8 (30)	65.48 (4.70)	0.79 (0.47 to 1.11)	5.52 (0.17)

CI: confidence interval

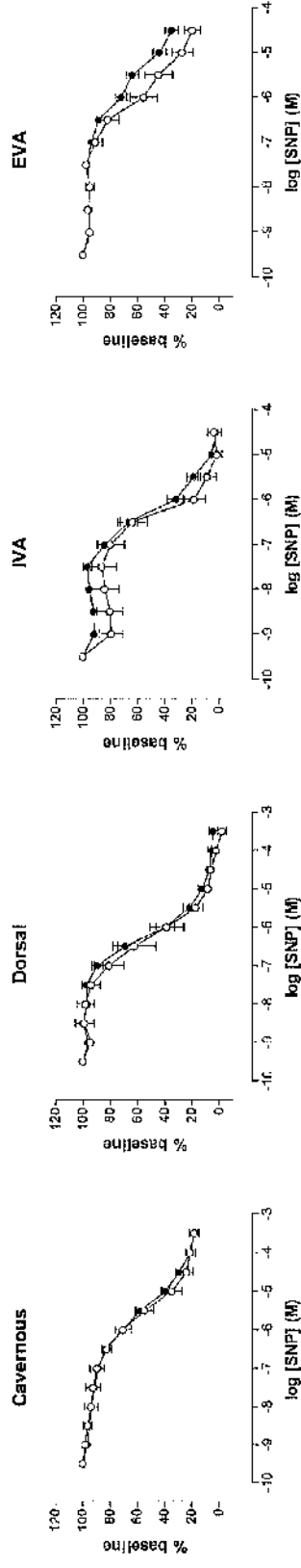


Figure 4-5: SNP-induced vasodilation in the genital arteries, 1st (●) and 2nd (○) controls, expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 4-3: SNP-induced vasodilation in the genital arteries, 1st and 2nd controls.

	n (N)	% Maximum Relaxation (s.e.mean)	-Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)
Cavernous	1 st Control	81.79 (3.76)	0.66 (0.54 to 0.78)	5.46 (0.08)
	2 nd Control	82.02 (6.74)	0.73 (0.45 to 1.01)	5.58 (0.14)
Dorsal	1 st Control	95.82 (2.55)	1.09 (0.82 to 1.36)	6.21 (0.12)
	2 nd Control	102.40 (5.43)	0.90 (0.49 to 1.32)	6.25 (0.12)
IVA	1 st Control	97.88 (1.28)	1.16 (0.84 to 1.48)	6.24 (0.06)
	2 nd Control	98.61 (3.73)	2.02 (0.59 to 3.44)	6.30 (0.10)
EVA	1 st Control	65.48 (4.70)	0.79 (0.47 to 1.11)	5.52 (0.17)
	2 nd Control	80.51 (5.89)	0.92 (0.40 to 1.45)	5.93 (0.16)

UK 343,664 – PDE-5 Inhibitor

A 30 minute incubation with the PDE-5 inhibitor, UK 343,664 (3 to 30 nM) did not significantly potentiate or inhibit SNP-induced vasodilation in any genital arterial preparation ($P > 0.05$, $n(N) = 2-8(2-9)$, Figure 4-6, Table 4-4).

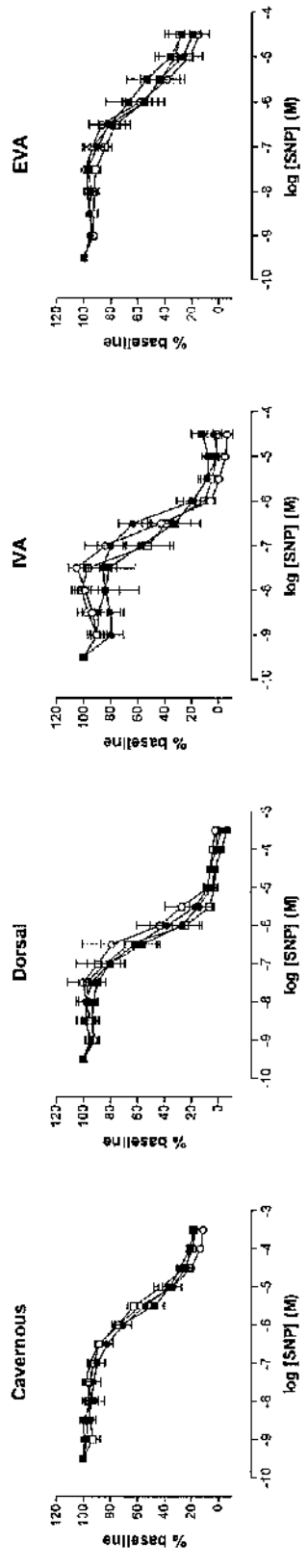


Figure 4-6: SNP-induced vasodilation in the genital arteries in the absence (○) or presence of UK 343,664, 3 nM (◻), 10 nM (◼) or 30 nM (◾) expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 4-4: SNP-induced vasodilation in the genital arteries in the absence or presence of UK 343,664.

		n (N)	% Maximum Relaxation (s.e.mean)	-Hill Slope (95% Confidence Interval)	pEC₅₀ (s.e.mean)
Cavernous	Control	3 (3)	82.02 (6.74)	0.73 (0.45 to 1.01)	5.58 (0.14)
	3 nM	2 (2)	88.76 (1.98)	0.83 (0.73 to 0.94)	5.44 (0.04)
	10 nM	3 (3)	81.34 (3.43)	1.04 (0.72 to 1.36)	5.75 (0.07)
	30 nM	3 (3)	82.44 (5.68)	0.80 (0.55 to 1.04)	5.35 (0.11)
Dorsal	Control	4 (4)	102.40 (5.43)	0.90 (0.49 to 1.32)	6.25 (0.12)
	3 nM	4 (4)	98.03 (7.41)	1.14 (0.33 to 1.95)	6.04 (0.15)
	10 nM	4 (4)	106.63 (4.82)	0.98 (0.54 to 1.43)	6.32 (0.11)
	30 nM	4 (4)	99.05 (4.29)	1.47 (0.69 to 2.24)	6.32 (0.09)
IVA	Control	7 (9)	98.61 (3.73)	2.02 (0.59 to 3.44)	6.30 (0.10)
	3 nM	6 (6)	106.34 (3.76)	1.85 (0.89 to 2.82)	6.54 (0.06)
	10 nM	5 (5)	92.42 (4.46)	1.05 (0.01 to 2.12)	6.87 (0.23)
	30 nM	6 (6)	98.39 (1.92)	1.21 (0.48 to 1.93)	6.80 (0.12)
EVA	Control	8 (9)	80.51 (5.69)	0.92 (0.40 to 1.45)	5.93 (0.16)
	3 nM	7 (7)	85.45 (7.31)	1.18 (0.40 to 1.97)	5.89 (0.14)
	10 nM	6 (6)	72.58 (9.82)	0.79 (0.03 to 1.62)	5.73 (0.39)
	30 nM	6 (6)	71.01 (11.32)	0.87 (0.09 to 1.65)	6.09 (0.26)

Electrical Field Stimulation

To demonstrate the presence of functional nitrergic neurons in the genital tissues, EFS protocols were performed on vessels mounted on a small vessel wire myograph. NOS inhibitors were tested against vasodilations induced by electrical stimulation of nerves and the consequent release of endogenous neurotransmitters.

Control Responses

To allow the study of EFS-induced vasodilation, vessels were incubated with guanethidine (10 μ M) for 1.5 hours to inhibit adrenergic neurons. NA (10 μ M) was used to raise tone in all preparations. Sufficient time was allowed for tone to reach a stable plateau before experimental protocols were begun. Following the establishment of stable tone, frequency-response curves were constructed using frequencies from 0.5 to 64 Hz, increasing two-fold with each step. EFS parameters were pulse duration 0.3 ms, current 35 mA, train duration 15 s with a delay of 5 minutes between trains. As previously described in Chapter 3 – Adrenergic Mechanisms, TTX was tested against the EFS parameters chosen for these studies and was found to inhibit, but not abolish, EFS-induced responses.

In many experiments involving EFS protocols, tone was found to decrease substantially over the course of a frequency-response curve, making comparisons of EFS-induced relaxation difficult (Figure 4-7). Incubation with a combination of cyclooxygenase inhibitor, indomethacin (1 μ M), and K^+ channel blockers, apamin (Apa, 100 nM) and charybdotoxin (ChTX, 100 nM), was found to maintain the stability of NA-induced tone over the course of a frequency-response curve without affecting EFS-induced relaxations (Figure 4-8). Therefore, during some EFS protocols, controls were performed both with and without cyclooxygenase and K^+ channel blockers before the effects of other inhibitors were determined.

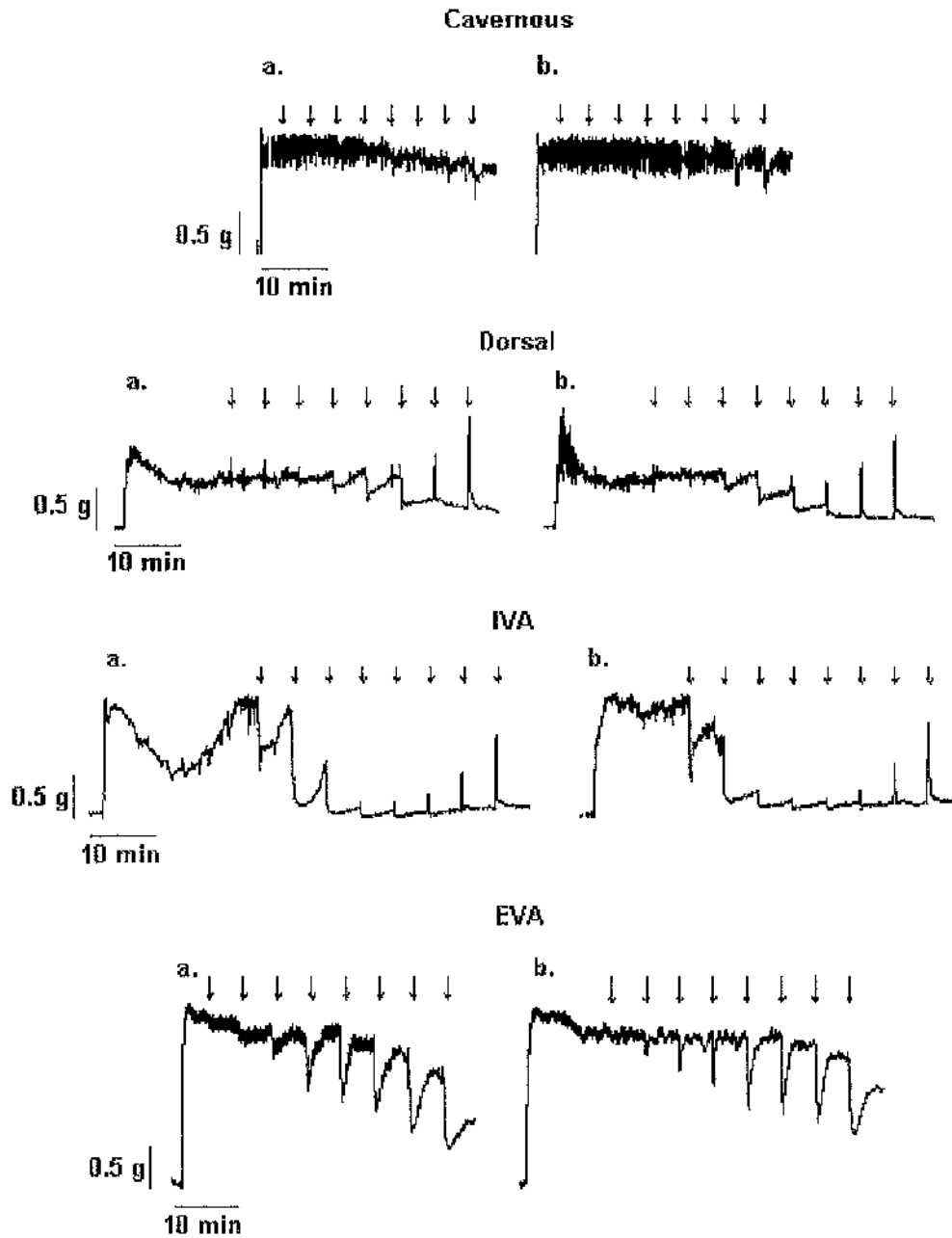


Figure 4-7: Frequency-response curves (arrows, 0.5 to 64 Hz) in the genital arteries following incubation with guanethidine ($10 \mu\text{M}$) and tone raised with NA ($10 \mu\text{M}$), 1st (a.) and 2nd (b.) controls. Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

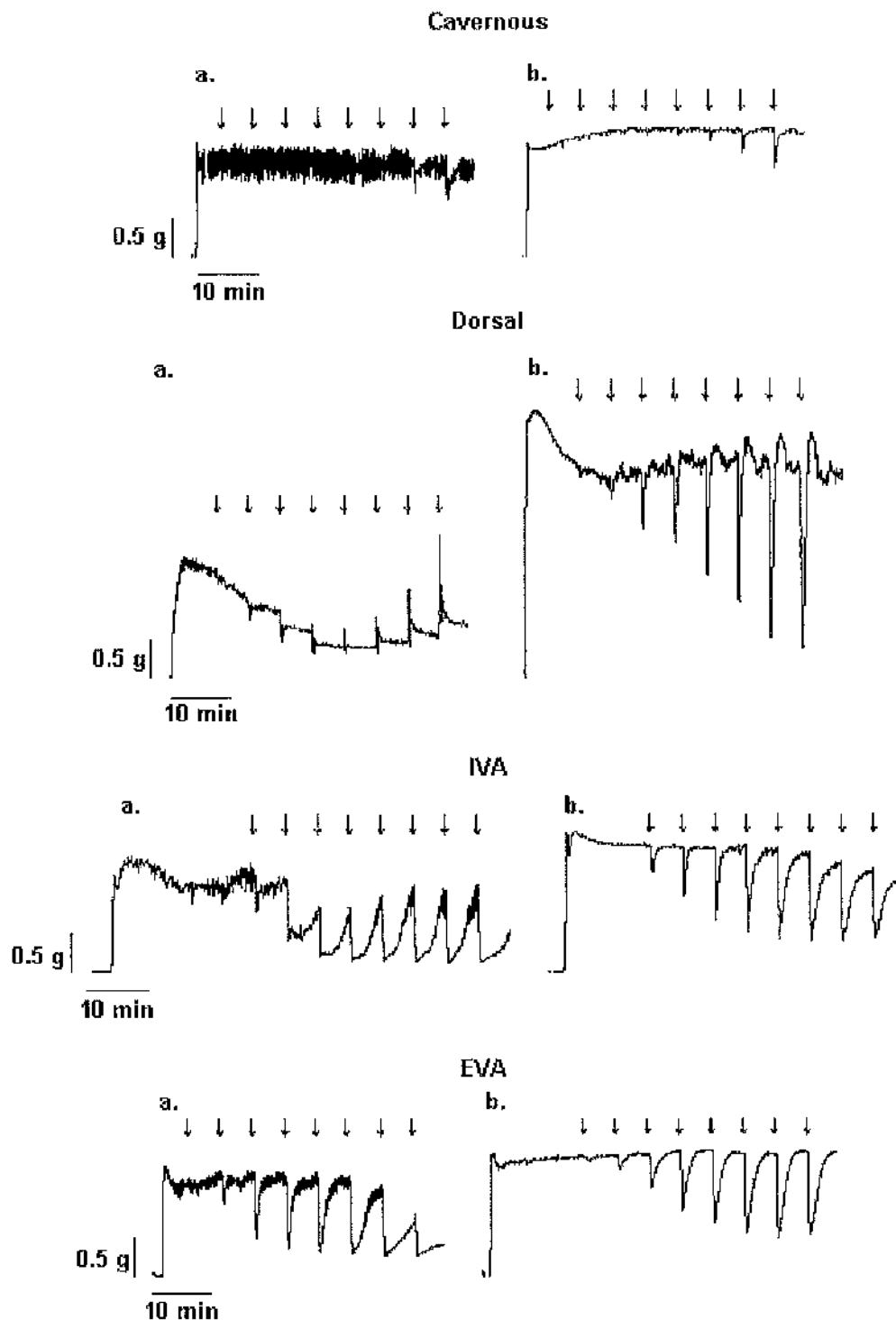


Figure 4-8: Frequency-response curves (arrows, 0.5 to 64 Hz) in the genital arteries following incubation with guanethidine (10 μ M) and tone raised with NA (10 μ M), in the absence (a.) or presence (b.) of indomethacin (1 μ M), Apa (100 nM) plus ChTX (100 nM). Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

Nitric Oxide Synthase Inhibition

To determine the effect of incubation with L-NAME (100 μ M) on EFS-induced responses a preliminary exploratory experiment was performed in male genital arteries. Initial experiments were carried out early in the study, prior to the establishment of EFS parameters and so a different set had been used: frequency 0.5 Hz, pulse width 0.1 ms, current 150 mA, train duration 30 s. In this experiment, EFS-induced contractions in male genital arteries were substantially increased following 40 minute L-NAME incubation (100 μ M, Figure 4-9).

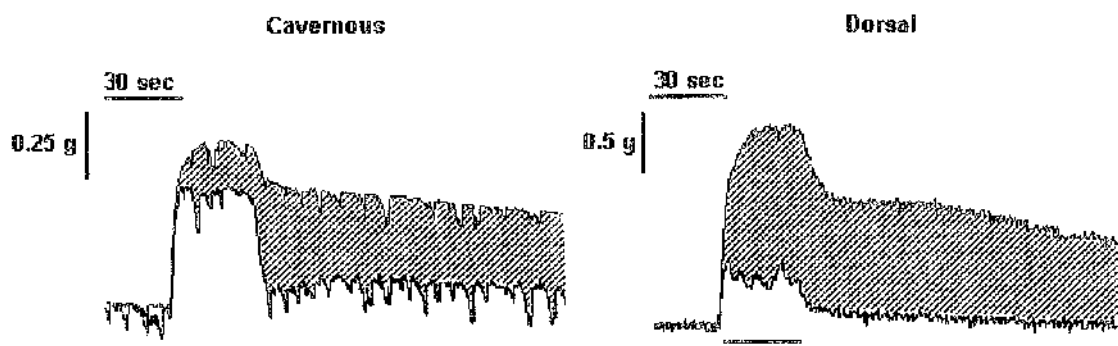


Figure 4-9: EFS-induced contraction in male genital arteries in the absence (black line) or presence (blue line) of L-NAME (100 μ M). Hatched areas indicate change in responses, stimulation period indicated by bar under traces. Raw traces obtained with the EFS parameters frequency 0.5 Hz, current 150 mA, pulse width 0.1 ms and train duration 30 s.

Further experiments were performed to investigate the effect of NOS inhibition on EFS-induced vasodilation using an established set of EFS parameters, see section Control Responses. To maintain NA-induced tone, all responses were measured in the presence of cyclooxygenase (indomethacin) and K^+ channel (Apa and ChTX) blockers.

In cavernous arteries, L-NAME (100 μ M) had very little effect on EFS-induced vasodilation (Figure 4-10). In three of five vessels the magnitude of EFS-induced relaxations remained unchanged, while in two of five vessels, relaxations in response to low (0.5 to 4 Hz) frequencies were increased. Mean data demonstrated no significant changes in maximal vasodilations at any frequency ($P > 0.05$, Figure 4-12, Table 4-5).

Vasodilation in the dorsal arteries was reduced by L-NAME (100 μ M) in three of seven vessels, particularly to low (0.5 to 4 Hz) frequencies (Figure 4-10). In a further three of seven vessels L-NAME had no effect on responses. In a single vessel EFS-induced

vasodilation was increased by incubation with L-NAME. Mean data demonstrated no significant changes in EFS-induced vasodilation at any frequency ($P > 0.05$, Figure 4-12, Table 4-5).

In the IVA, vasodilation was decreased by incubation with L-NAME (100 μM), particularly to low frequencies (1 to 4 Hz), in six of seven vessels (Figure 4-11). However, the overall effect was complicated by changes in both baseline tone and contractile components of the response. At low frequencies (1 to 4 Hz) maximal relaxations were significantly decreased by L-NAME ($P < 0.05$, $n(N) = 3(3)$, Figure 4-12, Table 4-5) but maximal relaxations to higher frequencies (8 to 64 Hz) were not affected ($P > 0.05$).

In all EVA preparations, six of six vessels, EFS-induced vasodilations were decreased by incubation with L-NAME (100 μM , Figure 4-11). At low (0.5 to 4 Hz) frequencies, relaxations were almost abolished with less than 9% relaxation remaining after incubation with NOS inhibitor. At frequencies of 2 Hz to 64 Hz, EFS-induced relaxation was significantly inhibited by L-NAME ($P < 0.05$, $n(N) = 3(3)$, Figure 4-12, Table 4-5) but an L-NAME resistant relaxation of approximately 35% remained.

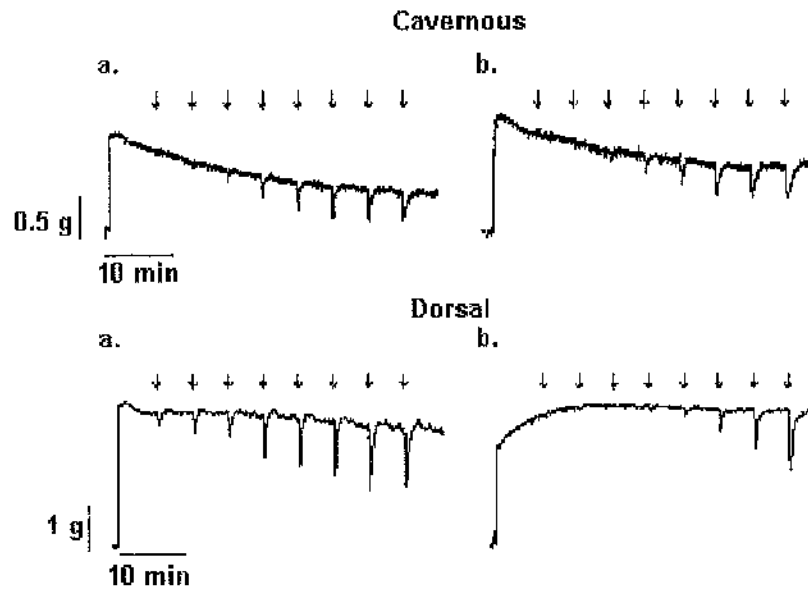


Figure 4-10: Frequency-response curves (arrows, 0.5 to 64 Hz) in the male genital arteries following incubation with guanethidine ($10 \mu\text{M}$), indomethacin ($1 \mu\text{M}$), Apa (100 nM) plus ChTX (100 nM) and tone raised with NA ($10 \mu\text{M}$). Curves in the absence (a.) or presence (b.) of L-NAME ($100 \mu\text{M}$). Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

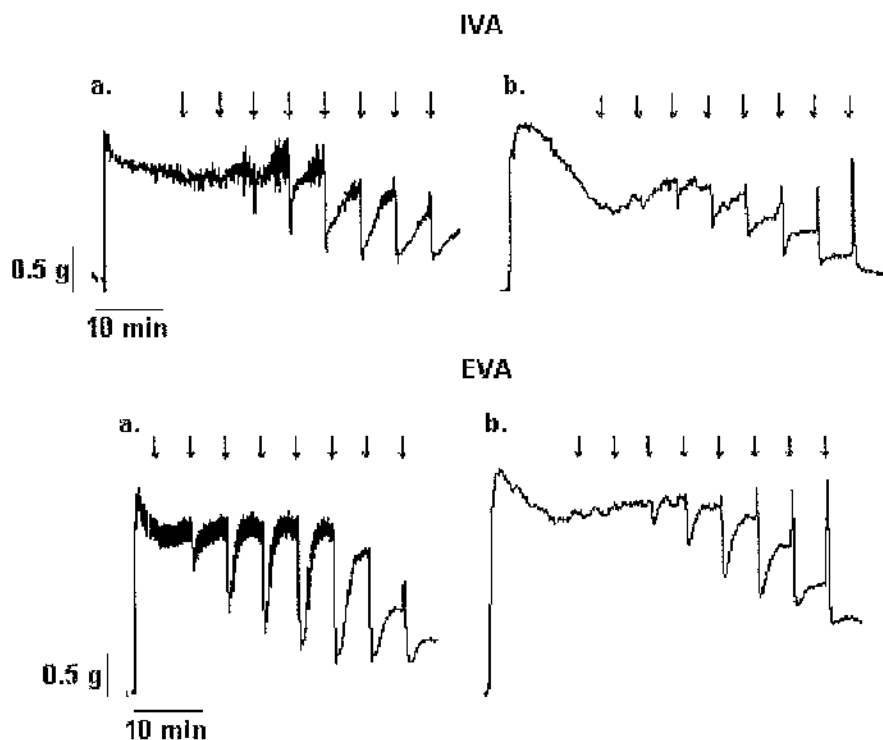


Figure 4-11: Frequency-response curves (arrows, 0.5 to 64 Hz) in the female genital arteries following incubation with guanethidine ($10 \mu\text{M}$) and tone raised with NA ($10 \mu\text{M}$), in the absence (a.) or presence (b.) of L-NAME ($100 \mu\text{M}$). Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

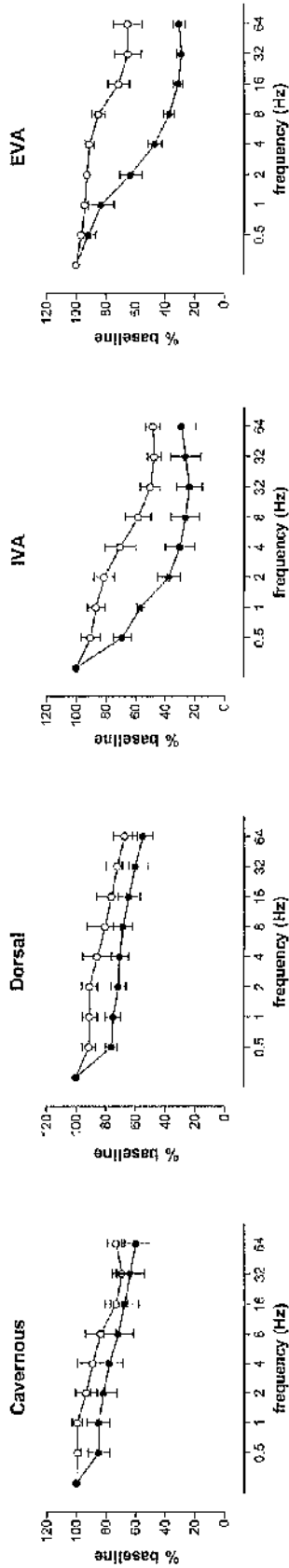


Figure 4-12: EFS-induced relaxation in the genital arteries in the absence (●) or presence (○) of L-NAME (100 μM). All responses performed in the presence of guanethidine (10 μM), indomethacin (1 μM), Apa (100 nM) and ChTX (100 nM) and expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 4-5: EFS-induced relaxation in the genital arteries in the absence or presence of L-NAME (100 μM).

	n (N)	0.5 Hz	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz	64 Hz	
Caverrous	Control	5 (18)	15.08 (7.36)	15.01 (7.77)	18.41 (9.03)	22.37 (9.12)	28.51 (10.05)	32.46 (9.65)	36.13 (9.46)	40.14 (9.44)
	L-NAME	4 (4)	0.96 (2.61)	0.74 (3.53)	6.75 (7.31)	11.01 (10.23)	16.53 (10.36)	26.83 (7.43)	30.60 (6.55)	26.74 (5.83)
Dorsal	Control	4 (16)	23.93 (3.95)	25.10 (5.22)	28.75 (5.04)	29.59 (6.04)	31.48 (6.78)	35.79 (7.43)	39.93 (8.88)	45.17 (6.86)
	L-NAME	3 (3)	8.66 (4.66)	9.23 (5.03)	9.28 (5.38)	14.49 (9.76)	19.54 (12.17)	23.98 (10.05)	27.92 (7.88)	32.85 (8.05)
IVA	Control	3 (3)	31.03 (6.01)	43.66 (2.82)	62.43 (7.61)	69.79 (9.81)	73.66 (9.58)	76.23 (8.85)	73.75 (10.38)	70.96 (10.05)
	L-NAME	3 (3)	9.66 (6.46)	13.46 (5.97)	18.61 (6.97)	29.55 (10.52)	41.67 (8.63)	49.94 (6.73)	52.50 (4.52)	51.71 (4.80)
EVA	Control	3 (3)	8.08 (5.03)	16.57 (8.66)	36.71 (7.47)	53.02 (4.86)	62.73 (3.64)	68.85 (3.14)	71.06 (2.96)	69.53 (3.99)
	L-NAME	3 (3)	3.38 (1.01)	5.69 (1.71)	7.13 (2.42)	8.99 (2.93)	14.97 (4.65)	28.59 (7.28)	34.94 (9.22)	34.94 (9.84)

Discussion

In all genital arterial preparations, inhibitory neurotransmission was demonstrated which was greater in female (approximately 62% and 70% relaxation in IVA and EVA respectively) than male (approximately 40% and 45% relaxation in cavernous and dorsal arteries respectively) genital arteries.

Nitregic neurotransmission has been well described in male penile tissues and NO is considered to be the main inhibitory neurotransmitter in erectile tissues. Using the NO donor SNP to directly stimulate vascular smooth muscle, male penile arteries demonstrated NO-induced vasodilations that were greater both in magnitude and potency in dorsal arteries. However, it is not known whether the physiological source of NO in male penile arteries is via activation of nNOS or eNOS.

In contrast to accepted knowledge, in the current study EFS-induced vasodilation was less successfully blocked by L-NAME in male than female genital arteries. Dorsal arteries demonstrated a degree of nitregic inhibition at low frequencies (0.5 to 2 Hz); an inhibition that was not found to be significant in mean data. However, vasodilation in male penile arteries was generally found to be poor in comparison to vasodilation in female arteries. In addition, without the inclusion of indomethacin, Apa and ChTX, responses in IVA, EVA and dorsal arteries demonstrated a substantial loss of baseline tone and particularly variable EFS-induced responses complicating the analysis of EFS-induced relaxation. It was found that using cyclooxygenase (indomethacin) and K⁺ channel (Apa and ChTX) blockers, both considered to be inhibitors of endothelium-derived relaxing factors (EDRFs), gave a maintained baseline from which to compare EFS-induced vasodilations. Further analysis of these experiments continued to give no significant evidence for nitregic vasodilation in male genital arteries. EFS-induced relaxation of cavernous arteries was largely unaffected by L-NAME but in two cases was actually increased by NOS inhibition. This suggests that NO pathways may have other, perhaps modulatory effects, on neurogenic vasodilator mechanisms in male penile arteries.

Nitregic neurotransmission has been less extensively studied in female genital tissues. Following a particular study by Ziessen *et al.* (2002), which showed that NANC neurotransmission in female rabbit vaginal tissues could not be completely blocked by NOS inhibitors, the opinion was formed that NO has only a partial role in the relaxation of vaginal tissues.

This study represents the first investigation of EFS-induced relaxation in isolated vaginal arteries. EFS-induced vasodilation in female vaginal arteries was significantly reduced by L-NAME in both IVA and EVA. Experiments in both vaginal artery preparations demonstrated a greater inhibition of low frequency responses; in the EVA responses were virtually abolished by L-NAME. Similarly, Ziessen *et al.* (2002) demonstrated that inhibition of NANC vasodilations in vaginal tissues by L-NAME or the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was more effective at low frequencies. Oh *et al.* (2003) demonstrated that partial inhibition of TTX-sensitive, EFS-induced relaxation of female rabbit vaginal tissue could be achieved using either L-NAME or δ -VIP, a VIP receptor antagonist. In addition, neuronal NO and VIP were co-localised in bovine, porcine and avian vaginal tissues by Majewski *et al.* (1995) and Costagliola *et al.* (1997). Further investigations would be warranted to determine whether the remaining vasodilation to high frequencies in female vaginal arteries were due to a neuropeptide, potentially VIP.

While inhibition of EFS-induced responses by L-NAME in female genital arteries was greater in EVA than IVA, responses to the NO donor SNP were both of greater magnitude and potency in IVA than EVA. In accordance with these findings, Traish *et al.* (2003) suggested that NOS activity in rabbit vaginal tissue was greater in proximal (approximating to the IVA) than distal (approximating to the EVA) vaginal regions. However, in the same year Kim *et al.* (2003) found no significant difference in NOS activity in proximal or distal regions of the rabbit vagina. This discrepancy between the relative predominance of L-NAME-sensitive EFS-induced vasodilation in IVA and EVA compared to their relative sensitivity to the NO donor, SNP, may be due to differences in nitrenergic innervation. If EVA were closely innervated by nitrenergic neurons, resulting in regular NO-induced smooth muscle relaxation, NO mechanisms may become down-regulated or less sensitive to subsequent activation. In contrast, IVA, which demonstrated a smaller L-NAME-sensitive component of EFS-induced vasodilation, may be less frequently exposed to NO and remain more sensitive to NO activation.

Curiously, despite the demonstration in both male corpus cavernosum (Gibson & Mirzazadeh, 1989; Mirzazadeh *et al.*, 1991; Rajfer *et al.*, 1992; Jeremy *et al.*, 1997; Ballard *et al.*, 1998) and female vaginal tissues (Giraldi *et al.*, 2002; Uckert *et al.*, 2005a) of a PDE-5-induced increase in cGMP and EFS-induced vasodilations, this study demonstrated no inhibition of SNP-induced vasodilation in any isolated genital artery preparation by the PDE-5 inhibitor UK 343,664 (3 to 30 nM, Wareing *et al.*, 2004). This area requires further investigation to determine the effect of PDE-5 inhibitors on EFS-

induced vasodilation, ACh-induced vasodilation and directly on genital arteries. Little or no PDE-5 involvement in the NO pathway in female genital arteries may explain the lack of success of PDE-5 inhibitors in the treatment of FSD; sildenafil citrate has proven to have a very limited effect on general FSD (Kaplan *et al.*, 1999; Basson *et al.*, 2002), despite the demonstration in this study of substantial L-NAME-sensitive, EFS-induced vasodilation.

It was interesting to note that in comparing responses to SNP between the genital arteries, parallels were seen between IVA and dorsal arteries, EVA and cavernous arteries. During further investigations it would be worthwhile to observe whether these parallels remain consistent. If this is the case, parallels in the treatment of male and female sexual dysfunction may be indicated.

Summary

In summary, both male and female genital arteries displayed concentration-dependent vasodilation in response to the NO donor, SNP, which was not inhibited by the PDE-5 inhibitor UK 343,664 (Table 4-6). Female genital vessels alone demonstrated significant inhibition of EFS-induced responses by L-NAME, suggesting a greater nitrgic innervation in female than male genital arteries. This is interesting since the use of PDE-5 inhibitors has been shown to be ineffective in the treatment of FSD but is widely used in the treatment of male ED. EFS-induced relaxation of female vaginal arteries demonstrated an L-NAME resistant component at high frequencies. Therefore, inhibitory innervation of male and female genital arteries may be due to a combination of nitrgic and an unknown neurotransmitter, potentially cholinergic, such as ACh, or peptidergic, for example VIP.

The lack of L-NAME-sensitive EFS-induced vasodilation in male arteries could be due to experimental conditions, which gave relatively poor and variable responses in controls. However, since good responses, which could be significantly inhibited by L-NAME were achieved in female arteries under the same conditions, this suggests that nitrgic relaxation of male genital arteries was poorer than would be expected from previous studies in corpus cavernosum strips. This may reflect a genuine difference in the relaxation of whole tissues and isolated arteries. If an alternative inhibitory neurotransmission is found to be of more importance than nitrgic neurotransmission in male penile arteries, this would have significant implications for the future treatment of ED.

Table 4-6: Summary of nitrenergic data in the genital arteries and the effect, increase (↑) or decrease (↓), of antagonists and blockers on vasodilation.

Agonist	Drugs	Cavernous	Dorsal	IVA	EVA
SNP (0.01 to 300 μM)	Control (E _{max} , % vasodilation)	82%	96%	98%	66%
	UK 343,664 (3 to 30 nM)	-	-	-	-
EFS-induced vasodilation	Control (% vasodilation at 64 Hz)	40%	45%	71%	70%
	L-NAME (100 μM)	-	-	↓*	↓*

(1 to 4 Hz) (2 to 64 Hz)

Significance vs controls, *: $P < 0.05$

References

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Chapter 5

Endothelium-Dependent Relaxation

Introduction

Endothelium-Dependent Relaxation

The advent of drugs such as PDE-5 inhibitors to treat male erectile dysfunction has driven research focusing on the vasodilator pathways of genital vasculature and tissues. Penile smooth muscle relaxation in response to increased parasympathetic activity is central to the erectile process and is under the control of both NANC neurons and the vascular endothelium (reviewed by Andersson & Wagner, 1995). Many studies have demonstrated the involvement of the neuronal NO pathway (reviewed by Burnett, 1997) but less is known about endothelium-dependent relaxation in genital tissues. Endothelial dysfunction has been associated with aging and diseases such as hypertension, diabetes and hypercholesterolemia (Martin-Morales *et al.*, 2001), all of which are also associated with a high prevalence of erectile dysfunction (Johannes *et al.*, 2000). Hence, endothelium-dependent vasodilation is an important area for consideration in the development of novel therapies for erectile dysfunction.

With scientific interest beginning to shift to the treatment of FSD it has become important to extend our knowledge of vasodilator pathways to include similarities and differences in tissue from the two sexes. Sildenafil citrate has proven to have very limited effect on general FSD (Kaplan *et al.*, 1999; Basson *et al.*, 2002) suggesting that pathways other than those involving NO may be of more significance in females. However, nothing is known of the mechanisms of endothelium-dependent vasodilation in female genital tissues.

In blood vessels in general, endothelium-dependent relaxation of smooth muscle, induced by agonists such as ACh, bradykinin or substance P, has been attributed to three endothelium-derived relaxing factors. Prostacyclin (PGI₂) is synthesised from arachidonic acid by the action of cyclooxygenases. NO is produced via activation of endothelial nitric oxide synthase (eNOS). The third and least well elucidated pathway involves the production of an unknown endothelium-derived hyperpolarising factor (EDHF). Which particular factor/s are released from endothelial cells varies with the agonist used, the vascular bed and the size of the vessel (Shimokawa *et al.*, 1996; Schrage *et al.*, 2005).

Prostaglandins

Along with contractile prostanoids such as $\text{PGF}_{2\alpha}$ and thromboxane A_2 , male erectile and female vaginal tissues have been demonstrated to synthesise and metabolise relaxatory prostanoids. These include prostaglandins E_1 , E_2 and I_2 (PGE_1 , PGE_2 and PGI_2) in males (Roy *et al.*, 1984, 1989; Jeremy *et al.*, 1986; Daley *et al.*, 1996a, 1996b) and PGE_1 and PGE_2 in females (Asboth *et al.*, 1985; Dubin *et al.*, 1985; Fichorova & Anderson, 1999).

Prostaglandins, prostacyclin and thromboxane are synthesised from arachidonic acid by the action of cyclooxygenases and are inactivated by prostaglandin-specific enzymes found in high concentrations in the lungs. The production of prostaglandins has been shown to be regulated by oxygen tension, which causes inhibition of cyclooxygenase activity. This may be an important factor in male penile tissues where oxygen tension varies with the erectile state (Daley *et al.*, 1996a). In human corpus cavernosum tissue synthesis, and release of PGI_2 has been demonstrated to occur in response to muscarinic receptor stimulation (Jeremy *et al.*, 1986).

Prostaglandins have been shown to have relaxation effects in both male and female erectile tissues. PGE_1 and PGE_2 caused relaxation of corpus cavernosum tissue, corpus spongiosum tissue, cavernous artery and retractor penis muscle in several species (Luducna & Grigas, 1972; Klinge & Sjostrand, 1974, 1977; Hedlund & Andersson, 1985). *In vivo* administration of PGE_1 was demonstrated to increase intracavernous pressure and erection in dog (Cahn *et al.*, 1996), human and monkey (Aboseif *et al.*, 1993). In human erectile tissue cyclooxygenase inhibitors were shown to decrease both spontaneous vasoconstrictions and basal tone (Christ *et al.*, 1990). In female vaginal tissues PGE_1 was demonstrated to decrease the contractility of human vaginal wall strips (Czekanowski, 1975) and *in vivo* administration of PGE_1 increased vaginal blood flow in dogs (Leffler & Amberson, 1982).

The receptors responsible for the actions of PGE and PGI are EP and IP receptors respectively. In erectile tissues four EP receptor subtypes have been demonstrated, EP_1 – EP_4 . The presence of EP receptors was demonstrated in male human and monkey corpus cavernosum tissue (Aboseif *et al.*, 1993). In addition, in female tissue, EP receptors have been demonstrated in human and rabbit cultured vaginal smooth muscle cells and hen vaginal tissue (Asboth *et al.*, 1985; Traish *et al.*, 1999). Stimulation of receptors by PGE leads to relaxation of smooth muscle via activation of AC and an increase in cAMP concentration in male and female tissues (Traish *et al.*, 1999; Uckert *et al.*, 2004).

Nitric Oxide

The main source of NO in male erectile tissues has been demonstrated to be via nitrenergic neurotransmission and the activation of nNOS. However, both constitutive isoforms of NOS, nNOS and eNOS, are present in penile erectile tissues. While most studies have demonstrated eNOS in vascular endothelium (Dail *et al.*, 1995; Bloch *et al.*, 1998; Hedlund *et al.*, 1999; Vanhatalo *et al.*, 2000), Bloch *et al.* (1998) also put forward evidence of eNOS in trabecular smooth muscle.

Female genital tissues have been shown to be innervated by NOS immunoreactive neurons (Grozdanovic *et al.*, 1994; Hoyle *et al.*, 1996) and both nNOS and eNOS are expressed in vaginal tissues. As with male penile tissues, eNOS has been demonstrated to be localised both in vaginal epithelium and smooth muscle cells (Chatterjee *et al.*, 1996; Uckert *et al.*, 2005). Activation of eNOS may occur via pathways involving cholinergic neurotransmission, activation of ET_B receptors or as a result of shear stress.

The significance of nNOS as the main catalyst for NO production in erectile tissue was thrown into doubt by the generation of homozygous nNOS-knockout mice which proved to be both viable and fertile (Huang *et al.*, 1993; Burnett *et al.*, 1996). These mice showed normal mating behaviour and erectile responses to nerve stimulation but also demonstrated a reduced fecundity. It is possible that these mice express a variant form of nNOS similar to penile nNOS (PnNOS) isoforms found in the rat which contain a 102 base pair insert (Magee *et al.*, 1996; Gonzalez-Cadavid *et al.*, 2000). Alternatively, maintained viability may be due to trans-splice variants, termed nNOS_α, nNOS_β and nNOS_γ, which produce shorter but functional proteins and persist in nNOS knockout mice (Eliasson *et al.*, 1997). A third explanation may be the upregulation of compensatory factors; for example Burnett *et al.* (1996) demonstrated that eNOS was significantly increased in penile vasculature and sinusoids of nNOS knockout mice compared to their wild type controls.

Therefore, while nNOS is thought to be the main source of NO in penile tissues, a significant role for NO production via eNOS may exist; particularly in the event of reduced nitrenergic neurotransmission. The downstream actions of NO, whether produced via cholinergic or shear stress activation of eNOS or via nitrenergic neurotransmission, are identical and lead ultimately to relaxation of both male and female genital tissues.

Endothelium-Derived Hyperpolarising Factor

There are many potential candidates for the identity of EDHF which all have studies to support or refute them (reviewed by Coleman *et al.*, 2004). In various studies EDHF has been suggested to be the potassium ion (K^+ , Edwards *et al.*, 1998), C-type natriuretic peptide (CNP, Chauhan *et al.*, 2003), hydrogen peroxide (H_2O_2 , Matoba *et al.*, 2000) or arachidonic acid derivatives including metabolites (e.g. the endogenous cannabinoid, arachidonylethanolamide (anadamide), Randall *et al.*, 1996) or products of cytochrome P450 epoxygenases (e.g. epoxyeicosatrienoic acid (EET), Azadzo *et al.*, 1992). It is therefore likely that more than one form of EDHF may exist and contribute to endothelium-dependent relaxation and the involvement of different factors may vary depending on the vascular bed and species studied.

The proposed mechanisms of action of EDHF are almost as varied as the candidates for its identity. An initial agonist-induced increase in intracellular endothelial calcium concentration is thought to lead to activation of small- and/or intermediate-conductance calcium-activated potassium channels (SK_{Ca} and IK_{Ca} respectively) on the endothelium. This results in hyperpolarisation of endothelial cells which spreads passively through myo-endothelial gap junctions to cause hyperpolarisation of smooth muscle cells and relaxation. Evidence for this pathway lies in the use of connexin mimetics to block gap junctions (Chaytor *et al.*, 1998; Mather *et al.*, 2005) and the association of the incidence of myo-endothelial electrical coupling with EDHF-mediated relaxation (Sandow & Hill, 2000). Alternatively, activation of K_{Ca} channels may lead to extrusion of potassium from the cell causing the extracellular concentration of potassium to increase. This in turn could activate inward rectifying potassium channels (K_{IR}) and/or sodium potassium ATPase (Na^+/K^+ ATPase) on the smooth muscle cells resulting in hyperpolarisation and relaxation. In many tissues complete abolition of EDHF-induced relaxation has been achieved using a combination of Apa (SK_{Ca} blocker) and ChTX (IK_{Ca} , K_V and large conductance calcium-activated potassium channel, BK_{Ca} blocker) but not with either inhibitor alone (Pettersson *et al.*, 1997; Doughty *et al.*, 1999; Angulo *et al.*, 2003b). Further studies have inhibited the EDHF pathway using barium (K_{IR} blocker) and/or ouabain (Na^+/K^+ ATPase blocker) (Edwards *et al.*, 1998; Scotland *et al.*, 2005).

A second hypothesis suggests that EET causes activation of BK_{Ca} on the smooth muscle leading to hyperpolarisation and relaxation. Blockers such as iberiotoxin (BK_{Ca} blocker) and tetraethylammonium (BK_{Ca} and some K_V channel blocker), however, have had limited success in inhibiting EDHF-induced relaxations (Pettersson *et al.*, 1997; Chaytor *et al.*,

1998; Archer *et al.*, 2003). It is likely that arachidonic acid metabolites such as EET affect EDHF-induced relaxation in a modulatory manner in certain tissues but are not involved in others (reviewed by Griffith, 2004).

Mechanisms of EDHF action may not be mutually exclusive and, as with the identity of EDHF itself, there may be multiple pathways/compounds which act simultaneously and potentially synergistically. The consensus of opinion is that while the precise mechanism has not yet been elucidated a hallmark of EDHF-induced relaxation is its abolition using a combination of Apa and ChTX. Therefore, in this study a combination of Apa and ChTX were used to block the action of EDHF and it is likely that this occurs via blockade of SK_{Ca} and IK_{Ca} channels respectively.

Aims

Due to a lack of research in this area, little is known about endothelium-dependent relaxation of isolated male and female genital arteries. The aims of this chapter were as follows.

- To investigate the contribution of prostaglandins, NO and EDHF to endothelium-dependent relaxation of male and female rabbit genital arteries.
- To compare the mechanisms of endothelium-dependent relaxation in male and female rabbit genital arteries.

Results

Endothelium

Endothelial Antibody

The primary antibody used to identify endothelial cells was a goat polyclonal antibody raised against mouse platelet/endothelial cell adhesion molecule-1 (PECAM). PECAM is a glycoprotein expressed on the cell surfaces of monocytes, neutrophils, platelets and some T cells that is involved in cell-cell interactions during growth. This antibody would be expected to bind to endothelial cells present in the tissues studied. In negative controls, the primary antibody was omitted and replaced with PBS since normal goat IgG was not available.

The secondary antibody used was a donkey anti-goat antibody conjugated to biotin. Vectastain[®] ABC Kit, mouse (Vector Laboratories, Inc) and Liquid DAB + Substrate Chromogen System (Vector Laboratories, Inc) were used to label positive structures with horseradish peroxidase (HRP) followed by peroxidase substrate (DAB). Using this system, negative structures were stained blue with a haematoxylin nuclear counterstain, while positive structures stained brown and were visualised under a light microscope.

Vaginal sections prepared for immunohistochemical analysis contained the entire vaginal vestibule and surrounding tissues. On staining these sections the vaginal lumen could be identified as a large space in the centre of the section surrounded by a convoluted wall. Vaginal tissue beyond the vaginal wall contained numerous blood vessels, nerve ganglia and blood filled cavities identified as sinuses (Figure 5-1).

In female vaginal tissues endothelial cells of both arteries and arterioles within the vaginal tissue stained positive using PECAM (Figures 5-1 and 5-2). In addition, positive staining in vaginal sinuses demonstrated a layer of non-vascular endothelium. The wall of the vaginal lumen demonstrated diffuse staining that may be attributable to non-specific staining. Nerve ganglia did not demonstrate positive staining using PECAM (Figure 5-2). Positive structures demonstrated using the endothelial antibody, PECAM, did not match any staining obtained using PGP 9.5, a pan-neuronal antibody.

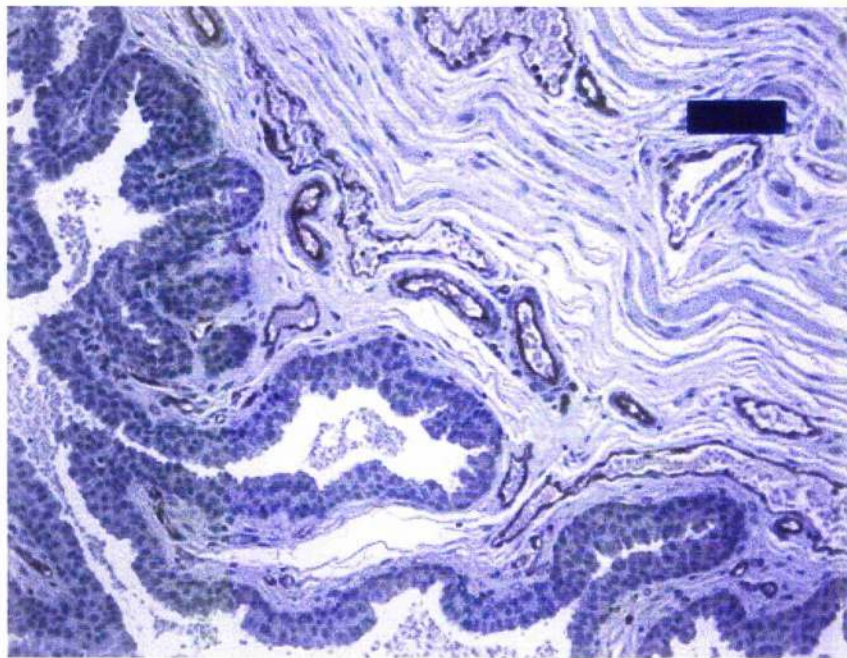
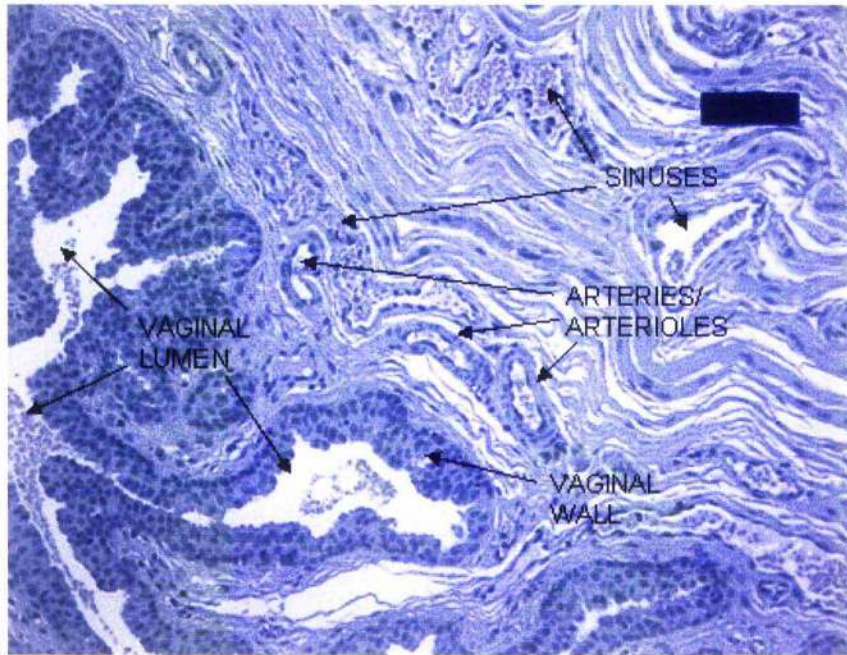


Figure 5-1: PECAM (1:100 dilution) staining in female vaginal tissue. A: Control, B: Brown staining positive for PECAM. Scale bar = 100 μ m.

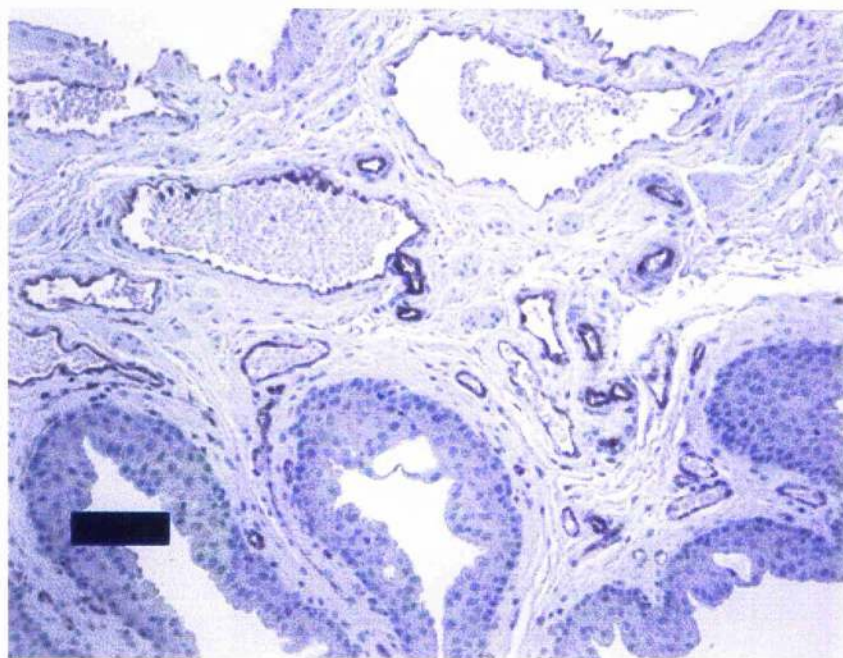
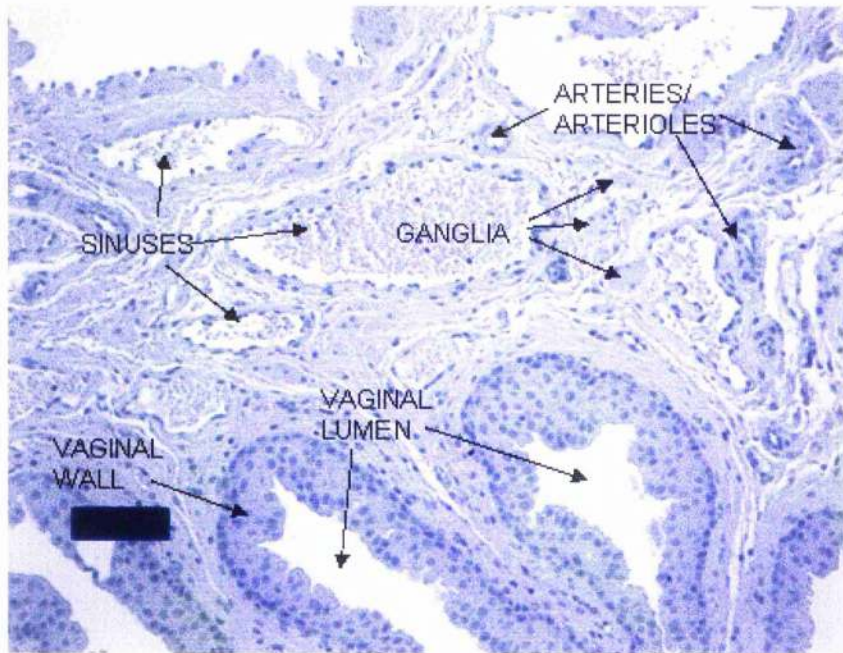


Figure 5-2: PECAM (1:100 dilution) staining in female vaginal tissue. A: Control, B: Brown staining positive for PECAM. Scale bar = 100 μ m.

Endothelium-Dependent Vasodilation

Noradrenaline-Induced Vasoconstriction

NA (10 μ M) was used to induce tone in all preparations as described previously in Chapter 4 – Nitroergic Mechanisms. Sufficient time was allowed for tone to reach a stable plateau before experimental protocols were begun. Sustained tone was measured and vasodilation responses taken as a decrease from this level of tone.

Incubation with a combination of K⁺ channel blockers, Apa (100 nM) plus ChTX (100 nM), and NOS inhibitor, L-NAME (100 μ M), significantly increased the magnitude of NA-induced vasoconstrictions in the dorsal artery ($P < 0.001$), EVA and IVA ($P < 0.01$).

Acetylcholine-Induced Vasodilation

From a stable NA-induced tone, ACh (1 nM to 30 μ M) caused reproducible concentration-dependent vasodilation in all four arteries (Figure 5-3, Table 5-1). ACh responses were maximal at 10 to 30 μ M and were greatest in IVA = dorsal artery > EVA > cavernous artery. The magnitude of ACh vasodilations were IVA: $98.2 \pm 1.0\%$ (n(N) = 15(30)), dorsal artery: $96.2 \pm 1.3\%$ (n(N) = 13(34)), EVA: $75.8 \pm 4.4\%$ (n(N) = 16(30)) and cavernous artery: $71.0 \pm 3.4\%$ (n(N) = 11(29)). ACh potency was not significantly different in IVA and dorsal arteries but there were significant differences in potency between all other arteries ($P < 0.05$). The rank order of agonist potency for ACh responses was; dorsal artery = IVA > cavernous artery > EVA.

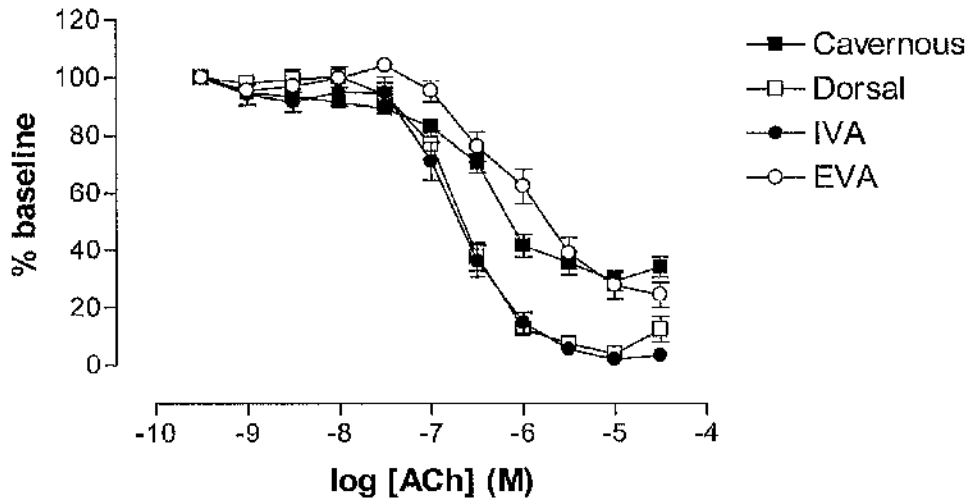


Figure 5-3: ACh-induced vasodilation in the genital arteries expressed as a percentage of NA (10 μ M) induced baseline tone \pm s.e.mean.

Table 5-1: Comparison of ACh-induced vasodilation between vessels.

	n (N)	% Maximum Relaxation (s.e.mean)	-Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
Cavernous	11 (29)	70.96 (3.40)	1.32 (0.95 to 1.69)	6.42 (0.05)
Dorsal	13 (34)	96.23 (1.28)	1.63 (1.24 to 2.03)	6.70 (0.04)
IVA	15 (30)	98.17 (1.02)	1.42 (1.03 to 1.81)	6.68 (0.05)
EVA	16 (30)	75.78 (4.39)	1.06 (0.69 to 1.44)	6.03 (0.09)

CI: confidence interval

Prostaglandins

In both male and female genital arterics NA-induced vasoconstriction and ACh-induced vasodilation were unaffected by incubation with the cyclooxygenase inhibitor indomethacin (1 μ M, $P > 0.05$, Figure 5-4, Table 5-2). Nevertheless, indomethacin was included in all experiments, alongside incubation with inhibitors and/or blockers, as part of a standard protocol to exclude the involvement of prostacyclin in ACh responses.

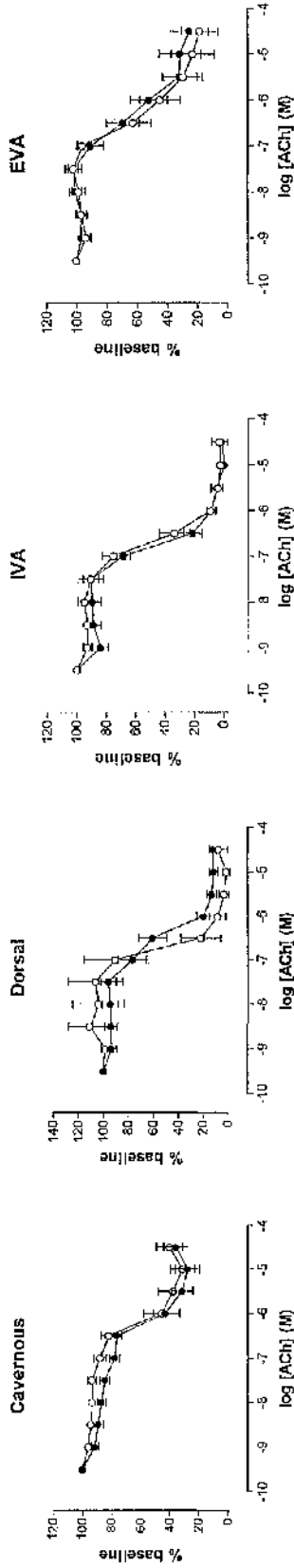


Figure 5-4: ACh-induced vasodilation in the genital arteries in the absence (●) or presence (○) of indomethacin (1 μM) expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 5-2: ACh-induced vasodilation in the genital arteries in the absence or presence of indomethacin (1 μM).

	n (N)	% Maximum Relaxation (s.e.mean)	-Hill Slope (95% Confidence Interval)	pEC ₅₀ (s.e.mean)
Cavernous	Control	72.22 (8.27)	1.72 (0.62 to 2.82)	6.29 (0.10)
	Indomethacin	4 (4)	68.47 (8.27)	2.33 (0.52 to 4.15)
Dorsal	Control	88.52 (3.19)	1.47 (0.72 to 2.23)	6.47 (0.09)
	Indomethacin	4 (4)	98.60 (3.24)	2.99 (0.24 to 5.73)
IVA	Control	100.47 (1.83)	2.05 (1.28 to 2.82)	6.78 (0.05)
	Indomethacin	5 (5)	98.40 (3.16)	1.87 (1.23 to 2.51)
EVA	Control	74.61 (12.72)	1.18 (0.23 to 2.13)	6.31 (0.17)
	Indomethacin	6 (6)	81.64 (12.24)	1.34 (0.32 to 2.36)

Nitric Oxide

In all four preparations incubation with L-NAME (100 μM) exposed a NOS-independent component of ACh-induced vasodilation (Figure 5-5, Table 5-3). The maximum size of this potentially EDHF-induced vasodilation was not significantly different amongst the four vessels, approximately 25% to 60% relaxation. However, the change from the control curve varied; particularly in the EVA at lower concentrations of ACh, below vessel pEC_{50} , where incubation with L-NAME had less effect on ACh-induced vasodilation.

Incubation of vessels with a combination of NOS inhibitor (L-NAME, 100 μM) and K^+ channel blockers (Apa, 100 nM and ChTX, 100 nM) greatly reduced ACh-induced vasodilation in all preparations ($P < 0.01$, Figure 5-5, Table 5-3). Maximal relaxations were decreased to approximately 4.9% to 9.5% demonstrating that the NOS-independent component of ACh-induced vasodilation was largely removed by additional incubation with K^+ channel blockers.

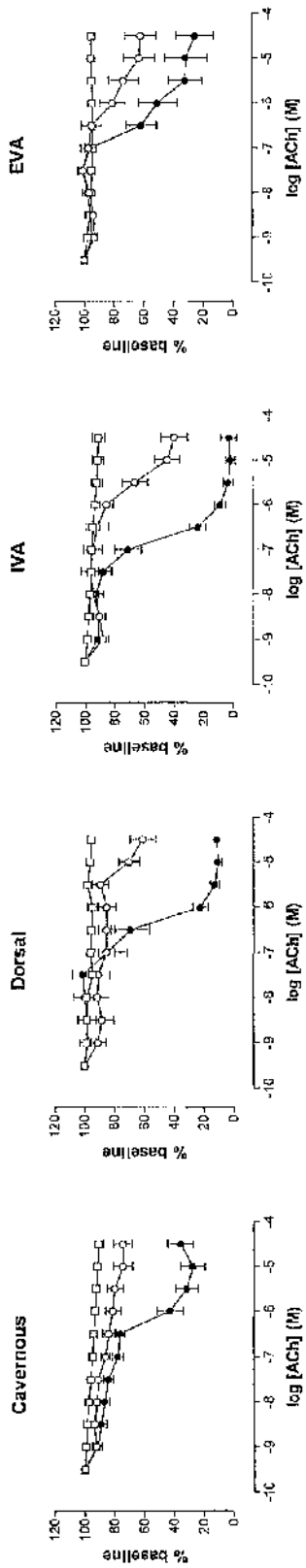


Figure 5-5: ACh-induced vasodilation in the genital arteries in the absence (●) or presence of 100 μM L-NAME (○), or a combination of 100 μM L-NAME plus K⁺ channel blockers Apa and ChTX, 100 nM (□) expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 5-3: ACh-induced vasodilation in the genital arteries in the absence or presence of L-NAME (100 μM), or a combination of L-NAME (100 μM) plus K⁺ channel blockers Apa and ChTX (100 nM).

	% Maximum Relaxation (s.e.mean)					
	n (N)	Control	n (N)	L-NAME	n (N)	L-NAME + Apa + ChTX
Cavernous	7 (7)	72.22 (8.27)	9 (9)	25.48 (6.23)	4 (4)	9.38 (2.68)
Dorsal	7 (7)	89.53 (2.88)	7 (7)	39.03 (8.68)	4 (4)	4.71 (2.80)
IVA	5 (5)	100.47 (1.83)	6 (7)	60.43 (9.06)	6 (8)	9.26 (4.15)
EVA	5 (7)	74.61 (12.72)	11 (14)	37.80 (10.59)	6 (6)	4.75 (1.81)

Endothelium-Derived Hyperpolarising Factor

To investigate EDHF involvement further, K⁺ channel blockers Apa (100 nM) and ChTX (100 nM) were tested alone against ACh-induced vasodilation.

ACh-induced vasodilation of cavernous arteries was partially inhibited by K⁺ channel blockers (Figure 5-6, Table 5-4). In these arteries the effects of NOS inhibitor and K⁺ channel blockers appear to be additive suggesting proportionate contributions of approximately NO, 67% and EDHF, 33 %.

In the dorsal arteries ACh-induced vasodilation was unaffected by K⁺ channel blockers: note that in these arteries, L-NAME resistant vasodilation occurred at only the highest two concentrations of ACh used, 10 and 30 μ M (Figure 5-6, Table 5-4). EDHF, therefore, had a very minimal role in the vasodilation of dorsal arteries while production of NO was the predominant mechanism of ACh-induced vasodilation.

Maximal vasodilation to 10 and 30 μ M ACh in IVA preparations was unaffected by K⁺ channel inhibition (Figure 5-6, Table 5-4). However, at lower concentrations of ACh, Apa and ChTX did significantly inhibit ACh-induced vasodilation ($P < 0.05$) in the IVA in contrast to dorsal arteries. Hence both EDHF and NO can be implicated in responses of the IVA to ACh.

In the EVA, vasodilation was greatly reduced by K⁺ channel blockers at all concentrations of ACh ($P < 0.01$, Figure 5-6, Table 5-4). Uniquely, in this vessel, inhibition by K⁺ channel blockers was greater than that by L-NAME suggesting that EDHF was the predominant factor involved in ACh-induced vasodilation of this vessel.

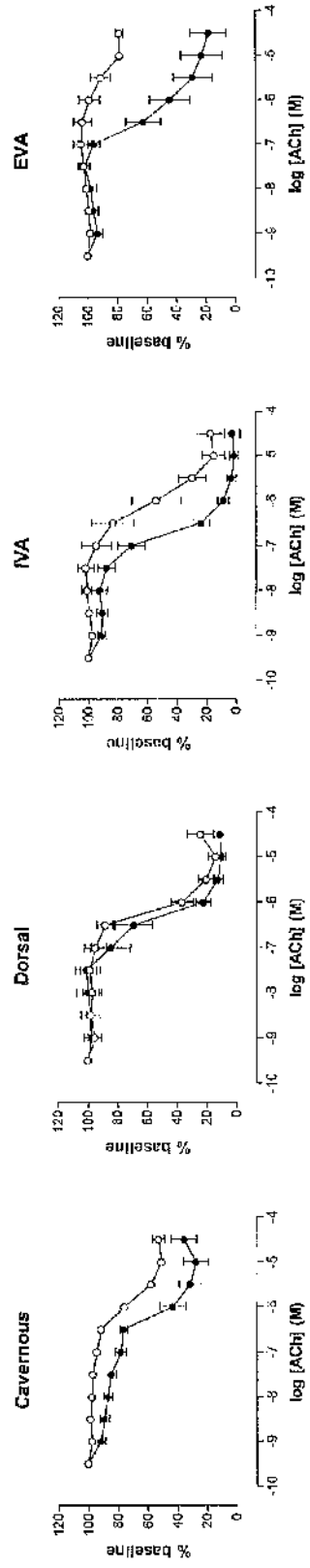


Figure 5-6: ACh-induced responses in the genital arteries in the absence (●) or presence (○) of K⁺ channel blockers Apa (100 nM) plus ChTX (100 nM) expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 5-4: ACh-induced responses in the genital arteries in the absence or presence of K⁺ channel blockers Apa (100 nM) plus ChTX (100 nM).

	% Maximum Relaxation (s.e.mean)		
	n (N)	Control	n (N) Apa + ChTX
Cavernous	7 (7)	72.22 (8.27)	4 (4) 49.02 (1.81)
Dorsal	7 (7)	89.53 (2.88)	5 (6) 85.73 (4.62)
IVA	5 (5)	100.47 (1.83)	6 (6) 84.59 (7.78)
EVA	5 (7)	74.61 (12.72)	6 (6) 21.38 (2.59)

Discussion

Endothelium

In male erectile tissues the three main events leading to erection are increased arterial inflow, relaxation of trabecular tissue and decreased venous outflow. The first two of these events require relaxation of both vascular and trabecular smooth muscle. Trabecular sinuses in the corpus cavernosum are lined by endothelium and, in addition to the vascular endothelium, provide a significant source of factors important in the relaxation of genital tissues. Immunohistochemical analysis of female vaginal tissues demonstrated that, similar to male erectile tissues, sinuses were present throughout this tissue. In addition, sinuses in female vaginal tissues were lined by an endothelial layer which may have an important role in relaxation. The vascular endothelium of arteries and arterioles in female genital tissues was also demonstrated.

Staining of endothelial cells by PECAM was not found in the same regions as previous staining with the pan-neuronal antibody, PGP 9.5. This demonstrates that neuronal innervation does not penetrate through to endothelial layers of arteries and arterioles. Indeed, PGP 9.5 staining demonstrated a plexus of neurons on the outer edge of vascular smooth muscle. This is an important consideration when investigating endothelial-dependent relaxation mechanisms. The agonist used in this study to investigate endothelial-dependent relaxation was ACh. Physiologically, the source of ACh may be either via release from parasympathetic cholinergic neurons acting on smooth muscle cells or circulating ACh acting on endothelial cells. However, ACh can cause vasoconstriction when it binds directly to cholinergic receptors on arterial smooth muscle and the presence of acetylcholinesterases leads to rapid breakdown of ACh in the blood. Therefore, the initial stimulus for endothelium-dependent relaxation is uncertain but may include many factors such as circulating factors in the blood, the effect of shear stress or the coordination of stimuli via myo-endothelial gap junctions. In both males and females, vascular and non-vascular (trabecular sinus) endothelial cells may have an important role in the relaxation of genital tissues; allowing increased arterial inflow during sexual arousal.

Endothelium-Dependent Relaxation

This study is the first comparison of the balance of NO and EDHF contributes to endothelium-dependent relaxation of isolated male and female genital arteries.

The lack of any effect of indomethacin on ACh-induced relaxation in male and female genital arteries demonstrated no involvement of prostacyclin. The inclusion of indomethacin in all experiments ensured no further prostacyclin involvement.

Inhibition of NOS demonstrated that an L-NAME-resistant vasodilation was present in all genital arteries studied. What was most striking, however, was the much greater involvement of EDHF in the female upstream artery, the EVA. This follows the pattern of sex differences found in rat and mouse mesenteric beds (McCulloch & Randall, 1998; Scotland *et al.*, 2005) and rat tail artery (Pak *et al.*, 2002) where EDHF was found to contribute relatively more in females. In the present study, NO was clearly involved in all four arteries, as shown by the effect of L-NAME on its own or with Apa and ChTX. The striking difference amongst the arteries was the effect of Apa and ChTX, which had a very clear effect on the female inflow artery (EVA), a very small effect on the male dorsal artery and an intermediate effect on the smaller arteries from both sexes.

There is a known pattern of greater EDHF involvement in smaller, downstream arteries (Miura *et al.*, 1999; Coats *et al.*, 2001; Angulo *et al.*, 2003a) and this corresponds to the present finding with the male arteries: there was a greater involvement of EDHF in ACh-induced relaxation of cavernous arteries compared with dorsal arteries. Interestingly, this was not the case with the female vaginal arteries; in which the upstream example, EVA, clearly had a greater EDHF involvement than the downstream IVA. Since vasodilation of inflow arteries is crucial to the onset of sexual arousal this is a significant point.

The clear role for NO in endothelium-mediated vasodilation in genital tissue arteries from both sexes suggests that it is not necessarily a relatively greater involvement of NO in the male that explains the success of sildenafil in the treatment of sexual dysfunction in males but not in females. However, involvement of EDHF in vasodilation in both sexes and a relatively greater role in the female may point to an alternative approach to sexual dysfunction in both sexes, but particularly in the female. Faulty EDHF mechanisms could lead to sexual dysfunction and, if this was the source of the problem in the female then enhancing the NO signalling pathway by blocking PDE-5 would not necessarily be effective. This could also be a factor in male subjects who are not responsive to sildenafil. Evidence is emerging concerning the failure of the EDHF system in general. The effectiveness of EDHF has been shown to decline with increasing age (Sandow, 2004) and in models of hypertension (Fujii *et al.*, 1992; Vanhoutte, 1996).

The current study showed that in male penile arteries there was a greater involvement of EDHF in ACh-induced relaxation of cavernous arteries compared with dorsal arteries. In dorsal arteries vasodilation could be almost entirely attributed to NO since no inhibition of relaxation was demonstrated using K⁺ channel blockers, with or without L-NAME. This demonstrated that NO was more dominant in larger calibre male genital arteries. Similarly, relaxations of human and horse penile helicine arteries, branches of the cavernous artery, have been shown to be mediated by a combination of NO and EDHF (Prieto *et al.*, 1998; Angulo *et al.*, 2003a) while that of bovine dorsal arteries was mediated largely by NO (Liu *et al.*, 1991). Interestingly, while isolated cavernous arteries appear to utilise both NO and EDHF (this study and Angulo *et al.*, 2003a), relaxation of human and rabbit corpus cavernosum tissue was shown to be almost completely mediated by NO (Azadzoi *et al.*, 1992; Angulo *et al.*, 2003a); demonstrating the diverging results that can be obtained by looking at whole tissue or isolated vessel preparations.

The inversion of the concept of NO upstream, EDHF downstream, in the female is, as far as we know unprecedented. However, it does not appear to have been studied, so may reflect a general trend in other female vasculature.

Summary

In summary, male and female genital arteries demonstrated significant gender differences in endothelium-dependent relaxation (Table 5-5). In the female inflow artery, EVA, EDHF predominated but both NO and EDHF contributed to overall function. In the male dorsal artery, NO mechanisms predominated and the involvement of EDHF appeared to be minimal. In the smaller genital arterics, the cavernous artery in males and IVA in females, both NO and EDHF were shown to contribute to relaxation while NO was proportionately more dominant than EDHF. The hypothesis proposed by other studies of increased EDHF involvement with decreasing vessel size was found to be consistent for data in male arteries but was reversed in female arteries where EDHF contribution was greater in the larger EVA. Gender differences and the relative contributions of NO and EDHF to the relaxation of male and female genital resistance arteries described in this study provide an insight into the mechanisms of sexual function in both males and females, which could be used to direct future investigations into the aetiology and hence the successful treatment of sexual dysfunction. While therapies for male sexual dysfunction have been directed towards NO pathways with the successes of drugs such as the PDE-5 inhibitors Viagra[®], Cialis[®] and Levitra[®], this study shows that a method of enhancing EDHF-induced relaxation could be more beneficial in the treatment of FSD. Since EDHF has also been

demonstrated to be involved in the relaxation of cavernous arteries a combination of treatments targeting both the NO and EDHF pathways could potentially have a greater effect on ED in males. In accordance with this hypothesis, Vick *et al.* (2002) demonstrated that a K⁺ channel opener had potential as a treatment for ED. Therefore, an additional area of study would be to fully elucidate the physiological mechanisms and pathology of EDHF-induced relaxation and hence the possible targets for drug therapies.

Table 5-5: Summary of endothelium-dependent relaxation data in the genital arteries and the effect, increase (↑), decrease (↓) or no change (-), of antagonists and blockers on responses.

Activity	Drugs	Cavernous	Dorsal	IVA	EVA
NA (10 μM)	Control (E _{max})	0.9 g	2.0 g	1.8 g	2.9 g
ACh (0.001 to 30 μM)	Control (E _{max} , % vasodilation)	71%	96%	98%	76%
	Indomethacin (1 μM)	-	-	-	-
	L-NAME (100 μM)	↓*	↓**	↓*	-
	K ⁺ channel blockers (Apa, 100 nM and ChTX, 100 nM)	-	-	↓*	↓**
	L-NAME plus K ⁺ channel blockers	↓**	↓***	↓***	↓*

Significance vs controls, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$

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Chapter 6

Peptidergic and Purinergic Mechanisms

Introduction

In this chapter investigations are made into some alternative candidates for sympathetic and parasympathetic neurotransmitters in male and female genital tissues. While adrenergic and NO mechanisms are often considered as the main excitatory and inhibitory pathways respectively, results presented in previous chapters have shown that this may not always be the case in the genital arteries being studied. The present chapter will review the role of peptides or purines as putative neurotransmitters within the genital arteries.

Adenosine 5'-Triphosphate

ATP is abundant in almost all tissues in the body but was not initially considered by many as a potential neurotransmitter (Williams, 1987). ATP release from both nerves and endothelium has since been shown to be involved in the control of vascular tone. ATP may act either via a direct action of ATP on P_{2X} or P_{2Y} receptors, or through the breakdown of ATP to adenosine, which acts via A₁, A_{2A}, A_{2B} and A₃ receptors (reviewed by Burnstock, 2002; Boeynaems *et al.*, 2005). There are currently seven known subtypes of the P_{2X} receptor and eight of the P_{2Y} receptor.

Vasoconstriction

The receptor thought to be responsible for the neurotransmitter actions of ATP is the P_{2X} purinoceptor. This is a ligand-gated ion channel whose actions include smooth muscle contraction, synaptic responses, bone formation, pain and visceral perception (Rang *et al.*, 2002; Vial *et al.*, 2004; Boeynaems *et al.*, 2005).

The presence of P_{2X} receptors has been demonstrated in both male and female rat genital tissues (Bardini *et al.*, 2000; Lee *et al.*, 2000). However, very few studies have reported a contractile role for ATP in the genital tissues. In males, ATP and adenosine have been shown to cause contraction of rabbit cavernosal smooth muscle *in vitro* (Wu *et al.*, 1993), while *in vivo* adenosine decreased erectile responses in rats (Sharifzadeh *et al.*, 1995). In females, no studies have shown a direct vasoconstrictor effect of either ATP or adenosine.

Vasodilation

In addition to P_{2X} receptors, mRNA for the P_{2Y} receptor has been demonstrated in both male and female genital tissues (Obara *et al.*, 1998; Min *et al.*, 2003). The P_{2Y} purinoceptor

is a G-protein coupled receptor linked to either AC or hydrolysis of phosphoinositide (Rang *et al.*, 2002). ATP was first shown to cause vasodilation of male dog penile arteries by Bowman & Gillespie (1983). Since this time, ATP, adenosine and the P_{2Y} agonist adenosine 5'-O-(2-thiodiphosphate) (ADPβS) have been demonstrated to cause relaxation of pre-contracted genital tissues *in vitro*. Adenosine- and ATP-induced relaxations were antagonised by the A_{2A} receptor antagonists CGS21680 and ZM-241385 (Mantelli *et al.*, 1995; Filippi *et al.*, 1999; Noto *et al.*, 2001) but the P₂ antagonists suramin and reactive blue-2 failed to inhibit responses to ATP (Ragazzi *et al.*, 1996; Filippi *et al.*, 1999; Noto *et al.*, 2001). In contrast, the action of purines in human corpus cavernosum has been suggested to be via activation of endothelial P_{2Y} receptors leading to NO production and subsequent relaxation (Shalev *et al.*, 1999). In support of this hypothesis, Chiang *et al.* (1994) demonstrated that adenosine-induced relaxation of rabbit genital tissues was decreased by endothelium removal. In addition, demonstration of P_{2Y} receptor mRNA in male tissues by Obara *et al.* (1998) found it to be localised in the endothelium of cavernous sinuses and blood vessels but *not* smooth muscle. However, many studies have shown that adenosine- and ATP-induced relaxation in rabbit, dog and human genital tissues is both endothelium- (Tong *et al.*, 1992) and NO-independent (Levin *et al.*, 1994; Mantelli *et al.*, 1995; Ragazzi *et al.*, 1996; Kaya *et al.*, 1998; Filippi *et al.*, 1999; Noto *et al.*, 2001). *In vivo*, ATP and adenosine were shown to increase intracavernous pressure and cause erection in dogs (Takahashi *et al.*, 1992; Noto *et al.*, 2001).

On the basis of these experiments it was postulated that ATP is a NANC inhibitory neurotransmitter in corpus cavernosum tissue, indicating a purinergic involvement in penile erection. This view has since been questioned due to the inability of ATP or other purines to affect EFS responses in dogs or rabbits or penile tumescence in humans (Wu *et al.*, 1993; Filippi *et al.*, 2000; Noto *et al.*, 2001). Current opinion suggests that the role of ATP may be as a modulator of NANC and/or adrenergic neurotransmission.

A similar conclusion was reached by Ziessen & Cellek (2002) when investigating the involvement of purines in the relaxation of female genital tissues. In their study ATP, adenosine and various other purines were shown to relax rabbit vaginal tissue. Similar to results in male penile tissues, ATP-induced relaxation was not inhibited by a P₂ antagonist, suramin, while adenosine-induced relaxation was blocked by an A_{2A} antagonist, ZM-241385. The authors concluded that while purinoceptors were present in the vaginal tissue, purines are not involved in EFS-induced relaxation.

Vasoactive Intestinal Polypeptide

The limited success of sildenafil in the treatment of FSD (Basson *et al.*, 2002) has led to the suggestion that, in contrast to male genital tissues, NO may not be the main inhibitory neurotransmitter in female genital tissues. Many peptides have been demonstrated to be present in nerves innervating vaginal tissues including VIP, pituitary adenylate cyclase activating peptide (PACAP), peptide histidine methionine (PHM), peptide histidine valine (PHV), calcitonin gene-related peptide (CGRP), helospectin-I and -II and substance P (Palle *et al.*, 1989; Majewski *et al.*, 1995; Hoyle *et al.*, 1996). However, the endogenous release of peptides as neurotransmitters in vaginal tissues has yet to be proven.

VIP has been detected in nerves innervating both vascular and non-vascular smooth muscle in human (Lynch *et al.*, 1980; Palle *et al.*, 1989; Uckert *et al.*, 2005), bovine, porcine (Majewski *et al.*, 1995) and avian (Costagliola *et al.*, 1997) vaginal tissues. Neurogenic VIP was described as abundant and co-localised with other inhibitory neurotransmitters, such as NO (Majewski *et al.*, 1995; Costagliola *et al.*, 1997).

The functional significance of VIP in genital tissues has also been demonstrated. *In vitro*, VIP inhibited vaginal smooth muscle activity (Palle *et al.*, 1989; Bredkjoer *et al.*, 1997), while EFS-induced relaxation of rabbit vaginal tissue was partially inhibited by d-VIP, a VIP receptor antagonist (Oh *et al.*, 2003). *In vivo*, VIP increased vaginal blood flow, which is a physiological indicator of sexual arousal (Ottesen *et al.*, 1983; Palle *et al.*, 1990).

VIP causes relaxation of smooth muscle via activation of G_s-coupled VIP receptors, VPAC₁ and VPAC₂, leading to activation of AC (Fahrenkrug, 1993). In turn, this results in an elevation of cAMP levels and consequent activation of cAMP-dependent protein kinase (PKA); lowering the cytosolic Ca²⁺ concentration (McDonald *et al.*, 1998). In female rabbit genital tissues, VIP caused a rise in cAMP concentration (Ziessen *et al.*, 2002), while in human vaginal tissue the cAMP specific phosphodiesterase, PDE-4, was found to be co-localised with VIP-IR neurons (Uckert *et al.*, 2005).

Aims

Female genital tissues are well innervated by VIP-immunoreactive neurons and it has been shown that VIP inhibitory neurotransmission causes relaxation of vaginal smooth muscle. However, little is known about the pharmacology of VIP responses in the vaginal tissue and no studies have determined the effect of VIP on isolated vaginal arteries.

In addition, a role for purinergic neurotransmission in the function of genital tissues from both sexes remains unclear. While the effects of purines on male penile tissues have been investigated in more depth our current knowledge of purine pharmacology in female genital tissues is provided by a single study.

The aims of this chapter were as follows.

- To verify the presence of VIP- immunoreactive neurons in female rabbit vaginal tissues.
- To characterise responses to exogenous VIP and ATP in female rabbit vaginal arteries.
- To determine the involvement of peptides as neurotransmitters in rabbit genital arteries from both sexes.

Results

Peptidergic Innervation

Vasoactive Intestinal Polypeptide Antibody

The primary antibody used to identify VIP neurons in female rabbit vaginal tissues was a sheep polyclonal antibody specific for VIP. Primary antibody was used at a dilution of 1:2500, which was within the manufacturers' recommended dilution range of 1:2500 to 1:5000. The secondary antibody used was a donkey anti-sheep antibody conjugated to FITC at a concentration of 1:50, again within the manufacturers' guidelines of 1:50 to 1:200. Negative controls consisted of slides where primary antibody was omitted and replaced with PBS since normal sheep IgG was not available. These slides were incubated with secondary antibody as for experimental slides. Using these antibodies, positive structures were stained fluorescent green and were visualised under a fluorescence microscope using a HQ525/40nm (chroma technology) filter.

Slides stained using the VIP primary antibody demonstrated fluorescence in an area surrounding arteries that was similar to the plexus of nerves visualised using PGP 9.5 (Figure 6-1). However, no ganglia or veins within the genital tissues were observed as staining positive for VIP. A large amount of non-specific staining was observed in connective tissues and in the endothelial layer of arteries but not of veins.

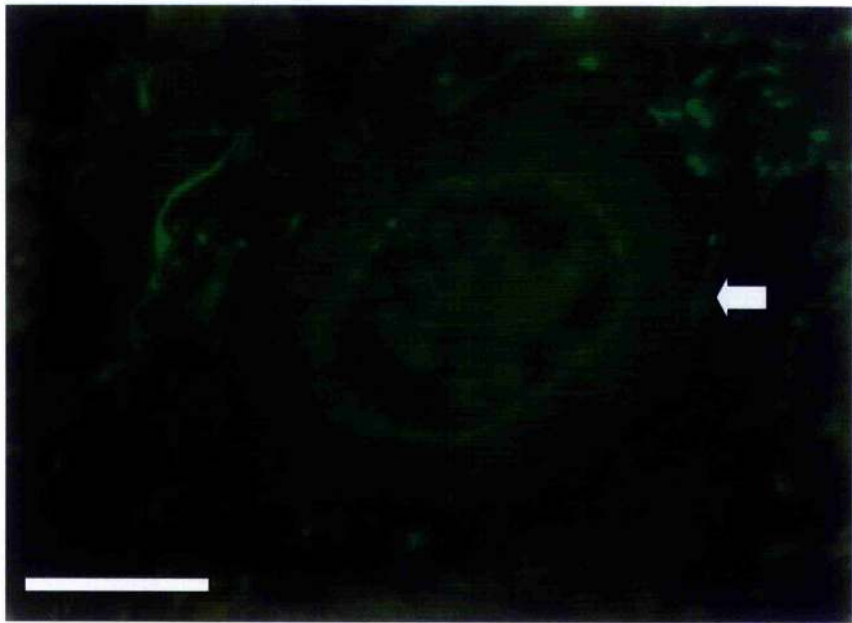
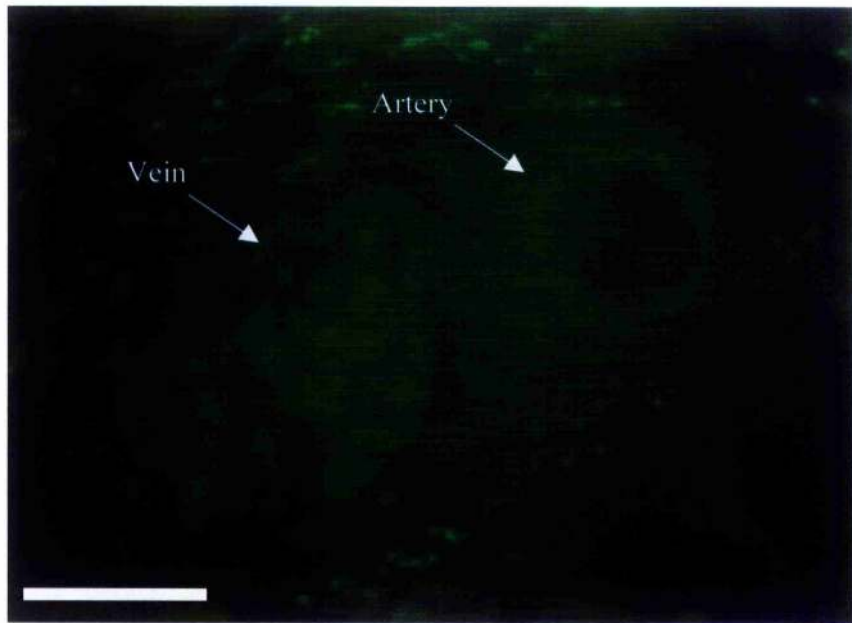


Figure 6-1: VIP (1:2500 dilution) staining the female vaginal tissue. A: Control, B: Green fluorescent staining (FITC 1:50) positive for VIP indicated by arrow. Scale bar = 50 μm .

Purines and Vasoconstriction

Adenosine 5'-Triphosphate

ATP induced concentration-dependent vasoconstriction in both female arterial preparations, as illustrated by the responses of two IVA and two EVA vessels (Figure 6-2). Responses amounted to $46.8 \pm 1.4\%$ ($n(N) = 5(10)$) and $17.2 \pm 11.5\%$ ($n(N) = 5(12)$) of NA ($10 \mu\text{M}$) sighting responses in the IVA and EVA respectively (Figure 6-3, Table 6-1). ATP responses demonstrated Hill slopes that were not significantly different from unity in either vaginal artery.

Alpha, Beta, Methylene Adenosine 5'-Triphosphate

In both female artery preparations, α, β , methylene adenosine 5'-triphosphate ($\alpha, \beta, \text{mATP}$) a selective P_{2X} purinoceptor agonist, induced vasoconstriction. The characteristics of these contractions are demonstrated by the responses of two IVA and two EVA vessels (Figure 6-4). No contraction was observed in response to $\alpha, \beta, \text{mATP}$ concentrations of $0.01 \mu\text{M}$ or lower. At $0.1 \mu\text{M}$, $\alpha, \beta, \text{mATP}$ induced a small, non-maximal response. Upon further stimulation with $1 \mu\text{M}$ $\alpha, \beta, \text{mATP}$ a large, maximal vasoconstriction was observed in both vaginal artery preparations. This was followed by desensitisation of ATP receptors leading to a small residual response at $10 \mu\text{M}$ and complete abolition of $\alpha, \beta, \text{mATP}$ -induced contraction at $100 \mu\text{M}$.

Analysis of mean data (Figure 6-5, Table 6-2) demonstrated that, in common with ATP-induced responses, vasoconstrictions to $\alpha, \beta, \text{mATP}$ were greater in IVA, $74.7 \pm 4.7\%$ ($n(N) = 2(4)$) NA ($10 \mu\text{M}$) sighting, than EVA, $33.0 \pm 12.0\%$ ($n(N) = 1(2)$) NA ($10 \mu\text{M}$) sighting, ($P < 0.05$). In IVA but not EVA, maximal responses to $\alpha, \beta, \text{mATP}$ were significantly greater than responses to ATP ($P < 0.05$).

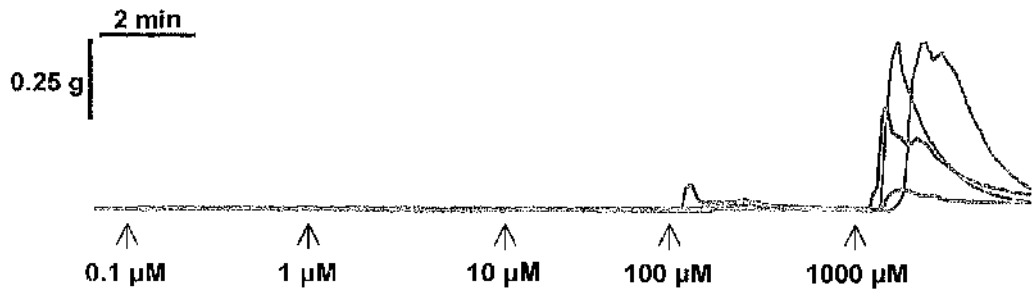


Figure 6-2: Raw traces of responses to ATP (0.1 to 1000 μM , arrows) in the female genital arteries. IVA: pink and green lines. EVA: blue and red lines.

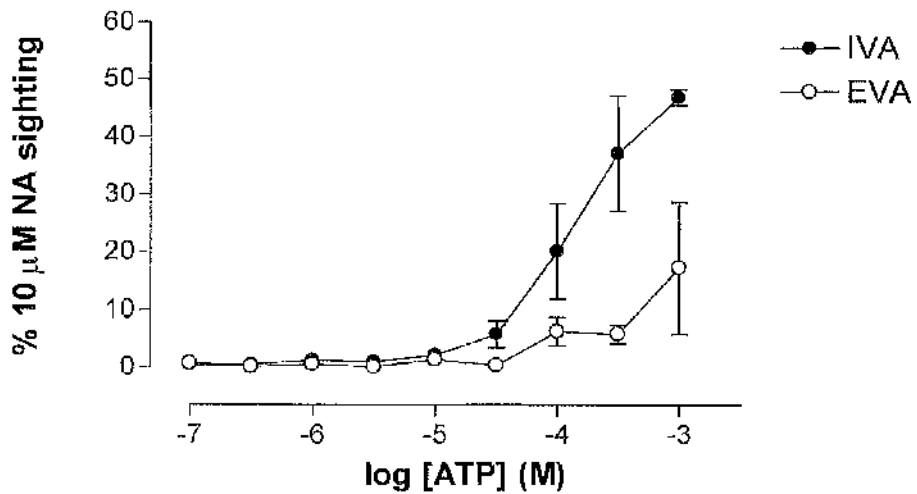


Figure 6-3: ATP-induced vasoconstriction in the female genital arteries expressed as a percentage of the NA ($10 \mu\text{M}$) sighting response \pm s.e.mean.

Table 6-1: Comparison of ATP-induced vasoconstriction between vessels.

	n (N)	E_{max} grams (s.e.mean)	E_{max} % NA Sighting (s.e.mean)	Hill Slope (95% CI)	pEC_{50} (s.e.mean)
IVA	5 (10)	0.59 (0.08)	46.80 (1.38)	1.39 (-0.44 to 3.22)	nd
EVA	5 (12)	0.28 (0.21)	17.22 (11.49)	0.59 (-1.04 to 2.23)	nd

CI: confidence interval, nd: not determined

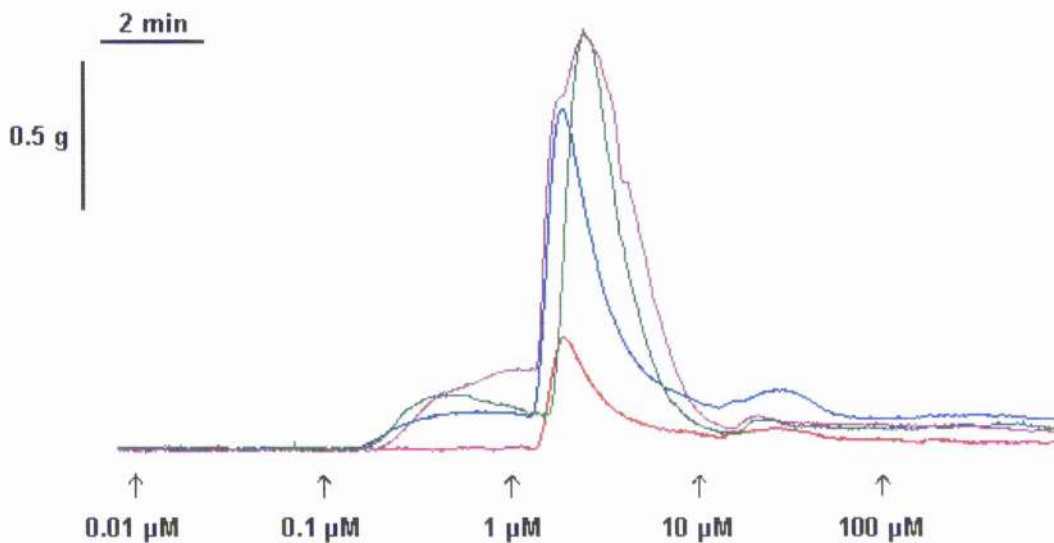


Figure 6-4: Raw traces of responses to $\alpha,\beta,mATP$ (0.01 to 100 μM , arrows) in the female genital arteries. IVA: pink and green lines. EVA: blue and red lines.

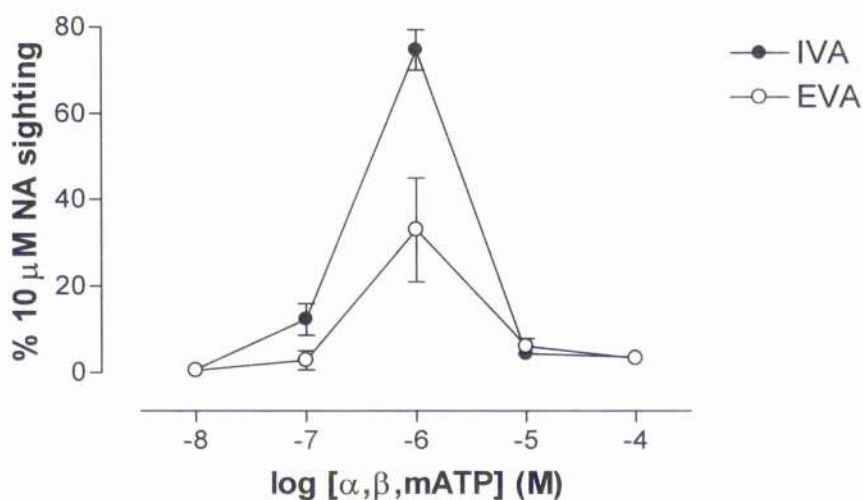


Figure 6-5: $\alpha,\beta,mATP$ -induced vasoconstriction in the female genital arteries expressed as a percentage of the NA (10 μM) sighting response \pm s.e.mean.

Table 6-2: Comparison of $\alpha,\beta,mATP$ -induced vasoconstriction between vessels.

	n (N)	E_{max} grams (s.e.mean)		E_{max} % NA Sighting (s.e.mean)	
		0.1 μM	1 μM	0.1 μM	1 μM
IVA	2 (4)	0.15 (0.03)	0.74 (0.20)	12.32 (3.68)	74.71 (4.65)
EVA	1 (2)	0.05 (0.04)	0.51 (0.26)	2.82 (2.16)	32.97 (11.96)

Peptides, Purines and Vasodilation

Vasoactive Intestinal Polypeptide

VIP induced concentration-dependent vasodilation of both female arterial preparations (Figure 6-6, Table 6-3). Maximal relaxation to VIP was greatest in IVA, $87.5 \pm 3.2\%$ ($n(N) = 14(24)$), compared to EVA, $69.2 \pm 3.2\%$ ($n(N) = 14(23)$, $P < 0.001$). Responses to VIP were also significantly more potent in IVA, $pEC_{50} 8.11 \pm 0.06$, compared to EVA, $pEC_{50} 7.59 \pm 0.05$ ($P < 0.001$).

When compared to ACh-induced vasodilation in the same artery, $98.2 \pm 1.0\%$ ($n(N) = 15(30)$), maximal responses to VIP in the IVA were significantly smaller ($P < 0.01$, Figure 6-7, Table 6-3). However, VIP-induced vasodilation in the EVA was not significantly different from ACh, $75.8 \pm 4.4\%$ ($n(N) = 16(30)$, $P > 0.05$). In both female genital artery preparations the potency of VIP responses was found to be significantly greater than those to ACh by approximately 1.5 log steps ($P < 0.001$). In the IVA the potency of VIP and ACh responses were $pEC_{50} 8.11 \pm 0.06$ ($n(N) = 14(24)$) compared to $pEC_{50} 6.68 \pm 0.05$ ($n(N) = 15(30)$), while in the EVA potencies were $pEC_{50} 7.59 \pm 0.05$ ($n(N) = 14(23)$) and 6.03 ± 0.09 ($n(N) = 16(30)$), respectively.

Vasodilation induced by VIP was reproducible following an extended two hour wash out period between first and second CRCs. Subsequent curves to VIP were well conserved with no significant changes in either potency or maximal relaxations ($P > 0.05$, Figure 6-8, Table 6-4).

VIP (6-28) – VIP Receptor Selective Antagonist

VIP fragment (6-28) ($10 \mu\text{M}$), a VIP receptor antagonist, significantly inhibited VIP-induced vasodilation in the IVA but not the EVA (Figure 6-9, Table 6-4). No decrease was observed in the maximal response in IVA, $80.6 \pm 11.5\%$ relaxation ($n(N) = 5(5)$) compared to control, $87.8 \pm 4.6\%$ relaxation ($n(N) = 5(5)$, $P > 0.05$). A parallel shift of the CRC occurred giving a pEC_{50} of 7.37 ± 0.24 compared to control $pEC_{50} 8.11 \pm 0.14$ ($P < 0.05$).

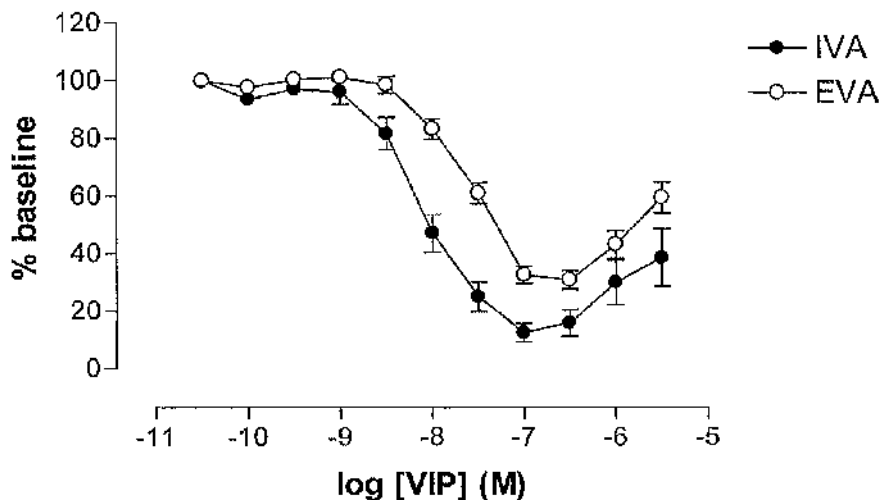


Figure 6-6: VIP-induced vasodilation in the female genital arteries expressed as a percentage of NA (10 μ M) induced baseline tone \pm s.e.mean.

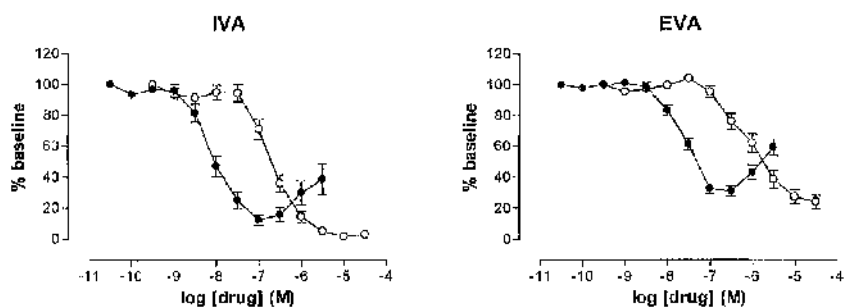


Figure 6-7: Comparison of VIP- (●) and ACh- (○) induced vasodilation in the female genital arteries expressed as a percentage of NA (10 μ M) induced baseline tone \pm s.e.mean.

Table 6-3: Comparison of VIP- and ACh-induced vasodilation between vessels.

		n (N)	% Maximum Relaxation (s.e.mean)	-Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
IVA	VIP	14 (24)	87.50 (3.22)	1.57 (0.99 to 2.14)	8.11 (0.06)
	ACh	15 (30)	98.17 (1.02)	1.42 (1.03 to 1.81)	6.68 (0.05)
EVA	VIP	14 (23)	69.19 (3.23)	1.48 (1.07 to 1.90)	7.59 (0.05)
	ACh	16 (30)	75.78 (4.39)	1.06 (0.69 to 1.44)	6.03 (0.09)

CI: confidence interval

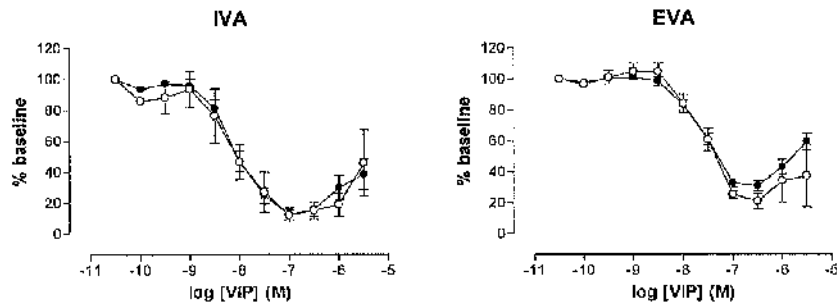


Figure 6-8: VIP-induced vasodilation in the female genital arteries, 1st (●) and 2nd (○) controls, expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

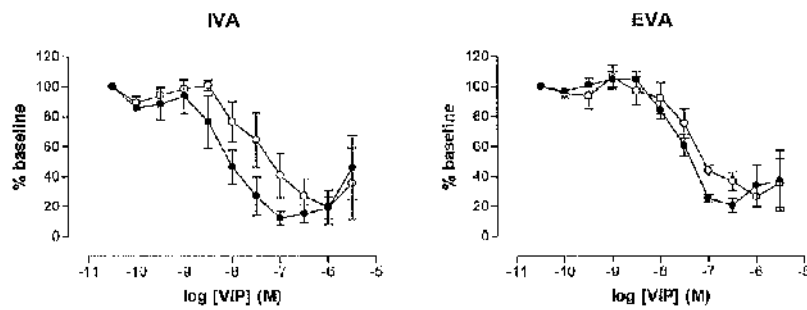


Figure 6-9: VIP-Induced vasodilation in the female genital arteries in the absence (●) or presence (○) of VIP (6-28) (10 μM), expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 6-4: VIP-induced vasodilation in the female genital arteries in the absence, 1st and 2nd controls, or presence of VIP (6-28) (10 nM).

	n (N)	% Maximum Relaxation (s.e.mean)	-Hill Slope (95% CI)	pEC ₅₀ (s.e.mean)
IVA	1 st Control	14 (24) 87.50 (3.22)	1.57 (0.99 to 2.14)	8.11 (0.05)
	2 nd Control	5 (5) 87.81 (4.64)	1.49 (0.22 to 2.76)	8.11 (0.14)
	VIP (6-28) (10 μM)	5 (5) 80.55 (11.54)	1.06 (0.02 to 2.10)	7.37 (0.24)
EVA	1 st Control	14 (23) 69.19 (3.23)	1.48 (1.07 to 1.90)	7.59 (0.05)
	2 nd Control	6 (6) 79.31 (5.00)	1.58 (0.81 to 2.35)	7.52 (0.07)
	VIP (6-28) (10 μM)	6 (6) 73.49 (7.08)	1.52 (0.36 to 2.69)	7.32 (0.13)

CI: confidence interval

Adenosine 5'-Triphosphate

Experiments were performed to determine if ATP could induce relaxation of NA-contracted female artery preparations. Following the establishment of stable tone using NA (10 μ M), ATP was added either as a single dose or in increasing concentrations during a CRC.

Little evidence was found in support of ATP-induced relaxation in either vaginal artery preparation. In the IVA, ATP (30 μ M) was found to cause a degree, approximately 20% to 38%, of relaxation in two of 16 vessels (Figure 6-10a) while in the EVA, ATP (0.1 to 1 mM) induced 5% to 26% relaxation in three of 11 vessels (Figure 6-11a). In the majority of vessels, no vasodilation occurred in response to ATP concentrations ranging from 10 nM to 1 mM (Figures 6-10b and 6-11b).

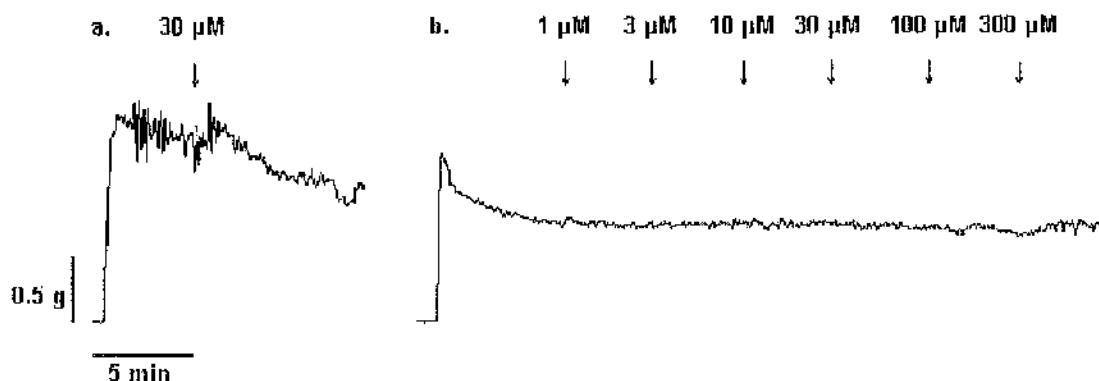


Figure 6-10: Raw traces demonstrating the effect of ATP in the IVA in the presence of guanethidine (10 μM) and NA-induced tone (10 μM). a. Relaxation in response to single dose ATP (30 μM, arrow). b. No relaxation in response to an ATP CRC (1 to 300 μM, arrows).

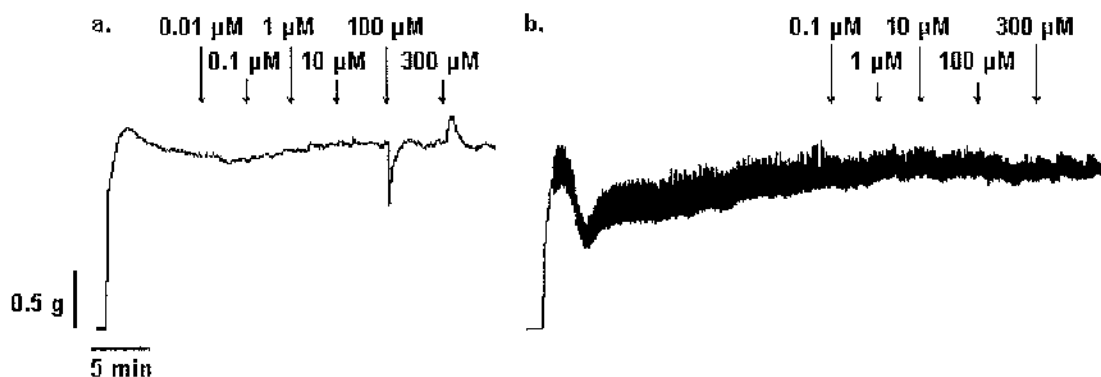


Figure 6-11: Raw traces demonstrating the effect of ATP in the EVA in the presence of guanethidine (10 μM) and NA-induced tone (10 μM). a. Relaxation to 100 μM ATP but contraction to 300 μM (0.01 to 300 μM, arrows). b. No relaxation to increasing concentrations of ATP (0.1 to 300 μM, arrows).

Purines and EFS-induced Vasoconstriction

Control Responses

As previously described in Chapter 3 – Adrenergic Mechanisms, EFS-induced vasoconstriction was found to be highly reproducible in response to the parameters frequency 0.5 to 64 Hz, pulse duration 0.3 ms, current 35 mA, train duration 15 s with a train delay of 5 minutes. Control responses are not further described in this chapter.

Adenosine 5'-Triphosphate

An initial exploratory experiment was performed in male genital arteries with EFS parameters frequency 0.5 Hz, pulse width 0.1 ms, current 150 mA and in the presence of the NOS inhibitor L-NAME. This experiment demonstrated that long train (30 s) responses could be substantially inhibited by α,β,mATP . As previously described in this chapter, α,β,mATP is a selective P_{2X} purinoceptor agonist, which desensitises the P_{2X} receptor following stimulation. α,β,mATP (1 μM) was applied to the bath 10 minutes prior to EFS and caused a large vasoconstriction, which was allowed to return to baseline before electrical stimulation of the tissues. In this experiment, inhibition of the P_{2X} receptor by α,β,mATP decreased the rate of rise and magnitude of the initial phase of the male artery response in addition to the magnitude of the second phase (Figure 6-12).

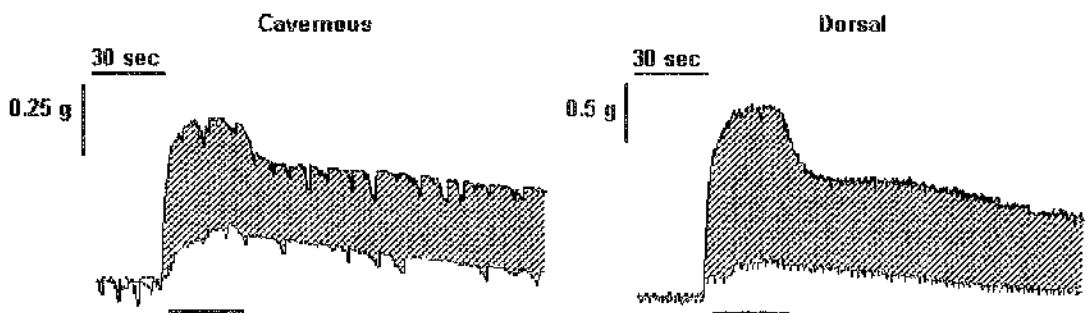


Figure 6-12: EFS-induced (0.5 Hz, 30 s) vasoconstriction in the male genital arteries in the presence of L-NAME (100 μM , black line) or L-NAME (100 μM) plus α,β,mATP (1 μM , blue line). Hatched areas indicate change in responses, stimulation period indicated by bar under traces. Raw traces obtained with the EFS parameters frequency 0.5 Hz, current 150 mA, pulse width 0.1 ms and train duration 30 s.

In all genital arteries, the selective P_{2X} receptor antagonist 2',3'-O-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate (TNP-ATP, 1 μM) was tested against EFS-

induced vasoconstriction in combination with AR inhibition with prazosin (10 nM) and rauwolfscine (10 nM). The effect of a combination of adrenergic and purinergic inhibition was compared to relevant controls in the presence of AR inhibition alone.

In the cavernous artery, EFS-induced vasoconstriction was reduced by TNP-ATP (1 μ M) alone in a single vessel by 9% at the highest frequency, 64 Hz, and by a combination of adrenergic and purinergic inhibition in three of four vessels by between 5% and 69% (Figure 6-13). Analysis of mean data obtained in the presence of adrenergic antagonists (10 nM prazosin plus 10 nM rauwolfscine) or a combination of adrenergic plus purinergic (1 μ M TNP-ATP) inhibition demonstrated no additional contribution of purinergic inhibition ($P > 0.05$, $n(N) = 4(4)$, Figure 6-14, Table 6-5).

EFS-induced vasoconstriction in dorsal arteries was decreased by a combination of adrenergic and purinergic inhibitors in six of six vessels by between 17% and 50% at the highest frequency, 64 Hz (Figure 6-13). Comparison of mean data with either adrenergic inhibition alone or a combination of adrenergic and purinergic inhibition did not demonstrate any change due to the addition of TNP-ATP (1 μ M, $P > 0.05$, $n(N) = 5(5)$, Figure 6-14, Table 6-5).

In the female IVA, the effect of TNP-ATP (1 μ M) on EFS-induced vasoconstriction was variable. Alone, TNP-ATP decreased contractions in one of three vessels by 2% at the highest frequency, 64 Hz (Figure 6-13). A combination of purinergic and adrenergic inhibition increased contractions by 8% in one vessel and decreased contractions by 19% in another at 64 Hz. When the combination of adrenergic and purinergic inhibition was compared to adrenergic inhibition alone no change due to the addition of TNP-ATP was observed ($P > 0.05$, $n(N) = 3(3)$, Figure 6-14, Table 6-5).

TNP-ATP (1 μ M) reduced EFS-induced vasoconstriction in a single EVA vessel by 5% at the highest frequency, 64 Hz (Figure 6-13). In contrast, a combination of adrenergic and purinergic inhibition decreased contractions by between 8% and 53% in three of four vessels. Analysis of mean data demonstrated no change in EFS-induced contractions was given by addition of purinergic inhibition compared to adrenergic inhibition alone ($P > 0.05$, $n(N) = 4(4)$, Figure 6-14, Table 6-5).

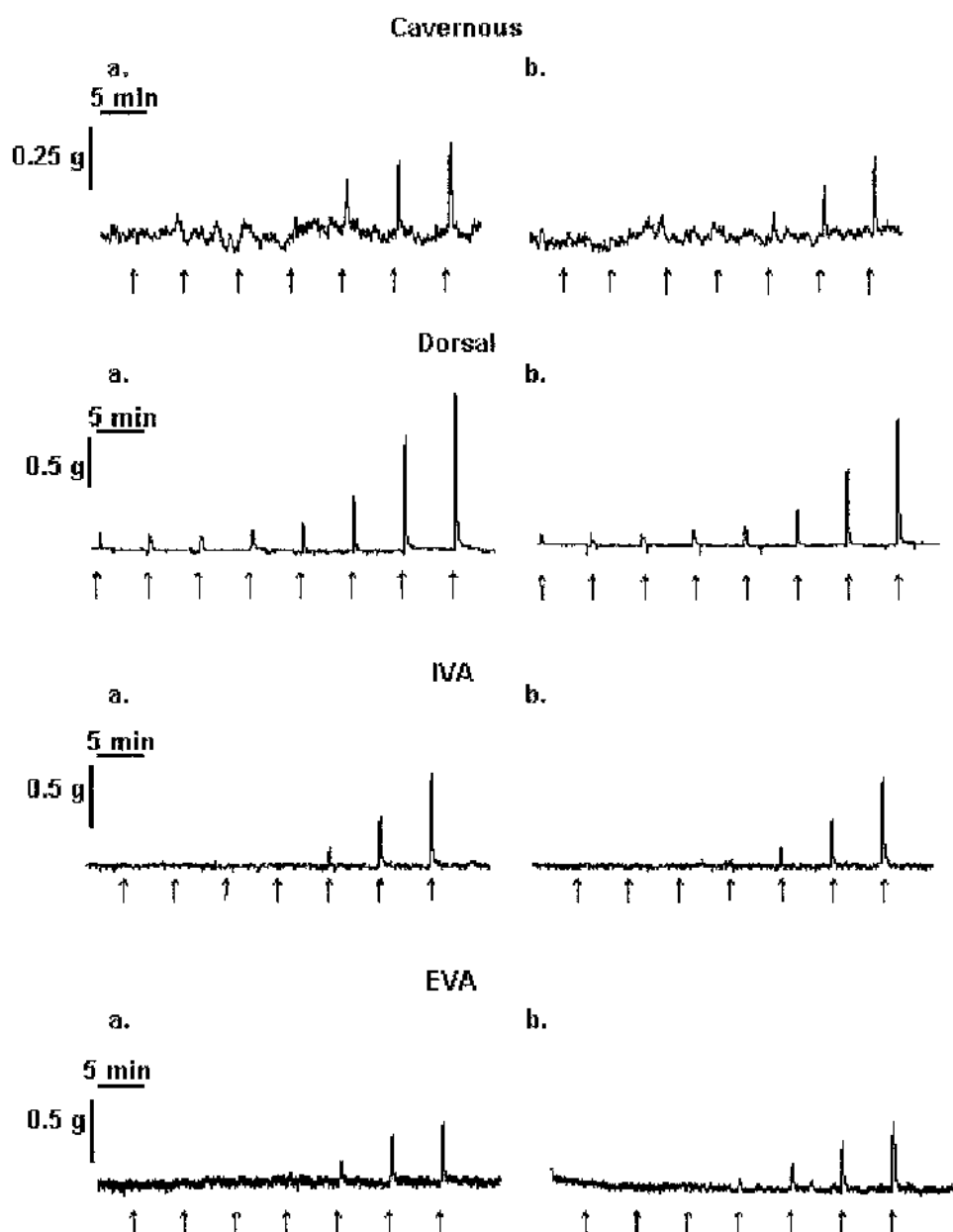


Figure 6-13: Frequency-response curves (arrows, 0.5 to 64 Hz) in the genital arteries in the absence (a.) or presence (b.) of TNP-ATP ($1 \mu\text{M}$). Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

Peptides, Purines and EFS-induced Vasodilation

Control Responses

To allow the study of EFS-induced vasodilation, vessels were incubated with guanethidine (10 μ M) for 1.5 hours to inhibit adrenergic contractile responses. In all preparations tone was induced using NA (10 μ M) and sufficient time allowed for tone to reach a stable plateau before experimental protocols were begun. As previously described in Chapter 4 – Nitroergic Mechanisms, incubation with cyclooxygenase (indomethacin) and K^+ channel (Apa and ChTX) blockers was found to maintain the stability of NA-induced tone over the course of a frequency-response curve without having a noticeable affect on EFS-induced relaxations. Therefore, during EFS protocols involving vasodilator responses, controls were performed both with and without K^+ channel blockers before the effects of antagonists were determined.

Vasoactive Intestinal Polypeptide Receptor Antagonism

EFS-induced vasodilation in female genital arteries was predominantly decreased by L-NAME (100 μ M) as detailed in Chapter 4 – Nitroergic Mechanisms, at low frequencies; responses were virtually abolished in EVA at 0.5 to 4 Hz, while at higher frequencies vasodilation was more resistant to L-NAME. VIP (6-28), a VIP receptor antagonist, was tested against the L-NAME-resistant component to determine if VIP was involved in female genital artery neurotransmission.

In the IVA, EFS-induced vasodilation at 64 Hz, $71.0 \pm 10.1\%$ relaxation ($n(N) = 3(3)$), was unaffected by incubation with L-NAME, $51.7 \pm 4.8\%$ relaxation ($n(N) = 3(3)$, $P > 0.05$). A combination of nitroergic (NOS) and peptidergic (VIP receptor) inhibition caused no further inhibition of EFS-induced vasodilation, $41.5 \pm 3.5\%$ relaxation at 64 Hz ($n(N) = 3(3)$, $P > 0.05$, Figure 6-15). In contrast to L-NAME, which significantly inhibited EFS-induced relaxation at low frequencies, analysis of mean data demonstrated that no additional inhibition of relaxation was provided by incubation with VIP (6-28) at any frequency used ($P > 0.05$, Figure 6-16, Table 6-6).

In the EVA, a combination of nitroergic and peptidergic inhibition greatly reduced vasodilation at all EFS frequencies in three of three vessels (Figure 6-15). Analysis of mean data showed a clear trend whereby at high frequencies (16 to 64 Hz) greater inhibition occurred of EFS-induced vasodilation using a combination of VIP (6-28) plus L-

NAME, $17.6 \pm 2.9\%$ ($n/N = 3/3$), than by L-NAME alone, $34.9 \pm 9.8\%$ ($n/N = 3/3$), Figure 6-16, Table 6-6). However, this trend did not represent a significant change ($P > 0.05$).

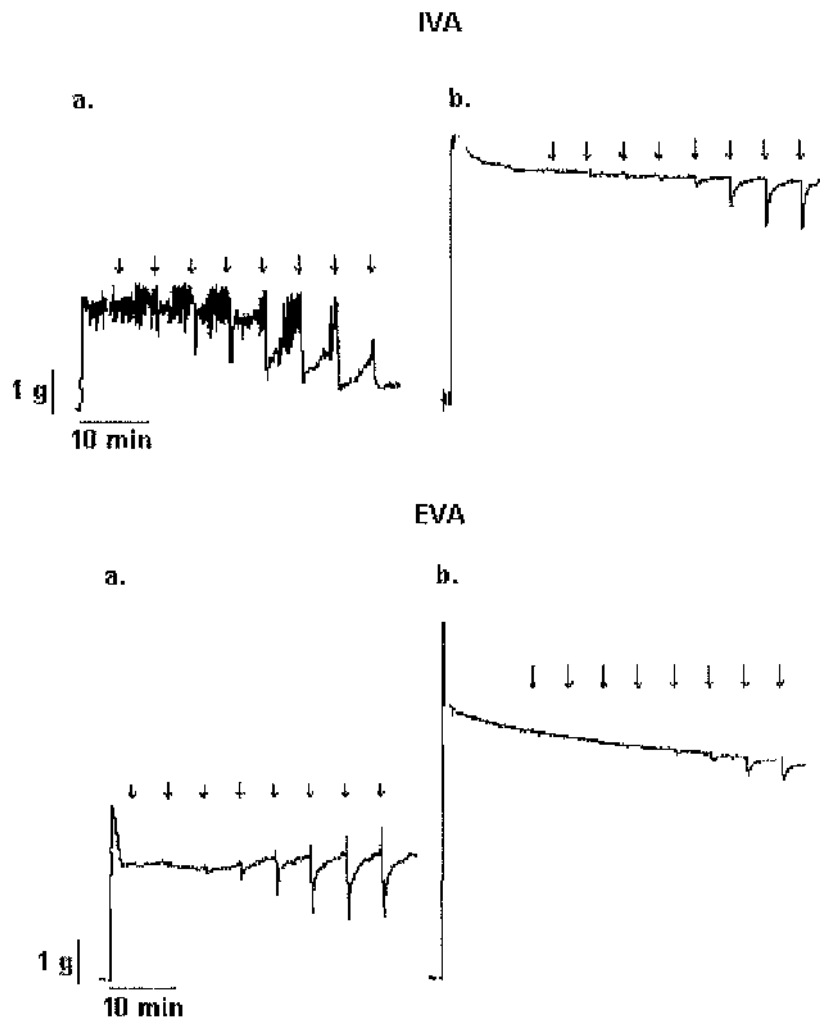


Figure 6-15: Frequency-response curves (arrows, 0.5 to 64 Hz) in the genital arteries following incubation with guanethidine ($10 \mu\text{M}$) and tone raised with NA ($10 \mu\text{M}$), in the absence (a.) or presence (b.) of indomethacin ($1 \mu\text{M}$), Apa (100 nM), ChTX (100 nM), L-NAME ($100 \mu\text{M}$) plus VIP (6-28) ($1 \mu\text{M}$). Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

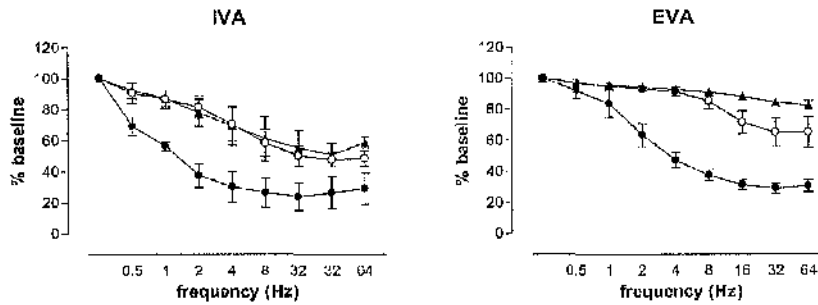


Figure 6-16: EFS-induced relaxation in the female genital arteries in the absence (●) or presence of 100 μM L-NAME (○) or 100 μM L-NAME plus 1 μM VIP (6-28) (▲) expressed as a percentage of NA (10 μM) induced baseline tone ± s.e.mean.

Table 6-6: EFS-induced relaxation in the genital arteries in the absence (control) or presence of L-NAME (100 μM) or L-NAME (100 μM) plus VIP (6-28) (1 μM).

		% Maximum Relaxation (s.e.mean)								
		n (N)	0.5 Hz	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz	64 Hz
IVA	Control	3 (3)	31.03 (6.01)	43.66 (2.82)	62.43 (7.61)	69.79 (9.81)	73.66 (9.58)	76.23 (8.85)	73.75 (10.38)	70.96 (10.05)
	L-NAME	3 (3)	9.66 (6.46)	13.46 (5.97)	18.61 (6.97)	29.55 (10.52)	41.67 (8.63)	49.94 (6.73)	52.50 (4.52)	51.71 (4.80)
	L-NAME + VIP (6-28)	3 (3)	7.88 (5.07)	13.01 (5.16)	22.21 (8.69)	30.55 (12.57)	38.90 (14.40)	44.86 (11.38)	49.43 (7.61)	41.45 (3.50)
EVA	Control	3 (3)	8.08 (5.03)	16.57 (8.66)	36.71 (7.47)	53.02 (4.86)	62.73 (3.64)	68.85 (3.14)	71.06 (2.96)	69.53 (3.99)
	L-NAME	3 (3)	3.38 (1.01)	5.69 (1.71)	7.13 (2.42)	8.99 (2.93)	14.97 (4.65)	28.59 (7.28)	34.94 (9.22)	34.94 (9.84)
	L-NAME + VIP (6-28)	3 (3)	3.14 (0.08)	4.88 (0.52)	5.97 (0.78)	7.71 (0.61)	9.22 (0.72)	12.03 (0.81)	15.77 (2.14)	17.56 (2.91)

Adenosine 5'-Triphosphate

In male genital arteries the selective P_{2Y} receptor antagonist 2'-deoxy- N^6 -methyladenosine 3',5'-bisphosphate (MRS 2179, 1 μ M) was tested against EFS-induced relaxations. The effect of MRS 2179 was tested on a background of cyclooxygenase (indomethacin) and K^+ channel (Apa and ChTX) inhibition to maintain NA-induced tone in the male genital arteries. Since no inhibition of EFS-induced relaxation in male genital arteries had been previously shown using L-NAME, the effect of TNP-ATP was tested in isolation.

In the cavernous artery, vasodilation induced by EFS was increased by incubation with MRS 2179 (1 μ M) in three of three vessels by between 4% and 23% at the highest frequency used, 64 Hz (Figure 6-17). Analysis of mean data demonstrated no significant change in the magnitude of EFS-induced vasodilation in the presence or absence of MRS 2179 ($P > 0.05$, Figure 6-18, Table 6-7).

As in cavernous arteries, MRS 2179 (1 μ M) increased EFS-induced vasodilations in the dorsal artery by between 6% and 22% in three of three vessels at the highest frequency used, 64 Hz (Figure 6-17). No significant change in EFS-induced relaxations was demonstrated by analysis of mean data ($P > 0.05$, Figure 6-18, Table 6-7).

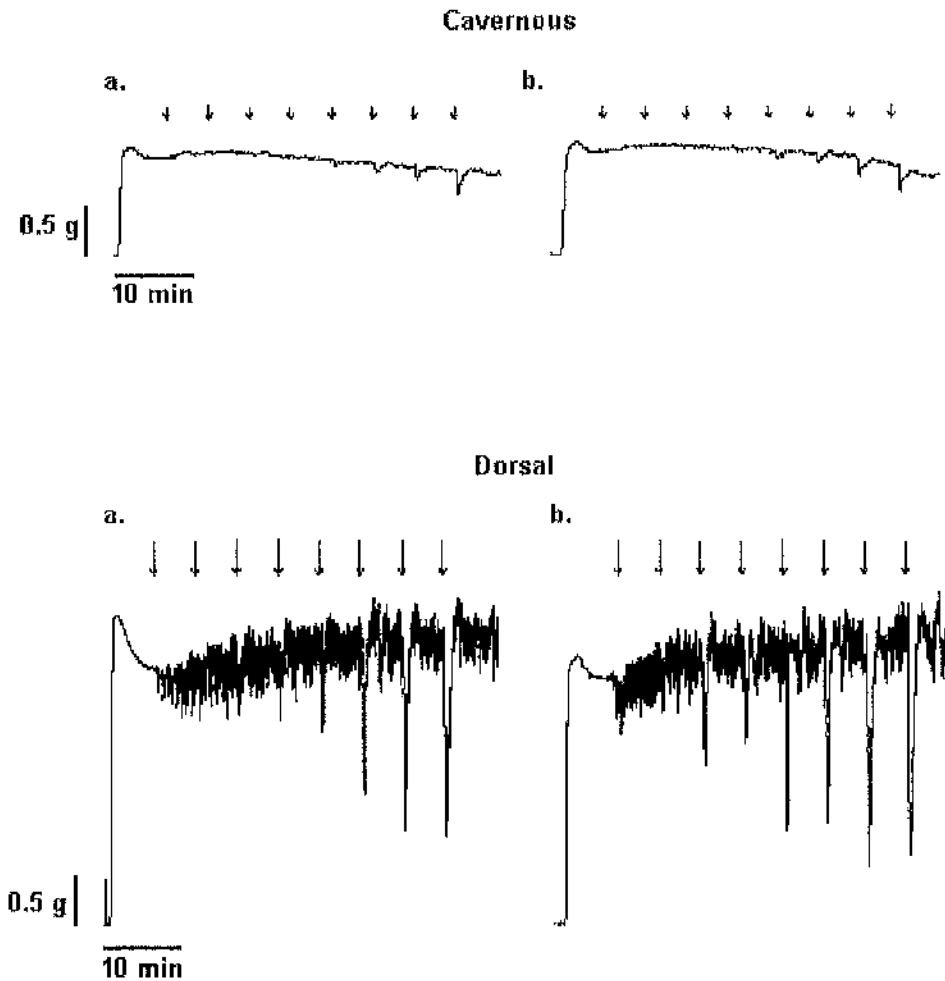


Figure 6-17: Frequency-response curves (arrows, 0.5 to 64 Hz) in the male genital arteries following incubation with guanethidine (10 μ M), indomethacin (1 μ M), Apa (100 nM) plus ChTX (100 nM) and tone raised with NA (10 μ M). Curves in the absence (a.) or presence (b.) of MRS 2179 (1 μ M). Raw traces obtained with the EFS parameters current 35 mA, pulse width 0.3 ms, train duration 15 s and train delay 5 minutes.

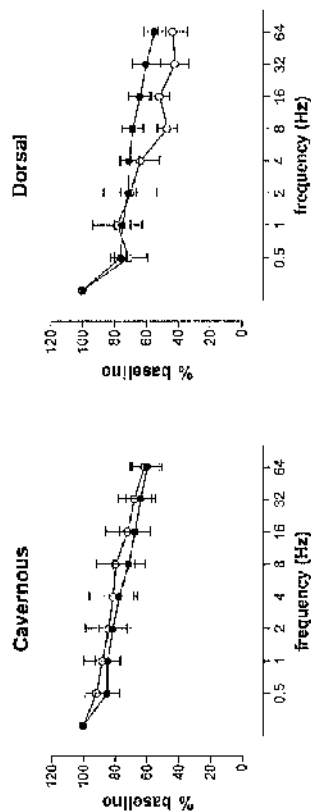


Figure 6-18: EFS-induced relaxation in the male genital arteries in the absence (●) or presence (○) of MRS 2179 (1 μ M). All responses performed in the presence of indomethacin (1 μ M), Apa (100 nM) and ChTX (100 nM) and expressed as a percentage of NA (10 μ M) induced baseline tone \pm s.e.mean.

Table 6-7: EFS-induced relaxation in the male genital arteries in the absence or presence of MRS 2179 (1 μ M).

		% Maximum Relaxation (s.e.mean)								
	n (N)	0.5 Hz	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz	64 Hz	
Cavernous	Control	5 (18)	15.08 (7.36)	15.01 (7.77)	18.41 (9.03)	22.37 (9.12)	28.51 (10.05)	32.46 (9.65)	36.13 (9.46)	40.14 (9.44)
	MRS 2179	3 (3)	8.29 (7.23)	11.75 (11.65)	15.76 (14.66)	18.61 (15.09)	20.16 (12.13)	27.78 (14.00)	32.21 (10.29)	38.58 (8.88)
Dorsal	Control	4 (16)	23.93 (3.95)	25.10 (5.22)	28.75 (5.04)	29.59 (6.04)	31.48 (6.78)	35.79 (7.43)	39.93 (8.88)	45.17 (6.86)
	MRS 2179	3 (3)	29.03 (11.61)	21.87 (15.53)	30.13 (16.70)	36.45 (12.26)	53.35 (6.14)	48.31 (6.17)	57.78 (8.91)	56.50 (9.34)

Discussion

Adenosine 5'-Triphosphate

In contrast to the demonstration by Ziessen & Cellick (2002) of ATP-induced relaxation of female rabbit vaginal tissues, in the current study only a minority of vaginal arteries demonstrated ATP-induced vasodilation. In a single EVA vessel (Figure 6-11), vasodilation was observed that was replaced by a contractile response at a higher concentration. This suggests that contractile P_{2X} receptors comprise the predominant response in the vaginal arteries, while the P_{2Y} receptor has little or no role in the control of vascular tone.

When the ability of MRS 2179, a P_{2Y} receptor antagonist, to decrease EFS-induced vasodilation in male genital arteries was investigated, no significant effect was shown. Indeed the only observed effect of P_{2Y} inhibition in individual vessels appeared to be an *increase* in EFS-induced vasodilation, not a decrease. This is in agreement with the consensus of opinion that while purinoceptors are present in the male penile tissues, no involvement of purines in EFS-induced vasodilation has been demonstrated.

Contractile responses to both ATP and α,β,γ -mATP in female genital arteries demonstrated that functional purinoceptors, likely to be the P_{2X} receptor, were present in these tissues. α,β,γ -mATP is marketed as a P_2 agonist that is more potent at the P_{2X} purinoceptor than ATP itself (Sigma-Aldrich Co., St Louis, USA, A-7699). This was confirmed in the current study which demonstrated vasoconstrictions to α,β,γ -mATP in both IVA and EVA at concentrations (0.1 to 10 μ M) lower than those at which ATP had any effect (> 10 μ M). The subsequent action of α,β,γ -mATP was to function as an ATP receptor antagonist by causing desensitisation of the ATP P_{2X} receptors. Vasoconstriction in response to long train EFS in male arteries was reduced dramatically by α,β,γ -mATP (1 μ M) but frequency-response curves were not reduced by the selective P_{2X} antagonist TNP-ATP (1 μ M). The lack of effect of TNP-ATP on EFS-induced vasoconstriction may be due either to a low potency of TNP-ATP or a lack of purinergic involvement in EFS-induced vasoconstriction. No studies have investigated the effect of purinergic inhibitors on EFS-induced vasoconstrictions in male or female genital tissues. The current study suggests that purines are not involved nerve-mediated vasoconstriction of male and female genital arteries but further investigations would be necessary to determine the effect of higher concentrations

of TNP-ATP and of α,β ,mATP in the absence of L-NAME on EFS-induced vasoconstriction.

Vasoactive Intestinal Polypeptide

As previously described in Chapter 4 – Nitrenergic Mechanisms, EFS-induced vasodilation in female genital arteries was decreased by the NOS inhibitor L-NAME but a resistant component remained at high frequencies, 8 to 64 Hz. Oh *et al.* (2003) showed that EFS-induced relaxation of rabbit vaginal wall tissue could be partially inhibited by the VIP receptor antagonist, dVIP. VIP positive staining was shown in a plexus surrounding arteries but not veins during the current study, suggesting that peptidergic innervation may be involved in the control of vaginal arteries. To determine if the L-NAME-resistant component in the current study could be attributed to peptidergic neurotransmission, both NOS inhibition alone and in combination with VIP receptor inhibition were tested against EFS-induced vasodilation in the vaginal arteries.

Addition of the VIP receptor antagonist, VIP (6-28), did not affect EFS-induced vasodilation in the IVA but demonstrated a non-significant reduction in EFS vasodilations at high frequencies in the EVA. While this trend proved, at this stage, to be non-significant the effect was clear and it may be found that with increased repetitions a significant role for VIP in EFS-induced vasodilation of the EVA could be demonstrated.

The functional significance of VIP in vaginal arteries was further demonstrated by pharmacological characterisation of responses to exogenous VIP. Female vaginal arteries were significantly more sensitive to VIP than to ACh, either reflecting a difference in the binding affinities of these two compounds to their respective receptors or a reduction in the effective concentration of ACh in the genital arteries by their rapid breakdown by acetylcholinesterases. Inhibition of EFS-induced vasodilation by VIP (6-28) in EVA but not IVA suggested that peptidergic, VIP mediated neurotransmission appears to have a greater role in upstream regions of the vaginal artery. In contrast, exogenous VIP demonstrated a greater affinity and was only inhibited by VIP (6-28) in the IVA. VIP has been shown to have a similar affinity at both VPAC₁ and VPAC₂ (Harmar *et al.*, 1998) and therefore the potency of VIP responses in the vaginal arteries provides no information about the VIP receptor subtype involved. A role for the VIP receptor in the IVA was indicated by the effect of VIP (6-28) but responses in the EVA may not be VIP receptor mediated.

An extended recovery period of 2 hours was required between VIP CRCs to achieve reproducible responses. In addition, vasodilation to high concentrations of VIP, 0.3 μM to 3 μM , demonstrated a suppression of maximal relaxation responses. Therefore, either VIP receptors were becoming desensitised at these high concentrations or, once bound VIP caused activation of a receptor followed by a latent period during which the receptor cannot be reactivated. This is in accordance with the demonstration in cultured cells (CHO, COS7 and HEK293 cell lines) of a rapid agonist-induced internalisation of VIP receptors and their re-expression at the cell surface within 2 hours (McDonald *et al.*, 1998; Langlet *et al.*, 2004). A greater degree of desensitisation of the VIP response was demonstrated compared to any other agonist used during this study, including ACh and SNP. Depending on the relative predominance of peptidergic neurotransmission in the female this may correspond to a latency period in the excitability of vaginal tissues via stimulation of VIP receptors.

There are currently few reliable antagonists of the VIP receptors. Vasoactive intestinal peptide fragment 6-28 (VIP (6-28)) is a carboxy-terminal fragment of the VIP molecule found to be two-fold more potent than any previously used antagonists (Fishbein *et al.*, 1994). However, the current study only demonstrated inhibition of VIP responses in the IVA using VIP (6-28) at a high, potentially non-selective, concentration of 10 μM . The development of more selective and potent inhibitors of VIP receptors would further assist the characterisation of VIP responses in the genital arteries.

Summary

In summary, the L-NAME-resistant component of EFS-induced vasodilation was not convincingly identified as either purinergic or due to VIP (Table 6-8). A role for VIP in EVA neurotransmission was indicated and would require further investigation to determine if VIP as a neurotransmitter could fully account for L-NAME-resistant vasodilation. Neither inhibition of VIP nor ATP receptors decreased EFS-induced vasodilation in IVA, cavernous or dorsal genital arteries, suggesting that an additional, unknown component remains unidentified in these arteries. The role of peptides in EFS-induced vasodilation could be investigated using peptidase inhibitors to determine if submaximal vasodilations can be increased.

The presence both of functional peptidergic, VIP, and purinergic, $\text{P}_{2\text{X}}$, receptors was demonstrated in female genital arteries. The studies presented within this thesis indicate that in female tissues, VIP may be of more importance in the upstream region of the

vaginal artery. In addition, the role of P_{2X} purinoceptors appears more predominant than P_{2Y} purinoceptors in both sexes. A role for purinergic neurotransmission, via P_{2X} purinoceptors, in vasoconstriction of male penile arteries could not be excluded.

Table 6-8: Summary of peptidergic data in the genital arteries and the effect, increase (↑), decrease (↓) or no change (-), of antagonists and blockers on responses.

Activity	Drugs	Cavernous	Dorsal	IVA	EVA
ATP (0.1 to 1000 μ M)	Control (E_{max})	nd	nd	0.6 g	0.3 g
α_2 ,mATP (1 μ M)	Control (E_{max})	nd	nd	0.7 g	0.5 g
VIP (0.1 to 3000 nM)	Control (E_{max} , % vasodilation)	nd	nd	88%	69%
	VIP (6-28) (10 μ M)	nd	nd	↓*	-
EFS-induced vasoconstriction	Control (E_{max} at 64 Hz)	0.3 g	1.2 g	0.9 g	0.5 g
	TNP-ATP (1 μ M)	-	-	-	-
EFS-induced vasodilation	Control (E_{max} at 64 Hz, % vasodilation)	40%	45%	71%	70%
	L-NAME (100 μ M)	nd	nd	↓*	↓*
	L-NAME (100 μ M) plus VIP (6-28) (10 μ M)	nd	nd	↓*	↓***
	MRS 2179 (1 μ M)	-	-	nd	nd

Significance vs controls, *: $P < 0.05$, ***: $P < 0.001$, nd: not determined

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Chapter 7

Spontaneous Activity

Introduction

Myogenic Tone and Vasomotion

Myogenic tone is a response shown by arterial smooth muscle to a change in transmural pressure; contraction to increased pressure and relaxation to decreased pressure (Hughes & Bund, 2002). It is not dependent on vascular endothelium or innervation but can be modulated by the endothelium, in particular by the action of shear stress (Hughes & Bund, 2002; Segal, 2005). Myogenic contraction of vascular smooth muscle cells may also translate into vasomotion which is rhythmic activity of vascular smooth muscle (Griffith *et al.*, 2005). Myogenic tone is thought to be involved in the regulation of peripheral resistance and hence blood pressure (Hughes & Bund, 2002) and in the oxygenation of tissues (Segal, 2005). In addition, changes in myogenic activity have been implicated in the aetiology of hypertension (Hughes & Bund, 2002).

Aims

Various incidences of spontaneous activity were noted throughout previous experimental protocols. These observations were frequent and interesting enough to warrant a detailed examination of their characteristics. Since myogenic tone may be important in the regulation of blood flow through the genital tissues the aims of this chapter were as follows.

- To describe the characteristics of spontaneous/myogenic activity observed in male and female rabbit genital arteries.
- To determine the underlying basal mechanisms active in each genital artery studied.

Results

Spontaneous Activity

During equilibration periods, vessels were occasionally observed to contract in the absence of agonists or antagonists. While this induction of endogenous tone was seen intermittently in each artery group, the most common vessel type to exhibit spontaneous vasoconstriction was the cavernous artery; $n(N)$ of $n(N)$ = cavernous, 58(144) of 100(422); dorsal, 13(17) of 101(361); IVA, 10(15) of 49(226) and EVA, 10(14) of 44(209). This equated to 13% to 58% of animals and 5% to 34% of vessels. Spontaneous vasoconstrictions could generally be returned to the pre-activity baseline by multiple changes of bathing media but in some instances would recur following the wash.

Effect of α -Adrenoceptor Antagonists

As previously mentioned, spontaneous vasoconstrictions were observed during equilibration periods either prior to or between experimental protocols. Experimental protocols often included incubation of antagonists during equilibration periods between 1st and 2nd CRCs to an agonist. On occasion, the addition of antagonists coincided with the occurrence of spontaneous activity within a vessel. In these instances it could be noted whether the antagonist in use had any effect on endogenous tone.

In the cavernous artery, a small percentage of vessels, seven of 144, demonstrated spontaneous vasoconstrictions whose timing corresponded with the addition of AR antagonists. In these experiments, inhibition of endogenous tone by prazosin (1 to 100 nM) and rauwolscine (0.1 to 1 μ M) was observed (Figure 7-1). Similarly, in dorsal arteries inhibition of spontaneous vasoconstrictions in three of 17 vessels was given by prazosin (10 to 100 nM, Figure 7-2). No inhibition of endogenous tone by any other antagonists was observed.

In the IVA, the timing of spontaneous vasoconstrictions did not occur such that any were inhibited by antagonists while in the EVA a few small magnitude spontaneous vasoconstrictions, two of 15 vessels, were inhibited by prazosin (10 nM, Figure 7-3).

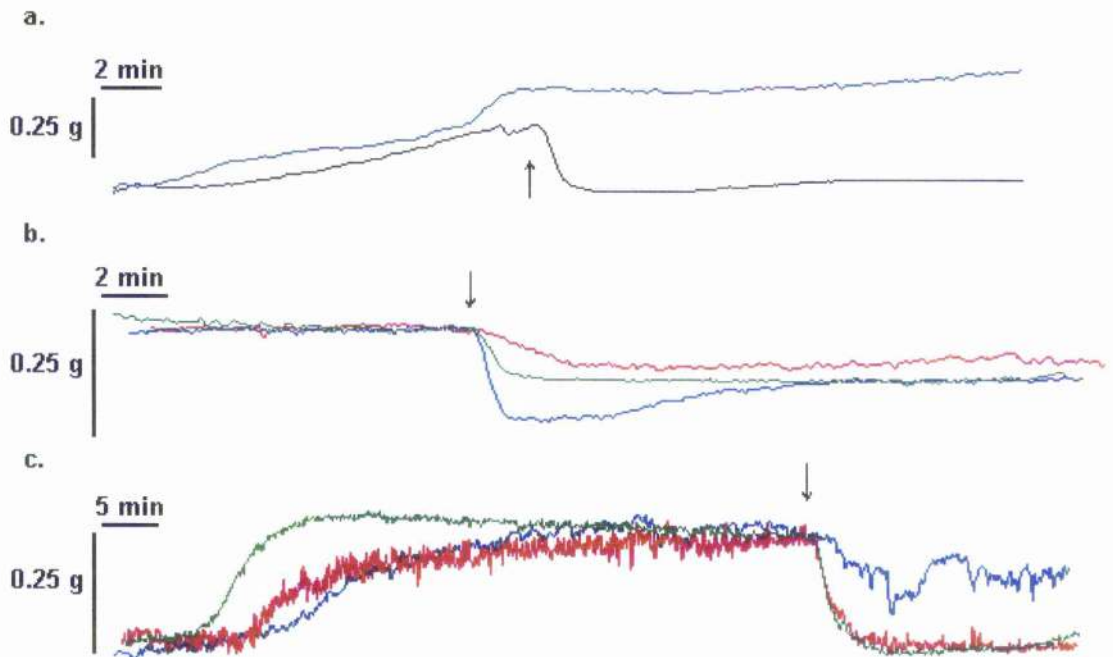


Figure 7-1: Raw traces demonstrating the effect of prazosin or rauwolscine (arrows) on spontaneous vasoconstrictions in the cavernous artery. Addition of a. Prazosin 100 nM (black) compared to an earlier spontaneous vasoconstriction in the same vessel (blue), b. Rauwolscine 0.1 μ M (green and blue) and 1 μ M (red), c. Prazosin 1 nM (blue), 10 nM (red) and 100 nM (green).

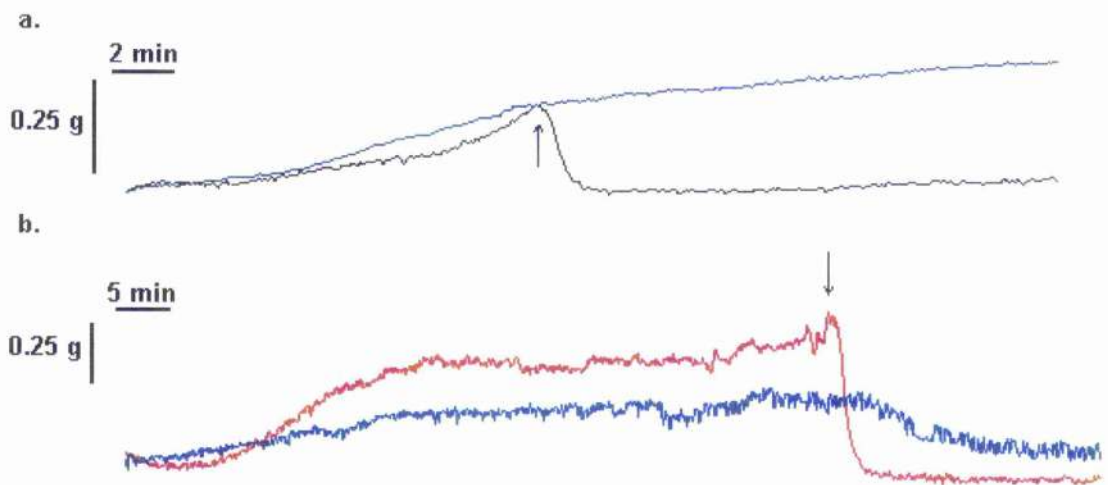


Figure 7-2: Raw traces demonstrating the effect of prazosin (arrows) on spontaneous vasoconstrictions in the dorsal artery. Addition of a. Prazosin 100 nM (black) compared to an earlier spontaneous vasoconstriction in the same vessel (blue), b. Prazosin 10 nM (blue) and 100 nM (red).

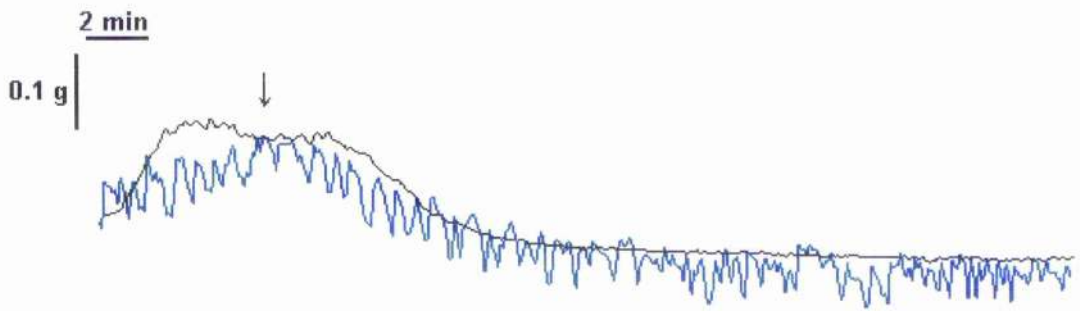


Figure 7-3: Raw traces demonstrating the effect of prazosin 10 nM (arrow, blue and black) on spontaneous vasoconstrictions in the EVA.

Effect of Noradrenaline Uptake Blockers

Vasoconstriction was observed in some vessels in the absence of agonists but during incubation with inhibitors (Figure 7-4, Table 7-1). Inhibitors which caused an increase in endogenous tone included the NA uptake blockers cocaine (3 μ M) and corticosterone (30 μ M).

As previously described, spontaneous vasoconstrictions in the absence of antagonists were predominantly observed in cavernous arteries, 34% of vessels. The induction of endogenous tone was significantly increased during incubation with NA uptake blockers, 0.38 ± 0.15 g, ($n(N) = 5(7)$), compared to controls, 0.06 ± 0.01 g ($n(N) = 23(61)$), $P < 0.001$).

In the IVA, EVA and dorsal arteries, few vessels exhibited spontaneous vasoconstrictions in the absence of agonists or antagonists, 5% to 7% of vessels. In contrast to cavernous arteries, NA uptake blockers did not significantly increase endogenous tone in the dorsal artery, IVA or EVA ($P > 0.05$).

Effect of Nitric Oxide Synthase and K^+ Channel Blockers

Increases in endogenous tone were also observed in some vessels in the absence of agonists but during incubation with the NOS inhibitor L-NAME (100 μ M) and the K^+ channel blockers Apa (100 nM) and ChTX (100 nM, Figure 7-4, Table 7-1).

Cavernous arteries demonstrated vasoconstriction during incubation with L-NAME, 0.13 ± 0.04 g ($n(N) = 15(28)$), K^+ channel blockers, 0.30 ± 0.09 g ($n(N) = 4(4)$) or a combination of L-NAME plus K^+ channel blockers, 0.29 ± 0.12 g ($n(N) = 4(4)$). However, only vasoconstriction during incubation with K^+ channel blockers alone was significantly greater than those in control vessels, 0.06 ± 0.01 g ($n(N) = 23(61)$), $P < 0.05$).

In the dorsal arteries, incubation with L-NAME, 0.15 ± 0.06 g ($n(N) = 17(30)$), $P < 0.01$), K^+ channel blockers, 0.12 ± 0.04 g ($n(N) = 5(6)$), $P < 0.01$), or a combination of L-NAME plus K^+ channel blockers, 0.68 ± 0.49 g ($n(N) = 6(7)$), $P < 0.001$), all caused vasoconstrictions of dorsal arteries significantly greater than those in control vessels, 0.03 ± 0.01 g ($n(N) = 25(67)$).

Similarly, in the IVA incubation with L-NAME, 0.17 ± 0.05 g ($n(N) = 5(9)$, $P < 0.001$), K^+ channel blockers, 0.08 ± 0.04 g ($n(N) = 6(6)$, $P < 0.01$), or a combination of L-NAME plus K^+ channel blockers, 0.91 ± 0.42 g ($n(N) = 6(8)$, $P < 0.001$), all caused vasoconstriction significantly greater than those in control vessels, 0.04 ± 0.01 g ($n(N) = 15(44)$).

Incubation with L-NAME, 0.39 ± 0.18 g ($n(N) = 17(30)$, $P < 0.001$), or a combination of L-NAME plus K^+ channel blockers, 0.67 ± 0.54 g ($n(N) = 6(7)$, $P < 0.001$), caused vasoconstrictions in the EVA significantly greater than controls, 0.02 ± 0.01 g ($n(N) = 25(67)$). Incubation with K^+ channel blockers alone caused no significant increase in endogenous tone, 0.06 ± 0.04 g ($n(N) = 6(6)$, $P > 0.05$).

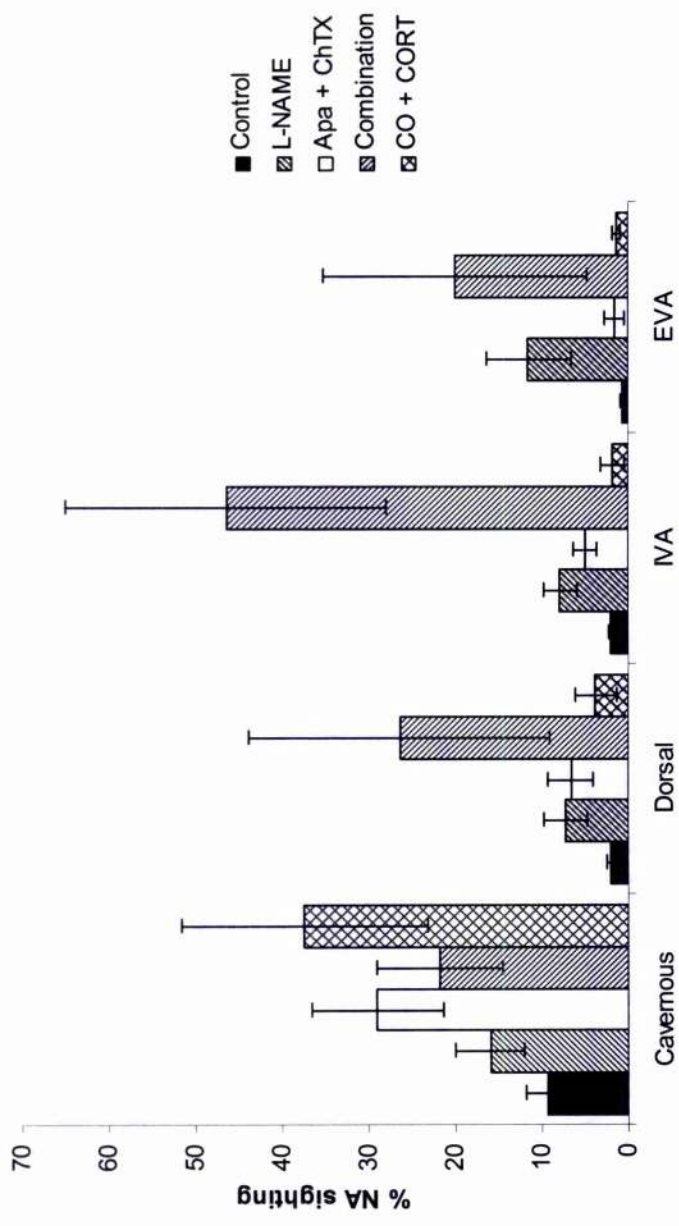


Figure 7-4: Increased endogenous tone observed in the genital arteries in the absence (control) or presence of NOS inhibitor (L-NAME, 100 μ M), K^+ channel blockers (Apa, 100 nM + ChTX, 100 nM), NOS inhibitor plus K^+ channel blockers (Combination) or NA uptake blockers cocaine plus corticosterone (CO, 3 μ M + CORT, 30 μ M). Expressed as a percentage of the NA (10 μ M) sighting response \pm s.e.mean.

Table 7-1: Increased endogenous tone observed in the genital arteries in the absence (control) or presence of NOS inhibitor (L-NAME, 100 μ M), K⁺ channel blockers (Apa, 100 nM + ChTX, 100 nM), NOS plus K⁺ channel blockers (Combination) or NA uptake blockers cocaine plus corticosterone (CO, 3 μ M + CORT, 30 μ M).

		n (N)	E _{max} grams (s.e.mean)	E _{max} % NA sighting (s.e.mean)	Significance vs Control
Cavernous	Control	23 (61)	0.06 (0.01)	9.40 (2.30)	-
	L-NAME	15 (28)	0.13 (0.04)	16.01 (4.07)	ns
	Apa + ChTX	4 (4)	0.30 (0.09)	29.04 (7.57)	<i>P</i> < 0.05
	Combination	4 (4)	0.29 (0.12)	21.78 (7.21)	ns
	CO + CORT	5 (7)	0.38 (0.15)	37.42 (14.28)	<i>P</i> < 0.001
Dorsal	Control	25 (67)	0.03 (0.01)	2.15 (0.45)	-
	L-NAME	17 (30)	0.15 (0.06)	7.25 (2.49)	<i>P</i> < 0.01
	Apa + ChTX	5 (6)	0.12 (0.04)	6.69 (2.54)	<i>P</i> < 0.01
	Combination	6 (7)	0.68 (0.49)	28.47 (17.30)	<i>P</i> < 0.001
	CO + CORT	6 (7)	0.09 (0.05)	3.77 (2.33)	ns
IVA	Control	15 (44)	0.04 (0.01)	1.94 (0.03)	-
	L-NAME	5 (9)	0.17 (0.05)	7.86 (1.96)	<i>P</i> < 0.001
	Apa + ChTX	6 (6)	0.08 (0.04)	4.96 (1.37)	<i>P</i> < 0.01
	Combination	6 (8)	0.91 (0.42)	46.47 (18.51)	<i>P</i> < 0.001
	CO + CORT	6 (6)	0.04 (0.03)	1.74 (1.37)	ns
EVA	Control	25 (67)	0.02 (0.01)	0.71 (0.24)	-
	L-NAME	17 (30)	0.39 (0.18)	11.48 (4.96)	<i>P</i> < 0.001
	Apa + ChTX	5 (6)	0.06 (0.04)	1.64 (1.08)	ns
	Combination	6 (7)	0.67 (0.54)	20.07 (15.24)	<i>P</i> < 0.001
	CO + CORT	6 (7)	0.04 (0.02)	1.36 (0.34)	ns

Spontaneous Activity in Pre-contracted Tissues

Rhythmic activity or vasomotion, characterised by a continual cycle of vasoconstriction and vasodilation around a stable baseline of agonist-induced tone, was the most commonly observed type of spontaneous activity. Rhythmic activity occurred in all artery groups (Figure 7-5), being most frequent in dorsal artery preparations; $n(N)$ of $n(N)$ = cavernous, 61(138) of 100(422); dorsal, 94(232) of 101(361); IVA, 41(110) of 49(226), and EVA, 24(44) of 44(209). This equated to 55% to 93% of animals and 21% to 64% of vessels. Spontaneous activity of this type was only observed following an agonist-induced increase in tone and therefore may not strictly speaking be considered 'spontaneous'. Rhythmic activity occurred randomly and when present appeared to be dependent on the level of tone, being more prevalent at higher tensions. Therefore, this activity shall be referred to simply as rhythmic activity.

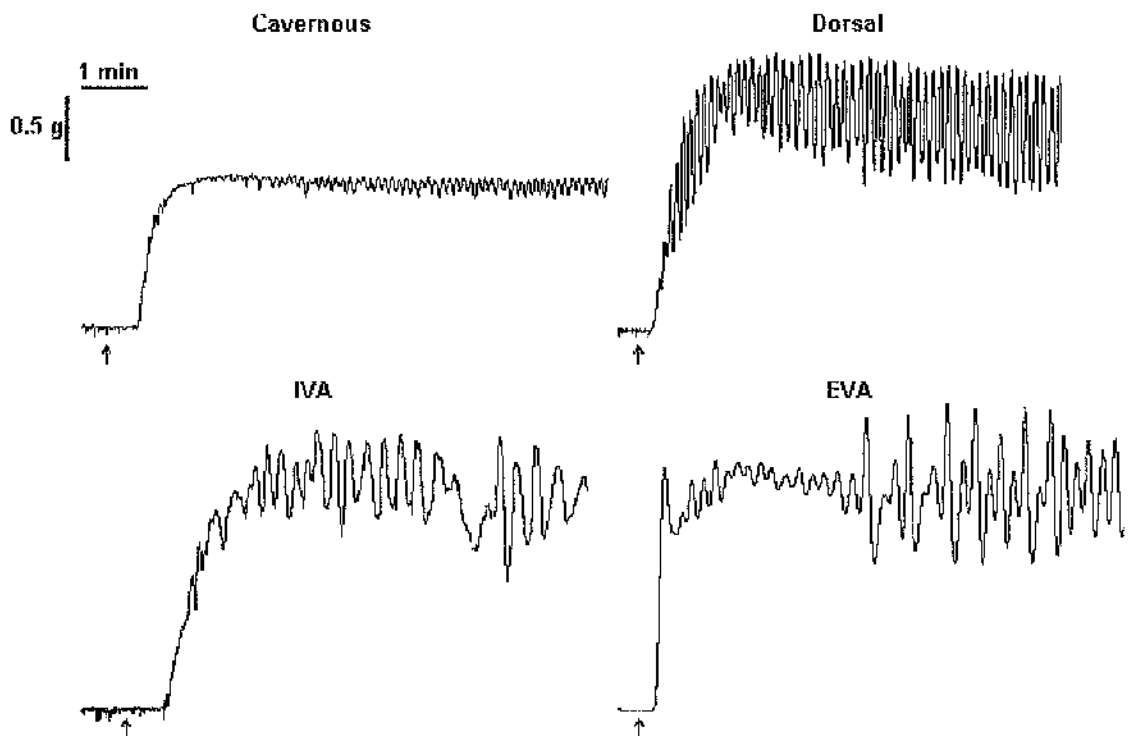


Figure 7-5: Raw traces demonstrating typical examples of rhythmic activity in the genital arteries following an agonist-induced increase in tone (NA, 10 μ M, arrows).

In the cavernous artery, following tone raised with NA or PE, rhythmic activity occurred in the presence of various adrenergic antagonists, including prazosin (10 nM), phentolamine (100 nM) and rauwolscine (10 nM). In addition, rhythmic activity was not inhibited in four of four vessels by the cyclooxygenase inhibitor indomethacin (1 μ M), nor, in four of five vessels, by the NOS inhibitor L-NAME (100 μ M). In a single case, rhythmic activity was

observed prior to, but not following, a 40 minute incubation with L-NAME (100 μ M). Rhythmic activity was also removed in three of three vessels following a 30 minute incubation with K^+ channel blockers Apa (100 nM) plus ChTX (100 nM) (Figure 7-6) or a combination of K^+ channel blockers plus L-NAME.

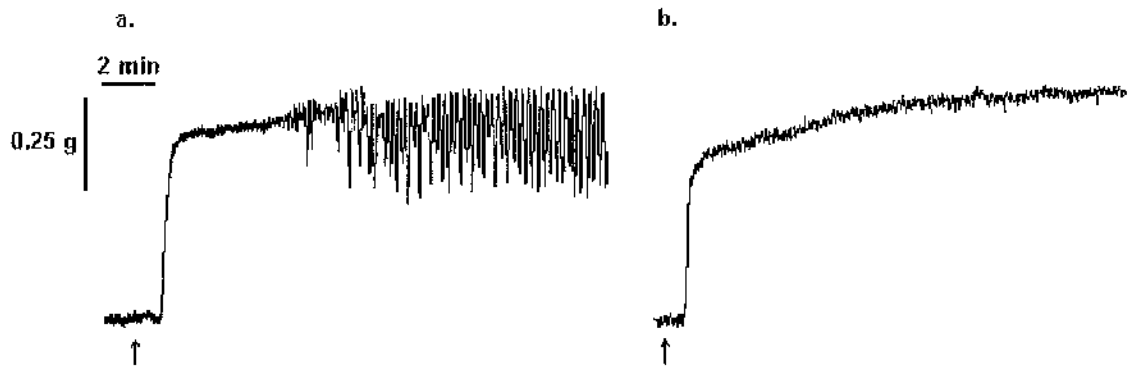


Figure 7-6: Raw traces demonstrating rhythmic activity in the cavernous artery following tone raised with NA (10 μ M, arrows); present prior to (a.) but not following (b.) a 30 minute incubation with K^+ channel blockers Apa (100 nM) plus ChTX (100 nM).

In the dorsal artery, rhythmic activity was observed in the presence of adrenergic antagonists, including prazosin (10 nM), phentolamine (100 nM) and rauwolscine (10 nM), and was not blocked by the cyclooxygenase inhibitor indomethacin (1 μ M) in two of two vessels. In contrast to cavernous arteries, rhythmic activity in the dorsal artery was not affected by K^+ channel blockers Apa (100 nM) and ChTX (100 nM) in five of five vessels. However, rhythmic activity was decreased or abolished in six of nine vessels by the NOS inhibitor L-NAME (100 μ M) (Figure 7-7) and in four of six vessels by a combination of K^+ channel blockers and L-NAME.

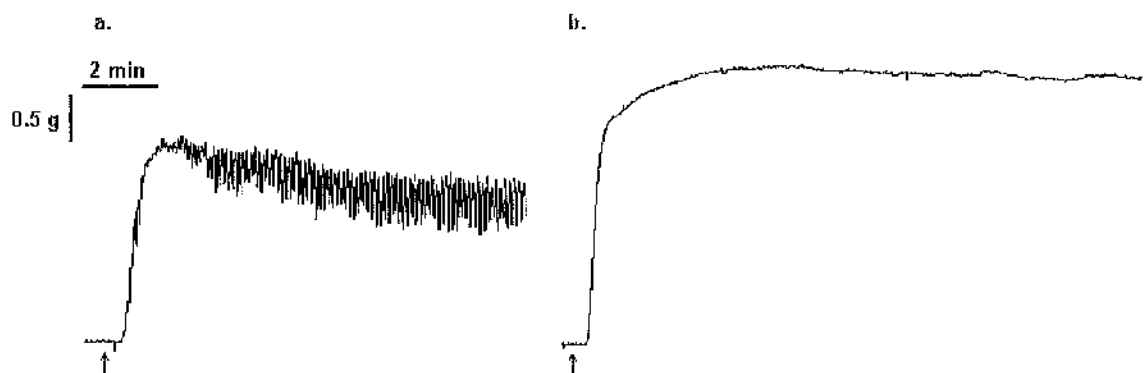


Figure 7-7: Raw traces demonstrating rhythmic activity in the dorsal artery following tone raised with NA (10 μ M, arrows); present prior to (a.) but not following (b.) a 40 minute incubation with L-NAME (100 μ M).

In female vaginal arteries, rhythmic activity was not affected by adrenergic antagonists, including prazosin (10 nM) and rauwolscine (10 nM), nor by the cyclooxygenase inhibitor indomethacin (1 μ M) in two of two vessels. In addition, in IVA and EVA rhythmic activity was not blocked by K^+ channel blockers Apa (100 nM) and ChTX (100 nM) in three of three vessels, nor by L-NAME (100 μ M) in four of four vessels. However, in both IVA and EVA rhythmic activity was abolished by a combination of K^+ channel blockers Apa (100 nM) and ChTX (100 nM) plus NOS inhibitor L-NAME (100 μ M) in three of three vessels (Figures 7-8 and 7-9).

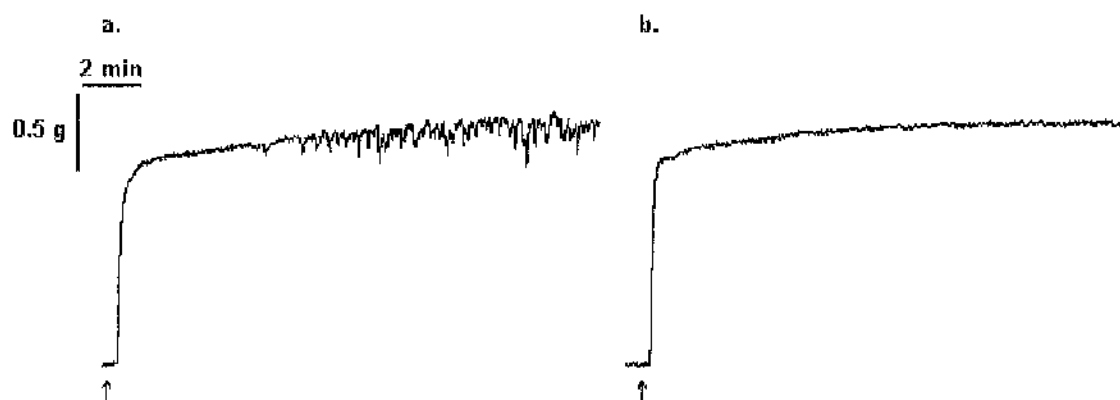


Figure 7-8: Raw traces demonstrating rhythmic activity in the IVA following tone raised with NA (10 μ M, arrows); present prior to (a.) but not following (b.) a 30 minute incubation with a combination of Apa (100 nM) plus ChTX (100 nM) plus L-NAME (100 μ M).

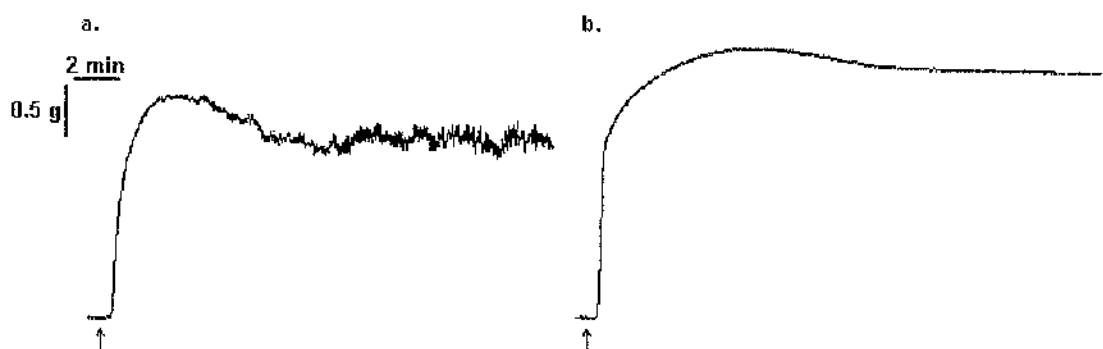


Figure 7-9: Raw traces demonstrating rhythmic activity in the EVA following tone raised with NA (10 μ M, arrows); present prior to (a.) but not following (b.) a 30 minute incubation with a combination of Apa (100 nM) plus ChTX (100 nM) plus L-NAME (100 μ M).

Vasodilation

Large, pronounced relaxations of AR agonist-induced tone (NA, 10 μ M or PE, 10 μ M) were evident in the EVA. In this artery distinct vasodilations of varying magnitude, from 12% to 95% of agonist-induced tone, were observed in 7% of vessel preparations (30% of animals), $n(N)$ of $n(N) = 13(15)$ of $44(209)$. Vasodilations differed from previously described rhythmic activity in that they did not occur as small oscillatory, spontaneous cycles but rather as a discreet vasodilation followed by a return to previous tone in less frequent regularity.

Spontaneous vasodilations occurred during the performance of various protocols involving an elevation in tone (NA, 10 μ M). It was noted that this type of vasodilation was not blocked by incubation with the VIP receptor antagonist, VIP (6-28) (10 nM or 3 μ M) in three of three vessels. However, in six vessels, spontaneous vasodilations coincided with a 40 minute incubation with the NOS inhibitor, L-NAME (100 μ M). In all six vessels, spontaneous vasodilations present prior to L-NAME incubation were completely abolished following the equilibration period (Figure 7-10). In two vessels (Figures 7-11 and 7-12), the occurrence of spontaneous vasodilations were progressively abolished over a shorter timeframe by the addition of L-NAME (100 μ M) without a wash-out of NA-induced tone.

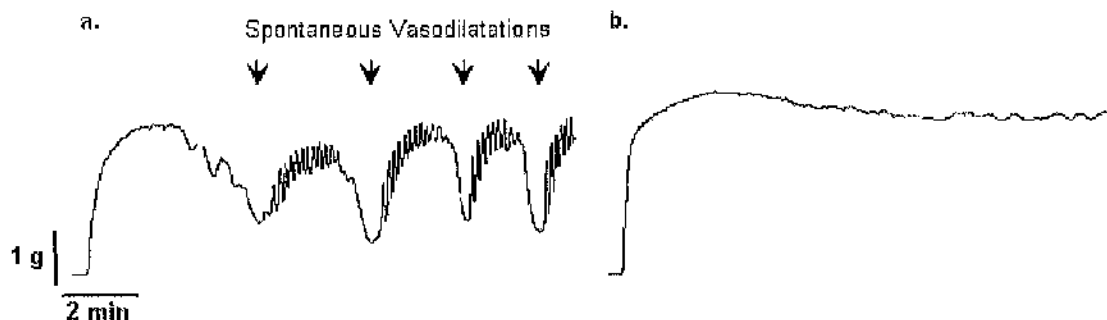


Figure 7-10: Raw traces demonstrating spontaneous vasodilations (arrows) in the EVA following the induction of tone using NA (10 μ M); present prior to (a.) but not following (b.) a 40 minute incubation with L-NAME (100 μ M).

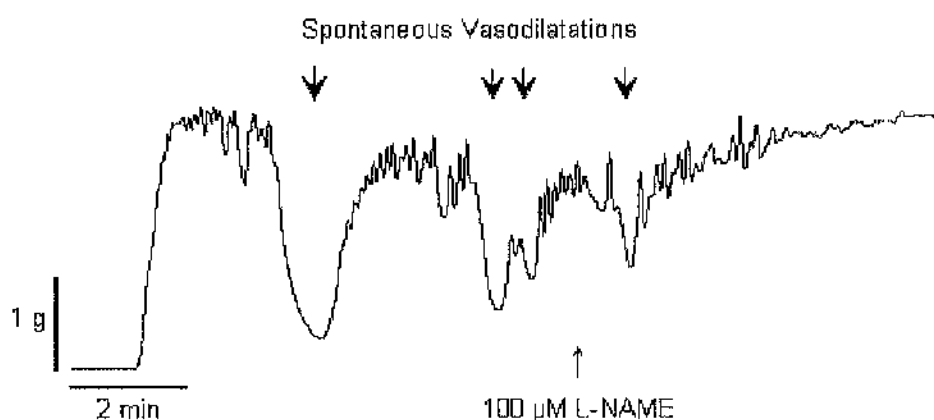


Figure 7-11: Raw traces demonstrating the effect of L-NAME (100 μ M, upward arrow) on spontaneous vasodilations (downward arrows) in the EVA following the induction of tone using NA (10 μ M).

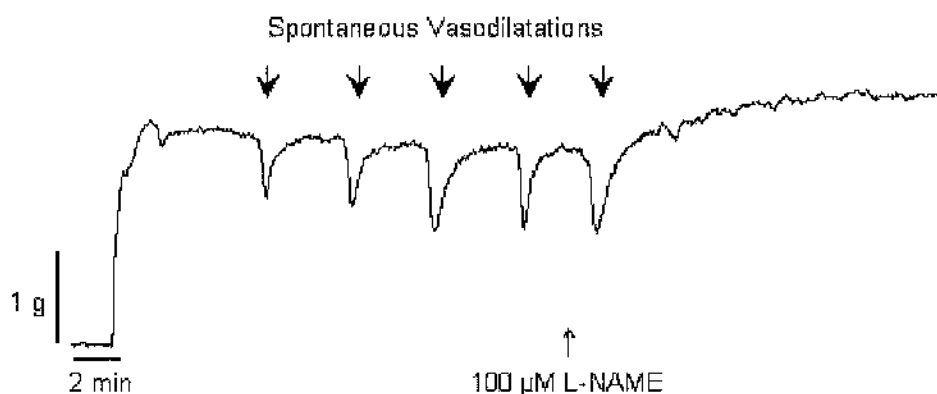


Figure 7-12: Raw traces demonstrating the effect of L-NAME (100 μ M, upward arrow) on spontaneous vasodilations (downward arrows) in the EVA following the induction of tone using NA (10 μ M).

Discussion

Spontaneous activity of blood vessels or tissues is a phenomenon that is either rarely observed or rarely reported. In the current study, many incidences of spontaneous activity with differing characteristics occurred and an examination of this activity provided a novel insight into the basal control of vascular function.

Spontaneous increases in endogenous tone were observed in all genital arterial preparations which could be returned to a pre-contractile baseline by multiple changes of the bathing media; suggesting that the release of endogenous factors was responsible for triggering spontaneous activity. Endogenous tone could also be returned to a pre-contractile baseline by α -AR antagonists in all vessels except the IVA. Additionally in the cavernous artery, endogenous tone was increased by incubation with NA uptake blockers; signifying that basal tone was due to NA acting at vascular smooth muscle ARs. If endogenous tone were due to spontaneous myogenic activity, this would not involve the activation of ARs by extracellular NA and so neither uptake blockers nor AR antagonists would be expected to have an effect. In turn, release of endogenous NA implies that tonic activity of adrenergic neurons was responsible for subsequent contraction of the smooth muscle; a hypothesis which could be tested using TTX to block neurogenic activity. This supports literature suggesting that erections induced by injection of α -AR antagonists and the association of priapism (prolonged erection) following treatment with α_1 -AR selective antagonists are due to a basal tonic sympathetic activity in penile tissues (Brindley, 1986; Saenz de Tejada *et al.*, 1991; Munoz *et al.*, 1994; Zorogniotti, 1994; Becker *et al.*, 1998; Marquer & Bressolle, 1998). However, blockade of receptors or neural mechanisms involved in adrenergic transmission does not universally lead to erection, or in this study reduction of endogenous tone, so perhaps spontaneous myogenic activity was partly responsible. Myogenic activity is frequently due to release of Ca^{2+} from internal stores (Griffith *et al.*, 2005), a pathway which could be tested using nifedipine, an L-type Ca^{2+} channel blocker.

It is interesting to note that spontaneous increases in endogenous tone were more frequently observed in the cavernous artery than in any other genital artery preparation. The cavernous tissues, and in particular the cavernous artery, have a pivotal role in penile erection since increased arterial inflow via this artery is one of the first stages of an erectile response. Therefore, basal vasoconstriction of the cavernous artery is particularly important in maintenance of penile flaccidity in the resting state. NA uptake blockers did

not increase endogenous tone in IVA, EVA or dorsal arteries and reduction of spontaneous endogenous tone by α -AR antagonists was less often observed in the female arteries. This suggests that basal release of endogenous NA is of less significance in female arteries than males and the action of α -AR antagonists in reducing endogenous tone may be via an inverse agonist action, i.e. inhibition of constitutively active receptors.

In opposition to endogenous contractile factors, the release of NO occurred in all genital arterial preparations, as shown by increased endogenous tone in the presence of L-NAME. Therefore, not only was NO a predominant inhibitory factor in dorsal arteries but it was also basally released to maintain resting tone. With the available information it was not possible to determine whether basal NO release was due to endothelial or nerve activity, and therefore eNOS or nNOS activity respectively. In the IVA, a combination of NOS and K^+ channel inhibitors was required to reveal maximal increases in endogenous tone while incubation with K^+ channel blockers increased endogenous tone in all vessels except the EVA. This suggests that basal K^+ channel activity, potentially due to release of endogenous EDHF, is also responsible for the maintenance of basal tone in the genital arteries. In addition in the IVA, NO and EDHF are each capable of partially compensating for a loss of the other in maintaining basal tone in these arteries.

Spontaneous rhythmic activity, induced by pre-contraction with α -AR agonists, was blocked by L-NAME in the dorsal artery, K^+ channel blockers in the cavernous artery and a combination of both in female vaginal arteries. This provides further evidence for the importance of a basal NO release in the dorsal arteries. In the cavernous arteries, K^+ channel activity predominated in the regulation of basal tone while a combination of endogenous factors, including NO and EDHF, mediated endogenous tone in the female vaginal arteries. Agonist-induced rhythmic activity was also observed in rabbit vaginal strips (Oh *et al.*, 2003), being more prevalent in the upper and middle than lower regions, but no evidence was provided that this activity could be blocked.

The novel finding of large relaxations of the EVA which could be blocked by L-NAME demonstrated the physiological importance of NO in these arteries.

Summary

In summary, evidence was provided for spontaneous basal activity in all genital arteries studied (Table 7-2). The existence of spontaneous activity prior to and coinciding with antagonist addition also provided some insights into underlying basal mechanisms

responsible. Factors controlling spontaneous vasoconstriction and vasodilation of the genital arteries may originate from neurons, smooth muscle or endothelial cells. Tonic sympathetic nerve activity has previously been implicated in the maintenance of basal tone in male cavernous tissues and it would, therefore, be reasonable to assume that tonic activity of other nerves, e.g. parasympathetic nitrergic nerves, may also act to oppose smooth muscle contraction. Myogenic activity has been suggested to be responsible for the regulation of peripheral resistance and oxygenation; a role that may be of particular importance in the penile tissues. In addition, results obtained in the current study implicated a spontaneous basal release of endothelial factors.

Basal tone in both EVA and dorsal arteries was maintained by a balance of NO release and AR activity. These factors may be due to basal neurogenic activity involving both NA and NO release from adrenergic and nitrergic neurons respectively. Equally, NO may be released spontaneously from the endothelium and ARs may be constitutively active. In EVA, the unique occurrence of spontaneous vasodilation suggests that relaxation mechanisms predominate in this vessel, predisposing the artery to vasodilation.

In the IVA, data suggests that basal levels of spontaneously released endothelial factors maintain tone in this vessel. Both basal K^+ channel activity, potentially due to EDHF release, and NO release exist and some pathway redundancy was shown to occur between NO and EDHF in this respect. The nature of the opposing vasoconstrictor factor could not be determined on the evidence available.

Basal tone in the cavernous artery was maintained by a balance of EDHF and adrenergic nerve activity. In this artery basal adrenergic nerve activity appeared to predominate, predisposing the artery to vasoconstriction; an attribute that would be of particular physiological relevance in the cavernous tissue.

Further investigation would be required to further elucidate the mechanisms behind basal tone of genital arteries. In particular, protocols would be designed to include time and vehicle controls in addition to further investigations into the effects of blocking nerve activity or Ca^{2+} channels. Basal mechanisms of vasoconstriction and vasodilation may have an important role in genital tissue circulation and may, therefore, be an important consideration in the pathology of sexual dysfunction.

Table 7-2: Summary of spontaneous activity in the genital arteries and the effect, increase (↑), decrease (↓) or no change (-), of antagonists and blockers on spontaneous activity.

Activity	Drugs	Cavernous	Dorsal	IVA	EVA
Increase in endogenous tone	Control (% of vessels)	34%	5%	7%	7%
	Prazosin (1 to 100 nM)	↓	↓	-	↓
	Rauwolfscine (0.1 to 10 μM)	↓	-	-	-
	Uptake blockers (Cocaine, 3 μM and Corticosterone, 30 μM)	↑***	-	-	-
	L-NAME (100 μM)	↑	↑**	↑***	↑***
	K ⁺ channel blockers (Apa, 100 nM and ChTX, 100 nM)	↑*	↑**	↑**	-
Tone-induced rhythmic activity	L-NAME plus K ⁺ channel blockers	↑	↑***	↑***	↑***
	Control (% of vessels)	33%	64%	49%	21%
	Indomethacin (1 μM)	-	-	-	-
	L-NAME (100 μM)	-	↓	-	-
	K ⁺ channel blockers (Apa, 100 nM and ChTX, 100 nM)	↓	-	-	-
	L-NAME plus K ⁺ channel blockers	↓	↓	↓	↓
Pronounced vasodilation	Control (% of vessels)	-	-	-	7%
	L-NAME (100 μM)	-	-	-	↓

Significance vs controls, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

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Chapter 8

General Discussion

Pharmacological and physiological data on vasoconstrictor and vasodilator responses of genital arteries is extremely limited. In the current study, small vessel wire myography combined with EFS and a plethora of agonists, antagonists and blocking agents were used to enhance current understanding of AR-mediated vasoconstriction, NO- peptide- and purine-mediated and endothelium-dependent vasodilation in isolated genital arteries from male and female New Zealand White rabbits. By improving our current understanding of genital vascular function a more coherent and sophisticated approach to the treatment of both male and female sexual dysfunction will be made possible.

While current therapies for male ED have enjoyed the recent successes of oral PDE-5 inhibitors, even these do not provide a definitive answer for all patients. In particular, patients with diabetes do not respond well to PDE-5 inhibitors and the use of PDE-5 inhibitors is contra-indicated in any patients taking nitrates or with a high cardiovascular risk (Reffelmann & Kloner, 2005; Wyllie, 2006). Therefore, the current study may provide the rationale for alternative approaches, e.g. targeting peptide or endothelial pathways, which could help to treat a further subset of both male and female patients. In addition, while this study has considered the peripheral mechanisms involved in sexual function of male and female tissues, it has not considered the central mechanisms thought to be of particular importance in the female. A combination of peripherally and centrally acting pharmacotherapies may provide a more effective approach; but whether this is a route which major leading manufacturers of compounds used to treat sexual dysfunction wish to pursue remains to be seen.

Adrenoceptor-Mediated Responses

Several important vasoconstrictor and vasodilator pathways in the genital arteries were investigated in detail during the current study. Male and female arteries were shown to be innervated by sympathetic adrenergic nerves using immunohistochemistry and/or EFS. During the investigation of adrenoceptor-mediated pathways, NA-induced vasoconstriction and active NA uptake mechanisms were demonstrated in both penile and vaginal arteries. AR-induced vasoconstriction was mediated by a combination of α_{1A} -ARs and α_2 -ARs, potentially the α_{2A} -AR subtype, in male penile arteries; providing clarification of α_1 -AR pharmacology in a field containing conflicting evidence for all three α_1 -AR candidates. The α_2 -AR was shown to be of particular importance in the vasoconstriction of dorsal penile arteries; reflected by the potency order of AR agonists, UK 14,304 > Medetomidine > NA = PE. α_2 -ARs have previously been shown to mediate NA-induced vasoconstriction

of cavernous arteries (Hedlund & Andersson, 1985a) and in the current study, while α_2 -ARs could not be excluded, a clearer role for α_{1A} -ARs was demonstrated in the cavernous arteries. Therefore, when considering treatments for male sexual dysfunction a combination of α_1 - and α_2 -AR antagonists may provide a more efficient approach to inhibition of sympathetic nerve-mediated vasoconstriction.

In female vaginal arteries the presence of functional α_1 -ARs was confirmed. However, α_2 -ARs were shown to make little or no contribution to AR-mediated responses in isolated vaginal arteries but had previously been demonstrated in vaginal tissue strips. The lack of α_2 -AR-mediated responses highlights a clear gender difference between male and female rabbits that was reflected in the order of agonist potencies in female vaginal arteries, NA = UK 14,304 = PE = Medetomidine. This study was the first investigation of AR-mediated responses in isolated vaginal arteries and provides a rationale for the use of α_1 - but not α_2 -AR antagonists as a potential therapy to enhance vaginal blood flow.

Evidence was found for basal AR activity, either via spontaneous NA release from nerves or constitutive AR activity, which could be involved in the maintenance of endogenous tone in both male and female genital arteries. To date, constitutively active receptors have only been shown in cell cultures (McLean *et al.*, 2002) and their significance, or indeed presence, in *in vitro* or *in vivo* environments is unknown. The presence of basal tone has implications for the development of novel treatment strategies since this would need to be overcome to allow vasodilation of genital arteries during sexual arousal.

Nitric Oxide-Mediated Responses

The contribution of NO to endothelium-dependent, direct smooth muscle, and nerve mediated relaxation was investigated during the current study. Considering the wealth of data proposing NO as the main NANC neurotransmitter in males using strips of penile tissue, surprisingly little evidence was found for NO involvement in EFS-induced relaxation in isolated male penile arteries. In female vaginal arteries, a greater inhibition of EFS-induced relaxation by blockade of NOS activity using L-NAME was demonstrated than in male penile arteries. In female arteries, L-NAME predominantly inhibited EFS-induced responses at low frequencies while an L-NAME-resistant component persisted at high stimulation frequencies.

Direct stimulation of smooth muscle by the NO donor drug, SNP, caused vasodilation of both male and female genital arteries. However, this relaxation was not potentiated by the

PDE-5 inhibitor, UK 343,664. These findings directly contradict previous data from cavernous arteries showing increased NO-mediated vasodilation (exogenous NO added as acidified sodium nitrate (NaNO_2)) in the presence of sildenafil (Simonsen *et al.*, 2001) and are a major consideration for the use of PDE-5 inhibitors in male ED. Further research is required to determine the PDE type involved in NO-mediated vasodilation of genital arteries. In particular, the effect of UK 343,664 on baseline tone, EFS-induced vasodilation and ACh-induced vasodilation would be studied to fully characterise UK 343,664 responses in the genital arteries. In addition, a comparison of UK 343,664 with alternative PDE-5 inhibitors (e.g. sildenafil) and inhibitors of different PDE isoforms (e.g. PDE-1, 2, 4, 9 or 10) would provide further information about the phosphodiesterases important to the function of male and female genital arteries. NO was found to contribute significantly more to endothelium-dependent relaxation of male than female genital arteries; which begs the question of whether PDE-5 inhibitors potentiate a neurogenic or endothelium-dependent increase in NO production. The main synthase activity in isolated male genital arteries, therefore, appears to be eNOS while in female vessels NO production is more likely to be via activity of nNOS. This finding may be of considerable significance when determining future approaches aimed to potentiate vasodilation of genital blood vessels in both genders.

ACh-induced, endothelium-dependent relaxation of male and female genital arteries was mediated by a combination of NO and K^+ channel activity. K^+ channel activity was demonstrated by inhibition of K^+ channels using Apa and ChTX and was, therefore, potentially due to release of EDHF. While prostaglandins were previously found to have direct vasodilator effects in penile and vaginal tissues, no role was demonstrated in ACh-induced relaxation of male or female genital arteries in the current study. Relative contributions of NO and K^+ channels (EDHF-like vasodilation) to endothelium-dependent vasodilation demonstrated significant gender differences. Vasodilation was predominantly mediated by NO in male dorsal arteries, K^+ channels in female EVA and a combination of both in smaller IVA and cavernous arteries. These findings confirm data that has previously concluded that a combination of NO and K^+ channels are responsible for endothelium-dependent relaxation of human and horse cavernous arteries (Prieto *et al.*, 1998; Angulo *et al.*, 2003). The greater contribution of EDHF than NO to relaxation of female genital arteries provides an explanation for the lack of effect of PDE-5 inhibitors in the treatment of FSD. Contrary to previous studies, the involvement of EDHF was demonstrated to be greater in upstream sections of the vaginal artery (EVA) compared to smaller downstream sections (IVA). Due to a lack of literature in this area, it is difficult to ascertain if this is a common trend in female systemic circulation or specific to the

genitalia. This study was the first to determine the relative contributions of EDHF and NO to endothelium-dependent relaxation of male and female genital arteries. Uniquely, the importance of EDHF was highlighted in female arteries, along with a partial role in male arteries.

Basal release of NO was found to contribute significantly to the balance of factors maintaining endogenous tone in both male and female genital arteries. The unique observation of L-NAME-sensitive, pronounced, spontaneous vasodilations in the EVA demonstrated the importance of NO in controlling vascular tone in this artery. Basal K^+ channel activity, thought to be due to a basal release of EDHF, also influenced endogenous tone in all genital arteries. In the IVA, NO and EDHF pathways showed some degree of compensation in that blockade of either pathway alone was significantly less effective than blockade of both pathways together.

Peptide- and Purine-Mediated Responses

L-NAME-resistant, EFS-induced vasodilation was shown not to be due to ATP in the female genital arteries. In contrast to published data in vaginal tissues, no direct vasodilator effect of ATP was demonstrated in isolated female vaginal arteries. However, functional P_{2X} receptors (ATP-induced vasoconstrictions) were demonstrated; suggesting that ATP may be involved in sympathetic, excitatory neurotransmission. Until now, no previous studies have investigated this role for ATP in isolated genital arteries.

Functional VIP receptors were demonstrated in female genital arteries and the presence of VIPergic neurons was confirmed by immunohistochemical studies. A role for VIP as a neurotransmitter in the EVA could not be excluded but the concept could not be fully tested due to the lack of selective antagonists. This peptide was previously thought to be compatible with a neurotransmitter role (Hedlund & Andersson, 1985b) but to date no studies have managed to conclusively delineate the physiological function of VIP. However, an additional, unknown vasodilator neurotransmitter remained unidentified in both male and female genital arteries and identification of this compound may solve the characterisation of L-NAME-resistant, non-adrenergic, non-cholinergic neurotransmission.

Gender Differences

One of the aims of this study was to provide a balanced investigation of vasoconstrictor and vasodilator mechanisms important in male and female genital arteries. This provided

the unique opportunity to make a direct comparison of mechanisms important to vascular function in both sexes.

When comparing the magnitude and potency of responses to various vasoconstrictor, adrenoceptor agonists, including NA, PE, UK 14,304 and medetomidine, clear differences became apparent. Vasoconstrictions in the EVA consistently gave the greatest magnitude but were least potent, while vasoconstrictions in the cavernous artery gave the smallest magnitude but were most potent. The size and potency of responses in the IVA and dorsal arteries lay between. Due to the volume of this data and its dispersion throughout previous chapters, it is summarised here in the following figure and corresponding table (Figure 8-1, Table 8-1). This data suggested that IVA and dorsal arteries may be comparable in function. However, while dorsal arteries demonstrated a significant contribution from both α_{1A} - and α_2 -ARs to adrenoceptor mediated vasoconstriction, α_2 -ARs were found not to have a significant role in female arteries.

Responses of IVA and dorsal arteries to vasodilator agonists, including SNP, ACh and VIP, were similar in potency and magnitude. Responses to vasodilator agonists are summarised in the following figure and corresponding table (Figure 8-2, Table 8-1). In addition, EVA and cavernous artery vasodilator responses were similar in magnitude and potency. It is interesting that EVA and cavernous arteries were comparable in vasodilator but not vasoconstrictor responses, where they represented opposite ends of an extreme. EVA and cavernous arteries were the most comparable in physiological function, being the main inflow arteries in both sexes, and therefore may have similar requirements in regard to vasodilation. However, detailed investigation of vasodilator pathways in the genital arteries demonstrated significant gender differences. Endothelium-dependent relaxation of genital arteries particularly demonstrated a greater involvement of EDHF in females and NO in males. In contrast, EFS-induced relaxation of genital arteries demonstrated a greater involvement of NO in females than in males.

This study demonstrates that while male and female genital systems may show some general similarities, the specifics of signal transduction pathways are often different. In this respect the function of female genital tissues may not be directly inferred from the more extensively studied function of male genital tissues. Consequently, simply using available data from males does not provide a suitable approach to understanding the physiology of female tissues. It is my opinion that each gender should be studied in isolation and that these studies may provide the basis for different approaches to sexual dysfunction in men and women.

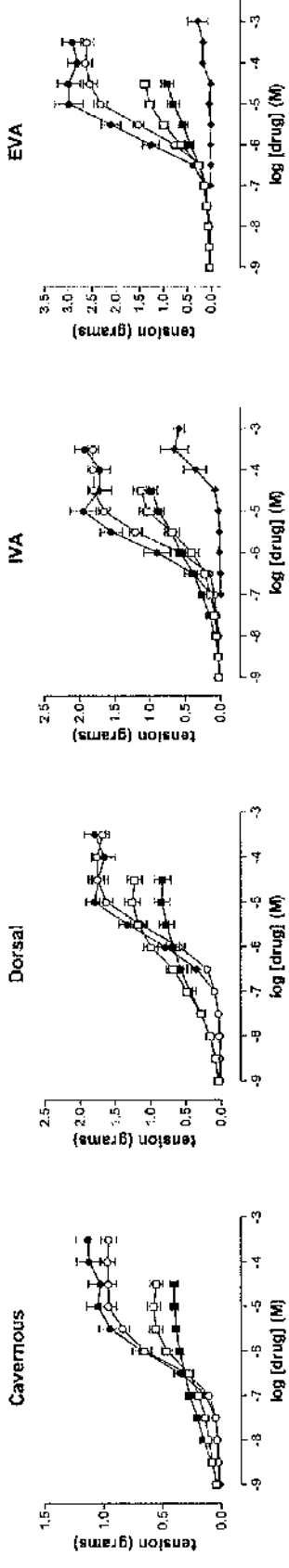


Figure 8-1: Comparison of vasoconstrictor agonists in the genital arteries, NA (●), PE (○), UK 14,304 (■), medetomidine (□) and ATP (◆) expressed as grams tension ± s.e.mean.

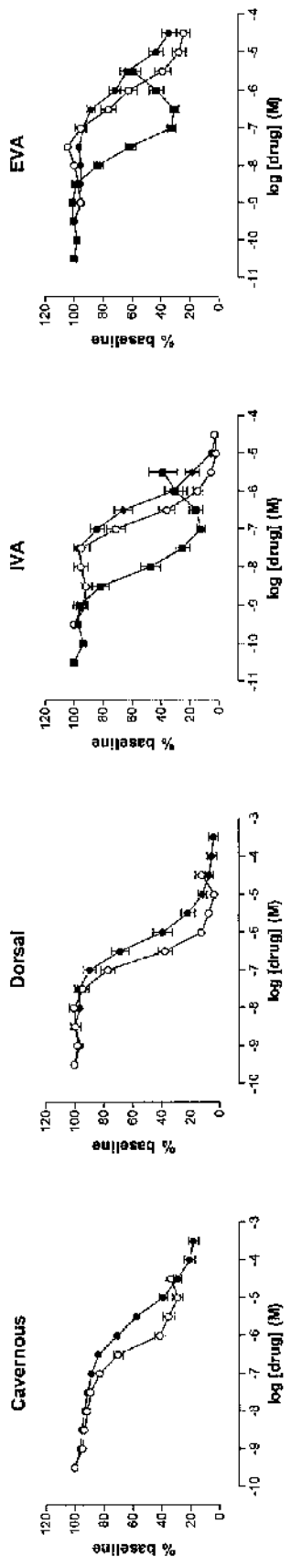


Figure 8-2: Comparison of vasodilator agonists in the genital arteries, SNP (●), ACh (○) and VIP (■) expressed as a percentage of NA (10 μm) induced sighting response ± s.e.mean.

Table 8-1: Comparison of maximal responses and potencies of vasoconstrictor and vasodilator agonists between genital arteries.

	Cavernous			EVA		
	Dorsal	IVA	EVA	Dorsal	IVA	EVA
Vasoconstrictor agonists	E_{max} grams (s.e.mean)			pEC₅₀ (s.e.mean)		
NA	1.08 (0.11)	1.87 (0.12)	1.81 (0.12)	2.88 (0.25)	6.27 (0.07)	5.92 (0.08) 5.81 (0.19) 5.94 (0.10)
PE	0.97 (0.08)	1.85 (0.10)	1.69 (0.15)	2.55 (0.23)	6.28 (0.04)	5.74 (0.05) 5.73 (0.18) 5.84 (0.19)
UK 14,304	0.43 (0.01)	0.88 (0.02)	0.98 (0.08)	0.92 (0.13)	7.86 (0.16)	7.18 (0.07) nd nd
Medetomidine	0.55 (0.06)	1.28 (0.11)	1.13 (0.11)	1.39 (0.10)	6.43 (0.10)	6.47 (0.14) nd nd
Vasodilator agonists	% Maximum Relaxation (s.e.mean)			pEC₅₀ (s.e.mean)		
SNP	81.79 (3.76)	95.82 (2.55)	97.88 (1.28)	65.48 (4.70)	5.46 (0.08)	6.21 (0.12) 6.24 (0.06) 5.52 (0.17)
ACh	70.96 (3.40)	96.23 (1.28)	98.17 (1.02)	75.78 (4.39)	6.42 (0.05)	6.70 (0.04) 6.68 (0.05) 6.03 (0.09)
VIP	nd	nd	87.50 (3.22)	69.19 (3.23)	nd	nd 8.11 (0.06) 7.59 (0.05)

nd: not determined

Future Studies

While the current study has made significant advances into the understanding of genital artery pharmacology, several areas have been identified that would benefit from further investigation.

In particular, the use of EFS raised almost as many questions as it answered. These included the demonstration of EFS-induced, TTX-resistant responses. The stimulation parameters used would be expected to lead to a nerve-induced response and would not be expected to result from direct smooth muscle stimulation. Direct nerve varicosity depolarisation, TTX-resistant Na^+ channels or a Na^+ channel-independent neurotransmission have previously been hypothesised to account for TTX-resistant neurotransmission (Campbell, 1993; Jackson *et al.*, 2002; Ikeda *et al.*, 2005).

Investigation into the role of NO and peptides in vasodilator neurotransmission revealed a potential role for VIP in the EVA and the presence of an unknown vasodilator neurotransmitter in all arteries that both require further investigation. In addition, further studies would be required to investigate the role of purinoceptors in EFS-induced vasoconstriction, a role that could not be excluded in the current study and that has not previously been shown.

Significant volumes of data have been presented herein describing the ARs involved in vasoconstriction of isolated male and female genital arteries, including full subtyping of α_1 -ARs, demonstration and partial subtyping of α_2 -ARs in male penile arteries and characterisation of the role of α_1 - and α_2 -ARs in female vaginal arteries. However, due to the constraints of time and resources, the α_1 - (female) and α_2 -AR (male and female) subtype/s responsible for vasoconstriction were not fully tested.

Many interesting points were raised as to the exact role of NO in vascular function of male and female genital arteries. In particular the role of PDE-5 in both male and females was questioned and this area would require further investigation to determine the PDE subtype responsible for the breakdown of cGMP in isolated genital arteries. Determination of the specific NO pathways, including identification of important NOS and PDE subtypes, would provide a more coherent rationale for the use of drugs such as PDE-5 inhibitors in the treatment of male and female sexual dysfunction.

The identification of spontaneous activity in genital arteries was made during the current study. This indicated the basal release of endogenous vasoconstrictor and vasodilator factors in both genders. Since these observations were made during the course of studies, sufficient time was not available to assess this phenomenon in isolation. Therefore, only suggestions could be made as to the mechanisms responsible for spontaneous activity in genital arteries. Basal mechanisms could be rigorously examined using protocols designed to investigate the time course of responses and their dependence on tonic nerve activity, constitutive receptors and/or myogenic activity.

A brief immunohistochemical analysis of female vaginal tissues was carried out during this study. While some interesting observations were made, the findings presented here would be enhanced further by studies to identify other nerve classes and their proportionate representation in the vaginal tissues. The presence of specific neurons and their location may provide an indication of the mechanisms likely to contribute to the control of both vascular and non-vascular smooth muscle.

The current study demonstrated the successful use of wire myography to examine the function of isolated genital arteries. This provided information concerning one of the three main events of penile erection; which include increased arterial inflow, decreased venous outflow and trabecular smooth muscle relaxation. In addition, the function of corpus cavernosum strips, and therefore trabecular smooth muscle, has previously been investigated in some depth. A few studies have begun to consider the role of active venous regulation, in addition to the veno-occlusive mechanism, in the control of venous outflow (Kirkeby *et al.*, 1991; Holmquist *et al.*, 1992; Segarra *et al.*, 1998; Recio *et al.*, 2004). Small vessel wire myography could be applied to examine the role of vasoconstrictor and vasodilator pathways controlling male and female genital veins.

Pathologies including heart failure, diabetes, hypercholesterolaemia and aging have all been implicated in the development of sexual dysfunction. A natural progression from the studies presented here on the normal vascular function of genital arteries would be to study these same mechanisms in animal models of pathological states.

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