Mechanisms of Peripheral Nervous Control of Rat Iris Arterioles

by

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STATEMENT

The contents of this thesis, except where referenced, are original and were planned and carried out by the author, with guidance from Dr C. Hill. The immunohistochemistry in Chapters 5 and 7 was done by Dr. C. Hill. The protocol for RT-PCR in Chapter 7 followed the protocol devised by Dr. M. Vidovic. The autoradiography in Chapter 7 was done in collaboration with Dr. E. Burcher and Mr. J. Strigas from the University of New South Wales, Sydney. The CGRP binding study was performed by Mr. J. Strigas.

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ABSTRACT

It is important to understand the mechanisms which control arteriolar diameter, as these small diameter vessels provide the greatest resistance to peripheral blood flow. The aim of this thesis was to characterize the peripheral nerve-mediated control of the diameter of arterioles found within the stromal layer of the rat iris, using an *in vitro* preparation. The overall results of this thesis indicate that neurogenic responses of iris arterioles are not voltage-dependent, but rather result from the activation of intracellular messenger systems to influence smooth muscle tone.

Sympathetic perivascular nerves release noradrenaline to produce vasoconstriction and, in most arterial vessels, a voltage-dependent non-adrenergic mechanism is responsible for all or part of the response. This thesis, however, demonstrates that in iris arterioles there is a purely α-adrenoceptor mediated response to nerve stimulation (10 Hz, 1 s) thus indicating that not all arterial vessels show a non-α-adrenergic component to the response produced by sympathetic nerve stimulation. The contractile response in iris arterioles is initiated by the release of intracellular calcium, with a contribution to contraction at supramaximal stimulation being due to the influx of calcium through voltage-dependent calcium channels. This is consistent with neurogenic α-adrenoceptor mediated contractile responses previously recorded in other tissues.

Repetitive nerve stimulation of iris arterioles activates sensory-motor nerves which release CGRP to act post-junctionally to inhibit sympathetic nerve-mediated vasoconstriction. This occurs independently of membrane hyperpolarization but rather via an intracellular mediated mechanism involving cyclic AMP-dependent activation of nitric oxide which is proposed to lead to a reduction in levels of intracellular calcium. Thus the mechanism for this inhibition biochemically opposes the mechanism for constriction. This interaction may serve to increase blood flow to areas where sensory nociceptor afferents have been activated. It was further demonstrated that, unlike the iris arterioles, the dilator muscle does not receive sensory-motor innervation. It is proposed that sensory-motor nerves may only innervate a tissue where there may be some

purpose for an interaction between second messenger systems mediating opposing neurogenic responses.

ABBREVIATIONS

The following is a list of abbreviations used in this thesis:

ATP, adenosine triphosphate; [^{125}I]-BH, Bolton Hunter; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; CEC, chloroethylclonidine; cyclic AMP, cyclic adenosine-3,5-monophosphate; cyclic GMP, cylic guanosine monophosphate; dibutyryl cyclic AMP, 3':5'-cyclic monophosphate; D-NAME, N^G-nitro-D-arginine methyl ester; dNTP, deoxyribonucleoside triphosphate; DOCC, depletion-operated calcium channel; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; G_S, adenylate cyclase stimulatory GTP-binding protein; IBMX, 3-iso-butyl-1-methyl-xanthine; IC_{50}, half maximal inhibitory concentration; IP_3, inositol trisphosphate; IUPHAR, International Union of Pharmacology; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-nitro-L-arginine methyl ester; NANC, non-adrenergic, non-cholinergic; mRNA, messenger RNA; NOS, nitric oxide synthase; NPY, neuropeptide Y; PBS, phosphate-bufffered saline; PHI, peptide histidine isoleucineamide; RNA, ribonucleic acid; RT-PCR, reverse-transcriptase polymerase chain reaction; SarSP, [sar^9,$Met(O_2)^{11}$]substance P; SCG, superior cervical ganglia; VIP, vasointestinal peptide; WB4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane].

PAPERS RESULTING FROM THIS THESIS

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CHAPTER 1.

GENERAL INTRODUCTION

INTRODUCTION

The circulatory system carries out a variety of functions in order to maintain homeostasis. These include the delivery of nutrients and oxygen to tissues, the removal of waste products and the facilitation of the processes of food absorption and waste excretion in specialized organs (Johnson, 1978).

The extent of contraction or relaxation of vascular smooth muscle determines the diameter and hence blood flow within an arterial vessel (Johnson, 1978). Arterial smooth muscle tone is subject to external influences, such as metabolic regulation or vascular nerve activity, as well as possessing intrinsic myogenic ability (Johnson, 1978). In addition, endothelial cells lining arterial vessels release vasoactive factors in response to circulating hormones, changes in intravascular pressure, tissue injury or disease conditions (Husain and Moss, 1988; Burnstock and Ralevic, 1994).

Moment-to-moment control of vascular tone is largely achieved through regulatory mechanisms which become apparent when local conditions, for example, tissue metabolism, change (Johnson, 1978). Neurotransmitters released from sympathetic and parasympathetic fibres of the autonomic nervous system nerves can act locally to regulate arterial tone. Efferent collaterals of sensory-nerves may also release neurotransmitters which cause vasodilatation (Holzer, 1992). Finally, systemically circulating hormones act to synergize with local mechanisms to adjust vascular reponses throughout the whole body.

The integration of these various influences is achieved through the electrical and chemical coupling of smooth muscle cells. Electrotonic potentials generated by extracellular or intracellular current injection can be recorded at a distance much greater than one cell length away, and in the case of arterial smooth muscle several hundred μms, from the source (Abe and Tomita, 1968; Hirst and Neild, 1978). This property of smooth muscle allows the propagation of local responses not only along arterial vessels

but also through arterial walls, which may consist of several layers of smooth muscle cells (Rhodin , 1980).

In this chapter, the various peripheral nervous influences on arterial diameter are reviewed.

STRUCTURE OF ARTERIAL VESSELS

The walls of arterial vessels are made up of three different layers (Rhodin , 1980):

1) *tunica intima*, or innermost layer, which contains the layer of endothelial cells which line the vessel wall and a thin basal lamina. Large elastic arteries, such as the aorta of some species, also contain a subendothelial layer of the intima which is composed of collagenous bundles, elastic fibrils and some smooth muscle cells.

2) *tunica media*, or middle layer of the vascular wall, which contains the smooth muscle cells, bundles of collagenous fibrils and networks of elastic fibrils. The thickness of this layer varies between vascular beds and with orders of branching of a vessel.

3) *tunica adventitia*, or the outermost, layer. The thickness of this layer also varies depending on the type and location of the vessel.

Rhodin (1967) divided small arterial vessels into three groups according to the diameter and structure of the vessel wall. Arterioles were defined as being small diameter vessels (50-100 μm) which have a medial layer only a few smooth muscle cells thick. In small dermal vessels of the rabbit, which had a media three to four smooth muscle layers thick, the outer most layer was arranged spirally and the innermost layers were arranged transverse to the long axis.

Arterioles less than 50 μm in diameter were defined as terminal arterioles (sometimes called metarterioles). The media of these vessels was only one, sometimes two, smooth muscle cells thick. Individual smooth muscle cells in the walls of terminal arterioles had an average length of 40 μm, width of 5 μm near the nucleus and were oriented circumferentially around the vessel. Each smooth muscle cell had a nucleus, sarcoplasmic reticulum and several mitochondria. Their membranes were surrounded by

a basal lamina and were not smooth but contain invaginations or calveoli. Adjacent smooth muscle cells were frequently connected end to end as well as side to side. Membranous contacts were reported to decrease with increasing vessel diameter. Gap junctions (15-19 nm) between smooth muscle cells provide low resistance pathways for the rapid spread of a signal and are proposed to be the sites of electrical coupling between these cells (Rhodin , 1980).

The endothelial cells of the terminal arterioles were flat, approximately 2 μm in height at the nucleus and reached 50 μm in length. The plasma membranes of endothelial cells were very smooth, with occasional microvilli. The basal surface of the endothelial cells differentiated into footlike processes which made contact with smooth muscle cells. It was suggested that these structures may mechanically stabilize the vessel wall. Alternatively these myoepithelial junctions may be a pathway for the movement of humoral substances from the endothelial cells to the smooth muscle cells (Rhodin , 1967).

Capillaries arose from terminal arterioles (Rhodin , 1967) and at these points the smooth muscle cells were arranged in a circular fashion and hence were referred to as precapillary sphincters. Precapillary sphincters were reported to have internal diameters of 7 μm, just wide enough to allow one red blood cell through at a time. The smooth muscle cell layer only extended 20-30 μm down the capillary beyond the precapillary sphincter. Unlike other arterial vessels, capillaries only had a tunica intima layer making up the vessel wall (Rhodin , 1967).

AUTONOMIC INNERVATION OF ARTERIAL VESSELS

SYMPATHETIC NERVES

Sympathetic vasoconstriction

Von Euler and colleagues (von Euler, 1956) were the first to identify noradrenaline as the neurotransmitter being released from the sympathetic nerves. Noradrenaline acts upon adrenoceptors, which were historically categorized as α and ß (Ahlquist, 1948). Additionally, α-adrenoceptors are further subdivided into α_1– and α_2–adrenoceptors and α_1-adrenoceptors have been further subdivided into α_{1A}, α_{1B}

and α_{1D} subtypes (Hieble *et al.,* 1995), while α_2–adrenoceptors have been divided into α_{2A}, α_{2B} and α_{2C} (Bylund *et al.,* 1994). Noradrenaline can modify vascular tone by 1) the activation of α_1– or α_2–adrenoceptors on arterial smooth muscle to produce vasoconstriction (Medgett and Ruffolo, 1988; McGrath, Flavahan and McKean, 1982); 2) the activation of α_2–adrenoceptors on endothelial cells to produce vasodilatation (Cocks and Angus, 1983); 3) the inhibition of neurotransmitter release by the activation of prejunctional α_2–adrenoceptors (Rand *et al.,* 1980); 4) the activation of postjunctional ß-adrenoceptors to cause vasodilatation (Itoh, Izumi and Kuriyama, 1982) and 5) the facilitation of neurotransmitter release by the activation of prejunctional ß-adrenoceptors (Medgett *et al.,* 1980).

Sympathetic nerve stimulation produces vasoconstriction of most vascular beds (Hirst and Edwards, 1989). Umbilical vessels are reported to be devoid of any nerve supply (Lachenmayer, 1971), as are the aorta and pulmonary arteries of some species (McLean, Twarog and Bergofsky, 1985). Sympathetic innervation of the rat mesentery extends to the terminal arterioles but not to the capillaries (Furness and Marshall, 1974), and some fine pial arteries are not innervated (Hill *et al.,* 1986).

In vivo, there is some evidence for differences between the rates of discharge of sympathetic nerves supplying resistance and capacitance vessels (Hadjiminas and Oberg, 1968). Sympathetic responses of resistance vessels in the skeletal hindlimb mucles are maximal at 25 to 30 Hz, whereas 6 to 8 Hz stimulation produces maximal responses of the capacitance vessels (Mellander, 1960).

As the stimulation of sympathetic nerves leads to the release of noradrenaline, then the activation of α–adrenoceptors could be expected to play an important role in the control of vascular tone, and hence blood flow. However, this does not appear to be the general case. For instance, α–adrenoceptor antagonists do not affect the sympathetic nerve-evoked vasoconstriction of the rabbit hindlimb *in vivo* (Hirst and Lew, 1987).

Intracellular recordings from smooth muscle cells of many arterial vessels show that sympathetic nerve stimulation produces rapid excitatory junction potentials which are resistant to α–adrenoceptor antagonists (see Table 1.1). Low frequency nerve stimulation evokes excitatory junction potentials which show facilitation. Higher

Table 1.1

Some arterial vessels from which α-adrenoceptor antagonist resistant sympathetic nerve-evoked excitatory junction potentials have been recorded

Vessel	Species	Reference
basilar artery	canine	Fujiwara et al, 1982
	guinea pig	Karashima and Kuriyama, 1981
	rat *	Hirst et al, 1982
	rabbit	Suprenant et al, 1987
ear artery	guinea pig *	Kajiwara et al, 1981
		Karishama and Kariyama, 1981
	rabbit	Suzuki and Kou, 1983
cerebral artery	canine *	Fujiwara et al, 1982
middle cerebral artery	canine	Kou et al,1982
	guinea pig	Karashima & Kurryama, 1981
mesenteric artery	canine	Kou et al, 1982
	guinea pig	Kuriyama & Makita, 1983
	rat (mature)	Hill et al, 1983
		Angus et al, 1988
	rabbit *	Holman & Suprenant, 1979
saphenous artery	guinea pig *	Cheung & Fujioka, 1986
submucosal arteriole	guinea pig *	Hirst & Neild, 1977
tail artery	rat	Cheung, 1984
uterine artery	guinea pig *	Bell, 1969

* indicates vessels where the sole electrical response to sympathetic nerve stimulation is insensitive to α-adrenoceptor antagonist

frequency stimulation causes summation of excitatory junction potentials and initiation of muscle action potential which is associated with arterial constriction (Hirst and Edwards, 1989).

In some vessels an excitatory junction potential has been the sole electrical response recorded following nerve stimulation (see Table 1.1). In thermoregulatory vessels, such as the rat tail artery (Cheung, 1984) and rabbit ear artery (Suzuki and Kou, 1983), the response to nerve stimulation consists of a rapid excitatory junction potential which is resistant to α–adrenoceptor antagonists, followed by a slower α–adrenoceptor mediated depolarization. In the guinea pig pulmonary artery (Suzuki, 1983) and the immature mesenteric artery (Hill, Hirst and van Helden, 1983), however, the electrical responses to nerve stimulation are totally abolished by α–adrenoceptor antagonists.

Nerve stimulation of short segments of submucosal arterioles produced excitatory junction potentials which were insensitive to α–adrenoceptor antagonists (Hirst and Neild, 1980a). Two types of responses have been recorded in response to ionophoretically applied noradrenaline on this preparation (Hirst and Neild, 1980a). A localized α–adrenoceptor mediated constriction of the arteriole, with no change in membrane potential, was the most common response recorded. A less frequent response to noradrenaline was a non-α-noradrenergic depolarization which was similar to the response to nerve stimulation. When the depolarization initiated an action potential, arteriolar constriction was recorded. Thus, noradrenaline produced responses which mimicked responses to nerve stimulation only when applied to certain areas. It was suggested that specialized junctional non-α–adrenoceptors were activated by neurally-released noradrenaline (Hirst and Neild, 1980a). These were named γ–adrenoceptors according to the nomenclature put forward by Ahlquist (Hirst and Neild, 1980c; Ahlquist, 1948). Traditional α–adrenoceptors were suggested to be extrajunctional and not associated with neuroeffector transmission (Hirst and Neild, 1980a).

A later study (Hirst and Neild, 1981) showed that in the majority of cases, ionophoretically applied noradrenaline produced depolarization of mesenteric arteries only when it was applied within 5 μm of varicose fluorescently-detected sympathetic nerve bundles on the surface of the vessel. The authors suggested that the receptors

activated by neurally-released noradrenaline were concentrated in regions beneath the sympathetic nerve varicosity (Hirst and Neild, 1981).

From the above, there is substantial evidence for the existence of postsynaptic non-α–adrenoceptors at sympathetic neuromuscular junctions. However, unlike α–adrenoceptors, there is no molecular equivalent described for the γ–adrenoceptor, and a selective antagonist for these receptors is yet to be found. In addition to being an antagonist for α–adrenoceptors and serotonin receptors dihydroergotamine is also an antagonist for γ–adrenoceptors (Muller-Schweinitzer and Weidman, 1978). Sympathetic nerve stimulation produced a depolarization of sinus venosus cells of the toad, which was abolished by α, ß-methylene ATP and dihydroergotamine. In the presence of α– and ß-adrenoceptor antagonists, dihydroergotamine abolished depolarizations of these cells which were produced by the exogenous application of the neurotransmitter for this synapse, adrenaline, but not those produced by applied ATP (Bramich, Edwards and Hirst, 1990).

Dihydroergotamine also blocks sympathetic vasoconstriction (Thulin, 1976). Morris (1994b) demonstrated that in the presence of an α-adrenoceptor antagonist, dihydroergotamine abolished vasoconstriction produced by local application of noradrenaline in third and fourth order cutaneous ear arteries. Sympathetic constrictions of small ear arteries are also significantly reduced by dihydroergotamine (Morris, 1994c). Dihydroergotamine did not significantly affect constrictions produced by applied ATP (Morris, 1994b). Dihydroergotamine is therefore able to distinguish between vasoconstrictions produced by γ-adrenoceptor activation and those produced by purinoceptor activation. Providing α–adrenoceptor and serotonin-mediated responses are excluded, dihydroergotamine is therefore the only selective antagonist described for junctional γ-adrenoceptors.

More recently, low affinity prazosin vascular α_1–adrenoceptors have been described (Flavahan and Vanhoutte, 1986) and denoted as α_{1L}- or α_{1N}-adrenoceptors (Muramatsu *et al.*, 1990). According to this nomenclature, the previously described α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors are proposed to belong to the α_{1H}-adrenoceptor family, that is they show a high affinity for prazosin (Muramatsu, Kigoshi and Oshita, 1990). The pharmacological, molecular and biochemical characterization of these

proposed low affinity α_1–adrenoceptors is, at present, insufficient for the International Union of Pharmacology (IUPHAR) to recognize them as additional α_1–adrenoceptor subtypes (Hieble *et al.*, 1995). However it will be interesting when more information is available about these receptors. It is tempting to speculate that the γ-adrenoceptors described by Hirst and Neild (1980a) may correlate with one of the low prazosin affinity α_1–adrenoceptor subtypes more recently described.

The relative contribution of different receptors mediating sympathetic vasoconstriction may vary along the length of a vessel. Morris (1994c) demonstrated that sympathetic vasoconstriction of the main guinea-pig ear artery was 50 to 70% blocked by the α_1–adrenoceptor antagonist, prazosin, and the remaining constriction was abolished by the non-selective receptor antagonist, suramin. However, sympathetic vasoconstriction of small ear arteries was significantly abolished by the γ-adrenoceptor antagonist, dihydroergotamine (Morris, 1994c).

The contribution of α– and non-α–adrenoceptors to postsynaptic responses following sympathetic nerve stimulation is highly dependent on the stimulus parameters applied (Kennedy, Saville and Burnstock, 1986). Low frequencies and short pulse stimulus trains reportedly favour non-α–adrenoceptor responses, whereas higher frequencies and longer pulses trains favour α–adrenoceptor mediated responses. One interpretation of these results is that neurotransmitter released by short, low frequency stimuli activates junctional γ-adrenoceptors (Hirst and Neild, 1980a). Following stimulus trains of several hundreds of pulses, neurotransmitter can overflow from the neuromuscular cleft (Morris and Gibbins, 1992). With these longer stimulus trains, noradrenaline would overflow onto extrajunctional α–adrenoceptors, as proposed by Hirst and Neild (1980a).

Sympathetic nerve stimulation evokes excitatory junction potentials in smooth muscle cells of the basilar artery and these potentials are insensitive to α–adrenoceptor antagonists (Hirst, Neild and Silverberg, 1982). Only at high concentrations does noradrenaline (0.2 to 10 mM) cause a depolarization of this vessel. This depolarization is also insensitive to α–adrenoceptor antagonists (Hirst, Neild and Silverberg, 1982).

Dopamine, L- and D-noradrenaline (1 mM) were equipotent in producing depolarization of the basilar artery. Considering that, compared to L-noradrenaline, dopamine and D-noradrenaline are poor agonists for α–adrenoceptors (Altura, 1978), the receptor being activated by these agonists did not have an agonist order of potency defined for an α–adrenoceptor. Therefore, the authors concluded that the basilar artery contained only junctional γ-adrenoceptors. These junctional receptors required high concentrations of noradrenaline for activation. Such high concentations of noradrenaline are reported to occur locally after the neural release of noradrenaline (Bell and Vogt, 1971; Bevan *et al.*, 1984).

Benham and Tsien (1988) reported that activation of a non-α–, non-ß-adrenoceptor by noradrenaline increased a dihydropyridine sensitive, L-type calcium channel in isolated smooth muscle cells. Although the concentration of noradrenaline used in this study was lower than concentrations previously used to activate γ-adrenoceptors (Hirst, Neild and Silverberg, 1982), it is possible that this current was due to activation of a γ-adrenoceptor. This would be consistent with the finding that γ-adrenoceptor activation only produces a contraction when voltage-dependent conductance changes occur (Hirst, 1977).

Concentrations of α_2–adrenoceptor agonists which do not themselves cause constriction have been shown to have an inhibitory effect on sympathetic nerve-mediated constriction, for example, in the rabbit ear artery (Xiao and Rand, 1989) and rabbit mesenteric artery (von Kugelgen and Starke, 1985). Cavero et al (1979) showed that the α–adrenoceptor antagonist, phentolamine, increased heart rate and increased the release of ^{3}H-noradrenaline measured in the dog coronary vein during sympathetic nerve stimulation. The α_2–adrenoceptor agonist, clonidine, was shown to inhibit sympathetically-nerve evoked ^{3}H-noradrenaline release. Electrical field stimulation for longer than 10 s produces a biphasic constriction of ileocolic artery (Bulloch and Starke, 1990), the first component being non-α–adrenoceptor mediated and the second being α–adrenoceptor mediated. An α_2–adrenoceptor antagonist potentiated both components of the vasoconstriction in response to these longer periods of stimulation, but had no effect on the monophasic vasoconstriction produce by shorter trains of stimulation

(Bulloch and Starke, 1990). In this vessel, then, presynaptic α_2–adrenoceptor activation affects both α– and non-α–adrenoceptor mediated sympathetic vasoconstriction.

Adler-Granshinsky and Langer (1975) were the first to show that prejunctional ß-adrenoceptors may have a positive feedback on the release of noradrenaline from sympathetic nerve endings. A facilitatory effect of presynaptic ß-adrenoceptor activation on stimulation-evoked noradrenaline outflow has also been demonstrated in human omental and pulmonary arteries (Gothert and Hentrich, 1985). Cholera toxin permanently activates of G_S, the G-protein to which ß-adrenoceptors are coupled (Watson and Girdlestone, 1994). Cholera toxin increased sympathetic neurotransmitter release in mesenteric artery, as did a ß-adrenoceptor agonist (Nozaki and Sperelakis, 1991), providing further evidence for pre-junctional facilitatory ß-adrenoceptors on sympathetic nerves.

Sympathetic neuromuscular junction

Sympathetic noradrenaline-containing nerves form a plexus at the adventitia–medial border of arterial vessels (Norberg and Hamberger, 1964). It has been reported that sympathetic nerves enter the tunica media of some arteries, for example, the main branch of the pulmonary artery of several species (Cech and Dolezel, 1967; Knight *et al.*, 1981).

The density of the noradrenergic plexus depends upon the vascular region and on the size of the blood vessel. The responsiveness of different branching segments of an arterial vessel to sympathetic nerve stimulation has been the subject of a number of studies. For example, in the ear artery, the constrictor responses to nerve stimulation increases as the artery divides to first and second order arterioles, before diminishing in the terminal arterioles, parallelling neuronal density (Owen *et al*, 1983).

The terminal regions of sympathetic axons are varicose (Gordon-Weeks, 1988). Terminal varicosities predominantly contain small granular vesicles (30-60 nm diameter) which have an electron-dense core. Large granular vesicles (90-120 nm diameter) with a dense core and mitochondria are also present. Noradrenaline is stored in both types of vesicles (Gordon-Weeks, 1988).

The presence of specialized sympathetic neuromuscular junctions on arterial vessels is supported by the reconstruction of individual varicosities and adjacent arterial smooth muscle cells from serial sections (Luff, McLachlan and Hirst, 1987; Luff and McLachlan, 1989). Schwann cells wrap around bundles of terminal axons, however many varicosities are only partly enclosed by Schwann cells (Luff, McLachlan and Hirst, 1987). In the majority of cases (84% and 94% in submucosal arterioles of the guinea pig intestine, (Luff, McLachlan and Hirst, 1987), the part of the varicosity which is not covered by Schwann cells comes into close contact with the arterial smooth muscle cell. At these sites of close apposition, the basal lamina of the varicosity and the smooth muscle fuse to form a single layer. At these contact regions, small granular storage vesicles are concentrated near the varicosity membrane facing the smooth muscle cell and the distance between the smooth muscle cells and the varicosity is less than 100 nm.

The presence of sympathetic neuroeffector junctions on arterial vessels is consistent with the concept of specialized areas on arterial vessels which contain synaptic γ-adrenoceptors, as suggested by Hirst and Neild (1980a). While at first it appeared that the presence of neuromuscular junctions could correlate with specialized postsynaptic γ-noradrenergic responses, this is not always the case (Luff and McLachlan, 1989). In some arteries where non-α noradrenergic responses are recorded following nerve stimulation, for example the rabbit and guinea pig basilar artery, no neuromuscular junctions have been found (Luff and McLachlan, 1989). Conversely, in vessels which show a purely α–adrenoceptor mediated response to nerve stimulation, such as the rat mesenteric veins (van Helden, 1988), similar studies making reconstructions from serial sections have shown that the majority of sympathetic terminal varicosities form neuromuscular junctions (Klemm, van Helden and Luff, 1993). Therefore, it is not possible to make a global generalization about the relationship between a particular response recorded in a blood vessel and the structural relationship between sympathetic nerve terminals and vascular smooth muscle cells.

Co-transmission in the sympathetic nervous system

An alternative explanation which has been put forward for the inability of α–adrenoceptor antagonists to inhibit sympathetic nerve-mediated vasoconstriction, or excitatory junction potentials, is that noradrenaline may not be the only neurotransmitter released following nerve stimulation. It was originally proposed by Dale that each branch of the same nerve makes and releases the same neurotransmitter, however, since the 1970's it has been recognised that more than one neurotransmitter may be released from sympathetic nerves (Burnstock and Kennedy, 1986). It should be noted that not all sympathetic nerves contain the same repertoire of cotransmitters and that discrete populations of sympathetic nerves containing different combinations of potential neurotransmitters innervate particular organs within a species (Morris and Gibbins, 1992).

Adenosine Triphosphate (ATP)

It has been proposed that ATP and noradrenaline are co-transmitters in the sympathetic nervous system, one reason for this being that noradrenaline and ATP are stored together in sympathetic neurones (Burnstock and Kennedy, 1986).

ATP is rapidly dephosphorylated by smooth muscle ectonucleotidases (Olsson and Pearson, 1990). Burnstock (1978) first divided purinoceptors into P_1-purinoceptors as being activated by adenosine and P2-purinoceptors as being activated by ATP. Since then, P_1-purinoceptors have been divided into A_1 and A_2 subtypes and P_2-purinoceptors into P_{2x} and P_{2y} subtypes (Watson and Girdlestone, 1994).

Furchgott (1966) was the first to demonstrate that ATP could produce vasodilatation or vasoconstriction. DeMey and Vanhoutte (1981) showed that the vasodilatation produced by ATP required an intact endothelium. In most vessels studied since then, ATP activates P_{2y}-purinoceptors on endothelial cells to produce vasodilatation (Olsson and Pearson, 1990). Following the removal of the endothelium, the activation of P_{2x}-purinoceptors on smooth muscle cells by ATP produces vasoconstriction (Burnstock and Kennedy, 1986).

Adenosine produces vasodilatation by activating A_2-adenosine receptors on arterial smooth muscle cells (Olsson and Pearson, 1990). Both ATP and adenosine can

inhibit the release of noradrenaline from perivascular nerves (Hedqvist and Fredholm, 1976; Shinozuka, Bjur and Westfall, 1988). Adenosine activates prejunctional A_1-purinoceptors to inhibit noradrenaline release (von Kugelgen, 1994). However, in the rat tail artery it has been suggested that adenosine and ATP act through a common "hybrid" prejunctional P_3-purinoceptor (Shinozuka, Bjur and Westfall, 1988). More recently, preliminary studies on the rabbit ear and saphenous artery suggest that facilatatory prejunctional purinoceptors exist on these vessels (Ishii *et al.,* 1995; Todorov, Bjur and Westfall, 1994).

The excitatory junction potentials produced by a brief application of ATP have been shown to mimic those produced by nerve stimulation (Suzuki, 1985). Excitatory junction potentials can be recorded in some tissues taken from animals pretreated with reserpine to deplete noradrenaline stores (Burnstock and Holman, 1966; Sneddon and Westfall, 1984), however very little of the sympathetic vasoconstrictor response in the spleen remains after reserpine treatment (Dixon, Mosimann and Weiner, 1979). Excitatory junction potentials can also be recorded after the inhibition of noradrenaline synthesis with α–methyl p-tyrosine (Cunnane and Manchanda, 1989), suggesting that they are purinergic in origin.

Nerve-evoked α–adrenoceptor resistant excitatory junction potentials are often abolished by a, ß-methylene ATP (Meldrum and Burnstock, 1983; Burnstock and Kennedy, 1986). Meldrum and Burnstock (1983) demonstrated that α, ß-methylene ATP did not affect α–adrenoceptor mediated contractions, but abolished constrictions due to ATP, suggesting that this compound desensitizes P_2-purinoceptors.

The selectivity of this compound has, however, been questioned. Byrne and Large (1986) demonstrated in the basilar artery that depolarizations produced by noradrenaline activation of γ–adrenoceptors or depolarizations produced by ATP were both significantly reduced by α, ß-methylene ATP. The depolarization produced by sympathetic nerve stimulation was also abolished by α, ß-methylene ATP. This study demonstrated that α, ß–methylene ATP will not distinguish between excitatory junction potentials produced by P_2-purinoceptor activation or γ–adrenoceptor activation.

Additionally, *in vivo* α, ß-methylene ATP may reduce the release of NPY from sympathetic nerves (Lacroix *et al.*,1989).

One explanation put forward by Byrne and Large (Byrne and Large, 1986) for the non-selectivity of α, ß-methylene ATP is that it may desensitize the channel which is utilized by both γ-adrenoceptors and by P_2-purinoceptors, and so may inhibit responses mediated by both receptors. However it has been argued that this is not the case, as the mechanism of producing vasoconstriction is different for the two receptors. (Edwards *et al.,* 1989). ATP increases the calcium permeability of isolated arterial smooth muscle cells (Benham *et al.,* 1987) and so may produce vasoconstriction with only small changes in membrane potential, while γ-adrenoceptor activation causes contriction after depolarizations of smooth muscle which activate voltage-dependent conductances (Hirst, 1977). Thus it would appear that α, ß-methylene ATP is not a selective tool to demonstrate purinergically-mediated, compared to γ-adrenoceptor-mediated, responses.

Another line of evidence in favour of ATP as a cotransmitter with noradrenaline is that sympathetic nerve stimulation produces tetrodotoxin- and guanethidine-sensitive release of preloaded ^{3}H-labelled purines (Burnstock and Kennedy, 1986). In some cases this overflow of ATP may, however, come from non-neuronal cells. For example, intracellular stores of purines can be released by both vascular smooth muscle and endothelial cells (Burnstock and Kennedy, 1986). In the rabbit aorta, most of the overflow of ATP and purines elicited by nerve stimulation disappeared after removal of the endothelium (Sedaa *et al.,* 1990). It was found that the activation of α–adrenoceptors on the endothelial cells lead to the release of ATP. The authors suggested that only 30% of the electrically-evoked ATP was actually neurally-released (Sedaa *et al.,* 1990). Thus, when investigating ^{3}H-labelled purine overflow it is important to distinguish between purines released from nerve endings and those released from post-junctional sites (von Kugelgen *et al.,* 1994).

In conclusion, a number of lines of evidence have been put forward in the literature to suggest that ATP may be a co-transmitter with noradrenaline in arterial

vessels. In the absence of a selective antagonist for synaptic purinoceptors, this is still controversial.

Neuropeptide Y (NPY)

NPY is a 36 amino acid peptide which was isolated from the porcine brain (Tatemoto, Carlquist and Mutt, 1982). NPY has been shown to be stored in, and released from, noradrenergic sympathetic nerves (Alm and Lundberg, 1988; Fleming *et al.,* 1989; Lundberg *et al.*, 1984a, Lundberg *et al.,* 1989; Lundberg 1990b; Morris and Murphy, 1988; Uddman and Edvinsson, 1989). At low frequencies of nerve stimulation, noradrenaline exerts an inhibitory pre-junctional effect on the release of NPY, via the activation of α_2–adrenoceptors (Lundberg, Rudehill and Sollevi, 1989; Lacroix *et al.,* 1989). At the electron-microscopic level, NPY-like immunoreactive nerve terminals were found to be close to the smooth muscle cells of the medial layer in mesenteric and uterine arteries (Edvinsson *et al.,* 1989; Fallgren, Ekblad and Edvinsson, 1989).

NPY acts upon at least three different types of NPY receptor (Grundemar and Hakanson, 1993). The vascular effects of NPY are mediated by Y_1- and Y_2-NPY receptors (Grundemar and Hakanson, 1993). NPY activates Y_1-NPY receptors, and in some vessels Y_2-NPY receptors, to produce vasoconstriction. Activation of postjunctional Y_1-NPY receptors may also potentiate the effects of other vasoconstrictors, whereas activation of pre-junctional Y_2-NPY receptors suppresses neurotransmitter release from sensory-motor nerves, and the release of both NPY and noradrenaline from sympathetic nerves (Grundemar and Hakanson, 1993; Wong-Dusting and Rand, 1988). As yet there are no commercially available specific antagonists for NPY receptors, although benextramine has been shown to have a low potency in reversibly antagonizing NPY receptors (Doughty *et al.,* 1990). The development of specific antagonists for NPY receptors will be a valuable tool for confirming the vascular roles of NPY.

NPY produces vasoconstriction of numerous vascular beds, being particularly potent in pial arteries (Hanko *et al.,* 1986). The ability of NPY to produce vasoconstriction varies along consecutive lengths of some vascular beds. For instance,

NPY produces a weaker constriction of the proximal segment of the superior mesenteric artery than the more distal finer ramifications of this vessel which are close to the intestine (Edvinsson *et al.,* 1989). In skeletal muscle vascular beds, the arterioles but not the supplying arteries, are sensitive to NPY (Lundberg *et al.,* 1987).

In the cutaneous vasculature of the guinea-pig, tyrosine hydroxylase-like immunoreactive fibres associated with arteries, but not arterioles, also showed NPY-like immunoreactivity (Gibbins and Morris, 1990). The ability of NPY to cause vasoconstriction correlated with the presence of NPY-like immunoreactive fibres (Morris, 1994a).

NPY-mediated sympathetic vasoconstriction varies between different vascular beds, even though they may have similar diameters. The activation of postsynaptic Y_1-NPY receptors contributes to sympathetic vasoconstriction of 3^o mesenteric arteries, which have internal diameters of 370 μm, but not of femoral arteries, internal diameter of 670 μm, even though Y_1-NPY receptors are present on both vessels (Angus *et al.,* 1995).

The vasoconstriction produced by NPY is described as being slower than that produced by noradrenaline (Lacroix *et al.,* 1988; Morris and Murphy, 1988; Lundberg *et al.,* 1989). In some arteries, a slow component of sympathetic vasoconstriction is non-noradrenergic and has a time-course similar to constriction produced by NPY (Lundberg *et al.,* 1984a; Morris and Murphy, 1988). Both the slow component of sympathetic vasoconstriction and the slow constriction produced by applied NPY are reduced, or abolished, by desensitizing NPY receptors or by treatment with the endopepetidase, trypsin (Morris and Murphy, 1988). This provides some good evidence for NPY acting as a co-transmitter for sympathetic vasoconstriction.

At concentrations below which NPY itself does not cause constriction, NPY may potentiate nerve-induced vasoconstriction (Edvinsson *et al.,* 1985; Pernow, Saria and Lundberg, 1986; Vu, Budai and Duckles, 1989). Both the non-α− and α− noradrenergic components of nerve-induced vasoconstriction may be potentiated by NPY (Saville, Maynard and Burnstock, 1990).

The contribution of NPY to sympathetic-evoked vasoconstriction also depends upon the stimulus parameters applied, being more pronounced at higher frequencies of stimulation (Morris and Murphy, 1988; Pernow *et al.,* 1989). It was suggested by these authors that the release of large vesicles containing both noradrenaline and NPY were favoured by higher frequencies of stimulation. However, consideration of the organization of the sympathetic neuromuscular junction as described above may suggest that NPY receptors are located extrajunctionally on some vessels. NPY receptors would therefore only be activated by neurotransmitter overflow from the neuromuscular cleft following longer, or higher frequency, conditions of stimulation (Morris and Gibbins, 1992).

NPY vasodilatation

Sympathetic ganglia contain different populations of noradrenergic and peptide containing neurons and, in many cases, the combinations of neuropeptides in these neurons correlates well with their specific peripheral projection; this has been termed "chemical coding" (Costa, Furness and Gibbins, 1986; Furness *et al.,* 1989).

In lumbar sympathetic ganglia, vasoconstrictor neurons contain tyrosine hydroxylase-like or tyrosine hydroxylase-like and NPY-like immunoreactivity (Gibbins, 1992). A second population of non-adrenergic NPY-like immunoreactive neurons which also contain vasointestinal peptide (VIP)-like immunoreactivity, project primarily to small arteries supplying skeletal muscle (Gibbins, 1992) to cause vasodilatation. Axons which project to the cerebral arteries from the superior cervical ganglia (SCG) also contain these two different populations of NPY-like immunoreactive axons (Gibbins and Morris, 1988). Additionally, in the paracervical ganglia, 90-95 % of parasympathetic neurons contain neuropeptides (Morris and Gibbins, 1987). Of these, three populations of noradrenergic neurons and eight populations of non-noradrenergic neurons have been identified (Morris and Gibbins, 1987). Two of these non-noradrenergic populations, in which the neuropeptide content in both includes NPY-like and VIP-like immunoreactive neurons, project mainly to the uterine artery (Morris *et al.,* 1985). Electrical stimulation of these non-noradrengic nerves causes

vasodilation of this artery which is proposed to be mediated by VIP and nitric oxide (Morris, 1993). Sympathetic vasodilatory neurons innervate arteries feeding intramuscular vascular beds but do not innervate the distal arterioles (Gibbins, 1992).

NPY may therefore be contained in both sympathetic vasoconstrictor and in vasodilator parasympathetic nerves. NPY in vasoconstrictor nerves is proposed to mediate the slow component of sympathetic vasoconstriction (Morris and Murphy, 1988). The significance of NPY in vasodilatory nerves is still unclear. Morris (1990) suggested that as exogenous NPY inhibited exogenous VIP-induced vasodilatation in the guinea-pig uterine artery, the role of NPY in vasodilator nerves may be to modify the potency of primary vasodilatory neurotransmitters.

Auto-regulation of neurotransmitter release

Neurotransmitter release may be regulated by the activation of presynaptic receptors (Starke, 1977; Langer, 1982; Story *et al.,* 1990). In this context, auto-inhibitory feedback refers to the mechanism where neurotransmitter released from a nerve acts upon pre-synaptic receptors to inhibit further release of neurotransmitter from that nerve. Some examples of the presynaptic receptors which can be activated on sympathetic nerves have already been given.

Presynaptic regulation of neurotransmitter release has been proposed to be one mechanism for preventing the accumulation of neurotransmitter at the neuromuscular cleft (Bevan *et al.,* 1984). The physiological relevance of this mechanism should be considered. Sympathetic nerves obey the "vesicle hypothesis", that is, that neurotransmitters are secreted in quanta equal to the content of a single varicosity, as first proposed by de Castillo and Katz (1955). The release of noradrenaline from an individual varicosity is intermittent, due to a low probability of release (Hirst and Neild, 1980b; Brock and Cunnane, 1987; Astrand and Stjarne, 1989). That is, the average varicosity will release a vesicular amount of noradrenaline in response to 1 or 2 out of 100 nerve pulses. With such a low probability of release, it seems unlikely that neurotransmitter released from one site would act locally to inhibit subsequent release from the same or nearby site. Stimulation of nerves for long periods, or at high

frequencies, is required for neurotransmitter to overflow from the active synapse (Morris and Gibbins, 1992).

It has been suggested that autoinhibition is a lateral rather than local effect (Brock and Cunnane, 1991). Following the release of noradrenaline from a particular vesicle, the probability of subsequent release is, if anything, slightly enhanced (Brock and Cunnane, 1991). The spread of noradrenaline to varicosities not yet activated could stimulate other prejunctional α receptors, thus decreasing the probability of release from other varicosities (Brock and Cunnane, 1991). This mechanism would function to reduce the overall magnitude of transmitter release from a nerve terminal network (Starke, 1987; Stjarne, 1989).

PARASYMPATHETIC NERVES

Parasympathetic nerve stimulation

Acetylcholine was the first parasympathetic neurotransmitter identified (von Euler, 1959). Acetylcholine activates at least five types of muscarinic receptors on effector tissues, these being M_1-, M_2-, M_3-, M_4- and M_5- muscarinic receptors (Watson and Girdlestone, 1994).

Parasympathetic nerve stimulation causes vasodilatation in some, but not all, vascular beds. Parasympathetic nerve-evoked dilatations are described in vascular beds such as those supplying the nasal mucosa and tongue (Lundberg *et al.,* 1990a; Izumi and Karita, 1994), the submandibular and submaxillary glands (Fazekas *et al.,* 1987; Bhoola *et al.,* 1965; Thulin, 1976). Thus parasympathetic vasodilatation has been predominantly observed in tissues involved in exocrine functions, however this is not strictly so as cervical neurons supply a cholinergic vasodilator innervation to the uterine artery (Bell, 1989).

Interestingly, parasympathetic nerve stimulation has been reported to produce constriction of vessels in the pulmonary circulation (Donald, O'Shea and Lillywhite, 1990).

Parasympathetic neuromuscular junction

Cholinergic nerves form a plexus around the blood vessels they innervate. A superficial cholinergic nerve plexus has been localized in the outer adventitial layer and a deeper cholinergic plexus localized in the adventitial-medial junction of the human pulmonary artery (Amenta *et al.,* 1983). In smaller pulmonary arteries, a single cholinergic nerve plexus was observed at the adventitial-medial junction (Amenta *et al.,* 1983).

Cholinergic neuroeffector junctions, as well as noradrenergic junctions, have been described following reconstruction of serial sections at the electron microscope level in the sinus venosus of the toad and the guinea pig sinoatrial node (Steele and Choate, 1994; Klemm, Hirst and Campbell, 1992). Cholinergic varicosities were defined as having an axon profile with diameter greater than 0.3 μm diameter and containing chromaffin negative synaptic vesicles (Gordon-Weeks, 1988). A large portion (82/96) of exposed varicosities (ie. part or all of the surface was free of Schwann cell wrapping) came into close contact with nearby sino-atrial node cells. The regions of close apposition showed similar characteristics as have been described for sympathetic neuromuscular junctions (Luff and M^{c}Lachlan, 1989).

Cotransmission in the parasympathetic nervous system

VIP and Peptide Histidine Isoleucineamide (PHI)

VIP is a 28 amino acid peptide that was first shown to be a vasodilator substance present in extracts of porcine intestine (Carlquist, Mutt and Jornvall, 1979). PHI is a 27 amino acid peptide which has 50% sequence homology with VIP, and is proposed to be an endogenous ligand of VIP and related receptors (Edvinsson and McCulloch, 1985; Huang *et al.,* 1989). Recent reports have described the cloning of two VIP receptors, VIP_1 and VIP_2 (Ishihara *et al.,* 1992; Lutz *et al.,* 1993), however at the moment there are few pharmacological or biochemical tools available to distinguish between these two subtypes (Watson and Girdlestone, 1994).

VIP-like immunoreactive nerves lie in the adventitial layer near the adventitial-medial border of major arteries and regional vascular beds, in a similar

distribution to acetylcholinesterase-like immunoreactive fibres (Della *et al.,* 1983; Barnes *et al.,* 1986; Tatemoto and Mutt, 1981; Jansen *et al.,* 1986; Edvinsson *et al.,* 1987). The mesenteric and pelvic vascular beds are most densely supplied by VIP-like immunoreactivity in the guinea-pig (Della *et al.,* 1983).

VIP, like other neuropeptides, is produced in nerve cell bodies and axonally transported to nerve terminals (Lundberg, Fahrenkrug and Brimijoin, 1981). In the cat submandibular gland, where both VIP and PHI are proposed to contribute to the atropine-resistant component of parasympathetic vasodilation, parasympathetic nerve stimulation releases VIP and PHI in a 1:1 ratio (Lundberg *et al.,* 1984b).

The peptide content of parasympathetic vasodilator nerves is different for different vascular beds and between species. Parasympathetic vasodilator neurons innervating guinea-pig pelvic vasculature contain VIP, NPY and dynorphin, and many contain somatostatin (Morris *et al.,* 1985). VIP only is present in some, but not all, guinea-pig cranial parasympathetic neurons (Della *et al.,* 1983; Hottenstein *et al.,* 1992), but co-exists with NPY in the rat cranial parasympathetic neurons (Leblanc and Landis, 1988).

Interestingly, VIP-like immunoreactive fibres have been found to be associated with arteries but not their distal segments. VIP-like immunoreactive fibres are abundant around blood vessels in the trachea but are sparse around blood vessels of the peripheral bronchi of the pig (Martling *et al,* 1990) . VIP-like immunoreactive sympathetic fibres are associated with arteries supplying the hindlimb skeletal muscle, although they are less dense than NPY-like and tyrosine hydroxylase-like immunoreactive fibres. These VIP-like immunoreactive fibres do not project to the intramuscular arterioles (Anderson *et al.,* 1995).

Parasympathetic nerve stimulation produces a biphasic vasodilatation in the submandibular and submaxillary glands (Fazekas *et al.,* 1987; Darke and Smaje, 1972; Lundberg, Anggard and Fahrenkrug, 1982). The ability of the muscarinic receptor antagonist, atropine, to inhibit parasympathetic vasodilatation is frequency-dependent in these tissues. Atropine inhibits the initial phase of vasodilatation which is more pronounced at low stimulus frequencies, but not the secondary, prolonged

vasodilatation which is more pronounced following higher stimulus frequencies or repetitive stimulation (Darke and Smaje, 1972; Thulin, 1976).

VIP, and possibly PHI, are proposed to mediate the atropine-insensitive sustained component of parasympathetic nerve-evoked vasodilatation (Lundberg *et al.*, 1981; Lundberg, Anggaard and Fahrenkrug, 1981a; Lundberg, Anggard and Fahrenkrug, 1981b; Lundberg, Anggard and Fahrenkrug, 1982; Lundblad, Anggard and Lundberg, 1983; Lundberg *et al.*, 1984b; Fazekas *et al.*, 1987; Ito *et al.*, 1987). These reports demonstrate several lines of evidence to indicate this. These include: 1) the presence of VIP-like immunoreactive parasympathetic fibres; 2) VIP release following parasympathetic nerve stimulation. This release was frequency dependent, thus correlating with the appearance of the atropine-insensitive vasodilatation; 3) both the non-cholinergic vasodilatation and the release of VIP were abolished by the nicotinic antagonist, hexamethonium, indicating that they both arise from the activation of postganglionic parasympathetic nerves and 4) the prolonged vasodilatation produced by VIP was similar to the slower component of the parasympathetic vasodilatation. The development of specific VIP receptor antagonists will enable the confirmation of the role of VIP as a cotransmitter with acetylcholine in the parasympathetic nervous system.

Auto-regulation of parasympathetic neurotransmitter release

Parasympathetic neurotransmitters may act prejunctionally to regulate parasympathetic neurotransmitter release. For example, acetylcholine may inhibit the release of VIP and PHI via muscarinic autoreceptors on fibres innervating the vasculature of the submandibular gland (Lundberg *et al.*, 1984a).

To summarize, the autonomic sympathetic and parasympathetic nerves innervating mammalian arterial vessels contain 'classical' neurotransmitters such as noradrenaline and acetylcholine, respectively. The release of these transmitters produces vasoconstriction and vasodilatation respectively and undergoes auto-regulation. The classical neurotransmitters generally produce the initial response to

autonomic nerve stimulation. In addition, neuropeptides present in autonomic nerves are proposed to mediate responses to sympathetic or parasympathetic nerve stimulation which occur following longer, or higher frequency, stimulus trains. This may be due to the presence of peptide receptors away from the synaptic sites or due to variable release mechanisms. When required, these neuropeptides are thus able to mediate longer lasting effects of nerve stimulation.

SENSORY-MOTOR NERVES

Non-adrenergic, non-cholinergic vasodilatation

After noradrenergic and cholinergic transmission are blocked, perivascular nerve stimulation may evoke a vasodilation. Stricker (1876) was the first to observe that stimulation of the peripheral ends of cut dorsal roots induced vasodilation in the skin area supplied by the transected afferent nerve-fibres and it is now believed that upon activation, sensory nerve fibres send orthodromic impulses to the spinal cord and antidromic impulses are sent along collaterals from the sensory nerves to produce peripheral vasodilation (see Holzer, 1992). These collaterals have been called "sensory-motor nerves" to distinguish them from the other afferent fibres which have a purely sensory role and whose terminals contain few vesicles (Burnstock, 1990). The peripheral effect of sensory-motor efferents can be induced by electrical stimulation or by direct activation of the sensory nerve terminals by stimuli such as capsaicin (Maggi and Meli, 1988).

Vasodilatation produced by sensory-motor nerves is predominant in the cutaneous vasculature, where it plays an important part in inflammation (Holzer, 1992). Sensory-motor nerves also produce vasodilatation of other vascular beds such as those in the nasal mucosa (Stjarne *et al.,* 1991), airway (Matran, 1991), mesenteric artery (Li and Duckles, 1992) and arterioles in striated muscle (Porszasz and Szolcsanyi, 1994). Sensory-motor nerves play an important role in gastric mucosal protection. When the barrier to gastric acid back-diffusion is disrupted, activation of these fibres leads to an increase in gastric mucosal blood flow to help remove the back diffusing acid (Guth, 1992).

Capsaicin

The term "capsaicin-sensitive" nerve refers to afferent neurons which are transiently excited by the acute application of capsaicin, and permanently damaged by long term treatment of capsaicin (Holzer, 1991a). These neurons comprise most of the primary afferent neurons with small cell bodies and unmyelinated axons (C fibre) and some afferent neurons with thin myelinated axons (Aδ fibre). Histochemical and biochemical evidence suggests that parasympathetic and sympathetic nerves remain unaffected after capsaicin treatment (Holzer, 1991a).

Primary sensory neurons may contain tachykinins, calcitonin gene-related peptide (CGRP), somatostatin, cholecystokinin, bombesin, oxytocin, opioid peptides and galanin (Hokfelt *et al.,* 1993). Substance P and CGRP are co-localized in the same large granular vesicles in trigeminal and dorsal root sensory ganglia (Merighi *et al.,* 1988).

Substance P- and CGRP-like immunoreactive nerve fibres have a similar distribution around guinea-pig blood vessels (Stjarne *et al.,* 1989). Gibbins, Furness and Costa (1987) demonstrated that nerves containing certain combinations of peptides showed distinctive peripheral projections in the guinea pig. For example, neurons containing substance P-, CGRP- and cholecystokinin-like immunoreactivity were distributed to small blood vessels in skeletal muscle, and neurons containing substance P- and CGRP-like immunoreactivity were found mainly in the heart and systemic blood vessels and vessels of the abdominal viscera and the airways.

Capsaicin treatment destroys all the substance P-like immunoreactive sensory neurons in neonatal rats (Holzer, 1991a). CGRP-like immunoreactive neurons in sensory ganglia of the rat are divided into two subgroups: small-medium sized neurons which contain substance P in the same soma, and large neurons which lack substance P (Lee *et al.,* 1985; Matsuyama *et al.,* 1986). While neonatal capsaicin treatment abolishes the small-medium CGRP-like and substance P-like immunoreactive neurons of trigeminal sensory ganglia, the large CGRP-like immunoreactive neurons are unaffected (Matsuyama *et al.,* 1986). On the other hand, both CGRP- and substance P-like immunoreactive fibres associated with the cerebral artery were destroyed by

capsaicin treatment. Therefore, although not all CGRP-immunoreactive sensory fibres are sensitive to capsaicin, in the rat and guinea-pig, all CGRP- and substance P-like immunoreactive fibres associated with blood vessels are capsaicin-sensitive (Wharton *et al.,* 1986).

CGRP

CGRP is a 37 amino acid peptide which is generated from the calcitonin gene by alternative splicing (Amara *et al.,* 1982). CGRP receptors have been divided into $CGRP_1$ and $CGRP_2$ (Watson and Girdlestone, 1994). $CGRP_2$ receptors on sensory-motor nerve terminals may mediate a negative feedback on the release of CGRP from these nerves (Nuki *et al.,* 1994). CGRP is probably inactivated by enzymes, at least one of which also breaks down substance P (Nuki *et al.,* 1994).

The distribution on blood vessels of sensory-motor nerve fibres containing CGRP is similar to that of substance P containing fibres, although in the rat the content of CGRP is reported to be greater than that of substance P (Wharton *et al.,* 1986). CGRP-like immunoreactivity is also higher in peripheral arteries than in the major blood vessels (Brain and Williams, 1985). Pial arteries show a well developed supply of CGRP-like immunoreactive fibres (Hanko *et al.,* 1985).

In the skin of the rat foot pad, CGRP-like immunoreactive fibres formed bundles which were smooth (Ishida-Yamamoto, Senba and Tohyama, 1989). When individual CGRP-like immunoreactive fibres left a bundle to innervate a blood vessel they became varicose (Ishida-Yamamoto, Senba and Tohyama, 1989). Examination of serial sections indicated that CGRP-like immunoreactive fibres did not form synaptic contacts with the underlying blood vessels (Ishida-Yamamoto, Senba and Tohyama, 1989).

Vessels where CGRP mediates capsaicin-sensitive vasodilation include the mesenteric artery (Han, Naes and Westfall, 1990), the rabbit skin (Hughes and Brain, 1991), rat hepatic artery (Bratveit and Helle, 1991) and the cerebral artery (Saito *et al.,* 1989).

Substance P

Substance P was the first member of the neuropeptide tachykinin family to be isolated by von Euler and Gaddum in 1931, and was sequenced in the early 1970's. Between 1983 and 1984 several groups reported the isolation of the other two mammalian tachykinins, neurokinin A and neurokinin B; and all three tachykinins all share a common C terminal sequence of Phe-Xaa-Gly-Leu-MetNH_2 (see Maggi, 1995).

Tachykinin synthesis occurs in the cell bodies of primary afferent neurons in the dorsal root ganglia (Too, Cordova and Maggio, 1989a; Helke *et al.,* 1990). Tachykinins synthesized by dorsal root ganglia neurons are transported to their peripheral nerve terminals to produce their sensory-motor effects (Maggi *et al.,* 1993). Neurokinin B levels in peripheral tissues are very low or, in many cases, undetectable (Too, Cordova and Maggio, 1989a; Tateishi *et al.,* 1990; Moussaoui *et al.,* 1992).

The receptors for the tachykinins have been divided according to their order of sensitivity of activation by the endogenous ligands, although all three tachykinin receptors may be activated by all the tachykinins. The NK_1-tachykinin receptor is preferentially activated by substance P, NK_2-tachykinin receptor by neurokinin A and NK_3-tachykinin receptor by neurokinin B (Maggi, 1995).

Vasodilatation is produced by the intravenous application of tachykinins *in vivo* or their application to precontracted blood vessels (Maggi, 1995). This vasodilatation is mediated by the activation of tachykinin receptors on endothelial cells (Saito *et al.,* 1990; Saito *et al.,* 1991).

Several studies have addressed the issue of whether tachykinins released from sensory-motor nerves gain access to receptors located on the endothelial cells. Neurokinins, but not CGRP, produce an endothelium-dependent vasodilatation of guinea-pig pulmonary arteries (Maggi *et al.*, 1990). Although sensory-motor nerves release both CGRP and neurokinins, the vasodilatation following stimulation of these nerves is not endothelium-dependent and is mediated by CGRP (Maggi *et al.,* 1990). Similar results have been found in coronary arteries (Franco-Cereceda, Rudehill and Lundberg, 1987), rat perfused mesenteric artery (Han, Naes and Westfall, 1990; Li and Duckles, 1992). In these vessels therefore, endothelial tachykinin receptors are not

affected by neurally-released peptide but rather CGRP mediates the neurogenic vasodilatation.

It is possible that in smaller blood vessels, which have thinner walls, neurally-released tachykinins may reach endothelial receptors to produce vasodilatation. For instance, sensory-motor vasodilatation of submucosal arterioles is produced by both CGRP and substance P (Vanner, 1994; Galligan *et al.,* 1990).

Several peptidases terminate the physiological effects of tachykinins. Endopeptidase 24.11 (also known as neutral endopeptidase or enkephalinase) inactivates all three tachykinins (Hooper, Kennedy and Turner, 1985; Nau *et al*, 1986). Peptidyl dipeptidase A (also known as angiotensin converting enzyme) inactivates substance P (Hooper, Kennedy and Turner, 1985), while aminopeptidases inactivate neurokinin A (Hooper, Kennedy and Turner, 1985).

Endopeptidase 24.11 also inactivates CGRP (Hooper, Kennedy and Turner, 1985). Peptidase inhibitors may potentiate the effects of neurally-released neurokinins. For example, in the guinea pig perfused lungs capsaicin increased the release of CGRP, but also further increased the release of neurokinin A and CGRP in the presence of the enkephalinase inhibitor, phosphoramidon, suggesting that the two peptides are broken down by a similar peptidase (Kroll *et al.,* 1990).

Capsaicin-sensitive, substance P-like immunoreactive fibres are localized in the advential-medial border of large peripheral arteries. No correlation was found between the degree of innervation of blood vessels, as detected histochemically, and the ability of substance P to change vascular tone, which led the authors to suggest that the substance P fibres were purely sensory in nature and did not subserve a vasomotor function (Barja, Mathison and Huggel, 1983).

NERVES CONTAINING NITRIC OXIDE

Li and Rand (1991) proposed the term 'nitrergic' to describe nitric oxide-mediated neurotransmission. Recent reviews indicate that nitric oxide meets the criteria defining a neurotransmitter: nitric oxide synthesis is found within neurons, nitric oxide

is released during non-adrenergic, non-cholinergic nerve stimulation, exogenous nitric oxide mimicks the effect of nerve stimulation and the removal of nitric oxide occurs as a consequence of its rapid breakdown (Rand, 1992; Sanders and Ward, 1992).

L-citrulline and nitric oxide are formed by the oxidation of a guanidinonitrogen atom of L-arginine (Moncada, 1992). This is at least a two step reaction which is catalysed by nitric oxide synthase (NOS). Two types of NOS have been identified: constitutive and inducible; both have binding sites for nicotinamide-adenine dinucleotide phosphate, flavin adenine nucleotide, flavin mononucleotide and a recognition site for calmodulin. The constitutive form of NOS consists of two isoforms: one found in endothelial cells and one found in neurons. The activity of this type of NOS is dependent on the concentration of calcium. Inducible NOS is found in macrophages after cytokine treatment. As calmodulin is always tightly bound to this type of NOS, this type of enzyme is calcium-independent (Leone *et al.,* 1991).

A number of L-arginine analogues, such as L-nitroarginine, L-N^G-monomethyl arginine (L-NMMA) and N^G-nitro L-arginine methyl ester (L-NAME), are widely used as competitive inhibitors of nitric oxide synthesis as their effects are reversed to some degree by an excess of L-arginine (Rees *et al.,* 1990; Liu *et al.,* 1992; Greenblatt, Loeb and Longnecker, 1993). The selectivity of these compounds for the L-arginine-nitric oxide pathway has been questioned (Buxton *et al.,* 1993; Thomas and Ramwell, 1992), however these compounds are still commonly used by investigators to investigate the action of nitric oxide.

Loesch, Belai and Burnstock (1994) determined the ultrastructural distribution of the neuronal isoform of NOS in the rat basilar artery. Axon varicosities showing NOS-like immunoreactivity were characterised by the presence of spherical agranular vesicles with diameters of 40-50 nm. NOS-like immunoreactive axons innervating the lingual artery form direct neuromuscular junctions at the outer surface of the tunica media and make direct contacts with non-varicose and varicose segments of non-NOS reactive axons (Kummer and Mayer, 1993).

Penile and cerebral arteries have been described as having nitrergic innervation (Rand, 1992). Neuronal NOS is located in nerve fibres innervating coronary arteries and pulmonary vessels (Klimaschewski *et al.,* 1992; Nozaki *et al.,* 1993). Thin NOS-immunoreactive fibres as well as many NOS-immunoreactive thicker nerve fibres and bundles occur in the outer media of monkey and canine cerebral, mesenteric and temporal arteries (Yoshida, Okamura and Toda, 1994). NOS-like immunoreactive fibres are also found in the adventitia of the human uterine artery (Toda *et al.,* 1994). In both the guinea pig and the pig, the distribution of NOS-like immunoreactivity matches the presence of VIP/cholinergic postganglionic vasodilator fibres (Edvinsson *et al.,* 1980; Gibbins, Brayden and Bevan, 1984; Ceccatelli *et al.,* 1994), although some cholinergic vasodilator fibres, for example to the tongue, lack NOS-immunoreactivity (Kummer *et al.,* 1992). Numerous NOS-containing fibres occur within the porcine pterygopalatine and trigeminal ganglion, while sensory ganglia of the vagal nerve also comprised a smaller number of fibres (Sienkiewicz *et al.,* 1995). Damaging the pterygopalatine ganglia, which contains numerous parasympathetic and sensory fibres, abolished NOS-like fibres in the wall of the ipsilateral middle cerebral artery (Yoshida, Okamura and Toda, 1994; Ehinger, Sundler and Uddman, 1983).

Non-adrenergic, non-cholinergic (NANC) nerve stimulation of bovine basilar and monkey cerebral arteries, temporal arteries and human uterine artery strips, which have been endothelium-denuded, produces a vasodilatation which is abolished by inhibition of nitric oxide synthesis (Yoshida, Okamura and Toda, 1994; Stubbs *et al.,* 1992). In the bovine ciliary artery, part of the NANC vasodilatation is mediated by CGRP released from capsaicin-sensitive sensory nerves, but 85% of the relaxation is abolished by inhibition of neuronal nitric oxide synthesis (Wiencke *et al.,* 1994).

Work undertaken for the past 20 years by Toda and colleagues supports the hypothesis that the activation of nicotinic receptors on nitrergic vasodilator nerves increases the transmembrane influx of calcium and activates NOS and thus produces neuronal nitric oxide (Toda and Okamura, 1992). Nicotine induces a vasodilatation of cat middle cerebral and canine saphenous arterial strips which is totally abolished by inhibiting both nitric oxide production and CGRP receptors (Okamura and Toda, 1994).

The literature therefore shows a good correlation between the presence of NOS-containing nerves and a functional nitrergic innervation. Neuronal nitric oxide appears to be found in postganglionic parasympathetic and sensory, but not sympathetic, nerves (Modin, 1994).

Nitric oxide is a very labile and freely diffusible molecule. A simple mechanism has been suggested to account for nitrergic neurotransmission (Sanders and Ward, 1992). As previously mentioned, neuronal NOS is calcium-dependent and the influx of calcium into nitrergic varicosities following a nerve impulse would increase the production of nitric oxide. The nitric oxide produced could easily diffuse from the varicosity and produce effects on nearby muscle cells.

Recently it was demonstrated that 3 days after ligating the vagus nerve, NOS-like immunoreactivity accumulates on the ganglion side of the ligation (Lumme, Vanhatalo and Soinila, 1996). VIP-like immunoreactivity was co-localized with this NOS-like immunoreactivity, indicating that NOS may be transported in parasympathetic nerves. Some of the fibres accumulating NOS were also substance P-like immunoreactive, suggesting that presumable sensory neurons of the nodose ganglia also transport NOS (Lumme, Vanhatalo and Soinila, 1996).

While it has been demonstrated that NOS may be axonally transported, Stark *et al.* (1993) provided evidence that nitrergic neurotransmission does not depend upon the release of stored nitric oxide. In the jejunum, the inhibitory effects of L-NMMA on nitrergic inhibitory junction potentials occured within 10 min. There would only be a small amount of neurotransmitter stored if synthesis inhibition had such a quick time course. The time course was not affected by electrical field-stimulation during the L-NMMA incubation period. If nitric oxide was stored, such stimulation would shorten the time course by accelerating depletion of neurotransmitter stores.

EFFECTS OF NEUROTRANSMITTERS ON VASCULAR ENDOTHELIUM

In response to stimulation of the appropriate receptors, the vascular endothelium releases a number of molecules which can cause either vasoconstriction or vasodilatation.

Endothelium-dependent vasodilatation

Furchgott and Zawadzki (1980) were the first to report that the vasodilatory response to acetylcholine was impaired when the endothelium was removed, and suggested this was because the enothelial cells release a relaxing factor which diffuses to the smooth muscle cells to produce vasodilation (EDRF). Other agonists which produce an endothelium-dependent vasodilatation include substance P, ATP, serotonin and histamine (Mione, Ralevic and Burnstock, 1990). Receptors for these agonists have been located on endothelial cells in various blood vessels (Loesch and Burnstock, 1988; Milner *et al.,* 1989).

Nitric oxide has been identified as one EDRF. Palmer and co-workers (1988) were the first to report that L-arginine was the physiological precursor for the formation of nitric oxide and that nitric oxide release can account for the biological activity of EDRF.

Nitric oxide formed by endothelial NOS rapidly diffuses within the endothelial cell and across membranes to adjacent smooth muscle cells where it activates soluble guanylate cyclase to cause vasodilation (Palmer, Ashton and Moncada, 1988). In this way nitric oxide can convert a signal in endothelial cells, such as elevated intracellular calcium following receptor activation, to a response in neighbouring smooth muscle cells.

Under physiological conditions, the activation of constitutive NOS in endothelial cells by shear stress means that a continuous release of nitric oxide keeps the vasculature in a permanent state of vasodilation. Inhibition of synthesis of nitric oxide with L-NMMA *in vivo* causes a rapid, sustained elevation in blood pressure (Rees, Palmer and Moncada, 1989; Greenblatt, Loeb and Longnecker, 1993; Gardiner *et al.,* 1990). Infusion of L-NMMA in conscious rats decreased blood flow and increased vascular resistance in the cerebrum, heart, kidney, spleen, gastrointestinal tract, skin, ear and white fat. The magnitude of increased vascular resistance was not uniform, the maximal effect being in the brown fat and the least in the heart. Interestingly, L-NMMA

infusion produced an increase in blood flow and decreased vascular resistance in the hepatic artery (Greenblatt, Loeb and Longnecker, 1993). The effect of inhibiting nitric oxide synthesis was greater in the large arteries of the cerebral vasculature (diameter of 275 μm) than in the arterioles (diameter of 62 μm; Faraci, 1991). Close intra-arterial infusion of L-NAME also reduces basal blood flow in the rabbit knee joint (Najafipour and Ferrell, 1993).

The endothelium releases an inhibitory factor which is not related to nitric oxide and has been termed EDHF (Taylor and Weston, 1988). The effects of EDHF and EDRF can been differentiated by comparing the effect of acetylcholine with the effect of the guanylate cyclase inhibitor, nitroprusside, and the K^+ channel opener, cromakalim, which hyperpolarizes smooth muscle independently of cyclic AMP or cyclic GMP (Taylor and Weston, 1988). Acetylcholine produces an endothelium-dependent increase in cyclic GMP levels, a transient hyperpolarization and a long-lasting vasodilatation (Plane, Pearson and Garland, 1995). The hyperpolarization was closely linked with an increase in the rate of efflux of K^+, which was mimicked by cromakalim, but not nitroprusside (Taylor *et al.,* 1988). The acetylcholine-induced increase in cyclic GMP was mimicked by nitroprusside but not cromakalim (Taylor *et al.,* 1988). Haemoglobin, which inactivates EDRF, prevented the increase in cyclic GMP, but had no effect on the hyperpolarization (Chen, Suzuki and Weston, 1988). Therefore, EDRF stimulates cyclic GMP formation, whereas EDHF-mediated hyperpolarizations are insensitive to inhibitors of nitric oxide, and result from the activation of K^+ channels.

The contribution of EDHF to endothelium-dependent vasodilatation has been questioned (Taylor and Weston, 1988). In the rat aorta and pulmonary artery, 75-80 % of the relaxation produced by acetylcholine was abolished by inhibiting nitric oxide synthesis, while the smooth muscle hyperpolarization was unaffected (Chen, Suzuki and Weston, 1988). The hyperpolarization or repolarization produced by endothelium-dependent vasodilatory agonists is transient compared to the sustained dilatation (Plane, Pearson and Garland, 1995). The effects of EDRF and EDHF are usually studied on vessels which have been preconstricted, which means the smooth muscle cells may be

in a depolarized state. It has been suggested that EDHF provides the primary drive for relaxation, by hyperpolarizing or repolarizing the membrane potential and EDRF maintains the relaxation (Taylor and Weston, 1988; Plane, Pearson and Garland, 1995).

In order to study endothelium-dependent vasodilatation, blood vessels are usually preconstricted. Recently it was demonstrated that the nature of acetylcholine-induced vasodilatation clearly depends upon the agent used to preconstrict (Hayashi *et al.,* 1994). Acetylcholine produced a sustained vasodilatation of renal afferent arterioles preconstricted with KCl or noradrenaline, but produced a transient dilatation followed by a small residual dilatation of arterioles preconstricted by elevated perfusion pressure.

Following KCl-induced preconstriction, acetylcholine vasodilatation was prevented by inhibiting both cyclo-oxygenase, using indomethacin, and NOS, using nitro-L-arginine. The acetylcholine-induced transient vasodilatation of myogenically pre-constricted vessels was not due to cyclo-oxygenase products or nitric oxide. The small residual vasodilatation was sensitive to indomethacin, and possibly mediated by prostaglandin E_2 (Hayashi *et al.,* 1994). Nitro-L-arginine inhibited the sustained acetylcholine-induced vasodilatation of noradrenaline pre-constricted vessels, leaving an initial transient dilatation which was insensitive to indomethacin. The mediator of the transient vasodilatation produced by acetylcholine was not identified in this study, although it had a time course similar to the previously reported effects of EDHF (Chen, Suzuki and Weston, 1988). Therefore, different mechanisms may mediate agonist-induced vasodilatation of a vessel, depending upon the nature of the underlying tone.

Endothelium-dependent vasoconstriction

Endothelial cells may also release vasoconstrictor agents, such as endothelin, a 22 amino acid peptide discovered in 1988 (Yanagisawa *et al.,* 1988). Endothelial-derived angiotensin II and thromboxane A_2 may also produce constriction of vascular smooth muscle cells (see Kuriyama, Kitamura and Nabata, 1995).

MODULATION OF VASOCONSTRICTION AND VASODILATATION

There is now much evidence for interactions between nerves subserving different functions. Such effects may occur post-junctionally in the effector cells or pre-junctionally to modify neurotransmitter release.

Postjunctional effects

Capsaicin pretreatment augments sympathetic vasoconstriction of isolated mesenteric arteries (Kawasaki *et al.,* 1990a; Remak, Hottenstein and Jacobson, 1990; Li and Duckles, 1992). Neither capsaicin nor the sensory-motor neurotransmitter CGRP inhibit ^{3}H-noradrenaline release following sympathetic nerve stimulation (Han, Naes and Westfall, 1990; Kawasaki *et al.,* 1990a; Maynard and Burnstock, 1989). This suggests a postjunctional inhibitory effect of sensory nerves on sympathetic vasoconstriction.

Interactions between sympathetic and sensory-motor nerves also occur *in vivo*. Periarterial nerve stimulation reduces blood flow of the mesenteric artery, but peak vasoconstriction is followed by a recovery of blood flow, despite continuing nerve stimulation (Remak, Hottenstein and Jacobson, 1990). This autoregulation of sympathetic vasoconstriction was inhibited by capsaicin (Remak, Hottenstein and Jacobson, 1990), suggesting the involvement of sensory-motor nerves.

Prejunctional effects

Activation of prejunctional 5HT, H_1- or H_3-histamine or opioid receptors may inhibit sympathetic vasonconstriction (Schlicker *et al.,* 1994; Molderings *et al.,* 1987).

While sensory-motor neurotransmitters act postjunctionally to inhibit sympathetic vasoconstriction, sympathetic neurotransmitters act prejunctionally to inhibit neurotransmitter release from sensory-motor nerves. For example, non-cholinergic vasodilatation of the isolated mesenteric artery is only observed when α–adrenoceptors and sympathetic neurotransmission are blocked (Kawasaki *et al.,* 1990b). In this artery, noradrenaline, adenosine and NPY act upon afferent nerve endings to inhibit the sensory-motor release of CGRP (Kawasaki *et al.,* 1991; Mantelli *et al.,* 1993;

Li and Duckles, 1991). Activation of prejunctional α_2–adrenoceptors also reduces sensory-motor nerve relaxation of the pulmonary artery (Butler *et al.,* 1993).

Activation of μ opioid receptors on sensory-motor nerve terminals may also inhibit sensory-motor vasodilatation of the mesenteric artery (Li and Duckles, 1991; Ralevic, Rubino and Burnstock, 1994). In reserpine pretreated rats, naloxone facilitated sensory-motor nerve evoked vasodilatation at low frequency of stimulation (Ralevic, Rubino and Burnstock, 1994). The authors suggested that sympathetic nerves were a source of opioids, although opioids may also be released from enteric nerves supplying these vessels (Lindh, Hokfelt and Elfvin, 1988).

Cholinergic and noradrenergic nerve terminals may be in close association with each other (Esterhuizen *et al.,* 1968). This has implications for possible prejunctional interactions between sympathetic vasoconstrictor mechanisms and parasympathetic and/or nitrergic vasodilator mechanisms. Sympathetic nerve stimulation attenuates the nasal vasodilator response to subsequent parasympathetic nerve stimulation by the activation of prejunctional Y_2-NPY receptors (Lacroix, Ulman and Potter, 1994). In the carotid artery, activation of prejunctional M_1-muscarinic receptors facilitates sympathetic neurotransmitter release (Casado, Marin and Salaices, 1992). However, the influence of prejunctional muscarinic receptors on sympathetic vasoconstriction appears to be vessel-dependent as, in the femoral artery, activation of sympathetic prejunctional M_3-muscarinic receptors inhibits noradrenaline release (Fernandes *et al.,* 1991).

In conclusion, auto-inhibition is not the only mechanism by which neurotransmission may be modified. A balance between vasoconstrictor and vasodilator nerves provides an additional level of control of vascular tone. This control is important for maintaining homeostasis. For example, an imbalance between vasodilator and vasoconstrictor neurons may have pathophysiological relevance for the etiology of hypertension (Holzer, 1992).

ARTERIAL RESISTANCE VESSELS

The greatest contribution to peripheral resistance in the body is provided by the small arteriolar vessels. In the 1820's, Poiseuille studied blood flow and the relationship

between flow of fluids through tubes of different internal diameter and lengths. A modified version of the equation Poiseuille derived for the resistance to flow is:

$$R = 8\eta/\pi.r^4$$

where R is the resistance to fluid flow, η is the fluid viscosity and r is the radius of the tube. This model shows that the resistance to flow is inversely proportional to r^4, thus a given degree of shortening of the smooth muscle in the vessel wall (ie. constriction) in a smaller diameter vessel will produce a much greater resistance to flow than in a larger diameter vessel. Conversely, dilatation of arterioles results in much greater change in flow than does dilatation of arteries. In fact, arterioles often have non-uniform diameters which means the resistance to flow in these vessels will be slightly greater than for a uniform tube used in the above model (Sugihara Seki, Minamiyama and Hanai, 1989). It is therefore important to understand the mechanisms which control arteriolar tone.

IRIS VASCULATURE

A transverse section through the dilator region of the albino rat iris shows that there are four layers comprising the iris tissue (Olson *et al.*, 1988). These are (anterior to posterior): a thin squamous epithelial-like layer of cells, the stromal layer, a single layer of myoepithelial cells of the dilator muscle and a cuboid epithelial cell layer. The relatively thick stromal layer of loose connective tissue contains chromatophores, blood vessels and nerve fibres. Large nerve bundles containing both myelinated and unmyelinated nerve fibres are found amongst the blood vessels of the iris stroma.

The eye has a dual vascular supply (Bill and Nilsson, 1985). The blood vessels of the uvea are distributed within the choroid, the ciliary body and the iris. The retinal blood vessels supply the inner parts of the retina.

In the iris two arterial circles arise from the ciliary arteries (Freddo and Raviola, 1982). The major arterial circle is formed from branches of the posterior ciliary arteries. These vessels are predominantly related to iris functions. The intramuscular circle is derived from branches of the anterior ciliary arteries. These vessels seem to be related functionally to the aqueous outflow and eye accommodation. A series of radial arterioles leaves the major circle of the iris, decreasing in diameter, to terminate in an

incomplete ring of arteriovenous anastomosis, known as the minor circle of the iris (Ojima and Matsuo, 1985; Rohen and Funk, 1994). The arterial vessels show a bended spiral course. The collecting venules running towards the iris root appear quite straight and drain the capillaries of the two capillary layers (Rohen and Funk, 1994).

INNERVATION

Vasoconstriction

The iris receives a rich sympathetic noradrenergic plexus (Olson *et al.*, 1988). The adrenergic nerves to the iris are derived from sympathetic nerve fibres originating from the ipsilateral superior cervical ganglion (Olson *et al.*, 1988). Stimulation of the cervical sympathetic chain reduces blood flow in all parts of the uvea, including the iris (Bill, 1975). Continuous stimulation at 10 Hz produced a steady-state near maximal reduction in total blood flow.

The distribution of NPY-like immunoreactive nerves closely mirrors the distribution of the catecholamine-containing sympathetic fibres in the eye (Stone, Kuwayama and Laties, 1987). Approximately 99% of these NPY-like immunoreactive fibres disappear after removal of the superior cervical ganglion (Olson *et al.*, 1988). A rich innervation of NPY-like immunoreactive fibres supplies the choroidal blood vessels of the albino rat, with a lower density around anterior uveal blood vessels (Stone, Kuwayama and Laties, 1987).

Interestingly, electrical stimulation of the oculomotor nerve (3rd cranial nerve) causes a redistribution of blood flow in the uvea, with a reduction of iris blood flow but an increase in choroidal blood flow (Stjernschantz, Alm and Bill, 1976).

Vasodilatation

Stimulation of the trigeminal ganglia causes an irritation response in the rabbit eye (Andersson and Bill, 1989). Neonatal capsaicin treatment abolishes the small-medium CGRP- and substance P-like immunoreactive neurons of trigeminal sensory ganglia; the large CGRP-immunoreactive neurons are unaffected (Matsuyama *et al.,* 1986).

Stimulation of the facial nerve produces a non-cholinergic increase in local blood flow of the choroid, and to a lesser extent, the uvea in monkeys, cats and rabbits, with near maximal effects obtained at 10 Hz (Bill and Nilsson, 1985). This increased blood flow was most likely to be due to vasodilatation as there was no associated change in mean arterial blood pressure (Bill and Nilsson, 1985).

VIP is proposed to mediate the non-cholinergic vasodilatation in the choroid (Nilsson and Bill, 1984). Intravenous adminisitration of VIP or PHI increases local blood flow in the choroid, but not the anterior uvea (Nilsson and Bill, 1984). VIP-like immunoreactive nerves have been demonstrated to supply arterioles of the choroid and ciliary body (Andersson and Bill, 1989; Terenghi *et al.,* 1982). The rat iris contains a relatively sparse irregular network of VIP-like immunoreactive nerves and the distribution of PHI-like immunoreactive nerves closely parallels that of VIP, suggesting the two peptides are co-localized (Olson *et al.,* 1988). There is no apparent association of VIP-like/PHI-like immunoreactive nerves with blood vessels of the rat iris, but VIP-like immunoreactive fibres have been found in association with the larger vessels in the choroid (Olson *et al.,* 1988). Extirpation of the pterygopalatine ganglion results in the loss of intra-ocular VIP-like immunoreactive nerve fibres, indicating that these nerve fibres were in the facial nerve (Andersson and Bill, 1989).

Nitric oxide plays a role in canine basal occular blood flow *in vivo*. Infusion of an inhibitor of the synthesis of nitric oxide, nitro-L-arginine, significantly reduced porcine and canine iris blood flow (Deussen, Sonntag and Vogel, 1993; Vogel and Sonntag, 1994). In the rat, NOS-like immunoreactivity has been demonstrated in the endothelium of intraocular blood vessels, particularly those in the choroid, iris and retina (Yamamoto *et al.,* 1993).

The posterior ciliary artery of the pig supplies both the uvea and the choroid (Su *et al.,* 1994). Electrical field stimulation of this vessel produced a NANC vasodilatation. This vasodilatation was endothelium-independent and reduced to half by inhibition of nitric oxide synthesis (Su *et al.,* 1994). This suggests that nitrergic nerves may innervate some ocular blood vessels and contribute to intra-ocular NANC vasodilatation.

NOS-like immunoreactivity has been localized in nerve fibres supplying the rat choroidal blood vessels, with the anterior uvea being sparsely innervated (Yamamoto *et al.,* 1993). Many neurons in the pterygopalatine ganglion showed NOS-like immunoreactivity and virtually all of these showed VIP-like immunoreactivity (Yamamoto *et al.,* 1993). Pterygopalatine ganglionectomy significantly reduced the number of peripheral NOS-like immunoreactive fibres in the eye, indicating these fibres were mainly in the facial nerve (Yamamoto *et al.,* 1993).

Taken together, the above results indicate that VIP, PHI and nitric oxide act as a co-transmitters for non-cholinergic vasodilatation in the choroid, but not the uvea, of the rat eye. In addition, the iris of the rat receives a rich supply of sensory nerves but they have no apparent close association with iris blood vessels (Olson *et al.,* 1988).

THESIS OUTLINE

The aim of this thesis was to characterize the nerve-mediated control of the diameter of arterioles found within the mature rat iris *in vitro*. The iris of the albino rat was chosen for the present study as it is well supplied with small arterial vessels which are readily visualized, thus making it a suitable preparation for *in vitro* physiology experiments. The study has been divided into the following chapters:

Chapter 2. An outline of the methods and materials used in this thesis.

Chapter 3. The role of sympathetic nerves in producing vasoconstriction.

Chapter 4. The mechanism of sympathetic vasoconstriction.

Chapter 5. The role of sensory-motor nerves in modulating vasoconstriction.

Chapter 6. The mechanism of action of sensory-motor nerve modulation of sympathetic vasoconstriction.

Chapter 7. The potential role of sensory-motor nerves in modulating other

sympathetic synapses.

Chapter 8. General discussion arising from the results of this thesis.

CHAPTER 2.

MATERIALS AND METHODS

ANIMALS

An unpigmented strain of Wistar rats (4 to 6 weeks old, of either sex) was used to allow visualization of arterioles in whole mount preparations of the iris for *in vitro* physiology experiments. The rats had access to water and food pellets *ad libitum*.

IN VITRO PREPARATION

Rats were killed with an overdose of anaesthetic ether. Immediately afterwards, both eyes were dissected out, the lens removed and the iris placed in a Krebs solution of the following composition (mM): NaCl, 119.8; KCl, 5.0; $NaHCO_3$ 25.0; NaH_2PO_4, 1.0; $CaCl_2$ 2.5; $MgCl_2$ 2.0; glucose 22.0, which was gassed with 95% O_2/5% CO_2. Hyoscine hydrochloride (1 μM) was added to the Krebs solution to prevent the possible activation of muscarinic receptors following transmural nerve stimulation.

Each iris contained, on average, four main arterioles. The iris was cut in half so that at least one arteriole was in each preparation. Half an iris was pinned out flat, anterior surface uppermost, by pins along the corneal and sphincter edges and along both cut edges of the dilator, in a 1 ml recording chamber, the base of which consisted of a coverslip coated with silicon resin (Sylgard 184, Dow Corning Corporation, Midland, USA). Figure 2.1 shows a diagramatic representation of a typical iris whole mount. For experiments examining elevated cyclic AMP, SarSP and capsaicin, the sphincter muscle was cut off before pinning out the preparation to prevent movement of the preparation, as these drugs cause constriction of the sphincter muscle. Preparations were viewed with an inverted compound microscope (Zeiss, Germany). The recording chamber was continuously perfused at 3 $ml.min^{-1}$ with carbogenated Krebs solution which was maintained at 33 to 34 ^{o}C, pH of 7.4. For experiments involving changes in bath temperature, the temperature was continuously monitored using a probe which was connected to a digital thermometer (HH71 thermometer, Omega Engineering Inc., Stamford, USA).

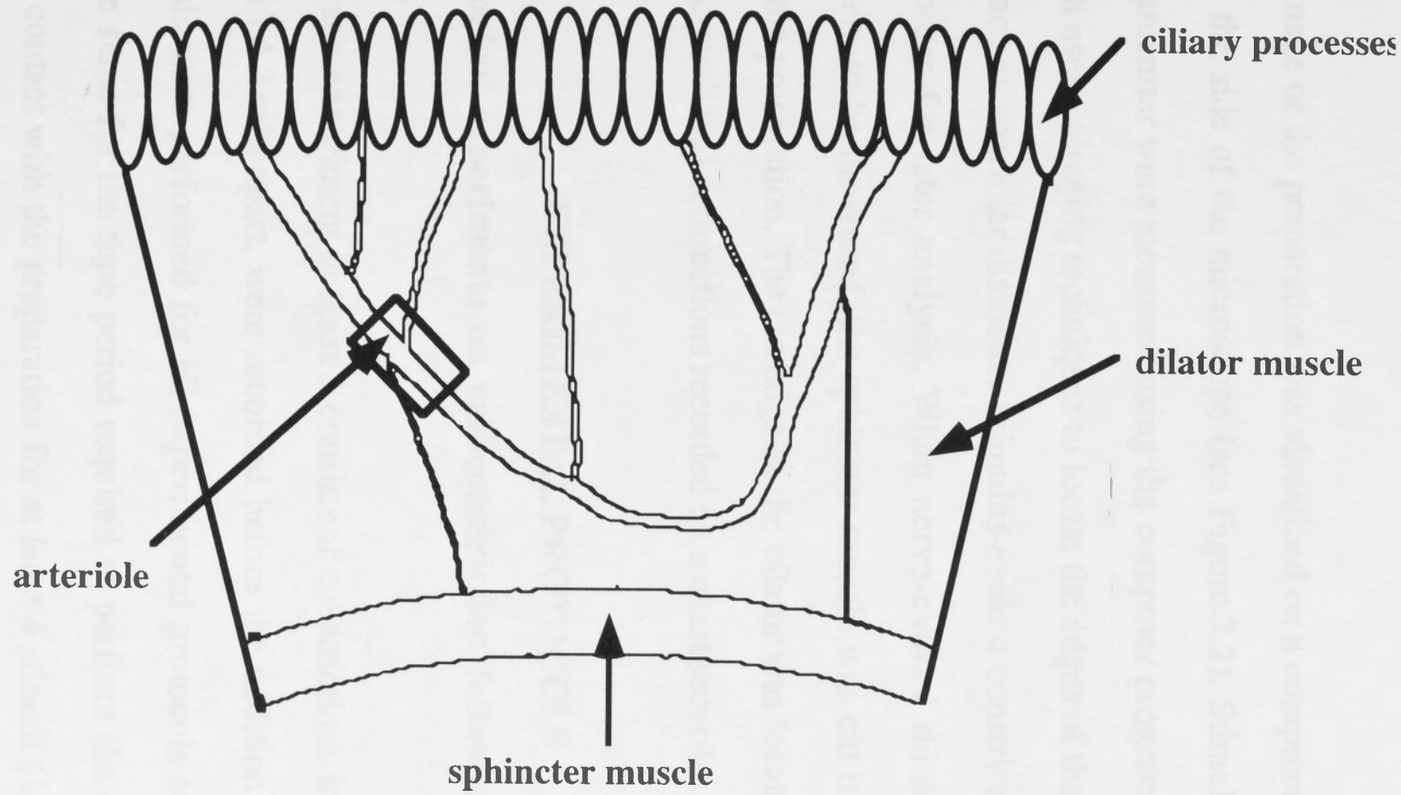


Figure 2.1 Diagram showing a typical iris whole mount preparation. Box indicates the general site of the arteriole on which experiments were done.

The preparations were equilibrated for 30 minutes before commencing nerve stimulation. Transmural nerve stimulation was applied to the iris preparation via two platinum electrodes positioned in the silicon resin, near the iris sphincter and corneal edges. Stimulation parameters were: pulse duration 0.1-0.15 ms, 10 Hz for 1 s every 3 min, 50-60 V or 100 mA. A section of arteriole was chosen, at the site indicated in Figure 2.1, where the vessel diameter in the stretched preparation was in the range of 30 to 50 μm.

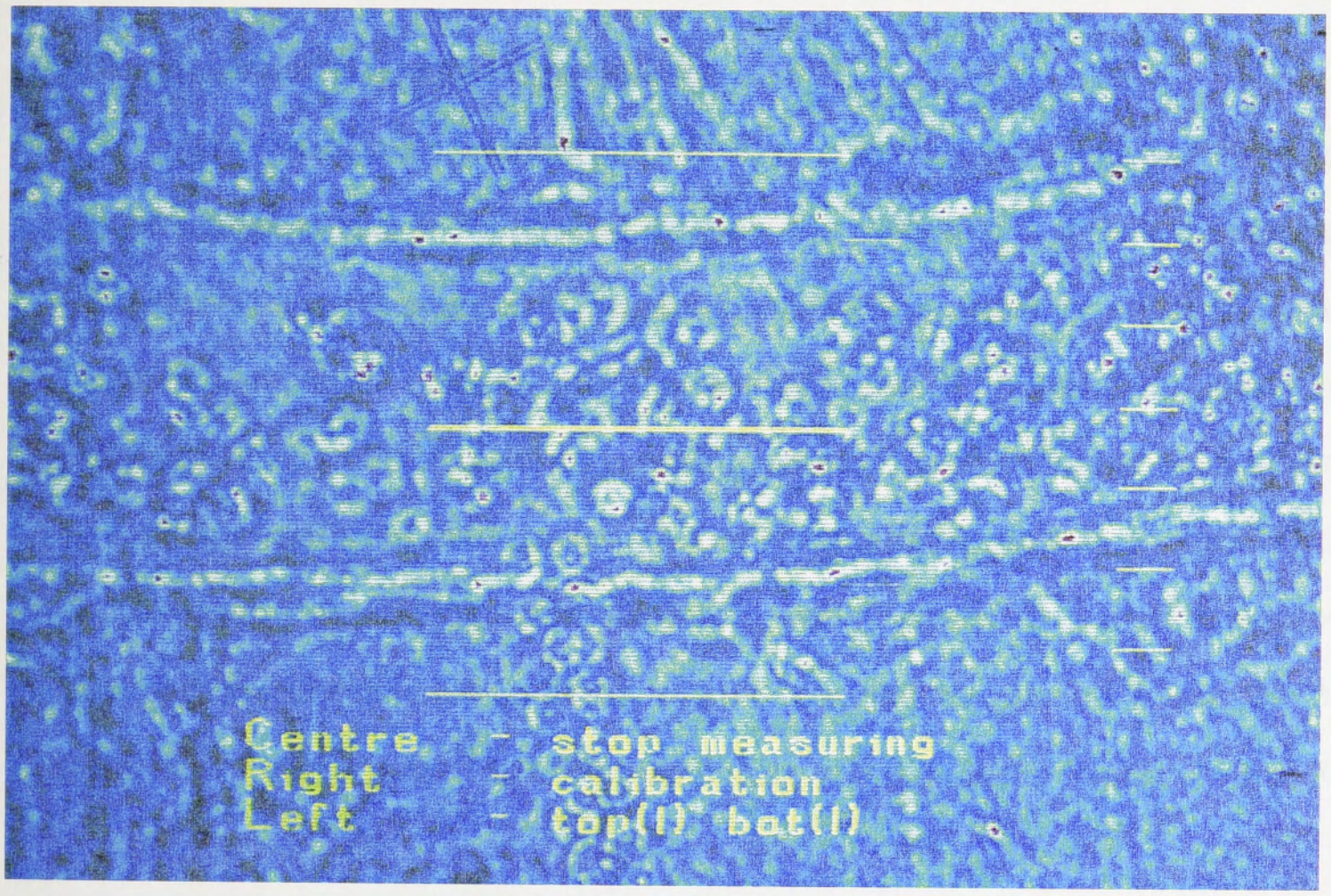
An image of the preparation was visualized on a computer monitor via a camera mounted on the side of the microscope (see Figure 2.2). Stimulus-evoked changes in arteriolar diamemter were measured using the computer program DIAMTRAK (Neild, 1989), which uses averaging techniques to locate the edges of the arterioles and records their difference as arteriolar diameter. Stimulus-evoked constrictions were recorded on a chart recorder for later analysis. When nerve-evoked dilator muscle contractile responses were to be measured, the sphincter muscle was cut off from the iris before pinning out the preparation. The cut edge of the dilator was located using DIAMTRAK and the nerve-mediated contractions recorded on a chart recorder.

EXPERIMENTAL PROTOCOLS

Pharmacological experiments on vasoconstriction following nerve stimulation every 3 min

For each experiment at least 3 consistent constrictions in response to trains of stimuli, applied 3 min apart, were recorded before the addition of any drug solution. Time controls were performed for all experimental protocols to show that responses remained the same for the time period required to perform the experiment. All drugs remained in contact with the preparation for at least 4 stimuli (12 min) or were left in, for up to 30 min, until the maximal response to the drug was obtained and responses became consistent with successive stimuli. Only one concentration of a drug was added to each preparation to prevent complications arising from possible desensitization of responses.

Figure 2.2 Photograph showing the segment of an iris arteriole corresponding to the marked site in Figure 2.1. The computer program, DIAMTRAK, uses the yellow dashes on the edges of the arterioles to measure arteriole diameter. Separation between calibration lines equals 10 μm.


Centre - stop measuring
Right - calibration
Left - top(l) bat(l)

The α–adrenoceptor antagonist, benextramine, acts by irreversibly alkylating the receptor and may also have other reversible actions (Melchiorre *et al.,* 1978; Plotek and Atlas, 1983). The effect of benextramine was therefore tested by perfusing the chamber with benextramine until the maximal effect on the nerve-mediated responsewas achieved and then returning to control Krebs for 10 to 15 min.

In experiments testing the effect of forskolin in the presence of capsaicin, L-NAME or D-NAME, these last 3 drugs were present in the Krebs for the entire experiment. After 30 min equilibration in the drug and after obtaining 3 to 4 responses to nerve stimulation, forskolin was added.

Exogenous noradrenaline experiments

The maximum magnitude of the constriction produced by perfused noradrenaline after 1 to 3 min of application was expressed as a % of the resting vessel diameter prior to the addition of noradrenaline. To investigate the effect of extracellular calcium removal on responses to noradrenaline, 3 to 4 control contractions to nerve stimulation were obtained before perfusing with calcium-free Krebs. Stimulation of the preparation continued until the response to nerve stimulation was gone, indicating that the removal of the calcium from the Krebs was effective. At this point noradrenaline was perfused in calcium-free Krebs, and the maximal constriction within 1 to 3 min of application was expressed as a % of the resting vessel diameter in calcium-free Krebs.

Capsaicin treatment

Newborn rats were injected with capsaicin (50 $mg.ml^{-1}$ in 10 % ethanol, 10 % Tween 80 in saline) on post-natal days 2, 10 and 17. Animals were visually monitored and kept warm until recovery from the injection. This treatment has been reported to lead to the long-term loss of substance P-containing nerves from the iris of rats (Hill and Vidovic, 1989). Control littermates were injected following the same regime with vehicle only. At 6 to 8 weeks of age the rats were killed and both irides removed. Contractile responses to trains of stimuli every 3 min and every 15 s were recorded from arterioles taken from vehicle- and capsaicin-treated rats.

Removal of superior cervical ganglia

Littermate weaner Wistar rats were anaesthetised (Ketamine hydrochloride 25 $mg.kg^{-1}$, Rompen 8 $mg.kg^{-1}$, i.p.). Rats were laid ventral side up and neck region shaven before removing both superior cervical ganglia, via an incision made in the skin. The incision was sewn up and the rats were monitored until recovery from the anaesthetic. Rats were killed 7-12 days after surgery and both eyes removed and used for *in vitro* experiments. One preparation from each experimental group was processed for catecholamine fluorescence (Faglu, Furness *et al*, 1978) to demonstrate that the iris was denervated. Preparations taken from unoperated age matched rats served as controls for these experiments.

Exogenous capsaicin experiments

The acute effect of capsaicin was studied on untreated rats. Control responses to nerve stimulus trains every 3 min and every 15 s were recorded. The preparations were then perfused with capsaicin and stimulated every 3 min. When the vasoconstrictor responses had returned to precapsaicin levels, the preparations were stimulated with trains of pulses every 15 s. Control experiments over comparable time periods showed vehicle had no effect on the loss of nerve stimulation with trains of pulses every 15 s. Contractile responses in the above experiments were expressed as a % of the resting vessel diameter.

Effect of antagonists on responses to nerve stimulation every 15 s

The effect of a number of receptor antagonists was tested on the contractile response elicited by trains of stimuli every 15 s. Three responses to trains of stimuli every 3 min were recorded in control Krebs and 3 min later, six trains of stimuli were applied every 15 s. Five min after the last pulse of this stimulus, trains of stimuli recommenced every 3 min. When responses to stimulus trains every 3 min were the same magnitude as control responses, the antagonist was added and stimulation every 3 min continued in the presence of the antagonist.

L-NAME, a pseudo-substrate for NOS; D-NAME, the inactive isomer of L-NAME; or the $CGRP_1$-receptor antagonist, $CGRP_{8-37}$; were perfused for 20 min, and RP 67580, a NK_1-tachykinin receptor antagonist; and L-659877, a NK_2-tachykinin receptor antagonist; either singly or together, were perfused for 30 min, before stimulating with six trains of stimuli every 15 s.

L-NAME (10 μM) significantly reduced the vessel diameter (80 + 3 % of control diameter, n=5, compared with 95 + 2 %, n=3, in the presence of D-NAME, unpaired t-test). This occurred within 10 to 15 min after the addition of L-NAME and indicated L-NAME was present and effective within the tissue.

RP 67580 has been shown to be a highly selective competitive antagonist for NK_1-tachykinin receptors, and at 1 μM is reported to have no cross reactivity with NK_2- or NK_3-tachykinin receptors (Garrett *et al.*, 1991). L-659877 has been similarly

shown to be selective for NK_2-tachykinin receptors (Maggi *et al.*, 1991; McKnight *et al.*, 1991) and was used at 1 μM in this study.

Intracellular recordings

Irides for intracellular recording experiments were taken from 3 to 4 week old Wistar rats, of either sex, as it was easier to make stable impalements of arterioles taken from rats at this age, compared to arterioles from 4 to 5 week old rats. The sphincter muscle was cut off before pinning out the iris to assist in the immobilization of the arteriole. Experiments were undertaken in an electrically shielded room. Intracellular recordings were made using conventional techniques with fine borosilicate glass electrodes (1.5 mm O.D x 0.86 mm I.D., Clark Electromedical Instruments, England), filled with 0.5 M KCl and having resistances of 100-200 MΩ. The earth electrode consisted of a covered Ag-AgCl plug attached to a silver wire.

Membrane potential was measured with an Axoclamp 2A (Axon Instruments, Foster City, California, USA). Impalements were achieved by gentle tapping of the microelectrode manipulator. Successful impalements were associated with a downward deflection on the voltage record. Deflections of less than 40 mV were considered to represent poor impalements. Sometimes after a deflection of less than 40 mV the membrane potential would continue decreasing over the next few minutes and the electrode would "seal" in a cell. Stable intracellular recordings could be maintained for up to 2.5 hours. The resting membrane potential was obtained by the difference between the potential of the electrode in the cell and the potential when the electrode was withdrawn from the cell, and was determined for each impalement. All membrane potential records were low-pass filtered with a cut-off frequency of 1 KHz.

Simultaneous changes in membrane potential and changes in vessel diameter, in the region where the cell was impaled, in response to nerve stimulation were stored on computer disk for later analysis.

In one series of experiments the individual responses to 1 to 10 pulses at 10 Hz were recorded for cells showing depolarizing responses. In other experiments, the effect of a drug on nerve-mediated contraction and depolarization was measured by recording

3 to 4 control responses and then changing from the control solution to Krebs containing the appropriate concentration of the drug. Throughout all of these experiments, the responses to nerve stimulation (10 Hz) were measured every 2 to 3 min. Drugs were perfused for at least 10 min, or until the maximal effect on the contraction and depolarization was recorded.

DATA ANALYSIS

Pharmacological experiments on nerve-evoked vasoconstriction

For each experiment, the 3 to 4 consistent responses to nerve stimulation obtained before the addition of a drug were averaged to obtain a mean control response. When the effect of a drug was maximal, 3 contractile responses to nerve stimulation were averaged and expressed as a % of the control response. For each drug, this % value was obtained for a number (n) of preparations from different animals and the combined results expressed as a mean ± s.e.m. Some of the drugs tested caused a change in resting vessel diameter. In order to standardize for the drug-induced change in diameter, in addition to the effects on nerve-mediated contractions, the nerve-mediated contractile response in drug solution was expressed as a ratio of the resting vessel diameter in drug solution prior to the stimulus, and then as a % of the response in control solution, where the latter control contraction was also expressed as a ratio of the resting vessel diameter. In the case of noradrenaline, the maximum magnitude of the contractile response was expressed as a % of the resting vessel diameter.

Intracellular recordings

The magnitude of the depolarization and the time to maximum depolarization from the first pulse of the stimulus were measured. Contractions were analysed for the magnitude of the contraction, the time from the first pulse of the stimulus to the onset of contraction and the time from the first pulse of the stimulus to peak contraction. The maximal effect of a drug on the nerve-evoked depolarization was expressed as a % of the nerve-evoked depolarization in control solution. Results are expressed as mean ± s.e.m. of a number of preparations.

Statistics

Where appropriate, statistical significance of a drug effect was tested using Student's t-test. If multiple comparisons were made, an analysis of variance with 95% confidence limits was performed to show a difference between treatment groups, followed by Student's t-tests with Bonferroni correction for multiple groups. A probability, P, of <0.05 was taken as statistically significant.

The half maximal inhibitory concentration (IC_{50}) for α–adrenoceptor antagonists was determined from responses lying between 20 % and 80 % on a fitted linear regression.

IMMUNOHISTOCHEMISTRY

Freshly dissected irides were stretched, pinned over sylgard and fixed in Zamboni fixative (2 % paraformaldehyde, 0 2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) for 1 h at 4 ^{o}C. Picric acid was removed by washes in 80% ethanol. The whole mounts were then treated with dimethyl sulphoxide (3 x 10 min), washed in phosphate buffered saline (PBS), preincubated in PBS containing 0.2% Triton-X and 7 $mg.ml^{-1}$ carrageenan for 10 min before incubating in rabbit antibodies against SP (1:400, Auspep Pty. Ltd., Australia) or CGRP (1:600, Milab, Sweden) for visualizing sensory nerves, or mouse monoclonal antibodies against tyrosine hydroxylase (1:10, Boehringer-Mannheim) for visualizing adrenergic nerves. Substance P and CGRP were detected following incubation in FITC-anti-rabbit IgG (1:100, Wellcome) and tyrosine hydroxylase following sequential incubation in biotinylated anti-mouse IgG (1:200, Vector Labs) and Texas Red conjugated to streptavidin (1:200, Amersham). Irides were also fixed overnight before sectioning on a cryostat at 10-μm. Sections were stained as above with the antibody for substance P (1:1500) and the antibody against tyrosine hydroxylase (1:40).

Preparations were mounted in buffered glycerol and viewed with an Olympus BH microscope with appropriate blue and green filter combinations (barrier filters 530 and 610 nm respectively) or with a Leica TCS-4D confocal microscope fitted with

Photographs were taken using Ektachrome 160 ASA film in camera mounted on Olympus microscope. Reconstructed confocal images were stored as PICT files and photographs developed by the Photography Unit at John Curtin School of Medical Research.

Omnichrome Krypton laser. No fluorescent structures were seen in preparations for which the primary antibodies were omitted, or in preparations exposed to primary antibody solutions which had been previously incubated at 4 ^{o}C overnight with the appropriate antigen.

REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR)

RNA isolation

Total cellular RNA was extracted from freshly dissected iris tissue using a single-step protocol for RNA isolation ('RNAzol B', Cinna/Biotecx). Both the sphincter muscle and the ciliary processes were dissected from the iris, leaving the dilator muscle and stroma. The manufacturer's recommended protocol for isolation of RNA was followed. Briefly, tissue was collected into RNAzol solution, homogenized at room temperature and the homogenate separated into two phases. The RNA was precipitated from the aqueous phase with isopropanol and the RNA pellet dissolved in water. Optical density readings were made to estimate the total amount of RNA before it was used in reverse transcription-polymerase chain reaction (RT-PCR).

Oligonucleotide primers

Oligodeoxynucleotide primers for the 3 tachykinin receptors for use in RT-PCR were synthesized using an Applied Biosystems Model 380B Nucleic Acid Synthesizer and solid phase synthesis. At the time of these experiments, CGRP receptors had not been sequenced and so their expression could not be investigated. Oligonucleotides for the rat NK_1-tachykinin receptor were designed from published sequences of cDNA isolated from rat brain cDNA library (Yokota *et al.*, 1989); the forward primer 5'-CACGGTCATCGTGGTGACTTCCGT-3' and reverse primer 5'-ACAGTGTACGCGTAGCCGATCACC-3' were used to generate a 537 base pair

fragment. The cDNA sequence for the rat NK_2-tachykinin receptor, derived from RNA isolated from the rat stomach (Sasai and Nakanishi, 1989), was used to design the forward 5'-ATCAACCTGGCCTTGGCGGACCTC-3' and reverse primer 5'-ATGACACTGTAAGCCCCGAACATC-3' which generated a 443 base pair fragment. For the NK_3-tachykinin receptor, the forward primer 5'-GCAGTCTTCGGAAACCTCATCGTT-3' and reverse primer 5'-CAGTACACCAGGATGATCACAATG-3' were designed from the sequence obtained from rat brain cDNA (Shigemoto *et al.*, 1990) and generated a 476 base pair fragment.

The three sets of primers were designed to span introns to avoid false positives being generated from the RT-PCR due to any genomic DNA present in the extracted RNA. The ability of the primer sets to amplify the appropriate message was tested by using cDNA reverse-transcribed from RNA isolated from the rat cerebral cortex. A cDNA fragment of the predicted size was generated by each set of primers.

RT-PCR

RNA (3.3 μg) was reverse transcribed with oligo dT (100 $ng.\mu l^{-1}$) or random primer (100 $ng.\mu l^{-1}$). Samples were incubated for 10 min at 65 ^oC, cooled at room temperature and the following reagents added: 5 μl 10X first strand buffer, 1 μl RNase block, 2 μl 25 mM dNTP and 1 μl reverse transcriptase (StrataScript RNase H-, 50 U/μl). The reaction was carried out in a volume of 50 μl and 2 μl from this cDNA pool was used in each PCR reaction using either the NK_1-, NK_2- and NK_3-tachykinin receptor primers.

The reaction mixture for the PCR comprised 10 mM Tris-HCl, pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, 0.1% Triton X-100, 200 μM of each of the deoxyribonucleoside triphosphates (dNTPs) dATP, dCTP, dGTP and dTTP, 20 pmole of forward and reverse primer and 0.2 U Supertaq DNA polymerase per 20 μl reaction (P.H. Stehelin and Cie, Ag, Basel, Switzerland). Samples were heat sealed and 30 cycles of PCR carried out in a capillary tube thermal cycler (Corbett Research, Sydney, Australia). The first cycle consisted of denaturation at 94 ^oC for 1 min, annealing at 63 ^oC for 1 min, and extension at 72 ^oC for 1 min, the remaining cycles consisted of

94 °C for 10 s, 63 °C for 10 s and 72 °C for 59 s. The amplification products were separated in 2 % agarose gel, stained with ethidium bromide and visualized using UV light.

AUTORADIOGRAPHY

Freshly dissected iris whole mounts were stretched on glass slides, which had been coated with a 2 % solution of 3-aminopropyltrithoxysilane, air dried and stored frozen at -70 °C until used. Iris tissue was frozen rapidly in mounting medium (CryO-M-bed, Bright Instrument Company Limitied, Huntingdon, England) and 10 µm sections cut on a cryostat, thaw mounted onto coated slides and stored dry at -70 °C.

For labelling with [^{125}-I]-Bolton-Hunter-[Sar^{9}, Met(O_2)^{11}]SP (BHSarSP), slides were preincubated in 3 x 5 min washes of 50 mM Tris-HCl buffer containing 0.02 % bovine serum albumin (BSA, pH 7.4, 25 °C). Slides were then incubated at 25 °C for 60 min in 50 mM Tris-HCl buffer containing 0.02 % BSA (pH 7.4, 25 °C), 3 mM $MnCl_2$, 1 µM phosphoramidon and 4 µg.ml^{-1} chymostatin at pH 7.4 with 75 pM BHSarSP. Non-specific binding was determined on some slides by co-incubating with 1 µM unlabelled [Sar^{9}, Met(O_2)^{11}]SP (SarSP).

For [^{125}I]-CGRP labelling, slides were preincubated in 2 x 5 min washes in 50 mM Tris-HCl buffer containing 0.02 % BSA (pH 7.4, 25 °C) followed by a third 5 min wash in the same buffer containing 40 µg.ml^{-1} bacitracin. Slides were then incubated for 60 min in 50 mM Tris-HCl buffer containing 0.02 % BSA (pH 7.4, 25 °C), 100 mM NaCl, 1 µM phosphoramidon, 40 µg.ml^{-1} bacitracin, 4 µg.ml^{-1} chymostatin and 60 pM [^{125}I]-CGRP (pH 7.4, 25 °C). Non-specific binding was determined on some slides by co-incubation with 1-µM CGRP.

After incubation, all slides were washed (4 x 4 min for sections; 3 x 10 min for whole mounts) in 50 mM Tris-HCl buffer (pH 7.4 at 4 °C) containing 0.02 % BSA, fixed in ice-cold 4 % paraformaldehyde in phosphate buffered saline (PBS), rinsed in ice-cold distilled water and dried rapidly. Labelled slides were dipped into liquid photographic emulsion (LM-1, Amersham) at 42 °C and allowed to expose in the dark at 4 °C for 13 days (BHSarSP) or 8 days ([^{125}I]-CGRP). Slides were then developed

(developer from Kodak, Australasia), stained with pyronin (pH 7.4) and viewed using light and dark field microscopy (Burcher *et al.*, 1986). Adjacent sections were stained with haematoxylin and eosin.

MATERIALS

Drugs

The following drugs were purchased commercially:

isethionic acid, sodium salt (Acros Organics, New Jersey, USA); caffeine (Ajax Chemicals, Sydney Australia); $CGRP_{1\text{-}37}$ (rat), $CGRP_{8\text{-}37}$ (rat), [Sar^{9}, Met $(O_2)^{11}$]-substance P (Auspep, Melbourne, Australia); capsaicin (Fluka Chemie, Switzerland); paraformaldehyde (ICN Biomedical, USA); mecamylamine hydrochloride (Merck, Sharp and Dome, South Granville, NSW, Australia); chloroethylclonidine (CEC), L-659877, phenylephrine hydrochloride, [2-(2,6-dimethoxyphenoxyethyl)-minomethyl-1,4-benzodioxane] (WB4101) (Research Biochemicals International, Natick, USA); dideoxyadenosine, N^{G}-nitro-D-arginine methyl ester hydrochloride (D-NAME), N^{G}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sapphire Bioscience Pty Ltd, Sydney, Australia); (-)-arterenol bitartrate (noradrenaline), bacitracin, benextramine tetrachloride, captopril, cholera toxin, chymostatin, N^{6},2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dibutyryl cyclic AMP), dantrolene sodium salt, diltiazem hydrochloride, forskolin, flufenamic acid, hexamethonium chloride, (-)-hyoscine (scopolamine) hydrochloride, 3-iso-butyl-1-methyl-xanthine (IBMX), nifedipine, phosphoramidon, prazosin hydrochloride, thapsigargin, verapamil hydrochloride, yohimbine hydrochloride (Sigma Chemical Co, St Louis, USA).

RP 67580 was kindly supplied by Rhone-Poulenc Rorer, France. All other reagents and chemicals were of analytical grade.

Drug solutions

All drugs were made up daily as 1000 times stocks. Stock solutions for capsaicin (10 mM), flufenamic acid (250 mM), forskolin (50 mM), IBMX and nifedipine (1 mM, 10 mM) were dissolved in absolute alcohol. Noradrenaline (10 mM) stock was dissolved in 2 %

ascorbic acid. Prazosin was dissolved in methanol (10 % volume) and made up to volume with water. RP 67580 was dissolved in 5 μM methanesulfonic acid, L-659877 was dissolved in 0.05 % dimethylformamide. Control experiments using Krebs containing 1 part per 1000 of diluent showed there was no effect of any of these diluents on arteriolar responses.

Caffeine and dibutyryl cyclic AMP were dissolved directly into Krebs by stirring and gentle heating. All other drugs were dissolved in deionized distilled water as stock solutions (1000 x).

Peptides were dissolved in deionized distilled water, aliquoted and stored at -20 ^{o}C until used. Concentrated solutions of peptides used for autoradiography (500 μM) were stored in 10 mM acetic acid (containing 1 % ß-mercaptoethanol) at -20 ^{o}C until use.

All stock solutions of drugs were diluted into Krebs to give the appropriate bath concentration. Solutions of light-sensitive drugs were protected from light using aluminium foil and illuminating preparations with only long wavelength (>610 nm) light.

For calcium-free Krebs, the calcium was omitted from the Krebs. For low chloride experiments, the NaCl in the Krebs was replaced with an equimolar quantity of sodium methyl sulfate or sodium isethionate. For 50 mM KCl, the NaCl concentration in the Krebs was reduced by the corresponding equimolar amount.

[^{125}I] Bolton Hunter reagent was purchased from Amersham, U.K. The radioligands were prepared by Mr. J. Strigas as previously described (Burcher *et al*, 1986).

purified as previously described (Burcher and Buckley) by Mr. J. Strigas.

CHAPTER 3.

SYMPATHETIC VASOCONSTRICTION

INTRODUCTION

Activation of sympathetic nerves plays an important role in the regulation of arterial resistance and blood flow (Osswald and Guimaraes, 1983). The terminal regions of sympathetic axons bear small swellings, or varicosities, in which noradrenaline is stored (Gordon-Weeks, 1988). Electron microscopical studies of reconstructed serial sections of blood vessels have shown synaptic neuroeffector specializations where varicosities come into close contact with the smooth muscle (Luff and McLachlan, 1989). Neurotransmitter released by sympathetic nerve stimulation is proposed to act upon synaptic receptors, whereas circulating catecholamines act upon extrajunctional receptors (Hirst *et al.,* 1992).

Pharmacological studies on nerve-mediated contractile responses of arteries have illustrated differences in the receptors(s) mediating constriction in different vessels. Postjunctional α_1–adrenoceptors are activated by noradrenaline released from sympathetic nerves to produce vasoconstriction in vessels such as the pulmonary artery (Suzuki, 1983) and the immature rat mesenteric artery (Hill, Hirst and van Helden, 1983), while α_2-adrenoceptors reportedly mediate neurogenic vasopressor responses in cutaneous vessels (Flavahan *et al.,* 1984; Medgett and Ruffolo, 1987). Specialized junctional γ-adrenoceptors have been implicated in nerve-mediated responses in vessels such as the rat basilar artery (Hirst, Neild and Silverberg, 1982), the rat mesenteric artery (Hill, Hirst and van Helden, 1983) and the vasculature of the rabbit hindlimb (Hirst and Lew, 1987). Other proposed mediators of sympathetic vasoconstriction include NPY or ATP, both of which have been implicated as sympathetic nerve co-transmitters with noradrenaline (Hanko *et al.,* 1986; Burnstock, 1972).

Activation of junctional γ-adrenoceptors produces excitatory junction potentials, which summate upon repetitive nerve stimulation to cause the opening of voltage-dependent calcium channels for the influx of calcium and vasoconstriction (Hirst and

Edwards, 1989). Only small membrane potential changes are, however, associated with α–adrenoceptor mediated constriction of the rat tail artery (Cheung, 1984). Furthermore, as the constriction precedes the slow depolarization for this α–adrenoceptor mediated response, it has been suggested that the calcium required for α–adrenergic contraction is released from intracellular or membrane-bound calcium stores (Hirst and Edwards, 1989).

The iris of the albino rat has a relatively rich vascular supply (Bill and Nilsson, 1985). The iris arterioles have a plexus of catecholamine-containing nerve fibres over their surfaces (Olson *et al.*, 1988). This chapter describes the sympathetic vasoconstriction of these iris arterioles to firstly, characterize pharmacologically the synaptic receptor type and secondly, to identify the source of calcium responsible for the constriction.

RESULTS

General observations

The main arterioles of the iris project at an angle from the ciliary edge out towards the sphincter before bending and returning to the ciliary edge (Figure 2.1). Smaller vessels arise from these arterioles and these give rise to capillaries which ultimately feed into radially collecting venules. The venules of the iris were generally of greater diameter than the arterioles and did not respond to nerve-stimulation. The vessels described in this thesis can been identified morphologically as arterioles, as defined by (Rhoden, 1967), as the vessel wall contains only a single layer of smooth muscle cells (S. Sandow, personal communication, 1995).

In all preparations, the arteriole showed some degree of pulsatile activity after the 30 min equilibration period, as observed by the rhythmic movement of red blood cells. In some preparations the arterioles showed regular spontaneous contractions (with a frequency of between 0.07 and 0.1 Hz). The venules showed no spontaneous activity.

The *in vitro* experiments were undertaken on arterioles in stretched preparations of iris. The diameter of these arterioles was between 20 to 40 μm. From observations of these arterioles at the ultrastructural level (S. Sandow, personal communication, 1995) it can be estimated that the measured diameter in stretched preparations would be approximately 20 % greater than the actual diameter *in vivo*.

As the arterioles project away from the ciliary body they become smaller in diameter. When the arteriolar diameter was less than approximately 20 μm the arteriole did not usually respond to nerve stimulation. Sphincters were frequently detected at the sites of origin of the smaller side branches from the main arteriole. Nerve stimulation caused these sphincters, and sometimes a short (5 μm) segment of the side branch, to constrict down to occlude blood flow down the side branch.

Response evoked by transmural sympathetic nerve stimulation

Transmural electrical stimulation (10 Hz, 1 s every 3 min) produces a consistent constriction of iris arterioles. Tetrodotoxin (1 μM; n=3) abolished these constrictions (Figure 3.1), indicating they were due to nerve stimulation (Adams and Swanson, 1994)

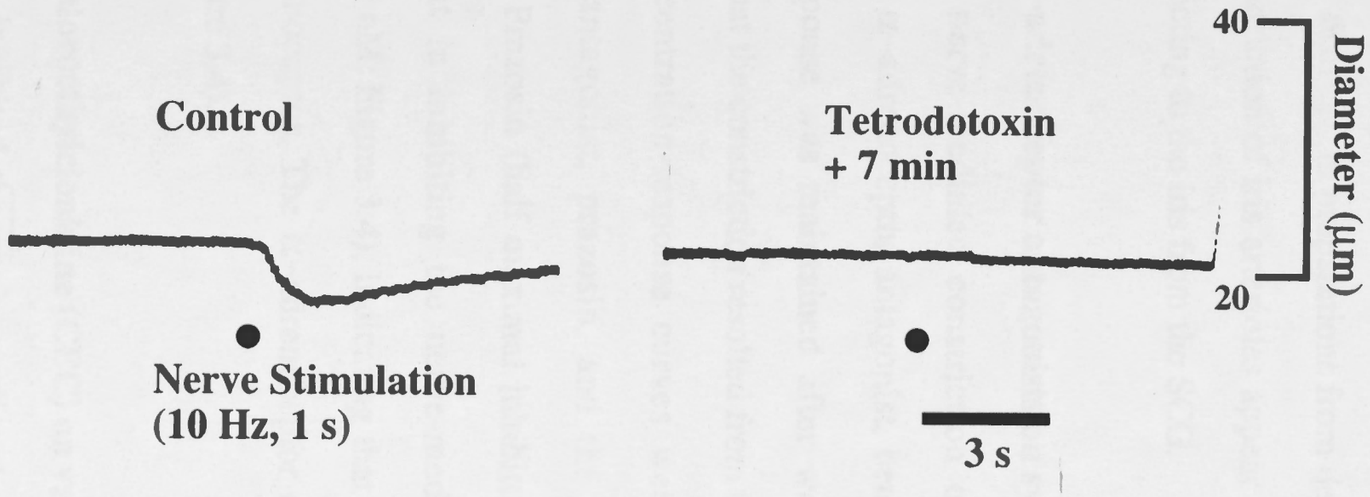


Figure 3.1 Chart recording showing the effect of tetrodotoxin (1 μM) on the nerve-mediated constriction of an iris arteriole. Dots indicate the onset of nerve stimulation (10 Hz, 1 s).

and not a result of direct muscle stimulation. Arterioles taken from rats killed 7 to 12 days after surgical removal of both superior cervical ganglia (SCG) showed no constriction in response to transmural nerve stimulation, although a long, slow vasodilatation was recorded in these arterioles (Figure 3.2 B). Nerve-mediated constrictions were recorded from iris arterioles taken from littermate control rats (Figure 3.2 A) and these were sometimes followed by long, slow vasodilatation although less often than in preparations from denervated animals (Table 3.1). Thus, nerve-evoked constriction of iris arterioles appear to be due to the activation of sympathetic nerves projecting to the iris from the SCG.

Effect of α–adrenoceptor antagonists on sympathetic vasoconstriction

The nerve-mediated constriction of iris arterioles was abolished by the irreversible α–adrenoceptor antagonist, benextramine (10 μM, n=3; Figure 3.3). The loss of response was maintained after washout with control Krebs (Figure 3.3), indicating that the constriction resulted from the activation of an α–adrenoceptor.

Concentration-response curves were constructed for the α_1–adrenoceptor preferring antagonist, prazosin, and the α_2–adrenoceptor preferring antagonist, yohimbine. Prazosin (half maximal inhibitory concentration = IC_{50} = 0.74 nM) was more potent in inhibiting the nerve-mediated vasoconstriction than yohimbine (IC_{50} = 260 nM; Figure 3.4), indicating that the response was due to the activation of an α_1–adrenoceptor. The α–adrenoceptor antagonist, WB4101 showed an IC_{50} of 14 nM (Figure 3.4).

Effect of Chloroethylclonidine (CEC) on vasoconstriction

CEC inhibited the nerve-mediated contraction of iris arteroles (Table 3.2), suggesting the involvement of α_{1B}-adrenoceptors.

A postjunctional action of CEC was confirmed by studying vasoconstriction to noradrenaline. Perfusion of the bath with noradrenaline produced a concentration-dependent arteriolar constriction (Figure 3.5 A). Noradrenaline (1 μM; n=3) produced a constriction which was not significantly different from that produced by nerve-

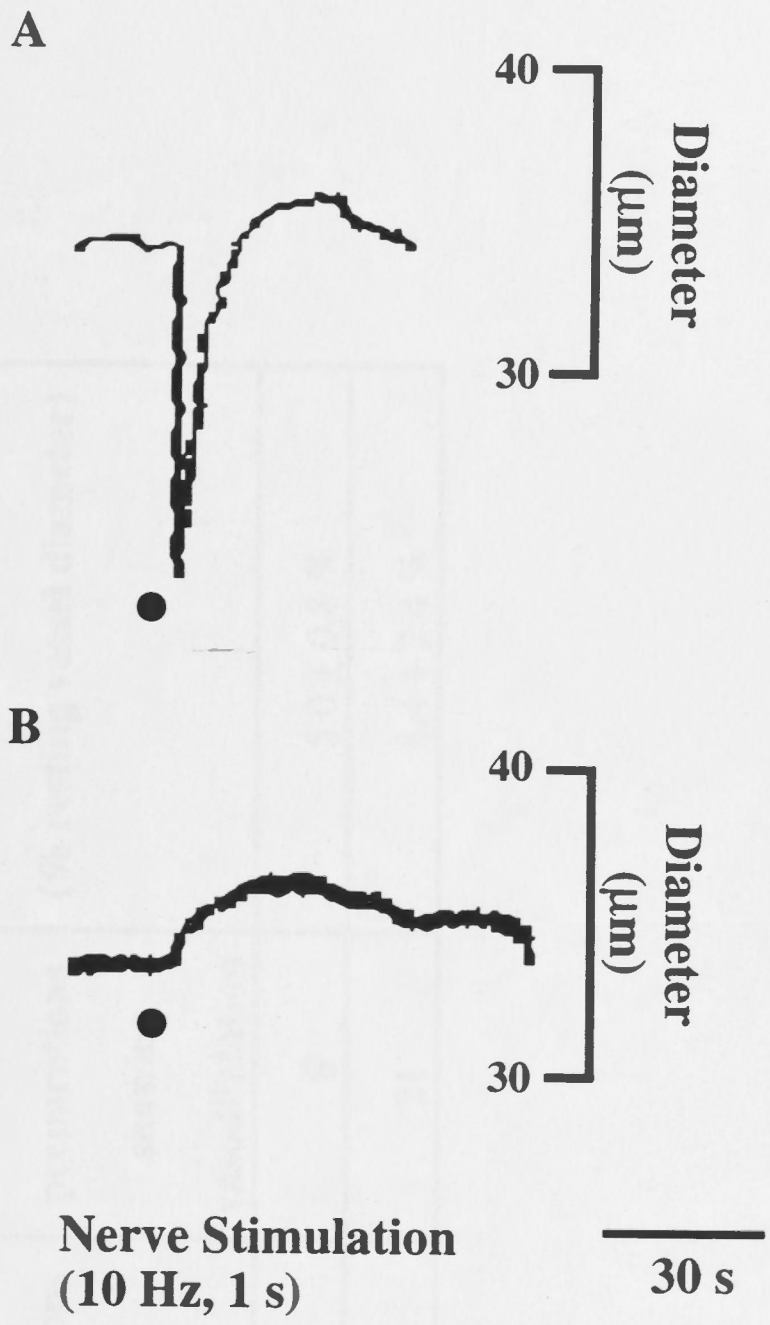


Figure 3.2

A. Chart recording showing the response to nerve stimulation of an iris arteriole taken from a control littermate.

B. Representative chart recording showing the response to nerve stimulation of an iris arteriole taken from a rat 7 to 12 days after the surgical removal of both SCGs.

Dots indicate the onset of nerve stimulation (10 Hz, 1 s every 3 min).

Table 3.1

Quantitation of vasodilatation measured after nerve-stimulation in litter-matched control unoperated and double-denervated animals.

Animal group	**Total # of preparations**	**# of preparations showing vasodilatation**	**Magnitude of vasodilation (% resting vessel diameter)**
Control unoperated (12)	21	6	5.0 ± 0.8 %
Double denervation (11)	29	21	8.4 + 2.4 %

( ) indicates number of animals in each group.

Values are expressed as mean ± s.e.m.

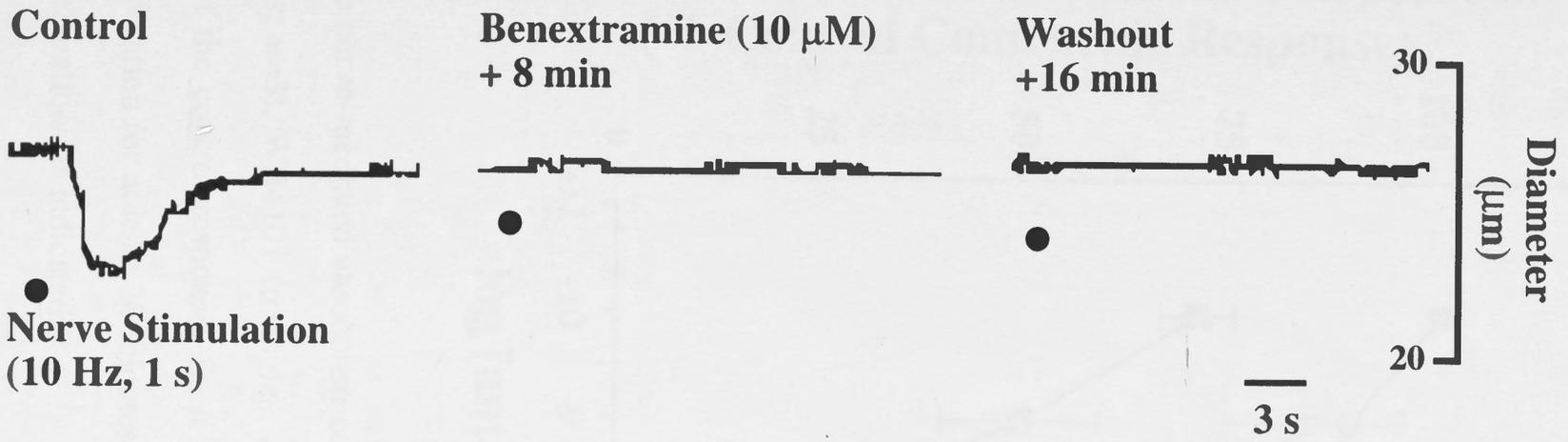


Figure 3.3 Traces showing the nerve-evoked contractile responses of an iris arteriole in control Krebs, in the presence of benextramine (1 μM) and after washout of benextramine. Dots indicate the onset of nerve stimulation (10 Hz, 1 s).

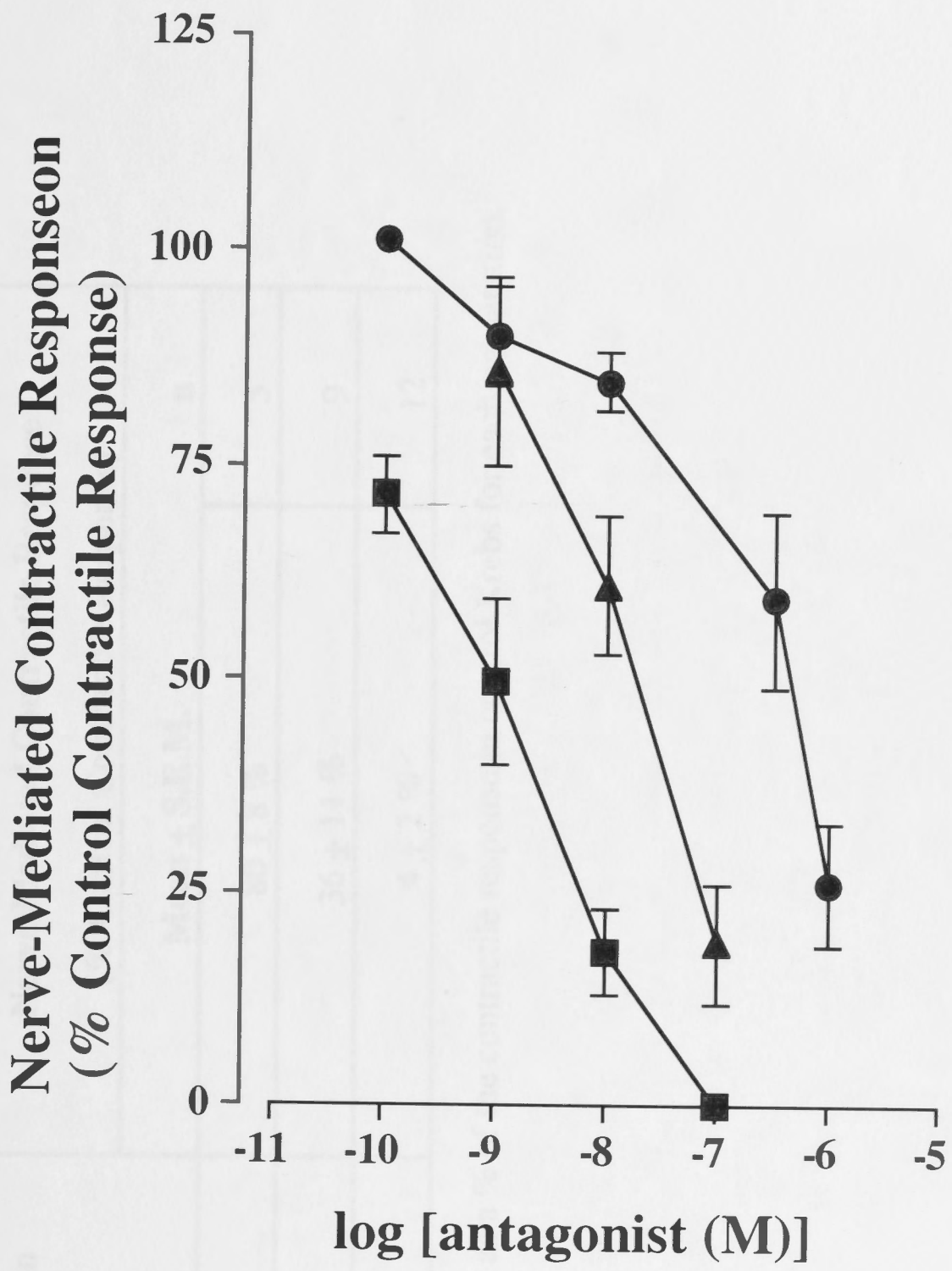


Figure 3.4 Average nerve-mediated vasoconstriction of iris arterioles in the presence of prazosin (squares; n=3), WB4101 (triangles; n=5) and yohimbine (circles; n=5) expressed as a % of the control response. Each concentration of antagonist was in contact wtih the preparation for at least 20 minutes. Points represent the mean ± s.e.m. for the number of preparations as indicated.

Table 3.2

Effect of CEC on nerve-evoked contraction of iris arterioles.

Concentration (μM)	**Nerve-Mediated Contractile Response (% Control Contractile Response)**	
	Mean ± S.E.M.	**n**
1	80 ± 8 %	5
3	36 ± 11 %	9
10	4 ± 2 %	12

Results are expressed as a % of the contractile response in control Krebs for each preparation.

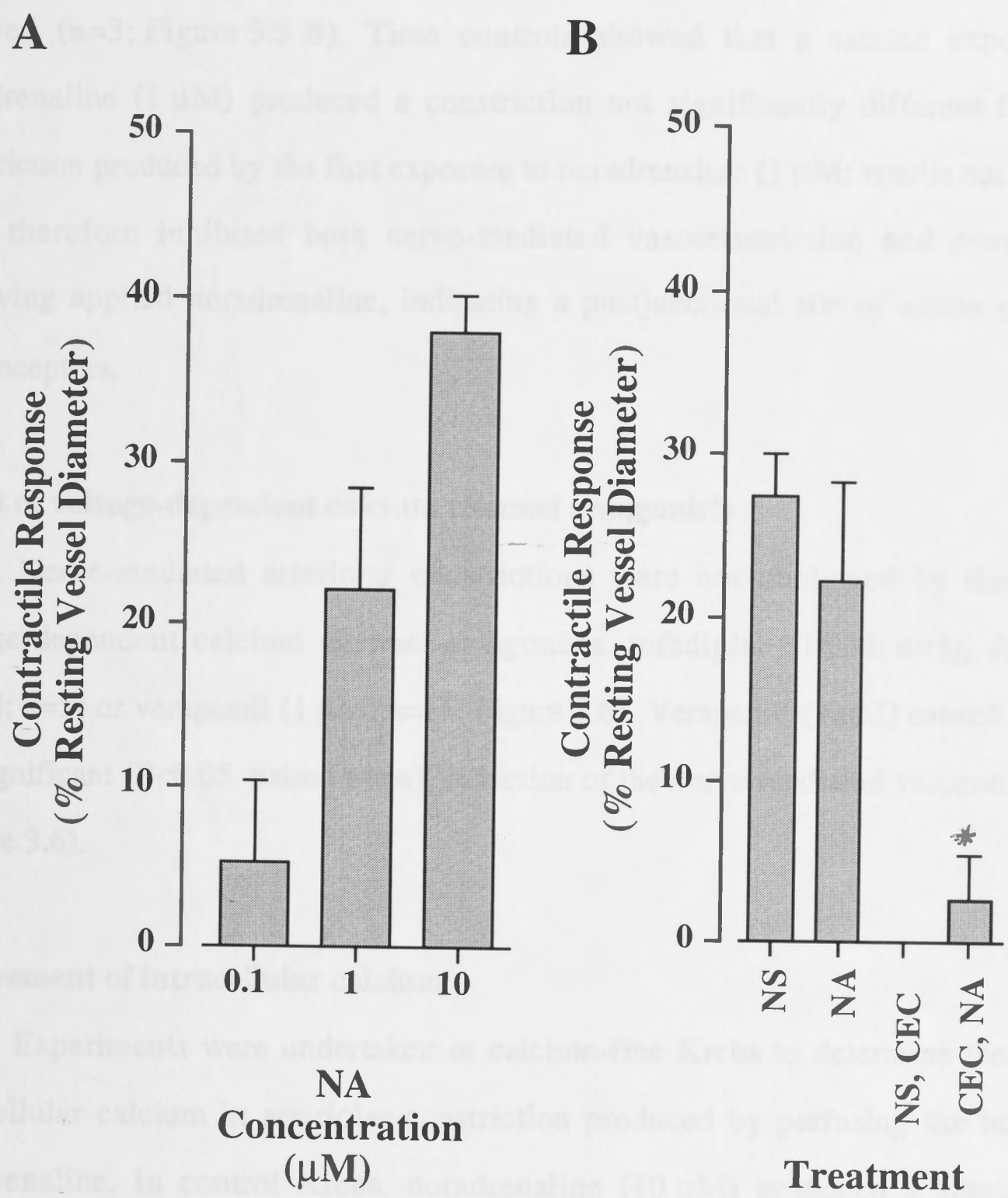


Figure 3.5 Iris arteriolar responses to nerve stimulation and noradrenaline.

A. Arteriolar constriction produced by different concentrations of noradrenaline.

B. Control arteriolar contractile responses are shown as an average of 3 contractions produced by nerve stimulation (NS) or as the maximal constricted diameter recorded within 3 min contact with noradrenaline (NA; 1 μM). After returning to control solution, CEC (10 μM) was added to the bath and 15 min later, when the response to nerve stimulation was blocked (CEC, NS), NA (1 μM) was added (CEC, NA). Columns represent the mean and s.e.m. (vertical bars) of 3 to 4 preparations.

* indicates significantly different from NA response in the absence of CEC (t-test with Bonferroni correction for Figure 3.5 B).

stimulation (Figure 3.5 B). When CEC (10 μM) had abolished the nerve-mediated vasoconstriction, the constriction produced by noradrenaline (1 μM) was significantly reduced (n=3; Figure 3.5 B). Time controls showed that a second exposure to noradrenaline (1 μM) produced a constriction not significantly different from the constriction produced by the first exposure to noradrenaline (1 μM; results not shown). CEC therefore inhibited both nerve-mediated vasoconstriction and constriction following applied noradrenaline, indicating a postjunctional site of action on α_{1B}-adrenoceptors.

Effect of voltage-dependent calcium channel antagonists

Nerve-mediated arteriolar constrictions were not abolished by the L-type voltage-dependent calcium channel antagonists, nifedipine (1 μM; n=3), diltiazem (1 μM; n=5) or verapamil (1 μM; n=11; Figure 3.6). Verapamil (1 μM) caused a small, but significant ($P<0.05$, paired t-test), reduction of the nerve-mediated vasoconstriction (Figure 3.6).

Involvement of intracellular calcium

Experiments were undertaken in calcium-free Krebs to determine the role of intracellular calcium in arteriolar constriction produced by perfusing the bath with noradrenaline. In control Krebs, noradrenaline (10 μM) produced a significantly ($P <0.05$, paired t-test) greater vasoconstriction than that produced by nerve-stimulation (n=4; Figure 3.7 A). In the presence of calcium-free Krebs, the nerve-mediated vasoconstriction was abolished and the addition of noradrenaline (10 μM) produced a constriction of the arteriole which was not significantly different to the constriction produced in control Krebs (n=4; Figure 3.7 B).

The contribution of intracellular calcium release to nerve-mediated constriction of iris arterioles was further investigated using caffeine, which releases a subpopulation of intracellular calcium stores (Lipscombe *et al.,* 1988) and may activate the ryanodine receptor and inhibit the inositol trisphosphate (IP_3) receptor (Ehrlich et al, 1994), dantrolene, which inhibits ryanodine-sensitive calcium-induced-calcium-release in

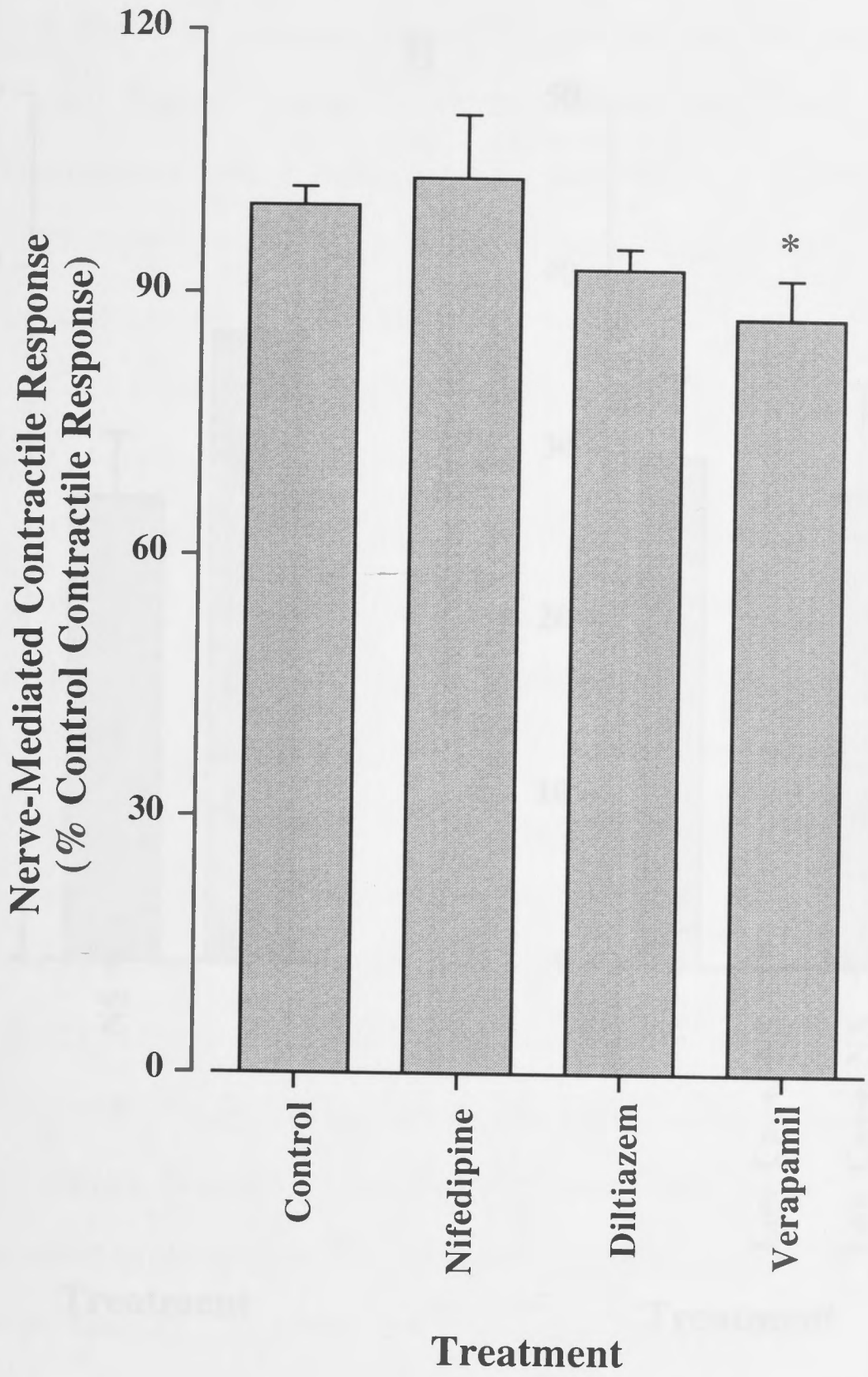


Figure 3.6 Effect of the L-type calcium channel antagonists nifedipine (1 μM; n=3), diltiazem (1 μM; n=5) or verapamil (1 μM; n=11) on the arteriolar contractile response to nerve stimulation. Results are expressed as a % of the contractile response in control Krebs. Control bar represents the variability of the control response in all the preparations (n=19). Each column represents the mean and s.e.m. (vertical bars) of the number of preparations indicated. * indicates differs significantly from control ($P<0.05$).

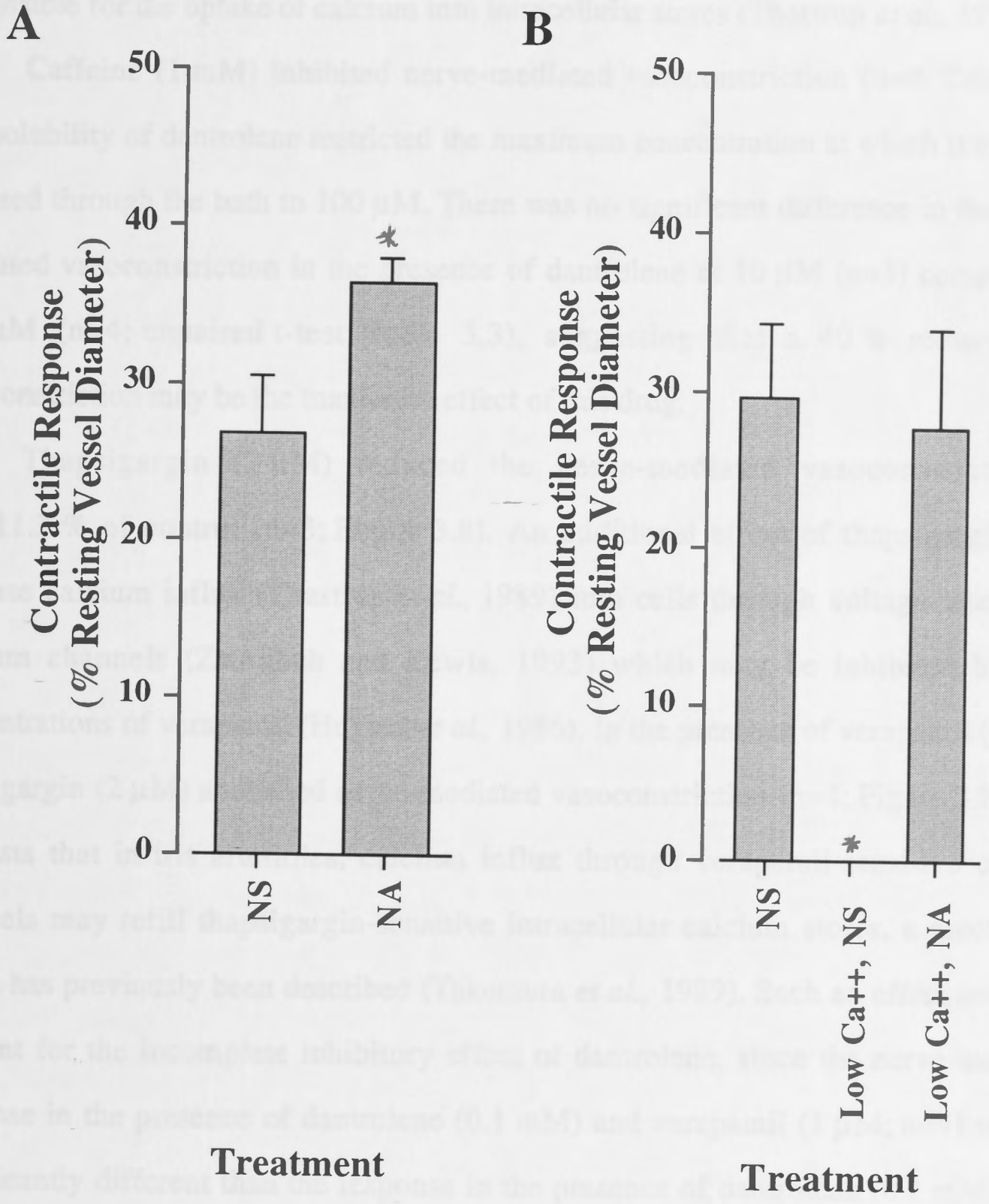


Figure 3.7 Arteriolar contractile responses to the perfusion of noradrenaline (10 μM) in the presence and absence of extracellular calcium. **A**. Control arteriolar contractile response is shown as an average of 3 contractions produced by nerve stimulation (NS). Perfusion of the preparation with noradrenaline (NA, 10 μM) constricted the arteriole. **B.** An average of 3 contractile responses to NS were recorded before perfusing the preparation with calcium-free Krebs. When the contractile response to NS was gone (Low Ca++, NS), NA (10 μM) was added (Low Ca++, NA).

Each column represents the mean and s.e.m. of 4 preparations.

* indicates significantly different from NS (t-test with Bonferroni correction for Figure 3.7 B).

skeletal muscle and longitudinal muscle of the intestine (Kuemmerle, Murthy and Makhlouf, 1994), and thapsigargin, which inhibits the calcium-ATPase pump responsible for the uptake of calcium into intracellular stores (Thastrup *et al.,* 1990).

Caffeine (1 mM) inhibited nerve-mediated vasoconstriction (n=4; Table 3.3). The solubility of dantrolene restricted the maximum concentration at which it could be perfused through the bath to 100 μM. There was no significant difference in the nerve-mediated vasoconstriction in the presence of dantrolene at 10 μM (n=5) compared to 100 μM (n=4; unpaired t-test, Table 3.3), suggesting that a 40 % reduction in vasoconstriction may be the maximum effect of this drug.

Thapsigargin (2 μM) reduced the nerve-mediated vasoconstriction to 61 ± 11.3 % of control (n=3; Figure 3.8). An additional effect of thapsigargin is to increase calcium influx (Thastrup *et al.,* 1989) into cells through voltage-insensitive calcium channels (Zweifach and Lewis, 1993) which may be inhibited by high concentrations of verapamil (Hughes *et al.,* 1986). In the presence of verapamil (1 μM), thapsigargin (2 μM) abolished nerve-mediated vasoconstriction (n=4; Figure 3.8). This suggests that in iris arterioles, calcium influx through verapamil-sensitive calcium channels may refill thapsigargin-sensitive intracellular calcium stores, a mechanism which has previously been described (Takemura *et al.,* 1989). Such an effect could not account for the incomplete inhibitory effect of dantrolene, since the nerve-mediated response in the presence of dantrolene (0.1 mM) and verapamil (1 μM; n=4) was not significantly different than the response in the presence of dantrolene (0.1 mM) alone (n=4; Figure 3.8).

Table 3.3

Effect of caffeine and dantrolene on nerve-evoked contractions of iris arterioles.

Drug (μM)	**Nerve-Mediated Contractile Response (% Control Contractile Response)**	
	Mean ± S.E.M.	**n**
Caffeine		
10	87.0 ± 6.0	4
100	60.4 ± 9.8	5
1000	0.0 ± 0.0	4
Dantrolene		
1	100.1 ± 2.4	3
10	77.2 ± 8.2	5
100	60.8 ± 8.2	4

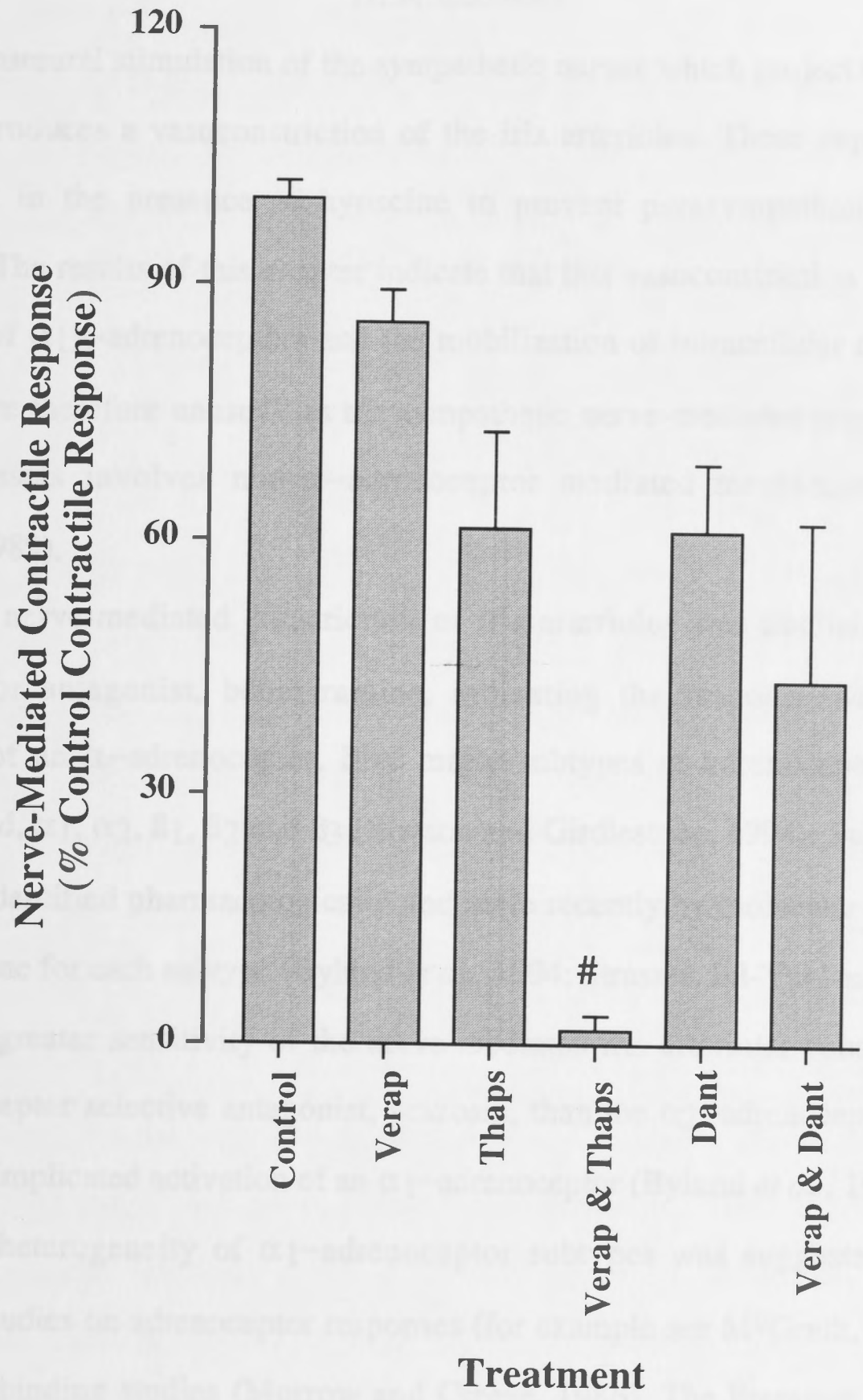


Figure 3.8 Effect of a combination of a L-type calcium channel antagonist, verapamil (Verap), and an intracellular calcium mobilizer, thapsigargin (Thaps) or dantrolene (Dant), on nerve stimulated arteriolar contractile response. Control bar represents the variability of the response in control Krebs in all of the preparations examined (n=15). Treatments are (left to right): Verap (1 μM; n=8), Thaps (2 μM; n=3), Verap (1 μM) then addtion of Thaps (2 μM; n=4), Dant (0.1 mM; n=4), Verap (1 μM) then addition of Dant (100 μM; n=4). Each column represents the mean and s.e.m. (vertical bar) of the number of preparations indicated in parenthesis. # indicates significantly different from thapsigargin alone ($P<0.05$).

DISCUSSION

Transmural stimulation of the sympathetic nerves which project to the iris from the SCG produces a vasoconstriction of the iris arterioles. These experiments were undertaken in the presence of hyoscine to prevent parasympathetic, cholinergic responses. The results of this chapter indicate that this vasoconstriction was due to the activation of α_{1B}-adrenoceptors and the mobilization of intracellular calcium. These arterioles are therefore unusual, as the sympathetic nerve-mediated responses of most arterial vessels involves non-α–adrenoceptor mediated mechanisms (Hirst and Edwards, 1989).

The nerve-mediated constriction of iris arterioles was abolished by the α–adrenoceptor antagonist, benextramine, indicating the response was due to the activation of an α–adrenoceptor. Five major subtypes of adrenoceptors have been characterized, α_1, α_2, $ß_1$, $ß_2$ and $ß_3$ (Watson and Girdlestone, 1994). Subtypes of each have been identified pharmacologically and more recently by molecular cloning of the cDNA or gene for each subtype (Bylund *et al.,* 1994; Strasser, Ihl-Vahl and Marquetant, 1992). The greater sensitivity of the nerve-mediated iris arteriolar constriction to the α_1–adrenoceptor selective antagonist, prazosin, than the α_2–adrenoceptor antagonist, yohimbine, implicated activation of an α_1–adrenoceptor (Bylund *et al.,* 1994).

The heterogeneity of α_1–adrenoceptor subtypes was suggested initially by functional studies on adrenoceptor responses (for example see McGrath, 1982), and by radioligand binding studies (Morrow and Creese, 1986). The literature regarding the characterization of α_1–adrenoceptors has been quite confusing in the past few years, however at present the consensus is that three native α_1–adrenoceptors have been identified: α_{1A}, α_{1B} and α_{1D} (Hieble *et al.,* 1995). In addition, α_1–adrenoceptors are proposed to show a high (α_{1H}) or a low (α_{1L}) affinity for prazosin (Flavahan and Vanhoutte, 1986). Oshita *et al.* (1992) suggested that the native receptors belong to the α_{1H}-group of adrenoceptors.

A number of receptor antagonists distinguish between α_1–adrenoceptor subtypes. It was originally demonstrated that CEC inactivates a subpopulation of α_1–adrenoceptors, and that the CEC-sensitive and -insensitive receptors correlated with

WB4101-insensitive and -sensitive receptors, which were designated α_{1B}- and α_{1A}-adrenoceptors respectively (Minneman, Han and Abel, 1988). Since then it has been determined that the α–adrenoceptor antagonist, WB4101, is more than 10 times less potent on α_{1B}-adrenoceptors (affinity 7-8) than on α_{1A}- or α_{1D}-adrenoceptors (affinity 9-10; Hieble *et al.,* 1995; Bylund *et al.,* 1994). The order of sensitivity to inactivation of α_1–adrenoceptors by CEC is α_{1B}- being most sensitive, α_{1D}- being less sensitive and α_{1A}-adrenoceptor being relatively insensitive (Hieble *et al.,* 1995; Bylund *et al.,* 1994). The receptor antagonists 5-methylurapidil (affinity 9), (±)-niguldipine (affinity 9-10) and SNAP, each show 30- to 100-fold more selectivity for the α_{1A}-adrenoceptors than the other two subytpes (Bylund *et al.,* 1994). More recently, the receptor antagonists BMY 7378 and SK&F 105854 have been demonstrated to show 50- to 100-fold more selectivity for the α_{1D}- than the α_{1A}- or α_{1B}-adrenoceptor subytpes (Hieble *et al.,* 1995). The low sensitivity of the nerve-mediated constriction of iris arterioles to WB4101 (IC_{50} of 14 nM), and the abolition of the neurogenic response by CEC are indicative of activation of an α_{1B}-adrenoceptor.

Recently, an additional presynaptic α_2–adrenoceptor agonist effect of CEC has been described (Bultmann and Starke, 1993). Such an effect would reduce neurotransmitter release and hence reduce nerve-mediated constriction. In the present study, however, the arteriolar nerve-mediated vasoconstriction was less sensitive to CEC than was the effect on neurotransmitter release as previously reported; in the vas deferens, 1 μM CEC reduced neurotransmitter release by 80 % (Bultmann and Starke, 1993), but at this concentration CEC only reduced the arteriolar vasconstriction by 20 %. Thus the sensitivity of CEC in the present study is more consistent with the higher concentrations reported to inactivate α_{1B}-adrenoceptors. Furthermore, it was confirmed that CEC does have a postsynaptic effect on iris arterioles, by demonstrating that CEC abolished the vasoconstriction produced by exogenous noradrenaline.

It has been demonstrated using RT-PCR that the iris tissue, which comprises both dilator muscle and iris arterioles but not the sphincter muscle, expresses only mRNA for the cloned α_{1b}- (cloned by Cotecchia *et al.,* 1988) and α_{1c}-adrenoceptor (cloned by Schwinn *et al.,* 1990) subtypes (Gould, Vidovic and Hill, 1995). According

to the lastest concensus on α_1–adrenoceptor nomenclature (Hieble *et al.,* 1995), the two receptor subtypes detected in this tissue correspond to the native α_{1B}- and α_{1A}-adrenoceptor subtypes respectively. The sensitivity of the nerve-mediated vasoconstriction to CEC indicates that the receptor is not an α_{1A}-adrenoceptor. Thus this mRNA expression confirms the pharmacological identification of the α_{1B}-adrenoceptor as mediating sympathetic vasoconstriction in iris arterioles.

Smooth muscle contraction occurs as a result of an increase in intracellular calcium (Hirst and Edwards, 1989). It was originally proposed that the pathway for increasing intracellular calcium following α_1–adrenoceptor activation was subtype-specific (Han, Abel and Minneman, 1987; Minneman, 1988). Alpha$_{1A}$-adrenoceptor activation was associated with calcium influx through G-protein coupling to voltage-dependent calcium channels, while α_{1B}-adrenoceptors coupled to the G-protein activation of phospholipase C and the release of intracellular calcium through the production of IP_3 (Minneman, 1988). Consistent with this, the α_{1A}-adrenoceptor-mediated component of vasoconstrictor responses to periarterial nerve-stimulation in the rat kidney is abolished by dihydropyridines (Blue Jr., Vimont and Clarke, 1992).

However, all three expressed, cloned α_1–adrenoceptor subtypes may couple to IP_3-mediated increase intracellular calcium (Perez, DeYoung and Graham, 1993; Han, Esbenshade and Minneman, 1992; Schwinn *et al.,* 1991). In addition, from receptor expression studies in cell lines, it has been demonstrated that activation of the α_{1B}-adrenoceptor subtype may activate several distinct effector pathways. For instance, activation of α_{1B}-adrenoceptors in COS-1 cells causes an accumulation of IP_3, an increase in cyclic AMP and an increase in arachadonic acid levels (Perez, DeYoung and Graham, 1993). The adrenaline-stimulated α_{1B}-adrenoceptor-mediated arachadonic acid release was abolished by dihydropyridines but the increase in IP_3 was not (Perez, DeYoung and Graham, 1993).

Studies in cell lines (Schwinn *et al.,* 1991) and isolated tissue (Burt, Chapple and Marshall, 1995) have demonstrated that the activation of α_{1B}-adrenoceptors leads to the mobilization of intracellular calcium and the influx of calcium through voltage-insensitive calcium channels. L-type voltage-dependent calcium channel blockers did

not abolish nerve-mediated vasoconstriction of iris arterioles, although the intracellular calcium mobilizer caffeine did not abolish the response. The constriction produced by exogenous noradrenaline was also independent of extracellular calcium. These results suggest that the contraction of iris arterioles is dependent on the release of intracellular calcium and the influx of calcium through voltage-dependent calcium channels was not essential for the initiation of arteriolar constriction.

Dantrolene inhibits ryanodine-sensitive calcium channels (Kuemmerle, Murthy and Makhlouf, 1994). These stores demonstrate calcium-induced-calcium-release (Ehrlich *et al.*, 1994). Dantrolene produced a 40 % decrease in the nerve-mediated vasoconstriction and the presence of verapamil did not alter the magnitude of the inhibitory effect of dantrolene on the contractile response. Caffeine totally abolished the neurogenic contractile response. In addition to inducing intracellular calcium release (Endo *et al.*, 1990), caffeine activates the ryanodine receptor-calcium release channel in skeletal muscle and has an inhibitory effect on the IP_3 receptor in excitable and non-excitable cells (Ehrlich *et al.*, 1994). IP_3-induced calcium release is inhibited by caffeine in smooth muscle cells of the rat portal vein (Loirand, Gregoire and Pacaud, 1994). Therefore, in iris arterioles, the release of intracellular calcium from caffeine-sensitive stores following α_{1B}-adrenoceptor activation appears to activate calcium-induced intracellular calcium release, which contributes some calcium for the arteriolar contraction. Such a mechanism for calcium propagation occurs in cultured vascular smooth muscle cells, where IP_3-induced release of calcium spreads through the cell to activate calcium-induced calcium release (Blatter and Wier, 1992).

Measurements of calcium in the smooth muscle cell line, DDT_1, have shown that noradrenaline induced a biphasic response comprising a transient component, which was dependent on intracellular calcium, and a sustained component, which was dependent on extracellular calcium (Han, Esbenshade and Minneman, 1992). CEC abolished both of these components and also eliminated IP_3 responses to noradrenaline. This, and other pharmacological data, suggested that the DDT_1 cell line contained exclusively the α_{1B}-adrenoceptor subtype (Han, Esbenshade and Minneman, 1992). While the activation of α_{1B}-adrenoceptors in these cells may lead to the influx of

extracellular calcium, this does not occur through voltage-sensitive calcium channels (Reynolds and Dubyak, 1985; Burt, Chapple and Marshall, 1995).

Three main mechanisms of voltage-independent calcium influx have been described (Felder, Singer Lahat and Mathes, 1994): firstly, the activation of receptor-operated calcium channels which are coupled tightly to the receptor and activation of which occurs independently of intracellular messengers. Secondly, depletion-operated calcium channels (DOCC), activation of which provides a source of calcium for refilling of calcium stores and is regulated by the concentration of calcium in the store. Michell (1975) first suggested that empty IP_3-sensitive stores recruit plasma membrane calcium influx for the refilling of calcium pools. Putney proposed the capacitative model for this current, whereby receptor-generated IP_3 binds to its receptor on calcium stores to trigger the release of calcium (Putney, 1986; Putney, 1991). The depletion of the store triggers the release of a messenger which then activates the DOCC on the plasma membrane. Thapsigargin may lead to the activation of these channels (Felder, Singer Lahat and Mathes, 1994). Thirdly, a second messenger-operated calcium channel, activation of which is regulated by calcium itself, or second messengers released following receptor activation. Activation of this channel occurs independently of the filled state of the calcium store. In addition to activating the release of intracellular calcium, IP_3 may act alone, or in conjunction with IP_4, to activate this channel (Irvine, 1992). It has not been established whether these channels are involved in the refilling of intracellular calcium pools (Felder, Singer Lahat and Mathes, 1994).

According to Putney's capacitative model (Putney, 1986; Putney, 1991), refilling of the arteriolar smooth muscle intracellular calcium stores would occur in response to the depletion of the caffeine-sensitive stores. High concentrations of verapamil inhibit calcium influx through calcium channels other than voltage-sensitive ones (Hughes *et al.,* 1986). Thapsigargin, which inhibits the calcium-ATPase responsible for maintaining calcium in intracellular organelles (Inesi and Sagara, 1992), discharges agonist-sensitive intracellular calcium stores and subsequently stimulates the influx of extracellular calcium in acinar cells (Bird *et al.,* 1992; Takemura *et al.,* 1989), reduced the arteriolar contractile response by 40 %. In the presence of verapamil, the contractile

response of the iris arterioles was abolished by thapsigargin. Thus, activation of α_{1B}-adrenoceptors on the iris arterioles appears to result in the release of calcium from internal stores, and that these stores are refilled by calcium influx through verapamil-sensitive calcium channels, thus supporting Putney's hypothesis of receptor-regulated calcium entry. Therefore, verapamil may be inhibiting extracellular calcium influx through DOCC in iris arterioles.

The arteriolar constriction produced by perfusion of the bath with a submaximal concentration of noradrenaline was totally inhibited by CEC, suggesting that only CEC-sensitive adrenoceptors are present on this vessel. Since the RT-PCR studies have shown that the only other α_1–adrenoceptor expressed in the iris is the α_{1A}-adrenoceptor (Gould, Vidovic and Hill, 1995), which is insensitive to CEC (Hieble *et al.*, 1995), these results suggest that only α_{1B}-adrenoceptors exist on the surface of the iris arterioles.

CHAPTER 4.

MEMBRANE POTENTIAL CHANGES ASSOCIATED WITH SYMPATHETIC NEUROEFFECTOR TRANSMISSION

INTRODUCTION

In most arterial vessels, the postjunctional response to sympathetic nerve stimulation is due to the activation of non-α–adrenoceptors alone, or both α– and non-α–adrenoceptors (Hirst and Edwards, 1989). Contraction following non-α–adrenoceptor activation occurs as a result of membrane depolarization (Hirst and Edwards, 1989). In arteries such as the adult rat mesenteric artery (Hill, Hirst and van Helden, 1983), a single brief supramaximal electrical stimulus produces a rapid excitatory junction potential which is resistant to α–adrenoceptor antagonists and which does not initiate a contraction. With repetitive nerve stimulation, successive excitatory junction potentials summate to produce a larger depolarization which, upon reaching the threshold for the opening of voltage-dependent calcium channels, results in calcium influx and contraction. On the other hand, α–adrenoceptor mediated vasoconstriction is accompanied by postjunctional voltage changes which do not appear to be essential for the constriction. In the rat tail artery, for example, these voltage changes are much slower than the non-α–adrenoceptor mediated excitatory junction potentials and are preceded by α–adrenoceptor mediated constriction (Cheung, 1984).

In contrast to the above results, studies in veins (Holman *et al.*, 1968; van Helden, 1991) and in the pulmonary artery (Suzuki, 1983) have found that the responses to sympathetic nerve stimulation are mediated purely by α–adrenoceptors. In the previous chapter it was also shown that the vasoconstriction of arterioles found within the stromal layer of the rat iris is also a purely α–adrenoceptor mediated response, which is due to the release of intracellular calcium, and is not totally dependent upon the entry of calcium through voltage-dependent calcium channels.

This chapter addresses the question of whether the nerve-mediated constriction of these iris arterioles is accompanied by any membrane potential changes and, if so, how these potentials compare with those previously described to accompany α– and non-α–adrenoceptor responses in other vessels.

RESULTS

Intracellular responses to transmural nerve stimulation

Two groups of cells could be identified in iris arterioles on the basis of their resting membrane potential values. Within the first group of cells, which had an average resting membrane potential of -74 ± 1 mV (n=69), the majority of cells showed no membrane potential change (average resting membrane potential of -74 ± 1 mv; n=56) in response to nerve stimulation (10 pulses at 10 Hz; Figure 4.1 A). In some cells, however, a small hyperpolarization was recorded following the first train of nerve stimulation, although subsequent stimuli often failed to elicit a response (average resting membrane potential of -73 ± 2 mv; n=13).

The impression was gained that cells of the first group were impaled when it was more difficult to make recordings, for example, in the earlier experiments, or in later experiments when the preparation seemed to get tough and more force was required to impale the cell. For these reasons these cells are proposed to lay deeper in the preparation than the second group of cells from which recordings were more commonly made, particularly in the later experiments.

The second group of cells had an average resting membrane potential value of -64 ± 1 mV (n=93) and responded to nerve stimulation with a depolarization. Within this group of cells, two subgroups could be identified on the basis of the size and shape of the depolarization. Following nerve stimulation (10 Hz for 1 s) some cells showed a small, smooth depolarization of 8 + 1 mV (average resting membrane potential of -61 ± 2 mV; n=28) and others showed a large, biphasic depolarization of 40 ± 1 mV (average resting membrane potential of -65 + 1 mV; n=65; Figure 4.1 B).

In the earlier experiments, the two types of depolarizing cells were recorded from with approximately an equal frequency, however, as time went by, recordings were made more often from the cells showing the large depolarizations, to a point where recordings were rarely recorded from the cells with the small depolarizations. These latter cells may represent either damaged cells or another type of cell in deeper cell layers of the iris. For these reasons, the remainder of the experiments were

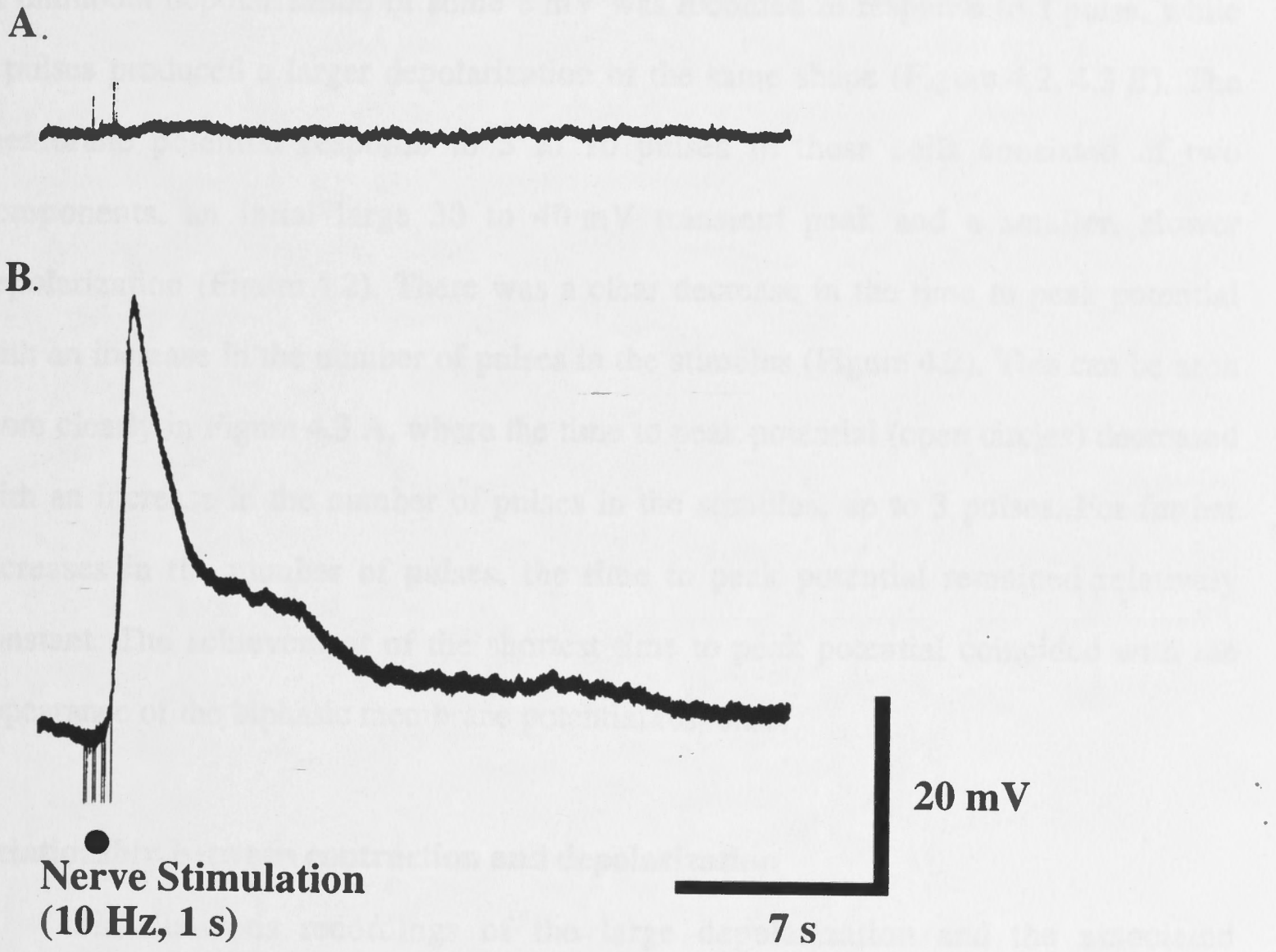


Figure 4.1 Intracellular recordings from cells of iris arteriole showing **A**. no membrane response or **B**. a depolarization in response to nerve stimulation (dot; 10 Hz, 1 s).

conducted only on the cells showing the large, biphasic depolarizations in response to nerve stimulation.

Membrane potential changes were recorded in the group of depolarizing cells when the number of pulses in a train at 10 Hz was increased from 1 to 10 (Figure 4.2). A unimodal depolarization of some 8 mV was recorded in response to 1 pulse, while 2 pulses produced a larger depolarization of the same shape (Figure 4.2, 4.3 B). The membrane potential response to 3 to 10 pulses in these cells consisted of two components, an intial large 30 to 40 mV transient peak and a smaller, slower depolarization (Figure 4.2). There was a clear decrease in the time to peak potential with an increase in the number of pulses in the stimulus (Figure 4.2). This can be seen more clearly in Figure 4.3 A, where the time to peak potential (open circles) decreased with an increase in the number of pulses in the stimulus, up to 3 pulses. For further increases in the number of pulses, the time to peak potential remained relatively constant. The achievement of the shortest time to peak potential coincided with the appearance of the biphasic membrane potential response.

Relationship between contraction and depolarization

Simultaneous recordings of the large depolarization and the associated contraction of the arteriole showed that the peak depolarization occured at the same time as the onset of the contraction (Figure 4.3 A). This relationship held for stimulus trains containing from 1 to 10 pulses (Figure 4.3 A). The peak amplitude of the contraction occured consistently later than the peak depolarization (Figure 4.3 A).

The amplitude of the depolarization showed the same relationship to the number of pulses in the stimulus as did the amplitude of the contraction; both increased to reach a maximum amplitude by 3 to 4 pulses (Figure 4.3 B).

Confirmation of an α–adrenoceptor-mediated response to nerve stimulation

Both the depolarization and the contraction were abolished by tetrodotoxin (1 μM; n=2; Figure 4.4), indicating they resulted from nerve activation (Adams and Swanson, 1994). The neurogenic depolarization and contraction were also abolished by

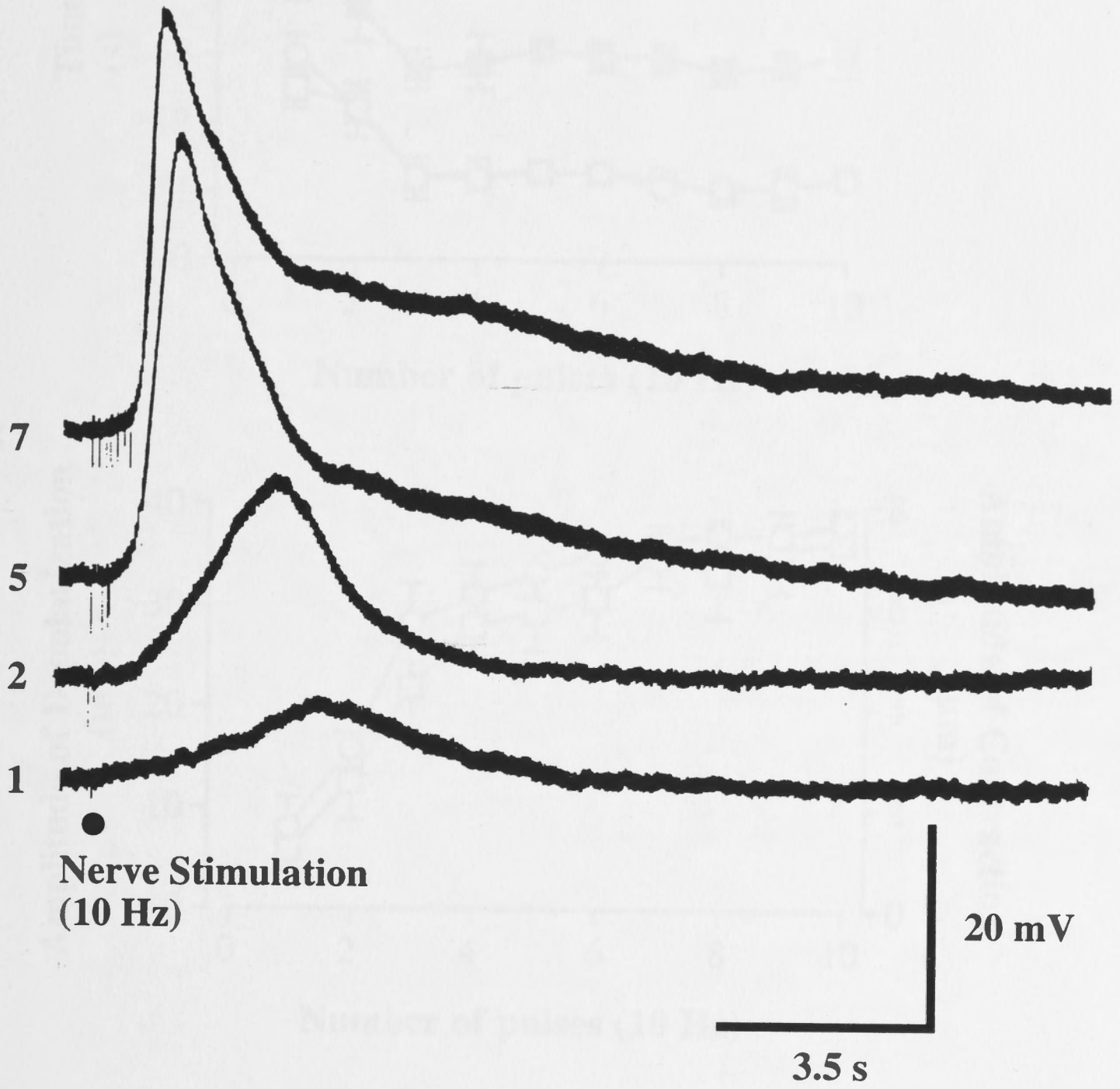


Figure 4.2 Intracellular responses to increasing numbers of pulses in the stimulus train, recorded from an iris arteriole cell showing a large, biphasic depolarization. Dot indicates onset of stimulation with 1, 2, 5 or 7 pulses at 10 Hz.

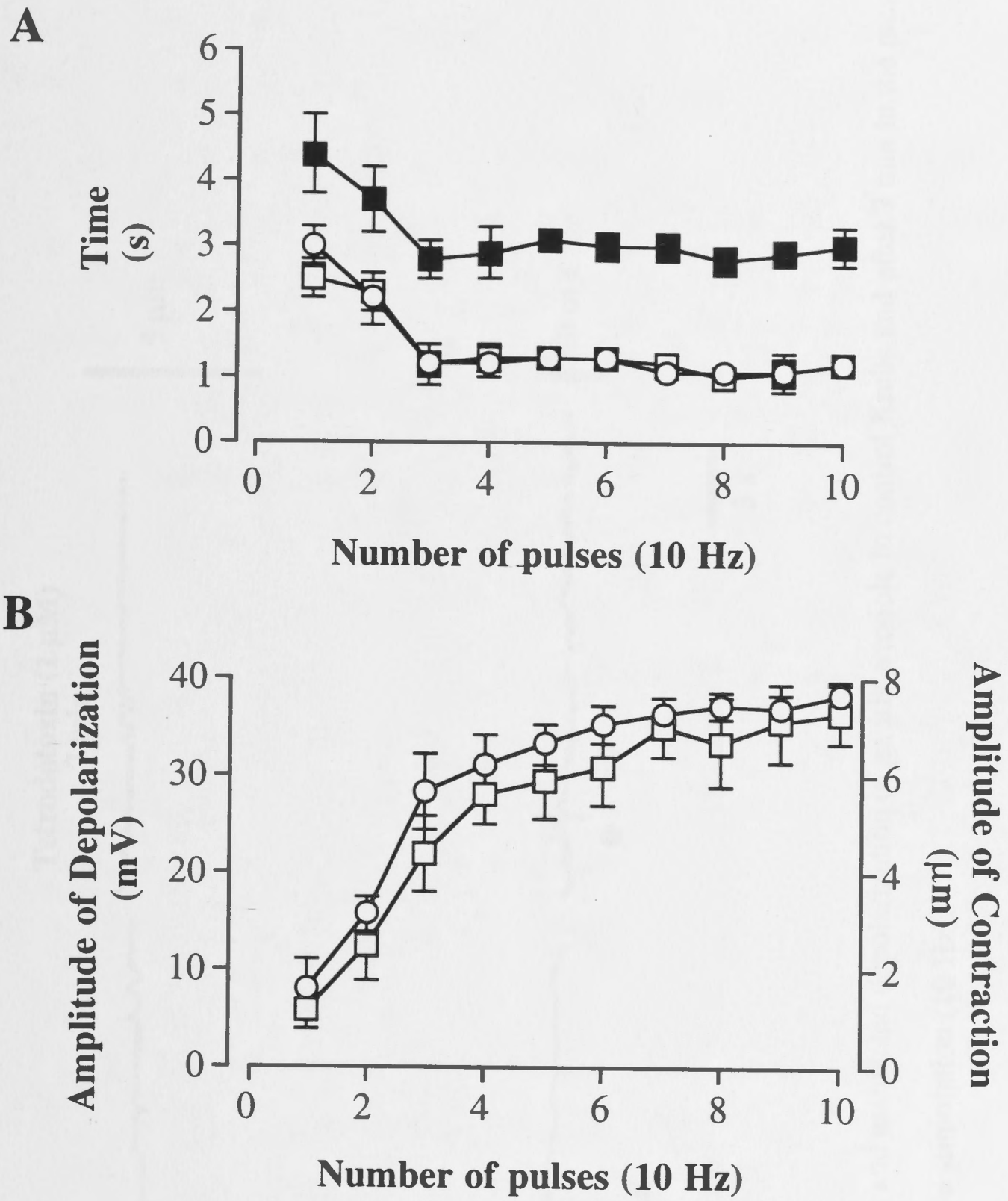


Figure 4.3 Comparison of the characteristics of the contraction of the iris arterioles and the large, biphasic depolarizations recorded in response to increasing number of pulses in the stimulus train (10 Hz). Panel A shows the changes in the time to onset of contraction (open squares), the time to peak contraction (closed squares) and the time to peak depolarization (open circles) with increasing number of pulses in the stimulus train. Panel B shows the change in amplitude of the contractions (squares) and the depolarizations (circles) with increasing number of pulses in the stimulus train.

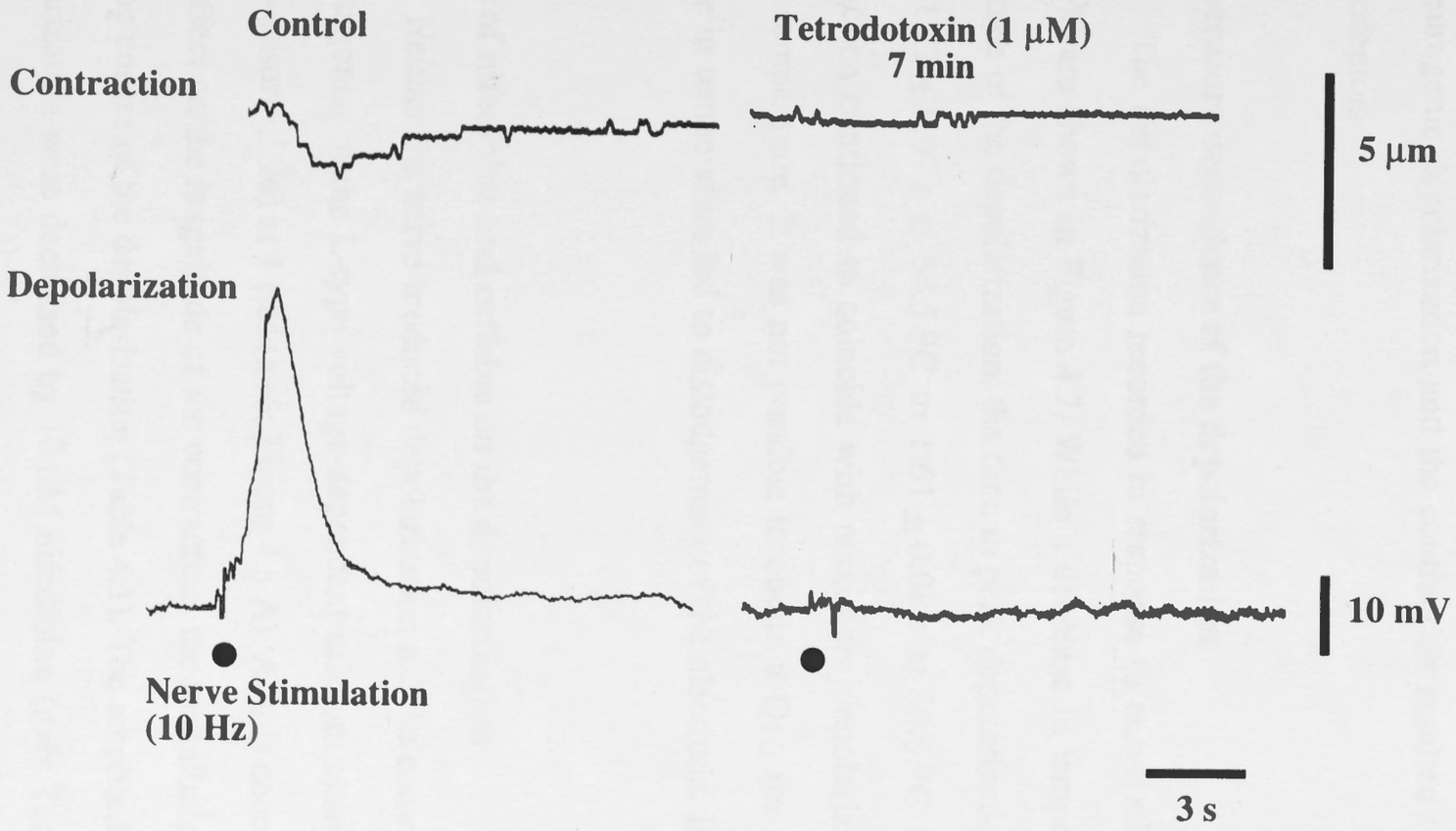


Figure 4.4 Traces showing nerve-evoked contraction and associated depolarization of an iris arteriole in control Krebs and after 7 min in the presence of tetrodotoxin (1 μM). Dots indicate the onset of nerve stimulation (10 Hz).

the irreversible α–adrenoceptor antagonist, benextramine (10 μM; n=7, Figure 4.5) and the α–adrenoceptor antagonist, prazosin (0.1 μM; n=3; Figure 4.6). Following return to control solution after benextramine, a small depolarization was sometimes recorded (19 ± 1 % control, n=3), the reason for this was not clear. In none of the experiments did the contraction return after washout in control Krebs. These observations indicate that both the neurogenic depolarization and the contraction resulted from the activation of α–adrenoceptors.

Temperature dependence of the depolarization

The depolarization recorded in response to nerve stimulation at 30.6 °C and 34.5 °C are shown in Figure 4.7. While a decrease in temperature did not affect the amplitude of the depolarization, the time to peak depolarization increased significantly from 1.13 ± 0.07 s at 34.5 °C to 1.61 ± 0.08 s at 30.6 °C (n=3). The onset of the contraction continued to coincide with maximum depolarization irrespective of the actual temperature. It was not possible to obtain a Q_{10} for the response, as a larger change in temperature led to dislodgement of the electrode. It is unclear why this was so.

Effect of nifedipine and caffeine on the depolarization

Neither the nerve-mediated depolarization nor the contraction were abolished by the antagonist of the L-type voltage-dependent calcium channels, nifedipine (Adams and Swanson, 1994) at 1 μM (n=6; Figure 4.8 A). At this concentration, nifedipine had little effect on the magnitude of the contraction, the magnitude of the depolarization or the time course of the depolarization (Table 4.1). The amplitudes of the contraction and depolarization were decreased by 10 μM nifedipine (n=6; Table 4.1; Figure 4.8 B).

In the presence of 10 μM nifedipine, the contraction in response to 50 mM KCl was reduced by 73.0 ± 2.2 % (n=3). In these experiments, the arterioles were preincubated in 10 μM benextramine to prevent the effects of KCl-

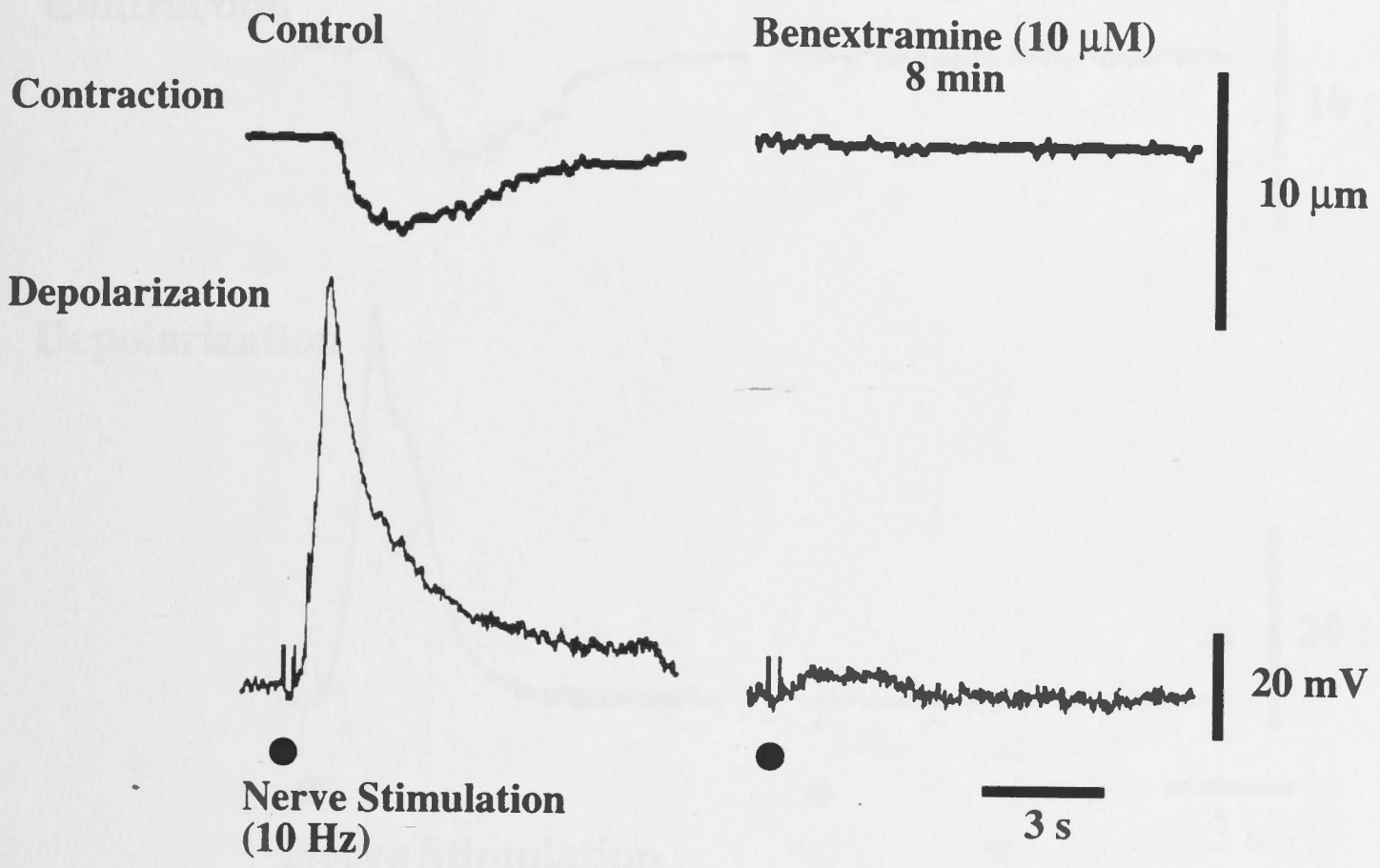


Figure 4.5 Traces showing cell depolarization and associated constriction of an iris arteriole in control Krebs and in the presence of benextramine (10 μM). Dots indicate the onset of nerve stimulation (10 Hz).

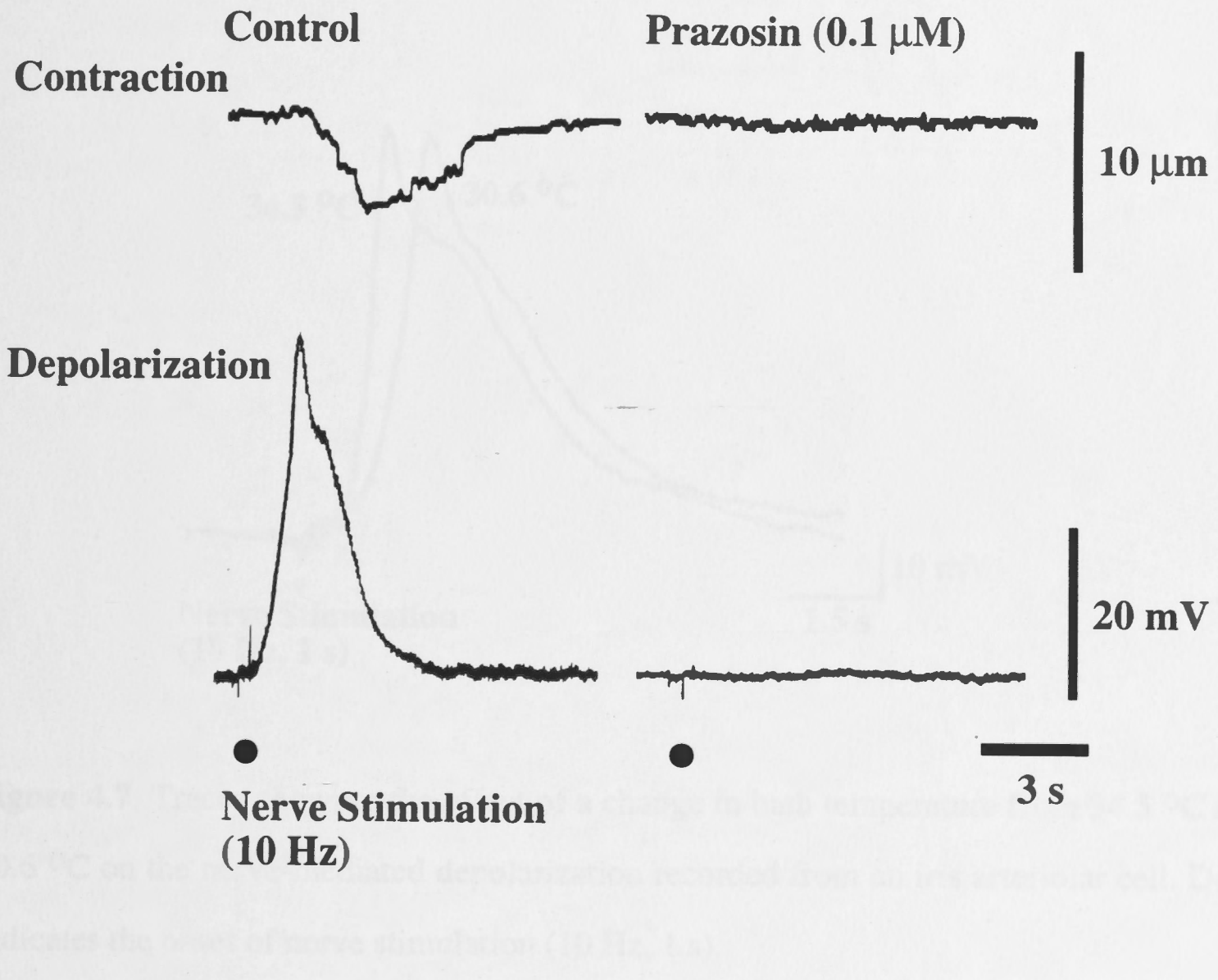


Figure 4.6 Traces showing nerve-evoked cell depolarization and associated constriction of an iris arteriole in control Krebs and in the presence of prazosin (0.1 μM). Dots indicate the onset of nerve stimulation (10 Hz).

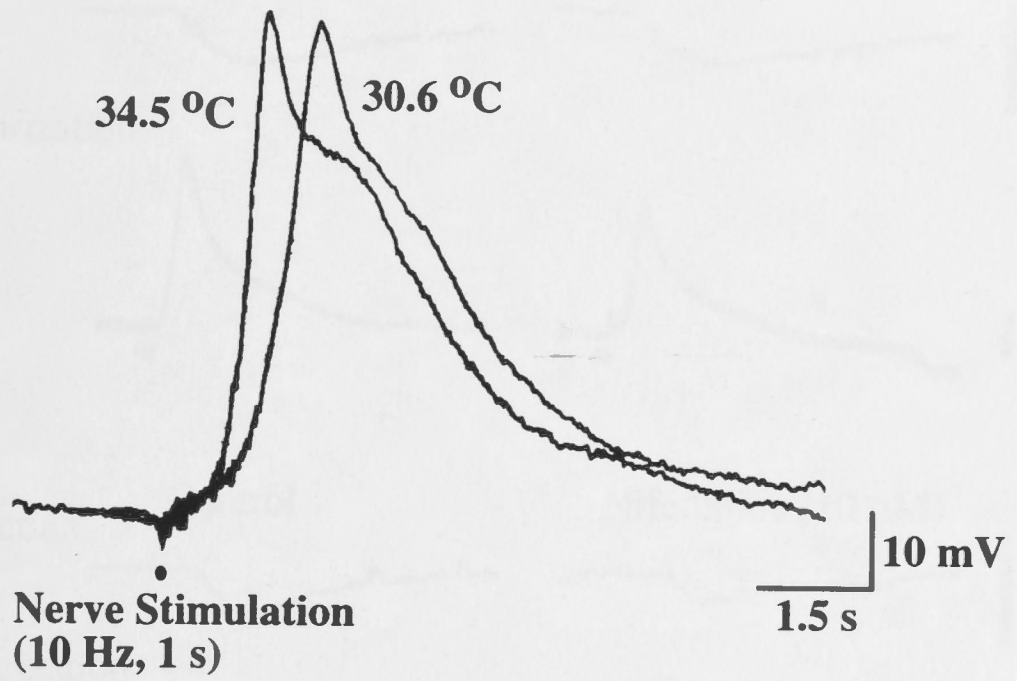


Figure 4.7 Traces showing the effect of a change in bath temperature from 34.5 °C to 30.6 °C on the nerve-mediated depolarization recorded from an iris arteriolar cell. Dot indicates the onset of nerve stimulation (10 Hz, 1 s).

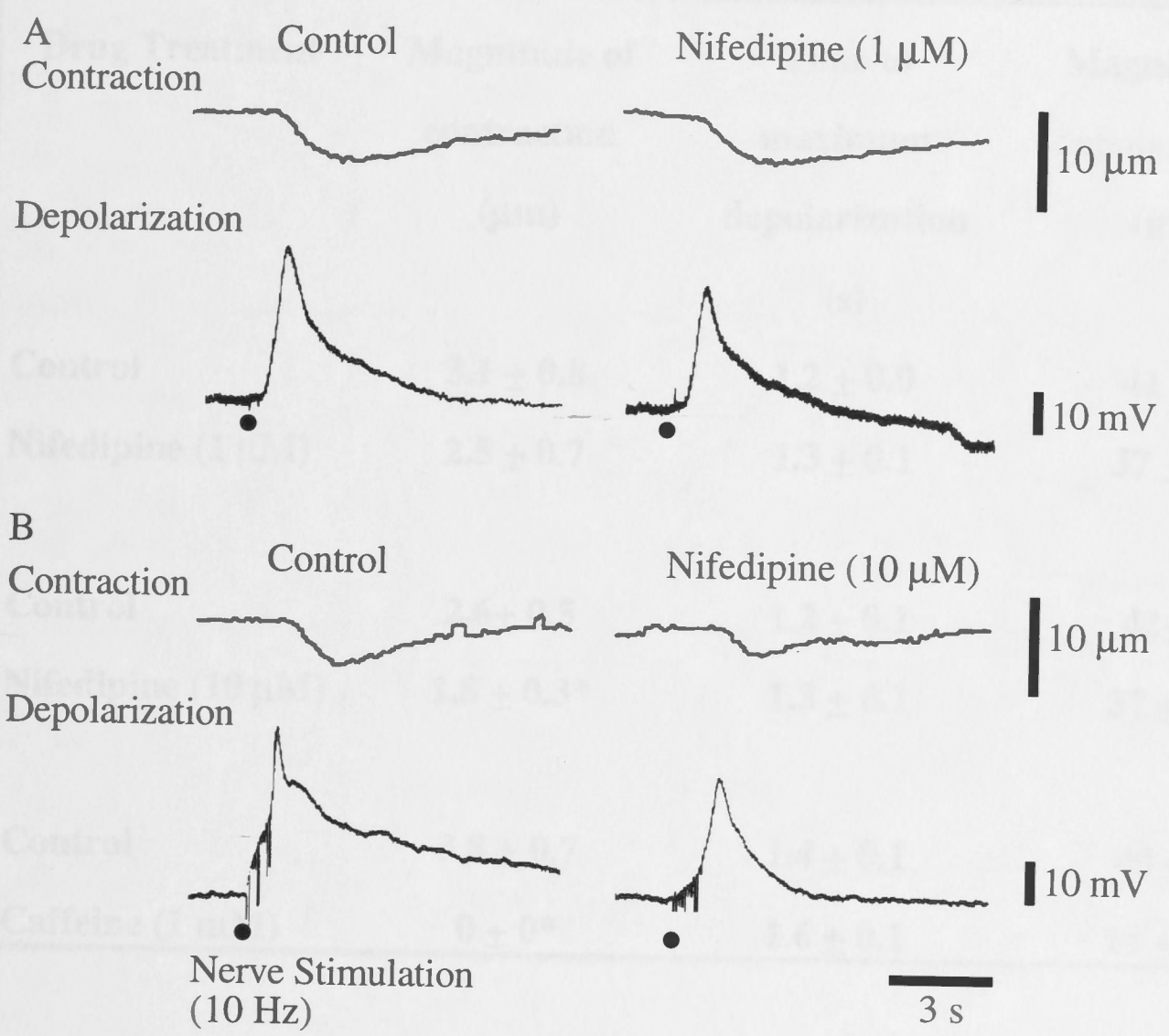


Figure 4.8 Traces showing cell depolarization and associated constriction in control Krebs and in the presence of **A.** 1 μM nifedipine or **B.** 10 μM nifedipine. Dots indicate the onset of nerve-stimulation (10 Hz).

Table 4.1 Effect of nifedipine and caffeine on nerve-mediated depolarization recorded from an arteriolar cell and associated contractile response.

Drug Treatment	Magnitude of contraction (μm)	Time to maximum depolarization (s)	Magnitude of depolarization (mV)
Control	3.1 ± 0.8	1.2 ± 0.0	41 ± 2
Nifedipine (1 μM)	2.5 ± 0.7	1.3 ± 0.1	37 ± 3*
Control	2.6± 0.5	1.2 ± 0.1	42± 1
Nifedipine (10 μM)	1.8 ± 0.3*	1.3 ± 0.1	37 ± 1*
Control	3.8 ± 0.7	1.4 ± 0.1	40 ± 3
Caffeine (1 mM)	0 ± 0*	1.6 ± 0.1	19 ± 3*

Results are expressed as the mean ± s.e.m. of 6 preparations.

* indicates significant difference from paired control (t-test with Bonferroni correction)

induced release of noradrenaline. The residual 27 % of the contraction presumably results from the KCl-induced release of other neurotransmitters, for example, NPY. Possible effects of acetylcholine were prevented by the presence of hyoscine hydrochloride (1 μM).

In contrast to nifedipine, the intracellular calcium mobilizer caffeine (1 mM) abolished the nerve-mediated contraction and substantially reduced the magnitude of the depolarization (n=6; Figure 4.9). In the presence of caffeine (1 mM), the time to maximum depolarization was longer than in control (Table 4.1), indicating the loss of the peak component of the depolarization.

Ionic current underlying the depolarization

Replacement of sodium chloride in the Krebs with sodium methyl sulfate (120 mM) reduced the magnitude of the depolarization to 8 ± 5 % of control (n=4; Figure 4.10) and abolished the contraction. This effect could be reversed by washing out the drug. A similar effect on the depolarization and contraction was produced by chloride replacement with sodium isethionate (120 mM; data not shown). Flufenamic acid, which is reported to block calcium-dependent chloride channels in Xenopus oocytes (White and Aylwin, 1990), blocked both the depolarization and the contraction (250 μM; n=6; Figure 4.11). In the presence of these drugs which interfere with chloride channels the contraction was abolished before the drugs had their maximal effect on the depolarization (Figure 4.10, 4.11).

Spontaneous depolarizations in iris arterioles

Spontaneous depolarizations were recorded in some iris arterioles (n=33). The frequency of these depolarizations was quite constant between different preparations, being about 1 per 10 s. Large spontaneous depolarizations were usually associated with spontaneous contractions of the iris arteriole (Figure 4.12 A). In Figure 4.12 A, the depolarizations were accompanied by spontaneous contractions of the arteriole throughout the record. The depolarizations become progressively smaller in amplitude as the cell membrane itself depolarized. In other preparations, in which small

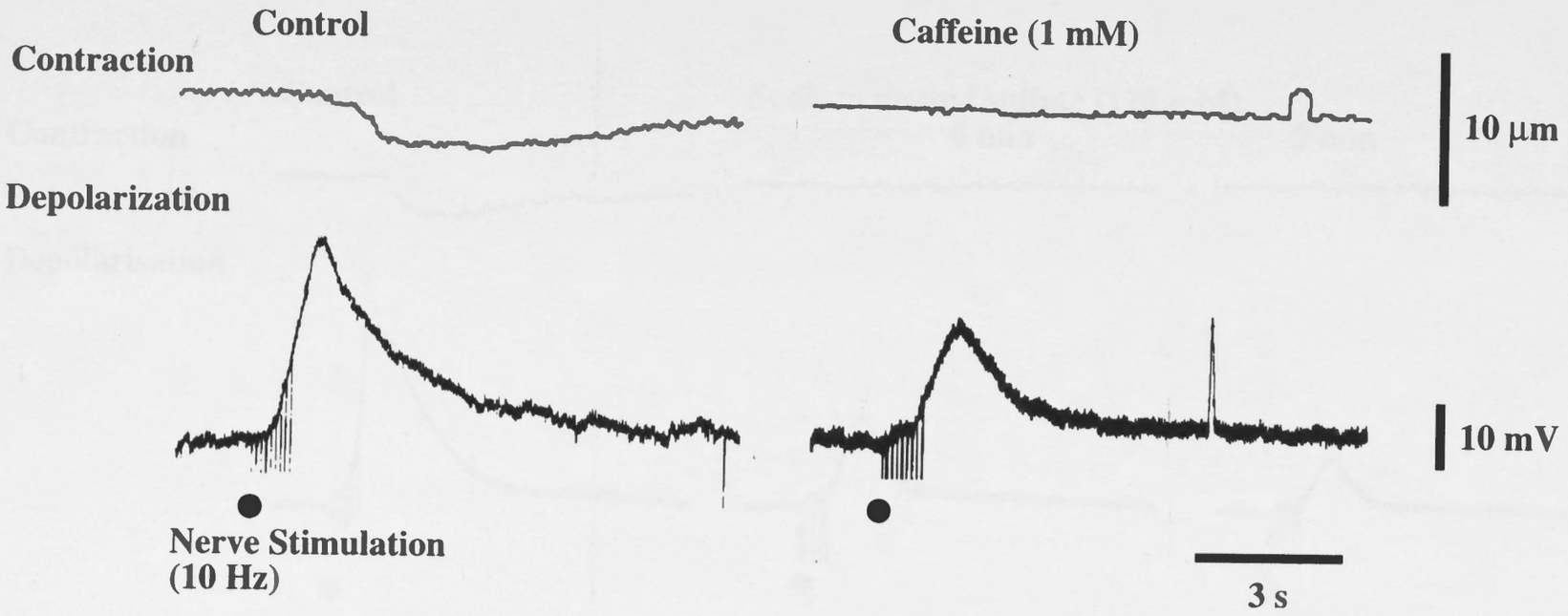


Figure 4.9 Traces showing the effect of caffeine (1mM) on cell depolarization and associated arteriolar constriction evoked by nerve stimulation (10 Hz). Dots indicate the onset of nerve stimulation (10 Hz).

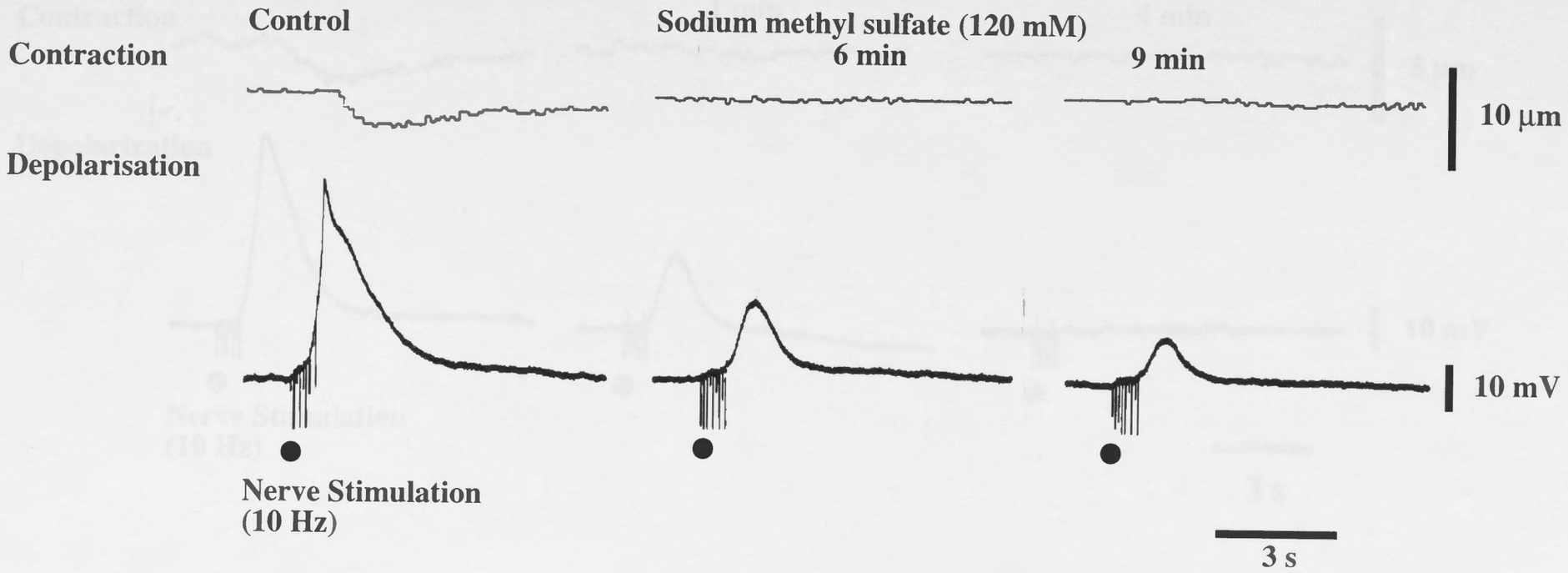


Figure 4.10 Traces showing nerve-mediated cell depolarization and associated constriction of an iris arteriole in control Krebs and after lowering external chloride with sodium methyl sulfate (120 mM) for 6 min, when the contraction was abolished, and 9 min, showing the maximal effect on the depolarization. Dots indicate the onset of nerve stimulation (10 Hz).

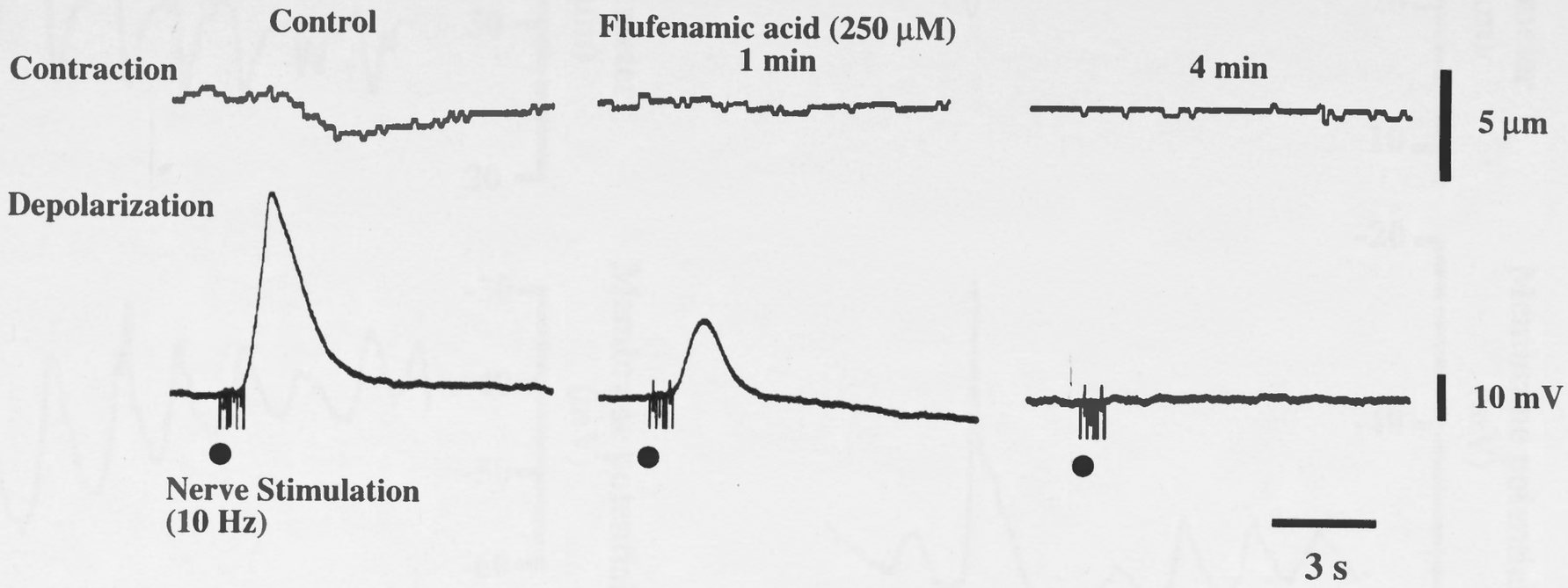


Figure 4.11 Traces showing nerve-evoked cell depolarization and associated constriction of an iris arteriole in control Kreb and in the presence of flufenamic acid (250 μM) for 1 min, when the contraction was abolished,and for 4 min, when the depolarization was abolished. Dots indicate onset of nerve stimulation (10 Hz).

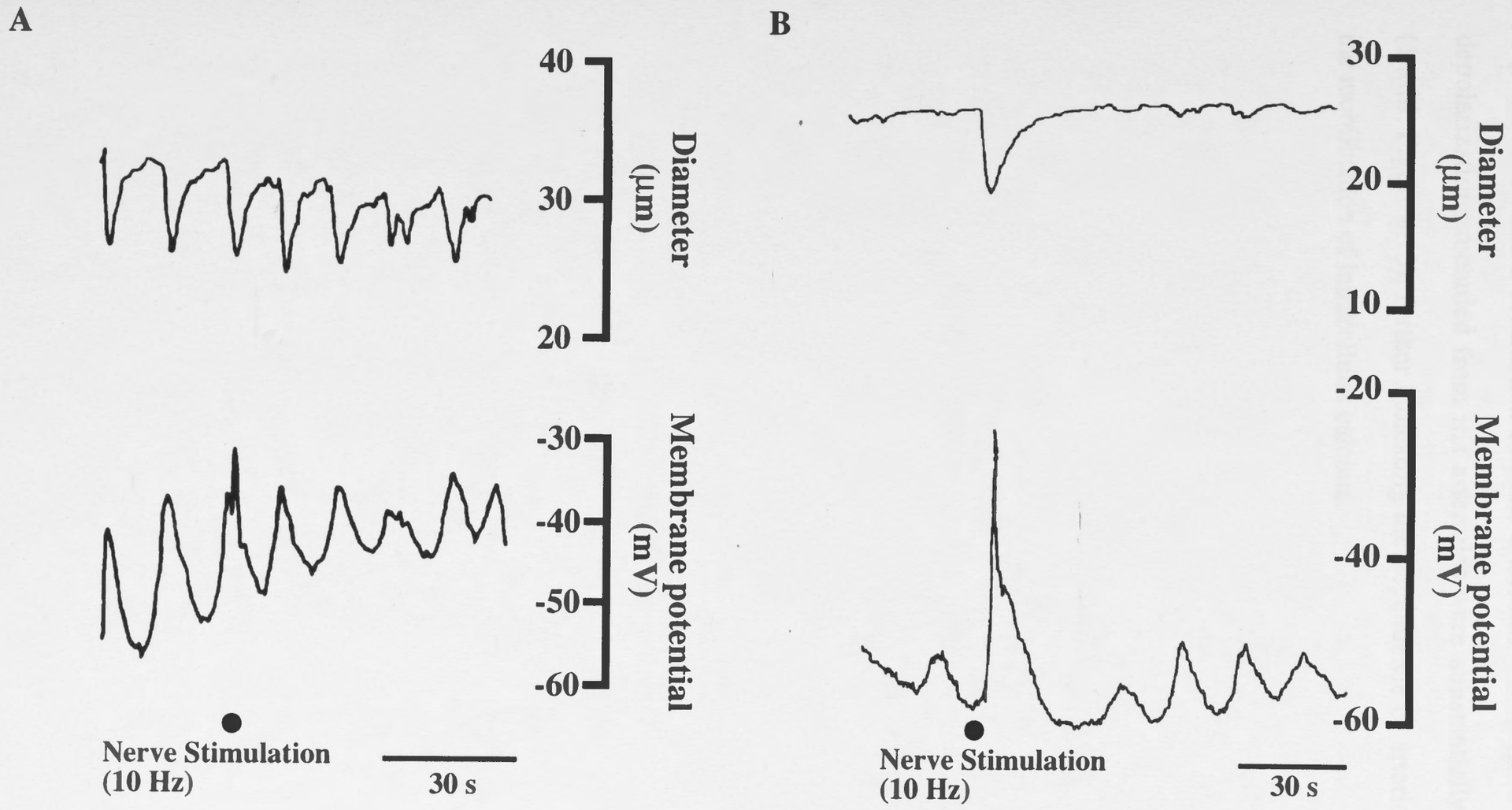


Figure 4.12 Spontaneous depolarizations recorded from iris arteriolar cells. In A, the depolarizations are large and are consistently accompanied by spontaneous contractions of the arteriole. Note also that, while the individual depolarizations are becoming smaller with time, the cell membrane is gradually depolarizing. In B, the spontaneous depolarizations are smaller and are not accompanied by contractions. Nerve stimulation does, however, evoke a contraction. Dots indicate onset of nerve stimulation (10 Hz).

spontaneous depolarizations of the arteriole were recorded, there was no significant spontaneous contraction of the arteriole, although nerve-stimulation produced a large depolarization and a contraction of these arterioles (Figure 4.12 B). Spontaneous depolarizations recorded from iris arterioles were substantially reduced by caffeine (1 mM; Figure 4.13), further indicating the dependence of arteriolar depolarization on the mobilization of intracellular calcium.

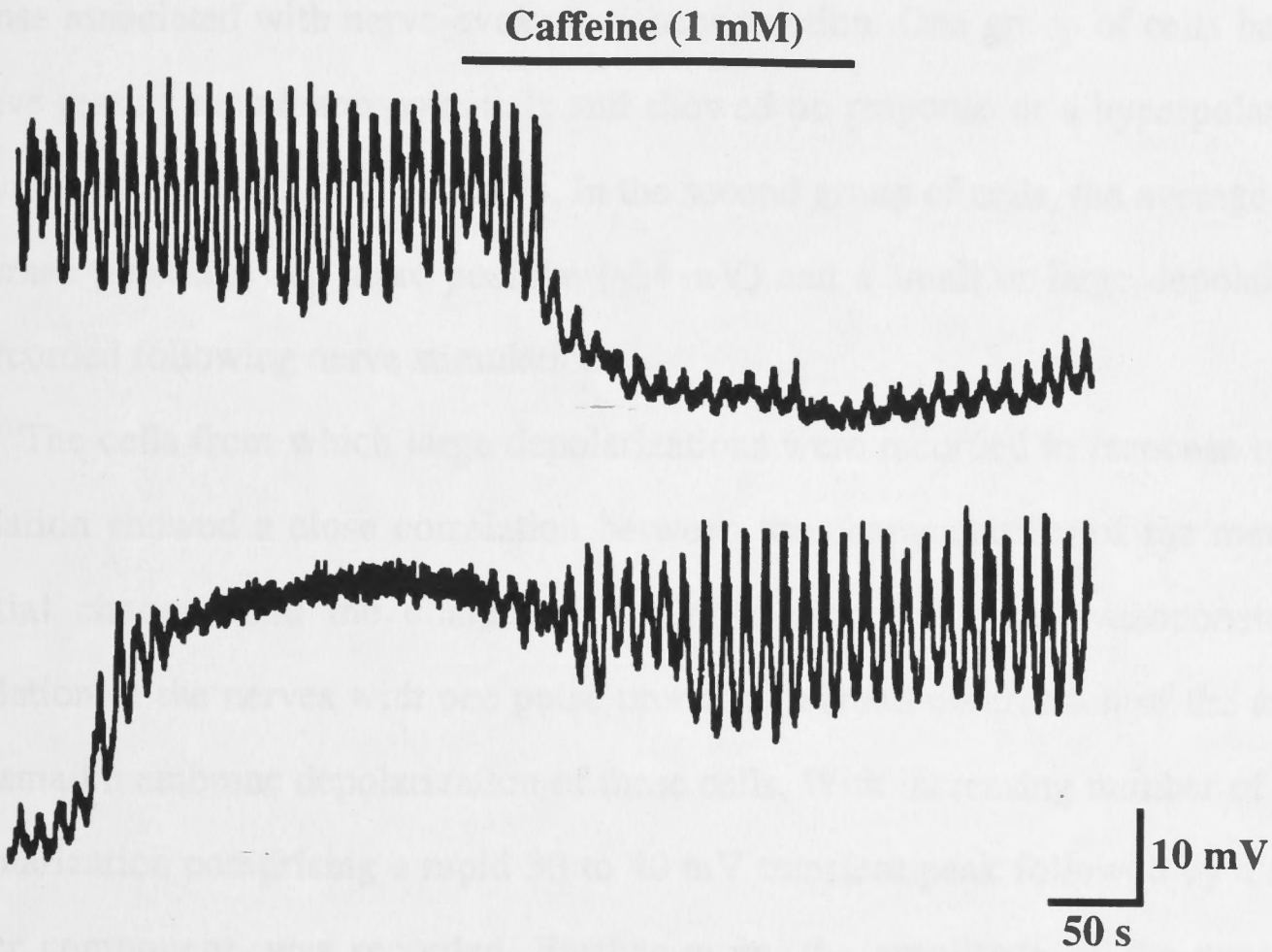


Figure 4.13 Trace showing spontaneous depolarizations recorded from an iris arteriolar cell. Bar indicates perfusion of the bath with caffeine (1 mM). Note that the spontaneous depolarizations return within 10 min of returning to Krebs from caffeine.

DISCUSSION

Transmural nerve-stimulation of the isolated iris of the rat causes a vasoconstriction due to the activation of α_{1B}-adrenoceptors. Using the intracellular recording technique, two distinct types of cells in the iris arterioles have been identified on the basis of both average resting membrane potential and the membrane potential response associated with nerve-evoked vasoconstriction. One group of cells had quite negative resting membrane potentials and showed no response or a hyperpolarization following nerve-stimulation (-74 mV). In the second group of cells, the average resting membrane potential was more positive (-64 mV) and a small or large depolarization was recorded following nerve stimulation.

The cells from which large depolarizations were recorded in response to nerve stimulation showed a close correlation between the characteristics of the membrane potential changes and the characteristics of the nerve-evoked vasoconstriction. Stimulation of the nerves with one pulse produced a small contraction of the arteriole and a small membrane depolarization of these cells. With increasing number of pulses, a depolarization comprising a rapid 30 to 40 mV transient peak followed by a slower, smaller component, was recorded. Further more, the amplitude of the contraction mirrored that of the depolarization. Recordings from this group of cells is therefore proposed to represent recordings from the smooth muscle cells of the arteriole. It is of interest, then, that the average resting membrane potential of these cells lies within the ranges previously reported for arterial smooth muscle (Holman, 1969; Hirst and Edwards, 1989; Kuriyama, Kitamura and Nabata, 1995).

Studies using two microelectrodes, one to inject current and one to measure the current reaching another cell, confirm that the proposed smooth muscle cells of the iris arterioles,that is those in which a large depolarization was recorded following nerve stimulation, are electrically coupled in the iris arterioles (Hirst et al, 1996, submitted for publication).

In addition, the cells in which either no response or a small hyperpolarization were recorded in response to nerve stimulation are proposed to represent the endothelial cells of the iris arteriole. Activation of K^+ channels in endothelial cells is reported to

lead to hyperpolarization (Revest and Abbott, 1992). The average resting membrane potential of these cells is significantly more negative than those of the proposed smooth muscle cells. This difference, and the difference in response to nerve stimulation between the two cell groups, suggests that the endothelial cells and the smooth muscle cells are not electrically coupled. Studies using two microelectrodes have indeed confirmed that these two cell layers are not electrically coupled in the iris arterioles (Hirst et al, 1996, submitted for publication). In two other vessels, the resting membrane potential of the endothelial cells has been reported to be more positive than the values in the present study (Marchenko and Sage, 1993; Xia, Little and Duling, 1995). In contrast to the iris arterioles, in both of these vessels, it has been shown that the endothelial cells and smooth muscle cells are electrically coupled (Xia, Little and Duling, 1995; Marchenko and Sage, 1994).

Both the nerve-evoked vasoconstriction and the large depolarization result from the activation of an α–adrenoceptor by neurally-released neurotransmitter. The time to maximum depolarization of approximately one second is also consistent with the time course of neurogenic α–adrenoceptor mediated depolarizations previously reported, for example, in the rat anococcygeus muscle (Creed, Gillespie and Muir, 1975; Byrne and Large, 1984) and the rat tail artery (Cheung, 1984). The iris arterioles are unusual in that with the stimulus parameters used, nerve stimulation failed to produce a rapid excitatory junction potential but instead the membrane potential changes showed a purely α–adrenoceptor mediated response. Slow α–adrenoceptor mediated depolarizations and contractions in response to nerve stimulation have been recorded from veins (Holman *et al.*, 1968; van Helden, 1991). Nerve stimulation of most mammalian arterial vessels, with parameters comparable to those used in this study, evokes an excitatory junction potential only, or an excitatory junction potential as well as a slower, α–adrenoceptor mediated component (Hirst and Edwards, 1989). To our knowledge, the only other arterial vessels where nerve-mediated depolarization occurs soley as a result of α–adrenoceptor activation only are the guinea–pig pulmonary artery (Suzuki, 1983) and the immature mesenteric artery of the rat (Hill, Hirst and van Helden, 1983).

Nerve-evoked constriction of iris arterioles is mediated by an α_{1B}-adrenoceptor (Chapter 3). Activation of this receptor leads to the formation of IP_3 which acts to liberate intracellular calcium, which in turn activates the contractile apparatus for contraction (Han, Abel and Minneman, 1987). The sensitivity of the time course of the large depolarization, and the contraction, to a change in temperature of only 4 ^oC further suggests that the synaptic α_{1B}-adrenoceptor is activating an intracellularly-mediated response. Neither the nerve-evoked large depolarization, nor the contraction, was abolished by the L-type voltage-dependent calcium channel antagonist nifedipine. At 10 μM, nifedipine reduced the magnitude of the contraction and abolished the transient peak of the depolarization. Caffeine abolished the nerve-evoked vasoconstriction, abolished the the transient peak of the depolarization and reduced the magnitude of the slower component of the depolarization, in addition to virtually abolishing spontaneous depolarizations. Caffeine acts intracellularly to inhibit IP_3-mediated liberation of intracellular calcium, either by depleting calcium stores or interfering with IP_3 receptor activation (Parker and Ivorra, 1991). It is possible that, at 1 mM, caffeine does not release all of the intracellular calcium stores and consequently the depolarization would not be totally abolished. Higher concentrations of caffeine, which have been used in other studies (Lamb, Volk and Shibata, 1994; Robinson and Burgoyne, 1991; Hogg *et al.*, 1993; Klockner, 1993; Lamb, Volk and Shibata, 1994) have an additional effect in the heart, in that they reduce the release of transmitter (3 mM; H. Cousins, personal communication, 1995), and so were not used in this study. The results of these experiments are consistent with the release of intracellular calcium which simultaneously activates the constriction and initiates the depolarization of iris arterioles. The results further suggest that the influx of calcium through a voltage-dependent mechanism contributes only a small component of the increase in intracellular calcium and that this additional component augments, but does not initiate, the contraction.

In smooth muscle cells of blood vessels, elevated intracellular calcium has been reported to activate a depolarizing membrane ionic current through the opening of calcium-dependent chloride channels. Calcium-dependent chloride conductances have

been demonstrated in smooth muscle cells of the rat portal vein (Pacaud *et al.*, 1989), rabbit ear artery (Amedee, Large and Wang, 1990), human mesenteric artery (Klockner, 1993) and rabbit coronary artery (Lamb, Volk and Shibata, 1994). The slow α–adrenoceptor mediated depolarization recorded in venous smooth muscle cells following noradrenaline perfusion (Byrne and Large, 1988; van Helden, 1991; Pacaud *et al.*, 1989; Pacaud *et al.*, 1991) or iontophoresed noradrenaline (van Helden, 1991), is due to an increase in a calcium-dependent chloride conductance. The α–adrenoceptor mediated depolarization of the rat iris dilator muscle and anococcygeus muscle is abolished by chloride ion replacement (Hill *et al.*, 1993; Large, 1984). In the present study, the nerve-mediated depolarization of the iris arterioles was reduced substantially by replacement of the chloride ions in the perfusion solution with sodium methyl sulfate or with sodium isethionate. Furthermore, flufenamic acid, which blocks the calcium-dependent chloride current activated by elevated intracellular calcium in Xenopus oocytes (White and Aylwin, 1990), blocked the nerve-evoked depolarization in iris arterioles. These results suggest that the neurogenic α–adrenoceptor mediated depolarization of iris arterioles is produced by intracellular calcium, liberated from caffeine sensitive stores, activating a calcium-sensitive chloride conductance. Thus, there would appear to be two simultaneous responses to elevated intracellular calcium in these arterioles; one being the activation of the contractile apparatus and vasoconstriction, the other being activation of cell membrane chloride channels, and chloride outflow to produce a depolarization.

The changes in the amplitude and time to maximum depolarization recorded in the iris arteriolar smooth muscle cells in response to an increasing number of stimulus pulses are consistent with the non-linear relationship between increasing levels of photo-released IP_3 and membrane chloride currents recorded in Xenopus oocytes (Parker and Miledi, 1989; Parker and Ivorra, 1992) or hepatocytes (Ogden *et al.*, 1990). The magnitude of the chloride current is reported to be proportional to the rate of rise of the intracellular calcium rather than the absolute concentration following release from IP_3-sensitive stores (Parker and Yao, 1994). With increasing intensity of illumination of caged IP_3 in Xenopus oocytes, the membrane chloride currents recorded became larger,

more transient and had shorter latencies (Parker and Miledi, 1989). Rapid elevation of IP3 in Xenopus oocytes evokes a chloride current consisting of an initial large transient spike and a slower, more sustained component (Gillo *et al.,* 1987) reminiscent of the two component voltage changes recorded in the cells in the present study.

Surprisingly, the nerve-mediated vasoconstriction in the present study was also abolished by chloride replacement and flufenamic acid. It is unlikely that these drugs interfere with neurotransmitter release as preliminary experiments showed that they also prevent vasoconstriction produced by perfusion of phenylepherine (data not shown). In the rat anococcygeus muscle, iontophoretic application of either noradrenaline or phenylephrine produced a depolarization of the smooth muscle membrane and a biphasic contraction. Reduction of the external chloride ion concentration lead to a loss of the depolarization and a loss of the initial rapid phase of the contraction (Hogg, Wang and Large, 1994). One explanation for these effects on arteriolar contraction, then, is that these drugs which interfere with chloride channels may be interacting with internal chloride channels, possibly on the sarcoplasmic reticulum, and thereby affecting intracellular calcium release. Indeed, flufenamic acid is alcohol soluble and may therefore permeate cell membranes to act intracellularly. The localization of chloride channels with caffeine-sensitive calcium stores has been suggested in the rat pulmonary artery (Hogg *et al.,* 1993). In rat parotid acinar cells, a model has also been proposed whereby IP3-sensitive calcium release sites are co-localized with chloride channels (Hassoni and Gray, 1994; Hassoni and Gray, 1994). Chloride channels are present on the sarcoplasmic reticulum membrane of cardiac and skeletal muscle (Kawano and Hiraoka, 1993), where it is proposed that chloride movement into the cytosol is required to maintain charge neutrality across the sarcoplasmic reticulum following the release of intracellular calcium. This movement of chloride ions, through internal chloride channels, into the cytosol would increase the intracellular chloride concentration. Elevated intracellular calcium in cardiac myocytes is sequestered into internal stores by a calcium pump and extruded from the cell via the sodium/calcium exchanger (Vassort *et al.,* 1978; Somlyo *et al.,* 1985; Crespo, Grantham and Cannell, 1990). The increased intracellular chloride concentration could

be lowered by movement of chloride ions out of the cells through calcium-activated chloride channels, resulting in the depolarization recorded in the present study following α–adrenoceptor activation in iris arterioles.

Drugs which interfere with chloride channels abolished the contraction before having their maximal effect on the depolarization. It is possible, then, that the chloride channel in the cell membrane is more sensitive to calcium than is the contractile apparatus, implying that the contractile apparatus has a higher threshold for activation by calcium than does the chloride channel in the cell membrane. Further support for this hypothesis is derived from cells showing regular spontaneous depolarizations. Large spontaneous depolarizations were associated with spontaneous contractions of the arteriole but small spontaneous depolarizations were not. It is interesting that the spontaneous release of calcium from IP3-sensitive stores has been reported to occur with similar frequencies as those for the spontaneous depolarizations reported here (Berridge and Galione, 1988; Terrar and White, 1989). Thus, spontaneously released calcium from intracellular stores may activate a cell membrane chloride channel and depolarization. If the intracellular calcium oscillations were sufficiently large, they would also activate the contractile apparatus and produce spontaneous contractions.

In summary, intracellular recordings have been used to investigate membrane potential changes associated with nerve-mediated constriction of iris arterioles. The results of these experiments suggest that both the nerve-mediated constriction and depolarization are due to the activation of α–adrenoceptors by neurally-released transmitter. Influx of calcium through voltage-dependent calcium channels following chloride-channel-mediated depolarizations, can augment vasoconstriction, but is not responsible for the initiation of the contraction.

CHAPTER 5.

MODULATION OF SYMPATHETIC VASOCONSTRICTION BY SENSORY-MOTOR NERVES

INTRODUCTION

Non-cholinergic vasodilatation following nerve stimulation has been observed in a number of vascular beds and may result from the release of substance P and/ or CGRP from capsaicin-sensitive sensory-motor nerves (Holzer, 1992).

Several lines of evidence suggest that mutual interactions occur between these peptidergic sensory-motor nerves and the sympathetic vasoconstrictor nerves. Both noradrenaline and NPY, which are co-released from sympathetic nerves, have been reported to act presynaptically on sensory-motor nerves to inhibit release of neuropeptides (Lindgren *et al.,* 1987; Matran, Martling and Lundberg, 1989; Kawasaki *et al.,* 1990b; Kawasaki *et al.,* 1991; Fuder and Selbach, 1993). On the other hand, in the rabbit central ear artery and in rat mesenteric arteries, neurotransmitters released from sensory-motor nerves have been reported to act postsynaptically to inhibit vasoconstriction produced by exogenous noradrenaline or sympathetic nerve stimulation (Kawasaki *et al.,* 1990a; Maynard, Saville and Burnstock, 1990; Remak, Hottenstein and Jacobson, 1990). This latter effect was mediated via CGRP and not substance P (Han, Naes and Westfall, 1990; Li and Duckles, 1992; Maynard, Saville and Burnstock, 1990). In mesenteric vessels, the nerve-mediated vasoconstriction results from the entry of calcium through voltage-dependent calcium channels (Hill, Hirst and van Helden, 1983). In these vessels, the inhibitory effect of CGRP has been suggested to be due to sensory-motor nerve-induced hyperpolarization and a corresponding reduction in adrenergic excitatory junction potentials (Meehan, Hottenstein and Kreulen, 1991). Indeed, CGRP has been shown to activate ATP-sensitive K^+ channels and cause a hyperpolarization of arterial smooth muscle cells (Saito *et al.,* 1989; Nelson *et al.,* 1990).

Sympathetic vasoconstriction of iris arterioles is mediated by α_{1B}-adrenoceptor activation and the mobilization of intracellular calcium. In these arterioles, blockade of voltage-dependent calcium channels did not prevent the contraction (Chapter 3). Thus, vasoconstriction may be seen to be largely independent of depolarization of the muscle membrane. This chapter investigates whether sensory modulation of sympathetic vasoconstriction is also found in these arterioles which do not require the operation of voltage-dependent calcium channels for the initiation of contraction.

RESULTS

Effect of time interval between stimulus trains

Stimulation of the intramural nerves at 10 Hz for 1 s every 3 min produced contractions which were consistent in amplitude (Figure 5.1). When the interval between stimulus trains was reduced to 15 s, however, the responses rapidly decreased in size and were frequently abolished by the third stimulus (Figure 5.1). When intervals between stimulus trains of 15 s to 3 min were tested, it was found that contractile responses were attenuated when trains of stimuli were separated by less than 2 min, the greatest attenuation occurring for stimuli separated by 15 s, which was the minimum time tested (Figure 5.2).

In preparations in which the blood vessels were spontaneously active, transmural stimulation elicited vasoconstriction which was followed by a silent period during which time the spontaneous contractions were absent (Figure 5.3).

Effect of capsaicin treatment

When the responses of iris blood vessels from capsaicin-treated rats were tested to stimulus trains separated by 15 s, it was found that the contractions persisted at a constant amplitude while those of blood vessels from vehicle-treated littermates were rapidly lost. Pooled results from preparations from capsaicin-treated and vehicle-treated rats are shown in Figure 5.4. Note that the first contractile response in arterioles from capsaicin-treated rats was slightly greater than in arterioles from control rats. While the contractions in the preparations from the control rats were eliminated by the third stimulus, those in the preparations from the capsaicin treated rats persisted for as long as the stimuli were presented (significantly different for the 2nd to 6th stimulus pulse, t-test with Bonferroni correction).

Treatment of control preparations with capsaicin (10 μM) led to an initial rapid loss of nerve mediated vasoconstriction (Figure 5.5) but over the next 15 min, the contractile response to nerve stimulation recovered. These observations correspond with the previously documented effects of acute capsaicin, i.e. massive release of neuropeptides from sensory-motor nerves followed by a prolonged loss of sensory-motor function due to desensitization and a blockade of nerve conduction (Holzer,

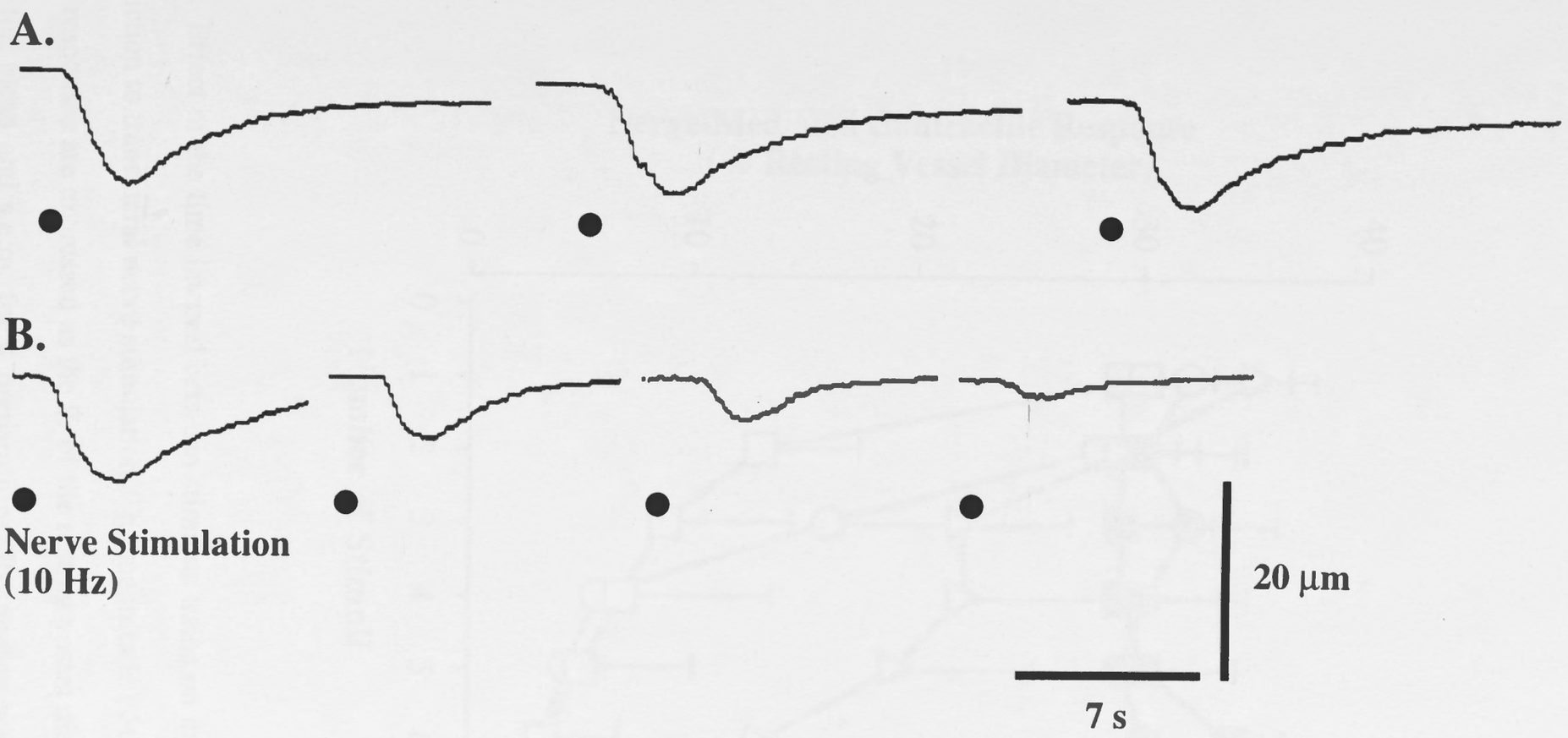


Figure 5.1 Representative traces showing contraction of an iris arteriole in response to transmural nerve stimulation at 10 Hz for 1 s either every 3 min (Panel A.) or every 15 s (Panel B). Note that the contractions remain constant in amplitude when stimulus trains are separated by 3 min but are rapidly desensitized when trains are separated by only 15 s.

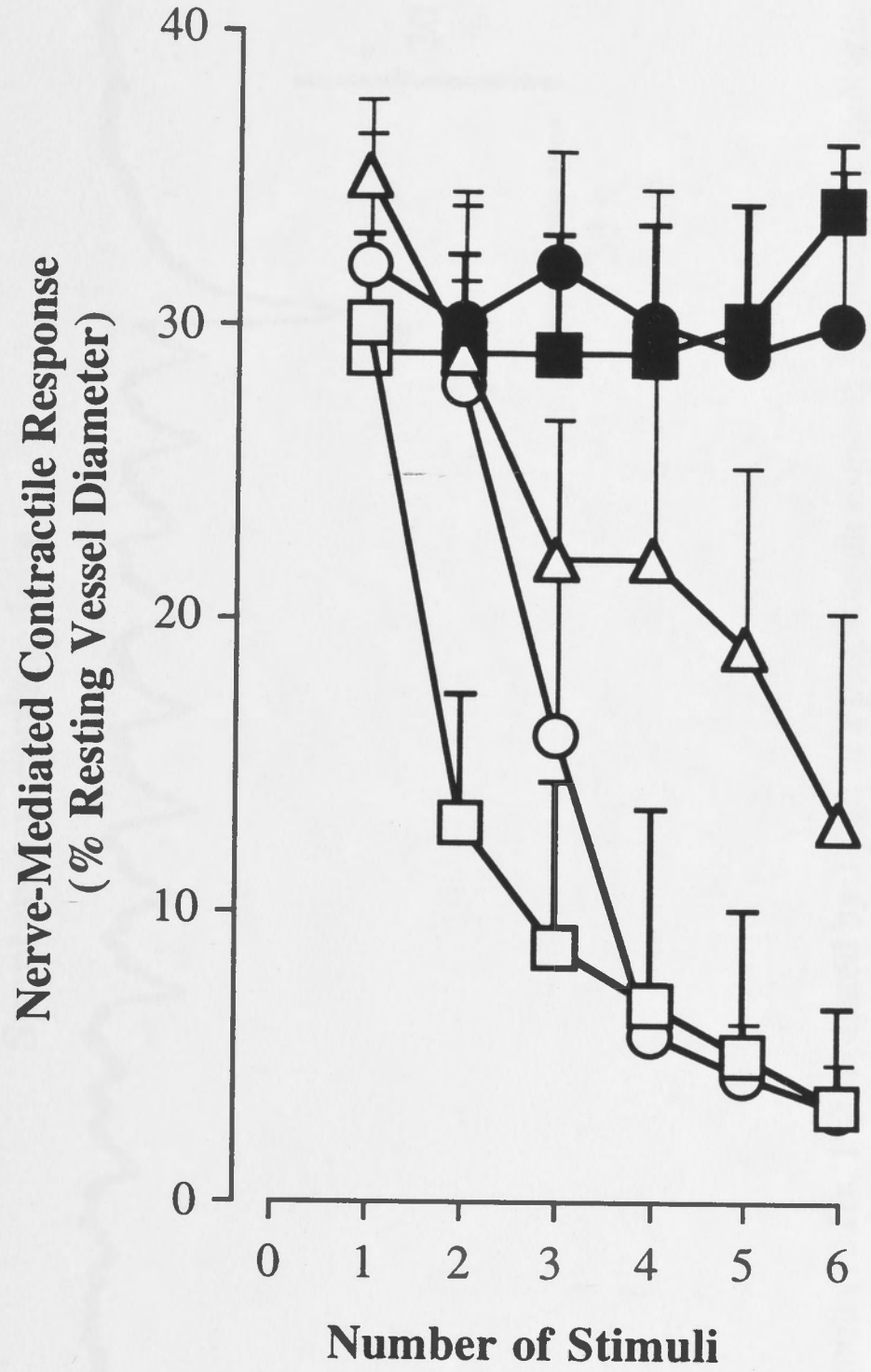


Figure 5.2 Effect of the time interval between stimulus trains on the amplitude of the vasoconstriction to transmural nerve stimulation. The magnitude of the nerve-mediated contractile responses are expressed as the % of the resting vessel diameter. Each point represents the mean and s.e.m. for 4 preparations. Responses were recorded for 6 consecutive stimulus trains where the individual trains were separated by 15 s (open squares), 30 s (open circles), 1 min (open triangles), 2 min (closed circles) and 3 min (closed squares).

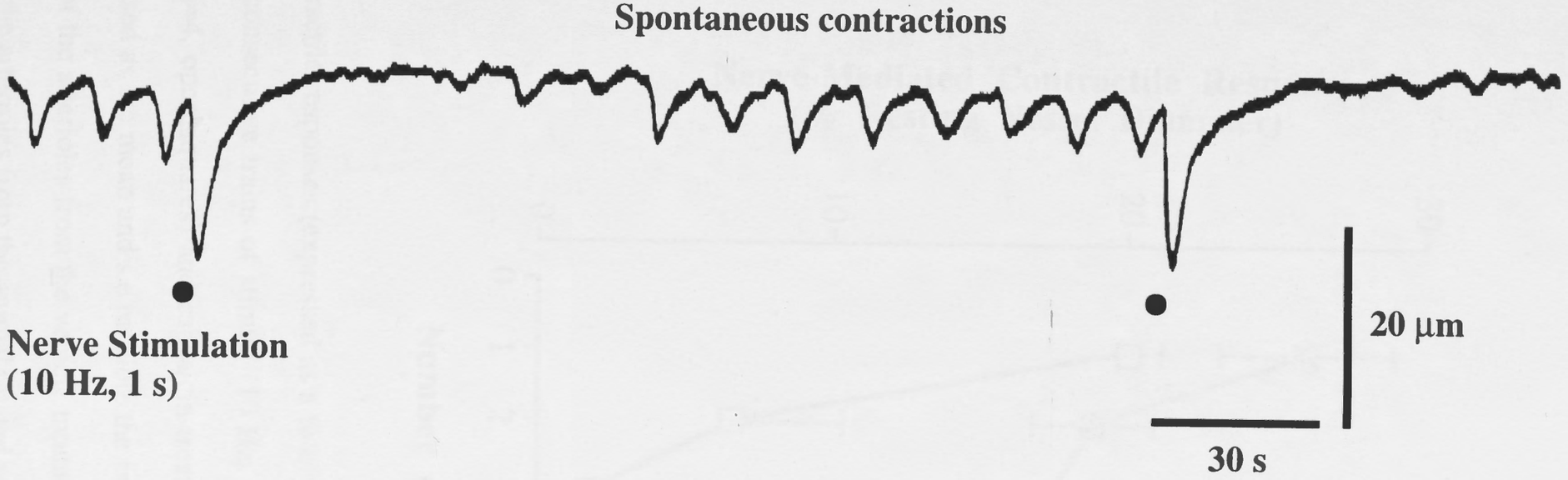


Figure 5.3 Contractile responses to trains of stimuli (10 Hz, 1 s) separated by 3 min in a preparation showing spontaneous contractions every 13 s. Note that a larger contraction immediately follows the nerve stimulation and that, for the next 40 to 50 s, the spontaneous contractions are abolished.

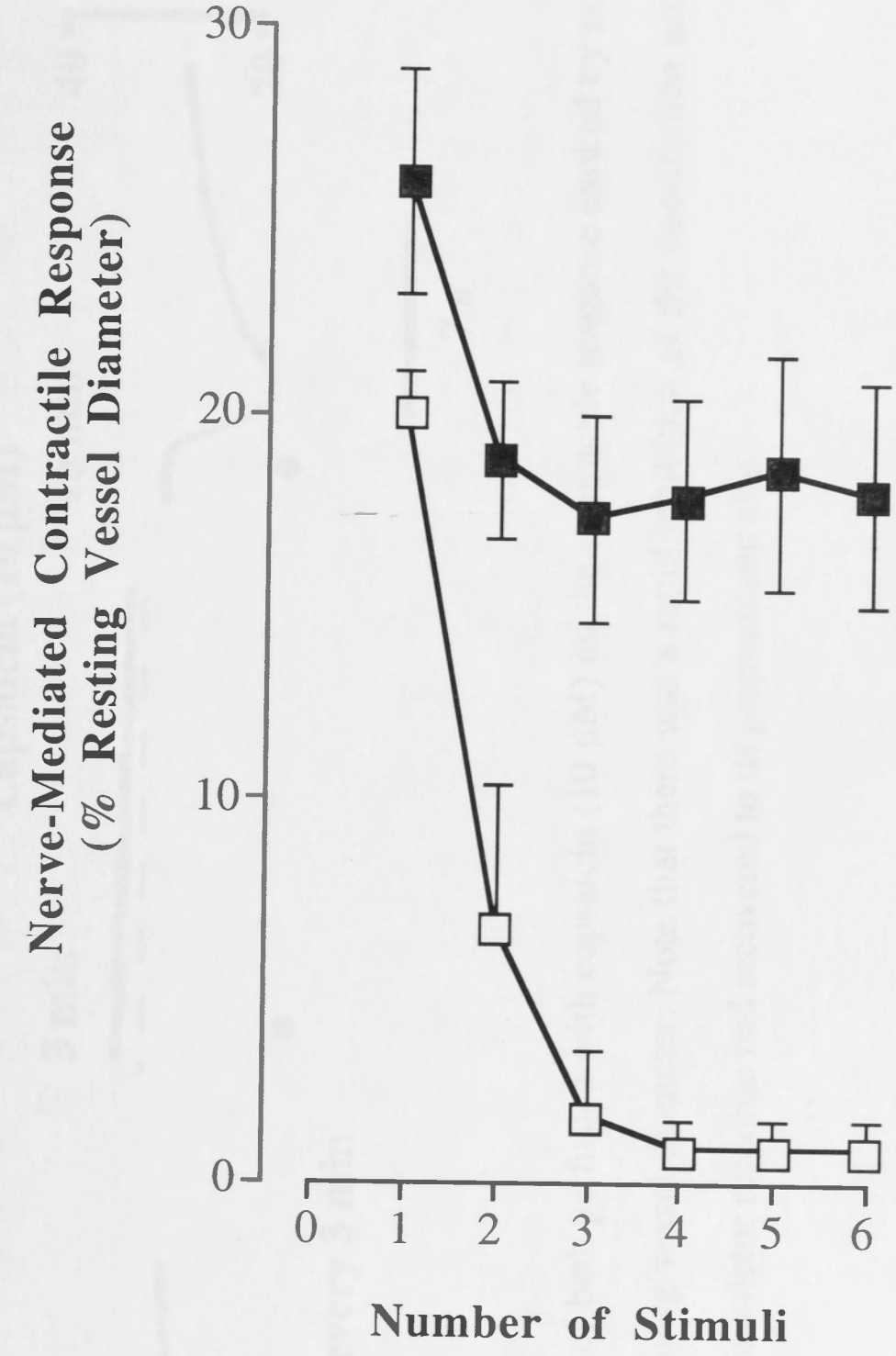


Figure 5.4 Contractile responses (expressed as a % of the resting vessel diameter) of iris arterioles to consecutive trains of stimuli (10 Hz, 1 s) separated by 15 s from a vehicle-treated (n=4, open squares) and capsaicin-treated (n=7, closed squares) rats. Values are expressed as the mean and s.e.m. Note the rapid loss of the nerve-mediated vasoconstriction of the arterioles from the vehicle treated animal but the persistence of the contraction in the arterioles from the animal treated neonatally with capsaicin ($50\ mg.kg^{-1}$).

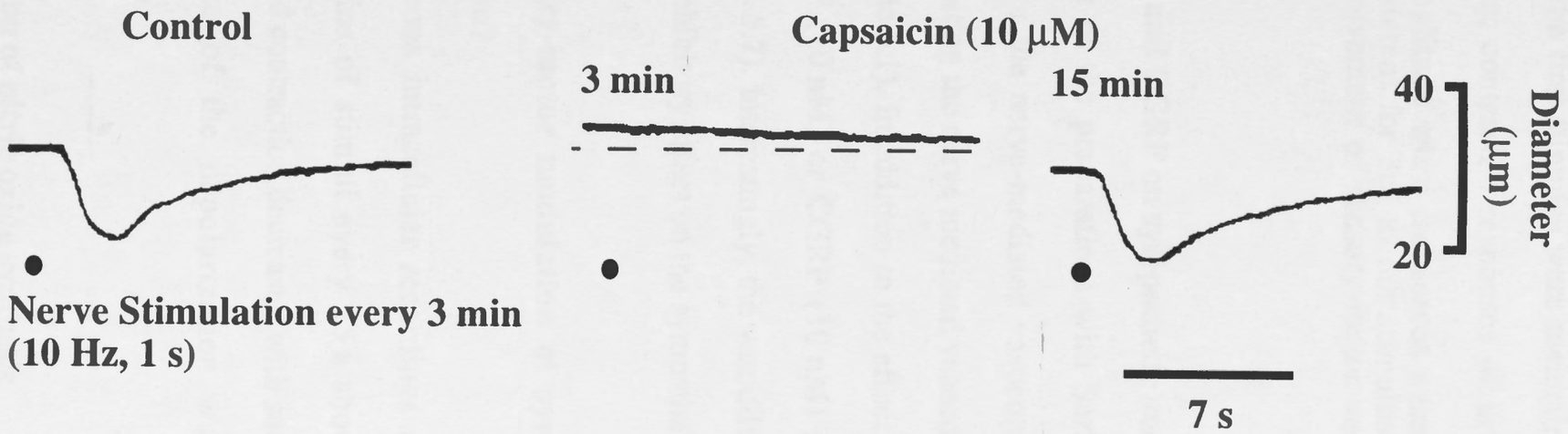


Figure 5.5 Traces showing the immediate effect of bath perfusion with capsaicin (10 μM) on the contractile response evoked by nerve stimulation every 3 min (10 Hz, 1s). Dotted line indicates resting vessel diameter. Note that there was a rapid inhibition of the constriction accompanied by a vasodilatation. Within 15 min, however, the vasoconstrictor response had recovered to its pretreatment size.

1991a). When the contractile response had returned to its pretreatment level, the preparations were stimulated every 15 s. In the presence of the capsaicin, the contractile responses after the first stimulus were maintained at constant amplitude (Figure 5.6). Before capsaicin, contractile responses of arterioles in these preparations rapidly decreased in amplitude when the nerves were stimulated every 15 s. These results suggest the involvement of sensory-motor nerves in the inhibition of sympathetic vasoconstriction.

Effect of SarSP and CGRP on sympathetic vasoconstriction

Perfusion of the preparations with SarSP led to a rapid and concentration-dependent loss of the nerve-mediated vasoconstriction (Table 5.1). CGRP was also effective in inhibiting the nerve mediated vasoconstriction, although slightly less potent than SarSP (Table 5.1). In addition to the effect on nerve stimulation, perfusion of the bath with SarSP (10 nM) or CGRP (10 nM) produced a vasodilatation of the iris arterioles (Figure 5.7). Interestingly, the vasodilatation produced by CGRP was longer-lasting than the inhibitory effect on the sympathetic vasoconstriction (result not shown).

Does the sensory-motor modulation of sympathetic vasoconstriction involve hyperpolarization?

Simultaneous intracellular recordings and measurement of the contractile responses to trains of stimuli every 15 s showed that the magnitude of both the depolarization and contraction decreased with successive stimuli (n=3; Figure 5.8). The decrease in size of the depolarization was, however, not accompanied by hyperpolarization.

Effect of inhibition of nitric oxide synthesis

The effect of inhibition of nitric oxide synthesis on the loss of nerve-mediated vasoconstriction during repetitive stimulation was tested using L-NAME. Preincubation of preparations for 20 min in the inactive isomer, D-NAME (10 μM), had no effect on the loss of nerve-mediated vasoconstrictions following nerve stimulation every 15 s

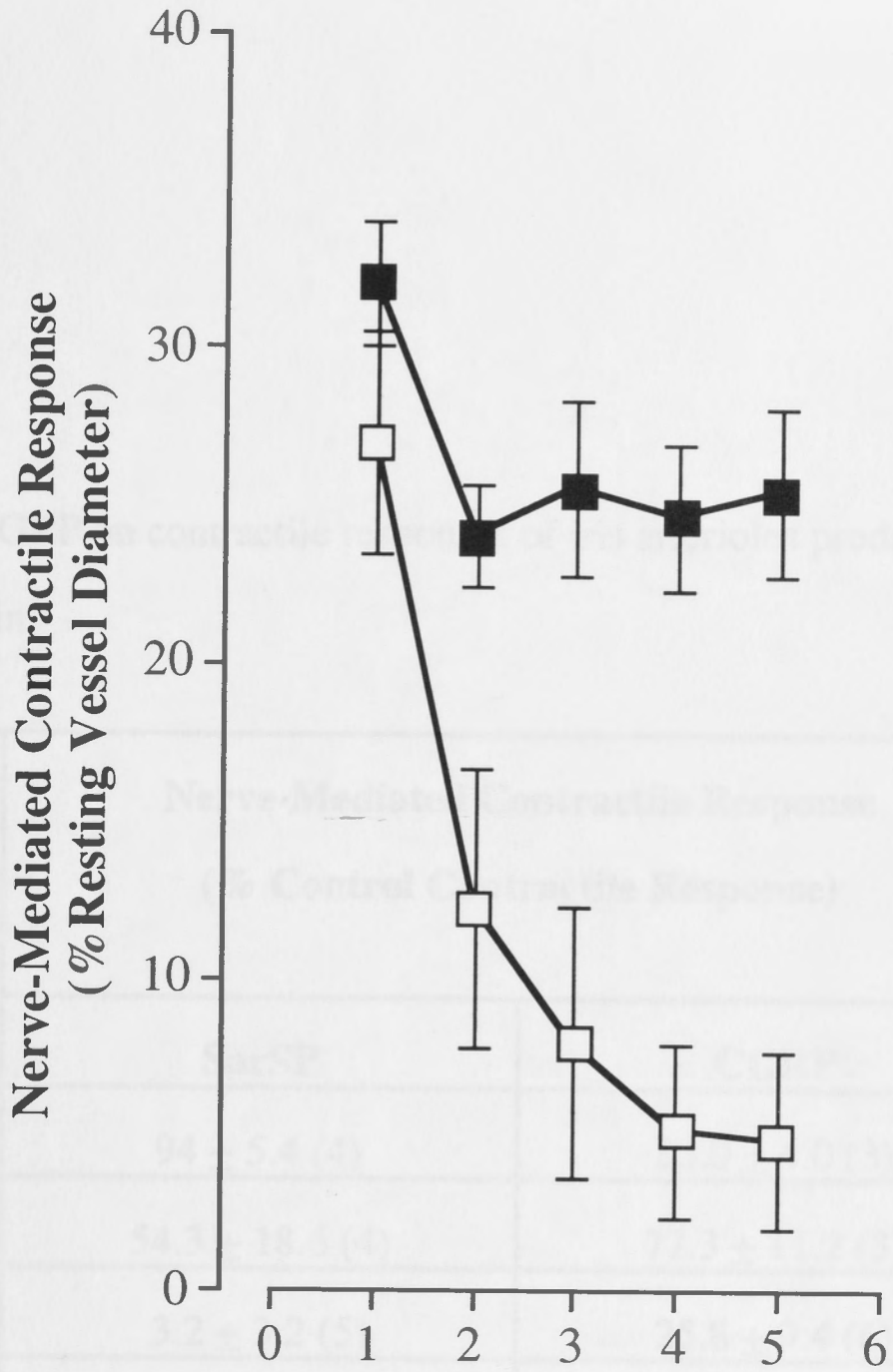


Figure 5.6 Effect of capsaicin on the desensitization of the vasoconstrictor response following repetitive nerve stimulation when the individual trains of stimuli (10 Hz, 1 s) were separated by 15 s. Points represent the mean and s.e.m. for 4 preparations which were repetitively stimulated in control Krebs, before the addition of capsaicin (open squares) and after the addition of capsaicin to the perfusion solution at a time when the vasoconstrictions had returned to pretreatment levels (closed squares). Note that the responses rapidly desensitized before the addition of capsaicin but remained constant in amplitude when repetitively stimulated after capsaicin had been perfusing through the recording chamber for at least 15 min.

Table 5.1

Effect of SarSP and CGRP on contractile responses of iris arterioles produced by nerve stimulation every 3 min.

Agonist concentration (n M)	**Nerve-Mediated Contractile Response (% Control Contractile Response)**	
	SarSP	**CGRP**
0.1	94 ± 5.4 (4)	85.0 ± 4.0 (3)
1	54.3 ± 18.6 (4)	77.3 ± 11.2 (3)
10	3.2 ± 3.2 (5)	25.8 ± 9.4 (6)
100	1.5 ± 1.5 (7)	19.9 ± 9.5 (4)

Results are expressed as mean ± s.e.m. of the number of preparations in parenthesis.

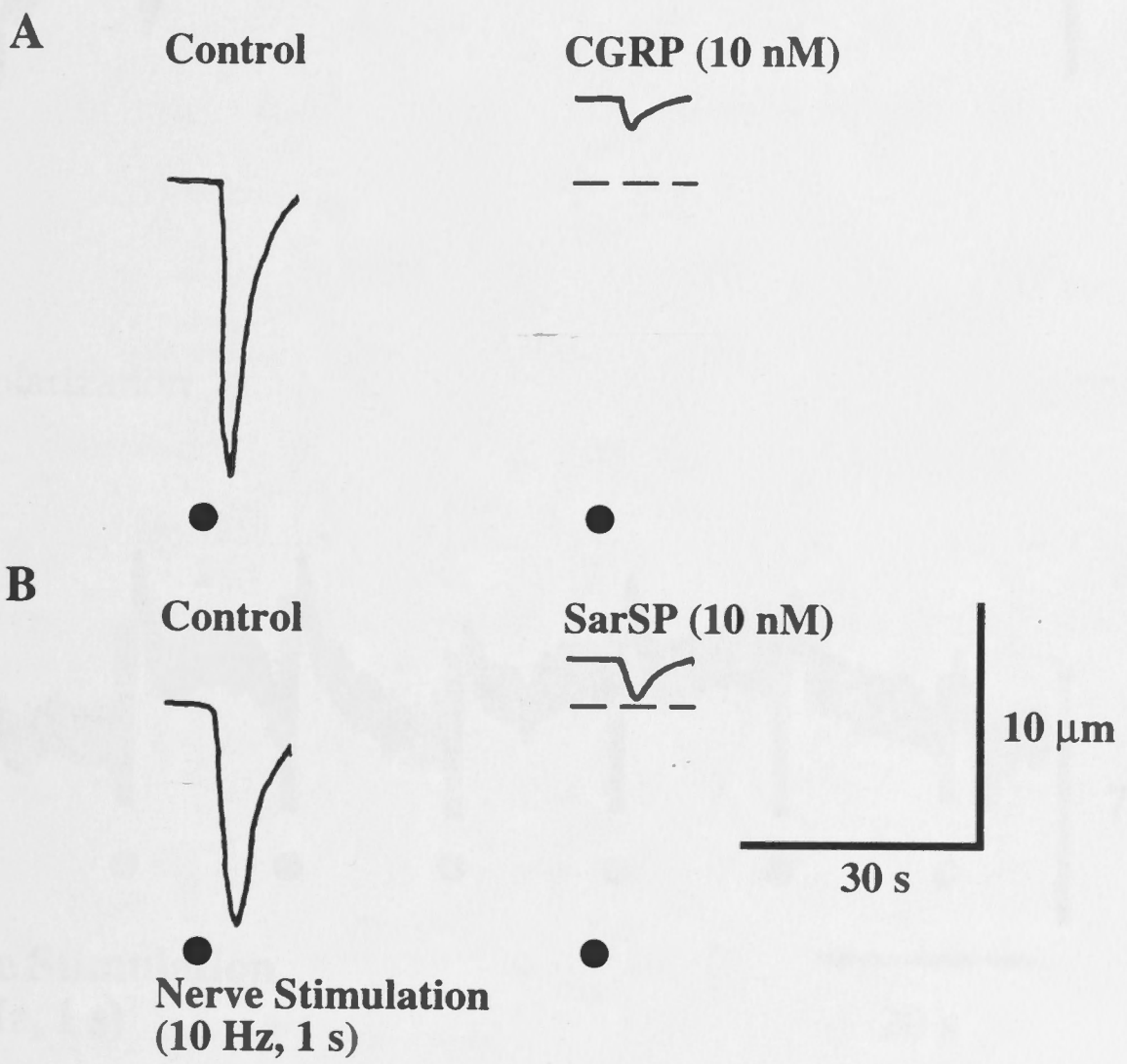


Figure 5.7 Traces showing the effect of SarSP (10 nM; Panel A) and CGRP (10 nM; Panel B) on the arteriolar contractile response to nerve stimulation every 3 min. Dots indicate the onset of nerve stimulation (10 Hz, 1 s).

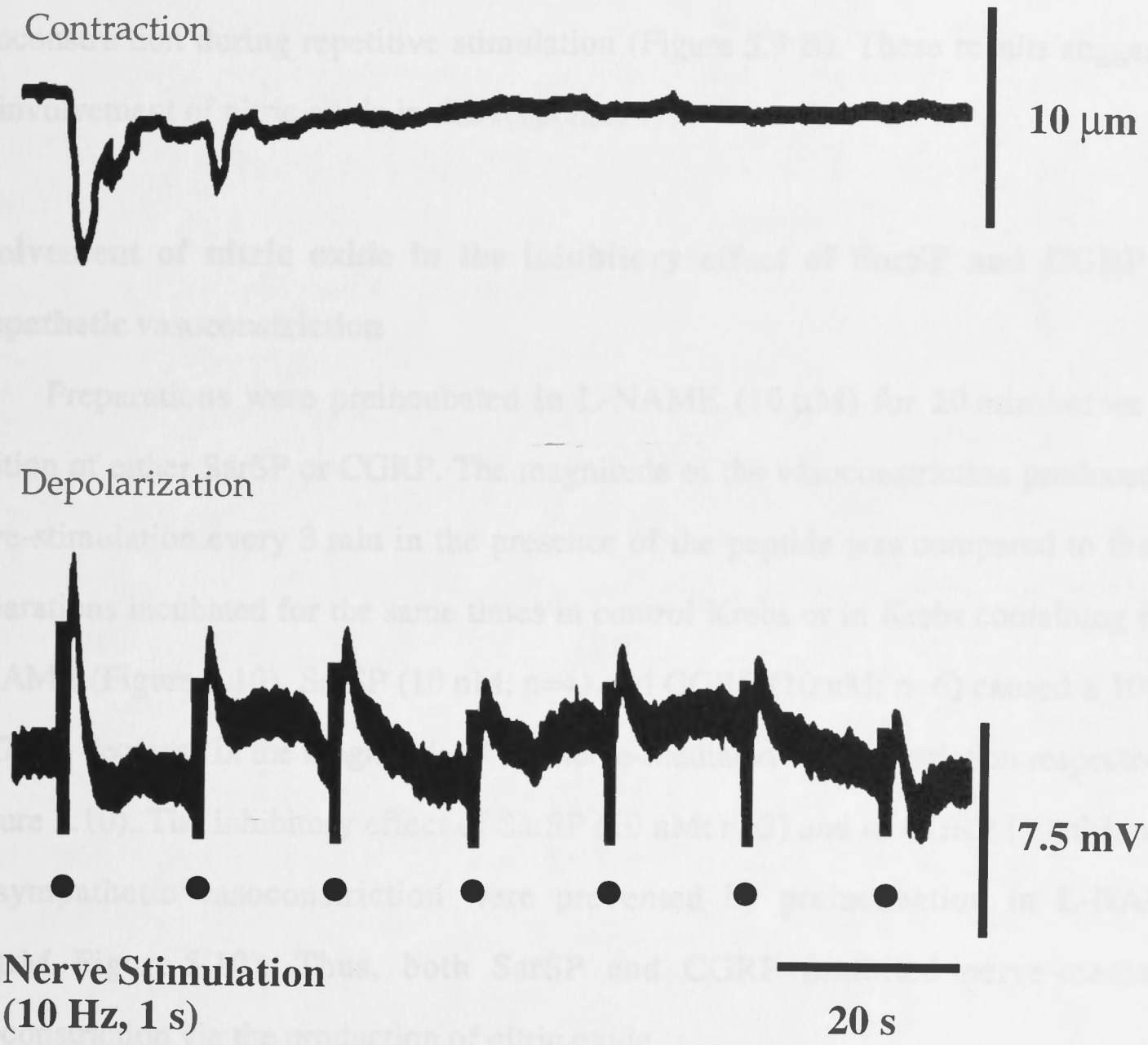


Figure 5.8 Traces showing simultaneous recording from an iris arteriole of intracellular responses and associated arteriolar contractile responses to nerve stimulation (10 Hz, 1 s) every 15 s.

(n=3; Figure 5.9 A). Preincubation in L-NAME (10 μM) led to a decrease in resting vessel diameter suggesting the involvement of nitric oxide in the maintenance of basal tone of the vessels. This change in basal tone had no effect on subsequent responses to nerve-stimulation (Figure 5.10). L-NAME prevented the loss of nerve-mediated vasoconstriction during repetitive stimulation (Figure 5.9 B). These results suggested the involvement of nitric oxide in this response.

Involvement of nitric oxide in the inhibitory effect of SarSP and CGRP on sympathetic vasoconstriction

Preparations were preincubated in L-NAME (10 μM) for 20 min before the addition of either SarSP or CGRP. The magnitude of the vasoconstriction produced by nerve-stimulation every 3 min in the presence of the peptide was compared to that in preparations incubated for the same times in control Krebs or in Krebs containing only L-NAME (Figure 5.10). SarSP (10 nM; n=4) and CGRP (10 nM; n=6) caused a 100 % and 75 % decrease in the magnitude of the nerve-mediated vasoconstriction respectively (Figure 5.10). The inhibitory effect of SarSP (10 nM; n=3) and of CGRP (10 nM; n=4) on sympathetic vasoconstriction were prevented by preincubation in L-NAME (10 μM, Figure 5.10). Thus, both SarSP and CGRP inhibited nerve-mediated vasoconstriction via the production of nitric oxide.

Effect of antagonists to neuropeptide receptors

In order to determine whether substance P or CGRP was involved in the loss of the vasoconstrictor responses, the effect of antagonists to the NK_1- and NK_2-tachykinin receptors, RP 67580 (1 μM; n=4) and L-659877 (1 μM; n=3) respectively, and the antagonist for $CGRP_1$ receptors, $CGRP_{8-37}$ (1 μM; n=4) were tested. Neither the NK_1- nor NK_2-tachykinin receptor antagonist was able to prevent the loss of the vasoconstriction in response to nerve stimulation every 15 s (Figure 5.11; closed squares; closed triangles respectively), nor were they able to prevent the effect when present in combination (n=4; Figure 5.11, closed circles). On the other hand, the

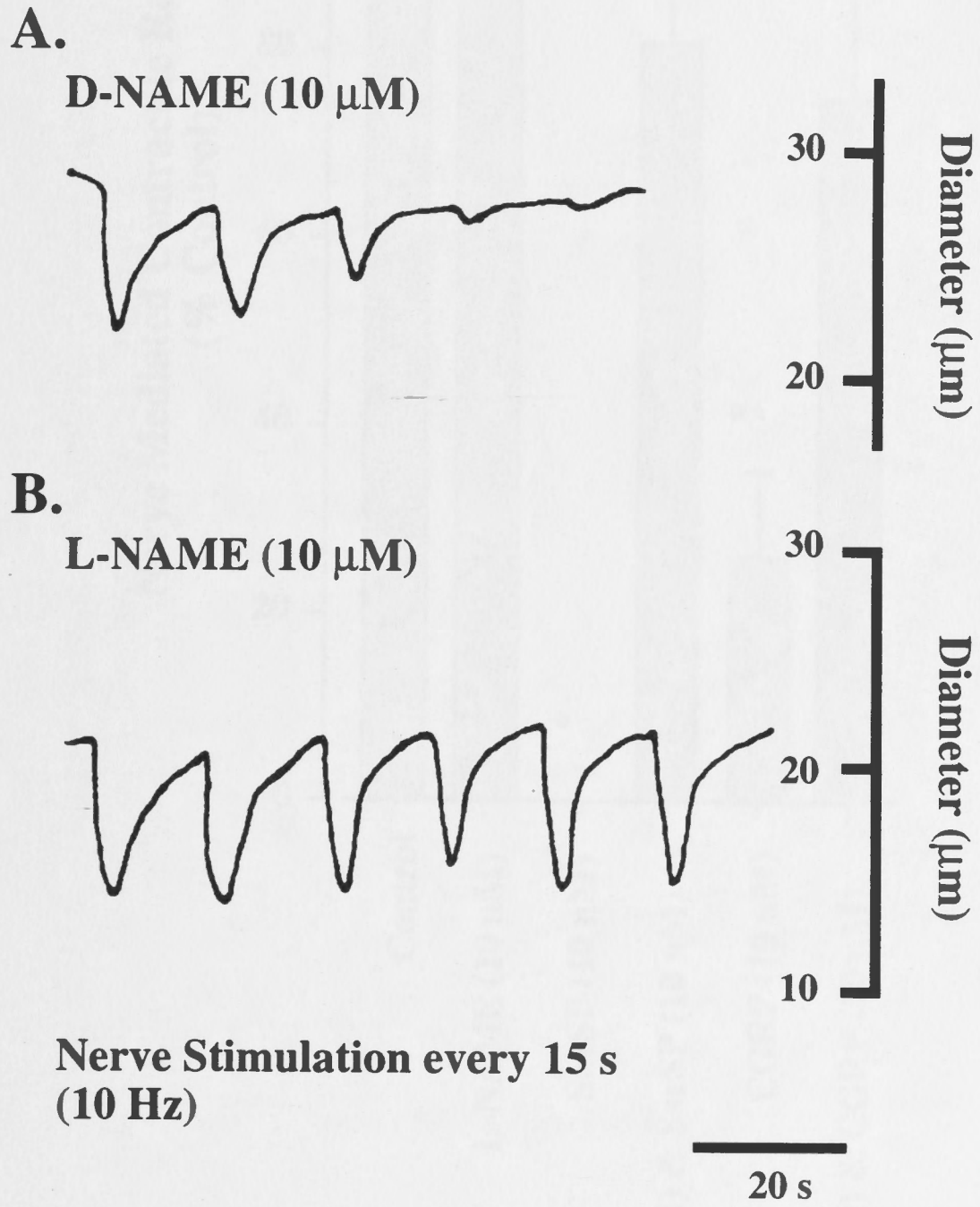


Figure 5.9 Effect of preincubation in **A**. D-NAME (10 μM) and **B.** L-NAME (10 μM) on an iris arteriole contractile response to nerve stimulation (10 Hz, 1 s) every 15 s. Note that the resting vessel diameter has been reduced in the presence of L-NAME.

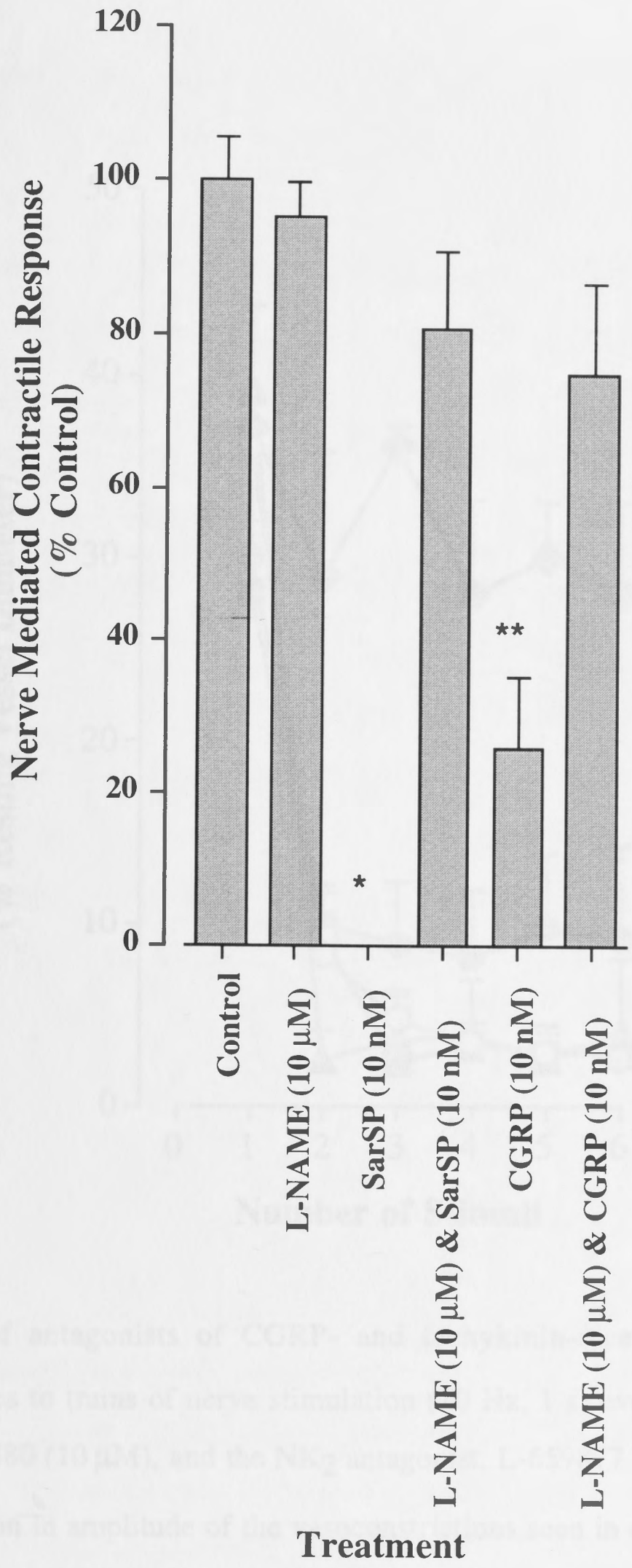


Figure 5.10 Effect of preincubation in L-NAME (10 μM) on the inhibition of nerve-mediated vasoconstriction produced by SarSP (10 nM) and CGRP (10 nM). Note that preincubation in L-NAME prevented the effect of both SarSP and CGRP. * indicates significantly different from L-NAME and SarSP; ** indicates significantly different from L-NAME and CGRP.

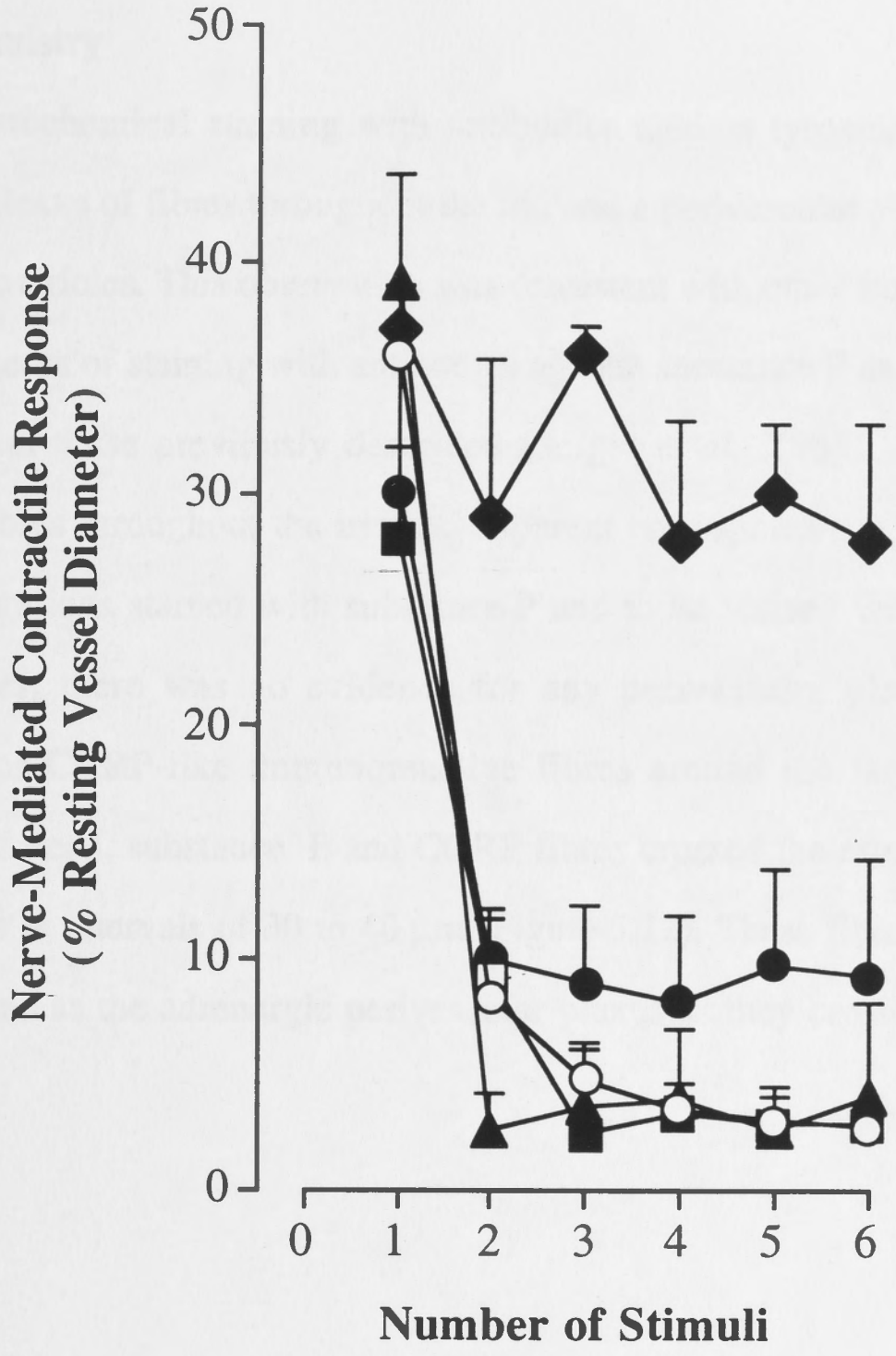


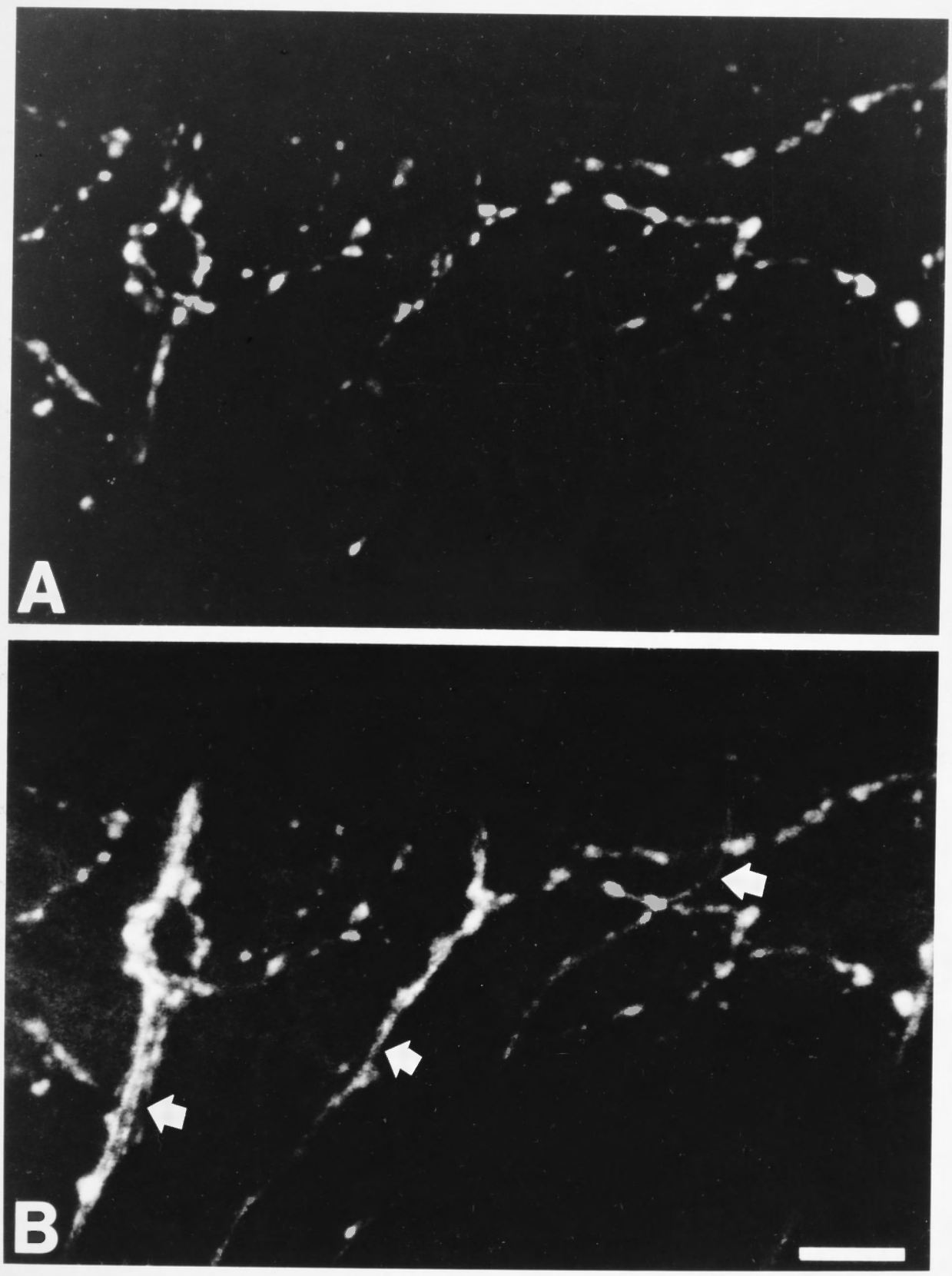
Figure 5.11 Effect of antagonists of CGRP- and tachykinin-receptors on the vasoconstrictor responses to trains of nerve stimulation (10 Hz, 1 s) every 15 s. The NK_1 antagonist, RP 67580 (10 μM), and the NK_2 antagonist, L-659877 (10 μM), had no effect on the reduction in amplitude of the vasoconstrictions seen in control Krebs (open circles), when present either singly (closed squares and closed triangles, respectively) or in combination (closed circles). The $CGRP_1$ antagonist, $CGRP_{8-37}$ (10 μM, closed diamonds), however, prevented the desensitization of the vasoconstrictions. Points represent the mean and s.e.m. for 3 to 4 preparations.

$CGRP_1$-receptor antagonist did prevent the loss of nerve-mediated vasoconstriction due to repetitive nerve stimulation (Figure 5.11, closed diamonds).

Immunohistochemistry

Immunohistochemical staining with antibodies against tyrosine hydroxylase revealed a dense plexus of fibres throughout the iris and a perivascular plexus of fibres around the larger arterioles. This observation was consistent with other studies (Olson et al, 1988). The patterns of staining with antibodies against substance P and CGRP were also consistent with those previously described (Seiger *et al.,* 1985). A moderately dense plexus of fibres throughout the iris and apparent correspondence in this plexus between the preparations stained with substance P and those stained with CGRP was observed. However, there was no evidence for any perivascular plexus of either substance P-like or CGRP-like immunoreactive fibres around the larger arterioles described above. Instead, substance P and CGRP fibres crossed the arterioles at right angles to their axis at intervals of 30 to 40 μm (Figure 5.12). These fibres appeared in the same focal plane as the adrenergic perivascular plexus as they crossed the vessels (Figure 5.12).

Figure 5.12 Immunohistochemistry of an arteriole from an iris treated simultaneously with antibodies against substance P to visualize sensory fibres and with tyrosine hydroxylase to visualize sympathetic fibres. Panel A shows the perivascular plexus formed by the varicose, adrenergic sympathetic fibres around the arteriole. Panel B shows the same adrenergic plexus with superposition of the fibres showing substance P-like immunoreactivity (arrows). This was reconstructed from eight optical sections, using a confocal microscope and appropriate filters to demonstrate the Texas Red-stained sympathetic fibres and the FITC-stained sensory fibres. Note how close together the varicosities of the two fibre types lie. Also note that, although the fibres showing substance P-like immunoreactivity do not form a perivascular plexus, they do lie in the same focal plane as the sympathetic fibres. Magnification bar represents 10 μm.


A
B

DISCUSSION

Transmural nerve stimulation of the rat iris at 10 Hz for 1 s every 3 min produced a consistent vasoconstriction of the iris arterioles. When the nerves were stimulated repetitively, with the interval between successive trains of stimuli being less than 2 min, these contractile responses showed a rapid desensitization. Similarly, in arterioles showing spontaneous contractions, nerve stimulation caused a vasoconstriction which was followed by a silent period of some 40 to 50 s during which the spontaneous activity was absent. These results demonstrate that repetitive stimulation of non-cholinergic nerves produces an immediate vasoconstriction followed by a long lasting inhibition of contractile activity. This could result from either, down regulation of the adrenergic receptors or molecules involved in their second messenger pathways, prejunctional inhibition of sympathetic nerve activity or an interaction with sensory-motor nerves.

The iris of the rat is richly supplied with nerve fibres of sympathetic, parasympathetic and sensory origin (Olson *et al.*, 1988). In the present study, the actions of the cholinergic, parasympathetic nerves have been prevented by the addition to the perfusion medium of hyoscine hydrochloride (10 μM). The influence of the sensory-motor fibre activation on the sympathetic vasoconstriction of iris arterioles was determined by treating newborn rats with multiple injections of capsaicin, which has been reported to selectively destroy the small, substance P-, CGRP-containing primary afferent sensory-motor neurones (Holzer, 1991a). Such treatment leads to the loss of the substance P-containing nerves within the iris (Hill and Vidovic, 1989). When arterioles from capsaicin-treated animals were tested for contractile responses to nerve stimulation it was found that the sympathetic nerve-induced vasoconstrictions were significantly larger than those in the vehicle-treated control animals. Similar results have been described in the mesenteric arteries from capsaicin-treated animals and have been attributed to the absence of sensory-motor nerve-mediated vasodilatation (Kawasaki *et al.*, 1990a; Remak, Hottenstein and Jacobson, 1990; Li and Duckles, 1992). Permanent ablation of capsaicin-sensitive neurons can lead to an increase in the transmitter content and/or the innervation density of sympathetic nerve endings (Terenghi *et al.*, 1986;

Luthman *et al.*, 1989; Hill and Vidovic, 1989). Reciprical interactions between sensory-motor and sympathetic nerves may explain the decreased blood flow observed in adult rats which have been treated as neonates with capsaicin (Hottenstein *et al.*, 1992).

When the trains of nerve stimuli were separated by shorter intervals, for example, 15 s, the contractile responses of iris arterioles from capsaicin-treated animals remained constant in amplitude rather than being rapidly reduced as occurred in the animals injected with the vehicle alone. Thus the loss of the vasoconstrictor response following rapid, repetitive nerve stimulation in normal animals appears to be due to activation of sensory-motor vasodilator nerves and not due to desensitization of the adrenergic receptors themselves. This phenomenon then corresponds to the sensory-motor nerve-mediated recovery of blood flow in the superior mesenteric artery that occurs with persistent sympathetic nerve stimulation (Remak, Hottenstein and Jacobson, 1990).

The prolonged inhibitory effect of such sensory-motor nerve stimulation seen in the present study, lasting up to 2 min, is consistent with the long lasting vasodilatation observed after stimulation of afferent nerves in previous studies (Low and Westerman, 1989; Koltzenburg, Lewin and McMahon, 1990). This suggests that either the peptides themselves are long lived, or that the effect of receptor activation is associated with a more profound change than occurs following sympathetic nerve stimulation.

Further support for the involvement of sensory-motor nerves in inhibiting sympathetic vasoconstriction was derived from experiments in which capsaicin was applied to the *in vitro* iris preparation. Immediate effects of capsaicin include excitation due to release of neuropeptides, followed by prolonged desensitization and a block of nerve conduction in the sensory C-fibres and Aδ-fibres (Holzer, 1991a). In the iris arterioles, the immediate effect of capsaicin was to cause a vasodilatation and an abolition of the sympathetic vasoconstriction. This provided further support for the ability of the sensory-motor nerves to modulate the action of the sympathetic nerves in this tissue. Within the next 15 min, the sympathetic vasoconstriction returned to precapsaicin levels presumably due to either desensitization of the neuropeptide receptors or a block in the conduction of the sensory-motor nerves. At this time, rapid,

repetitive transmural nerve stimulation caused a contraction which was maintained at a constant amplitude for as many stimuli as were delivered. Thus, in both the capsaicin treated animals and in the preparations treated *in vitro* with capsaicin, prevention of the release of neuropeptides from the sensory-motor nerves enabled the sympathetic nerve-induced vasoconstriction to be maintained at a constant amplitude even following rapid repetitive stimulation.

The sensory nerves affected by capsaicin have been reported to contain both substance P and CGRP, as well as neurokinin A, galanin, VIP and somatostatin (Holzer, 1991a). In the mesenteric arteries and in the rabbit central ear artery, vasodilatation and the inhibition of the sympathetic vasoconstriction have been reported to be mediated by CGRP (Han, Naes and Westfall, 1990; Kawasaki *et al.,* 1990a; Maynard, Saville and Burnstock, 1990; Li and Duckles, 1992). CGRP in these arteriolar beds is extremely potent as a vasodilator with effective concentrations in the sub-nanomolar range (Kawasaki *et al.,* 1988; Han, Naes and Westfall, 1990; Kawasaki *et al.,* 1990a), while substance P is relatively ineffective (Kawasaki *et al.,* 1988; Kawasaki *et al.,* 1990a; Li and Duckles, 1992). In the arterioles of the rat iris, both the NK_1-neurokinin receptor agonist, SarSP, and CGRP were effective in inhibiting sympathetic vasoconstriction.

It was observed that the vasodilatation produced by CGRP was longer-lasting than than the inhibitory effect on the sympathetic constriction. This suggests that CGRP is producing these effects by two different mechanisms. Further experiments are required to examine this vasodilatation and to determine if the vasodilatation can be inhibited independently of the inhibitory effect on the constriction.

Experiments using antagonists for the neurokinin receptor subtypes, NK_1 and NK_2, and for $CGRP_1$ receptors indicated that CGRP released from the sensory-motor nerves may inhibit sympathetic vasoconstriction of the arterioles of the rat iris. Since the NK_1-neurokinin receptor selective agonist, SarSP, was also capable of inhibiting sympathetic vasoconstriction in the same arterioles, these results further suggested that, either substance P is not co-released with CGRP from the sensory-motor nerves, or that the tachykinin concentration is insufficient to activate neurokinin receptors on the arterioles, possibly due to rapid degradation. This latter hypothesis could be tested by

comparing responses to trains of stimuli every 15 s in the presence of peptidase inhibitors to prevent the breakdown of neurokinins, and then testing the effect of the tachykinin-receptor antagonists, alone and in the presence of the CGRP receptor antagonist.

Anatomically, arteries have been said to be innervated by particular nerve types according to the presence of perivascular networks of fibres. Thus, the main mesenteric arteries are described as being densely innervated by peptide-containing fibres while the precapillary arterioles and veins are sparsely innervated (Furness *et al.,* 1982; Barja, Mathison and Huggel, 1983; Uddman *et al.,* 1986). Similarly, the larger of the arterioles of the rat iris have been described as receiving a sympathetic, adrenergic innervation (Olson *et al.,* 1988). On the other hand, immunohistochemical studies of substance P-containing fibres in the rat iris have found no obvious association of these fibres with blood vessels (Seiger *et al.,* 1985).

The present study, has demonstrated that capsaicin-sensitive sensory-motor nerve fibres have a vasodilatory effect on these arterioles. Substance P-like and CGRP-like immunoreactive fibres do not form the perivascular plexuses over the iris arterioles, as the adrenergic fibres do. Instead the fibres cross the arterioles at right angles to the axis of the vessels. Using the confocal microscope, however, it was demonstrated that the peptidergic fibres lie as close to the smooth muscle cells as do the fibres of the adrenergic perivascular plexus and frequently, as they cross the arterioles, their varicosities are in close association with the varicosities of the adrenergic fibres. The paucity of peptidergic fibres and the demonstration of their functional interaction with the blood vessels suggests that either the peptides are capable of diffusing over quite long distances before inactivation, or that the second messenger molecules activated by the peptides must be capable of extensive diffusion.

The effect of CGRP in inhibiting sympathetic vasoconstriction in the present study could be either prejunctional and due to a reduction in noradrenaline release, or postjunctional and due to an action within the smooth muscle of the arterioles themselves. The immunohistochemical data provides evidence for a close association between the sympathetic and sensory-motor varicosities. Such a relationship is

consistent with prejunctional actions of the sensory-motor fibres on the sympathetic or vice versa. On the other hand, anatomical and physiological considerations would favour a postsynaptic mechanism. The adrenergic fibres are present in far greater density on the vessel surface than are the sensory fibres. This fact, coupled with the reported low probability of neurotransmitter release from individual sympathetic varicosities (Hirst and Neild, 1980b; Brock and Cunnane, 1988; Lavidis and Bennett, 1993), suggests that the likelihood of the release of noradrenaline from a sympathetic varicosity lying in close proximity to a sensory fibre would be low. Indeed, in the mesenteric arteries and the central ear artery, CGRP has been shown to have no effect on the release of noradrenaline (Maynard and Burnstock, 1989; Han, Naes and Westfall, 1990; Kawasaki *et al.,* 1990a) and so, in these arteries, CGRP has been proposed to operate postsynaptically. A more reasonable explanation, then, would be that in the iris arterioles, the neuropeptides act postsynaptically to cause the production of a second messenger molecule, for example nitric oxide, which could diffuse rapidly between the coupled smooth muscle cells.

Sympathetic vasoconstriction in the mesenteric and submucosal arteries and the basilar artery is resistant to α–adrenoceptor antagonists and results from the summation of individual excitatory junction potentials, the initiation of a muscle action potential and the influx of calcium through voltage dependent calcium channels (Hirst, 1977; Hirst, Neild and Silverberg, 1982; Morgan, 1983). In these vessels non-adrenergic, non-cholinergic (NANC) nerve stimulation produces inhibitory junction potentials in association with vasodilatation, which are both inhibited by capsaicin (Meehan, Hottenstein and Kreulen, 1991; Saito *et al.,* 1989), thus indicating sensory-motor nerve activity. In addition, the sensory-motor neurotransmitter in these vessels, CGRP, causes a vasodilatation via an increase in cyclic AMP, which in turn activates K^+ channels (Kitazono, Heistad and Faraci, 1993; Quayle *et al.,* 1994; Nelson *et al.,* 1990). The resulting hyperpolarization would thus antagonize the sympathetic nerve-induced depolarization and reduce calcium influx through voltage-dependent calcium channels. It appears then, that sensory-motor modulation of sympathetic-vasoconstriction occurs

via a hyperpolarization in arteries where the constriction occurs as a consequence of non-α-adrenoceptor activation and voltage-dependent events.

In the present study, CGRP acts to inhibit vasoconstriction of these arterioles via nitric oxide production and the inhibitory effect of repetitive nerve stimulation on neurogenic vasoconstriction is also dependent on the synthesis of nitric oxide. Furthermore, there was no hyperpolarization associated with repetitive stimulation of iris arterioles every 15 s, but rather the magnitude of the depolarization decreased with each train of stimulus. In other vessels where α–adrenoceptors and voltage-independent events mediate sympathetic vasonstriction, sensory-motor modulation of this constriction also appears to be via the action of nitric oxide and independent of hyperpolarization. In the rat aorta, the effects of CGRP on vasodilatation are due to the production of nitric oxide (Fiscus *et al.,* 1991; Gray and Marshall, 1992a; Hao *et al.,* 1994). Nitric oxide has also been reported to inhibit sympathetic vasoconstriction of blood vessels in the knee joint (Najafipour and Ferrell, 1993), and in the pulmonary artery (Cederqvist *et al.,* 1991). In these latter vessels, sympathetic vasoconstriction is mainly mediated by α–adrenoceptors (Lee *et al.,* 1988; Khoshbaten and Ferrell, 1993; Suzuki, 1983). In the pulmonary artery, nitric oxide had no effect on the release of ^{3}H-noradrenaline from sympathetic nerves, indicating that this modulation occurs postjunctionally (Cederqvist *et al.,* 1991).

One explanation for the nitric oxide-mediated effect of CGRP is that the intracellular calcium available for the α_{1B}-adrenoceptor-mediated constriction of the arterioles is reduced. This hypothesis could be tested by imaging cytosolic calcium during repetitive nerve stimulation. In aortic smooth muscle, CGRP-stimulated vasodilatation was associated with a reduction in the concentration of intracellular calcium (Ishikawa, Ouchi and Orimo, 1993). Endothelial nitric oxide may produce relaxation of vascular smooth muscle via the production of cyclic GMP (Moncada, 1992). In turn, cyclic GMP may inhibit IP_3-induced mobilization of intracellular calcium (Murthy *et al.,* 1993) by phosphorylating the IP_3 receptor (Komalavilas and Lincoln, 1994), or may lower the concentration of stored intracellular calcium in

smooth muscle cells by activating the calcium extrusion pump in the sarcoplasmic reticulum (Itoh *et al.*, 1985).

This chapter demonstrates that in isolated iris arterioles, where intracellular calcium release mediates sympathetic vasoconstriction, sensory-motor nerves can exert an inhibitory effect on the vasoconstriction via the release of CGRP and the synthesis of nitric oxide, through a mechanism that is independent of membrane hyperpolarization.

CHAPTER 6.

MECHANISM OF SENSORY-MOTOR MODULATION OF SYMPATHETIC VASOCONSTRICTION

INTRODUCTION

Previous studies indicate that mutual interations occur between sympathetic and sensory-motor perivascular nerves. While sympathetic neurotransmitters may act prejunctionally to inhibit the release of CGRP from perivascular sensory-motor nerves (Lindgren *et al.,* 1987; Matran, Martling and Lundberg, 1989; Kawasaki *et al.,* 1990a; Kawasaki *et al.,* 1991; Li and Duckles, 1991; Fuder and Selbach, 1993), it has been shown in the rabbit central ear artery and rat mesenteric vessels that CGRP does not alter the sympathetic nerve-evoked efflux of ^{3}H-noradrenaline, thus implicating a postjunctional site of inhibition of sympathetic vasoconstriction (Kawasaki *et al.,* 1990a; Maynard and Burnstock, 1989).

The predominant effector pathway for CGRP receptor activation is the stimulation of adenylate cyclase (Chatterjee *et al.,* 1993; Watson and Girdlestone, 1994). Intracellular interactions between receptor-activated changes in the level of cyclic AMP have been shown to alter the activity of the second messenger pathway involving IP3 (Abdel-Latif, 1989; Abdel-Latif, 1991). For example, elevation of cyclic AMP, with isoproterenol or forskolin, inhibits muscarinic receptor activated IP3 formation in the iris sphincter muscle (Tachado, Akhtar and Abdel-Latif, 1989; Kharbanda, Nakamura and Kufe, 1993). Both cyclic AMP and cyclic GMP have recently been shown to inhibit IP3-dependent intracellular calcium release due to agonist stimulation (Murthy *et al.,* 1993). Thus it has been suggested that interaction between these two second messenger pathways may be one mechanism for regulating contraction and relaxation of smooth muscle (Abdel-Latif, 1991).

The previous chapter demonstrated that in iris arterioles, sensory-motor nerves release CGRP which acts via the production of nitric oxide to inhibit sympathetic

vasoconstriction. In vessels such as the rat aorta, vascular relaxation by elevated cyclic AMP has been shown to occur indirectly via the production of nitric oxide (Gray and Marshall, 1992a; Gray and Marshall, 1992b).

This chapter investigates further the mechanism by which CGRP, released from sensory-motor nerves, inhibits sympathetic vasoconstriction of iris arterioles, in particular the possible involvment of cyclic AMP, as well as nitric oxide.

RESULTS

Effect of increasing intracellular cyclic AMP

Three agents were used to test the effect of increasing intracellular cyclic AMP on the nerve-mediated contractile responses of the iris arteriole: dibutyryl cyclic AMP (1 mM; n=3), IBMX, a phosphodiesterase inhibitor (100 μM; n=3) and forskolin, a direct activator of adenylate cyclase (50 μM; n=3) . Increasing cyclic AMP with any of these three agents abolished the nerve-mediated constriction of the arterioles (Figure 6.1 B.). In addition, increasing cyclic AMP caused a dilatation of the arterioles (38.5 ± 1.1 μm, n=3, in forskolin, 50 μM; compared to 33.3 ± 1.2 μm, n=3, in control; $P<0.05$; Figure 6.1 A.). Forskolin(0.1 μM) also inhibited nerve-mediated depolarization associated with sympathetic vasoconstriction (Figure 6.2), as well as inhibiting spontaneous depolarizationsof iris arterioles (Figure 6.3).

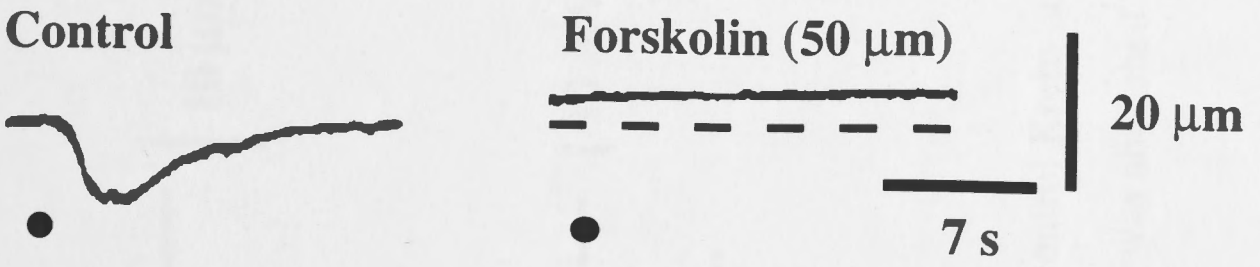
Does the effect of increased cyclic AMP in the arterioles occur in the arteriolar cells or in the sensory-motor nerves?

After inhibiting sensory-motor nerve transmission by treatment with capsaicin (10 μM) for 15 min, forskolin (50 μM) still caused a vasodilatation and abolished vasoconstriction due to nerve stimulation every 3 min (n=3; Figure 6.4). This suggests that the effect of cyclic AMP is postjunctional and not prejunctional.

Involvement of the GTP-binding protein, G_S

To investigate the physiological relevance of the effect of cyclic AMP on sympathetic nerve-mediated responses, the involvement of the GTP-binding protein, G_S, was examined using cholera toxin, which causes ADP ribosylation and permanent activation of G_S (Cassel and Selinger, 1977; Milligan and Mitchell, 1993). Cholera toxin (2 $\mu g.ml^{-1}$) treatment for 1.5 hours caused a dilatation of the arterioles and prevented the nerve-mediated vasoconstriction (n= 3; Figure 6.5).

A.



B.

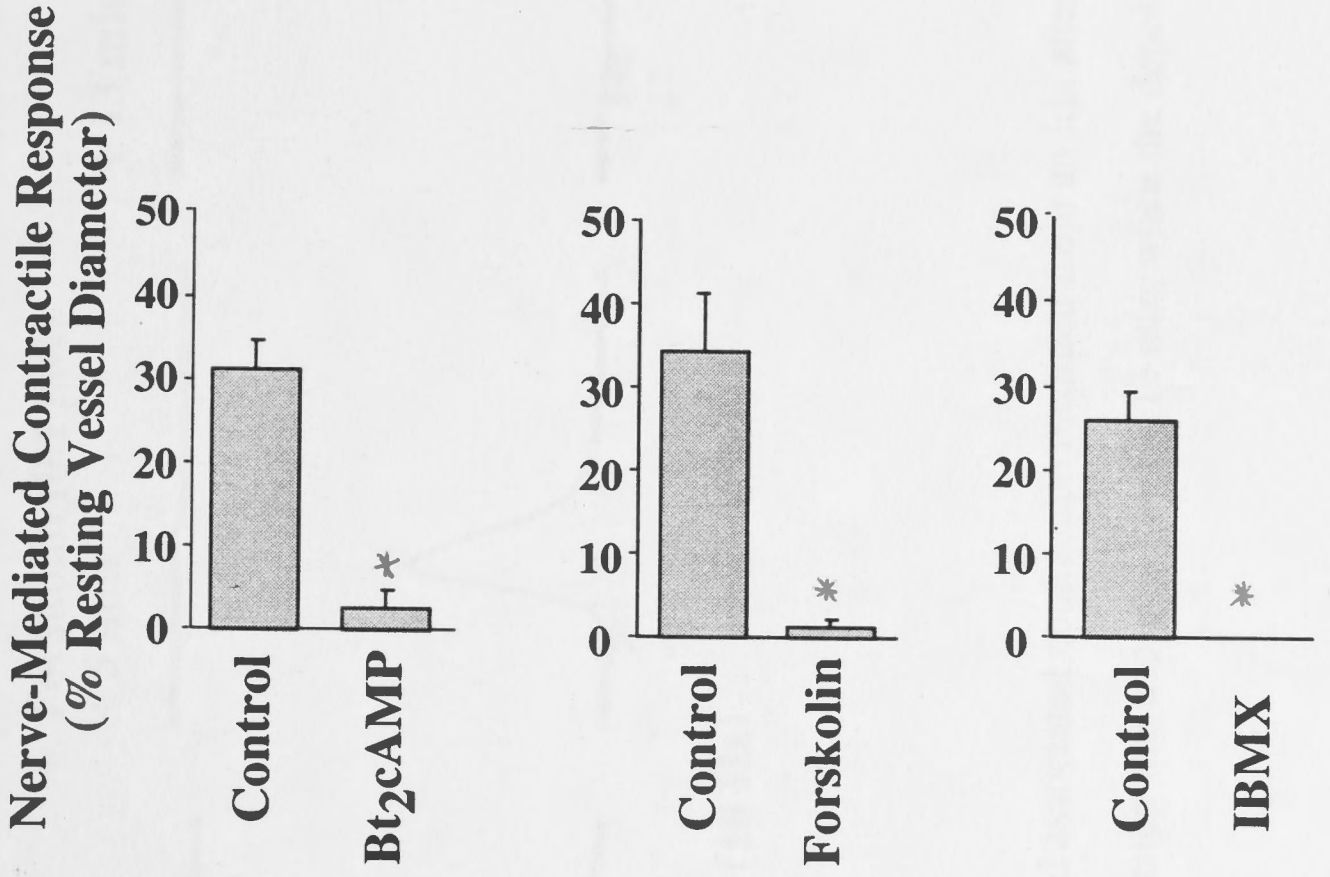


Figure 6.1

A. Trace illustrating the effect of forskolin (50 μM) on the nerve-mediated contractile response of an iris arteriole. Dots indicate onset of nerve stimulation (10 Hz, 1 s). Dotted line represents resting vessel diameter of arteriole. Note the vasodilatation in the presence of forskolin (50 μm).

B. Effect of (left to right) dibutyryl cyclic AMP (Bt_2cAMP; 1 mM), forskolin (50 μM) or isobutylmethylxanthine (IBMX; 100 μM) on the contractile respone evoked by nerve-stimulation (10 Hz, 1 s). Columns represent the mean and vertical bars represent the s.e.m. of 3 preparations.

* indicates significant difference from paired control (t-test with Bonferroni correction).

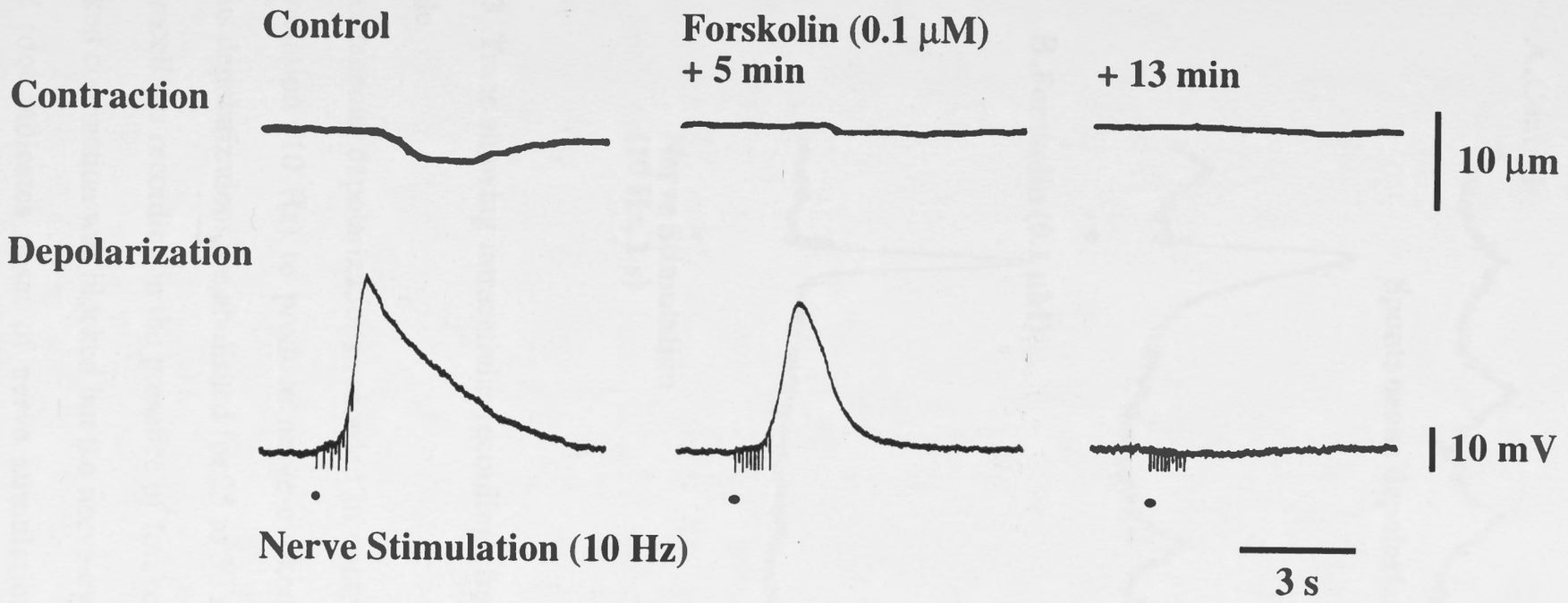


Figure 6.2 Trace showing nerve-evoked depolarization and associated contraction recorded from an iris arteriole in control Krebs and after perfusing the bath with forskolin (0.1 μM) for 5 min, when the contraction was abolished, or for 13 min, when the depolarization was abolished.
Dots indicate onset of nerve stimulation (10 Hz).

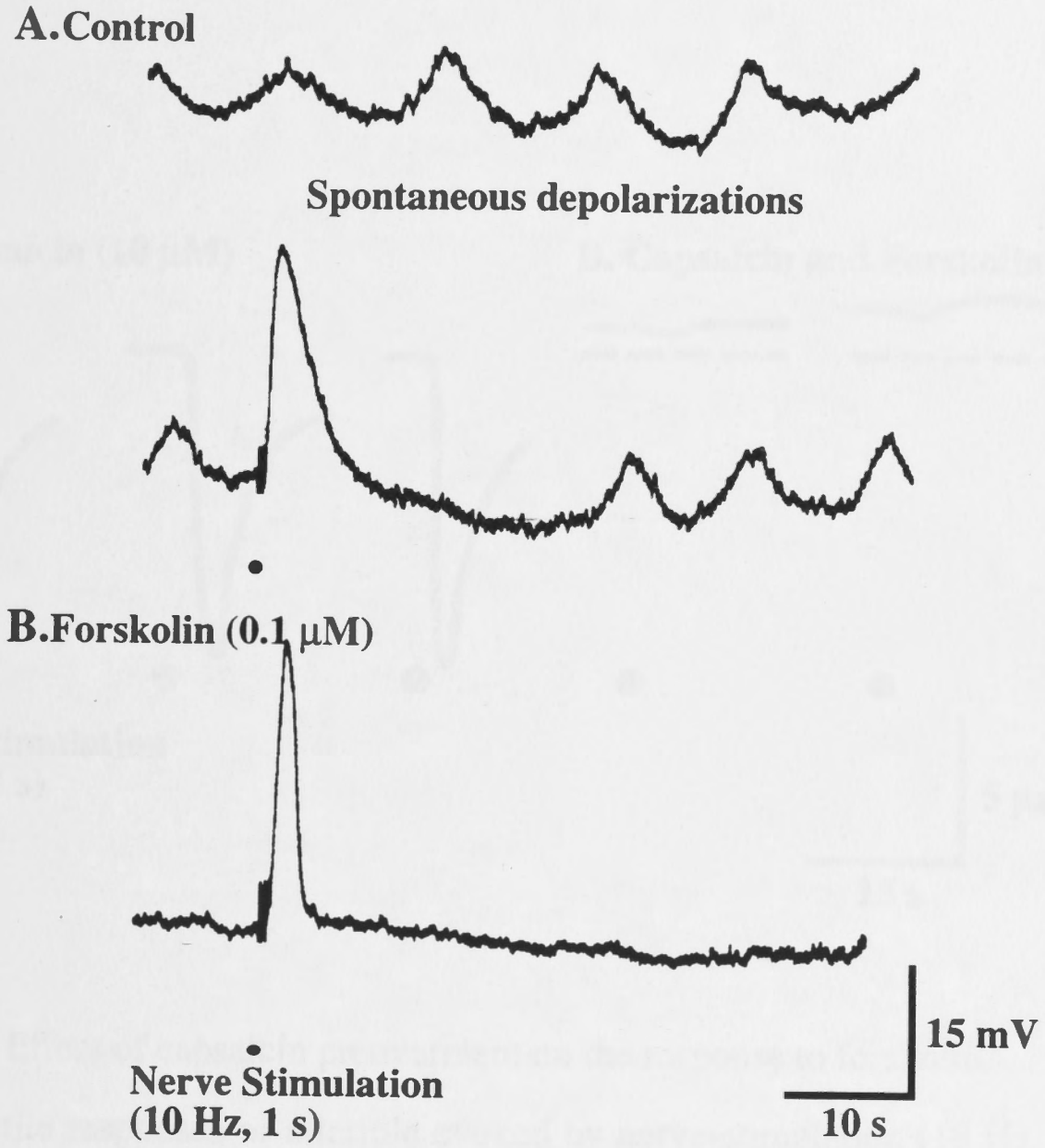


Figure 6.3 Trace showing intracellular recordings from a spontaneously depolarizing iris arteriole.

A Spontaneous depolarizations recorded in control Krebs. Dot indicates time of nerve stimulation (10 Hz) to produce nerve-evoked depolarization. Note that the spontaneous depolarizations are abolished for 25 to 30 s after nerve stimulation.

B Intracellular recording in the presence of forskolin (0.1 μM) at a time when the nerve-evoked contraction was blocked but the nerve-evoked depolarization was largely unaffected (dot indicates onset of nerve stimulation). Note that the spontaneous depolarizations are abolished in the presence of forskolin.

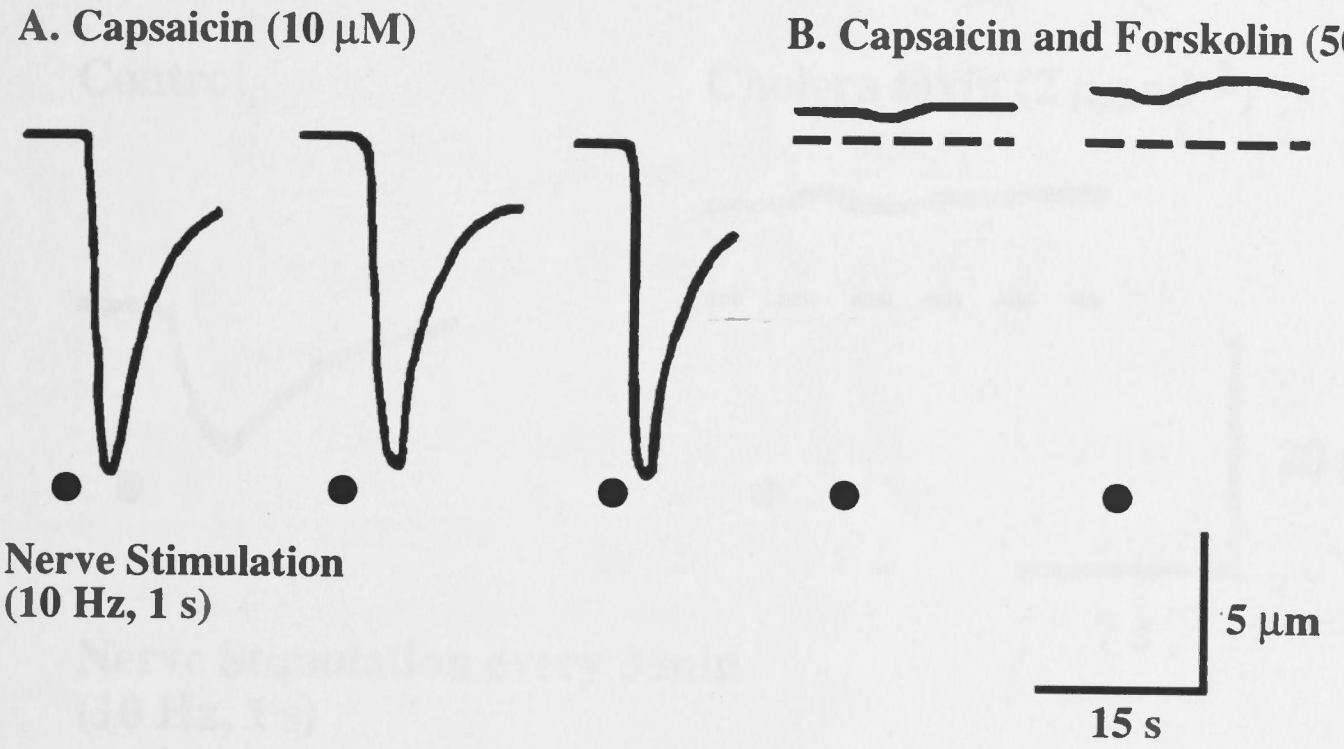


Figure 6.4 Effect of capsaicin pretreatment on the response to forskolin.

A. Contractile responses of arteriole evoked by nerve-stimulation (10 Hz, 1 s every 3 min) after perfusion of chamber with capsaicin (10 μM) for 15 min.

B. The effect of subsequent addition of forskolin (50 μM) on the nerve-mediated contractile response of the arteriole in the continued presence of capsaicin (10 μM). Dotted line indicates resting vessel diameter of arteriole in capsaicin alone. Dots indicate time of onset of nerve stimulation (10 Hz, 1 s).

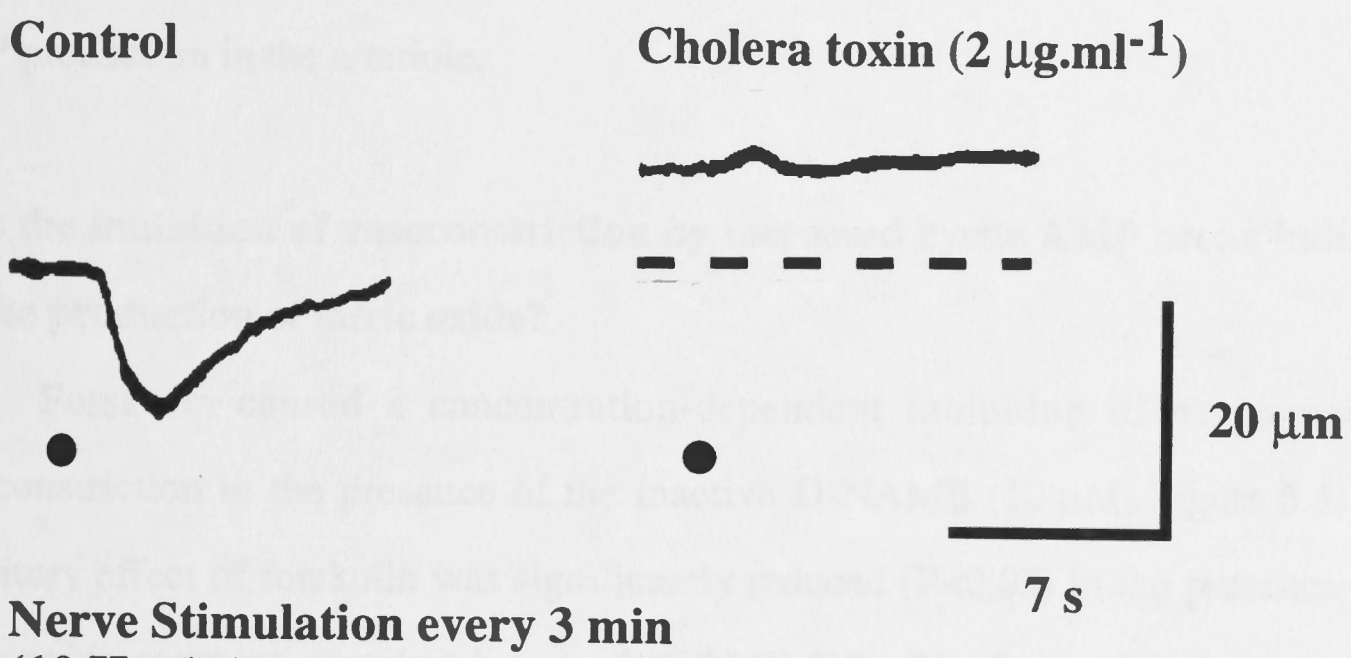


Figure 6.5 Trace illustrating the effect of cholera toxin (2 $\mu g.ml^{-1}$) on the nerve-mediated contractile response of an iris arteriole. Dots indicate onset of nerve stimulation (10 Hz, 1 s every 3 min). Note the vasodilation in the presence of cholera toxin.

Involvement of cyclic AMP in sensory-motor modulation of sympathetic vasoconstriction

The GTP-binding protein, G_S, stimulates adenylate cyclase to catalyse the production of cyclic AMP. Inhibition of adenylate cyclase with dideoxyadenosine (Schulze, 1982; 1 mM; n=8) prevented the loss of vasoconstriction in response to trains of stimuli every 15 s (Figure 6.6). Incubation in dideoxyadenosine (1 mM) caused a significant decrease in the resting diameter, indicating there is a basal level of cylic AMP production in the arteriole.

Does the inhibition of vasoconstriction by increased cyclic AMP occur indirectly via the production of nitric oxide?

Forskolin caused a concentration-dependent inhibition of nerve-mediated vasoconstriction in the presence of the inactive D-NAME (10 μM, Figure 6.5). This inhibitory effect of forskolin was significantly reduced ($P<0.05$) in the presence of the nitric oxide synthase pseudosubstrate, L-NAME (10 μM), for each concentration of forskolin tested (Figure 6.7). In the absence of forskolin, there was no significant difference between the size of the control nerve-mediated constriction, when expressed as a percentage of the resting vessel diameter, recorded in the presence of L-NAME (28 ± 3.1 %; n=10) or D-NAME (25.5 ± 2.2 %; n=10) alone.

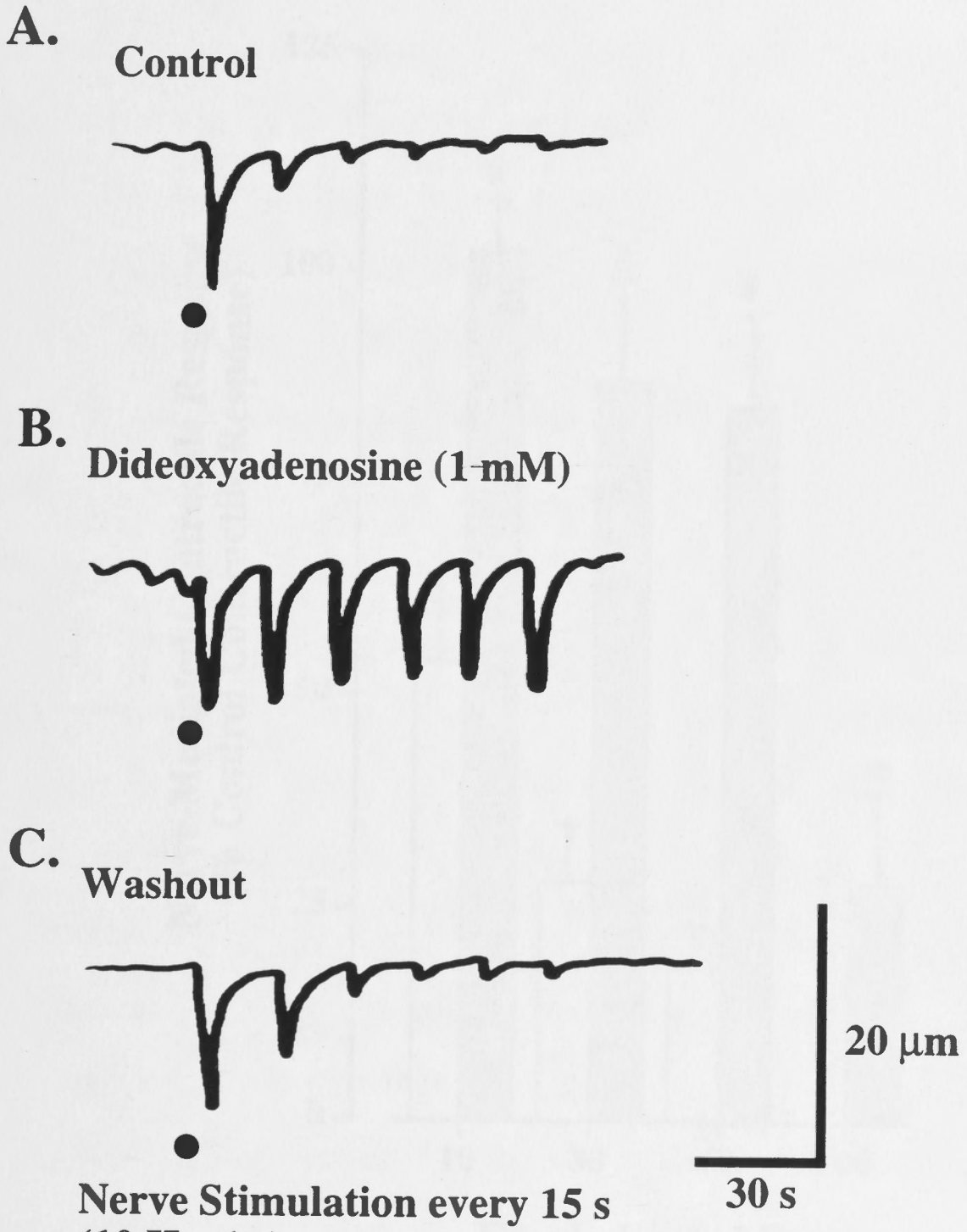


Figure 6.6 Effect of dideoxyadenosine (1 mM) on contractile responses evoked by nerve-stimulation (10 Hz, 1 s) every 15 s. Dot indicates the first of 6 pulses of stimuli applied every 15 s. **A.** Control contractile responses to nerve stimulation, **B.** contractile responses in the presence of dideoxyadenosine (1 mM) and **C.** contractile responses after washout in control Krebs.

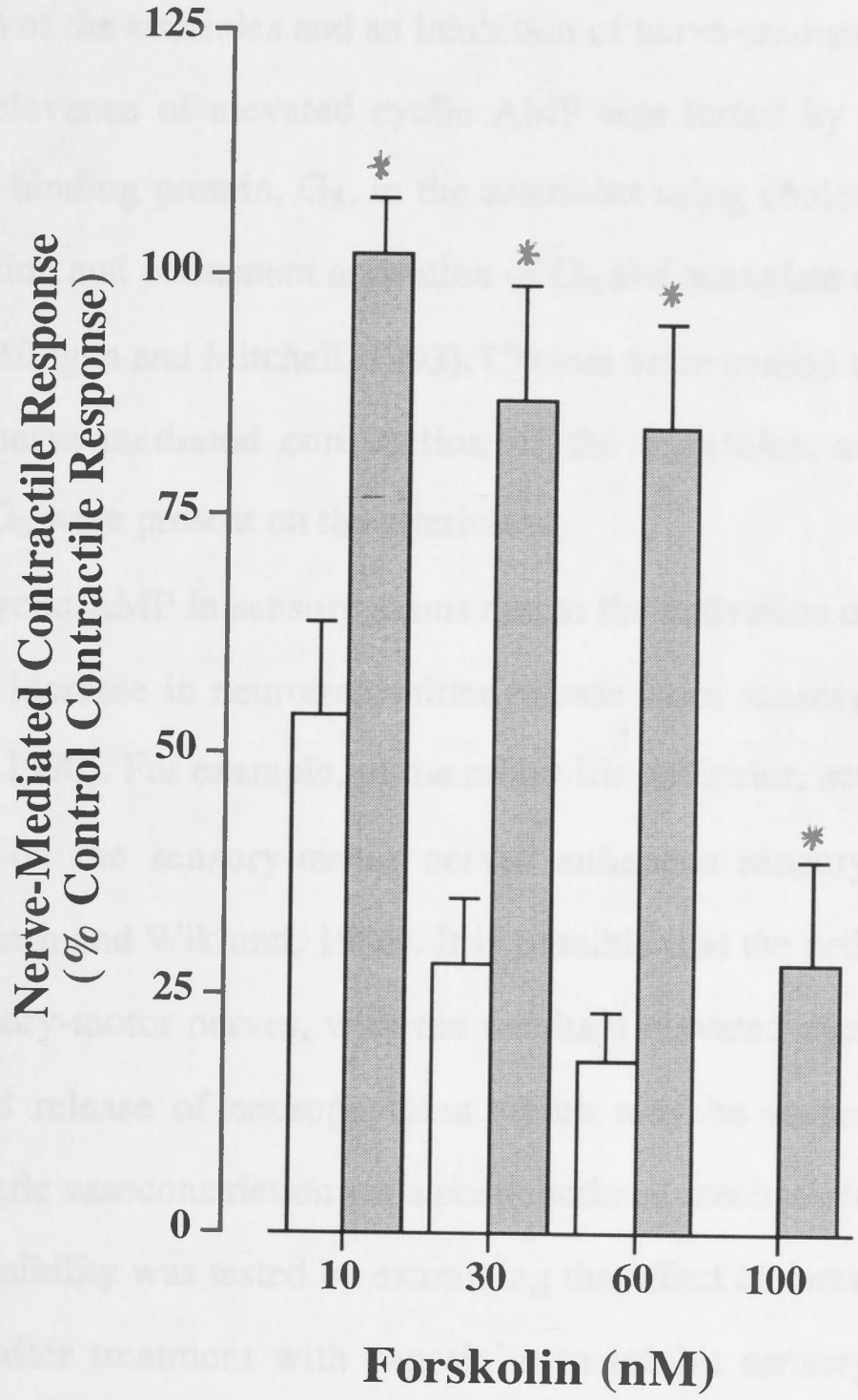


Figure 6.7 Effect of forskolin on nerve-mediated contractile response (10 Hz, 1 s every 3 min) in the presence of D-NAME (10 μM, open columns) and L-NAME (10 μM; stippled columns). Columns and vertical bars represent mean and s.e.m. of 3 to 4 preparations.

* indicates significant difference between D-NAME and L-NAME for the same concentration of forskolin (t-test with Bonferroni correction).

DISCUSSION

The predominant effector pathway following CGRP receptor activation is the elevation of cyclic AMP (Chatterjee *et al.,* 1993; Watson and Girdlestone, 1994). In the present study, elevation of intracellular cyclic AMP, using three different agents, produced a relaxation of the arterioles and an inhibition of nerve-mediated constriction. The physiological relevance of elevated cyclic AMP was tested by examining the presence of the GTP binding protein, G_S, in the arterioles using cholera toxin, which causes ADP ribosylation and permanent activation of G_S and adenylate cyclase (Cassel and Selinger, 1977; Milligan and Mitchell, 1993). Cholera toxin caused a vasodilatation and prevented the nerve-mediated constriction of the arterioles, suggesting that receptors coupled to G_S were present on the arterioles.

Increases in cyclic AMP in sensory axons due to the activation of prejunctional receptors leads to an increase in neurotransmitter release from sensory-motor nerves (Rhoden and Barnes, 1990). For example, in the rabbit iris sphincter, activation of A_2-adenosine receptors on the sensory-motor nerves enhances sensory-motor nerve transmission (Gustafsson and Wiklund, 1986). It is possible that the activation of such receptors on the sensory-motor nerves, with the resultant elevated cyclic AMP, may produce an increased release of neuropeptides which may be responsible for the inhibition of sympathetic vasoconstriction via a postjunctional mechanism not involving cyclic AMP. This possibility was tested by examining the effect of forskolin on nerve-mediated responses after treatment with capsaicin, to inhibit sensory-motor nerve transmission (Holzer, 1991b). In these experiments, forskolin still inhibited sympathetic vasoconstriction as well as producing a relaxation of the arterioles independently of sensory-motor nerve activity. These results thus show that the ability of cyclic AMP to inhibit arteriolar constriction is a postjunctional event and is not due to a prejunctional effect on sensory-motor nerves.

CGRP has been identified as the neurotransmitter which mediates the inhibitory effect of sensory-motor nerves on sympathetic nerve-mediated constriction in iris arterioles (Chapter 5). In the present study, inhibition of adenylate cyclase with dideoxyadenosine also prevented the loss of sympathetic vasoconstriction produced by

trains of stimuli every 15 s. These results provide evidence for the involvement of adenylate cyclase and cyclic AMP in the sensory-motor interaction with sympathetic vasoconstriction.

In the previous chapter it was demonstrated that the inhibitory effect of CGRP on sympathetic vasoconstriction was due to the production of nitric oxide. Endothelial nitric oxide produces vasodilatation of vascular smooth muscle by activating the production of cyclic GMP (Moncada *et al.,* 1991). In blood vessels such as the rat aorta and porcine palmar lateral vein, elevated cyclic AMP levels produce an increase in cyclic GMP, suggesting that an interaction occurs between these two second messengers (Gray and Marshall, 1992a; Gray and Marshall, 1992b; Wright, Amirchetty-Rao and Kendall, 1994). In the rat aorta, effects of increased cyclic AMP following activation of either ß-adrenoceptors or CGRP-receptors are mediated indirectly via the production of nitric oxide by the endothelium (Gray and Marshall, 1992a; Gray and Marshall, 1992b). In the present study, inhibition of nitric oxide synthesis with L-NAME prevented the inhibition of sympathetic nerve-mediated vasoconstriction produced by forskolin. This suggests that in the arterioles, elevation of cyclic AMP also inhibits nerve-mediated vasoconstriction indirectly via the production of nitric oxide. The activity of NOS has been reported to be affected by phosphorylation by a number of cellular kinases (Bredt, Ferris and Snyder, 1992). A phosphorylation site for the cyclic AMP-dependent protein kinase A on NOS has been identified (Bredt, Ferris and Snyder, 1992) and this may provide a mechanism for the above response in the arterioles.

Elevation of intracellular cyclic AMP caused an inhibition of both spontaneous and neurogenically mediated depolarizations. One explanation for this is that cyclic AMP pathway is acting intracellularly to reduce the release of calcium. This is consistent with the cyclic AMP-dependent activation of nitric oxide to inhibit sympathetic vasoconstriction as described in the previous chapter.

The present results indicate a postjunctional site for the inhibition of sympathetic vasoconstriction by neurally-released CGRP. This interaction occurs as a result of the sequential activation of G_S, adenylate cyclase and NOS.

CHAPTER 7.

SENSORY-MOTOR MODULATION OF SYMPATHETIC NERVE-EVOKED CONTRACTION - A COMMON PHENOMENON?

INTRODUCTION

In the previous two chapters it was demonstrated that neurotransmitter released from sensory-motor nerves causes the production of second messengers, including cyclic AMP, which act to inhibit sympathetic constriction of the iris arterioles. In bovine iris sphincter muscle there is also a recipricol effect between muscarinic receptor-activated IP_3 formation and cyclic AMP formation (Tachado, Akhtar and Abdel-Latif, 1989; Tachado *et al.*, 1992).

This chapter examines whether an interaction of such second messenger systems activated by sympathetic and sensory-motor nerves occurs in other sympathetic nerve target tissues, and could therefore be considered to represent a general regulatory mechanism. The rat iris dilator muscle was chosen for this study since α_{1B}-adrenoceptor activation mediates the sympathetic nerve-evoked contraction of both the iris dilator muscle and the arterioles found within the stromal layer of the iris (Takayanagi, Shiraishi and Kokubu, 1992; Hill *et al.*, 1993; see Chapter 1.).

RESULTS

Immunohistochemistry

Double-labelling immunohistochemical studies were designed to investigate the anatomical relationship of both the sympathetic and sensory-motor nerve fibres with the dilator muscle, using antibodies against tyrosine hydroxylase for the sympathetic fibres and antibodies against substance P for the sensory fibres. In relation to the iris dilator muscle, the sympathetic fibres formed a fine, varicose network over the anterior surface of the muscle (Figure 7.1 A), while the sensory fibres were not found in this focal plane (Figure 7.1 B). The sensory fibres were, however, distributed through the stroma anterior to the dilator layer (Figure 7.1 C) where the sympathetic fibres could also be found (Figure 7.1 D). These results were confirmed in iris sections, where the substance P-like immunoreactivity could be seen in the stroma but rarely in contact with the dilator muscle, while the tyrosine hydroxylase-like immunoreactive fibres were found along the anterior surface of the dilator muscle as well as in the stroma. Thus, the sympathetic and sensory fibres did not demonstrate the same anatomical relationship with the dilator muscle as they did with the iris arterioles (see Figure 5.11).

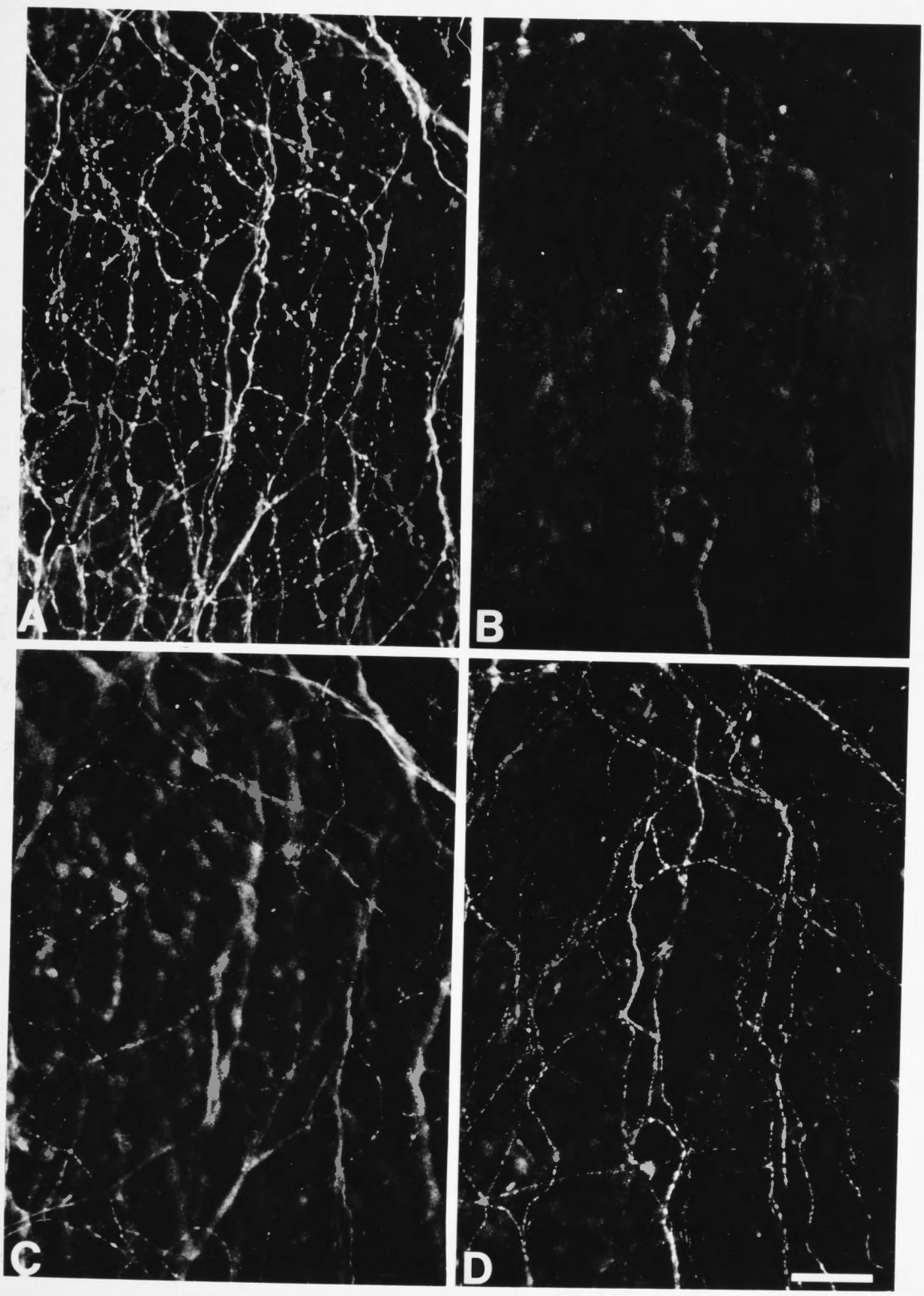
Effect of interval between stimulus trains

A consistent contraction of the dilator muscle was produced by stimulation of the transmural nerves at 10 Hz for 1 s every 3 min or every 15 s (n=3; Figure 7.2). These results suggest that activation of the sympathetic nerves produced a contraction of the iris muscle which was not altered by rapid stimulation, as it was in the iris arterioles (see Figure 5.1).

Expression of tachykinin receptors in the iris

In order to determine whether the genes for the receptors for the sensory-motor neuropeptides were expressed in the iris tissue, RT-PCR was performed using receptor-specific primers for the tachykinin receptor subtypes. The expression of CGRP receptors was not examined, as at the time of these experiments their sequence was unknown.

Figure 7.1 Double-labelling immunohistochemistry showing sensory, substance P (Panel B, D) and sympathetic, adrenergic (Panel A, C) nerve fibres within the stroma of the rat iris. A dense network of varicose sympathetic fibres showing immunoreactivity for tyrosine hydroxylase can be seen along the anterior surface of the dilator muscle (Panel A). At the same focal plane, the sensory fibres are out of focus (Panel B). By contrast, when the sensory fibres within the stroma are in focus (Panel D), the sympathetic fibres can be seen in the stroma (Panel C). Calibration bar represents 50 μm.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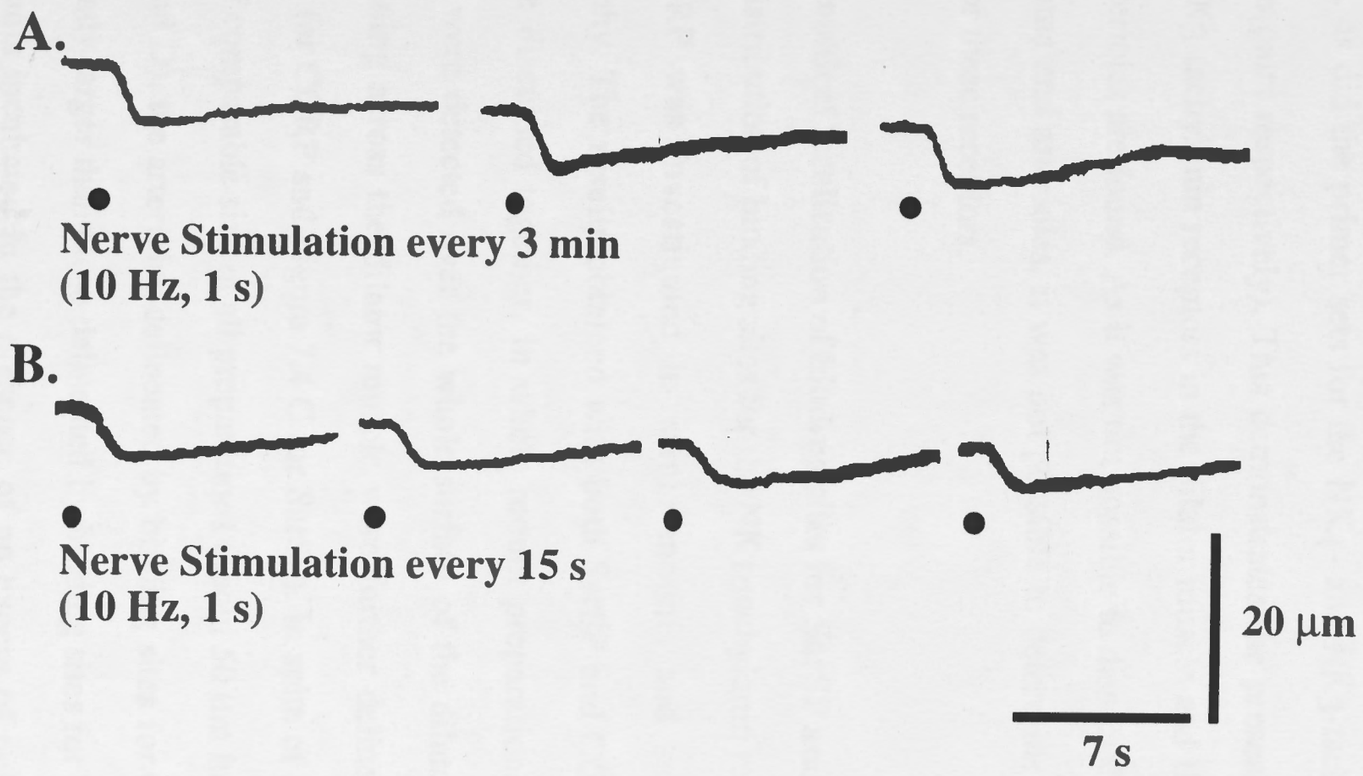


Figure 7.2 Traces showing the dilator muscle contractile responses to trains of nerve-stimulation (10 Hz, 1 s) A. every 3 min and B. every 15 s. Dots indicate onset of nerve stimulation.

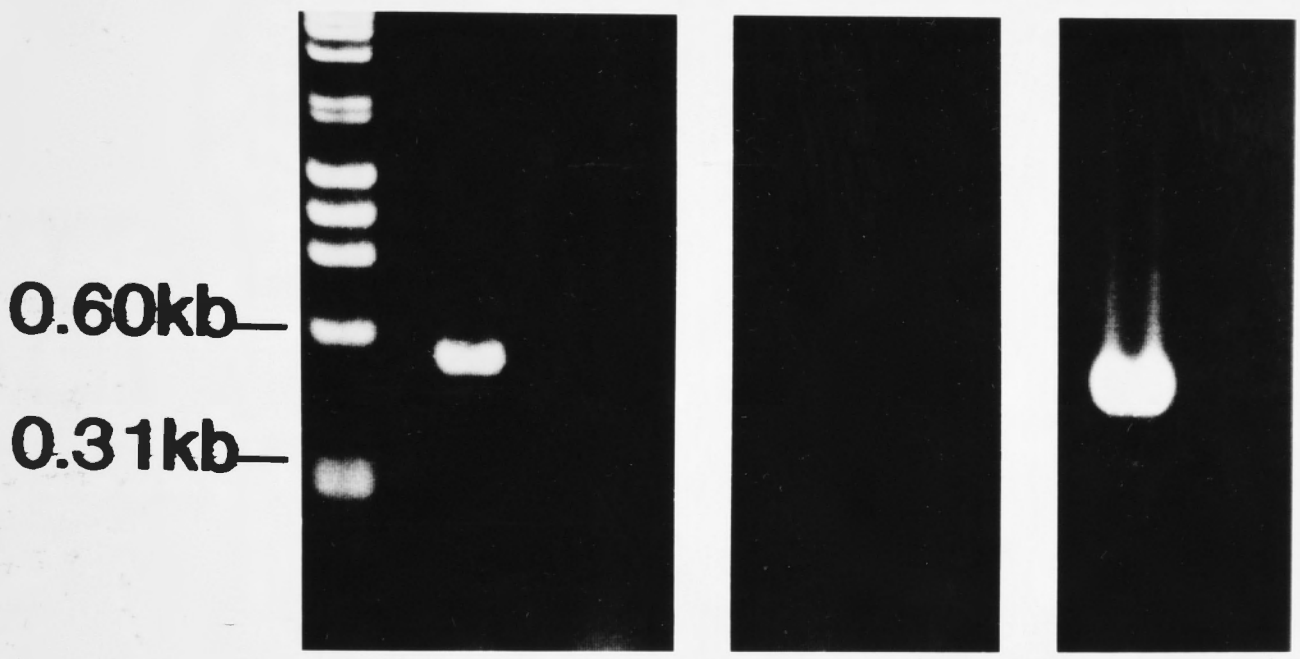
Both NK_1- and NK_3-tachykinin receptor primers generated PCR products of the appropriate size; 537 base pairs for the NK_1-tachykinin receptor and 476 base pairs for the NK_3-tachykinin receptor primer sets (Figure 7.3). Under similar conditions and using cDNA from the same RT pool, no PCR products were detected by the NK_2-tachykinin receptor primers in the iris tissue (Figure 7.3). The NK_2-tachykinin receptor primers did, however generate a product of appropriate size (443 base pairs) from rat brain cDNA, as did the primer sets for the NK_1- and NK_3-tachykinin receptors (537 and 476 base pairs respectively). This demonstrates the presence of mRNA for both NK_1- and NK_3-tachykinin receptors in the dilator muscle and the iris stromal layer in which the arterioles are found. As it was not possible to dissect the dilator muscle away from the stroma and arterioles, it was not possible to determine the exact location for the mRNA for these receptors.

Autoradiographical localization of binding sites for SarSP and CGRP

The distribution of binding sites for the NK_1-tachykinin receptor agonist, SarSP, and for CGRP was investigated in whole mounts and sections of iris using autoradiography. The results obtained with both SarSP and CGRP were similar and hence will be discussed together. In whole mount preparations of the iris, specific binding sites were detected over the whole surface of the dilator muscle. The larger arterioles running across the dilator muscle were further delineated by silver grains (Figure 7.4 A for CGRP and Figure 7.4 C for SarSP). In spite of the arterioles in these areas being of comparable size in all preparations (about 50 μm in diameter, see arrows in Panels B and D), the arterioles delineated by binding sites for CGRP (Figure 7.4 A) were consistently larger than those delineated by binding sites for SarSP (Figure 7.4 C). In whole mounts incubated in the presence of an excess of unlabelled ligand, non-specific binding of labelled ligands was seen as a lower density of silver grains over the surface of the dilator muscle. Blood vessels were no longer highlighted by silver grains (Figure 7.4 B, D, arrows).

In order to determine the exact location of the neuropeptide binding seen in the iris whole mounts, in relation to the surface of the dilator muscle, transverse sections of

Figure 7.3 Agarose gel electrophoresis of RT-PCR products amplified with receptor-specific primers for NK_1- (lanes 2,3), NK_2- (lanes 4,5) and NK_3- (lanes 6,7) tachykinin receptors. RNA used in RT-PCR was isolated form iris dilator and stromal tissue. A specific band of 537 base pairs (lane 2) was generated with the NK_1 primer pair and a 476 base pair product (lane 6) from the NK_3 primer pair. No PCR product was generated from the same cDNA pool with the NK_2 receptor specific primers (lane 4). No PCR products were seen in the receptor-specific control experiments which contain no reverse transcriptase in the RT reaction (lanes 3, 5, 7). DNA size markers (X174/HaeIII) appear in lane 1.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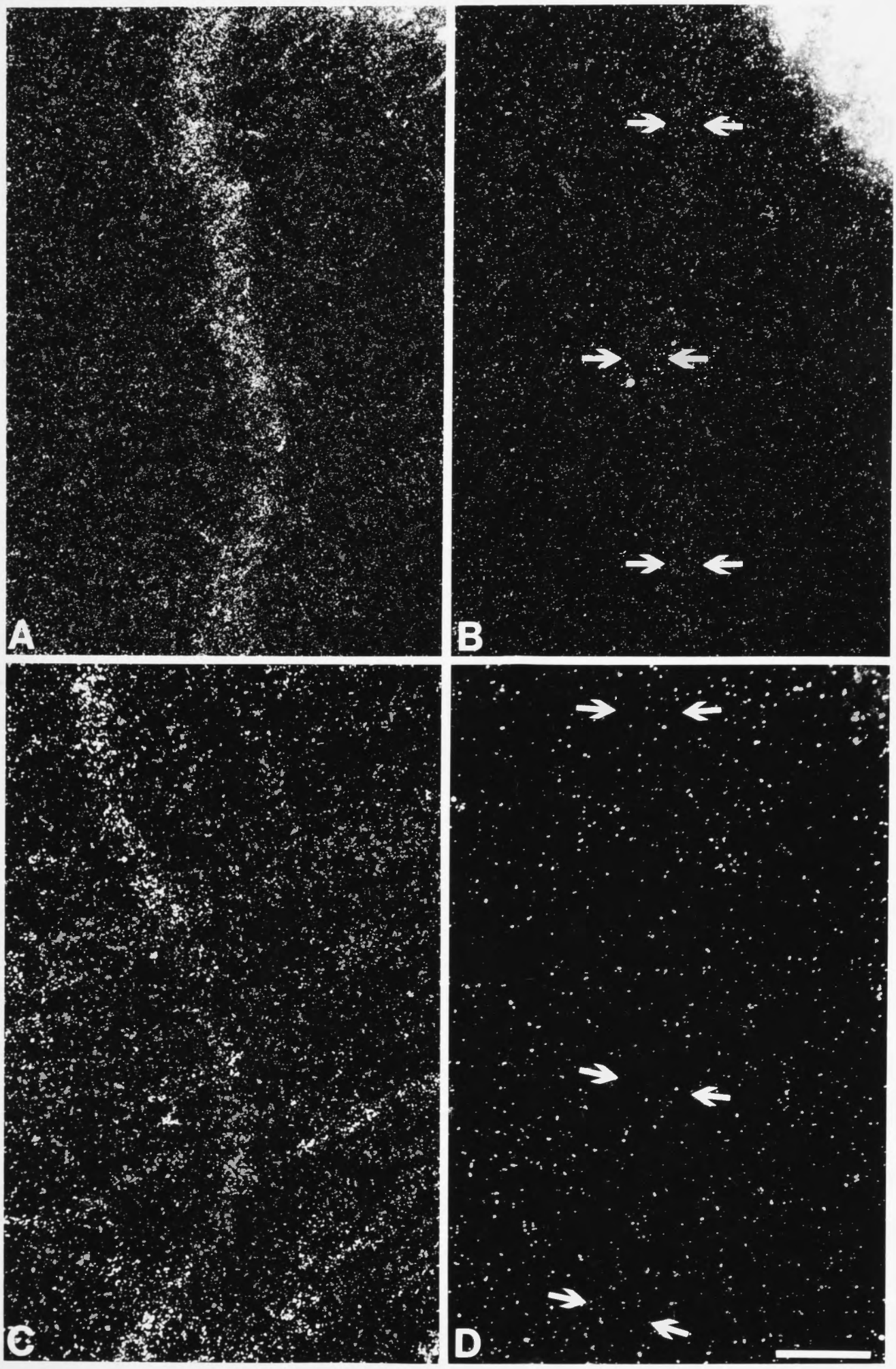
0.31kb
1 2 3 4 5 6 7

Figure 7.4 Distribution of binding sites for BHSarSP and [^{125}I]CGRP over whole mounts of the rat iris. Whole mounts were incubated with either 60 pM [^{125}I]CGRP (Panel A, B) or 75 pM BHSarSP (Panel C, D). Whole mounts are oriented with the ciliary processes at the upper right corner and the sphincter muscle beyond the lower left corner. Non-specific binding was defined in the presence of an excess (1 μM) of cold ligands (Panel B, D respectively). Arrows indicate the edges of the arteriole in these whole-mounts, as determined from overexposed prints of the same negative. Note that the specific binding for both ligands was associated with both the dilator muscle and the arteriole of the iris. Calibration bar represents 150 μM for Panels A, B and 75 μm for Panels C, D.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B
C
D

the iris were incubated with BHSarSP. In these sections, silver grains were found throughout the stroma rather than being specifically associated with the anterior surface of the dilator muscle (Figure 7.5 A, see also Figure 7.5 C for iris morphology, arrow marks the posterior surface of the dilator muscle). Non-specific binding in adjacent sections was low and could be seen over the whole of the iris sections (Figure 7.5 B, D). Thus, binding sites for the NK_1-tachykinin receptor agonist, SarSP, and for CGRP were distributed over the surface of the arterioles and throughout the stromal tissue of the iris, but did not appear to be specifically associated with the surface of the dilator muscle.

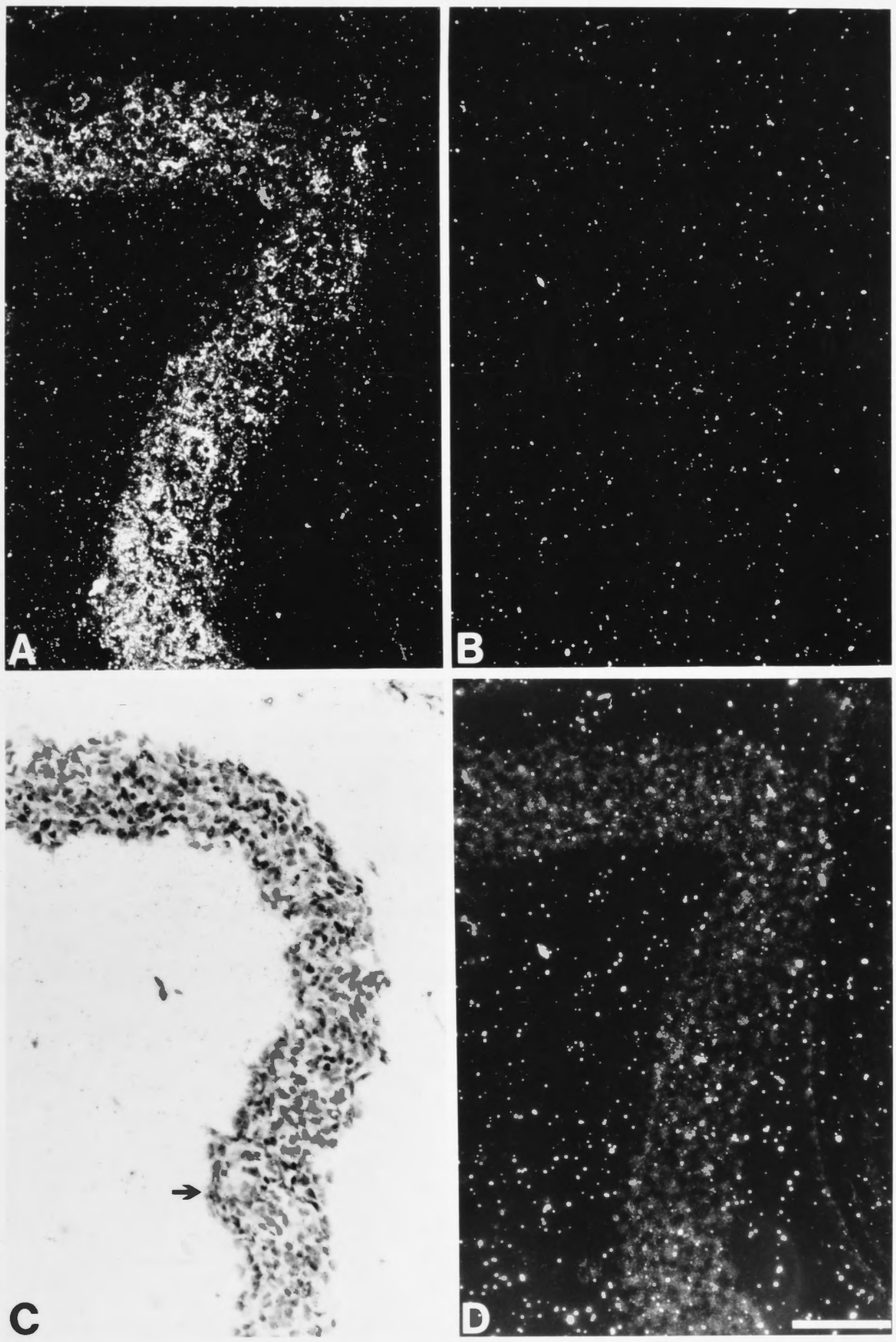
Effect of SarSP and CGRP on sympathetic nerve-evoked contraction

In order to determine a possible efferent functional role of the sensory-motor nerves on the iris dilator muscle, the effect of the the NK_1-tachykinin receptor agonist, SarSP and the effect of CGRP on sympathetic contraction were tested. Neither SarSP (10 nM; n=3) nor CGRP (10 nM; n=3) altered the resting tone of the dilator muscle, nor did they have any effect on the size of the contraction following transmural nerve stimulation (Figure 7.6). In contrast, these concentrations of SarSP and CGRP produced a dilatation and an inhibition of nerve-mediated constriction of the iris arterioles (see Figure 5.7; Table 5.1).

Effect of increasing intracellular cyclic AMP

Three agents were used to test the effect of increasing intracellular cyclic AMP on the nerve-mediated contractile response of the iris dilator muscle: dibutyryl cyclic AMP (1 mM; n=3), IBMX (100 μM; n=3), a phosphodiesterase inhibitor, and forskolin (50 μM; n=3), a direct activator of adenylate cyclase. Increasing cyclic AMP with any of these three agents produced a relaxation of the dilator muscle and a concomittent potentiation of the nerve-evoked contraction (Figure 7.7 A, B).

Figure 7.5 Binding sites for BHSarSP in transverse sections of the rat iris. Total and non-specific binding were seen in sections incubated with BHSarSP in the absence (Panel A) or presence (Panel B) of an excess of unlabelled ligand. Panel B is printed so that the background is comparable to that in Panel A, while Panel D is deliberately overexposed to demonstrate the position of the iris section. A similar iris section is stained with haematoxylin and eosin in Panel C with an arrow marking the position of the posterior surface of the iris, along which lie the two epithelial layers, the inner of which comprises the myoepithelial cells of the dilator muscle. Calibration bar represents 75 μm.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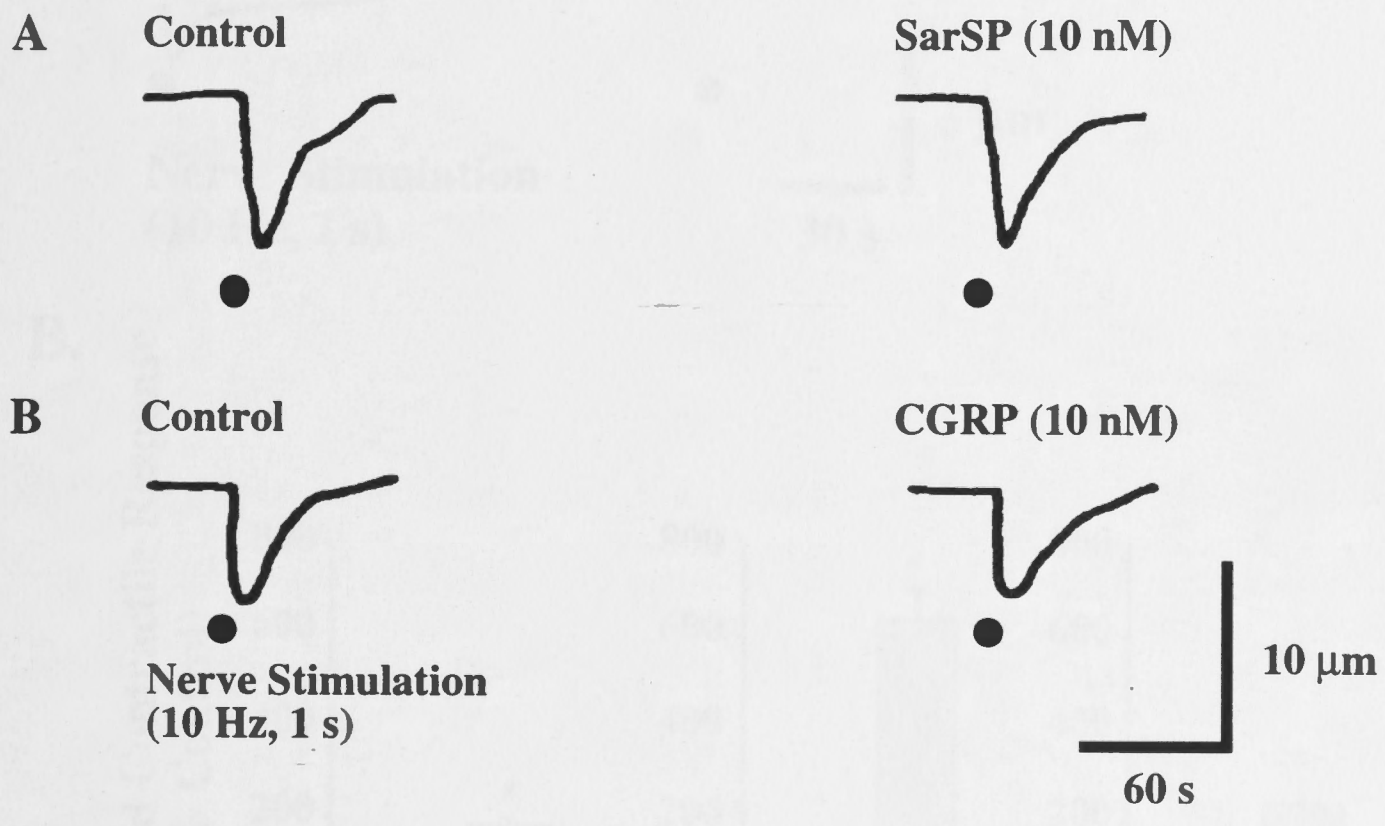
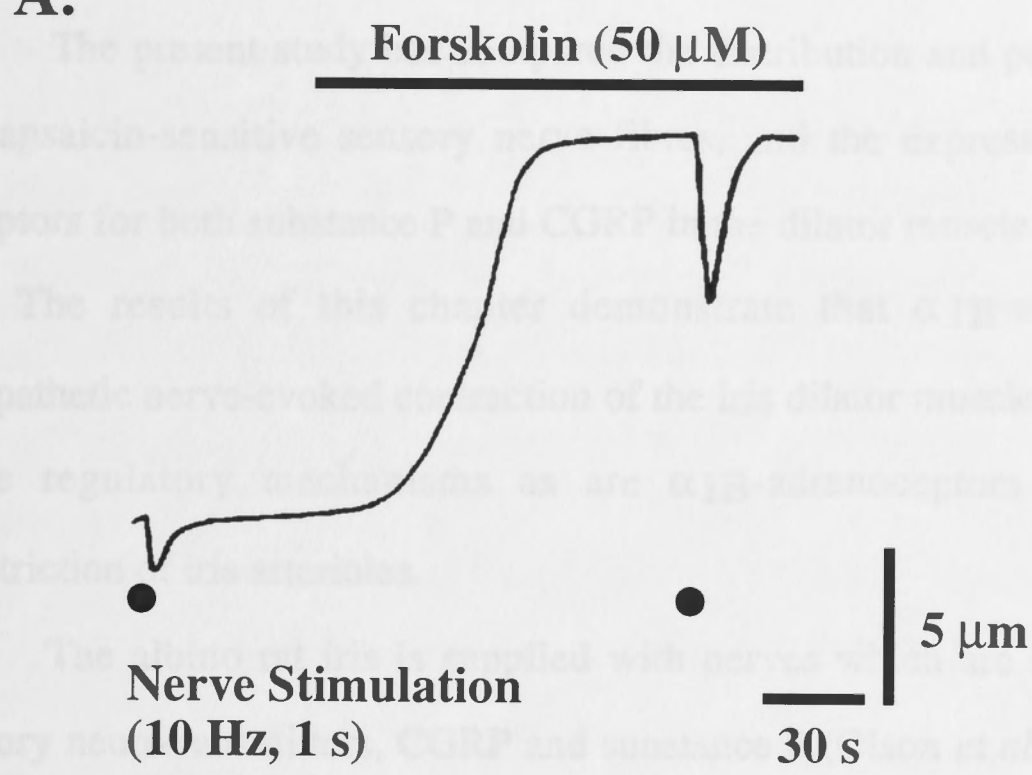


Figure 7.6. Traces showing the effect of SarSP (10 nM; Panel A) and **B** CGRP (10 nM; Panel B) on the dilator muscle contractile response to nerve stimulation every 3 min. Dots indicate the onset of nerve stimulation (10 Hz, 1 s).



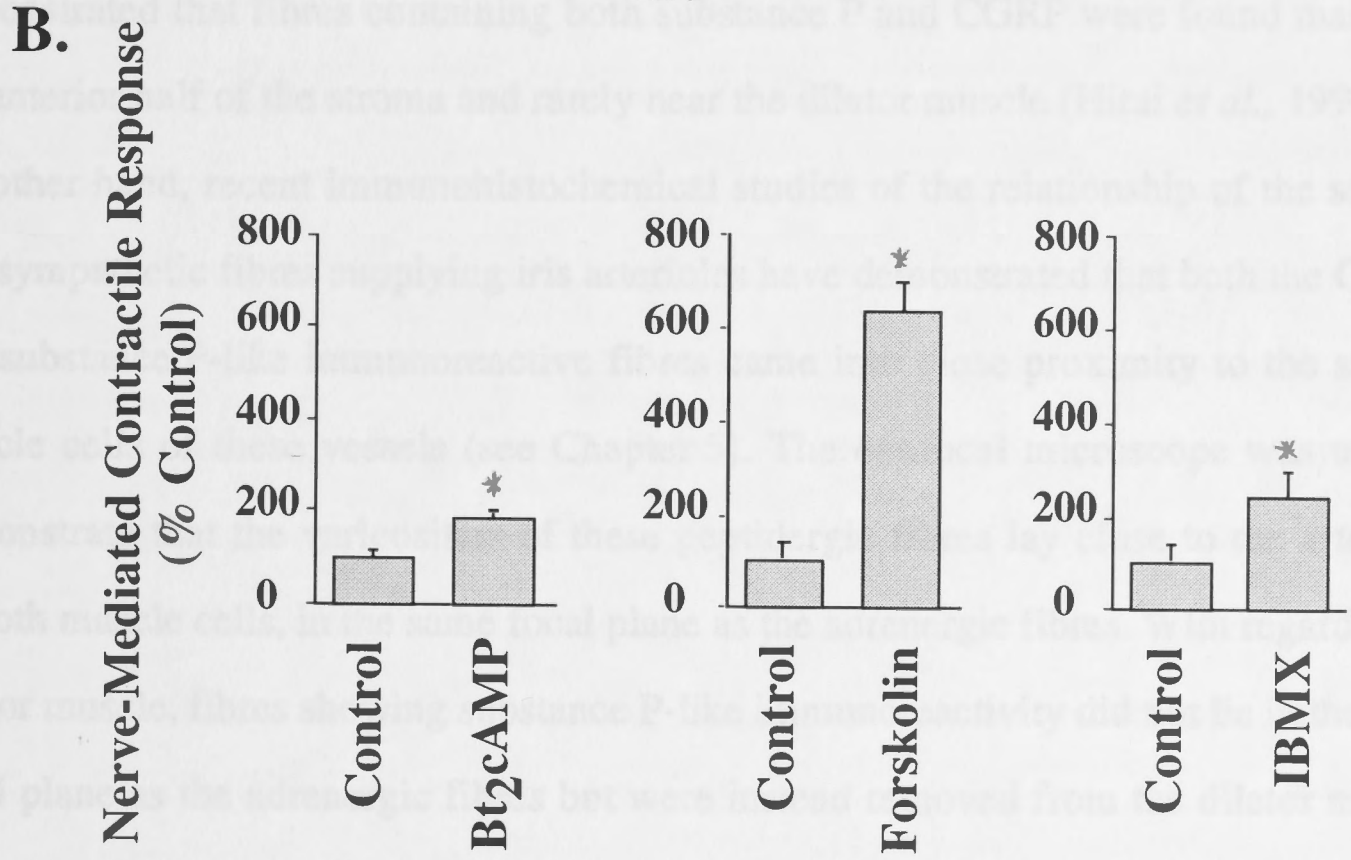


Figure 7.7

A. Trace illustrating the effect of forskolin (50 μM) on the nerve-mediated contractile response of the dilator muscle. Dots indicate onset of nerve stimulation (10 Hz, 1 s).

B. Effect of (left to right) dibutyryl cyclic AMP (Bt_2cAMP; 1 mM; n=3), forskolin (50 μM; n=3) or 3-iso-butyl-1-methyl-xanthine (IBMX; 100 μM; n=3) on the dilator muscle contractile response evoked by nerve stimulation (10 Hz, 1 s). Columns represent the mean and vertical bars respresent the s.e.m. of the number of preparations indicated in parenthesis.

* indicates significant difference from paired control (t-test with Bonferroni correction).

DISCUSSION

The present study has compared the distribution and possible efferent function of capsaicin-sensitive sensory nerve fibres, and the expression and distribution of receptors for both substance P and CGRP in the dilator muscle and the arterioles of the iris. The results of this chapter demonstrate that α_{1B}-adrenoceptors mediating sympathetic nerve-evoked contraction of the iris dilator muscle are not subjected to the same regulatory mechanisms as are α_{1B}-adrenoceptors mediating sympathetic constriction of iris arterioles.

The albino rat iris is supplied with nerves which are immunoreactive for the sensory neurotransmitters, CGRP and substance P (Olson *et al.,* 1988). It was recently demonstrated that fibres containing both substance P and CGRP were found mainly in the anterior half of the stroma and rarely near the dilator muscle (Hirai *et al.,* 1994). On the other hand, recent immunohistochemical studies of the relationship of the sensory and sympathetic fibres supplying iris arterioles have demonstrated that both the CGRP- and substance P-like immunoreactive fibres came into close proximity to the smooth muscle cells of these vessels (see Chapter 5). The confocal microscope was used to demonstrate that the varicosities of these peptidergic fibres lay close to the arteriolar smooth muscle cells, in the same focal plane as the adrenergic fibres. With regard to the dilator muscle, fibres showing substance P-like immunoreactivity did not lie in the same focal plane as the adrenergic fibres but were instead removed from the dilator muscle. Although the sensory fibres did not lie very close to the dilator muscle, it was still possible that they could release transmitter from a distance to influence the dilator muscle activity.

The distribution of binding sities for the NK_1-tachykinin receptor agonist, SarSP, and for CGRP in the iris were similar. Binding sites were distributed throughout the stroma but did not appear to be specifically associated with the anterior surface of the dilator. Thus the dilator muscle does not appear to have binding sites for these sensory neuropeptides. In contrast, specific binding sites for both SarSP and CGRP were associated with the arterioles of the iris. Furthermore, it was observed that the arterioles delineated by the CGRP ligand appeared to be larger in diameter than the

blood vessels delineated by the SarSP ligand. Previous studies have demonstrated that the vasodilatory actions of substance P are endothelium-dependent (Maggi *et al.,* 1990; Stephenson, Burcher and Summers, 1986; Saito *et al.,* 1990), as is the effect of CGRP on the iris arterioles (see Chapter 5). The apparently larger size of the arterioles following incubation with CGRP suggests that the CGRP binding sites are located on both the endothelial and smooth muscle cells. Unfortunately it was not possible to remove the endothelial cells from these arterioles to clarify this point.

RT-PCR supported the autoradiographical results in demonstrating that mRNA for the NK_1-neurokinin receptor is expressed in the iris. In addition, these studies detected mRNA for NK_3-, but not NK_2-, tachykinin receptors in the iris. While it has been previously demonstrated that the NK_3-tachykinin receptor is expressed in the whole eye (Shigemoto *et al.,* 1990), this finding was unusual as neurokinin B levels in peripheral tissues are very low or, in many cases, undetectable (Hua *et al.,* 1985; Too, Cordova and Maggio, 1989b). Functional studies in the rabbit iris sphincter found that NK_1- and NK_3-, but not NK_2-, tachykinin receptor selective agonists were potent contractile agents (Hall, Mitchell and Morton, 1993; Hall, Mitchell and Morton, 1991; Wang and Hakanson, 1993). However, the tissue used in the present experiments contained only the dilator muscle and the connective tissue stroma containing the blood vessels; the ciliary processes and the sphincter muscle having been removed prior to the RNA extraction. Thus the observed expression of mRNA for the NK_3-neurokinin receptor could not be due to the inclusion of the sphincter muscle. There appear to be no previous reports in the rat iris for the presence of neurokinin B, the endogenous preferred ligand for the NK_3-neurokinin receptor (Maggi, 1995), although the receptor could be activated by less potent endogenous neurokinin ligands or the mRNA may not be translated into protein. Further studies are required to answer these questions.

Neither CGRP nor the NK_1-tachykinin receptor agonist, SarSP, at 10 nM, affected the tone of the dilator muscle nor the ability of the sympathetic fibres to elicit contaction of the dilator muscle. At this concentration the inhibitory effect of these two peptides on iris arteriolar constriction is maximal (Table 5.1), while their vasodilatory effects in other blood vessels are maximal at much lower concentrations (Han, Naes and

Westfall, 1990; Kawasaki *et al.,* 1990a; Kawasaki *et al.,* 1988; Withrington, 1992). Furthermore, stimulus conditions which led to the activation of sensory-motor nerves in the arterioles failed to influence the sympathetic nerve-evoked contraction of the iris dilator muscle. These results support the binding studies which suggested that there are few, if any, receptors for substance P and CGRP on the rat dilator muscle surface.

Previous studies indicate that substance P and CGRP released from sensory-motor nerves mediate the ocular responses to injury, that is, miosis, conjunctive hyperaemia and an aqueous flare response (see Unger *et al.,* 1985). The present study demonstrates that neither CGRP nor SarSP alter the tone of the iris dilator muscle. Consistent with this, substance P has also been shown to have no effect on the dilator muscle of rabbits and cows, even up to 10^{-4} µM (Soloway, Stjernschantz and Sears, 1981; Suzuki and Kobayashi, 1983), a concentration which causes contraction of the iris sphincter muscle in several species (Tachado *et al.,* 1991). The intravitreal injection of CGRP into the rabbit eye does not cause miosis, but causes vasodilatation and the aqueous flare response. Intracameral injection of substance P causes miosis, increase in intro-ocular pressure and an aqueous flare response (Oksala, Stjernschantz and von Dickhoff, 1989). The above results together indicate that the miotic pupil response occurs as a result of the release of substance P to cause contraction of the sphincter muscle, without a contribution from the dilator muscle, as the current results show it does not receive a functional sensory-motor innervation.

In the dilator muscle, the absence of sensory nerve fibres was well correlated with the absence of of binding sites for SarSP and CGRP and the absence of responsiveness of the tissues to applied neuropeptide. Such a correlation suggests that the nerve fibres may be important for the expression of the receptors.

In small cutaneous arterioles, there is a good correlation between the presence of NPY fibres and the ability of these arterioles to respond to exogenous NPY (Morris, 1994a). In the iris arterioles, however, there was a paucity of sensory fibres and an abundance of binding sites for SarSP and CGRP, suggesting that there is not always such a close correlation between the presence of the nerves and the level of receptor expression. Mismatches between receptor localizations and neurotransmitter types have been

described in the brain (Herkenham, 1987) and removal of the cholinergic neurones innervating the sweat glands of the rat foot, either during development or in the adult, failed to produce any change in the expression of the muscarinic receptors in that tissue (Grant and Landis, 1991; Grant, Landis and Siegel, 1991).

The presence of NK_1-tachykinin and CGRP receptors within the stroma suggest that the sensory fibres may also be performing an efferent function perhaps in addition to their more conventional afferent role. Antigen-presenting cells derived from the bone marrow have been demonstrated in the iris and ciliary body of mouse eyes (Williamson, Bradley and Streilein, 1989) and these cells form a network within the stromal layer of the iris (Knisely *et al.,* 1991). These cells may well bear on their surfaces the binding sites for neuropeptides demonstrated in the present chapter, since a number of neuropeptides, including substance P and CGRP, have been shown to positively and negatively modulate macrophage effector functions (Nong *et al.,* 1989; Peck, 1987).

In the present study, not only was there no evidence for a functional sensory fibre innervation of the dilator muscle, but the response to cyclic AMP was different than in the arteriole. Elevated intracellular cyclic AMP caused a relaxation of the dilator muscle and a concomitant potentiation of the nerve-mediated contraction. This latter effect most likely occurred as a result of the change in resting tone of the tissue due to elevated cyclic AMP. Thus, an increase in cyclic AMP had the opposite effect on the sympathetic nerve-mediated contraction of the iris dilator muscle to that of iris arterioles, in spite of apparently identical receptor subtypes mediating both responses. The interaction between α_{1B}-adrenoceptors and receptors coupled to increases in cyclic AMP in iris arteriole occurs as a result of the cyclic AMP-dependent activation of NOS (see Chapter 6). Since constitutive NOS is found in endothelial cells and not smooth muscle cells (Leone *et al.,* 1991), the failure of cyclic AMP to inhibit the sympathetic nerve-evoked contraction of the dilator muscle appears to be due to the absence of NOS in the dilator muscle cells.

In conclusion, there is no evidence that the dilator muscle receives a sensory-motor innervation, nor that activation of these nerves can modulate sympathetic nerve-induced contraction. While sensory-motor nerve activity produces a loss of sympathetic

vasoconstriction with repetitive nerve-stimulation thereby acting to maintain the blood flow to the iris *in vivo*, activation of sensory-motor nerves does not affect the contractile response of the dilator muscle produced by sympathetic nerve-stimulation thus permitting sustained pupil dilation. Furthermore, cyclic AMP does not interact with the sympathetic contractile response of the dilator muscle, as it does in the iris arterioles. It is therefore unlikely that activation of other receptors on the iris dilator muscle which are coupled to elevated cyclic AMP, such as ß-adrenoceptors, could modulate the response to sympathetic nerve stimulation, as do CGRP receptors on the arterioles.

CHAPTER 8.

GENERAL DISCUSSION

Arterial smooth muscle tone is subject to physical, hormonal and nervous influences, all of which act to maintain blood flow within the body (Johnson, 1978). It is important to understand the mechanisms which control arteriolar diameter, as these small diameter vessels provide the greatest contribution to the resistance to peripheral blood flow (see Chapter 1). This thesis has investigated the peripheral nervous control of such an arteriole in the rat iris.

Sympathetic nerve stimulation produces a constriction of iris arterioles which is due to the activation of postjunctional α_{1B}-adrenoceptors. In contrast to previous studies where sympathetic neurotransmission in arterial vessels involves non-α–adrenergic mechanisms (see Table 1.1), the results of this thesis suggest that non α–adrenergic mechanisms do not exist in all resistance vascular beds. The arterioles in the current study are not being perfused, but rather superfused, with oxygenated Krebs. Non-α adrenergic neurogenic responses have been recorded in mesenteric arterioles (Hirst and Neild, 1980a) and the basilar artery (Hirst, Neild and Silverberg, 1982) under the same experimental conditions, and using similar stimulus parameters, so the purely α–adrenergic neurogenic responses recorded in the iris arterioles are not likely to be an artefact of the present experimental conditions.

Smooth muscle contraction occurs following the elevation of intracellular calcium, which may occur as a result of the release of stored intracellular calcium or the influx of extracellular calcium (Hirst and Edwards, 1989). The contractile response of iris arterioles was only partly dependent on the influx of calcium through voltage-dependent calcium channels, thus suggesting an initial voltage-independent mechanism. The findings of this thesis indicate that the sympathetic contractile response of iris arterioles occurs primarily as a result of the release of intracellular calcium, followed by the influx of extracellular calcium.

Hashimoto *et al.* (1986) demonstrated that α_1–adrenoceptor pharmacomechanical coupling, that is, contraction occurring independently of depolarization (Somlyo and Somlyo, 1968), in vascular smooth muscle occurs as a result of stored intracellular calcium released following IP3 production. Consistent with this, the changes in amplitude and time to maximum depolarization recorded from iris arteriolar smooth muscle cells in response to increasing numbers of pulses in the stimulus train are consistent with the non-linear relationship between increases in the levels of IP3 and evoked membrane chloride currents (Parker and Miledi, 1989; Miledi and Parker, 1989). Further indications for the utilization of IP3-mediated intracellular calcium release for iris arteriole contraction came from preparations where spontaneous contractions and depolarizations were recorded. These had frequencies in the range previously described for oscillations in intracellular calcium from intracellular stores (Berridge and Galione, 1988). Such oscillations may occur as a result of a second messenger, such as IP3, acting at the sarcoplasmic reticulum to produce periodic uptake and release of calcium (Berridge and Galione, 1988). Caffeine abolished both the nerve-evoked contraction and depolarization as well as substantially reducing the spontaneous depolarizations. Furthermore, the spontaneous depolarizations of iris arterioles were abolished by elevation of cyclic AMP, which has been shown to reduce levels of stored intracellular calcium in other smooth muscle tissues (Abe and Karaki, 1989; Ishikawa, Ouchi and Orimo, 1993).

From the above, we would predict that the spontaneous contractions and depolarizations are being initiated by changes in intracellular calcium and would therefore be insensitive to inhibition of voltage-dependent calcium channels. The role of IP3 in the neurogenic contraction could be tested by determining whether the responses to exogenous IP3 mimic the nerve-evoked contraction and depolarizations. In addition, experiments on arteriolar neurogenic contractions in combination with the imaging of cytosolic calcium using pharmacological agents which interfere with the IP3 pathway, for example inhibitors of phospholipase C, or those which may lower stored intracellular calcium, for example cyclic AMP or cyclic GMP would further characterize how changes in intracellular calcium relate to the neurogenic contractile

response of the iris arterioles. These experiments could be done in the presence of voltage-dependent calcium channel antagonists to prevent any influx of calcium which occurs with supramaximal stimulus.

While non-α adrenergic mechanisms appear to activate voltage-dependent contractile responses, α–adrenoceptors appear to mediate all the voltage-independent contractile responses that have been observed in response to sympathetic nerve stimulation (present study; see Hirst and Edwards, 1989 for review). In the anococcygeus muscle and rat tail artery, it has been reported that α–adrenoceptor mediated contraction preceded the depolarization, suggesting no cause and effect relationship between the depolarization and contraction (Byrne and Large, 1985; Cheung, 1984). Further evidence for the voltage-independence of neurogenic α–adrenoceptor mediated contractions is that nerve-evoked α–adrenergic depolarization can produce contraction when the membrane potential is at a more hyperpolarized value than the threshold potential for an action potential (Cheung, 1984). Similarly, a small contraction of iris arterioles was observed in response to one stimulus pulse, which was associated with a change in membrane potential which would not be expected to open voltage-dependent calcium channels. Rather than being voltage-dependent events, α–adrenoceptor mediated contractions and depolarizations can be seen to reflect changes in intracellular calcium (present study; Hirst and Edwards, 1989; Parker and Miledi, 1989).

With larger stimulus strengths applied to the iris arterioles, the resulting depolarization was sufficiently large enough to open voltage-dependent calcium channels and allow calcium influx through these channels to contribute to the depolarization and contraction. Thus, while neurogenic α–adrenoceptor contractions are not voltage-initiated, with sufficient stimulus, initiation of an action potential may superimpose over the associated depolarization to potentiate the level of intracellular calcium and cause an additional increment in the size of the contraction (see also Hirst and Edwards, 1989).

Arterial smooth muscle cells are electrically coupled to form a syncitium (see Hirst and Neild, 1978). The smooth muscle cells of the iris arterioles show resting

membrane potentials and electrical coupling characteristics (Hirst et al, 1996, submitted for publication) comparable to those of other arterial vessels (Hirst and Edwards, 1989; Kuriyama, Kitamura and Nabata, 1995). The electrical properties of the arterioles therefore do not provide an explanation for the lack of a non-α adrenergic component of sympathetic neuroeffector transmission in iris arterioles.

The only other mature arterial vessel where a purely α–adrenoceptor mediated contraction and depolarization have been recorded in response to sympathetic nerve stimulation is the pulmonary artery (Suzuki, 1983), however, α–adrenoceptor mediated sympathetic nerve-evoked responses occur in veins (Holman *et al.*, 1968; Cheung, 1985; van Helden, 1988). It has therefore been suggested that α–adrenoceptors mediate sympathetic neuroeffector transmission in vessels which are subject to low perfusion pressures whereas in systemic arteries, which are exposed to higher perfusion pressures, the responses to sympathetic nerve stimulation are resistant, to some degree, to α–adrenoceptor antagonists (Hirst and Edwards, 1989). This may provide an explanation for the purely α–adrenoceptor mechanism for sympathetic neuro-effector transmisssion in the iris arterioles. It has been determined that, in cats, the blood pressure at the origin of the ciliary arteries is about 19 mmHg lower than in the common carotid artery and about 15 mmHg lower than in the femoral artery (Bill, 1963; Bill, 1966). The blood flow in the eye in rabbits has been estimated to be 0.32 $mg.ml^{-1}.gm^{-1}$, which is 46 % of the cerebral blood flow (Bill and Nilsson, 1985). The blood flow in the eye is therefore lower than in other parts of the circulatory system. Within the eye itself, the blood flow in the iris is almost one tenth of that in the choroid (Stjernschantz, Alm and Bill, 1976). It may be that non-α–adrenoceptor mechanisms are sensitive to the blood flow *in vivo*, their influence being down-regulated by a low blood flow or perfusion pressure.

The activation of ß-adrenoceptors causes hyperpolarization of the mesenteric and coronary arteries (Mulvany, Nilsson and Flatman, 1982; Itoh, Izumi and Kuriyama, 1982). If a ß-adrenoceptor blocker is not present in the perfusing solution then any depolarization produced by noradrenaline may be reduced by a ß-adrenergic effect. It has been demonstrated that the depolarization dose-response curve for noradrenaline on the basilar artery is shifted to the left in the presence of a ß-adrenoceptor antagonist

(Harder, Abel and Hermsmeyer, 1981). However, an α–adrenoceptor antagonist abolished all responses to nerve stimulation. In addition, a ß-adrenoceptor antagonist did not increase the magnitude of neurogenic vasoconstriction (results not shown), which would indicate the unmasking of an additional effect of noradrenaline. It is therefore unlikely that a voltage-dependent non-α–adrenergic response was being masked by ß-adrenoceptor activation in this preparation.

The present study has only investigated arteriolar neurogenic contraction under one set of stimulus parameters, that is, 10 Hz for 1 s. It is yet to be determined whether the mechanism for iris arteriolar contraction varies with different stimulus frequencies. For example, the contribution of NPY to sympathetic-evoked vasoconstriction is reported to be more pronounced following longer trains of, or higher frequency, stimulation (Morris and Murphy, 1988; Pernow *et al.*, 1989). NPY-like immunoreactivity has been located in tyrosine hydroylase-like immunoreactive sympathetic fibres associated with blood vessels in the rat iris (Olson *et al.*, 1988), suggesting that under certain stimulus conditions NPY may play a role in sympathetic vasoconstriction. In the presence of benextramine, to inhibit postjunctional α–adrenoceptor responses to depolarization-evoked release of noradrenaline, KCl-induced contraction is reduced by 70 % in the presence of nifedipine (10 μM, Chapter 4). The remaining 30 % of the contraction may be produced by the depolarization-evoked neural release of NPY. This further suggests that with sufficient stimulus strength NPY may be released from sympathetic nerves supplying the iris arterioles.

NPY Y_1 receptors, which are reported to mediate vasoconstricton and to potentiate constrictor response to noradrenaline (Grundemar and Hakanson, 1993), are coupled to a reduction in cyclic AMP (Watson and Girdlestone, 1994). In addition to producing hyperpolarization, ß-adrenoceptor activation produces elevated cyclic AMP levels (Watson and Girdlestone, 1994), which could counteract, and possibly mask, any postjunctional responses that may have been produced by neurally-released NPY. However, as mentioned previously we have no evidence for the activation of ß-adrenoceptors by neurally-released noradrenaline. Elevation of cyclic AMP by the activation of A_2-adenosine or $CGRP_1$ receptors present on the iris arterioles (Watson

and Girdlestone, 1994; Gould, Vidovic and Hill, 1995, Chapter 5) might however counteract postjunctional NPY Y_1 receptor-mediated contraction.

Alpha-adrenoceptor subtypes may be sensitive to physical conditions such as pH. For instance, the exogenous noradrenaline curve on the rabbit aorta is competitively shifted to the right by acidosis (pH 7.24-6.51). The maximum tension produced by noradrenaline was no different between pH 7.24-6.90 or during alkalosis (pH 7.61-8.04), but was decreased by 30 % at pH 6.51 (Stokke *et al.*, 1984). The authors concluded that high concentration of hydrogen ions may exert α–adrenoceptor antagonist properties. A differential sensitivity of α_1– and α_2–adrenoceptors to acidosis has been demonstrated in the pithed rat, where α_2–adrenoceptor responses are favoured in acidosis (Grant, McGrath and O'Brien, 1985). This may be due to an inhibitory effect on the adrenoceptor, as proposed above, or due to differences in the ionization state, and therefore the selectivity of the receptor agonist for different receptor subtypes with different pH. While the gas bubbling rate in the present experiments maintained the pH at 7.4, the arteriolar neurogenic response of iris arterioles might show a different α–adrenergic pharmacology under different pH conditions.

The *in vivo* blood flow at any given time will reflect a balance between vasoconstrictor and vasodilatory nervous influences. For instance, in the present study it was demonstrated that activation of sensory-motor nerves inhibits the sympathetic vasoconstriction of iris arterioles. This interaction occurs postjunctionally, and as a result of an interaction between the two neuroeffector pathways. In the rat, sensory-motor vasodilatation is associated with an increase in vascular permeability (Holzer, 1992). An increase in blood flow to an injured tissue would facilitate the delivery of macromolecules and leucocytes to the tissue to provide resistance to further damage by noxious stimuli, and to assist repair to the injury (Jancso, Jancso Gabor and Szolcsanyi, 1967; Holzer, 1992). These sensory-motor nerves therefore play an important role in increasing blood flow to an injured site and combatting further tissue injury.

In the iris arterioles the sensory-motor nerves are not acting via a hyperpolarization to antagonize sympathetic vasoconstriction but are acting via the cyclic AMP-dependent production of nitric oxide, which is proposed to be acting

intracellularly to reduce the release of calcium utilized for contraction. As discussed in Chapter 6, sensory modulation of sympathetic vasoconstriction appears to occur via the production of nitric oxide in arteries where voltage-independent events mediate sympathetic constriction, but via hyperpolarization in arteries where the constriction is voltage-dependent. This may represent a general principal for the interaction between neural vasoconstriction and vasodilatation; the mechanism for vasodilatation acts to oppose the mechanism of sympathetic vasoconstriction. In mesenteric and basilar arteries, where the sympathetic vasoconstriction occurs as a consequence of voltage-dependent conductance changes (Hill, Hirst and van Helden, 1983; Hirst, Neild and Silverberg, 1982), acetylcholine produces an endothelium-dependent hyperpolarization which is responsible for the vasodilatation (Khan, Mathews and Meisheri, 1993; Rand and Garland, 1992). However in the pulmonary artery where α–adrenoceptors mediate sympathetic vasoconstriction (Suzuki, 1983), most of the vasodilatation produced by acetylcholine was abolished by inhibiting nitric oxide synthesis, but the hyperpolarization was unaffected, indicating that the hyperpolarization was not associated with the vasodilatation (Chen, Suzuki and Weston, 1988).

The sensory-motor neurotransmitter, CGRP, acts postjunctionally to inhibit sympathetic vasoconstriction of isolated iris arterioles as a result of cyclic AMP-dependent activation of nitric oxide synthesis. Using stimulus conditions which evoked sensory-motor activity in the iris arterioles, no functional sensory-motor activity was found in the iris dilator muscle. Despite both neurogenic contractile responses being mediated by the same adrenoceptor, the lack of the ability of cyclic AMP to inhibit neurogenic contraction in the dilator muscle was attributed to a lack of constitutive NOS in the dilator muscle cells. Thus, the dilator muscle is one example of a sympathetic target which does not receive functional sensory-motor innervation. It may be that sensory-motor nerves only innervate tissues where they may also be required to counteract the opposing neurogenic response.

The physiological consequences of differences in the sensory-motor innervation of the iris arterioles and dilator muscle should be considered. Upon injury to the eye, activation of sensory-motor nerves to the arterioles causes dilatation to promote blood

flow to the iris for reasons described previously. In addition, sensory-motor activity causes constriction of the pupil by contracting the sphincter muscle (Tachado *et al.*, 1991). Pupil dilation occurs following sympathetic nerve stimulation. In some situations it may be necessary to maintain this dilatation for many minutes, however the delivery of oxygen to the iris would be severely affected if vasoconstriction was sustained for extended periods. Sensory-motor nerve activity, brought on by changes in pH or pO_2 that may occur locally in the iris arterioles with repetitive sympathetic nerve-stimulation, would counteract the vasoconstriction of the arterioles, but not the dilator constriction, thus providing a mechanism for the maintenance of blood flow to the iris during pupil dilation.. Whether such an interaction between sympathetic and sensory-motor nerves occurs in the iris sphincter muscle has not yet been detemined.

At the two highest concentrations tested, CGRP produced a vasodilatation of the iris arterioles which was considerably longer-lasting than the inhibitory effect on the vasoconstriction (unpublished observations). This suggests that these two effects of CGRP may be produced via two different mechanisms. Time did not permit investigation of the mechanism for vasodilation. It may be that in the iris arterioles, $CGRP_1$-receptor activation leads to the elevated production of cyclic AMP which causes vasodilatation and also activates nitric oxide synthesis in endothelial cells to inhibit sympathetic vasoconstriction. It is tempting to speculate that pretreating with dideoxyadenosine, to inhibit adenylate cyclase, would inhibit CGRP-mediated vasodilatation, but a vasodilatation would still be observed after inhibition of nitric oxide synthesis. In order to examine CGRP-induced vasodilatation the arterioles would need to be preconstricted, preferably with an α–adrenoceptor antagonist to mimic sympathetic vasoconstriction, as it has recently been demonstrated that the mechanism for vasodilation is influenced by the preconstricting agent (Hayashi et al, 1994).

It is yet to be determined whether the iris arterioles receive a functional parasympathetic innervation. Preliminary experiments in which the effect of sympathetic and sensory-motor nerves had been inhibited with guanethidine and capsaicin respectively, suggested that in unconstricted arterioles there is no parasympathetic innervation (results not shown), however in order to measure

vasodilatation these experiments need to be repeated in arterioles which have been preconstricted. A vasodilatation may only be consistently observed in preconstricted arterioles as indicated by the fact that a sensory-motor vasodilatation was not observed in all arterioles taken from sympathetically-denervated rats, although all arterioles received a functional sensory-motor innervation (Chapter 1, Chapter 5).

In conclusion, this study has demonstrated a number of features of neurogenic constriction of iris arterioles, the first being that, unlike most other arterial vessels, the contractile response and depolarization are both purely due to activation of an α–adrenoceptor. This suggests that not all resistance vessels utilize non-α–adrenoceptor mechanisms for constriction. The α–adrenoceptor arteriolar response occurs primarily as a result of the release of intracellular calcium, with a component at supramaximal stimulation being due to the influx through voltage-sensitive calcium channels. Sensory-motor activation releases CGRP which acts postjunctionally to inhibit sympathetic constriction independently of hyperpolarization, via cyclic AMP-dependent activation of nitric oxide synthesis, which suggests that the mechanism of neurogenic inhibition biochemically opposes the mechanism for sympathetic contraction. Finally, it was demonstrated that an interaction between sympathetic and sensory-motor nerves does not occur in all tissues. It may be that sensory-motor nerves only innervate a tissue where there may be some purpose for an interaction with the second messenger system mediating an opposing neurogenic response.

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