Biphasic neurogenic vasodilatation in the bovine intraocular long posterior ciliary artery

A thesis submitted for the degree of Doctor of Philosophy in Faculty of Biomedical and Life Sciences at the University of Glasgow

By

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SUMMARY

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Summary

1. Previous research in the bovine intraocular long posterior ciliary artery (LPCA) has suggested that its response to electrical field stimulation (EFS) is a uniphasic vasodilatation, mediated jointly by the neurotransmitters nitric oxide (NO) and calcitonin gene related-peptide (CGRP).
2. This study of the bovine intraocular LPCA, where a short period of EFS (10 s) was employed, demonstrated that the vasodilator response to EFS was in fact biphasic. The first and second components of the response peaked separately at 10 s and 50 s following the onset of stimulation.
3. Both components of vasodilatation to EFS were abolished by tetrodotoxin (TTX), confirming their neurogenic origin.
4. Inhibition of the first component of the vasodilatation to EFS by the nitric oxide synthase (NOS) inhibitor, L-NAME, and the inhibitor of soluble guanylate cyclase, ODQ, confirmed the involvement of NO from nitrergic nerves. Experiments involving treatment with capsaicin and the CGRP antagonist, $CGRP_{8-37}$, failed to produce any evidence of involvement of CGRP in the second component of neurogenic vasodilatation.
5. A number of other potential neurotransmitter candidates including substance P, vasoactive intestinal polypeptide (VIP) and adenosine triphosphate (ATP) were investigated, but there was no convincing evidence to suggest that they play a role in mediating the second component of neurogenic vasodilatation.
6. As the transmitter responsible for mediating the second component of neurogenic vasodilatation could not be identified, the mechanism by which it operated was also investigated.
7. Although the first component of neurogenic vasodilatation was clearly mediated by NO, inhibitors of PDE5, PKG and the soluble guanylate cyclase/cGMP/NO pathway had no effect on the second component of vasodilatation.
8. Furthermore, various K^+ channel blockers, or inhibitors of the PKA/cAMP pathway did not inhibit the second component of neurogenic vasodilatation. Therefore, neither the identity of the neurotransmitter responsible for the second component, nor its mechanism of action could be determined.
9. N^G-substituted analogues of L-arginine are routinely used to inhibit the NOS family of enzymes. The first of these to be introduced, N^G-methyl-L-arginine (L-NMMA), is generally reported to inhibit all three isoforms of NOS. Despite this, however, L-

NMMA does not inhibit nitrergic nerve-mediated relaxation in other bovine tissues including the retractor penis muscle or penile artery.

10. In this study of the bovine intraocular LPCA, L-NMMA was found to inhibit the endothelium-dependent dilatation mediated by eNOS, but not the nitrergic vasodilatation by nNOS. Indeed, the ability of L-NMMA to protect nitrergic dilatation against blockade by L-NAME in a manner similar to L-arginine, suggests it might act as an alternative substrate for nNOS in this tissue.
11. Isoform-selective inhibitors of NOS are of great interest, both as investigational tools and potential therapeutic agents. Two nNOS-specific inhibitors have recently been identified: N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidine tris (trifluoroacetate) salt (AAAN) and N^G-propyl-L-arginine.
12. AAAN failed to affect vasodilatation induced either by nitrergic nerves or by bradykinin-induced, endothelium-derived NO in the bovine intraocular LPCA.
13. Although N^G-propyl-L-arginine did inhibit nitrergic vasodilatation, it also blocked the bradykinin-induced, endothelium-dependent vasodilatation mediated by NO. It thus failed to exhibit the expected selectivity for nNOS over eNOS in the bovine intraocular LPCA.
14. These findings with AAAN and N^G-propyl-L-arginine are a reminder that it is not always possible to extrapolate findings in biochemical assays to functional responses in intact tissues.
15. In conclusion, these findings show that neurogenic vasodilatation in the bovine LPCA involves two components: a fast, transient component mediated by NO from nitrergic nerves, and a second slower, more sustained component mediated by an as yet unidentified second neurotransmitter.

CONTENTS

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Table of Contents

Chapters

LIST OF FIGURES

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List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

ABBREVIATIONS

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Abbreviations

4-AP	4-aminopyridine
7-NI	7-nitroindazole
AAAN	N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidine tris(trifluoroacetate) salt
ACh	acetylcholine
ANP	atrial natriuretic peptide
ANS	autonomic nervous system
ATP	adenosine triphosphate
$BaCl_2$	barium chloride
BH_4	(6R)-5,6,7,8-tetrahydrobiopterin
BK_{Ca}	large conductance calcium-sensitive K^+ channel
BNP	brain natriuretic peptide
BRP	bovine retractor penis
Ca^{2+}	calcium ion
cAMP	cyclic adenosine 3',5'-monophosphate
CBS	cystathionine β-synthase
cGMP	cyclic guanosine 3',5'-monophosphate
CGRP	calcitonin gene-related peptide
$CGRP_{8-37}$	calcitonin gene-related peptide fragment$_{8-37}$
CNP	c-type natriuretic peptide
CNS	central nervous system
CO	carbon monoxide
CO_2	carbon dioxide
COX	cyclo-oxygenase
CSE	cystathionine γ-lysase
DMSO	dimethyl sulfoxide
EC_{50}	half maximal effective concentration
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
EETs	epoxyeicosatrienoic acids
EFS	electrical field stimulation
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinulceotide
FMN	flavin mononucleotide
H_2S	hydrogen sulphide
i.d.	internal diameter
IC_{50}	half maximal inhibitory concentration
IF	inhibitory factor
IK_{Ca}	intermediate conductance calcium-sensitive K^+ channel
iNOS	inducible nitric oxide synthase
IP_3	inositol trisphosphate
K^+	potassium ion
K_{ATP}	ATP-sensitive K^+ channel
K_{IR}	inward rectifying K^+ channel
K_V	voltage-dependent K^+ channel

L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
L-NOARG	N^G-nitro-L-arginine
LPCA	long posterior ciliary artery
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
NA	noradrenaline
NADPH	nicotinamide-adenine-dinucleotide phosphate
NANC	non adrenergic non cholinergic
NK	neurokinin
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
ODQ	1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PACAP	pituitary adenylate cyclase activating polypeptide
PAR	protease activated receptor
PDE	phosphodiesterase
PGI_2	prostacyclin
PHI	peptide histidine isoleucine
PHM	peptide histidine methionine
PKA	protein kinase A
PKG	protein kinase G
PPADS	4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3 benzenedisulfonic acid tetrasodium salt
RB2	reactive blue 2
Rp-8-Br-cAMPS	8-bromoadenosine-3',5'-cyclic monophosphorothioate Rp-isomer
Rp-8-Br-PET-cGMPS	Rp-8-Bromo-β-phenyl-1,N^2-ethenoguanosine 3':5'-cyclic monophosphorothioate sodium salt
SK_{Ca}	small conductance calcium-sensitive K^+ channel
SMC	smooth muscle cell
TEA	tetraethylammonium
TTX	tetrodotoxin
U46619	9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin$F_{2\alpha}$
VIP	vasoactive intestinal polypeptide
VSM	vascular smooth muscle

PUBLICATIONS

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Publications

OVEREND, J., WILSON, W.S. & MARTIN, W. (2005). Biphasic neurogenic vasodilatation in the bovine intraocular long posterior ciliary artery: involvement of nitric oxide and an additional unidentified neurotransmitter. *Br. J. Pharmacol.,* **145**, 1001-1008.

OVEREND, J. & MARTIN, W. (2007). Differential effects of nitric oxide synthase inhibitors on endothelium-dependent and nitrergic nerve-mediated vasodilatation in the bovine ciliary artery. *Br. J. Pharmacol.*, **150**, 488-493.

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DECLARATION

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I declare that this thesis has been composed by myself and is a record of my work performed by myself (except where indicated). It has not been previously submitted for a higher degree.

The research was carried out in the Institute of Biomedical and Life Sciences, in the Division of Neuroscience and Biomedical Systems, University of Glasgow, under the supervision of Professor William Martin.

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01 August 2007

CHAPTER 1.

INTRODUCTION

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1 Introduction

1.1 The Eye

The eye is the organ of vision and operates by detecting light. Only the anterior portion of the eye is visible (Figure 1.1a), the remainder sits within a bony fossa of the skull, which separates the eye from the cranial cavity. This fossa protects the delicate structures of the eye and provides a pathway for ocular nerves and blood vessels. The anatomy of the eye is dedicated to focusing light onto the photosensitive cells of the retina. The wall of the eyeball can be divided into three layers: the fibrous tunic, the vascular tunic and the nervous tunic.

1.1.1 Fibrous Tunic

The fibrous tunic is the outermost layer and provides the eyeball with shape, whilst protecting the delicate inner structures of the eye. It consists of the anterior cornea, which covers one-sixth of the surface area, and the posterior sclera, which covers the remaining five-sixths. The cornea is an avascular, curved, transparent coat that covers the iris and helps to focus light entering the eye. In the bovine eye, the cornea is roughly pear shaped (Prince *et al.*, 1960). The sclera is better known as the "white" of the eye and is a protective coat of dense connective tissue. This makes the eye more rigid and also serves as an attachment for extraocular muscles that move the eye (Figure 1.1b).

1.1.2 Vascular Tunic

The vascular tunic or uvea is the middle layer of the eyeball and is made up of the choroid, the ciliary body and the iris. The choroid is a vascular layer that lines the sclera and nourishes the retina. It is highly pigmented and the bovine choroid appears almost black in colour. The ciliary body connects the choroid with the iris and is located just behind the iris. Attached to the ciliary body are tiny fibres called zonules and these fibres hold the large, crystalline lens of the eye in place. The ciliary body produces a fluid known as aqueous humor, which nourishes both the cornea and lens and gives shape to the eye. Another function of the ciliary body is to change the shape of the lens (accommodation) to enable the eye to focus on both near and distant objects. However, in the bovine eye there is no real evidence of functional activity of the ciliary body, with respect to the accommodation process (Prince *et al.*, 1960).

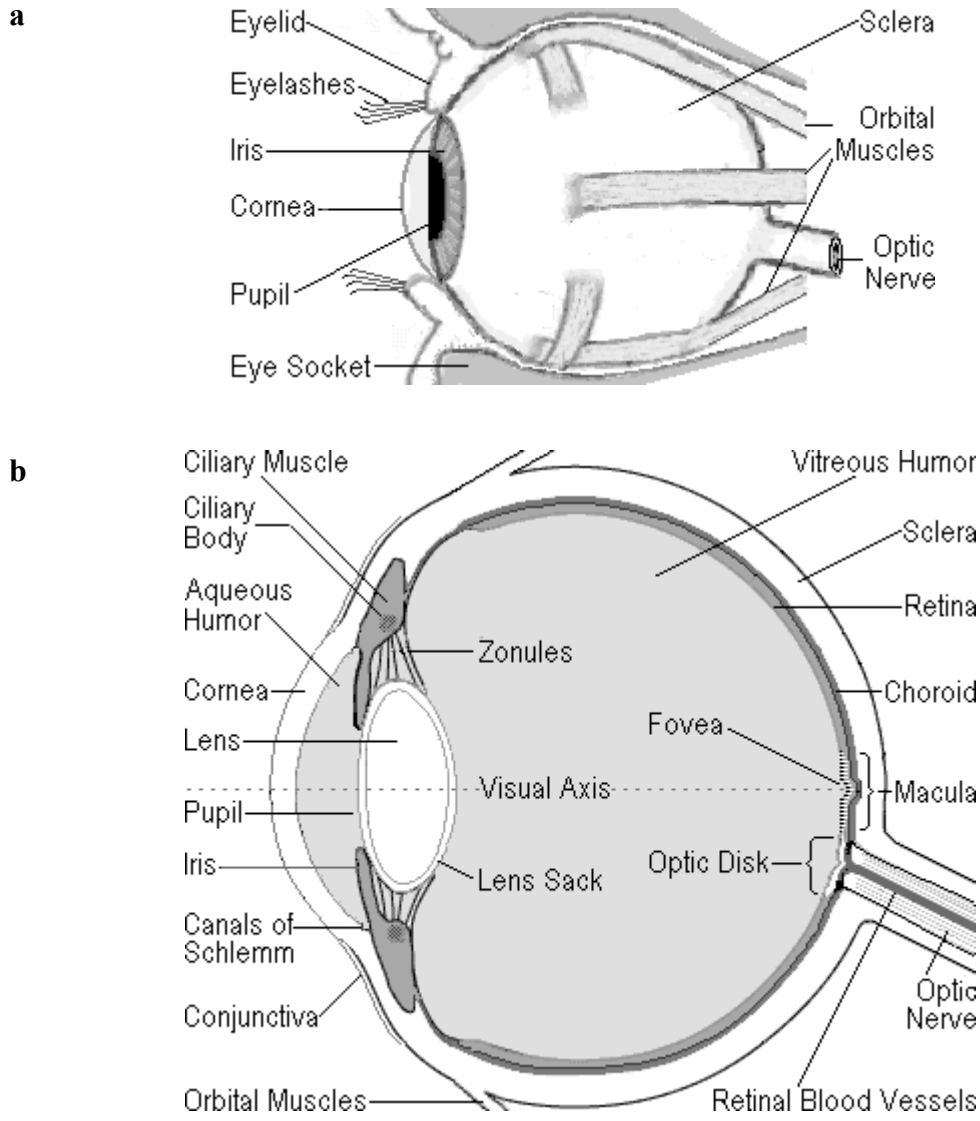


Figure 1.1 Schematic diagrams showing (a) the external and (b) the internal structures of the mammalian eye. Taken from www.99main.com/~charlief/Blindness.htm

The iris is a circular, pigmented diaphragm that divides the space between the cornea and the lens into two chambers, the anterior and posterior, both of which are filled with aqueous humor. A third chamber exists between the lens and the retina and this is filled by a more viscous liquid, vitreous humor. The opening in the middle of the iris is known as the pupil. In the bovine eye, this is oval in shape and becomes almost circular upon dilation (Prince *et al.*, 1960). The size of the pupil determines the amount of light that enters the eye, with pupil size controlled by the dilator and sphincter muscles of the iris (Figure 1.1b).

1.1.3 Nervous Tunic

The innermost layer of the eye, or nervous tunic, is more commonly known as the retina. The retina itself is comprised of many layers, made up of two types of photoreceptor cells, known as rods and cones. As these are neural cells it is essential that they, like the brain, receive a constant oxygen supply. Cones are situated in a central area of the retina, known as the macula. More specifically, they are densely packed within the central area of the macula, the fovea. Cones operate best in bright light and allow us to detect colour. The rods are spread throughout the retina and operate best in dim light, giving us "night vision". Nerve impulses are carried by the optic nerve from the cells of the retina to the brain. The outer layers of the retina receive nutrients and oxygen from the choroid, while the retinal blood vessels nourish the inner layers (Figure 1.1b).

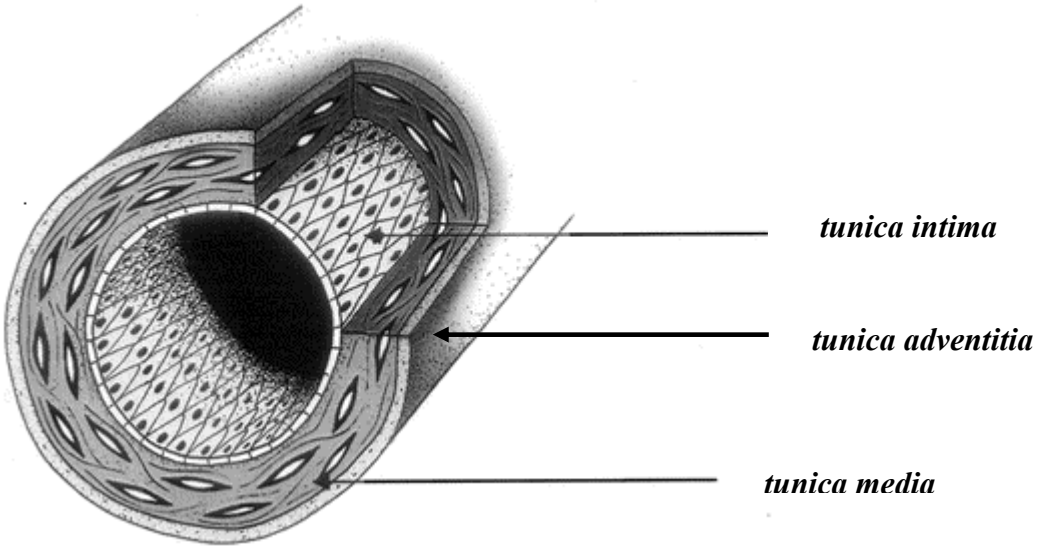
1.2 Structure and Function of Arteries

Arteries are the vessels of the vascular system responsible for transporting blood from the heart to all of the tissues in the body. However, they are not passive "tubes" that simply allow blood to flow through the body. The largest artery is the aorta, which progressively divides into smaller elastic arteries, conduit or muscular arteries, terminal arteries, arterioles and then capillaries. Together the terminal arteries and arterioles are known as the resistance vessels or small arteries and have a diameter of 100 – 500 μm (Bloom & Fawcett, 1968). All blood vessels, apart from capillaries, have a wall comprising of three morphologically distinct layers.

1.2.1 Tunica Intima

The *tunica intima* is the innermost layer, which surrounds the lumen. It consists of a flat layer of endothelial cells, which rest on a bed of collagen and proteoglycans, and is bound

a



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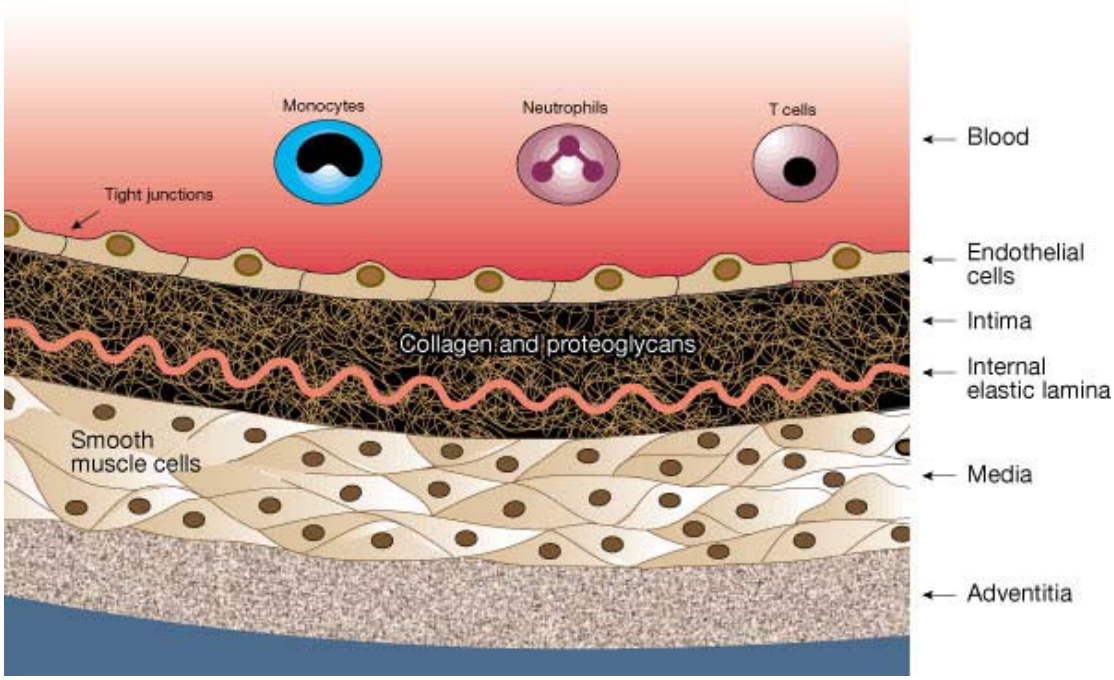


Figure 1.2 Schematic diagram of (a) the structure of a typical artery, showing the three distinct layers, the *tunica intima,* the *tunica media* and the *tunica adventitia* (adapted from Bryan *et al.,* 2005), and (b) arterial structure in more detail (taken from Lusis, 2000).

by a sheet of elastic fibres called the internal elastic lamina (Figure 1.2a and 1.2b). Until quite recently, the endothelium was considered merely to be a barrier between the blood and vascular smooth muscle (VSM). The discovery by Furchgott & Zawadzki (1980) that the endothelium influences the underlying vascular smooth muscle by synthesising and secreting endothelium-derived relaxing factor (EDRF), now regarded as nitric oxide (NO; Palmer *et al*., 1987), was a catalyst for further, intense study of the endothelium. The discovery that it releases not only NO, but a number of other vasoactive substances, such as endothelium-derived hyperpolarising factor (EDHF; Chen *et al*., 1988) and prostacyclin (PGI_2; Moncada *et al*., 1976), which cause smooth muscle relaxation, and endothelin (De May & Vanhoutte, 1981), a potent vasoconstrictor, has meant that the endothelium is now recognised as a vital regulatory component of the cardiovascular system. As well as local vascular control, the endothelium is also involved in transport of metabolites and signalling molecules between blood and tissues (Baldwin & Thurston, 2001) and inhibition and activation of platelet aggregation (Wu & Thiagarajan, 1996). The loss of endothelial function is implicated in pathophysiological states, such as atherosclerosis (Lusis, 2000).

1.2.2 Tunica Media

The *tunica media*, or middle layer, consists of spindle-shaped smooth muscles cells (SMC) arranged in a helical pattern, surrounded by a matrix of collagen and elastin and bound by the external elastic lamina (Figure 1.2a and 1.2b). The degree of contraction of this VSM controls the diameter of the lumen and therefore the blood flow through it. The contractile unit within the cell is composed of actin and myosin filaments. Agents that elicit contraction of VSM increase intracellular calcium (Ca^{2+}) concentration ($[Ca^{2+}]_i$) and those that elicit vasodilatation reduce $[Ca^{2+}]_i$ or desensitise the contractile apparatus to Ca^{2+}. In SMC, Ca^{2+} and calmodulin regulate the activity of the enzyme myosin-light chain kinase (MLCK), which phosphorylates the regulatory light chains of myosin (MLC). Phosphorylation of MLC allows the myosin ATPase to be activated by actin and the muscle to contract (Levick, 2000). The enzyme MLC phosphatase (MLCP) is responsible for dephosphorylation of MLC. Dephosphorylation of MLC promotes relaxation (Pfitzer, 2001).

1.2.3 Tunica Adventitia

The outer layer, or *tunica adventitia*, is less structured than the other two layers and is a sheath of connective tissue interspersed with collagen bundles, elastin fibres and fibroblasts, which loosely tethers the artery in place (Figure 1.2a and 1.2b). In larger

vessels, the *adventitia* also contains smaller blood vessels known as the *vasa vasorum,* which penetrate into the *media* and nourish the SMC. Recent evidence suggests that the *adventitia* is also much more physiologically relevant than perhaps was first thought. It may act as a biological processing centre for retrieval, integration, storage and release of key regulators of vessel wall function. In particular, the adventitial fibroblast seems to have an important role in the response to vascular stress such as hypoxia or over-distension of the vessel. Once activated, the fibroblast may undergo phenotypic changes such as proliferation, differentiation and release factors that affect neighbouring smooth muscle tone and growth. They also stimulate recruitment of inflammatory and progenitor cells to the vessel wall (Stenmark *et al*., 2006).

1.3 Regulation of Vascular Tone

The active tension exerted by VSM in a segment of wall is called vascular tone. Arteries and arterioles have a degree of tone (i.e. are partially constricted) at all times, known as basal tone. In order to undergo vasodilatation, a vessel must initially have some degree of basal tone. Vessel tone can be influenced by a number of mechanisms. As mentioned in section 1.2.1, the endothelium releases the vasodilators NO, PGI_2 and EDHF and endothelin, which causes vasoconstriction. In addition to this, nerves that innervate arteries release a variety of vasoactive transmitters capable of causing vasodilatation or vasoconstriction.

Furthermore, in tissues such as skeletal muscle, the heart and the brain, an increase in metabolic activity results in metabolic hyperaemia. This is an increase in blood flow to the tissue caused by vasodilatation of the arteries and arterioles in response to the products of metabolism. Metabolic activity results in the accumulation of carbon dioxide (CO_2), lactate, potassium (K^+) and adenosine, which results in vasodilatation (Madden, 1993; Clifford & Hellsten, 2004).

Circulating hormones also play a role in vascular tone, For example, catecholamines released from the adrenal gland (Wong, 2003), vasopressin from the pituitary gland (Treschan & Peters, 2006) and the renin-angiotensin-aldosterone system (Seravalle *et al.,* 1993) promote vasoconstriction in the vast majority of tissues, while natriuretic peptides (atrial natriuretic peptide; ANP, brain natriuretic peptide; BNP and cardiac natriuretic peptide; CNP) released from atrial and ventricular myocytes and the endothelium, respectively, promote vasodilatation (Ahluwalia *et al.,* 2004).

Finally, myogenic tone, a contributing factor to basal tone, is the result of the contraction of VSM in response to the mechanical distension caused by an increase in pressure within the vessel. This mechanism, discovered by Sir William Bayliss, is important in stabilising blood flow in response to changes in blood pressure (Levick, 2000).

1.4 Neurogenic Control of Vascular Tone

Blood vessels are innervated by a variety of different types of nerves. These nerves are capable of mediating vasoconstriction, and in a small number of vessels vasodilatation, depending on the neurotransmitter released. They run along the adventitial-medial border of the blood vessel and do not directly innervate the inner part of the media. The neurotransmitter is released from the nerve terminal into a cleft between the nerve and VSM, known as the neuroeffector junction. This cleft can be as small as 60 nm in some arterioles or as great as 2 μm in some large elastic arteries (Burnstock, 1993).

1.4.1 Sympathetic Constrictor Nerves

1.4.1.1 Anatomy of Sympathetic Constrictor Nerves

Sympathetic nerves are part of the autonomic nervous system (ANS). The main difference between somatic and autonomic nervous systems is that the ANS consists of a chain of two neurons, while in the somatic system, one neuron connects the central nervous system (CNS) to the skeletal muscle. The two neurons in the autonomic pathway are known as the preganglionic and the postganglionic fibres and they synapse in an autonomic ganglion. This lies outwith the CNS and contains the nerve endings of the preganglionic fibre and the cell body of the postganglionic fibre (Tortora, 1999). Sympathetic preganglionic neurons have their cell bodies in the lateral horn of the grey matter of the thoracic and lumbar segments of the spinal cord. The fibres leave the spinal cord in spinal nerves as the thoracolumbar sympathetic outflow. They leave the spinal cord as filaments that run to the paravertebral chain of sympathetic ganglia (bilaterally on either side of spinal cord). These ganglia contain the cell bodies of the postganglionic sympathetic neurons, the axons of which rejoin the spinal nerve (Tortora, 1999). Many of the postganglionic sympathetic fibres reach their destination via branches of the spinal nerve. The nerve terminals of sympathetic fibres, which innervate all blood vessels, end in nodules known as varicosities. Each varicosity is filled with many synaptic vesicles, which are the site of synthesis and release of the main sympathetic neurotransmitter, noradrenaline (NA; Tortora, 1999).

1.4.1.2 Pharmacology of Sympathetic Constrictor Nerves

NA released from sympathetic varicosities binds to various adrenoceptors on VSM. Ahlquist (1948) identified two classes of adrenoceptors, which he named α and β based upon differential responses to various catecholamines such as adrenaline, NA and isoprenaline. Some years later, Langer (1974) suggested subclassification of α-receptors into α_1 and α_2 subtypes. Cloning of adrenergic receptors, has subsequently revealed at least 9 subtypes of adrenoceptors (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3; Insel, 1996). On the whole, vasoconstriction is mediated by activation of α_1-adrenoceptors on the membrane of VSM, via activation of the inositol trisphosphate (IP_3) pathway and release of Ca^{2+} stored within the sarcoplasmic reticulum. Although α_2-adrenoceptors mediate vasoconstriction in cutaneous arterioles, they more commonly occur on nerve fibres, as prejunctional receptors (also known as autoreceptors). Activation of these prejunctional receptors by NA inhibits further NA release from the nerve terminal. Until the 1970s, the theory that nerves released a single transmitter, commonly known as Dale's Principle, was widely accepted. The suggestion by Burnstock (1976) that adenosine 5' triphosphate (ATP) may be released from adrenergic nerves, along with NA, has since been confirmed, initially with evidence from the guinea-pig vas deferens (Fedan *et al*., 1981; Sneddon & Burnstock, 1984b). Purinergic cotransmission was later described in the rat tail artery (Sneddon & Burnstock, 1984a) and in the rabbit saphenous artery (Burnstock & Warland, 1987a). ATP mediates vasoconstriction by activating postjunctional P2X purinergic receptors. Another co-transmitter released with NA, especially at high stimulation frequencies, is neuropeptide Y (NPY; Lundberg *et al*., 1986). Neuropeptide Y actives the Y_1 receptor on VSM to initiate vasoconstriction.

1.4.2 Sympathetic Vasodilator Nerves

In some species, such as cats and dogs (Bulbring & Burn, 1935; Uvnäs, 1966), small arteries that serve the skeletal muscle are innervated by sympathetic vasodilator nerves, as well as sympathetic vasoconstrictor nerves. These nerves utilise acetylcholine (ACh) as their neurotransmitter and excitation of sympathetic cholinergic nerves causes relaxation of VSM and increased muscle blood flow, as part of the defence response (Abrahams *et al*., 1964). The presence of these nerves in the arteries of human skeletal muscle is highly contentious. Proponents of the theory have suggested that these nerves are activated at times of emotional or physical stress, akin to the defence mechanism in animals. Much of the evidence has arisen from an observation that there is marked skeletal muscle

vasodilatation in the forearm, when the subject is under mental stress or at the time of fainting, which is absent following surgical sympathectomy (Barcroft & Edholm, 1945; Blair *et al*., 1959). However, there is no histochemical evidence to demonstrate sympathetic cholinergic vasodilator nerves in the human forearm and most researchers believe that the vasodilatation is a result of circulating catecholamines and locally released NO (Reed *et al*., 2000).

1.4.3 Parasympathetic Vasodilator Nerves

Most blood vessels lack parasympathetic vasodilator nerves. However, they can be found in a small number of tissues, most commonly in the brain (Biesold *et al*., 1989), reproductive system (Toda *et al*., 2005), eye (Toda *et al*., 1997) and coronary circulation (Broten *et al*., 1992; Feigl, 1969).

1.4.3.1 Anatomy of Parasympathetic Vasodilator Nerves

Parasympathetic nerves arise from two separate regions of the CNS. The cranial outflow consists of preganglionic fibres in certain cranial nerves (e.g. vagus, glossopharyngeal, oculomotor and facial nerves; Tortora, 1999). Ganglia lie close to the target organ, with short postganglionic fibres compared to the sympathetic system. Parasympathetic fibres destined for pelvic and abdominal viscera emerge from the sacral outflow from the spinal cord (nervi erigentes). They synapse in pelvic ganglia, with short postganglionic fibres that run to the bladder, genitalia, etc. Although the parasympathetic branch of the ANS utilises ACh as its primary postganglionic neurotransmitter (Tortora, 1999), this does not appear to be the case with parasympathetic vasodilator nerves.

1.4.3.2 Pharmacology of Parasympathetic Vasodilator Nerves

Early studies suggested parasympathetic vasodilator nerves did exist in the cerebral circulation, utilising ACh as a neurotransmitter (Biesold *et al*., 1989; Busija & Heistad, 1984). Indeed there was anatomical and physiological evidence to support this theory, including a dense network of choline acetyltransferase (ChAT)-immunoreactive fibres (Saito *et al*., 1985; Yu *et al*., 1992) and measurable ChAT activity in the cerebral vessels (Florence & Bevan, 1979). However, in the early 1980s, Lee (Lee 1980; 1982) suggested that nerve-derived ACh did not induce vasodilatation in the cat cerebral artery. In fact, he provided evidence that ACh was a vasoconstrictor neurotransmitter in this vessel. It is now widely accepted that although exogenously applied ACh can initiate vasodilatation by

activating muscarinic receptors on endothelial cells, ACh released from parasympathetic nerves does not induce vasodilatation. Instead, NO released from nitrergic nerves is responsible for the vasodilatory effects of parasympathetic nerve stimulation (Lee *et al.,* 2001). However, ACh either co-released from these nerves or from separate cholinergic nerves does have a role to play in the regulation of vascular tone in monkey, porcine and canine ciliary arteries through presynaptic inhibition of release of NO and noradrenaline from nitrergic and adrenergic nerves, respectively (Toda *et al.*, 1997; 1998; 1999).

1.4.4 Non-Adrenergic Non-Cholinergic (NANC) Nerves

For many years it was thought that only adrenergic and cholinergic systems of autonomic nerve transmission existed and this became known as the autonomic paradigm. However, from the end of the 19th century until the discovery of adrenergic neuron blocking agents, nerve mediated responses that did not easily fit the autonomic paradigm were reported, but their significance not fully understood. For example, the research group led by Burnstock (Burnstock *et al*., 1963; Burnstock *et al*., 1964) reported that transmural stimulation of guinea pig taenia coeli produced inhibitory junction potentials in smooth muscle cells, which were associated with relaxation and persisted in the presence of atropine and guanethidine. As the number of specific adrenergic blocking agents grew, it became apparent that NANC nerves did indeed exist and played an important role in the relaxation of smooth muscle in the gastrointestinal (Hata *et al*., 2000), respiratory (Linden, 1996) and urogenital tracts (Andersson & Holmquist, 1994). The theory of NANC transmission is now widely accepted and a number of NANC transmitters have been identified: NO, calcitonin gene related peptide (CGRP), substance P, vasoactive intestinal polypeptide (VIP), NPY and ATP.

1.4.5 Nitrergic Nerves

Much of the research that suggested NO was a putative NANC transmitter was carried out in the anococcygeus and retractor penis (RP) muscles of various species, as these muscles receive dual innervation consisting of a motor adrenergic component from the lumbar sympathetic output and an inhibitory NANC component from the sacral parasympathetic output (Gillespie, 1971; Klinge & Sjostrand, 1974). A labile compound, termed inhibitory factor (IF) was extracted from the bovine RP (BRP) and rat anococcygeus muscle (Gillespie *et al*., 1981; Gillespie & Martin, 1980), which was able to replicate the relaxation witnessed upon stimulation of NANC nerves. At around this time it was also

noted that IF (now identified as an NO-releasing s-nitrosothiol) bore a striking resemblance to the newly identified vasodilator, EDRF (Furchgott & Zawadzki, 1980). The discovery that oxyhaemoglobin blocked both relaxation to electrical field stimulation (EFS) in the BRP and rat anococcygeus muscle (Bowman & Gillespie, 1982) and also EDRF in the rabbit aorta (Martin *et al*., 1985), further suggested involvement of a common substance. In addition to this, the relaxant effects of EDRF, IF and NANC nerves in the BRP were all associated with an increase in cyclic guanosine 3',5'-monophosphate (cGMP; Rapoport & Murad, 1983), were blocked by the inhibitor of soluble guanylate cyclase, methylene blue (Martin *et al*., 1985), and potentiated by the selective phosphodiesterase 5 (PDE5) inhibitor, zaprinast (Bowman & Drummond, 1984). The identification of EDRF as NO, the elucidation of the NO synthesis pathway and subsequent development of NOS inhibitors, such as N^G-monomethyl-L-arginine (L-NMMA), eventually resulted in the inhibition of NANC relaxation in the rat (Gillespie *et al*., 1989) and mouse (Gibson *et al*., 1990) anococcygeus muscle. Furthermore, although L-NMMA did not block NANC relaxation in the BRP, it was blocked by another NOS inhibitor, N^G-nitro-L-arginine (L-NOARG), confirming the role of the L-arginine-NO pathway in NANC transmission in this tissue also (Liu *et al*., 1991). Moreover, Gillespie & Sheng (1990) continued to investigate the hypothesis that NANC nerves released NO in these tissues and utilised drugs capable of disabling NO by generating superoxide anions. They examined the effects of pyrogallol and hydroquinone on the BRP and rat anococcygeus muscle response to NANC nerve stimulation. Both drugs significantly reduced the NANC response to stimulation in the rat anococcygeus muscle but not the BRP muscle. In rat anococcygeus, the action of pyrogallol was reversed by superoxide dismutase, suggesting the inhibition by pyrogallol was due to the generation of superoxide anions. This was taken as further evidence that NO may have a neurotransmitter function, at least in some tissues.

Parasympathetic, NANC vasodilator nerves were first discovered in dog cerebral arteries (Toda, 1975). He suggested that nicotine caused a transient relaxation in strips of isolated canine cerebral arteries by activating a specific nicotinic receptor and that β-adrenergic and cholinergic mechanisms did not have a large role to play in this process. Fifteen years later Toda & Okamura (1990) suggested that NO could act as a neurotransmitter in VSM, following further experimentation with dog cerebral artery strips. EFS of such strips resulted in relaxation, which was abolished by tetrodotoxin (TTX). The NOS inhibitor L-NMMA inhibited relaxation in a concentration-dependent manner. The actions of L-NMMA were reversed by L-arginine, but not D-arginine. In endothelium-denuded tissue, responses to EFS and L-NMMA were not significantly different from tissues with intact

endothelium. Toda & Okamura also measured what they termed as NO_x, this being NO or its breakdown products (NO_2^- and NO_3^-) and found that the level of NO_x in the perfusate of tissues increased significantly following EFS. From this evidence they hypothesized that NO or an NO related compound had a role in transmitting information from nerve to VSM in this tissue.

It is now widely recognised that NO transmission is an important control mechanism in many autonomically innervated tissues. In the literature nerves that operate via the release of NO as a neurotransmitter have been called both nitroxidergic (Toda & Okamura, 1991) and nitrergic (Rand, 1992). The issue was resolved by the NO Nomenclature Committee of the International Union of Pharmacology, which chose "nitrergic" as the official term (Moncada *et al*., 1997).

1.4.5.1 NO Synthesis

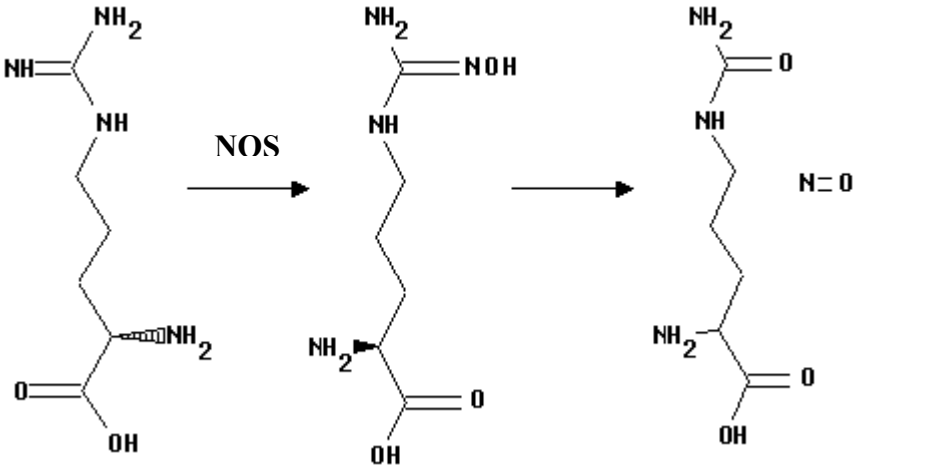
NO is synthesised by the NO synthase (NOS) family of enzymes, from the amino acid L-arginine (Palmer *et al*., 1988). This two-step process, which also utilises O_2, results in the formation of L-citrulline and NO via the intermediate product N^G-hydroxy-L-arginine (Figure 1.3). L-arginine is obtained from the extracellular space via a cation amino acid transporter system (Closs *et al.,* 2006) or by synthesis from L-citrulline inside the cell via argininosuccinate synthetase and argininosuccinate lysase (Mori & Gotoh, 2000).

Three distinct forms of NOS exist and all three have been purified, characterised and cloned:

1. Neuronal NOS, also known as nNOS, ncNOS, bNOS or NOS I (referred to as nNOS in the present study). nNOS was the first member of the NOS family to be purified, from the rat cerebellum (Bredt & Snyder, 1990). However, nNOS has also been identified in a number of other tissues, including skeletal muscle (Nakane *et al*., 1993) and epithelial cells of rat lungs, uterus and stomach (Schmidt *et al*., 1992). Human nNOS is 161 kDa in size (Nakane *et al*., 1993).

2. Inducible NOS, also known as iNOS, mNOS and NOS II (referred to as iNOS in the present study). iNOS was first identified in murine macrophages (Hevel *et al*., 1991; Stuehr *et al*., 1991) and has since been identified in murine and human lung epithelial cells (Asano *et al*., 1994; Robbins *et al*., 1994) and the pancreatic islets of

diabetic rats (Kleemann *et al*., 1993). iNOS is slightly smaller than nNOS and is 131 kDa in size (Charles *et al*., 1993).



L-arginine **N^G-hydroxy-L-arginine** **L-Citrulline + NO**

Figure 1.3 Schematic diagram showing the synthesis of NO from the amino acid L-arginine. NO is synthesised by the nitric oxide synthase (NOS) family of enzymes, in a two-step process, which also utilises O_2 and results in the formation of L-citrulline and NO via the intermediate product N^G-hydroxy-L-arginine.

3. Endothelial NOS, also known as eNOS, ecNOS and NOS III (referred to as eNOS in the present study). The third member of the NOS family was initially discovered in endothelial cells of the bovine aorta (Pollock *et al*., 1991). eNOS has also been identified in syncytiotrophoblasts of human placenta (Myatt *et al*., 1993) and kidney tubular epithelial cells (Tracey *et al*., 1994). eNOS is similar in size to iNOS and has been characterised as 133 kDa (Marsden *et al*., 1992).

All three isoforms require the cofactors nicotinamide-adenine-dinucleotide phosphate (NADPH), (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and contain an iron protoporphorin IX (haem) prosthetic group. eNOS and nNOS are constitutively expressed in cells (Bredt & Snyder, 1990) and synthesise NO in response to an increase in intracellular Ca^{2+} concentration (Busse & Mülsch, 1990) and other Ca^{2+}-independent processes, such as shear stress (Fleming *et al*., 1998). In contrast, iNOS is not constitutively expressed and is induced by inflammatory cytokines (interleukin-1, tumor necrosis factor-α, interferon-γ) and bacterial endotoxins (Hierholzer *et al*., 1998). However, Dweik *et al*. (1998) reported that iNOS is expressed constitutively in lung epithelial cells, where it controls generation of NO in proportion to the inspired oxygen concentration.

1.4.5.2 Inhibitors of NOS

Inhibition of NOS, especially of specific NOS isoforms, is of great pharmacological and therapeutic interest. For example nNOS has been implicated in the pathophysiology of many neurodegenerative diseases, such as Parkinsons disease (Hantraye *et al*., 1996) and iNOS has been implicated in autoimmune and inflammatory disorders such as rheumatoid arthritis (Evans *et al*., 1995). Specific and non-specific NOS inhibitors have already been identified and these are usually classified by their mechanism of action.

The discovery that L-arginine was the substrate for NOS led to the identification of a number of L-arginine analogues, which occupy the substrate binding site on NOS and prevent NO formation. The first L-arginine analogue identified was L-NMMA (Hibbs *et al*., 1987), which inhibits all NOS isoforms. Another commonly used L-arginine analogue, L-NOARG (Moore *et al*., 1990) is commonly administered in prodrug form as N^G-nitro-L-arginine methyl ester (L-NAME; Hobbs & Gibson, 1990). L-arginine analogues are generally reported as being non-specific NOS inhibitors (Rees *et al*., 1990). N^G-propyl-L-

arginine (Zhang *et al*., 1997a) is a putative nNOS-specific inhibitor, that is reported to exhibit ~150-fold selectivity for bovine nNOS over eNOS in enzyme assays (Zhang *et al*., 1997b).

Another putative nNOS-selective inhibitor is N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidine tris(trifluoroacetate) (AAAN), a nitroarginine-containing dipeptide amide, which has a ~2,500-fold greater selectivity for rat nNOS over bovine eNOS (Hah *et al*., 2001). Other NOS inhibitors do not bind to the substrate-binding site, but instead attach to another part of the NOS molecule. For example, imidazole and compounds that contain this structure, bind to the haem site and are weak NOS inhibitors, which are relatively iNOS selective (Chabin *et al*., 1996). The indazole family are also NOS inhibitors. The best-studied member of this group is 7-nitroindazole (7-NI). In isolated enzyme studies 7-NI is an equipotent inhibitor of all three enzymes (Alderton *et al*., 2001), however, when administered *in vivo*, it has also been described as an nNOS specific inhibitor (Southan & Szabo, 1996).

1.4.5.3 Mechanism of NO-Mediated Vasodilatation

NO-induced vasodilatation is mediated via the second messenger, cGMP, which plays an important role in many cellular signal transduction pathways. The level of cGMP within a cell is determined by the rate of synthesis, by guanylate cyclase, and the rate of hydrolysis by cyclic nucleotide phosphodiesterases (PDEs).

1.4.5.3.1 cGMP Synthesis

cGMP can be synthesised by two different forms of guanylate cyclase, particulate guanylate cyclase and soluble guanylate cyclase. Particulate guanylate cyclase is a membrane bound enzyme and is activated by the binding of peptide ligands, such as ANP and BNP, to membrane bound receptors that have transmembrane domains coupled with guanylate cyclase. Soluble guanylate cyclase is located within the cytoplasm of the cell and is an intracellular target of NO (Figure 1.4). NO activates soluble guanylate cyclase by binding to a haem moiety in the structure of the enzyme and inducing a conformational change. This, in turn, activates the catalytic site of the enzyme to produce cGMP (Ignarro *et al*., 1984).

1.4.5.3.2 Activation of cGMP-Dependent Protein Kinase

An increase in intracellular cGMP serves to activate cGMP-dependent protein kinase (PKG). A number of forms of PKG exist, mainly a soluble form (type I, α and β) and a membrane-bound form (type II). Both type Iα and Iβ have been identified in VSM (Keilbach *et al*., 1992). As discussed earlier, VSM contracts when $[Ca^{2+}]_i$ is increased and dilates when $[Ca^{2+}]_i$ is decreased. PKG activation has been reported to mediate vasodilatation by reducing cytosolic Ca^{2+} in a number of ways:

1. Inhibition of Ca^{2+} influx through voltage-gated (L-type) Ca^{2+} channels and by promotion of Ca^{2+} uptake into the sarcoplasmic reticulum (Clapp & Gurney, 1991) via phosphorylation of phospholamban (Cornwell *et al*., 2006).

2. Activation of the Na^+/Ca^{2+} exchanger (Furukawa *et al*., 1991).

3. Activation of the plasma membrane Ca^{2+}-ATPase (Yoshida *et al*., 1991).

4. Inhibition of inositol phosphate formation, which is important for Ca^{2+} release (Hirata *et al*., 1990).

PKG activation is reported to induce vasodilatation by triggering phosphorylation of MLCK, which leads to decreased Ca^{2+} sensitivity (Himpens *et al.,* 1989) and also by activating K_{Ca} channels, leading to hyperpolarisation of the SMC (Robertson *et al*., 1993). However, NO can also directly activate K_{Ca} channels in the absence of cGMP (Bolotina *et al*., 1994).

1.4.5.3.3 The PDE Family

The PDE group is a large family of enzymes that catalyse hydrolysis of the 3'-phosphoester bond of both cGMP and cyclic adenosine 3', 5'-monophosphate (cAMP). Currently, at least 11 distinct PDE families have been identified (Soderling & Beavo, 2000). Several genes encode each family and there are over 50 different PDE enzymes. Although each family differs in structure, distribution, regulation and function, all PDEs have a core of ~ 270 highly conserved amino acids, which make up the catalytic domain (Charbonneau, 1990). PDEs within each family are at least 65% homologous but between families this homology drops to 40%, with most of the similarity in the catalytic domain (Beavo, 1995). Each PDE family hydrolyses cGMP (PDE5, PDE6 and PDE9), cAMP

(PDE4, PDE7 and PDE8) or both cyclic monophosphates (PDE1, PDE2, PDE3, PDE10 and PDE11).

Not all of the PDE isoforms have been reported in smooth muscle and to date it is unknown whether PDE6, PDE8, PDE9, PDE10 and PDE11 are expressed in the vascular system. PDE1 has been identified in a number of VSM sources, including bovine (Lugnier *et al*., 1986), canine (Pagani *et al*., 1992) and human (Lugnier *et al*., 1986) aorta. Low PDE2 activity has been detected in extracts of porcine aorta (Saeki & Saito, 1993). PDE3 has a high affinity for both cAMP and cGMP and has been identified in cultured rat (Liu & Maurice, 1998) and human (Palmer & Maurice, 2000) VSM cells. cAMP-specific PDE4, has been identified in the bovine (Prigent *et al*., 1988) and porcine (Saeki & Saito, 1993) aorta and the human pulmonary artery (Rabe *et al*., 1994). PDE5 is cGMP specific and is abundant in VSM (Corbin & Francis, 1999). PDE5 is perhaps the most familiar member of the PDE family, thanks to the development of the PDE5 inhibitor, sildenafil (Viagra; Boolell *et al*., 1996), which is used to treat male erectile dysfunction. PDE7 has been identified in VSM, but not endothelial cells, of the human pulmonary artery (Smith *et al*., 2003). Meanwhile, PDE8 - 11 are fairly recent discoveries (Soderling & Beavo, 2000) and consequently much less is known about their distribution and function.

1.4.5.4 Mechanism of Nitrergic Neurotransmission

The gaseous nature of NO has led to fierce debate over the actual mechanism of nitrergic transmission. NO must be synthesised as required and is not stored in vesicles, like traditional transmitters. Toda & Okamura (2003) proposed a mechanism of nitrergic transmission to cerebral arteries, which is illustrated in Figure 1.4. As has already been discussed, NO is synthesized from L-arginine by nNOS, which is activated by Ca^{2+} in the presence of calmodulin and other cofactors (Bredt & Snyder, 1990). The response to electrical stimulation is abolished by removal of extracellular Ca^{2+} and also by ω-conotoxin GVIA, an N-type Ca^{2+} channel blocker (Toda *et al*., 1995), but not by nicardipine, a blocker of L-type Ca^{2+} channels (Toda & Okamura, 1992). They suggest that Ca^{2+} entry into nerve terminals via N-type Ca^{2+} channels, upon arrival of generated action potentials, is responsible for activation of nNOS and NO synthesis (Toda *et al*., 1995).

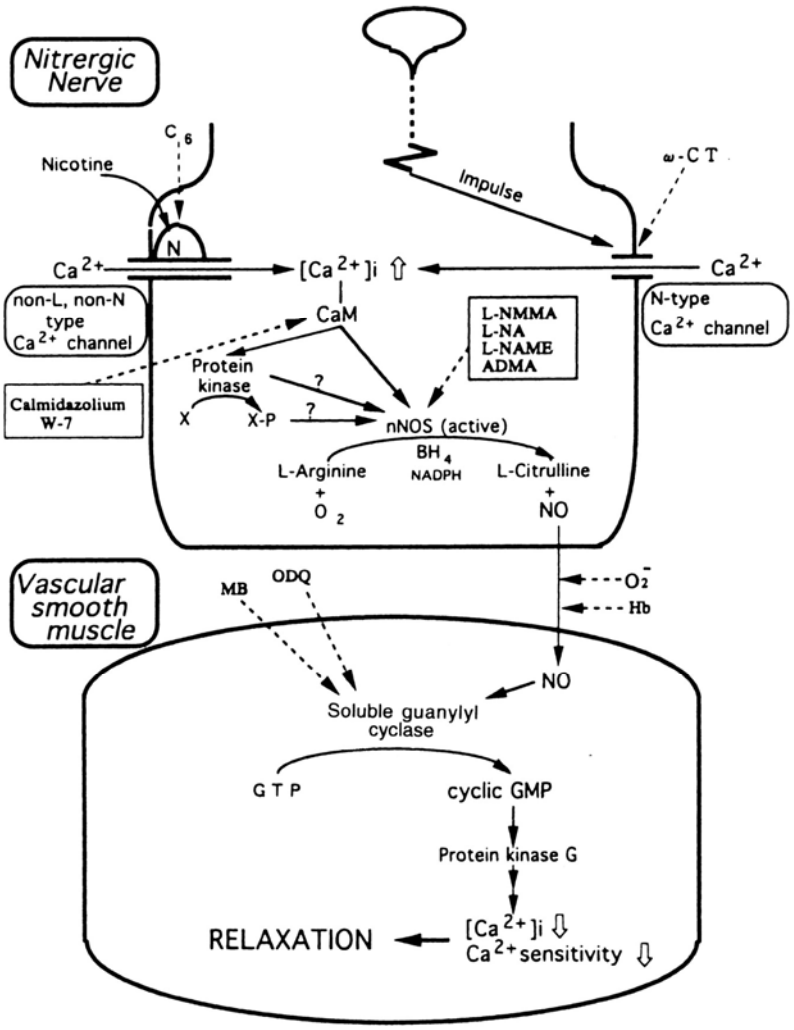


Figure 1.4 Schematic diagram summarising a proposed mechanism of neurotransmission from nitrergic nerves to smooth muscle (adapted from Toda & Okamura, 2003). A solid line indicates stimulation and a dotted line indicates inhibition. ω-CT, ω-conotoxin GVIA; N, nicotinic receptor; X, unknown protein that contributes to nNOS activation upon phosphorylation (X-P); O_2^-, superoxide anion; MB, methylene blue; Hb, haemoglobin; C_6, hexamethonium; CaM, calmodulin; ODQ, 1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; GTP, guanosine triphosphate; ADMA, asymmetric dimethylarginine; L-NA, L-nitroarginine; W-7, a calmodulin antagonist.

1.4.6 VIP-Containing Nerves

VIP was first isolated in the porcine duodenum in 1970 (Said & Mutt, 1970) and contains 28 amino acid residues (Mutt & Said, 1974). The human VIP amino acid sequence is identical to the bovine, ovine, canine, porcine, goat, rat and rabbit peptide (Nussdorfer & Malendowicz, 1998). VIP-like immunoreactivity was quickly described within nerve cell bodies and axons, particularly in the cerebral circulation of a number of species (Edvinsson *et al*., 1980).

1.4.6.1 Synthesis of VIP

VIP is derived from prepro-VIP, cleavage of which yields not only VIP, but also peptide histidine isoleucine/methionine (PHI/PHM). PHI /PHM are structurally related to VIP and can mimic many of the actions of VIP but are less potent (Nussdorfer & Malendowicz, 1998). VIP is synthesised in the neuronal cell body and transported to the axon terminal (Gilbert *et al*., 1980).

1.4.6.2 VIP Receptors

The VIP receptor belongs to a family of G-protein coupled receptors (GPCR), which are also activated by pituitary adenylate cyclase activating polypeptide (PACAP), PHI and PHM (Harmar *et al*., 1998). Two subtypes of VIP receptor, VPAC1 and VPAC2 have been cloned (Adamou *et al*., 1995; Sreedharan *et al*., 1993). In the porcine basilar artery, both VPAC1 (endothelium) and VPAC2 (smooth muscle) can mediate vasodilatation, via different mechanisms. Another VIP-activated receptor, the natriuretic peptide clearance receptor (NPR-C), can also mediate vasodilatation in this vessel (Grant *et al*., 2006).

1.4.6.3 VIP Inhibition

The VIP fragments, VIP_{6-28} and $VIP_{10-28,}$ the VIP derivative [4-Cl-D-Phe^{6}, Leu^{17}]VIP and a growth hormone releasing factor derivative are effective, non-specific, antagonists against VIP-mediated relaxation of the gut in a preparation of mouse stomach (Mulè & Serio, 2003) and guinea-pig taenia coli (Grider & Rivier, 1990). However other mechanisms of VIP inhibition have also been reported. The protease α-chymotrypsin (Mulè & Serio, 2003) and VIP antiserum (Matthew *et al*., 1997) have also been used successfully to inhibit neurally-mediated vasodilatation.

1.4.6.4 Mechanism of VIP-Mediated Vasodilatation

VIP is known to mediate vasodilatation via a number of mechanisms. In rat cerebral vessels and the rabbit mesenteric artery (Huang & Rorstadt, 1983; Itoh *et al*., 1985), VIP-mediated neurogenic vasodilatation is mediated by activation of adenylate cyclase (AC) and cAMP.

There are nine recognised isoforms of AC to date (AC-I to AC-IX; Houslay & Milligan, 1997). However, only AC-III has been identified in VSM (Zhang *et al*., 1997c). All $_{AC}$ isoforms are coupled to GPCR and differ from each other in their activation or inhibition by Ca^{2+}/calmodulin, phosphorylation by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) and differences in the structure of the G-protein (Hurley, 1998). An increase in intracellular cAMP serves to activate PKA, which promotes vasodilatation in a number of ways:

1. Phosphorylation of MLCK, decreasing the sensitivity of the contractile apparatus to Ca^{2+} (de Lanerolle *et al*., 1984).

2. Stimulation of Ca^{2+}-ATPase in the plasma membrane and sarcoplasmic reticulum, resulting in decreased $[Ca^{2+}]_i$ (Kimura *et al*., 1982).

3. Phosphorylation of K_{Ca} channels, resulting in hyperpolarisation and vasodilatation (Price *et al*., 1996; Taguchi *et al*., 1995).

However, in the human uterine artery (Jovanovic *et al*., 1998), VIP mediated vasodilatation is endothelium-dependent and is produced by NO, leading to stimulation of guanylate cyclase and an increase in intracellular cGMP. Furthermore, VIP is reported to produce smooth muscle hyperpolarisation via activation of ATP-sensitive K^+ (K_{ATP}) channels, which in turn leads to vasodilatation (Standen *et al*., 1989).

1.4.6.5 Breakdown of VIP

VIP is hydrolysed by a number of enzymes including neutral endopeptidase (Goetzl *et al*., 1989) and mast cell chymase and tryptase (Caughey *et al*., 1988). As previously mentioned, degradation of VIP by protease enzymes, is often used as an investigational tool.

1.4.7 Vascular Sensory Nerves

Release of the neuropeptide transmitters calcitonin gene-related peptide (CGRP) and substance P from the terminals of sensory nerves can result in vasodilatation. In addition to release by nerve stimulation, the irritant capsaicin, extracted from chilli peppers, induces the release of CGRP and substance P from sensory neurons. This results in a profound depletion of CGRP and substance P within the nerves (Wharton *et al*., 1986).

1.4.7.1 CGRP

CGRP is a single chain 37 amino acid peptide, which is highly conserved in different mammalian species (Collyear *et al*., 1991). There are two forms of CGRP in humans: α-CGRP (Morris *et al*., 1984) and β-CGRP (Alevizaki *et al*., 1986), which differ by 3 amino acids. Immunoreactivity to CGRP has been demonstrated in many tissues but is concentrated in unmyelinated C-fibres and Aδ fibres of the peripheral nervous system and the dorsal horn of the spinal cord (Rosenfeld *et al*., 1983).

1.4.7.1.1 CGRP Receptors

Two CGRP receptor subtypes have been described, $CGRP_1$ and $CGRP_2$. Classification is based upon the antagonist properties of the CGRP inhibitor $CGRP_{8-37}$ (Chiba *et al*., 1989). Receptors that can be inhibited by $CGRP_{8-37}$ have been designated $CGRP_1$ receptors, and those unaffected are known as $CGRP_2$ receptors (Dennis *et al*., 1990). $CGRP_{8-37}$ is an effective CGRP antagonist, both *in vivo* (Gardiner *et al*., 1990) and in response to EFS *in vitro* in the canine lingual artery (Kobayashi *et al*., 1995).

1.4.7.1.2 Mechanism of CGRP-Mediated Vasodilatation

CGRP is a potent vasodilator (Brain *et al*., 1986) and mediates neurogenic vasodilatation in the rat mesentery (Kawasaki *et al*., 1988), the canine lingual artery (Kobayashi *et al*., 1995) and small mesenteric artery of the rabbit (Kakuyama *et al*., 1998). CGRP-induced vasodilatation can be both endothelium-dependent and in some cases endothelium-independent. In rat aorta, dilatation in response to CGRP is endothelium-dependent and can be inhibited by both NOS inhibitors and $CGRP_{8-37}$. This suggests that dilatation to CGRP is mediated via NO in this tissue (Gray & Marshall, 1992). However in cat cerebral arteries, human skeletal muscle arteries and rabbit mesenteric arteries (Nelson *et al*., 1990),

CGRP-induced vasodilatation is endothelium-independent and may be the result of activation of K_{ATP} channels.

1.4.7.2 Substance P

Substance P is part of the tachykinin family of peptides, along with neurokinin A, neurokinin B, neurokinin K and neuropeptide γ. Although substance P was initially isolated from equine brain and intestine in the 1930s (Von Euler & Gaddum, 1931), the structure was not determined until the 1970s (Chang *et al*., 1971) when it was discovered that substance P was an undecapeptide. It was demonstrated by Hökfelt *et al*. (1975) that substance P is present in sensory neurons.

1.4.7.2.1 Substance P Receptors

Three receptor types mediate the effects of the tachykinins: neurokinin 1 (NK_1), neurokinin 2 (NK_2) and neurokinin 3 (NK_3; Buck *et al*., 1984; Lee *et al*., 1982). Each peptide can act as a full agonist at all 3 receptors, but substance P is more selective for NK_1 receptors (Bell & McDermott, 1996). Substance P-induced vasodilatation is mediated via NK_1 receptors. NK_1 antagonists such as L-733,060 (Seabrook *et al*., 1996) are available to inhibit responses mediated by this receptor.

1.4.7.2.2 Mechanism of Substance P-Mediated Vasodilatation

In a number of vascular beds, substance P-induced vasodilatation is mediated via NO (Persson *et al*., 1991; Whittle *et al*., 1989). However, in other tissues substance P can evoke vasodilatation in the presence of NOS inhibitors (Kerezoudis *et al*., 1993). In some blood vessels, such as the rabbit jugular vein (Nantel *et al*., 1990), substance P evokes a vasoconstrictor response.

1.4.8 Purinergic Nerves

ATP, first identified by Fiske & Subbarow (1929) from muscle extract, is a purine nucleotide, which can be found in every cell of the body, as a source of energy. In addition to this critical role in cellular metabolism, extracellular ATP exerts pronounced effects in a variety of biological processes including neurotransmission, muscle contraction, cardiac function and vasodilatation. The first to propose ATP as a neurotransmitter was the research group led by Burnstock (Burnstock *et al*., 1970). Although the concept met with

resistance for many years, purinergic transmission and co-transmission involving ATP (Burnstock, 1971) are now widely accepted.

1.4.8.1 Synthesis of ATP

The ATP molecule is comprised of three different chemical structures: an adenine ring formed through purine biosynthesis, a ribose molecule formed in the pentose phosphate pathway and a triphosphate chain. Nerve terminals take up glucose from the extracellular surroundings and some ATP is generated from glycolysis and the citric acid cycle. However, the majority of ATP is formed by oxidative phosphorylation in the mitochondria of the nerve terminal (Sperlagh & Vizi, 1996). ATP utilised in neurotransmission, as opposed to cellular metabolism, is stored in a variety of synaptic vesicles. ATP has been identified within cholinergic synaptic vesicles (Dowdall *et al*., 1974) and in those containing catecholamines (Douglas & Poisner, 1966).

1.4.8.2 ATP Receptors

ATP receptors are members of the purinoceptor family. Initially two isoforms of purinoceptor were identified, P1 (the adenosine receptor) and P2 (ATP and ADP receptor; Burnstock, 1978). The nomenclature of these receptors has been adapted, with the discovery of new receptor isoforms. The current system for P2 receptors was suggested by Abbracchio & Burnstock (1994), who proposed that receptors be classified as either P2X (ligand-gated ion channel receptors) or P2Y (G-protein-coupled receptors). Currently seven P2X and eight P2Y receptor subtypes exist (Burnstock, 2006). P2Y receptors mediate vasodilatation in the rabbit portal vein (Brizzolara *et al*., 1993) and lamb coronary arteries (Simonsen *et al*., 1997).

1.4.8.3 ATP Inhibition

The large number of purinoceptor subtypes has led to a vast array of specific and non-specific purinoceptor antagonists being developed. Previous research has reported that the non-specific inhibitors, suramin and reactive blue 2 (RB2), inhibit neurogenic, ATP-mediated vasodilatation in lamb coronary arteries (Simonsen *et al*., 1997). Another novel inhibitor, 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3 benzenedisulfonic acid tetrasodium salt (PPADS), effectively inhibits neurogenic ATP in the rabbit vas deferens (Lambrecht *et al*., 1992).

1.4.8.4 Mechanism of ATP-Mediated Vasodilatation

ATP can mediate vasodilatation by activation of P2Y receptors on the endothelium or VSM and is believed to mediate neurogenic vasodilatation in the rabbit portal vein and mesenteric artery (Brizzolara *et al*., 1993; Kakuyama *et al*., 1998) and lamb coronary arteries (Simonsen *et al*., 1997). Activation of $P2Y_1$ and $P2Y_2$ receptors on the endothelium, results in activation of NOS and production of NO. PGI_2 production can also be stimulated in this way but plays a minimal role in vasodilatation (Ralevic & Burnstock, 1998). Activation of $P2Y_1$ receptors on VSM is also reported in rabbit mesenteric and hepatic arteries (Brizzolara & Burnstock, 1991; Mathieson & Burnstock, 1985).

1.4.8.5 Breakdown of ATP

ATP released from neurons and non-neuronal cells can be broken down by a number of enzyme families, known collectively as the ectonucleotidases. ATP is broken down to adenosine, which is then capable of activating P1 receptors. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatases (E-NPPases), alkaline phosphatases, ecto-5'-nucleotidase and ecto-nucleoside diphosphokinase (E-NDPK) are all capable of breaking down extracellular ATP (Zimmerman, 2001).

1.4.9 Other Potential Vasodilator Neurotransmitters

NO, although now firmly established, challenged the traditional definition of a neurotransmitter. Recently another two gases, carbon monoxide (CO) and hydrogen sulphide (H_2S), have been proposed as putative neurotransmitters.

1.4.9.1 CO

CO is generated by the enzyme haem oxygenase (HO) and is able to stimulate soluble guanylate cyclase (Stone & Marletta, 1994). Three isoforms of HO have been identified, one highly inducible (HO1; Shibahara *et al*., 1985) and two constitutive forms (HO2 and HO3; McCoubrey *et al*., 1997; Rotenberg & Maines, 1990).

Evidence that CO plays a role in neurotransmission is based upon studies of NANC transmission in the enteric nervous system. In the gut, HO2 is co-localised with nNOS and NANC transmission is reduced by 50% in nNOS-deficient mice and also in HO2-deficient mice (Battish *et al*., 2000; Ny *et al*., 1997; Zakhary *et al*., 1997). In mice lacking both

nNOS and HO2, very little NANC transmission is observed (Xue *et al*., 2000). cGMP levels are depleted in a similar manner in both knockout mice (Zakhary *et al*., 1997).

CO also has guanylate cyclase-independent effects. In rat-tail arteries, a combination of cGMP inhibitors and inhibitors of large conductance calcium-activated K^+ channel are needed to fully inhibit CO-induced vasodilatation (Wang *et al*., 1997).

1.4.9.2 H_2S

H_2S, a potent SMC relaxant, is formed from cysteine by the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lysase (CSE; Stipanuk & Beck, 1982). CBS is the dominant enzyme in the brain (Abe & Kimura, 1996), but both isoforms are present in SMC (Hosoki *et al*., 1997).

The mechanism of H_2S-mediated SMC relaxation is unknown at the present time. In the rat aorta it is not blocked by inhibitors of the cGMP pathway and seems to be partly mediated by the endothelium and partly by the VSM (Zhao & Wang, 2002). Additional studies in the rat aorta suggest that H_2S may activate K_{ATP} channels in the VSM, resulting in hyperpolarisation and vasodilatation (Zhao *et al*., 2001). However, H_2S-mediated relaxation of the guinea-pig ileum is not blocked by inhibitors of NOS, cyclo-oxygenase (COX) or K_{ATP} channels (Teague *et al*., 2002).

1.5 Endothelial Control of Vascular Tone

1.5.1 NO

1.5.1.1 Identification of Endothelium Derived-Relaxing Factor as NO

As has already been discussed, until the early 1980s the common belief was that the endothelium of blood vessels acted merely as a barrier and did not contribute to vascular tone. However, Furchgott & Zawadzki (1980) demonstrated that vasodilatation in response to ACh was endothelium-dependent and that the endothelium did contribute to vascular tone by releasing a vasodilator substance. The term EDRF was coined to describe this labile compound released from the endothelium. A number of groups investigated EDRF, in order to identify it. Bioassays were utilised to show that EDRF had a very short half-life (Griffith *et al*., 1984) and that it was released under basal conditions and after stimulation with ACh (Griffith *et al*., 1984; Martin *et al*., 1985). EDRF was also inhibited by Hb,

methylene blue (Martin *et al*., 1985) and hydroquinone (Griffith *et al*., 1984). The effects of EDRF appeared to be mediated by stimulation of soluble guanylate cyclase and an increase in intracellular cGMP concentration (Rapoport & Murad, 1983).

EDRF was identified in 1987 (Ignarro *et al*., 1987; Palmer *et al*., 1987), when evidence from different groups supported the proposal that EDRF was NO. NO can be measured using chemiluminesence and using this technique it was shown that the concentration of bradykinin that induced EDRF release in cultured porcine aortic endothelial cells also caused a concentration-dependent release of NO (Palmer *et al*., 1987). Comparison of the physiological actions of EDRF and NO on vascular strips (Hutchinson *et al*., 1987; Palmer *et al*., 1987) and platelets (Radomski *et al*., 1987) showed that they were indistinguishable from each other. The actions of NO and EDRF on vascular strips and platelets were potentiated by superoxide dismutase (SOD) and cytochrome c and inhibited by iron (Fe^{2+}; Hutchinson *et al*., 1987; Palmer *et al*., 1987). As EDRF and NO appeared to have identical chemical properties and biological actions it was widely accepted, although not by all groups (Dusting *et al*., 1988), that EDRF was indeed NO.

1.5.1.2 Mechanism of Endothelium-Dependent, NO-Mediated Vasodilatation

Shear stress and activation of membrane bound receptors on the vascular endothelium, by mediators such as bradykinin or ACh, results in an influx of Ca^{2+} into the endothelial cell. The resulting increase in intracellular Ca^{2+} stimulates eNOS to generate NO, which diffuses into surrounding SMC (Moncada & Higgs, 1993). This stimulates the soluble guanylate cyclase pathway described earlier (Figure 1.5). However, it has also been suggested that in the rabbit aorta and rat middle cerebral arteries, NO may mediate vasodilatation via activation of K_{Ca} channels (Bolotina *et al*., 1994; Sun *et al*., 2000).

1.5.2 PGI_2

PGI_2, a member of the prostanoid family, was the first EDRF to be discovered, when Moncada *et al*. (1976) described an agent that had platelet anti-aggregating properties and was also capable of relaxing VSM.

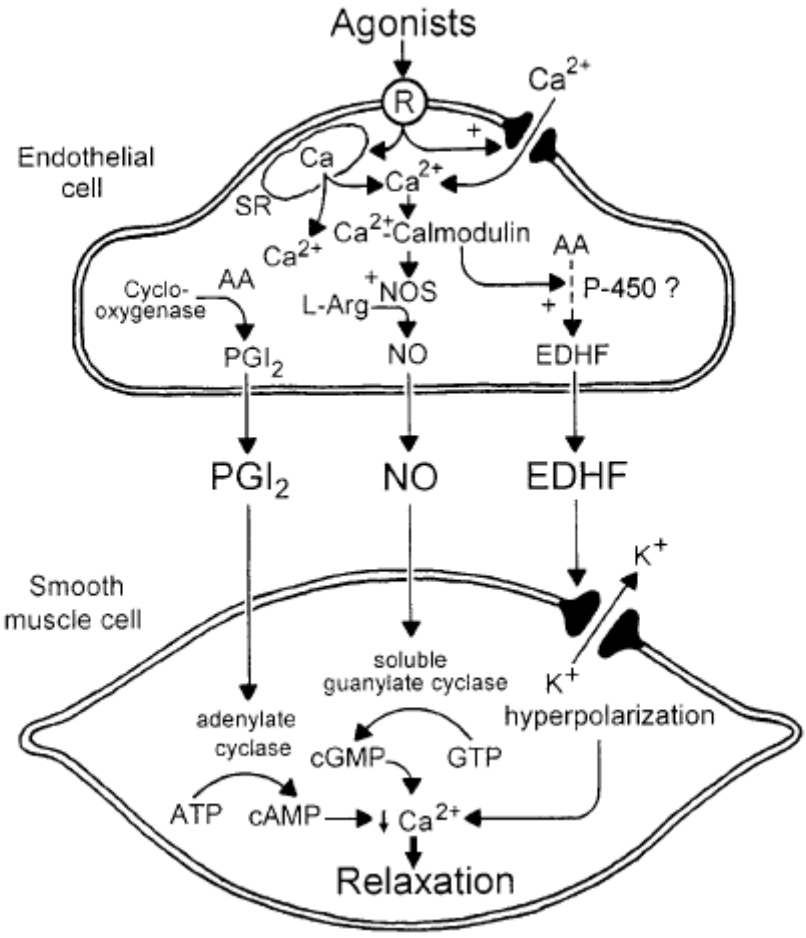


Figure 1.5 Schematic diagram showing the mechanisms of smooth muscle relaxation employed by the endothelium-derived vasodilators NO, PGI_2 and EDHF. R, receptor; SR, sarcoplasmic reticulum; AA, arachidonic acid; P-450, cytochrome P-450. Adapted from Momboulil & Vanhoutte, 1999.

1.5.2.1 PGI_2 Synthesis

PGI_2 is synthesised, like a number of other vasoactive products, from arachidonic acid released from membrane bound phospholipids by phospholipases. The COX family of enzymes converts arachidonic acid into prostaglandins G_2 and H_2 and these are, in turn, converted into various vasoactive prostanoids such as PGI_2, prostaglandins D_2, E_2 and $F_{2\alpha}$ and thromboxane A_2. The endothelium synthesises mainly PGI_2 and platelets synthesise thromboxane A_2 (Moncada & Vane, 1979).

1.5.2.2 Receptors for PGI_2

Five isoforms of prostanoid receptor have been classified. These are DP, EP, FP, IP and TP and are specific for prostaglandins D_2, E_2 and $F_{2\alpha}$, PGI_2 and thromboxane, respectively (Coleman *et al*., 1994). As expected, IP receptors have been localised in the VSM and on platelets (Oliva & Nicosia, 1987).

1.5.2.3 Mechanism of PGI_2-Mediated Vasodilatation

Activation of IP receptors, which are coupled to $_{AC}$, increases the cAMP level within the VSM cell and initiates vasdilatation (Kukovetz *et al*., 1979). However, PGI_2 can also elicit hyperpolarisation in VSM, leading to vasodilatation (Parkington *et al*., 1995). Furthermore, PGI_2 can also stimulate release of NO from endothelial cells, which in turn, results in vasodilatation (Shimokawa *et al*., 1988).

1.5.2.4 Inhibitors of PGI_2

Non-steroidal anti-inflammatory drugs (NSAID), such as aspirin, commonly prescribed for acute and chronic pain, inhibit the COX family of enzymes (Vane, 1971). There are two recognised COX isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells of the body and is known as the "housekeeping" isoform as it regulates the synthesis of prostaglandins used for homeostatic purposes, such as vasodilatation and blood clotting. In contrast, COX-2 is an inflammatory, inducible enzyme (Dubois *et al*., 1998). The discovery of the role of COX-2 in inflammatory diseases led to a surge in research into a specific COX-2 inhibitor, which could be used as an anti-inflammatory, without affecting the activity of COX-1. A number of COX-2 inhibitors such as celecoxib and rofecobix have been introduced in recent years (Wallace, 1999).

1.5.3 EDHF

In the late 1980s it became apparent that there was a third endothelium-dependent mediator of vasodilatation, unaffected by inhibitors of NO and PGI_2. This became known as EDHF. The identity of EDHF is still under investigation, as there are a number of mechanisms by which hyperpolarisation of VSM can occur and a number of candidate molecules that can initiate the hyperpolarisation process. Therefore, Félétou & Vanhoutte (2006) proposed that "once an endothelial mediator has been identified properly, it should be adequately named (i.e., epoxyeicosatrienoic acid [EET], H_2O_2) and no longer denominated with the acronym EDHF".

The characteristics of an EDHF-mediated vasodilatation have been described as (Campbell & Harder, 2001):

1. Endothelium-dependent

2. Distinct from a vasodilatation mediated by endothelium-derived NO or COX metabolites

3. Results in hyperpolarisation of VSM

4. Involves activation of K^+ channels and may be inhibited by the K^+ channel blockers, charybdotoxin and apamin (Waldron & Garland, 1994)

1.5.3.1 Hyperpolarisation and K^+ Channels

The membrane potential of arterial smooth muscle has an important role to play in arterial tone. K^+ channels are important regulators of membrane potential; the opening of K^+ channels leads to K^+ efflux, causing membrane potential hyperpolarisation, which in turn closes voltage-dependent Ca^{2+} channels, decreasing Ca^{2+} entry, resulting in vasodilatation (Nelson & Quayle, 1995). Four types of K^+ channel have been reported in VSM, voltage-dependent K^+ (K_V) channels, Ca^{2+}-activated K^+ (K_{Ca}) channels, inward rectifier K^+ (K_{IR}) channels and K_{ATP} channels.

1.5.3.1.1 K_V Channels

K_V channels are activated by membrane depolarisation and can be inhibited by 4-aminopyridine (4-AP; Okabe *et al*., 1987) and also charybdotoxin (Zygmunt *et al*., 1997).

1.5.3.1.2 K_{Ca} Channels

Three categories of K_{Ca} channels have been identified and classified according to their conductance: large-conductance, 100–300 pS (BK_{Ca}; Marty, 1981), intermediate-conductance, 25–100 pS (IK_{Ca}; Ishii *et al*., 1997), and small-conductance, 2–25 pS (SK_{Ca}; Blatz & Magleby, 1986) Ca^{2+}-activated potassium channels. These channels can be activated by membrane depolarisation and intracellular Ca^{2+}. BK_{Ca} channels are inhibited by iberiotoxin (Latorre *et al*., 1992) and charybdotoxin (Miller *et al*., 1985). Charybdotoxin also inhibits IK_{Ca} and K_V channels, as mentioned previously, while apamin is reported to inhibit SK_{Ca} (Blatz & Magleby, 1986).

1.5.3.1.3 K_{IR} Channels

The K_{IR} channel conducts inward current at membrane potential negative to the K^+ equilibrium potential (E_K; ~-85 mV) and smaller outward currents at membrane potentials positive to E_K. K_{IR} channels are sensitive to blockade by $BaCl_2$ (Quayle *et al*., 1993).

1.5.3.1.4 K_{ATP} Channels

K_{ATP} channels were initially discovered in cardiac muscle, but have also been described in VSM (Standen *et al*., 1989). They are closed by intracellular ATP and have been reported to react to a number of endogenous vasodilators, such as CGRP (Nelson *et al*., 1990) and VIP (Standen *et al*., 1989). K_{ATP} channels are blocked by sulfonylurea drugs such as glibenclamide (Schmid-Antomarchi *et al*., 1987).

Some of the putative EDHF candidates include K^+ ions, gap junctions, arachidonic acid metabolites and CNP.

1.5.3.2 K^+ Ions

K^+ ions were described as EDHF by Edwards *et al*. (1998) in the rat hepatic and mesenteric arteries. They hypothesised that the activation of K_{Ca} channels in the membrane of

endothelial cells led to efflux of K^+ from the cell. This, in turn, led to an increase in extracellular K^+ and activated VSM Na^+/K^+ATPase and K_{IR} channels, leading to a net loss of positive charge and hyperpolarisation of the cell.

However, a number of studies, also in the rat mesenteric artery, have refuted the claims of Edwards *et al*. (1998). Both Doughty *et al*. (2000) and Lacy *et al*. (2000) report that the response to K^+ does not mimic the response to EDHF in this vessel.

1.5.3.3 Arachidonic Acid Metabolites

One product of arachidonic acid metabolism, EETs, has also been described as an EDHF. The mechanism of EETs-mediated vasodilatation is dependent on activation of endothelial receptors. This gives rise to an increase in cytoplasmic free Ca^{2+}, which in turn releases arachidonic acid from membrane phospholipids. The arachidonic acid is converted to EETs by an epoxygenase enzyme. EETs then diffuse from the endothelium to VSM cells where it activates and opens BK_{Ca} channels. The resulting K^+ flux from the SMC leads to hyperpolarisation and dilatation (Bryan *et al*., 2005).

A number of separate observations have led to EETs being described as an EDHF. Selective inhibition of epoxygenases by antisense oligonucleotide transfection blocked EDHF-mediated dilatation in resistance arteries from the hamster gracilis muscle (Bolz *et al*., 2000), as did an EETs antagonist in bovine coronary arteries (Gauthier *et al*., 2002). EETs also increased the open state probability of BK_{Ca} channels and hyperpolarised VSM in bovine coronary arteries (Campbell *et al*., 1996). Furthermore, agents that increase expression of cytochrome P450 epoxygenase, also increased EDHF-mediated dilatation in the porcine coronary arteries (Fisslthaler *et al*., 1999).

However, those who oppose the EETs theory argue that the specific BK_{Ca} blocker, iberiotoxin, does not inhibit EDHF-mediated vasodilatation in the rat hepatic (Zygmunt & Högestätt, 1996) and mesenteric (Vanheel & Voorde, 1997) and guinea-pig basilar arteries (Petersson *et al*., 2006).

1.5.3.4 Gap Junctions

Gap junctions are intracellular channels that allow small water-soluble molecules, but not proteins, to pass between cells. Gap junctions are comprised of protein subunits called connexins and six connexins are required to form a connexon or hemichannel. A gap

junction is formed when the hemichannels of two adjacent cells dock (Christ *et al*., 1996). Gap junctions between endothelial cells and smooth muscle cells are called myoendothelial gap junctions (Sandow & Hill, 2000). These myoendothelial gap junctions may be involved in the EDHF response, by relaying the dilator signal from the endothelium to SMC.

Gap junctions can be blocked by peptide inhibitors, known as gap peptides and the EDHF-mediated response in the rabbit superior mesenteric artery can be inhibited in this way. Further evidence for the involvement of gap junctions in the EDHF response was provided by Sandow *et al*. (2002) who compared the structure of arteries that did and did not have an EDHF-mediated dilatation. The rat mesenteric artery, which does undergo EDHF-mediated dilatation was found to contain myoendothelial gap junctions and had tight electrical coupling between the endothelium and smooth muscle cells. However, in the rat femoral artery, which does not have an EDHF-mediated dilatation, myoendothelial gap junctions were absent and there was no electrical coupling between the endothelium and smooth muscle cells.

Although there is much evidence for the involvement of gap junctions in the EDHF-mediated response, the identity of the substance conducted between the endothelium and the smooth muscle remains unknown.

1.5.3.5 CNP

CNP is a member of the natriuretic peptide family, homologous to both ANP and BNP, originally isolated in the porcine brain (Sudoh *et al.,* 1990). CNP contains 22 amino acids, 17 of which form a ring structure that is highly conserved in all 3 natriuretic peptides (Sudoh *et al.,* 1990). CNP is also highly conserved between species with almost 100% homology between the human and porcine form (Ogawa *et al*., 1992).

CNP binds to the natriuretic peptide receptors (NPR) and is a potent vasodilator. Evidence that CNP is EDHF is provided by the observation that CNP-induced vasodilatation is inhibited by a variety of K^+ channel blockers (Honing *et al*., 2001; Wei *et al*., 1994). Furthermore, in the rat mesenteric artery, EDHF and CNP-induced hyperpolarisation and relaxation of the VSM are both attenuated in the presence of high $[K^+]$ and a combination of Ba^{2+} and ouabain. The responses were also mimicked by the NPR-C agonist $cANF^{4-23}$, indicating that the effects of CNP were mediated by NPR-C (Chauhan *et al*., 2003).

However, in the porcine coronary artery, the characteristics of the hyperpolarisation evoked by CNP were not comparable to EDHF-mediate hyperpolarisation (Barton *et al*., 1998). In addition to this, the mechanism of hyperpolarisation suggested by Chauhan *et al*. (2003) relies upon activation of GIRK channels and to date there is no evidence that CNP can activate GIRK in any cell type (Félétou & Vanhoutte, 2006).

1.5.4 Endothelin

In addition to the vasodilators NO, PGI_2 and EDHF, the endothelium also releases a vasoconstrictor substance, endothelin. De May & Vanhoutte, (1981) initially reported that in canine femoral arteries, the endothelium was essential for vascular contraction. Endothelin (known today as ET-1) was eventually characterised by Yanagisawa *et al*. (1988) from the endothelial cells of pig aorta. Another two endothelins, ET-2 and ET-3, have since been discovered.

ET-1 is a 21 amino acid peptide and has been localised in non-vascular tissues, such as the brain and kidney, in addition to the endothelium (Sakurai *et al*., 1991). ET-2 and ET-3 can also be detected in other tissues, but not the vascular endothelium. The endothelins are synthesised from precursors known as preproendothelins (preproET). PreproET undergo cleavage by endopeptidases to form intermediate products, proendothelins (proET), also known as big endothelins (big ET). Big ET is then processed by a specific endothelin converting enzyme (ECE) to form active endothelin (Ortega Mateo & de Artinano, 1997).

ET receptors exist in both the endothelium and VSM. Two isoforms of ET receptors, ET_A and ET_B, exist, with ET_A showing affinity for ET-1 over ET-2 and ET-3 and ET_B showing no selectivity for any form of ET (Hosoda *et al*., 1991). The vasoconstrictor response of ET is usually mediated by ET_A receptors on the VSM, however in the rabbit jugular vein, ET_B also plays a role (Sumner *et al*., 1992). In addition to this, activation of endothelial ET_B receptors can sometimes also mediate vasodilatation, via the NO-cGMP and COX pathways (Iwasaki *et al*., 1999; Tirapelli *et al*., 2005). Activation of both ET-receptor subtypes is thought to be coupled to an activation of phospholipase C and this in turn leads to production of phosphoinositol and diacylglycerol and an increase in free cytosolic Ca^{2+} and therefore contraction of the VSM (Takuwa *et al*., 1989; 1990).

1.6 Vasculature of the Bovine Eye

The blood vessels that supply the bovine eye stem from the carotid artery (Prince *et al.*, 1960), with the external carotid artery continuing on to become the internal maxillary artery. The largest branch of the internal maxillary artery is the external ophthalmic artery and this supplies the orbit of the eye. The external ophthalmic artery generates many branches such as the lateral rectus muscle artery and the lacrimal artery, which nourish distinct parts of the eye. It eventually branches into the ethmoidal and supraorbital arteries, with some branches of the supraorbital artery becoming some of the dorsal anterior arteries (Figure 1.6).

Importantly, some of the branches of the external ophthalmic artery form a network of vessels known as the external rete and this is the origin of the ciliary artery. The ciliary artery bifurcates into the large medial and smaller lateral ciliary artery, which run parallel to each other and are loosely tethered to the optic nerve. Both arteries branch before reaching the globe of the eye and then each further subdivides into many short posterior ciliary arteries. Two branches of the large medial ciliary artery each form a long posterior ciliary artery (LPCA), one of which travels along the medial side of the globe and the other travels along the lateral side. The LPCA enter the sclera superficially at first but penetrate fully before the equator is reached. These vessels anastamose with anterior ciliary arteries and form the major arterial circle of the iris, which supplies both the iris and the ciliary body and is well developed in the bovine eye (Prince *et al.*, 1960).

Within the bovine eye there are three main venous chains that allow blood to be carried away from the eye: the supraorbital vein, which is the largest intraorbital vein, the lateral orbital vein and the inferior orbital vein (Prince *et al.*, 1960).

1.6.1 The Bovine Intraocular LPCA

Figure 1.7 shows a transverse section of the bovine intraocular LPCA, which was used throughout this study. It was dissected out and then fixed, processed and stained for light microscopy by Dr Ian Montgomery, a histologist in this department. Sections were then stained with haematoxylin and eosin (H&E), staining the cell nuclei blue and other structures, such as smooth muscle and endothelial cells, deep pink. Images of the vessel, at a magnification of x12, were then captured. The internal diameter of the vessel is approximately 400 μm and some erythrocytes (stained cherry red) are present in the lumen. A thin layer of endothelial cells can be observed surrounding the lumen, resting on a bed of

SMC. These SMC are long, thin cells with elongated nuclei and the *tunica media* appears to consist of 5-6 layers of spindle-shaped cells, arranged concentrically. It is clear from this image that the *tunica adventitia* is much less structured than both the *tunica intima* and the *tunica media.* The fibroblasts and elastin fibres of the *adventitia* appear to be interspersed with darkly pigmented areas, which may be cells from the choroid, as this structure lies adjacent to the intraocular LPCA.

1.7 Regulation of Ocular Vascular Tone

1.7.1 Neural Mechanisms Regulating Ocular Vascular Tone

1.7.1.1 Adrenergic Innervation of the Ocular Vasculature

Adrenergic nerves have a role to play in regulating the ocular circulation. It has been reported that blood flow to the retina and the choroid is increased by approximately 30% after superior cervical ganglionectomy and is decreased by approximately 50% after stimulation of sympathetic nerve fibres in the cat (Weiter *et al*., 1973). The central retinal artery of the rat (Bergua *et al*., 2003) and the bovine ophthalmic artery (Yoshitomi & Ito, 1994) both contain adrenergic nerve fibres. In the bovine ophthalmic artery, neurogenic contractions are sensitive to guanethidine but not α- or β-adrenergic blocking agents, leaving the authors to speculate that ATP or NPY released as a cotransmitter may mediate contraction (Yoshitomi & Ito, 1994).

1.7.1.2 Cholinergic Innervation of the Ocular Vasculature

Histochemical studies have demonstrated the presence of nerves containing acetylcholinesterase in the adventitia of porcine, monkey and dog ciliary arteries (Toda *et al*., 1997, 1998, 1999). Functional studies in porcine and monkey ciliary arteries have suggested that ACh released from cholinergic nerves acts prejunctionally to inhibit vasodilatation mediated by nitrergic nerves (Toda *et al*., 1997; Toda *et al*., 1998). However, in the dog ciliary artery, ACh interferes with release of NA from sympathetic nerves by acting on prejunctional muscarinic receptors (Toda *et al*., 1999).

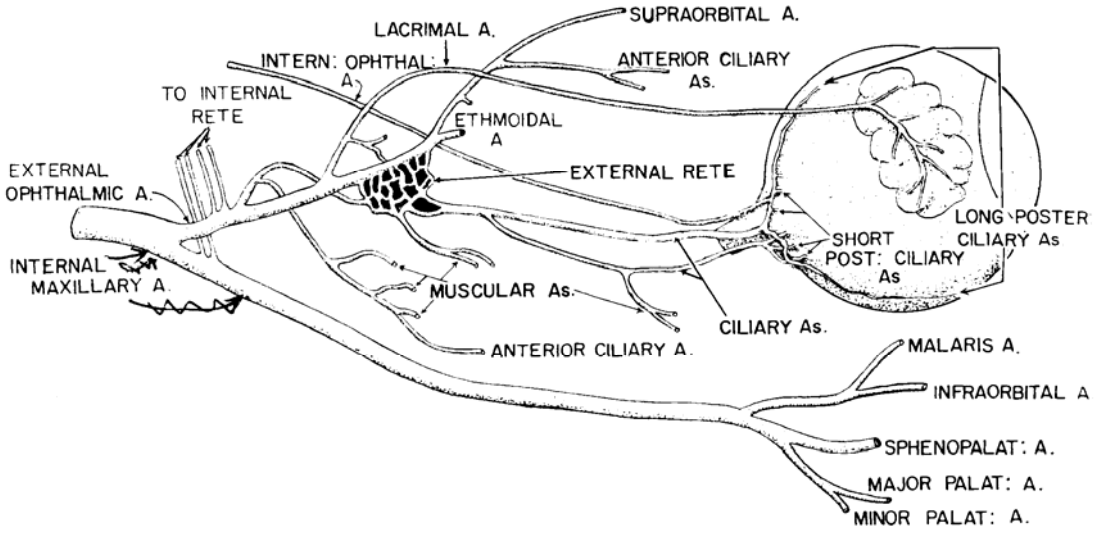


Figure 1.6 Schematic diagram of the vascular system of the bovine eye. The bovine intraocular long posterior ciliary artery (LPCA) originally stems from the internal maxillary artery, which branches to form external ophthalmic artery and then in turn forms the ciliary arteries. Two LPCAs run towards either pole of the globe of the eye but penetrate the sclera before reaching the equator to form the intraocular LPCA (adapted from Prince *et al.,* 1960).

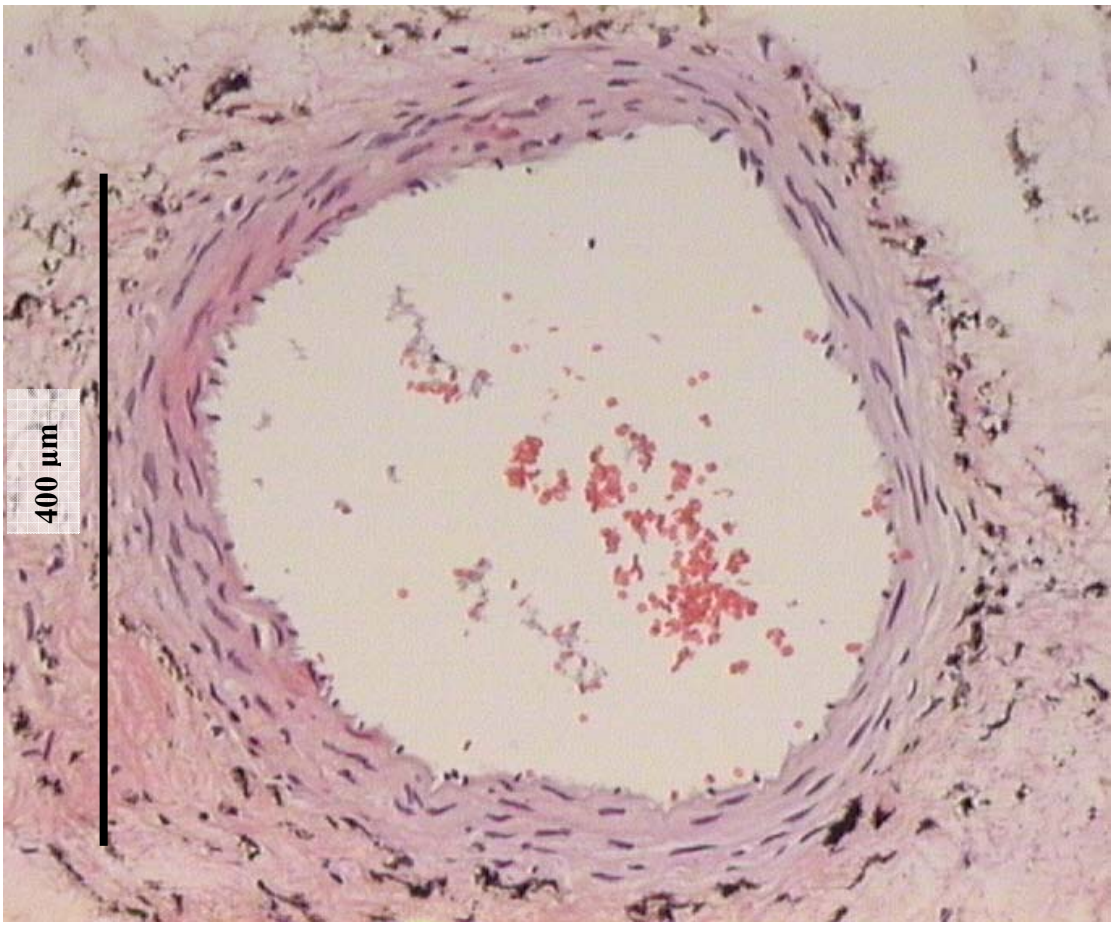


Figure 1.7 An image of the bovine intraocular LPCA histochemically stained with H&E. Cell nuclei are stained blue and other structures, such as smooth muscle and endothelial cells, deep pink. The internal diameter (i.d.) of the vessel is approximately 400 µm.

1.7.1.3 Nitrergic Innervation of the Ocular Vasculature

Prior to the development of specific antibodies for the each of the NOS subtypes, NADPH diaphorase was used to localise nerve tracts containing NOS enzymes histochemically. Positive NADPH reactions have been seen in dog ophthalmic and retinal arteries (Toda *et al*., 1993) and the posterior ciliary arteries of pigs and monkeys (Toda *et al*., 1997; Toda *et al*., 1998).

Evidence from canine retinal (Toda *et al*., 1993) and monkey ophthalmic (Ayajiki *et al*., 2000) arteries, suggests that these nitrergic nerves are likely to stem from the pterygopalatine ganglion. Ethanol was injected into the vicinity of the unilateral pterygopalatine ganglion, to determine if damage to the ganglion would impair the response to nitrergic nerve stimulation in the canine cerebral and retinal arteries. One week after treatment with ethanol, NADPH diaphorase activity was examined in the ganglia and in the arteries. In the ganglia, NADPH diaphorase staining in the cell bodies and nerve fibres was markedly decreased, compared to control tissues, and in the arteries there was no positive staining at all. In functional tests of the retinal artery, control tissues responded to nicotine with a transient contraction, followed by a dilatation. This vasodilatation was abolished by inhibition of NOS and restored by L-arginine. In ethanol treated tissues, a number of vessels did not respond to nicotine and the remainder showed a contraction. In birds, however, it seems that the source of nitrergic nerves innervating the ocular vasculature may be the ciliary ganglion (Bergua *et al*., 1996; Sun *et al*., 1994).

1.7.1.4 Other Innervation of the Ocular Vasculature

Gaspar *et al*. (2004) reported the presence of CGRP, NK1 and NK2 receptors in rabbit retinal arteries, which mediated CGPR and substance P-induced vasodilatation, respectively, although it is not clear if these have functional relevance. Nerve fibres showing VIP-like immunoreactivity have been described in human (Flügel *et al*., 1994) and duck choroid (Bergua *et al*., 1996) and monkey (Ye *et al*., 1990) and rat central retinal arteries (Bergua *et al*., 2003), however, again, the presence of these immunoreactive fibres is not indicative of a functional role in neurogenic vasodilatation.

1.7.2 Endothelial Mechanisms Regulating Ocular Vascular Tone

1.7.2.1 NO

Immunohistochemical and functional studies have both indicated that in a number of species, basal production and release of NO provides tonic vasodilator tone in the ocular vasculature. NADPH diaphorase staining in rat, rabbit pig and cat choroidal vessels suggests the presence of NOS (Flügel *et al*., 1994; Yamamoto *et al*., 1993). Furthermore, NOS inhibition in isolated segments of the porcine and human ophthalmic arteries leads to contraction of the vessel (Haefliger *et al*., 1993; Yao *et al*., 1991).

1.7.2.2 PGI_2

Indomethacin inhibits a NO-independent vasodilatation in the porcine ciliary artery (Quinn *et al*., 2003), suggesting that a product of COX is responsible for this response. In the porcine ophthalmic microcirculation (Meyer *et al*., 1993) and the retinal and choroidal circulation of the newborn pig (Hardy *et al*., 1998), there is evidence that prostaglandins may be involved in endothelin and NO-mediated vasodilatation, respectively. However, Benedito *et al*. (1991) reported that there was no role for PGI_2 in vasodilatation in the bovine small retinal arteries.

1.7.2.3 EDHF

Little work has been carried out to identify the significance of EDHF–mediated vasodilatation in the eye. However, the majority of current research has been in the bovine eye, where EDHF is an important vasodilator mechanism (McNeish *et al*., 2001). In the bovine ciliary vascular bed it mediates the endothelium-dependent vasodilator response induced by ACh and bradykinin. In these vessels NO mediates only a small tonic vasodilator action. EDHF-mediated vasodilatation in this vascular bed is blocked in a time and concentration-dependent manner by ascorbate (McNeish *et al*., 2002). In addition, an EDHF-mediated vasodilatation has recently been described in the porcine ciliary artery (Cleary *et al*., 2005).

1.8 Neurogenic Control of the Ciliary Artery

Neurogenic vasodilatation has been specifically investigated in the ciliary artery of a number of species.

1.8.1 Bovine LPCA

Wiencke *et al*. (1994) have previously described a uniphasic neurogenic vasodilatation in the bovine intraocular LPCA. In this tissue, neurogenic vasodilatation was inhibited by the NOS inhibitor L-NOARG which was partly reversed by addition of surplus L-arginine. However, the authors also reported that part of the response persisted in the presence of L-NOARG. This was sensitive to incubation with capsaicin and the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$. The authors suggested that CGRP released from trigeminal sensory fibres was responsible for the capsaicin-sensitive component of the response and that NO released from nitrergic nerves mediated the L-NOARG-sensitive component.

1.8.2 Porcine LPCA

Su *et al*. (1994) reported on neurogenic vasodilatation in the porcine LPCA. Nerve stimulation resulted in a frequency-dependent contraction, which was abolished by TTX. Addition of guanethidine abolished the contractile response to EFS and uncovered a relaxant response. Incubation of the vessel with L-NAME reduced the magnitude of the relaxation by 52%. Relaxation was restored on addition of excess L-arginine. The authors concluded that two neurotransmitters mediated relaxation, one of which was probably NO. They hypothesised that the other transmitter might be a neuropeptide such as VIP or CGRP. However, although Toda and colleagues (Toda *et al*., 1997) described VIP-like immunoreactive fibres in the porcine ciliary artery, they concluded that only nitrergic nerves were responsible for neurogenic vasodilatation in this vessel.

1.8.3 Canine Ciliary Artery

Toda *et al*. (1999) investigated neurogenic vasodilatation in the canine ciliary artery. In this vessel the response to EFS was abolished by the NOS inhibitor, L-NA, and the inhibitor of soluble guanylate cyclase, ODQ, and could be restored by L-arginine. They concluded that neurogenic relaxation was mediated solely by NO in this vessel.

1.8.4 Monkey Ciliary Artery

Toda and co-workers (Toda *et al*., 1998) also investigated neurogenic vasodilatation in ciliary arteries from monkeys. They observed slight contractions in response to EFS in a small number of tissues, but relaxation in the majority. These responses were inhibited by TTX. The NOS inhibitor, L-NA, either abolished the relaxation, or in some cases reversed

the relaxation to a contraction. They concluded that a nitrergic vasodilator nerve was heavily involved in the regulation of vascular tone in this vessel.

1.9 Aims

The main aims of this investigation were as follows:

1. To confirm the presence of vasodilator and vasoconstrictor nerves in the bovine intraocular LPCA.

2. To identify the neurotransmitters responsible for vasodilatation in this vessel and therefore confirm or dispute the findings of Wiencke *et al.* (1994).

3. To identify the mechanisms by which the neurotransmitters induce vasodilatation in the bovine intraocular LPCA.

4. To establish whether the NOS inhibitor, L-NMMA, inhibits both endothelium-dependent and nitrergic nerve-mediated vasodilatation in the bovine intraocular LPCA.

5. To investigate whether the putative nNOS-specific inhibitors, AAAN and N^G-propyl-L-arginine, specifically block the neuronal but not the endothelial isoform of NOS in the bovine intraocular LPCA.

CHAPTER 2.

METHODS & MATERIALS

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2 Methods and Materials

2.1 Myography

2.1.1 Historical Background of Myography

The mechanical, morphological and pharmacological properties of small arteries are of great interest, as they are implicated in the physiological control of blood pressure and blood flow as well as the pathophysiology of hypertension. Small arteries are prearteriolar vessels with a diameter of <500 μm (Bloom & Fawcett, 1968). Depending on the vascular bed examined, at least 50% of peripheral resistance lies proximal to vessels with diameters of 100 μm (Mulvany & Aalkjaer, 1990).

However, until Mulvany & Halpern (1976) adapted a technique suggested by Bevan & Osher (1972), information about small arteries was restricted to that obtained from perfusion experiments and histological examination, as traditional organ bath techniques were too traumatic to be successfully applied to vessels of this size. This technique, known as wire myography, was further developed in subsequent years (Mulvany *et al*., 1978; Mulvany & Halpern, 1977) and allows ring preparations of small arteries with an internal diameter of 100 – 400 μm to be investigated.

Technological advances in recent years, have allowed the technique to be adapted for EFS experiments, with platinum electrodes embedded in the mounting jaws of the myograph (Angus *et al*., 1988).

2.1.2 The Myograph

The model of myograph used in the present study was the multi myograph model 610 M (Danish Myo Technology, Aarhaus, Denmark). This system has four separate chambers, which allows four vessels to be studied simultaneously. Each chamber can be detached from the myograph to allow the vessel to be mounted under a microscope. Each myograph unit consists of a round, acid-resistant, stainless steel bath, which holds a maximum volume of 8 ml (Figure 2.1). Inside the bath, two plastic mounting jaws are positioned on opposite sides of the chamber. One of the mounting jaws is fixed in place and connected via a pin to an isometric force transducer. The other jaw is moveable and is connected to a

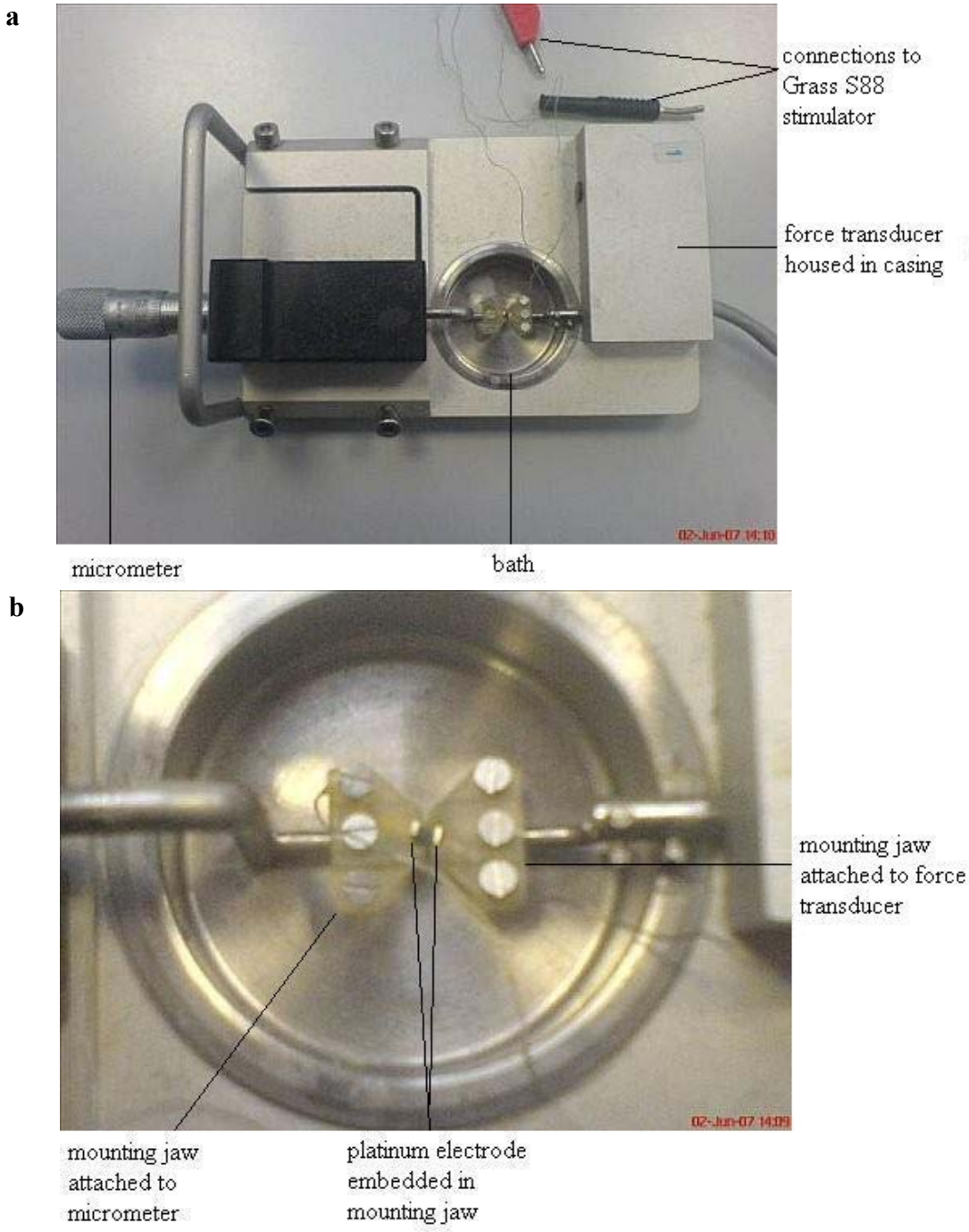

Figure 2.1 Colour photographs showing (a) an individual myograph unit and (b) a view of the mounting jaws. The main features of the myograph, such as: the bath, the micrometer, the force transducer unit and the connections to the Grass 88 stimulator are highlighted in photograph (a). Photograph (b) displays a close-up view of the bath, containing the mounting jaws and the platinum electrodes.

micrometer, which allows the distance between jaws to be adjusted and the internal diameter of the vessel determined (Figure 2.1). Embedded within each of the plastic jaws is a small platinum electrode, which was connected to an S88 stimulator (Grass, Quincy, U.S.A.). These jaws, although specifically adapted for EFS experiments, can also be used for experiments that do not require nerve stimulation. Each unit also has a gas supply and suction facility mounted on a swivelling arm, which allows the bathing medium to be gassed with an appropriate gas mix and washed out when necessary. Both of these functions are controlled by a system of valves, which allows each bath to be monitored individually. However, bath temperature is controlled centrally and all baths are maintained at a pre-determined temperature (37 ^{o}C in all experiments in this study). The system also contains an external thermometer, which can be inserted into the baths to verify the temperature. Chamber covers were used to keep the temperature and conditions of the mounted rings as stable as possible. There are holes in the covers to accommodate gassing and suction apparatus, and to allow the addition of fresh bathing medium and drugs. Each myograph unit was connected to a separate input port of a Powerlab 4/20 data acquisition system (ADInstruments, Hastings, U.K.) and this was linked to a PC. Data was displayed on the PC using Chart v5 software (ADInstruments, Hastings, UK).

2.1.3 Calibration of the Myograph

The myograph was calibrated regularly, especially after non-routine maintenance, as per the owner's manual supplied by Danish Myo Technology (Aarhaus, Denmark). In summary, a 40 μm wire was mounted on the jaw connected to the force transducer, in the usual manner. The bath was filled with 5 ml of Krebs solution and the temperature set to 37 ^{o}C. The calibration bridge, balance and weight were placed on the myograph, to be warmed up, together with the myograph unit, for a period of 30 min. The balance was then placed over the bath, resting upon the bridge. The tip of the transducer arm on the balance was placed in the gap between the mounting wire and the jaw. The bridge was then adjusted carefully so that the tip of the transducer arm was hanging freely in the gap and was not touching either the mounting wire or the jaw. The balance was left in this position for a further 30 min. The 2 g weight was then placed on the balance, in order that the force applied on the transducer mimicked the stretch created by the contraction of a mounted ring preparation. This calibrated the force transducer to an output of 9.81 mN. This process was then repeated for the other three myograph units.

2.1.4 Maintenance of the Myograph

2.1.4.1 General Cleaning

The myograph chamber and surroundings were cleaned after each experiment. The myograph bath was filled with a 10 % acetic acid solution. The acid was left for a few minutes to dissolve salt build-up. A cotton swab was then used to clean all chamber surfaces. The acetic acid was drained away and the myograph bath and supports were rinsed 10 times with distilled water.

2.1.4.2 Myograph Chamber Pipes

To prevent the pipes from being blocked by salt deposits after an experiment, the chamber covers were removed and the vacuum pump turned on for approximately 10 seconds. Gas flow was not turned off until the vacuum pump was switched off. Sometimes, however, a blockage due to salt accumulation did occur in the gas inlet pipe. On such occasions, the rubber tubing section of the gas inlet pipe was disconnected and a thin piece of wire entered into the lumen of the metal section of the pipe. The wire was gently rotated within the pipe and this was enough to dislodge the blockage. The rubber tubing was then reconnected.

2.1.4.3 Force Transducer

As it is the most fragile component of the myograph, the force transducer must be handled with great care. Particular attention was taken to ensure that buffer from the bath did not enter the transducer housing. Consequently, the area where the force transducer arm enters the housing unit was sealed with high vacuum grease (Danish Myo Technology, Aarhus, Denmark). The seal was checked on a daily basis and replaced at least once a week

2.2 Dissection of the Bovine Intraocular LPCA

Bovine eyes were obtained from a local abattoir and transported to the laboratory within 60 minutes of killing. Eyes were stored in Krebs solution containing (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11.5; which had been gassed with O_2 containing 5 % CO_2, at 4^{o}C until required.

To dissect the intraocular LPCA, all excess fat and skeletal muscle was removed from the globe of the eye. An incision was made with a scalpel at the equator of the globe and scissors were used to cut around the equator. The posterior segment of the eye was discarded, along with the vitreous humour and lens. The choroid of the anterior portion of the eye was separated from the sclera and the intraocular LPCA located, running along the surface of the sclera. The area of sclera surrounding the vessel was cut away from the rest of the eye and carefully pinned to a dissecting dish (a large petrie dish with a Sylguard-covered base, suitable for securing tissue samples) containing cold, gassed Krebs solution.

Dissection was carried out under a microscope (Vickers, York, UK) with an external light source (Leica, Wetzlar, Germany) at x10 magnification. To avoid damage to the vessel, the artery was manoeuvred by gripping the surrounding connective tissue gently with fine forceps (Dumont #5, Interfocus, Haverhill, UK). The vessel was cut at the proximal (closest to the cornea) end using fine, spring-mounted dissection scissors, with a straight, sharp edge (FST, Interfocus, Haverhill, UK). The cut end of the vessel was lifted gently and the underlying connective tissue, tethering the vessel to the sclera, was cut away. By progressively cutting away the underlying tissue, whilst lifting the freed section of the vessel without stretching it, removal of the vessel from the sclera was fairly simple. The distal end of the vessel was cut, just as it entered into the sclera. The whole vessel was carefully removed and stored in gassed Krebs solution at 4^{o}C until required.

2.3 Mounting Procedure

The bovine intraocular LPCA was placed in a dissecting dish containing Krebs solution. A 2 mm segment was cut using spring-mounted dissection scissors, for mounting on the myograph. The vessel was held in position using fine forceps, grasped in the left hand. To avoid damage to the vessel during the mounting procedure, the artery was held in place by gripping the surrounding connective tissue and never the vessel itself. A 5 cm length of 40 μm tungsten wire (Danish Myo Technology, Aarhaus, Denmark) was held by another pair of fine forceps in the right hand. The tip of the wire was inserted into the proximal end of the artery lumen and gently guided through the lumen until the tip exited the distal end. The wire was fed through the lumen, until the vessel sat approximately half way along the wire. Care was taken to ensure that the wire remained in the centre of the lumen at all times, so that the endothelium remained intact. In some vessels, access to the lumen was not possible because opposing vessel walls had become stuck together. In such cases the

wire was used to gently tease apart the vessel walls. An individual myograph chamber was placed under the microscope with the micrometer on the left hand side and 5 ml of Krebs solution was added to the bath.

The stages of mounting the vessel are shown in Figure 2.2 and are described as follows: (a) The wire skewering the lumen was carefully lifted from the dissection dish using forceps and placed between the jaws of the myograph, ensuring that the segment of intraocular LPCA sat in the gap between the prongs of the jaws. (b) The jaws were brought together until the wire was held securely without additional help from the forceps. (c) The distal section of the wire was then bent to the left, towards the micrometer and wrapped in an anti-clockwise manner around the distal fixing screw. The screw was then tightened. (d) The proximal section of wire was bent to the left and wrapped in a clockwise manner around the proximal fixing screw, which was then tightened. (e) The jaws were opened slightly and a second 5 cm length of tungsten wire was carefully passed through the lumen, in parallel to the existing wire. Care was taken not to stretch the vessel wall, damage the endothelium or cross the wires. The jaws were then closed again, holding the second wire in place. (f) The distal section of the wire was bent to the right, towards the force transducer and wrapped in a clockwise fashion around the fixing screw. The screw was then tightened. (g) The proximal section of the wire was bent to the right and wrapped around the fixing screw in an anti-clockwise manner and the screw tightened. All superfluous lengths of tungsten wire were trimmed and removed from the bath. The jaws were then adjusted so a small but distinct space was visible between the intralumenal wires (Figure 2.3).

The chamber was then returned to the myograph unit and plugged into the appropriate myo interface. A chamber cover was placed over the bath and the gas supply/suction unit placed into the bath. This process was repeated with the three remaining chambers. Prior to mounting the vessels, the temperature of the unit was set to 37 ^{o}C. The tissues were then left for 30 minutes to equilibrate.

2.4 Setting Transmural Stretch

All experiments were conducted at a level of stretch equivalent to a transmural pressure of 100 mmHg, as this is equivalent to the pressure experienced by the bovine intraocular LPCA *in vivo*. The degree of stretch was determined for each vessel prior to the beginning of experiments, utilising La Place's law of pressure within a sphere.

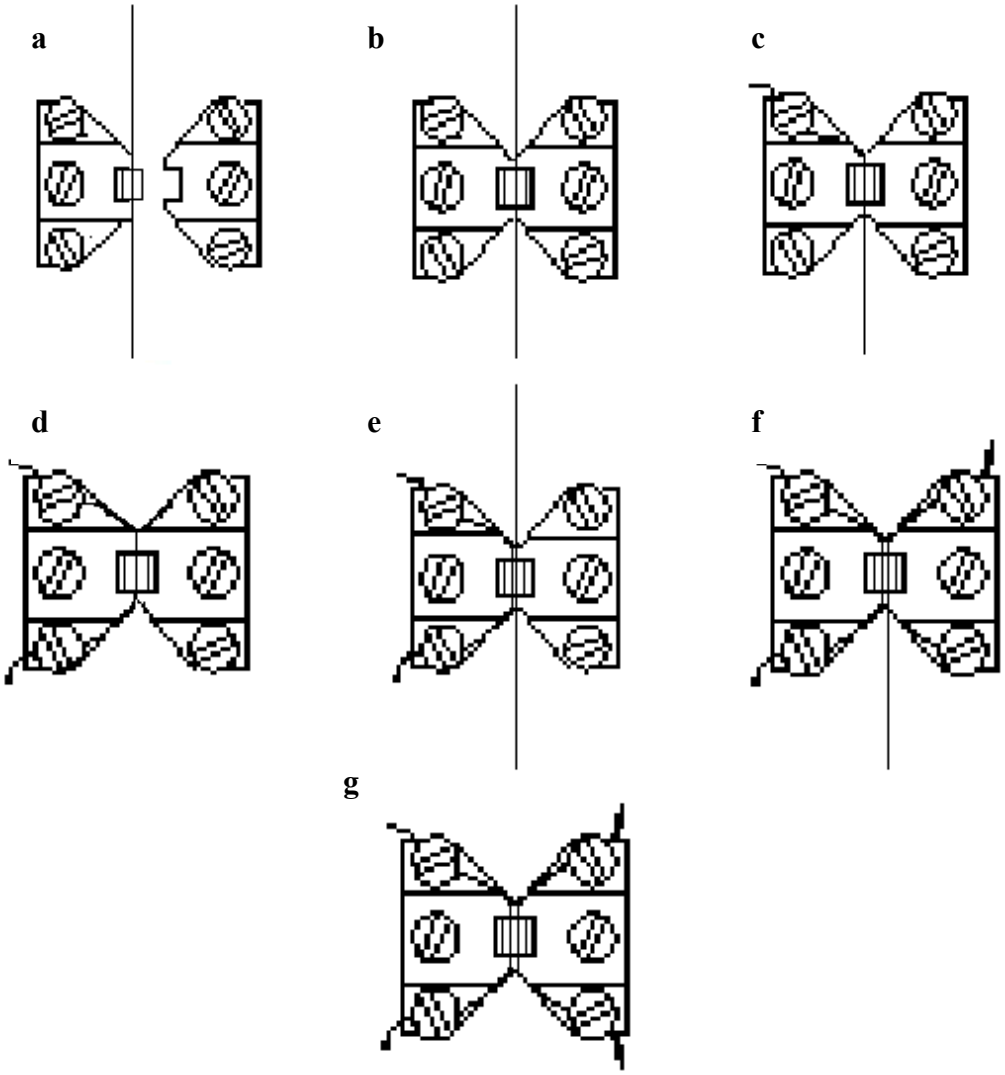

Figure 2.2 Schematic visualising the stages of mounting a 2 mm section of bovine intraocular LPCA on a wire myograph, as described in the text. Adapted from the multi myograph 610 owners manual (Danish Myo Technology, Aarhaus, Denmark).

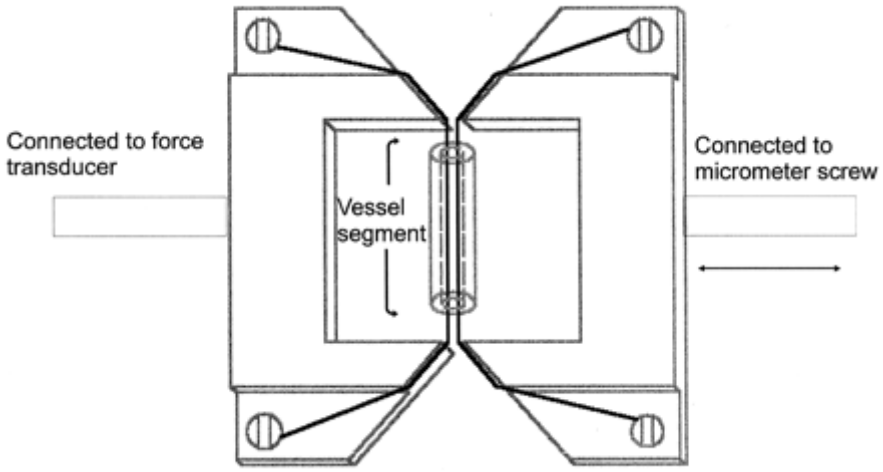

Figure 2.3 Schematic showing final stage of the mounting process (stage g in Figure 2.2). At this time, all superfluous lengths of tungsten wire had been trimmed and removed. The jaws were adjusted so a small but distinct space was visible between the intralumenal wires (adapted from Wang *et al*., 2000).

LaPlace's law states that the pressure (*P)* within a sphere is proportional to wall tension (*T*) and is inversely proportional to the internal radius (*r*):

$$P = \frac{2T}{r}$$

T is defined as stress (*S*) multiplied by wall thickness (*w*). Stress is the force per unit cross sectional area of wall. Consequently, LaPlace's law can also be written as:

$$P = \frac{2Sw}{r}$$

Therefore, LaPlace's law states that as the radius of a sphere decreases, the magnitude of the inward component of the wall stress increases. This, in turn, increases pressure generated within the sphere.

An expanded version of this equation was used to determine the degree of stretch to apply in order to obtain the required pressure equivalent (*Pi*). The *Pi* is an estimate of the force needed to extend the vessel to the internal circumference (*IC*) and set pressure, as seen *in vivo*:

$$Pi = \text{wall tension}/IC/(2x\pi)$$

To determine the *IC* of each vessel, the *IC* of the vessel at point X_0 (the point when the intralumenal wires are touching) was first established. As each wire had a diameter of 40 μm, the following calculation was employed:

$$(2 + \pi) \times 40$$

Therefore, at X_0 the *IC* was 205.6 μm. A note of the micrometer reading at X_0 was also made. The *IC* was further dependent on point X_i, which is the point where the wires are apart and initial tension has been applied to the vessel. The micrometer reading at X_i was noted. *IC* was finally calculated using the following equation:

$$IC = 2(\text{micrometer reading at } X_i - \text{micrometer reading at } X_0) + IC \text{ at } X_0$$

The *IC* value was then converted from μm into mm in order to calculate *Pi*. Wall tension (*T*) can be described as force (*F*) divided by wall length (*L*). In this situation wall length is doubled to account for the upper and lower wall, so:

$$T = \frac{F}{2L}$$

$$Pi = \text{wall tension}/IC/(2x\pi)$$

$$Pi = \frac{(2x\pi)xT}{IC}$$

$$Pi = \frac{(2x\pi)xF}{2LxIC}$$

The *Pi* was calculated in KPa and given that the pressure exerted by 1 mm column of mercury (1 mmHg) is 133 Pa, the *Pi* was divided by 0.1333 to calculate the pressure in mmHg. If the tension was too low, more tension was applied by moving the jaws further apart. The *Pi* value was then recalculated, until a value of approximately 100 mmHg was obtained. An excel spreadsheet was established based on these principles, to automatically calculate the *Pi* for a given set of X_0 and X_i values.

2.5 Experimental Protocols

2.5.1 Stimulation

Rings of bovine intraocular LPCA were mounted, as previously described, on myograph jaws containing a pair of parallel platinum electrodes for EFS. EFS, consisting of square wave pulses (10 - 15 V, 0.3 ms pulse width, 10 s train length, 0.5 - 32 Hz), was delivered from an S88 stimulator (Grass, Quincy, U.S.A.). Stimulation parameters were designed to evoke maximal neurogenic and minimal myogenic response. TTX; (Narahashi *et al*., 1964; 0.1 μM) was used to confirm the neurogenic origin of responses obtained. All experiments were carried out in the presence of tone induced by the thromboxane A_2 mimetic, U46619 (0.1 – 1 μM), and adrenergic neurone blockade using guanethidine (Page & Dustan, 1959; 30 μM). The concentration of U46619 used was chosen to give a submaximal level of tone (60 – 80% of maximal tone). Prior to performing the control frequency-response, a

voltage-response curve (10 – 15 V) was constructed for each ring of bovine intraocular LPCA, to determine the optimal stimulation strength for that tissue.

2.5.2 Stimulation in the Absence of Tone

Early in the project, frequency-response curves were generated in a small number of rings, in the absence of U46619-induced tone, to investigate if a contractile response to EFS could be obtained in the bovine intraocular LPCA. A control frequency-response curve was obtained, followed by a curve generated in the presence of the NOS inhibitor, L-NAME (Hobbs & Gibson, 1990; 100 μM), to determine if NO released spontaneously from endothelial cells or from nerves by EFS suppressed neurogenic constriction. A final curve was then obtained in the presence of the adrenergic neurone blocker, guanethidine (30 μM), and L-NAME (100 μM) to determine if neurogenic contraction was adrenergic.

2.5.3 Endothelium-Denudation

The role of the endothelium in both, bradykinin-mediated vasodilatation and neurogenic vasodilatation was investigated. To remove the endothelium, a human hair was passed through the lumen of the isolated vessel and rotated gently for 2 minutes. The vessel was then mounted on the myograph jaws as normal. As the endothelium had to be removed prior to mounting, a different set of bovine intraocular LPCA rings had to serve as control tissues. These endothelium-denuded rings then followed the normal protocol for generating frequency-response curves (as described below), or in the presence of U46619-induced tone, were subjected to bradykinin (1 μM).

2.5.4 Frequency-Response Curves

A control frequency-response curve (0.5 – 32 Hz) was obtained for each vessel before a second curve was generated in the presence of the appropriate blocking agent. A period of at least 5 minutes, or until the vessel had regained a stable level of tone, was observed between each stimulation. In time-matched controls, where the second curve was also generated under control conditions, there was no statistically significant difference between the first and second curves.

2.5.5 Stimulation at a Single Frequency of 16 Hz

In some groups of experiments, a full frequency-response curve (0.5 – 32 Hz) was not performed and instead tissues were stimulated at a single frequency of 16 Hz only. This allowed a large number of potential blocking agents to be screened, without being a time-consuming process. Stimulation was carried out at 16 Hz and not the optimal stimulation value of 32 Hz, as repeated stimulation at the higher value often led to a loss in vessel tone because the level of tone did not return to pre-stimulation level following EFS induced-vasodilatation. As before, all experiments were carried out in the presence of tone induced by the thromboxane A_2 mimetic, U46619 (0.1 – 1 μM), and adrenergic neurone blockade using guanethidine (30 μM). The same stimulation parameters were also used (10 - 15 V, 0.3 ms pulse width, 10 s train length). In these experiments, EFS was delivered at the single frequency of 16 Hz at 15 min intervals. When reproducible neurogenic vasodilatation had been obtained the appropriate blocking agent was then added and subsequent effects on neurogenic vasodilatation noted.

2.5.6 Concentration-Response Curves and Desensitisation of Receptors

In experiments where concentration-response curves were produced, they were generated in cumulative fashion. Protocols involving desensitisation of receptors were used to investigate the involvement of various putative neurotransmitter substances in the response to EFS. This was carried out in a number of ways. In order to investigate the potential role of VIP in neurogenic vasodilatation, 0.3 μM VIP was introduced to the myograph bath to promote vasodilatation and the tone was allowed to stabilise following loss of the dilator response. The same concentration of the peptide was added again and lack of response was taken as an indication that receptors had become desensitised (Toda, 1982). In contrast, desensitisation to substance P (Fukuhara *et al*., 1998) was established by constructing a concentration response curve (0.1 nM – 0.1 μM) to the peptide, during which complete desensitisation occurred. The P2X agonist, α-β-methyleneATP, was used to desensitise P2X receptors and therefore determine if they were involved in ATP-mediated dilatation of the bovine intraocular LPCA (Corr & Burnstock, 1994). 10 μM α-β-methyleneATP was introduced to the myograph bath, which promoted vasoconstriction. Following this, the tone was allowed to stabilise and this process was repeated until a lack of constrictor response was taken as an indication that receptors had become desensitised. This usually occurred within three additions of α-β-methyleneATP (30 μM in total).

2.6 Role of NO in Neurogenic Vasodilatation

Initially, frequency-response curves were carried out in the presence of L-NAME (100 μM) and ODQ (Garthwaite *et al*., 1995; 0.3 μM), to determine the role of NO and soluble guanylate cyclase, respectively, in neurogenic vasodilatation. To investigate the mechanism of EFS-induced vasodilatation, frequency-response curves were also performed in the presence of the PDE5 inhibitors, zaprinast (Bowman & Drummond, 1984; 10 μM) and sildenafil (Boolell *et al*., 1996; 1 μM), to determine if cGMP played a role. In addition to this, single stimulation experiments were carried out using the PKG inhibitors, Rp-8-Br-PET-cGMPS (Butt *et al*., 1995; 30 μM) and KT5823 (Wyatt *et al*., 1991; 10 μM), and the PKA inhibitors, Rp-8-Br-cAMPS (Gjertsen *et al.,* 1995; 50 – 100 μM) and H89 (Combest *et al*., 1988; 10 μM), to determine the role of PKG and PKA, respectively.

Additionally, in a separate series of experiments to assess the ability of various NOS inhibitors to block nitrergic nerve-mediated vasodilatation in the bovine intraocular LPCA, frequency-response curves were generated in the presence of the non-specific NOS inhibitor, L-NMMA (Hibbs *et al*., 1987), and the putative nNOS inhibitors, AAAN (Hah *et al*., 2001) and N^G-propyl-L-arginine (Zhang *et al*., 1997a). In some experiments, the concentration dependence of the ability of NOS inhibitors to inhibit neurogenic vasodilatation was examined. In such experiments, EFS was delivered at a single frequency of 16 Hz (as previously described in section 2.5.5). When reproducible neurogenic vasodilatation had been obtained, the effects of increasing concentrations of L-NAME (0.1 – 100 μM), L-NMMA (10 μM – 1 mM) or N^G-propyl-L-arginine (0.1 – 100 μM) were examined. Other experiments were conducted to determine if L-arginine or L-NMMA could inhibit the blockade of neurogenic vasodilatation induced by L-NAME. In these experiments, reproducible neurogenic vasodilatation at 16 Hz, was first obtained in the presence of either L-arginine or L-NMMA (both 1 mM) before examining the effects of increasing concentrations of L-NAME (0.1 μM – 1 mM).

2.7 Role of CGRP in Neurogenic Vasodilatation

A frequency-response curve, following incubation of the vessels for approximately 20 min with the sensory nerve excitotoxin, capsaicin (Jancsó *et al*., 1967; 1 μM), which causes the release and depletion of both CGRP and substance P from nerve terminals was utilised to determine if either neuropeptide was involved in neurogenic vasodilatation. In addition to

this a concentration-response curve to CGRP (0.1 – 300 nM) was performed in the presence of the $CGRP_1$ receptor antagonist $CGRP_{8-37}$ (Chiba *et al*., 1989; 1 and 5 μM) to determine if $CGRP_{8-37}$ could successfully inhibit CGRP-induced vasodilatation in the bovine intraocular LPCA. Following this, frequency-response curves were generated in the presence of $CGRP_{8-37}$ (1 and 5 μM), to explore the involvement of CGRP in neurogenic vasodilatation.

2.8 Role of Substance P in Neurogenic Vasodilatation

Following the experiments carried out in the presence of capsaicin, a frequency-response curve was generated in the presence of receptors desensitised to substance P (as described in section 2.5.6) to further determine the role of this neuropeptide in neurogenic vasodilatation. Additionally, a frequency-response curve was obtained in the presence of the NK_1 receptor antagonist, L-733,060 (Seabrook *et al*., 1996; 0.3 μM).

2.9 Role of VIP in Neurogenic Vasodilatation

A number of strategies were employed to examine the role of VIP in neurogenic vasodilatation. Initially a frequency-response curve was generated in the presence of receptors desensitised to VIP (as described in section 2.5.6), to determine whether neurogenic vasodilatation was still possible, when dilatation in response to exogenous VIP was inhibited. A separate group of vessels were incubated with the protease, α-chymotrypsin (Morris, 1993; 10 U ml^{-1}), in order to break down VIP present in the nerve terminals, and then a frequency-response curve generated. Furthermore, concentration-response curves to VIP (0.1 – 300 nM) in the presence of the K_{ATP} channel blocker, glibenclamide (Schmid-Antomarchi *et al*., 1987; 10 μM), and VIP antiserum (raised in rabbit; active against rat, cat, porcine, bovine, monkey and human VIP; Matthew *et al*., 1997; dilution 1:250) were performed, to determine if either inhibited VIP-mediated dilatation in the bovine intraocular LPCA. Following this, frequency-response curves were also obtained in the presence of VIP antiserum (dilution 1:250, incubated for 2 hr) and glibenclamide (10 μM) to investigate the involvement of VIP in neurogenic vasodilatation.

2.10 Role of ATP in Neurogenic Vasodilatation

Concentration-response curves to ATP (10 nM - 30 μM) were carried out in the presence of the P2Y receptor antagonists, suramin (Dunn & Blakeley, 1998), PPADS (Lambrecht *et al*., 1992), and reactive blue 2 (Burnstock & Warland, 1987b) in order to determine if they

blocked ATP-induced vasodilatation in the bovine intraocular LPCA. A concentration-response curve to ATP was also carried out in the presence of receptors desensitised to α-β-methyleneATP (as described in section 2.5.6) to establish if P2X receptors mediated ATP-induced vasodilatation in this vessel. A frequency response curve was also constructed in the presence of suramin (500 μM), to explore the involvement of ATP in neurogenic vasodilatation.

2.11 Role of K^+ Channels in Neurogenic Vasodilatation

A number of specific and non-specific K^+ channel inhibitors were utilised to determine if K^+ channels were involved in the mechanism of neurogenic vasodilatation in the bovine intraocular LPCA. Frequency-response curves were generated in the presence of the inhibitor of large conductance K_{Ca} channels, iberiotoxin (Latorre *et al*., 1992; 0.1 μM), the inhibitor of large and intermediate K_{Ca} channels and K_V channels, charybdotoxin (Grissmer *et al*., 1994; Miller *et al*., 1985; 0.1 μM), the inhibitor of small conductance K_{Ca} channels, apamin (Blatz & Magleby, 1986; 0.1 μM), and the K_{ATP} channel inhibitor, glibenclamide (Schmid-Antomarchi *et al*., 1987; 10 μM). Single stimulations at 16 Hz were carried out in the presence of the non-specific K^+ channel blocker, TEA (Nelson & Quayle, 1995; 10 μM) and the inhibitor of K_{IR} channels, $BaCl_2$ (Quayle *et al*., 1993; 30 μM), to determine if the aforementioned K^+ channels played a role in neurogenic vasodilatation.

2.12 Ability of NOS Inhibitors to Block Endothelium-Dependent, NO-Mediated Vasodilatation

In order to assess the ability of the non-specific NOS inhibitiors, L-NAME and L-NMMA, and the putative nNOS inhibitors, N^G-propyl-L-arginine and AAAN to inhibit eNOS, in addition to nNOS, in the bovine intraocular LPCA, endothelium-dependent dilatation was evoked using bradykinin (1 μM) in tissues with U46619 (0.1 μM)-induced tone. To ensure that only the nitric oxide-mediated vasodilatation was investigated, the tissues were treated with apamin (0.1 μM) and charybdotoxin (0.1 μM) to block EDHF and indomethacin (10 μM) to inhibit cyclo-oxygenase. The effects of NOS inhibitors L-NAME, L-NMMA, AAAN and N^G-propyl-L-arginine (all 100 μM) were then examined on bradykinin-induced, NO-mediated dilatation.

In a further group of experiments, the concentration dependence of the ability of N^G-propyl-L-arginine to inhibit endothelium-dependent, nitric oxide-mediated vasodilatation

was examined. In the presence of U46619 (0.1 μM)-induced tone, endothelium-dependent dilatation was induced using bradykinin (1 μM). To ensure that only the NO-mediated vasodilatation was investigated, the tissues were treated with apamin (0.1 μM) and charybdotoxin (0.1 μM) to block EDHF and indomethacin (10 μM) to inhibit cyclo-oxygenase. The effects of increasing concentrations of N^G-propyl-L-arginine (0.1 – 100 μM) were then examined on bradykinin-induced, NO-mediated dilatation.

2.13 Use of Papaverine

At the end of all experimental protocols, papaverine (500 μM) was added to determine the level of tone that had been present and all relaxations were expressed as a percentage of this active tone.

2.14 Histological Staining

Samples of bovine intraocular LPCA were dissected out as described previously (section 2.2) and then fixed, processed and stained for light microscopy using the following schedule. Sections were stained with H&E. This stained cell nuclei blue and other structures, such as smooth muscle and endothelial cells, deep pink.

2.14.1 Fixation of Bovine Intraocular LPCA Samples

Vessels were bathed in 10% formalin for 18 hours at room temperature and then washed in running tap water for a few minutes. The vessels were then placed in 70% ethanol for two thirty-minute periods, 90% ethanol for four thirty-minute periods and 100% ethanol for a further four thirty-minute periods. The next stage was to incubate the vessels in Tissue-Clear (Sakura Finetek, Zoeterwoude, Netherlands) for two fifteen-minute periods. The tissues were then placed in liquid paraffin wax for two periods of two hours duration. The tissues were then embedded in cassettes using fresh paraffin wax.

2.14.2 Sectioning of Bovine Intraocular LPCA Samples

Embedded specimens were trimmed and then cooled on ice before sectioning at 5 μm using a Leica RM2035 microtome (Leica, Wetzlar, Germany) with Accu-Edge low profile microtome blades (Sakura Finetek, Zoeterwoude, Netherlands). The sections were floated on distilled water at 36 ^oC, and then mounted on poly-L-lysine coated microscope slides (Sigma, Poole, UK). The slides were then dried overnight at 36 ^oC.

2.14.3 H&E Staining of Bovine Intraocular LPCA.

The slides were placed in Tissue-Clear for three two-minute periods and then placed in 100% ethanol for three two-minute periods, 90% ethanol for three two-minute periods and 70% ethanol for two minutes. They were then washed in gently running tap water for several minutes and placed in haematoxylin (Mayer, 1903) for five minutes and rinsed in running tap water for a further five minutes. The slides were then bathed in a 1% aqueous eosin solution for two minutes and rinsed briefly in running tap water. They were then briefly rinsed in 70% ethanol and then 90% ethanol, before being placed in 90% ethanol for two periods of two minutes. The slides were then bathed in 100% ethanol for three two-minute periods. The final stage was to place the slides in Tissue-Clear for three two-minute periods and then mount them with the synthetic mounting media D.P.X. (Sigma, Poole, UK).

2.15 Drugs and Solvents

α-chymotrypsin, α-β-methyleneATP (α-β-methyleneadenosine-5'triphosphate lithium salt), apamin (from bee venom), AAAN (N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidine tris(trifluoroacetate) salt , ATP (adenosine-5'triphosphate disodium), barium chloride dihydrate crystalline, bradykinin triacetate, capsaicin (8-methyl-N-vanillyl-*trans*-6-nonenamide), $CGRP_{8-37}$, DMSO (dimethyl sulfoxide), guanethidine sulphate, indomethacin, L-arginine, L-NAME (N^G-nitro-L-arginine methyl ester), L-NMMA (N^G-monomethyl-L-arginine acetate salt), papaverine hydrochloride, PPADS (4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3 benzenedisulfonic acid tetrasodium salt), reactive blue 2 (1-amino-4-[[4-[[4-chloro-6-[[3 (or 4)-sulfophenyl]amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid), Rp-8-Br-cAMPS (8-bromoadenosine-3',5'-cyclic monophosphorothioate Rp-isomer), Rp-8-Br-PET-cGMPS (Rp-8-Bromo-β-phenyl-1,N^2-ethenoguanosine 3':5'-cyclic monophosphorothioate sodium salt), substance P, suramin, tetraethylammonium chloride (TEA), U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin$F_{2\alpha}$), VIP, VIP antiserum (rabbit anti-vasoactive intestinal peptide) and zaprinast (M&B 22948) were all obtained from Sigma (Poole, U.K.). CGRP, H89 dihydrochloride and KT5823 were all obtained from Merck Biosciences (Nottingham, U.K.). Charybdotoxin and iberiotoxin were obtained from Latoxan (Valence, France). Ethanol (absolute) was purchased from Fisher Scientific (Loughborough, UK). Glibenclamide was purchased from Tocris (Avonmouth, UK). L-733,066 ((2s,3s)-3-[(3,5-bis(trifluoromethyl)phenyl)methoxy]-2-phenylpiperidine hydrochloride) was purchased

from Sigma (Schnelldorf, Germany). Levcromakalim was a gift from GlaxoSmithKline (Harlow, UK). ODQ (1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) and N^G-propyl-L-arginine were obtained from Alexis Biochemicals (Nottingham, U.K.). Sildenafil (Viagra) was a gift from Pfizer (Sandwich, UK). TTX was purchased from Biomol (Plymouth Meeting, U.S.A.).

All drugs were dissolved and diluted in 0.9% saline, with the following exceptions: capsaicin (1 mM), glibenclamide (1 mM) and N^G-propyl-L-arginine (100 mM) in 100% ethanol; levcromakalim (10 mM) in 70% ethanol; U46619 (1 mM) in 50% ethanol; indomethacin (1 mM) in Na_2CO_3 (1 M); TTX (1 mM) in 0.1 M acetic acid; CGRP (300 μM) and $CGRP_{8-37}$ (300 μM) in 1% acetic acid; ODQ (1 mM), L-733,060 (100 μM), H-89 (1 mM) and KT5823 (1 mM) in DMSO.

2.16 Statistical Analysis

Results are expressed as the mean ± s.e.mean of *n* observations, each from a separate vessel from a different eye. Statistical comparisons were made using one-way analysis of variance (ANOVA) and the Bonferroni post-hoc test and confirmed using the Kruskal-Wallis test. When necessary, further statistical analysis was carried out using the repeated measures General Linear Model test and the Wilcoxon Signed Ranks test. All statistics were performed with the aid of a computer program, Prism (GraphPad, San Diego, U.S.A.). A probability (P) less than or equal to 0.05 was considered significant.

CHAPTER 3.

RESULTS: IDENTIFICATION OF NEUROTRANSMITTERS MEDIATING NEUROGENIC VASODILATATION

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3 Identification of Neurotransmitters Mediating Neurogenic Vasodilatation

3.1 Determination of Stimulation Parameters

Preliminary experiments were carried out to determine the stimulation parameters required to obtain a neurogenic response in the bovine intraocular LPCA. The following initial experiment was carried out using untested parameters, merely to establish if EFS evoked dilatation in the bovine LPCA.

In the presence of submaximal U46619 (0.1 μM)–induced tone and the adrenergic neurone blocker, guanethidine (30 μM), EFS (15 V, 16 Hz, 0.3 ms pulse width, 10 s train length) of bovine intraocular LPCA rings evoked dilatation that was biphasic in nature (Figure 3.1). The optimal times for the first and second components of dilatation to occur were 10 s and 50 s, respectively. Therefore all subsequent work involved two measurements, made at 10 s and 50 s, so that pharmacological analysis of each component was possible.

3.1.1 Voltage-Response Curve

As EFS in the presence of U46619-induced tone evoked dilatation, the next stage was to establish the optimum stimulus strength required to obtain maximum dilatation. A voltage response curve (10 - 18 V, 16 Hz, 0.3 ms pulse width, 10 s train length) was constructed in the presence of guanethidine (30 μM). Dilatation occurred optimally at 18 V (Figure 3.2), with responses of 16 ± 2% and 27 ± 4% (n=16) for the first and second components, respectively. Furthermore, to determine that the response to EFS was entirely neurogenic, a second voltage response curve (5 – 25 V, 16 Hz, 0.3 ms pulse width, 10 s train length) was carried out in the presence of the neurotoxin TTX, (Narahashi *et al*., 1964; 0.1 μM). Both first and second components were completely inhibited in the presence of TTX when the stimulus strength was between 5 and 15 V (n=8, Figure 3.3). However, between 15 and 20 V a non-neurogenic, vasodilator response developed (Figure 3.3) and first (4 ± 5%) and second (24 ± 5%) components were observed. Therefore, the maximum stimulus strength employed in all subsequent experiments was 15 V and all experimental protocols were designed to include a voltage-response curve for each set of tissues.

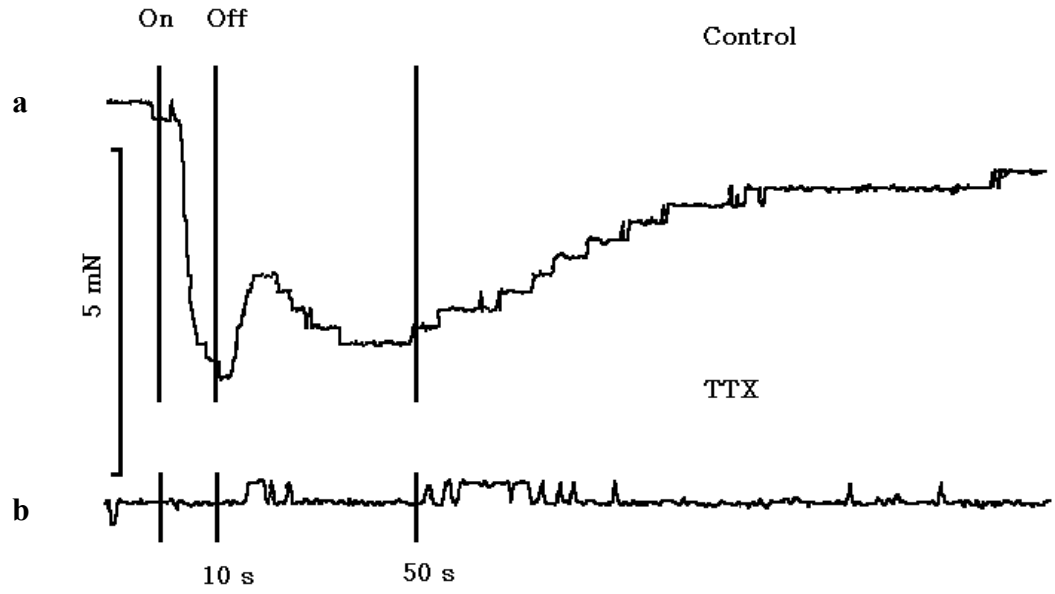

Figure 3.1 The biphasic relaxant response of bovine intraocular LPCA rings to EFS (15 V, 16 Hz, 0.3 ms pulse width, 10 s train length). The response was generated in the presence of U46619 (0.1 μM) and guanethidine (30 μM). The first solid line marker indicates the onset of stimulation; the second (at 10 s) represents both the end of the stimulation period and the peak of the first component of dilatation. The third line (at 50 s) indicates the peak of the second component of relaxation. (a) The control biphasic response. (b) Both components of dilatation were abolished by TTX (0.1 μM).

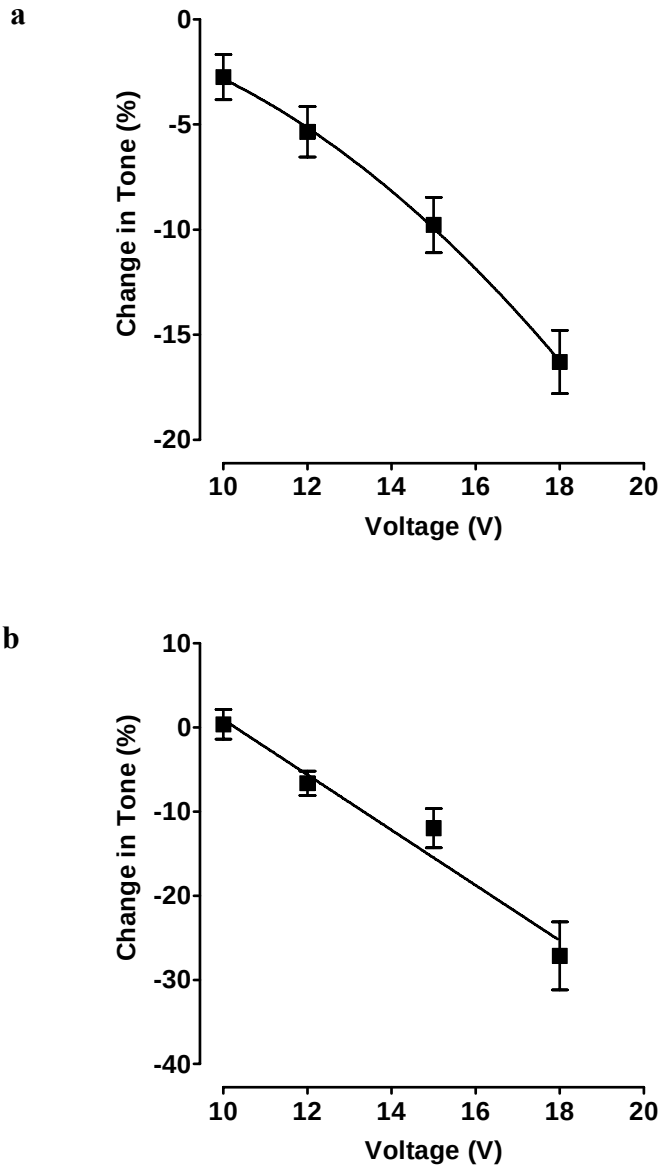

Figure 3.2 Voltage - response curves to EFS (10 – 18 V, 16 Hz, 0.3 ms pulse width, 10 s train length) in control bovine intraocular LPCA rings. The response was generated in the presence of U46619 (0.1 μM) and guanethidine (30 μM). The first (a) and second (b) components measured at 10 s and 50 s, respectively, both increased in magnitude in a voltage-dependent manner, optimally at 18 V. Data are mean±s.e. mean; n=16.

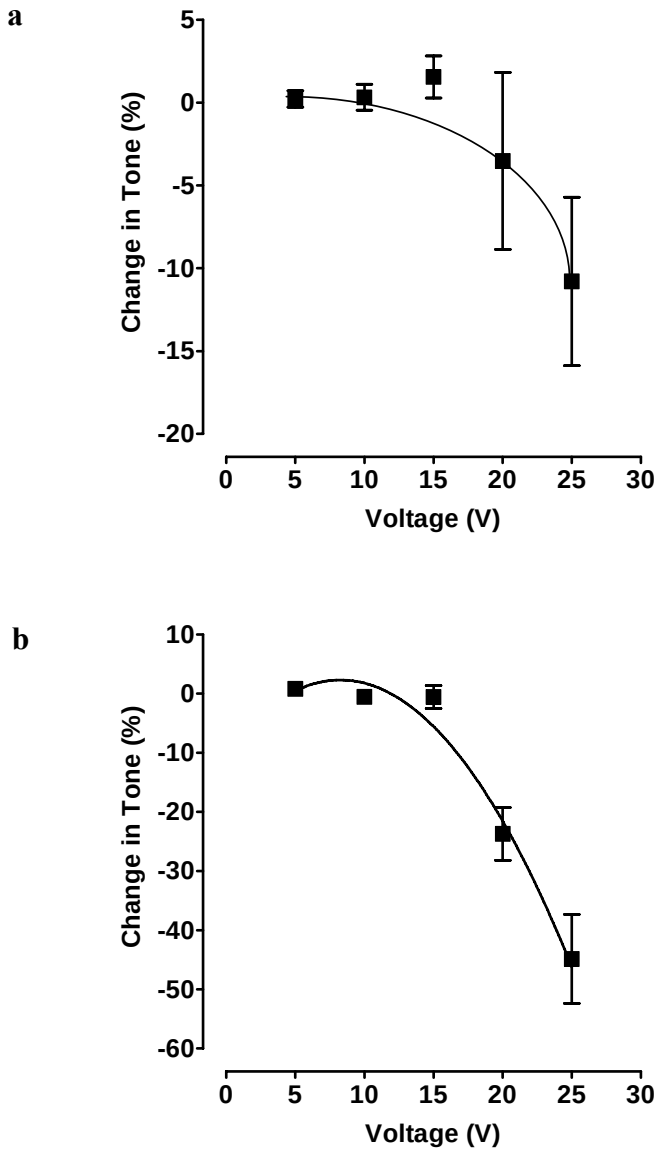

Figure 3.3 Voltage - response curves to EFS (5 – 25V, 16 Hz, 0.3 ms pulse width, 10 s train length) in bovine intraocular LPCA rings in the presence of TTX (0.1 μM). The response was generated in the presence of U46619 (0.1 μM) and guanethidine (30 μM). The first (a) and second (b) components of dilatation measured at 10 s and 50 s respectively, were inhibited in the presence of TTX at lower voltages (5 – 15 V). However, at voltages above 15 V a non-neurogenic dilatation was observed. For this reason, the stimulation strength was never increased above 15 V for the remainder of the EFS experiments. Data are mean±s.e. mean; n=8.

3.1.2 Frequency-Response Curve

A frequency-response curve was generated (10 - 15 V, 0.5 - 32 Hz, 0.3 ms pulse width, 10 s train length) to determine the optimum frequency for stimulation (n=21, Figures 3.4 and 3.5). The dilatation was maximally observed for both first (10 s) and second components (50 s) at 32 Hz (32 ± 3% and 21 ± 2%, respectively).

3.1.3 Constrictor Response to EFS in the Absence of Tone

A frequency-response curve (10 - 15 V, 0.5 - 32 Hz, , 0.3 ms pulse width, 10 s train length) was carried out in the absence of tone to determine if a constrictor response could be obtained in the bovine LPCA (Figure 3.6). Although control tissues were unresponsive to stimulation at any frequency (n=6, response at 32 Hz was 0.02 ± 0.10 mN), incubation with the inhibitor of NOS, L-NAME (Hobbs & Gibson, 1990; 100 μM), uncovered a small, frequency-dependent constrictor response. The optimum frequency for contraction was 32 Hz (0.79 ± 0.12 mN, n=11) and this was significantly different to the control response ($P<0.001$). In the presence of guanethidine (30 μM) this constrictor response was inhibited (0.17 ± 0.09 mN, n=9) and was undistinguishable from the control response.

3.1.4 Effect of TTX on Neurogenic Vasodilatation

To determine if dilator responses to EFS were neurogenic at all frequencies, frequency response curves (0.5 – 32 Hz) were generated in the absence and presence of TTX (0.1 μM,). Both components of dilatation to EFS were abolished at all frequencies. At 32 Hz, the first and second components were 25 ± 3% and 19 ± 3% (n=8), respectively in control tissues and 1 ± 1 % and 1 ± 1% (n=8) in rings treated with TTX ($P<0.001$). This confirmed that both components of dilatation were neurogenic in origin at all frequencies (Figures 3.1 and 3.7).

3.1.5 *Effect of Removal of the Endothelium on Neurogenic Vasodilatation*

The endothelium-dependent relaxant, bradykinin (1 μM), evoked powerful dilatation in control tissues (58 ± 4%, n=8) and this was virtually abolished in endothelium-denuded tissues (5 ± 1%, n=8, $P<0.001$, Figure 3.8). Endothelium denudation had no effect, however on the magnitude of the first or second components of dilatation to EFS at any frequency (Figure 3.9). The first and second components at the optimal frequency of 32Hz

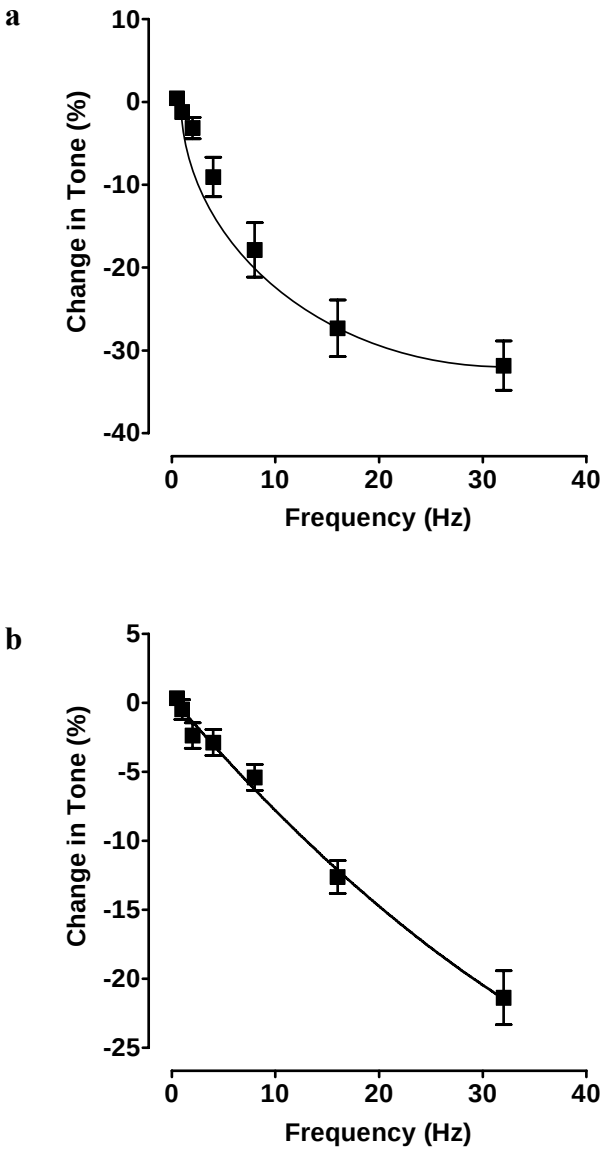

Figure 3.4 Frequency-response curves to EFS (15V, 0.5 - 32 Hz, 0.3 ms pulse width, 10 s train length) in control bovine intraocular LPCA rings, generated in the presence of U46610 (0.1 μM) and guanethidine (30 μM). Both (a) the first component of dilatation, measured at 10 s and (b) the second component, measured at 50 s dilate in a frequency-dependent manner, optimally at 32 Hz. Data are mean±s.e. mean; n≥20.

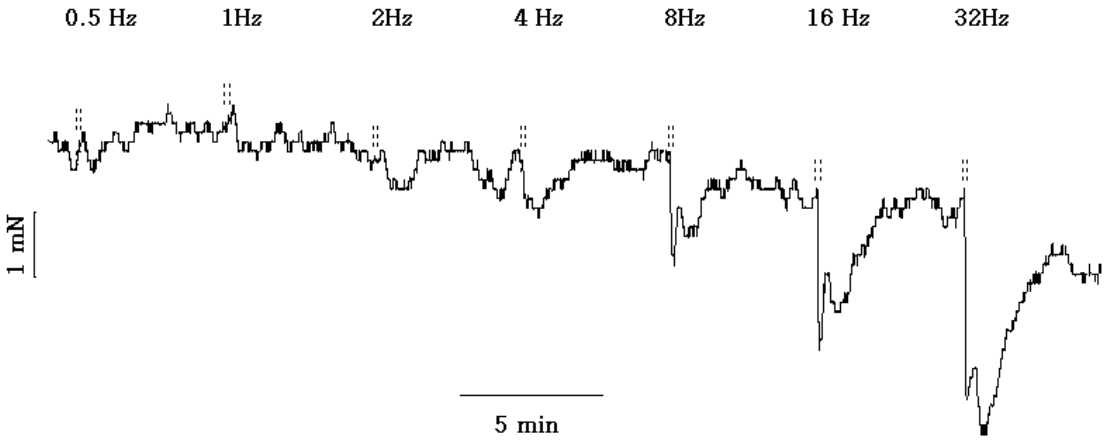

Figure 3.5 The relaxant response of bovine intraocular LPCA rings to EFS (15 V, 0.3 ms pulse width, 10 s train length) over a range of frequencies from 0.5 – 32 Hz, in the presence of U46619 (0.1 μM) and guanethidine (30 μM). The dotted markers indicate the onset of stimulation and end of the stimulation period. A clear biphasic response can be seen at frequencies of 8 Hz and greater. A 5 minute recovery period was observed between stimulations.

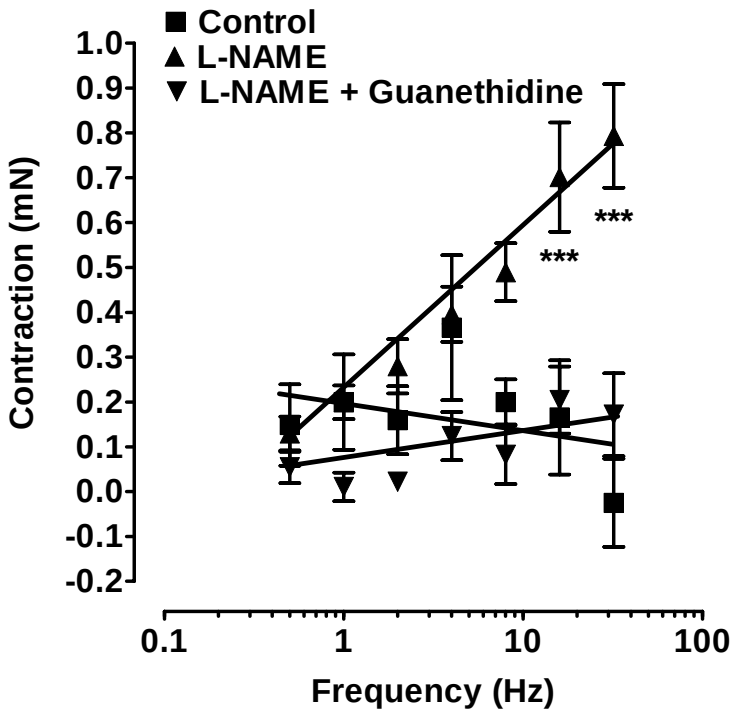

Figure 3.6 Frequency-response curves showing constriction to EFS (0.5 - 32 Hz) in the absence of tone, in control bovine intraocular LPCA rings and in those treated with the inhibitor of NOS, L-NAME (100 μM) or L-NAME (100 μM) and the adrenergic neurone blocker, guanethidine (30 μM). There was little response to stimulation at any frequency in control tissues. In the presence of L-NAME, a small frequency–dependent contraction was uncovered. This contraction was inhibited in the presence of guanethidine. The x-axis is displayed in a log scale to facilitate interpretation of the data points from 0.5 to 8 Hz. Data are mean±s.e. mean; n≥6; **P<0.01 and ***P<0.001 indicate differences from control.

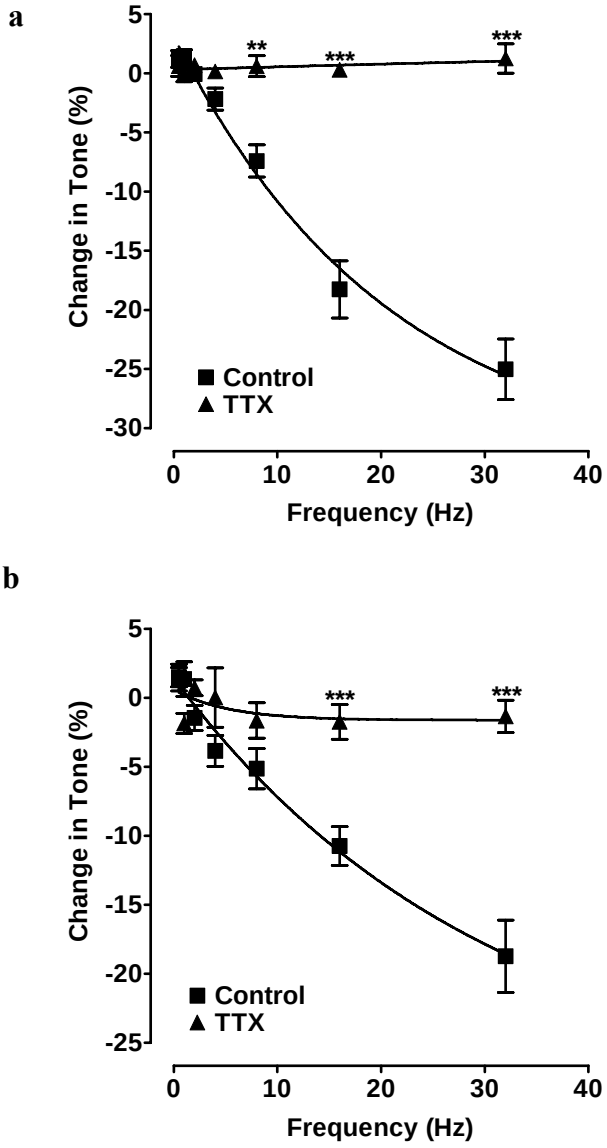

Figure 3.7 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those treated with the neurotoxin, TTX (0.1 μM). Both (a) the first component of dilatation, measured at 10 s, and (b) the second component, measured at 50 s, were abolished by TTX at all frequencies. Data are mean±s.e. mean; n=8.

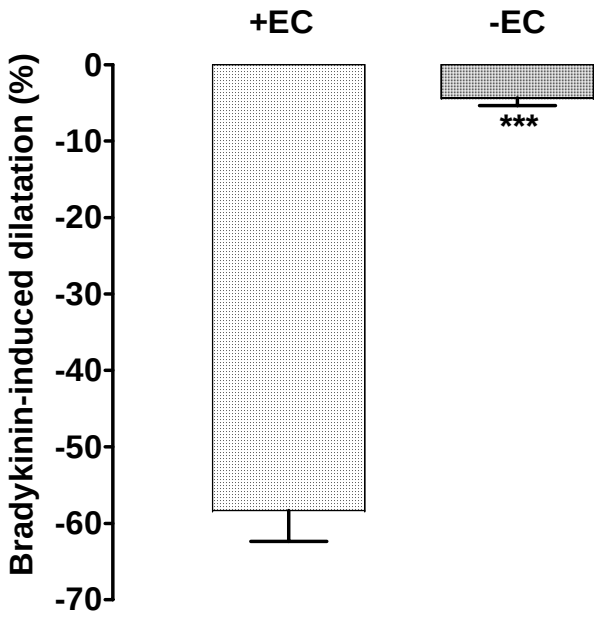

Figure 3.8 In the presence of U46619, the addition of bradykinin (1 μM) induced powerful dilatation of control bovine intraocular LPCA rings. In rings where the endothelium had been denuded by rubbing with a human hair, relaxation to bradykinin (1 μM) was virtually abolished. +EC and –EC indicate the presence and absence of the endothelium, respectively. Data are mean±s.e. mean; n=8; ***P<0.001 indicates a difference from endothelium-containing rings.

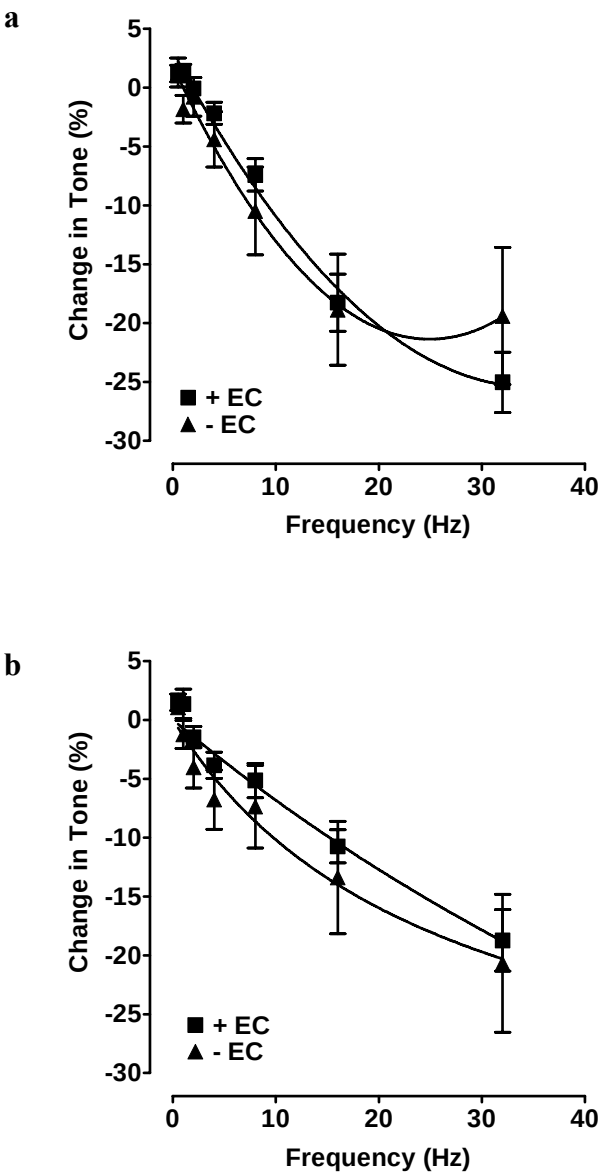

Figure 3.9 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in endothelium denuded tissues. Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was affected by endothelial denudation. +EC and –EC indicate the presence and absence of the endothelium, respectively. Data are mean±s.e. mean; n=8.

were 25 ± 3% and 19 ± 3% (n=8), respectively, in control tissues and 19 ± 6% and 21 ± 6% (n=8) in endothelium-denuded tissues. These data suggest that the responses to EFS are not influenced by the presence of the endothelium.

3.2 Role of NO in Neurogenic Vasodilatation

NO has previously been identified as a neurotransmitter that mediates dilatation in response to EFS in the bovine intraocular LPCA (Wiencke *et al*., 1994). Therefore, we initially attempted to inhibit the response to EFS using known blockers of the NO-cGMP pathway.

3.2.1 Effect of L-NAME on Neurogenic Vasodilatation

The first component of dilatation to EFS at all frequencies was abolished and even reversed to a small contraction by the NOS inhibitor, L-NAME (100 μM), but the second component remained entirely unaffected. Control dilatations at 32 Hz were 36 ± 5% and 24 ± 3% (n=12) for first and second components, respectively. A contraction of 9 ± 4% ($P<0.001$) and dilatation of 25 ± 3% (n=12) were obtained in L-NAME treated tissues (Figures 3.10 and 3.11). These data suggested that the first, but not second, component of dilatation was mediated by NO released from nitrergic nerves.

3.2.2 Effect of ODQ on Neurogenic Vasodilatation

The inhibitor of soluble guanylate cyclase, ODQ (Garthwaite *et al*., 1995; 0.3 μM), also abolished the first component of dilatation at all frequencies (Figure 3.12). At the optimum frequency of 32 Hz a contraction of 9 ± 3% was obtained, compared with a dilatation of 26 ± 5% in control tissues ($P<0.001$, n=7). However, ODQ had no effect on the magnitude of the second component which was 31 ± 6% in control tissues and 33 ± 5% in treated rings, respectively (n=7). These data suggest that the first, but not the second component of dilatation, is mediated via soluble guanylate cyclase.

3.3 Role of CGRP in Neurogenic Vasodilatation

CGRP has also previously been proposed as a neurotransmitter that mediates dilatation in response to EFS in the bovine intraocular LPCA (Wiencke *et al*., 1994). In order to test

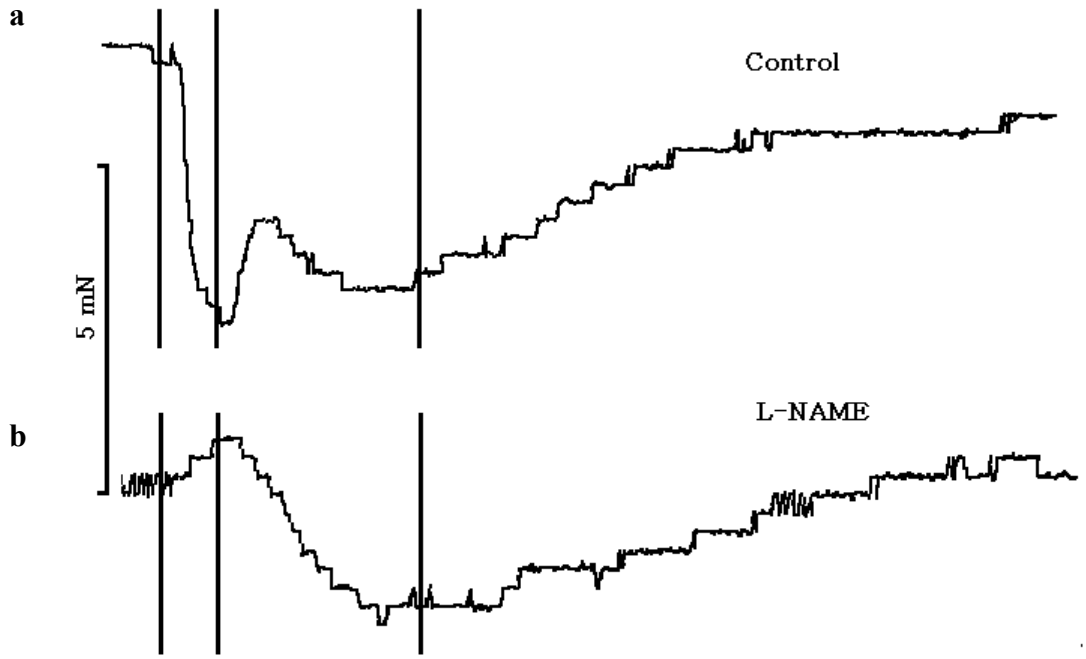

Figure 3.10 The biphasic dilator response of bovine intraocular LPCA rings to EFS (15 V, 0.3 ms pulse width, 10 s train length, 16 Hz). The first solid line marker indicates the onset of stimulation; the second (at 10 s) represents both the end of the stimulation period and the peak of the first component of dilatation. The third line (at 50 s) indicates the peak of the second component of dilatation. (a) The control biphasic response. (b) The first component is abolished by the NOS inhibitor, L-NAME (100 μM), but the second component is unaffected.

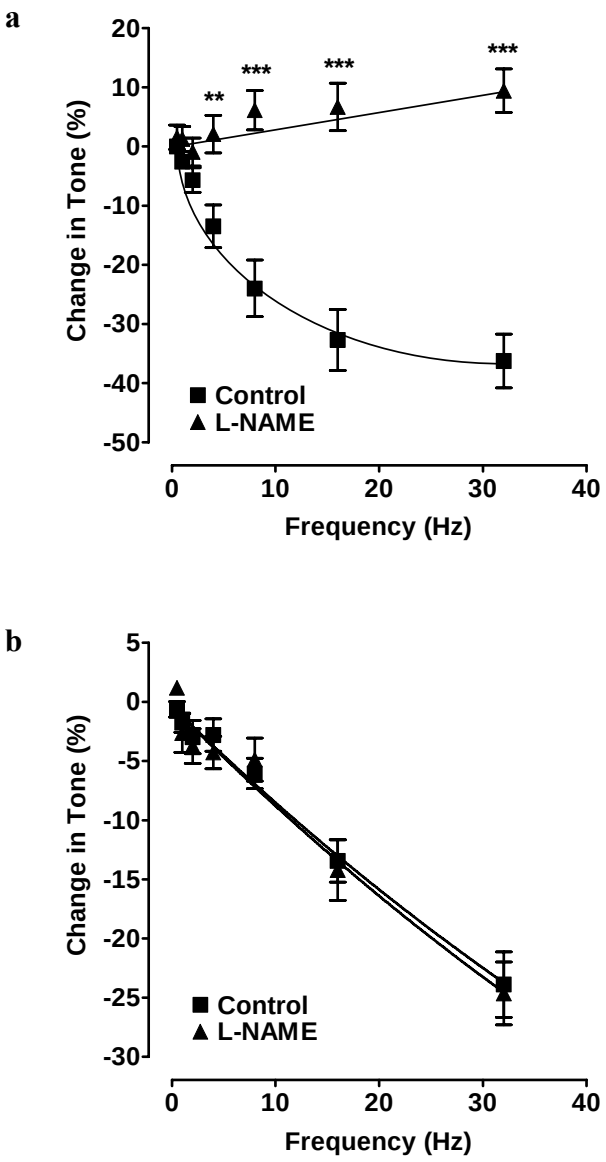

Figure 3.11 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA and in those treated with the NOS inhibitor, L-NAME (100 μM). (a) The first component of dilatation, measured at 10 s, was abolished by L-NAME at all frequencies. (b) The second component, measured at 50 s, was unaffected by L-NAME. Data are mean±s.e. mean; n=12; **P<0.01 and ***P<0.001 indicate differences from control.

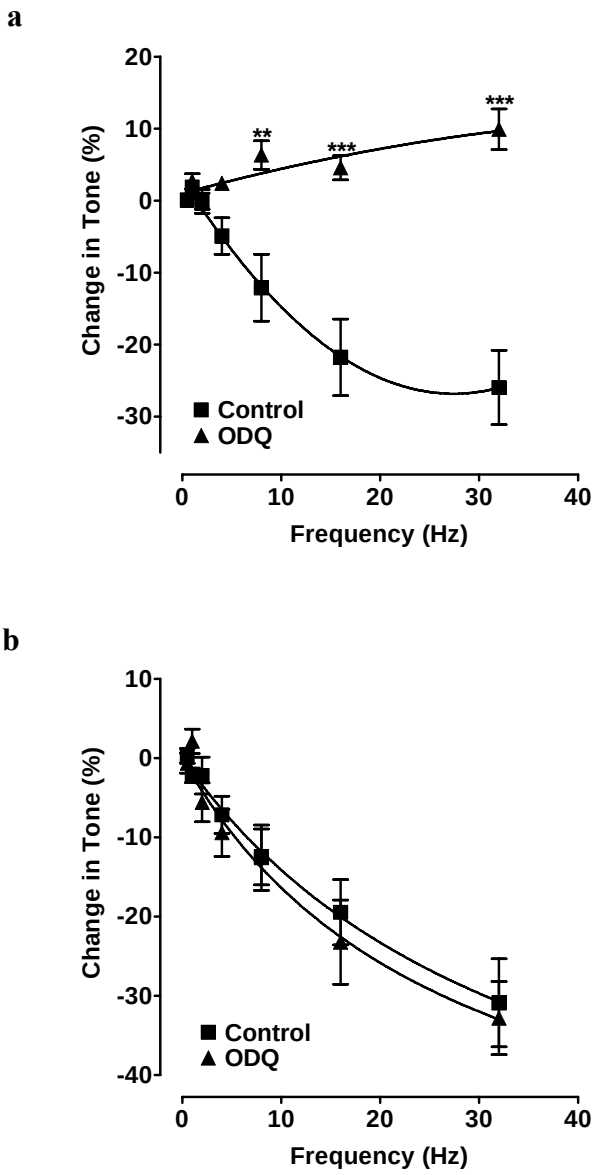

Figure 3.12 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those treated with the inhibitor of soluble guanylate cyclase, ODQ (0.3 μM). (a) The first component of dilatation, measured at 10 s, was abolished by ODQ at all frequencies. (b) The second component, measured at 50s, was unaffected by ODQ. Data are mean±s.e. mean; n=7; **P<0.01 and ***P<0.001 indicate differences from control.

this hypothesis a number of strategies, including inhibiting release of CGRP from sensory nerves and utilising a $CGRP_1$ receptor antagonist, were employed.

3.3.1 Effect of capsaicin and $CGRP_{8-37}$ on Neurogenic Vasodilatation

The sensory nerve excitotoxin, capsaicin (Jancsó *et al*., 1967; 1 μM), induced a transient fall in U46619 (0.1 μM)- induced tone of bovine intraocular LPCA rings (15 ± 6%, n=8, Figure 3.13a), which recovered to pre-drug levels within 15 min (Figure 3.13b). A second addition of capsaicin (1 μM) failed to induce dilatation, but instead promoted a small contraction (19 ± 5%, n=8).

Furthermore, desensitisation with capsaicin (1 μM), as above, for 1 hr prior to EFS had no effect on either the first or second component of dilatation at any frequency (Figure 3.14): first and second components at 32 Hz were 28 ± 3% and 18 ± 2% (n=12), respectively, in control tissues and 25 ± 1 % and 18 ± 3% in rings treated with capsaicin (n=12). From these data, it seems unlikely that CGRP mediates either the first or second component of neurogenic dilatation in this tissue.

In the presence of U46619 (0.1 μM)- induced tone in bovine intraocular LPCA, CGRP (0.1 – 300 nM, n=10) induced concentration-dependent dilatation, with a maximally observed dilatation of 90 ± 2% and a pEC_{50} of 8.90 ± 0.04 (Figure 3.15).

In the presence of the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (Chiba *et al*., 1989; 1 and 5 μM, n≥8), the CGRP concentration-response curve was shifted to the right, with an apparent pK_B of 6.65, but the maximally observed response was unaffected (Figure 3.15). Furthermore, pretreatment of vessels that had not been previously exposed to capsaicin with the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (1 μM), abolished the capsaicin (1 μM)-induced dilatation (reversed to a small contraction of 6 ± 7%, n=15, Figure 3.13). It is therefore likely that the capsaicin-induced dilatation is mediated through release of CGRP.

The first and second components of dilatation to EFS remained unaffected at all frequencies in ciliary artery rings pretreated with $CGRP_{8-37}$ (1 μM, Figure 3.16): control dilatations at 32 Hz were 25 ± 4% and 27 ± 4% (n=10) for first and second components, respectively, and dilatations of 24 ± 3% and 25 ± 4% (n=10) were obtained in $CGRP_{8-37}$ treated tissues. This experiment was repeated using 5 μM $CGRP_{8-37}$ (n=8). Again $CGRP_{8-37}$

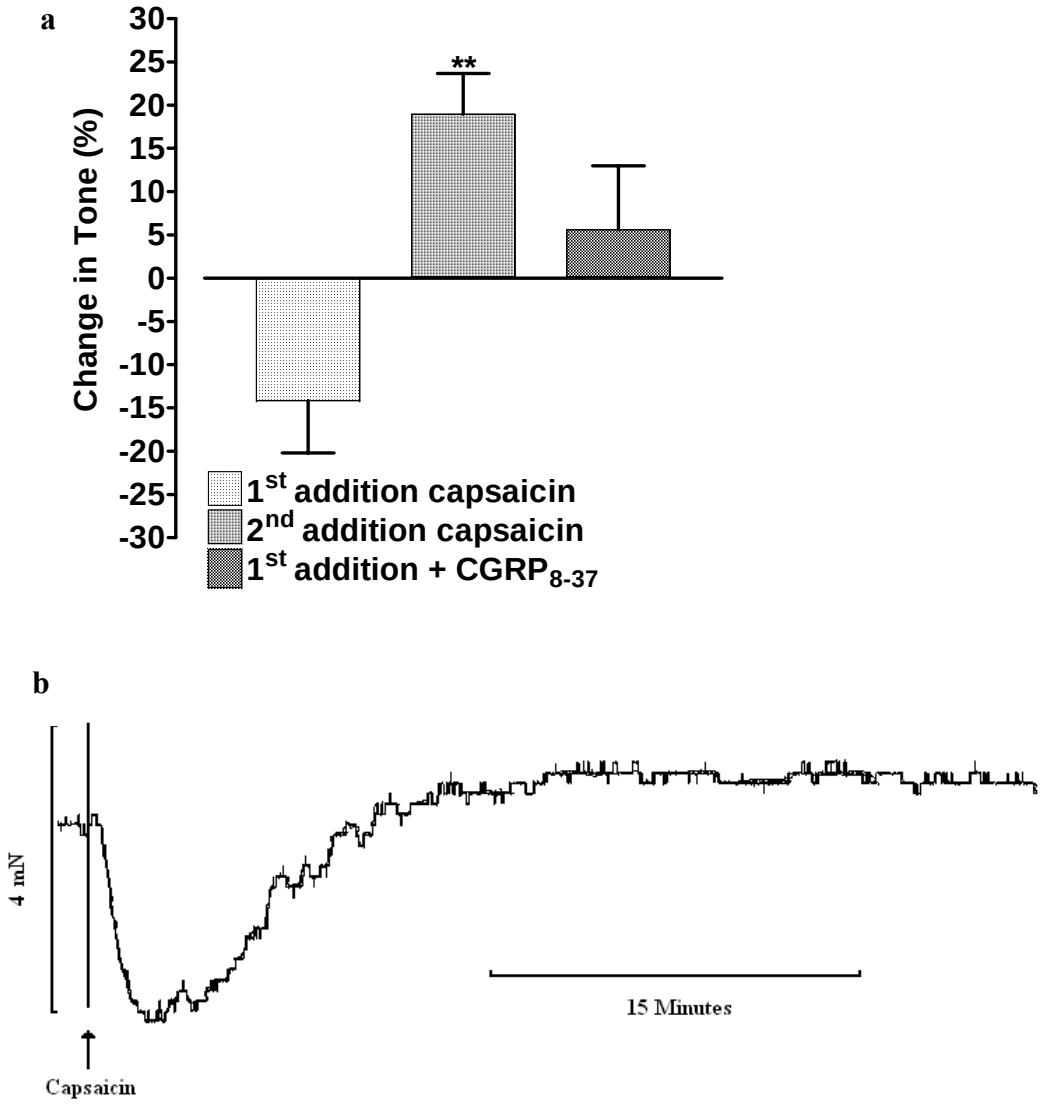

Figure 3.13 The response of bovine intraocular LPCA rings to capsaicin. (a) A first addition of capsaicin (1 μM) induced dilatation of bovine intraocular LPCA rings that was transient in nature. When the response had completely resolved, a second addition of capsaicin (1 μM) failed to elicit relaxation, but produced a slight contraction. Furthermore, pretreatment with the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (1 μM), inhibited the relaxant response to a first addition of capsaicin. (b) The response to capsaicin (1 μM) was transient and resolved within approximately 15 minutes. Data are mean±s.e. mean; n≥8; **P<0.01 indicates a difference from the first addition of capsaicin.

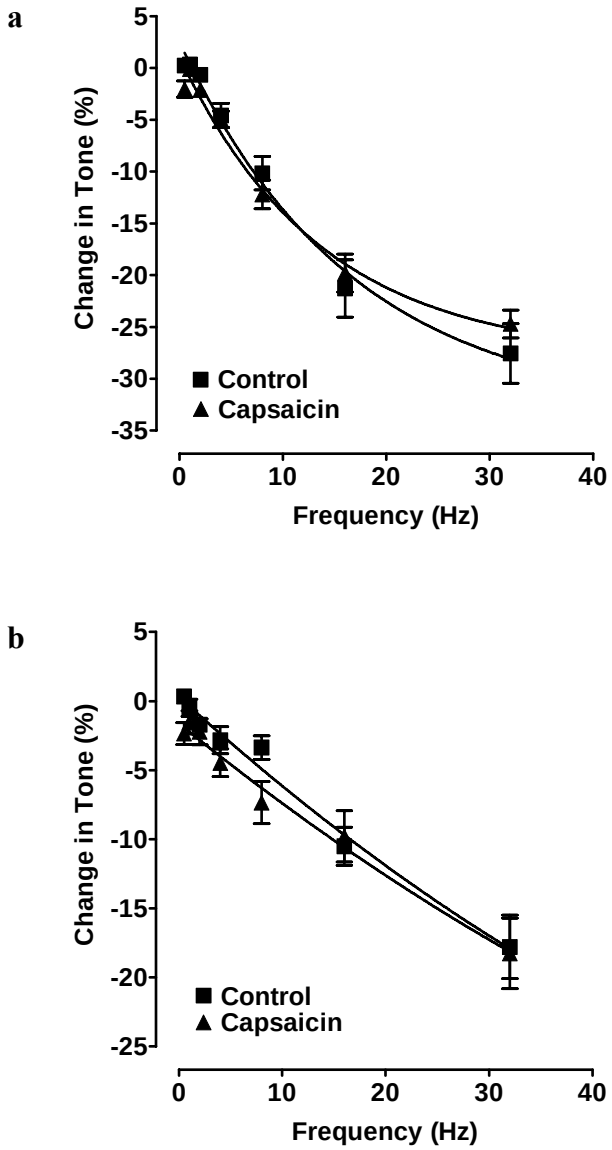

Figure 3.14 Frequency-response curves to EFS (0.5-32 Hz) in control bovine intraocular LPCA rings and in those pretreated with capsaicin (1 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited in the presence of capsaicin. Data are mean±s.e. mean; n=12.

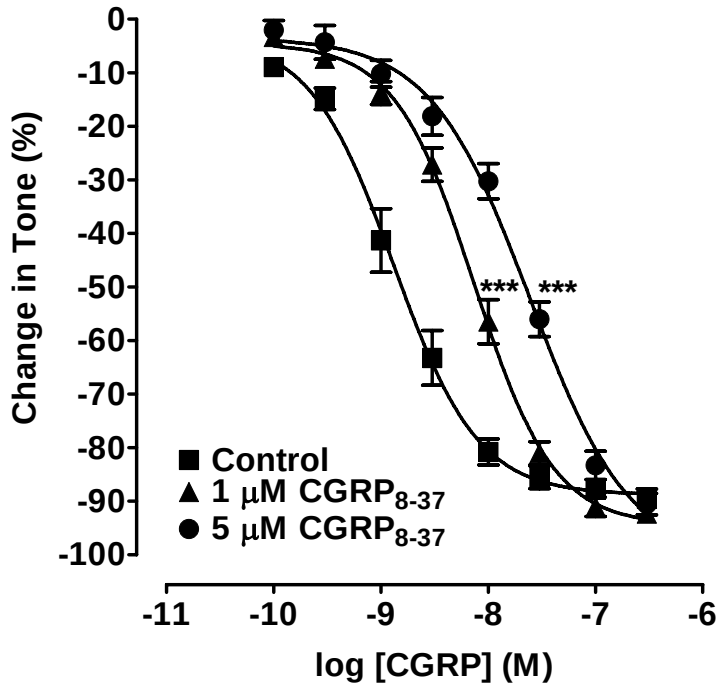

Figure 3.15 CGRP (0.1-300 nM) induced concentration-dependent relaxation of bovine intraocular LPCA rings and this was inhibited following pretreatment with the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (1 and 5 μM). Data are mean±s.e. mean; n≥8. ***P<0.001 indicates a difference in pEC_{50} from control.

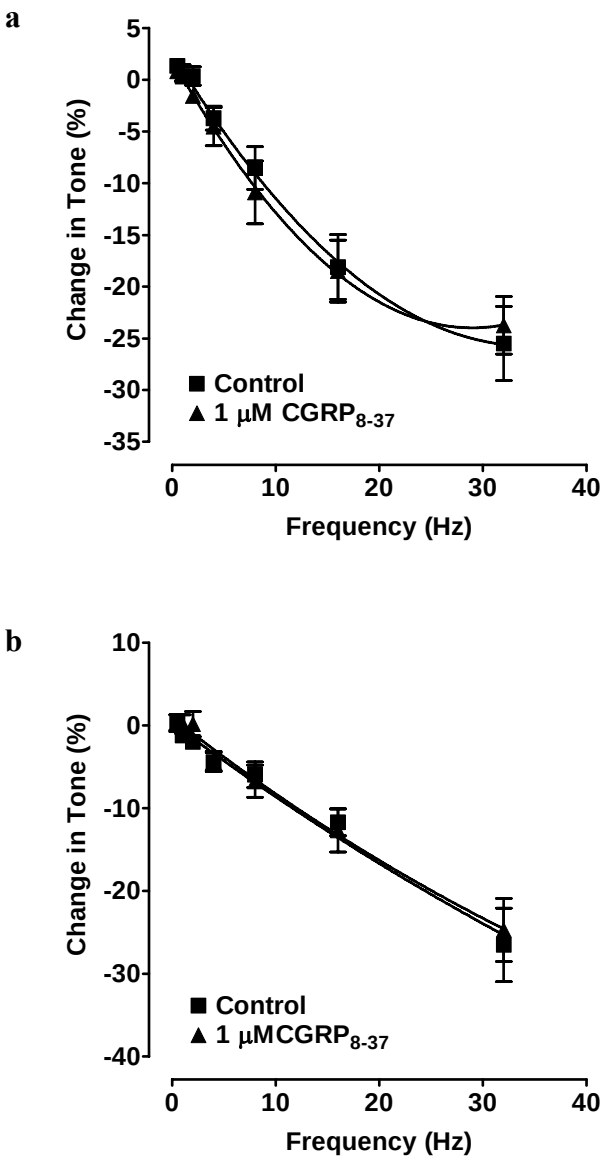

Figure 3.16 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those pretreated with the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (1 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was affected by $CGRP_{8-37}$. Data are mean±s.e. mean; n=10.

failed to inhibit EFS-induced dilatation, with first and second components at 32 Hz of 28 ± 4% and 21 ± 3%, respectively, in control tissues and 30 ± 4% and 22 ± 4% in rings treated with antagonist (Figure 3.17). These experiments were also repeated in the presence of L-NAME (100 μM) to remove the first component (Figure 3.18). Once more, $CGRP_{8-37}$ (5μM) did not inhibit the second component of dilatation to EFS. In fact, the maximally observed response at 32 Hz was slightly potentiated (16 ± 3% in control tissues and 24 ± 2% in those treated with both L-NAME and $CGRP_{8-37,}$ $P<0.01$, n=11).

The above data make it improbable that CGRP contributes to either the first or second components of neurogenic dilatation in the bovine intraocular LPCA.

3.4 Role of Substance P in Neurogenic Vasodilatation

Once we determined that the transmitter mediating the second component of dilatation to EFS was not CGRP, we sought to establish if it was another neuropeptide, substance P.

3.4.1 Effect of Substance P Desensitisation on Neurogenic Vasodilatation

Substance P (0.1 nM - 0.1 μM) induced concentration-dependent dilatation of ciliary artery rings, with a maximally observed dilatation of 18 ± 4% (n=8, Figure 3.19). However, rapid desensitisation occurred, such that it was difficult to complete a concentration-response curve (Figures 3.19 and 3.20).

Following the development of complete desensitisation to substance P (0.1 μM), the first and second components of dilatation to EFS remained unaffected at all frequencies (Figure 3.21). Control responses of 19 ± 4% and 22 ± 3% (n=11) were obtained for the first and second components, respectively, at 32 Hz. In tissues desensitised to substance P, responses of 22 ± 4% and 27 ± 3% (n=11), respectively, were obtained.

3.4.2 Effect of L733,060 on Neurogenic Vasodilatation

In the presence of the NK_1 antagonist, L733,060 (Seabrook *et al*., 1996; 0.3 μM), the relaxant response to exogenous substance P (0.1 μM) was reduced from 16 ± 4% to 3 ± 2% ($P<0.01$, n=10, Figure 3.22), confirming that the antagonist is effective in this tissue. Nevertheless, in the presence of L733,060 (0.3 μM), the response to EFS at all frequencies was unchanged (n=4, Figure 3.23). At the optimum frequency of 32 Hz, control values of of 33 ± 4% and 40 ± 4% (n=8) were obtained for the first and second components,

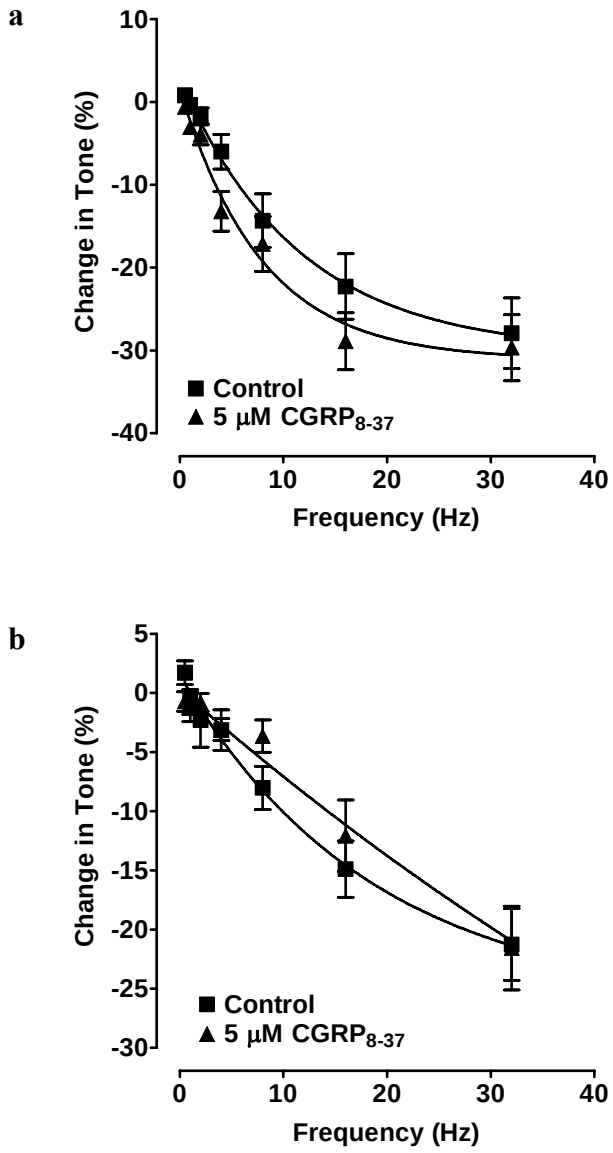

Figure 3.17 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those pretreated with the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (5 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by $CGRP_{8-37}$. Data are mean±s.e. mean; n=8.

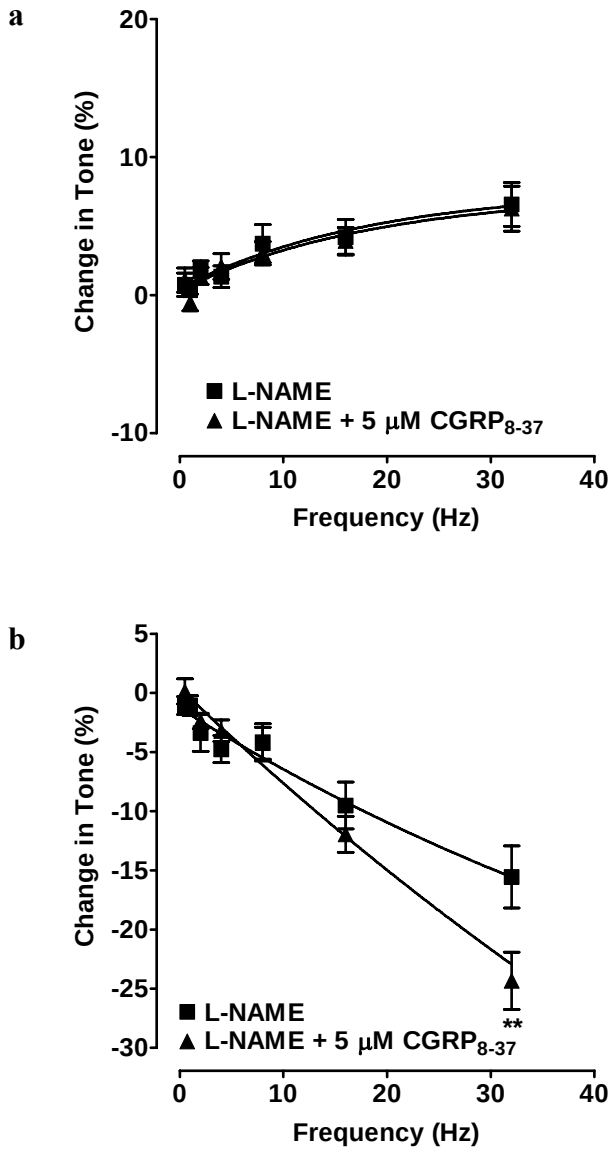

Figure 3.18 Frequency-response curves to EFS (0.5 - 32 Hz) in bovine intraocular LPCA rings pretreated with the NOS inhibitor, L-NAME (100 μM), or a combination of L-NAME (100 μM) and the $CGRP_1$ receptor antagonist, $CGRP8_{-37}$ (5 μM). (a) As expected, the first component of dilatation, as measured at 10 s, was abolished by L-NAME alone and by the combination of L-NAME and $CGRP_{8-37}$, at all frequencies. (b) The second component, as measured at 50 s, was unaffected by L-NAME or the combination of L-NAME and $CGRP_{8-37}$, apart from at 32 Hz, where the response appeared to be potentiated. Data are mean±s.e. mean; n=11; **P<0.01 indicates a difference from control.

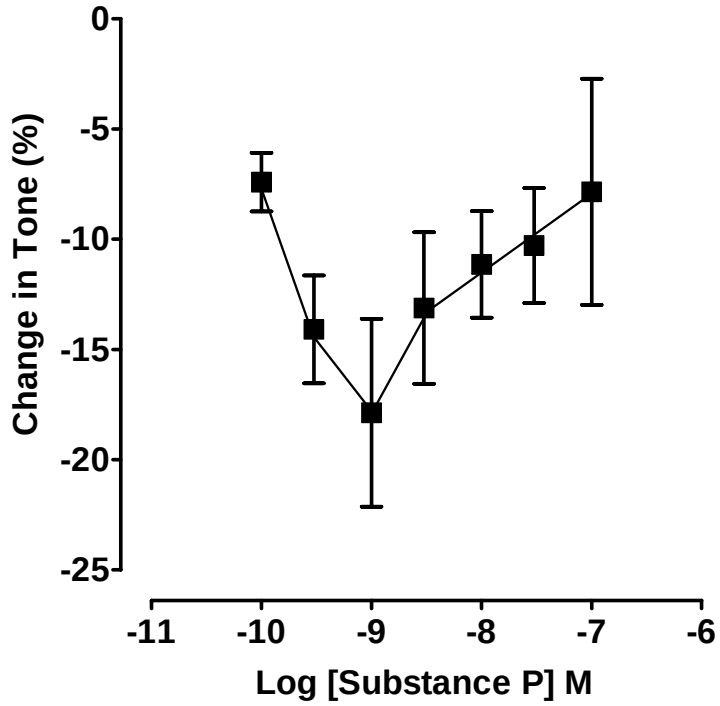

Figure 3.19 Substance P (0.1-100 nM) induced concentration-dependent relaxation of bovine intraocular LPCA rings. However, desensitisation occurred very quickly, such that further addition of Substance P after a concentration of 1 nM failed to promote relaxation. Data are mean±s.e. mean; n=8.

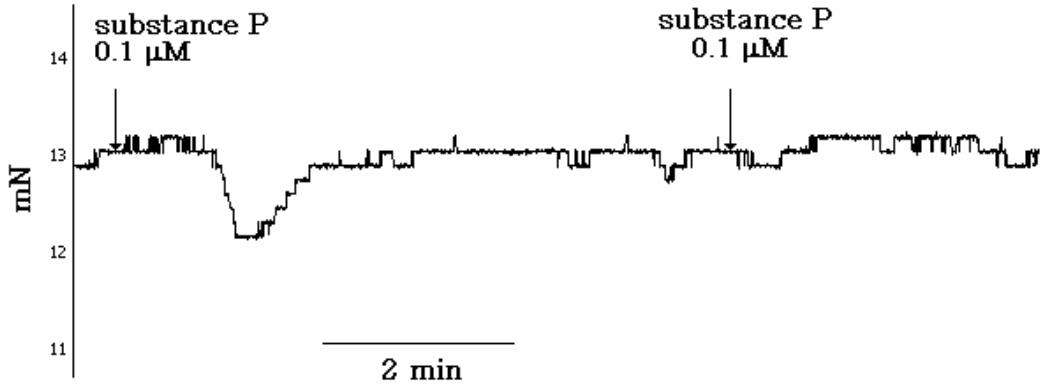

Figure 3.20 Substance P (0.1 µM) induced dilatation of bovine intraocular LPCA rings. However, desensitisation set in quickly, such that the response was lost within 2 min and a second addition of substance P (0.1 µM) failed to promote further relaxation.

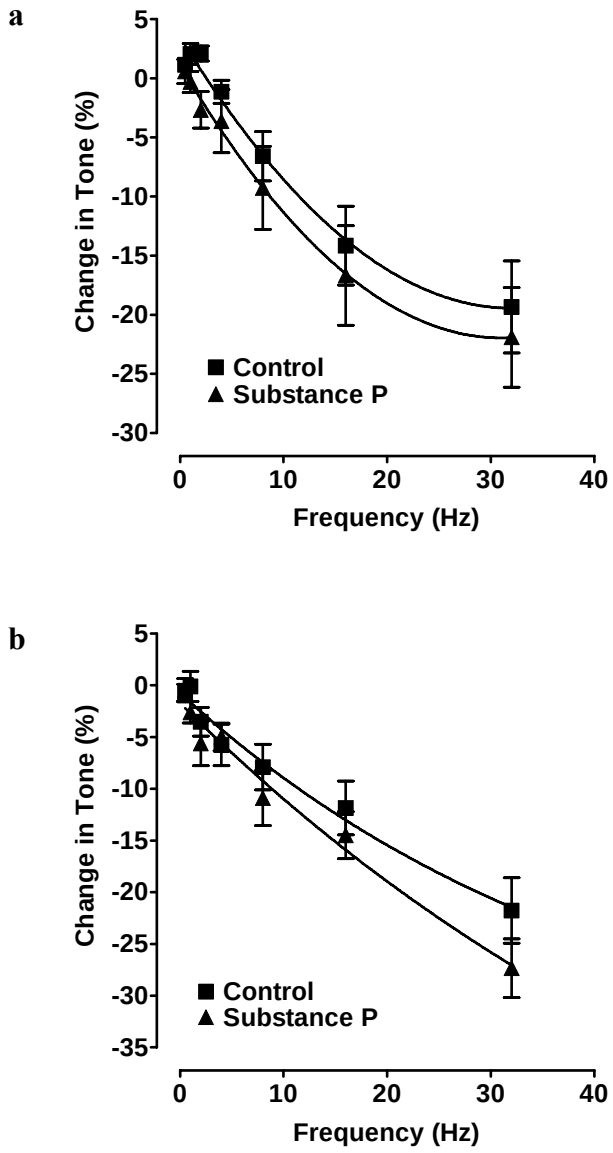

Figure 3.21 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those desensitised to substance P (0.1 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by desensitisation to substance P. Data are mean±s.e. mean; n=11.

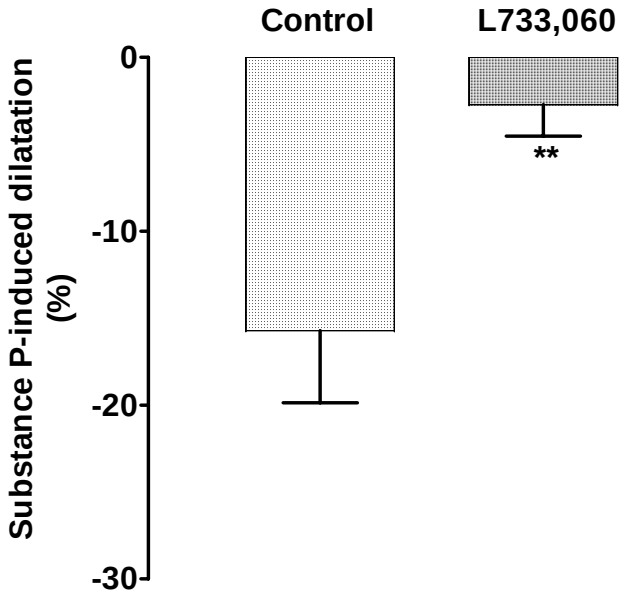

Figure 3.22 Addition of substance P (0.1 μM) induced dilatation of bovine intraocular LPCA rings. Pretreatment with the NK_1 antagonist, L733,060 (0.3 μM), inhibited the dilator response to substance P (0.1 μM). Data are mean±s.e. mean; n=10; **P<0.01 indicates a difference from control.

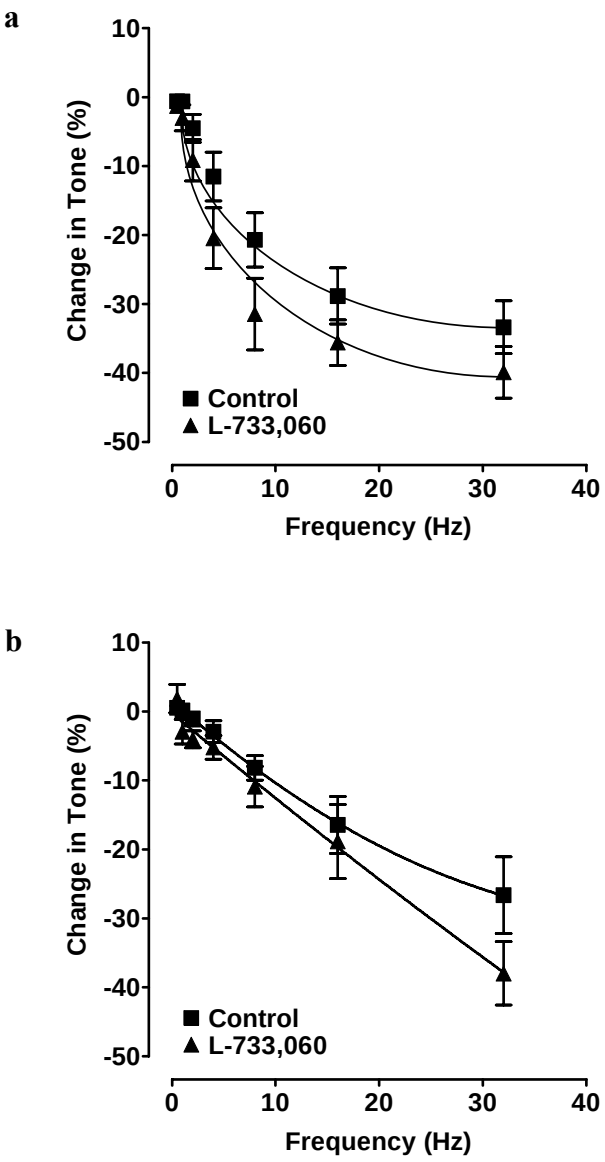

Figure 3.23 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those pretreated with NK_1 antagonist, L-733,060 (0.3 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by L-733,060. Data are mean±s.e. mean; n=8.

respectively. Values of 27 ± 6% and 38 ± 5% (n=8) were obtained for first and second components respectively, in rings treated with L733,060. Persistence of dilatation, both in the presence of L733,060 and in tissues desensitised to substance P, suggests that this neuropeptide does not contribute to either component of neurogenic dilatation.

3.5 Role of VIP in Neurogenic Vasodilatation

VIP is another neurotransmitter that is known to promote vasodilatation in response to EFS in certain tissues. Consequently, we utilised a desensitisation protocol, a protease, VIP antiserum and glibenclamide to determine if VIP contributed to neurogenic vasodilatation in the bovine intraocular LPCA.

3.5.1 Effect of VIP desensitisation on Neurogenic Vasodilatation

VIP (0.1 nM – 0.3 μM) induced concentration-dependent dilatation, with a maximal dilatation of 77 ± 3% and a pEC_{50} of 8.47 ± 0.11 (Figure 3.24, n=7). Addition of VIP (0.3 μM) induced dilatation (73 ± 2%, n=12) in control rings (Figure 3.25). Approximately 25 min later this dilatation had declined to 30 ± 2% (n=12) and a second addition of VIP (0.3 μM) failed to promote any further dilatation (Toda, 1982; Figure 3.25). In this desensitised state, further U46619 (0.2 - 0.5 μM) was added to re-establish tone to the original level, but the first and second components of dilatation to EFS remained unaffected at all frequencies (Figure 3.26). First and second components at 32 Hz were 30 ± 3% and 17 ± 2%, respectively (n=11), in control tissues. In rings partially desensitised to VIP, first and second components were 34 ± 3 % and 14 ± 2% (n=11), respectively. These data suggest that VIP does not contribute to either component of dilatation to EFS.

3.5.2 Effect of α-chymotrypsin on Neurogenic Vasodilatation

The protease, α-chymotrypsin, was used in an attempt to degrade endogenous VIP that might contribute to the EFS-induced dilatation (Morris, 1993). However, addition of α-chymotrypsin (10 U ml^{-1}) to LPCA rings led to a pronounced fall in tone (43 ± 3%, n=7), which did not recover spontaneously. Additional U46619 (0.2 μM) was used in an attempt to restore tone, but it continued to fall, thus preventing full frequency-response curve from being constructed. Nevertheless, in the continued presence of α-chymotrypsin, the first and second components of dilatation at a single frequency (16 Hz, Figure 3.27) remained unaffected: first component 13 ± 2% and 16 ± 2% for control and treated,

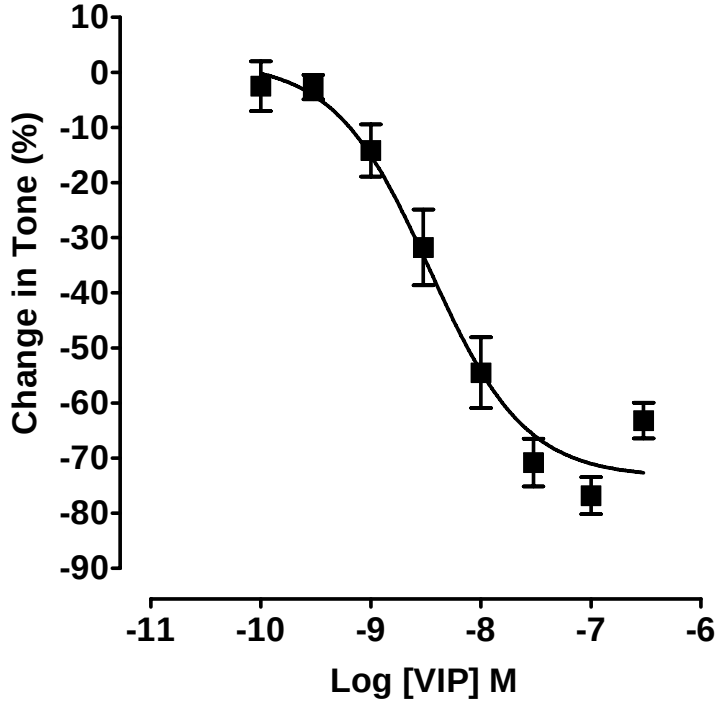

Figure 3.24 VIP (0.1-300 nM) induced concentration-dependent dilatation of bovine intraocular LPCA rings. Data are mean±s.e. mean; n=7.

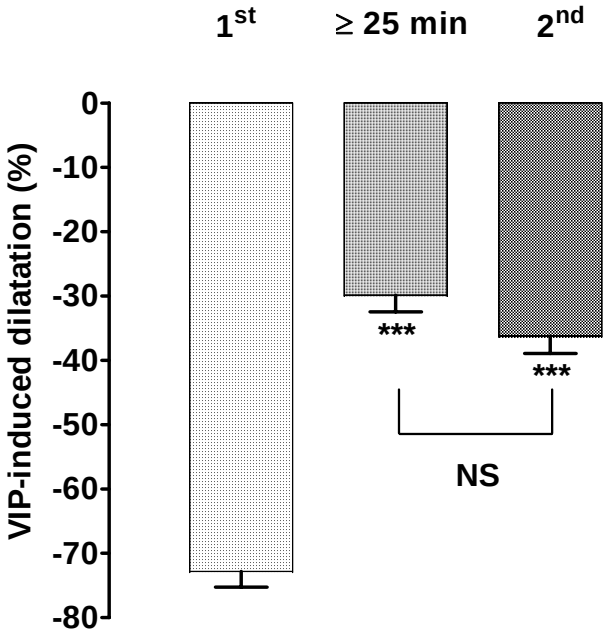

Figure 3.25 VIP (0.3 μM) induced powerful relaxation of bovine intraocular LPCA rings, which decayed to a lower but stable level within ~25 min. In this state, a second addition of VIP failed to promote further relaxation. Data are mean±s.e. mean; n=12; ***P<0.001 indicates differences from the maximally observed relaxation induced by VIP. NS indicates no significant difference.

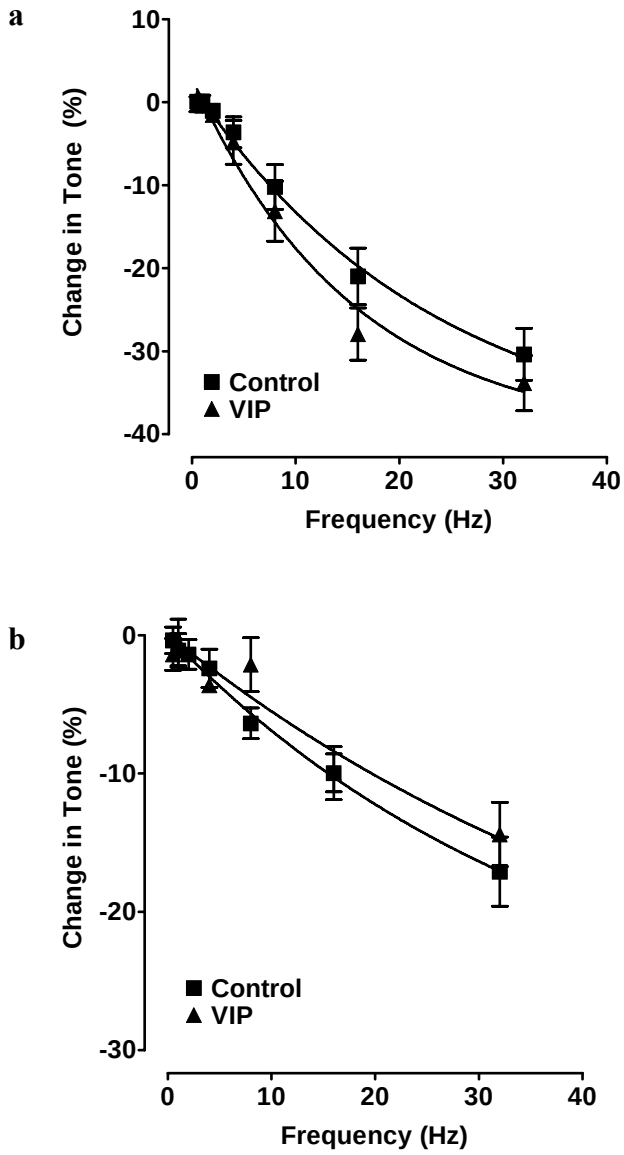

Figure 3.26 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those partially desensitised to VIP (0.6 μM). Desensitisation to VIP did not inhibit (a) the first component of dilatation, as measured at 10 s, or (b) the second component of dilatation, measured at 50 s. Data are mean±s.e. mean; n=9.

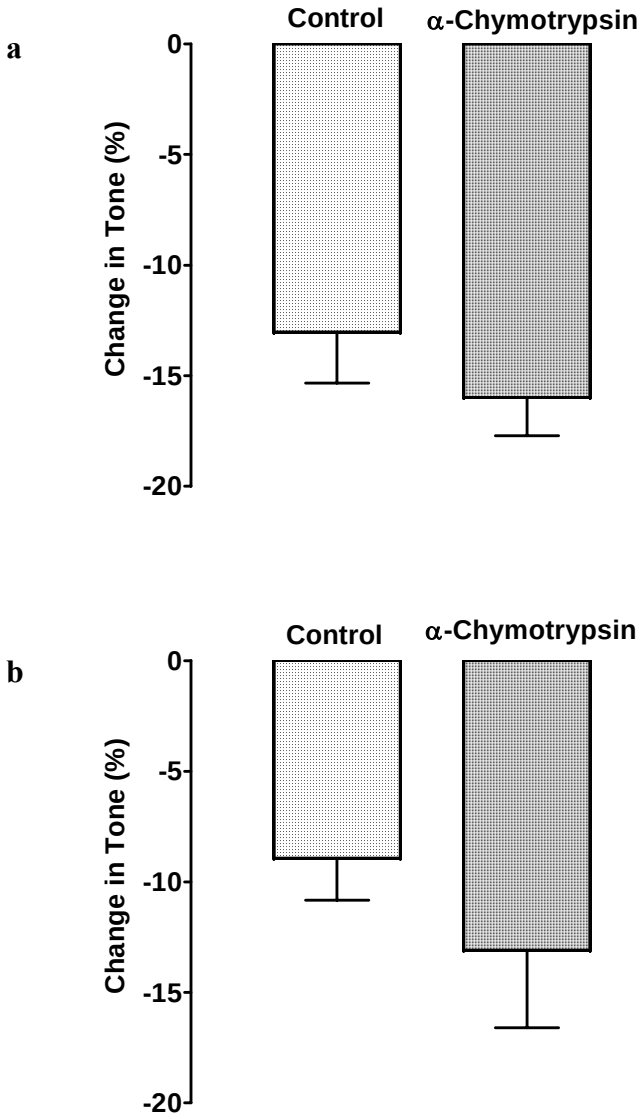

Figure 3.27 Response to a single stimulation at 16 Hz in control bovine intraocular LPCA rings and in those pretreated with α-chymotrypsin (10 U ml^{-1}). Incubation with α-chymotrypsin had no effect on either (a) the first component of dilatation, measured at 10s or (b) the second component, measured at 50 s. Both responses appeared to be potentiated, but this was not statistically significant. Data are mean±s.e. mean; n=5.

respectively (n=5); and second component 9 ±.2% and 13 ± 4% for control and treated, respectively (n=5). The large fall in tone and resultant small responses to stimulation at 16 Hz meant the outcome of incubation with α-chymotrypsin was unsatisfactory. However, it remains unlikely that VIP is involved in EFS-induced vasodilatation in the bovine intraocular LPCA.

3.5.3 Effect of VIP antiserum on Neurogenic Vasodilatation

VIP antiserum (Matthew *et al*., 1997; dilution 1:250) failed to block dilatation to exogenous VIP (0.1 nM – 0.3 μM) in a satisfactory manner (n=7, Figure 3.28). Although the maximum response was significantly reduced from 77 ± 3% in control tissues, to 52 ± 3% in the presence of VIP antiserum ($P<0.01$), the respective pEC_{50} values (8.47 ± 0.11 and 8.67 ± 0.10) were not significantly different. The lower and upper portions of the VIP concentration-response curve were, however, unaffected by it. The addition of VIP antiserum also induced a sustained fall in tone of ciliary artery rings (25 ± 6%, n=7), which was difficult to restore to the original level with further U46619. The presence of antiserum also promoted a great deal of frothing in the myograph chamber making responses difficult to discern. In spite of these difficulties, a frequency curve (0.5 – 32 Hz) was constructed in the presence of VIP antiserum (Figure 3.29). The first and second components of dilatation remained unaffected, however: at 32 Hz the first components were 22 ± 2% and 12 ± 5%, for control and treated tissues, respectively (n=8); and second components of 28 ± 5% and 24 ± 6%, for control and treated tissues respectively (n=8).

3.5.4 Effect of glibenclamide on Neurogenic Vasodilatation

Results obtained with the desensitisation protocol, α-chymotrypsin and with VIP antiserum were somewhat unsatisfactory, so another strategy was employed to determine if VIP contributed to the neurogenic relaxation of the bovine intraocular LPCA. As VIP is reported to mediate dilatation via K_{ATP} channels (Standen *et al*., 1989), the effects of the K_{ATP} channel blocker glibenclamide were investigated. Prior to doing this, however, experiments were conducted on the bovine intraocular LPCA to determine if glibenclamide inhibited dilatation to the K_{ATP} channel opener, levcromakalim. Indeed, in this tissue, glibenclamide (10 μM) powerfully inhibited dilatation to levcromakalim (0.1 nM - 30 μM, Figure 3.30, n=6). Thus glibenclamide was clearly an effective K_{ATP} channel blocker in the

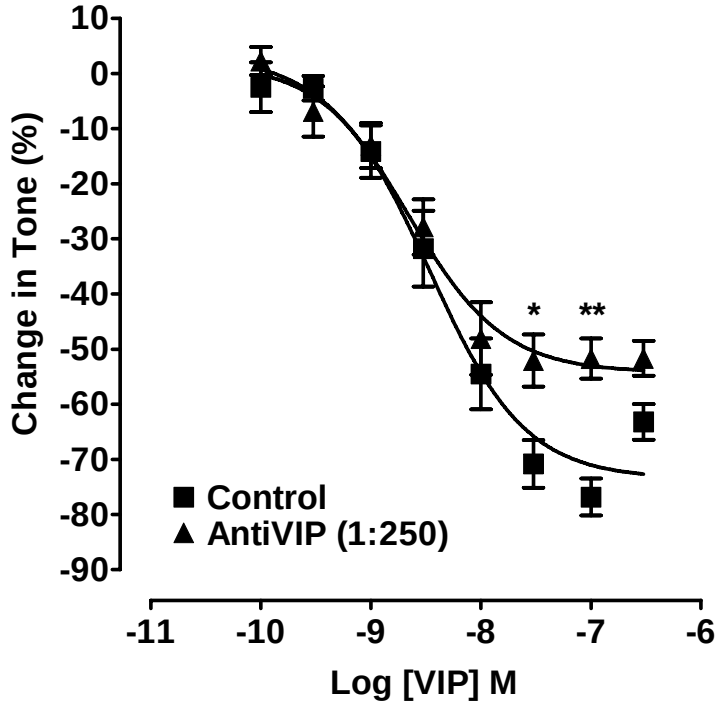

Figure 3.28 VIP (0.1-300 nM) induced concentration-dependent relaxation of bovine intraocular LPCA rings. This was slightly inhibited at high concentrations of VIP (30 and 100 nM only) by incubation with antiVIP (1:250). Data are mean±s.e. mean; n=7; *P<0.01 and **P<0.001 indicate differences from control.

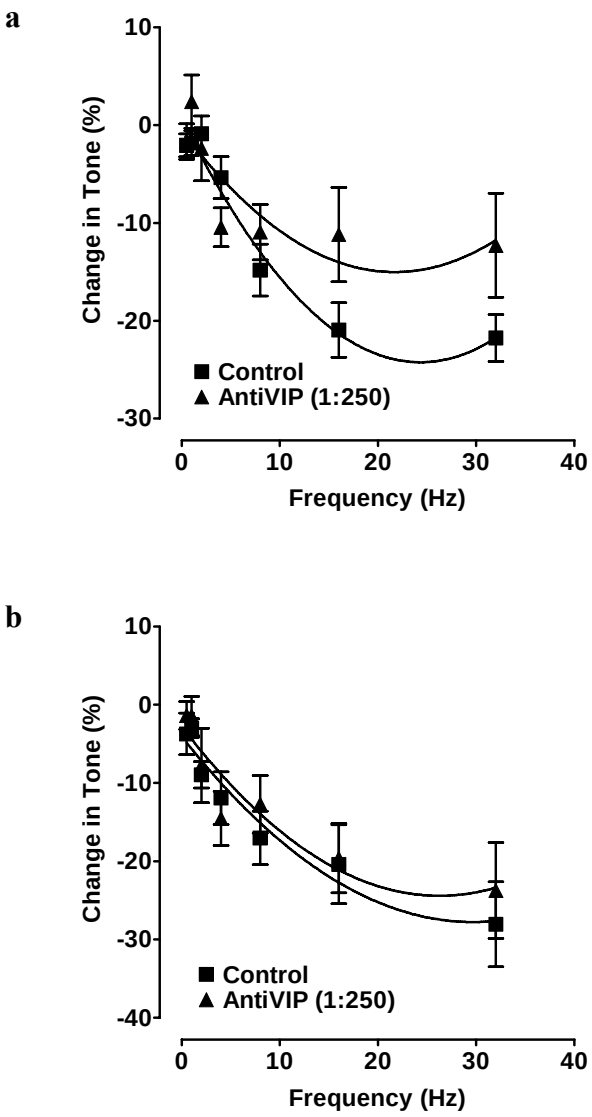

Figure 3.29 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those pretreated with antiVIP (1:250 dilution). Incubation with antiVIP had no effect on either (a) the first component of dilatation, measured at 10 s or (b) The second component of dilatation, measured at 50 s. Data are mean±s.e. mean; n=8.

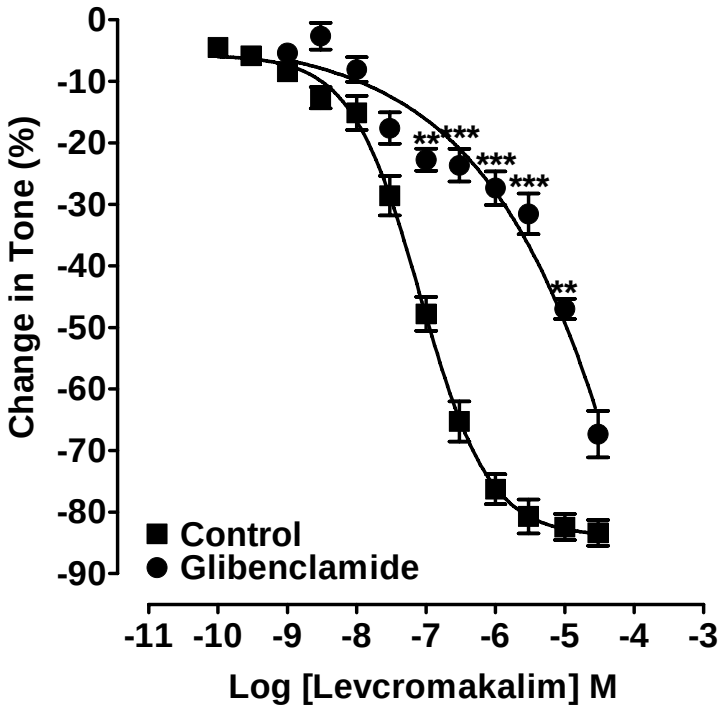

Figure 3.30 The K_{ATP} channel opener, levcromakalim (0.1 nM–30 μM), induced powerful relaxation of bovine intraocular LPCA rings. As expected, incubation with glibenclamide (1 μM), which blocks K_{ATP} channels, shifted the concentration response curve to the right and also reduced the maximally observed relaxation. Data are mean±s.e. mean; n=6; **P<0.01 and ***P<0.001 indicate differences from control.

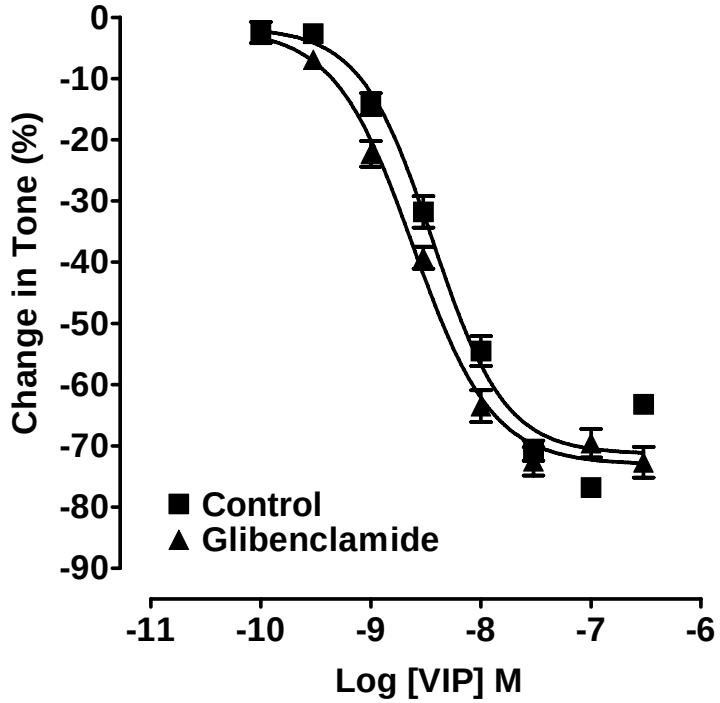

Figure 3.31 VIP (0.1-300 nM) induced concentration-dependent dilatation of bovine intraocular LPCA rings. Incubation with glibenclamide (10 μM), which blocks K_{ATP} channels, did not affect relaxation to VIP. Data are mean±s.e. mean; n=6.

bovine LPCA. Despite this, dilatation to exogenous VIP (0.1 nM – 0.3 μM) was unaffected in the presence of glibenclamide (10 μM, Figure 3.31): pEC_{50} values of 8.47 ± 0.11 and 8.61 ± 0.06 for control and treated tissues, respectively. Although glibenclamide did not block dilatation to exogenous VIP, we assessed whether it could inhibit neurogenic relaxation of the bovine intraocular LPCA. Glibenclamide (10 μM) failed to inhibit the response to EFS at all frequencies (Figure 3.32) and actually seemed to potentiate the second response, although this was not significant: first and second components at 32 Hz were 32 ± 4% and 32 ± 5% (n=6), respectively, in control tissues and 34 ± 4 % and 40 ± 6% (n=6) in rings treated with glibenclamide.

The above data make it unlikely that VIP contributes to either the first or second components of neurogenic dilatation in the bovine intraocular LPCA.

3.6 Role of ATP in Neurogenic Vasodilatation

The final neurotransmitter candidate investigated for involvement in neurogenic vasodilatation in the bovine intraocular LPCA was ATP. A number of established ATP receptor antagonists and other modulators were employed to test this possibility.

3.6.1 Effect of Suramin

Exogenous ATP (10 nM – 30 μM) induced concentration-dependent dilatation, with a maximally observed dilatation of 71 ± 6% (n=7, Figure 3.33) and a pEC_{50} of 6.38 ± 0.06.

A concentration-response curve to ATP was performed in the presence of the P2Y purinoceptor antagonist, suramin (Dunn & Blakeley, 1998; 500 μM). However, suramin did not block dilatation to exogenous ATP (Figure 3.33): pEC_{50} values in control and treated tissues were 6.38 ± 0.06 and 6.14 ± 0.03, respectively (n=8).

Furthermore, suramin (500 μM) did not inhibit neurogenic dilatation in the bovine intraocular LPCA (Figure 3.34). If anything, the response was slightly potentiated in the presence of suramin, but this was not significant. At 32 Hz, control responses for the first and second components were 22 ± 6% and 34 ± 5% (n=8), respectively. In the presence of suramin, the equivalent values were 29 ± 9% and 38 ± 4% (n=8). These data suggest that ATP does not contribute to either component of neurogenic dilatation. However, as suramin did not block dilatation to exogenous ATP in the bovine intraocular LPCA, other

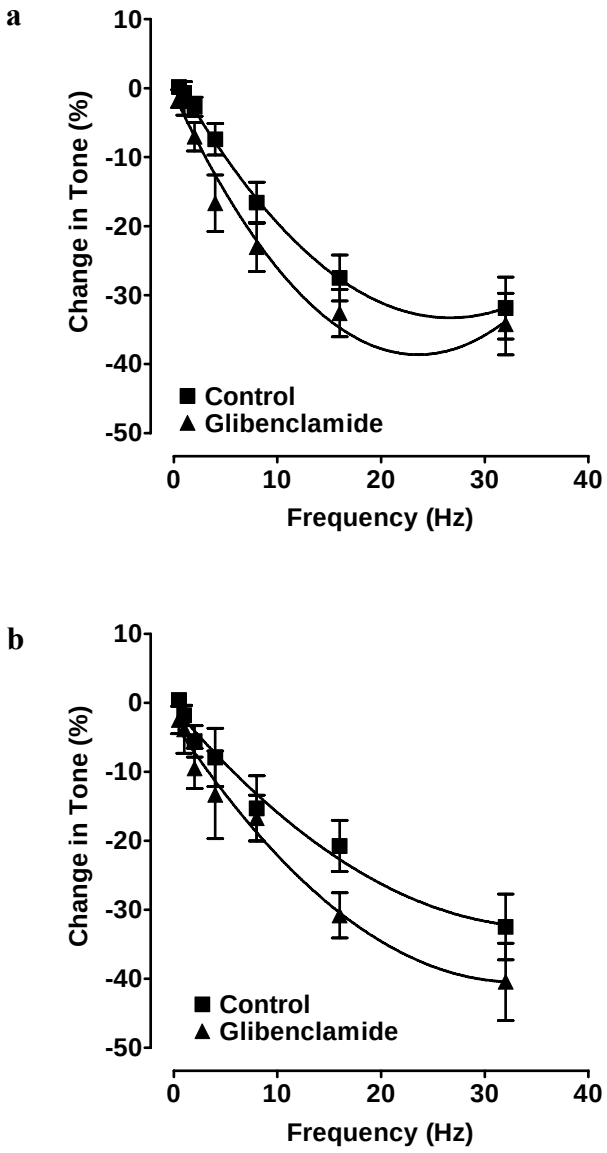

Figure 3.32 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those pretreated with the K_{ATP} channel inhibitor, glibenclamide (10 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by glibenclamide. Data are mean±s.e. mean; n=6.

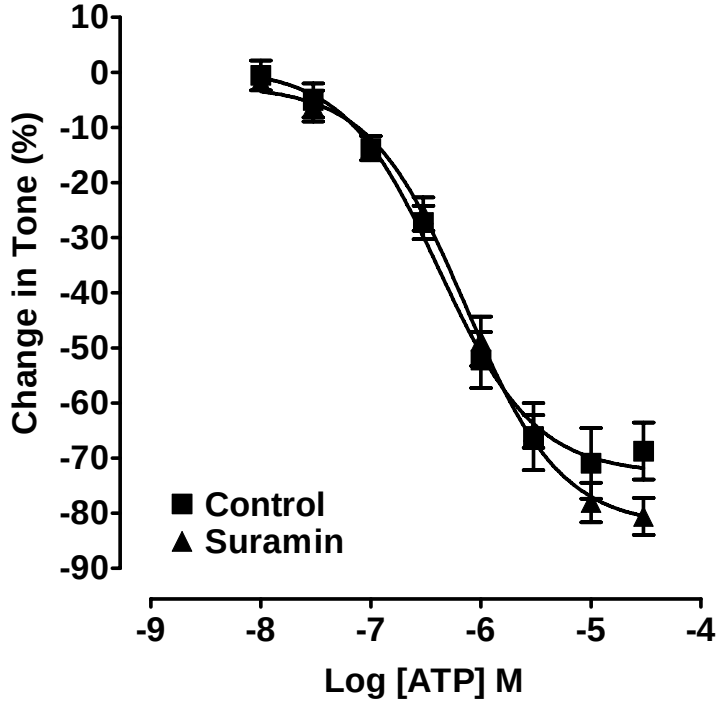

Figure 3.33 ATP (10 nM-30 μM) induced concentration-dependent dilatation of bovine intraocular LPCA rings, which was unaffected following pretreatment with the P2Y receptor antagonist, suramin (500 μM). Data are mean±s.e. mean; n=8.

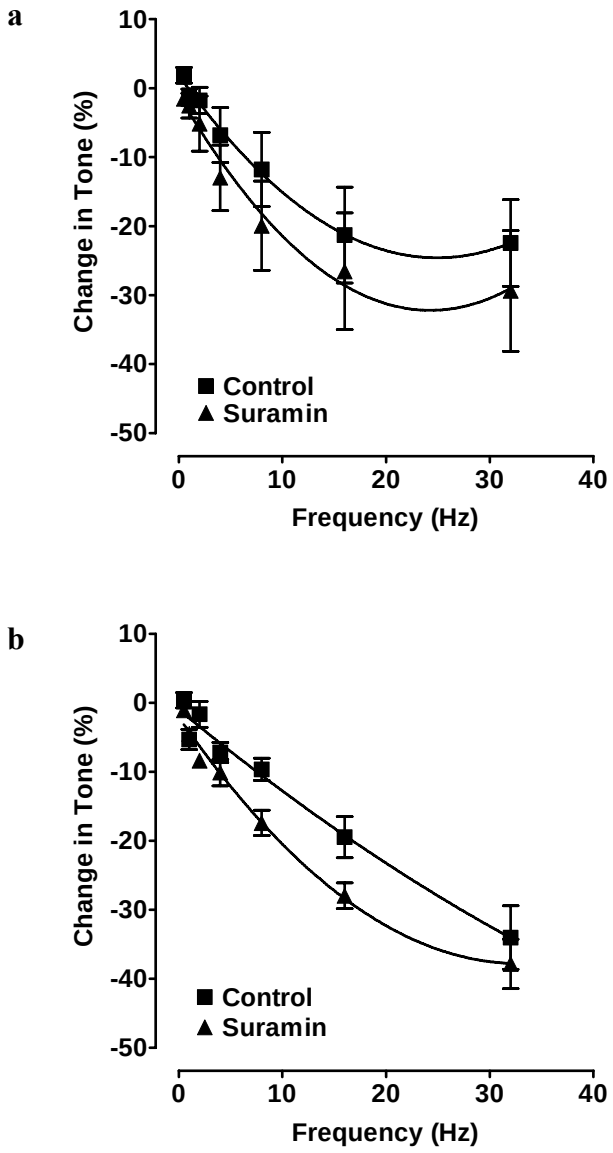

Figure 3.34 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those pretreated with the P2Y receptor antagonist, suramin (500 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by suramin. It appeared to potentiate both, however this was not statistically significant. Data are mean±s.e. mean; n=8.

ATP receptor antagonists were employed to determine the role of ATP.

3.6.2 Effect of PPADS

A concentration-response curve to ATP (10 nM – 30 μM) was performed in the presence and absence of the P2Y receptor antagonist, PPADS (Lambrecht *et al*., 1992; 100 μM). As with suramin, PPADS did not block dilatation to exogenous ATP (Figure 3.35): the pEC_{50} values in control and treated tissues were 6.38 ± 0.06 and 6.42 ± 0.05, respectively (n=10). These data suggests that PPADS is not an effective antagonist to ATP in the bovine intraocular LPCA. Experiments were therefore not conducted to determine the effects of this agent on neurogenic vasodilatation in the bovine intraocular LPCA.

3.6.3 Effect of RB2

Similarly, another P2Y receptor antagonist, reactive blue 2 (Burnstock & Warland, 1987b; 300 μM) did not inhibit dilatation to exogenous ATP (10 nM – 30 μM, Figure 3.36). Although there was a slight rightward shift of the concentration-response curve, the pEC_{50} values in control and treated tissues (6.38 ± 0.06 and 5.91 ± 0.06, respectively) were not significantly different (n=8). Consequently, we did not investigate effects of reactive blue 2 on neurogenic vasodilatation in the bovine intraocular LPCA.

3.6.4 Effect of α,β-methyleneATP

A further concentration-response curve to ATP was performed in the presence of P2X receptor agonist, α,β-methyleneATP (Corr & Burnstock, 1994; 30 μM; Figure 3.37). In these experiments α,β-methyleneATP acted as an antagonist at the P2X receptors by desensitising the receptor. On the whole the response to exogenous ATP was unaffected by pretreatment with α,β-methyleneATP: the pEC_{50} values in control and treated tissues were 6.38 ± 0.06 and 6.22 ± 0.04, respectively. However, the response to 30 μM ATP appears to have been slightly potentiated in the presence of α,β-methyleneATP (n=7, $P<0.05$)

As we were unable to find a suitable blocking agent for ATP in the bovine intraocular LPCA, we were unable to fully rule out the involvement of ATP in neurogenic vasodilatation in this tissue

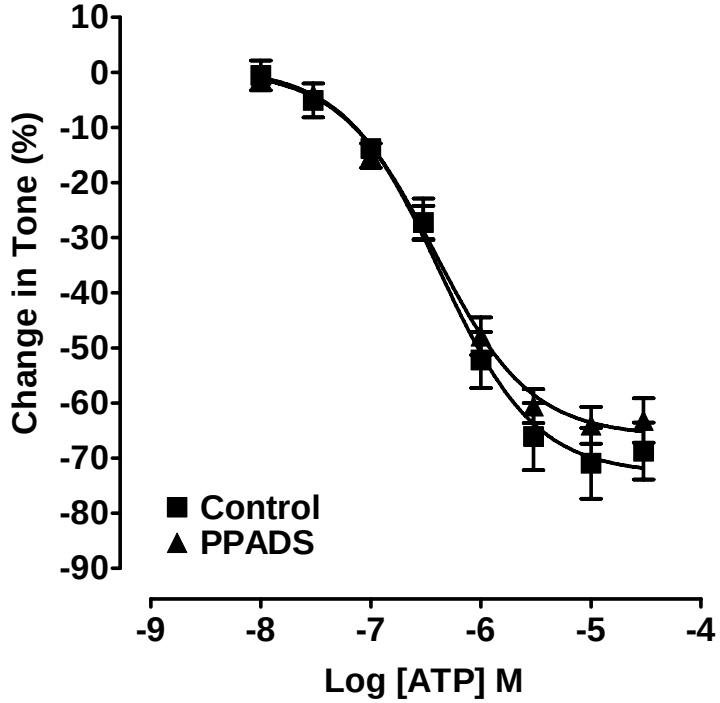

Figure 3.35 ATP (10 nM-30 μM) induced concentration-dependent relaxation of bovine intraocular LPCA rings, which was unaffected following pretreatment with the P2Y receptor antagonist, PPADS (100 μM). Data are mean±s.e. mean; n=10.

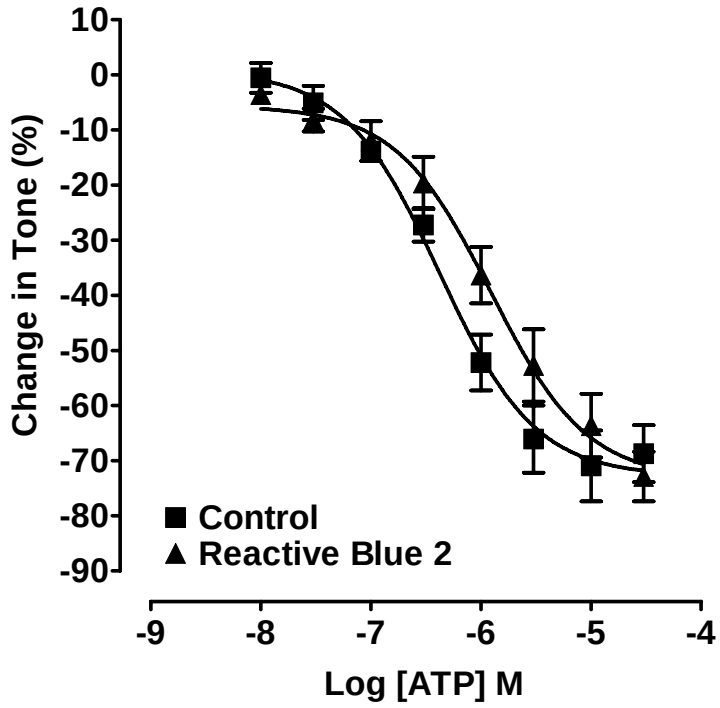

Figure 3.36 ATP (10 nM-30 μM) induced concentration-dependent dilatation of bovine intraocular LPCA rings, which was unaffected following pretreatment with the P2Y receptor antagonist, RB2 (300 μM). Data are mean±s.e. mean; n=8.

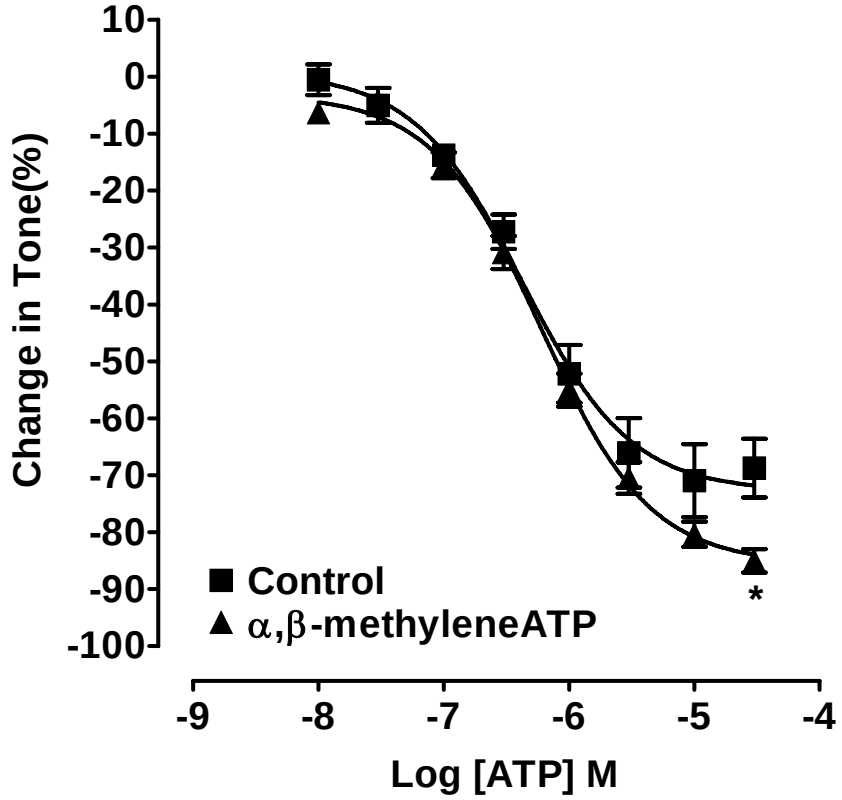

Figure 3.37 ATP (10 nM-30 μM) induced concentration-dependent dilatation of bovine intraocular LPCA rings. Generally, this was unaffected by pretreatment with the P2X receptor agonist, α,β-methyleneATP (30 μM). However the response to 30 μM ATP appears to have been slightly potentiated in the presence of α,β-methyleneATP. Data are mean±s.e. mean; n=7; *P<0.05 indicates difference from control.

CHAPTER 4.

RESULTS: IDENTIFICATION OF THE MECHANISM MEDIATING NEUROGENIC VASODILATATION

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4 Identification of the Mechanism Mediating Neurogenic Vasodilatation

In the previous chapter, it was established that neurogenic vasodilatation in the bovine intraocular LPCA was biphasic in nature and that the first component appeared to be mediated by NO released from nitrergic nerves. However, the identity of the transmitter that mediated the second component of dilatation remained unclear because it was not blocked using conventional neurotransmitter inhibitors and desensitisation protocols. Consequently, an attempt was made to determine the mechanism by which the unknown transmitter promoted dilatation.

4.1 Role of K^+ Channels in Neurogenic Vasodilatation

As has already been discussed, vasodilatation can be facilitated by hyperpolarisation of the VSM, through the opening of K^+ channels, or by activation of the cAMP or cGMP pathways. Consequently, an attempt was made to inhibit or potentiate these pathways using appropriate antagonists and other modulators, to determine if they were responsible for mediating the second component of dilatation. The first strategy adopted was to block a variety of K^+ channels to determine if they were involved.

4.1.1 Effect of TEA on Neurogenic Vasodilatation

The non-specific K^+ channel blocker, TEA (Nelson & Quayle, 1995; 10 mM) increased U46619-induced tone of intraocular LPCA rings by 0.82 ± 0.19 mN (Figure 4.1). TEA also appeared to potentiate the response to EFS when bovine intraocular LPCA rings were stimulated at a single frequency of 16 Hz (Figure 4.2): the magnitude of the first component of dilatation increased from 15 ± 3% to 22 ± 3% (n=12) and the second component from 16 ± 3% to 21 ± 3% (n=12), however neither increase was statistically significant. These data suggest that non-specific block of K^+ channels did not inhibit neurogenic vasodilatation, therefore a number of specific K^+ channels were blocked using selective inhibitors.

4.1.2 Effect of $BaCl_2$ on Neurogenic Vasodilatation

The inhibitor of K_{IR} channels, $BaCl_2$ (Quayle *et al*., 1993; 30 μM), caused an increase in U46619-induced tone of 0.39 ± 0.16 mN (Figure 4.1), upon addition to the bath. $BaCl_2$ failed, however, to affect either component of dilatation in response to stimulation at 16 Hz (Figure 4.3). Again, there was a slight, but statistically insignificant, increase in the

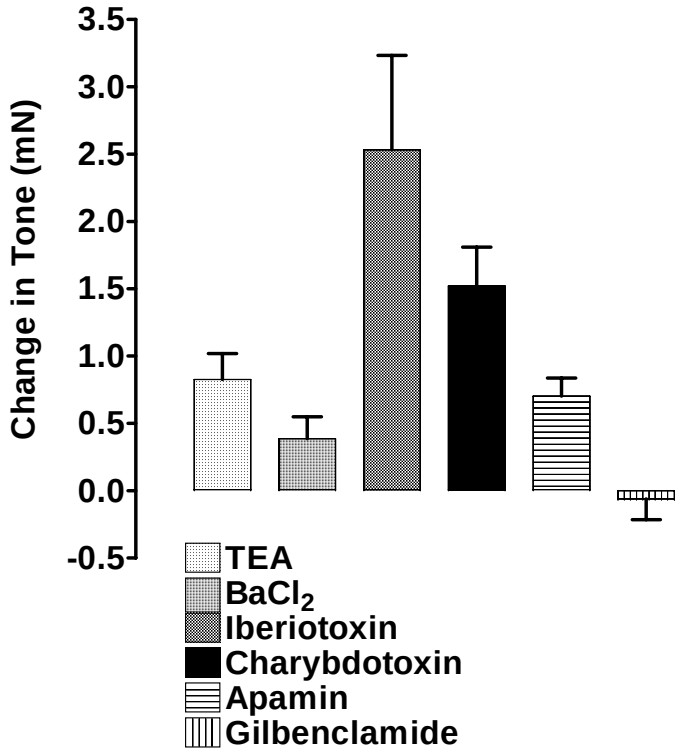

Figure 4.1 A rise in U46619-induced tone was observed upon addition of a number of K^+ channel inhibitors: the non-specific K^+ channel blocker, TEA (10 mM), the inhibitor of inward rectifier K^+ (K_{IR}) channels, $BaCl_2$ (30 μM), the inhibitor of large conductance K_{Ca} channels, iberiotoxin (0.1 μM), the inhibitor of large and intermediate K_{Ca} channels and K_V channels, charybdotoxin (0.1 μM), and the small conductance K_{Ca} channel inhibitor, apamin (0.1 μM). All induced a rise in tone. Only the K_{ATP} channel inhibitor, glibenclamide (10 μM) did not induce a rise in tone. Data are mean±s.e. mean; n≥6.

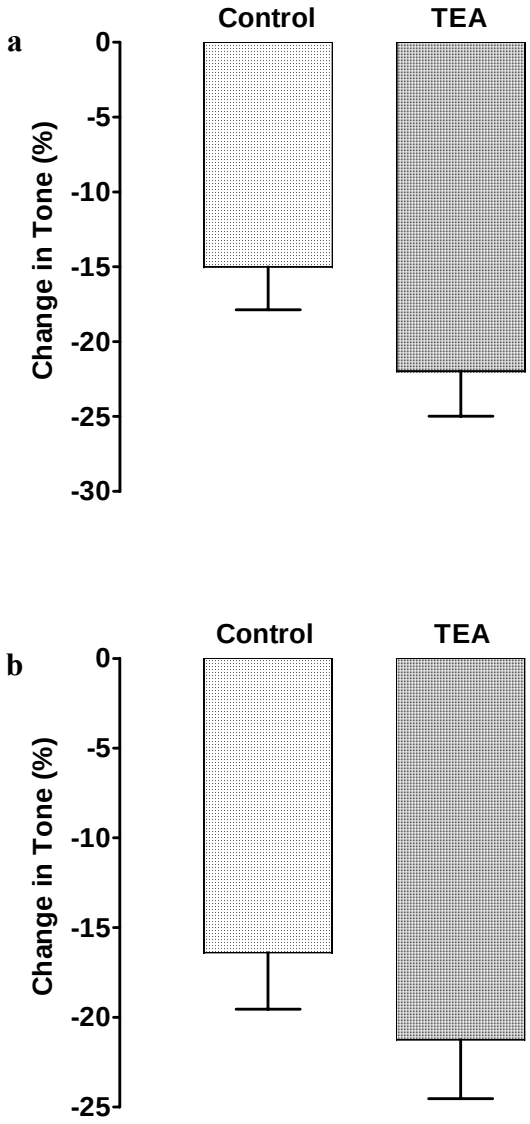

Figure 4.2 Dilatation in response to EFS at a single frequency (16 Hz) in control bovine intraocular LPCA rings and in those incubated with the K^+ channel blocker, TEA (10 mM). Pretreatment with TEA appeared to potentiate both (a) the first component, measured at 10s and (b) the second component, measured at 50 s, but neither increase was statistically significant. Data are mean±s.e. mean; n=12.

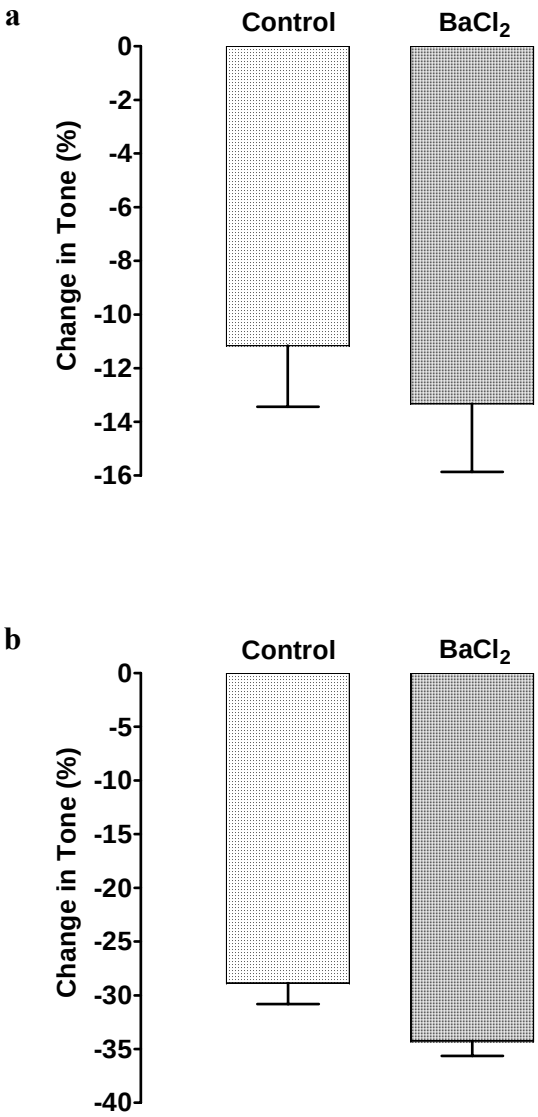

Figure 4.3 Dilatation in response to EFS at a single frequency (16 Hz) in control bovine intraocular LPCA rings and in those pretreated with the $K_{(IR)}$ channel blocker, $BaCl_2$ (30 μM). Incubation with $BaCl_2$ appeared to potentiate both (a) the first component, measured at 10s and (b) the second component, measured at 50 s, but neither increase was statistically significant. Data are mean±s.e. mean; n=6.

magnitude of the responses: the magnitude of the first component increased from 11 ± 6% to 13 ± 6% (n=6) and the second component from 29 ± 5% to 34 ± 3% (n=6). These data suggest that K_{IR} channels do not play a role in neurogenic vasodilatation in the bovine intraocular LPCA.

4.1.3 Effect of Iberiotoxin on Neurogenic Vasodilatation

A frequency-response curve was generated in the presence of the BK_{Ca} channel inhibitor, iberiotoxin (Latorre *et al*., 1992; 0.1 μM). Addition of iberiotoxin to the bath led to an increase in U46619-induced tone of 2.53 ± 0.70 mN (Figure 4.1). However, iberiotoxin blocked neither the first nor the second component of neurogenic vasodilatation, at any frequency (Figure 4.4). Control dilatations at 32 Hz were 25 ± 4% and 18 ± 4% (n=8) for first and second components, respectively, and dilatations of 22 ± 4% and 21 ± 4% (n=8), respectively, were obtained in iberiotoxin-treated tissues. These data suggest that large conductance K_{Ca} channels do not play a role in neurogenic vasodilatation in the bovine intraocular LPCA.

4.1.4 Effect of Charybdotoxin on Neurogenic Vasodilatation

The inhibitor of BK_{Ca}, IK_{Ca} and K_V channels, charybdotoxin (Grissmer *et al*., 1994; Miller *et al*., 1985; 0.1 μM), also caused an increase in U46619-induced tone when added to the bath (1.52 ± 0.29 mN, Figure 4.1). However, charybdotoxin failed to affect neurogenic vasodilatation at any frequencies (Figure 4.5). At the optimum frequency of 32 Hz, control responses for the first and second components were 25 ± 4% and 24 ± 3% (n=12), respectively. In the presence of charybdotoxin, the responses were unaffected and were 22 ± 3% and 19 ± 2% (n=12), respectively. These data suggest that large and intermediate conductance K_{Ca} and K_V channels do not play a role in neurogenic vasodilatation in the bovine intraocular LPCA.

4.1.5 Effect of Apamin on Neurogenic Vasodilatation

The small conductance K_{Ca} channel inhibitor, apamin (Blatz & Magleby, 1986; 0.1 μM), caused a small increase in U46619-induced tone when it was added to the bath (0.70 ± 0.13 mN, Figure 4.1). The first and second components of neurogenic vasodilatation remained unaffected at all frequencies in bovine intraocular LPCA rings pretreated with apamin. At

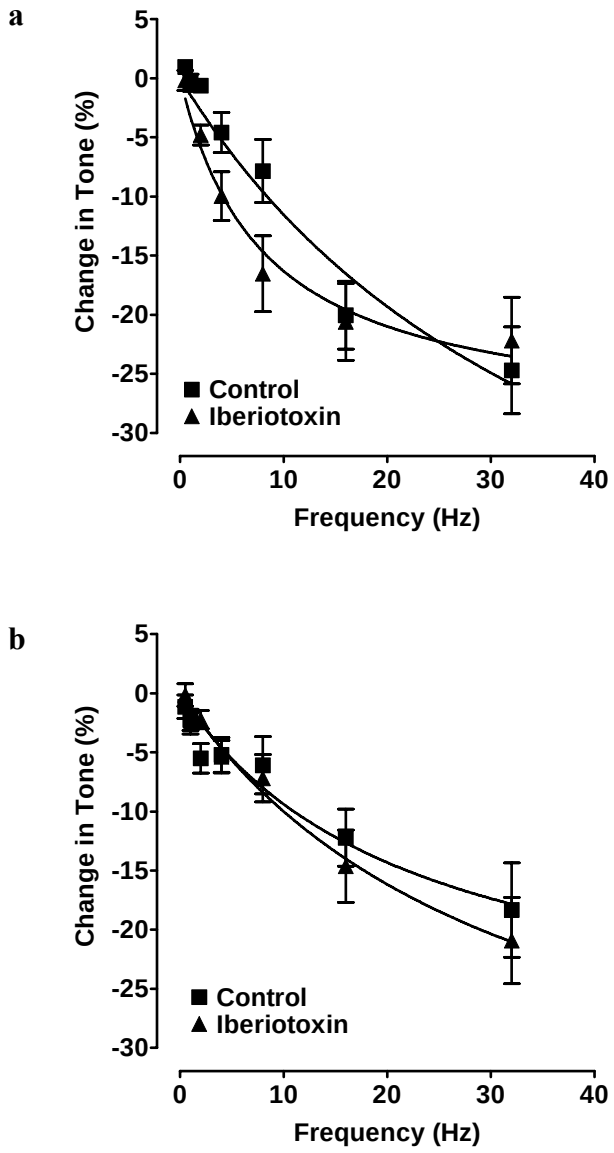

Figure 4.4 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those incubated with the inhibitor of large conductance K_{Ca} channels, iberiotoxin (0.1 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by iberiotoxin. Data are mean±s.e. mean; n=8.

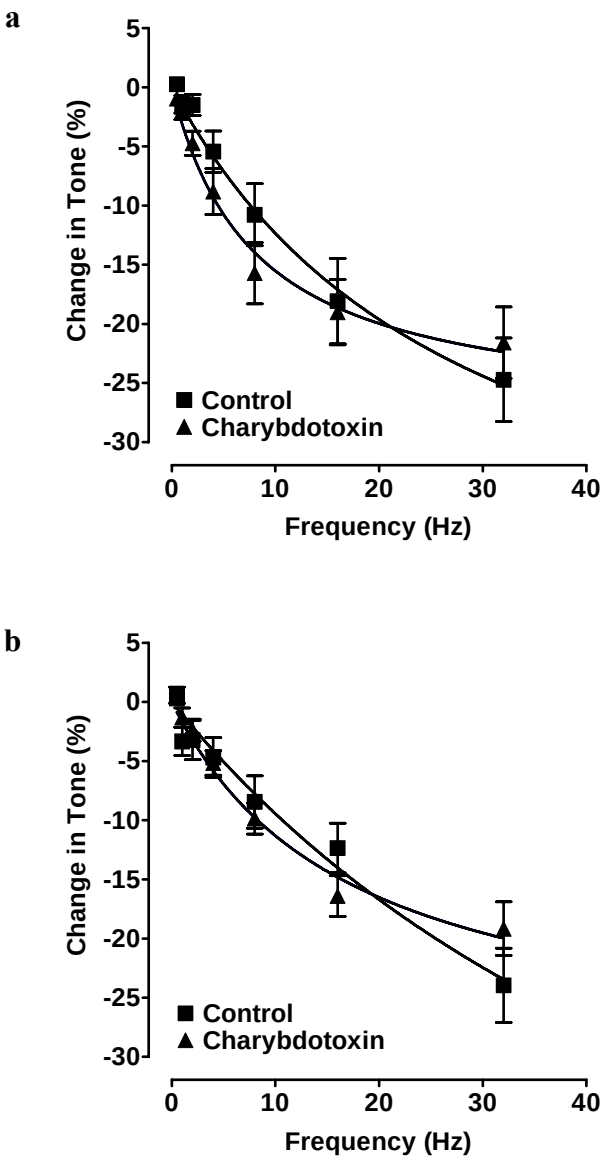

Figure 4.5 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those incubated with the inhibitor of large and intermediate conductance K_{Ca} and K_V channels, charybdotoxin (0.1 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by charybdotoxin. Data are mean±s.e. mean; n=12.

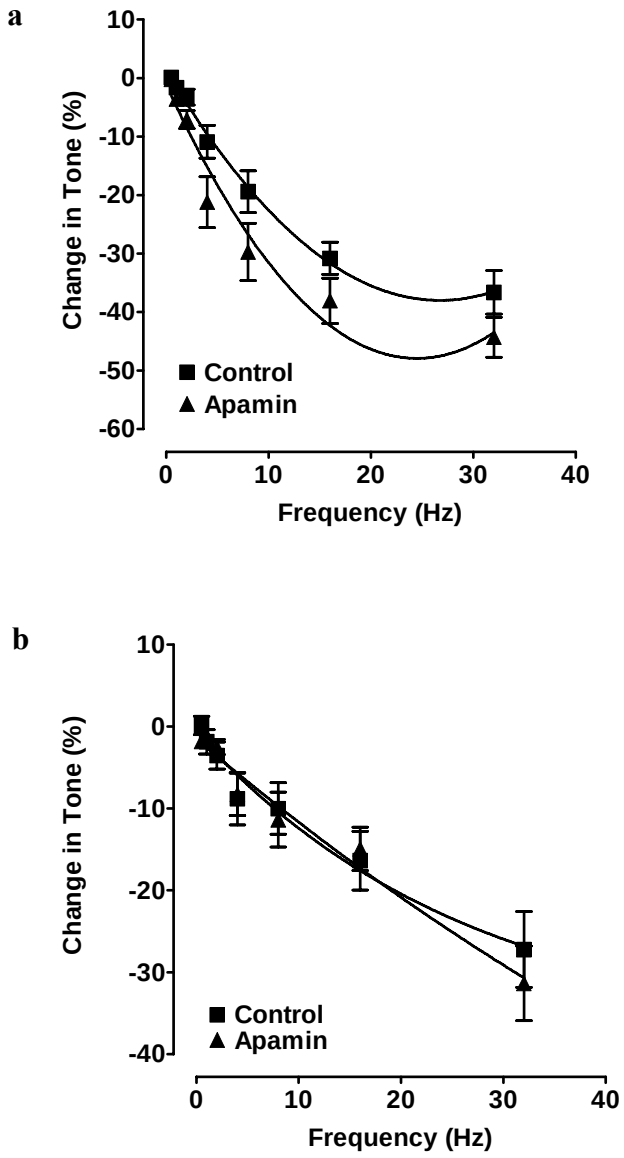

Figure 4.6 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those incubated with the small conductance K_{Ca} channel inhibitor, apamin (0.1 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by apamin. Data are mean±s.e. mean; n=6.

32 Hz control responses for the first and second components were 37 ± 4% and 27 ± 5% (n=6), respectively (Figure 4.6). In the presence of apamin the responses were 44 ± 3% and 31 ± 5% (n=6), respectively. These data suggest that small conductance K_{Ca} channels do not play a role neurogenic vasodilatation in the bovine intraocular LPCA.

4.1.6 Effect of Glibenclamide on Neurogenic Vasodilatation

The K_{ATP} channel inhibitor, glibenclamide (Schmid-Antomarchi *et al*., 1987; 10 μM), had no affect on U46619-induced tone (0.07 ± 0.15 mN, Figure 4.1). Glibenclamide also failed to inhibit neurogenic vasodilatation at all frequencies, as was described in the previous chapter (Figure 3.32) and seemed to potentiate the second response, although this was not statistically significant. Control dilatations at 32 Hz were 32 ± 4% and 32 ± 5% (n=6) for first and second components, respectively, and 34 ± 4 % and 40 ± 6% (n=6) in rings treated with glibenclamide. These data suggest that K_{ATP} channels do not play a role in neurogenic vasodilatation in the bovine intraocular LPCA.

4.2 Role of cGMP in Neurogenic Vasodilatation

As none of the specific or non-specific K^+ channel inhibitors affected either the first or second components of neurogenic vasodilatation, the cGMP pathway was investigated for its involvement. From earlier observations in the presence of L-NAME and ODQ, it was likely that the NO and soluble guanylate cyclase systems mediated the first but not the second component of dilatation.

4.2.1 Effect of PDE5 Inhibition on Neurogenic Vasodilatation

Addition of the PDE5 inhibitor, zaprinast (Bowman & Drummond, 1984; 10 μM), to the bath led to a large drop in U46619-induced tone (3.18 ± 0.66 mN) and additional U46619 (0.1 – 0.5 μM) was used to restore it. Zaprinast potentiated the first component of neurogenic vasodilatation at 16 and 32 Hz (both $P<0.05$, Figure 4.7). At 32 Hz the response increased from 22 ± 8% in control tissues to 31 ± 8% in treated tissues (n=5). The second component was, however, unchanged in the presence of zaprinast, with responses at 32 Hz of 38 ± 8% in control tissues and 41 ± 10% in treated rings (n=5).

Another PDE_5 inhibitor, sildenafil (Boolell *et al*., 1996; 1 μM), also produced a fall in tone and again additional U46619 (0.1 – 0.5 μM) was used to reinstate it to the previous level. However, in contrast to zaprinast, sildenafil did not potentiate the first component of

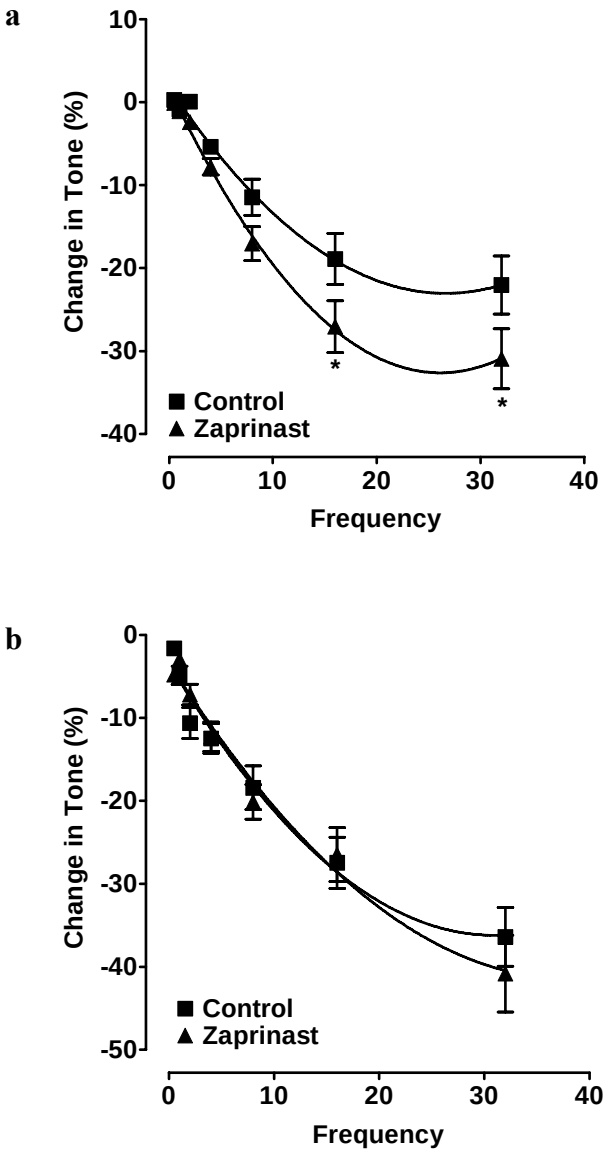

Figure 4.7 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those incubated with the PDE5 inhibitor, zaprinast (10 μM). (a) Pretreatment with zaprinast significantly potentiated the first component of vasodilatation at 16 & 32 Hz, as measured at 10 s. (b) The second component of relaxation, measured at 50 s, was not affected by treatment with zaprinast. Data are mean±s.e. mean; n=5. *P<0.05 indicates a difference from control.

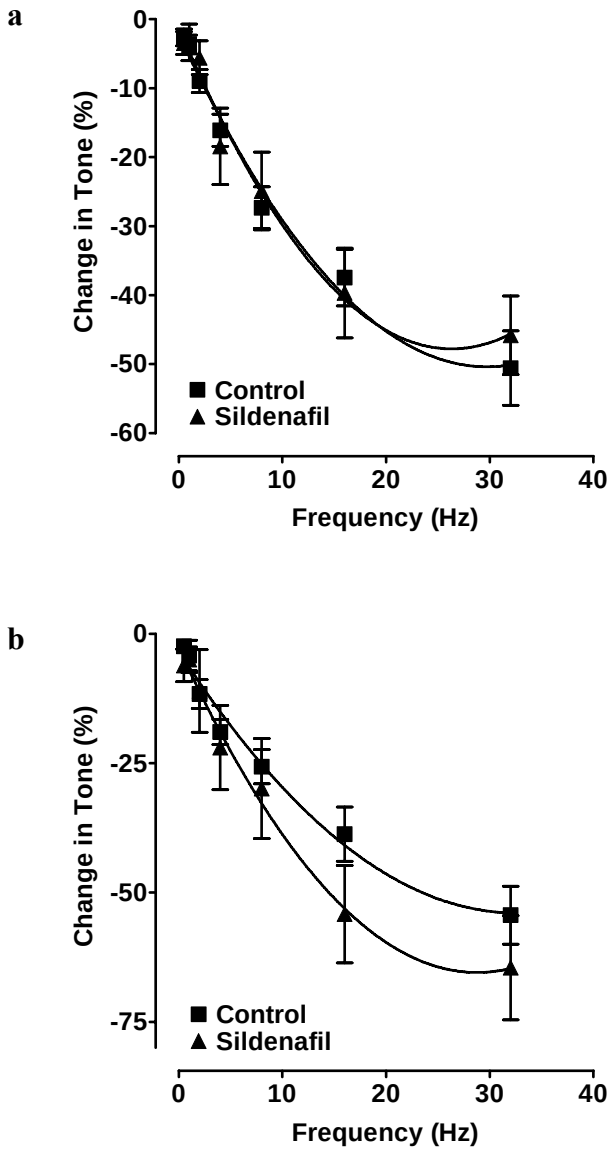

Figure 4.8 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those incubated with the PDE5 inhibitor, sildenafil (1 μM). (a) Incubation with sildenafil did not affect the first component of the relaxation, measured at 10 s. (b) The second component of relaxation, measured at 50 s, appeared to be potentiated at frequencies of 16 and 32 Hz in the presence of sildenafil, but this was not statistically significant. Data are mean±s.e. mean; n=7.

dilatation (Figure 4.8): the magnitude of the first component in control tissues was 51 ± 5%, compared with 46 ± 6% in rings incubated with sildenafil (n=7). Furthermore, incubation with sildenafil appeared to increase the magnitude of the second component of dilatation at 16 and 32 Hz, but this was not statistically significant: at 32 Hz the response was 54 ± 6% in control tissues and 65 ± 10% in treated rings (n=7). Thus zaprinast potentiated the magnitude of the first component, as expected, but surprisingly this was not the case for sildenafil. Furthermore, there is no evidence to suggest that the cGMP pathway mediates the second component of dilatation.

4.2.2 Effect of PKG Inhibition on Neurogenic Vasodilatation

As the results obtained with the PDE5 inhibitors were inconsistent, another stage of the cGMP pathway was blocked by inhibiting PKG. Incubation with the PKG inhibitor, Rp-8-Br-PET-cGMPS (Butt *et al*., 1995; 30 μM), caused an increase in U46619-induced tone of 0.44 ± 0.07 mN. Stimulation at a single frequency of 16 Hz in the presence of Rp-8-Br-PET-cGMPS reduced the magnitude of the first and second component of dilatation. However only the first component was significantly reduced ($P<0.05$; Figure 4.9). The first component of the response was reduced from 26 ± 4% to 17 ± 3% (n=6) and the second component from 32 ± 10% to 21 ± 7% (n=6), in the presence of Rp-8-Br-PET-cGMPS.

Although Rp-8-Br-PET-cGMPS did slightly inhibit the first component of dilatation, the blockade was fairly poor and so the effects of another PKG inhibitor, KT5823 (Wyatt *et al*., 1991; 10 μM), were examined (Figure 4.10). The addition of KT5823 to the bath, led to a decrease in tone of 1.02 ± 0.30 mN, however, this recovered spontaneously and did not require further addition of U46619. The first and second components of neurogenic vasodilatation remained unaffected at all frequencies in bovine intraocular LPCA rings pretreated with KT5823: first and second components were 22 ± 5% and 22 ± 6% (n=6), respectively, in control tissues and 21 ± 6 % and 20 ± 6% (n=6) in rings treated with the PKG inhibitor.

The PKG inhibitors utilised, like the PDE5 inhibitors, had limited success in inhibiting the first and second components of EFS-mediated vasodilatation.

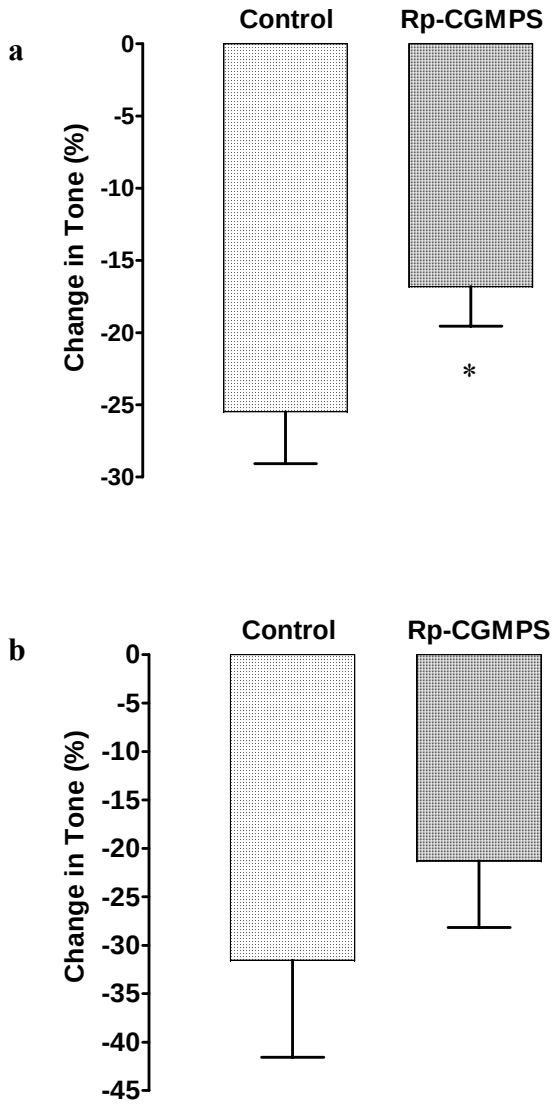

Figure 4.9 Dilatation in response to EFS at a single frequency (16 Hz) in control bovine intraocular LPCA rings and in those pretreated with the PKG inhibitor, Rp-8-Br-PET-cGMPS (30 μM). Incubation with Rp-8-Br-PET-cGMPS appeared to reduce the magnitude of both (a) the first component, measured at 10 s, and (b) the second component, measured at 50 s. However only the reduction measured at 10 s was statistically significant ($P<0.05$). Data are mean±s.e. mean; n=6.

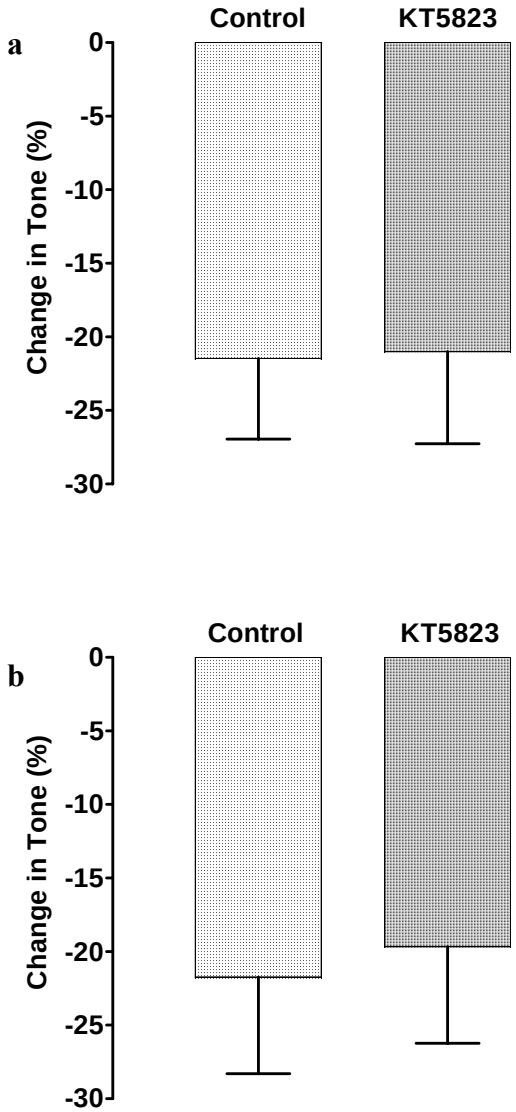

Figure 4.10 Dilatation in response to EFS at a single frequency (16 Hz) in control bovine intraocular LPCA rings and in those pretreated with the PKG inhibitor, KT5823 (10 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by KT5823. Data are mean±s.e. mean; n=6.

4.3 Role of cAMP in Neurogenic Vasodilatation

As inhibition of PDE and PKG failed to provide information on the mechanisms underlying the first and second components of neurogenic dilatation in the bovine intraocular LPCA, the possible involvement of the cAMP pathway was investigated.

4.3.1 Effect of PKA Inhibition on Neurogenic Vasodilatation

The PKA inhibitor, Rp-8-Br-cAMPS (Gjertsen *et al*., 1995; 50 μM) caused a rise in U46619-induced tone of 0.72 ± 0.24 mN, when added to the bath. Stimulation at a single frequency of 16 Hz in the presence of Rp-8-Br-cAMPS appeared to significantly reduce the magnitude of the second ($P<0.05$), but not the first component of dilatation (Figure 4.11). The second component was reduced from 29 ± 3% to 17 ± 4% (n=4) in the presence of Rp-8-Br-cAMPS. The first component remained unchanged and control responses were 38 ± 7%, compared with 35 ± 6% (n=4) in treated tissues.

To determine if the second component could be further inhibited, a second group of tissues was incubated with a higher concentration of Rp-8-Br-cAMPS (100 μM, Figure 4.12). Incubation with the higher concentration of Rp-8-Br-cAMPS appeared to reduce the magnitude of both the first and second components of dilatation, but neither reduction was significant. First and second components at 16 Hz were 18 ± 6% and 29 ± 5% (n=7), respectively, in control tissues and 9 ± 7% and 17 ± 3% (n=7) in rings treated with the Rp-8-Br-cAMPS.

A second PKA inhibitor, H89 (Combest *et al*., 1988; 10 μM), was also used in an attempt to block neurogenic vasodilatation (Figure 4.13). Incubation with H89 resulted in a fall in U46619-induced tone of 1.79 ± 0.29 mN and it was difficult to recover this to the original level, even with the use of additional U46619. However, H89 had no effect on either the first or second components of dilatation after stimulation at a single frequency of 16 Hz. First and second components at 16 Hz were 20 ± 8% and 25 ± 7% (n=6), respectively, in control tissues and 23 ± 4 % and 23 ± 4% (n=6) in rings treated with H89.

On the basis of the above experiments, it was not possible to determine if PKA was involved in either the first or second components of neurogenic vasodilatation in the bovine intraocular LPCA.

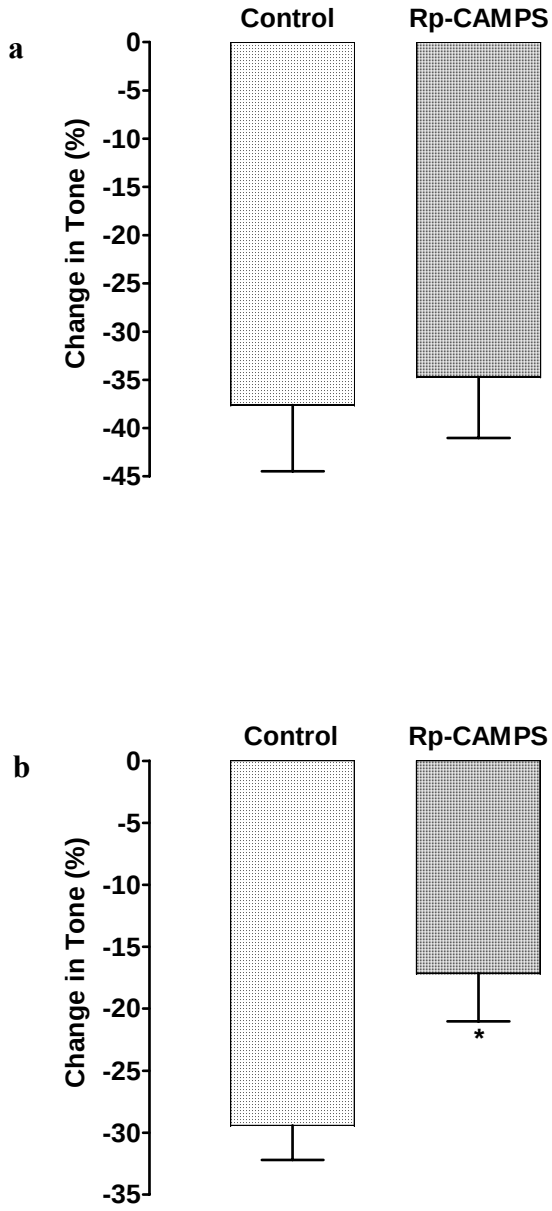

Figure 4.11 Dilatation in response to EFS at a single frequency (16 Hz) in control bovine intraocular LPCA rings and in those pretreated with the PKA inhibitor, Rp-8-Br-cAMPS (50 μM). (a) The first component of dilatation, measured at 10 s, was unaffected by Rp-8-Br-cAMPS. However, (b) the second component, measured at 50 s, was partially inhibited by Rp-8-Br-cAMPS. Data are mean±s.e. mean; n=4; *P<0.05 indicates a difference from control.

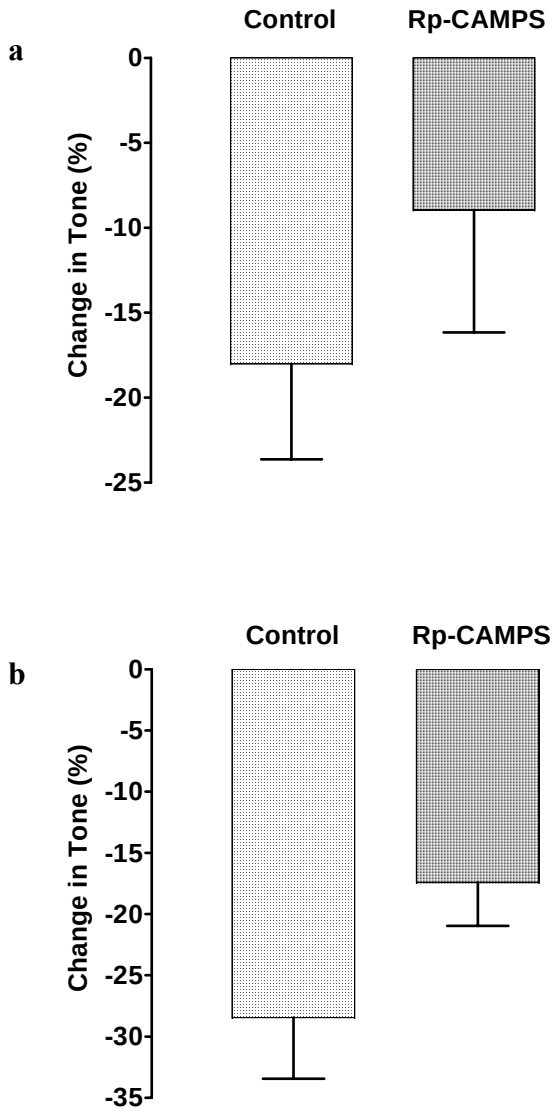

Figure 4.12 Dilatation in response to EFS at a single frequency (16 Hz) in control bovine intraocular LPCA rings and in those pretreated with the PKA inhibitor, Rp-8-Br-cAMPS (100 μM). Incubation with Rp-8-Br-cAMPS appeared to reduce the magnitude of (a) the first component, measured at 10 s and (b) the second component, measured at 50 s, but neither reduction was statistically significant. Data are mean±s.e. mean; n=7.

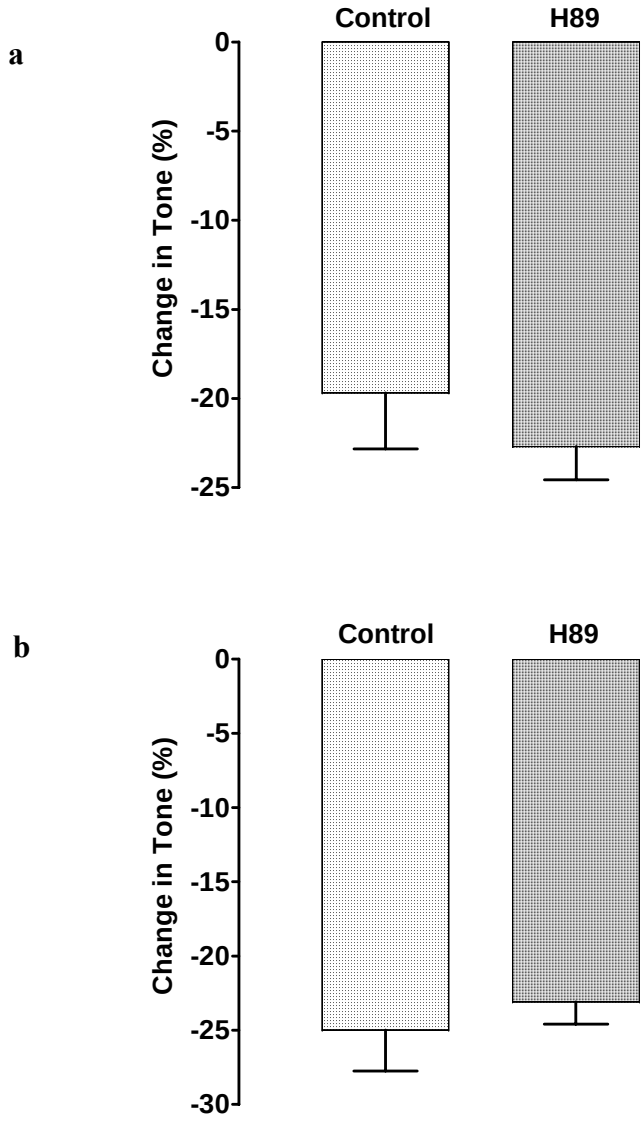

Figure 4.13 Dilatation in response to EFS at a single frequency (16 Hz) in control bovine intraocular LPCA rings and in those pretreated with the PKA inhibitor, H89 (10 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by H89. Data are mean±s.e. mean; n=6.

CHAPTER 5.

RESULTS: EFFECT OF NOS INHIBITORS ON NEUROGENIC AND ENDOTHELIUM-DEPENDENT VASODILATATION

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5 Effect of NOS Inhibitors on Neurogenic and Endothelium-Dependent Vasodilatation

N^G-substituted analogues of L-arginine are routinely used to inhibit the NOS family of enzymes (Hobbs *et al*., 1999). The first of these to be introduced, L-NMMA (Hibbs *et al*., 1987), is generally reported to inhibit all three isoforms of NOS in standard enzyme assays (Moore *et al.,* 1996). However, there have been a number of anomalous findings regarding the specificity of L-NMMA. Although L-NMMA blocks nitrergic nerve mediated-relaxation in the rat anococcygeus (Gillespie *et al.,* 1989) and mouse anococcygeus (Gibson *et al.,* 1990) muscle it does not inhibit nitrergic relaxation in the BRP muscle (Martin *et al*., 1993). Furthermore, in the BRP muscle, L-NMMA actually protects against block by the NOS inhibitors L-NOARG and L-NAME (Martin *et al*., 1993). In the bovine penile artery, L-NMMA also fails to inhibit nitrergic transmission but does block endothelium-dependent, NO-mediated vasodilatation (Liu *et al.*, 1991). Furthermore, in the rat aorta, L-NMMA inhibits basal release, but not agonist-stimulated release of NO from the endothelium (Frew *et al.,* 1993). Therefore, the aim of these experiments was to determine the effects of L-NMMA, L-NAME and the putative nNOS-specific inhibitors, AAAN (Hah *et al*., 2001) and N^G-propyl-L-arginine (Zhang *et al*., 1997a), on endothelium-dependent and nitrergic nerve-mediated dilatation in the bovine intraocular LPCA.

5.1 Effect of NOS Inhibitors on Neurogenic Vasodilatation

5.1.1 L-NAME

As has been established in chapter 3, the first component of neurogenic vasodilatation, measured at 10 s, was abolished at all frequencies by the NOS inhibitor, L-NAME (Hobbs & Gibson, 1990; 100 μM), but the second component, measured at 50 s, was unaffected (Figure 3.11). Control dilatations at 32 Hz were 36 ± 5% and 24 ± 3% (n=12) for first and second components, respectively. In contrast, a contraction of 9 ± 4% ($P<0.001$) and dilatation of 25 ± 3% (n=12) were obtained in L-NAME-treated tissues.

5.1.2 L-NMMA

A frequency-response curve (10 - 15 V, 0.5 - 32 Hz, 0.3 ms, 10 s train length) was also generated in the presence of the L-arginine analogue, L-NMMA (Hibbs *et al*., 1987; 100

μM). In contrast to L-NAME, L-NMMA did not inhibit either the first or second component of neurogenic vasodilatation at any frequency (Figure 5.1): at 32 Hz responses of 30 ± 7% and 42 ± 9% (n=8) were obtained for the first and second components in control tissues, respectively, and in the presence of L-NMMA the responses were 35 ± 4% and 34 ± 5% (n=8), respectively. These data show that L-NMMA does not inhibit nitrergic nerve-mediated dilatation in the bovine intraocular LPCA.

5.1.3 Blockade of L-NAME action by L-NMMA and L-arginine

EFS at a single frequency of 16 Hz in the presence of L-NAME (0.1 – 100 μM) demonstrated that inhibition was concentration-dependent (Figure 5.2). Complete inhibition was obtained using 100 μM L-NAME and a pEC_{50} value of 5.74 ± 0.16 was generated (n=13). In comparison, L-NMMA (10 μM – 1 mM) failed to inhibit dilatation at any of the concentrations examined (Figure 5.2, n=8). Pre-incubation with L-arginine (1 mM) protected against blockade by L-NAME at all concentrations but the maximally observed response was unaffected. In the presence of L-arginine the new apparent pEC_{50} for L-NAME was 4.07 ± 0.11 (n=8), which was significantly different ($P<0.001$) from that of L-NAME alone (Figure 5.2). Furthermore, pre-incubation with L-NMMA (1 mM) also protected against blockade by L-NAME, with the maximally observed response again unaffected. A new apparent pEC_{50} of 3.50 ± 0.26 (n=5) for L-NAME was obtained, which was significantly different ($P<0.001$) from that of L-NAME alone (Figure 5.2). L-NMMA appeared to protect against blockade by L-NAME with greater potency than L-arginine, but a comparison of EC_{50} values demonstrated that this was not statistically significant. These data show that L-NMMA does not inhibit nitrergic vasodilatation in the bovine intraocular LPCA at any concentration but instead acts like the endogenous substrate, L-arginine, in preventing blockade by L-NAME.

5.1.4 AAAN

Frequency-response curves were generated in the presence of the putative nNOS-specific inhibitor AAAN (Hah *et al*., 2001; 100 μM). AAAN had no effect on either the first or second components of dilatation: at 32 Hz dilatations of 30 ± 5% and 38 ± 4% (n=11) were obtained, for first and second components in control tissues, respectively, and in the presence of AAAN, the responses were 21 ± 4% and 38 ± 6% (n=11), respectively (Figure 5.3). These data suggest that AAAN does not inhibit nitrergic vasodilatation in the bovine intraocular LPCA.

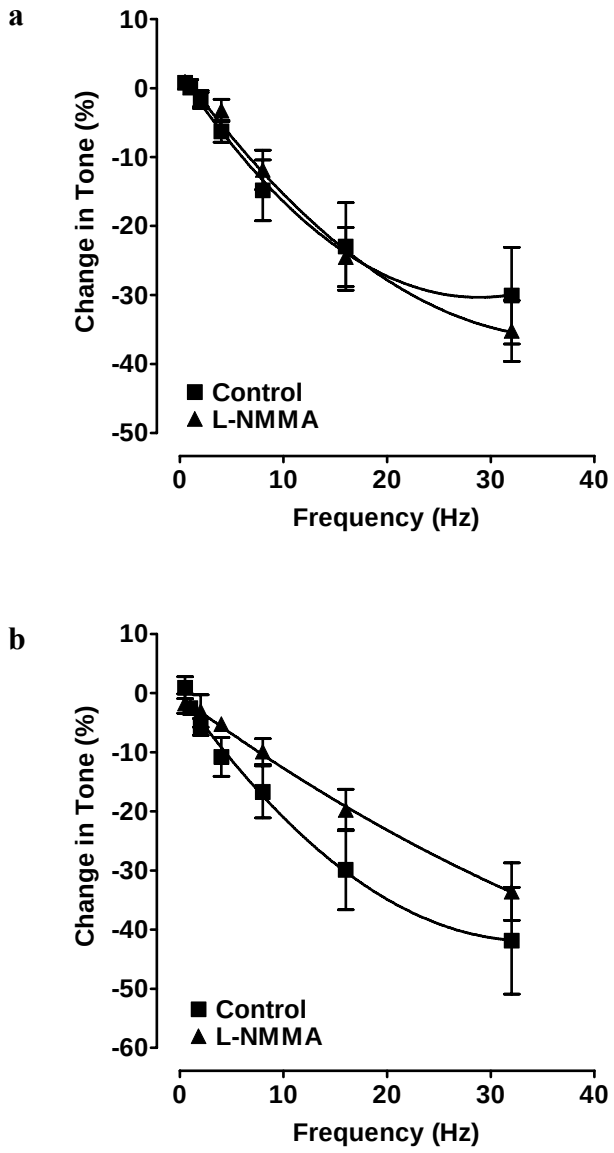

Figure 5.1 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those treated with the NOS inhibitor, L-NMMA (100 μM). Both (a) the first component of dilatation, measured at 10 s, and (b) the second component, measured at 50 s, were unaffected by L-NMMA. Data are mean±s.e. mean; n=8.

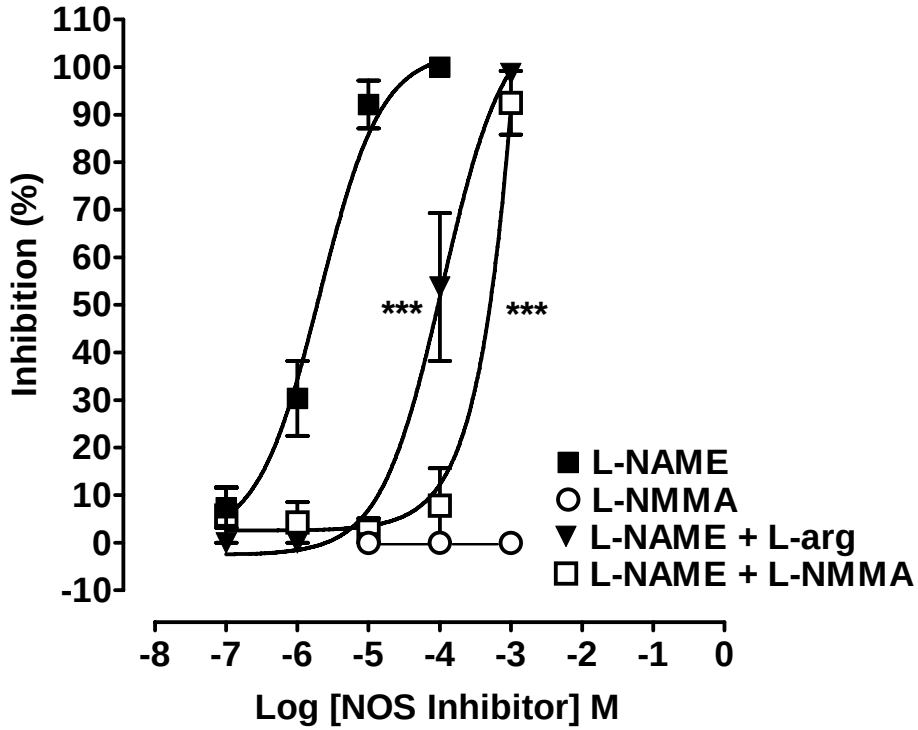

Figure 5.2 The NOS inhibitor, L-NAME (0.1-100 μM), blocked, in a concentration-dependent manner, the first component of dilatation in response to EFS at a single frequency (16 Hz) in bovine intraocular LPCA rings. However, another NOS inhibitor, L-NMMA (10 μM – 1 mM), did not inhibit neurogenic dilatation at any concentration. Furthermore, pretreatment with both L-NMMA and L-arginine (both 1 mM), protected against blockade by L-NAME at all concentrations. L-NMMA seemed to protect against blockade by L-NAME with greater potency than L-arginine, but this was not statistically significant. Data are mean±s.e. mean; n≥5; ***P<0.001 indicate a difference in pEC_{50} from L-NAME alone.

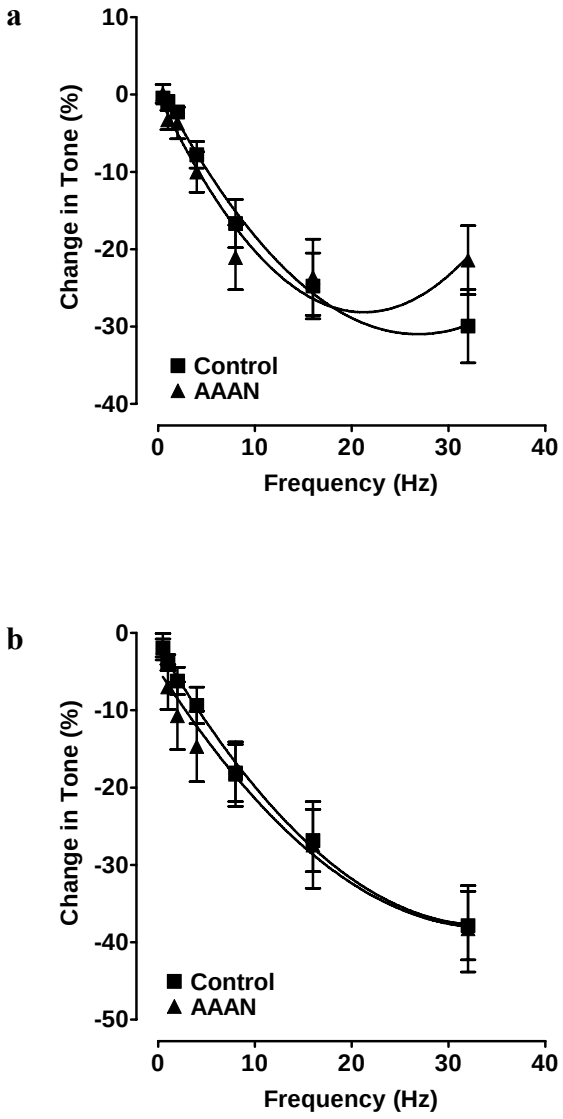

Figure 5.3 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine LPCA rings and in those pretreated with nNOS inhibitor, AAAN (100 μM). Neither (a) the first component of dilatation, measured at 10 s, nor (b) the second component, measured at 50 s, was inhibited by AAAN. Data are mean±s.e. mean; n=11.

5.1.5 N^G-Propyl-L-Arginine

Frequency-response curves were also generated in the presence of another putative nNOS-specific inhibitor, N^G-propyl-L-arginine (Zhang *et al*., 1997a). In contrast to AAAN, N^G-propyl-L-arginine abolished the first component of neurogenic vasodilatation at all frequencies. Indeed, at 32 Hz the first component was transformed from a dilatation of 23 ± 3% to a small contraction of 6 ± 4% (n=9, P<0.001). The second component was unaffected and was 34 ± 4% in control tissues and 33 ± 3% in treated tissues (n=9, Figure 5.4). Furthermore, inhibition by N^G-propyl-L-arginine was concentration-dependent, with a maximally observed inhibition of 81 ± 9% and a pEC_{50} value of 4.95 ± 0.42 (n=12, Figure 5.5). These data show that N^G-propyl-L-arginine is an effective inhibitor of nitrergic vasodilatation in the bovine intraocular LPCA.

5.2 Effect of NOS Inhibitors on Endothelium-Dependent, NO-Mediated Vasodilatation

5.2.1 L-NAME

In the presence of submaximal U46619 (0.1 μM)–induced tone, bradykinin (1 μM) elicited a dilatation of 58 ± 4% (n=6) in control rings of bovine intraocular LPCA (Figure 5.6). Pre-treatment with L-NAME alone resulted in an apparent small reduction of the bradykinin-induced dilatation to 46 ± 5% (n=11, Figure 5.6), but this was not statistically significant. In the presence of the EDHF blockers, apamin and charybdotoxin (Waldron & Garland, 1994; Zygmunt & Högestätt, 1996; both 0.1 μM), and the COX inhibitor, indomethacin (10 μM), dilatation was also reduced slightly to 45 ± 8% (n=6, Figure 5.6), but again this was not statistically significant. However, following blockade of EDHF and COX, dilatation was significantly reduced by L-NAME to 13 ± 4% (P<0.001, n=6, Figure 5.6). These data suggest that L-NAME can powerfully block the endothelium-dependent, NO-mediated component of vasodilatation in the bovine intraocular LPCA, but that in order to observe this clearly, other endothelium-derived vasodilators must first be removed.

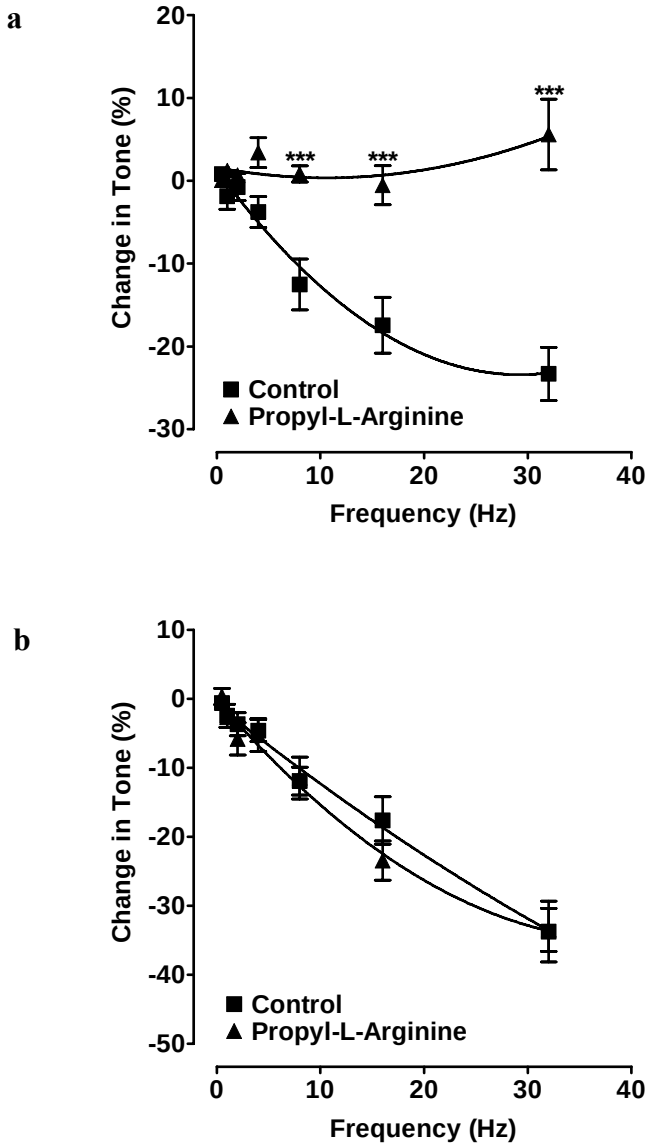

Figure 5.4 Frequency-response curves to EFS (0.5 - 32 Hz) in control bovine intraocular LPCA rings and in those treated with the nNOS inhibitor, N^G-propyl-L-arginine (100 μM). (a) The first component of dilatation, measured at 10 s, was abolished by N^G-propyl-L-arginine at all frequencies. (b) The second component, measured at 50 s, was unaffected by N^G-propyl-L-arginine. Data are mean±s.e. mean; n=9; ***P<0.001 indicate differences from control.

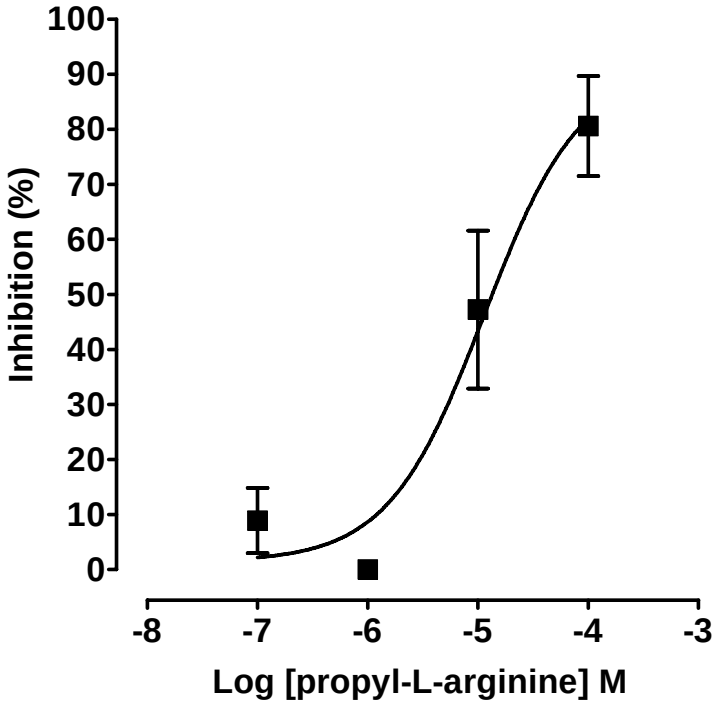

Figure 5.5 The nNOS-specific inhibitor, N^G- propyl-L-arginine (0.1-100 μM), inhibited, in a concentration-dependent manner, dilatation of bovine intraocular LPCA rings in response to EFS at a single frequency (16 Hz). Data are mean±s.e. mean; n≥6.

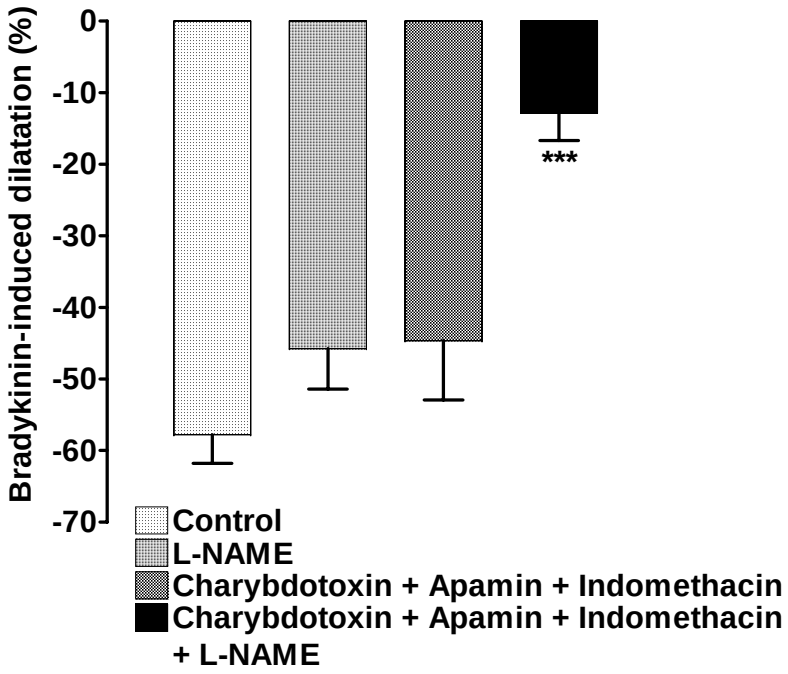

Figure 5.6 Bradykinin (1 μM) induced powerful dilatation of bovine intraocular LPCA rings. This was inhibited slightly by the NOS inhibitor, L-NAME (100 μM), alone but this was not statistically significant. Pre-treatment with the EDHF blockers, apamin and charybdotoxin (both 0.1 μM), and the COX inhibitor, indomethacin (10 μM), also appeared to cause a small inhibition, but again this was not statistically significant. However, in the presence of L-NAME, following blockade of EDHF and COX, the NO-mediated component of bradykinin-induced dilatation was significantly reduced. Data are mean±s.e. mean; n≥6; ***P<0.001 indicates a difference from control.

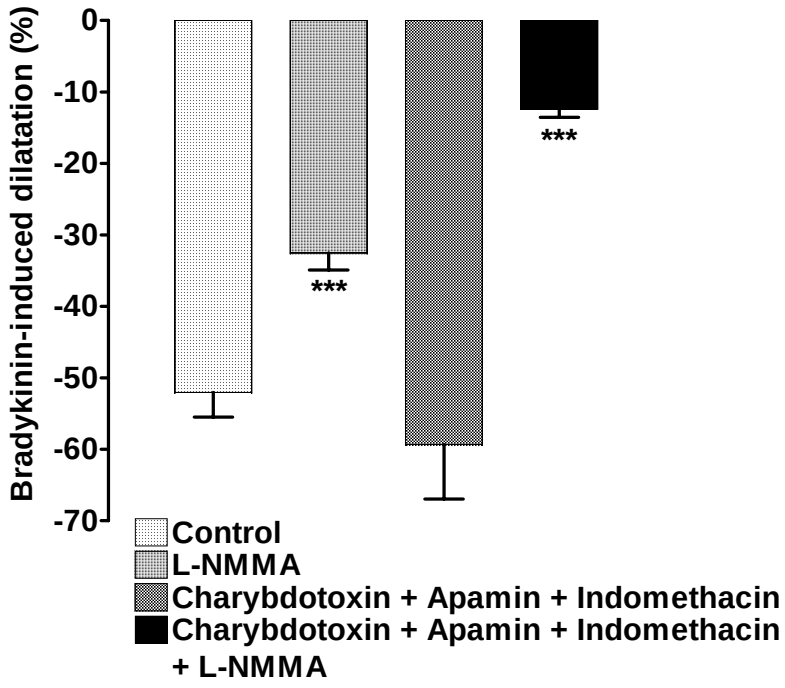

Figure 5.7 Bradykinin (1 μM) induced powerful dilatation of bovine intraocular LPCA rings. This was significantly inhibited in the presence of the NOS inhibitor, L-NMMA (100 μM) alone. In the presence of the blockers of EDHF, apamin and charybdotoxin (both 0.1 μM), and the COX inhibitor, indomethacin (10 μM), bradykinin-induced dilatation was apparently increased, but this was not statistically significant. However, in the presence of L-NMMA, following blockade of EDHF and COX, the NO-mediated component of bradykinin-induced dilatation was further reduced. Data are mean±s.e. mean; n≥6; ***P<0.001 indicate differences from control.

5.2.2 L-NMMA

In the presence of submaximal U46619 (0.1 μM)–induced tone, bradykinin (1 μM) elicited a dilatation of 52 ± 3% (n=10), in control rings of bovine intraocular LPCA (Figure 5.7). Pre-treatment with L-NMMA alone reduced the bradykinin-induced dilatation to 33 ± 2% (n=10, Figure 5.7) and this was significantly different from the control response ($P<0.001$). In the presence of the blockers of EDHF, apamin and charybdotoxin (both 0.1 μM), and the COX inhibitor, indomethacin (10 μM), bradykinin-induced dilatation was apparently increased to 59 ± 7% (n=6, Figure 5.7), but this was not statistically significant from the control dilatation. However, following blockade of EDHF and COX, dilatation was further reduced by L-NMMA to 12 ± 1% ($P<0.001$, n=10, Figure 5.7). These data show that L-NMMA powerfully blocks endothelium-dependent, NO-mediated vasodilatation in the bovine intraocular LPCA, but as with L-NAME, inhibition of other endothelium-derived vasodilators is necessary to observe this clearly.

5.2.3 AAAN

In the presence of submaximal U46619 (0.1 μM)–induced tone, bradykinin (1 μM) elicited a dilatation of 68 ± 6% (n=8) in control rings of bovine intraocular LPCA (Figure 5.8). In the presence of blockers of EDHF, apamin and charybdotoxin (both 0.1 μM), and the COX inhibitor, indomethacin (10 μM), dilatation was apparently reduced to 58 ± 5% (n=8), but this was not statistically significant. AAAN (100 μM) had no effect on the NO-mediated component of bradykinin-induced dilatation (55 ± 5%, n=8) in the presence of inhibitors of EDHF and COX. These data suggest that AAAN does not inhibit endothelium-dependent, NO-mediated vasodilatation in the bovine intraocular LPCA.

5.2.4 N^G- Propyl-L-Arginine

In the presence of submaximal U46619 (0.1 μM)–induced tone, bradykinin (1 μM) elicited a dilatation of 68 ± 6% (n=6) in control rings of bovine intraocular LPCA (Figure 5.9). In the presence of the blockers of EDHF, apamin and charybdotoxin (both 0.1 μM), and the COX inhibitor, indomethacin (10 μM), dilatation was unaffected and was 66 ± 7% (n=6). In contrast to AAAN, pretreatment N^G-propyl-L-arginine inhibited the endothelium-dependent, NO-mediated component of vasodilatation, seen in the presence of inhibitors of EDHF and COX: it was reduced to 22 ± 4%, which was

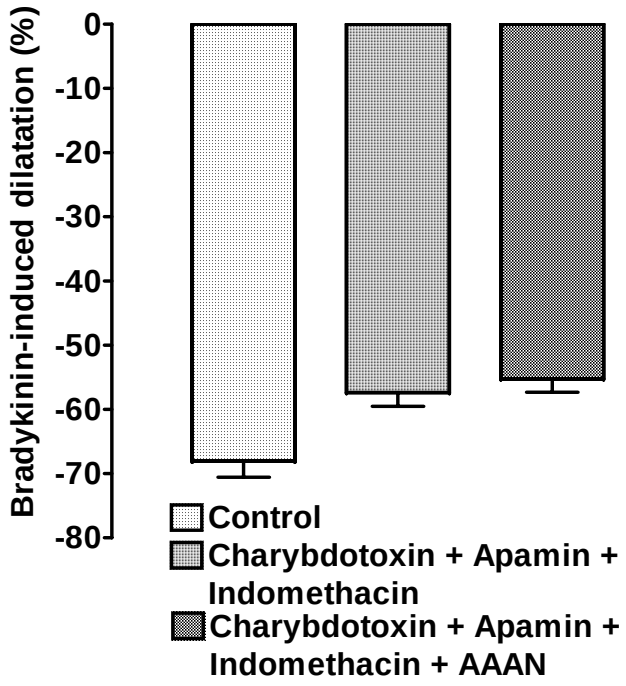

Figure 5.8 Bradykinin (1 μM) induced powerful dilatation of bovine intraocular LPCA. In the presence of the EDHF blockers, apamin and charybdotoxin (both 0.1 μM), and the COX inhibitor, indomethacin (10 μM), the dilatation was reduced but this was not statistically significant. AAAN (100 μM) had no effect on the NO-mediated component of bradykinin-induced dilatation in the presence of inhibitors of EDHF and COX. Data are mean±s.e. mean; n=8.

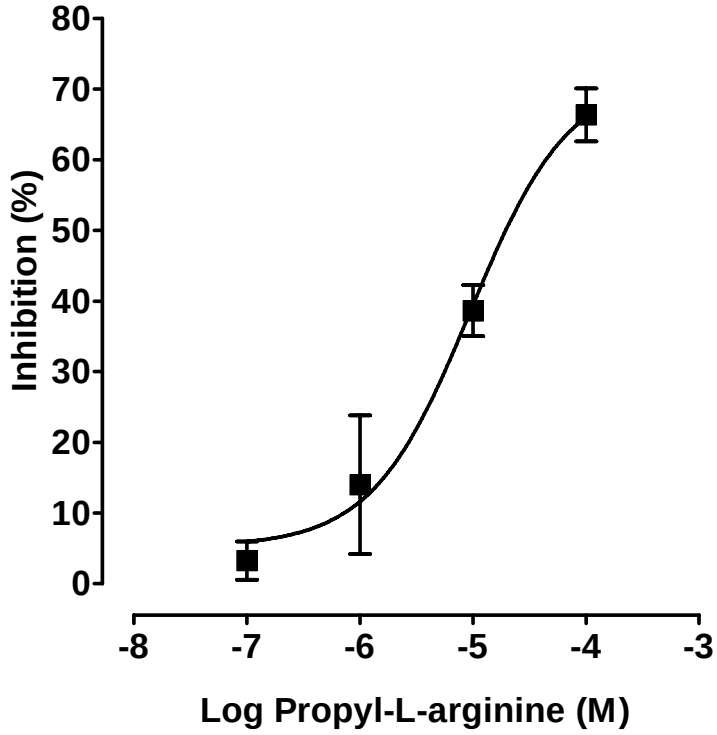

Figure 5.9 nNOS-specific inhibitor, N^G- propyl-L-arginine (0.1-100 μM), inhibited bradykinin (1 μM)-induced relaxation of bovine intraocular LPCA rings, in a concentration-dependent manner. Data are mean±s.e. mean; n≥4.

statistically significant ($P<0.001$).

N^G-propyl-L-arginine inhibited the endothelial-dependent, NO-mediated component of vasodilatation induced by bradykinin in a concentration-dependent manner, with a pEC_{50} of 5.03 ± 0.14 (Figure 5.10): a maximum inhibition of 63 ± 4% (n=6) was obtained using 100 μM N^G-propyl-L-arginine. Consequently, these data show that N^G-propyl-L-arginine is not a powerful inhibitor of endothelium-dependent, NO-mediated vasodilatation in the bovine intraocular LPCA. Surprisingly, N^G-propyl-L-arginine inhibited the endothelium-dependent and nitrergic nerve-mediated vasodilatation with equal potency in the bovine intraocular LPCA (Figure 5.10), with pEC_{50} values of 5.03 ± 0.14 and 4.95 ± 0.42, respectively.

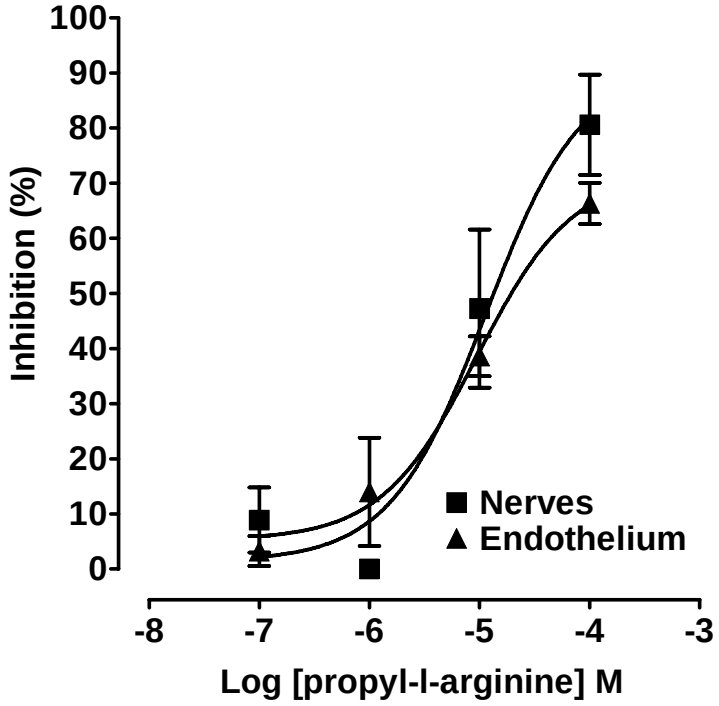

Figure 5.10 The putative nNOS-specific inhibitor, N^G-propyl-L-arginine (0.1-100 μM) inhibited dilatation of bovine intraocular LPCA rings in response to EFS at a single frequency (16 Hz) in a concentration-dependent manner. Furthermore, N^G-propyl-L-arginine inhibited the endothelium-dependent, NO-mediated dilatation induced by bradykinin (1 μM) over a similar concentration range. Data are mean±s.e. mean; n≥4.

CHAPTER 6.

DISCUSSION

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6 Discussion

6.1 Neurogenic Vasodilatation in the Bovine Intraocular LPCA

The main aim of this study was to investigate neurogenic vasodilatation in the bovine intraocular LPCA. However, before examining this process in detail, it was important to determine the optimal stimulation parameters for this tissue and determine if a constrictor innervation, which could be observed optimally at low tone, was present in the tissue.

In the absence of the thromboxane A_2-mimetic, U46619-induced tone, control tissues were unresponsive to EFS at any frequency. A small, frequency-dependent neurogenic vasoconstriction was uncovered only in the presence of the NOS inhibitor, L-NAME (Hobbs & Gibson, 1990), and was subsequently blocked by the adrenergic neurone blocking drug, guanethidine (Page & Dustan, 1959). This would seem to suggest that a small population of adrenergic vasoconstrictor nerves does exist in the bovine intraocular LPCA but that constriction is blocked by NO released from nitrergic nerves. Therefore, all future experiments were carried out in the presence of guanethidine to ensure that only unopposed dilator responses were observed.

In the presence of U46619-induced tone and blockade of adrenergic vasoconstriction with guanethidine, EFS with a train length of 10 s elicited vasodilator responses in the bovine intraocular LPCA and these were clearly resolved into two separate components, which peaked at 10 s and 50 s, respectively. It is likely that response is biphasic in nature because the first component of the compound response decayed rapidly upon termination of EFS and the latency of the second (~12 s, observed in the presence of L-NAME to block the first component) was sufficiently long to have begun after the first had decayed substantially. The two components of the biphasic response to EFS were both voltage- and frequency-dependent, with the latter maximally observed at 32 Hz, while the optimal voltage for stimulation was specific to individual tissues. Stimulation strength was capped at a maximum of 15 V, however, as TTX-resistant, non-neurogenic dilatations were observed at voltages greater than 15 V.

Previous research by Wiencke *et al*. (1994) had concluded that the response to EFS in the bovine intraocular LPCA was monophasic dilator response that decayed with either a fast or a slow time course and was mediated in part by NO and in part by CGRP. However, these authors used an EFS train length of 20 s, in comparison to the train length of 10 s used in the current study. The clear temporal separation of the two components, obtained in

the current study, provided better conditions for pharmacological analysis than when the two components were fused together in a single response using a longer train length (Wiencke *et al*., 1994).

The neurogenic nature of the biphasic response was confirmed by the use of the neurotoxin, TTX. TTX has been recognised as a poisonous substance from the ovaries and liver of the pufferfish, since the time of the early Egyptian and Chinese dynasties. It was eventually extracted and named in the early 1900s (Tahara, 1910) and is now known to block voltage-gated sodium channels, thus preventing action potential generation and therefore inhibiting neurotransmission (Narahashi *et al*., 1964). Some sodium channels are TTX-insensitive (Ritter & Mendell, 1992), however these are mostly found in sensory neurons involved in the nociception pathway (Gold, 1999). Both components of relaxation following EFS in the bovine intraocular LPCA were inhibited in the presence of TTX, confirming they were neurogenic in origin. Furthermore, endothelial denudation, which abolished dilatation to bradykinin, did not inhibit neurogenic vasodilatation in this tissue, suggesting that endothelial cells are not involved in either inducing or modulating the nerve-evoked dilatation.

6.1.1 Role of NO in Neurogenic Vasodilatation in the Bovine Intraocular LPCA

The NOS inhibitor, L-NAME (Hobbs & Gibson, 1990), abolished the first component of neurogenic vasodilatation at all frequencies examined, but had no effect on the second. Therefore, the first component of neurogenic vasodilatation appears to be nitrergic, supporting the earlier reported involvement of nitric oxide in neurogenic vasodilatation in this vessel (Wiencke *et al*., 1994). Furthermore, the inhibitor of soluble guanylate cyclase, ODQ (Garthwaite *et al*., 1995), also abolished the first component of relaxation, but did not affect the second. This would seem to confirm the involvement of the NO-guanylate cyclase-cGMP pathway in the first component of neurogenic vasodilatation. As has already been discussed in chapter 1, previous reports describe neurogenic vasodilatation mediated solely by nitrergic nerves in retinal and ciliary arteries from a range of species, including pig, monkey and dog (Toda *et al*., 1997; Toda *et al*., 1999; Toda *et al*., 1994; Toda *et al*., 1998). However, in contrast to Toda *et al*. (1997), who reported only nitrergic vasodilatation in the porcine ciliary artery, Su *et al*. (1994) described only a partial inhibition of neurogenic vasodilatation by L-NAME and speculated that another transmitter, possibly a peptide, was responsible for the L-NAME-insensitive part of the response.

Thus, as the transmitter responsible for mediating the first component had been identified as NO from nitrergic nerves, the search to identify the mediator responsible for the second component began.

6.1.2 Role of CGRP in Neurogenic Vasodilatation in the Bovine Intraocular LPCA

In contrast to the earlier report of Wiencke *et al*. (1994), this study found no evidence to support a role for CGRP in neurogenic vasodilatation of the bovine intraocular LPCA. The $CGRP_1$ receptor antagonist, $CGRP_{8-37}$, inhibited the dilator actions of exogenous CGRP with the expected pK_B of ~6.7 (Brain *et al*., 2002), but it had no effect on either component of neurogenic dilatation, at any frequency. Furthermore, in the presence of L-NAME, to rule out the possibility that the nitrergic component may have inadvertently contributed to the second component of neurogenic vasodilatation and masked any effect of CGRP inhibition, the second component was again entirely unaffected by $CGRP_{8-37}$.

It is, however, likely that CGRP-containing sensory nerves are present in the bovine intraocular LPCA, since capsaicin, which activates vanilloid VR1 receptors on C and Aδ fibres (Akerman *et al*., 2003; Joó *et al*., 1969) induced a small transient dilatation that was inhibited by pretreatment with $CGRP_{8-37}$. Why EFS did not appear to activate these nerves in this study is not clear. It is possible that the simulation parameters used in the present study did not activate CGRP-containing sensory nerves. Previous findings on dural vasculature show that neurogenic (EFS-induced) vasodilatation involves only Aδ fibres, whereas neurogenic inflammation, which is associated with prolonged, high intensity stimulation, additionally involves C fibres (Akerman *et al*., 2003; Williamson *et al*., 1997a; Williamson *et al*., 1997b). It is therefore possible in the bovine LPCA that CGRP is present exclusively in C fibres that can be activated by capsaicin but not by the EFS parameters used in the present study or by those used by Wiencke *et al*. (1994). Indeed, further evidence against a role for CGRP in neurogenic relaxation was obtained when complete desensitisation to capsaicin had been obtained through prior exposure to the excitotoxin. Under these conditions, where prolonged inhibition of C fibre transmitter release occurs (Jancsó *et al*., 1967; Lynn, 1990), both the first and second components of neurogenic vasodilatation remained entirely unaffected at all frequencies. Thus, although CGRP-containing sensory fibres sensitive to capsaicin appear to be present, the separation of the two components of neurogenic vasodilatation in bovine intraocular LPCA provides no evidence of a role for CGRP, in contrast to the report by Wiencke *et al*. (1994).

6.1.3 Role of Substance P in Neurogenic Vasodilatation in the Bovine Intraocular LPCA

The inability of capsaicin desensitisation to affect neurogenic vasodilatation in the bovine intraocular LPCA also rules against a role for substance P, since it too is present in sensory fibres. Nevertheless, substance P is known to be involved in neurogenic responses in other blood vessels, including rat mesenteric veins (Ahluwalia & Vallance, 1997; Claing *et al*., 1992). Although substance P was able to promote dilatation of the bovine intraocular LPCA, complete desensitisation to this agent occurred within minutes, thus making it an unlikely transmitter candidate for the neurogenic vasodilator response, as the latter responses are highly reproducible upon successive stimulations. Moreover, when complete desensitisation to substance P had been obtained or when the NK_1 receptor antagonist, L-733,060 (Seabrook *et al*., 1996), was present, neither the first nor the second components of neurogenic vasodilatation was affected. Substance P is therefore unlikely to contribute to neurogenic vasodilatation in the bovine intraocular LPCA.

6.1.4 Role of VIP in Neurogenic Vasodilatation in the Bovine Intraocular LPCA

The presence of VIP-containing perivascular nerves has been demonstrated histochemically in ciliary arteries from a wide range of species, including pig, rat and human (Bergua *et al*., 2003; Flügel *et al*., 1994; Toda *et al*., 1997). Nevertheless, all studies to-date agree that VIP has no functional role in the ciliary artery and that neurogenic vasodilatation in these tissues is mediated solely by nitrergic nerves. VIP powerfully relaxed the bovine intraocular LPCA and high concentrations led to partial desensitisation to the peptide. In this partially desensitised state, which has been used previously to rule out involvement of VIP in the neurogenic vasodilatation in dog and monkey cerebral arteries (Toda, 1982), the first and second components of neurogenic vasodilatation were completely unaffected.

Moreover, treatment with a protease, α-chymotrypsin, a strategy previously employed to successfully establish the role of a peptide in the neurogenic vasodilatation in guinea pig uterine and bovine mesenteric arteries (Leckström *et al*., 1993; Morris, 1993), failed to inhibit either the first or second components of the neurogenic response in the bovine intraocular LPCA. Although VIP is an unlikely candidate to mediate the second component of neurogenic vasodilatation, the pronounced fall in tone generated by α-

chymotrypsin in the intraocular LPCA made it difficult to dismiss it completely. This fall in tone may have been mediated by protease-activated receptors (PARs), a class of GPCR activated by proteolytic cleavage. Four members of the PAR family have been identified: PAR1 (thrombin receptor), PAR2, PAR3 and PAR4. PAR2 (Nystedt *et al*., 1994) is activated by trypsin and has been identified on endothelial and SMC of a number of vascular tissues.

A role for VIP in neurogenic vasodilatation was previously established in sheep cerebral artery with the use of VIP antiserum (Matthew *et al*., 1997). In the bovine intraocular LPCA, however, VIP antiserum failed to convincingly block dilatation to exogenous VIP and caused such a profound fall in tone and frothing in the myograph baths, that its effects on neurogenic vasodilatation could not be tested. Furthermore, although VIP is reported to mediate vasodilatation via activation of K_{ATP} channels in the rabbit cerebral artery (Standen *et al*., 1989), the K_{ATP} channel inhibitor, glibenclamide, failed to block relaxation to exogenous VIP and had no effect on either the first or second components of neurogenic vasodilatation. In addition, as activation of K_{ATP} channels is also reported to mediate vasodilatation by CGRP in a number of VSM preparations (Luu *et al*., 1997; Nelson *et al*., 1990), the inability of glibenclamide to block neurogenic vasodilatation further suggests that CGRP does not mediate the second component of neurogenic vasodilatation in this vessel. Therefore, none of the experimental approaches used in this study provided any evidence that VIP contributes to neurogenic vasodilatation in the bovine intraocular LPCA.

6.1.5 Role of ATP in Neurogenic Vasodilatation in the Bovine Intraocular LPCA

The final transmitter candidate investigated in detail was ATP, since this nucleotide is believed to mediate neurogenic vasodilatation together with NO in the rabbit portal vein and mesenteric artery (Brizzolara *et al*., 1993; Kakuyama *et al*., 1998). ATP-induced vasodilatation is mediated mainly via $P2Y_1$ receptors and $P2Y_2$ receptors. For example $P2Y_1$ receptors mediate vasodilatation in rat middle cerebral arteries (You *et al*., 1999) and rabbit pulmonary artery (Qasabian *et al*., 1997), while $P2Y_2$ receptors mediate vasodilatation in rat middle cerebral arteries (You *et al*., 1999) and in the mouse aorta. Furthermore, UTP mediates vasodilatation in the rabbit ear artery by activation of $P2Y_4$ receptors (Saiag *et al*., 1998) and in the mouse aorta by activation of $P2Y_6$ receptors (Guns *et al*., 2006). Suramin, RB2 and PPADS all block more than one P2Y receptor subtype (von Kugelgen, 2006). Suramin inhibits $P2Y_1$ and $P2Y_2$ receptors with PK_B values of 5.7 and 4.3, respectively, but does not inhibit $P2Y_4$ receptors (Charlton *et al*., 1996) and is a

poor inhibitor of $P2Y_6$ receptors (von Kugelgen, 2006). RB2 generally inhibits agonist-induced responses at all P2Y receptors, although weakly at $P2Y_4$ receptors (von Kugelgen, 2006). PPADS is an effective inhibitor of $P2Y_1$ and $P2Y_6$ receptors, but a poor inhibitor of $P2Y_4$ receptors and does not inhibit $P2Y_2$ receptors at all (von Kugelgen, 2006).

ATP did relax the bovine intraocular LPCA, but this was completely unaffected by the P2Y receptor antagonists, suramin (Dunn & Blakeley, 1998), PPADS (Lambrecht *et al*., 1992) or RB2 (Burnstock & Warland, 1987b), at concentrations that are effective in other tissues, or by the desensitising P2X receptor agonist, α,β-methyleneATP (Simonsen *et al*., 1997). Thus, lack of a suitable blocking agent prevented further investigation into the possible role of ATP in the neurogenic vasodilatation of the bovine intraocular LPCA.

The lack of availability of selective P2Y antagonists has hampered the identification of specific receptors within tissues. Recently, a number of subtype-specific antagonists have been suggested and these could, in future studies, be of use in determining the receptor ATP acts on to produce dilatation in the bovine intraocular LPCA. For example, the bisphosphate analogue MRS2179 (Kaiser & Buxton, 2002) has been reported as a $P2Y_1$ receptor-specific antagonist in the guinea-pig aorta, and the diisothiocyanate derivative of 1,2-diphenylethane, MRS2567, has been described as a $P2Y_6$ receptor-specific antagonist in both human and rat astrocytes (Mamedova *et al*., 2004). However, until a subtype-specific inhibitor is available that is also effective at blocking ATP-induced dilatation in the bovine intraocular LPCA, it may be difficult to determine whether this nucleotide has any role in neurogenic vasodilatation in this tissue.

6.1.6 CO and H_2S as Potential NANC Neurotransmitters

The gasotransmitters, CO and H_2S, have been reported to regulate cardiovascular function and both were considered as candidate molecules for the unidentified transmitter that mediates the second component of neurogenic vasodilatation in the bovine intraocular LPCA. However, neither gas was examined directly as part of the current investigation.

6.1.6.1 CO

CO-induced vasodilatation was originally demonstrated in the rat coronary artery (McGrath & Smith, 1984) and has since been observed in a vast number of tissues; rat and rabbit thoracic aorta (Furchgott & Jothianandan, 1991; Lin & McGrath, 1988), canine

carotid, coronary and femoral arteries (Vedernikov *et al*., 1989), rat tail artery (Wang *et al*., 1997) and piglet mesenteric artery (Villamor *et al*., 2000). However, the response to CO is not universal as it does not induce vasodilatation in canine or rabbit basilar or middle cerebral arteries (Brian *et al*., 1994). Furthermore, in the rat gracilis muscle arterioles it actually causes vasoconstriction (Kozma *et al*., 1999). CO-induced vasodilatation can be mediated by activation of both specific K^+ channels and the cGMP pathway. For example, CO induced a concentration-dependent, endothelium-independent vasodilatation in the rat tail artery that appeared to be mediated by both the cGMP pathway and BK_{Ca} channels (Wang *et al*., 1997). Inhibitors of BK_{Ca} channels have also been found to inhibit CO-mediated vasodilatation in piglet pial arterioles (Leffler *et al*., 1999) and hypoxic rat mesenteric arterioles (Naik & Walker, 2003). Although CO was not directly examined, the suggested mechanisms of CO-mediated vasodilatation have been investigated in the course of the present study. Neither L-NAME nor ODQ, which inhibit components of the cGMP pathway or iberiotoxin, charybdotoxin or TEA, which inhibit BK_{Ca} channels, were capable of inhibiting the second component of neurogenic vasodilatation. Therefore, it would seem that CO does not appear to be involved in the second component of neurogenic vasodilatation in the bovine intraocular LPCA. Further investigation, perhaps using an inhibitor of haem oxygenase, the enzyme that generates CO, such as zinc protoporphyrin IX (ZnPPIX; Naik *et al*., 2003) is required to determine if CO does indeed have a role in neurogenic vasodilatation in the bovine intraocular LPCA.

6.1.6.2 H_2S

More recently, H_2S has also been reported as a putative gasotransmitter. H_2S-induced vasodilatation was first observed in the rat aorta (Hosoki *et al*., 1997). The vascular effects of H_2S have been shown *in vivo* and *in vitro* in the rat, where vasodilatation is reportedly mediated via K_{ATP} channels as it is blocked by the K_{ATP} channel blocker, glibenclamide, and mimicked by pinacidil, a K_{ATP} channel opener (Zhao *et al*., 2001). However, in rat, the decrease in blood pressure observed upon bolus i.v. injection of H_2S was transient and lasted approximately 30 s (Zhao *et al*., 2001), whereas in the present study, the time course for the second component of neurogenic relaxation was of much greater duration. In addition, although H_2S was not investigated directly in the present study, the involvement of K_{ATP} channels was examined by the use of glibenclamide. As this K_{ATP} channel blocker was unable to inhibit the second component of vasodilatation, it would seem unlikely that H_2S mediates neurogenic vasodilatation in the bovine intraocular LPCA. However, as the mechanism of H_2S-induced vasodilatation has not been fully elucidated, further

investigation is required to determine if H_2S does indeed have a role in neurogenic vasodilatation in the bovine intraocular LPCA. One possible strategy for this would be to utilise inhibitors of the two enzymes which generate H_2S, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). Amino-oxyacetic acid (Braunstein *et al*., 1971) and propargylglycine (Uren *et al*., 1978) inhibit CBS and CSE, respectively, and may be of use in determining the role, if any, of H_2S in neurogenic vasodilatation in the bovine intraocular LPCA.

6.1.7 Other Putative NANC Neurotransmitters

In addition to the gasotransmitters, two neuropeptide NANC transmitters, which could potentially mediate the second component of neurogenic vasodilatation in bovine intraocular LPCA, but were not investigated in the present study, are PACAP and PHI, which are both structurally-related to VIP. PACAP and PHI-containing fibres have been identified in the rat cerebral circulation (Ceccatelli *et al*., 1991) using immunohistochemistry. Functionally, they have been described as NANC inhibitory transmitters in the rat and guinea-pig gastric fundus (Curro *et al*., 2002; Katsoulis *et al*., 1996). Although in theory, the treatment with α-chymotrypsin in this study should have inhibited any vasodilatation mediated by a peptide such as PACAP or PHI, the large fall in tone generated by the protease in the bovine intraocular LPCA made investigation difficult. It is therefore not possible to rule out PACAP or PHI as a neurotransmitter in this vessel. The PACAP receptor antagonist, $PACAP_{6-38}$ and anti-PHI serum have both been used successfully to inhibit EFS-induced relaxant responses in the mouse (Baccari & Calami, 2001) and rat (Curro *et al*., 2002) gastric fundus, respectively, and are potential tools for further study in the bovine intraocular LPCA.

6.2 Identification of the Mechanisms of the Second Component of Neurogenic Vasodilatation in the Bovine Intraocular LPCA

Although it had been established that neurogenic vasodilatation in the bovine intraocular LPCA was biphasic in nature and that the first component appeared to be mediated by NO released from nitrergic nerves and acting via soluble guanylate cyclase, the identity of the transmitter that mediated the second component of dilatation remained unclear. As this vasodilatation could not be blocked using conventional neurotransmitter inhibitors or desensitisation protocols, an attempt was made to determine the mechanism by which the

unknown transmitter promoted dilatation. Three potential mechanisms of vasodilatation; hyperpolarisation-induced, cGMP-induced and cAMP-induced were investigated.

6.2.1 Hyperpolarisation-Induced, Neurogenic Vasodilatation in the Bovine Intraocular LPCA

The membrane potential of VSM cells, which is highly influenced by K^+ channels, plays an important role in arterial tone. The opening of K^+ channels in the cell membrane leads to increased K^+ efflux and this, in turn, causes membrane hyperpolarisation. The resultant hyperpolarisation closes voltage-dependent Ca^{2+} channels, decreasing Ca^{2+} entry and this leads to vasodilatation. Conversely, inhibition of K^+ channels causes membrane potential depolarisation and vasoconstriction (Nelson & Quayle, 1995). Four types of K^+ channel have been identified in VSM; K_{IR}, K_V, K_{ATP} and K_{Ca} (of which there are 3 subtypes; BK_{Ca}, IK_{Ca} and SK_{Ca}). NO (Bolotina *et al*., 1994), CGRP (Nelson *et al*., 1990), VIP (Standen *et al*., 1989), substance P (Frieden *et al*., 1999) and PACAP (Kishi *et al*., 1996), have all been shown to mediate vasodilatation via activation of a variety of K^+ channels, resulting in the hyperpolarisation of the SMC.

Addition of the non-specific K^+ channel blocker, TEA (Nelson & Quayle, 1995), the inhibitor of K_{IR} channels, $BaCl_2$ (Quayle *et al*., 1993), the BK_{Ca} channel inhibitor, iberiotoxin (Latorre *et al*., 1992), the inhibitor of BK_{Ca}, IK_{Ca} and K_V channels, charybdotoxin (Grissmer *et al*., 1994; Miller *et al*., 1985; Desai *et al*., 2006) and the small conductance K_{Ca} channel inhibitor, apamin (Blatz & Magleby, 1986), but not the K_{ATP} channel inhibitor, glibenclamide (Schmid-Antomarchi *et al*., 1987), to the myograph bath caused an increase in U46619-induced tone, suggesting that K_{IR}, $BK_{Ca,}$ $IK_{Ca,}$ SK_{Ca} and K_V channels but not K_{ATP} channels are tonically active in the membrane of VSM cells in the bovine intraocular LPCA.

6.2.1.1 Effect of Non-Specific Inhibition of K^+ Channels on Neurogenic Vasodilatation in the Bovine Intraocular LPCA

The non-specific K^+ channel blocker, TEA (Nelson & Quayle, 1995), has previously been shown to inhibit NANC-mediated relaxations in the smooth muscle of the human colon (Sahin *et al*., 2001). However, incubation with TEA appeared to potentiate the magnitude of neurogenic vasodilatation in bovine intraocular LPCA rings stimulated at a single frequency of 16 Hz. TEA has also been shown to potentiate EFS-elicited vasodilatation in the rabbit facial vein (Tanaka *et al*., 2003). In the facial vein the potentiated response was

attributed to enhanced transmitter release from the nerve terminals, linked to functional coupling between prejunctional TEA-sensitive K^+ channels and Q-type Ca^{2+} channels. Furthermore, there is evidence showing that K^+ channel inhibitors, such as TEA and 4-aminopyridine, can enhance transmitter output from a variety of nerves by prolonging the nerve action potential. In the bullfrog sympathetic ganglia (Kumamoto & Kuba, 1985) and the guinea-pig inferior mesenteric ganglia (King & Szurszewski, 1988) enhanced ACh released was noted in the presence of TEA. Therefore, it is possible that in bovine intraocular LPCA, interaction between prejunctional TEA-sensitive K^+ channels and unidentified Ca^{2+} channels or a TEA-mediated increase in action potential duration contributes to an enhanced level of transmitter output and subsequently an increase in the magnitude of neurogenic vasodilatation.

6.2.1.2 Effect of Inhibition of K_{IR} Channels on Neurogenic Vasodilatation in the Bovine Intraocular LPCA

Low concentrations of the inhibitor of K_{IR} channels, $BaCl_2$ (Quayle *et al*., 1993), were found to block the inhibitory action of CGRP in the guinea-pig ureter SMC (Maggi *et al*., 1995). Furthermore, K_{IR} channels are important in K^+-induced dilatations in cerebral (Edwards & Hirst, 1988) and coronary (Knot *et al*., 1996) arteries. Indeed, it has been suggested that K^+ may be the elusive vasodilator agent EDHF (Edwards *et al*., 1998). Edwards *et al.* (1998) suggested that K^+ efflux through charybdotoxin and apamin-sensitive K^+ channels on endothelial cells resulted in an increase in myoendothelial K^+ concentration, which hyperpolarised adjacent SMC by activating K_{IR} channels and Na^+/K^+ ATPase, resulting in vasodilatation. Therefore, the role of these channels in EFS-induced vasodilatation in the bovine intraocular LPCA was examined. However, $BaCl_2$ failed to affect either component of dilatation in response to stimulation at16 Hz, suggesting that K_{IR} channels do not play a role in neurogenic vasodilatation in the bovine intraocular LPCA.

6.2.1.3 Effect of Inhibition of K_{Ca} and K_V Channels on Neurogenic Vasodilatation in the Bovine Intraocular LPCA

It is known that in the bovine isolated perfused eye, charybdotoxin alone, which blocks BK_{Ca}, IK_{Ca} and K_V channels (Grissmer *et al*., 1994; Miller *et al*., 1985), and the combination of charybdotoxin and apamin, which blocks SK_{Ca} channels (Blatz & Magleby, 1986), can be used to block ACh-mediated vasodilatation (McNeish *et al*., 2001). These findings suggested that this vasodilatation was mediated by an EDHF. Therefore, as these

channels are present within the vasculature of the bovine eye, it is plausible that these same channels could be involved in the mechanism of neurogenic vasodilatation in the bovine intraocular LPCA. In addition to this: CGRP relaxes precontracted rat coronary arteries via activation of BK_{Ca} channels (Sheykhzade & Berg Nyborg, 2001); VIP and PACAP are involved in NANC inhibitory responses of longitudinal muscle of the rat distal colon via activation of charybdotoxin and apamin-sensitive K^+ channels, respectively (Kishi *et al*., 1996); VIP dilates the porcine coronary by activation of both BK_{Ca} and K_V channels (Kawasaki *et al*., 1997), while in the same tissue, substance P is reported to activate SK_{Ca} channels (Frieden *et al*., 1999) and finally, NO activates charybdotoxin-sensitive channels in the rabbit aorta (Bolotina *et al*., 1994). However, in the bovine intraocular LPCA, charybdotoxin, apamin and the inhibitor of BK_{Ca} channels, iberiotoxin (Latorre *et al*., 1992), do not affect either the first or the second components of neurogenic vasodilatation, suggesting that $BK_{Ca,}$ IK_{Ca}, SK_{Ca} and K_V channels play no role in neurogenic vasodilatation in this vessel.

6.2.1.4 Effect of Inhibition of K_{ATP} Channels on Neurogenic Vasodilatation in the Bovine Intraocular LPCA Inhibition

K_{ATP} channel activation has previously been suggested as the mechanism by which a number of endogenous vasodilators, especially CGRP, mediate vasodilatation. CGRP has been shown to activate K_{ATP} channels in the rabbit mesenteric (Nelson *et al*., 1990) and porcine coronary arteries (Miyoshi & Nakaya, 1995). In addition, the K_{ATP} channel inhibitor, glibenclamide, has been used to inhibit CGRP-mediated vasodilatations in the rat cerebral (Hong *et al*., 1994), rabbit mesenteric (Nelson *et al*., 1990) and human uterine arteries (Nelson *et al*., 1993). However in a number of cases, glibenclamide only partially inhibited the CGRP-mediated dilatation (Kitazono *et al*., 1993; Maggi *et al*., 1994) and in the porcine coronary artery, it failed completely (Kageyama *et al*., 1993). This would suggest that additional mechanisms, such as activation of K_{Ca} channels, are in operation at the same time. Another neuropeptide, VIP, has been show to elicit glibenclamide-sensitive hyperpolarisation of the membrane potential of rabbit cerebral arteries (Standen *et al*., 1989). In addition, K_{ATP} channels have been found to play a role in adenosine-mediated vasodilatation in the pig coronary artery (Dart & Standen, 1993), as well as vasodilatation mediated by NO in the rabbit mesenteric artery (Murphy & Brayden, 1995). Although the K_{ATP} channel inhibitor, glibenclamide, was able to block dilatation in response to the K_{ATP} channel opener, levcromakalim, in the bovine intraocular LPCA, it failed to inhibit neurogenic vasodilatation at all frequencies. Furthermore, it did not inhibit relaxation induced by exogenous VIP. This would suggest that K_{ATP} channels do not play a role in

either the first or second components of neurogenic vasodilatation in the bovine intraocular LPCA.

As inhibition of K^+ channels had no effect on neurogenic vasodilatation in the bovine intraocular LPCA, the cGMP/PKG pathway was investigated.

6.2.2 *Effect of PDE5 Inhibition on Neurogenic Vasodilatation in the Bovine Intraocular LPCA*

Prior to the discovery of nitrergic transmission, the PDE5 inhibitor, zaprinast, was used to demonstrate that guanylate cyclase/cGMP pathway played a role in neurogenic relaxation in the BRP muscle (Bowman & Drummond, 1984) and the mouse anococcygeus muscle (Gibson & Mirzazadeh, 1989). In the BRP, zaprinast elevated the basal cGMP content and also potentiated both the magnitude of the relaxation and the rise in cGMP produced by NANC nerve stimulation. Similarly, in the mouse anococcygeus muscle, zaprinast was shown to increase relaxations induced by NANC nerve stimulation and NO donors, but to have no effect on relaxations to VIP, papaverine or 3-isobutyl-1-methylxanthine. Following the identification of NO as a NANC transmitter, zaprinast has been shown to potentiate nitrergic neurotransmission in a variety of tissues such as the corpus cavernosum of man (Bush *et al*., 1992; Rajfer *et al*., 1992) and rabbit (Bush *et al*., 1992) and the gastric fundus of the cat (Barbier & Lefebvre, 1995), pig (Lefebvre *et al*., 1995) and rat (Williams & Parsons, 1995). In addition, another PDE5 inhibitor, sildenafil, has been shown to enhance neurogenic NO-mediated and soluble guanylate cyclase-dependent vasodilatation in the bovine penile small arteries (Simonsen *et al*., 2001) and relaxation of the human corpus cavernosum (Ballard *et al*., 1998). In the bovine intraocular LPCA zaprinast potentiated the first component of neurogenic vasodilatation but not the second. This would seem to confirm that the first, but not the second component is mediated by cGMP. However, in contrast to zaprinast, sildenafil did not potentiate the first component of dilatation and instead appeared to increase the magnitude of the second component, although this was not statistically significant. It is unclear why zaprinast, but not sildenafil was able to potentiate the magnitude of the first component, as sildenafil is a highly potent inhibitor of PDE5, with an IC_{50} of 3.5 nM against PDE5 extracted from the human corpus cavernosum (Ballard *et al*., 1998). Furthermore, in the same series of experiments, Ballard *et al*. (1998) were able to determine that sildenafil was >200 times more potent than zaprinast as an inhibitor of PDE5 from the human corpus cavernosum. It is possible that sildenafil is not as effective against the isoform of PDE5 found in the bovine intraocular

LPCA. However, the data capture method utilised in this study did not allow changes in duration of the respose to EFS to be captured. It is therefore possible that if sildenafil potentiated the duration of the relaxation but did not potentiate the magnitude of it, as has been shown by Hallen *et al*. (2007), this would not have been recorded. Experimental design should take this factor into account when using PDE5 inhibitors to investigate the guanylate cyclase/cGMP pathway in the future. Another method which could be used to confirm the participation of the guanylate cyclase/cGMP pathway in the first, but not second component of neurogenic dilatation in the bovine LPCA, would be to utilise the PDE5 inhibitors vardenafil and tadalafil, which were not examined in the present study. Both vardenafil and tadalafil have been found to prolong nerve-induced relaxation in the rabbit corpus cavernosum (Hallen *et al*., 2007) and pig urethra (Werkstrom *et al*., 2006) and may be useful tools for clarifying the situation in the bovine intraocular LPCA.

6.2.3 Effect of PKG Inhibition on Neurogenic Vasodilatation in the Bovine Intraocular LPCA

As the results obtained with the PDE5 inhibitors were inconsistent, another stage of the cGMP-mediated vasodilatation pathway was blocked by inhibiting PKG. PKG is reported to facilitate vasodilatation by reducing the cytosolic [Ca^{2+}] in a number of ways, including: inhibition of Ca^{2+} mobilisation through voltage gated Ca^{2+} channels (Clapp & Gurney, 1991) and activation of both the Na^{+}/Ca^{2+} exchanger (Furukawa *et al*., 1991) and the plasma membrane Ca^{2+}-ATPase (Yoshida *et al*., 1991). Therefore, in theory, inhibition of PKG should block cGMP-mediated vasodilatation. Incubation with the PKG inhibitor, Rp-8-Br-PET-cGMPS (Butt *et al*., 1995) did significantly inhibit the first but not the second component of neurogenic vasodilatation. However, although Rp-8-Br-PET-cGMPS is described as a potent and selective competitive inhibitor of PKG, with an apparent K_i of 0.03 μM (Butt *et al*., 1995) and competitively suppressed the cGMP-induced current in xenopus rod photoreceptors with an IC_{50} of 25 μM (Wei *et al*., 1996), the level of blockade observed in the bovine intraocular LPCA was only marginally significant.

As neurogenic vasodilatation was not fully inhibited by Rp-8-Br-PET-cGMPS, the effects of another PKG inhibitor, KT5823 (Wyatt *et al*., 1991), was examined on the response to EFS. Although this compound has a K_i of 234 nM *in vitro*, the first and second components of neurogenic vasodilatation remained unaffected in the presence of KT5823. While this compound has been used extensively to determine the role of PKG in numerous cell signalling processes, such as proliferation of SMC (Chiche *et al*., 1998), proliferation of neuronal cells (Firestein & Bredt, 1998) and in regulation of ion channels (Darvish *et*

al., 1995), it has also been reported that it is an ineffective inhibitor in intact cells (Burkhardt *et al*., 2000). Burkhardt *et al*. (2000) suggested a number of reasons, such as compartmentalisation of the drug and insufficient incubation time for the drug to pass through the cell membrane, for the disparity in efficacy between *in vitro* experiments utilising KT5823 and those involving intact cells. It is possible that this may be the reason why KT5823 does not inhibit the first component of neurogenic vasodilatation in the bovine intraocular LPCA, and suggests that KT5823 should not be used in isolation to determine the role of PKG in cell signalling pathways.

Therefore, although results with L-NAME and ODQ, demonstrate the involvement of the cGMP-mediated pathway in neurogenic vasodilatation in the bovine intraocular LPCA, this could not be confirmed convincingly by either the use of inhibitors of PDE5 or PKG.

6.2.4 cAMP-Induced, Neurogenic Vasodilatation in the Bovine Intraocular LPCA

As inhibition of PDE5 and PKG failed to assist with characterisation of the mechanisms underlying the first and second components of neurogenic dilatation in the bovine intraocular LPCA, the cAMP-mediated vasodilatation pathway was investigated by inhibiting PKA.

6.2.4.1 Effect of PKA Inhibition on Neurogenic Vasodilatation in the Bovine Intraocular LPCA

PKA is reported to mediate vasodilatation in a number of ways, including: decreasing the sensitivity of myosin light chain kinase to the Ca^{2+}-calmodulin complex (de Lanerolle *et al*., 1984), reducing cytosolic [Ca^{2+}] by stimulating the Ca^{2+}-ATPase in the plasma membrane and sarcoplasminc reticulum (Kimura *et al*., 1982) and phosphorylation of K_{Ca} channels, resulting in hyperpolarisation (Price *et al*., 1996; Taguchi *et al*., 1995). Promisingly, blockade of this kinase with the PKA inhibitor, Rp-8-Br-cAMPS (Gjertsen *et al*., 1995; 50 μM), appeared to significantly reduce the magnitude of the second, but not the first component of neurogenic dilatation in the bovine intraocular LPCA. However, when a second group of tissues was incubated with a higher concentration of Rp-8-Br-cAMPS (100 μM) to determine if the second component could be further inhibited, the magnitude of both the first and second components of dilatation was reduced, but neither reduction was significant.

In an attempt to determine if neurogenic vasodilatation could indeed be blocked by inhibition of PKA, a second PKA inhibitor, H89 (Combest *et al*., 1988), was also utilised. H89, which has an IC_{50} of 135 nM against PKA (Davies *et al*., 2000), had no effect on the magnitude of either the first or second component of neurogenic dilatation in the bovine intraocular LPCA.

Although initial results indicated that the second component of neurogenic relaxation in the bovine intraocular LPCA could be blocked by inhibition of PKA by Rp-8-Br-cAMPS, this could not be confirmed using a higher concentration of Rp-8-Br-cAMPS or a second inhibitor, H89. Again, it is difficult to conclude whether the cAMP/PKA pathway is or is not involved in either component of neurogenic vasodilatation in the bovine intraocular LPCA, or whether both Rp-8-Br-cAMPS and H89 are unsuitable for use in this tissue and another inhibitor of the cAMP pathway should be examined in this vessel. Although Rp-8-Br-cAMPS was selected from a panel of other Rp-cAMPS analogues, as it was more resistant to breakdown by PDEs and also penetrated into cells because of its lipophilic nature (Gjertsen *et al*., 1995), the strong ionic nature of the cyclic nucleotide phosphate group still limits membrane permeability (Kruppa *et al*., 1997). Perhaps poor tissue permeability or a lack of specificity of both Rp-8-Br-cAMPS and H89, for the isoform of PKA found within the bovine intraocular LPCA was the problem.

Therefore, both the identification and the mechanism of action of the second component of neurogenic relaxation in the bovine intraocular LPCA remain unidentified.

6.2.5 Interaction of NO and the Rho Kinase Pathway

Although it has been clearly established that NO induces vasodilatation via cGMP/PKG-dependent mechanisms already discussed, it has also been suggested recently that NO can promote vasodilatation via a second signalling pathway, known as the RhoA/Rho kinase pathway. As has been discussed in chapter 1, contraction of VSM is initiated by an increase in the concentration of intracellular Ca^{2+}, which in turn, combines with calmodulin. The Ca^{2+}-calmodulin complex activates MLCK to phosphorylate MLC, enabling the cycling of myosin cross bridges with actin and smooth muscle contraction. Therefore, the contractile state of the smooth muscle is determined by the level of MLC phosphorylation (for review see Somlyo & Somlyo, 2000). Although this pathway is activated by receptor-mediated or mechanical (stretch) stimulation, a low level of MLC phosphorylation is maintained in the absence of stimuli in some tissues and this is known

as myogenic tone. The level of MLC phosphorylation is also regulated by MLC phosphatase, which dephosphorylates MLC and results in relaxation of the smooth muscle. MLC phosphatase consists of three subunits, the largest of which is the myosin binding subunit. If this subunit becomes phosphorylated, MLC phosphatase is inhibited, MLC remains phosphorylated and smooth muscle contraction persists (Somlyo & Somlyo, 2000). Rho kinase, which is activated by a G-protein known as Rho A, phosphorylates MLC phosphatase and therefore plays a major role in maintaining contraction. As there are data in the literature that suggests inhibition of RhoA/Rho kinase induces vasodilatation, it is possible to speculate that NO and other endogenous vasodilators may induce vasodilatation by inhibiting the Rho kinase pathway. Although this has not been fully investigated, there is some initial evidence to support this theory. Sauzeau *et al*. (2000) reported that PKG can phosphorylate and therefore inhibit RhoA and that the NO donor, sodium nitroprusside, caused the inactivation of RhoA by uncoupling it from the cell membrane, concluding that the inactivation of RhoA contributes to the vasodilator action of NO. Chitaley & Webb (2002) utilised the Rho kinase inhibitor, Y-27632, in rings of rat aorta and found that in tissues where NO was inhibited using L-NNA or ODQ, the Y-27632 was markedly less effective at inhibiting phenylephrine-induced contraction. Furthermore, addition of sodium nitroprusside restored the effect of Y-27632 and allowed the blockade of phenylephrine-induced contraction. They concluded that reduction in efficacy of Y-27632 was due to increased Rho kinase activity in the absence of NO.

Therefore, although at the moment evidence is limited, it is a possibility that NO could mediate, at least in part, the first component of vasodilatation in the bovine intraocular LPCA via the RhoA/Rho kinase pathway. It remains unlikely that the second component of neurogenic vasodilatation is mediated in this way, as both NOS inhibitors and ODQ fail to inhibit the vasodilatation. However, as the ability to inhibit neurogenic vasodilatation in the bovine intraocular LPCA using PDE, PKG and PKA inhibitors was very limited, the next step, not attempted in the present study, may be to utilise inhibitors of the RhoA/Rho kinase pathway.

6.3 Comparison of the Effect of NOS inhibitors on Neurogenic and Endothelium-Dependent Vasodilatation in the Bovine Intraocular LPCA

The final aim of this investigation was to determine the differential effects of a series of non-specific NOS inhibitors (L-NAME and L-NMMA) and putative nNOS-specific (AAAN and N^G-propyl-L-arginine) inhibitors on both nitrergic nerve-mediated and endothelium-dependent vasodilatation of the bovine intraocular LPCA.

6.3.1 Effects of L-NAME and L-NMMA on Neurogenic Vasodilatation in the Bovine Intraocular LPCA

As has already been discussed, L-NAME (Hobbs & Gibson, 1990), abolished the first component of neurogenic vasodilatation at all frequencies examined, but had no effect on the second. In the present study, L-NMMA (Hibbs *et al*., 1987) failed to block nitrergic transmission in the bovine intraocular LPCA, but acted like L-arginine in inhibiting blockade by L-NAME. Both L-NAME and L-NMMA, along with a third N^G-substituted analogue of L-arginine, L-NOARG (Moore *et al*., 1990), are routinely used experimentally as non-isoform selective inhibitors of NOS. Although L-NMMA inhibits all three isoforms of NOS in standard enzyme assays (Moore *et al*., 1996), many reports demonstrate anomalous findings with this agent, especially in bovine tissue. For example, in the bovine penile artery, L-NMMA fails to block nitrergic transmission, despite being able to inhibit the endothelium-dependent, NO-mediated dilatation induced by ACh (Liu *et al*., 1991). Furthermore, L-NMMA does not block nitrergic transmission in the BRP muscle, but acts like the endogenous substrate, L-arginine, to inhibit blockade by L-NOARG or L-NAME (Liu *et al*., 1991; Martin *et al*., 1993). In addition, in the rat aorta and pulmonary artery, L-NMMA, enhances rather than inhibits the production of NO, assessed by chemiluminescence detection, and inhibits the basal but not agonist-stimulated, endothelium-dependent dilatation produced by NO (Archer & Hampl, 1992; Frew *et al*., 1993). In addition to the evidence provided by Liu *et al*. (1991), who suggested that in the BRP muscle L-NMMA acts like the endogenous substrate, L-arginine, biochemical analysis of murine macrophage iNOS has revealed that L-NMMA does not behave as a simple competitive inhibitor. Instead, it acts as an alternative substrate and mechanism-based inhibitor of the enzyme (Olken & Marletta, 1993). According to this scheme, L-NMMA is metabolised initially to N^G-hydroxy-N^G-methyl-L-arginine, and finally to NO and L-citrulline, but with the intermediate production of a "suicide inhibitor" that slowly and irreversibly blocks the enzyme. These findings can therefore account for the seemingly anomalous ability of L-NMMA to augment NO production at some sites while blocking it at others.

6.3.2 Effects of L-NAME and L-NMMA on Endothelium-Dependent Vasodilatation in the Bovine Intraocular LPCA

The examination of the actions of L-NMMA and L-NAME on endothelium-dependent, NO-mediated dilatation in the bovine ciliary artery were initially confounded because of

the simultaneous activation of other endothelium-derived factors. The application of L-NAME alone did not inhibit NO-mediated, bradykinin-induced vasodilatation, although L-NMMA did partially inhibit this response. In addition, inhibition of other endothelium-derived factors that can mediate vasodilatation, EDHF and COX (not in the presence of L-NAME or L-NMMA), did not inhibit NO-mediated, bradykinin-induced vasodilatation either. A compensatory mechanism seemed to be operating, whereby if one mediator of endothelium-dependent vasodilatation was inhibited, the other ensured that the vasodilatory capability of the vessel was maintained. This has been reported in the mesenteric resistance arteries of eNOS knockout mice (Scotland *et al*., 2001) and *in vivo* in rats who were subjected to chronic inhibition of NOS (Desai *et al*., 2006). Following blockade of EDHF with apamin and charybdotoxin (Waldron & Garland, 1994; Zygmunt & Högestätt, 1996) and COX with indomethacin, vasodilator responses mediated solely by endothelium-derived NO could be elicited with bradykinin. These dilator responses, unlike those produced by the nitrergic nerves, were blocked by L-NMMA. They were also blocked by L-NAME. Thus, in the bovine ciliary artery L-NMMA blocks endothelium-dependent, NO-mediated dilatation but not that produced by nitrergic nerves, and this is in keeping with earlier findings on the bovine penile artery (Liu *et al*., 1991).

6.3.3 Selective Inhibition of nNOS

Since the discovery of NO, great effort has been expended in developing isoform-selective inhibitors of NOS, both as investigational tools and potential therapeutic agents. Excess NO, usually produced by either iNOS or nNOS, is thought to play a detrimental role in a wide range of conditions such as septic shock (Feihl *et al*., 2001), schizophrenia (Das *et al*., 1995), Alzheimer's disease (Dorheim *et al*., 1994) and in the over-production of osteoclasts, leading to disorders of the bone and joints, such as osteoporosis and rheumatoid arthritis (MacIntyre *et al*., 1991). However, although inhibiting excess NO production may be therapeutically beneficial in patients suffering from these diseases, it is important that the inhibitory action does not effect eNOS activity and its role in maintaining blood pressure and flow.

Two nNOS-specific inhibitors have recently been identified: AAAN, which has a ~2,500-fold greater selectivity for rat nNOS over bovine eNOS (Hah *et al*., 2001), and N^G-propyl-L-arginine, which is ~150-fold more selective for bovine nNOS than eNOS (Zhang *et al*., 1997a; Zhang *et al*., 1997b). The effects of these were examined against neurogenic and endothelium-dependent vasodilatation in the bovine intraocular LPCA.

6.3.4 Effects of AAAN and N^G-propyl-L-arginine on Neurogenic and Endothelium-Dependent Vasodilatation in the Bovine Intraocular LPCA

In the bovine intraocular LPCA, AAAN at concentrations up to 100 μM, failed to affect vasodilatation induced either by nitrergic nerves or by bradykinin-induced endothelium-derived NO. A lack of effect on endothelium-dependent vasodilatation might have been expected, given its poor K_i for bovine eNOS (314 μM; Hah *et al*., 2001). Nevertheless, the failure to block nitrergic transmission was surprising, in view of its K_i of 0.12 μM for rat nNOS. Whether the failure of AAAN to block nitrergic transmission in the bovine intraocular LPCA results from poor tissue penetration, a major difference in K_i values for bovine and rat nNOS, or some other factor, remains to be established. Equally disappointing were the actions of N^G-propyl-L-arginine (Zhang *et al*., 1997a; Zhang *et al*., 1997b) on the bovine intraocular LPCA. Although N^G-propyl-L-arginine did inhibit nitrergic vasodilatation, it failed to exhibit the ~150-fold selectivity for nNOS over eNOS seen in enzyme assays (Zhang *et al*., 1997a). Indeed, N^G-propyl-L-arginine blocked endothelium-dependent, NO-mediated vasodilatation over the same concentration range as for blockade of nitrergic transmission. These findings with AAAN and N^G-propyl-L-arginine therefore serve as a reminder that it is not always possible to extrapolate findings in biochemical assays to functional responses in intact tissues.

Debate over the reported specificity of a NOS inhibitor is not uncommon. Another example of a discrepancy between biochemical assays and studies in an intact system involves a member of the indazole family of NOS inhibitors, 7-NI. In isolated enzyme studies 7-NI is an equipotent inhibitor of all three enzymes; with IC_{50} values of 9.7 μM, 8.3 μM and 11.8 μM for iNOS, nNOS and eNOS, respectively (Alderton *et al*., 2001). However, it is described as an nNOS specific inhibitor *in vivo* in models of brain injury (Yoshida *et al*., 1994), where application of 7-NI did not alter the systemic arterial blood pressure or inhibit the dilation of pial arterioles in response to ACh, but did greatly reduce NOS activity within the cerebral cortex and reduced the size of the infarct in response to cerebral artery occlusion. Selectivity for nNOS *in vivo* may be due to different uptake rates of the drug into endothelial cells and neurons (Southan & Szabo, 1996). However, Zagvazdin *et al*. (1996) suggest that 7-NI does suppress basal endothelial NO formation *in vivo* in rats and that this effect is masked by the use of anaesthetics in other studies.

6.4 Conclusions

Although this investigation into neurogenic vasodilatation in the bovine intraocular LPCA has led to some interesting discoveries about the innervation of the vessel, many questions remain unanswered. It is quite clear that in the presence of U46619-induced tone and blockade of adrenergic vasoconstriction with guanethidine, EFS elicits a biphasic vasodilator response and it is likely that the first component is mediated by the neurotransmitter, NO. However in contrast to previous findings by Wiencke *et al*. (1994), the current report could find no evidence that the neuropeptide, CGRP, mediates the second component of neurogenic vasodilatation. In addition to this, substance P and VIP are unlikely to play a role. The involvement of ATP has not been discounted, due to lack of suitable antagonists, but the identity of the neurotransmitter that mediates the second component of neurogenic vasodilatation remains a mystery.

Furthermore, an extensive investigation into the mechanism of vasodilatation employed by the mediator of the second component, also failed to provide conclusive answers as to how the transmitter operates. It is likely that the first component is mediated via the NO/cGMP/PKG pathway, although promising results with the PDE5 inhibitor zaprinast were not confirmed by the use of another PDE5 inhibitor, sildenafil, or a number of PKG inhibitors. Inhibiting various K^+ channels to prevent hyperpolarisation of the cell membrane potential or the cAMP/PKA pathway did not yield any clues as to the possible mechanism of the mediator of the second component of neurogenic vasodilatation. Therefore the mechanism responsible for the second pathway remains unconfirmed.

Interestingly, nitrergic vasodilatation in the bovine intraocular LPCA, like many other bovine tissues, is not inhibited by the NOS inhibitor L-NMMA. Instead L-NMMA acts like L-arginine in inhibiting neurogenic blockade by L-NAME. L-NMMA and L-NAME are both successful in blocking endothelium-derived NO, serving as a warning that although generally mooted as a non-specific NOS inhibitor, L-NMMA should not be used as the sole tool when defining nitrergic neurotransmission. Further care should be taken with compounds such as AAAN and N^G-propyl-L-arginine, which are portrayed as putative nNOS-specific inhibitors. In the bovine intraocular LPCA, AAAN was found to inhibit neither nitrergic transmission nor endothelium-derived NO. N^G-propyl-L-arginine did appear to inhibit nNOS, but with the same potency as for eNOS, rendering it a non-specific NOS inhibitor in this tissue. Again, care should be taken when utilising these compounds.

In conclusion, although many aspects of this study have not been fully resolved, it has thrown up some interesting questions: what is the identity of the second neurotransmitter? What is the mechanism of neurotransmission employed? Why does L-NMMA not inhibit nitrergic neurotransmission in bovine tissues? There is certainly scope for future research in this tissue, should anyone wish to continue it.

CHAPTER 7.

REFERENCE LIST

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7 Reference List

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