CHARACTERISATION OF NON-ADRENERGIC, NON-CHOLINERGIC RESPONSES IN THE VAGINAL WALL

A thesis submitted for the degree of

Doctor of Philosophy from the University of London by:

Tom Ziessen

Registered at: The Wolfson Institute for Biomedical Research,

Cruciform Building, University College London,

Gower Street, London. WC1E 6BT

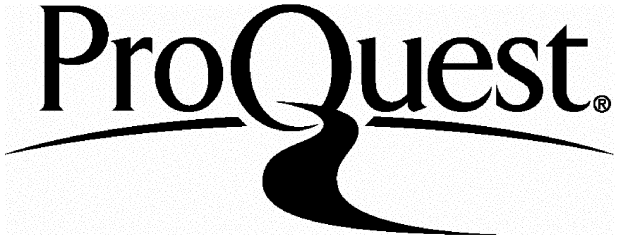
The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without the prior written consent of the author.

ProQuest Number: U641973

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.



ProQuest U641973

Published by ProQuest LLC(2015). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code.
Microform Edition © ProQuest LLC.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

This thesis describes experiments to characterise non-adrenergic, non-cholinergic (NANC) relaxation responses in the rabbit vaginal wall. Specifically the roles of nitric oxide (NO), neuropeptides, purines and pyrimidines as mediators of these responses were investigated.

The results of this investigation suggest that after inhibition of adrenergic and cholinergic responses, electrical field stimulation (EFS) of pre-contracted vaginal wall strips results in NANC relaxations. These relaxations are partially mediated by NO acting via soluble guanylate cyclase to increase levels of the second messenger cyclic guanosine-3', 5'-monophosphate (cGMP). However the major proportion of these responses is not mediated by NO and is not accompanied by increased cGMP or cyclic adenosine-3', 5'-monophosphate (cAMP).

Vasoactive intestinal peptide (VIP)-related neuropeptides are able to concentration-dependently induce relaxation responses in the vaginal wall. However, my results suggest that these neuropeptides are not involved in NANC relaxations, since they are associated with increased cAMP and are inhibited by α-chymotrypsin, whilst NANC relaxations are unaffected. Other neuropeptides investigated did not induce relaxations, and so are unlikely to be involved in mediating NANC responses in the vaginal wall.

Purines and pyrimidines were also found to induce relaxation responses in the vaginal wall. Furthermore adenosine- and ATP-induced responses are associated with increased cAMP levels. However the fact that inhibitors of these responses have no effect on NANC relaxations and the fact that NANC relaxations are not associated with increased cAMP levels suggest that purines and pyrimidines are also not mediators of NANC responses in this tissue.

NANC relaxations of vaginal wall strips from ovariectomized rabbits were also investigated, but were not found to differ greatly from those from control animals.

In conclusion, NANC relaxation responses in the rabbit vaginal wall from both normal and ovariectomized animals are in part mediated by NO, whilst the mediator of the major component of these responses remains unidentified.

ACKNOWLEDGEMENTS

I would like to thank Selim Cellek and Salvador Moncada for their support, supervision and enthusiasm in my work.

I also want to thank Pfizer for their sponsorship, in particular to Dr. Rob Wallis for his support and interest in my work, not to mention a few free lunches.

I would also like to thank Dr. Andrew Batchelor for a large amount of help, supervision and for many useful discussions in my first year.

I would like to thank Dr. Brian King for generously giving his time to discuss my results and for his help in getting to grips with the field of purine and pyrimidine signalling.

I would also like to thank Dr. Adrian Hobbs, Prof. John Garthwaite and Annie Higgs for generously giving their time to read and comment on this thesis.

I am grateful to the entire pharmacology wing of the Wolfson Institute for Biomedical Research for their help and support and for putting up with my bad moods. In particular to Linda Connelly for her friendship and cheerfulness, and for reminding me that there was an end in sight.

Lastly I want to thank my family for their continued support and love, and for being proud of my work in a field that many (less enlightened) people might find a little embarrassing.

TABLE OF CONTENTS

ABBREVIATIONS

ABBREVIATION	FULL NAME
ACh	Acetylcholine
ADP	Adenosine 5'-diphosphate
AEA	Arachidonylethanolamide
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
cAMP	Cyclic adenosine-3', 5'-monophosphate
cGMP	Cyclic guanosine-3', 5'-monophosphate
CB	Cannabinoid
CGRP	Calcitonin gene-related peptide
ChTX	Charybdotoxin
CO	Carbon monoxide
CRC	Concentration response curve
ω-CTX	ω-conotoxin GVIA
DAG	Diacylglycerol
2'3'-DDA	2', 3'-Dideoxyadenosine
2'5'-DDA	2', 5'-Dideoxyadenosine
DETA-NONOate	(2,2'-hydroxynitrosohydrazino)*bis*-ethanamine
EDRF	Endothelium derived-relaxing factor
EFS	Electrical field stimulation
FSD	Female sexual dysfunction
FSAD	Female sexual arousal disorder
GDPβS	Guanosine 5'-O-(2-thiodiphosphate)
GPCRs	G-protein coupled receptors
Hb	Haemoglobin

IbTX	Iberiotoxin
IP_3	Inositol 1,4,5 trisphosphate
i.v.	Intra-venous
α, β-methylene ATP	α, β-meATP
β, γ-methylene ATP	β, γ-meATP
2-methythio ATP	2MeSATP
LDV	Large dense-cored vesicle
L-NA	N^G-nitro-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl L-arginine
L-NIO	N-iminoethyl-L-ornithine
MALDI-TOF MS	Matrix assisted laser desorption ionisation-time of flight mass spectrometry
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphorylase
MRLC	Myosin regulatory light chain
NANC	Non-adrenergic, non-cholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NPY	Neuropeptide Y
ODQ	1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one
PACAP	Pituitary adenylate cyclase activating peptide
PBS	Phosphate buffered saline
PCA	Perchloric acid

PHM	Peptide histidine methionine
PHI	Peptide histidine isoleucine
PHV	Peptide histidine valine
PLC	Phospholipase C
PPADS	Pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid
8-PT	8-Phenyltheophylline
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
TEA	Tetraethylammonium chloride
TTX	Tetrodotoxin
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate
VIP	Vasoactive intestinal peptide
VOCC	Voltage-operated calcium channel
ZnPP	Zinc (II) protoporphyrin IX

LIST OF FIGURES AND TABLES

CHAPTER I

INTRODUCTION

I: 1 GENERAL INTRODUCTION

a) History of the study of female sexual function

Before 1950 the consensus opinion of female sexuality was based on the psychoanalytical works of Freud. In his essays on sexuality he claims that the vagina is the locus of female eroticism in the mature female, with the clitoris being the locus of female eroticism in the infantile girl, only playing the function of transmitting the sexual excitation to the adjacent sexual parts in the mature woman (Freud, 1901). This 'understanding' of female sexuality was however not supported by any anatomical evidence until 1950 when Grafenberg described an erogenous zone on the anterior wall of the vagina (Grafenberg, 1950). This was refuted a few years later when evidence was presented that the clitoris was highly sensitive to tactile stimulation, whilst the vagina was much less sensitive (Kinsey *et al.*, 1953). Krantz published a histological study in 1958 supporting this claim when he described the sparse innervation of the vagina with corpuscular sensory receptors compared to the skin of the clitoris (Krantz, 1958).

Studies of the physiological changes that occur during sexual response cycles were studied in far greater detail by Masters and Johnson, who studied over 7,500 complete cycles of sexual responses in females (Masters & Johnson, 1966). They described the sexual response by defining four periods during the sexual responses in which characteristic changes could be observed. These phases were the excitement phase, the plateau phase, the orgasmic phase and the resolution phase. During the excitement phase they described the labia minora (see Figure 1) as increasing in diameter by two to

three times, accompanied by a vivid colour change that accompanies the vasocongestion. These colour changes were termed the "sex skin" and no premenopausal woman was observed to develop this colour change and not go on to experience orgasm, presuming the stimulation continued without major alteration. Their studies also showed that the Bartholin's glands (see Figure 1) located in each side of the minor labia (see Figure 1), which had previously been assumed to be responsible for vaginal lubrication, do secrete a mucoid material in response to sexual stimulation, but that this was found to be minimally involved in lubrication responses. Instead they found that vaginal lubrication occurs as a transudate through the walls of the vagina as a result of massive localized vasocongestion, early in the excitement phase. Also during the excitement phase they noted a lengthening and distension of the vaginal barrel, particularly the inner two-thirds. As the plateau phase starts some distension occurs in the outer vagina, as well as marked localized vasocongestion.

As well as these observations Masters and Johnson also made various assertions including that the 'primary focus' for the female sexual response is the clitoris. This dogma of vaginal insensitivity reported by Krantz and supported by Masters and Johnson continued until the mid-70s, despite several authors defending the erotic relevance of the vagina (Fox & Fox, 1969; Fisher, 1973).

In 1978 however a histological study found numerous nerve endings in the sub-epithelial region of the vaginal lamina propria (see Figure 2), refuting the earlier study by Krantz (Burgos & Roig de Vargas Linares, 1978). In 1980 Hoch provided systematic

experimental evidence that vaginal stimulation could bring women to the 'plateau phase' of Masters and Johnson (Hoch, 1980a; Hoch, 1980b). This research was supported by Perry and Whipple in 1982 (Perry & Whipple, 1982) when they described the presence of an erogenous zone on the anterior vaginal wall (see Figure 2), which was later named the G (Grafenberg) spot in honour of the first author describing this 'zone' almost 30 years previously (Hoch, 1983). The presence of a discrete 'spot' has been a point of contention, as the posterior wall has also been found to be an erogenous zone in a significant minority of the subjects of Perry and Whipple, as well as in studies by Alzate and Londoño (Alzate & Londoño, 1984).

At the end of the 70s as the debate over the relative importance of the vagina in sexual arousal continued, work started to take place to quantitatively assess the haemodynamic changes that take place in the genitalia during sexual arousal using the non-invasive techniques of photoplethysmography and the heated oxygen electrode. Photoplethysmography measures blood flow by shining a light into the tissue, and measuring the amount scattered or reflected back (Challoner, 1979). This technique allows measurement of pulsatile changes and changes in the total blood volume in the illuminated area, and thereby allows quantitative measurements of arousal-induced vasocongestion and vasodilatation. However the technique suffers from the disadvantage that tissue movement may lead to artefacts, which can be a problem in measuring changes in vaginal haemodynamics (Wagner & Levin, 1978).

The heated oxygen electrode works by placing a heated electrode (usually at 43°C) on the surface of the tissue. Heat is removed from the electrode by conduction into the blood and the power needed to keep the electrode at the set temperature is directly related to local blood flow under the electrode (Huch *et al.*, 1979). The advantage of this technique is that it is not influenced to such a degree as photoplethysmography by movement artefacts since the electrode is held in one place by a suction cup (Levin *et al.*, 1982).

Measurements using these techniques allowed objective confirmation of the changes described by Masters and Johnson in 1966. The genital response, initiated by arousal consists of a local vasocongestive response and orgasm. The vasocongestive phase was termed the "lubrication-swelling" phase (Kaplan, 1974). In this phase the vagina becomes engorged, and a lubrication response occurs. Using photoplethysmography and the heated oxygen electrode it has been shown that there is low blood flow and a very low oxygen tension in the vagina, in the absence of sexual arousal (Wagner & Ottesen, 1980; Levin *et al.*, 1982). During sexual arousal vaginal blood flow was found to be increased with accompanying increased oxygenation (Wagner & Levin, 1978). This increased blood flow that led to engorgement of the vaginal tissue suggested that the increased arterial inflow is not initially matched by the venous drainage. The resulting increased pressure leads to elevated transudation, leading to increased vaginal lubrication. The three physiological components of the female genital sexual response: vaginal wall engorgement, increased lubrication, and clitoral erection are all associated with changes in smooth muscle tone. Smooth muscle

relaxation can be mediated by a number of different pathways including local neurogenic release of vasorelaxant substances or changes in levels of circulating hormones or other vasorelaxant substances. However the onset of the vasocongestive and lubrication responses in the vaginal wall take place within seconds of initiation of arousal, suggesting that this process is mediated by neural mechanisms (Levin, 1980). Other evidence that also points to neurogenic control of vaginal engorgement and lubrication comes from paraplegic women who have electrodes implanted on the sacral nerves (parasympathetic innervation of the vagina arises from sacral spinal segments 2, 3, and 4) to assist in bladder and rectal control. In these women stimulation of these nerves (to empty their bladders) leads to vaginal lubrication (Brindley, 1988), and in a study with a single patient stimulation of sacral spinal segments 2 and 3 increased vaginal blood flow as measured by photoplethysmography (Levin & MacDonagh, 1993).

As with other organs of the urogenital tract the tone of vaginal and the clitoral corpus cavernosum smooth muscle is controlled by adrenergic, cholinergic and non-adrenergic non-cholinergic (NANC) neurotransmitters. Adrenergic nerves release noradrenaline, which causes contraction of smooth muscle via α-adrenoceptors and can cause relaxation via β-adrenoceptors. α-adrenoceptor-mediated contractions have been demonstrated in both the vaginal wall and the clitoral corpus cavernosum in the rabbit by the contractile effect of α-adrenoceptor agonist phenylephrine (Cellek *et al.*, 1999; Cellek & Moncada, 1998). β-adrenoceptor-mediated relaxations have not been demonstrated in these tissues, but have been shown in the human penile corpus cavernosum (Adaikan &

Karim, 1981). Cholinergic nerves release acetylcholine (ACh) and early studies to identify the neurotransmitter responsible for mediating vaginal smooth muscle relaxation focused on ACh as the putative neurotransmitter. However, it was found that blockade of muscarinic sites by administration of atropine has no effect on vaginal lubrication (Riley & Riley, 1983). The role of NANC neurotransmitters as mediators of relaxation of vaginal wall smooth muscle is the subject of this thesis.

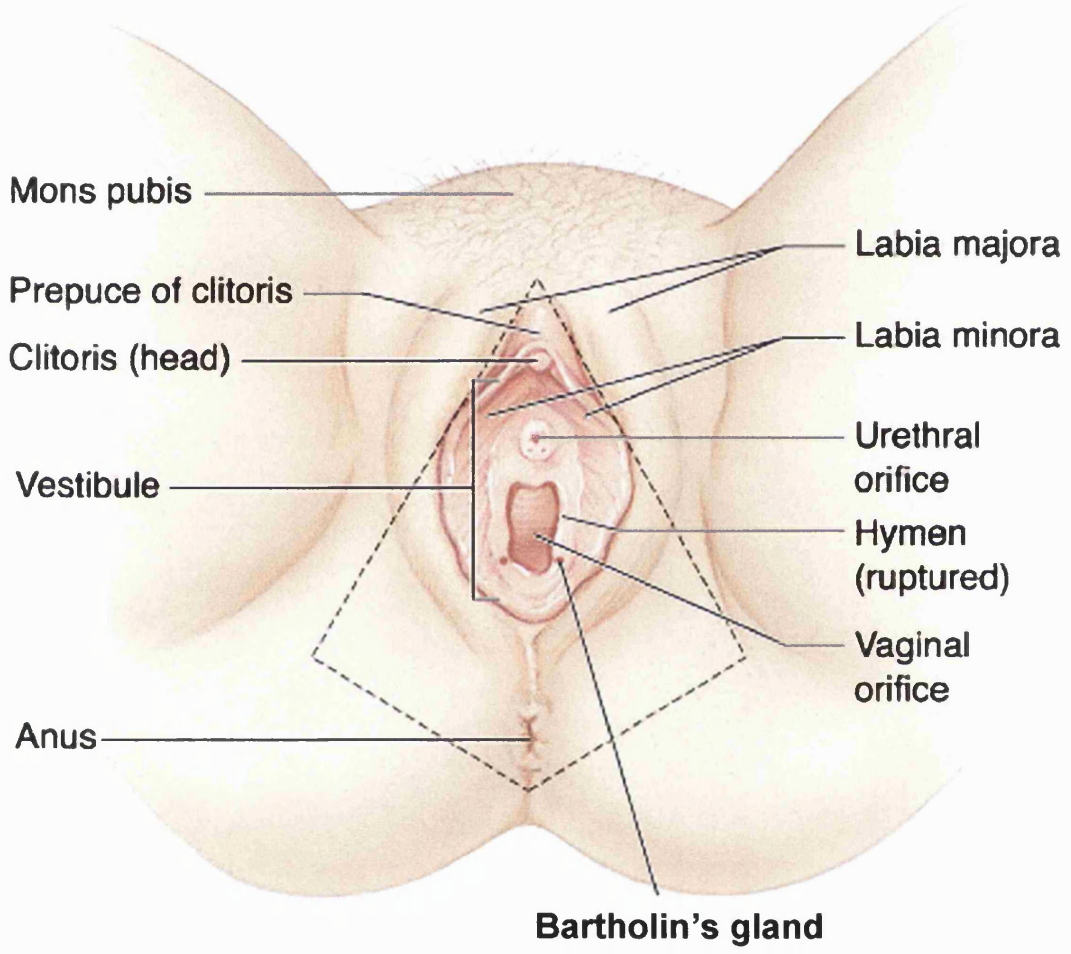


Figure 1: Human female external genitalia.

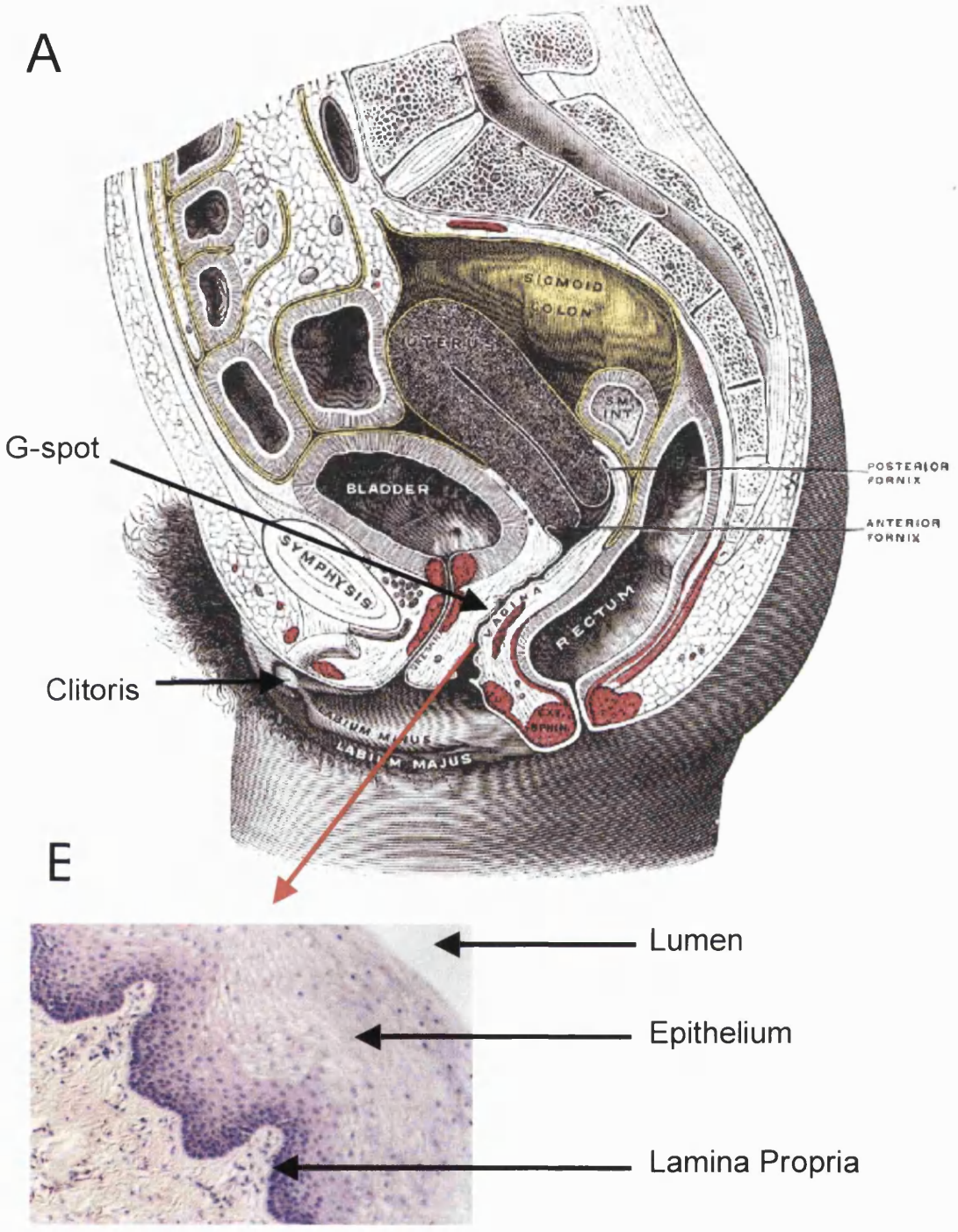


Figure 2: Human female internal genitalia. A: Cross-section through the human female abdomen showing structures of the genitalia. **B:** Microscopic section of the vaginal wall showing epithelium and lamina propria.

b) Inhibitory NANC neurotransmission

Over a century ago in 1895 it was shown that responses induced by stimulation of some parasympathetic nerves innervating the bladder of dogs, cats and rabbits were resistant to blockade by atropine (Langley & Anderson, 1895). However in 1914 Dale described the peripheral action of ACh as a vasodilatation that mimicked the effects of stimulating nerves of the parasympathetic nervous system. He found that these effects were abolished by atropine, and proposed ACh as a likely candidate for the neurotransmitter of the peripheral parasympathetic fibres (Dale, 1914). Earlier, post-ganglionic sympathetic nerve stimulation of the ileo-coccal sphincter had been shown to cause contractions that were mimicked by adrenaline, or a related substance (Elliott, 1904). These observations led Dale to propose the terminology "adrenergic" and "cholinergic", based on the chemical function of the adrenaline related substance that was later found to be noradrenaline, and the choline related substance that had already been suggested to be ACh. He further suggested that postganglionic sympathetic fibres are predominantly, though not entirely, adrenergic and that postganglionic parasympathetic fibres were predominantly, and perhaps entirely cholinergic (Dale, 1933).

This view predominated for some time, and the non-cholinergic responses did not lead to a great deal of further study until far more recently when it was shown that stimulation of some inhibitory autonomic nerves induced responses that were not mediated by either noradrenaline or ACh. In the guinea-pig taenia caecum it was shown that transmural electrical stimulation after atropine revealed relaxations, which were only partially inhibited by guanethidine (which blocks adrenergic sympathetic transmission by

inhibiting release of noradrenaline from postganglionic adrenergic neurons). These relaxations could be inhibited by the local anaesthetics cinchocaine and procaine indicating a neuronal origin. These relaxation responses persisted after 4 days of storage at 4°C, and it was suggested that this was due to the survival of inhibitory neurons that were intramural, and therefore protected from damage (Burnstock *et al.*, 1966). Similar responses were also shown in the rat anococcygeus muscle, where blockade of adrenergic neurons with guanethidine revealed inhibitory responses that were atropine-resistant, and tetrodotoxin-sensitive (Gillespie, 1972). These responses were termed NANC responses since they were not mediated by noradrenaline or ACh.

These early studies on NANC relaxation responses have led to a massive increase in the study of these responses, and to an appreciation of the importance of NANC neurotransmitters in the control of many biological systems including the cardiovascular system (see Ahluwalia & Cellek, 1997 for review), the gastrointestinal tract (see Curro & Preziosi, 1998 for review) and the urogenital tract (for review see Andersson & Holmquist, 1994; Andersson & Persson, 1995).

c) Criteria for NANC neurotransmitter candidates

It has been proposed and generally acknowledged that to be accepted as a neurotransmitter, candidate substances must fulfil several criteria (Eccles, 1964). These are that

- For every chemical synapse, the neurotransmitter substance must be present in sufficient quantities in the presynaptic terminals to account for the action on the postsynaptic cell. The presynaptic nerve must also contain the enzymes required to synthesize the transmitter.
- The neurotransmitter must be released into the synaptic cleft in response to the arrival of an action potential at the presynaptic element, or endfoot.
- The neurotransmitter then must bind with a receptor molecule on the outer surface of the postsynaptic membrane to produce its characteristic effects.
- Finally, the neurotransmitter substance must be removed from the synapse, either by returning the molecule to the presynaptic element or by inactivating the molecule through a biochemical transformation.

In order to identify the neurotransmitters involved in the inhibitory NANC responses we must bear these criteria in mind. Firstly we need to ensure that the proposed neurotransmitters are present and that the enzymes necessary for their synthesis are present in the neurons.

Secondly blockade of the action potential should inhibit the neurogenic response. The sodium channel inhibitor tetrodotoxin (TTX) selectively blocks the voltage-gated sodium channels on the axon. Blockade of presynaptic sodium channels with TTX prevents propagation of the action potential, so the endfoot of the nerve does not become depolarised and no neurotransmitter is released. Depolarization of the endfoot causes voltage operated calcium channels (VOCCs) to open, and the influx of calcium leads to calcium dependent release of neurotransmitters. Blockade of N-type VOCCs using ω-conotoxin GVIA can be used to block this calcium entry, and so provides another way of preventing neurotransmitter release.

The third characteristic can be tested for by exogenous application of the proposed neurotransmitter. The exogenously applied substance should be able to mimic responses seen when the neurons are stimulated, for it to be considered as a candidate neurotransmitter for the neurogenic responses observed.

The last criteria can be tested for pharmacologically if the mechanism of removal of the proposed neurotransmitter is already known. In this case blockade of the enzymes responsible for the removal of the neurotransmitter should potentiate responses whether generated by exogenous application of neurotransmitter or by stimulation of the nerves.

I: 2 NITRIC OXIDE AS A NANC NEUROTRANSMITTER

a) Discovery of nitric oxide as a signalling molecule

Nitric oxide (NO) has been used therapeutically for over 100 years. In 1867 amyl nitrate, a compound that had been recently synthesised was suggested as a treatment for angina, as it reduced angina pain, as well as reducing blood pressure (Brunton, 1867). However, this drug proved to lose its efficacy with repeated treatment. In 1879 a more effective therapy using the nitrovasodilator, nitroglycerin, was suggested as a treatment for angina (Murrell, 1879). However, the mechanism of action of these drugs (donation of NO) and the physiological significance of NO has only recently become apparent, through various observations.

Firstly, during experiments to study guanylate cyclase, azide was added to inhibit GTP hydrolysis, but this was found to activate the enzyme soluble guanylate cyclase (sGC), leading to increased cGMP in homogenized liver or in brain slices (Kimura *et al.*, 1975a; Kimura *et al.*, 1975b). It was found that nitrovasodilators including nitroglycerin and sodium nitruprusside produced similar smooth muscle relaxations, guanylate cyclase activation and an increase in the intracellular second messenger cGMP (Katsuki & Murad, 1977; Katsuki *et al.*, 1977).

Endothelium-derived relaxing factor (EDRF) was discovered in 1980 when rings of rabbit thoracic aorta were observed to relax in response to administration of ACh (Furchgott & Zawadzki, 1980). Previously ACh had been mainly observed to cause

contractions when used *in vitro*, and this was found to be due to loss of endothelium during tissue preparation, since rubbing of the intimal surface removed the relaxant effect. Demonstration of the relaxant factor was achieved by sandwiching endothelium intact longitudinal strips (which had no contractile effects in the experiments due to the orientation of the smooth muscle) with endothelium-denuded circular strips. Application of ACh in these transfer experiments caused relaxation of the endothelium-denuded strip, and it was concluded that this was due to ACh stimulating the release of an unidentified substance, that was later termed EDRF (Furchgott *et al.*, 1984). These endothelium-dependent relaxations were also found to be associated with increased levels of cGMP (Rapoport & Murad, 1983). In contrast, nitrovasodilator induced relaxations were found to be endothelium independent, but were also associated with increased levels of cGMP (Rapoport *et al.*, 1983).

In 1986 Furchgott and Ignarro independently suggested that EDRF might be NO at a meeting at the Mayo Clinic in Rochester, Minnesota. However, it was not until 1987 that the first direct evidence that EDRF was NO was provided when bradykinin-induced EDRF release from cultured endothelial cells was shown via chemiluminescence to be NO, and that these amounts of EDRF could cause relaxation of vascular strips, that was identical to that caused by NO (Palmer *et al.*, 1987). The precursor to this NO was then shown to be L-arginine in two ways. Firstly, depriving the cultured endothelial cells of L-arginine decreased the amount of EDRF that was released, and this effect could be reversed by the addition of L-, but not D-arginine; secondly, incubation of the cells with

^{15}N labelled L-arginine gave rise to ^{15}NO, also showing that the nitrogen was derived from the terminal guanidino nitrogen atom of L-arginine (Palmer *et al.*, 1988).

b) Nitric oxide as a neurotransmitter

Evidence for neuronal NO synthesis was obtained before EDRF had been identified as NO. In 1982 it was found that L-arginine, but not D-arginine stimulated sGC activity in a fraction of the homogenate from a neuroblastoma cell line (Deguchi & Yoshioka, 1982). Subsequently after EDRF was shown to be identical to NO, and at the same time as NO was being shown to be synthesised from L-arginine, EDRF was suggested to have a role as a neurotransmitter when it was found to be released from neurons in the cerebellum due to calcium influx following activation of NMDA receptors with glutamate (Garthwaite *et al.*, 1988). This was followed by further evidence that NO was a neurotransmitter when it was shown that in the cerebellum glutamate stimulated conversion of L-arginine to citrulline, that this conversion was stoichiometric with regards to increases in cGMP, and that both were blocked by an analogue of L-arginine that had previously been shown to inhibit EDRF production, N^G-monomethyl L-arginine (L-NMMA; Bredt & Snyder, 1989; Garthwaite *et al.*, 1989).

Before these studies showing NO to be a neurotransmitter in the central nervous system an investigation was carried out comparing EDRF-induced responses and neurogenic NANC responses. It was found that both EDRF-induced relaxations in the aorta and neurogenic relaxations in the bovine retractor penis were abolished by an

inhibitor of sGC, methylene blue, but that the relaxations in the bovine retractor penis were not endothelium-dependent (Bowman *et al.*, 1986). Subsequently, further evidence was provided that EDRF, by then identified as NO, was the mediator of NANC relaxation responses in several tissues from different species. It was found that NANC relaxation responses in the rat anococcygeus were inhibited by L-NMMA (Gillespie *et al.*, 1989), and that this was reversed by L-arginine (Li & Rand, 1989). In the mouse anococcygeus NANC relaxations were shown to be inhibited by L-NMMA and another inhibitor of NO synthesis, N^G-nitro-L-arginine (L-NA), and this inhibition was also reversed by L-arginine (Gibson *et al.*, 1990). In the bovine retractor penis NANC relaxations were reduced by L-NMMA but abolished by L-NA, both effects being reversed by L-arginine (Liu *et al.*, 1991).

NO is now a well-characterised neurotransmitter in the peripheral nervous system and mediates the NANC relaxation responses in many tissues in the genital tract including those of the clitoral (Cellek & Moncada, 1998) and penile (Ignarro *et al.*, 1990; Kimura *et al.*, 1992; Cellek & Moncada, 1997a) corpora cavernosae and the vaginal wall (Cellek *et al.*, 1999; Giraldi *et al.*, 2001). Nerves that release NO, and NO-mediated neurotransmission are now known as nitrergic (Li & Rand, 1991; Moncada *et al.*, 1997). Nitric oxide is generated in the nitrergic nerves by activation of the neuronal nitric oxide synthase (nNOS) which catalyses the oxidation of the terminal guanidino nitrogen of L-arginine leading to the production of NO and citrulline. NO diffuses into the smooth muscle and activates sGC, producing an increase in the intracellular cGMP concentration, leading to relaxation.

c) Pharmacology of nitrergic neurotransmission

The use of pharmacology to study physiological processes requires an idea of the biochemical pathways involved and secondly requires drugs that are both potent and specific inhibitors of the enzymes involved in those pathways.

In order to study the role of NO as a NANC neurotransmitter we need to keep in mind the criteria for neurotransmitters discussed in section I: 1c. NO is an uncharged gaseous molecule that can freely cross biological membranes, and for this reason cannot be stored within vesicles as for classical neurotransmitters. It is generally considered that NO is not stored in the neuron, but is synthesised *de novo* upon nitrergic nerve stimulation (Snyder, 1992). However it has been suggested that NO could be stored as a nitrosothiol compound, which is more stable and cannot freely diffuse across biological membranes (Thornbury et al., 1991; Sanders & Ward, 1992). This compund, stored within vesicles could then be released upon nerve stimulation as for classical neurotransmitters. Whether NO is synthesised upon nerve stimulation, or whether it is first synthesised, and stored as a nitrosothiol to be released upon subsequent stimulation, its synthesis requires the presence of nNOS.

Thus if NO is to be considered as a potential mediator of NANC responses then nNOS must be present in the presynaptic neurons. If present then its involvement in NANC responses can be studied by the use of NOS inhibitors. Inhibition of NOS will prevent synthesis of NO and NANC responses mediated by NO will be abolished if inhibition is complete. The NOS inhibitors N^G-nitro-L-arginine methyl ester (L-NAME),

N^G-nitro-L-arginine (L-NA) and L-NMMA are all chemically modified derivatives of the NOS substrate L-arginine, and along with a structurally closely related compound, N-iminoethyl-L-ornithine (L-NIO) all non-selectively inhibit NO synthesis by all NOS isoforms. As previously mentioned L-NMMA has been used to demonstrate the involvement of NO in NANC relaxation responses in the rat anococcygeus (Gillespie *et al.*, 1989; Li & Rand, 1989), and L-NMMA and L-NA were used to show the presence of nitrergic responses in the mouse anococcygeus (Gibson *et al.*, 1990). Furthermore the involvement of NO in NANC relaxation responses in various tissues of the genital tract has been shown using these inhibitors. In isolated human and rabbit penile corpus cavernosum strips NANC responses have been shown to be inhibited by L-NMMA (Kim *et al.*, 1991). L-NA has also been shown to reduce NANC relaxations in the human penile corpus cavernosum (Pickard *et al.*, 1991). L-NAME and L-NIO have also been shown to inhibit NANC relaxation responses in the rabbit penile corpus cavernosum (Teixeira *et al.*, 1998). Furthermore, nitrergic NANC relaxations in the rabbit clitoral corpus cavernosum are abolished by L-NAME (Cellek & Moncada, 1998).

The inhibition by these NOS inhibitors is competitive with L-arginine, so the concentrations required to effectively abolish NOS activity will be dependent on L-arginine concentration. For this reason caution should be taken in drawing conclusions when lack of complete inhibition of responses is observed. An observation such as this could mean that another neurotransmitter is responsible for the remaining response, or it could mean that the concentration of inhibitor relative to L-arginine is not sufficient to abolish activity. Another consideration with using these NOS inhibitors is that L-NMMA

has been found to be metabolised to L-arginine in vascular endothelial cells (Hecker *et al.*, 1990), and furthermore has been found to be ineffective at inhibiting nitrergic relaxations in the bovine retractor penis, actually reversing the inhibitory effect of L-NA in this tissue (Martin *et al.*, 1993). Also in the rabbit anococcygeus L-NA, but not L-NMMA, has been shown to inhibit EFS-induced nitrergic relaxations (Cellek & Moncada, 1997b). Thus, L-NMMA is not a suitable NOS inhibitor for studying the role of NO in NANC responses.

The second criterion for proposed neurotransmitters is that they should be released into the synaptic cleft or, in the case of smooth muscle innervation, into the neuromuscular junction. If NO is released then this can be detected by direct or indirect means. Nitric oxide can be detected directly using a NO-selective electrode. This method has been used to detect NO release from cultured endothelial cells (Tsukahara *et al.*, 1993), but has not been used to detect neurogenic NO release from smooth muscle preparations. Nitric oxide is highly labile making direct detection difficult, however in oxygenated solution it is converted to the more stable oxidation products nitrite and nitrate. These can then be detected in the solution by the Greiss reaction and this has been used as evidence for the involvement of NO in the rabbit penile corpus cavernosum and anococcygeus (Ignarro *et al.*, 1990; Kasakov *et al.*, 1995).

The passage of released NO from the nerve to the muscle provides a second point for possible pharmacological intervention. EDRF- and nitrovasodilator-induced responses were found to be inhibited by oxyhaemoglobin (Hb; Martin *et al.*, 1985), and this was

because NO reacts with Hb with high affinity to form methaemoglobin and NO_3^- (Kelm & Schrader, 1990). Because of this Hb can be used to inhibit NO-mediated responses. Indeed Hb has been shown to reduce EFS-induced NANC relaxation responses in the bovine retractor penis and the rat anococcygeus muscle (Bowman & Gillespie, 1982; Gillespie & Sheng, 1989). Furthermore Hb has been shown to reduce NANC relaxation responses in both human and rabbit penile corpus cavernosum strips (Kim *et al.*, 1991). However inhibition by this method requires sufficient Hb concentrations in the neuromuscular space to bind NO before it reaches its target cells. Therefore use of Hb as a tool for examining the role of NO in NANC responses is limited, since lack of complete inhibition may represent limited accessibility of Hb in sufficient quantity to bind NO, rather than suggesting that NO is not involved in the response.

The third criterion for a neurotransmitter, that the neurotransmitter must bind with a cell surface postsynaptic receptor to effect its target cells, has had to be amended with the identification of NO as a neurotransmitter, since NO can freely diffuse into its target cells. The main receptor for NO is sGC, which is activated by NO to synthesise the second messenger cGMP. This allows a further point for pharmacological intervention, since sGC inhibitors such as ODQ and methylene blue can be used to inhibit NO-induced responses. However, methylene blue has sGC-independent effects on NO mediated responses, and has been shown *in vitro* to be a direct inhibitor of purified cerebellar NOS at lower concentrations than those required for inhibition of S-nitrosoglutathione-induced sGC activity (Mayer *et al.*, 1993). Methylene blue has also been shown to inhibit rat hippocampal NOS activity *in vivo* (Volke et al., 1999). Furthermore methylene blue has

non-selective effects on other pathways such as inducing muscarinic contraction of the guinea-pig trachea (Hwang *et al.*, 1998). In light of these findings, results obtained with this compound cannot be considered to be due to sGC inhibition. ODQ on the other hand is a selective inhibitor of sGC and has been shown to be an effective tool for investigating the role of NO in the genital tract. ODQ has been shown to inhibit EFS-induced NANC relaxations in the rabbit anococcygeus (Cellek *et al.*, 1996). It has also been found to inhibit nitrergic NANC relaxations in isolated rabbit penile (Teixeira *et al.*, 1998) and clitoral corpus cavernosum strips (Cellek & Moncada, 1998) as well as human penile corpus cavernosum strips (Teixeira *et al.*, 2001).

Further evidence that NO mediates a response can be obtained by mimicking the response using exogenously applied NO, NO-donors and NO-independent sGC activators. Use of NO-independent sGC activators provides limited useful information since the time-course for a response mediated by a NO-independent sGC activator will depend on entry into the target cell, mechanism of activating sGC and kinetics of its deactivation, all of which are likely to differ from that of NO, thus these compounds have limited use in the study of NO-mediated responses. Also, until recently the only widely used NO-independent sGC activator was YC-1, and this has been found to have non-specific effects including inhibition of phosphodiesterases (PDEs; Galle *et al.*, 1999), and inhibition of extracellular calcium entry through a cGMP-independent mechanism (Wang *et al.*, 2001). Two sGC activators, structurally related to YC-1, BAY 41-2272 and BAY 41-8543, have recently been reported to activate sGC independently of NO (Stasch *et al.*, 2001; Stasch et al., 2002). These compounds may prove to be selective for sGC, and may

have uses in the study of nitrergic responses, but are still unlikely to mimic the time-course of endogenous NO-induced responses for the reasons mentioned above.

NO and NO-donors may be more effective at mimicking endogenous NO-induced responses. Indeed, in the identification of NO as EDRF the reproduction of the relaxation responses induced by endogenously released EDRF by exogenously applied NO, as well as the fact that both EDRF and NO had dimishing effects on successive tissue strips as they passed down a bioassay cascade provided strong evidence that EDRF was in fact NO (Palmer *et al.*, 1987). However, it is technically difficult to produce solutions of NO since in oxygenated solutions NO is converted to nitrite and nitrate. For this reason NO donors are more commonly used as sGC activators. Thus NO donors such as DETA NONOate can be used to mimic endogenous responses and provide evidence that NO acting via sGC activation may be the mechanism for endogenous responses.

NO-induced responses are terminated by the removal of NO, and its second messenger molecule cGMP. NO can be removed by many mechanisms including being sequestered by oxyhemoglobin, or by oxidation to nitrite or nitrate as previously mentioned. Cyclic nucleotides are degraded by PDEs, with the 3',5'-cyclic phosphate moiety cleaved to the corresponding 5'-nucleotide. To date eleven PDE families have been identified and characterised, these differ in their tissue distribution and substrate specificity for cGMP and cAMP (See Francis *et al.*, 2001 for review). Some of the members of the PDE1 family, as well as all known members of the PDE5, 6 and 9 families are specific for cGMP. And in tissues where these are involved in terminating

the NO-induced response, inhibitors of these enzymes can be used to study nitrergic neurotransmission since, in the presence of these compounds, NO-induced responses should be potentiated. Indeed in the bovine retractor penis zaprinast, an inhibitor of PDEs 1, 5, 6, 9 and 11, has been shown to potentiate NANC relaxation responses (Bowman & Drummond, 1984). Furthermore in the rat anococcygeus and in the human penile corpus cavernosum zaprinast has been shown to increase nitrergic NANC relaxations (Mirzazadeh *et al.*, 1991; Rajfer *et al.*, 1992). In the female genital tract both zaprinast and sildenafil, a PDE5-selective inhibitor, have been shown to potentiate NANC responses in isolated rabbit clitoral corpus cavernosum strips (Cellek & Moncada, 1998; Vemulapalli & Kurowski, 2000), suggesting that these compounds are effective at potentiating nitrergic responses in this tissue.

d) Summary

It is now generally accepted that NO is a physiological signalling molecule. It has been shown to be EDRF as well as being the mediator of NANC relaxation responses in various tissues. Nitrergic neurotransmission accounts for the NANC relaxation responses in the penile and clitoral corpus cavernosum, and appears be involved in the NANC relaxation responses of the vaginal wall.

There are many pharmacological tools available for use in the study of nitrergic neurotransmission, for example:

- NOS inhibitors can be used to inhibit NO production
- NO can be sequestered between the nerve and the effector cell using Hb
- The second messenger system used by NO can be inhibited with inhibitors of sGC

These tools can be used to investigate the role of NO in NANC relaxation responses in the genital tract.

I: 3 NEUROPEPTIDES AS NANC NEUROTRANSMITTERS

a) Isolation and characterisation of peptides

Vasoactive intestinal peptide (VIP) was first isolated from porcine intestine, and was found to be a 28 amino acid peptide with a wide range of biological activity, including the ability to cause systemic vasodilatation (Said & Mutt, 1970). VIP was later sequenced and was found to have a degree of homology with glucagon and secretin (Mutt & Said, 1974) and, due to this, was originally considered to be a gastrointestinal hormone. However, immunohistochemical and radioimmunochemical studies indicated VIP was localised to the nerves fibres innervating both the gastrointestinal tract and the hypothalamus, suggesting that VIP was in fact a neuropeptide with a wide distribution in the central and peripheral nervous systems (Larsson *et al.*, 1976; Fahrenkrug & Schaffalitzky de Muckadell, 1978), including nerves innervating the female genitourinary tract in multiple different species (Larsson *et al.*, 1977). In addition plasma VIP was shown to be increased following electrical stimulation of the vagal nerve in anaesthetized pigs (Schaffalitzky de Muckadell *et al.*, 1977). Subsequently *in vivo* experiments were performed in the presence of atropine in the feline gastrointestinal tract. In these experiments vagal nerve stimulation resulted in relaxation of the stomach and stimulation of the pelvic nerves to the colon resulted in vasodilatation of the veins from the large bowel. Both effects were accompanied by increased venous VIP concentrations from the stimulated organs and, since neurogenic gastric fundus relaxation was known to be mediated by NANC neurotransmission, it was suggested that VIP may have a physiological role as a NANC neurotransmitter (Fahrenkrug *et al.*, 1978). Direct evidence

that VIP can mediate inhibitory NANC responses was later obtained by *in vivo* experiments with the opossum lower oesophageal sphincter where relaxations induced by exogenous VIP or electrical stimulation of the vagal nerve were reduced by infusion of VIP antiserum (Goyal *et al.*, 1980).

VIP is derived from a 170 amino acid precursor peptide (prepro-VIP), which contains a second active sequence (residues 81-107) known as peptide histidine methionine (PHM). PHM was first discovered in the porcine upper intestine, as peptide histidine isoleucine (PHI) (Tatemoto & Mutt, 1980), and was later characterised and found to be a 27 amino acid peptide with high sequence homology with VIP (48% identity, Figure 3), secretin and glucagon (Tatemoto & Mutt, 1981). It was later found that the human orthologue of PHI differed in sequence by two amino acids, and unfortunately, from a terminology point of view, one of these alterations was that the N-terminal residue was methionine (Itoh *et al.*, 1983), and so the human peptide was designated PHM which has 44% sequence identity with VIP (Figure 3).

A larger form of PHM with unknown sequence and pharmacology was found to be present in other tissues including the genitalia (Yiangou *et al.*, 1986). This was later purified, and after characterisation, identified as a peptide derived from prepro-VIP (residues 81-122) and in line with earlier terminology it was designated peptide histidine valine (PHV) (Yiangou *et al.*, 1987).

VIP has been the most widely studied of a larger group of related peptides belonging to a family of similar peptides known as the secretin-glucagon-VIP family of peptides. Another peptide in this family is pituitary adenylate cyclase activating peptide (PACAP). PACAP was first isolated from the ovine hypothalamus and was found to be a 38-residue peptide whose NH_2-terminal sequence showed 68% identity with VIP (Figure 3). Its biological activity was tested in cultured anterior pituitary cells where, as its name suggests, it was found to stimulate adenylate cyclase activity. It was also tested in anaesthetised rats and was found to induce comparable vasodilatation to that induced by VIP (Miyata *et al.*, 1989). It was later found that PACAP-38 exists with the same sequence in human tissue and can also exist in a COOH-terminally truncated form, with only 27 residues, PACAP-27 (Kimura *et al.*, 1990; Miyata *et al.*, 1990). Shortly after their discovery both forms of PACAP were shown to have endothelium-independent vasorelaxant activity in the rabbit aorta, and vasodilatory action in humans (Warren *et al.*, 1991; Warren *et al.*, 1992).

Two other members of the secretin-glucagon-VIP family of peptides are helospectin I and II, first discovered and sequenced from Gila monster (Heloderma suspectum) venom (Parker *et al.*, 1984). Helospectin I is a 38-amino acid peptide, and helospectin II is identical apart from lacking serine-38 (Parker *et al.*, 1984). The NH_2-terminal region of these peptides shows 54% identity with VIP, but unlike the other VIP-related peptides the helospectins do not have amidated C-termini (Figure 3). Both of these peptides were shown to bind VIP receptors in rat liver membranes and were found to activate adenylate cyclase in these preparations (Vandermeers *et al.*, 1987). The

biological activity of the helospectins was later compared to VIP on isolated rat femoral arteries, and they were found to be able to relax these tissues to the same extent, but with a lower potency than VIP (Grundemar & Hogestatt, 1990). However it was not until 1992 that the presence of these peptides was shown by immunocytochemistry in nerve fibres in rodent and human gastrointestinal tract tissues (Absood *et al.*, 1992a).

Another peptide is generated by alternative processing of the mRNA for the hormone calcitonin. This was first observed in the rat hypothalamus and the peptide product produced was named calcitonin gene-related peptide (CGRP; Amara *et al.*, 1982). The observation that this alternate processing was tissue-specific and occurred in neural tissues suggested that CGRP was a novel neuropeptide, and further work showed it to be present in nerves throughout the central and peripheral nervous system (Rosenfeld *et al.*, 1983). In 1984 a related peptide with 89% homology was isolated from human medullary thyroid carcinoma tissue, and was named human CGRP (hCGRP). The human CGRP was shown to be a 37-residue peptide with a C-terminal amide and a secondary structure containing a disulphide bridge (Morris *et al.*, 1984). CGRP given intracerebroventricularly has been shown to stimulate sympathetic outflow, increasing plasma noradrenaline and causing vasoconstriction (Fisher *et al.*, 1983), however a role for CGRP as a mediator of vasodilatation has also been suggested, since intravenous CGRP lowers mean arterial pressure in rats, relaxes rat aorta in an endothelium-dependent, cyclooxygenase-independent manner, and decreases perfusion pressure in rat and rabbit mesenteric vasculature (Fisher *et al.*, 1983; Brain *et al.*, 1985; Marshall *et al.*, 1986). Human CGRP exits in two forms, hCGRP-I and hCGRP-II, which differ in

positions 3, 22 and 25. Neither shows significant homology with the VIP-related peptides mentioned above (Figure 3).

Another group of neuropeptides are the tachykinins, which include substance P, which was first identified in the intestine as a substance that could stimulate atropine-resistant contractions of rabbit ileum, but also lower arterial pressure, which was presumed to be due to vasodilatation (Euler & Gaddum, 1931). Forty years after its discovery it was isolated and purified, and found to be an 11-amino acid peptide (Chang & Leeman, 1970). Substance P was later shown to be released from rat spinal cord and it was proposed as an excitatory neurotransmitter in afferent fibers (Otsuka & Konishi, 1976; Otsuka *et al.*, 1976).

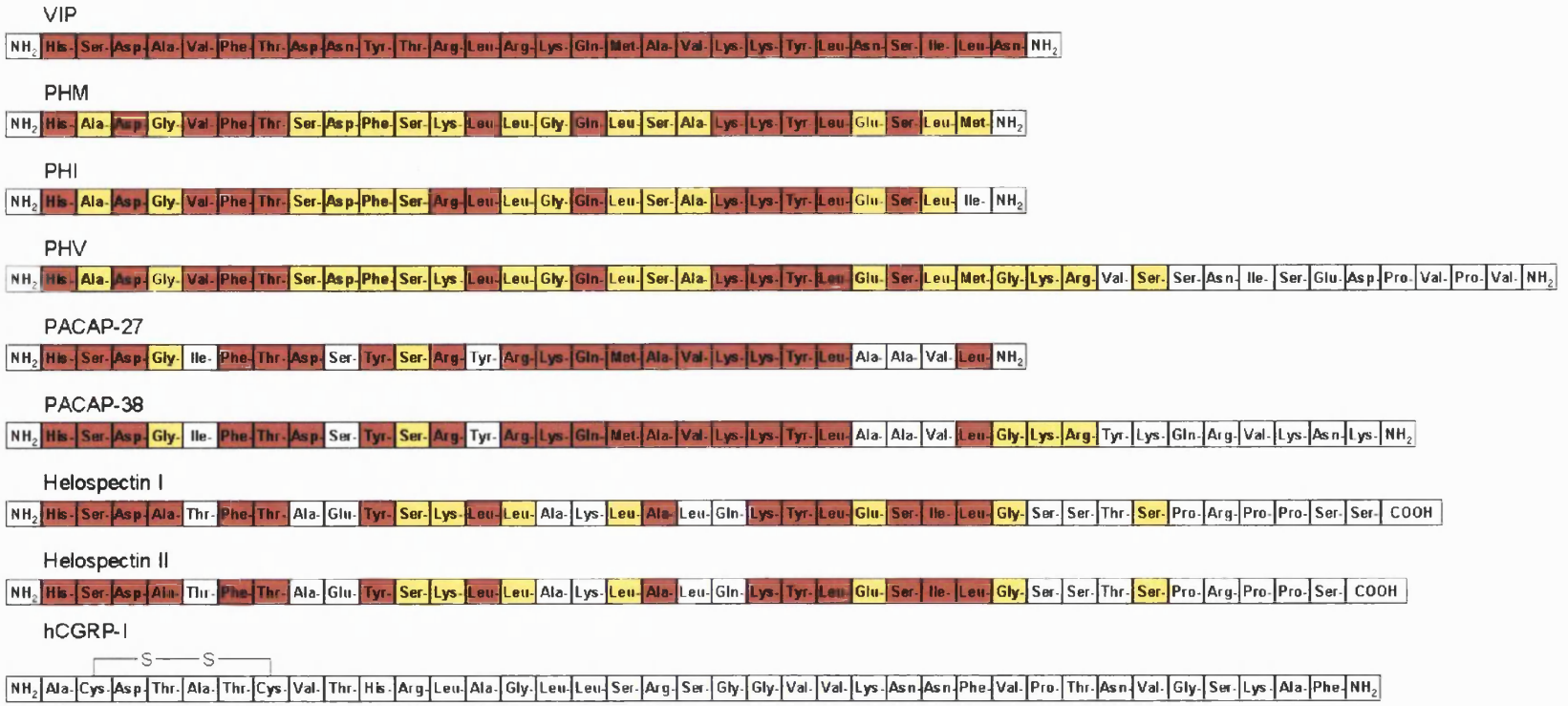


Figure 3: Comparison of peptide sequence of VIP, PHM, PHI, PHV,PACAP-27, PACAP-38, helospectin I, helospectin II and human CGRP (hCGRP-1). Amino acid residues in red indicate identity with VIP, those in yellow indicate sequence identity with PHV.

b) Peptide signalling pathways

All neuropeptides are produced by cleavages from a prepropeptide containing a N-terminal signal that targets it to the rough endoplasmic reticulum (RER). As it enters the RER the signal sequence is cleaved to leave the propeptide. This is shuttled through the Golgi apparatus, and is packaged into secretory granules at the *trans* face of the Golgi. Within these granules, called large dense-cored vesicles (LDVs), the final post-translational processing occurs, with peptides excised from their propeptides by endoproteases, and modifications such as C-terminal amidation occurring. This final modification and processing takes place on the LDV's journey from the cell body to the nerve terminals (Burks, 1994).

Once in the nerve terminal the LDVs await action potentials signalling their release. In 1985 Lundberg & Hökfelt proposed that peptides were responsible for slow onset, long duration responses, and suggested that peptide release required higher frequency stimulation than classical neurotransmitters (Lundberg & Hökfelt, 1985).

When neuropeptides are released from the presynaptic terminal they diffuse to the post-synaptic cell, and interact with cell surface receptors. So far all identified receptors for the secretin-glucagon-VIP family of peptides are G-protein coupled receptors (GPCRs) (see Ulrich *et al.*, 1998 for review). GPCRs consist of seven transmembrane helices connected by three intracellular, and three extracellular loops, with an extracellular N-terminal peptide, and a C-terminal cytoplasmic domain. CGRP and

related peptides are thought to act via receptor activity modifying proteins, which also have intracellular effects mediated through GPCRs (see Chu *et al.*, 2001 for review)

VIP interacts with high affinity to two GPCRs, originally named VIP_1 and VIP_2, currently known as $VPAC_1$ and $VPAC_2$ due to their ability to bind both VIP and PACAP with high affinity (Harmar *et al.*, 1998). $VPAC_1$ was first isolated from rat lung and expressed in mouse COP cells. In membrane preparations from these cells it was found that the receptor had similar binding affinities for VIP (IC_{50}=3nM), PACAP-27 (IC_{50}=2.5nM), PACAP-38 (IC_{50}=1nM) and PHM (IC_{50}=6nM), and that these peptides all induced cAMP production (Ishihara *et al.*, 1992). The $VPAC_1$ receptor has no known splice variants, but there is considerable species variability, with the human receptor (cloned by Couvineau *et al.*, 1994) having a far lower affinity for PHI and PHV than the rat when expressed in COS cells (IC_{50}=1000nM and 3000nM respectively). $VPAC_2$ was first cloned from the rat olfactory bulb (Lutz *et al.*, 1993), and subsequently from a human cell line (Svoboda *et al.*, 1994). As for $VPAC_1$ no splice variants of $VPAC_2$ have been described to date. In cell lines both human and rat $VPAC_2$ receptors show similar binding affinities for VIP (IC_{50}=3-4nM), PACAP-27 (IC_{50}=10nM), PACAP-38 (IC_{50}=2nM), PHI (IC_{50}=10nM) and PHV (IC_{50}=30nM), with all the peptides inducing cAMP production (Lutz *et al.*, 1993; Svoboda *et al.*, 1994). Both $VPAC_1$ and $VPAC_2$ have very low affinity for the VIP-related peptide, secretin (IC_{50}=1,500-30,000nM).

A third receptor having a far greater specificity for PACAP than the other VIP-related peptides has also been cloned from the rat pancreatic acinar carcinoma cell line

(Pisegna & Wank, 1993). The receptor was shown to have a high degree of homology with $VPAC_1$ (50% identity, 68% similarity) suggesting it was a member of the same GPCR subfamily. This receptor is now classified as the PAC_1 receptor (Harmar *et al.*, 1998). When expressed in cell lines human and rat PAC_1 receptors show similar affinity for PACAP-27 and PACAP-38 (both IC_{50}=1nM), and a similar lack of affinity for VIP (IC_{50}=1000nM). PHM (or PHI) and PHV bind the PAC_1 receptor with even lower affinity (Ciccarelli *et al.*, 1995). Unlike $VPAC_1$ and $VPAC_2$, PAC_1 receptors have six possible splice variants (Spengler *et al.*, 1993; Pantaloni *et al.*, 1996), which may differ both in their selectivity for PACAP-27 verses PACAP–38 and in their G-protein coupling and second messenger mechanisms (Journot *et al.*, 1995; Van Rampelbergh *et al.*, 1996). Four splice variants have also been described for the human PAC_1 receptor (Pisegna & Wank, 1996).

All the receptors for VIP-related peptides have significant sequence homology, possess a large extracellular NH_2-terminal domain with six conserved cysteine residues, and can all exist positively coupled to adenylate cyclase via G_s, although it should be noted that some of them might be coupled to other effectors such as phospholipase C (Spengler *et al.*, 1993; Van Rampelbergh *et al.*, 1997). The receptors have conserved basic amino acids in the third intracellular loop, which may be important in the coupling to G_s.

All the peptides of the VIP family and CGRP can cause elevation of cAMP, and intravenous injection of equal concentrations of these peptides has been shown to induce

increased plasma cAMP concentrations in mice (Absood *et al.*, 1992b). In contrast substance P did not induce any increase in cAMP levels (Absood *et al.*, 1992b).

The main target receptor for substance P is the NK-1 receptor, which binds substance P with affinity several orders of magnitude greater than the other tachykinins (Kage *et al.*, 1995). In the absence of active tone substance P has been found to induce contractile responses in rabbit pulmonary artery strips, but induced relaxant responses when the tone was raised (Obara *et al.*, 1989). Exogenously applied substance P has been shown to induce relaxation of precontracted rat tracheal smooth muscle, however the relaxation was inhibited by L-NAME or indomethacin indicating a role for NO or prostaglandin synthesis in mediating these responses (Mhanna *et al.*, 1999). The involvement of prostaglandins in substance P-induced relaxation responses was also suggested in intrapulmonary bronchi from rats since substance P was found to induce release of prostaglandin E2, and that this was inhibited by incubation with a NK-1 receptor antagonist (RP-67580) or the cyclooxygenase inhibitor meclofenamate (Szarek *et al.*, 1998).

c) Pharmacology of peptide signalling

Peptides to be considered as possible mediators of NANC responses must be present in the neurons innervating the tissue of interest in order to satisfy the first criterion for a neurotransmitter, and exogenous application of peptides must induce relaxation responses if they are to be considered candidates for inhibitory NANC neurotransmitters. Inhibition of the synthesis of the peptides does not represent a practical method for determining the involvement of peptides in NANC responses, since it is not possible to selectively inhibit the synthesis of potential neuropeptides without also inhibiting protein synthesis in general, which would obviously have far reaching general effects in all cell types studied. However various pharmacological agents are available to aid in the study of these potential neurotransmitters.

Firstly peptides can be depleted from the neurons, so that subsequent action potentials will have no stored peptide to release. This is possible if the peptides are contained within sensory neurons. The peptides CGRP and substance P have been shown to be localised to sensory neurons, and thus the sensory neurotoxin, capsaicin, can be used to study their roles in NANC neurotransmission. Treatment with capsaicin induces release of stored neuropeptides from sensory neurons, following which these nerves are desensitised. It has been shown in rat mesenteric resistance vessels that treatment with capsaicin both diminished CGRP containing nerves, and inhibited NANC relaxation responses to periarterial nerve stimulation (Kawasaki *et al.*, 1988). Furthermore capsaicin treatment has been found to specifically deplete CGRP and substance P, but not VIP from

nerves of guinea-pig cerebral arteries (Buck & Burks, 1986; Saito *et al.*, 1986). Substance P-induced prostaglandin release has also been shown to be mimicked by capsaicin treatment in rat intrapulmonary bronchi where capsaicin-induced responses were also shown to be inhibited by an antagonist of the NK-1 receptor (Szarek *et al.*, 1998).

Once released from the neuron pharmacological intervention can be used to influence peptide concentration in the synaptic cleft. The serine protease α-chymotrypsin cleaves peptides on the C-terminal side of the bulky aromatic amino acids tyrosine, tryptophan or phenylalanine, giving potential cleavage sites for all the peptides mentioned above. Indeed this peptidase has been used to successfully inhibit nerve induced relaxation responses in the guinea-pig trachea, however the inhibition observed was only partial (Moffatt *et al.*, 1999). Any inhibition suggests that the responses are mediated by peptides, however a lack of inhibition does not necessarily show that responses are not peptide-mediated. The large size of the enzyme may limit accessibility into the neuromuscular junction, and even with access the rate of peptide cleavage may not be fast enough to prevent released peptides reaching their receptors. Thus evidence for non-involvement of peptides arising from a lack of inhibition by α-chymotrypsin, should be interpreted cautiously, until there is sufficient supporting evidence to back up such a conclusion.

Peptides can also be sequestered using antisera raised against them, as was mentioned in section I: 3a when VIP antiserum was used to block neurogenic relaxations in the opossum lower oesophageal sphincter (Goyal *et al.*, 1980). However this type of

pharmacological intervention has the same limitation as the use of α-chymotrypsin, in that the large size of the antibodies makes it difficult to obtain access in sufficient concentrations in the neuromuscular space to bind all released peptide before it reaches its target receptors.

Another option is to attempt to enhance the amount of peptide presented to their receptors to try and potentiate the response. Neurogenic peptide signals are terminated by the breakdown of the peptides by endopeptidase enzymes. Inhibition of these endopeptidases should reduce peptide degradation and enhance the peptide-induced response. This type of pharmacological intervention has been used to enhance peptide induced responses in several tissues. In isolated guinea-pig tracheal smooth muscle the endopeptidase inhibitor phosphoramidon was found to increase the relaxant potency of peptide component 5-27 of atrial natriuretic factor (Fernandes *et al.*, 1992), and in guinea-pig colon smooth muscle the endopeptidase inhibitor DL-thiorphan prolonged and enhanced non-nitrergic NANC relaxation responses (Maggi & Giuliani, 1996). The advantage of this type of intervention is that the inhibitors are smaller than α-chymotrypsin or antibodies, and so have greater chance of reaching effective concentrations in the neuromuscular space. Also their target, the active sites of the endopeptidases can be blocked in advance of stimulation, so any effect does not require a chance interception of peptide between the neuron and muscle cell.

A fourth way to affect peptide-induced responses is to use specific receptor agonists and antagonists. With peptide signalling many reported antagonists available are

either inactive fragments of the endogenous agonist, or peptide agonists with substituted residues. These antagonists compete with agonist peptides to inhibit responses by blocking the receptor-binding site. Their advantage is that if exogenous agonist peptide causes a response then a smaller antagonist peptide is unlikely to have any access problems to the sites of the receptors. This type of pharmacological intervention has been used to demonstrate a role of peptides in several tissues. In the porcine retractor penis both VIP- and electrical field stimulation (EFS)-induced relaxations were reduced by VIP fragments 6-28 and 10-28 (La *et al.*, 2001) and in the rat distal colon VIP- and EFS-induced responses were inhibited by VIP fragment 10-28 (Kishi *et al.*, 1996).

Peptide fragments or peptides containing substitutions can also be useful in attempting to identify receptor subtypes. An example of this would be with VIP receptors. [Acetyl-His^{1}, D-Phe^{2}, Lys^{15}, Arg^{16}]VIP(3-7)GRF(8-27)-NH_2 has been reported to be a selective antagonist of both human and rat $VPAC_1$ receptors (Gourlet *et al.*, 1997). However no selective antagonists have been described for the $VPAC_2$ receptor. Maxadilan, a 61 residue peptide, which has no sequence homology with PACAP, nevertheless acts as a potent agonist at the PAC_1 receptor, whilst being inactive at $VPAC_1$ and $VPAC_2$ receptors (Moro & Lerner, 1997), and furthermore a fragment of this peptide acts as a selective antagonist at PAC_1 receptors (Uchida *et al.*, 1998). The PACAP fragment PACAP(6-38) is a potent PAC_1 receptor antagonist with a K_i of 14nM, and has significant affinity for $VPAC_2$ receptors, but does not interact with $VPAC_1$ (Dickinson *et al.*, 1997). Another peptide antagonist has been described for CGRP receptors, CGRP 8-37, which is a fragment of CGRP missing its first 7 residues, and hence also its di-

sulphide bond. This peptide fragment has been shown to inhibit both CGRP-induced relaxations and those induced by the neurosecretagogue α-latrotoxin in the rat hepatic artery (Hogestatt *et al.*, 2000). However, in other studies it was found that CGRP 8-37 was a potent antagonist at inhibiting CGRP-induced cAMP accumulation in SK-N-MC cells, but a weak antagonist of CGRP responses in guinea-pig atria and rat vas deferens (Longmore *et al.*, 1994).

A fifth way to affect peptide-induced responses is to target their second messenger pathways. As mentioned in section I: 3c, CGRP and VIP-related peptides have been shown to stimulate adenylate cyclase. Thus inhibitors of adenylate cyclase can be used to investigate the responses induced by these peptides. Indeed in the canine gastric fundus it has been shown that the adenylate cyclase inhibitor MDL-12, 330A was able to reduce VIP- and forskolin-induced relaxation as well as EFS-induced NANC relaxations suggesting that in this tissue NANC responses were partially mediated by cAMP, and possibly by VIP (Bayguinov *et al.*, 1999). In the rat adrenal gland PACAP-38 has been shown to increase cAMP levels and aldosterone secretion (Mazzocchi *et al.*, 2002). Another adenylate cyclase inhibitor, SQ 22536 was found to reduce the PACAP-38-induced aldosterone secretion from this tissue suggesting that PACAP-38 used cAMP as a second messenger to induce aldosterone secretion (Mazzocchi *et al.*, 2002). A third adenylate cyclase inhibitor 2', 5'-dideoxyadenosine (2'5'-DDA) has been shown to reduce CGRP-mediated increases in cAMP in the rabbit iris dilator muscle (Yousufzai & Abdel-Latif, 1998).

d) Summary

VIP, VIP-related peptides, CGRP and substance P have a role as signalling molecules. VIP and its related peptides are considered to be NANC neurotransmitters responsible for mediating smooth muscle relaxation in various tissues. CGRP and substance P also have effects on smooth muscle tone, and are thought to have a physiological role as neurotransmitters in sensory neurons.

There are many pharmacological tools available for use in the study of peptidergic neurotransmission, for example:

- Peptides can be depleted from sensory neurons by capsaicin
- Peptides can be targeted between the nerve and the effector cell by degrading them with peptidases or by potentiating their effects by inhibiting endogenous peptidases
- Many peptide receptor antagonists are available to inhibit peptide-induced responses in their effector cells

These tools can be used to investigate the roles of peptides in NANC relaxation responses in the genital tract.

I: 4 PURINES AND PYRIMIDINES AS NANC NEUROTRANSMITTERS

a) Discovery of purines and pyrimidines as signalling molecules

As early as 1929 it was found that adenine compounds had physiological effects in the cardiovascular system. It was shown that adenosine, at doses that did not reduce heart rate, caused aortic dilatation in dogs that was unaffected by atropine (Drury & Szent-György, 1929). Shortly afterwards it was also found that ATP had biological activity that was distinct from that of adenosine, implying the effects were mediated by different receptors (Gillespie, 1934).

The first indication that purines may be neurotransmitters was a study showing that following stimulation of the auricular nerve to induce vasodilatation in the ear capillary bed ATP was detected in the venous perfusate in sufficient quantities to induce dilatation of the ear arteries (Holton & Holton, 1953). However it was not until 1972 that the concept of purinergic nerves arose when Burnstock proposed that ATP may be the neurotransmitter responsible for NANC relaxations in the smooth muscle of the gastrointestinal tract and the bladder, influencing its target cells through receptors that he termed purinoceptors (Burnstock, 1972). He later proposed a pharmacological basis for differentiating between different types of purinoceptors, and proposed that the term P1 purinoceptor be used for receptors with high selectivity for adenosine as their natural ligand, and P2 purinoceptor for receptors at which ATP had high agonist potencies (Burnstock, 1978). This distinction was also based on the fact that methylxanthines

inhibited the actions of adenosine such as inhibiting vasodilatation of vessels in the dog heart (Afonso, 1970) and adenosine-induced accumulation of cAMP in guinea-pig cerebral cortex slices without inhibiting those of ATP (Sattin & Rall, 1970).

Further subclassification of the receptors soon followed, with the identification of two different adenosine receptors (P1 purinoceptors) that either inhibited or activated adenylate cyclase (van Calker *et al.*, 1979; Londos *et al.*, 1980). Four P1 purinoceptors have now been identified and cloned (Linden *et al.*, 1991; Pierce *et al.*, 1992; Salvatore *et al.*, 1993) with distinct molecular structures, pharmacological characteristics and tissue distribution. The consensus nomenclature for these P1 receptors is now A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm *et al.*, 1997). A_{2A} receptors have been shown to be involved in mediating inhibitory responses in smooth muscle structures via stimulation of adenylate cyclase (Haynes, 2000). A_1 and A_3 receptors are also coupled to adenylate cyclase, but inhibit its activity rather than stimulate it. A_{2B} receptors also stimulate adenylate cyclase, however the physiological roles for this receptor are currently difficult to elucidate due to a lack of commercially available antagonists, although the synthesis of selective antagonists has been recently reported (Kim *et al.*, 2000).

Subclassification of P2 purinoceptors into P2X and P2Y purinoceptors was suggested in 1985, based largely on the pharmacological activity profiles of different ATP analogues (Burnstock & Kennedy, 1985). A plethora of P2 subtypes that did not fit the pharmacological profiles defining the P2X and P2Y purinoceptor subtypes were described in the following years. These included P2T purinoceptors, which were ADP-

selective, and found on platelets (Gordon, 1986), P2Z purinoceptors, which were found on mast cells and were preferentially activated by a tetrabasic form of ATP, ATP^{4-} (Gordon, 1986) and P2U purinoceptors, which were activated equally by ATP and the pyrimidine UTP (O'Connor *et al.*, 1991) amongst others. A biochemical basis for the difference between P2X and P2Y subtypes emerged in 1991, when it was shown that P2X purinoceptors were ion-gated channels, and P2Y purinoceptors were G-protein-coupled receptors (Dubyak, 1991). The confusing list of other proposed P2 purinoceptor subtypes was based on pharmacological selectivity of the receptors to different agonists, but variability in expression systems and between species made identification of specific receptors equivocal. However, all P2 receptors identified so far have fallen into the broad categories of either ion-gated channels or G-protein-coupled receptors. This has led to revisions in the nomenclature whereby P2 purinoceptors are now named P2 receptors (to take into account the activity of pyrimidines on some subtypes) and are P2X receptors if they are ion-gated and P2Y receptors if they are G-protein-coupled receptors (Abbracchio & Burnstock, 1994; Fredholm *et al.*, 1997). To date seven mammalian P2X receptors ($P2X_{1-7}$) and seven mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$) have been cloned and characterised (Ralevic & Burnstock, 1998; Hollopeter *et al.*, 2001; Communi *et al.*, 2001).

The purines ATP, ADP, and adenosine as well as the pyrimidines UTP and UDP are considered to be signalling molecules in both neural, and vascular and non-vascular smooth muscle preparations (for review see Ralevic & Burnstock, 1998), influencing target cells through the receptors discussed above.

b) Purines and pyrimidines as mediators of smooth muscle relaxation

It is well documented that adenosine can induce relaxation responses in a variety of tissues. Adenosine has been shown to cause concentration-dependent relaxation of isolated lobes of the rat prostate gland, with maximal relaxation responses at 10mM adenosine (Preston *et al.*, 2000). Similarly in the guinea-pig isolated heart exogenous adenosine induced vasodilatation as measured by increased coronary conductance, however in this tissue maximal responses to adenosine were observed at 10μM (Erga *et al.*, 2000).

It is well documented that ATP can cause contraction of smooth muscle preparations through activation of P2X-purinoceptors, and can cause relaxation via P2Y-purinoceptors (Burnstock & Warland, 1987; McMurray *et al.*, 1998; Simonsen *et al.*, 1997). However, it has recently been shown that there are exceptions to this rule when it was shown that ATP-induced relaxation responses in isolated rat pylorus and ileum strips were mediated by P2X receptors (Ishiguchi *et al.*, 2000; Storr *et al.*, 2000a). As a result of these findings relaxation responses to ATP should not be considered as demonstrating the presence of P2Y receptors, without further supporting evidence.

Relaxation responses to ATP, ADP and UTP have been observed in human placental cotyledons in which the tone had been raised by prostaglandin $F_{2\alpha}$ (Ralevic *et al.*, 1997). However these responses were endothelium–dependent and were abolished by incubation with L-NAME, suggesting that these relaxations were mediated by purine and pyrimidine-induced stimulation of EDRF, rather than by direct relaxant effects on the

smooth muscle.

c) Pharmacology of purine and pyrimidine signalling

For purines and pyrimidines to be considered as possible mediators of NANC relaxation responses they must be present in the neurons innervating the tissue of interest in order to satisfy the first criterion for a neurotransmitter. However, these are ubiquitous molecules, present in all cells with roles in many intracellular processes, thus their presence tells us nothing about their possible role as extracellular signalling molecules.

In order to be considered as candidates for mediators of NANC relaxation responses they must be capable of inducing relaxation responses when applied exogenously. This provides one of the points in which drugs can be used to study these responses since extracellular signalling effects of purines and pyrimidines all occur via P1 or P2 receptors.

The classification of P1 and P2 receptors as well as the subclassification of P2 receptors into P2X and P2Y receptors was originally based on the pharmacological profiles of these different receptors. Although receptor classification is now based on their molecular structure the characteristic pharmacological profiles of these receptors remains an important means of identifying receptor subtypes.

Whilst obviously P1 receptors are selectively activated by adenosine as their natural ligand a number of subtype-specific agonists and antagonists have been developed which greatly facilitate the study of the roles of these receptors. P1 receptors are antagonised by methylxanthines at concentrations below those that inhibit phosphodiesterases. This inhibition is non-selective, and so does not give any information of the P1 receptor subtype involved in the responses. Another non-selective P1 receptor antagonist is 8-phenyltheophylline (8-PT). This has been shown to cause partial inhibition of adenosine induced relaxation responses in isolated lobes of the rat prostate gland (Preston *et al.*, 2000). A non-xanthine inhibitor of A_{2A} receptors, ZM 241385, has been described that was shown to inhibit A_{2A} receptor agonist-mediated guinea-pig coronary vasodilatation, and inhibit adenosine-induced relaxation of the guinea-pig aorta (Poucher *et al.*, 1995). These antagonists can be used to pharmacologically distinguish between adenosine-mediated responses and those mediated by other pathways.

The original subclassification of P2 receptors into P2X and P2Y receptors was largely based on the observation that distinct populations were selectively activated by different stable analogues of ATP. Receptors at which potent activation was achieved by α, β-methylene ATP (α, β-meATP) and β, γ-methylene ATP (β, γ-meATP) were termed P2X, and those which were most potently activated by 2-methylthio ATP (2MeSATP), and weakly activated by α, β-meATP and β, γ-meATP were P2Y receptors. Desensitization of P2X receptors using α, β-meATP was also observed (Burnstock & Kennedy, 1985). These pharmacological distinctions between the receptors provide useful tools to discriminate between the receptor subtypes involved in mediating

responses; however 2MeSATP has also been found to activate P2X receptors in some tissues.

In the rabbit mesenteric artery α, β-meATP and ATP have both been shown to cause concentration-dependent contractions at basal tone, and 2MeSATP and ATP have been shown to induce relaxations when the tissue tone was raised with noradrenaline (Burnstock & Warland, 1987). However in this tissue 2MeSATP was also able to induce contractions at basal tone at lower concentrations than ATP, although at higher concentrations than α, β-meATP (Burnstock & Warland, 1987). Thus 2MeSATP does not appear to be a selective agonist of P2Y receptors and, if used, its efficacy cannot be used to conclude that responses are mediated by P2Y receptors.

The natural ligand for all P2X receptors is ATP, and these receptors are not activated by ADP or pyrimidine nucleotides. Thus, if responses are elicited by ADP or pyrimidine nucleotides this strongly suggests the responses are mediated by P2Y receptors, whilst if responses are elicited by α, β-meATP this strongly suggests that responses are mediated by P2X receptors.

In addition to the use of different activities of agonists to study P2 receptors there are also number of non-selective P2 antagonists such as suramin and cibacron blue (the active component of reactive blue 2, which was previously used as a P2 antagonist). Whilst these compounds do not give any reliable information about the P2 receptor subtype mediating a response they can be useful to distinguish P2 mediated responses

from responses mediated by other pathways. For example, in isolated rabbit mesenteric artery strips precontracted with noradrenaline ATP- and 2MeATP-induced relaxation responses were abolished by reactive blue 2, whilst ACh- and adenosine-induced relaxation responses were unaffected (Burnstock & Warland, 1987).

Since the current distinction between P2X and P2Y receptors is based on the fact that P2X receptors are ligand-gated ion channels and P2Y receptors are GPCRs, this can be used as a further point for pharmacological intervention. When activated, GPCRs bind their associated G-protein stimulating the release of GDP from the G-protein α-subunit, and allowing GTP to bind. The α-subunit with bound GTP then dissociates to interact with its effector enzyme. GDPβS stabilises G proteins in their inactive state, and thus can be used to inhibit P2Y, but not P2X receptor-mediated responses. Indeed, in the marmoset urinary bladder, ATP has been found to elicit both contractile and relaxant responses. However whilst α, β-meATP only elicited contractile responses, the relaxation responses to ATP were inhibited by GDPβS (McMurray *et al.*, 1998).

d) Summary

It is generally accepted that purines and pyrimidines have physiological roles as extracellular signalling molecules in a variety of tissues. Furthermore ATP is thought to act as a NANC neurotransmitter in some tissues.

Although P1 and P2 receptors were originally recognised based on their different pharmacological properties over 20 years ago the study of the physiological effects of purines and pyrimidines is still hampered by a lack of potent selective compounds to interact with purine and pyrimidine signalling pathways.

Pharmacological intervention to study the role of these compounds is limited to the use of agonists and antagonists to interact with receptors on the effector tissue. However, studying the second messenger pathways utilised by these compounds can also help to identify their mechanisms of action, and to determine if they may be involved in NANC responses in the genital tract.

I: 5 NEUROTRANSMITTERS IN THE FEMALE GENITAL TRACT

Even before it had been proposed as a NANC neurotransmitter, VIP was shown to be present in nerves of the vagina of pigs, cats, rats and mice, as well as other organs of the female genitourinary tract. The number of VIP-positive nerves was shown to be particularly high in the vagina, uterus and ureters, and these nerves were mainly located in the smooth muscle wall of the organs (Larsson *et al.*, 1977). These findings were confirmed when VIP immunoreactivity was further shown to be present in the female genital tract of cats, goats, pigs, rabbits and rats, with the highest concentration of VIP found in the cervix (Ottesen *et al.*, 1981). Also at this time VIP was found to inhibit spontaneous contractile activity of cat, goat, pig and rabbit myometrium, with a lesser effect in rats (Ottesen *et al.*, 1981).

In 1983 it was shown that substance P increased contractile activity in rabbit and cat uterine smooth muscle, and that this was reversed by VIP, but that both substances were able to increase blood flow in this organ (Ottesen *et al.*, 1983a). Neither of these effects were inhibited by blockers of adrenergic or cholinergic signalling, and it was suggested that they may mediate NANC responses in the uterus (Ottesen *et al.*, 1983a). In another study substance P was also shown to have a contractile effect on human uterine smooth muscle, and again this could be reversed by VIP (Ottesen *et al.*, 1983b). It was also shown that intravenous injection of VIP increased vaginal blood flow in humans (Ottesen *et al.*, 1983c). These studies showing its presence and effects led to the proposal that VIP was a NANC neurotransmitter in the female genital tract (Ottesen, 1983).

Further studies followed using immunochemistry and radioimmunoassay techniques to examine the occurrence and distribution of possible peptide mediators of NANC responses in the female genital tract. Using these techniques VIP, PHM and neuropeptide Y (NPY) were shown to be present in the human female genital tract, with high concentrations localized to neurons innervating the vagina and cervix, and particularly high density of VIP containing neurons found beneath the vaginal epithelium (Blank *et al.*, 1986). Also CGRP was shown to be present, and localized to sensory nerves in the female rat genitalia (Inyama *et al.*, 1986). The abundance of VIP and PHM, in the human vagina and cervix was confirmed in 1989 when it was also shown that PHM was co-localised with VIP, and that a C-terminally extended form of PHM was also present (Palle *et al.*, 1989). In a subsequent study this was shown to be PHV, with PHV concentrations at 50-70% of the VIP concentrations, and it was found that in all regions of the human female genital tract the molar ratio of VIP to PHM/PMV was close to 1:1 (Palle *et al.*, 1992). The presence of PACAP-27 and –38 containing neurons throughout the female genital tract was later shown, highest concentrations of PACAP-38 containing neurons were found to be in the ovaries and the proximal vaginal wall, and whilst PACAP-27, was ubiquitously expressed its concentration was found to be only 1- 5% of that of PACAP-38 in all regions (Steenstrup *et al.*, 1995). PACAP- and helospectin-like immunoreactivity was also found to be co-localized with VIP, but to a lesser extent than that of PHM, which was entirely co-localized with VIP-immunoreactivity throughout the female genital tract. Two-thirds of the VIP-containing nerves in the lamina propria and vessels of the smooth muscle layer showed PACAP-like immunoreactivity, as well as

half of the VIP-containing nerves running parallel to the mucosal epithelium. All nerve fibers that stained positively for PACAP also showed VIP-immunoreactivity (Graf *et al.*, 1995).

In 1994 it was found that much of the female mouse genital tract was also innervated by NOS containing neurons, with no nitrergic innervation of the ovaries and fallopian tubes, and the densest innervation to the vagina and cervix (Grozdanovic *et al.*, 1994). NOS co-localization with VIP and SP immunoreactive neurons was subsequently shown in the porcine and bovine vaginal walls (Majewski *et al.*, 1995). In 1996 this was extended to the human vagina, when it was shown that NOS, CGRP, substance P and NPY where all present, but that substance P innervation was sparse and that the relative density of immunoreactive fibres innervating the vagina was NPY>VIP>>NOS>CGRP>SP (Hoyle *et al.*, 1996). In 1997 nNOS containing neurons were also found in the glans and corpora cavernosa of the human clitoris, with highest concentrations in the corpora cavernosa (Burnett *et al.*, 1997).

Thus the NANC neurotransmitter candidates NO, VIP, VIP-related peptides, CGRP and substance P have all been shown to fulfil the first criterion for a neurotransmitter in the vaginal wall.

I: 6 AIMS OF THIS THESIS

Sexual function plays an important part in maintaining healthy relationships, and sexual dysfunction can lead to emotional problems including low self-esteem and depression. Female sexual dysfunction (FSD) can result from loss of desire, decreased arousal, lack of orgasm and coital pain. These subtypes can be psychologically linked to each other with, for example, decreased arousal leading to lack of orgasm, and this can lead to loss of desire.

The genital physiological changes that occur in normal female sexual arousal include clitoral tumescence, which is analogous to penile erection, vaginal engorgement and vaginal lubrication. These processes are mediated by smooth muscle relaxation in these structures, and the mediators of these responses have received limited attention. If these physiological changes are diminished this can lead to female sexual arousal disorder (FSAD). Without a full understanding of the control of these changes, effective treatment of FSAD will be hampered by a lack of specific targets to potentiate the normal physiological response.

NANC innervation of the vagina may be responsible for mediating vaginal engorgement and vaginal lubrication, and the aim of this thesis is to more fully characterise the neurotransmitters that mediate NANC relaxations in this organ to provide possible targets for the future treatment of FSD.

CHAPTER II

METHODS

II: 1 TISSUE PREPARATION

a) Studies with rabbit vaginal wall and clitoral corpus cavernosum

Female New Zealand white rabbits (weight 3.59 ± 0.03kg; range 2.9 – 5.2kg, N=187, Harlan, UK) were sacrificed by an overdose of pentobarbitone (Euthatal, Rhône Merieux, UK) injected into the ear marginal vein. The vaginal canal including the clitoris was excised down to the pubic bone (see Figure 4) and transferred to modified Krebs solution (for composition see section II: 7) kept at room temperature and gassed with 95% O_2/5% CO_2. The inducible isoform of NOS (iNOS) can be induced by trace amounts of endotoxin in the buffer (Rees *et al.*, 1990). Since I wanted to study nNOS in isolation dexamethasone (10μM) was added to the Krebs solution to prevent induction of iNOS. I also added the cyclooxygenase inhibitor indomethacin (10μM) to prevent synthesis of prostaglandins, since these can cause non-neurogenic relaxations (Daniel *et al.*, 1979).

The vaginal canal was carefully opened by cutting along the posterior wall. The clitoral body was removed leaving two strips of vaginal wall tissue. For experiments using longitudinal strips of vaginal wall smooth muscle two strips (2 x 8mm) were dissected free of connective tissue one from the proximal and one from the distal part of each of these tissue strips (see Figure 4). For experiments using circular strips of vaginal wall four circular strips (2 x 6mm) were dissected free of connective tissue all from the distal sections of the vaginal canal. For experiments involving the clitoral corpus cavernosum, the tunica albuginea of the clitoral body was cut open to reveal the corpus cavernosum. Two pieces of cavernous tissue (2 x 6mm) were isolated using fine scissors.

The ends of the strips were tied with silk suture and mounted horizontally between two platinum electrodes in superfusion chambers continually perfused at 1ml/min with modified Krebs solution at 37°C (Figure 5).

b) Studies with rat vaginal wall

For experiments using strips of rat vaginal wall, mature female Sprague-Dawley rats (weight 267 ± 37g, range 205 - 400g, N=5 Charles River, UK), were sacrificed by stunning followed by cervical dislocation. The vaginal canal was excised and placed in modified Krebs solution as described in section II: 1a. Longitudinal vaginal wall strips (1 x 5mm) or circular vaginal strips (1 x 7mm) were removed and dissected free of connective tissue. The strips were tied and mounted in the perfusion chambers as for the rabbit tissue strips.

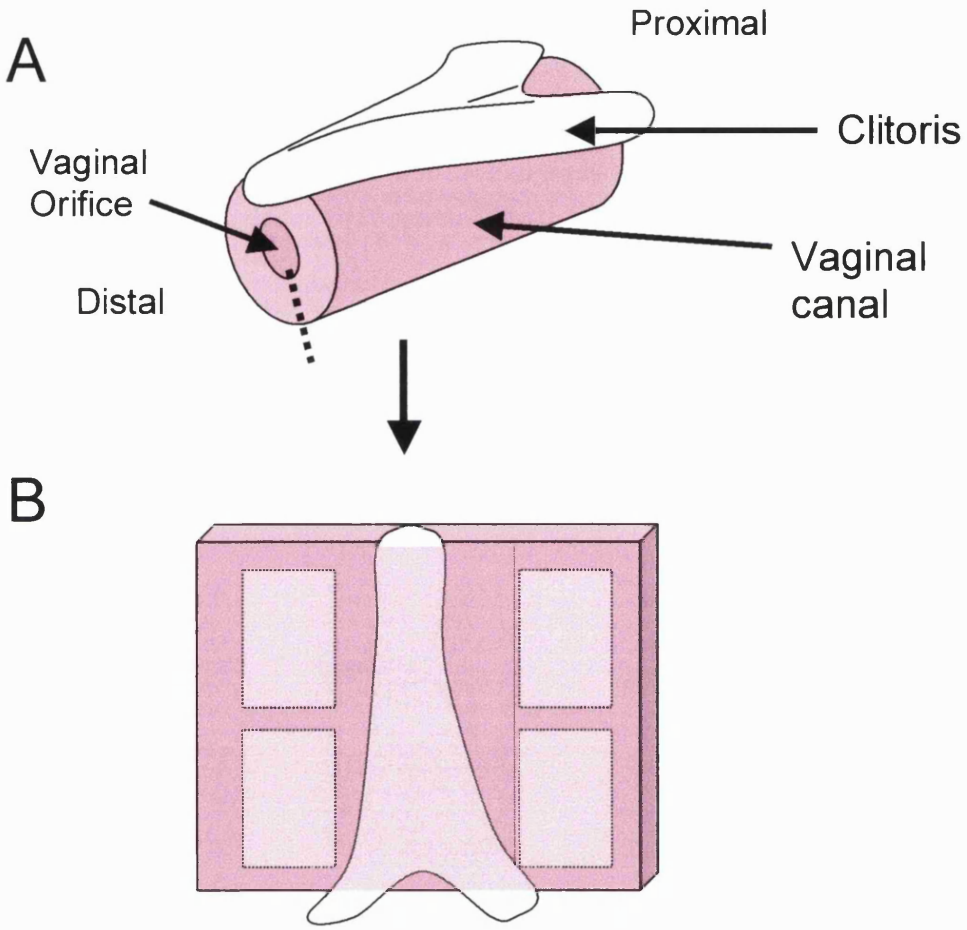


Figure 4: Dissection of longitudinal rabbit vaginal wall strips from the vaginal canal and clitoris. A: The vaginal canal and clitoris were removed from the rabbit, and the vaginal canal was dissected open along the posterior wall indicated by the dashed line. The clitoris was then dissected from the opened structure (**B**), and four longitudinal strips (2 x 8mm) of vaginal wall were removed as indicated by the dashed rectangles.

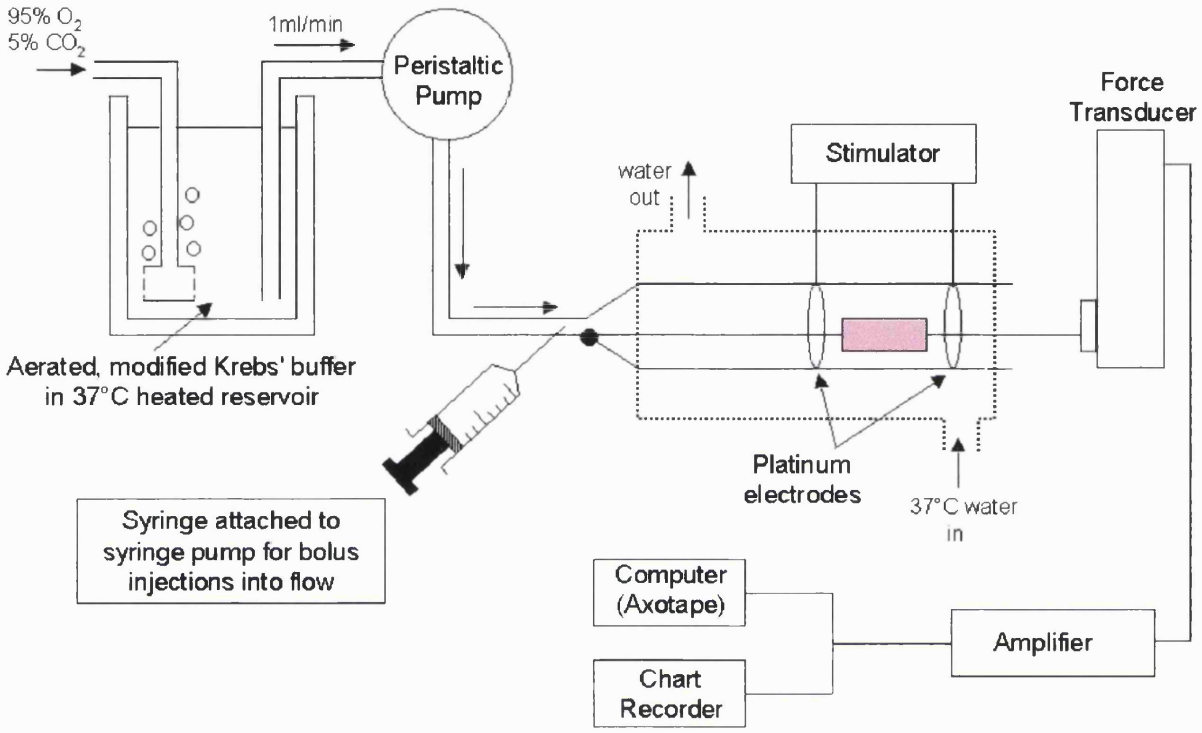


Figure 5: Perfusion chamber apparatus. Tissue strips were tied to two lengths of silk suture and held between platinum electrodes within a jacketed chamber at 37°C by attaching one length of suture to the entrance of the chamber using the inlet tubing. The other suture was attached to a force transducer, which allowed measurement of tension changes through an amplifier attached to two recording devices (computer and chart recorder). Aerated, modified Krebs buffer was pumped from a reservoir to the chamber inlet and superfused over the tissue strip. Bolus injections of drugs were pumped into the inlet tube just prior to entry into the chamber.

II: 2 MEASUREMENT OF MECHANICAL RESPONSES

The silk suture attached to one end of the preparation was anchored to the entrance of the chamber by the Krebs inflow tubing, whilst the other end was tied to a force-displacement transducer (FT03C, Grass, USA) connected to a chart recorder (Linearcorder WR3101, Graphtec, UK) for measurement of isometric changes in tension. The mechanical responses were also recorded on a computer running specialized software for later analysis (Axotape, Axon Instruments, USA). The preparations were stretched to approximately their *in situ* length by applying tension of 0.4g for longitudinal and circular strips of rabbit vaginal wall, 0.6g for strips of rabbit clitoral corpus cavernosum and 0.4g for longitudinal and circular strips of rat vaginal wall, and were then allowed to equilibrate for 90min. The preparations were either stimulated by infusion of drugs or by electrical field stimulation (EFS).

Short term EFS was applied as 5-second trains of rectangular pulses of 50V, 0.3ms pulse duration, 1 - 50Hz, delivered by stimulators (S88, Grass, USA). These stimulation parameters were chosen as they induced neurogenic responses whilst not inducing myogenic responses. Furthermore, these parameters have been previously utilised in the study of nitrergic responses in the male and female corpora cavernosae and in the rabbit vaginal wall using the perfusion chamber apparatus used in this study (Cellek & Moncada, 1997a; Cellek & Moncada, 1998; Cellek *et al.*, 1999). Long term EFS was applied as for short term EFS, but with longer trains of pulses as described in text.

Drugs were introduced either by addition to the reservoir feeding the superfusion chamber or, for short-term exposure, by infusion into the perfusate by injection into the tubing at the entrance to the perfusion chamber at a rate of 100µl/min using a syringe pump (Harvard Apparatus Model '22', UK).

II: 3 MEASUREMENT OF INTRACELLULAR CYCLIC NUCLEOTIDE CONCENTRATIONS

a) Freezing tissue strips

Modified perfusion chambers in which the tissues were accessible from above (Cellek *et al.*, 1996; Ziessen *et al.*, 2002; Figure 6), were used in experiments to evaluate intracellular concentrations of cyclic adenosine-3', 5'-monophosphate (cAMP) or cyclic guanosine-3', 5'-monophosphate (cGMP). The tissues were set up as described in section II: 2 for closed superfusion chambers. After NANC relaxation responses were revealed with guanethidine, scopolamine and phenylephrine, relaxation responses to EFS or drugs were elicited to give a time-course for the response. After recovery to basal tone the tissues were incubated for 20 minutes with the non-specific phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 1mM) to prevent breakdown of cAMP and cGMP. IBMX inhibited tissue tone, so when EFS or drugs were applied subsequently any relaxation responses could not be observed. Instead tissues were freeze-clamped either under basal conditions or at the estimated time of the peak of their response to EFS or drugs determined by the time course of the response prior to incubation with IBMX. The frozen samples were then homogenized in a stainless steel pestle and mortar on dry ice.

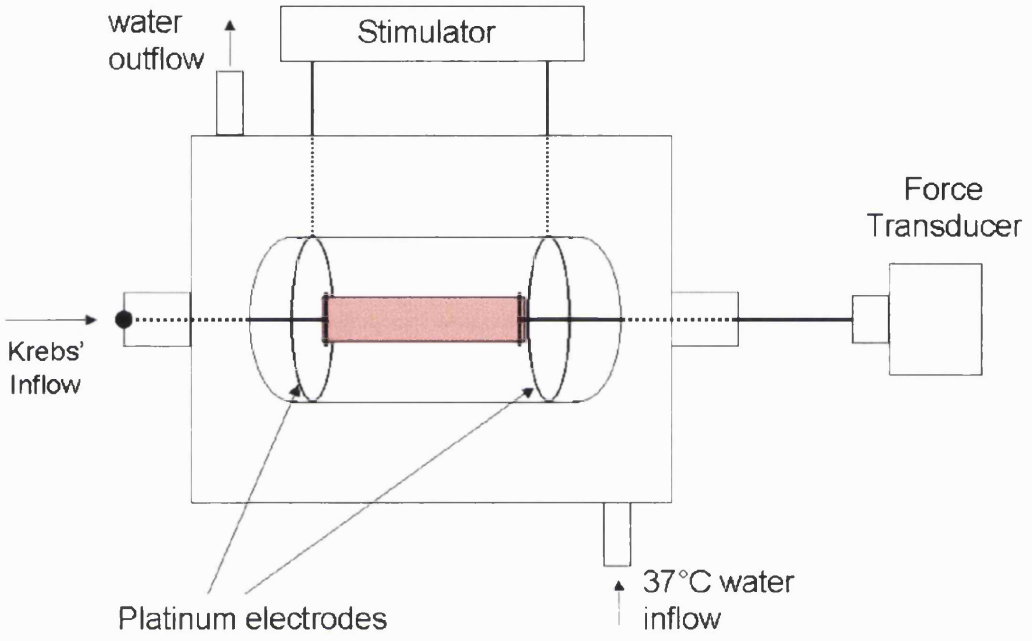


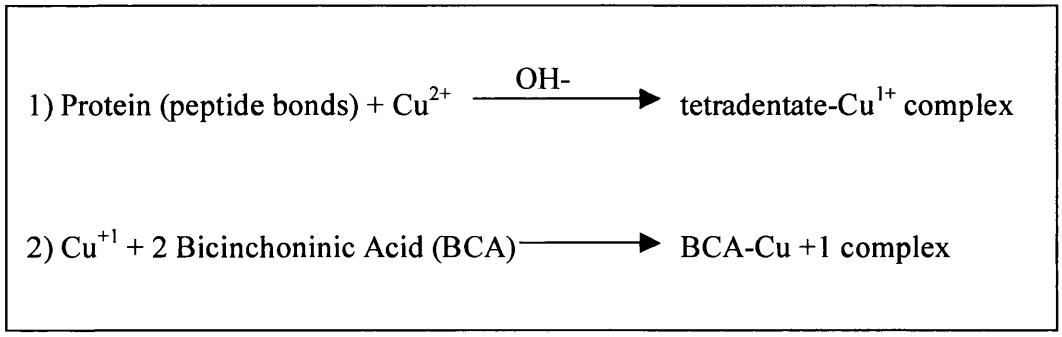
Figure 6: Modified perfusion chamber apparatus viewed from above. Tissue strips were placed in chamber in central bath area between electrodes. Tissues were accessible from above enabling them to be freeze-clamped. For experiments to analyse peptide release from tissue strips (see section II: 5), superfusing modified Krebs solution was withdrawn by pipette from adjacent to the tissue.

b) Extraction of cyclic nucleotides

Each homogenized sample was incubated in 1ml of 0.5M perchloric acid (PCA) for 1 hour on ice then sonicated for 5s at 4°C (18 microns using Soniprep-150, Sanyo-Gallenkamp, UK). The samples were centrifuged (10min, 10,000g, 4°C), and 2 x 450µl aliquots of the supernatant were removed for cyclic nucleotide assays. The remaining supernatant was used to measure soluble protein content (see section II: 3c). To prepare samples for measurement of intracellular cyclic nucleotide concentrations, each 450µl supernatant aliquot was neutralised with 300µl of 1M K_3PO_4. The sample was centrifuged (8,000g, 5min, 4°C); the supernatant was recovered and lyophilised using a Heto Drywinner (Heto-Holten, Denmark) and assayed for cAMP and cGMP content.

c) Measurement of soluble protein content

Soluble protein content of samples prepared above was calculated using BCA protein assay kit (Pierce, USA). In this assay copper ions (Cu^{2+}) form a complex with the peptide bonds within proteins, reducing the copper to Cu^{1+} by the biuret reaction. Cu^{1+} then forms a purple coloured complex with bicinchoninic acid (BCA), which can be measured by its absorbance at 562nm. The reaction scheme for this is:



The biuret reaction requires alkaline conditions, so I needed to ensure the amount of PCA in the samples did not prevent measurement of protein concentration. Protein standard curves were generated in which bovine serum albumin protein standards (Pierce, USA) were diluted with either phosphate buffered saline (PBS, Life Technologies, UK) or PCA. Both diluents allowed the reaction to proceed, enabling protein concentration to be calculated; however the profile of the curves differed (Figure 7). For this reason protein standards were diluted using PCA for all estimations of protein concentration.

d) Measurement of cAMP and cGMP content

Samples prepared as above were assayed for cAMP or cGMP content using specific enzyme immunoassay (EIA) kits (Amersham Pharmacia, UK). The acetylation EIA protocol was used to give maximum sensitivity (detection range of 2 – 128fmol/well for cAMP assay and 2-512 fmol/well for cGMP assay). In this assay lyophilised samples were dissolved in 1ml assay buffer (see section II: 7). Samples and standards (see section II: 7) were then acetylated by addition of acetylation reagent (25µl for cAMP assay, 100µl for cGMP assay; see section II: 7) and immediately vortexing tubes. 50µl duplicates of standards and samples were then placed in wells coated with donkey anti-rabbit IgG, and pre-incubated with rabbit antiserum to bind the acetylated cyclic nucleotides. The antiserum is raised against acetylated cyclic nucleotides and thus binds acetylated cyclic nucleotides with higher affinity than non-acetylated cyclic nucleotides. After incubation (1 hour for cAMP assay, 2 hours for cGMP assay) at 4°C cyclic nucleotide conjugated to peroxidase was added to compete with un-conjugated cyclic nucleotide for 1 hour. Wells were then washed and incubated with 3,3', 5'5'-tetramethylbenzidine (TMB)/hydrogen peroxide at 37°C for 30 minutes. The reaction was then stopped with 1M sulphuric acid. A yellow colour was produced in proportion to the amount of cyclic nucleotide-conjugate present, which is restricted by the cyclic nucleotide content of the sample (Figure 8). With these assays the intra-assay variation was low as evidenced by the small range of variation seen between duplicate samples (Figure 8). To control for inter-assay variation at least one basal sample, cAMP positive sample (forskolin treated) and one cGMP positive (DETA-NONOate treated) sample were included on each assay plate.

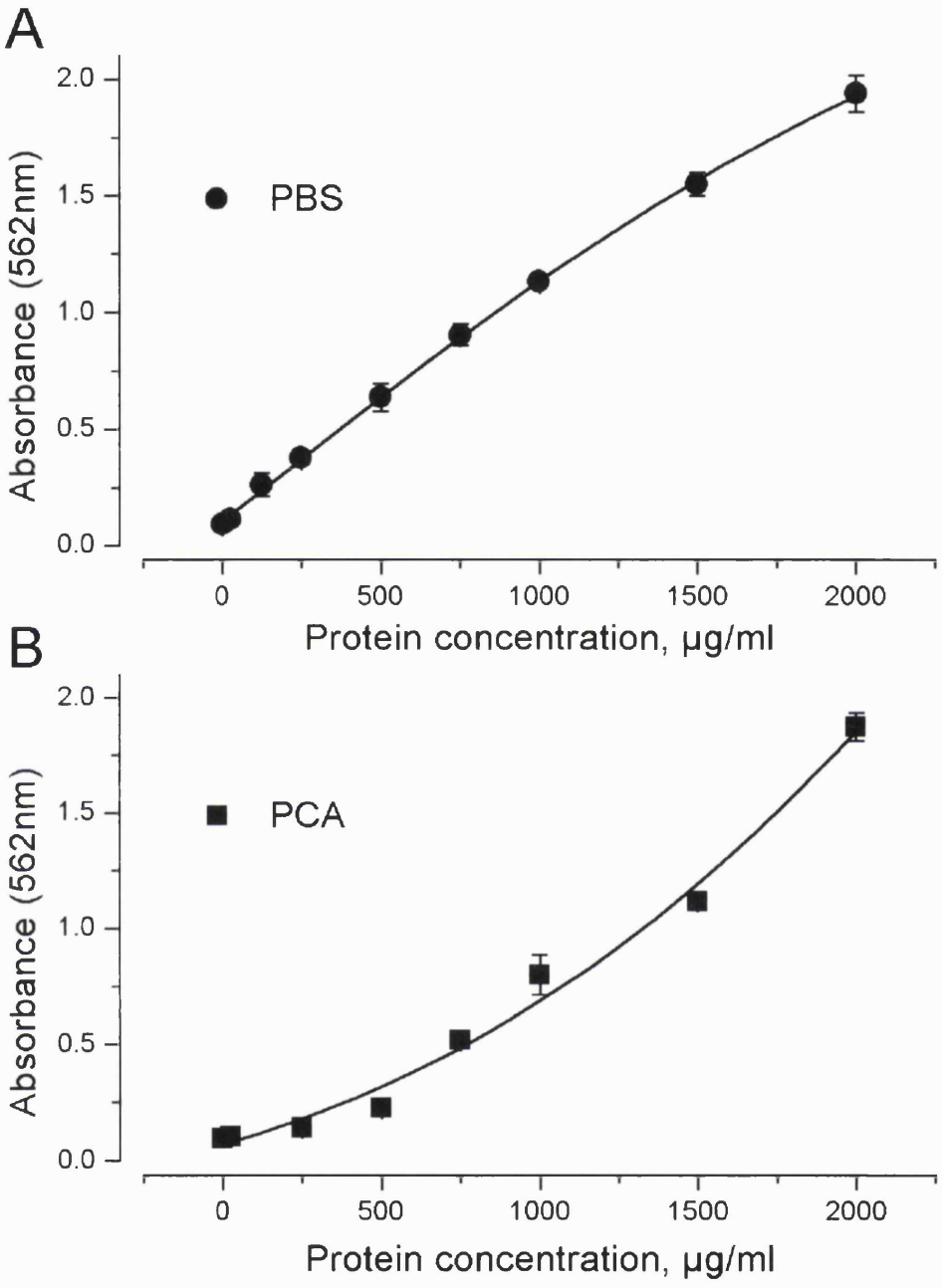


Figure 7: Typical protein standard curves. Curves were generated using BCA protein assay kit with BSA protein standards diluted with either phosphate buffered saline (PBS, ●, **A**) or perchloric acid (PCA, ■, **B**). Data points represent mean ± range for duplicate samples in a single experiment.

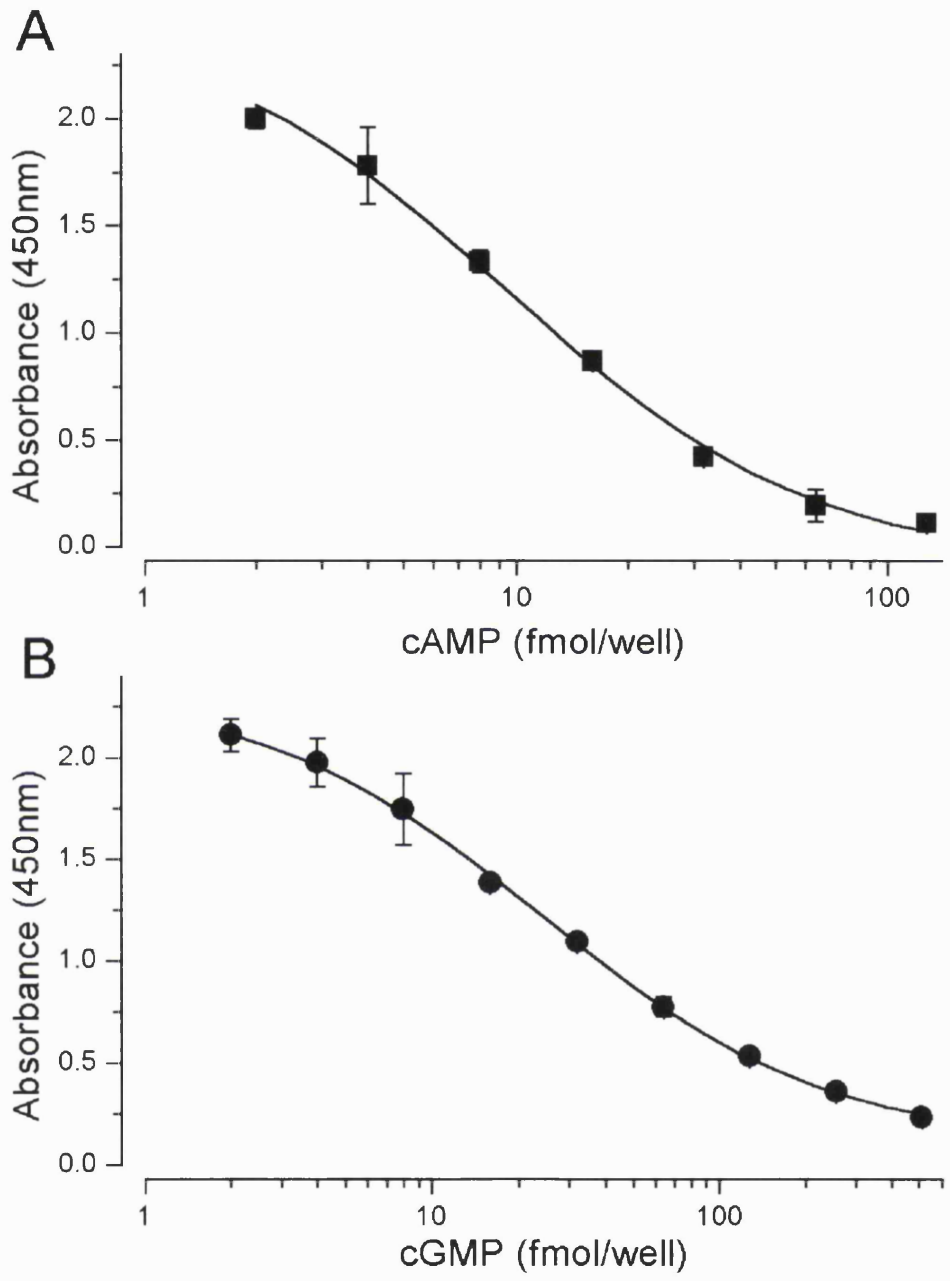


Figure 8: Typical cAMP and cGMP standard curves. Standard absorbance curves generated using cAMP (**A**) and cGMP (**B**) EIA kits. Data points represent mean ± range for duplicate samples in a single experiment.

II: 4 BIOASSAY STUDIES TO EXAMINE TRANSFERABILITY OF NEUROTRANSMITTERS BETWEEN DIFFERENT TISSUES

In experiments to detect biological effects of substances released from a donor tissue strip on a recipient tissue strip, both were prepared and tied with silk suture as described in section II: 1a. Strips were then placed within the superfusion chamber apparatus with the donor strip placed between electrodes and the recipient strip placed between the second electrode and the outside of the chamber (Figure 9). Both strips were anchored by silk suture fixed at the entrance to the chamber by the inflow tubing. The other end of the donor suture was tensioned until the strip was its approximate *in situ* length (this was assessed visually), and the suture was then anchored to the outside of the chamber. The other end of the recipient suture was attached to a force-displacement transducer (FT03C, Grass, USA) and tensioned for measurement of mechanical responses as described in section II: 2.

In some bioassay experiments either 1 in 1000 dilution of a protease cocktail (for composition see section II: 7) or a combination of the aminopeptidase inhibitor bestatin (3μM) and the endopeptidases inhibitors phosphoramidon (1μM) and DL-thiorphan (1μM) was added into the superfusing Krebs solution. The purpose of this was to attempt to reduce breakdown of any peptides released by the donor tissue to increase the potential amount of peptide reching the recipient tissue.

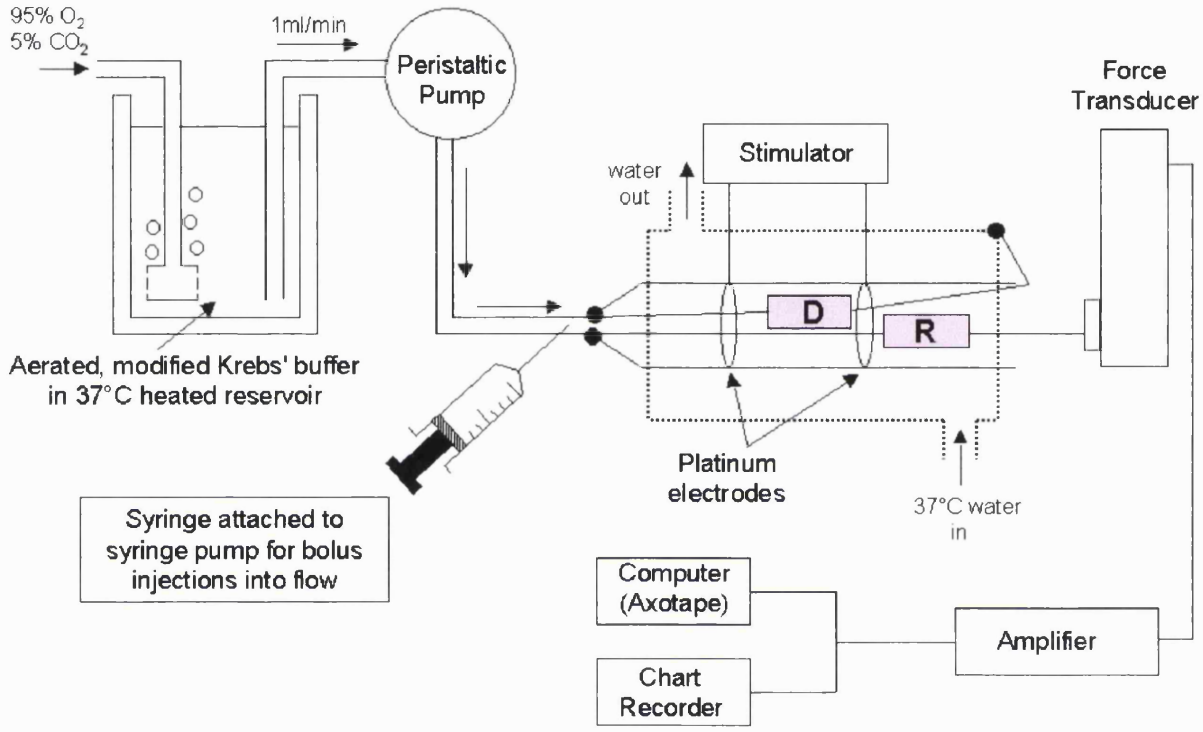


Figure 9: Transfer experiment apparatus. Aerated modified Krebs solution is pumped through a jacketed, heated (37°C), chamber containing two pieces of tissue. The donor tissue (**D**) is held between electrodes by silk suture with its contractile responses unrecorded. The recipient tissue (**R**) is attached at one end to a force transducer and at the other by the tubing attachment at the entrance of the chamber.

II: 5 STUDIES TO IDENTIFY TISSUE RELEASED NEUROPEPTIDES

a) Preparation of samples

Strips of vaginal wall were mounted in modified chambers in which the tissues were accessible from above (as described in section II: 3a). The tissues were stimulated with EFS rectangular pulses of 50V, 0.3ms pulse duration, 50Hz, delivered by S88 stimulators (Grass, USA). Perfusing modified Krebs solution was collected (10 x 0.5ml samples) from adjacent to the tissue under basal conditions and during stimulation. These samples were passed over 2ml columns made from sephadex (G25 fine, Amersham pharmacia, UK) in water, in order to remove excess salts. This desalting method uses size-exclusion chromatography to separate large molecules from a solution with a high salt composition. The larger molecules cannot enter the sephadex beads (molecular weight cut-off for G25 beads is 5000) and thus have a smaller volume available to them in the column. These larger molecules thus pass through the column more quickly than the salts, which pass through the beads. Desalted solutions were then lyophilised using a Heto Drywinner (Heto-Holten, Denmark).

Samples were dissolved in 1ml tricine sample buffer (for composition see section II: 7). Pre-stained molecular weight standards (ovalbumin, 43,000; carbonic anhydrase, 29,000; beta-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6,200; insulin (alpha and beta chains), 2,850; Life Technologies, UK) were diluted 1:1 with tricine sample buffer, and 60µl of all samples and standards were placed in 1.5ml tubes at 95°C for 10 minutes.

10-20% Tris-Tricine ready gels (Biorad) were placed in a gel tank facing each other and the reservoir was filled Tris/Tricine running buffer (for composition see section II: 7). 10µl of molecular weight standards and 25µl samples were placed in wells and gels were run for 70min at 100V.

Gels were then transferred from gel tanks to staining baths and rinsed in water, then covered with coomassie blue staining solution (for composition see section II: 7) at room temperature, on a shaker platform for 60 minutes. The methanol and acetic acid in the staining solution causes any proteins to precipitate, fixing their position in the gel, whilst the coomassie blue stains them. After incubation in staining solution the gel was rinsed with destaining solution (for composition see section II: 7), then covered with destaining solution and incubated at room temperature on the shaker platform for 24 hours.

b) Analysis of samples

Bands were excised and analysed by M-Scan Ltd, UK. For this, excised bands were subjected to in-gel digestion using trypsin, then purified using a C_{18} cartridge. C_{18} cartridges concentrate and separate proteins and peptides by reversed phase chromatography. Briefly, samples are loaded onto columns, which contain hydrophobic octadecyl hydrocarbon ligands bound to silica beads. The proteins and peptides bind to the hydrophobic surface via nonpolar-nonpolar interactions or van der Waals forces. After this concentrating step they are eluted from the column, and are separated based on differing hydrophobicity. This is acheived by an increasing organic solvent (most commonly acetonitrile) concentration, which displaces the proteins.

Purified samples were then analysed by matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). For this technique samples are dissolved in a matrix of sinapic acid and crystallized on a plate. The plate is placed in the TOF mass spectrometer and the sample is then bombarded with a high-energy pulse from a laser source at a wavelength that is readily absorbed by the matrix, but not by the protein, causing desorption and ionisation of the sample molecules by protonation from the matrix. Using this technique most ionised molecules only receive a single charge. This occurs in an electric field, which accelerates the ionised sample towards a detector. This allows calculation of the TOF as the exact time of the ionising laser pulse and time of detection are known. TOF is proportional to mass and charge, but since this technique produces mainly singly charged molecules, TOF is proportional to

mass, and so mass/charge signals observed give mass peaks and once trypsin auto-digestion peaks and any contaminating peaks have been eliminated remaining peaks can be cross-referenced against a database of known proteins and peptides. Protein identification results from identifying the mass fragments as those that would arise from tryptic digestion of known proteins. A schematic diagram of this process is shown in Figure 10.

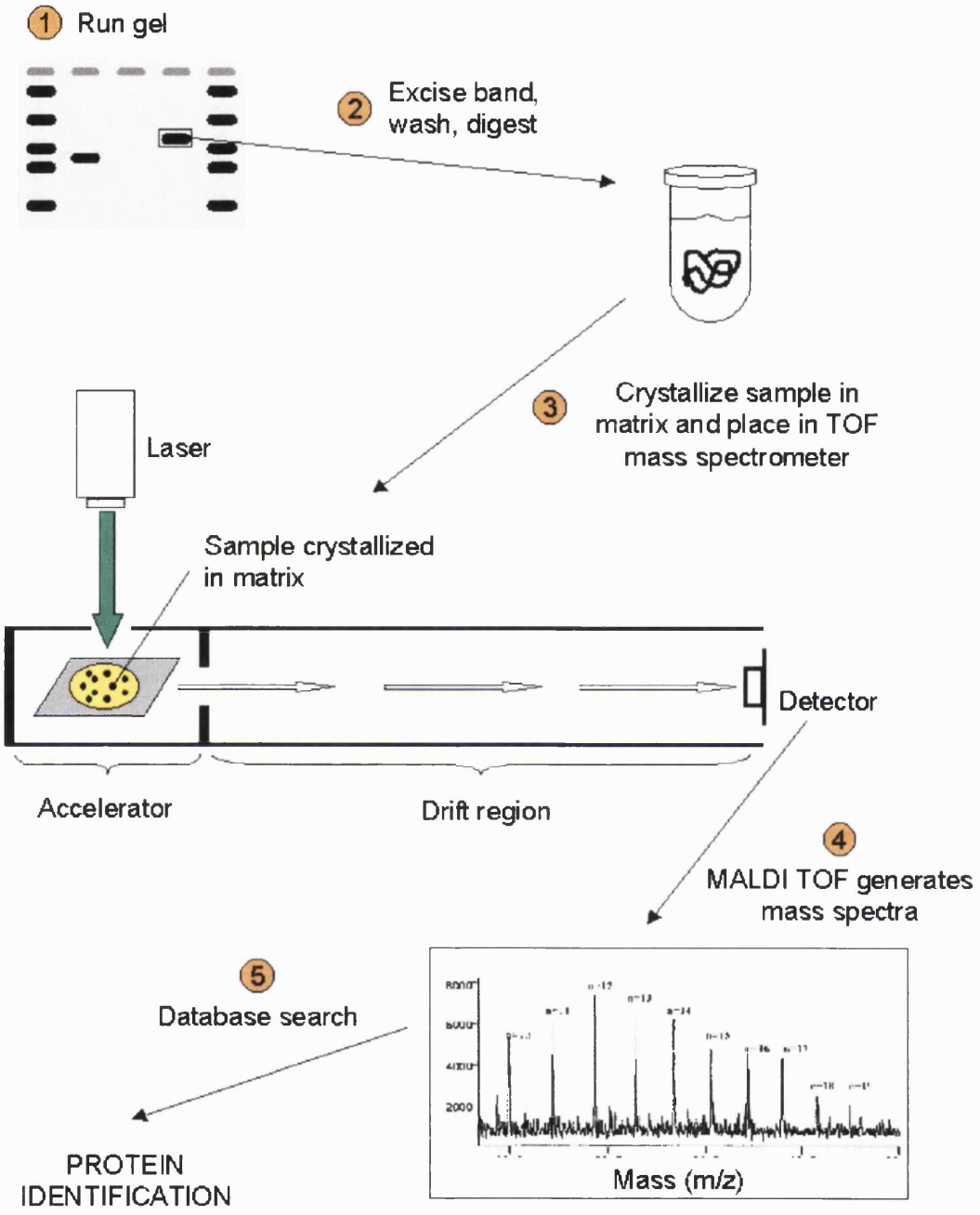


Figure 10: Schematic diagram of protein identification by MALDI-TOF MS. 1) Protein to be identified is run on a gel. 2) Protein band of interest is excised and in-gel digestion with trypsin is performed. 3) Tryptic fragments are crystallized in matrix. 4) Sample is then analysed by MALDI-TOF MS. 5) Mass peaks observed correspond to tryptic fragments of known proteins in database, identifying protein in sample.

II: 6 STUDIES USING OVARIECTOMIZED RABBITS

a) Ovariectomy procedure

Female New Zealand white rabbits (3.4 – 4.0kg, N=6 Harlan, UK) were sedated by injection of hypnorm (fentanyl Citrate (0.315mg/ml) and fluanisone (10mg/ml), National Veterinary Services Ltd., UK) into thigh muscle (0.3ml/kg). After 15 minutes anaesthesia was induced by injection of diazepam (5mg/ml, National Veterinary Services Ltd., UK) (two thirds of 2mg/kg) via a 25-gauge butterfly inserted in the ear marginal vein, and taped in place. At this point a subcutaneous injection of the antibiotic baytril (2.5%, National Veterinary Services Ltd., UK) was given into the tented scruff of the neck (0.2ml/kg). Once under anaesthesia the abdomen was shaved from 6cm below the sternum to 4cm above the clitoris. The rabbit was then placed on a heated mat to maintain body temperature, and a pulse oximeter was attached to the base of the tail to monitor blood oxygen saturation and heart rate. The last third of the diazepam was then injected and the butterfly flushed through with 300µl sterile saline. Anaesthesia was maintained by inhalation of halothane via a mask placed over the nose of the rabbit, and depth of anaesthesia checked every five minutes by checking for reflex action to painful stimulus. The shaved area was swabbed with chlorohexane and a 6cm mid-line incision was made to the abdomen. The ovaries were located and, after tying off fallopian tubes with No.3 non-absorbable suture, were removed. The incision was closed using subcutaneous stitching, and a subcutaneous injection of the vetergesic bupreorpine (0.3mg/ml, National Veterinary Services Ltd., UK) was administered (0.03mg/kg). Further subcutaneous injections of bupreorpine (0.03mg/kg) and baytril (0.2ml/kg) were given 24 and 48 hours

post-op. The animals were then kept for 12 (N=2) or 24 weeks (N=4) post-op before being used. During this time they were fed *ad libitum* standard rabbit diet.

b) Detection of ovarian tissue

Successful removal of the ovaries was established by a gonadotrophin releasing hormone (GnRH) stimulation test (Kanayama *et al.*, 1995). For this a basal blood sample of 2ml was taken from the rabbit ear marginal vein. This was followed by an i.v. injection of 0.32μg of the synthetic nonapeptide analogue of human GnRH, Buserelin (Receptal, Hoechst, UK). After 3 hours a further 2ml blood sample was taken from the ear marginal vein. Measurement of blood oestrodiol levels was performed by Surrey Diagnostics, UK. Complete removal of ovaries was assumed if tests revealed blood oestradiol concentrations of <10 pM in both pre- and post- GnRH stimulation blood. Sham-operated NZW rabbits have previously been shown to have blood oestradiol concentrations of 144 ± 31pM (N=8; Rouleu *et al.*, 2001).

II: 7 CHEMICALS

Adenosine free base, adenosine hemisulphate, adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), arachidonylethanolamide (AEA), bestatin, calcitonin gene-related peptide (CGRP), calcitonin gene-related peptide fragment 8-37 (CGRP 8-37), (3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulphonate (CHAPS), charybdotoxin, α-chymotrypsin type I, α-chymotrypsin type II-S, cibacron blue, ω-conotoxin GVIA (ω-CTX), dexamethasone, DL-3-mercapto-2-benzylpropanoyl-glycine (DL-thiorphan), guanosine 5'-O-(2-thiodiphosphate) (GDPβS), guanethidine, indomethacin, isoprenaline, N^G-nitro-L-arginine (L-NA), N^G-nitro-L-arginine methyl ester (L-NAME), N-iminoethyl-L-ornithine (L-NIO), (5α)-4, 5-epoxy-3, 14-dihydro-17-(2-propenyl)morphinan-6-one (naloxone), (5α)-17-(cyclopropylmethyl)-4, 5-epoxy-3, 14-dihydromorphinan-6-one (naltrexone), 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ), ouabain, phenylephrine, pituitary adenylate cyclase-activating peptide (PACAP-27 and PACAP-38), 8-phenyltheophylline (8-PT), N-(α-rhamnopyranosyloxy-hydroxyphosphinyl)-Leu-Trp (phosphoramidon), scopolamine, sodium nitroprusside (SNP), somatostatin, tetraethylammonium chloride (TEA), uridine 5'-diphosphate (UDP), uridine 5'-triphosphate (UTP), vasoactive intestinal peptide (VIP), vasoactive intestinal peptide fragment 6-28 (VIP 6-28), and vasoactive intestinal peptide fragment 10-28 (VIP 10-28) were purchased from Sigma, UK.

Apamin, (E)-N-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide (capsaicin), 2', 3'-dideoxyadenosine (2'3'-DDA), 2', 5'-dideoxyadenosine (2'5'-DDA), 9, 11-dideoxy-9α, 11α-methanoepoxyprostaglandin $F_{2\alpha}$ (U-46619), (2,2'-hydroxynitrosohydrazino) bis-ethanamine (DETA NONOate), forskolin, isobutylmethylxanthine (IBMX), *cis*-N-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A), neuropeptide Y, pertussis toxin, pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS), substance P, suramin, 9-(tetrahydro-2'-furyl)adenine (SQ 22536), tetrodotoxin (TTX) and [Arg^8]-vasopressin were purchased from Calbiochem, UK.

Peptide histidine methionine-27 (PHM) was purchased from Bachem Ltd., UK.

Glibenclamide was purchased from Alexis Corporation Ltd., UK.

4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385), 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) and zinc (II) protoporphyrin IX (ZnPP) were purchased from Tocris Cookson Ltd, UK.

Iberiotoxin (IbTX) was purchased from RBI, UK.

Helospectin I, helospectin II and peptide histidine valine (PHV) were synthesized by the Scientific Support Services in the Wolfson Institute for Biomedical Research.

Stock solutions of drugs were made up as follows, and then diluted as necessary in modified Krebs solution:

Adenosine free base (200mM in 0.3M NaOH), AEA (50mM in DMSO), apamin (1mM in 5% acetic acid), bestatin (10mM in water), capsaicin (200mM in ethanol), CGRP (1mM in water), CGRP 8-37 (1mM in water), charybdotoxin (100μM in water), ω-CTX (1mM in water), 2'3'-DDA (10mM in DMSO), 2'5'-DDA (10mM in DMSO), DETA NONOate (50mM in water), dexamethasone (10mg/ml in ethanol), DPCPX (1mM in DMSO), forskolin (100mM in DMSO), glibenclamide (100mM in DMSO), guanethidine (10mM in water), helospectin I (1mM in water), helospectin II (1mM in water), IbTX (100μM in water), IBMX (500mM in DMSO), indomethacin (5mg/ml in 5% $NaHCO_3$), L-NA (100mM in 0.1M NaOH), L-NIO (500mM in water), MDL-12,330A (3mg/ml in water), naloxone (100mM in water), naltrexone (100mM in water), neuropeptide Y (1mM in water), ODQ (10mM in DMSO), PACAP-27 (1mM in water), PACAP-38 (1mM in water), pertussis toxin (0.1mg/ml in water), PHM (1mM in water), phosphoramidon (10mg/ml in water), PHV (1mM in water), PPADS (10mM in water), 8-PT (25mM in 80% methanol / 20% 0.2M NaOH) scopolamine (10mM in water), somatostatin (1mM in water), SNP (100mM in water, substance P (1mM in water), suramin (100mM in water), DL-thiorphan (2.5mM in 50% ethanol/ 50% water), TTX (3mM in water), [Arg^8]-vasopressin (1mM in water), VIP (1mM in water), VIP 6-28 (1mM in water), VIP 10-28 (1mM in water), ZM-241385 (100mM in DMSO) and ZnPP (100mM in 0.2M NaOH).

Modified Krebs solution consisted of (mM): NaCl 136.9, KCl 2.7, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, $CaCl_2$ 1.8, glucose 12.5, dexamethasone 0.01, indomethacin 0.01.

The protease inhibitor cocktail used in transfer experiments was purchased from Sigma (P 8340) and contained 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF; 104mM), aprotinin (80μM), pepstatin A (1.5mM), trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane (1.4mM), bestatin (3.6mM), and leupeptin (2.1mM), dissolved in DMSO.

Tris/Tricine running buffer consists of 900ml water and 100ml Tris/Tricine/SDS (Bio-Rad, UK). Tris sample buffer was made up on the day of use from 9.8ml tricine sample buffer (Bio-Rad, UK) and 200μl β-mercaptoethanol.

Coomassie blue staining solution contained water (50%), methanol (45%), glacial acetic acid (5%) and coomassie blue (0.1%). The staining solution was filtered prior to use. Destaining solution contained water (87.5%), methanol (5%) and glacial acetic acid (7.5%).

Cyclic nucleotide assay buffer contained sodium acetate (0.05M), bovine serum albumin (0.02%; w/v) and preservative (0.01% w/v for cAMP assay; 0.5% w/v for cGMP

assay) at pH 5.8. Acetylation reagent consisted of acetic acid and triethylamine (mixed with a ratio of 1:2) prepared immediately before use.

cAMP cyclic nucleotide standards were prepared by reconstituting lyophilised cAMP, by adding 4 ml assay buffer giving a concentration of 32pmol/ml. 50µl of this led to a final cGMP content of 128fmol/well. 64fmol/well standard was prepared by mixing 1ml assay buffer with 1ml 32pmol/ml cGMP. Standards for 2, 4, 8, 16, and 32 fmol/well were prepared by repetition of this dilution process.

cGMP cyclic nucleotide standards were prepared by reconstituting 1.28nmol lyophilised cGMP, by adding 2.5ml assay buffer giving a concentration of 10.24pmol/ml. 50µl of this led to a final cGMP content of 512fmol/well. 256fmol/well standard was prepared by mixing 1ml assay buffer with 1ml 10.24pmol/ml cGMP. Standards for 2, 4, 8, 16, 32, 64 and 128 fmol/well were prepared by repetition of this dilution process.

II: 8 ANALYSIS OF THE RESULTS

a) Measurement of responses

In experiments to establish frequency dependence of the responses, duration and magnitude of relaxation were taken into account by measuring the relaxation as the area above the trace from the start of stimulation until the tissue returned to resting tone and expressing it as a percentage of the maximum relaxation to 50Hz EFS-induced relaxation (calculated using Clampfit Software, Axon Instruments, USA).

Over long periods, loss of phenylephrine-induced tone was observed in the vaginal wall. To enable us to compare responses over these periods, EFS-induced relaxations were measured as a percentage of the tone at the time of the relaxation. The effects of inhibitors were expressed as percentage of control relaxation prior to addition of the inhibitor. The following equation was used:

Percent of relaxation unaffected by inhibitor = 100 – ((magnitude of relaxation at time t (mN) ÷ tone at time t (mN)) ÷ (magnitude of control relaxation (mN) ÷ control tone (mN)) x 100)

Concentration-response curves (CRCs) to purines and pyrimidines were constructed by injecting each concentration of the drug after first eliciting an EFS-induced relaxation response. The magnitudes of the purine- or primidine-induced

responses were expressed as a percentage of the EFS-induced response prior to each injection. The tissue was allowed to recover tone between successive stimulations or injections. To examine the effect of antagonists on these CRCs the antagonists were applied for 30min before the construction of a second CRC. In the absence of antagonist, repeated CRCs gave lower maximal responses. Therefore each CRC was compared to the initial response to 3mM agonist and CRCs constructed after application of antagonist were compared with time-matched CRC controls.

Calculations of EC_{50} values were performed using Origin software (OriginLab Corporation, USA). Mean values for EC_{50} were expressed as pEC_{50} (-log[half maximum effective concentration (M)]). Tension changes in tissue strips were measured in mN.

b) Statistical analysis

Results are expressed as mean values ± standard error (s.e.) of mean from a number (n/N) of tissue strips, where small n denotes the number of tissue strips used, and capital N denotes the number of animals used for each set of experiments. Statistical analyses were performed using Prism v3.0 software (GraphPad Software Inc, USA). Data were compared as appropriate by Student's unpaired t-test for comparison of two means, by one-way analysis of variance (ANOVA) followed by Dunnett test for comparison of multiple means and by two way ANOVA followed by a Bonferroni post-test for either comparison of CRCs in the presence or absence of drugs or for comparison of the effect of the same drug in different tissues.

CHAPTER III

RESULTS

III: 1 CHARACTERISING AND ELICITING THE EFS-INDUCED NANC RELAXATION RESPONSE IN THE RABBIT VAGINAL WALL

a) Responses to 5Hz EFS in rabbit longitudinal vaginal wall strips

After longitudinal vaginal wall strips were equilibrated EFS (5s train, 0.3ms pulses, 50V at 5Hz) was applied every 2 mins. Tissues either maintained basal tone (47.2% of strips, n=580; Figure 11A) or relaxed slightly during stimulation (52.8% of strips, n=580; Figure 11B). Following termination of stimulation a contractile response was observed, with tension returning to baseline levels within 2 mins (Figure 11).

Responses to endogenous noradrenaline and ACh were blocked by incubation of vaginal wall strips with guanethidine (10μM), which inhibits release of noradrenaline from postganglionic adrenergic neurons and scopolamine (10μM), a muscarinic cholinergic antagonist. This abolished contractile responses and induced a slight increase in tone (Figure 12).

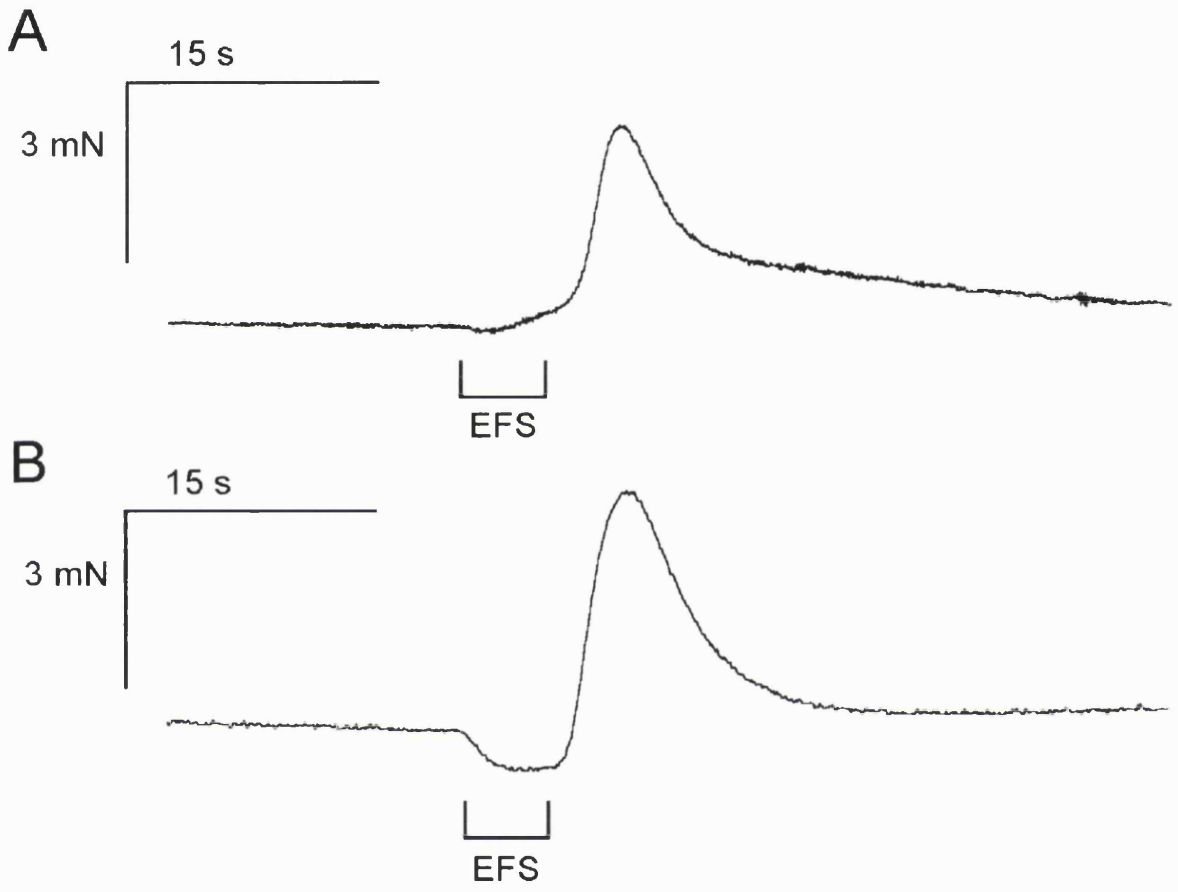


Figure 11: Typical responses to electrical field stimulation (EFS) in longitudinal strips of rabbit vaginal wall. Tissue either maintains tone (**A**), or relaxes (**B**) during stimulation (5s train, 0.3ms pulses, 50V at 5Hz), and a transient contraction occurs after stimulation is terminated. Mechanograms are original recordings of single tissue strips and are representative of all experiments in this series (n=580).

b) NANC relaxation responses elicited after application of phenylephrine

After inhibiting noradrenergic and cholinergic pathways addition of phenylephrine caused concentration-dependent increases in tissue tone (pEC_{50}=6.12 ± 0.10; Figure 12). In subsequent experiments, 1μM phenylephrine was used to contract tissues. This contracted the tissue by 7.5 ± 0.5mN above basal tone, peaking at 16.1 ± 0.9 minutes after addition of phenylephrine (n/N=30/17). After the contraction had peaked phenylephrine-induced tone decreased over time. The rate at which phenylephrine-induced tone diminished was not constant and also decreased over time (Figure 13). EFS (5Hz as above) after application of guanethidine (10μM), scopolamine (10μM) and phenylephrine (1μM) induced non-adrenergic, non-cholinergic (NANC) relaxation responses. NANC relaxation responses were elicited in this way in all subsequent experiments unless otherwise stated. The responses seemed to consist of two responses, a fast relaxation response, followed by a slower onset relaxation response. These two responses overlapped to varying extent to give three different relaxation profiles (Figure 14). These profiles all consisted of an initial fast relaxation with a rapid recovery. Following this fast relaxation a slower relaxation response was observed, with the two sometimes separated by a contraction. Different response profiles occurred depending on the speed of onset of the second, slow relaxation response. If this response had a slow onset, and reached a peak after the initial relaxation had recovered then the two responses appeared to be separated by a contraction (49.8% of tissue strips, n/N=512/140; Figure 14A and 14B). If the peak of the second relaxation occurred during the recovery from the fast response then the profile was of a two-phased relaxation response (32.0% of tissue strips, n/N=512/140; Figure 14C). If the slow response peaked during the fast relaxation

response then the two became merged to give the appearance of a single relaxation response (18.2% of tissue strips, n/N=512/140; Figure 14D).

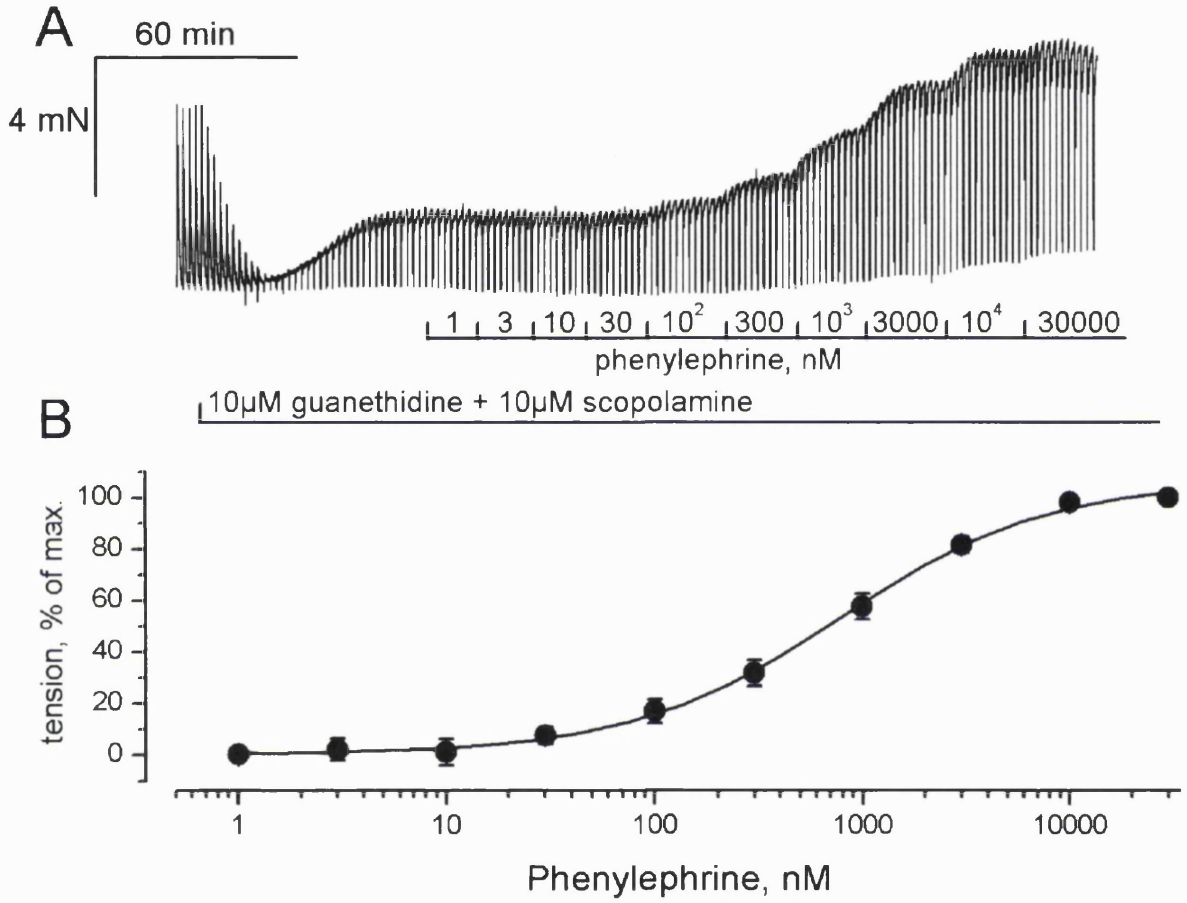


Figure 12: Effect of guanethidine, scopolamine and phenylephrine in rabbit longitudinal vaginal wall strips. EFS (5Hz, 0.3ms pulses, 50V, 5sec train every 2min)-induced contractile responses in the vaginal wall are abolished after treatment with guanethidine (10μM) and scopolamine (10μM). Subsequent incubation with phenylephrine induces concentration-dependent increases in tone. EFS-induced NANC responses are revealed in strips with raised tone. Mechanogram is an original recording of a single tissue strip and is representative of all experiments in this series (**A**, n/N=5/4). **B:** Concentration-dependence of phenylephrine-induced tone is expressed as a percentage of that induced by the maximum concentration used (30μM). Data points represent mean ± s.e. mean.

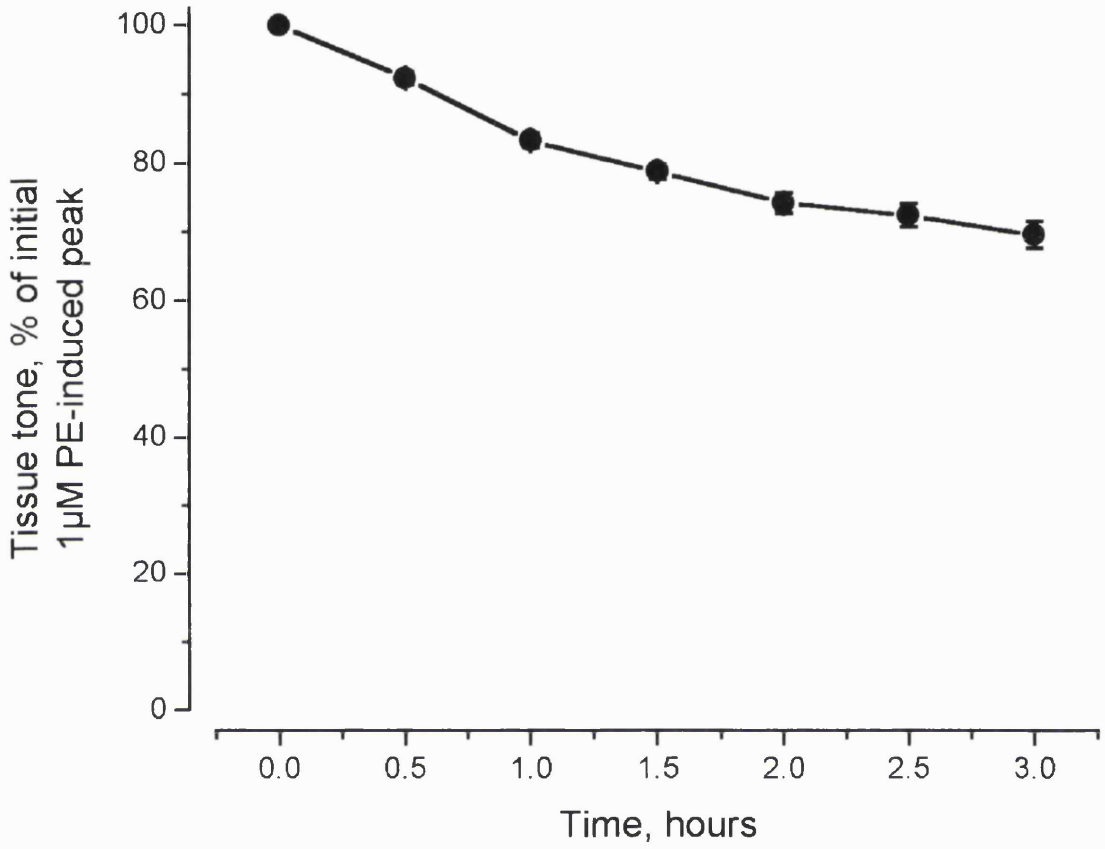


Figure 13: Time-course of loss of phenylephrine-induced tone in rabbit longitudinal vaginal wall strips. Phenylephrine-induced tone decreases over time after reaching an initial peak of contraction (time=0) in rabbit longitudinal vaginal wall strips. Data points represent mean ± s.e. mean (n/N=10/7).

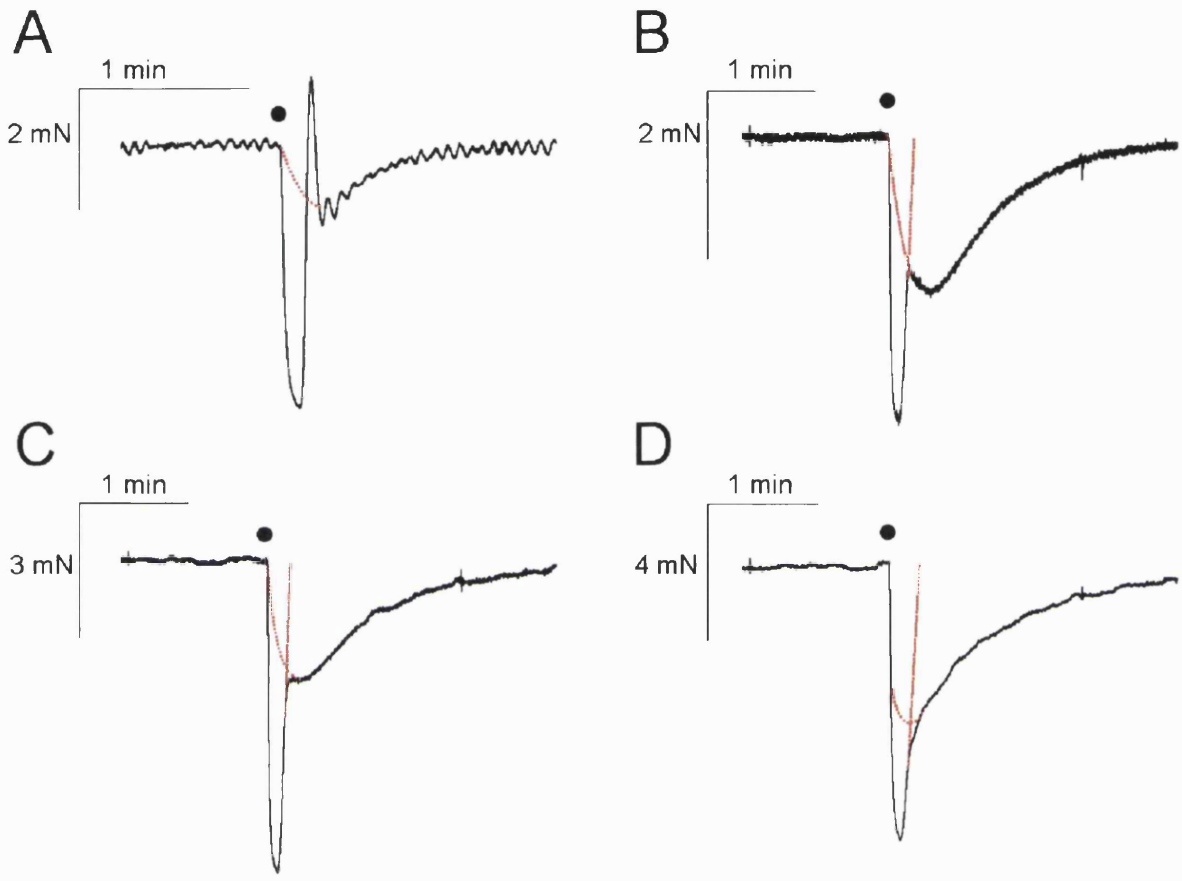


Figure 14: Fast and slow relaxation responses in rabbit longitudinal vaginal wall strips. Typical relaxation responses to 5Hz EFS (●, 0.3msec pulse, 50V, 5sec train) following incubation of vaginal wall strips with guanethidine (10μM), scopolamine (10μM) and phenylephrine (1μM). Responses consist of a fast relaxation response and a slow response that can peak either following the fast response, separated by a contractile response (**A** and **B**), as the tissue is recovering from the fast relaxation, giving a biphasic relaxation profile (**C**), or during the fast relaxation, giving the impression of a single relaxation response (**D**). Mechanograms are original recordings of single tissue strips and are representative of all experiments in this series (n/N=512/140). Red dashed lines indicate the presumed course of the fast and slow responses if viewed in isolation.

c) Frequency-dependence of NANC relaxation responses

EFS-induced relaxation responses in phenylephrine pre-contracted strips were frequency-dependent (Figures 15 & 16). Both fast and slow responses increased in magnitude and duration as frequency increased, regardless of the relaxation profile seen, reaching a maximal response at 50Hz (Figure 15). Measurement of this frequency dependence was achieved by measuring the area of the relaxation response and expressing it as a percentage of that induced by 50Hz EFS (Figure 16). In all further experiments 5Hz was used unless otherwise stated, as this frequency produced 52.4 ± 1.7% of the maximum response. Also, unless otherwise stated, in further experiments the magnitude of the fast relaxation response was used to measure the effects of drugs for two reasons. Firstly, since the variability in the relaxation profile seemed to occur in the slow response this gave inconclusive results when trying to assess the effect of drugs. Secondly, the magnitude of the slow response could not be accurately assessed if the response had the profile type shown in Figure 14D or 15A.

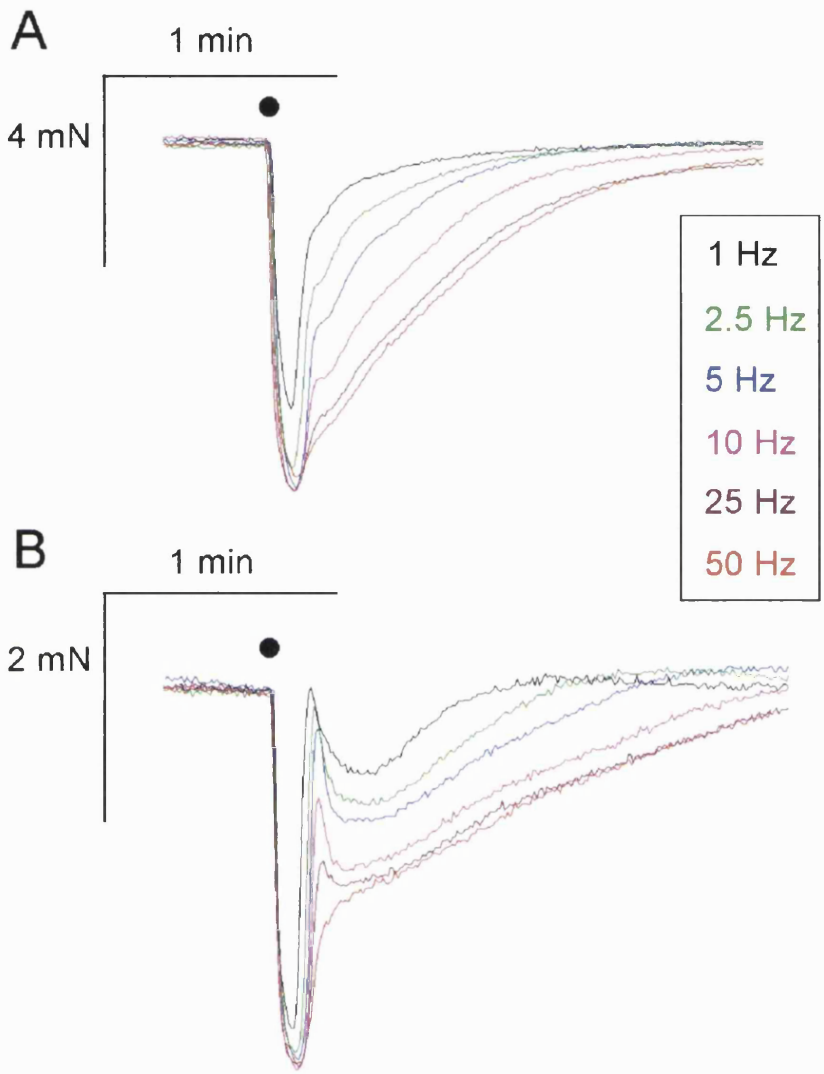


Figure 15: Typical traces of frequency dependence of EFS-induced relaxation responses in rabbit longitudinal vaginal wall strips. EFS (●, 1-50 Hz, 0.3msec pulse, 50V, 5sec train)-induced relaxations increase in both magnitude and duration as frequency of stimulation is increased. Responses in two different strips are shown (**A** and **B**). In each responses are overlaid so that the point of stimulation at each frequency is in the same place. Mechanograms are original recordings of single tissue strips and are representative of all experiments in this series.

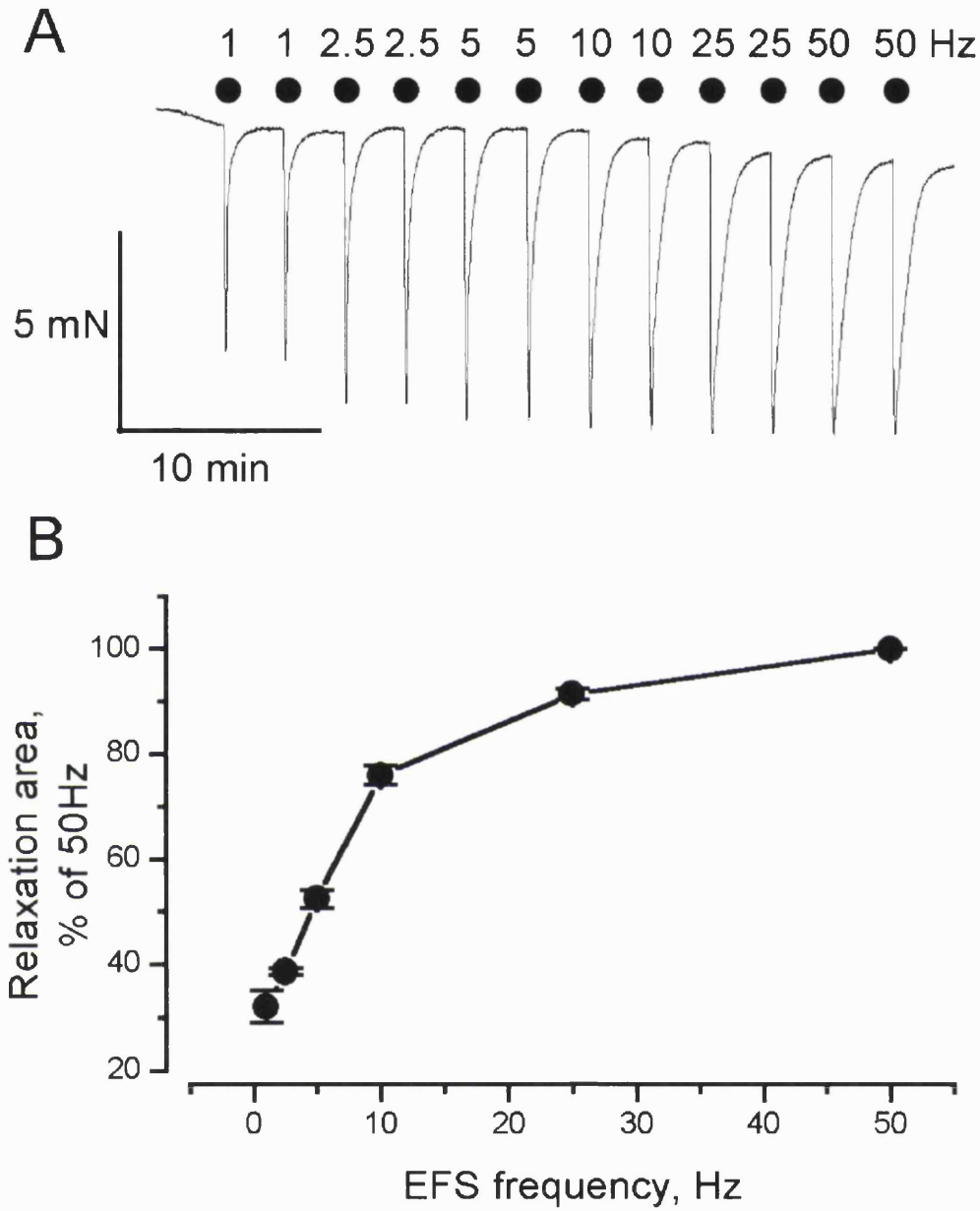


Figure 16: Frequency dependence of EFS-induced relaxation responses in rabbit longitudinal vaginal wall strips. NANC relaxation responses elicited by EFS (indicated by dots) were frequency-dependent. The mechanogram (**A**) is an original recording of a single tissue preparation and is representative of all the experiments in this series (n/N=6/6). The frequency-dependence of the relaxation magnitude and duration is expressed as the area of the relaxation as a percentage of relaxation elicited by the maximum frequency used (50 Hz). Data points represent mean ± s.e. mean (**B**).

d) Comparison of longitudinal and circular strips of rabbit and rat vaginal wall

Circular strips of rabbit vaginal wall, longitudinal strips of the rat vaginal wall, and circular strips from the distal part of rat vaginal wall (not from more proximal regions, more than 2mm from the external opening) were contracted by EFS. Rabbit circular vaginal wall strips contract during 5s trains of 5Hz EFS, followed by a further transient contraction on termination of stimulation, with the tissue then returning to basal tone (n/N=2/2). Rat circular distal vaginal wall strips had varying responses to 5Hz EFS with the tissues either contracting during EFS (33% of tissue strips; Figure 17A), or transiently relaxing before contracting during EFS (67% of strips; Figure 17B). In both cases these responses were followed by a further transient contraction on termination of stimulation, then a return to basal tone. In longitudinal strips of rat vaginal wall a contractile response was seen during 5Hz EFS, with a return to basal tone observed on termination of stimulation (Figure 17C).

In all cases these contractions were inhibited by guanethidine (10μM) and scopolamine (10μM), as seen in longitudinal strips of rabbit vaginal wall. When rabbit circular strips and rat distal circular strips were incubated with phenylephrine (1μM) tissue tone was increased as for longitudinal strips of rabbit vaginal wall. Subsequent EFS induced similar frequency-dependent relaxation responses to those seen in rabbit longitudinal strips (Figure 18). The longitudinal strips and the proximal circular strips of rat vaginal wall did not give contractile responses to phenylephrine.

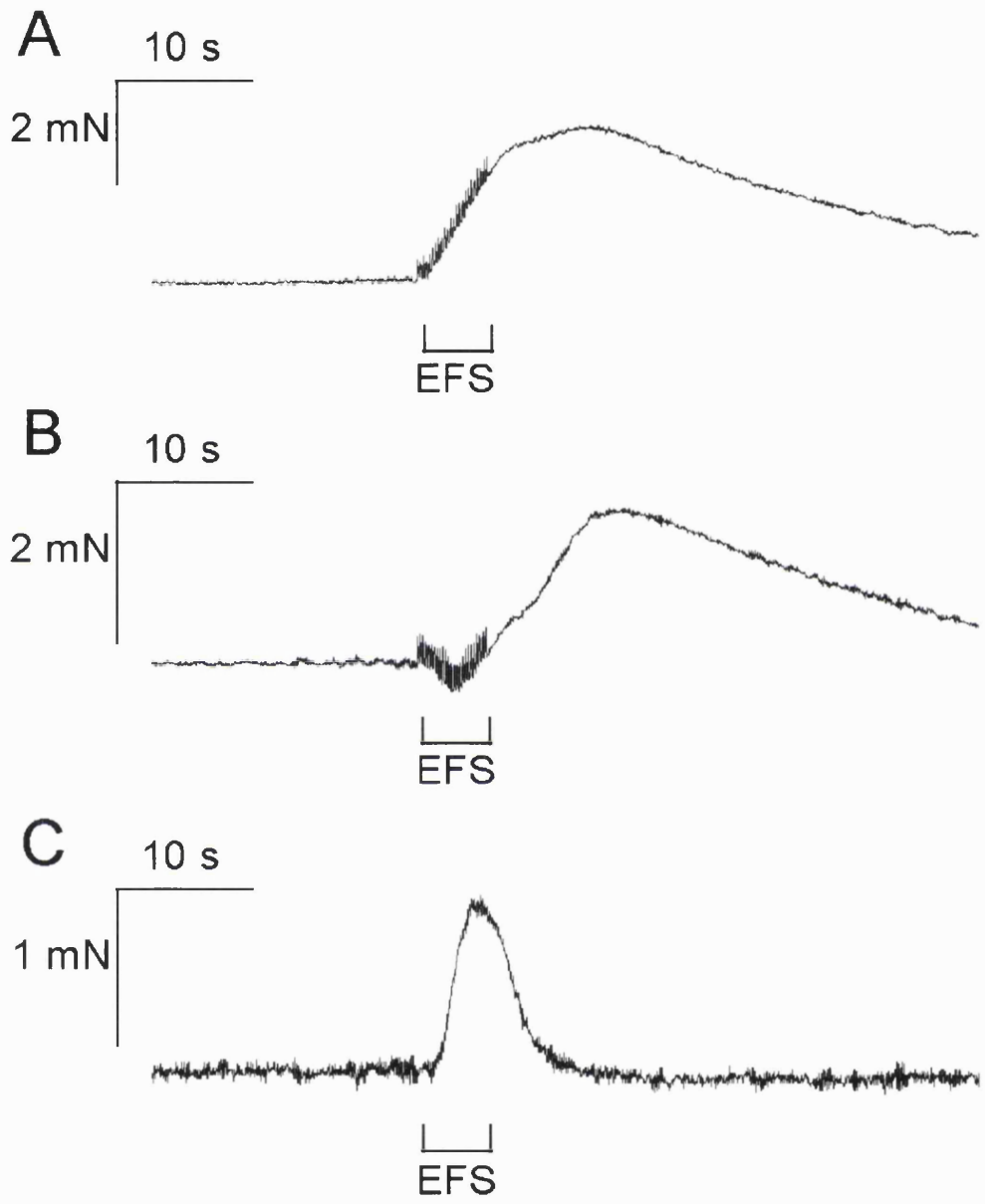


Figure 17: Typical responses to 5Hz EFS in rat circular and longitudinal vaginal wall strips. Circular strips of rat distal vaginal wall either contract (**A**) during a 5s train of 5Hz EFS or transiently relax followed by a contraction (**B**). Longitudinal strips of rat vaginal wall contract during stimulation, returning to basal tone on termination of EFS (**C**). Mechanograms are original recordings of single tissue strips and are representative of all experiments in this series (n/N=6/4 for rat circular strips, n/N=2/2 for rat longitudinal strips).

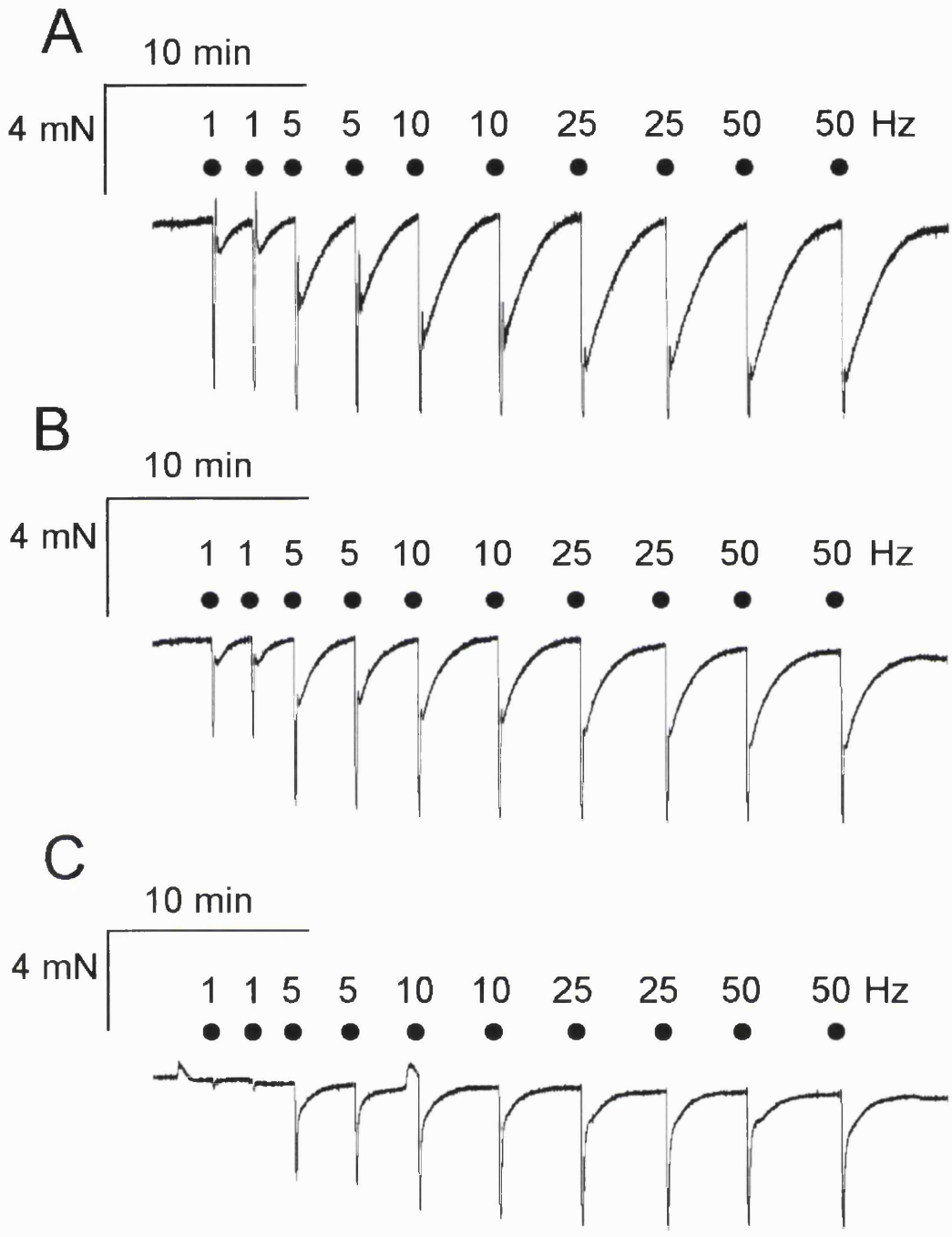


Figure 18: Comparison between frequency-dependent EFS(●)-induced NANC relaxation responses in rabbit longitudinal (A) and circular (B) vaginal wall strips and rat circular vaginal wall strips (C). The mechanograms are original recordings of single tissue preparations and are representative of all the experiments in this series (A: n/N=6/6; B: n/N=2/2; C: n/N=6/4)

III: 2 THE ROLE OF NITRIC OXIDE IN NANC RELAXATION RESPONSES

a) Effect of NOS inhibitors on EFS-induced NANC relaxation responses in rabbit longitudinal vaginal wall and clitoral corpus cavernosum strips

The NOS inhibitors L-NAME (1-500μM), L-NA (1-300 μM), and L-NIO (1-500μM) caused partial inhibition of EFS-induced NANC relaxations in a concentration-dependent manner (Figures 19, 20 & 21). However, a significant component of the EFS-induced relaxation responses were insensitive to blockade by these inhibitors with 61.4 ± 8.9%, 81.1 ± 2.1% and 74.4 ± 6.7% of the responses remaining after incubation with the highest concentrations of L-NAME, L-NA and L-NIO respectively in rabbit longitudinal vaginal wall strips.

As a control for the efficacy of the NOS inhibitors, the compounds were used simultaneously to inhibit NANC relaxation responses of the vaginal wall and clitoral corpus cavernosum from the same animal. Responses of the clitoral cavernosum were abolished by L-NAME (500μM; Figures 19 & 21), completely or almost completely abolished (18.3 ± 18.1% of control relaxations remaining) by L-NA (300μM; Figure 21), and reduced (35.4 ± 6.8% of control relaxations remaining) by L-NIO (500μM; Figure 20 & 21). The effect of the NOS inhibitors was significantly different between vaginal wall and clitoral corpus cavernosum strips with a probability of observing the differences by chance of 0.01%, 0.07% and 1.8% for L-NAME, L-NA and L-NIO respectively (Determined by two way ANOVA).

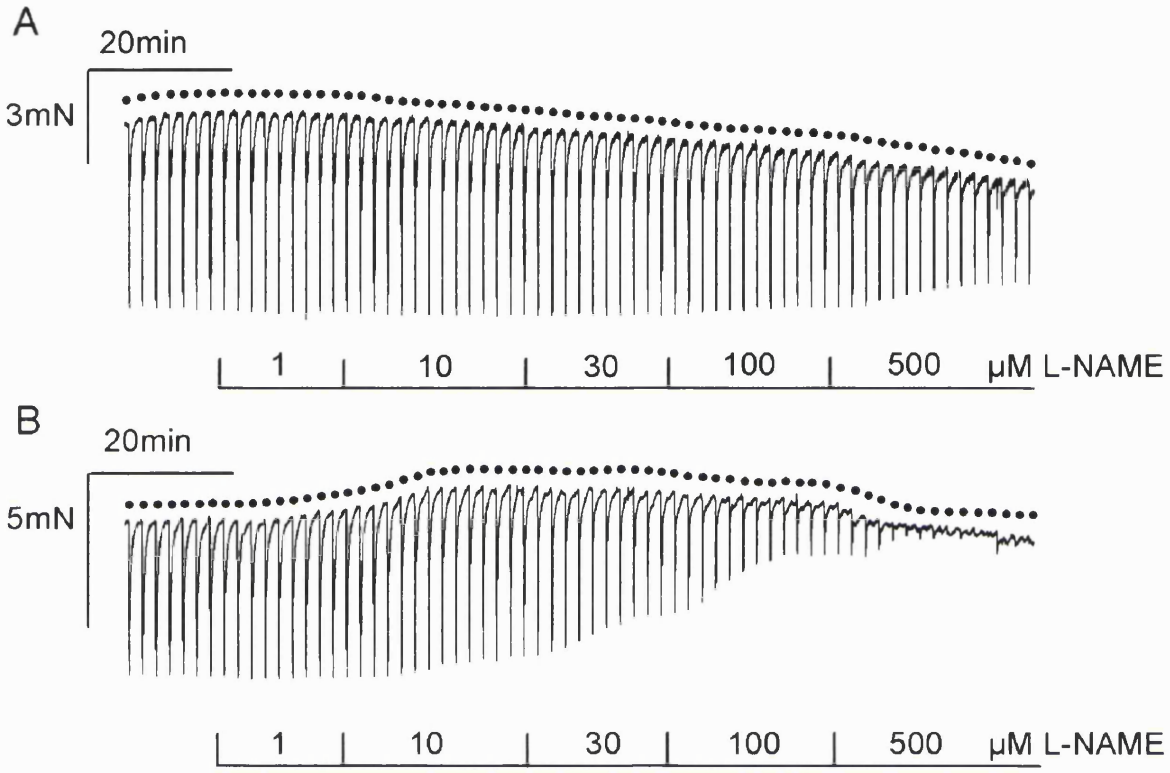


Figure 19: Effect of L-NAME on 5 Hz EFS-induced NANC relaxation responses in rabbit longitudinal vaginal wall and clitoral corpus cavernosum strips. Vaginal wall and clitoral corpus cavernosum strips from the same animals were treated simultaneously for comparison. NANC relaxation responses elicited by EFS (5 Hz, every 2 min, indicated by dots) were partially inhibited by L-NAME (1-500 µM) in the vaginal wall (**A**) but were abolished in the clitoral corpus cavernosum (**B**). The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series (n/N=4/4).

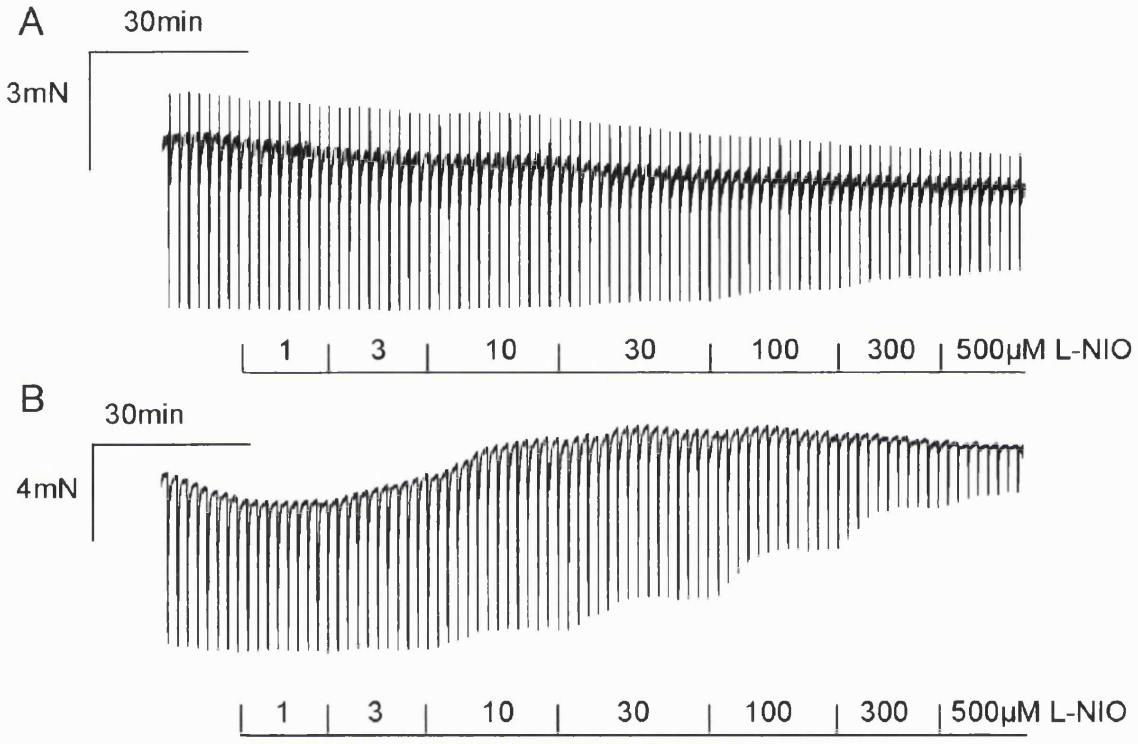


Figure 20: Effect of L-NIO on 5 Hz EFS-induced NANC relaxation responses in rabbit longitudinal vaginal wall and clitoral corpus cavernosum strips. Vaginal wall and clitoral corpus cavernosum strips from the same animals were treated simultaneously for comparison. NANC relaxation responses elicited by EFS (5 Hz, every 2 min) were partially inhibited by L-NIO (1-500 µM) in the vaginal wall (**A**) and to a greater extent in the clitoral corpus cavernosum (**B**). The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series (n/N=4/4).

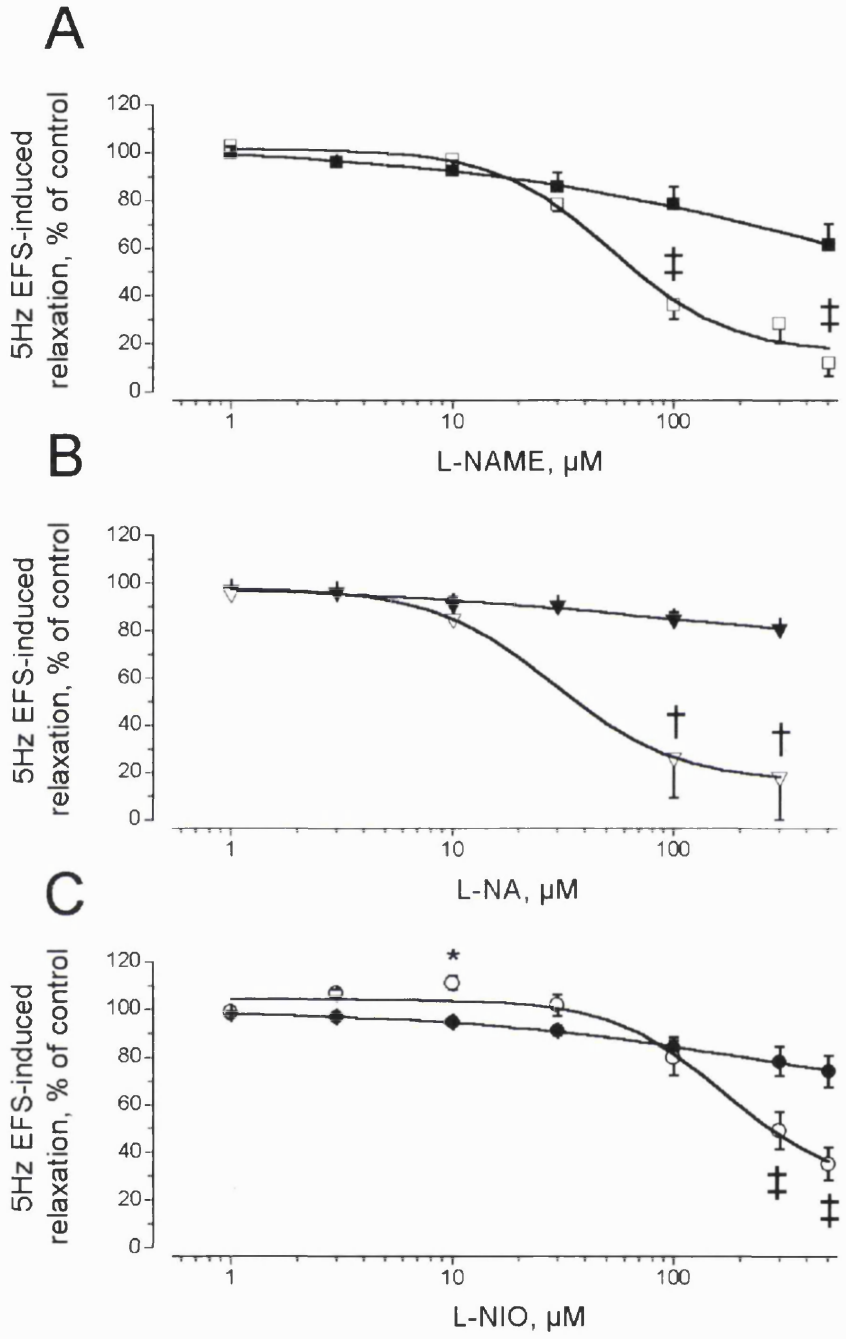


Figure 21: Concentration response curves to NOS inhibitors in rabbit longitudinal vaginal wall and clitoral corpus cavernosum strips. Comparison of inhibition of 5Hz EFS-induced relaxation responses in the rabbit vaginal wall (filled shapes) and clitoral corpus cavernosum (open shapes) by L-NAME (**A**, squares), L-NA (**B**, triangles) and L-NIO (**C**, circles). Data points represent mean ± s.e. mean (n/N=4/4 for each NOS inhibitor). Effect of NOS inhibitor concentration significantly different in vaginal wall and clitoral cavernosum strips, *=P<0.05, †=*P*<0.01, ‡=P<0.001. Statistical analyses performed using two way ANOVA followed by Bonferroni post-test.

In a separate series of experiments EFS-induced relaxations were elicited in rabbit longitudinal vaginal wall strips at different frequencies (1-50Hz) between successive incubations with increasing concentrations of L-NAME (3-500μM). The NOS-inhibitor was observed to be more effective at inhibiting the relaxation responses at low frequencies, with only 1 Hz EFS-induced relaxations being significantly reduced by 30μM L-NAME, and a greater degree of inhibition of 1 Hz EFS-induced relaxation seen at the highest concentration of 500μM L-NAME (Figure 22).

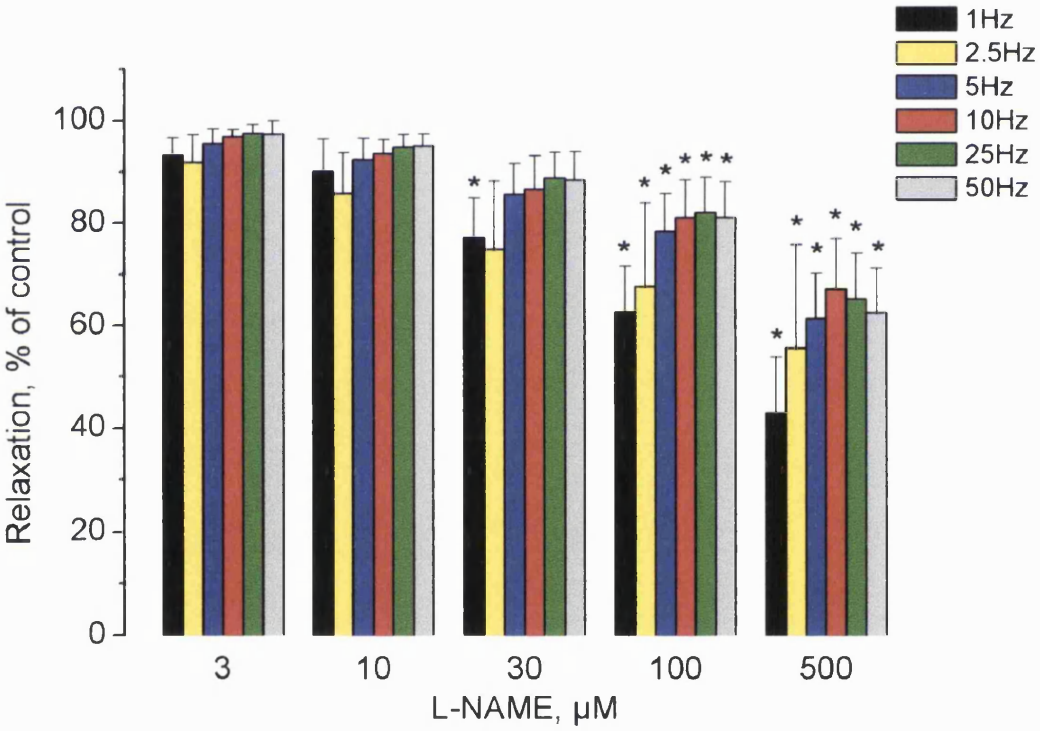


Figure 22: Degree of inhibition by L-NAME of relaxation responses to EFS is dependent on stimulation frequency in rabbit longitudinal vaginal wall strips. Control for each bar is that frequency stimulation in the absence of L-NAME , to enable comparison of frequencies that give initial responses of varying magnitude. Bars represent mean ± s.e. mean (n/N=4/4). *Significantly different from control responses for each frequency in the absence of L-NAME, $P<0.05$. Statistical analyses performed using ANOVA followed by Dunnett test.

b) Effect of ODQ and tetrodotoxin on EFS-induced NANC relaxation responses in the rabbit vaginal wall

As for the NOS inhibitors, the sGC inhibitor, ODQ (0.1-10μM) caused partial inhibition of the relaxation responses in a concentration-dependent manner. The degree of inhibition observed varied with stimulation frequency (Figure 23). 1Hz EFS-induced relaxations were significantly inhibited at lower concentrations of ODQ than those required to inhibit high frequency EFS-induced relaxations, and at the maximum concentration of ODQ used 1Hz EFS-induced relaxations were inhibited significantly more than those at higher (5-50Hz) frequencies (n/N=4/4, $P<0.05$).

When L-NAME and ODQ were used in combination, the resultant inhibition was no greater than the inhibition with either agent alone. After combination of L-NAME (500μM) and ODQ (1μM, n/N=9/4), L-NAME alone (500μM, n/N=57/23) or ODQ alone (10μM, n/N=5/4), 70.3 ± 4.0%, 68.0 ± 1.8%, and 68.7 ± 1.8% of the 5Hz EFS-induced relaxation responses were unaffected respectively ($P>0.05$). Treatment of the tissue with tetrodotoxin (TTX, 1μM) abolished EFS (5Hz)-induced non-nitrergic relaxation responses (Figure 24; n/N=4/4 for TTX after L-NAME+ODQ; n/N=8/4 for TTX after L-NAME alone).

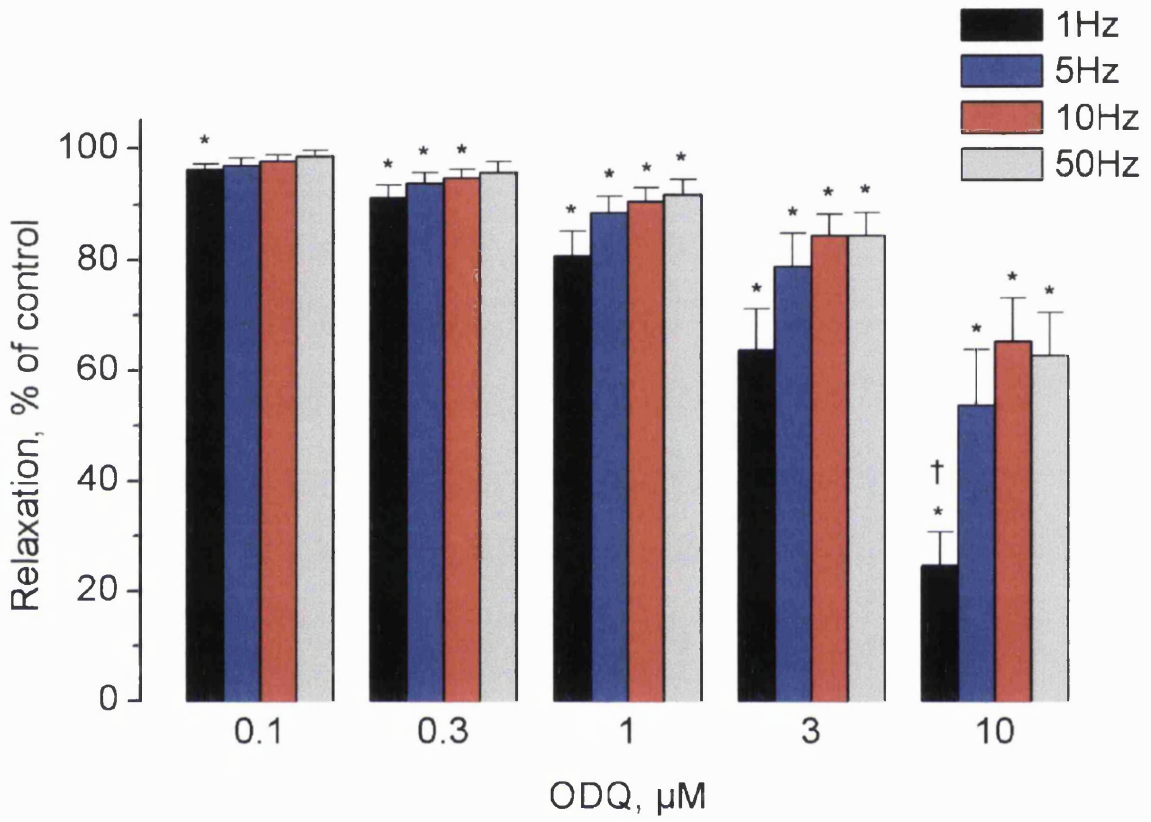


Figure 23: Degree of inhibition by ODQ of relaxation responses to EFS is dependent on stimulation frequency in rabbit longitudinal vaginal wall strips. Control for each bar is that frequency stimulation in the absence of ODQ, to enable comparison of frequencies that give initial responses of varying magnitude. Bars represent mean ± s.e. mean (n/N=4/4). *Significantly different from control responses for each frequency in the absence of ODQ, $P<0.05$. †Significantly different from other frequencies at same concentration of inhibitor, $P<0.05$. Statistical analyses performed using ANOVA followed by Dunnett test.

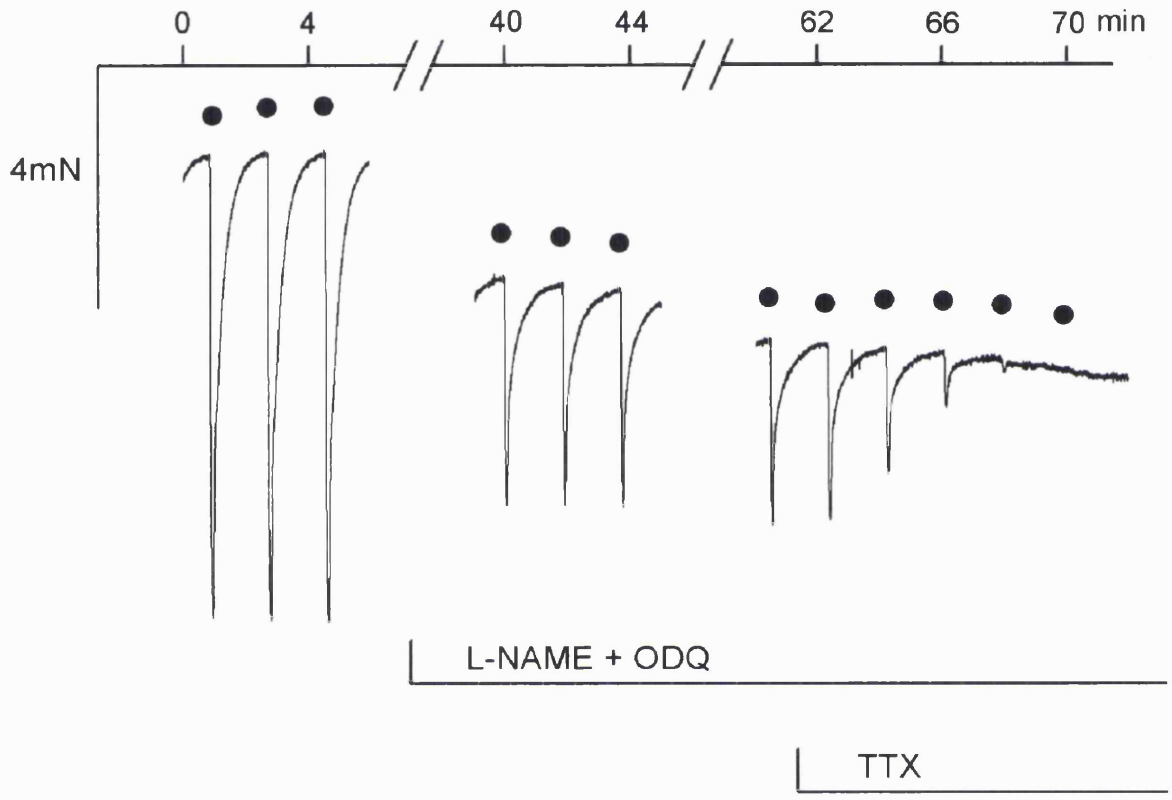


Figure 24: Partial inhibition of NANC responses by L-NAME and ODQ, and total inhibition by TTX in rabbit longitudinal vaginal wall strips. NANC responses elicited by EFS (5 Hz, every 2 min, indicated by dots) were partially inhibited by a combination of L-NAME (500 µM) and ODQ (1 µM). Addition of the sodium channel blocker TTX (1 µM) completely abolished the responses. The mechanogram is an original recording of a single tissue preparation and is representative of all experiments in this series (n/N=4/4).

c) Effect of L-NAME and neurotoxins on EFS-induced NANC relaxation responses in the rabbit vaginal wall and clitoral corpus cavernosum

As for concentration-dependent partial inhibition of NANC relaxation responses by NOS inhibitors (see section III: 2a), incubation of longitudinal vaginal wall strips with L-NAME at the maximum concentration used (500μM) caused slight inhibition of the fast relaxation (68.0 ± 1.8% of control responses remained, n/N=57/23; Figure 25A) it also caused partial inhibition of the slow relaxation responses (61.8 ± 6.2% of control responses remained, n/N=18/10; Figure 25A). Incubation with the voltage operated calcium channel (VOCC) blocker ω-conotoxin (ω-CTX, 100nM) also caused slight inhibition of the fast responses (89.4 ± 1.9% of control responses remained, n/N=10/6), but inhibited the slower responses to a greater extent than fast responses (42.5 ± 4.6% of control remaining, $P<0.05$, n/N=10/6). Using L-NAME and ω-CTX in conjunction caused a greater degree of inhibition of the fast responses than either treatment alone ($P<0.05$) and almost completely abolished the slow responses (44.8 ± 5.4% and 6.5 ± 1.6% of control remaining respectively; Figure 25A). Addition of 1μM TTX abolished remaining responses (Figure 25A).

In the clitoral corpus cavernosum 100 nM ω-CTX inhibited responses to 85.8 ± 2.1 % of control responses (n/N=10/6), whilst a combination of ω-CTX (100nM) and L-NAME (500μM) abolished the responses (4.5 ± 1.3 % of control responses remained; Figure 25B).

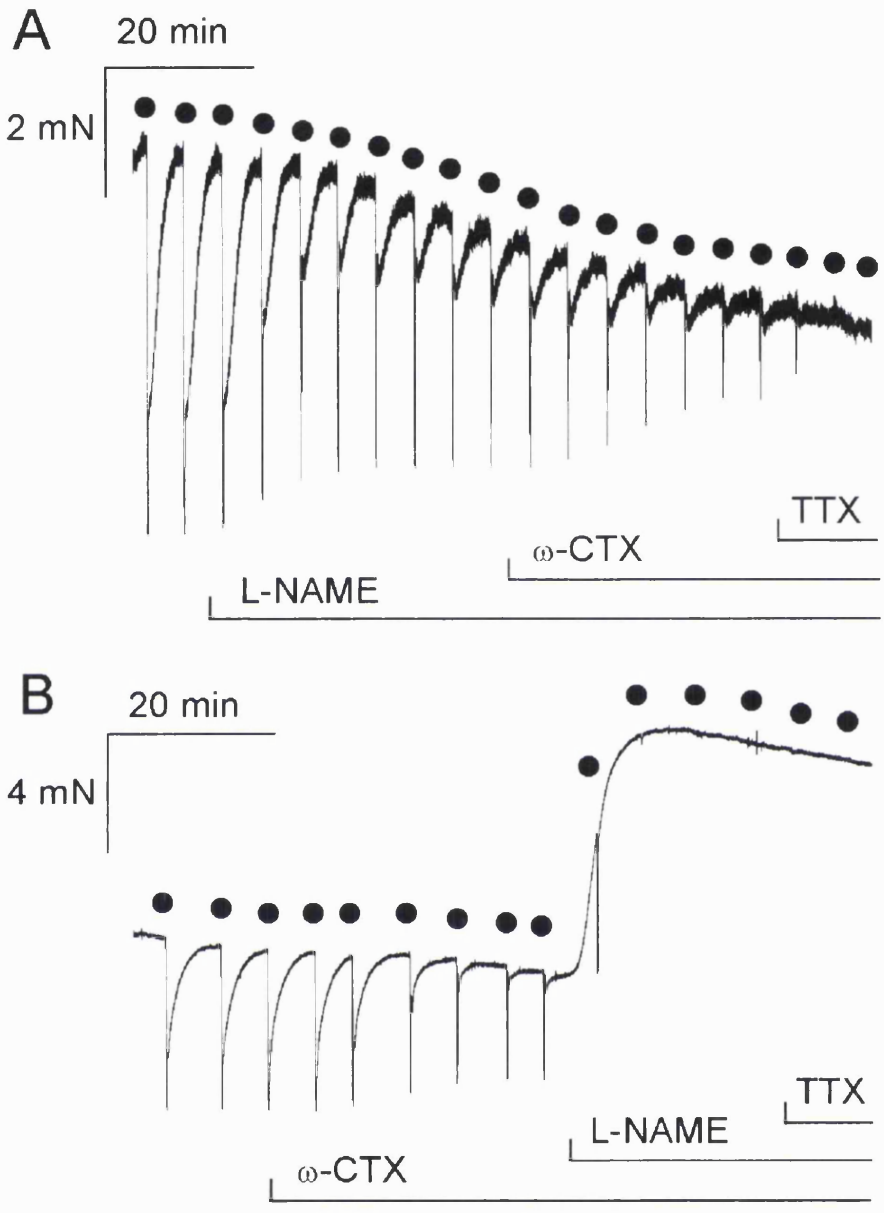


Figure 25: Effect of L-NAME, ω-CTX and TTX on NANC relaxation responses in rabbit longitudinal vaginal wall (A) and clitoral corpus cavernosum (B) strips. After treatment of tissue strips with guanethidine, scopolamine and phenylephrine 5Hz EFS (●)induced relaxation responses. In vaginal wall strips fast and slow relaxation responses to were partially inhibited by L-NAME (500μM), and were reduced to a greater extent following addition of ω-CTX (100nM). Remaining relaxation responses were abolished by TTX (1μM) (**A**: n/N=8/4). In the clitoral corpus cavernosum 5Hz EFS-induced responses were reduced in magnitude and duration by ω-CTX (100nM), and were abolished by subsequent addition of L-NAME (500μM; **B**: n/N=8/4).

d) Effect of L-NAME on circular strips of rat vaginal wall

After establishing the frequency-dependence of EFS-induced NANC relaxation responses in rat circular vaginal wall strips the tissues were incubated with L-NAME (500μM). In the rat vaginal wall the EFS-induced relaxations were reduced to a greater extent ($P<0.05$) than those in longitudinal rabbit vaginal wall strips (Table 1).

Table 1: Comparison of the inhibitory effect of 500μM L-NAME on EFS-induced relaxation responses in rat circular (n/N=3/3) and rabbit longitudinal (n/N=4/4) vaginal wall strips. *Significantly different from degree of inhibition in rabbit vaginal wall strips, $P<0.05$. Statistical analyses performed using two population, unpaired Student's *t*-test.

Stimulation Frequency	**Relaxations remaining after 500μM L-NAME (mean % of control ± s.e.)**	
Hz	**Rabbit**	**Rat**
1	43.0 ± 10.9	2.9 ± 1.6 *
5	61.4 ± 8.9	13.8 ± 5.6 *
10	67.2 ± 9.8	19.7 ± 6.1 *
25	65.3 ± 8.9	20.6 ± 6.2 *
50	62.6 ± 8.6	19.0 ± 6.5 *

III: 3 THE ROLE OF PEPTIDES IN NANC RELAXATION RESPONSES

a) Effect of VIP-related peptides on phenylephrine pre-contracted rabbit longitudinal vaginal wall strips

After eliciting NANC relaxation responses following incubation with guanethidine (10μM), scopolamine (10μM) and phenylephrine (1μM), EFS was stopped and subsequent addition of VIP (1nM - 3μM), PACAP-27 (1nM - 5μM), PACAP-38 (1nM - 3μM), PHM (1nM - 5μM), PHV (1nM - 3μM), helospectin I (1nM - 1μM) and helospectin II (1nM - 3μM) in cumulative concentrations induced relaxation responses in a concentration-dependent manner (Figure 26 and Figure 27A for helospectin II). The efficacy and potency of the different peptides (efficacy was measured as the relaxation induced by the highest concentration of peptide compared to that induced by 5Hz EFS-induced relaxation and potency was represented by pEC_{50} values) are compared in Table 2.

b) Effect of α-chymotrypsin on EFS-induced, and exogenous peptide-induced relaxations in rabbit longitudinal vaginal wall strips.

In the presence of α-chymotrypsin (2 units/ml), relaxation responses to cumulative concentrations of exogenous peptides were not observed (Figure 27B for helospectin II). The presence of α-chymotrypsin lowered the tone of the tissue (with 79.2 ± 1.0% of tone remaining after 30 mins incubation, n/N=20/13) but had no effect on EFS-induced non-nitrergic relaxations, with responses during incubation with the peptidase 97.5 ± 3.8% of control, ($P>0.05$, n/N=6/6; Figure 27C).

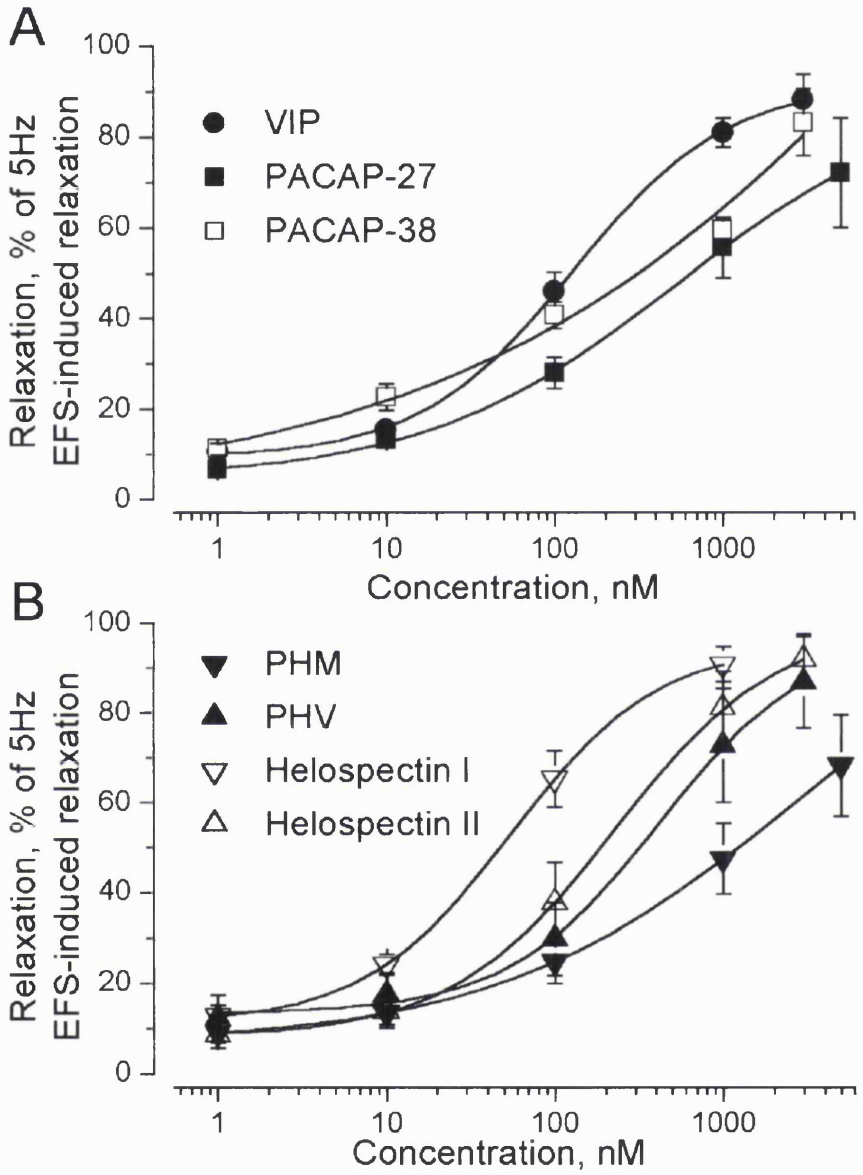


Figure 26: Concentration-response curves showing the relaxant effect of the VIP-related peptides in rabbit longitudinal vaginal wall strips. A: VIP (●), PACAP-27 (■), PACAP-38 (□) and **B**: PHM (▲), PHV (▼), Helospectin I (▽) and Helospectin II (△) induce concentration-dependent relaxation responses. Relaxations are expressed as a percentage of 5 Hz EFS-induced relaxations in the absence of peptides. Data points represent mean ± s.e. mean (n/N=4/4 for VIP, PACAP-38, PHV and helospectin II, n/N=5/4 for PACAP-27 and PHM, n/N=6/4 for helospectin I).

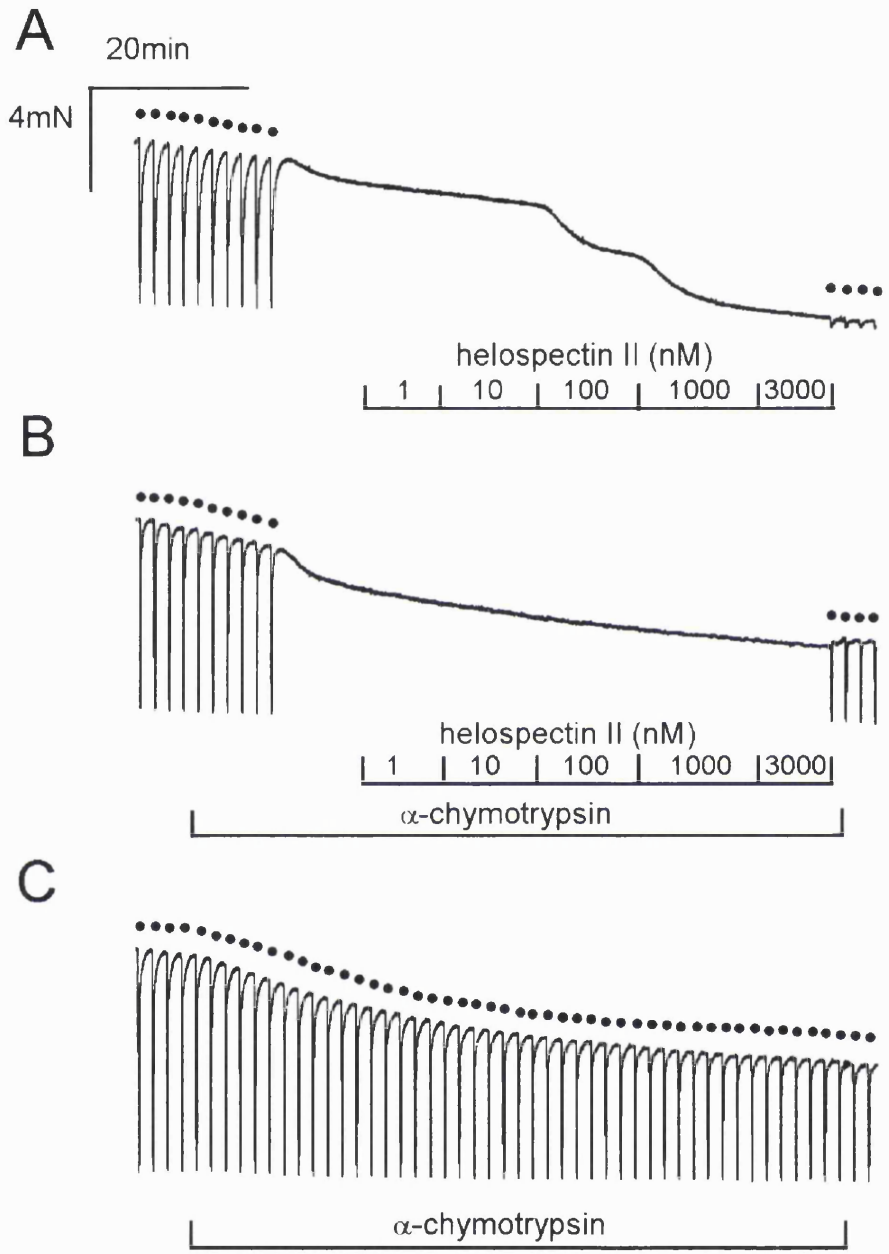


Figure 27: Effect of α-chymotrypsin on EFS- and helospectin II-induced relaxation responses in rabbit longitudinal vaginal wall strips. After treatment of the tissue with guanethidine, scopolamine, phenylephrine and L-NAME, exogenous application of cumulative concentrations of helospectin II (1 nM- 3 μM) resulted in relaxation responses (**A**). These responses were inhibited in the presence of α-chymotrypsin type II (2 units/ml, **B**). EFS- (5 Hz, every two minutes, indicated by dots) induced relaxations were not inhibited by α-chymotrypsin (**C**). The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series (n/N=4/4).

Table 2: Efficacy and potency of VIP-related peptides in producing relaxation responses in rabbit longitudinal vaginal wall strips. †= Significantly different from VIP-induced responses, P<0.01. Statistical analyses performed using two population, unpaired Student's *t*-test.

PEPTIDE	Concentration Range (μM)	Maximum relaxation (% of 5Hz EFS-induced relaxation ± s.e.)	pEC_{50} ± s.e. (M)	n/N
VIP	0.001 – 3	88.2 ± 5.5	6.89 ± 0.04	4/4
PACAP-27	0.001 – 5	72.0 ± 12.1	6.27 ± 0.09 †	5/4
PACAP-38	0.001 – 3	83.1 ± 7.3	6.68 ± 0.12	4/4
PHM	0.001 – 5	68.2 ± 11.3	5.50 ± 0.01 †	5/4
PHV	0.001 – 3	86.7 ± 10.2	6.38 ± 0.13 †	4/4
Helospectin I	0.001 – 1	90.8 ± 3.9	7.12 ± 0.18	6/4
Helospectin II	0.001 – 3	91.8 ± 5.8	6.64 ± 0.02	4/4

When the peptides VIP (300nM) and PACAP-27 (400nM) were added into the superfusion medium as an infusion, in the presence of L-NAME (500μM), a marked relaxation response was observed (Figure 28). These relaxations were completely abolished when the peptidase α-chymotrypsin was added into the superfusion medium (2

units/ml; Figure 28). After removal of the peptidase from the medium, further relaxations to VIP and PACAP were obtainable (Figure 28).

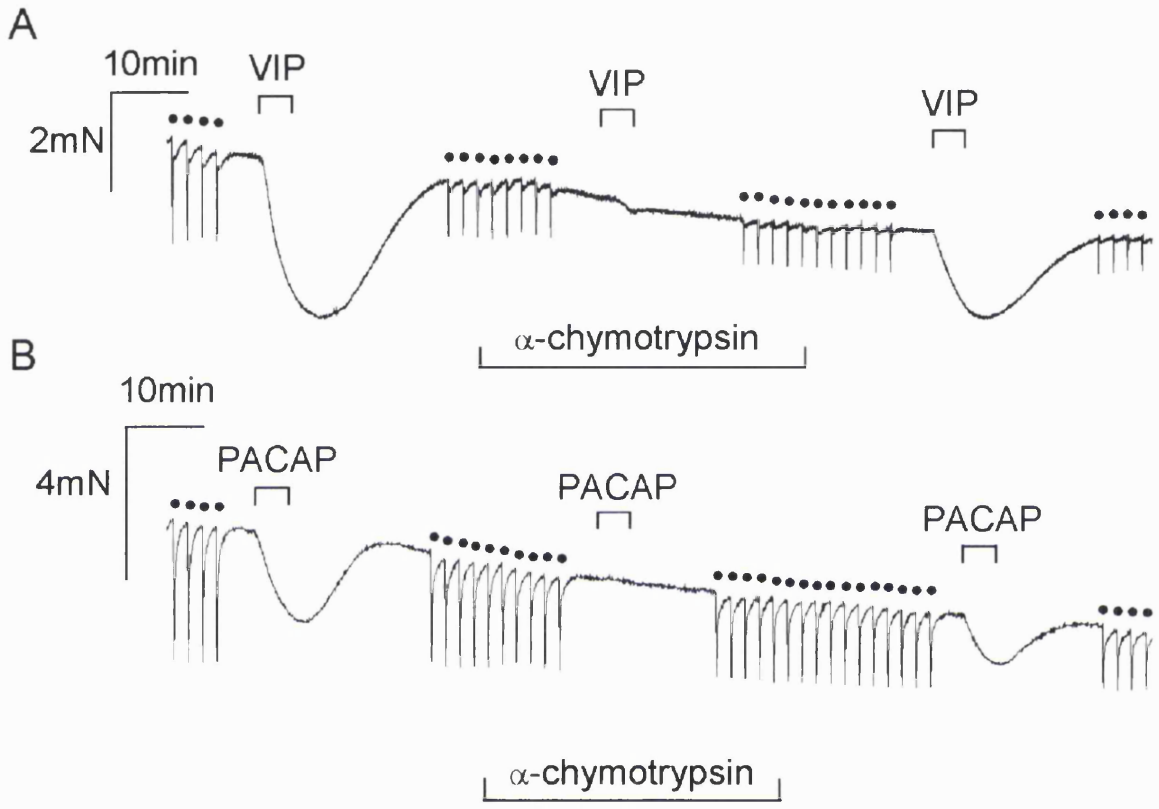


Figure 28: Inhibition of VIP- and PACAP-27-induced responses by α-chymotrypsin in rabbit longitudinal vaginal wall strips. After treatment of the tissue with guanethidine, scopolamine, phenylephrine and L-NAME, exogenous application of VIP (**A**, 300 nM for 4 min, n/N=5/4) or PACAP-27 (**B**, 400 nM for 4 min, n/N=4/4) produced relaxations that were completely inhibited by α-chymotrypsin type II (2 units/ml). Removal of the peptidase restored the relaxation responses. α-chymotrypsin did not affect EFS- (5 Hz, every two minutes, indicated by dots) induced non-nitrergic NANC relaxations. The mechanograms are original recordings of single tissue preparations and are representative of all experiments in this series.

c) Effect of VIP fragment 10 - 28 on VIP- and EFS-induced relaxation responses in rabbit longitudinal vaginal wall strips

Incubation with the VPAC receptor antagonist, VIP fragment 10 - 28 (1μM) in the presence of L-NAME (500μM) did not reduce 5Hz EFS-induced non-nitrergic NANC relaxation responses with 95.8 ± 2.1% of control responses remaining in the presence of the antagonist (P>0.05; Figure 29). In the presence of L-NAME, VIP (100nM)-induced relaxation responses were also unaffected by the presence of the antagonist (Figure 29).

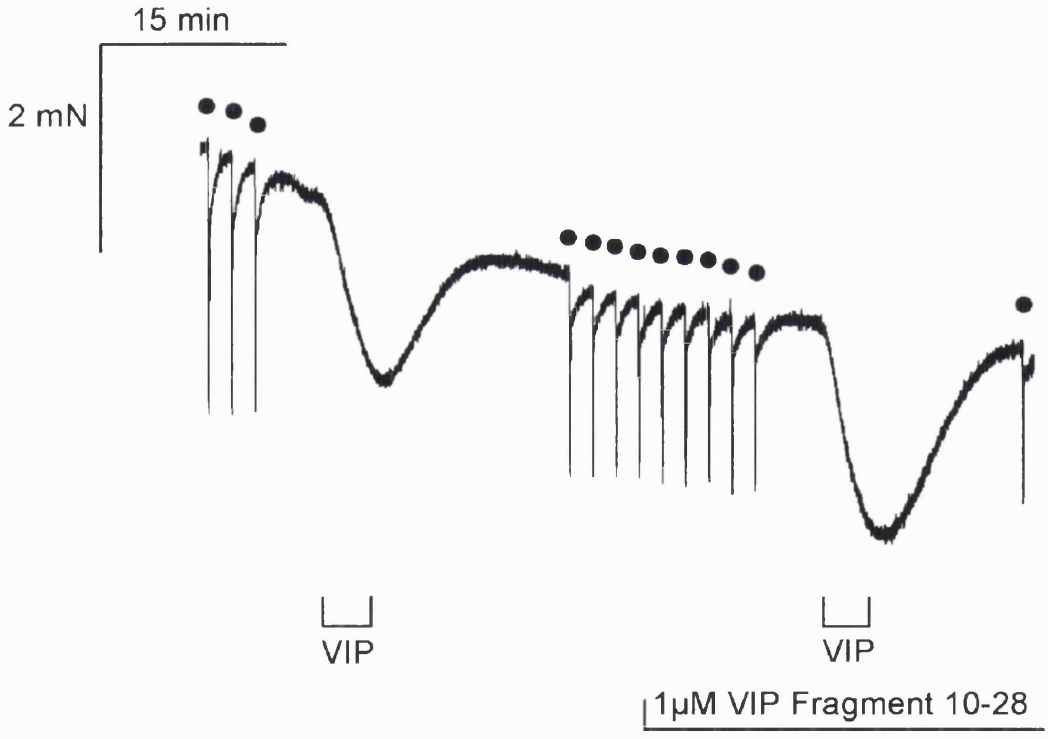


Figure 29: Effect of VIP fragment 10-28 on VIP and EFS-induced relaxations in rabbit longitudinal vaginal wall strips. In the presence of L-NAME (500μM) 5Hz EFS (●)-induced and VIP-induced relaxations were repeatable before and during incubation with VIP fragment 10-28 (1μM). The mechanogram is an original recording of a single tissue strip and is representative of all experiments in this series (n/N=4/4).

d) Effect of CGRP on phenylephrine pre-contracted rabbit longitudinal and rat circular vaginal wall strips and rabbit clitoral corpus cavernosum

Exogenous application of CGRP (0.1 – 1μM) had no effect on rabbit vaginal wall tension (Figure 30A). However in the rabbit clitoral corpus cavernosum CGRP (100nM) induced relaxation responses of 46.9 ± 12.6 % of 5 Hz EFS-induced relaxations (Figure 30B). As a further positive control for the efficacy of CGRP circular strips of rat vaginal wall were used as these have previously been shown to be relaxed by CGRP (Giraldi *et al.*, 2001). In this preparation, 200nM CGRP caused marked relaxation responses of 79.2 ± 20.2% of 5Hz EFS-induced relaxations (Figure 30C).

e) Effect of substance P on phenylephrine pre-contracted rabbit longitudinal vaginal wall and clitoral corpus cavernosum strips

Exogenous application of substance P (1μM) had no effect on longitudinal vaginal wall strips in the absence or presence of 500μM L-NAME (Figure 31A). In a single strip in the absence of L-NAME exogenous application of 10μM substance P also had no effect (Figure 31B).

In the clitoral corpus cavernosum exogenous application of substance P (1μM) induced a contractile response on top of the phenylephrine-induced tone in the absence or presence of 500μM L-NAME (Figure 31C)

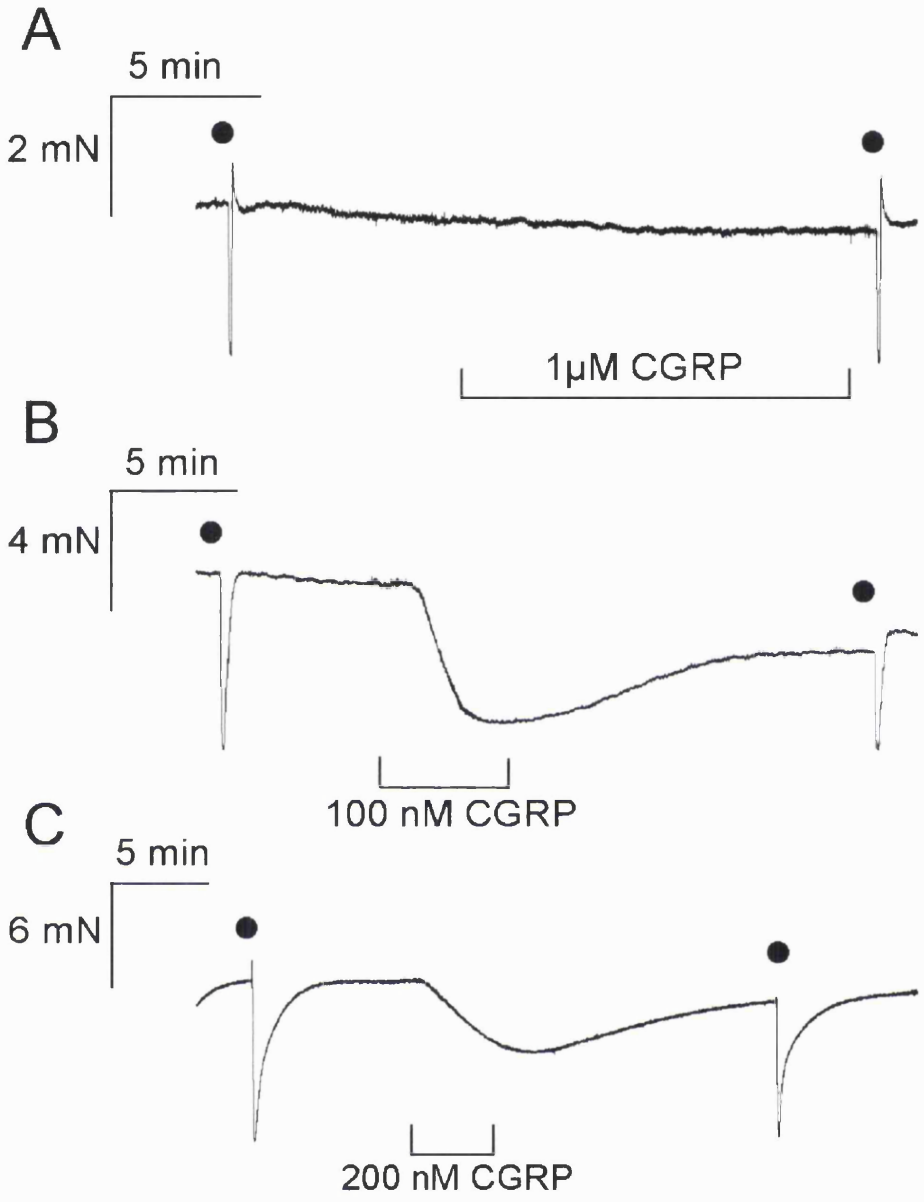


Figure 30: Effect of CGRP in rabbit longitudinal vaginal wall and clitoral corpus cavernosum strips and rat circular vaginal wall strips. CGRP (1µM) had no effect on rabbit vaginal wall strips (**A**), whilst 100nM and 200nM CGRP caused relaxation responses in rabbit clitoral corpus cavernosum (**B**) and rat vaginal wall (**C**) respectively. Mechanograms are original recordings of single tissue strips and are representative of all experiments in this series (n/N=7/5 for rabbit vaginal wall, n/N=6/4 for rabbit clitoral corpus cavernosum and rat vaginal wall). Dots indicate 5Hz EFS.

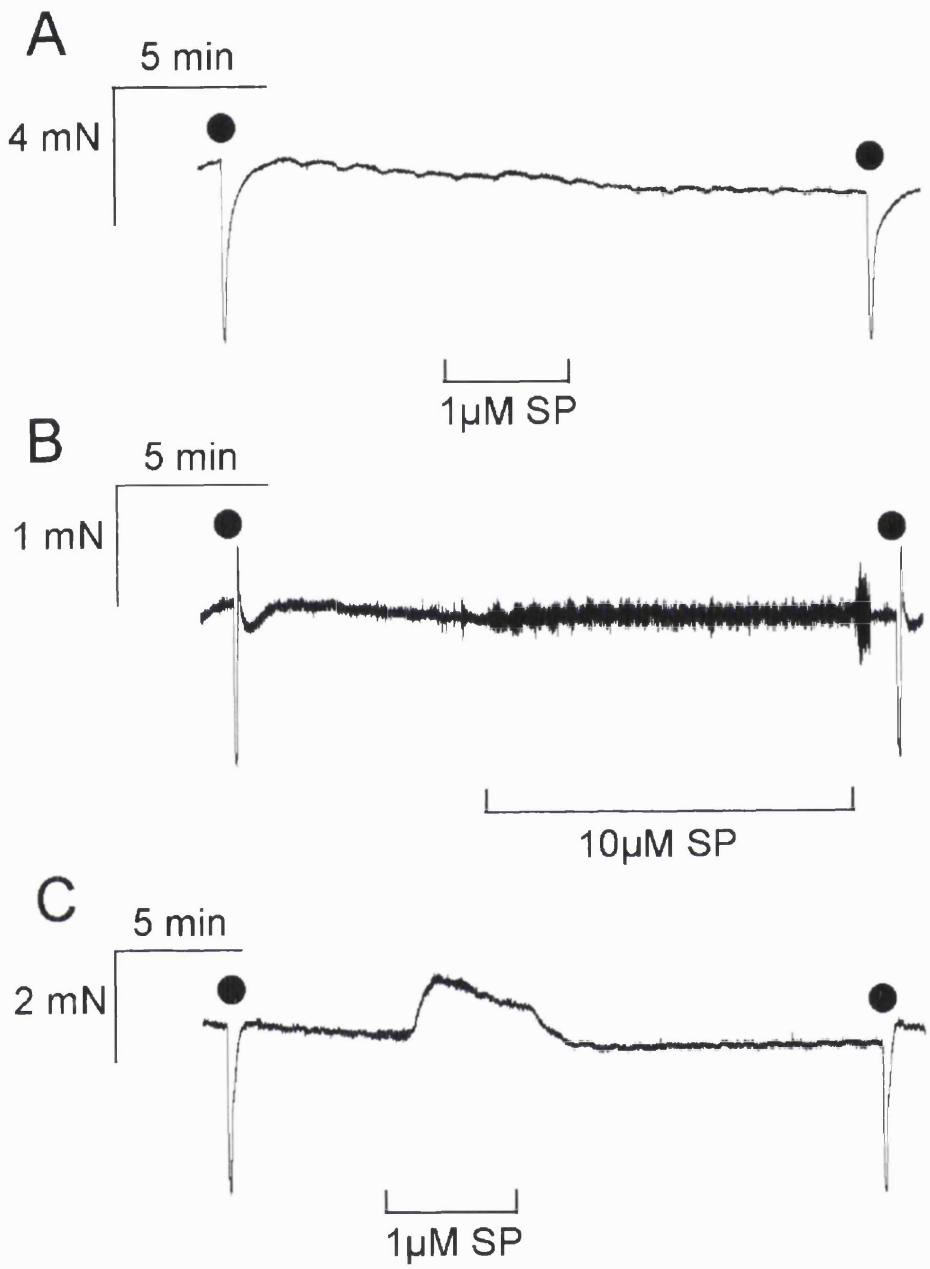


Figure 31: Effect of substance P (SP) in rabbit longitudinal vaginal wall and clitoral corpus cavernosum strips. SP has no effect in the vaginal wall when applied exogenously at concentrations of 1µM (**A**: n/N=9/5) or 10µM (**B**: n=1). Exogenous application of 1µM SP causes contractions in the clitoral corpus cavernosum in the absence of L-NAME (**C**: n/N=4/4). Mechanograms are original recordings of single tissue strips and are representative of all experiments in this series. Dots indicate 5Hz EFS.

III: 4 ROLE OF PURINES AND PYRIMIDINES IN NANC RELAXATION RESPONSES IN THE VAGINAL WALL

a) Effect of purines and pyrimidines in phenylephrine pre-contracted rabbit longitudinal vaginal wall strips

Once a stable tone was achieved, EFS was terminated and CRCs were constructed for relaxation responses to ATP (0.03-10mM), ADP (0.03-10mM), adenosine (0.03-10mM), UTP (0.03-10mM) or UDP (0.03-10mM). All these purines and pyrimidines caused concentration-dependent relaxation responses (Figure 32 and 33). There was a high degree of variability in the responsiveness to the nucleotides and to adenosine, and this is shown by the range of maximal responses seen. This range, and the efficacy and potency of the different purines and pyrimidines (efficacy was measured as the relaxation induced by the highest concentration of nucleotide or nucleoside compared to that induced by 5Hz EFS-induced relaxation and potency was represented by pEC_{50} values) are compared in Table 3.

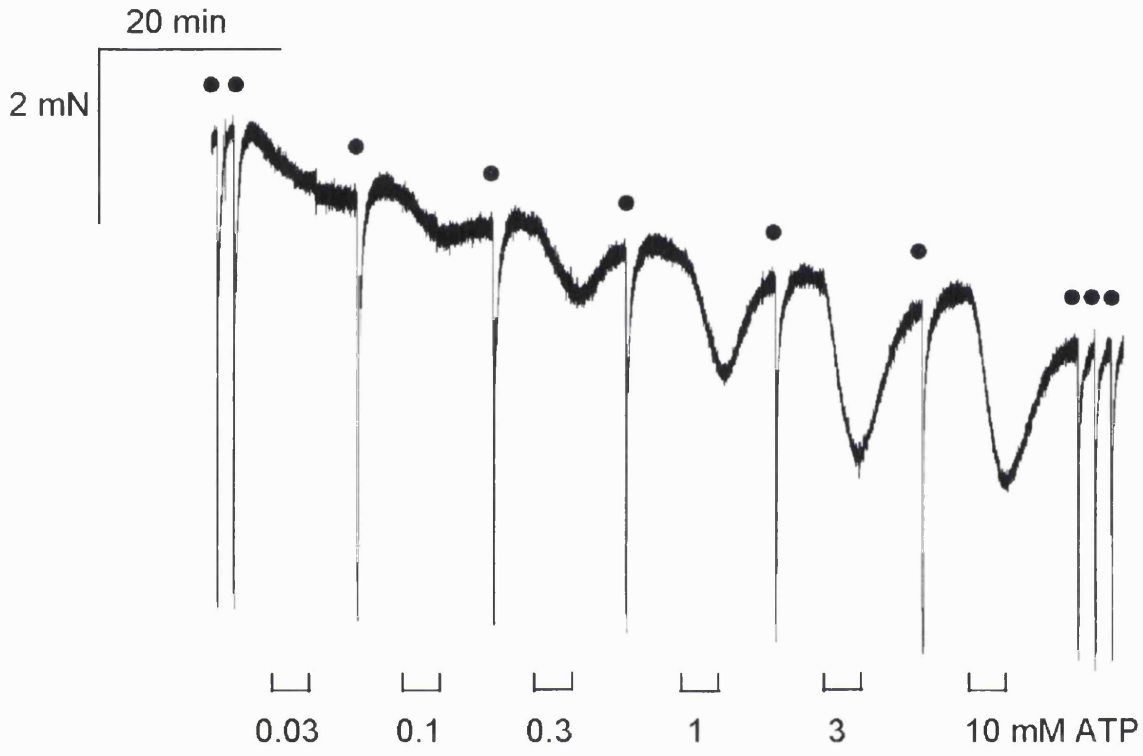


Figure 32: Effect of ATP in phenylephrine-precontracted rabbit longitudinal vaginal wall strips. Exogenous application of ATP causes concentration dependent relaxation responses. The tissue was stimulated by EFS (5Hz, 50V, 0.3msec pulse, 5sec train, indicated by dots) between successive applications of ATP. The mechanogram is an original recording of a single tissue preparation and is representative of all the experiments in this series (n/N=8/4).

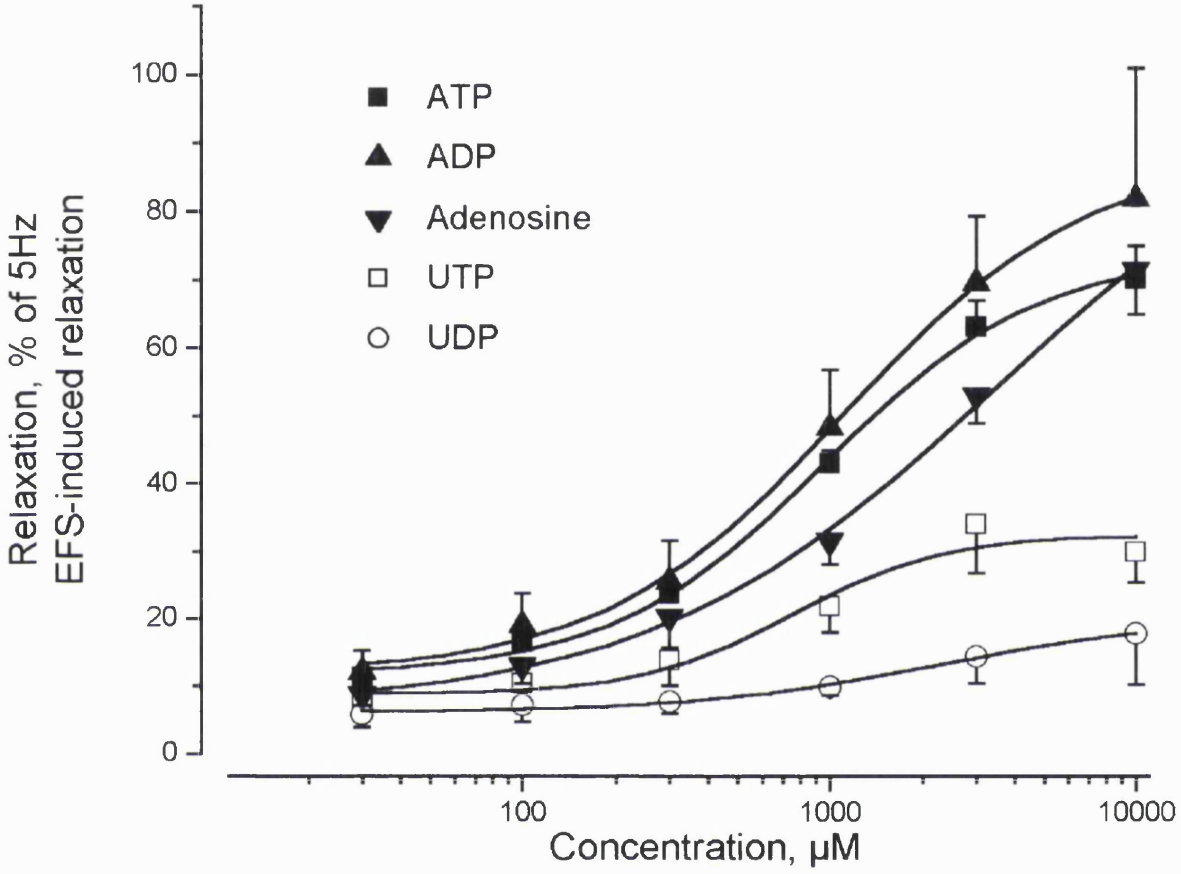


Figure 33: Concentration-response curves showing the relaxant effect of ATP (n/N=8/4), ADP (n/N=8/4), adenosine (n/N=10/4), UTP (n/N=8/4) and UDP (n/N=6/4) in rabbit longitudinal vaginal wall strips. Relaxations are expressed as a percentage of 5Hz EFS-induced relaxations in the absence of nucleotides. Data points represent mean ± s.e. mean.

Table 3: Efficacy and potency of purines and pyrimidines in producing relaxation responses in the rabbit vaginal wall.

	Concentration Range (mM)	**Maximum relaxation (% of 5Hz EFS-induced relaxation ± s.e.)**	**Maximum relaxation range (% of 5Hz EFS)**	**pEC_{50} ± s.e. (M)**	**n/N**
ATP	0.3 – 10	70.1 ± 4.9	52.4 – 83.3	3.02 ± 0.05	8/4
ADP	0.3 – 10	81.9 ± 19.0	25.2 – 105.0	2.96 ± 0.06	8/4
Adenosine	0.3 – 10	71.5 ± 6.5	47.6 – 100.0	2.48 ± 0.27	10/4
UTP	0.3 – 10	29.9 ± 4.6	17.6 – 67.2	3.12 ± 0.18	8/4
UDP	0.3 – 10	17.8 ± 7.6	6.0 – 34.6	2.63 ± 0.17	6/4

b) Effect of L-NAME and neurotoxins on purine- and pyrimidine-induced responses in phenylephrine pre-contracted rabbit longitudinal vaginal wall strips

Relaxations responses induced by adenosine (1mM), ATP (1mM), ADP (1mM), UTP (10mM) and UDP (10mM) were unaffected when repeated after 30 minutes incubation with 500μM L-NAME ($P>0.05$; Figure 34 and Figure 35 for adenosine and ADP).

Relaxation responses induced by 1mM ATP were unaffected by treatment of vaginal wall strips with a combination of L-NAME (500μM), ω-CTX (100nM) and TTX (1μM) with relaxations following incubation with the inhibitors 101.8 ± 4.3% of control ($P>0.05$; Figure 36A).

In the absence of L-NAME exogenous adenosine (1mM)-induced relaxations were not inhibited by incubation with ω-CTX (100nM) and TTX (1μM) with 97.0 ± 14.6% of control responses remaining after incubation with the neurotoxins ($P>0.05$; Figure 36B)

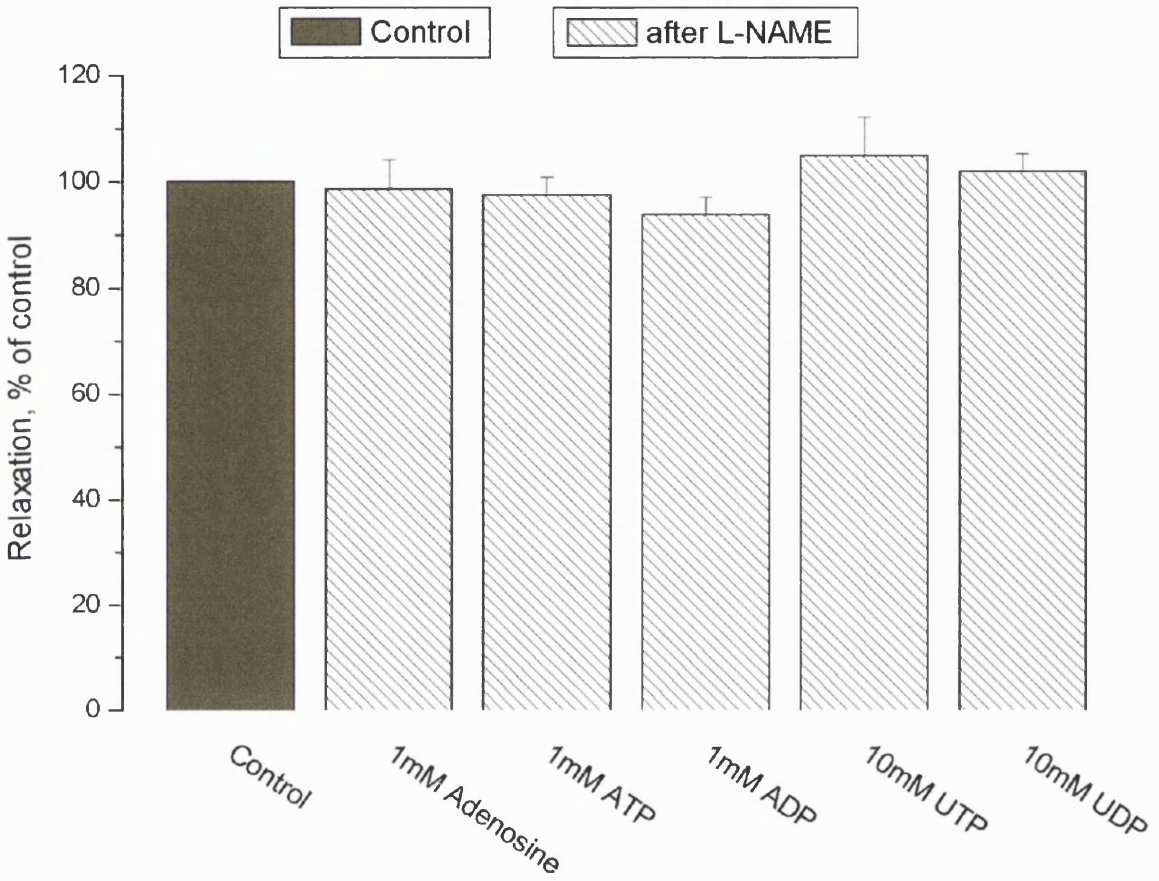


Figure 34: Effect of L-NAME on purine- and pyrimidine-induced relaxation responses in rabbit longitudinal vaginal wall strips. Relaxation responses to adenosine (1mM, n/N=6/3), ATP (1mM, n/N=4/4), ADP (1mM, n/N=4/4), UTP (10mM, n/N=6/3) and UDP (10mM, n/N=6/3) were not inhibited by incubation with L-NAME (500µM, P>0.05). Statistical analyses performed using Student's *t*-tests.

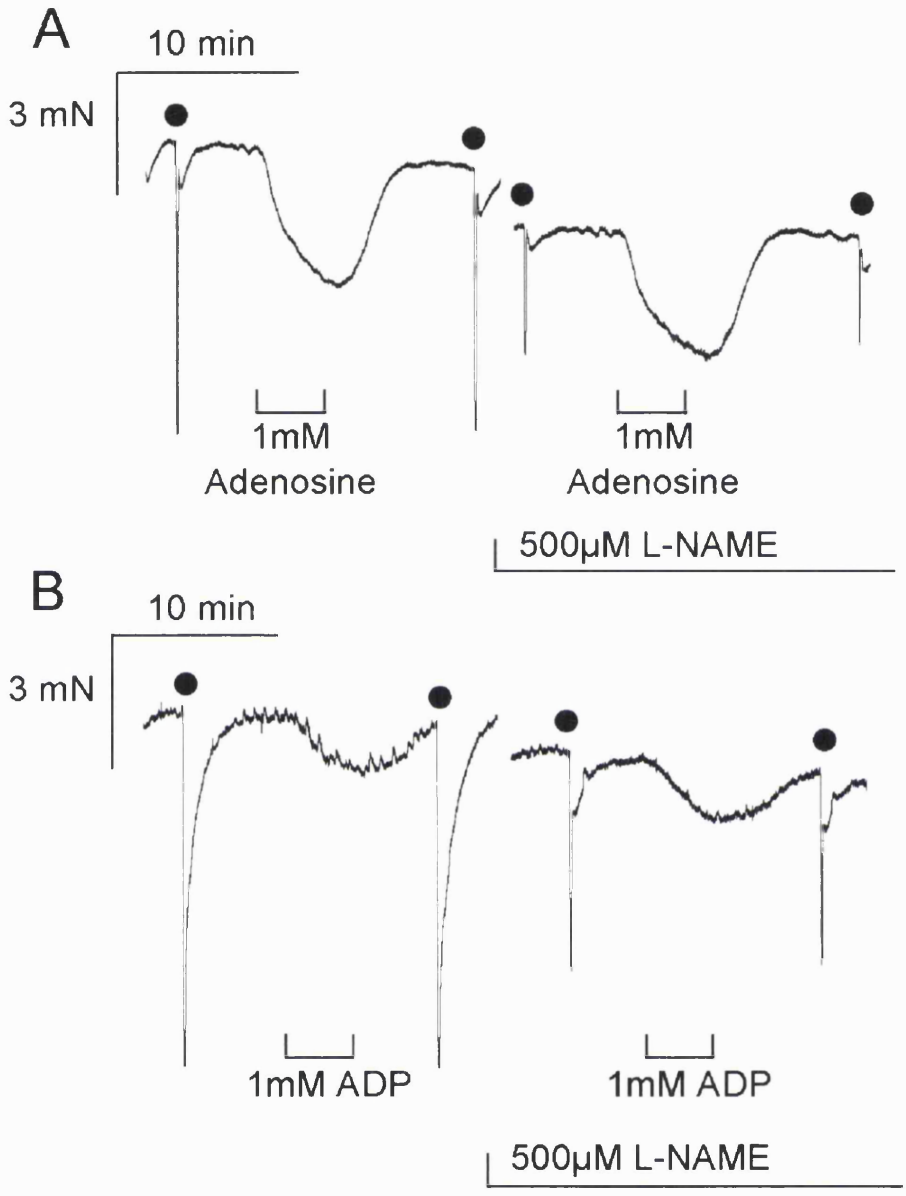


Figure 35: Effect of L-NAME on adenosine- and ATP-induced relaxation responses in rabbit longitudinal vaginal wall strips. Relaxation responses to exogenous application of adenosine (1mM, **A**) or ADP (1mM, **B**) are not affected by incubation with L-NAME (500µM). The mechanogram is an original recording of a single tissue preparation and is representative of all the experiments in this series (n/N=6/3 for adenosine and n/N=4/4 for ADP). Dots indicate 5Hz EFS.

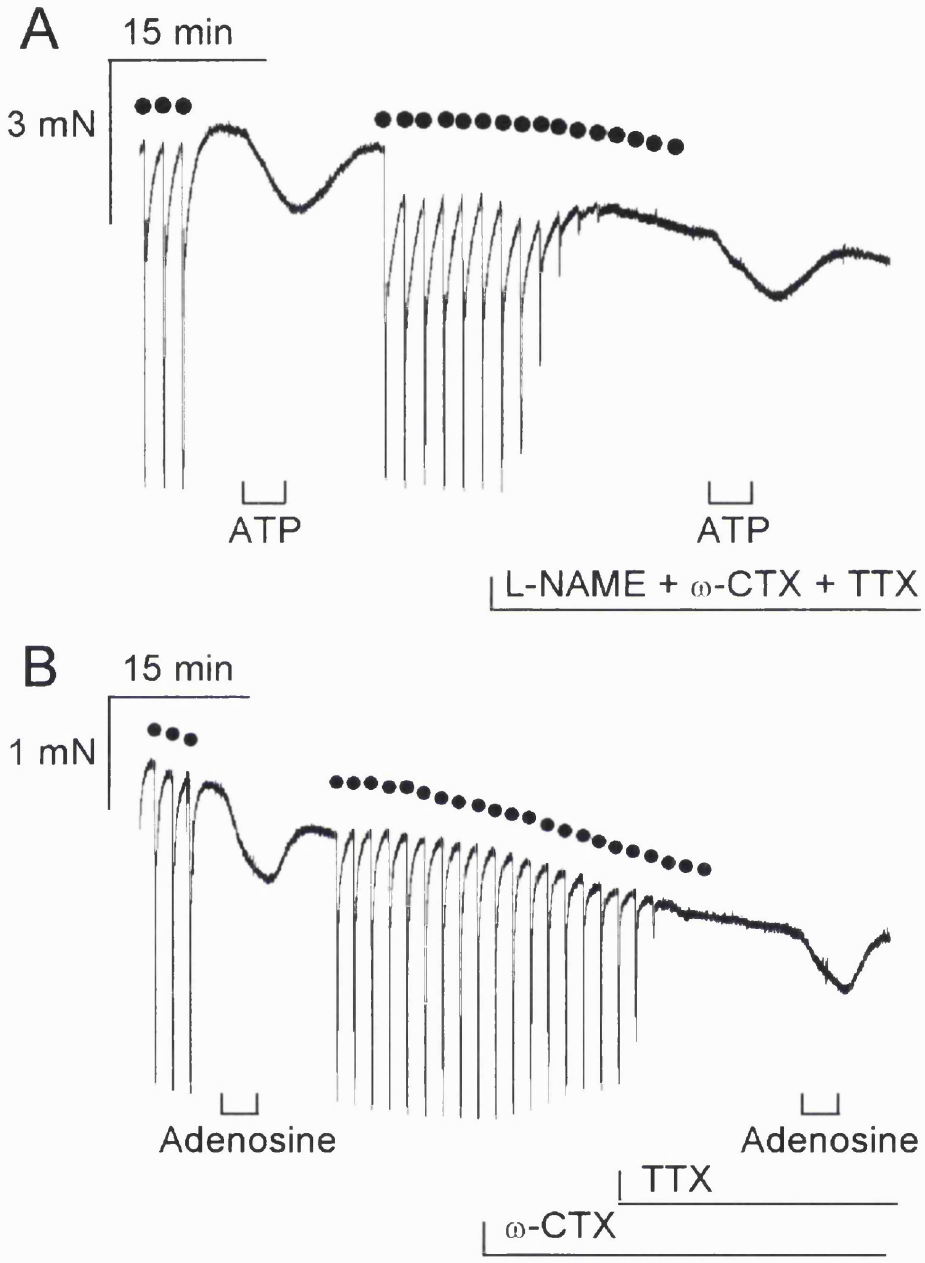


Figure 36: Effect of L-NAME and neurotoxins on ATP- and adenosine-induced relaxation responses in rabbit longitudinal vaginal wall strips. Relaxation response induced by ATP (1mM) is not inhibited by incubation with L-NAME (500µM), ω-CTx (100nM) and TTX (1µM; **A**, n/N=8/4). Relaxation response induced by adenosine (1mM) is not inhibited by incubation with ω-CTx (100nM) and TTX (1µM; **B**, n/N=4/2). EFS (●)-induced relaxations are entirely inhibited. The mechanograms are original recordings of single tissue preparations and are representative of all the experiments in this series.

c) Effect of P1 antagonists on adenosine- and ATP-induced responses in rabbit longitudinal vaginal wall strips

ZM-241385:

The role of P1 receptors was investigated by repeating the CRC to adenosine after 20 mins incubation with the A_{2A} antagonist ZM-241385 (30μM). In control experiments the CRC curve was repeated in the absence of ZM-241385. In the presence of ZM-241385 relaxation responses to adenosine were significantly reduced (Figure 37).

In the presence of L-NAME (500μM), exogenous application of a single, sub-maximal, dose of adenosine (500μM), caused relaxation responses that were abolished in the presence of ZM-241385 (30μM; Figure 38).

500μM ATP-induced relaxation responses in the presence of L-NAME were reduced by 25 mins incubation with ZM-241385 (30μM), with responses after incubation 61.6 ± 6.2% of those before addition of the antagonist ($P<0.05$, n/N=4/2).

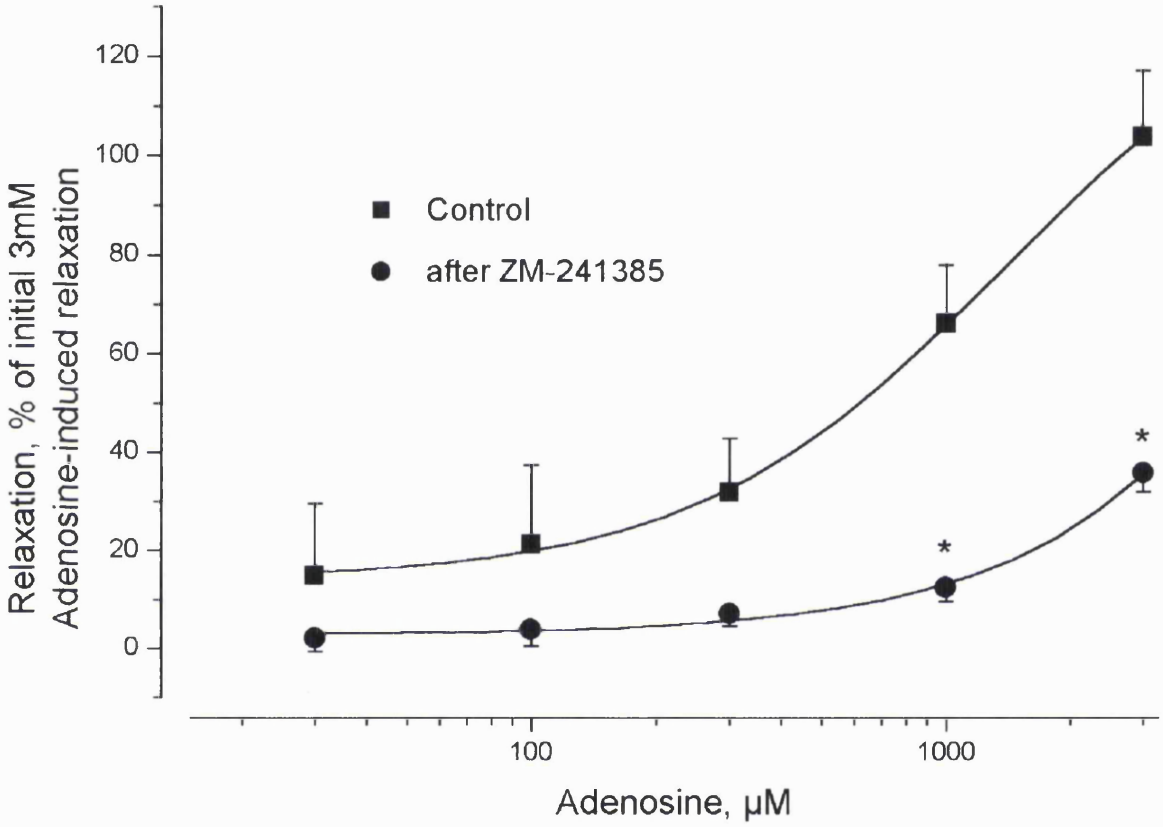


Figure 37: Effect of ZM-241385 on adenosine-induced relaxation responses in rabbit longitudinal vaginal wall strips. Relaxation responses elicited by exogenous application of adenosine were partially inhibited by ZM-241385 (30µM, n/N=6/3). Data points represent mean ± s.e. mean. Drug effect was stastically different, P<0.001. *Adenosine concentration effect significantly different from control, *P*<0.001. Statistical analysis performed using two way ANOVA with Bonferroni post-test.

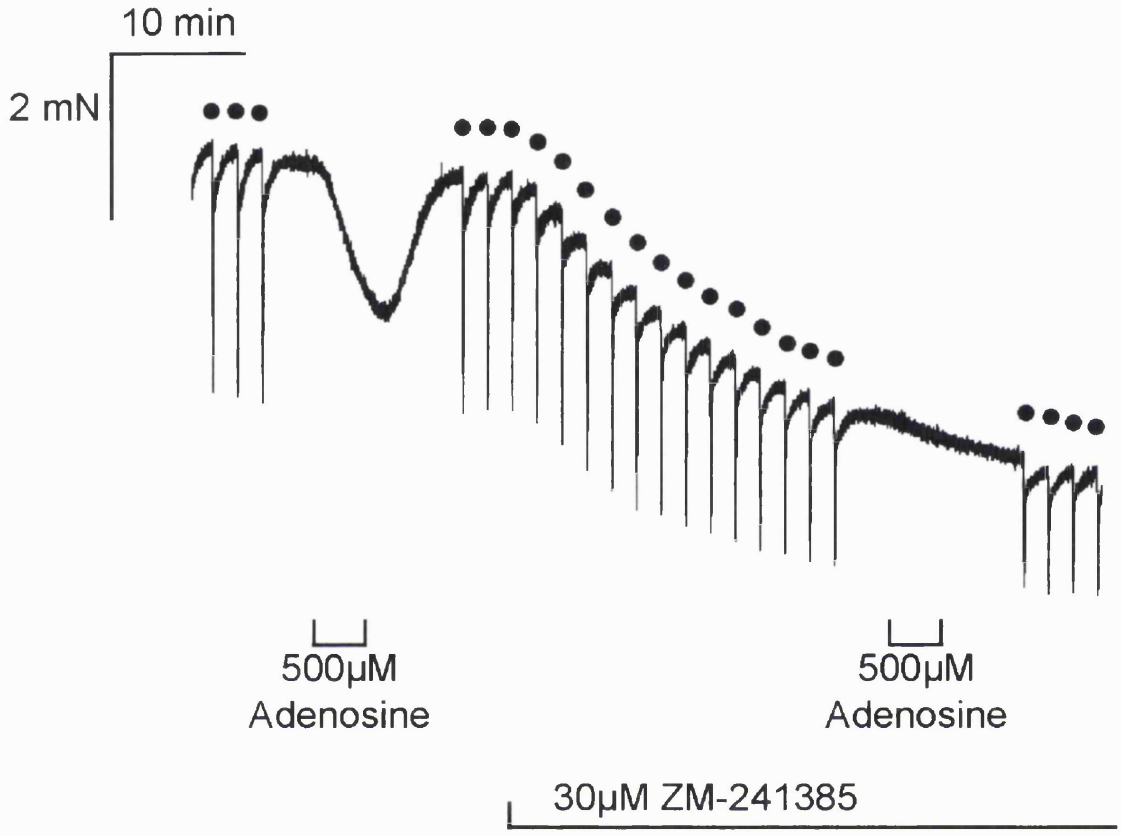


Figure 38: Effect of ZM-241385 on adenosine-induced relaxation responses in the presence of L-NAME in rabbit longitudinal vaginal wall strips. Relaxation response elicited by adenosine (500µM) after incubation with L-NAME (500µM) is completely inhibited by ZM-241385 (30µM), whilst EFS (●)-induced relaxations were not inhibited. The mechanogram is an original recording of a single tissue preparation and is representative of all the experiments in this series (n/N=4/4).

8-PT:

The effect of the P1 receptor antagonist 8-PT on adenosine- and ATP-induced relaxation responses was investigated in the presence of L-NAME (500μM). Neither adenosine (500μM)- nor ATP (500μM)-induced relaxations were significantly reduced by 25 mins incubation with 8-PT (100μM) with 86.4 ± 6.9% and 101.5 ± 9.0% of control responses remaining respectively ($P>0.05$, n/N=6/4 for adenosine, n/N=4/3 for ATP)

d) Effect of P2 antagonists on ATP-induced responses in rabbit longitudinal vaginal wall strips

Cibacron Blue and Suramin:

To investigate the role of P2 purinoceptors in mediating the relaxation responses to ATP, the CRC to ATP was repeated after 20 mins incubation with the non-selective P2 antagonists cibacron blue (500μM) or suramin (100μM). Cibacron blue significantly inhibited the response to ATP (Figure 39 and 40A), however, suramin potentiated the response to ATP (Figure 40B).

PPADS:

To assess the effect of the P2 antagonist PPADS 500μM ATP-induced relaxation responses were induced, then repeated in the presence of 10 and 50μM PPADS. ATP-induced responses were not inhibited in the presence of PPADS (50μM) with 103.8 ± 2.5% of control responses remaining after incubation with the inhibitor ($P>0.05$; Figure 41).

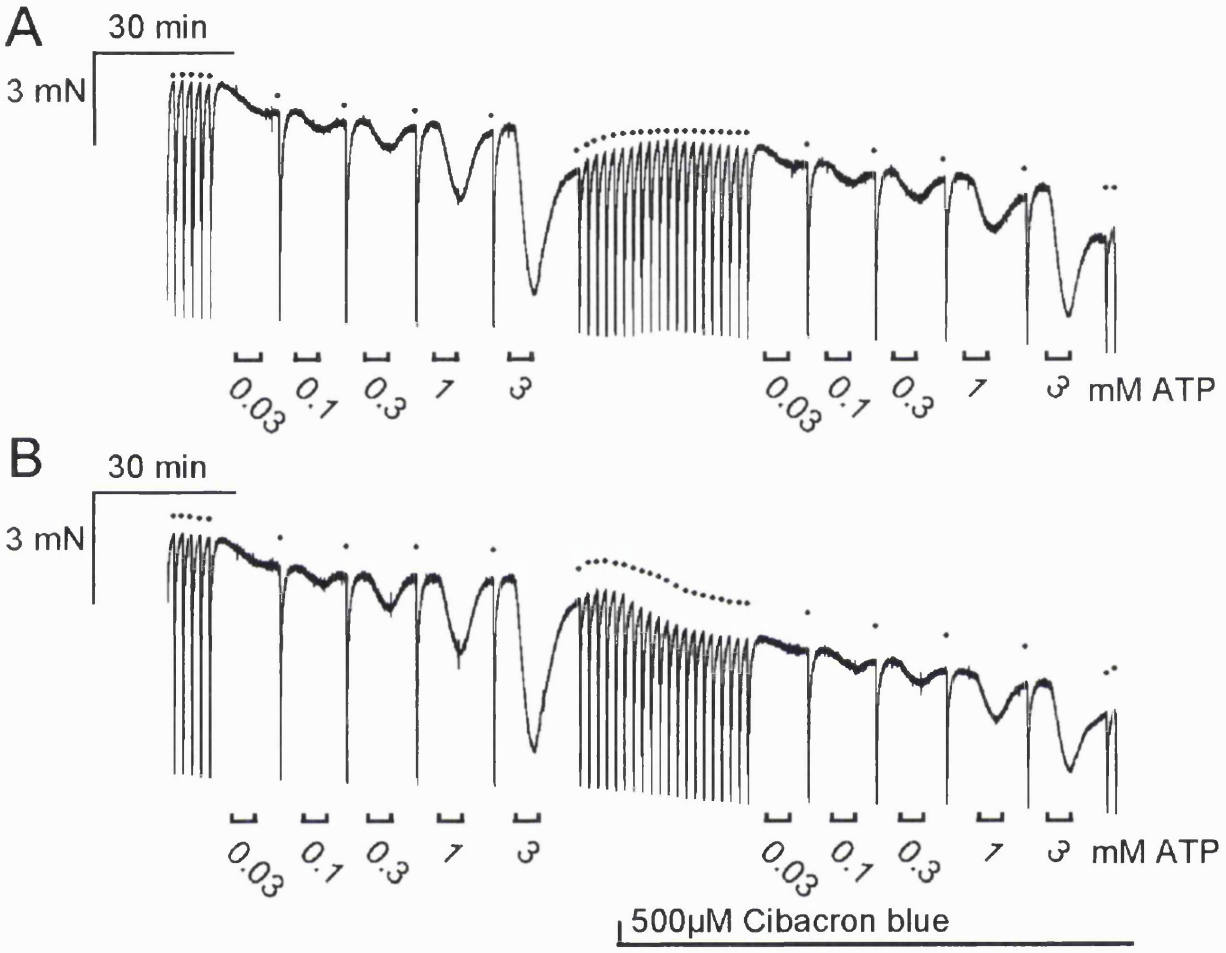


Figure 39: Effect of cibacron blue on ATP-induced relaxation responses in rabbit longitudinal vaginal wall strips. Exogenous application of ATP induced concentration-dependent relaxation responses. CRCs to ATP were repeated in the absence (**A**) and presence (**B**) of cibacron blue (500µM). 5s trains of 5Hz EFS (●) were applied every 2 min before construction of each CRC and a single train was applied between successive applications of ATP. The mechanograms are original recordings of single tissue preparations and are representative of all the experiments in this series (n/N=6/4).

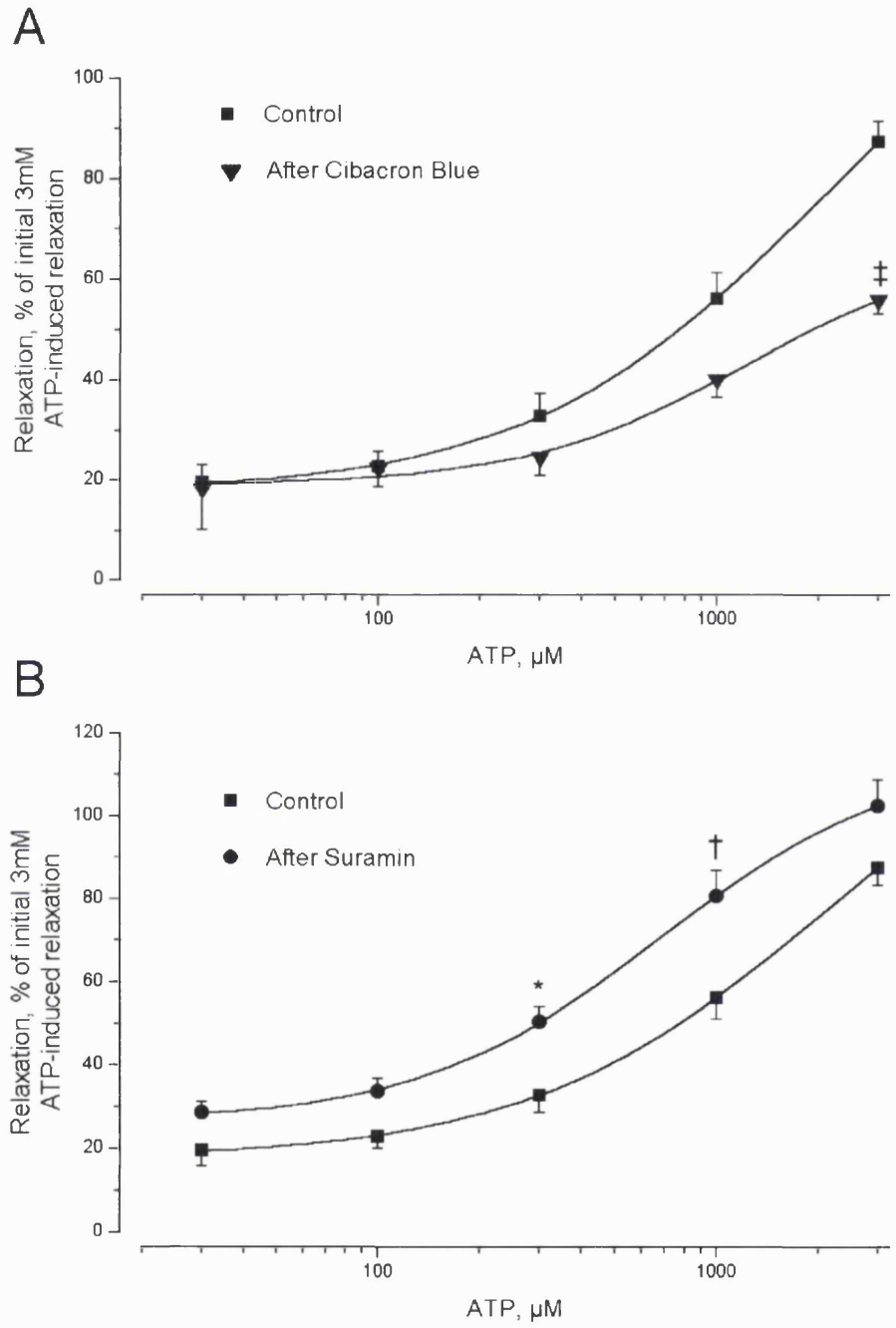


Figure 40: Effect of cibacron blue and suramin on ATP-induced relaxation responses in rabbit longitudinal vaginal wall strips. Relaxation responses elicited by exogenous application of ATP were (**A**) partially inhibited by cibacron blue (500μM, n/N=6/4) but were (**B**) potentiated by the presence of suramin (100μM, n/N=6/3). Data points represent mean ± s.e. mean. Both drug effects were significantly different, P<0.0001. ATP concentration effect significantly different from control, *=*P*<0.05, †=P<0.01, ‡=P<0.001. Statistical analyses performed using two way ANOVA with Bonferroni post-test.

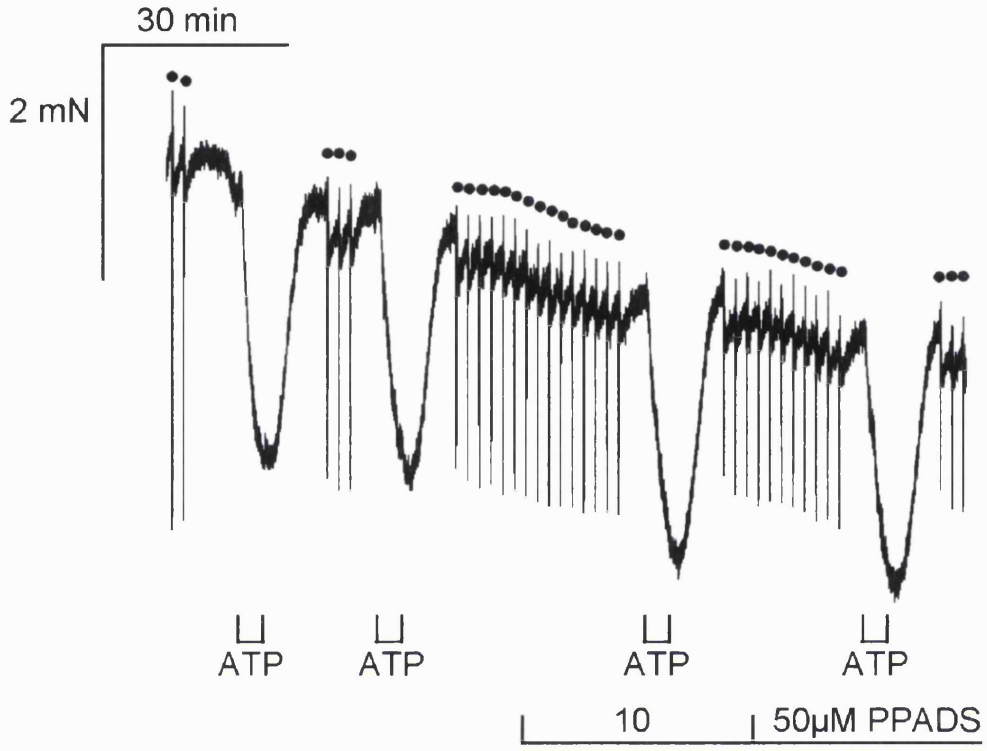


Figure 41: Effect of PPADS on relaxation responses to ATP in rabbit longitudinal vaginal wall strips. Relaxation responses to ATP (500µM) are reproducible, and are not inhibited by incubation with PPADS (10 and 50µM). EFS (●)-induced relaxations are also not affected by PPADS. The mechanogram is an original recording of a single tissue preparation and is representative of all experiments in this series (n/N=3/3).

e) Effect of P1 and P2 antagonists on NANC relaxation responses in rabbit longitudinal vaginal wall strips

ZM-241385 (30μM) caused a reduction in tissue tone with 75.7 ± 2.2% of tone prior to addition of the antagonist remaining after 35 mins of incubation in its presence (n/N=15/7; Figure 38), however it did not inhibit EFS-induced relaxation responses (94.0 ± 4.4% of time-matched control, P>0.05, n/N=6/3).

8-PT (100μM) did not significantly reduce non-nitrergic NANC relaxation responses with 93.3 ± 3.3% of control responses remaining after 20 minutes incubation with the antagonist (P>0.05, n/N=6/4).

Tissue tone was slightly reduced by cibacron blue (500μM), with 83.7 ± 1.8% of tone prior to incubation with the inhibitor remaining after 35 mins (n/N=5/3). EFS-induced NANC relaxation responses were unaffected by cibacron blue with 94.2 ± 2.5% of time-matched controls remaining (P>0.05; Figure 42) or suramin (97.6 ± 1.6% of time-matched control, P>0.05, n/N=6/3).

EFS-induced relaxations were also unaffected by incubation with PPADS (50μM), with 94.0 ± 1.4 % of control responses remaining (P>0.05; Figure 41).

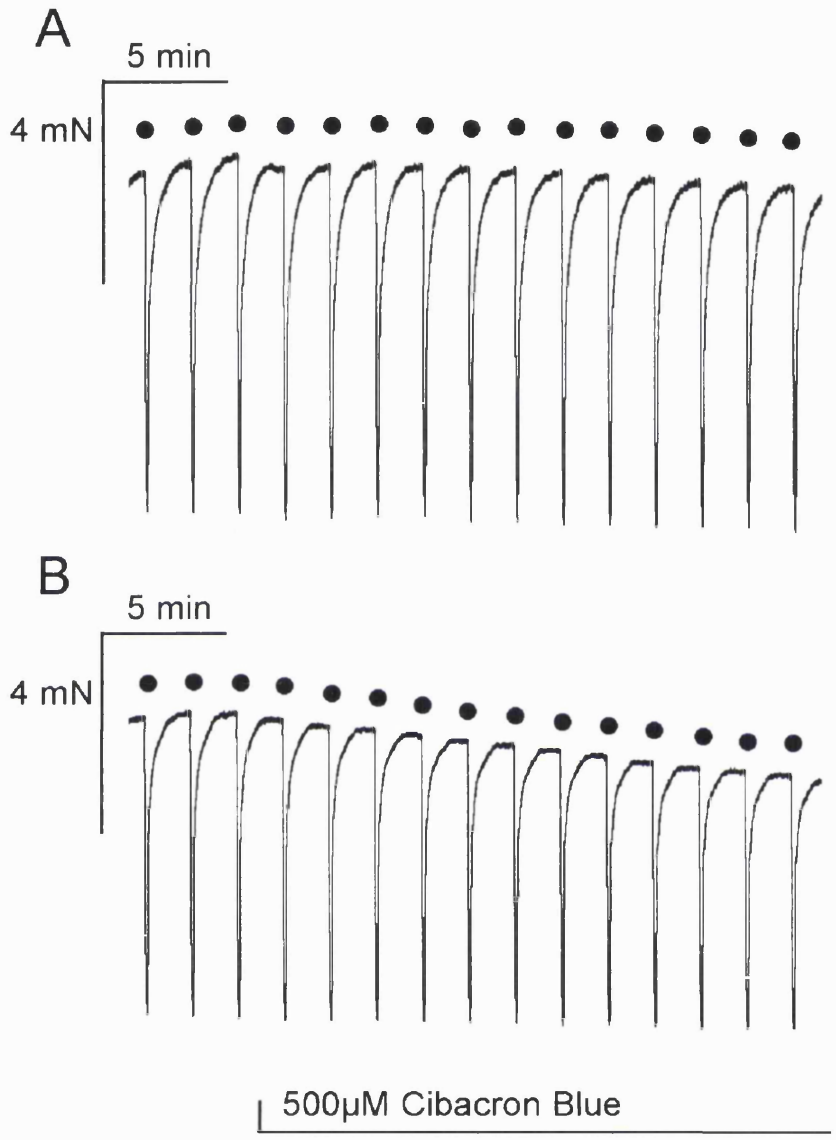


Figure 42: Effect of cibacron blue on EFS-induced relaxations in rabbit longitudinal vaginal wall strips. 5 Hz EFS (●)-induced relaxation responses are unaffected by cibacron blue (500µM, **B**). Tissue tone is reduced however compared to time-matched controls (**A**) The mechanograms are original recordings of single tissue strips and are representative of all the experiments in this series (n/N=6/4).

f) Effect of G protein inhibitors on ATP and ADP induced relaxation responses in rabbit longitudinal vaginal wall strips

GDPβS:

After eliciting relaxation responses to ATP or ADP (both 1mM) vaginal wall strips were incubated with 100μM GDPβS (which stabilizes G proteins in their inactive state) for 30 minutes. GDPβS caused a slight decrease in tone, and significantly inhibited relaxation responses to subsequent application of exogenous ADP ($P<0.05$; Figure 43). Relaxation responses to exogenous application of ATP were not significantly attenuated in the presence of GDPβS (Figure 43).

Pertussis toxin:

Incubation of vaginal wall strips with pertussis toxin (100ng/ml) for 60 mins, after eliciting an initial relaxation response to ATP or ADP (1mM), failed to inhibit relaxation responses to subsequent application of ATP or ADP (Figure 43).

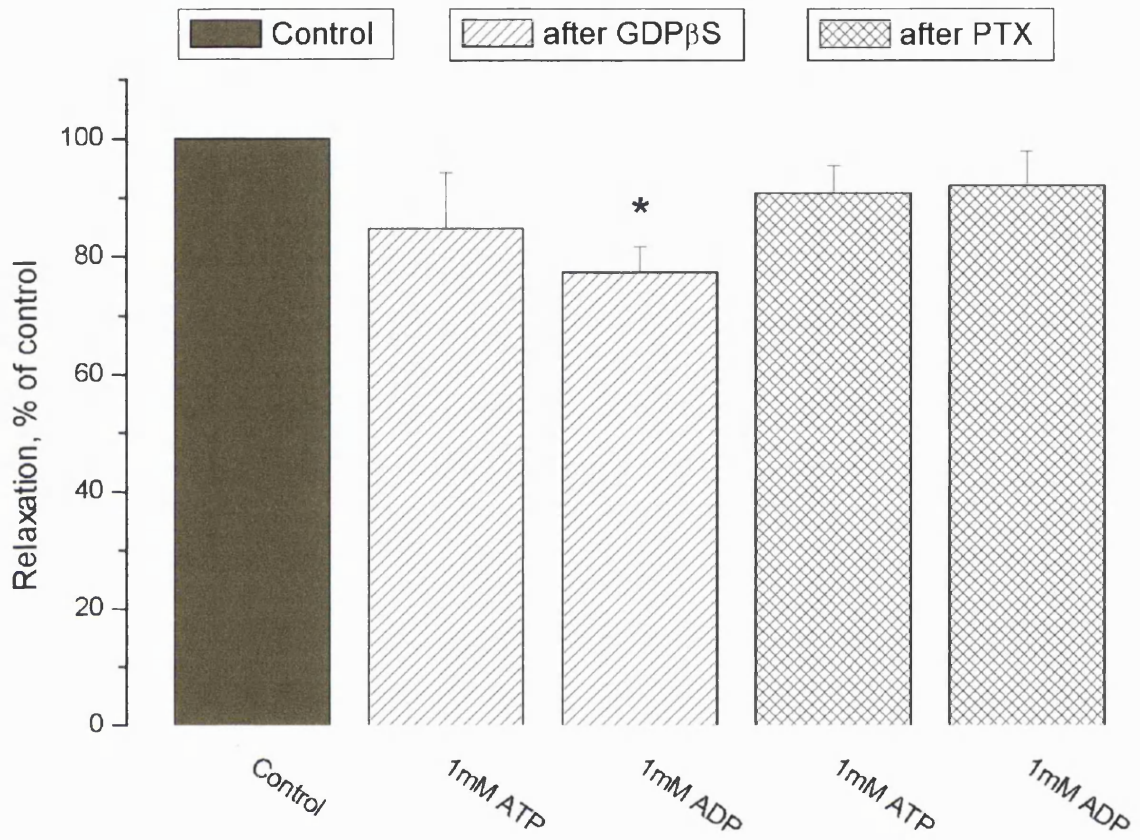


Figure 43: Effect of GDPβS and pertussis toxin on ATP- and ADP-induced relaxation responses in rabbit longitudinal vaginal wall strips. Relaxation responses to ATP (1mM) are not significantly inhibited after incubation with pertussis toxin (PTX, 100ng/ml, n/N=4/4) or GDPßS (100µM, n/N=4/4). Relaxation responses to ADP (1mM) were significantly reduced after incubation with GDPßS (n/N=4/4), but were unaffected by pertussis toxin (n/N=4/4). *Significantly different from control, $P<0.05$. Statistical analyses performed using Student's *t*-tests.

III: 5 STUDIES INTO SECOND MESSENGER PATHWAYS INVOLVED IN NANC RELAXATION RESPONSES

a) Measurement of changes in intracellular cyclic nucleotide content in rabbit longitudinal vaginal wall strips

Rabbit vaginal wall strips were frozen under basal conditions, when stimulated by EFS (5Hz) in the absence and presence of L-NAME (500 μM), in the presence of peptides (all at 400 nM, Figure 44), in the presence of ATP or adenosine (both at 500μM and 10mM, Figure 45), the NO donor DETA NONOate (250μM) or the adenylate cyclase activator forskolin (10μM). Measurement of changes in the intracellular content of cyclic nucleotides showed that cGMP content was unaltered except when stimulated by EFS (5Hz), or in the presence of DETA NONOate (250μM). EFS-induced increases in cGMP were abolished by L-NAME. EFS did not cause any changes in cAMP content, but VIP, PACAP-27, PACAP-38, PHM, PHV, helospectin I and II and forskolin all caused some increase in cAMP content (Figure 44). Both ATP and adenosine also caused a significant increase in intracellular cAMP content when applied at 10mM, but did not cause a significant increase at 500μM (Figure 45),

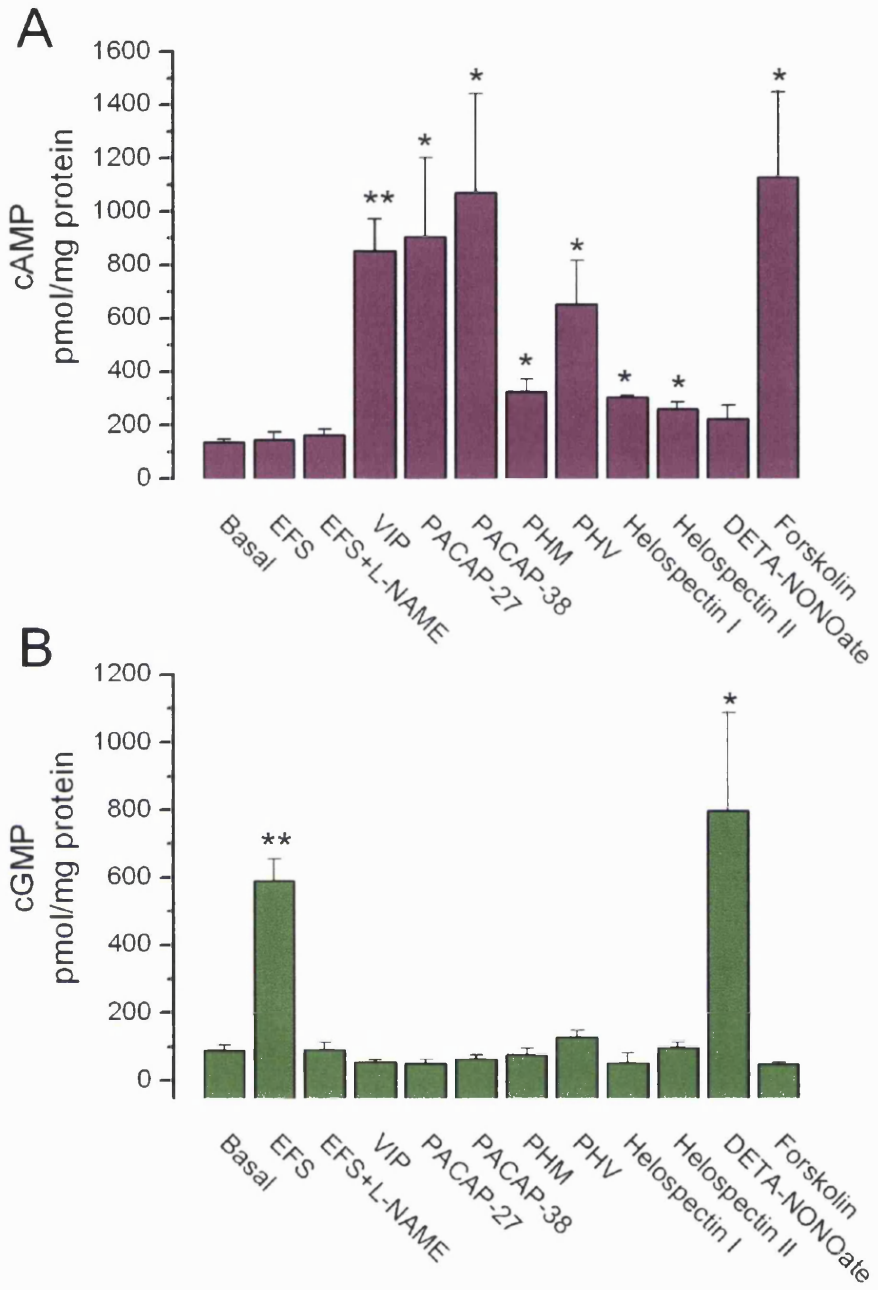


Figure 44: Effect of EFS and peptides on intracellular cAMP (A) and cGMP (B) content in rabbit longitudinal vaginal wall strips. Tissue strips frozen during EFS (5 Hz) showed a marked increase in cGMP content that was completely inhibited in the presence of L-NAME (500 μM). EFS did not cause an increase in cAMP content. Exogenous application of peptides (all 400 nM) caused no increase in cGMP, but caused marked increase in cAMP. DETA NONOate (250 μM) and forskolin (10 μM) produced significant increase in cGMP and cAMP content respectively. *Significantly different from basal, $P<0.05$. **Significantly different from basal, $P<0.001$. n/N=5/5 for basal, DETA-NONOate and forskolin treatment, n/N=4/4 for other treatments. Statistical analyses performed using ANOVA followed by Dunnett test.

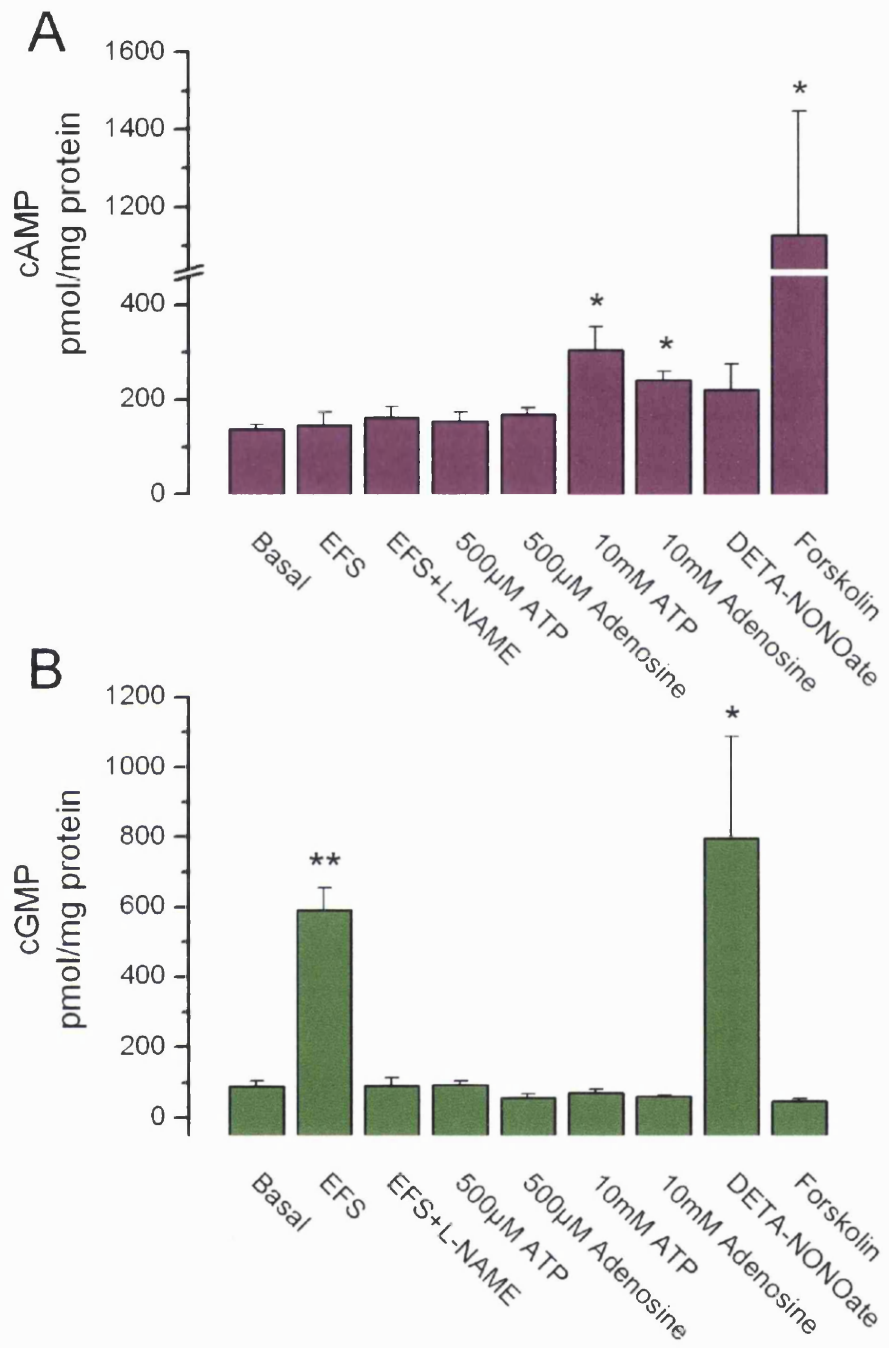


Figure 45: Effect of adenosine and ATP on intracellular cAMP (A) and cGMP (B) content in rabbit longitudinal vaginal wall strips. Adenosine and ATP caused significant increases in cAMP levels at 10mM, but not at 500µM. Neither adenosine nor ATP caused any increases in cGMP levels at either concentration. *Significantly different from basal, $P<0.05$. **Significantly different from basal, $P<0.001$. n/N=5/5 for basal, DETA-NONOate and forskolin treatment, n/N=4/4 for other treatments. Statistical analyses performed using ANOVA followed by Dunnett test.

b) Effect of potassium channel inhibitors on non-nitrergic NANC relaxations in the rabbit vaginal wall

Apamin and charybdotoxin:

After incubation of longitudinal vaginal wall strips with the small conductance Ca^{2+}-activated K^+ channel blocker apamin (1μM) or large conductance Ca^{2+}-activated K^+ channel blocker charybdotoxin (ChTX, 100nM) in the presence of L-NAME (500μM), EFS-induced responses were unaffected with 99.3± 1.9 % (P>0.05, n/N=5/4) and 105.0 ± 1.4 % (P>0.05, n/N=3/3) of control responses remaining respectively. Furthermore using a combination of apamin (1μM) and ChTX (100nM) in the presence of L-NAME (500μM) did not cause inhibition of EFS-induced relaxations with 97.2 ± 2.5 % of control relaxations remaining (P>0.05; Figure 46).

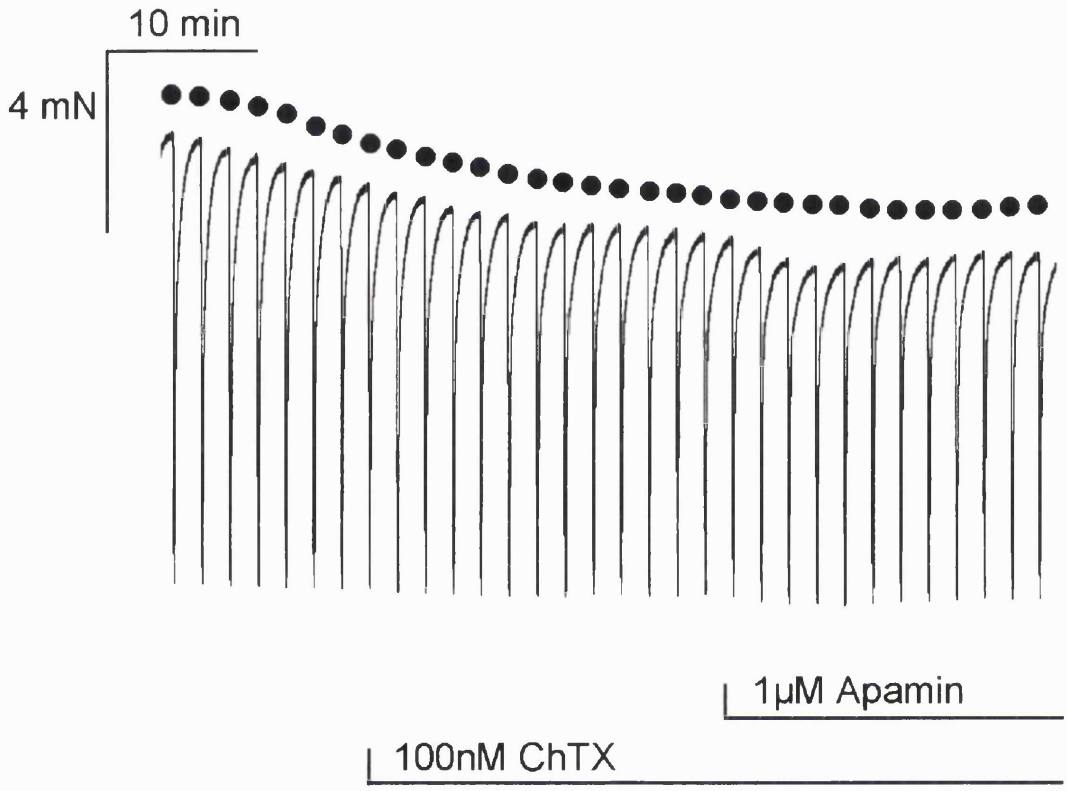


Figure 46: Effect of the potassium channel inhibitors charybdotoxin (ChTX) and apamin on 5Hz EFS-induced non-nitrergic NANC relaxation responses in rabbit longitudinal vaginal wall strips. 5Hz EFS (●)-induced relaxation responses in the presence of L-NAME (500µM) are not inhibited by a combination of ChTX (100nM) and apamin (1µM). The mechanogram is an original recording of a single tissue preparation and is representative of all experiments in this series (n/N=8/4).

III: 6 BIOASSAY STUDIES TO TRANSFER MEDIATORS OF NANC RELAXATIONS FROM STRIPS OF VAGINAL WALL

In this series of experiments longitudinal strips of vaginal wall (donor strip) were placed in superfusion chambers between the electrodes as usual, but with the silk suture holding it attached to the outside of the chamber. A second strip (recipient strip) of vaginal wall was placed beyond the electrodes and attached to the force transducer to measure tension.

In a pilot experiment to ensure a 4.5 minute stimulation duration would be effective at producing a sustained relaxation (and thus sustained release of the mediators NANC relaxations), donor strips were placed in chambers and recorded without recipient strips in place. EFS (50Hz, 0.3ms pulse duration, 50V) applied to donor strips for 4.5 mins after incubation with guanethidine (10μM), scopolamine (10μM) and phenylephrine (1μM) induced a sustained relaxation response followed by a contractile response and secondary relaxation response after stimulation was terminated. Incubation with L-NAME (500μM) reduced the initial peak of 50Hz relaxation responses to 86.3 ± 0.9% of control responses and abolished or reduced the contractile and secondary relaxation responses (n/N=2/2).

In a second pilot experiment to ensure recipient strips were not affected by EFS, strips of vaginal wall were placed in chambers outside electrodes with no donor strip

present. Strips were then incubated with guanethidine, scopolamine and phenylephrine. EFS (50Hz, 0.3ms pulse duration, 50V) for 4.5 mins had no effect on phenylephrine-induced tone (Figure 47A). Incubation with the NO donor sodium nitroprusside (SNP, 1μM) for 4.5 minutes caused a marked relaxation response followed by a return to basal phenylephrine-induced tone. The relaxation response elicited by SNP was reproducible over the course of the experiments (Figure 47A).

When donor strips of vaginal wall tissue were included in the superfusion chamber between the electrodes EFS (50Hz for 4.5 mins)-induced responses in recipient strips were highly variable with either no response (40% of strips, n/N=6/4) or a relaxation response (60% of strips, n/N=9/6) observed. In those experiments in which relaxation responses were observed they were either entirely inhibited by incubation with L-NAME (500μM, n/N=2/1) or were reduced (n/N=7/5; Figure 47B and 48). In experiments in which L-NAME resistant relaxations were observed these relaxations were resistant to incubation with α-chymotrypsin (2 units/ml, n/N=3/2), however in most cases these remaining transferred relaxation responses were also not inhibited by incubation with TTX (1μM) (n/N=6/4; Figure 47B). In only a single experiment were TTX-sensitive relaxation responses observed (Figure 48). Relaxation responses elicited by SNP were unaffected by incubation with L-NAME and TTX.

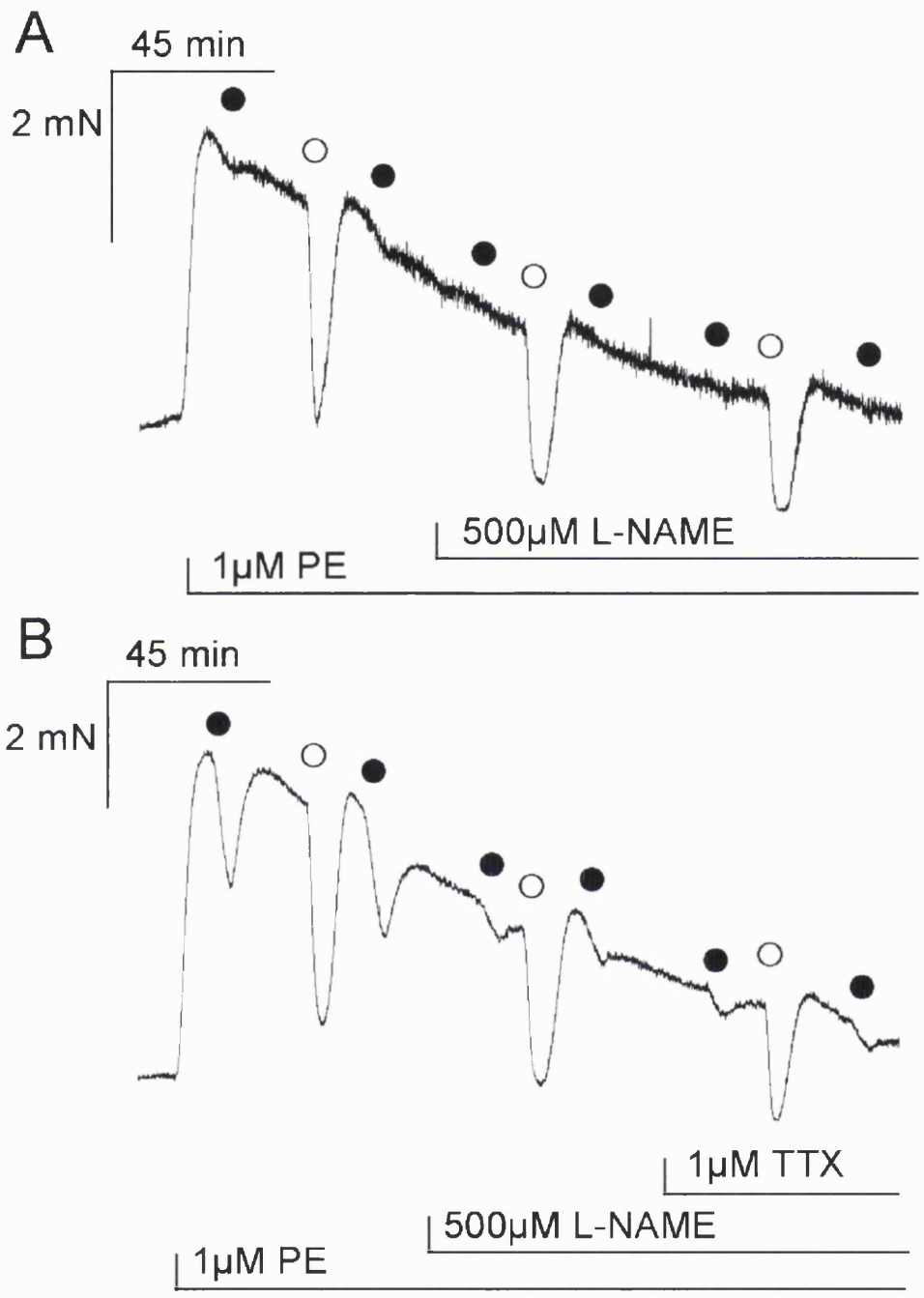


Figure 47: Effect of L-NAME and TTX in transfer experiments. EFS (●, 50Hz, for 4.5 minutes) had no effect on recipient tissue strips in the absence of a donor strip (**A**: n/N=2/2). In the presence of a donor strip EFS induced relaxation responses that were partially inhibited by L-NAME (500µM, **B**: n/N=6/4). Remaining relaxation responses were not affected by incubation with TTX (1µM). SNP(○, 1µM, for 4.5 minutes)-induced relaxation responses were not inhibited by L-NAME or TTX. Mechanograms are original recordings of single tissue preparations.

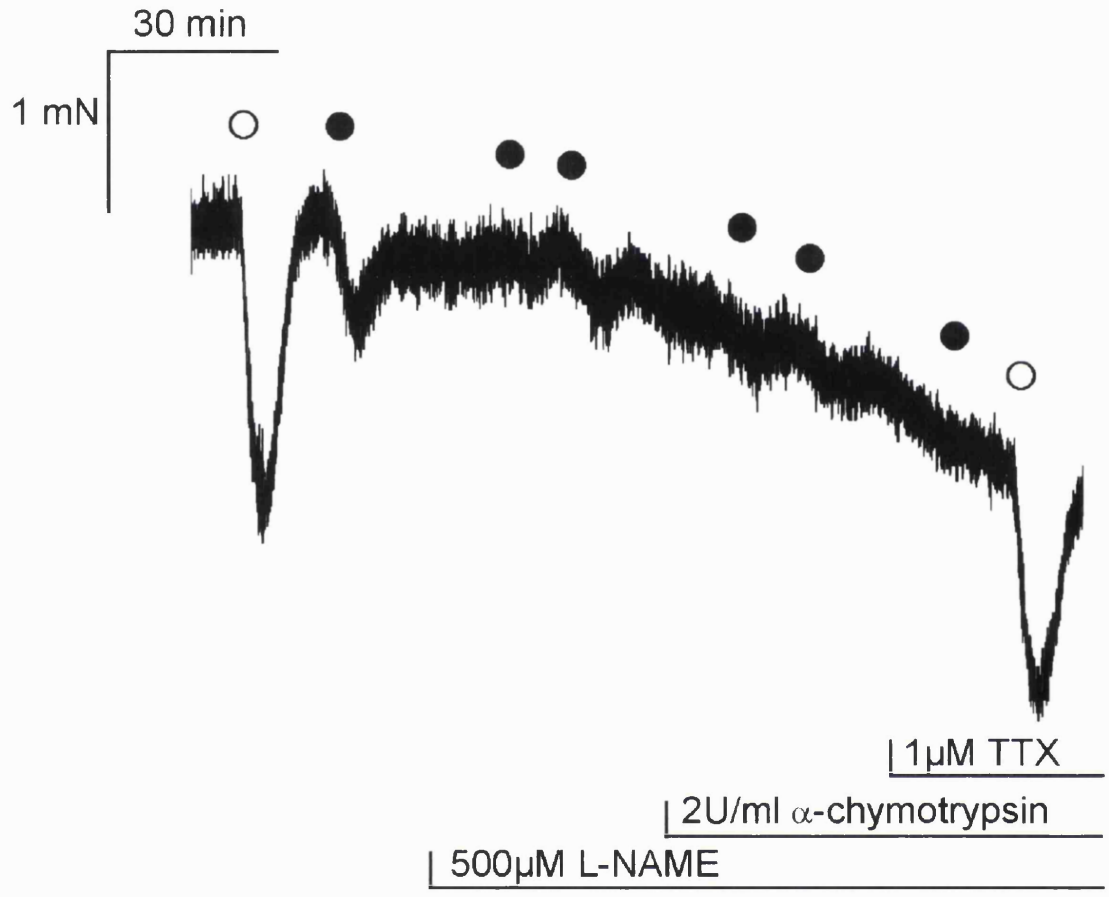


Figure 48: Lack of effect of α-chymotrypsin on L-NAME-resistant, TTX-sensitive relaxations in a single transfer experiment. Transferred EFS (●)-induced relaxations, partially inhibited by L-NAME, are unaffected by α-chymotrypsin, but are abolished by TTX. Relaxations induced by exogenous application of SNP (1μM, ○) are unaffected. Mechanogram is a single trace of vaginal wall recipient tissue, with vaginal wall donor tissue.

In experiments to attempt to increase the size of the transferred relaxations observed either a 1 in 1000 dilution of protease inhibitor cocktail (for composition see section II: 7, n/N=2/1) or a combination of the aminopeptidase inhibitor bestatin (3μM), and the endopeptidase inhibitors phosphoramidon (1μM) and DL-thiorphan (1μM; n/N=2/1) was added to superfusing Krebs solution, however, neither increased transferred relaxation responses.

In an attempt to increase transferred relaxations by positioning recipient tissue strips closer to donor strips the recipient strip was incubated in Krebs containing 3μM TTX before placing it in the superfusion chamber in a sandwich arrangement close, but not touching, the donor strip between the electrodes. Tissues were then treated as above with guanethidine, scopolamine and phenylephrine. However 5s trains of 5Hz EFS induced small relaxation responses in the recipient strip, and 4.5mins stimulation at 50Hz failed to induce transferred relaxation responses (Figure 49).

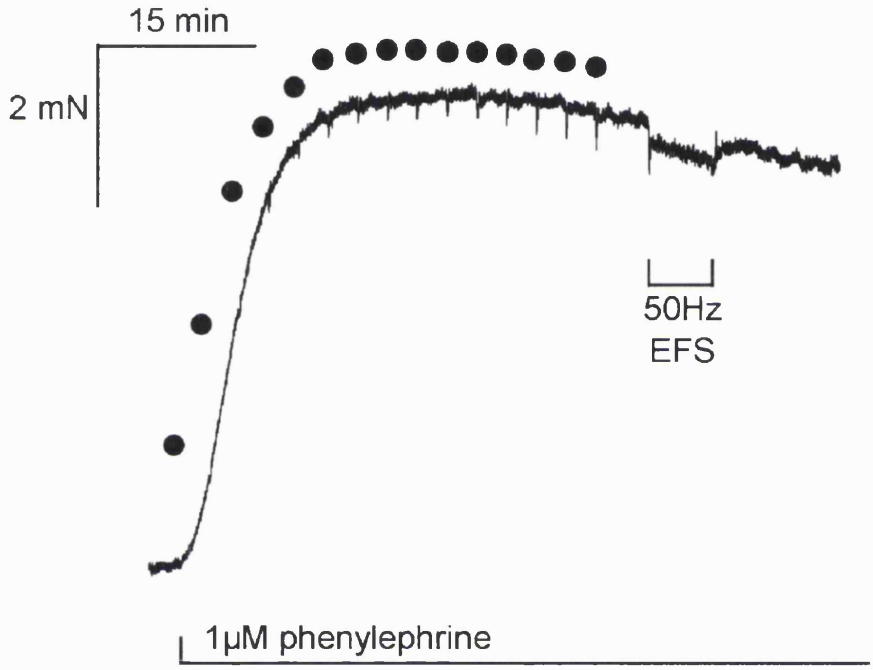


Figure 49: Effect of pre-incubation of recipient strip with TTX on transferability of mediators of NANC relaxations in rabbit longitudinal vaginal wall strips. In recipient strips incubated in TTX prior to placement in chamber 5Hz EFS (●, 0.3ms pulse, 50V, 5s train) induced small relaxation responses after raising tissue tone with phenylephrine (1µM). 50Hz EFS (0.3ms pulse, 50V, for 4.5 minutes) produced a small sustained relaxation during stimulation. No transferred relaxation was observed. The mechanogram is an original recording of a single tissue strip and is representative of all experiments in this series (n/N=2/1).

III: 7 STUDIES TO ISOLATE AND IDENTIFY THE MEDIATOR OF NON-NITRERGIC NANC RELAXATIONS IN THE VAGINAL WALL

In pilot experiments to ensure that long-term EFS produced sustained relaxations EFS (5Hz, 0.3ms pulse, 50V) was applied to longitudinal strips of vaginal wall tissue for 10 mins after incubation with guanethidine (10μM), scopolamine (10μM) and phenylephrine (1μM). A sustained relaxation response was observed, followed by a transient contraction and secondary relaxation response on termination of stimulation (Figure 50). After incubation with L-NAME (500μM) for 25 mins EFS was applied for 18 mins. A sustained relaxation response was again observed, with the initial peak magnitude reduced to 80.7 ± 6.3% of the initial relaxation and on termination of stimulation the contractile response followed by the secondary relaxation response was reduced or abolished (Figure 50). This suggested that the mediator or mediators of non-nitrergic NANC relaxations could be released over this period of time.

In experiments to collect superfusate non-nitrergic NANC relaxations were elicited in rabbit longitudinal vaginal wall strips after incubation with guanethidine (10μM), scopolamine (10μM), phenylephrine (1μM) and L-NAME (500μM) in superfusion chambers in which tissue strips were accessible from above. Once stable relaxation responses had been achieved stimulation was terminated and tissue was left to rest unstimulated for 10 mins before 10 x 0.5 ml samples of superfusing Krebs was pipetted from adjacent to the tissue strip at 60 s intervals, and immediately pooled and

placed at 4°C (basal samples). This was followed by a further 10 min period of rest before stimulating the tissue with EFS (50Hz, 0.3ms pulse duration, 50V). During stimulation samples of modified Krebs solution were removed from adjacent to tissue and stored (stimulated samples). Basal and stimulated samples were desalted, lyophilised, and run on Tris-tricine ready gels. In addition to protein marker standards, an additional control lane was included in gels containing a sample consisting of a mix of VIP (130μM, MW: 3325.8) and somatostatin (15μM, MW: 1637.9). Gels were subsequently stained with coomassie blue, revealing no proteins in basal lanes, and a single band at approximately 15kDa in lanes containing stimulated samples (Figure 51).

Further stimulated samples were taken and were analysed by MALDI-TOF MS by M Scan, UK. The band was identified as consisting of four proteins corresponding to rabbit haemoglobin α1 and α2 and haemoglobin β1 and β2 chains.

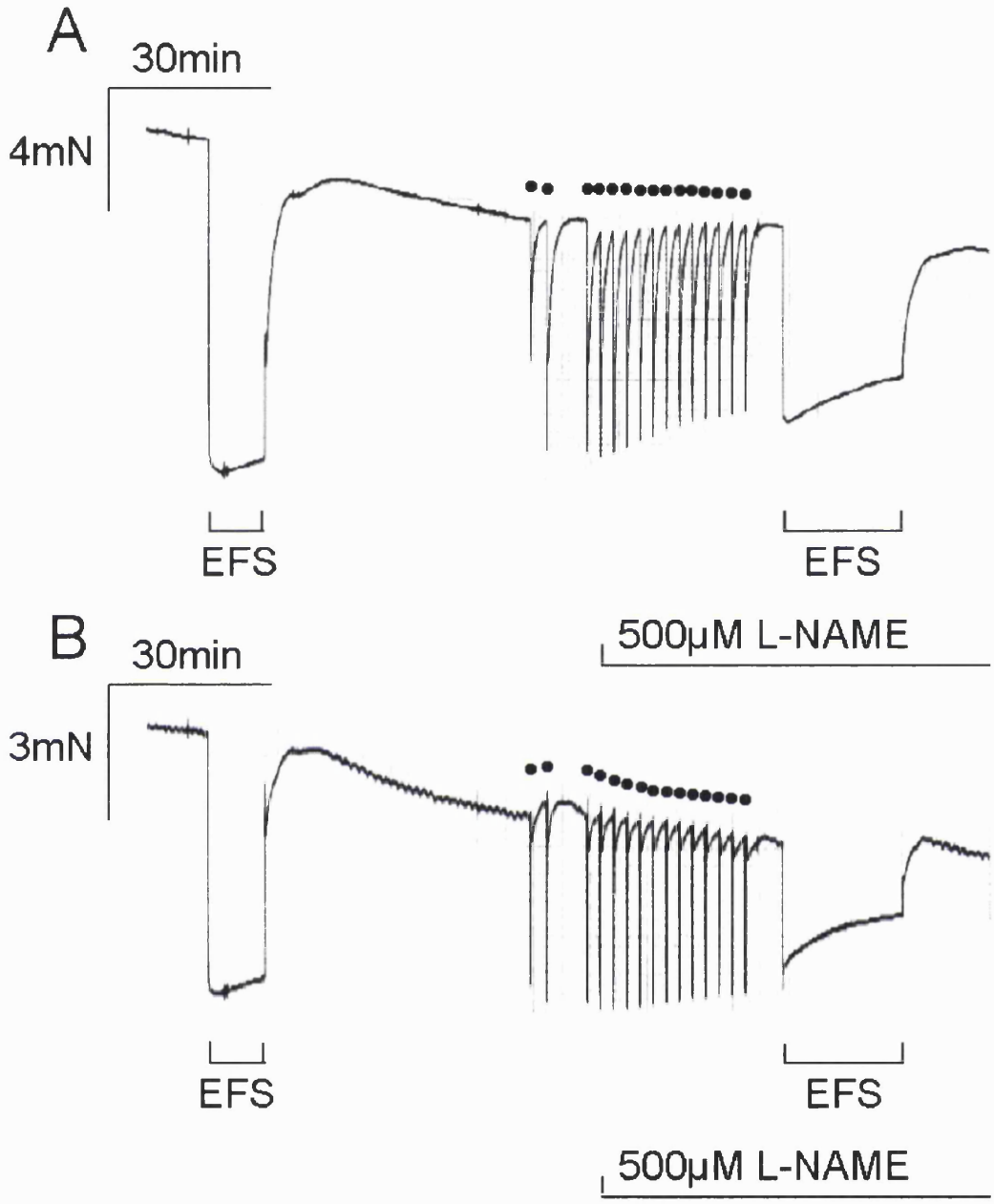


Figure 50: Effect of L-NAME on long term EFS in rabbit longitudinal vaginal wall strips. EFS (5Hz, 0.3msec pulses, 50V) applied for 10 minutes induced sustained relaxation responses, followed by a contraction and secondary relaxation response on termination of stimulation in the absence of L-NAME. After incubation with L-NAME (500µM) EFS was applied for 18 minutes. This induced a sustained relaxation response, with contractile and secondary relaxation responses abolished (**A**) or reduced (**B**). Mechanograms are original recordings of single tissue preparations (n/N=2/1).

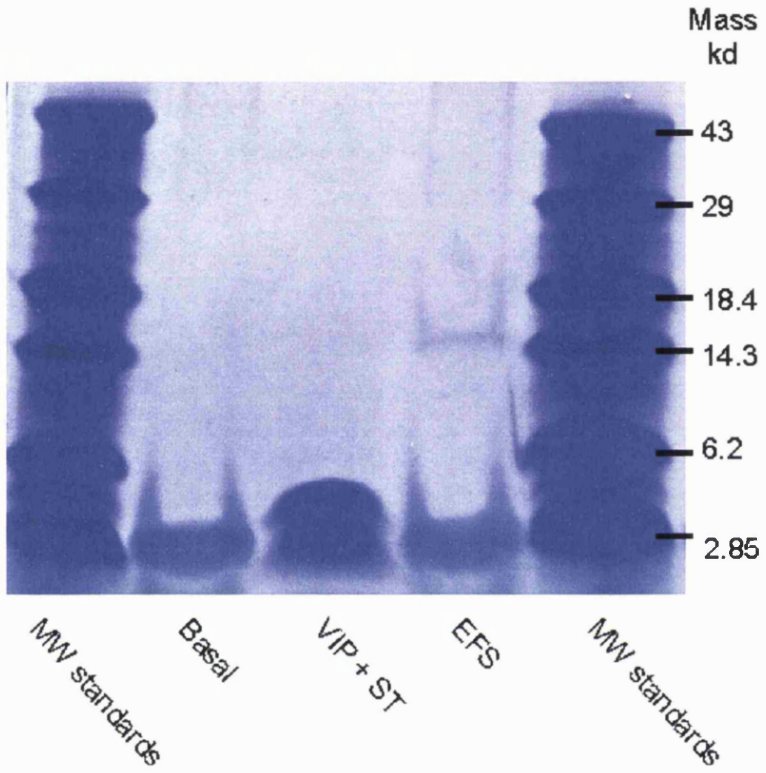


Figure 51: Representative coomassie-stained gel showing ~15kDa band in EFS-stimulated samples. Tris-tricine ready gel loaded with molecular weight (MW) standards, basal sample, sample containing VIP and somatostatin (VIP + ST), sample collected during stimulation (EFS), and a second lane containg MW standards. The gel was fixed and stained using coomassie blue. Sample collected during stimulation showed a band that was between 14.3 and 18.4kd that was not present in basal samples. Gel is representative of all experiments in this series (n=5).

III: 8 STUDIES ON THE EFFECT OF OVARY REMOVAL ON NANC RELAXATION RESPONSES IN THE RABBIT VAGINAL WALL AND CLITORAL CORPUS CAVERNOSUM

a) Effect of ovariectomy on oestradiol levels

All ovariectomized rabbits were successfully spayed as evidenced by the GnRH stimulation test in which all animals were found to have blood levels of oestradiol less than 10pM both pre- and post-stimulation (N=6).

b) Eliciting the NANC relaxation response in vaginal wall and clitoral corpus cavernosum strips from ovariectomized animals

In order to determine whether contractility was affected by ovariectomy absolute changes in tension induced by 5Hz EFS were examined at the peak of the 1μM phenylephrine-induced contraction following incubation with guanethidine (10μM) and scopolamine (10μM) in tissue strips from control animals and animals 24 weeks post-op. Using this as a measure of contractility no significant differences ($P>0.05$) in longitudinal vaginal wall strip or clitoral corpus cavernosum contractility were seen between control animals and ovariectomized animals with relaxations in strips from control animals of 6.87 ± 1.18mN and 9.81 ± 0.98mN observed in vaginal wall and clitoral corpus cavernosum strips respectively (n/N=8/6 for both), and relaxations in strips from ovariectomized animals of 9.32 ± 0.98mN in vaginal wall strips and 8.93 ± 1.47mN in clitoral corpus cavernosum strips (n/N=8/4 for both).

EFS-induced NANC relaxation responses, in vaginal wall strips from both control animals and 24 weeks post-op ovariectomized animals were frequency dependent. However 1 – 25Hz EFS-induced responses were significantly greater in strips from ovariectomized animals than those from control animals (as a percentage of 50Hz EFS-induced responses), with maximal responses observed at 10 Hz (Figure 52A).

EFS-induced NANC relaxation responses in clitoral corpus cavernosum strips were also frequency dependent, but there was no difference in these responses between strips from control and ovariectomized animals (Figure 52B).

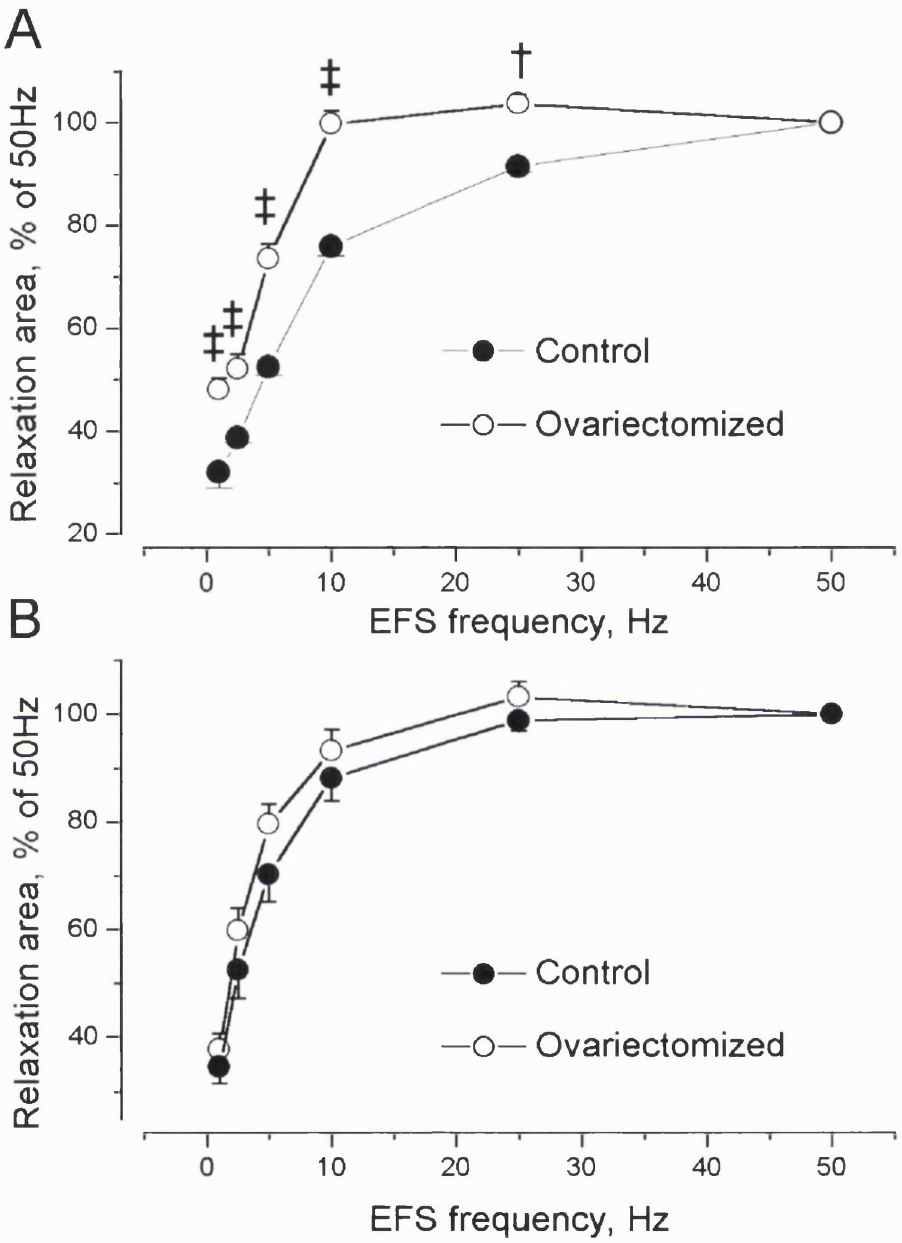


Figure 52: Comparison between frequency dependence of EFS-induced relaxation response in longitudinal rabbit vaginal wall strips (A) and clitoral corpus cavernosum strips (B) from ovariectomized and control animals. The frequency-dependence of the relaxation magnitude and duration is expressed as the area of the relaxation as a percentage of relaxation elicited by the maximum frequency used (50 Hz) from control animals (●, n/N=6/6) or spayed animals 24 weeks post-op (○, n/N=8/4). Effect of ovariectomy statistically different in the vaginal wall, P<0.0001. Frequency effect significantly different from control, †=*P*<0.01, ‡=P<0.001. Statistical analyses performed using two way ANOVA followed by Bonferroni post-test.

c) Effect of NOS inhibitors on EFS-induced relaxations in longitudinal vaginal wall strips from ovariectomized animals

Incubation with L-NAME (1 - 500μM) caused concentration-dependent partial inhibition of 1 - 50Hz EFS-induced relaxation responses in vaginal wall strips from 24 weeks post-op animals. There was no significant difference ($P>0.05$) in either the degree of inhibition, or the inhibition profile between ovariectomized and control animals at all stimulation frequencies (Figure 53). However, whilst the differences were not significant, the inhibition curves for L-NAME in vaginal wall strips from ovariectomized animals all lay above those from control strips.

Incubation with L-NA (1 - 300μM) also caused concentration-dependent partial inhibition of 1 – 50Hz EFS-induced NANC relaxations in vaginal wall strips from 24 weeks post-op animals. To achieve a similar degree of inhibition of EFS-induced relaxations at frequencies of 2.5 – 50Hz required significantly greater concentrations of L-NA in spayed animals than in control animals as determined by two way ANOVA with probabilities of observing these differences by chance of 0.26%, 0.4%, 0.58, 1.3% and 2.7% at 2.5, 5, 10, 25 and 50Hz respectively. Furthermore at 2.5Hz stimulation the degree of inhibition of EFS-induced responses was significantly greater in control animals than ovariectomized animals at 300μM L-NA (Figure 54).

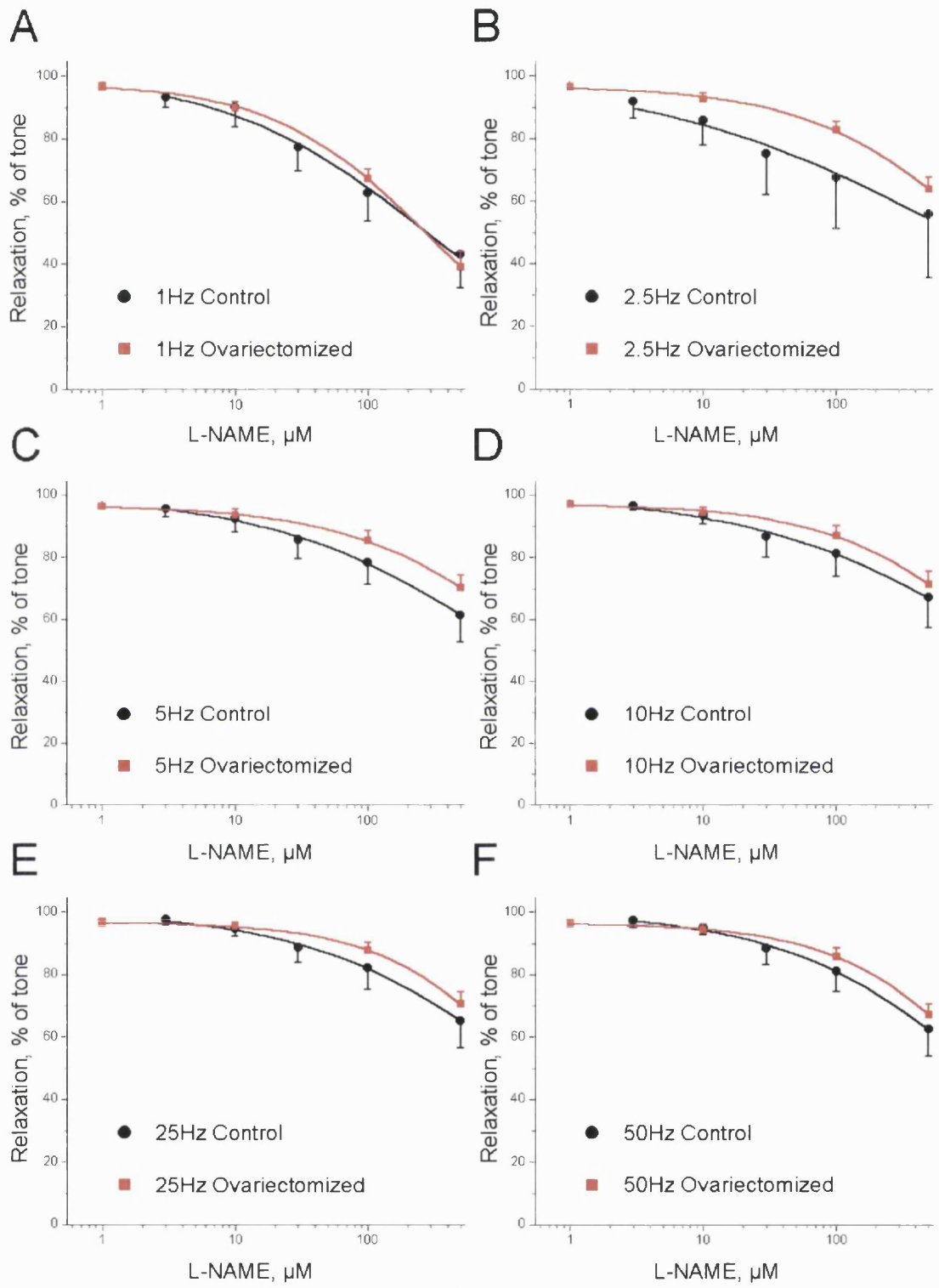


Figure 53: Effect of ovariectomy on inhibition of EFS-induced relaxations by L-NAME in rabbit longitudinal vaginal wall strips. n/N=4/4 for control; n/N=4/4 for 24 weeks post-ovariectomy. Statistical analyses performed using two way ANOVA followed by Bonferroni post-test. Data points represent mean ± s.e. mean.

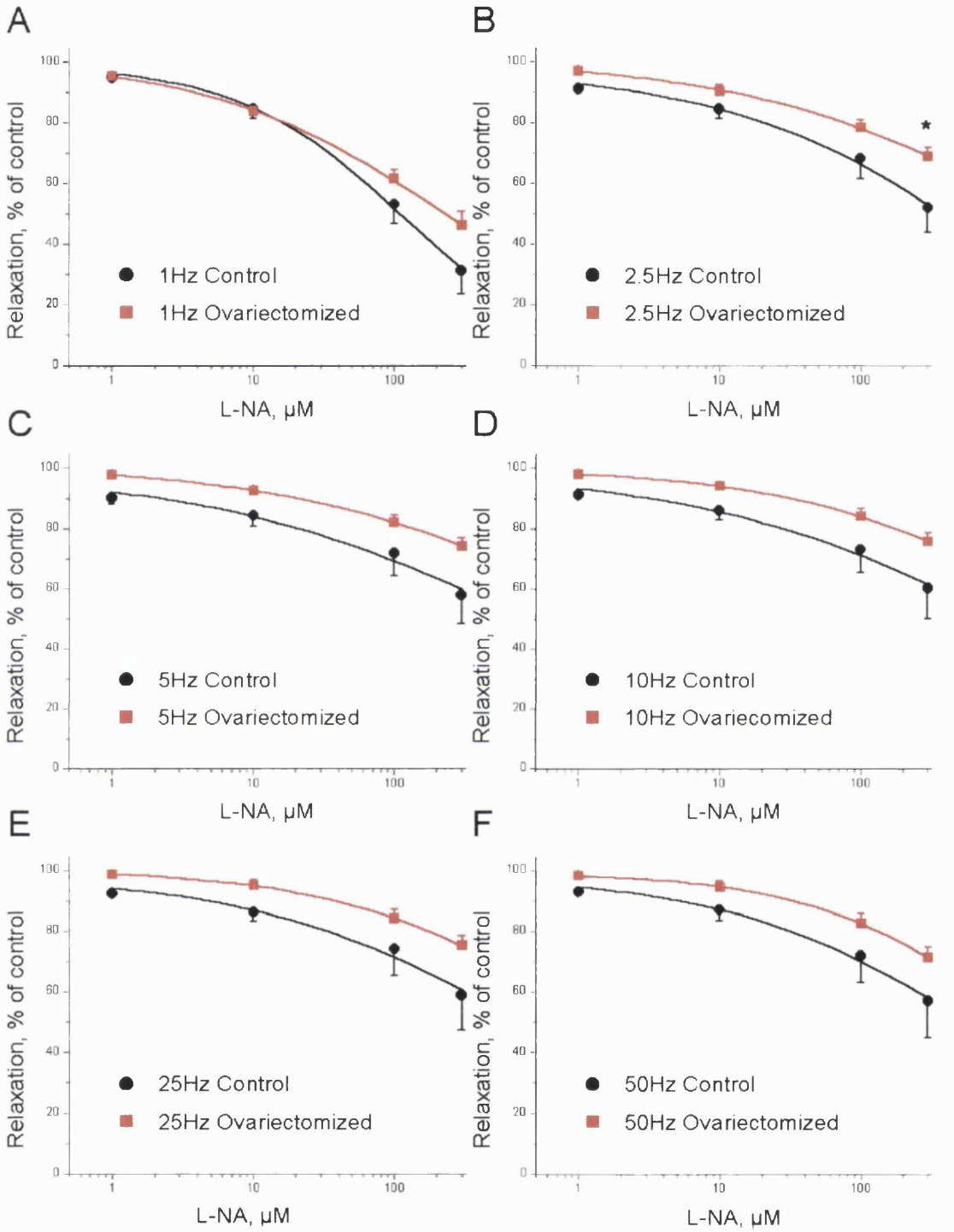


Figure 54: Effect of ovariectomy on inhibition of EFS-induced relaxations by L-NA in rabbit longitudinal vaginal wall strips *Significantly different from control $P<0.05$; n/N=4/4 for control; n/N=4/4 for 24 weeks post-ovariectomy. Statistical analyses performed using two way ANOVA followed by Bonferroni post-test. Data points represent mean ± s.e. mean.

d) Effect of NOS inhibitors on EFS-induced relaxation responses in clitoral corpus cavernosum strips from spayed animals

Incubation with L-NAME (1 – 500μM) caused concentration dependent inhibition of 1 - 50Hz EFS-induced NANC relaxation responses in clitoral corpus cavernosum from 24 weeks post-op ovariectomized animals. 1Hz EFS-induced responses were inhibited to the same extent and with a similar inhibitory profile in tissue strips from ovariectomized and control animals (Figure 55). At 100μM L-NAME, 2.5 - 25Hz EFS induced relaxation responses were inhibited to a significantly greater extent in tissue strips from control animals than ovariectomized animals ($P<0.05$; Figure 55). Also at 2.5 – 50Hz EFS higher concentrations of L-NAME were required to inhibit responses in strips from ovariectomized animals to the same extent as those from control animals as determined by two way ANOVA with probabilities of observing these differences by chance of 1.24%, 0.77%, 0.68%, 0.68% and 0.6% at 2.5, 5, 10, 25 and 50Hz respectively.

Incubation of clitoral corpus cavernosum strips from 24 weeks post-op ovariectomized animals with L-NA (1 – 300μM) also caused concentration dependent inhibition of 1 – 50Hz EFS-induced NANC relaxation responses (Figure 56). 1 and 2.5Hz EFS-induced relaxation responses were inhibited with similar inhibitory profiles in tissue strips from spayed and control animals. 5 and 10Hz EFS-induced responses were inhibited to a not quite significantly greater extent in control animals as determined by two way ANOVA with probabilities of observing these differences by chance of 9.14% and 6.28% at 5 and 10Hz respectively. EFS-induced responses to 25 and 50Hz, however, were inhibited to a significantly greater extent in clitoral corpus cavernosum strips from

control animals than ovariectomized animals with probabilities of observing these responses by chance of 4.39% and 2.97% at 25 and 50Hz respectively.

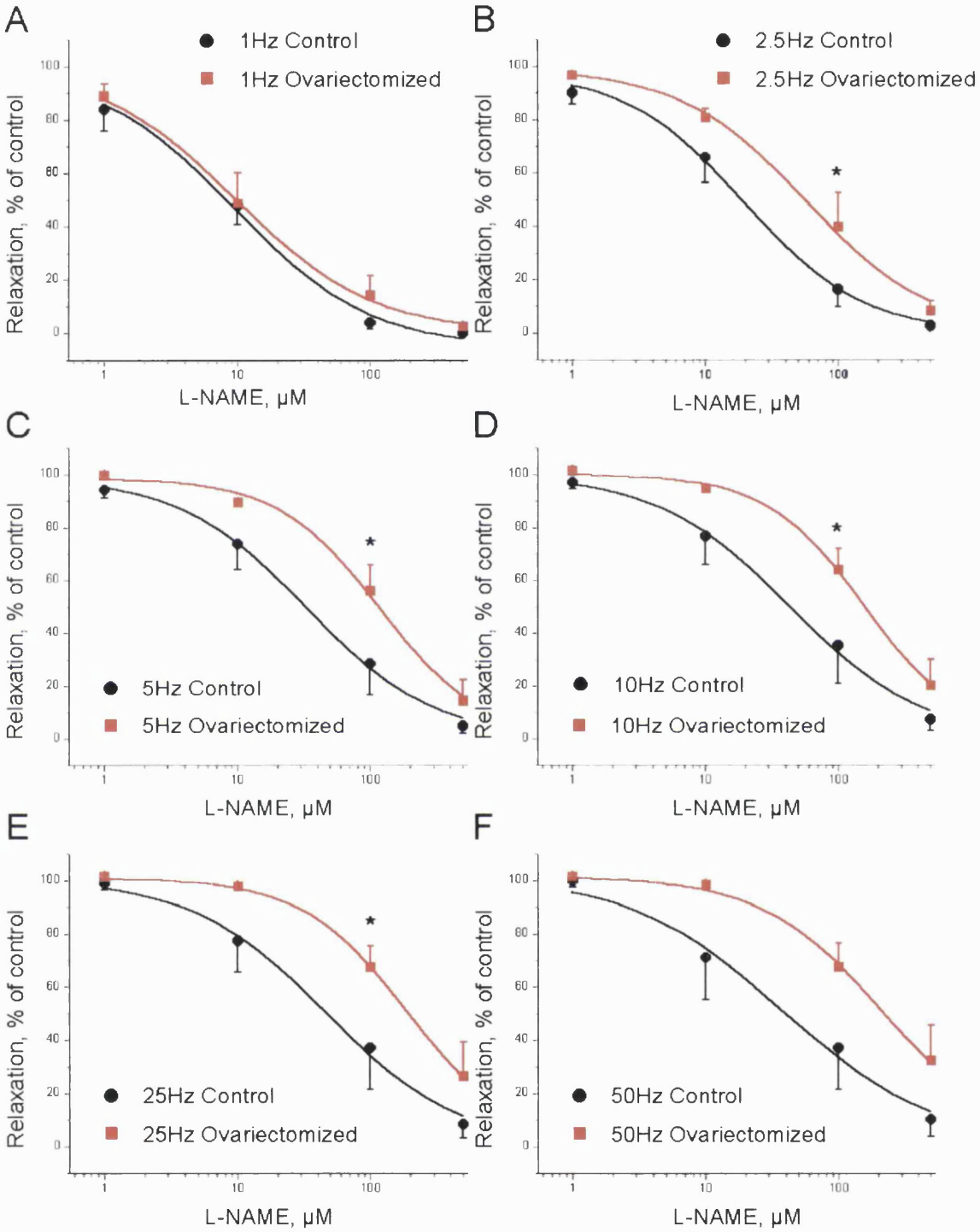


Figure 55: Effect of ovariectomy on inhibition of EFS-induced relaxations by L-NAME in rabbit clitoral corpus cavernosum strips. *Significantly different from control $P<0.05$; n/N=4/4 for control; n/N=4/4 for 24 weeks post-ovariectomy. Statistical analyses performed using two way ANOVA followed by Bonferroni post-test. Data points represent mean ± s.e. mean.

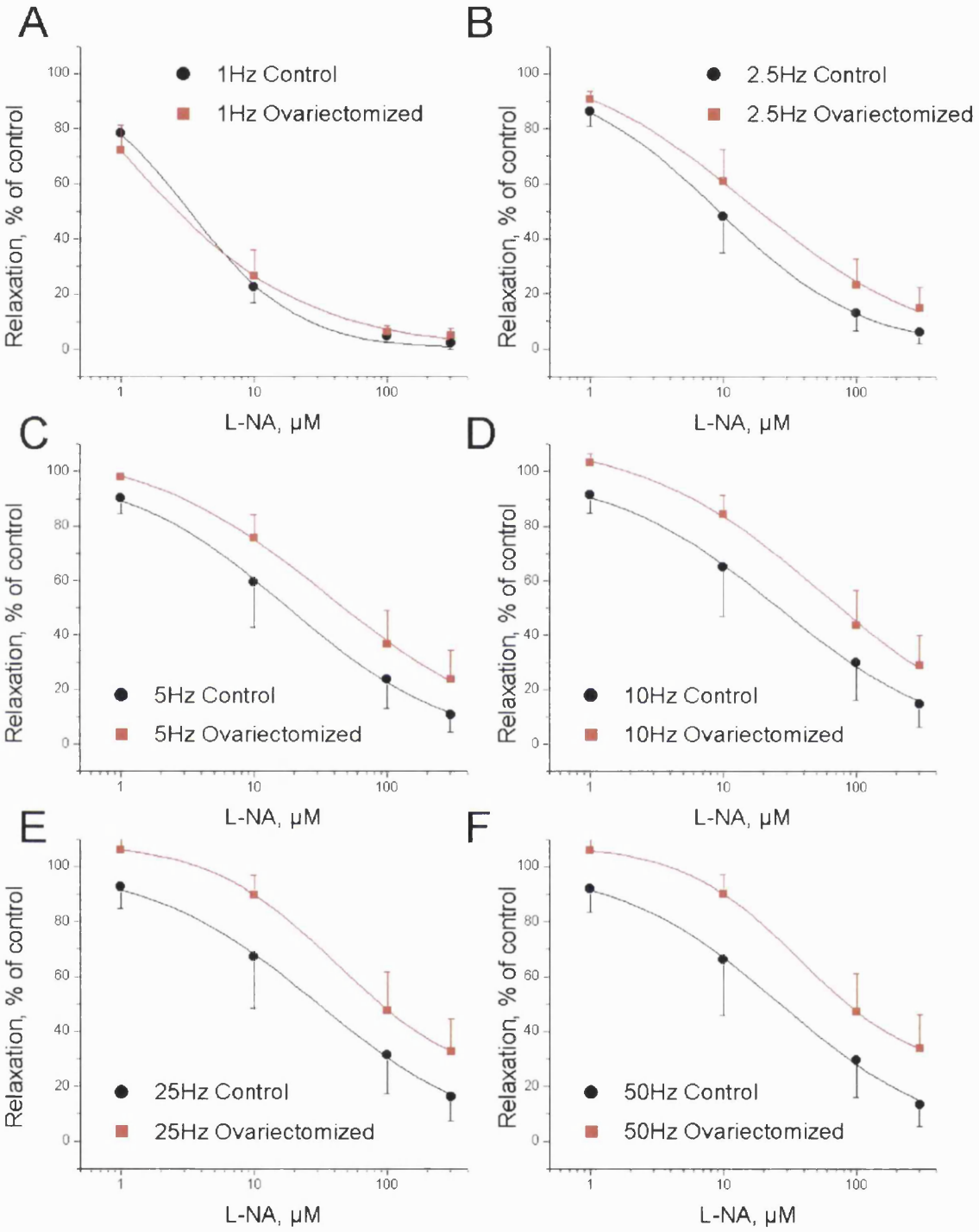


Figure 56: Effect of ovariectomy on inhibition of EFS-induced relaxations by L-NA in rabbit clitoral corpus cavernosum strips. n/N=3/3 for control; n/N=4/4 for 24 weeks post-ovariectomy. Statistical analyses performed using two way ANOVA followed by Bonferroni post-test. Data points represent mean ± s.e. mean.

CHAPTER IV

DISCUSSION

IV: 1 CHARACTERISATION OF NANC RELAXATION RESPONSES IN VAGINAL WALL STRIPS

a) Balance between adrenergic, cholinergic and NANC responses

Short-term stimulation of circular or longitudinal strips of tissue from rabbit or rat vaginal wall results in contractions that occur either during or after termination of stimulation. It has previously been shown in the human and rabbit penile corpus cavernosum that EFS does not induce contractile responses until after termination of stimulation, unless the NO-sGC-cGMP pathway is inhibited, in which case a contraction is observed as soon as stimulation commences (Cellek & Moncada, 1997a). Furthermore it has been shown that in the rat and mouse penile corpus cavernosum EFS-induced contractions begin during stimulation (Cellek & Moncada, 1997a). These results were interpreted as evidence that in this tissue there is a strong nitrergic innervation in the human and rabbit that controlled the noradrenergic contraction, whilst in the rat and mouse the balance of neurotransmitters means that the noradrenergic contraction is stronger than the nitrergic relaxation.

In the current study the fact that the contractile responses did not occur until termination of stimulation in rabbit longitudinal vaginal wall strips, and that in approximately 50% of the strips a relaxant response was observed during stimulation, indicates that in these strips when noradrenergic, cholinergic and NANC neurons are stimulated simultaneously the relaxant response overrides that of the contractile response (as shown in the human and rabbit penile corpus cavernosum by Cellek & Moncada,

1997a). In rat circular strips the balance between contractile and relaxant mechanisms may be more equal (as shown in the rat penile corpus cavernosum by Cellek & Moncada, 1997a), explaining why in these strips a contractile response is often observed during stimulation. In rabbit circular vaginal wall strips and rat longitudinal vaginal wall strips a contractile response was observed during stimulation in all cases, which would suggest that in these strips the contractile mechanism is stronger than the relaxant pathway.

In all cases contractile responses were inhibited after incubation with guanethidine and scopolamine, however incubation with scopolamine alone did not inhibit contractile responses in rabbit longitudinal vaginal wall strips (data not shown in results chapter since the effect of scopolamine alone was only examined in tissue strips from 2 animals) suggesting that contractions are mediated by noradrenaline. This is in agreement with other smooth muscle structures in the genital tract. For example, in isolated human penile corpus cavernosum strips noradrenaline, phenylephrine and the α_2-adrenoceptor agonist clonidine have been shown to induce concentration-dependent contractions, whilst the cholinergic agonists ACh and carbachol do not contract these preparations (Hedlund & Andersson, 1985). Also EFS-induced contractions in isolated human corpus cavernosum strips have been shown to be inhibited by bretylium, an inhibitor of noradrenaline release, or the α_1-adrenoceptor antagonist prazosin (Saenz de Tejada *et al.*, 1988). Furthermore it has been shown that EFS-induced contractions in both human and rabbit penile corpus cavernosum strips are attenuated by prazosin (Saenz de Tejada *et al.*, 1989). Also bretylium has been reported to inhibit EFS-induced

contractions in rabbit clitoral corpus cavernosum and vaginal wall strips (unpublished observations mentioned in Min *et al.*, 2001a).

The fact that rabbit longitudinal strips did not behave in the same way as circular strips was surprising. It may be that the smooth muscle running in the circular direction contains a larger amount of α-adrenoceptors, so that when NANC neurotransmitters and noradrenaline are released upon EFS the noradrenaline-induced contraction overrides the NANC relaxation response. Another possibility is that there are more noradrenergic neurons innervating the muscle running in the circular direction than the longitudinal direction. These possibilities could be investigated by immunohistochemical studies on the rabbit vaginal wall to determine if there is a difference in α-adrenoceptor distribution or adrenergic innervation between smooth muscle running in circular and longitudinal directions. In the current study I did not examine these possibilities, and therefore cannot determine the reason why a difference was observed between responses in rabbit circular and longitudinal strips.

The importance of overcoming the noradrenergic contractile response for female sexual arousal has been demonstrated in humans when clonidine was administered to healthy women. This was found to impair vaginal engorgement and lubrication (Meston et al., 1997). Also the α-adrenoceptor antagonist phentolamine has been shown to increase blood flow in the rabbit vaginal wall (Park et al., 1997).

b) Eliciting the NANC response

After inhibition of the noradrenergic contractile responses raising tissue tone with phenylephrine revealed NANC relaxation responses upon subsequent stimulation in all tissue strips with the exception of longitudinal and proximal circular strips from rat vaginal wall since tone could not be raised with phenylephrine in these strips. In rabbit longitudinal vaginal wall strips raising tissue tone with histamine (100μM) or the thromboxane A2 agonist U46619 (1μM) in the presence of guanethidine and scopolamine also revealed NANC relaxation responses upon subsequent EFS (data not shown since only 2 longitudinal strips of rabbit vaginal wall were tested for each drug).

Smooth muscle contraction is mediated by phosphorylation of myosin regulatory light chain (MRLC), which is regulated by the relative activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP; see Somlyo *et al.*, 1999 for review). This is mainly regulated by changes in intracellular calcium with increased calcium leading to activation of MLCK, which phosphorylates the MRLC leading to contraction (see Stull *et al.*, 1998 for review). Contraction can also occur by sensitisation of the regulatory proteins to calcium so that contraction can occur with smaller changes in calcium, or independently of changes in calcium levels (see Somlyo & Somlyo, 2000 for review). This mechanism is mainly thought to occur via activation of Rho-kinase, which phosphorylates MLCP, inactivating it. This then prevents de-phosphorylation of the MRLC, and thus leads to contraction.

Phenylephrine has been reported to act on α_1-adrenoceptors to activate the phospholipase C (PLC)/ inositol 1,4,5 trisphosphate (IP_3)/ diacylglycerol (DAG) pathway, with increased IP_3 levels leading to release of Ca^{2+} from intracellular stores and subsequent calcium entry through VOCCs to contract the smooth muscle in pig renal arteries (Eckert *et al.*, 2000). Histamine has also been reported to act through H_1 receptors to induce contractile responses via the PLC/ IP_3/ DAG pathway in the rabbit lingual artery and the guinea-pig trachea (Suyama *et al.*, 1994; Matyas *et al.*, 1995). The PLC/ IP_3/ DAG pathway can theoretically be involved in Ca^{2+} sensitisation as well as increasing intracellular Ca^{2+}, since the DAG produced by PLC activation can activate protein kinase C. This can then phosphorylate a protein called CPI-17, which when phosphorylated inhibits the catalytic subunit of MLCP (Li *et al.*, 1998). Indeed in the rabbit femoral artery, histamine has been shown to induce Ca^{2+}-sensitisation and CPI-17 phosphorylation (Kitazawa *et al.*, 2000). Also in this tissue phenylephrine was equally potent at inducing contractions, but with significantly lower accompanying CPI-17 phosphorylation (Kitazawa *et al.*, 2000). However PLC products are mainly thought to have either small and transient Ca^{2+}-sensitising effect (Iizuka *et al*,. 1999) or to have no role in G-protein mediated Ca^{2+}-sensitising effects (Jensen *et al.*, 1996; Walker *et al.*, 1998; Strassheim *et al.*, 1999). U46619 on the other hand is a potent Ca^{2+}-sensitising agonist, mediating its effects with little or no detectable Ca^{2+} release (Bradley & Morgan, 1987; Himpens & Somlyo, 1988). To complicate matters further it appears that several GPCRs that act via the PLC/ IP_3/ DAG pathway can activate the Rho-kinase Ca^{2+}-sensitising pathway depending on receptors and cell types involved (Katoh *et al.*, 1998; Croxton *et al.*, 1998; Klages *et al.*, 1999; Hirshman & Emala, 1999). Whilst in the current

study I did not examine the different pathways involved in contracting the vaginal wall by these different agonists, my results show that whatever mechanism is used does not affect the ability of NANC stimulation to induce relaxation responses in the rabbit vaginal wall.

c) Summary

In this study I have provided evidence that in the rabbit vaginal wall EFS induces contractions that are mediated by noradrenaline.

The degree to which the NANC relaxant responses control the noradrenergic responses vary with tissue and species, but in rabbit longitudinal vaginal wall strips NANC inhibitory neurotransmission has overriding control of the noradrenergic contraction.

Raising tissue tone after incubation with guanethidine and scopolamine reveals NANC relaxations regardless of agonist used to raise tone.

IV: 2 ROLE OF NITRIC OXIDE AS A MEDIATOR OF NANC RELAXATION RESPONSES IN THE VAGINAL WALL AND CLITORAL CORPUS CAVERNOSUM

a) Effect of NOS inhibitors

The NOS inhibitors L-NAME, L-NA and L-NIO all partially inhibited fast NANC relaxation responses in rabbit vaginal wall strips. The degree of inhibition varied dependent on frequency of stimulation and inhibitor used. I also found that these NOS inhibitors completely or almost completely abolished relaxation responses in the clitoral corpus cavernosum, with the exception of L-NIO, which reduced 5Hz EFS-induced responses to about 35% of control responses.

This study gives an overall rank order of potency for the NOS inhibitors used L-NA ≥ L-NAME > L-NIO. L-NA and L-NAME are derivatives of L-arginine and L-NIO is a derivative of L-ornithine. L-NA has been reported to have the greatest potency against nNOS however its solubility in water is limited. For this reason I also used L-NAME, which is slightly less potent than L-NA but more soluble, which may account for its greater efficacy *in vitro*. Indeed L-NA has been reported to have similar potency to L-NAME as an inhibitor of NANC relaxation responses of the rat anococcygeus muscle (Hobbs & Gibson, 1990). I also used L-NIO, which is less potent than L-NAME, in order to confirm my results with a non-arginine derivative NOS inhibitor.

In this study the NANC relaxation responses are a reversal of agonist-induced tone, rather than relaxation of a spontaneous resting tone. It has previously been shown that EFS- and SNP-induced relaxation responses varied in the mouse anococcygeus muscle depending on the agonist used to increase the tissue tone (Gibson *et al.*, 1994). However the maximal effects of these relaxant stimuli reduced agonist-induced tone by more than 90%, with the exception of high K^+ induced tone, which was only reduced to about 50% by SNP (Gibson *et al.*, 1994). The main mechanism for NO/cGMP-mediated smooth muscle relaxation is currently thought to be via activation of cGMP-dependent protein kinase causing a decrease in intracellular Ca^{2+} concentration and a loss of sensitivity of the contractile proteins to Ca^{2+} (Carvajal *et al.*, 2000). Thus the non-nitrergic component of the relaxations in the vaginal wall may be dependent on the use of phenylephrine to increase tissue tone. However, in experiments in rabbit longitudinal vaginal wall strips in which the tone was raised with either U46619 (100nM) or histamine (100μM), treatment with L-NAME still left the majority of the NANC relaxations unaffected. This provides further evidence of the importance of the non-nitrergic component of the NANC relaxations in, the rabbit vaginal wall.

In experiments with circular strips of phenylephrine-precontracted rabbit and rat vaginal wall 1 – 50Hz EFS-induced NANC relaxation responses were resistant to inhibition by L-NAME. These experiments indicate non-nitrergic responses are not species specific. However, the degree of inhibition was greater in the rat suggesting that the importance of NO as a mediator of NANC relaxation responses in the vaginal wall may be species-dependent. Experiments with human vaginal wall strips are needed to see

if in this tissue NANC relaxations are also mediated by both nitrergic and non-nitrergic mechanisms and, if so, which species best models the responses seen in the human.

b) Role of sGC in mediating NANC relaxation responses

In order to further study the role of NO as a mediator of NANC relaxation responses in longitudinal strips of rabbit vaginal wall the second messenger pathways involved in NANC relaxation responses were also studied. Incubation of tissue strips with the sGC inhibitor ODQ reduced 5Hz EFS-induced NANC relaxation responses to about 70% of control responses, which was similar to the degree of inhibition seen using L-NAME. Furthermore the relaxation responses were not inhibited to a greater degree by using a combination of L-NAME and ODQ. Also the inhibition profile for both drugs was similar with 1 Hz EFS-induced relaxation inhibited to a greater extent than those induced by higher frequency stimulation. Taken together these results suggest that the inhibitors may be blocking different points of the same relaxant pathway, suggesting that the NANC relaxations in the rabbit vaginal wall are partially mediated by the NO-sGC-cGMP pathway. Experiments to assess the effects of EFS on intracellular cGMP supported this hypothesis, with cGMP levels being significantly raised during stimulation. The increases in cGMP were abolished by the presence of L-NAME (500μM), supporting the concept that nitrergic relaxations are mediated by cGMP, and also providing evidence that L-NAME effectively abolished nitrergic responses.

The fact that both NOS inhibitors and ODQ were more effective at inhibiting low frequency EFS-induced responses suggests that as stimulation frequency increases there is a change in the balance of neurotransmitters released, with a greater proportion of the responses mediated by NO at low frequency stimulation, and another neurotransmitter having an increasing role as stimulation frequency is increased.

c) Role of NO as a mediator of slow relaxation responses

This study has mainly concentrated on the fast NANC relaxation responses to EFS. However, in longitudinal and circular tissue strips from rabbit vaginal wall EFS elicited fast and slow relaxation responses, both of which were frequency-dependent. EFS-induced NANC relaxation responses in rat distal circular vaginal wall strips were also frequency-dependent, but with no slow relaxation response evident. These results suggest that there is a species difference in NANC neurotransmitters released. Similar fast and slow relaxation responses have been reported in several tissues including the female pig urethra (Werkström *et al.*, 1995), the cat bronchiole (Fujisawa *et al.*, 1999) and the opossum lower esophageal sphincter (Uc et al., 1999). Moreover, in the opossum lower esophageal sphincter similar variation in responses was observed with contractions sometimes seen between the fast and slow relaxations (Uc et al., 1999). In these studies the fast components of the responses were abolished by NOS inhibitors, whilst the slow components were either unaffected or only slightly affected, indicating that NO was the neurotransmitter responsible for mediating the fast responses, and that another neurotransmitter was responsible for mediating the slow responses. In the current study

this was not the case since both fast and slow relaxations in the rabbit vaginal wall were only partially inhibited by L-NAME, suggesting that in this tissue NO is involved in mediating both fast and slow relaxations, but that another mediator is responsible for mediating the majority of both fast and slow responses.

d) Effect of neurotoxins on NANC relaxation responses

The release of classical neurotransmitters is induced following arrival of an action potential at the pre-synaptic terminal. The depolarisation caused by the action potential causes opening of VOCCs, allowing influx of calcium, promoting fusion of vesicle membranes and release of stored neurotransmitters into the synaptic cleft (Burks, 1994). Nitric oxide is generally not thought to be stored as with classical neurotransmitters, and instead is synthesised *de novo* (Snyder, 1992). However as for release of classical neurotransmitters nNOS activity is regulated by calcium. Increased cytosolic calcium following nerve stimulation stimulates nNOS through interaction with calmodulin, resulting in calcium-dependent release of NO (Knowles *et al.*, 1989).

In this study NANC relaxations remaining after incubation with L-NAME or L-NAME and ODQ in vaginal wall strips were abolished by incubation with the sodium channel blocker TTX, which prevents propagation of the action potential, indicating that these responses were neurogenic.

In the female pig urethra and the cat bronchiole where EFS induced fast and slow relaxations as discussed above, the non-nitrergic, slow component of the responses are markedly reduced or abolished by incubation with the N-type VOCC blocker ω-CTX GVIA (Werkström *et al.*, 1995; Fujisawa *et al.*, 1999). In my study however incubation with ω-CTX (100nM) only partially inhibited 5Hz EFS-induced responses in the rabbit longitudinal vaginal wall strips. Fast relaxation responses were only slightly inhibited with about 90% of control responses remaining and slow responses were inhibited to a greater extent with about 45% of control responses remaining. Incubation of rabbit clitoral corpus cavernosum strips with ω-CTX (100nM), also only caused partial inhibition of 5Hz EFS-induced responses with about 85% of control responses remaining. Increasing the concentration of ω-CTX to 200nM did not increase the degree of inhibition above that seen with 100nM ω-CTX indicating that 100nM ω-CTX is maximally effective (data not shown in results chapter due to low number of experiments performed; n/N=4/2 for both vaginal wall and clitoral corpus cavernosum strips). Incubation with ω-CTX (100nM) in combination with L-NAME (500μM) in vaginal wall strips caused a greater degree of inhibition of fast and slow responses than either agent alone with about 45% and 5% of fast and slow control responses remaining respectively, whilst a combination of ω-CTX and L-NAME abolished responses in the clitoral corpus cavernosum.

VOCCs can be divided into low- and high voltage-activated calcium channels (Sher *et al.*, 1991), and the high voltage-activated channels can be further subdivided into

L, N and P subtypes). N type VOCCs are known to be present in neurons, but absent in smooth muscle cells (Miller, 1987), thus effects of ω-CTX can be attributed to blockade of calcium entry into neurons. ω-CTX has been shown to be more effective at inhibiting classical neurotransmitter-induced responses, such as those to noradrenaline, than those to nitric oxide (Boeckxstaens *et al.*, 1993; Zygmunt *et al.*, 1993). In this study ω-CTX only partially inhibited NO-induced responses. The limited inhibition of NANC relaxations by ω-CTX in this study may indicate that other calcium channels are involved in mediating both nitrergic and non-nitrergic neurotransmitter release. P type VOCCs may mediate presynaptic calcium influx in the neurons as has been suggested in mouse motor nerve terminals (Uchitel *et al.*, 1992). Another VOCC-independent mechanism has been proposed whereby intracellular sodium accumulation due to the action potential may cause increased cytoplasmic calcium levels through release of calcium from intracellular stores via a Na^{+}/Ca^{2+} exchanger (Minchin, 1980; Sandoval, 1980). Whatever the mechanism involved in releasing both NO and the mediator of non-nitrergic relaxations from the presynaptic nerve terminals, the fact that the responses are entirely inhibited by TTX confirms that the responses are neurogenic in origin.

e) Summary

In this study I have provided evidence that NANC relaxations of rabbit longitudinal vaginal wall strips are partially mediated by NO.

The nitrergic NANC relaxations are:

- Inhibited by NOS inhibitors with a rank order of potency L-NA ≥ L-NAME > L-NIO
- Inhibited by the sGC inhibitor ODQ with similar efficacy to L-NAME
- Associated with increased intracellular cGMP content

In the rabbit vaginal wall the majority of the NANC relaxations are not mediated by NO, and these non-nitrergic NANC relaxations are:

- Neurogenic, as they are abolished by TTX
- Frequency dependent, with a greater proportion of the responses non-nitrergic at higher stimulation frequencies
- Observed regardless of agonist used to induce tone

In the rat vaginal wall I also found that the NANC responses are partially mediated by NO, with the non-nitrergic responses being frequency dependent as in the rabbit.

IV: 3 ROLE OF NEUROPEPTIDES AS MEDIATORS OF NANC RELAXATION RESPONSES IN THE VAGINAL WALL

a) Frequency dependence of responses

The existence of a non-nitrergic NANC neurotransmitter in the smooth muscle of another urogenital structure, the urethra, has been demonstrated (Bridgewater *et al.*, 1993; Werkström *et al.*, 1995) in the female pig. In this tissue, high frequency stimulation (>12 Hz) elicits non-nitrergic NANC relaxation responses (Werkström *et al.*, 1995). Similar responses have been reported in the guinea-pig trachea (Tucker *et al.*, 1990; Brave *at al.*, 1991; Moffatt *et al.*, 1999). This suggested that a neuropeptide might mediate these responses since neuropeptides are known to be released following high frequency stimulation (Lundberg, 1996). In my experiments non-nitrergic NANC responses in the rabbit vaginal wall were inhibited with TTX and were greater in magnitude and duration at high frequencies, thus I hypothesised that they were mediated by a neuropeptide. I therefore aimed to investigate possible candidate neuropeptides, which may be responsible for the non-nitrergic NANC responses.

Previous studies have demonstrated VIP, PACAP, PHM, PHV, CGRP, substance P and helospectin-I and -II immunoreactive nerve fibres in the human vagina (Graf *et al.*, 1995; Hoyle *et al.*, 1996) and human genital tract (Steenstrup *et al.*, 1995), and co-localisation of VIP with nNOS in nerve fibres in the porcine and bovine vagina (Majewski *et al.*, 1995). Furthermore, it has been shown that exogenously applied VIP inhibited spontaneous contractions in cat and rabbit myometrial strips and caused

increased vaginal blood flow and increased vaginal lubrication in humans (Ottesen *et al.*, 1981; Ottesen *et al.*, 1987), suggesting a possible role for this peptide in the female genital sexual response.

b) Effect of VIP-related peptides

I have shown in this study that exogenous application of VIP, PACAP-27, PACAP-38, PHM, PHV, helospectin-I and helospectin-II caused relaxation responses in the rabbit vaginal wall, with similar efficacy and potency, with the exception of PACAP-27, PHM and PHV, which had similar efficacy, but lower potency at inducing relaxations. This suggests that these responses were due to interaction with $VPAC_1$ or $VPAC_2$ receptors rather than PAC_1 receptors, since PAC_1-mediated responses would be expected to be mediated by the peptides with a potency order of PACAP-27=PACAP-38>>VIP>PHM=PHV=helospectin I=Helospectin II, as discussed in section I: 3b. The concentrations of VIP-related peptides that induced relaxation responses in the vaginal wall are several orders of magnitude higher than would be expected from binding studies with isolated receptors expressed in single cells (see section I: 3b), however this is likely to be due to binding studies giving a higher apparent affinity of ligands for their receptors. Furthermore, my findings agree with the effective concentrations from functional studies on the effects of VIP-related peptides in other tissues. For example submaximal relaxation responses were induced by 1μM VIP in isolated guinea-pig urethra strips (Werkström *et al.*, 1998). In the opposum internal anal sphincter, maximal

VIP-induced responses were observed at 3μM VIP, whilst relaxations induced by PACAP-38 had not plateaued at 10μM PACAP (Chakder & Rattan, 1998).

In this study relaxation responses to exogenous application of all peptides could be inhibited with the protease α-chymotrypsin. EFS-induced relaxations however were not affected by α-chymotrypsin. It has previously been reported that the peptidase α-chymotrypsin is able to block non-nitrergic EFS-induced relaxations in the guinea-pig trachea (Moffatt *et al.*, 1999) and in the female pig urethra (Werkström *et al.*, 1997) suggesting that the peptidase is capable of inhibiting responses to endogenously-released neuropeptides as well as inhibiting responses to exogenously-applied peptides. The fact that in this study the peptidase had no effect on EFS-induced relaxations provides evidence against any of these peptides being involved in the non-nitrergic component of the NANC relaxation in the rabbit vaginal wall.

c) VIP receptor antagonists

VIP-related peptides induce relaxations by binding to cell surface receptors. In this study I attempted to inhibit this binding using the VIP receptor antagonists VIP fragment 10-28 and VIP fragment 6-28 (data not shown for VIP fragment 6-28 since experiments using this antagonist were only performed in 2 longitudinal strips of rabbit vaginal wall). In the porcine retractor penis 100nM VIP-induced relaxations (as well as EFS-induced relaxations) were reduced by either 1μM VIP fragment 10–28 or 1μM VIP fragment 6– 28 (La *et al.*, 2001). In the current study both VIP fragments 10 - 28 and 6 -

28 (both at 1μM) failed to inhibit relaxation responses elicited by 100nM VIP. Both inhibitors also failed to affect 5Hz EFS-induced NANC relaxations, however the lack of effect of VIP fragment 10-28 and VIP fragment 6–28 on VIP-induced relaxations means that I can make no conclusions about the role of VIP in NANC relaxation from the observation that these antagonists do not inhibit NANC relaxations.

Variability in the efficacy of these antagonists has been observed in several smooth muscle preparations. VIP fragment 10–28 has been shown to inhibit both VIP- and EFS-induced relaxation responses in the rat distal colon (Kishi *et al.*, 1996), however in the female pig urethra VIP-induced responses (both exogenous and endogenous) were not inhibited by VIP fragment 10-28 (up to 10μM; Werkström *et al.*, 1997) and in the opossum lower esophageal sphincter VIP fragment 10-28 was found to inconsistently antagonise VIP-induced relaxations, with no effect on EFS-induced relaxations (Uc *et al.*, 1999). In the opossum lower esophageal sphincter VIP fragment 6–28 also failed to inhibit VIP-induced responses (Uc *et al.*, 1999).

In this study I also found the VIP antagonists [Lys^{1}, Pro$^{2,\ 5}$, Arg$^{3,\ 4}$, Tyr^{6}]VIP (0.5μM) and [D-p-Cl-Phe^{6}, Leu^{17}]VIP (1μM) to be without effect on non-nitrergic NANC relaxation responses (data not shown since experiments using these antagonists were only performed in 2 longitudinal strips of rabbit vaginal wall for each drug). However no conclusions can be made based on the lack of effect of these antagonists as these did not antagonise VIP-induced relaxations in the opossum lower esophageal sphincter (Uc *et al.*, 1999). The lack of effect of these antagonists on VIP-induced

responses in some preparations may be due to the antagonists only being effective at certain receptor subtypes.

Previous studies have shown that suramin, although considered to be a general P2 antagonist, is also an effective antagonist at VIP receptors. Suramin has been shown to inhibit radiolabelled VIP binding and block VIP-induced increases in cAMP in two human cancer cell lines (Bellan *et al.*, 1991), furthermore 200μM suramin has been shown to block VIP- and PACAP-27 (both at 100nM)-induced relaxation responses in the rat gastric fundus (Jenkinson & Reid, 2000) and 100μM suramin blocked VIP- and PACAP-27 (both at 100nM)-induced hyperpolarization in circular muscle strips of rat colon (Pluja *et al.*, 2000). Whilst in this study I did not attempt to inhibit VIP- or PACAP-induced relaxations with suramin, I did find that 100μM suramin did not affect non-nitrergic NANC relaxation responses, suggesting that these responses are not mediated by these peptides. However without further experiments to determine whether suramin is an effective VIP or PACAP receptor antagonist in this tissue I cannot use this lack of effect of suramin on NANC relaxations to make any conclusions about the involvement of VIP or PACAP in these responses.

d) Effect of endopeptidase inhibitors

Neuropeptide-induced responses are terminated by the breakdown of the peptides by neutral endopeptidases, thus to further investigate the possibility that neuropeptides

may mediate non-nitrergic NANC relaxation responses in the rabbit vaginal wall I used the neutral endopeptidase inhibitors phosphoramidon and DL-thiorphan.

Phosphoramidon has been shown to both increase magnitude and duration of VIP-induced relaxation responses and to increase the potency of atrial natriuretic factor (ANF)-induced relaxations in guinea-pig tracheal smooth muscle strips (Yoshihara *et al.*, 1998; Fernandes *et al.*, 1992). DL-thiorphan has been shown to enhance the PHI- and helodermin-induced mucus secretion in the rat trachea (Wagner *et al.*, 1998). Both phosphoramidon and DL-thiorphan have been shown to inhibit the degradation of several peptides by guinea-pig gastric muscle cells (Gu *et al.*, 1993). Furthermore DL-thiorphan (1μM) has been shown to enhance and prolong L-NA-resistant EFS-induced NANC relaxations in guinea-pig colon circular muscle strips (Maggi & Giuliani, 1996).

In this study the effects of phosphoramidon (100nM) and DL-thiorphan (1μM) were each tested in strips of rabbit longitudinal vaginal wall. When non-nitrergic NANC relaxations after addition of drugs were overlaid on relaxations elicited prior to incubation no differences were seen in response magnitude or duration (Figure 57). If the non-nitrergic NANC relaxation responses were mediated by a neuropeptide then an enhancement of relaxation magnitude and duration would be expected. Lack of effect of these inhibitors does not show that peptides are not involved in NANC responses, since we have no control to ensure that peptide degradation was indeed blocked.

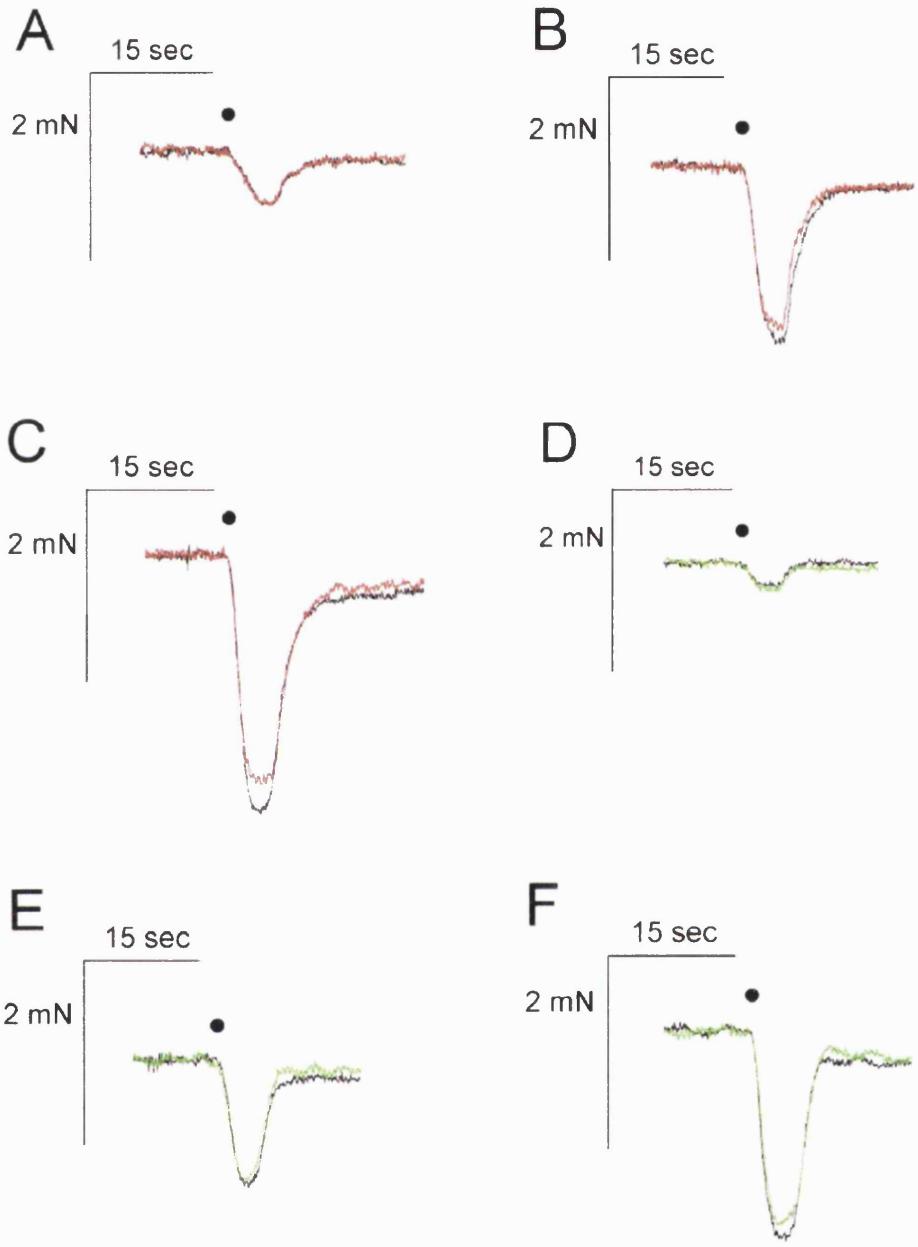


Figure 57: Effect of endonuclease inhibitors on EFS induced relaxation responses in rabbit longitudinal vaginal wall strips. Phosphoramidon (**A-C**, red traces) or DL-thiorphan (**D-F**, green traces) had no effect on 1 Hz (**A**+**D**), 2.5 Hz (**B**+**E**) or 5 Hz (**C**+**B**) EFS (5 sec train, start indicated by dots)-induced relaxation responses when start point of relaxations were overlaid. Black traces are of responses prior to addition of drugs. Mechanograms are original recordings of single tissue strips (n=1 for each drug).

e) Mechanisms of relaxation induced by VIP-related peptides

In sheep cerebral arteries and rat gastric fundus strips VIP has been reported to induce relaxation responses at least partially by stimulating NO production since VIP-induced relaxations were shown to be inhibited by L-NA, enhanced by L-arginine and were associated with a rise in intracellular cGMP in sheep cerebral arteries (Matthew *et al.*, 1997). Also VIP-induced relaxations were found to be reduced by the NOS inhibitor L-NMMA and enhanced by L-arginine in rat gastric fundus strips (Li & Rand, 1990). However, in the current study VIP-induced relaxations were unaffected by pre-incubation with L-NAME and did not induce increases in intracellular cGMP, suggesting a mechanism of action independent of NO, as in the guinea-pig trachea and opossum lower esophageal sphincter (Li & Rand, 1991; Uc *et al.*, 1999).

A NO-independent mechanism by which VIP can induce smooth muscle relaxation is by activation of adenylate cyclase. Binding of VIP-related peptides to their receptors activates adenylate cyclase via the G protein G_s leading to an increase in intracellular cAMP content (Hirata *et al.*, 1985; Warren *et al.*, 1991). In this study all the peptides that were capable of inducing relaxation when applied exogenously caused an increase in intracellular cAMP content, and did not affect intracellular cGMP content, suggesting that in the vaginal wall VIP-related peptides may be acting through this pathway. However EFS did not cause any increase in cAMP content in the absence or presence of L-NAME, suggesting that the mechanism leading to both nitrergic and non-nitrergic NANC relaxations is different from that induced by VIP, PACAP-27, PACAP-38, PHM, PHV, and helospectin- I and -II.

In this study I also examined the effects of the adenylate cyclase inhibitors MDL-12,330A, SQ 22536 , 2'3'-DDA and 2'5'-DDA on responses in rabbit longitudinal vaginal wall strips (data not shown in results chapter due to low number of experiments performed). MDL-12,330A (50μM) caused a slight reduction in tissue tone (n=2/1), but did not affect non-nitrergic relaxation responses (n=1). However MDL-12,330A was also ineffective at inhibiting relaxation responses induced by forskolin (5μM), indicating that in this tissue this inhibitor was not effective at inhibiting adenylate cyclase. I also found SQ 22536 (100μM) to reduce tissue tone, but to have no effect on non-nitrergic relaxation responses (n/N=2/1). This concentration of SQ 22536 has previously been shown to abolish iloprost-induced relaxations and increases in cAMP in isolated guinea-pig aorta strips (Turcato & Clapp, 1999). However in the current study 1μM isoprenaline-induced relaxation responses, which have been shown to be mediated by adenylate cyclase in the rabbit iris dilator smooth muscle (Yousufzai & Abdel-Latif, 1998) were also unaffected by SQ 22536 (n/N=2/1) suggesting that this compound is not effective as an adenylate cyclase inhibitor in this tissue. SQ 22536 has also been found to be an ineffective adenylate cyclase inhibitor in cultured human cancer cells. In these cells it failed to inhibit VIP- and PACAP-38-mediated increases in cAMP, that could be inhibited by MDL-12,330A (Csernus *et al.*, 1999). 2'3'-DDA (50μM) and 2'5'DDA (10μM) also reduced vaginal wall tone in the current study without inhibiting non-nitrergic NANC relaxation responses (n/N=2/1 for both). However 2'3'-DDA was not able to inhibit forskolin-induced relaxation responses. Therefore I concluded that MDL-12,330A, SQ 22536, 2'3'-DDA and 2'5'DDA are not suitable compounds to study the role of adenylate cyclase in the rabbit vaginal wall.

One mechanism by which VIP and PACAP-38 can induce relaxation responses involves activation of K^+ channels. It has been shown that VIP- and PACAP-induced relaxations are charybdotoxin- and apamin-sensitive respectively in rat colon (Kishi *et al.*, 1996). Furthermore the charybdotoxin-sensitive, VIP-induced relaxations are associated with increased cAMP and are reduced by an inhibitor of cAMP-dependent protein kinase (Kishi *et al.*, 2000). Thus the increase in cAMP induced by these peptides may induce smooth muscle relaxation via a mechanism involving activation of K^+ channels by cAMP-dependent protein kinase. In the current study however I have shown that EFS-induced non-nitrergic relaxations are charybdotoxin- and apamin-insensitive. Thus, if these pathways are involved in responses evoked by exogenous VIP, my results strongly suggest that these pathways are not involved in EFS-induced non-nitrergic relaxations.

f) Effect of CGRP and substance P

It has previously been reported that the human vagina stains positively for the peptides CGRP and substance P (Hoyle *et al.*, 1996). In this study I have demonstrated that CGRP and substance P did not cause any relaxation responses in the rabbit vaginal wall at concentrations at which they produced relaxation and contraction respectively in the clitoral corpus cavernosum. This suggests that they do not have a functional role as NANC neurotransmitters in the rabbit vaginal wall.

In tissues in which CGRP or substance P have been implicated as mediators of NANC relaxation responses the peptides are thought to originate from sensory neurons. In this study I found that capsaicin (1μM) had no relaxant effect on rabbit vaginal wall strips and also had no effect on non-nitrergic NANC relaxations (data not shown due to low number of experiments performed; n/N=4/2). Capsaicin (1μM) treatment of rabbit, rat and guinea-pig cerebral arteries has previously been shown to deplete CGRP-immunoreactivity from neurons (Saito & Goto, 1986). Furthermore acute capsaicin treatment in the guinea-pig right atrium has been shown to mimic the effects of electrically stimulated neurogenic NANC responses, whilst following capsaicin treatment NANC responses were abolished as was CGRP-immunoreactivity (Saito *et al.*, 1986). It has also been shown that substance P- and CGRP-mediated relaxations in rat intrapulmonary bronchi can be blocked by capsaicin. Furthermore in this tissue substance P and CGRP stimulated their relaxation responses in this tissue by stimulating prostaglandin synthesis in the epithelium, and it is the prostaglandin that is the ultimate mediator of these relaxations (Szarek et al., 1998). The lack of effect of capsaicin in my study adds further support to the conclusion that CGRP and substance P do not have a role in NANC relaxation responses in the rabbit vaginal wall, and furthermore suggests that sensory neurons are not involved.

CGRP has recently been shown to cause relaxation in the rat vagina (Giraldi *et al.*, 2001). In this study I have also shown that CGRP can induce relaxations in rat vaginal wall strips, but have found CGRP to be unable to cause relaxations in the rabbit vagina. It is possible that this is due to absence of CGRP receptors in the rabbit vagina,

which requires further investigation. Alternatively it is possible that in the rabbit vaginal wall CGRP and substance P are released by sensory neurons, but have no effect in my experiments since their mechanism of action may be via stimulation of prostaglandin synthesis as in the rat intrapulmonary bronchi. In all my experiments the modified Krebs solution contained the cyclooxygenase inhibitor indomethacin, which would prevent any prostaglandin synthesis, and this may be the reason why I saw no effect of CGRP or substance P. If this were the case then it would still imply a species difference between the rabbit and rat since indomethacin was present in my experiments with rat vaginal wall, suggesting that this is not the mechanism by which CGRP induces relaxation responses in this tissue. Further experiments in the absence of indomethacin should determine whether this is the case in the rabbit vaginal wall, but even if CGRP and substance P are able to induce prostaglandin synthesis they are still not involved in the non-nitrergic NANC relaxation responses described in this thesis.

g) Differences between peptide-induced responses in vaginal wall strips from rabbit and rat

VIP causes relaxation in the rabbit vagina (this study and Berman *et al.*, 1999a) however in the rat it has been reported not to induce relaxations (Giraldi *et al.*, 2001). Surprisingly I have observed VIP-induced relaxations in the rat vagina (data not shown due to low number of animals used; n/N=7/3). This discrepancy may be due to degradation of VIP in the study of Giraldi and colleagues, or due to differences in the responsiveness between our experimental models or the sensitivity between our

experimental apparatus. Thus, there seems to be a species-difference in the vaginal smooth muscle between rat and rabbit in respect to responses to CGRP, and possibly also VIP. Species specific responses to CGRP have been reported previously when it was found that CGRP increases the beat rate in rat isolated perfused heart, but not in the rabbit heart (Marshall *et al.*, 1986).

It should be noted that intravenous infusion of VIP in (pre-menopausal) humans has been shown to increase vaginal blood flow and lubrication (Ottesen *et al.*, 1987), which is likely to occur due to VIP-induced relaxation of the smooth muscle in the vascular and non-vascular structures of the vagina. However as yet no *in vitro* pharmacological studies have shown that VIP or CGRP induce a relaxation response in human vaginal smooth muscle. Further studies with human vaginal tissue should clarify which species most resembles human tissue.

In my experiments I used circular and longitudinal strips from both upper and lower parts of the rabbit vaginal wall, and achieved similar results in tissues from all regions. This may suggest that non-nitrergic NANC neurotransmission is important in all regions of the rabbit vaginal canal. In the rabbit stimulation of the pelvic nerves causes lengthening of the vaginal canal, as well decreasing the luminal pressure suggesting that longitudinal muscle is important in the responses (Park *et al.*, 1997). Therefore I mainly used longitudinal strips in this study.

h) Other peptides that may be involved in mediating NANC responses

In other experiments I tested the effects of various peptides when applied exogenously in an attempt to identify a possible candidate for the mediator of non-nitrergic NANC relaxation responses in the rabbit longitudinal vaginal wall. I found that [Arg^8]vasopressin (1μM) induced a contractile response in phenylephrine-precontracted longitudinal rabbit vaginal wall strips in the presence of L-NAME (data not shown due to low number of experiments; n/N=2/1). Neuropeptide Y (100nM) also induced a contractile response in the presence or absence of L-NAME (not shown since n=1) suggesting that neither of these peptides are likely to be involved in mediating relaxant responses in this tissue.

i) Summary

In this study the frequency-dependence of the non-nitrergic component of the NANC relaxation responses suggested a possible role for neuropeptides in mediating NANC relaxation responses. Also, circumstantial evidence from previous studies in humans suggested a possible role for VIP (or related peptides) in mediating the sexual response since sexual arousal is accompanied by increased plasma VIP, and injection of VIP induces vaginal lubrication.

This study provides evidence that VIP-related peptides are not mediators of the non-nitrergic NANC relaxation response since:

- Peptide-, but not EFS-induced responses were inhibited by α-chymotrypsion.
- Peptide-, but not EFS-induced responses were associated with increased intracellular cAMP content.
- NANC responses were not inhibited by apamin and charybdotoxin.

In this study I have also shown that CGRP, Substance P, NPY and [Arg^8]vasopressin are not mediators of the non-nitrergic NANC relaxation responses in the rabbit vaginal wall since none of these peptides induce relaxation responses when applied exogenously.

IV: 4 ROLE OF PURINES AND PYRIMIDINES AS MEDIATORS OF NANC RELAXATION RESPONSES IN THE VAGINAL WALL

ATP has been proposed as a NANC neurotransmitter candidate (Burnstock, 1972), and along with other purines and pyrimidines is generally accepted as a signalling molecule mediating diverse biological effects in many if not all organs of the body (for review see Ralevic & Burnstock, 1998). I decided to investigate the possibility that these may be involved in non-nitrergic NANC relaxations in the rabbit vaginal wall.

a) Effect of exogenous application of purines and pyrimidines

In this study I have demonstrated that purines (ATP, ADP and adenosine) and pyrimidines (UTP and, to a lesser extent UDP) are able to concentration-dependently induce relaxation responses in rabbit vaginal smooth muscle. However the concentrations (0.3 – 10mM) required to induce these relaxations were far in excess of those required to activate P1 or P2 receptors when expressed in a variety of cell types. For example 1μM adenosine induced maximal A_1 receptor-mediated contractions in dispersed cat esophageal smooth muscle cells (Shim *et al.*, 2002). ATP, UTP and UDP have been shown to stimulate inositol trisphosphate (IP_3) production in 1321N1 human astrocytoma cells (which do not express any endogenous P2 receptors) transfected with $P2Y_4$ receptors at concentrations as low as 10μM, with maximal responses observed at 100μM (Communi *et al.*, 1996a). ADP was maximally effective at stimulating potassium

channel-dependent currents and inhibited adenylate cyclase at 10μM via $P2Y_{12}$ receptors expressed in *Xenopus* oocytes (Hollopeter *et al.*, 2001).

There are a number of possible explanations to explain the discrepancy between the concentrations of purines and pyrimidines required to induce responses in this study and those required to activate recombinant receptors expressed in transfected cells, or endogenous receptors in dispersed cells. Firstly it is possible that transfected cells have far greater expression levels of receptors than those in the vaginal wall, and this may enable relatively small concentrations of agonists to have far greater effects than they would in cells that express the receptors endogenously. Indeed the concentrations of agonists required to induce relaxations in this study are similar to those in other studies on the effects of purines and pyrimidines in isolated tissue strips. EFS-induced contractions of isolated lobes of the rat prostate gland were inhibited by 10mM adenosine (Preston *et al.*, 2000) and this concentration was also required for maximal adenosine-mediated inhibition of phenylephrine-induced tone in rat epididymal smooth muscle (Haynes, 2000). In lamb coronary small arteries maximal inhibition of U46619-induced tone was achieved by 1mM ATP (Simonsen *et al.*, 1997). In marmoset urinary bladder strips ATP induced contractile and relaxation responses, however in this tissue the relaxant responses had not plateaued at 10mM ATP (McMurray *et al.*, 1998). ATP was found to induce relaxation responses in phenylephrine-precontracted human and rabbit penile corpus cavernosum strips that were maximal at 3mM ATP in the human strips, but that had not plateaued at 3mM in the rabbit strips (Filippi *et al.*, 1999).

Whilst the effective concentrations of agonists used in this study were similar to those in other studies there are other possible explanations for the high concentrations used compared to those required to activate P1 and P2 receptors in transfected cells and dispersed cells. In this study I used physiological agonists rather than metabolically stable analogues and have made no attempt to ensure that the agonists used were in their intended state. The vaginal wall strips have unknown ectonucleotidase activity which may have converted nucleotide triphospates to diphosphates, and such ectonucleotidase activity may be absent in single cell preparations. As well as this commercially available nucleotides often contain other nucleotides as by products.

The relaxant responses mediated by P1 or P2 receptors were not due to an indirect action on the smooth muscle via stimulation of intrinsic nerves leading to release of a secondary transmitter causing the smooth muscle to relax since inhibition of neurotransmission with the sodium channel blocker TTX in combination with the N-type VOCC inhibitor ω-CTX did not affect relaxation responses to adenosine or ATP. A TTX-resistant relaxant effect of ATP has previously been shown in the marmoset urinary bladder, and this was suggested as evidence that the ATP-induced relaxation was mediated via receptors on the smooth muscle cells (McMurray et al., 1998).

b) Involvement of P1 receptors in relaxation responses

The role of P1 receptors in mediating adenosine-induced relaxations was studied using P1 antagonists. Studies using the A_{2A} adenosine receptor antagonist ZM-241385

suggested that the adenosine-induced relaxations were at least partially mediated by these receptors since incubation of vaginal wall strips with 30μM ZM-241385 significantly reduced relaxation responses, with relaxation responses to 3mM adenosine reduced to about 35% of control responses and relaxations induced by 500μM adenosine abolished. As well as inhibiting adenosine-induced responses ZM-241385 also reduced 500μM ATP-induced relaxations to about 60% of control responses suggesting that metabolism of ATP to adenosine is likely to play a part in ATP-induced relaxation responses. ZM-241385 caused a slight decrease in tone, but did not inhibit the EFS-induced relaxation responses, suggesting that the EFS-induced relaxations are not mediated by adenosine. Thus whilst my experiments suggest that A_{2A} receptors exist in the rabbit vaginal wall, and are involved in mediating the relaxations induced by exogenous adenosine and ATP, they do not seem to be involved in mediating NANC relaxation responses.

The non-selective P1 antagonist 8-PT was found not to significantly reduce 500μM adenosine-induced relaxations with about 85% of control responses remaining following incubation with 8-PT (100μM), whilst 500μM ATP-induced relaxations were about 100% of control responses following incubation with 8-PT. EFS-induced NANC relaxations were not affected by this antagonist. The fact that 8-PT did not significantly reduce adenosine-induced responses in this tissue suggests that this compound is not a suitable tool for the investigation of the involvement of P1 receptors in responses in the rabbit vaginal wall.

Adenosine has been shown to induce contractile responses in the guinea-pig myometrium and cat esophageal smooth muscle cells via A_1 receptors (Smith *et al.*, 1988; Shim *et al.*, 2002). In the current study 40 minutes incubation with A_1 antagonist DPCPX (100nM) was found to have no effect on 500μM adenosine-, 500μM ATP- or 5Hz EFS-induced relaxation responses (data not shown due to low number of strips tested; n/N=2/1 for adenosine- and ATP-induced relaxations and n/N=4/1 for EFS-induced relaxations). This suggests that A_1 receptors are not involved in mediating adenosine, ATP or NANC relaxation responses, although this was expected since A_1 receptors are known to mediate inhibition rather than activation of adenylate cyclase as discussed in the introduction.

c) Involvement of P2 receptors in relaxation responses

In this study, ATP-induced relaxations were reduced by the P2 receptor antagonist cibacron blue, but enhanced by another P2 receptor antagonist, suramin. P2 receptors have been subclassified into P2X receptors and P2Y receptors. Purine and pyrimidine nucleotides have been shown to cause both contractile and relaxant effects in smooth muscle from various tissues in many different species. ATP-induced contractions are mainly mediated by P2X receptors, and ATP-induced relaxations are mainly mediated by P2Y receptors. However there are exceptions to this general rule. In the rat pylorus and ileum strips ATP and α, β-methylene ATP (α, β-meATP) induce relaxation responses that are inhibited by PPADS, suggesting that they are mediated by P2X receptors (Ishiguchi *et al.*, 2000; Storr *et al.*, 2000a). P2X receptors are activated by ATP and α, β-

meATP, but not by ADP, UTP or UDP. The ability of all the purine and pyrimidine nucleotides to induce relaxation responses, as well as the lack of effect of PPADS in the rabbit vaginal wall suggests the involvement of P2Y receptors in these responses.

To date seven mammalian P2Y receptors have been cloned and characterised: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ (Ralevic & Burnstock, 1998; Hollopeter *et al.*, 2001; Communi *et al.*, 2001). Three other P2Y receptors have been cloned from non-mammalian sources but these may be orthologues of mammalian receptors. The mammalian P2Y receptors have been reported to have a different selectivity for purines and pyrimidines as well as different selectivities for nucleotide di- and triphosphates. $P2Y_1$ receptors are selective for adenine nucleotides, and $P2Y_{11}$ receptors are selective for ATP (Nicholas *et al.*, 1996; Communi *et al.*, 1997). $P2Y_2$ receptors are activated by both purines and pyrimidines, but are selective for triphosphate nucleotides, and it has been shown that UDP and ADP do not activate this receptor (Nicholas *et al.*, 1996). $P2Y_4$ receptors show great species variability. The human $P2Y_4$ receptor shows strong selectivity for UTP over ATP, whilst the rat and mouse $P2Y_4$ receptors are activated equally by the nucleotides (Nicholas *et al.*, 1996; Bogdanov *et al.*, 1998; Lazarowski *et al.*, 2001). However, neither ADP nor UDP activate this receptor. $P2Y_6$ receptors, on the other hand, respond preferentially to the nucleoside diphosphate UDP, whilst ADP, ATP and UTP are less effective (Nicholas *et al.*, 1996; Communi *et al.*, 1996b). $P2Y_{12}$ receptors are selectively activated by ADP, but are only found in platelets (Hollopeter *et al.*, 2001). $P2Y_{13}$ receptors have a wide distribution, and whilst

they are predominately expressed in the brain and spleen, they are also present in the uterus (Communi *et al.*, 2001).

As well as having different agonist potencies the P2Y receptors have different affinities for antagonists. For example $P2Y_2$ receptors have been reported to be sensitive to suramin (Charlton *et al.*, 1996), whilst $P2Y_4$ receptors are suramin-insensitive (Communi *et al.*, 1996a). Cibacron blue has greater antagonist potency than suramin at the $P2Y_6$ receptor (Robaye *et al.*, 1997).

None of the receptor subtypes have a profile that matches the responses seen in the rabbit vaginal wall. It is possible that a new subtype of P2Y receptor is present in this tissue, but far more probable that co-expression of multiple types of receptor is responsible for the pharmacological profile seen. In this case, a combination of $P2Y_4$ and $P2Y_6$ may account for my observed results. $P2Y_4$ is probably present as this would account for the efficacy of cibacron blue, and the activity of ATP and UTP. $P2Y_6$ is also likely to be present as this would account for the activity of ADP and UDP, as well as further supporting the greater antagonist potency of cibacron blue over suramin.

Whilst the differing sensitivity of P2Y receptors to cibacron blue and suramin may explain the lack of inhibition observed with suramin, it does not explain the fact that suramin potentiated relaxation responses to ATP. It is possible that the smooth muscle of the vaginal wall has both P2X and P2Y receptors, and that exogenous application of ATP causes conflicting signals to the muscle to both contract and relax, although only the

dominant relaxation response is observed. If this were the case then suramin may enhance the relaxation responses by inhibition of the P2X component. However this explanation for the effect of suramin is unlikely since incubation of rabbit longitudinal vaginal wall strips with the P2X-selective antagonist PPADS (50μM) did not affect relaxation responses induced by 500μM ATP or EFS. If P2X receptors were present and inhibition of these receptors was the mechanism by which suramin was enhancing relaxation responses then PPADS would be expected to enhance the responses.

There is also another explanation for suramin enhancing the responses, which is that suramin has been reported to be an ectoATPase inhibitor at concentrations below that used in this study (Crack *et al.*, 1994; Yegutkin & Burnstock, 2000). If it is acting in this way in this system, the responses could be enhanced by an increase in the amount of agonist reaching the receptors. A high ectoATPase activity in the vaginal wall is also suggested by the high concentrations of nucleotides required to induce relaxations. Whatever the reason for these differences, the lack of effect of cibacron blue and suramin on EFS-induced NANC relaxations suggests that these relaxations are not mediated by nucleotides acting at P2Y receptors.

d) Mechanisms involved in purine- and pyrimidine-induced relaxations

P1 and P2Y receptors are G-protein coupled receptors. Adenosine receptors are coupled to adenylate cyclase and A_{2A} receptors are thought to induce relaxations via production of cAMP (Haynes, 2000). All P2Y receptors are G protein-coupled receptors,

and have been shown to couple to various signal transduction pathways including adenylate cyclase (Communi *et al.*, 1997; King *et al.*, 2000), phospholipase C (Communi *et al.*, 1997), Rho-dependent kinase (Sauzeau *et al.*, 2000) and MAP kinase pathways (Sellers *et al.*, 2001). In this study both ATP and adenosine increased cAMP levels at 10mM, suggesting that this the second messenger pathway stimulated by these agonists to induce relaxation involves stimulation of adenylate cyclase. However 500μM adenosine or ATP failed to significantly increase intracellular cAMP levels. This may suggest that increasing intracellular cAMP is only partially responsible for mediating purine-induced relaxations. Another explanation for this is that low concentrations of purines that induced submaximal relaxations produced increases in intracellular cAMP, but that these increases were too small to be detected by my assay. This could be explained by these increases only occurring in localized areas of the tissue, making them undetectable when analysing changes in intracellular cAMP in the whole tissue. Whatever the explanation for the lack of increase in cAMP observed with submaximal concentrations of purines, the increases induced by 10mM ATP or adenosine suggest involvement of adenylate cyclase in these relaxations. EFS-induced nitrergic and non-nitrergic relaxations on the other hand were not associated with increased cAMP suggesting that neither ATP nor adenosine is involved in NANC relaxation responses in the vaginal wall.

As P2Y receptors are coupled to G proteins, GDPßS, which stabilizes G proteins in their inactive state, should inhibit responses mediated by these receptors. Indeed responses to ADP were significantly reduced, suggesting that these responses are

mediated by P2Y receptors. Surprisingly I found there to be no significant inhibition of the responses to ATP when using GDPßS. The concentration used (100μM) was chosen since it inhibited relaxation responses to 1mM ATP in the marmoset urinary bladder smooth muscle and the incubation time in this study (30min) is longer than that required to inhibit responses in the marmoset urinary bladder (20min) (McMurray *et al.*, 1998). The fact that I have shown that ATP-induced relaxations are partially mediated via P1 receptors, all of which are GPCRs, suggests that the lack of effect of GDPßS is not due to these responses being mediated by receptors that are not GPCRs.

ATP- and ADP-induced relaxations were also resistant to inhibition by pertussis toxin. Adenosine-induced vasodilatation in porcine coronary arterioles has been shown to be sensitive to inhibition by pertussis toxin (Hein & Kuo, 1999), however in this tissue adenosine-induced responses were associated with NO production, were inhibited by NOS inhibitors and were dependent on an intact endothelium as was inhibition by pertussis toxin, suggesting a different mechanism from adenosine-induced relaxations in rabbit vaginal wall strips. The inactivity of both these inhibitors may not indicate that the receptors responsible for the relaxation responses in this tissue are coupled to pertussis toxin-insensitive G proteins. Instead it may be that accessory proteins are present in this tissue that stabilize the receptor-G protein complex, making it insensitive to inhibition by these compounds as has been suggested to be the case in the rat hippocampus (van der Ploeg *et al.*, 1992).

e) Role of purine and pyrimidine receptors in the vaginal wall

One question that remains to be answered is what is the purpose of P1 and P2 receptors in vaginal wall tissue if these receptors are not involved in NANC responses. One possibility is that these receptors are involved in relaxation of the tissue in response to non-neuronal release of purines or pyrimidines. Mechanically induced ATP release has been reported in response to shear stress, stretching, and osmotic swelling, and changes in blood flow have been reported to induce release of ATP from endothelial cells (see Burnstock, 1999 for review). Also release of both adenosine and ATP have been reported from non-neural sites, in the rat vas deferens (Fredholm *et al.*, 1982), with ATP release presumed to be from smooth muscle following α_1-adrenoceptor stimulation (Bultmann & Starke, 2001).

Thus P1 and P2 receptors may have a role in modulating the physiological changes that occur in the vagina in response to noradrenergic stimulation, such as hastening the return of the tissue to basal tone following a noradrenergic contraction. They may be involved in potentiating relaxation responses in response to the NANC mediated increases in blood flow that accompany sexual arousal. They may also be involved in assisting the vaginal wall relaxation during the stretching involved in coitus or parturition. However since in this study I did not attempt to investigate these possibilities, I cannot make any conclusions about the physiological role of these receptors in this tissue.

f) Summary

This study has shown that adenosine, ATP, ADP, UTP and UDP are capable of inducing relaxation responses in the rabbit vaginal wall. It has also shown that these responses are not mediated by NO since they are resistant to inhibition by L-NAME.

Adenosine-induced relaxations were found to be mediated by A_{2A} receptors, and were associated with increases in cAMP. ATP-induced relaxations were also associated with increases in cAMP and were partly mediated by A_{2A} receptors, presumably following degradation to adenosine.

The portion of ATP-mediated relaxations not mediated by A_{2A} receptors were presumably mediated by P2Y receptors since:

- ADP and ATP were equi-effective at mediating responses
- UTP and UDP also induced relaxation responses
- ATP-induced relaxations were reduced by the general P2 receptor antagonist cibacron blue, but not by the P2X receptor antagonist PPADS

The purines and pyrimidines studied do not appear to have a role in mediating non-nitrergic NANC responses in the rabbit vaginal wall since non-nitrergic NANC responses were not affected by P1 or P2 antagonists, and were not associated with increases in intracellular cAMP content.

IV: 5 ATTEMPTS TO IDENTIFY THE MEDIATOR OF NON-NITRERGIC NANC RELAXANT RESPONSES

a) Bioassay

In 1964 a pioneer in the field of bioassay, Jack Gaddum said "the pharmacologist has been a 'jack of all trades' borrowing from physiology, biochemistry, pathology, microbiology and statistics – but he has developed one technique of his own, and that is the technique of bioassay" (Gaddum, 1964).

Bioassay is the technique of testing the effects of a substance by applying it to a tissue and observing the effects. Indeed the early work leading to the identification of nor-adrenaline as a neurotransmitter in the sympathetic nervous system involved taking supra-renal extract and applying it to various different smooth muscles (Lewandowsky, 1899). This was followed by the finding that the supra-renal extract contracts or relaxes different smooth muscles as does stimulation of their sympathetic nerve supply (Langley, 1901). An advance in bioassay techniques followed 20 years later when the first experiments were done in which solution was taken from next to a donor organ, and transferred to a recipient organ to observe the effect of transferred substances. In these experiments frog hearts were exposed to Ringer solution that had been in contact with another frog heart. It was found that Ringer solution taken during vagus nerve stimulation of the donor heart causes a decrease in the heart beat of the recipient heart that is blocked by the presence of atropine, whilst Ringer solution taken from the donor heart without stimulation has no effect (Loewi, 1921). This type of transfer bioassay was possible in

part due to the use of frogs, and cold Ringer solution, which slowed the breakdown of the substance released from the vagus nerve (ACh) by cholinesterases enough so that it could be detected.

The use of transfer bioassay in pharmacology greatly increased due to the experiments of Gaddum in the 1950s, when he developed the technique of superfusion, defining superfusion as the technique of suspending a tissue strip in air and keeping it in good condition by running a suitable solution over its surface (by analogy with perfusion in which the fluid runs through the tissue; Gaddum, 1953). This technique provided improved sensitivity of detection, allowing transfer bioassay experiments to be more easily performed with mammalian tissues. Using this technique he was able to investigate the release of an unknown substance from rat intestine that contracted rat uterus which he termed Substance R. Substance R remained unidentified, but with these experiments he was able to rule out various candidates for Substance R such as ACh and bradykinin, and showed that the Substance R was not broken down by trypsin or chymotrypsin (Gaddum, 1953).

Since these early experiments bioassay has proven its importance, particularly following the development of the cascade superfusion bioassay apparatus (Vane, 1964). It has been crucial in the discovery of prostacyclin (PGI_2; Moncada *et al.*, 1976), and in the identification of EDRF as NO (Palmer *et al.*, 1987).

One of the advantages of the use of these types of bioassay is that they provide a means of detecting substances within seconds to minutes of their generation or release and allow detection of unstable compounds that would be lost in other extraction procedures. Another that was important to this study is that they maximize the chances of serendipitous discovery of unknown substances, since with other techniques different methods need to be employed to identify different classes of compounds. Another advantage of bioassay is that the comparison between transferred responses and exogenously applied compounds is easier since the transferred substances are presented to the recipient tissue as exogenous compounds. This means that the time-course of responses would be expected to be the same between responses to exogenous compounds and transferred substances if the substances are identical, applied at equal concentrations and for the same duration. Furthermore if transferred substances and exogenously applied compounds are different then use of drugs that effect exogenous drug-induced responses, but not endogenously released substances provides strong evidence that the substances are different. This is because effects of endogenous release of substances may not be affected by drugs due to lack of accessibility to the sites at which they work. For example if studying the role of peptides as mediators of a response using α-chymotrypsin, inhibition by the peptidase indicates involvement of peptides in the response. However a lack of effect could be explained by lack of accessibility of the peptidase at sufficient concentrations to breakdown the peptides responsible for the response, rather than proving that peptides are not involved. However, in a bioassay experiment, if the peptides are released from a donor tissue they can be degraded by the peptidase on their journey

from donor to recipient tissue as with exogenous application of peptides. This allows the experimenter greater confidence in his conclusions based on the effect of the drug.

In order to better characterise the properties of the mediator of non-nitrergic NANC relaxations I wanted to set up a bioassay system to transfer the released mediator to a detector tissue. I used the same experimental apparatus used to show the existence of the non-nitrergic NANC relaxations since it allowed stimulation of the donor tissue, without stimulating the recipient tissue, whilst enabling the recipient tissue to be positioned in close proximity to the donor tissue. The use of this apparatus however does have disadvantages. In the superfusion bioassay systems discussed above the test solution is perfused through the organ or cells releasing the test substance, maximising the concentration of the substance present. In the apparatus used in this study the modified Krebs solution is not forced to pass through the donor tissue, but passes around it, with some of it passively entering and leaving the tissue, and it is probable that some of the solution never even comes into contact with the donor strip. Thus any released substances are not as concentrated as they might be in the fluid that passes the donor strip. Also one of the advantages of Gaddum's superfusion technique where sensitivity is increased by the solution containing the unknown substance completely replacing the fluid covering the tissue is lost since the perfusing modified Krebs solution containing any released substances is not forced into contact with the recipient strip by replacing existing fluid, and instead washes past the tissue strip, with only a proportion of the fluid entering the tissue. Thus the released substance is diluted and any effects of a released substance will be reduced.

One of the principles of bioassay is the differential sensitivity and effects of substances on tissues from different organs. In this study I used vaginal wall as both donor and detector organ since in the absence of knowledge about the identity of the mediator of non-nitrergic NANC relaxations this was the only organ that I knew could be relaxed by this substance.

My attempts at transferring the mediator of non-nitrergic NANC relaxations were unsuccessful, and there was also poor reproducibility of those experiments where L-NAME sensitive relaxations were observed. The dilution of released substances discussed above may account for the lack of reproducibility of these experiments. Successful bioassay of neurogenic NANC NO release has been shown from the canine ileocolonic junction and the rat gastric fundus (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991). Unsuccessful attempts have been made to assay substances responsible for non-nitrergic bradykinin-induced relaxations by bioassay in porcine coronary arteries (Kauser & Rubanyi, 1992) suggesting that either the mediator of these relaxations was non-diffusible or that it was extremely labile. In my experiments the mediator of the non-nitergic relaxations may also be either non-diffusible or extremely labile, however I cannot conclude this since my inability to reproducibly transfer NO suggests that my bioassay system lacked the required sensitivity for such a conclusion to be drawn.

Interestingly a relaxant response that was resistant to blockade of NO and cyclooxygenase pathways, K^+ channel and beta adrenoceptor blockade and that was not

associated with increases in cAMP or cGMP has been shown to be detectable by bioassay. Rat urinary bladders were found to release this substance that relaxed rat thoracic aorta strips in response to carbachol (Fovaeus *et al.*, 1999). Whilst the non-nitrergic response in the vaginal wall was non-cholinergic these relaxant responses have similar properties.

Attempts to improve the sensitivity of the bioassay system that result in reproducible transfer of NO may enable me to make conclusions about whether or not the mediator of the non-nitrergic response is detectable. Improved sensitivity could be achieved by using a cascade bioassay apparatus since this would reduce dilution of bioactive substances between donor and recipient tissue. Increasing the amount of donor tissue and designing a chamber in which perfusing Krebs perfused through the donor tissue would also increase the concentration of released bioactive substances reaching the recipient tissue. However such an apparatus has other associated problems such as how to stimulate the nerve fibres in the donor tissue, and how to perfuse the Krebs buffer through the tissue.

The unsuccessful attempts to set up a bioassay system for the mediator of non-nitrergic relaxations in the current study were consistent with the possibility that the responses were mediated by a neuropeptide, since bioassay of neuropeptide-induced responses has not been observed from tissues or whole organs. There are several explanations for this. Firstly neuropeptides are degraded within the synaptic space by

endopeptidases, making them highly labile. In my experiments addition of peptidase inhibitors did not allow us to transfer the substance mediating the relaxation response, but the lack of sensitivity of the bioassay apparatus used could explain this. Secondly whilst peptides may reach the concentrations required to activate their receptors within the synaptic space, even if they are released without being degraded they will be greatly diluted by entering the extracellular volume of the whole tissue, and the resulting concentrations may be too small to subsequently activate their receptors, even without the extra dilution involved in transfer from one tissue to another.

b) Analysis of perfusate

In order to test the theory that a peptide was released by EFS, but that due to the reasons discussed above it was not entering the perfusing Krebs solution in high enough concentrations to be detected by bioassay, I decided to try and detect released peptide by another means. Perfusing Krebs solution was collected from adjacent to the tissue at rest and during stimulation. This was then desalted, and lyophilised before being resuspended in 1ml sample buffer. This step was to concentrate any peptides in the samples by a factor of 5. All of the neuropeptides that have been shown to be present in the vaginal wall have low molecular weights, with substance P having a molecular weight of 1347, VIP having a molecular weight of 3346, and CGRP having a molecular weight of 3807. The samples were run on 10-20% Tris-Tricine ready gels since these gels are reported by the manufacturer to be ideal for separating small molecular weight proteins, with molecular weights as low as 1000. Once the gels had been run they were stained with coomassie

blue to detect any peptides or proteins in the gel. The gels revealed a protein in stimulated samples that had a molecular weight of ~15kd, and that was not present in unstimulated samples. Coomassie blue has been reported to be able to detect levels of protein as low as 0.1-0.5μg (Spector, 1978). Silver staining of in-gel proteins has been reported to be able to detect protein levels of as low as 1ng, and I used this technique in a preliminary experiment to stain a single gel to determine if other protein bands were present at levels that could not be detected by coomassie blue (not shown). However this technique revealed only the same band at ~15kd that was observed in gels stained with coomassie blue. Since coomassie blue staining is simpler, less time consuming, and cheaper, I used coomassie blue staining for all subsequent gels.

The protein detected in stimulated samples was far larger than any of the candidate neuropeptides discussed in this thesis and sure enough analysis by MALDI-TOF MS revealed the band to be made up of proteins corresponding to the rabbit haemoglobin α1, α2, β1 and β2 chains. These proteins are obviously not signalling molecules, and they may have been present in the tissue due to ruptured blood cells within the tissue sample. Their presence in stimulated samples, but not those taken when the tissue was at rest may be due to muscle movement caused by stimulation dislodging the proteins, and causing them to be released into the perfusing Krebs solution.

Using this technique I was therefore unable to detect release of any neuropeptides from the vaginal wall strips. One explanation for this is that neuropeptides are not released from this tissue by EFS, and indeed I have found no evidence to suggest that

they are. However my experiments do not allow me to conclude that no neuropeptides are released since it is possible that they are released, but are extremely labile as discussed above. Alternatively the detection limit used in these experiments may still be too low to detect any released peptides. Future experiments could be done to attempt to increase possible peptide release. For example peptidase inhibitors could be included in the Krebs solution to reduce any peptide degradation. Also increasing the amount of tissue stimulated would increase the amount of peptide released. Larger tissue samples could not be used in the current experiments due to the size of the chamber in which the strips were stimulated.

Another way of improving the detection limit would be to improve the concentrating step. In the current study the maximum concentration of peptide achieved in the sample buffer would have been only 5 times the concentration in the perfusing Krebs solution. If peptides were present the actual concentration may have been lower since some peptide may have been lost due to adsorption to the chamber, and tubes used in desalting, and lyophilization steps. In another study neuropeptide release from the isolated, perfused dog adrenal medulla was detected following a concentration step where the perfusate was passed over C-18 reverse phase cartridges (Chritton *et al.*, 1997). Using this technique released neuropeptides absorb to the cartridge, and are later released by an eluting step. This technique removes the need to desalt samples, and large volumes of perfusate can be passed through the cartridges, effectively filtering out and concentrating any peptides.

c) Summary

In this study I have been unsuccessful in setting up a bioassay system for measuring release of the mediators of NANC relaxation responses from the rabbit vaginal wall.

I have also been unable to detect the presence of any neuropeptides in the perfusate following stimulation of vaginal wall strips.

Thus these preliminary experiments have not led to the identification of the mediator of non-nitrergic NANC responses. Refinement of both approaches used may lead to the future identification of this factor.

IV: 6 NANC RELAXATION RESPONSES IN OVARIECTOMIZED RABBIT VAGINAL WALL

The incidence of female sexual dysfunction is known to be significantly increased following menopause (Berman *et al.*, 1999b). Whilst the main effect of menopause on female sexual function is a decrease in desire, menopause is also associated with decreased arousal (Avis *et al.*, 2000). Since the physiological components of the female genital sexual response: vaginal wall engorgement, increased lubrication, and clitoral tumescence are all associated with changes in smooth muscle tone I compared contractile and relaxant properties in an animal model of menopause, the ovariectomized rabbit (Min *et al.*, 2001a; Yoon *et al.*, 2001).

a) Effects of ovarian hormones on NOS

The effects of menopause on sexual function are likely to be due to decreased production of ovarian steroid hormones, and the effect of hormone replacement following removal of the ovaries has been studied in various urogenital tract tissues from different species.

Oestrogen treatment has been shown to affect NOS transcription, expression and activity, however the effect of oestrogens varies between species and tissue. NOS (cytosolic, indicating nNOS) activity (as determined by citrulline assay) has been shown to be decreased in vaginal and uterine tissue in response to treatment with oestrogen in

ovariectomized rabbits (Batra & Al Hijji, 1998; Al Hijji *et al.*, 2000) and rats (Al Hijji *et al.*, 2001). On the other hand nNOS and eNOS transcription (determined by ribonuclease protection assay) and expression (determined by Western blotting) have been shown to be upregulated by oestrogen treatment in ovariectomized sheep myometrium (Zhang *et al.*, 1999).

These studies suggest that NOS activity is decreased by oestrogen, whilst expression is increased, however it is difficult to make any general conclusions about the effects of oestrogen and progesterone in the above studies, since methods of introducing the steroid hormones varied considerably between studies, as did the form of injected hormone for example in one study a single intramuscular injection of polyestradiol phosphate given 7 days before tissue removal was used as oestrogen treatment in rabbits (Al Hijji *et al.*, 2000), whilst in another study daily intravenous injections of estradiol-17β for 5 or 8 days was used for oestrogen treatment in sheep (Zhang *et al.*, 1999). Also all these studies have compared the effects of oestrogen or progesterone treatment of ovariectomized animals with untreated, ovariectomized control animals. This has the advantage that it allows the effects of the steroid hormones to be investigated in isolation, but has the disadvantage that supramaximal physiological concentrations of hormones are achieved with these treatments and so the effects of the hormones may differ from those due to physiological ovarian hormone release. In the present study I looked at changes between normal animals and ovariectomized animals. The advantage of this is that the control animals will have normal oestrogen and progesterone levels. The obvious downside to this is that any differences observed in ovariectomized animals due to

decrease in hormone levels cannot be attributed to the effects of a specific hormone. The problem of comparing ovariectomized controls with ovariectomized animals with hormone replacement is highlighted by a study in which eNOS and nNOS expression (as determined by immunohistochemical analysis) were studied in the rat vagina from control rats with intact ovaries, ovariectomized rats and ovariectomized rats following oestrogen replacement in which eNOS and nNOS expression were significantly greater in vaginal tissue from ovariectomized animals after oestrogen replacement than in tissue from untreated ovariectomized animals. However vaginal tissue from both untreated and oestrogen treated ovariectomized rats showed levels of eNOS expression within the expression range seen during the oestrous cycle of intact rats, and nNOS expression was reduced in ovariectomized animals at 14 days post-op, but not at 7 days post-op (Berman *et al.*, 1998). In one study in which sham operated rabbits were compared with ovariectomized animals and ovariectomized animals following oestrogen replacement both NOS activity and expression were studied (Yoon *et al.*, 2001). In this study clitoral and vaginal smooth muscle from ovariectomized animals were found to have greater NOS activity (determined by nitrite and nitrate concentrations) than sham operated animals, which had similar levels to the oestrogen-replacement group (Yoon *et al.*, 2001), thus supporting the idea that oestrogen decreases NOS activity. However in this study eNOS and nNOS expression (determined by Western blotting) were also elevated in vaginal and clitoral tissue from ovariectomized animals compared to control and oestrogen replacement groups, thus disagreeing with the findings of Zhang (Zhang *et al.*, 1999) and Berman (Berman *et al.*, 1998).

Another ovarian steroid hormone progesterone may also have functional effects on urogenital smooth muscle. In ovariectomized rabbits nNOS activity has been shown to be downregulated in the uterine horn from animals treated with progesterone as with oestrogen treatment, whilst vaginal nNOS activity was enhanced compared to untreated ovariectomized control animals (Al Hijji *et al.*, 2000). In ovariectomized rats on the other hand progesterone treatment had no significant effect on either eNOS or nNOS activity in the uterus or the vagina when compared to untreated ovariectomized control animals (Al Hijji *et al.*, 2001). These suggest that the effects of progesterone are both tissue and species specific.

b) Effect of ovariectomy on smooth muscle

As well as changes in NOS activity or expression previous studies have also shown removal of the ovaries to affect the structure of urogenital organs. Histological examination of the rabbit vagina has shown that there was thinning of the epithelial layers, decreased submucosal microvasculature, and diffuse fibrosis whilst in the clitoral corpus cavernosum there was a decrease in the percentage of smooth muscle from ovariectomized animals compared to intact animals or ovariectomized animals with (supramaximal) oestrogen replacement (Park *et al.*, 2001). In another study smooth muscle content was decreased and collagen content increased in the ovariectomized rabbit vagina and clitoral corpus cavernosum compared to sham operated animals (Yoon *et al.*, 2001). Interestingly (supramaximal) oestrogen replacement increased smooth muscle content of the vagina and clitoris to 160% and 200% of that in sham operated

animals, and reduced collagen content to 50% and 30% respectively (Yoon *et al.*, 2001). Also a study on the effects of ovariectomy on the rat vagina showed increased vaginal atrophy in ovariectomized animals 14 days post op that was reversed by 3 days oestrogen treatment (Berman *et al.*, 1998). In the current study I did not find there to be any difference in contractility of longitudinal vaginal wall strips or clitoral corpus cavernosum strips from control or ovariectomized animals 24 weeks post-op suggesting that removal of ovaries did not affect contractile function in these tissues. It is possible that structural changes occurred but that these did not affect the contractile responsiveness in these tissues. However I did not perform histological examinations on tissue strips and so cannot make any conclusions about any structural changes that may have occurred as a result of ovary removal.

Frequency dependence of NANC relaxation responses in vaginal wall strips differed between strips from control and ovariectomized animals with maximal responses observed at lower frequencies in strips from ovariectomized animals. Different stimulation frequencies may affect the type of neurotransmitters released from neurons with NO release observed at low frequency stimulation and peptide release occurring at higher frequency stimulation. The change in the frequency-response profile suggests that removal of the ovarian tissue changes the balance of neurotransmitters responsible for mediating the NANC relaxation response in this tissue. In the clitoral corpus cavernosum strips the frequency-dependent relaxation responses were similar in strips from control and ovariectomized animals. Since NO appears to be the sole neurotransmitter

responsible for mediating NANC relaxation responses in this tissue this is expected since there is no balance of different mediators to affect.

I examined the effects of L-NAME and L-NA on the frequency dependent NANC relaxations in both longitudinal vaginal wall and clitoral corpus cavernosum strips to determine if there was a difference between ovariectomized animals and intact animals. I found that both L-NAME and L-NA caused partial inhibition of NANC relaxation responses in the vaginal wall from ovariectomized animals, as with intact animals. With L-NAME I found there to be no significant difference in the degree of inhibition observed at any stimulation frequency or concentration of inhibitor used between inhibition in strips from intact or ovariectomized animals. However, with strips from ovariectomized animals the profile of the concentration response curves at 2.5–50Hz stimulation was always above that in control animals. When L-NA was used to inhibit NOS the inhibition was significantly greater in strips from control animals than ovariectomized animals at 2.5 – 50Hz stimulation. This may suggest that NO has a smaller role in mediating NANC relaxation responses in vaginal wall strips from ovariectomized animals, and a corresponding increase in the role of the mediator of non-nitrergic NANC relaxations. If this were the case then I would expect there to be a greater degree of inhibition at the highest concentrations of NOS inhibitor in intact animals than control animals, however no significant differences were observed. The lack of significance between responses observed at the highest concentrations of NOS inhibitors may not mean that this is not the case since the NO-mediated responses are not the major component of the NANC relaxations elicited by 2.5–50Hz EFS in control animals, thus

any reductions in this already minor component will be more difficult to observe. If I take as an example the 5Hz EFS-induced responses at the maximal concentration of L-NA, the mean responses remaining are 58% and 74% of control responses in control and ovariectomized animals respectively. With the variation between tissue strips seen in these experiments, for there to be significantly less inhibition in ovariectomized animals a mean value of 83% would be required with the same number of animals used, a difference of 25%. Increasing the number of animals may result in a reduced error, enabling any differences to be seen; however since no great differences were observed in these preliminary experiments, no further experiments have been done as yet. In the absence of further experiments then I cannot conclude that NO plays a smaller role in mediating NANC relaxation responses in the rabbit vaginal wall following ovariectomy, but the data I have suggests that this may be the case.

In clitoral corpus cavernosum strips from ovariectomized and intact rabbits NANC responses elicited by 1Hz stimulation were completely inhibited, with similar inhibition profiles, by L-NAME and L-NA, suggesting that ovariectomy has no effect on these responses. At higher frequency stimulation a difference in inhibition profile was seen with significantly different curves for inhibition by L-NAME of 2.5 – 50Hz EFS-induced relaxations, and significantly, or almost significantly different curves for inhibition by L-NA of 5 – 50Hz EFS-induced relaxations. Furthermore, at 100μM L-NAME, inhibition of 2.5 – 25Hz EFS-induced responses was significantly greater in clitoral corpus cavernosum strips from intact animals than from ovariectomized animals. If NO is the sole mediator of NANC relaxations in this tissue then the difference in

responses between control and ovariectomized animals is difficult to explain. It could be that NO is responsible for the great majority of the responses in control animals and that another mediator of NANC relaxations begins to be expressed following removal of the ovarian hormones. Another possibility could be that the loss of oestrogen in some way sensitises the relaxant pathways, so that a small amount of NO released in the presence of the NOS inhibitors has greater effect.

Another possible explanation for the difference in inhibition profiles observed in vaginal wall and clitoral corpus cavernosum strips from ovariectomized and control animals could be that the loss of sex hormones in ovariectomized animals influences L-arginine metabolism. L-arginine is synthesised by arginosuccinate synthase and can be broken down by the enzyme arginase (see Wu & Morris, 1998 for review). If in ovariectomized animals there is an increase in arginosuccinate synthase and/or a decrease in arginase expression or activity then the intracellular concentration of L-arginine would be higher. Indeed it has been shown that in ovariectomized rabbits oestrogen treatment leads to an increase in arginase activity in the vaginal wall (Min *et al.*, 2001b). Since L-NAME and L-NA compete with L-arginine to inhibit NOS, increased L-arginine concentrations would shift the inhibition curves to the right. Whilst I did not examine L-arginine concentrations in the current study there was a rightward shift of the inhibition curves to L-NAME and L-NA in both vaginal wall and clitoral corpus cavernosum strips from experiments with ovariectomised animals compared to control. Thus ovariectomy may increase L-arginine concentrations in the vaginal wall and the clitoral corpus

cavernosum, possibly by altering arginase expression or activity, however, this conclusion cannot be drawn without further investigation.

It has previously been shown that in postmenopausal women intravenous injection of VIP has no effect on vaginal blood flow at the same concentration that causes increased blood flow and vaginal lubrication in pre-menopausal women, and further that this effect of VIP is restored in women receiving hormone replacement therapy, suggesting that loss of ovarian hormones causes loss of VIP-receptor mediated responses (Palle *et al.*, 1991). If the ovariectomized rabbit models the changes that occur in humans then this would further suggest that the non-nitrergic NANC mediator is not VIP since if anything the non-nitrergic responses were enhanced in this study. Obviously without testing for the relaxant effect of VIP in vaginal tissue from ovariectomized animals I cannot make this conclusion. Further experiments to test for VIP-induced responses are therefore needed to find out how closely the ovariectomized rabbit models the menopausal human.

Whatever the explanation for the differences observed in NANC relaxation responses from control and ovariectomized animals, the differences are not great. NO is still only partially responsible for mediating NANC relaxation responses in the vaginal wall, with the major component probably the unidentified mediator that has been the main focus of this thesis. However I did not perform experiments to determine whether other neurotransmitters may be involved in the NANC relaxations in ovariectomized animals. Also in the clitoral corpus cavernosum whether another neurotransmitter

becomes involved in mediating NANC relaxations or the relaxant pathway becomes sensitised so that residual NO release after incubation with NOS inhibitors can still induce small relaxations, NO is still responsible for mediating the great majority of the responses in this tissue.

Should these results prove to be representative of the NANC responses that occur in the human clitoris and vagina, they have implications for the treatment of FSD in menopausal women. If FSD in a menopausal woman is due to an arousal disorder then similar treatment to that for arousal disorders in premenopausal women should be effective. However treatments based on enhancing the nitrergic pathway, such as use of PDE 5 inhibitors, may be marginally less effective.

c) Summary

This study has provided evidence that ovariectomy in the rabbit:

- Has no effect on the ability of either vaginal wall or clitoral corpus cavernosum to contract
- Causes a change in the frequency dependence of NANC relaxation responses in the vaginal wall, but not in the clitoral corpus cavernosum
- May cause a small, but not significant reduction in the role of NO as a mediator of NANC relaxation responses in both the vaginal wall and clitoral corpus cavernosum

These results suggest that some changes occur in the NANC responses in the vaginal wall or clitoral corpus cavernosum as a result of removing the ovaries. However these changes do not appear to have any great functional implications.

IV: 7 OTHER POSSIBLE MEDIATORS OF NON-NITRERGIC NANC RELAXANT RESPONSES

The evidence gathered in this study suggesting that VIP-related peptides, CGRP, substance P, purines or pyrimidines are not involved in non-nitrergic relaxations in the vaginal wall led me to consider other possible mediators of these responses.

a) Carbon monoxide

One possible candidate was another gaseous compound, carbon monoxide (CO), which has been suggested as a possible neural messenger and as a mediator of NANC relaxation responses in opossum internal anal sphincter smooth muscle (Verma *et al.*, 1993; Rattan & Chakder, 1993). CO can be generated by the enzyme heme oxygenase as a product of the breakdown of protoheme IX (Tenhunen *et al.*, 1968). CO generation by this enzyme has been shown to be inhibited *in vivo* and *in vitro* using the metalloporphyrin zinc (II) protoporphyrin IX (ZnPP; Hamori *et al.*, 1989; Vreman *et al.*, 1989). I made several attempts to use this inhibitor to see if it would affect NANC relaxation responses in rabbit longitudinal vaginal wall strips, however whilst ZnPP was soluble in a dilute alkaline solution, and remained in solution in the reagent reservoir due to the rapid mixing caused by bubbling the solution, the compound precipitated en route to the tissue strips, suggesting that this compound is not suitable for use in the horizontal perfusion chamber apparatus. Despite being unable to inhibit any potential CO synthesis in this study other evidence suggests that this compound is not the mediator of non-

nitrergic NANC relaxations in the rabbit vaginal wall. CO is thought to stimulate muscle relaxation by activation of sGC (Furchgott & Jothianandan, 1991; Verma *et al.*, 1993; Rattan & Chakder, 1993). This study has shown that NO mediated responses were mediated by sGC, and that inhibition of sGC by ODQ caused a similar degree of inhibition of NANC relaxation responses as that caused by NOS inhibition. If CO were involved in mediating NANC relaxation responses then the degree of inhibition by ODQ would be expected to be greater than that by NOS inhibitors. Also non-nitrergic relaxations were not associated with any changes in intracellular cGMP providing further evidence that CO is not a mediator of non-nitrergic relaxation responses in this tissue.

b) Histamine

Another possible candidate for the mediator of non-nitrergic NANC relaxations is histamine. Histamine has been shown to induce relaxation responses in the human arterial preparations either via H_1 receptors localized to the endothelium or via H_2 receptors on the smooth muscle (Van de Voorde *et al.*, 1998).

In the human internal mammary artery histamine-induced relaxations have been shown to be endothelium-dependent and are completely inhibited by inhibition of sGC or NOS, suggesting that the relaxant response is due to histamine-induced release of EDRF (NO) (Tsuda *et al.*, 2001). In the current study I cannot rule out endothelium-dependent responses, since the neurogenic mediator of non-nitrergic relaxations may act on the endothelium to induce release of a second substance that mediates the smooth muscle

response. In a single experiment (not shown) I exposed rabbit longitudinal vaginal wall strips to the detergent CHAPS (0.3% for 5 minutes), which is a concentration and duration commonly used to remove the endothelium from vascular smooth muscle preparations. In this experiment CHAPS had no effect on NANC relaxation responses, however I did not repeat this experiment and did not perform histological analysis of the strip to determine whether the endothelium had been removed. However I can rule out the possibility that histamine is involved in non-nitrergic NANC relaxations mediated via the endothelium since in histamine-induced endothelium dependent-relaxations the endothelium to smooth muscle signalling molecule is NO (Tsuda *et al.*, 2001).

Endothelium- and NO-independent smooth muscle relaxation by histamine has been reported in the rabbit penile dorsal artery (Martinez *et al.*, 2000). In this tissue histamine induced contractile responses, but in the presence of a H_1 receptor antagonist (mepyramine) histamine-induced contractions were inhibited and histamine-induced relaxation responses were revealed that were inhibited by incubation with a H_2 receptor antagonist (cimetidine). Also a H_2 receptor agonist (dimaprit) was found to mimic the relaxation responses to histamine. In this tissue the endothelium-independent relaxations were unaffected by L-NAME, indomethacin, apamin and charybdotoxin (Martinez *et al.*, 2000). Thus the H_2 receptor mediated histamine-induced relaxations in the rabbit penile dorsal artery show some similar characteristics to the non-nitrergic NANC relaxation responses in the rabbit vaginal wall. In the current study I found that histamine (0.1-100μM) induced concentration-dependent contractions in rabbit longitudinal vaginal wall strips (data not shown due to low number of experiments performed; n/N=2/1). However

I have not examined the effects of H_1 and H_2 receptor antagonists or agonists. Further experiments are required to investigate the possibility that histamine is the mediator of non-nitrergic NANC relaxations in the rabbit vaginal wall acting via H_2 receptors.

c) Cannabinoids

Another class of compounds that have been shown to induce relaxation responses in smooth muscle preparations are cannabinoids. Cannabinoids exert their effects via cannabinoid (CB) receptors, which are G protein-coupled receptors, and may induce vasodilatation by inhibition of neurotransmitter release by sympathetic neurons, by stimulating release of secondary transmitter substances from endothelial cells or by direct action on the smooth muscle (for review see Hillard, 2000). The CB1 receptor agonist arachidonylethanolamide (also known as anandamide) has been shown to induce endothelium-independent relaxation responses in rat coronary arteries with maximal relaxation observed with 1μM anandamide, whilst the CB2 agonist palmitoylethanolamide was without effect (White *et al.*, 2001). These anandamide-induced relaxations were abolished by the K^+ channel blockers tetraethylammonium (TEA, 1mM) or Iberiotoxin (IbTX, 50nM). In sheep coronary arteries anandamide also induced endothelium-dependent and -independent relaxation responses that were also resistant to L-NAME, but were reduced by indomethacin, TEA and IbTX (Grainger & Boachie-Ansah, 2001).

In the current study limited experiments were performed to determine the possible involvement of cannabinoids in non-nitrergic NANC relaxation responses. In two phenylephrine-precontracted longitudinal rabbit vaginal wall strips from a single animal anandamide (500nM) had no effect on the non-nitrergic NANC relaxation responses (not shown). This suggests that cannabinoids do not mediate relaxant responses in this tissue however the limited number of experiments mean that I cannot conclude that non-nitrergic NANC relaxant responses in the vaginal wall are not mediated by cannabinoids. However it is unlikely that cannabinoids mediate non-nitrergic NANC relaxation responses in the vaginal wall since apart from the fact that anandamide did not induce relaxant responses the non-nitrergic NANC relaxations were observed in the presence of indomethacin, and were also unaffected by TEA (1mM) or IbTX (300nM; data not shown due to low number of experiments performed; n/N=4/2 for TEA and n/N=2/1 for IbTX). Further experiments with anandamide and other cannabinoid receptor agonists and antagonists should give a clearer answer as to whether cannabinoids are involved in non-nitrergic NANC relaxation responses in the rabbit vaginal wall.

d) Opioids

A further class of compounds, the opioids, can produce relaxant effects, as well as various other effects in smooth muscle preparations. In rat oesophageal smooth muscle the endogenous μ-opioid agonists endomorphin-1 and –2 reduced neurogenic contractile responses, and this effect was reversed by the opioid antagonist naloxone (1μM) (Storr *et al.*, 2000b). The μ-opioid receptor agonist DAMGO has been shown to augment

isoproterenol and forskolin-induced relaxations in canine airway smooth muscle, which was reversible by naloxone (Tagaya *et al.*, 1995). The opioid agonist morphine has also been shown to augment noradrenaline-induced contractions in rat aortic smooth muscle, an effect that was also abolished by naloxone (Parra *et al.*, 1995). Naloxone has also been shown to enhance EFS-induced ACh and noradrenaline release (without altering contractile responses) and to enhance NANC relaxation responses in the guinea-pig colon (Marino *et al.*, 1993).

In this study phenylephrine-precontracted rabbit longitudinal vaginal wall strips were incubated with the opioid antagonists naltrexone (10μM) and naloxone (10μM) in the presence of L-NAME. I found that the neither antagonist affected EFS-induced non-nitrergic NANC relaxant responses (data not shown due to low number of experiments performed; n/N=4/1 for naltrexone and n/N=2/1 for naloxone). These preliminary experiments suggest that opioids are not involved in mediating non-nitrergic NANC relaxant responses in the vaginal wall, however as I did not investigate the effects of exogenously applied opioid agonists and as a limited number of experiments were performed with these antagonists, I cannot definitely conclude that non-nitrergic NANC relaxant responses in the vaginal wall are not mediated by opioids.

Interestingly an inhibitory role in sexual behaviour has been indicated for these compounds since the opioid antagonist naloxone has been shown to enhance consummatory sexual behaviour in castrated male Japanese Quails when applied centrally, but not peripherally (Riters *et al.*, 1999), and the opioid antagonist naltrexone

has been shown to increase arousal at orgasm and increase orgamic intensity (measured by subjective reports from participants) in human males (Sathe *et al.*, 2001), and to increase sexual receptivity (and food intake) in obese Zucker female rats (Marin-Bivens & Olster, 1999). Whilst these effects are likely to be due to the antagonism of effects of opioids on the central nervous system, a role in modulating peripheral responses has not been excluded. Whilst my preliminary data suggests that opioids do not have a role as mediators of NANC relaxations in the vaginal wall the modulatory effects of opioids in other smooth muscle preparations and their role in sexual behaviour suggest that further research into their effects on genital smooth muscle contractility may be warranted.

IV: 8 CONCLUSION

Female sexual dysfunction is a common disorder occurring in 22-43% of women that can have detrimental effects on the emotional and physical well being of the individual (Goldstein, 2000). One type of FSD is Female Sexual Arousal Disorder (FSAD), defined as persistent or recurrent inability to attain, or to maintain until completion of the sexual activity, an adequate lubrication-swelling response of sexual excitement causing personal distress (American Psychiatric Association, 2000). FSD arising from arousal disorders has symptoms including diminished vaginal lubrication, lack of vaginal wall engorgement and decreased clitoral tumescence (Goldstein, 2000), all of which result from a lack of smooth muscle relaxation.

In this thesis I have studied NANC relaxation responses of the rabbit vaginal wall. I have shown that these responses are partially mediated by NO and discussed evidence suggesting the presence of a non-nitrergic NANC relaxation response in this tissue.

I have demonstrated that receptors for various peptides as well as purines and pyrimidines are present in vaginal wall smooth muscle and that they can cause relaxation responses, and thus may provide targets for treatment of female sexual dysfunction. However I have provided evidence that neither these peptides nor the purines or pyrimidines mediate the non-nitrergic NANC relaxation responses in this tissue.

This has implications for the use of these relaxant pathways as targets for the treatment of FSD. Drugs targeted to stimulate these pathways may result in relaxation of

the vaginal smooth muscle leading to vaginal engorgement and lubrication. However a better treatment strategy is one that enhances a neurogenic signal originating in the brain due to desire (such as use of PDE 5 inhibitors). The evidence presented in this thesis suggests that the peptides studied as well as purines and pyrimidines are not involved in non-nitrergic NANC relaxation responses in this tissue, and thus do not provide targets for this type of treatment. Further studies are needed to identify the mediator of non-nitrergic NANC relaxation responses in the rabbit vaginal, and to determine if this mediator is involved in NANC responses in the human.

REFERENCE LIST

Abbracchio, M. P. and Burnstock, G. (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol.Ther.* **64**, 445 - 475.

Absood, A., Ekblad, E., Ekelund, M., Hakanson, R. and Sundler, F. (1992a) Helospectin-like peptides in the gastrointestinal tract: immunocytochemical localization and immunochemical characterization. *Neuroscience* **46**, 431 - 438.

Absood, A., Chen, D. and Hakanson, R. (1992b) Neuropeptides of the vasoactive intestinal peptide/helodermin/pituitary adenylate cyclase activating peptide family elevate plasma cAMP in mice: comparison with a range of other regulatory peptides. *Regul.Pept.* **40**, 311 - 322.

Adaikan, P. G. and Karim, S. M. (1981) Adrenoreceptors in the human penis. *J.Auton.Pharmacol.* **1**, 199 - 203.

Afonso, S. (1970) Inhibition of coronary vasodilating action of dipyridamole and adenosine by aminophylline in the dog. *Circ.Res.* **26**, 743 - 754.

Ahluwalia, A. and Cellek, S. (1997) Regulation of the cardiovascular system by non-adrenergic non- cholinergic nerves. *Curr.Opin.Nephrol.Hypertens.* **6**, 74 - 79.

Al Hijji, J., Larsson, B. and Batra, S. (2000) Nitric oxide synthase in the rabbit uterus and vagina: hormonal regulation and functional significance. *Biol.Reprod.* **62**, 1387 - 1392.

Al Hijji, J., Larsson, I. and Batra, S. (2001) Effect of ovarian steroids on nitric oxide synthase in the rat uterus, cervix and vagina. *Life.Sci.* **69**, 1133 - 1142.

Alzate, H. and Londoño, M. L. (1984) Vaginal erotic sensitivity. *J.Sex.Marital.Ther.* **10**, 49 - 56.

Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. and Evans, R. M. (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* **298**, 240 - 244.

American Psychiatric Association (2000) Female sexual arousal disorder. In Diagnostic and Statistical Manual of Mental Disorders. pp. 543 - 544. American Psychiatric Association, Washington, DC.

Andersson, K. E. and Holmquist, F. (1994) Regulation of tone in penile cavernous smooth muscle. Established concepts and new findings. *World.J.Urol.* **12**, 249 - 261.

Andersson, K. E. and Persson, K. (1995) Nitric oxide synthase and the lower urinary tract: possible implications for physiology and pathophysiology. *Scand.J.Urol.Nephrol.Suppl* **175**, 43 - 53.

Avis, N. E., Stellato, R., Crawford, S., Johannes, C. and Longcope, C. (2000) Is there an association between menopause status and sexual functioning? *Menopause.* **7**, 297 - 309.

Batra, S. and Al Hijji, J. (1998) Characterization of nitric oxide synthase activity in rabbit uterus and vagina: downregulation by estrogen. *Life.Sci.* **62**, 2093 - 2100.

Bayguinov, O., Keef, K. D., Hagen, B. and Sanders, K. M. (1999) Parallel pathways mediate inhibitory effects of vasoactive intestinal polypeptide and nitric oxide in canine fundus. *Br.J.Pharmacol.* **126**, 1543 - 1552.

Bellan, C., Pic, P., Marvaldi, J., Fantini, J. and Pichon, J. (1991) Suramin inhibits vasoactive intestinal peptide (VIP) binding and VIP- induced cAMP accumulation into two human cancerous cell lines. *Second.Messengers.Phosphoproteins.* **13**, 163 - 171.

Berman, J. R., McCarthy, M. M. and Kyprianou, N. (1998) Effect of estrogen withdrawal on nitric oxide synthase expression and apoptosis in the rat vagina. *Urology* **51**, 650 - 656.

Berman, J., Line, E., Kim, N. N., Goldstein, I., and Traish, A. (1999a) Effect of vasoactive agents in modulating vaginal smooth muscle contractility. In Proceedings of the Female Sexual Function Forum Meeting. Boston.

Berman, J. R., Berman, L. A., Werbin, T. J., Flaherty, E. E., Leahy, N. M. and Goldstein, I. (1999b) Clinical evaluation of female sexual function: effects of age and estrogen status on subjective and physiologic sexual responses. *Int.J.Impot.Res.* **11 Suppl 1**, S31 - S38.

Blank, M. A., Gu, J., Allen, J. M., Huang, W. M., Yiangou, Y., Ch'ng, J., Lewis, G., Elder, M. G., Polak, J. M. and Bloom, S. R. (1986) The regional distribution of NPY-, PHM-, and VIP-containing nerves in the human female genital tract. *Int.J.Fertil.* **31**, 218 - 222.

Boeckxstaens, G. E., Pelckmans, P. A., Bogers, J. J., Bult, H., De Man, J. G., Oosterbosch, L., Herman, A. G. and Van Maercke, Y. M. (1991) Release of nitric oxide upon stimulation of nonadrenergic noncholinergic nerves in the rat gastric fundus. *J.Pharmacol.Exp.Ther.* **256**, 441 - 447.

Boeckxstaens, G. E., De Man, J. G., Pelckmans, P. A., Cromheeke, K. M., Herman, A. G. and Van Maercke, Y. M. (1993) Ca^{2+} dependency of the release of nitric oxide from non-adrenergic non- cholinergic nerves. *Br.J.Pharmacol.* **110**, 1329 - 1334.

Bogdanov, Y. D., Wildman, S. S., Clements, M. P., King, B. F. and Burnstock, G. (1998) Molecular cloning and characterization of rat P2Y4 nucleotide receptor. *Br.J.Pharmacol.* **124**, 428 - 430.

Bowman, A. and Drummond, A. H. (1984) Cyclic GMP mediates neurogenic relaxation in the bovine retractor penis muscle. *Br.J.Pharmacol.* **81**, 665 – 674.

Bowman, A. and Gillespie, J. S. (1982) Block of some non-adrenergic inhibitory responses of smooth muscle by a substance from haemolysed erythrocytes. *J.Physiol.* **328**, 11 – 25.

Bowman, A., Gillespie, J. S. and Soares-da-Silva, P. (1986) A comparison of the action of the endothelium-derived relaxant factor and the inhibitory factor from the bovine retractor penis on rabbit aortic smooth muscle. *Br.J.Pharmacol.* **87**, 175 - 181.

Bradley, A. B. and Morgan, K. G. (1987) Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. *J.Physiol.* **385**, 437 - 448.

Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R. and MacIntyre, I. (1985) Calcitonin gene-related peptide is a potent vasodilator. *Nature* **313**, 54 - 56.

Brave, S. R., Hobbs, A. J. Gibson, A. and Tucker, J. F. (1991) The influence of L-N^G-nitro-arginine on field stimulation induced contractions and acetylcholine release in guinea pig isolated tracheal smooth muscle. *Biochem.Biophys.Res.Commun.* **179**, 1017 – 1022.

Bredt, D. S. and Snyder, S. H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc.Natl.Acad.Sci.U.S.A* **86**, 9030 - 9033.

Bridgewater, M., MacNeil, H. F. and Brading, A. F. (1993) Regulation of tone in pig urethral smooth muscle. *J.Urol.* **150**, 223 - 228.

Brindley, G. S. (1988) The actions of parasympathetic and sympathetic nerves in human micurition, erection and seminal emission, and their restoration in paraplegic pations by implanted electrical stimulators. *Proc.R.Soc.Lond.B:Biol.Sci.* **235**, 111 - 120.

Brunton, T. L. (1867) On the use of nitrite of amyl in angina pectoris. *Lancet.* **July 27**, 97 - 98.

Buck, S. H. and Burks, T. F. (1986) The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol.Rev.* **38**, 179 - 226.

Bult, H., Boeckxstaens, G. E., Pelckmans, P. A., Jordaens, F. H., Van Maercke, Y. M. and Herman, A. G. (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature*. **345**, 346 - 347.

Bultmann, R. and Starke, K. (2001) Nucleotide-evoked relaxation of rat vas deferens-a possible role for endogenous ATP released upon alpha(1)-adrenoceptor stimulation. *Eur.J.Pharmacol.* **422**, 197 - 202.

Burgos, M. H. and Roig de Vargas Linares, C. E. (1978) Ultrastructure of the vaginal mucosa. In The human vagina. Hafez, E. S. E. and Evans, T. N. (Eds.) Elsvier/North Holland, Amsterdam.

Burnett, A. L., Calvin, D. C., Silver, R. I., Peppas, D. S. and Docimo, S. G. (1997) Immunohistochemical description of nitric oxide synthase isoforms in human clitoris. *J.Urol.* **158**, 75 – 78.

Burks, T. F. (1994) Neurotransmission and neurotransmitters. In Physiology of the gastrointestinal tract. Johnson, L. R., Alpers, D. H., Christersen, J., Jacobsen, E. D. and Walsh, J. H. (Eds.) pp. 211 – 242. Raven Press, New York.

Burnstock, G. (1972) Purinergic nerves. *Pharmacol.Rev.* **24**, 509 - 581.

Burnstock, G. (1978) A basis for distinguishing two types of purinergic receptor. In Cell membrane receptors for drugs and hormones: A multidisciplinary approach. Straub, R. W. and Bolis, L. (Eds.) pp. 107 - 118. Raven Press, New York.

Burnstock, G. (1999) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J.Anat.* **194**, 335 – 342.

Burnstock, G. and Kennedy, C. (1985) Is there a basis for distinguishing two types of P2-purinoceptor? *Gen.Pharmacol.* **16**, 433 - 440.

Burnstock, G. and Warland, J. J. (1987) P2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P2y-but not the P2x-purinoceptor. *Br.J.Pharmacol.* **90**, 383 - 391.

Burnstock, G., Campbell, G. and Rand, M. J. (1966) The inhibitory innervation of the taenia of the guinea-pig caecum. *J.Physiol* **182**, 504 - 526.

Carvajal, J. A., Germain, A. M., Huidobro-Toro, J. P. and Weiner, C. P. (2000) Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J.Cell.Physiol.* **184**, 409 – 420.

Cellek, S. and Moncada, S. (1997a) Nitrergic control of peripheral sympathetic responses in the human corpus cavernosum: a comparison with other species. *Proc.Natl.Acad.Sci.U.S.A.* **94**, 8226 - 8231.

Cellek, S. and Moncada, S. (1997b) Modulation of noradrenergic responses by nitric oxide from inducible nitric oxide synthase. *Nitric.Oxide*. **1**, 204 – 210.

Cellek, S. and Moncada, S. (1998) Nitrergic neurotransmission mediates the non-adrenergic non-cholinergic responses in the clitoral corpus cavernosum of the rabbit. *Br.J.Pharmacol.* **125**, 1627 - 1629.

Cellek, S., Kasakov, L. and Moncada, S. (1996) Inhibition of nitrergic relaxations by a selective inhibitor of the soluble guanylate cyclase. *Br.J.Pharmacol.* **118**, 137 - 140.

Cellek, S., Ziessen, T. M. and Moncada, S. (1999) Non-adrenergic non-cholinergic responses in the rabbit vaginal wall are mediated by nitrergic neurotransmission. *Br.J.Pharmacol.* **126**, 98P.

Chakder, S. and Rattan, S. (1998) Involvement of pituitary adenylate cyclase-activating peptide in opossum internal anal sphincter relaxation. *Am.J.Physiol.* **275**, G769 - G777.

Challoner, A. V. J. (1979) Photoelectric plethysmography for estimating cutaneous blood flow. In Non-invasive physiological measurements. Rolfe, P. (Ed.) pp. 125 – 148. Saunders, W.B., Philadelphia.

Chang, M. M. and Leeman, S. E. (1970) Isolation of a sialogogic peptide from bovine hypothalamic tissue and its characterization as substance P. *J.Biol.Chem.* **245**, 4784 - 4790.

Charlton, S. J., Brown, C. A., Weisman, G. A., Turner, J. T., Erb, L. and Boarder, M. R. (1996) PPADS and suramin as antagonists at cloned P2Y- and P2U-purinoceptors. *Br.J.Pharmacol.* **118**, 704 - 710.

Chritton, S. L., Chinnow, S. L., Grabau, C., Dousa, M. K., Lucas, D., Roddy, D., Yaksh, T. L. and Tyce, G. M. (1997) Adrenomedullary secretion of DOPA, catecholamines, catechol metabolites, and neuropeptides. *J.Neurochem.* **69**, 2413 - 2420.

Chu, D. Q., Smith, D. M. and Brain, S. D. (2001) Studies of the microvascular effects of adrenomedullin and related peptides. *Peptides.* **22**, 1881 – 1886.

Ciccarelli, E., Svoboda, M., De Neef, P., Di Paolo, E., Bollen, A., Dubeaux, C., Vilardaga, J. P., Waelbroeck, M. and Robberecht, P. (1995) Pharmacological properties of two recombinant splice variants of the PACAP type I receptor, transfected and stably expressed in CHO cells. *Eur.J.Pharmacol.* **288**, 259 - 267.

Communi, D., Motte, S., Boeynaems, J. M. and Pirotton, S. (1996a) Pharmacological characterization of the human P2Y4 receptor. *Eur.J.Pharmacol.* **317**, 383 - 389.

Communi, D., Parmentier, M. and Boeynaems, J. M. (1996b) Cloning, functional expression and tissue distribution of the human P2Y6 receptor. *Biochem.Biophys.Res.Commun.* **222**, 303 - 308.

Communi, D., Govaerts, C., Parmentier, M. and Boeynaems, J. M. (1997) Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J.Biol.Chem.* **272**, 31969 - 31973.

Communi, D., Gonzalez, N. S., Detheux, M., Brezillon, S., Lannoy, V., Parmentier, M. and Boeynaems, J. M. (2001) Identification of a novel human ADP receptor coupled to G(i). *J.Biol.Chem.* **276**, 41479 - 41485.

Couvineau, A., Rouyer-Fessard, C., Darmoul, D., Maoret, J. J., Carrero, I., Ogier-Denis, E. and Laburthe, M. (1994) Human intestinal VIP receptor: cloning and functional expression of two cDNA encoding proteins with different N-terminal domains. *Biochem.Biophys.Res.Commun.* **200**, 769 - 776.

Crack, B. E., Beukers, M. W., McKechnie, K. C., Ijzerman, A. P. and Leff, P. (1994) Pharmacological analysis of ecto-ATPase inhibition: evidence for combined enzyme inhibition and receptor antagonism in P2X-purinoceptor ligands. *Br.J.Pharmacol.* **113**, 1432 - 1438.

Croxton, T. L., Lande, B. and Hirshman, C. A. (1998) Role of G proteins in agonist-induced Ca^{2+} sensitization of tracheal smooth muscle. *Am.J.Physiol.* **275**, L748 - L755.

Csernus, V., Schally, A. V. and Groot, K. (1999) Effect of GHRH and peptides from the vasoactive intestinal peptide family on cAMP production of human cancer cell lines in vitro. *J.Endocrinol.* **163**, 269 - 280.

Curro, D. and Preziosi, P. (1998) Non-adrenergic non-cholinergic relaxation of the rat stomach. *Gen.Pharmacol.* **31**, 697 - 703.

Dale, H. H. (1914) The action of certain esters and ethers of choline, and their relation to muscarine. *J.Pharmacol.Exp.Ther.* **6**, 147 - 190.

Dale, H. H. (1933) Nomenclature of fibers in the autonomic system and their effects. *J.Physiol.* **80**, 10P - 11P.

Daniel, E. E., Crankshaw, J. and Sarna, S. (1979) Prostaglandins and tetrodotoxin-insensitive relaxation of opossum lower esophageal sphincter. *Am.J.Physiol.* **236**, E153 - E172.

Deguchi, T. and Yoshioka, M. (1982) L-arginine identified as an endogenous activator for soluble guanylate cyclase from neuroblastoma cells. *J.Biol.Chem.* **257**, 10147 – 10151.

Dickinson, T., Fleetwood-Walker, S. M., Mitchell, R. and Lutz, E. M. (1997) Evidence for roles of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) receptors in modulating the responses of rat dorsal horn neurons to sensory inputs. *Neuropeptides* **31**, 175 - 185.

Drury, A. N. and Szent-György, A. (1929) The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J.Physiol (Lond)* **68**, 213 - 237.

Dubyak, G. R. (1991) Signal transduction by P2-purinergic receptors for extracellular ATP. *Am.J.Respir.Cell Mol.Biol.* **4**, 295 - 300.

Eccles, J. C. (1964) The physiology of synapses. Academic Press Inc., Publishers, New York.

Elliott, T. R. (1904) On the innervation of the ileo-colic sphincter. *J.Physiol* **31**, 157 - 168.

Erga, K. S., Seubert, C. N., Liang, H. X., Wu, L., Shryock, J. C. and Belardinelli, L. (2000) Role of A(2A)-adenosine receptor activation for ATP-mediated coronary vasodilation in guinea-pig isolated heart. *Br.J.Pharmacol.* **130**, 1065 - 1075.

Euler, U. S. v. and Gaddum, J. H. (1931) An unidentified depressor substance in certain tissue extracts. *J.Physiol (Lond)* **72**, 74 - 87.

Fahrenkrug, J. and Schaffalitzky de Muckadell, O. B. (1978) Distribution of vasoactive intestinal polypeptide (VIP) in the porcine central nervous system. *J.Neurochem.* **31**, 1445 - 1451.

Fahrenkrug, J., Haglund, U., Jodal, M., Lundgren, O., Olbe, L. and de Muckadell, O. B. (1978) Nervous release of vasoactive intestinal polypeptide in the gastrointestinal tract of cats: possible physiological implications. *J.Physiol.* **284**, 291 - 305.

Fernandes, L. B., Preuss, J. M. and Goldie, R. G. (1992) Epithelial modulation of the relaxant activity of atriopeptides in rat and guinea-pig tracheal smooth muscle. *Eur.J.Pharmacol.* **212**, 187 - 194.

Filippi, S., Amerini, S., Maggi, M., Natali, A. and Ledda, F. (1999) Studies on the mechanisms involved in the ATP-induced relaxation in human and rabbit corpus cavernosum. *J.Urol.* **161**, 326 - 331.

Fisher, L. A., Kikkawa, D. O., Rivier, J. E., Amara, S. G., Evans, R. M., Rosenfeld, M. G., Vale, W. W. and Brown, M. R. (1983) Stimulation of noradrenergic sympathetic outflow by calcitonin gene- related peptide. *Nature.* **305**, 534 - 536.

Fisher, S. (1973) Understanding the female orgasm. Penguin books, Harmondsworth, England.

Fovaeus, M., Fujiwara, M., Hogestatt, E. D., Persson, K. and Andersson, K. E. (1999) A non-nitrergic smooth muscle relaxant factor released from rat urinary bladder by muscarinic receptor stimulation. *J.Urol.* **161**, 649 - 653.

Fox, C. A. and Fox, B. (1969) Blood pressure and respiratory patterns during human coitus. *J.Reprod.Fertil.* **19**, 405 - 415.

Francis, S. H., Turko, I. V. and Corbin, J. D. (2001) Cyclic nucleotide phosphodiesterases: Relating structure and function. *Prog.Nucleic.Acid.Res.Mol.Biol.* **65**, 1 – 52.

Fredholm, B. B., Fried, G. and Hedqvist, P. (1982) Origin of adenosine released from rat vas deferens by nerve stimulation. *Eur.J.Pharmacol.* **79**, 233 - 243.

Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Dubyak, G. R., Harden, T. K., Jacobson, K. A., Schwabe, U. and Williams, M. (1997) Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol.Sci.* **18**, 79 - 82.

Freud, S. (1901) The standard edition of the complete psychological works of Sigmund Freud. The Hogarth Press, London.

Fujisawa, K., Onoue, H., Abe, K. and Ito, Y. (1999) Multiple calcium channels regulate neurotransmitter release from vagus nerve terminals in the cat bronchiole. *Br.J.Pharmacol.* **128**, 262 – 268.

Furchgott, R. F. and Zawadzki, J. V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* **288**, 373 - 376.

Furchgott, R. F. and Jothianandan, D. (1991) Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* **28**, 52 - 61.

Furchgott, R. F., Cherry, P. D., Zawadzki, J. V. and Jothianandan, D. (1984) Endothelial cells as mediators of vasodilation of arteries. *J.Cardiovasc.Pharmacol.* **6 Suppl 2**, S336 - S343.

Gaddum, J. H. (1953) The technique of superfusion. *Br.J.Pharmacol.* **8**, 321 - 326.

Gaddum, J. H. (1964) Drugs in our society. Talanay, P. (Ed.) pp. 17 - 26. John Hopkins Press, Baltimore.

Galle, J., Zabel, U., Hubner, U., Hatzelmann, A., Wagner, B., Wanner, C. and Schmidt, H. H. (1999) Effects of the soluble guanylyl cyclase activator, YC-1, on vascular tone, cyclic GMP levels and phosphodiesterase activity. *Br.J.Pharmacol.* **127**, 195 – 203.

Garthwaite, J., Charles, S. L. and Chess-Williams, R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature.* **336**, 385 - 388.

Garthwaite, J., Garthwaite, G., Palmer, R. M. and Moncada, S. (1989) NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur.J.Pharmacol.* **172**, 413 - 416.

Gibson, A., Mirzazadeh, S., Hobbs, A. J. and Moore, P. K. (1990) L-NG-monomethyl arginine and L-NG-nitro arginine inhibit non- adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle. *Br.J.Pharmacol.* **99**, 602 - 606.

Gibson, A., McFadzean, I., Tucker, J. F. and Wayman, C. (1994) Variable potency of nitrergic-nitrovasodilator relaxations of the mouse anococcygeus against different forms of induced tone. *Br.J.Pharmacol.* **113**, 1494 – 1500.

Gillespie, J. H. (1934) The biological significance of the linkages in adenosine triphosphoric acid. *J.Physiol (Lond)* **80**, 345 - 359.

Gillespie, J. S. (1972) The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br.J.Pharmacol.* **45**, 404 - 416.

Gillespie, J. S. and Sheng, H. (1989) A comparison of haemoglobin and erythrocytes as inhibitors of smooth muscle relaxation by the NANC transmitter in the BRP and rat anococcygeus and by EDRF in the rabbit aortic strip. *Br.J.Pharmacol.* **98**, 445 – 450.

Gillespie, J. S., Liu, X. R. and Martin, W. (1989) The effects of L-arginine and NG-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br.J.Pharmacol.* **98**, 1080 - 1082.

Giraldi, A., Persson, K., Werkström, V., Alm, P., Wagner, G. and Andersson, K. E. (2001) Effects of diabetes on neurotransmission in rat vaginal smooth muscle. *Int.J.Impot.Res.* **13**, 58 - 66.

Goldstein, I. (2000) Female sexual arousal disorder: new insights. *Int.J.Impot.Res.* **12 Suppl 4**, S152 - S157.

Gordon, J. L. (1986) Extracellular ATP: effects, sources and fate. *Biochem.J.* **233**, 309 - 319.

Gourlet, P., De Neef, P., Cnudde, J., Waelbroeck, M. and Robberecht, P. (1997) In vitro properties of a high affinity selective antagonist of the VIP1 receptor. *Peptides* **18**, 1555 - 1560.

Goyal, R. K., Rattan, S. and Said, S. I. (1980) VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones. *Nature.* **288**, 378 - 380.

Graf, A. H., Schiechl, A., Hacker, G. W., Hauser Kronberger, C., Steiner, H., Arimura, A., Sundler, F., Staudach, A. and Dietze, O. (1995) Helospectin and pituitary adenylate cyclase activating polypeptide in the human vagina. *Regul.Pept.* **55**, 277 - 286.

Grafenberg, E. (1950) The role of the urethra in female orgams. *Int.J.Sexol.* **3**, 145 - 148.

Grainger, J. and Boachie-Ansah, G. (2001) Anandamide-induced relaxation of sheep coronary arteries: the role of the vascular endothelium, arachidonic acid metabolites and potassium channels. *Br.J.Pharmacol.* **134**, 1003 - 1012.

Grozdanovic, Z., Mayer, B., Baumgarten, H. G. and Bruning, G. (1994) Nitric oxide synthase-containing nerve fibers and neurons in the genital tract of the female mouse. *Cell Tissue Res.* **275**, 355 - 360.

Grundemar, L. and Hogestatt, E. D. (1990) Vascular effects of helodermin, helospectin I and helospectin II: a comparison with vasoactive intestinal peptide (VIP). *Br.J.Pharmacol.* **99**, 526 - 528.

Gu, Z. F., Menozzi, D., Okamoto, A., Maton, P. N. and Bunnett, N. W. (1993) Neutral endopeptidase (EC 3.4.24.11) modulates the contractile effects of neuropeptides on muscle cells from the guinea-pig stomach. *Exp.Physiol.* **78**, 35 - 48.

Hamori, C. J., Vreman, H. J., Rodgers, P. A. and Stevenson, D. K. (1989) Zinc protoporphyrin inhibits CO production in rats. *J.Pediatr.Gastroenterol.Nutr.* **8**, 110 - 115.

Harmar, A. J., Arimura, A., Gozes, I., Journot, L., Laburthe, M., Pisegna, J. R., Rawlings, S. R., Robberecht, P., Said, S. I., Sreedharan, S. P., Wank, S. A. and Waschek, J. A. (1998) International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase- activating polypeptide. *Pharmacol.Rev.* **50**, 265 - 270.

Haynes, J. M. (2000) A(2A) adenosine receptor mediated potassium channel activation in rat epididymal smooth muscle. *Br.J.Pharmacol.* **130**, 685 - 691.

Hecker, M., Mitchell, J. A., Harris, H. J., Katsura, M., Thiemermann, C. and Vane, J. R. (1990) Endothelial cells metabolize NG-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. *Biochem.Biophys.Res.Commun.* **167**, 1037 - 1043.

Hedlund, H. and Andersson, K.E. (1985) Comparison of the responses to drugs acting on adrenoreceptors and muscarinic receptors in human isolated corpus cavernosum and cavernous artery. *J.Auton.Pharmacol.* **5**, 81 – 88.

Hein, T. W. and Kuo, L. (1999) cAMP-independent dilation of coronary arterioles to adenosine: role of nitric oxide, G proteins, and K(ATP) channels. *Circ.Res.* **85**, 634 - 642.

Hillard, C. J. (2000) Endocannabinoids and vascular function. *J.Pharmacol.Exp.Ther.* **294**, 27 - 32.

Himpens, B. and Somlyo, A. P. (1988) Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J.Physiol.* **395**, 507 - 530.

Hirata, Y., Tomita, M., Takata, S. and Fujita, T. (1985) Functional receptors for vasoactive intestinal peptide in cultured vascular smooth muscle cells from rat aorta. *Biochem.Biophys.Res.Commun.* **132**, 1079 - 1087.

Hirshman, C. A. and Emala, C. W. (1999) Actin reorganization in airway smooth muscle cells involves Gq and Gi-2 activation of Rho. *Am.J.Physiol.* **277**, L653 - 661.

Hobbs, A. J. and Gibson, A. (1990) L-NG-nitro-arginine and its methyl ester are potent inhibitors of non- adrenergic, non-cholinergic transmission in the rat anococcygeus. *Br.J.Pharmacol.* **100**, 749 - 752.

Hoch, Z. (1980a) The female orgasmic reflex - Its sensory arm. In Proceedings of the ninth world congress of gynecology and obstetrics. Sakamoto, S., Tojo, S., and Nakayama, T. (Eds.) pp. Excerpta Medica, Amsterdam.

Hoch, Z. (1980b) The sensory arm of the female orgasmic reflex. *J.Sex.Educ.Ther.* **6**, 4 - 7.

Hoch, Z. (1983) The G spot. *J.Sex.Marital.Ther.* **9**, 166 - 167.

Hogestatt, E. D., Johansson, R., Andersson, D. A. and Zygmunt, P. M. (2000) Involvement of sensory nerves in vasodilator responses to acetylcholine and potassium ions in rat hepatic artery. *Br.J.Pharmacol.* **130**, 27 - 32.

Hollopeter, G., Jantzen, H. M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R. B., Nurden, P., Nurden, A., Julius, D. and Conley, P. B. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature.* **409**, 202 - 207.

Holton, F. A. and Holton, P. (1953) The possibility that ATP is a neurotransmitter at sensory nerve endings. *J.Physiol (Lond)* **119**, 50 - 51.

Hoyle, C. H., Stones, R. W., Robson, T., Whitley, K. and Burnstock, G. (1996) Innervation of vasculature and microvasculature of the human vagina by NOS and neuropeptide-containing nerves. *J.Anat.* **188**, 633 - 644.

Huch, R., Huch, A. and Rolfe, P. (1979) Transcutaneous measurement of PO_2 using electrochemical analysis. In Non-invasive physiological measurements. Rolfe, P. (Ed.) pp. 313 – 331. Saunders, W.B., Philadelphia.

Hwang, T. L., Wu, C. C. and Teng, C. M. (1998) Comparison of two soluble guanylyl cyclase inhibitors, methylene blue and ODQ, on sodium nitroprusside-induced relaxation in guinea-pig trachea. *Br.J.Pharmacol.* **125**, 1158 - 1163.

Ignarro, L. J., Bush, P. A., Buga, G. M., Wood, K. S., Fukuto, J. M. and Rajfer, J. (1990) Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem.Biophys.Res.Commun.* **170**, 843 - 850.

Iizuka, K., Yoshii, A., Samizo, K., Tsukagoshi, H., Ishizuka, T., Dobashi, K., Nakazawa, T. and Mori, M. (1999) A major role for the Rho-associated coiled coil forming protein kinase in G-protein-mediated Ca^{2+}-sensitization through inhibition of myosin phosphatase in rabbit trachea. *Br.J.Pharmacol.* **128**, 925 - 933.

Inyama, C. O., Wharton, J., Su, H. C. and Polak, J. M. (1986) CGRP-immunoreactive nerves in the genitalia of the female rat originate from dorsal root ganglia T11-L3 and L6-S1: a combined immunocytochemical and retrograde tracing study. *Neurosci.Lett.* **69**, 13 - 18.

Ishiguchi, T., Takahashi, T., Itoh, H. and Owyang, C. (2000) Nitrergic and purinergic regulation of the rat pylorus. *Am.J.Physiol Gastrointest.Liver Physiol* G740 - G747.

Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K. and Nagata, S. (1992) Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron.* **8**, 811 - 819.

Itoh, N., Obata, K., Yanaihara, N. and Okamoto, H. (1983) Human preprovasoactive intestinal polypeptide contains a novel PHI-27- like peptide, PHM-27. *Nature.* **304**, 547 - 549.

Jenkinson, K. M. and Reid, J. J. (2000) The P(2)-purinoceptor antagonist suramin is a competitive antagonist at vasoactive intestinal peptide receptors in the rat gastric fundus. *Br.J.Pharmacol.* **130**, 1632 - 1638.

Jensen, P. E., Gong, M. C., Somlyo, A. V. & Somlyo, A. P. (1996) Separate upstream and convergent downstream pathways of G-protein and phorbol ester-mediated Ca^{2+}-sensitization of myosin light chain phosphorylation in smooth muscle. *Biochem.J.* **318**, 469 - 475.

Journot, L., Waeber, C., Pantaloni, C., Holsboer, F., Seeburg, P. H., Bockaert, J. and Spengler, D. (1995) Differential signal transduction by six splice variants of the pituitary adenylate cyclase-activating peptide (PACAP) receptor. *Biochem.Soc.Trans.* **23**, 133 - 137.

Kage, R., Hershey, A. D., Krause, J. E., Boyd, N. D. and Leeman, S. E. (1995) Characterization of the substance P (NK-1) receptor in tunicamycin- treated transfected cells using a photoaffinity analogue of substance P. *J.Neurochem.* **64**, 316 - 321.

Kanayama, K., Nariai, K., Sankai, T. and Endo, T. (1995) Superovulation induction by human menopausal gonadotrophin in rabbits. *J.Vet.Med.Sci.* **57**, 543 – 544.

Kaplan, H. S. (1974) The new sex therapy: active treatment of sexual dysfunctions. Baillière Tindall, London.

Kasakov, L., Cellek, S. and Moncada, S. (1995) Characterization of nitrergic neurotransmission during short- and long-term electrical stimulation of the rabbit anococcygeus muscle. *Br.J.Pharmacol.* **115**, 1149 – 1154.

Katoh, H., Aoki, J., Yamaguchi, Y., Kitano, Y., Ichikawa, A. and Negishi, M. (1998) Constitutively active $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ induce Rho-dependent neurite retraction through different signaling pathways. *J.Biol.Chem.* **273**, 28700 - 28707.

Katsuki, S. and Murad, F. (1977) Regulation of adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3',5'-monophosphate levels and contractility in bovine tracheal smooth muscle. *Mol.Pharmacol.* **13**, 330 - 341.

Katsuki, S., Arnold, W. P. and Murad, F. (1977) Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. *J.Cyclic.Nucleotide.Res.* **3**, 239 - 247.

Kauser, K. and Rubanyi, G. M. (1992) Bradykinin-induced, N omega-nitro-L-arginine-insensitive endothelium- dependent relaxation of porcine coronary arteries is not mediated by bioassayable relaxing substances. *J.Cardiovasc.Pharmacol.* **20 Suppl 12**, S101 - S104.

Kawasaki, H., Takasaki, K., Saito, A. and Goto, K. (1988) Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature.* **335**, 164 - 167.

Kelm, M. and Schrader, J. (1990) Control of coronary vascular tone by nitric oxide. *Circ.Res.* **66**, 1561 - 1575.

Kim, N., Azadzoi, K. M., Goldstein, I. and Saenz de Tejada, I. (1991) A nitric oxide-like factor mediates nonadrenergic-noncholinergic neurogenic relaxation of penile corpus cavernosum smooth muscle. *J.Clin.Invest.* **88**, 112 - 118

Kim, Y. C., Ji, X., Melman, N., Linden, J. and Jacobson, K. A. (2000) Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A(2B) adenosine receptors. *J.Med.Chem.* **43**, 1165 - 1172.

Kimura, H., Mittal, C. K. and Murad, F. (1975a) Increases in cyclic GMP levels in brain and liver with sodium azide an activator of guanylate cyclase. *Nature* **257**, 700 - 702.

Kimura, H., Mittal, C. K. and Murad, F. (1975b) Activation of guanylate cyclase from rat liver and other tissues by sodium azide. *J.Biol.Chem.* **250**, 8016 - 8022.

Kimura, C., Ohkubo, S., Ogi, K., Hosoya, M., Itoh, Y., Onda, H., Miyata, A., Jiang, L., Dahl, R. R., Stibbs, H. H., Arimura, A. and Fujino, M. (1990) A novel peptide which stimulates adenylate cyclase: molecular cloning and characterization of the ovine and human cDNAs. *Biochem.Biophys.Res.Commun.* **166**, 81 - 89.

Kimura, K., Tamura, M., Kawanishi, Y. and Kagawa, S. (1992) Nitric oxide (NO) as non-adrenergic non-cholinergic neurotransmitter in human corpus cavernosum. *Jpn.J.Pharmacol.* **58 Suppl 2**, 385P

King, B. F., Burnstock, G., Boyer, J., Boeynaems, J. M., Weisman, G. A., Kennedy, C., Jacobson, K. A., Humphries, R. G., Abbracchio, M. P., Gachet, C., and Miras-Portugal, M. T. (2000) P2Y receptors. In The IUPHAR compendium of receptor characterization and classification. Girdlestone, D. (Ed.) pp. 306 - 320. IUPHAR Media, London.

Kinsey, A. C., Pomeroy, W. B., Martin, C. E., and Gebhard, P. H. (1953) Sexual behaviour in the human female. Saunders, Philadelphia.

Kishi, M., Takeuchi, T., Suthamnatpong, N., Ishii, T., Nishio, H., Hata, F. and Takewaki, T. (1996) VIP- and PACAP-mediated nonadrenergic, noncholinergic inhibition in longitudinal muscle of rat distal colon: involvement of activation of charybdotoxin- and apamin-sensitive K+ channels. *Br.J.Pharmacol.* **119**, 623 - 630.

Kishi, M., Takeuchi, T., Katayama, H., Yamazaki, Y., Nishio, H., Hata, F. and Takewaki, T. (2000) Involvement of cyclic AMP-PKA pathway in VIP-induced, charybdotoxin-sensitive relaxation of longtitudinal muscle of the distal colon of Wistar-ST rats. *Br.J.Pharmacol.* **129**, 140 - 146.

Klages, B., Brandt, U., Simon, M. I., Schultz, G. and Offermanns, S. (1999) Activation of G_{12} / G_{13} results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J.Cell.Biol.* **14**, 745 - 754.

Knowles, R. G., Palacios, M., Palmer, R. M. and Moncada, S. (1989) Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc.Natl.Acad.Sci.U.S.A* **86**, 5159 - 5162.

Krantz, K. E. (1958) Innervation of the human vulva and vagina: A microscopic study. *Obstet.Gynecol.* **12**, 382 - 396.

La, J., Kim, T., Sung, T., Kang, T., Lee, J. and Yang, I. (2001) Involvement of nitric oxide and vasoactive intestinal peptide in the nonadrenergic-noncholinergic relaxation of the porcine retractor penis muscle. *Jpn.J.Pharmacol.* **86**, 236 - 243.

Langley, J. N. (1901) Observations on the physiological action of extracts of the supra-renal bodies. *J.Physiol.* **23**, 407 – 414.

Langley, J. N. and Anderson, H. K. (1895) The innervation of the pelvic and adjoining viscera: Part II. The Bladder. *J.Physiol.* **19**, 71 - 84.

Larsson, L. I., Fahrenkrug, J., Schaffalitzky de Muckadell, O. B., Sundler, F., Hakanson, R. and Rehfeld, J. R. (1976) Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. *Proc.Natl.Acad.Sci.U.S.A* **73**, 3197 - 3200.

Larsson, L. I., Fahrenkrug, J. and Schaffalitzky de Muckadell, O. B. (1977) Vasoactive intestinal polypeptide occurs in nerves of the female genitourinary tract. *Science.* **197**, 1374 - 1375.

Lazarowski, E. R., Rochelle, L. G., O'Neal, W. K., Ribeiro, C. M., Grubb, B. R., Zhang, V., Harden, T. K. and Boucher, R. C. (2001) Cloning and functional characterization of two murine uridine nucleotide receptors reveal a potential target for correcting ion transport deficiency in cystic fibrosis gallbladder. *J.Pharmacol.Exp.Ther.* **297**, 43 - 49.

Levin, R., Wagner, G. and Ottesen, B. (1982) Simultaneous monitoring of human vaginal haemodynamics by three independent methods during sexual arousal. In Sexology. Hoch, Z. and Lief, H. I. (Eds.) pp. 114 - 120. Excerpta Medica, Elsevier, Amsterdam.

Levin, R. J. (1980) The physiology of sexual function in women. *Clin.Obstet.Gynaecol.* 7, 213 - 252.

Levin, R. J. and MacDonagh, R. P. (1993) Increased vaginal blood flow induced by implant electrical stimulation of sacral anterior roots in the conscious woman: a case study. *Arch.Sex Behav.* **22**, 471 - 475.

Lewandowsky, M. (1899) Uber die wirkung des Nebennierenextractes auf die glatten Muskeln, in Besonderen des Auges. *Archiv fur Anatomie und Physiologie.*306 – 366.

Li, C. G. and Rand, M. J. (1989) Evidence for a role of nitric oxide in the neurotransmitter system mediating relaxation of the rat anococcygeus muscle. *Clin.Exp.Pharmacol.Physiol.* **16**, 933 - 938.

Li, C. G. and Rand, M. J. (1990) Nitric oxide and vasoactive intestinal polypeptide mediate non- adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur.J.Pharmacol.* **191**, 303 - 309.

Li, C. G. and Rand, M. J. (1991) Evidence that part of the NANC relaxant response of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide. *Br.J.Pharmacol.* **102**, 91 - 94.

Li, L., Eto, M., Lee, M. R., Morita, F., Yazawa, M. and Kitazawa, T. (1998) Possible involvement of the novel CPI-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle. *J.Physiol.* **508**, 871 – 881.

Linden, J., Tucker, A. L. and Lynch, K. R. (1991) Molecular cloning of adenosine A1 and A2 receptors. *Trends Pharmacol.Sci.* **12**, 326 - 328.

Liu, X. R., Gillespie, J. S., Gibson, I. F. and Martin, W. (1991) Effects of N^G-substituted analogues of L-arginine on NANC relaxation of the rat anococcygeus and bovine retractor penis muscles and the bovine penile artery. *Br.J.Pharmacol.* **104**, 53 - 58.

Loewi, O. (1921) Uber humorale Ubertragbarkeit der Herznervenwirkung. *Pflugers Archiv fur die Gesamte Physiologie Menschen un der Tiere.* **189**, 239 – 242.

Londos, C., Cooper, D. M. and Wolff, J. (1980) Subclasses of external adenosine receptors. *Proc.Natl.Acad.Sci.U.S.A* **77**, 2551 - 2554.

Longmore, J., Hogg, J. E., Hutson, P. H. and Hill, R. G. (1994) Effects of two truncated forms of human calcitonin-gene related peptide: implications for receptor classification. *Eur.J.Pharmacol.* **265**, 53 - 59.

Lundberg, J. M. (1996) Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol.Rev.* **48**, 113 - 178.

Lundberg, J. M. and Hökfelt, T. (1985) Coexistence of peptides and classical neurotransmitters. In Neurotransmitters in action. Bousfield, D. (Ed.) pp. 104 - 118. Elsevier Biomedical Press BV, Amsterdam.

Lutz, E. M., Sheward, W. J., West, K. M., Morrow, J. A., Fink, G. and Harmar, A. J. (1993) The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Lett.* **334**, 3 - 8.

Maggi, C. A. and Giuliani, S. (1996) Characterization of the apamin- and L-nitroarginine-resistant NANC inhibitory transmission to the circular muscle of guinea-pig colon. *J.Auton.Pharmacol.* **16**, 131 - 145.

Majewski, M., Sienkiewicz, W., Kaleczyc, J., Mayer, B., Czaja, K. and Lakomy, M. (1995) The distribution and co-localization of immunoreactivity to nitric oxide synthase, vasoactive intestinal polypeptide and substance P within nerve fibres supplying bovine and porcine female genital organs. *Cell Tissue Res.* **281**, 445 - 464.

Marin-Bivens, C. L. and Olster, D. H. (1999) Opioid receptor blockade promotes weight loss and improves the display of sexual behaviors in obese Zucker female rats. *Pharmacol.Biochem.Behav.* **63**, 515 - 520.

Marino, F., Creta, F., De Ponti, F., Giaroni, C., Lecchini, S. and Frigo, G. M. (1993) Opioid pathways exert a tonic restraint in the guinea-pig isolated colon: changes after chronic sympathetic denervation. *J.Pharm.Pharmacol.* **45**, 668 - 670.

Marshall, I., Al Kazwini, S. J., Holman, J. J. and Craig, R. K. (1986) Human and rat alpha-CGRP but not calcitonin cause mesenteric vasodilatation in rats. *Eur.J.Pharmacol.* **123**, 217 - 222.

Martin, W., Villani, G. M., Jothianandan, D. and Furchgott, R. F. (1985) Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J.Pharmacol.Exp.Ther.* 232, 708 – 716.

Martin, W., Gillespie, J. S. and Gibson, I. F. (1993) Actions and interactions of N^G-substituted analogues of L-arginine on NANC neurotransmission in the bovine retractor penis and rat anococcygeus muscles. *Br.J.Pharmacol.* **108**, 242 – 247.

Martinez, A. C., Garcia-Sacristan, A., Rivera, L. and Benedito, S. (2000) Biphasic response to histamine in rabbit penile dorsal artery. *J.Cardiovasc.Pharmacol.* **36**, 737 - 743.

Masters, W. H. and Johnson, V. E. (1966) Human sexual response. Little, Brown, Boston, Mass.

Matthew, J. D., Wadsworth, R. M. and McPhaden, A. R. (1997) Inhibition of vasodilator neurotransmission in the sheep middle cerebral artery by VIP antiserum. *J.Auton.Pharmacol.* **17**, 13 - 19.

Matyas, S., Pucovsky, V. and Bauer, V. (1995) Involvement of different Ca2+ sources in changes of responsiveness of guinea-pig trachea to repeated administration of histamine and acetylcholine. *Gen.Physiol.Biophys.* **14**, 51 – 60.

Mayer, B., Brunner, F. and Schmidt, K. (1993) Inhibition of nitric oxide synthesis by methylene blue. *Biochem.Pharmacol.* **45**, 367 – 374.

Mazzocchi, G., Malendowicz, L. K., Neri, G., Andreis, P. G., Ziolkowska, A., Gottardo, L., Nowak, K. W. and Nussdorfer, G. G. (2002) Pituitary adenylate cyclase-activating

polypeptide and PACAP receptor expression and function in the rat adrenal gland. *Int.J.Mol.Med.* **9**, 233 - 243.

McMurray, G., Dass, N. and Brading, A. F. (1998) Purinoceptor subtypes mediating contraction and relaxation of marmoset urinary bladder smooth muscle. *Br.J.Pharmacol.* **123**, 1579 - 1586.

Meston, C.M., Gorzalka, B.B. and Wright, J.M. (1997) Inhibition of subjective and physiological sexual arousal in women by clondine. *Psychosom.Med.* **59**, 399 – 407.

Mhanna, M. J., Dreshaj, I. A., Haxhiu, M. A. and Martin, R. J. (1999) Mechanism for substance P-induced relaxation of precontracted airway smooth muscle during development. *Am.J.Physiol.* **276**, L51 – L56.

Miller, R. J. (1987) Multiple calcium channels and neuronal function. *Science.* **235**, 46 - 52.

Min, K., O'Connell, L., Munarriz, R., Huang, Y-H., Choi, S., Kim, N., Goldstein, I. and Traish, A. (2001a) Experimental models for the investigation of female sexual function and dysfunction. *Int.J.Impot.Res.* **13**, 151 – 156.

Min, K., Munarriz, R., Kim, N., Goldstein, I. and Traish, A. (2001b) Effects of ovariectomy and estrogen and androgen treatment on vaginal nitric oxide synthase activity and smooth muscle. In Proceedings of the Female Sexual Function Forum Meeting. Boston.

Minchin, M. C. (1980) The role of Ca^{2+} in the protoveratrine-induced release of gamma-aminobutyrate from rat brain slices. *Biochem.J.* **190**, 333 - 339.

Mirzazadeh, S., Hobbs, A. J., Tucker, J. F. and Gibson, A. (1991) Cyclic nucleotide content of the rat anococcygeus during relaxations induced by drugs or by non-adrenergic, non-cholinergic field stimulation. *J.Pharm.Pharmacol.* **43**, 247 – 251.

Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uehara, A., Jiang, L., Culler, M. D. and Coy, D. H. (1989) Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem.Biophys.Res.Commun.* **164**, 567 - 574.

Miyata, A., Jiang, L., Dahl, R. D., Kitada, C., Kubo, K., Fujino, M., Minamino, N. and Arimura, A. (1990) Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochem.Biophys.Res.Commun.* **170**, 643 - 648.

Moffatt, J. D., Dumsday, B. and McLean, J. R. (1999) Characterization of non-adrenergic, non-cholinergic inhibitory responses of the isolated guinea-pig trachea: differences between pre- and post-ganglionic nerve stimulation. *Br.J.Pharmacol.* **128**, 458 - 464.

Moncada, S., Gryglewski, R., Bunting, S. and Vane, J. R. (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature.* **263**, 663 - 665.

Moncada, S., Higgs, A. and Furchgott, R. (1997) International Union of Pharmacology Nomenclature in Nitric Oxide Research. *Pharmacol.Rev.* **49**, 137 - 142.

Moro, O. and Lerner, E. A. (1997) Maxadilan, the vasodilator from sand flies, is a specific pituitary adenylate cyclase activating peptide type I receptor agonist. *J.Biol.Chem.* **272**, 966 - 970.

Morris, H. R., Panico, M., Etienne, T., Tippins, J., Girgis, S. I. and MacIntyre, I. (1984) Isolation and characterization of human calcitonin gene-related peptide. *Nature.* **308**, 746 - 748.

Murrell, W. (1879) Nitroglycerin as a remedy for angina pectoris. *Lancet.* **Jan 18**, 80 - 81.

Mutt, V. and Said, S. I. (1974) Structure of the porcine vasoactive intestinal octacosapeptide. The amino-acid sequence. Use of kallikrein in its determination. *Eur.J.Biochem.* **42**, 581 - 589.

Nicholas, R. A., Lazarowski, E. R., Watt, W. C., Li, Q., Boyer, J. and Harden, T. K. (1996) Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. *J.Auton.Pharmacol.* **16**, 319 - 323.

O'Connor, S. E., Dainty, I. A. and Leff, P. (1991) Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol.Sci.* **12**, 137 - 141.

Obara, H., Kusunoki, M., Mori, M., Mikawa, K. and Iwai, S. (1989) The effects of various peptides on the isolated pulmonary artery. *Peptides* **10**, 241 - 243.

Otsuka, M. and Konishi, S. (1976) Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. *Nature.* **264**, 83 - 84.

Otsuka, M., Konishi, S., Takahashi, T. and Saito, K. (1976) Substance P and primary afferent transmission. *Adv.Biochem.Psychopharmacol.* **15**, 187 - 191.

Ottesen, B. (1983) Vasoactive intestinal polypeptide as a neurotransmitter in the female genital tract. *Am.J.Obstet.Gynecol.* **147**, 208 - 224.

Ottesen, B., Larsen, J. J., Fahrenkrug, J., Stjernquist, M. and Sundler, F. (1981) Distribution and motor effect of VIP in female genital tract. *Am.J.Physiol* **240**, E32 - E36.

Ottesen, B., Gram, B. R. and Fahrenkrug, J. (1983a) Neuropeptides in the female genital tract: effect on vascular and non- vascular smooth muscle. *Peptides.* **4**, 387 - 392.

Ottesen, B., Sondergaard, F. and Fahrenkrug, J. (1983b) Neuropeptides in the regulation of female genital smooth muscle contractility. *Acta Obstet.Gynecol.Scand.* **62**, 591 - 592.

Ottesen, B., Gerstenberg, T., Ulrichsen, H., Manthorpe, T., Fahrenkrug, J. and Wagner, G. (1983c) Vasoactive intestinal polypeptide (VIP) increases vaginal blood flow and inhibits uterine smooth muscle activity in women. *Eur.J.Clin.Invest.* **13**, 321 - 324.

Ottesen, B., Pedersen, B., Nielsen, J., Dalgaard, D., Wagner, G. and Fahrenkrug, J. (1987) Vasoactive intestinal polypeptide (VIP) provokes vaginal lubrication in normal women. *Peptides.* **8**, 797 - 800.

Palle, C., Ottesen, B., Jorgensen, J. and Fahrenkrug, J. (1989) Peptide histidine methionine and vasoactive intestinal peptide: occurrence and relaxant effect in the human female reproductive tract. *Biol.Reprod.* **41**, 1103 - 1111.

Palle, C., Bredkjaer, H. E., Fahrenkrug, J. and Ottesen, B. (1991) Vasoactive intestinal polypeptide loses its ability to increase vaginal blood flow after menopause. *Am.J.Obstet.Gynecol.* **164**, 556 - 558.

Palle, C., Ottesen, B. and Fahrenkrug, J. (1992) Peptide histidine valine (PHV) is present and biologically active in the human female genital tract. *Regul.Pept.* **38**, 101 - 109.

Palmer, R. M., Ferrige, A. G. and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature.* **327**, 524 - 526.

Palmer, R. M., Ashton, D. S. and Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature.* **333**, 664 - 666.

Pantaloni, C., Brabet, P., Bilanges, B., Dumuis, A., Houssami, S., Spengler, D., Bockaert, J. and Journot, L. (1996) Alternative splicing in the N-terminal extracellular domain of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor modulates receptor selectivity and relative potencies of PACAP-27 and PACAP-38 in phospholipase C activation. *J.Biol.Chem.* **271**, 22146 - 22151.

Park, K., Goldstein, I., Andry, C., Siroky, M. B., Krane, R. J. and Azadzoi, K. M. (1997) Vasculogenic female sexual dysfunction: the hemodynamic basis for vaginal

engorgement insufficiency and clitoral erectile insufficiency [published erratum appears in Int J Impot Res 1998 Mar;10(1):67]. *Int.J.Impot.Res.* **9**, 27 - 37.

Park, K., Ahn, K., Lee, S., Ryu, S., Park, Y. and Azadzoi, K. M. (2001) Decreased circulating levels of estrogen alter vaginal and clitoral blood flow and structure in the rabbit. *Int.J.Impot.Res.* **13**, 116 - 124.

Parker, D. S., Raufman, J. P., O'Donohue, T. L., Bledsoe, M., Yoshida, H. and Pisano, J. J. (1984) Amino acid sequences of helospectins, new members of the glucagon superfamily, found in Gila monster venom. *J.Biol.Chem.* **259**, 11751 - 11755.

Parra, L., Perez-Vizcaino, F., Alsasua, A., Martin, M. I. and Tamargo, J. (1995) mu- and delta-opioid receptor-mediated contractile effects on rat aortic vascular smooth muscle. *Eur.J.Pharmacol.* **277**, 99 - 105.

Perry, J. D. and Whipple, B. (1982) Multiple components of the female orgasm. In Circumvaginal musculature and sexual function. Graber, B. (Ed.) Karger, Basel.

Pickard, R. S., Powell, P. H. and Zar, M. A. (1991) The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve-evoked relaxation of human cavernosal smooth muscle. *Br.J.Pharmacol.* **104**, 755 - 759

Pierce, K. D., Furlong, T. J., Selbie, L. A. and Shine, J. (1992) Molecular cloning and expression of an adenosine A2B receptor from human brain. *Biochem.Biophys.Res.Commun.* **187**, 86 - 93.

Pisegna, J. R. and Wank, S. A. (1993) Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. *Proc.Natl.Acad.Sci.U.S.A* **90**, 6345 - 6349.

Pisegna, J. R. and Wank, S. A. (1996) Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase C. *J.Biol.Chem.* **271**, 17267 - 17274.

Pluja, L., Fernandez, E. and Jimenez, M. (2000) Electrical and mechanical effects of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide in the rat colon involve different mechanisms. *Eur.J.Pharmacol.* **389**, 217 - 224.

Poucher, S. M., Keddie, J. R., Singh, P., Stoggall, S. M., Caulkett, P. W., Jones, G. and Coll, M. G. (1995) The in vitro pharmacology of ZM 241385, a potent, non-xanthine A2a selective adenosine receptor antagonist. *Br.J.Pharmacol.* **115**, 1096 - 1102.

Preston, A., Lau, W. A., Pennefather, J. N. and Ventura, S. (2000) Effects of adenine nucleosides and nucleotides on neuromuscular transmission to the prostatic stroma of the rat. *Br.J.Pharmacol.* **131**, 1073 - 1080.

Rajfer, J., Aronson, W. J., Bush, P. A., Dorey, F.J. and Ignarro, L. J. (1992) Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N.Engl.J.Med.* **326**, 90 – 94.

Ralevic, V. and Burnstock, G. (1998) Receptors for purines and pyrimidines. *Pharmacol.Rev.* **50**, 413 - 492.

Ralevic, V., Burrell, S., Kingdom, J. and Burnstock, G. (1997) Characterization of P2 receptors for purine and pyrimidine nucleotides in human placental cotyledons. *Br.J.Pharmacol.* **121**, 1121 - 1126.

Rapoport, R. M. and Murad, F. (1983) Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role of cyclic GMP. *J.Cyclic.Nucleotide.Protein Phosphor.Res.* **9**, 281 - 296.

Rapoport, R. M., Draznin, M. B. and Murad, F. (1983) Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature.* **306**, 174 - 176.

Rattan, S. and Chakder, S. (1993) Inhibitory effect of CO on internal anal sphincter: heme oxygenase inhibitor inhibits NANC relaxation. *Am.J.Physiol.* **265**, G799 - G804.

Rees, D. D., Cellek, S., Palmer, R. M. and Moncada, S. (1990) Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem.Biophys.Res.Commun.* **173**, 541 - 547.

Riley, A. J. and Riley, E. J. (1983) Cholinergic and adrenergic control mechanisms in human sexual response. In Psychopharmacology of sexual dysfunction. Wheatley, D. (Ed.) pp. 125 - 137. Oxford University Press, Oxfrod.

Riters, L. V., Absil, P. and Balthazart, J. (1999) Effects of naloxone on the acquisition and expression of appetitive and consummatory sexual behavior in male Japanese quail. *Physiol Behav.* **66**, 763 - 773.

Robaye, B., Boeynaems, J. M. and Communi, D. (1997) Slow desensitization of the human P2Y6 receptor. *Eur.J.Pharmacol.* **329**, 231 - 236.

Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. and Evans, R. M. (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature.* **304**, 129 - 135.

Rouleau, J. R., Dagnault, A., Simard, D., Lavallee, B., Belanger, A., Blouin, A., Kingma, J. G. Jr. (2001) Effect of estrogen replacement therapy on distribution of myocardial blood flow in female anesthetized rabbits. *Am.J.Physiol.Heart.Circ.Physiol.* **281**, H1407 – H1412.

Saenz de Tejada, I., Blanco, R., Goldstein, I., Azadzoi, K., de las Morenas, A., Krane, R.J. and Cohen, R.A. (1988) Cholinergic neurotransmission in human corpus cavernosum. I. Responses of isolated tissue. *Am.J.Physiol.* **254**, H459 – H467

Saenz de Tejada, I., Kim, N., Lagan, I., Krane, R. J. and Goldstein, I. (1989) Regulation of adrenergic activity in penile corpus cavernosum. *J.Urol.* **4**, 1117 – 1121.

Said, S. I. and Mutt, V. (1970) Polypeptide with broad biological activity: isolation from small intestine. *Science.* **169**, 1217 - 1218.

Saito, A. and Goto, K. (1986) Depletion of calcitonin gene-related peptide (CGRP) by capsaicin in cerebral arteries. *J.Pharmacobiodyn.* **9**, 613 – 619.

Saito, A., Kimura, S. and Goto, K. (1986) Calcitonin gene-related peptide as potential neurotransmitter in guinea pig right atrium. *Am.J.Physiol.* **250**, H693 - H698.

Salvatore, C. A., Jacobson, M. A., Taylor, H. E., Linden, J. and Johnson, R. G. (1993) Molecular cloning and characterization of the human A3 adenosine receptor. *Proc.Natl.Acad.Sci.U.S.A* **90**, 10365 - 10369.

Sanders, K. M. and Ward, S. M. (1992) Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am.J.Physiol.* **262,** G379 - G392

Sandoval, M. E. (1980) Sodium-dependent efflux of [3H]GABA from synaptosomes probably related to mitochondrial calcium mobilization. *J.Neurochem.* **35**, 915 - 921.

Sathe, R. S., Komisaruk, B. R., Ladas, A. K. and Godbole, S. V. (2001) Naltrexone-induced augmentation of sexual response in men. *Arch.Med.Res.* **32**, 221 - 226.

Sattin, A. and Rall, T. W. (1970) The effect of adenosine and adenine nucleotides on the cyclic adenosine 3', 5'-phosphate content of guinea pig cerebral cortex slices. *Mol.Pharmacol.* **6**, 13 - 23.

Sauzeau, V., Le Jeune, H., Cario-Toumaniantz, C., Vaillant, N., Gadeau, A. P., Desgranges, C., Scalbert, E., Chardin, P., Pacaud, P. and Loirand, G. (2000) P2Y(1), P2Y(2), P2Y(4), and P2Y(6) receptors are coupled to Rho and Rho kinase activation in vascular myocytes. *Am.J.Physiol Heart Circ.Physiol.* **278**, H1751 - H1761.

Schaffalitzky de Muckadell, O. B., Fahrenkrug, J. and Holst, J. J. (1977) Release of vasoactive intestinal polypeptide (VIP) by electric stimulation of the vagal nerves. *Gastroenterology.* **72**, 373 - 375.

Sellers, L. A., Simon, J., Lundahl, T. S., Cousens, D. J., Humphrey, P. P. and Barnard, E. A. (2001) Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis. *J.Biol.Chem.* **276**, 16379 - 16390.

Sher, E., Biancardi, E., Passafaro, M. and Clementi, F. (1991) Physiopathology of neuronal voltage-operated calcium channels. *FASEB J.* **5**, 2677 - 2683.

Shim, J. O., Shin, C. Y., Lee, T. S., Yang, S. J., An, J. Y., Song, H. J., Kim, T. H., Huh, I. H. and Sohn, U. D. (2002) Signal transduction mechanism via adenosine A1 receptor in the cat esophageal smooth muscle cells. *Cell Signal.* **14**, 365 - 372.

Simonsen, U., Garcia-Sacristan, A. and Prieto, D. (1997) Involvement of ATP in the non-adrenergic non-cholinergic inhibitory neurotransmission of lamb isolated coronary small arteries. *Br.J.Pharmacol.* **120**, 411 - 420.

Smith, M. A., Buxton, I. L. and Westfall, D. P. (1988) Pharmacological classification of receptors for adenyl purines in guinea pig myometrium. *J.Pharmacol.Exp.Ther.* **247**, 1059 - 1063.

Snyder, S. H. (1992) Nitric Oxide: first in a new class of neurotransmitters. *Science.* **257**, 494 - 496.

Somlyo, A.P. and Somlyo, A.V. (2000) Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J.Physiol.* **522**, 177 – 185.

Somlyo, A.P., Wu, X., Walker, L.A. and Somlyo, A.V. (1999) Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Rev.Physiol.Biochem.Pharmacol.* **134**, 201 – 234.

Spector, T. (1978) Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. *Anal.Biochem.* **86**, 142 - 146.

Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H. and Journot, L. (1993) Differential signal transduction by five splice variants of the PACAP receptor. *Nature.* **365**, 170 - 175.

Stasch, J. P., Becker, E. M., Alonso-Alija, C., Apeler, H., Dembowsky, K., Feurer, A., Gerzer, R., Minuth, T., Perzborn, E., Pleiss, U., Schroder, H., Schroeder, W., Stahl, E., Steinke, W., Straub, A. and Schramm, M. (2001) NO-independent regulatory site on soluble guanylate cyclase. *Nature.* **410**, 212 - 215.

Stasch, J. P., Alonso-Alija, C., Apeler, H., Dembowsky, K., Feurer, A., Minuth, T., Perzborn, E., Schramm, M. and Straub, A. (2002) Pharmacological actions of a novel NO-independent guanylyl cyclase stimulator, BAY 41-8543: in vitro studies. *Br.J.Pharmacol.* **135**, 333 – 343.

Steenstrup, B. R., Alm, P., Hannibal, J., Jorgensen, J. C., Palle, C., Junge, J., Christensen, H. B., Ottesen, B. and Fahrenkrug, J. (1995) Pituitary adenylate cyclase-activating polypeptide: occurrence and relaxant effect in female genital tract. *Am.J.Physiol.* **269**, E108 – E117.

Storr, M., Franck, H., Saur, D., Schusdziarra, V. and Allescher, H. D. (2000a) Mechanisms of alpha, beta-methylene atp-induced inhibition in rat ileal smooth muscle: involvement of intracellular Ca^{2+} stores in purinergic inhibition. *Clin.Exp.Pharmacol.Physiol.* **27**, 771 - 779.

Storr, M., Geisler, F., Neuhuber, W. L., Schusdziarra, V. and Allescher, H. D. (2000b) Endomorphin-1 and -2, endogenous ligands for the mu-opioid receptor, inhibit striated and smooth muscle contraction in the rat oesophagus. *Neurogastroenterol.Motil.* **12**, 441 - 448.

Strassheim, D., May, L. G., Varker, K. A., Puhl, H. L., Phelps, S. H., Porter, R. A., Aronstam, R. S., Noti, J. D. and Williams, C. L. (1999) M_3 muscarinic acetylcholine receptors regulate cytoplasmic myosin by a process involving RhoA and requiring conventional protein kinase C isoforms. *J.Biol.Chem.* **274**, 18675 - 18685.

Stull, J.T., Lin, P.J., Krueger, J.K., Trewhella, J. and Zhi, G. (1998) Myosin light chain kinase: functional domains and structural motifs. *Acta.Physiol.Scand.* **164**, 471 – 482.

Suyama, N., Todoki, K. and Okabe E. (1994) A mechanism underlying histamine-induced contraction in isolated rabbit lingual artery. *Nippon.Yakurigaku.Zasshi.* **103**, 175 – 186.

Svoboda, M., Tastenoy, M., Van Rampelbergh, J., Goossens, J. F., De Neef, P., Waelbroeck, M. and Robberecht, P. (1994) Molecular cloning and functional characterization of a human VIP receptor from SUP-T1 lymphoblasts. *Biochem.Biophys.Res.Commun.* **205**, 1617 - 1624.

Szarek, J. L., Spurlock, B., Gruetter, C. A. and Lemke, S. (1998) Substance P and capsaicin release prostaglandin E2 from rat intrapulmonary bronchi. *Am.J.Physiol.* **275**, L1006 – L1012.

Tagaya, E., Tamaoki, J., Chiyotani, A. and Konno, K. (1995) Stimulation of opioid mu-receptors potentiates beta adrenoceptor- mediated relaxation of canine airway smooth muscle. *J.Pharmacol.Exp.Ther.* **275**, 1288 - 1292.

Tatemoto, K. and Mutt, V. (1980) Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature.* **285**, 417 - 418.

Tatemoto, K. and Mutt, V. (1981) Isolation and characterization of the intestinal peptide porcine PHI (PHI-27), a new member of the glucagon--secretin family. *Proc.Natl.Acad.Sci.U.S.A* **78**, 6603 - 6607.

Teixeira, C. E., Bento, A. C., Lopes-Martins, R. A., Teixeira, S. A., von Eickestedt, V., Muscara, M. N., Arantes, E. C., Giglio, J. R., Antunes, E. and de Nucci, G. (1998) Effect of Tityus serrulatus scorpion venom on the rabbit isolated corpus cavernosum and the involvement of NANC nitrergic nerve fibres. *Br.J.Pharmacol.* **123**, 435 – 442.

Teixeira, C. E., Faro, R., Moreno, R. A., Rodrigues Netto, N. Jr., Fregonesi, A., Antunes, E. and De Nucci, G. (2001) Nonadrenergic, noncholinergic relaxation of human isolated corpus cavernosum induced by scorpion venom. *Urology*. **57**, 816 – 820.

Tenhunen, R., Marver, H. S. and Schmid, R. (1968) The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc.Natl.Acad.Sci.U.S.A* **61**, 748 - 755.

Thornbury. K. D., Ward, S. M., Dalziel, H. H., Carl, A., Westfall, D. P. and Sanders, K. M. (1991) Nitric oxide and nitrosocysteine mimic nonadrenergic, noncholinergic hyperpolarization in canine proximal colon. *Am.J.Physiol.* **261**, G553 - G557

Tsuda, A., Tanaka, K. A., Huraux, C., Szlam, F., Sato, N., Yamaguchi, K. and Levy, J. H. (2001) The in vitro reversal of histamine-induced vasodilation in the human internal mammary artery. *Anesth.Analg.* **93**, 1453 – 1459.

Tsukahara, H., Gordienko, D. V. and Goligorsky, M. S. (1993) Continuous monitoring of nitric oxide release from human umbilical vein endothelial cells. *Biochem.Biophys.Res.Commun.* **193**, 722 – 729.

Tucker, J. F., Brave, S. R., Charalambous, L., Hobbs, A. J. and Gibson, A. (1990) L-N^G-nitro arginine inhibits non-adrenergic, non-cholinergic relaxations of guinea-pig isolated tracheal smooth muscle. *Br.J.Pharmacol.* **100**, 663 – 664.

Turcato, S. and Clapp, L. H. (1999) Effects of the adenylyl cyclase inhibitor SQ22536 on iloprost- induced vasorelaxation and cyclic AMP elevation in isolated guinea-pig aorta. *Br.J.Pharmacol.* **126**, 845 - 847.

Uc, A., Oh, S. T., Murray, J. A., Clark, E. and Conklin, J. L. (1999) Biphasic relaxation of the opossum lower esophageal sphincter: roles of NO., VIP, and CGRP. *Am.J.Physiol* **277**, G548 - G554.

Uchida, D., Tatsuno, I., Tanaka, T., Hirai, A., Saito, Y., Moro, O. and Tajima, M. (1998) Maxadilan is a specific agonist and its deleted peptide (M65) is a specific antagonist for PACAP type 1 receptor. *Ann.N.Y.Acad.Sci.* **865**, 253 - 258.

Uchitel, O. D., Protti, D. A., Sanchez, V., Cherksey, B. D., Sugimori, M. and Llinas, R. (1992) P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. *Proc.Natl.Acad.Sci.U.S.A* **89**, 3330 - 3333.

Ulrich, C. D. 2nd, Holtmann, M. and Miller, L. J. (1998) Secretin and vasoactive intestinal peptide receptors: members of a unique family of G protein-coupled receptors. *Gastroenterology.* **114**, 382 – 397.

van Calker, D., Muller, M. and Hamprecht, B. (1979) Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J.Neurochem.* **33**, 999 - 1005.

Van de Voorde, J., Delaey, C., Depypere, H. and Vanheel, B. (1998) Mechanisms involved in the vasorelaxing influence of histamine on isolated human subcutaneous resistance arteries. *Eur.J.Pharmacol.* **349**, 61 - 66.

van der Ploeg, I., Parkinson, F. E. and Fredholm, B. B. (1992) Effect of pertussis toxin on radioligand binding to rat brain adenosine A1 receptors. *J.Neurochem.* **58**, 1221 - 1229.

Van Rampelbergh, J., Gourlet, P., De Neef, P., Robberecht, P. and Waelbroeck, M. (1996) Properties of the pituitary adenylate cyclase-activating polypeptide I and II receptors, vasoactive intestinal peptide1, and chimeric amino- terminal pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal peptide1 receptors: evidence for multiple receptor states. *Mol.Pharmacol.* **50**, 1596 - 1604.

Van Rampelbergh, J., Poloczek, P., Francoys, I., Delporte, C., Winand, J., Robberecht, P. and Waelbroeck, M. (1997) The pituitary adenylate cyclase activating polypeptide (PACAP I) and VIP (PACAP II VIP1) receptors stimulate inositol phosphate synthesis in transfected CHO cells through interaction with different G proteins. *Biochim.Biophys.Acta.* **1357**, 249 - 255.

Vandermeers, A., Gourlet, P., Vandermeers-Piret, M. C., Cauvin, A., De Neef, P., Rathe, J., Svoboda, M., Robberecht, P. and Christophe, J. (1987) Chemical, immunological and biological properties of peptides like vasoactive-intestinal-peptide and peptide-histidine-isoleucinamide extracted from the venom of two lizards (Heloderma horridum and Heloderma suspectum). *Eur.J.Biochem.* **164**, 321 - 327.

Vane, J. R. (1964) The use of isolated organs for detecting active substances in the circulating blood. *Br.J.Pharmacol.* **23**, 360 - 373.

Vemulapalli, S. and Kurowski, S. (2000) Sildenafil relaxes rabbit clitoral corpus cavernosum. *Life.Sci.* **67**, 23 – 29.

Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V. and Snyder, S. H. (1993) Carbon monoxide: a putative neural messenger. *Science* **259**, 381 - 384.

Volke, V., Wegener, G., Vasar, E. and Rosenberg, R. (1999) Methylene blue inhibits hippocampal nitric oxide synthase activity in vivo. *Brain.Res.* **826**, 303 – 305.

Vreman, H. J., Gillman, M. J. and Stevenson, D. K. (1989) In vitro inhibition of adult rat intestinal heme oxygenase by metalloporphyrins. *Pediatr.Res.* **26**, 362 - 365.

Wagner, G. and Levin, R. (1978) Oxygen tension of the vaginal surface during sexual stimulation in the human. *Fertility and Sterility* **30**, 50 - 53.

Wagner, G. and Ottesen, B. (1980) Vaginal blood flow during sexual stimulation. *Obstet.Gynecol.* **56**, 621 - 624.

Wagner, U., Bredenbroker, D., Storm, B., Tackenberg, B., Fehmann, H. C. and Von Wichert, P. (1998) Effects of VIP and related peptides on airway mucus secretion from isolated rat trachea. *Peptides.* **19**, 241 - 245.

Walker, L. A., Gailly, P., Jensen, P. E., Somlyo, A. V. and Somlyo, A. P. (1998) The unimportance of being (protein kinase C) epsilon. *FASEB.J.* **12**, 813 - 821.

Wang, J. P., Chang, L. C., Huang, L. J. and Kuo, S. C. (2001) Inhibition of extracellular Ca(2+) entry by YC-1, an activator of soluble guanylyl cyclase, through a cyclic GMP-independent pathway in rat neutrophils. *Biochem.Pharmacol.* **62**, 679 – 684.

Warren, J. B., Donnelly, L. E., Cullen, S., Robertson, B. E., Ghatei, M. A., Bloom, S. R. and MacDermot, J. (1991) Pituitary adenylate cyclase-activating polypeptide: a novel, long- lasting, endothelium-independent vasorelaxant. *Eur.J.Pharmacol.* **197**, 131 - 134.

Warren, J. B., Cockcroft, J. R., Larkin, S. W., Kajekar, R., Macrae, A., Ghatei, M. A. and Bloom, S. R. (1992) Pituitary adenylate cyclase activating polypeptide is a potent vasodilator in humans. *J.Cardiovasc.Pharmacol.* **20**, 83 - 87.

Werkström, V., Persson, K., Ny, L., Bridgewater, M., Brading, A. F. and Andersson, K. E. (1995) Factors involved in the relaxation of female pig urethra evoked by electrical field stimulation. *Br.J.Pharmacol.* **116**, 1599 - 1604.

Werkström, V., Persson, K. and Andersson, K. E. (1997) NANC transmitters in the female pig urethra - localization and modulation of release via alpha 2-adrenoceptors and potassium channels. *Br.J.Pharmacol.* **121**, 1605 - 1612.

Werkström, V., Alm, P., Persson, K. and Andersson, K. E. (1998) Inhibitory innervation of the guinea-pig urethra; roles of CO, NO and VIP. *J.Auton.Nerv.Syst.* **74**, 33 - 42.

White, R., Ho, W. S., Bottrill, F. E., Ford, W. R. and Hiley, C. R. (2001) Mechanisms of anandamide-induced vasorelaxation in rat isolated coronary arteries. *Br.J.Pharmacol.* **134**, 921 - 929.

Wu, G. and Morris, S.M. (1998) Arginine metabolism: Nitric oxide and beyond. *Biochem.J.* **336,** 1 - 17.

Yegutkin, G. G. and Burnstock, G. (2000) Inhibitory effects of some purinergic agents on ecto-ATPase activity and pattern of stepwise ATP hydrolysis in rat liver plasma membranes. *Biochim.Biophys.Acta* **1466**, 234 - 244.

Yiangou, Y., Requejo, F., Polak, J. M. and Bloom, S. R. (1986) Characterization of a novel prepro VIP derived peptide. *Biochem.Biophys.Res.Commun.* **139**, 1142 - 1149.

Yiangou, Y., Di, M., V, Spokes, R. A., Panico, M., Morris, H. R. and Bloom, S. R. (1987) Isolation, characterization, and pharmacological actions of peptide histidine valine 42, a novel prepro-vasoactive intestinal peptide- derived peptide. *J.Biol.Chem.* **262**, 14010 - 14013.

Yoon, H. N., Chung, W. S., Park, Y. Y., Shim, B. S., Han, W. S. and Kwon, S. W. (2001) Effects of estrogen on nitric oxide synthase and histological composition in the rabbit clitoris and vagina. *Int.J.Impot.Res.* **13**, 205 - 211.

Yoshihara, S., Linden, A., Kashimoto, K. and Watanabe, S. (1998) A novel long-acting VIP analog in the guinea pig trachea in vitro. *Peptides.* **19**, 593 - 597.

Yousufzai, S. Y. and Abdel-Latif, A. A. (1998) Calcitonin gene-related peptide relaxes rabbit iris dilator smooth muscle via cyclic AMP-dependent mechanisms: cross-talk between the sensory and sympathetic nervous systems. *Curr.Eye Res.* **17**, 197 - 204.

Zhang, J., Massmann, G. A., Mirabile, C. P. and Figueroa, J. P. (1999) Nonpregnant sheep uterine type I and type III nitric oxide synthase expression is differentially regulated by estrogen. *Biol.Reprod.* **60**, 1198 - 1203.

Ziessen, T., Moncada, S. and Cellek, S. (2002) Characterization of the non-nitrergic NANC relaxation responses in the rabbit vaginal wall. *Br.J.Pharmacol.* **135**, 546 - 554.

Zygmunt, P. M., Zygmunt, P. K., Hogestatt, E. D. and Andersson, K. E. (1993) Effects of omega-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the rabbit urethra and detrusor. *Br.J.Pharmacol.* **110**, 1285 - 1290.