THE RESPONSES OF AUTONOMICALLY-INNERVATED SMOOTH MUSCLE TO NERVE STIMULATION AND TO ~~DURGS~~ DRUGS.

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by

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SUMMARY

1. The intracellular electrical and mechanical responses of the rabbit ear artery, the rabbit superior mesenteric artery and the mouse vas deferens were examined in response to field stimulation of intramural nerves and to drugs. Each tissue demonstrated co-transmission involving noradrenaline (NA) and adenosine 5'-triphosphate (ATP), or a closely related nucleotide.

2. In the rabbit ear artery, the rabbit mesenteric artery and the mouse vas deferens the electrical response evoked by field stimulation consisted of excitatory junction potentials (e.j.p.s), which facilitated and summated to fire action potentials. These e.j.p.s were abolished by α,β-methylene ATP (α,βMeATP, 1-10 x 10^{-6}M), suggesting that they were mediated by ATP. Only in the rabbit ear artery was there an additional electrical event mediated by NA. This took the form of a small, slow membrane depolarization which followed the e.j.p.s and which was antagonized by the α-adrenoceptor antagonists phentolamine (1 x 10^{-6}M) or prazosin (1 x 10^{-7}M).

3. In each of these tissues, all electrical and mechanical responses to field stimulation were abolished by either tetrodotoxin (TTX, 1 x 10^{-6}M) or guanethidine (1 x 10^{-6}M), suggesting that they were due to transmitters released from sympathetic nerves.

4. In the mouse vas deferens and rabbit mesenteric artery, both transmitters (NA and ATP) played a role in the contractile response to field stimulation. In the rabbit ear artery, however, only NA appeared to mediate a contractile event.

5. Contractile responses to nerve-released ATP were accompanied by a change in membrane potential. Na^+ and K^+ appeared to be the main ions

underlying the e.j.p. and action potential. In contrast, contractile responses evoked by NA appeared to be mediated by both voltage-dependent and voltage-independent mechanisms, the relative contribution of each to the overall response varying from one tissue to another.

6. In the mouse vas deferens, exogenous application of ATP (1×10^{-4} - 1×10^{-3}M) or its stable analogue, α,βMeATP (1×10^{-6}M) by pressure ejection from a micro-pipette produced dose dependent depolarizations. NA (1×10^{-4} - 1×10^{-3}M), similarly applied produced no such change in membrane potential.

7. In the mouse vas deferens, local application of bradykinin (1-100 x 10^{-7}M) produced small, slow membrane hyperpolarizations. VIP (1-100 x 10^{-7}M), neuropeptide Y (1-100 x 10^{-7}M), substance P (1-100 x 10^{-7}M), leu-enkephalin (1-100 x 10^{-7}M), met-enkephalin (1-100 x 10^{-7}M), somatostatin (1-100 x 10^{-7}M) and bombesin (1-100 x 10^{-7}M), similarly applied, each produced no significant change in membrane potential. None of these peptides, it was concluded, appear to be the transmitter mediating the e.j.p.s in this tissue.

8. In the rat anococcygeus muscle, membrane potential changes recorded intracellularly following field (transmural) or extrinsic nerve stimulation were indistinguishable. Single stimuli usually produced a slow depolarization; trains of pulses produced a fast e.j.p. initially, followed by a slow depolarization similar to that produced by single pulses. The fast e.j.p.s, the slow depolarizations and the accompanying contractions were abolished by the α-adrenoceptor antagonists phentolamine (1×10^{-6}) or prazosin (1×10^{-7}M), by TTX (1×10^{-6}M), but were unaffected by α,βMeATP (1 - 10 x 10^{-6}M).

9. Application of NA (1×10^{-8} - 1×10^{-6}M) by pressure ejection from a micro-pipette to the rat anococcygeus muscle depolarized the membrane and produced localized contractions, both of which were abolished by phentolamine (1×10^{-6}M) or prazosin (1×10^{-7}M).

10. Application of ATP (1×10^{-4} - 1×10^{-3}M) by pressure ejection to the rat anococcygeus muscle produced small membrane depolarizations and localized contractions which were unaffected by phentolamine (1×10^{-6}M) or prazosin (1×10^{-7}M), but abolished by α,βMeATP (1×10^{-6}M).

11. Results from experiments on the rat anococcygeus muscle show that field or extrinsinc nerve stimulation released only one excitatory transmitter, namely NA, although receptors for both NA and ATP are present on this muscle and that there was no evidence for excitatory co-transmission in this tissue.

12. The pressor responses of tail arteries and mesenteric bed preparations from spontaneously-hypertensive rats (SHR) to field stimulation exceeded those from age-matched normotensive (WKY) animals.

13. In each artery (tail and mesenteric), pressor responses to exogenous catecholamines (NA and ADR) were potentiated in hypertensive (SHR) animals, whereas those to exogenous ATP were not.

14. In the tail artery and mesenteric bed preparations from spontaneously-hypertensive (SHR) and normotensive (WKY) rats, pressor responses were greatly reduced by the α-adrenoceptor antagonists phentolamine (1×10^{-6}M) or prazosin (1×10^{-7}M), but were unaffected by α,βMeATP (1 - 10×10^{-6}M). ATP appeared, therefore, to play no role in the contractile response evoked by field stimulation in either artery

in normotensive or hypertensive animals.

15. There was no significant difference in the [^{3}H] overflow evoked by field stimulation in SHR and WKY tail arteries pre-loaded with either [^{3}H]-NA or [^{3}H]-adenosine. Thus, the increased vascular responsiveness of hypertensive rat tail arteries did not appear to involve an increased NA or ATP release.

16. The density of the adrenergic innervation in tail and superior mesenteric arteries from normotensive (WKY) and spontaneously-hypertensive (SHR) rats was examined using Falck histochemistry. In each artery, there was no significant difference in the density of the innervation between the 2 groups of animals, suggesting that the increased responsiveness of the arteries from hypertensive (SHR) animals could not be attributed to alterations in the density of adrenergic innervation.

17. It also appeared unlikely that the increased vascular reactivity of arteries from spontaneously-hypertensive (SHR) rats could be attributed to structural changes in the vascular smooth muscle. In both the tail and the superior mesenteric artery, the smooth muscle cells and endothelial layer from normotensive (WKY) and spontaneously-hypertensive (SHR) rats were indistinguishable. There was, however, the suggestion of an increased wall-to-lumen ratio in the arteries from hypertensive rats.

18. α,βMeATP (3×10^{-6}M) had no significant inhibitory effect on the [^{3}H] release evoked by field stimulation in spontaneously-hypertensive (SHR) or normotensive (WKY) rat tail arteries pre-loaded with [^{3}H]-NA, suggesting that α,βMeATP has no inhibitory effects on transmitter release.

CHAPTER 1: INTRODUCTION

FOREWORD

The view, commonly attributed to Dale, that each neurone contains only one neurotransmitter, has been a traditional concept in neurobiology for the past 50 years and has provided a framework for an understanding of peripheral neuro-effector systems and the action of drugs thereon.

More recently, a growing body of evidence has suggested that, in addition to the classical neurotransmitters, noradrenaline (NA) or acetylcholine (ACh), many nerves release either a biologically-active purine or peptide (for reviews see Burnstock, 1972; 1979; 1981; 1986; Hökfelt et al., 1980a, d). Since these purines and peptides are synthesized and stored within nerves, released during nerve activity and interact with specific post-synaptic receptors to produce a change in post-synaptic activity, they are, by at least one definition, neurotransmitters (Burnstock, 1976).

It is now recognised, that in many cases, a nerve can release more than one substance in response to stimulation (Burnstock, 1976; 1982; 1985; 1986; Cuello, 1982; Osborne, 1983). This phenomenon of co-transmission, in which each substance can be demonstrated to have a transmitter function, has led to a reconsideration of the one nerve-one transmitter hypothesis embodied in Dale's Principle. However, there are in fact two versions of the so-called Dale's Principle. The original, put forward by Dale himself in 1935, proposed that a neurone contained and released the same transmitter from each axon terminal (Dale, 1935). The second version, a reinterpretation by Eccles, proposed that each neurone contained and secreted one, and only one neurotransmitter (Eccles et al., 1956). Ironically, the original definition, which implied a uniformity, rather than a singularity of transmitters released from each axon can be expanded to embrace the current concept

of co-transmission.

Since the number of neuronal systems in which co-transmission has been proposed to exist is continually increasing, the neurone of future years' investigation may subsequently be shown to release several neurotransmitters. Indeed a neurone with only one putative neurotransmitter may soon become an unusual finding.

The evidence for co-transmission, to follow, has come from work spanning the past three decades. Many of the earlier ideas on co-transmission have, with the development of new experimental techniques, failed to be confirmed. However, having introduced the idea of the multiple transmitter neurone, these theories have freed future investigators from the need to conform to a somewhat restrictive, though originally enlightened, view, and encouraged the development and expression of new ideas on peripheral neuroeffector transmission.

I. EARLY THEORIES ON NEUROTRANSMISSION

One of the early indications that more than one substance could be involved in the transmission process, came from a series of histochemical experiments carried out by Koelle in 1955. Using a modification of the thiocholine technique (Koelle & Friendenwald, 1949) as a means of locating ACh, tissues were stained for acetylcholinesterase (AChE), the enzyme involved in the hydrolysis of ACh. Fresh-frozen sections were incubated in a medium containing acetylthiocholine (AThCh), magnesium and copper glycinate. AThCh was hydrolysed by the tissue AChE and a white mercaptide salt precipitated at the site of enzymatic activity. This salt was subsequently converted into copper sulphide, which could be viewed microscopically (Koelle & Friendenwald, 1949). Using this technique, the distribution of AChE in the cells of ciliary and stellate ganglia from cats, rabbits and rhesus monkeys was described. As expected, high concentrations of AChE were localized in the cholinergic neurones of all species. Surprisingly, however, variable concentrations of the enzyme were also present in several adrenergic and sensory neurones (Koelle, 1955). To explain the presence of AChE in presumably non-cholinergic neurones, it was proposed that the terms 'cholinergic' and 'adrenergic' may define the predominant, but not necessarily the exclusive transmitter in a given neurone and that those neurones containing intermediate concentrations of AChE might liberate both ACh and another transmitter (Koelle, 1955).

Using the same technique, Abrahams et al. (1957) carried out a histochemical survey of the distribution of AChE in the dog hypothalamus in an attempt to localize the cholinergic pathways involved in the hypothalamic control of neurohypophyseal secretion. The only fibres found in the area of the hypothalamico-hypophyseal tracts which stained for AChE, however, were those assumed to release the hormones

(vasopressin and oxytocin). In order to explain these observations, they proposed that impulses conducted along the hypothalamico-hypophyseal neurosecretory fibres first liberated ACh at their terminals. This ACh then provided the stimulus for the subsequent liberation of oxytocin and vasopressin from the same nerve terminals.

This technique was used again a few years later (Koelle & Koelle, 1959) to examine the AChE distribution in the cat superior cervical ganglion. Surprisingly, the AChE was confined, almost entirely, to pre-synaptic membranes, in contrast to the situation at the skeletal neuromuscular junction where the enzyme was located almost exclusively post-junctionally (Couteaux & Taxi, 1952). In view of the primary function of AChE, in the rapid destruction of ACh after its activation of post-junctional receptors, its location at the neuromuscular junction seemed much more strategically favourable than that at the superior cervical ganglion. The implication of these results was significant. If AChE indicated the presence of ACh, what was its pre-junctional role?

Thus two inconsistencies regarding the function of neuronal AChE, in terms of the traditional concept of cholinergic transmission were observed (Grundfest, 1957): First, the presence of varying concentrations of AChE in, presumably, non-cholinergic neurones and secondly, the predominant localization of the enzyme in certain ganglia at pre-, rather than at post-synaptic sites.

By way of explanation, it was proposed (Koelle, 1961) that the ACh liberated by the nerve action potential, acted, initially on the same pre-synaptic terminal to liberate increased additional quanta of ACh. It was this additional, secondarily released ACh which acted post-synaptically to effect transmission. A similar mechanism was proposed for non-cholinergic neurones, where the initial liberation of ACh promoted the release of another neurotransmitter from the same nerve end-

ings (Koelle, 1961). Thus, the pre-synaptic AChE served to hydrolyse the pre-synaptically acting ACh and ACh was present in non-cholinergic neurones as an intermediary in the release process.

A 2-step mechanism in both sympathetic and parasympathetic nerves was postulated also by Burn and Rand (1959) to explain several pharmacological inconsistencies in the traditional view of autonomic neurotransmission. Their work concerned mainly post-ganglionic sympathetic nerves, which, they proposed, contained both ACh and NA. The primary event following depolarization of the nerve terminal was not the release of NA, but of ACh. This ACh then caused an increased influx of calcium ions into the nerve terminal and, in turn, the release of NA from intraneuronal vesicles. This became known as the "cholinergic-link" hypothesis.

The evidence for this theory had several components (for reviews see Burn & Rand, 1965; Burn, 1966):

A. EXISTENCE OF CHOLINERGIC FIBRES

Both functional and histochemical evidence for the existence of ACh itself or of cholinergic fibres in sympathetic nerves was available. Stimulation of sympathetic post-ganglionic fibres liberated NA and ACh from the nerves supplying various tissues, for example, the mucous membrane of the dog buccal cavity and lips (Von Euler & Gaddum, 1931), the hindleg of the dog and cat (Bülbring & Rand, 1935) and the accelerator nerves to the cat heart (Folkow et al., 1948).

Histochemical support for the 'cholinergic-link' hypothesis came, almost exclusively from experiments using the thiocholine technique (Koelle & Friendwald, 1949), where positive staining for AChE was observed in sympathetic ganglia (Koelle, 1955).

B. THE APPARENT SIMILARITIES OF THE EFFECTS OF ACh, NICOTINE AND SYMPATHETIC NERVE STIMULATION

ACh, nicotine and sympathetic nerve stimulation each appeared to produce similar effects in several organs. For example, contraction of the piloerector muscles in the skin of the cat tail was produced by ACh (Brücke, 1935) and nicotine (Coon and Rothman, 1940). In these experiments most of the hair, except for a few tufts, was removed from the cats tail and ACh or nicotine injected into the skin at the base of the tufts. Pilo-erection was observed. Normally, the pilomotor muscles contracted in response to sympathetic nerve stimulation, so it was assumed that ACh and nicotine were each acting on pre-synaptic nerve terminals to cause the release of NA which, in turn, caused piloerection.

Other examples of sympathomimetic effects allegedly caused by ACh or nicotine included the relaxation of the isolated kitten intestine by nicotine (Ambache & Edwards, 1951), the acceleration of the isolated atria of the rabbit (Kottegoda, 1953a) and the constriction of the vessels of the perfused rabbit ear artery (Kottegoda, 1953b).

C. THE EFFECT OF SUBSTANCES WHICH BLOCKED THE RELEASE OF NA

ACh and nicotine each block the release of NA from postganglionic fibres. This was first demonstrated by Brücke (1935) for ACh in the pilomotor muscles of the cat tail. Small amounts of ACh caused piloerection, larger doses a transient piloerection and subsequent abolition of the response to sympathetic nerve stimulation. On the assumption that nerve impulses released only NA, Burn and Rand argued that there was no existing explanation for Brücke's observations in the cat tail. Since ACh caused little or no piloerection in a reserpinized cat (Burn & Rand, 1959), the release of NA, they claimed, was responsible for the piloerection observed. It was therefore argued that larger doses of ACh blocked the release of NA by desensitization

of pre-synaptic nicotinic receptors and that the normal release of NA was mediated by ACh.

D. ACTION OF THE HEMICHOLINIUMS

Hemicholiniums interfere with the transport of choline to intra-neuronal sites and so prevent the synthesis of ACh (MacIntosh et al., 1956). The action is overcome by choline. Hemicholiniums (Brandon & Rand, 1961) abolished the splenic contractions to nerve stimulation, an action also reversed by choline. Since the splenic nerves were assumed to be noradrenergic, it was again argued that NA release was mediated by ACh.

E. ADRENERGIC NEURONE BLOCKING DRUGS

The adrenergic neurone blocking drugs bretylium and guanethidine, as well as high doses of ACh, blocked NA release from sympathetic nerves. These drugs, it was claimed (Burn & Rand, 1960), were active by virtue of their resemblance to ACh. As with ACh, each carries a large positive charge, bretylium on its quaternary nitrogen and guanethidine on its guanine group. It was therefore proposed that these adrenergic neurone blocking drugs desensitized the cholinergic component in the 'cholinergic-link' hypothesis and thus prevented NA release.

F. ACTION OF ANTICHOLINESTERASES

If ACh acts as an intermediate in the release of NA, anticholinesterases should increase the amount of NA released. This was subsequently claimed to be demonstrated in the rabbit ear artery, where vasoconstriction in response to nerve stimulation was potentiated by eserine (Burn & Rand, 1960).

Despite the plausibility of the evidence, the following objections were made to the hypothesis and have, with time, been sustained:-

A. NON-SPECIFICITY OF THE RESPONSES TO ACh AND NICOTINE

Just as ACh and nicotine played a major role in providing evi-

dence for the creation of the "cholinergic-link" hypothesis, further investigation of their effects were instrumental in its rejection. The effects of each lacked specificity. Thus, it was alleged (Ferry, 1963) that, in the cat spleen, close intra-arterial injection of ACh caused a vigorous antidromic spike discharge in post-ganglionic sympathetic splenic nerve fibres. This suggested that the sympathomimetic actions of ACh were indeed due to stimulation of sympathetic nerves. However the effects were non-specific, since a similar action of ACh was also observed in sensory nerves (Gray & Diamond, 1957), where no such transmitter role for ACh had been proposed.

B. HISTOCHEMICAL EVIDENCE

Evidence for the 'cholinergic-link' hypothesis relied on the ability to locate AChE histochemically in sympathetic ganglia (Koelle, 1955). Subsequent investigations however by other workers, (Sjökvist, 1963; Hamberger et al., 1965) failed to confirm the presence of the enzyme in post-ganglionic sympathetic neurones. Furthermore, AChE activity is now no longer regarded as the best indicator of cholinergic neurones. Choline acetylase, the enzyme involved in the synthesis of ACh, is now regarded as a better marker for cholinergic neurones. Thus many adrenergic neurones contain low or even moderate levels of AChE activity, but no measurable choline acetylase activity, and therefore no ACh (Buckley et al., 1967).

C. LACK OF POTENTIATING EFFECTS OF ANTICHOLINESTERASES

In the cat spleen, anticholinesterases, such as eserine, failed to prolong or potentiate the effects of splenic nerve stimulation or increase the amount of NA liberated (Blakeley et al., 1963), effects which might have been anticipated in any ACh-mediated NA release.

Today the general consensus of opinion now fails to accept these early views of Koelle and Burn and Rand on the mechanisms of neuro-

transmission. Notwithstanding, their proposals marked important milestones in the evolution of the neurotransmitter theory. By putting forward these hypotheses they were in effect challenging existing views and prompting ideas upon which are based our current concepts of neurotransmission.

II. CURRENT VIEWS ON THE CO-EXISTENCE OF ACETYLCHOLINE AND NORADRENALINE

While Koelle and Burn and Rand proposed the release of two substances from a single nerve terminal in response to stimulation, only one, it was assumed, produced an effect post-junctionally. The first substance acted pre-synaptically, merely to cause the release of the second which then exerted the transmitter function. In other words, neurotransmission was a 2-step process which involved only one true neurotransmitter. Recent years has seen a modification of this view. The co-transmission theory, as presently understood, suggests that 2 (or more) substances may be released simultaneously following nerve stimulation, and, in contrast to earlier views, that both substances may have a post-synaptic transmitter function.

The idea that autonomic neurotransmission involves the release of more than one substance from post-ganglionic nerve terminals becomes feasible when the development of the autonomic nervous system is examined from an embryological point of view. Neurones destined for all parts of the autonomic nervous system arise, it is believed (Weston, 1970), from a common origin - the neural crest. Certain of the neural crest cells migrate ventrally between the developing neural tube and the somite and remain there to form the sensory ganglia. Others continue to migrate ventrally and produce autonomic neurones in widely scattered locations, to form, for example, paravertebral, prevertebral and enteric ganglia (Weston, 1970). The final transmitter to be synthesized is substantially influenced by the chemical environment surrounding the nerves themselves during development (Weston, 1970; Johnston *et al.*, 1974; Noden, 1975; LeDouarin *et al.*, 1975).

Much of the evidence for the view that neurones have the potential to manufacture more than one neurotransmitter and that this deci-

sion is dependent on environmental factors has come from experiments using cell culture techniques. For example, when cells of the quail neural crest, normally destined to provide catecholamine-releasing cells of the adrenal medulla, were transplanted into the rostral regions of chick embryos, they subsequently provided cells which migrated to the intestine to form the intrinsic gut plexuses and exhibited cholinergic function (LeDouarin & Teillet, 1974; LeDouarin et al., 1975). Conversely, areas of the neural crest normally destined to become the cholinergic intrinsic gut plexuses, when placed in the mid-region of the chick embryo, provided catecholamine-synthesizing neurones for the adrenal medulla and sympathetic chain ganglia.

A similar effect has also been demonstrated in cultures of dissociated rat sympathetic neurones (Bray, 1970; Mains & Patterson, 1973). When grown in the virtual absence of other cell types, these neurones developed the expected adrenergic properties, such as the ability to synthesize, store and release NA (Claude, 1973; Rees & Bunge, 1974; Patterson et al., 1975). In contrast, when co-cultured with appropriate (see below) non-neuronal cells, the mixed neuronal cultures produced as much as a 1000-fold increase in ACh synthesis, compared with that from the neurones cultured alone (Patterson & Chun, 1974). Furthermore, the ACh was secreted at functioning cholinergic synapses (O'Lague et al., 1974; 1975; Johnson et al., 1976; Nurse & O'Lague, 1975; Furchpan et al., 1976).

In addition, there is compelling evidence for the idea that, under certain conditions in vitro, a single sympathetic neurone may, at different times, release NA, ACh or a mixture of both transmitters (Hill et al., 1976; Patterson et al., 1975; Bunge et al., 1978). This was particularly well demonstrated in a series of electrophysiological studies on single, isolated, sympathetic neurones grown on heart cells previously dissociated from new-born rats (Furshpan et al., 1976).

It was shown that some neurones inhibited, some excited, and others inhibited then excited the cardiac myocytes. There is pharmacological and electron microscopic evidence for the storage and secretion of ACh by the first group of neurones, catecholamines by the second, and both ACh and catecholamines by the third. It seemed likely that this represented a true reflection of the events that occurred in vivo during perinatal development (Hill & Hendry, 1977). On the basis of these results it appeared that a population of sympathetic cells having the potential to synthesize both ACh and NA was present at birth. These multi-potential cells require Nerve Growth Factor (NGF) to survive, to which they respond with an increased production of both choline acetyltransferase and tyrosine hydroxylase, the enzymes that synthesize respectively ACh and NA. At this stage they have a viable amine uptake pump. In the presence of appropriate environmental factors (for review see Patterson, 1978) most of the cells appear to differentiate into either noradrenergic or cholinergic neurones. Those which differentiate into cholinergic neurones gradually lose their ability to synthesize tyrosine hydroxylase and to take up catecholamines and become unresponsive to NGF. Those differentiating into noradrenergic neurones lose their ability to synthesize choline acetyltransferase. On the other hand, some neurones fail to become differentiated and retain their ability to synthesize, store and release both NA and ACh. Such neurones could provide the structural basis for our current view that 2 or more substances may co-exist within neurones.

Thus, there is much evidence that, under certain conditions, a developing neurone may release both NA and ACh. Much of this evidence has come from cell culture techniques. In vivo work, however, (Hill & Hendry, 1977) has demonstrated that such a multi-potential population of cells is present at birth, suggesting that such neurones are not merely an artifact of in vitro techniques.

III. NON-ADRENERGIC, NON-CHOLINERGIC NEUROTRANSMITTERS

In the course of their experiments on isolated sympathetic neurones, Furshpan et al. (1976) and Potter et al. (1986) observed a neuronally-evoked myocyte hyperpolarization which was atropine resistant. Further work (Furshpan et al., 1986) revealed that this hyperpolarization was purinergically mediated; it was antagonized by adenosine receptor blockers (8-phenyltheophylline, theophylline, 7-(2-chloroethyl) theophylline) and was attenuated by an enzyme (adenosine deaminase) that hydrolyses adenosine to pharmacologically inactive inosine. This led the authors to conclude that, in addition to NA and ACh co-release, many neurones also released a purine together with NA, ACh, or a combination of both classical transmitters.

This work presupposes the existence of transmitter substances distinct from the classical transmitters, NA and ACh. Increasing evidence suggests that these so-called non-adrenergic, non-cholinergic (NANC) transmitters, specifically purines and peptides, may be co-released with either or both of the classical transmitters (Burnsock, 1976; 1982; 1986; Höckfelt et al., 1977; 1980a; d; Cuello, 1982).

Before considering the merits of these substances as co-transmitters, it is necessary to examine them as neurotransmitters in their own right, being synthesized, stored and released from distinct NANC neurones.

Evidence that the autonomic nervous system may release substances other than NA and ACh came as early as 1895 when it was shown that the contractile responses of the urinary bladder to parasympathetic (pelvic) nerve stimulation were antagonized only partially by the cholinergic antagonist atropine (Langley & Anderson, 1895). Such observations were not restricted to contractile responses. In both the stomach (Langley, 1898) and small intestine (Bayliss & Starling, 1899), inhibi-

tory components of the vagus were discovered which were facilitated, rather than antagonized, by atropine and therefore could not be attributed to stimulation of cholinergic post-ganglionic fibres. At the time however, this atropine-resistance was 'explained away' within the traditional existing concepts of cholinergic and adrenergic neurotransmission by suggesting it that arose because of the existence of a so-called 'peripheral mechanism' (McSwiney & Wadge, 1928) in which the peripheral tissue itself could determine the response to nerve stimulation, irrespective of which division of the autonomic nervous system was being stimulated. If tone was low the tissue would contract and if high it would relax with either type of stimulation. Only in the intermediate tone range was there a distinctive difference between sympathetic and parasympathetic nerve stimulation. Alternatively, atropine-resistance was attributed to stimulation of inhibitory sympathetic nerves in the vagus (Harrison & McSwiney, 1936) or, in the case of penile erection, to an access problem, because the ACh receptors in the small neuro-effector junction were inaccessible to exogenous antagonists (Dale & Gaddum, 1930).

The first conclusive pharmacological evidence for the existence of NANC fibres came with the advent of adrenergic neurone blocking drugs such as guanethidine. Together with atropine, to abolish the effects of parasympathetic nerve stimulation, this meant that both divisions of the autonomic nervous system could now be blocked. Since these neurone blocking drugs acted on the nerve endings they did not suffer from the difficulties in access attributed to atropine by Dale and Gaddum. Subsequently, the inhibitory responses to vagal stimulation were shown to persist, while those to sympathetic nerve stimulation were blocked, in the presence of the adrenergic neurone blocking agents alone (Burnstock et al., 1966) or in combination with α- and β-adrenoceptor antagonists (Bucknell & Whitney, 1964).

NANC responses were not restricted to inhibitory nerves; they were also involved in contractile responses. In fact, the first tentative suggestions that all neurogenic responses could not be explained within the classical framework of autonomic neurotransmission were made in connection with a contractile event (Henderson & Roepke, 1934, 1935). It was shown that in the dog urinary bladder the contractile response to parasympathetic nerve stimulation was mediated partly by ACh and partly by a non-cholinergic, atropine-resistant mechanism. Since this response was also unaffected by the α-adrenoceptor antagonist phentolamine (Ambache & Zar, 1970) it was proposed that it was mediated by an as yet unknown NANC neurotransmitter. In addition to the urinary bladder, contractile responses resistant to both adrenergic and cholinergic antagonists have been demonstrated in a variety of tissues including the guinea-pig ileum (Ambache & Freeman, 1968; Bywater et al., 1981; Bauer & Kuriyama, 1982a), cat colon (Hulten & Jodal, 1969), chicken oesophagus (Hassan, 1969) and chick rectum (Bartlett & Hassan, 1971).

The mechanism underlying NANC responses was first investigated following the development of intracellular micro-electrode recording techniques. It was the mechanism underlying the inhibitory response which was first to be described. In the early 1960's, inhibitory junction potentials (i.j.p.s), resistant to both adrenergic and cholinergic blocking agents, but sensitive to tetrodotoxin (TTX), were recorded intracellularly from the guinea-pig taenia coli in response to field stimulation (Burnstock et al., 1963; 1964; Bennett et al., 1966a; Kuriyama et al., 1967). These intracellular recording techniques demonstrated, for the first time, fundamental differences between the membrane inhibitory responses produced by NANC and adrenergic nerve stimulation. Thus, in the taenia coli, perivascular sympathetic nerve stimulation with single pulses produced no electrical or mechanical

responses. Trains of pulses at low frequencies (below 5Hz) inhibited spontaneous spike activity and relaxed the muscle without a change in membrane potential. Only at higher stimulation frequencies was the relaxation accompanied by a hyperpolarization; this reached a maximum of some 16mV (Bennett et al., 1966a). In contrast, field stimulation of NANC nerves with a single pulse evoked an i.j.p. which could reach some 25mV and a relaxation (Bennett et al., 1966b). With trains of pulses, i.j.p.s summated to values of up to 30mV. While the maximum inhibitory response to sympathetic nerve stimulation was obtained above 10Hz, that to NANC nerve stimulation reached a maximum below 10Hz (Bennett et al., 1966a, b). Similar responses from so-called NANC nerves have since been demonstrated in a variety of gastro-intestinal smooth muscle preparations including the guinea-pig stomach (Kuriyama et al., 1970), the longitudinal muscle of the guinea-pig pig ileum (Kuriyama et al., 1967; Hidaka & Kuriyama, 1969), the guinea-pig and rabbit colon (Furness, 1969a, b) and also in other areas including the trachea (Coburn & Tomita, 1973), lung (Robinson et al., 1971), blood vessels (Hughes & Vane, 1967), and the accessory muscles of the reproductive system, namely the bovine retractor penis muscle (Klinge & Sjöstrand, 1974; Byrne & Muir, 1985), anococcygeus (Gillespie, 1972; Creed et al., 1975) and rectococcygeus (King & Muir, 1981). There is now convincing evidence that in many of these tissues, including the taenia coli (Bennett et al., 1963; Tomita, 1972) and jejunum (Hidaka & Kuriyama, 1969) the mechanism underlying the i.j.p.s is a selective increase in K^+ conductance.

Intracellular recording techniques have also been used to investigate the mechanisms underlying the contractile NANC responses in a variety of tissues including the urinary bladder (Ursillo, 1961; Creed et al., 1983), chicken rectum (Takewaki et al., 1977), guinea-pig ileum (Bywater et al., 1981; Bauer & Kuriyama, 1982b) and the circular muscle

of the rabbit jejunum (Kitamura, 1978). In these tissues, NANC nerve stimulation evoked excitatory junction potentials (e.j.p.s) which were graded with stimulus strength and summated to fire action potentials. The latency of the e.j.p.s varied considerably, from 5-15ms in the chicken rectum (Takewaki et al., 1977) to 350-900ms in the guinea-pig ileum (Bauer & Kuriyama, 1982a). This may be due to differences in the distance between nerve varicosities and smooth muscle cells, different transmitters, or variations in post-synaptic mechanisms. Here the ionic basis for the NANC-mediated electrical response remains to be fully elucidated. In the guinea-pig ileum, e.j.p.s were enhanced by passive hyperpolarization of the membrane potential and had a reversal potential of -27mV (Bauer & Kuriyama, 1982b). This may be the equilibrium potential for one ion, eg Cl^-, or the net reversal potential for a number of ions eg Na^+ and K^+.

Attempts have been made to obtain structural evidence for NANC nerves using electron microscopic techniques. This appeared to achieve some success following the report of 'large opaque vesicles' (LOV) in many NANC fibres. For example, in the toad lung, a tissue with a reported NANC innervation (Wood & Burnstock, 1967; Campbell, 1971) Robinson and co-workers observed a predominance of LOV in the nerve endings (Robinson et al., 1971). Furthermore, when the adrenergic nerves supplying the toad lung were destroyed with 6-hydroxydopamine (6-OHDA), both the NANC inhibitory response to vagal stimulation and the profiles containing a predominance of LOV remained unchanged (Robinson et al., 1971). Similar profiles have also been reported in the guinea-pig myenteric plexus (Gabella, 1972), avian gizzard (Burnstock, 1972), bovine retractor penis muscle (Eranko et al., 1976) and rat anococcygeus muscle (Gibbins & Haller, 1979), tissues all known to have an NANC innervation. It was thus proposed that the large opaque vesicles were the storage site for the NANC transmitter.

While this proposal stimulated much interest in the ultrastructure of NANC fibres (see Gibbins & Haller, 1979; Gibbins, 1982), it has not been universally accepted. Thus, while large opaque vesicles exist in many tissues which show NANC responses, the incidence of their occurrence was no greater than in cholinergically-innervated tissues which lacked NANC responses. For example, the profile of the NANC nerves in the rabbit anococcygeus muscle, hepatic portal vein and the toad lung were similar to those of the cholinergic nerves in the atria of the rabbit, guinea-pig and toad (Gibbins, 1982). In other words, the type of response could not be related to the presence of a particular vesicle profile in the nerve endings. It is therefore clear that NANC autonomic nerves were not uniquely represented by a population of large opaque vesicles.

Although the absence of a unique profile has proved a disappointment in establishing the existence of NANC transmitters in nerves, it has not diminished attempts to identify the substance(s) involved. Five criteria are generally regarded as necessary for establishing a substance as a neurotransmitter (Eccles, 1964), namely, (a) synthesis and storage in nerve terminals; (b) release during nerve stimulation; (c) post-junctional responses of exogenous transmitter which mimic responses to nerve stimulation; (d) enzymes which inactivate the transmitter and/or an uptake system for the transmitter or its metabolites; (e) drugs which produce parallel blocking or potentiating effects on the responses to both exogenous transmitter and nerve stimulation. With these criteria in mind, two main types of NANC transmitter substances have been proposed; (a) purines; predominantely ATP, which are released form 'purinergic nerves' and (b) peptides; a group including many biologically active polypeptides which are released from so-called 'peptidergic nerves' (for reviews see Burnstock, 1979; 1981; 1986; Hökfelt et al., 1977; 1980a, d).

PURINERGIC NERVES

There is now considerable evidence to support the existence of purinergic nerves. The first suggestion that adenosine 5'-triphosphate (ATP) or a related nucleotide may be involved in chemical transmission came much earlier from work on sensory nerve endings (Holton & Holton, 1953; 1954). These workers proposed that such substances may participate in capillary dilatation. This possibility was further supported by the demonstration of ATP release from the perfused rabbit ear following antidromic stimulation of the sensory nerves (Holton, 1958).

A formidable case for ATP as the neurotransmitter in certain NANC nerves has been put forward by Burnstock (for review see Burnstock, 1979; 1981; 1986). Each of the criteria set out for the establishment of a substance as a neurotransmitter (see above) has been considered;

(i) Presence in nerve fibres

The first step was to establish ATP's presence in nerves, although it was necessary to remember that ATP is present in all cells as an energy source and so its presence in storage vesicles within nerves does not automatically warrant its role as a putative transmitter. Notwithstanding, adenine nucleotides have been detected following nerve stimulation by radiochemical techniques (Su et al., 1971; Su, 1975; Westfall et al., 1978). It has been claimed that tissues such as the taenia coli (Su et al., 1971) and the vas deferens (Westfall et al., 1978) of the guinea-pig and the thoracic aorta, ear artery and portal vein of the rabbit (Su, 1975), each of which produce NANC responses, can accumulate [^{3}H]-adenosine and convert it into [^{3}H]-ATP, which can then be released following electrical stimulation. This implied that the enzyme required for the conversion of adenosine into ATP occurred in the nerves and that the synthesis of ATP could be performed in neuronal tissues. Both the [^{3}H] release and the mechanical responses induced by electrical stimulation of the guinea-pig taenia

coli (Su et al., 1971) vas deferens (Westfall et al., 1978) and rabbit vascular smooth muscles (Su, 1975) were abolished by TTX, suggesting that the tritiated purines were released form nerves.

The presence of ATP in nerves has also been shown microscopically by the use of the quinacrine technique. Quinacrine, it was claimed, bound to adenine nucleotides to produce a fluorescent complex (Irvin & Irvin, 1954) in both cell bodies and varicose fibres in Auerbach's plexus (Olson et al., 1976), urinary bladder (Burnstock et al., 1978c) and anococcygeus muscle (Burnstock et al., 1978a). Furthermore, quinacrine fluorescence in nerves was reduced by depolarization (Alund & Olson, 1979). These findings have been used as support for the view that quinacrine binds to a compound, presumably ATP, released by nerve activity.

(ii) Release by nerve stimulation

The first indication that ATP was released by nerve stimulation came from studies on the perfused stomach of the guinea-pig and toad. Stimulation of the vagus nerve of Auerbach's plexus in these tissues (Burnstock, 1970) produced the ATP breakdown products, adenosine and inosine in the perfusion fluid. Pre-incubation of the guinea-pig taenia coli with [^{3}H]-adenosine released [^{3}H]-ATP in response to nerve stimulation and this release was inhibited by TTX (Su et al., 1971). More recently, the luciferin-luciferase luminescence technique has been used as a sensitive means of assaying ATP (Strehler & Totter, 1952). The principle behind this method is the interaction of ATP with synthetic luciferin and with luciferase extracted from firefly tails, to produce luminescence. Using this technique, detection of ATP following field stimulation has been claimed in the guinea-pig taenia coli and urinary bladder and in the rabbit and rat anococcygeus muscles (Burnstock et al., 1978a, b, c). In each of these situations, the release of ATP was associated with inhibitory responses in the effector.

Because of its widespread occurrence and many functions, the possibility that ATP is not located within the nerve terminal, but arises from sources other than nerves has been considered. The nerve membrane, for example, is itself a potential source of ATP, e.g. during the propagation of an action potential (Burnstock et al., 1978c). However, the amount of nucleotide collected during stimulation of NANC nerves was estimated to be some 1000-fold greater than that released as a direct result of activation of the axon membrane during impulse propagation. The problem of whether the ATP released comes secondarily from muscle rather than nerve terminals may also have been resolved. While a 2- to 6-fold increase in ATP release from the guinea-pig taenia coli and urinary bladder occurred following activation of NANC nerves, no significant increase in ATP release occurred in response to direct muscle stimulation (Burnstock et al., 1978c).

(iii) Mimicry of nerve stimulation

One of the most important criteria in establishing a substance as a neurotransmitter is that the response to exogenous application of the substance should mimic that to NANC nerve stimulation.

Adenine nucleotides, particularly ATP, closely mimic both NANC nerve-mediated inhibitory and excitatory responses (Ambache & Zar, 1970; Burnstock et al., 1970; 1972; Burnstock, 1972; 1979; 1981). In the case of the inhibitory response, the latency and duration of the relaxations produced by ATP are similar to those produced by nerve stimulation. This is particularly well demonstrated in gastrointestinal smooth muscle, including the stomach, colon and ileum of the guinea-pig and rabbit, the rat gastric fundus, duodenum and colon, the mouse duodenum and colon (Burnstock et al., 1970; Okwuasaba et al., 1977; McKenzie et al., 1977; Huizinga & Den Hertog, 1980) and the guniea-pig internal anal sphincter (Crema et al., 1983; Lim & Muir, 1983). Characteristically, a rebound contraction also followed the

inhibitory responses to both ATP and NANC nerve stimulation in these preparations.

Mimicry of NANC nerve-mediated responses by ATP is also observed in vascular smooth muscle. Both nerve stimulation and ATP produced vasodilation in the guinea-pig uterine artery (Bell, 1976), rabbit portal vein (Hughes & Vane, 1967) and bull penile artery (Klinge & Sjöstrand, 1974).

Mimicry may extend not only to the mechanical events themselves but to the underlying electrical mechanisms. The electrical basis of the relaxation produced by both NANC nerve stimulation and ATP is an increase in potassium conductance in the guinea-pig taenia coli (Axelsson & Holmberg, 1969; Tomita & Watanabe, 1973), ileum (Bauer & Kuriyama, 1982b), stomach (Vladimirova & Shuba, 1978) and rabbit caecum (Small, 1974). In those tissues where the electrical response to NANC nerve stimulation is small, e.g. the anococcygeus muscle (Gillespie, 1982) and bovine retractor penis muscle (Byrne & Muir, 1984), that to ATP is also insignificant. These results may not prove that the NANC transmitter and ATP are identical, however, they demonstrate a close similarity between the two.

The ability of ATP to mimic NANC nerve-mediated mechanical excitatory responses has been observed in the guinea-pig urinary bladder (Ambache & Zar, 1970; Muir & Smart, 1983; MacKenzie & Burnstock, 1984), the chicken rectum (Bartlet & Hassan, 1971; Meldrum & Burnstock, 1985) and the guinea-pig ileum (Bauer & Kuriyama, 1982a). The responses to both NANC nerve stimulation and ATP consisted predominantly of a rapid phasic contraction which was not maintained despite continious stimulation. Furthermore, in the guinea-pig urinary bladder (Burnstock et al., 1978c) ATP produced contractions of a similar latency and rates of rise and decline to nerve stimulation.

(iv) Blockade of responses to NANC nerve stimulation and ATP

Antagonists of putative transmitters should also be effective against the response to NANC nerve stimulation. Selective antagonists have however proved difficult to find, a fact which has been a major stumbling block in the universal acceptance of the 'purinergic nerve' hypothesis.

Furthermore, as with many other peripherally acting neurotransmitters, it now seems likely that there is more than one type of purinoceptor (Burnstock, 1978). Based on the rank order of potency of agonists, purinergic receptors have been divided into 2 major types; P_1-purinoceptors, which were more sensitive to adenosine and adenosine 5'-monophosphate (AMP) than to ATP, and P_2-purinoceptors, which were more sensitive to ATP and adenosine 5'-diphosphate (ADP). Furthermore, P_1-receptor occupation was suggested to lead to changes in intracellular cyclic adenosine 5'-monophosphate (cAMP) levels. P_2-receptor occupation did not but in some cases evoked prostaglandin biosynthesis.

Methylxanthines are claimed to be competitive antagonists at the P_1-purinoceptor, while P_2-purinoceptors were antagonized (though non-specifically) by quinidine, 2-substituted imadazolines and 2'2-pyridylisatogen (Burnstock, 1978). The ability of methylxanthines to block post-synaptic P_1-purinoceptors in both vascular and non-vascular smooth muscle is well documented (see Burnstock, 1978). For example, both the vasodilatation in the brain (Oberdörster et al., 1975) and the vasoconstriction in the kidney (Osswald, 1975) produced by adenosine were antagonised by theophylline. Similarly in non-vascular smooth muscle, the relaxation of the trachea (Coleman, 1976) and ileum (Ally & Nakatsu, 1976) produced by adenosine were blocked by aminophylline and theophylline respectively.

However the ability of these compounds to block the responses to adenosine was greater than their ability to oppose those to NANC nerve

stimulation. For example, theophylline antagonised the responses to adenosine, but not to NANC nerve stimulation in the rabbit duodenum (Small & Weston, 1979), thus undermining the use of such compounds in the investigation of the nature of P_1 receptors and as specific antagonists for the NANC transmitter.

Subsequently, biochemical, pharmacolgical and receptor binding studies have led to a proposed sub-division of the P_1-purinoceptor into A_1 and A_2 receptors (Van Calker et al., 1979) or R_i and R_a receptors (Londos et al., 1980). The R_i receptor, occupation of which inhibits adenylate cyclase, appears to be analogous to the A_1 receptor and the R_a receptor, which activates adenylate cyclase, appears to be analogous to the A_2 receptor (see Kennedy & Burnstock, 1984). Furthermore, the R_a or A_2 receptor is more susceptible to 5'-carboxamide analogues of adenosine such as 5'-N-ethylcarboxamide adenosine (NECA) and less responsive to N^6-substituted analogues such as L-N^6-phenylisopropyl adenosine (L-PIA), whereas the reverse is true for R_i or A_1 receptors (Bruns et al., 1980).

Attempts to obtain specific P_2-purinoceptor antagonists have been even less successful. For example, the potential ATP antagonist, 2-2'pyridylisatogen tosylate also blocked ACh and histamine receptors at similar concentrations (Burnstock et al., 1978a). In the taenia coli, this drug reduced the inhibitory effects of ATP, but not those of NANC nerve stimulation (Spedding et al., 1975). Imidazolines, such as phentolamine, in a concentration exceeding that required to block α-adrenoceptors, also abolished the inhibitory response of the taenia coli to ATP, without antagonising that to field stimulation (Ambache et al., 1977a). These results implied a lack of specificity of these compounds for the receptors utilized by both the neuronally-released transmitter(s) and ATP.

Two recently developed substances have made important contribu-

tions to the problem of blockade of ATP-mediated responses. Arylazido aminopropionyl ATP ($ANAPP_3$; Hogaboom et al., 1980) was claimed to be a a specific P_2-purinoceptor antagonist. Indeed, $ANAPP_3$ abolished the contractile responses to ATP and NANC nerve stimulaion in the urinary bladder of the guinea-pig (Westfall et al., 1983) and cat (Thoebald, 1982) and in the guinea-pig vas deferens (Sneddon & Westfall, 1984). However, this antagonist failed to block the relaxant effects of both ATP and nerve stimulation in the guinea-pig stomach fundus strip (Frew & Lundy, 1982) and rabbit anococcygeus (Sneddon et al., 1982). This inability to block inhibitory, but not excitatory responses to ATP was also demonstrated with α,β-methylene ATP (α,βMeATP; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984), a stable analogue of ATP, which acts by desensitizing P_2-purinoceptors (Kasakov & Burnstock, 1983). Clearly there are differences between P_2-purinoceptors mediating relaxation and those producing contraction.

Thus it is becoming apparent that the P_2-purinoceptor also may not form a homogeneous group. Sub-division of the P_2-purinoceptor has been suggested on the basis of the action of apamin, a Ca^{2+}-operated K^+ channel antagonist, which non-specifically blocks some actions of ATP (Shuba & Vladimirova, 1980), or on anatomical locations (Su, 1981). More recently (for review see Burnstock & Kennedy, 1985), P_2 receptors have been subdivided on the basis of the rank order of potency of structural analogues of ATP, Thus: Subtype 1 (P_{2x}), α,βMeATP, β,γ-methyleneATP > ATP = 2-methylthioATP at the P_2-purinoceptor mediating contraction of the vas deferens and urinary bladder of the guinea-pig and rat; Subtype 2 (P_{2y}), 2-methylthioATP >> ATP > α,β-methyleneATP, β,γ-methyleneATP at the P_2-purinoceptor mediating relaxation of the guinea-pig taenia coli and rabbit portal vein. P_{2x}-receptors are anatgonized by $ANAPP_3$ and selectively desensitized by α,βmeATP, whereas P_{2y}-receptors show only weak antagonism with these drugs.

Thus, the actions of adenosine and ATP appear to be mediated via several sub-classes of purinoceptors. However, until selective antagonists of these receptors are developed, one of the main criteria for establishment of a substance as a neurotransmitter remains unfulfilled.

(v) Inactivation

The rapid recovery which follows both the excitatory and inhibitory responses to NANC nerve stimulation suggests the presence of an effective inactivation mechanism for the transmitter. ATP is believed to be de-phosphorylated by ecto-ATPases and a 5'-nucleotidase to adenosine (see Maguire & Satchell, 1979), which is then inactivated by either deamination to inosine, or uptake into smooth muscle or neurones. Form these observations, Burnstock (1972; 1979) proposed the 'purinergic nerve' hypothesis, according to which, ATP is synthesized and stored in nerve terminals. Following its release and the activation of purinergic receptors on the post-synaptic membrane, ATP is rapidly broken down by a magnesium-activated ATPase and a 5'-nucleotidase to adenosine. Adenosine is then taken up into the nerve terminals by a high affinity uptake system, converted into ATP, and reincorporated into physiological stores. Any adenosine not taken up in this way is broken down by adenosine deaminase to inosine, which is pharmacologically inactive, cannot be taken up by nerves, and leaks into the circulation.

Although this hypothesis of purinergic transmission was attractive it was not universally accepted. This was partly because other groups had difficulty in repeating some of the results, partly because some of the antagonists claimed to be selective for ATP were not so, but perhaps most of all because ATP's central role in cell metabolism made it difficult to prove its specific role as a neurotransmitter.

For example, while many of the preparations used by Burnstock et al. (1970) responded to ATP in a similar manner to NANC nerve stimula-

tion, many more including the pig stomach (Ohga & Taneika, 1977), opossum oesophageal sphincter (Daniel et al., 1979) and rat anococcygeus (Gillespie, 1972) did not. Furthermore, it was shown that the guinea-pig ileum (Weston, 1973a), taenia coli (Ambache et al., 1977a), urinary bladder (Ambache & Zar, 1970; Ambache et al., 1977b), rabbit duodenum (Weston, 1973b) and pig stomach (Ohga & Taneika, 1977) were all desensitized to ATP while simultaneously retaining their response to NANC nerve stimulation.

Gillespie and his colleagues have shown that the anococcygeus muscle of cat, rabbit and rat receives, in addition to excitatory nerves, a NANC innervation (Creed & Gillespie, 1977; Creed et al., 1977; Gillespie & McGrath, 1974; Gillespie & McKnight, 1978). Although these workers showed that ATP mimicked the NANC nerve-mediated inhibition of the anococcygeus in the cat and rabbit, they argued that it was unlikely to be the transmitter since it caused a contraction of the rat anococcygeus and the transmitter was likely to be the same in all 3 species. However, it was subsequently demonstrated (Burnstock et al., 1978a) that, in the presence of low concentrations of the prostaglandin synthesis inhibitor indomethacin, the ATP-induced contraction of the rat anococcygeus was converted to a relaxation. Since ATP induces prostaglandin synthesis (see Burnstock 1978a) it was argued that this could explain the anomalous result.

Several authors have raised objections about the low potency of ATP in some preparations supplied by NANC nerves. Work on the rat bladder (Brown et al., 1979) has suggested that rapid breakdown of ATP (which contracts the bladder) to AMP and adenosine (both of which relax the bladder) account for the low potency of exogenously applied ATP (Ambache et al., 1977a, b). This is supported by the finding that β,γ-methyleneATP, which is more resistant to degradation, is about 100-fold more potent than ATP and precisely mimics the NANC nerve-

mediated contractions.

Furthermore, objections have been raised against the proposed ATP antagonists (Burnstock, 1972), many of which, including theophylline and 2,2'-pyridylisatogen, have since been shown to be non-specific (Small & Weston, 1979; Spedding et al., 1975).

Considering all this evidence, much of it conflicting, I am inclined to believe that there is good evidence for ATP as a neurotransmitter in some, but by no means all, NANC nerves. Clearly, however, many gaps in the literature must be filled before the 'purinergic nerve' hypothesis can gain universal acceptance.

PEPTIDERGIC NERVES

The other major group of candidates for NANC neurotransmitters, particularly in the gut, are the neuropeptides. The term 'peptidergic neurone' was first proposed by Bargmann and co-workers in 1967 to describe those neurones in the hypothalamus involved in the secretion of peptide hormones. Since then a total of over 30 peptides, including substance P, neuropeptide Y, somatostatin, vasoactive intestinal polypeptide and bombesin have been proposed as neurotransmitters in both peripheral and central nervous systems (for reviews see Otsuka & Takahashi, 1977; Hökfelt et al., 1980a, d; Iversen 1983b).

Doubts have arisen not only about the need for so many peptide neurotransmitters, given that one excitatory and one inhibitory transmitter should be sufficient to operate the nervous system, but also because classical transmitters have always been small molecules (molecular weight ~ 200) whereas some of the peptides consist of up to 30 or more amino acids (molecular weight ~ 3000). Furthermore, the method of replenishment of peptide neurotransmitters in nerve endings seems to be different from that for the classical transmitters. For example, intraneuronal NA levels are kept constant by enzymatic synthesis in

nerve endings and reuptake from the extraneuronal space. Peptides, on the other hand, are probably produced only on the ribosomes of the cell soma, possibly in the form of a larger precursor molecule, without local synthesis in nerve endings or uptake mechanisms (for review see Höckfelt, 1980a). The amount of peptide released is much smaller than those of classical transmitters, however, receptors may be activated at very low concentrations, a situation which may compensate for an 'inefficient' replacement mechanism of released transmitter (for reviews see Hökfelt et al., 1980a, d; Iversen, 1983a).

Although many peptides have been proposed as neurotransmitters, only a few come anywhere near fulfilling the criteria (see above) required to establish a transmitter function.

(i) Presence in nerves

The presence of peptides in autonomic nerves has been shown by immunohistochemistry, radioimmunoassay and bioassay techniques.

Between 1975 and 1977, important new findings suggested that it was unlikely that all NANC nerves comprised a single population of neurones containing one transmitter. Systematic electron-microscopic studies (Cook & Burnstock, 1976) revealed up to nine morphologically-distinguishable neurones in the enteric plexus, including some nerve profiles containing a complex mixture of vesicles, suggesting that they may contain more than transmitter. Using these techniques to localize biologically-active peptides, autonomic nerves containing enkephalin, substance P, vasoactive intestinal polypeptide (VIP), neurotensin, somatostatin, gastrin releasing peptide (GRP), neuropeptide Y (NPT)/pancreatic polypeptide (PP), bombesin (BN), cholecystokinin (CCK), calcitonin gene-related peptide (CGRP), and most recently galanin (GAL) have been described (Höckfelt et al., 1980a, d; Furness & Costa, 1981; Melander et al., 1985).

Of these, VIP and substance P immunoreactive fibres are particu-

larly abundant in autonomic ganglia and in Auerbachs' plexus. For example, the longitudinal muscle of the taenia coli is innervated by fibres which are immunoreactive to both VIP and substance P (Jessen et al., 1980). Nerve cell bodies containing VIP in the myenteric plexus project in an anal direction to supply the circular muscle coat of the intestinal wall (Furness & Costa, 1980) and may mediate the descending inhibitory pathways of the peristaltic reflex (Hirst & McKirdy, 1974; Costa & Furness, 1976). Furthermore, immunohistochemical, radioimmunoassay and bioassay techniques have each demonstrated the preferential distribution of substance P in spinal dorsal roots, as opposed to ventral roots (Amin et al., 1954; Takahashi et al., 1974; Takahashi & Otsuka, 1975), leading these authors to propose that substance P is an excitatory transmitter in primary sensory neurones.

Studies combining light and electron microscopy with immunohistochemistry have indicated that large granular vesicles are a feature common to many peptidergic neurones (see Cuello, 1978). Such vesicles are now regarded as the storage site for peptide transmitters and form the structural evidence for peptidergic neurones.

(ii) Release by nerve stimulation

The release of VIP and substance P have each been demonstrated in vitro following nerve stimulation in several tissues. VIP was released into the venous outflow when NANC inhibitory nerves supplying the cat stomach were activated either reflexly (by distending the upper oesophagus) or by electrical stimulation (Fahrenkrug et al., 1978b). Release was also observed in other NANC nerves innervating gastrointestinal smooth muscles, including the lower oesophageal sphincter (Goyal & Cobb, 1981), taenia coli (Fahrenkrug et al., 1978a) and small intestine (Fahrenkrug et al., 1978b), and also from enteric vasodilator nerves (Fahrenkrug et al., 1978b). These relults suggested that VIP could be the neurotransmitter released by NANC inhibitory nerves in these tis-

sues.

Substance P, has beem claimed to be released by an action potential-dependent mechanism from intrinsic nerves in the intestine to contract smooth muscle (Franco et al., 1979) and has been proposed as an excitatory neurotransmitter in the intestine (Furness & Costa, 1980). Again, substance P has been detected in the gastric antrum following vagal stimulation (Uvnäs-Wallensten, 1978). Although these observations are in keeping with the view that substance P may have a transmitter function, it should be noted that at least a proportion of the substance P released may be [illegible] (Von Lembeck, 1953; Jessell & Iversen, 1977). stimulation of sensory nerves.

(iii) Mimicry of nerve stimulation

Certain polypeptides, including VIP and substance P mimic the responses to NANC nerve stimulation in some tissues. VIP is a powerful inhibitor of many smooth muscles including those of the alimentary tract (Furness & Costa, 1981), respiratory tract (Kitamura et al., 1980) and the urogenital tract (Sjöstrand et al., 1981).

The ability of close-arterial infusion of VIP to induce relaxation of the stomach (Eklund et al., 1979) and the concomitant release of VIP from the corpus-fundus of the stomach during vagal stimulation (Fahrenkrug et al., 1978a, b) strongly favoured VIP as the transmitter in this preparation. In the guinea-pig taenia coli however, the effects of exogenous VIP showed certain differences from the response to stimulation of NANC nerves. Nerve stimulation produced a response with a short latency which developed rapidly and was poorly maintained, whereas the response to VIP was slow in onset (10s or greater), slow to develop and persistent (Cocks & Burnstock, 1979). It could be argued that this was not sufficient evidence against VIP as a neurotransmitter, since access to receptors is limited for exogenous VIP compared to nerve-released transmitter. However, apamin failed to block the hyper-

polarization to VIP (Hills et al., 1983), but antagonised that to NANC nerve stimulation, suggesting that the ionic basis of the two responses differed.

In cat trachea, low concentrations on VIP (10^{-12}-10^{-10}M) relaxed the smooth muscle with no accompanying electrical change, unlike the response produced by inhibitory nerve stimulation. At higher concentrations (10^{-8}M) however, VIP produced relaxations and accompanying hyperpolarisations (Ito & Takeda, 1982).

There is also evidence that VIP is released from enteric vasodilator nerves and that infusion of VIP mimicked the effects of stimulation of these nerves (Fahrenkrug et al., 1978a, b; Eklund et al., 1979). Furthermore, the relaxations produced by both NANC nerve stimulation and VIP in the cat lower oesophageal sphincter were significantly reduced by VIP-antisera (Biancani et al., 1984), observations consistent with a view of VIP as a neurotransmitter at this site.

Substance P also mimicked NANC nerve-mediated contractions in certain intestinal smooth muscles (Franco et al., 1979). Longitudinal and circular muscles of the ileum, in which field stimulation of NANC nerves evoked e.j.p.s, were also depolarized by substance P. Moreover, the e.j.p.s were abolished during depolarization with substance P. These e[illegible] were not reversed by passive membrane hyperpolarization, suggesting that they and the substance P-evoked depolarizarion shared the same ionic basis (Bauer & Kuriyama, 1982a). Substance P also mimicked the motor response in both the colon and rectum to pelvic nerve stimulation (Andersson et al., 1983) and the rat parotid gland (Gallacher, 1983).

There is evidence therefore, that certain peptides can mimic NANC nerve-mediated responses at some sites. At such sites, they may have a transmitter function, however evidence for this is incomplete.

(iv) Blockade of responses to NANC nerve stimulation and peptides

Little success has been achieved with the development of peptide antagonists. This was probably due to the difficulties in obtaining pure preparations to identify their molecular structure. Apparently, the only means of antagonising the response to peptides is by desensitizating the receptors to the particular peptide under observation, or by using active anti-sera, where available. Blockade of the relaxation to both VIP and nerve stimulation by VIP-anti-serum has been demonstrated in the opossum oesophageal sphincter (Goyal et al., 1980), rabbit internal anal sphincter (Biancani et al., 1983) and cat trachea (Ito & Takeda, 1982). The effects appeared to be specific, at least in the oesophageal sphincter, as the relaxation to isoprenaline was not blocked (Goyal et al., 1980). This encouraging work was slightly overshadowed, however, by the observation by other workers (de Carle & Pye, 1982) that in human oesphageal and gastric muscles VIP-antiserum was ineffective in reducing the responses to NANC nerve stimulation while antagonising those to exogenous VIP.

Desensitization to the inhibitory effects of VIP on the cat trachea was accompanied by a marked reduction in the response to inhibitory nerve stimulation (Ito & Takeda, 1982). These results suggested that the receptors occupied by exogenously added VIP and the neuronally-released NANC transmitter were similar.

Several substances, including AMP, cystine di-β-napthylamide and trimethaphan camphorsulfonate have been reported to antagonize the actions of substance P on the guinea-pig ileum (Stern & Hykovic, 1961). Furthermore, baclofen (β-(4-chlorophenyl)-γ-aminobutyric acid) antagonized the depolarizing action of substance P on rat spinal motoneurones (Otsuka & Konishi, 1976). The depolarizing effects of l-glutamate, another excitatory transmitter candidate (Graham et al., 1967), were also found to be reduced, but to a smaller extent (Otsuka & Konishi,

1976). Since baclofen readily blocked the monosynaptic and polysynaptic reflexes, as well as the dorsal root potentials (Saito et al., 1975; Otsuka & Konishi, 1976), it was proposed by these authors that baclofen blocked primary afferent transmission by antagonizing the transmitter action of substance P.

Clearly the proposed substance P antagonists comprise a mixed group of compounds, none of which appear to be specific. Until such a specific antagonist for substance P is found, this criteria will remain unfulfilled.

Recently, some progress has been made in producing a synthetic analogue of substance P which has been claimed to possess receptor antagonistic properties (Leander et al., 1981). The compound, [D-Pro^2, D-Trp^{7,9}]-substance P has been shown to antagonize the effects of substance P in the guinea-pig taenia coli and urinary bladder, and in the rabbit iris sphincter pupillae muscle (Leander et al., 1981). The antagonistic effect of the compound on the nerve mediated responses however was not convincing. It abolished the NANC mediated contractions in the iris sphincter pupillae muscle, but was ineffective in the guinea-pig urinary bladder (Leander et al., 1981). These results suggested that in the rabbit iris sphincter pupillae muscle, but not the guinea-pig urinary bladder, the neuronally released transmitter and applied substance P shared the same receptors.

(v) Inactivation

The rapid recovery which follows both the excitatory and inhibitory responses to NANC nerve stimulation suggests the presence of an efficient inactivation mechanism for the transmitter(s). In general, this takes the form of either enzymatic breakdown or reuptake into nerves and/or muscle.

There is little evidence for the inactivation of peptides following their release from nerves. It has been suggested (Lee et al.,

1981) that a 'substance P degrading enzyme' exists, which inactivates the peptide by cleaving the phenylamine residues at positions 7 and 8. This enzyme has a high degree of specificity and is therefore a promising candidate for a physiological inactivation mechanism. At present, there is no evidence for a rapid inactivation mechanism for the termination of the VIP response. Consistent with this is the ability to identify the presence of VIP in venous outflow following nerve stimulation.

To date, no uptake mechanism has been reported for peptides (see Hökfelt et al., 1980a). This may be because peptides are probably produced only in the ribosomes of the cell soma, possibly in the form of a large precursor molecule (Gainer et al., 1977) without local synthesis in nerve endings. Hence replacement of released peptides from a nerve ending is probably via axonal transport. The relaxation produced by VIP is persistent (Cocks & Burnstock, 1979) and so compatible with the possibility of destruction by a peptidase with time, as opposed to rapid uptake, a theory which hardly strengthens its claims as a transmitter mediating the rapidly developing and short-lived inhibition of smooth muscle.

Although there is much evidence for the presence of several biologically active peptides in both central and peripheral nerve endings, few, if any, fulfil all the criteria required to establish a transmitter function. The lack of specific antagonists or a rapid inactivating mechanism leaves large gaps in the peptidergic nerve hypothesis. Furthermore, the abundant presence of substance P in sensory neurones also complicates matters.

Of all the peptides examined as neurotransmitters, VIP appears to be the most likely candidate, especially in the gastrointestinal tract. However, the observation that the response to VIP is slow in onset and long lasting does not fit well into the conventional pattern of action

of a neurotransmitter. These observations and VIP's ability to circulate in the local blood supply without immediate destruction would seem indicative of a more regulatory role usually associated with a hormone. It could thus be argued that some of the responses to VIP suggest its role would be better described as part of the neuroendocrine system. The major effect of VIP is on vascular smooth muscle, where it produces a powerful vasodilatory effect, especially in the gut. In the gut, VIP neurones are particularly densely distributed in blood vessels and the epithelium of the mucosa. VIP also causes secretion in the gut (for review see Said, 1980). Considering these effects, VIP could still be a neurotransmitter as these are slower yet supportive to the tissues directly involved, e.g. increasing blood flow to an actively secreting epithelium.

Clearly, there are many examples of non-adrenergic, non-cholinergic putative transmitters in the autonomic nervous system. There is however increasing evidence that, in addition to being present in separate neurones, many of these putative transmitter substances are stored together with the classical transmitters, either in the same, or in separate vesicles, and are co-released following stimulation of both sympathetic and parasympathetic nerves. The term 'co-existence' has been used to describe this symbiotic presence of 2, or more, transmitter substances in a single nerve terminal, the occurrence and possible physiological significance of which will be discussed in the following chapters.

IV. CO-EXISTENCE OF PEPTIDES WITH CLASSICAL TRANSMITTERS

The idea that peptides may be co-released from autonomic nerves is not surprising, if one considers the early observation (Abrahams et al., 1957) of AChE staining and hence presumably ACh in the areas of the hypothalamico-hypophyseal tract associated with release of oxytocin and vasopressin. However, the first clear evidence for the co-existence of biologically active peptides and classical transmitters came, not from neurones, but from endocrine cells (Pearce, 1969). Certain peripheral endocrine cells, particularly those located in the gastrointestinal tract, were shown to contain both a biogenic amine, for example 5HT or histamine and a peptide hormone, such as substance P, somatostatin or neurotensin (Pearce, 1969). These cells were part of what Pearce termed the APUD (Amine content or Precursor Uptake and Decarboxylation) system. Since neurones have the same embryonic origin as endocrine cells, it was proposed that they also might contain both a peptide and an amine (Pearce, 1969). Indeed, a population of guinea-pig sympathetic ganglion cells was found subsequently to be somatostatin-immunoreactive (Hökfelt et al., 1977). Several other cases of classical transmitter-peptide co-existence have been since reported in several tissues (see Table 1). From this table it is clear that co-existence involving peptides occurs in both central and peripheral neurones and involves ACh, gamma-amino butyric acid (GABA) and 5HT as well as each of the three physiologically occurring catecholamines (noradrenaline, adrenaline and dopamine). Furthermore, co-existence does not refer to only 2 transmitter substances; 3 or more have been found in some neurones (Erichsen et al., 1982).

From table 1 it is clear that although the same peptides and classical transmitters can be observed in several systems, different combinations exist. For example, somatostatin may co-exist with NA in

TABLE 1

CLASSICAL TRANSMITTER	PEPTIDE	TISSUE/REGION (SPECIES)	REFERENCE
DA	Enkephalin	Carotid body (cat)	Lundberg et al., 1979
	CCK	Ventral tegmental area (rat, man)	Hökfelt et al., 1980
NA	Somatostatin	Sympathetic ganglia (guinea pig)	Hökfelt et al., 1977
		SIF cells (cat)	Lundberg et al., 1980
	Enkephalin	Superior cervical ganglion (rat)	Schultzberg et al., 1979
		Adrenal medulla (several)	Schultzberg et al., 1978
	NPY, VIP & dynorphin	Uterine artery (guinea pig)	Morris et al., 1985
5HT	Substance P	Medulla oblongata (rat)	Chan-Palay et al., 1978
	TRH	Medulla oblongata (rat)	Johansson et al., 1981
	Enkephalin	Medulla oblongata (cat & rat)	Glazer et al., 1981
ACh	VIP	Autonomic ganglia (cat)	Lundberg, 1981
	Enkephalin	Cochlear nerve (guinea pig)	Altschuler et al., 1983
	Substance P & enkephalin	Ciliary ganglion (avian)	Erichsen et al., 1982
GABA	Somatostatin	Thalamus (cat)	Oertel et al., 1983
	Motilin	Cerebellum (rat)	Chan-Palay et al., 1981
ADR	Enkephalin	Adrenal medulla (various)	Schultzberg et al., 1978
	NPY	Adrenal medulla (cat)	Lundberg et al., 1986

guinea-pig sympathetic post-ganglionic neurones (Hökfelt et al., 1977), with GABA in the cat thalamus (Oertel et al., 1983) and with ACh in the toad heart (Campbell et al., 1982). Alternatively, a particular classical transmitter is not necessarily always stored with the same peptide. For example, NA may co-exist with somatostatin in the guinea-pig sympathetic ganglia (Hökfelt et al., 1977), with enkephalin in the rat superior cervical gangloin (Schultzberg et al., 1979) and with neuropeptide Y, VIP and dynorphin in the guinea-pig uterine artery (Morris et al., 1985). As far as can be ascertained, there seems to be no restriction on the possible combinations of classical transmitters and peptides that may co-exist.

Evidence that a neurone may contain more than one neurotransmitter has inevitably led to investigations concerning the packaging of the substances involved. In many tissues, the NANC transmitter substance appears to be stored in vesicles, separate from those holding the classical transmitter within the same nerve ending (Cook & Burnstock, 1976). This separate storage system for co-existing transmitters would appear to allow differential release of the co-transmitters at, for example, different impulse frequencies.

Much of the evidence to date for co-transmission involving peptides and classical transmitters has come from immunohistochemical techniques in which positive staining for a given peptide has been demonstrated in an autonomic neurone. The fact that a substance exists in a nerve however, does not necessarily imply that it is a neurotransmitter; post-synaptic effects must also be demonstrated.

Such a functional role for peptides has been perhaps best demonstrated in the cat submandibular salivary gland (Lundberg & Hökfelt, 1983). Here, parasympathetic nerves innervate both the blood vessels and the exocrine acinar elements (Garrett, 1974) and, upon stimulation, produce salivary secretion and an increased local blood

flow. Immunohistochemical analysis (Lundberg, 1981) strongly suggested that these parasympathetic nerves contained a VIP-like peptide, in addition to ACh. Confirmation of these results came from experiments which showed that electrical stimulation increased the overflow of both ACh and VIP into the venous effluent of the gland (Lundberg et al., 1982). Since both the secretion and the vasodilatation produced at low frequencies (2Hz) of nerve stimulation were potentiated by eserine and abolished by atropine, it appeared that ACh was a prerequisite not only for salivation but also for the increased blood flow observed at these frequencies. At higher (> 10Hz) frequencies, however, the vasodilatation was atropine-resistant. This effect was attributed to VIP, a well known vasodilatator (Lundberg et al., 1982) which, although it did not cause salivation alone, potentiated the ACh-induced salivary secretion, by increasing blood flow.

One advantage of such a mechanism could be that the co-transmitter is released in some demanding situations to enhance the action of the principal transmitter. This may occur in several ways:- by post-junctional enhancement of transmitter action, by pre-junctional enhancement of transmitter release or by a separate synergistic action on blood vessels which provides for the increased metabolic needs of the tissue (Burnstock, 1985). When the emergency is over, reduction of stimulus frequency by central control centres would reduce co-transmitter release.

Another peptide for which a possible physiological function has been proposed is neuropeptide Y (NPY). This peptide was first isolated from brain tissue in 1982 (Tatemoto et al., 1982) and has since been identified, using immunohistochemical techniques, in both the adrenal medulla (Lundberg et al., 1986) and in a variety of sympathetic neurones (Uddman et al., 1985; Morris et al., 1985). NPY is a potent vasoconstrictor in cat pial arteries (Edvinson et al., 1984), cat

salivary gland arteries (Lundberg & Tatemoto, 1982), guinea-pig uterine arteries (Morris et al., 1985) and rat tail arteries (Neild, 1987). At low concentrations, NPY potentiates the contractile effects of each artery to exogenous NA and perivascular nerve stimulation. These observations have led to the suggestion that NPY may participate in the physiological control of artery diameter (Neild, 1987), and thus in vascular tone.

Co-transmission may also have a functional role in the central nervous system. For example, a group of dopaminergic neurones in the nucleus accumbens and tuberculum olfactorium in rat and man are believed to co-release CCK (Hökfelt et al., 1980c, d). These mesolimbic dopaminergic systems have been associated with higher mental functions and, according to the so-called 'dopamine hypothesis', disturbances of this system may represent one component in the pathogenesis of schizophrenia (Pearce et al., 1977). If CCK is co-released with dopamine, the peptide could also be involved in the aetiology and symptomatology of schizophrenia. In fact, CCK has been shown to inhibit dopamine release in these regions of the brain (Hökfelt et al., 1980b, c) and thus may act in vivo to regulate dopamine release. An imbalance between peptide and amine may exist in schizophrenia, whereupon a loss or decrease in peptide would lead to an overactive dopaminergic system (Matthyasse & Kety, 1975).

Much of the evidence for ATP as a co-transmitter (see next section) has come from experiments using electrophysiological techniques. Such evidence is sparse for peptides, though a few examples do exist. In bullfrog sympathetic ganglia, ACh is the neurotransmitter contained in preganglionic fibres (Jan & Jan, 1983). Three types of synaptic responses are mediated by ACh; (a) a fast excitatory post-synaptic potential (EPSP), (b) a slow EPSP and (c) a slow inhibitory post-synaptic potential (IPSP). In addition, a fourth synaptic potential

has been identified (Nishi & Koketsu, 1968). This response, termed the late slow EPSP, lasts for several seconds, and is not mediated by ACh. New evidence now suggests that this late slow EPSP is mediated by a peptide transmitter which resembles mammalian lutenizing hormone-releasing hormone (LHRH) (Jan & Jan, 1983). LHRH has been identified in sympathetic ganglia using immunohistochemical techniques (Jan et al., 1979) and is released into the extracellular medium following pre-ganglionic stimulation (Jan et al., 1979). The most convincing evidence so far has come from electrophysiological experiments, in which the late slow EPSP was mimicked by local application of LHRH to the surface of the sympathetic neurone, via a brief pressure pulse (Jan & Jan, 1982; Jan et al., 1980; Katayama & Nishi, 1982). The LHRH-induced depolarization resembled the late slow EPSP in several ways; (a) both were associated with similar conductance changes, (b) their amplitudes varied in parallel as the membrane potential was shifted over a wide range, suggesting similar ionic mechanisms were involved, (c) both responses increased the excitibility of the neurone, (d) both responses were blocked by LHRH antagonists. Together this evidence suggests that LHRH is co-released with ACh from nerve terminals in the bullfrog sympathetic ganglia and interacts with post-synaptic receptors to produce a late slow EPSP.

A mammalian counterpart for the late slow EPSP observed in amphibian ganglia was first reported in the guinea-pig inferior mesenteric ganglia (Neild, 1978). Pharmacological analysis of the response has led to the suggestion that it is mediated by substance P (Dun & Karczmar, 1979); when applied locally, substance P produced a depolarization similar to that elicited by repetitive hypogastric nerve stimulation. Furthermore, following desensitization by continious application of substance P, pre-synaptic stimulation failed to elicit the late slow EPSP.

Clearly there are several examples of the co-existence of peptides and classical transmitters in a variety of neurones, both peripherally and centrally. Much of the evidence to date comes solely from localization of the peptide in neurones using immunohistochemical techniques, but the few examples where a physiological function is apparent gives credibility to the idea of peptides as co-transmitters.

V. CO-EXISTENCE OF ATP WITH CLASSICAL TRANSMITTERS

As with the peptides, the first evidence that purines co-existed with classical transmitters did not come from neurones. Relatively large amounts of ATP were demonstrated, first in adrenal medullary granules (Hillarp et al., 1955), and a few years later, in nerve granules (Schümann, 1958). Following the observation that ATP facilitated catecholamine uptake into both adrenal medullary granules (Kirshner, 1962; Carlsson et al., 1962; 1963) and adrenergic nerve terminals (Euler & Lishajko, 1963; Stjärne, 1964), it was suggested by these workers that the nucleotide acted in vivo as an energy source, for the re-uptake of catecholamines following stimulation-induced release.

The first evidence that ATP may, in addition to being an energy source, act together with other substances to augment their effects came again from work on the adrenal chromaffin cells (Douglas & Poisner, 1966; Douglas, 1968; Stevens et al., 1972). Evidence was presented that the cat adrenal gland could accumulate and phosphorylate [^{3}H]-adenosine, and that stimulation of the gland with carbachol resulted in the appearance of [^{3}H]-ATP in the perfusate solution. This ATP, it was proposed (Van Dyke et al., 1977) acted locally within the medulla as a 'co-agonist' along with the simultaneously released catecholamines, acting either directly on receptors, or indirectly via transformation into cyclic nucleotides.

Extension of this ancillary role of the nucleotide to neuronal function was first proposed by Su following the demonstration that stimulation of periarterial adrenergic nerves in the guinea-pig taenia coli released tritium from tissues pre-incubated with [^{3}H]-adenosine (Su et al,. 1971) . This effect was abolished by the adrenergic neurone blocking drug guanethidine, indicating release of the purine from adrenergic, rather than purinergic nerves.

Support from investigations in other tissues soon followed. ATP, it was claimed, was co-released with NA from the sympathetic nerves supplying the cat nictitating membrane (Langer & Pinto, 1976), guinea-pig vas deferens (Westfall et al., 1978; Fedan et al., 1981), rabbit aorta and portal vein (Su, 1975; 1978), rabbit ear artery (Head et al., 1977), and dog basilar artery (Muramatsu et al., 1981). Having established that ATP could be released following stimulation of many sympathetic nerves, it was necessary to demonstrate a functional role for the nucleotide before it could be accepted as a neurotransmitter.

It is perhaps in the rodent vas deferens that the functional significance of the co-existence of purines and classical transmitters is most fully understood and where a functional role for each transmitter is apparent. The biphasic nature of the contractile response of the rodent vas deferens to field stimulation and the inability of α-adrenoceptor antagonists to abolish the contraction, led to the suggestion that nerve stimulation may release two transmitter substances (Swedin, 1971; Ambache & Zar, 1971). In the vas deferens, field stimulation produced a biphasic contractile response, comprising an initial rapid twitch, followed by a slower, tonic contraction which lasts throughout the stimulation period (McGrath, 1978). The initial rapid twitch contraction appeared to be mediated by ATP. It was mimicked by exogenous ATP and blocked by the P_2-purinoceptor antagonist arylazido aminopropionyl ATP ($ANAPP_3$) (Fedan et al., 1981; Sneddon & Westfall, 1984) and by desensitization of the P_2-purinoceptor by α,β-methylene ATP (α,βMeATP) (Meldrum & Burnstock, 1983). The twitch phase was unaffected by α-adrenoceptor antagonists, or by NA depletion with reserpine. On the other hand, the slower phase of the contraction was mimicked by NA, and blocked by α-adrenoceptor antagonists (Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984). Both phases were blocked by guanethidine or chemical sympathectomy using 6-OHDA, indicating that

both transmitters were released from adrenergic nerves, rather than separate adrenergic and purinergic nerves (Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984).

Co-existence of ATP with NA has not been restricted to non-vascular smooth muscle. ATP release has been demonstrated from sympathetic nerves innervating, for example, the rabbit ear artery (Head et al., 1977), aorta and portal vein (Su, 1978) and dog basilar artery (Muramatsu et al., 1981).

Evidence for ATP as a co-transmitter has also been demonstrated in nonadrenergic nerves. The nucleotide is stored together with ACh in the synaptic terminals of nerves supplying the electric organ of torpedo rays (Bohan et al., 1973); ATP is released together with ACh from phrenic nerves in the rat diaphragm (Silinsky & Hubbard, 1973; Silinsky, 1975) and is a strong contender for the NANC transmitter in the guinea-pig urinary bladder (MacKenzie et al., 1982).

VI. AIMS OF THESIS

Notwithstanding the widespread occurrence and many functions of adenine nucleotides, their biochemical and physiological role has, until recently, been restricted to that of a modulator of metabolism and in the storage of neurotransmitters (e.g. noradrenaline).

More recently, evidence from studies of the autonomic nervous system makes it clear that adenine nucleotides, particularly ATP, may have a more direct role to play in neurotransmission, as transmitters in their own right. Confirmation of this view would have far reaching consequences for medical science in two important directions:-

(i) It would necessitate a revision of the classical view of transmission in the autonomic nervous system, from one comprising two antagonistic, sympathetic and parasympathetic components, each releasing but one transmitter, to a more complex view which recognises, in addition, non-adrenergic, non-cholinergic transmitters and co-transmitters in effector control.

(ii) Significance for medicine. Just as the modification of noradrenergic and cholinergic transmitter activity has provided a most useful source of clinically-active drugs, the application of a similar approach to the manipulation of co-transmitter control, assuming the availability of selective antagonists, would result in the development of a new range of potentially clinically-useful drugs.

In these considerations, establishment of the mechanism by which ATP acts is of paramount importance. This thesis is an attempt to contribute in this respect. In particular it sought, by the use of intracellular micro-electrode recording techniques, to analyse the effects of co-transmitters and to correlate their electrical and the mechanical responses to nerve stimulation in a variety of tissues. This approach has been particularly successful in other situations involving NANC

inhibitory nerves (e.g. in the taenia coli, see Bennett et al., 1966a, b) in elucidating transmitter mechanisms. Indeed, evidence for its successful application to the problem of co-transmission already exists in both vascular (Cheung, 1982; 1984; Suzuki et al., 1984; Suzuki & Kou, 1983) and non-vascular (Sneddon & Westfall, 1984; Burnstock & Sneddon, 1984) smooth muscle.

The work described in this thesis was intended to broaden the application of micro-electrode techniques to co-transmission in a variety of vascular and non-vascular tissues, including the mouse vas deferens, rat anococcygeus muscle, rat tail and mesenteric arteries, rabbit ear and mesenteric arteries.

The thesis also aimed to examine one potential clinical role for co-transmission - i.e. the proposed role of ATP released as a co-transmitter in hypertension (see Vidal et al., 1986). Such a role, were it to be proven, would contribute not only to our understanding of the underlying mechanism of the disease but also to its treatment.

CHAPTER 2: MATERIALS AND METHODS

In this investigation, several smooth muscle preparations, both vascular and non-vascular, were examined.

All tissues were removed as quickly as possible and transferred to a petri dish containing oxygenated Krebs solution. Connective tissue was then removed with the aid of a dissecting microscope and tissues prepared, as described below, for either mechanical, intracellular electrical or radio-chemical recording. All tissues were left to equilibrate for at least 30min before starting each experiment and all antagonists were left in contact with the tissues for at least 20min before their effects were investigated.

I. DISSECTION OF TISSUES

A. MOUSE VAS DEFERENS

Porton strain male mice (30-40g) were killed by a blow to the head and subsequent exsanguination. The abdominal cavity was opened by a midline incision and the testicles pushed out of the scrotum. Holding the epididymus with forceps, the connection with the vas was severed and a thread tied round this end of the tissue. It was then possible to dissect the preparation free of connective tissue and fat, before severing the organ at the prostatic end.

In tissues in which hypogastric nerve stimulation was to be employed, a large area of surrounding connective tissue was dissected out along with the vas. The preparation was then transferred to a perti dish and the extrinsic (hypogastric) nerves identified and ligated under microscopic control.

B. RAT ANOCOCCYGEUS MUSCLE

Adult male albino Wistar strain rats (200-300g) were killed by a blow to the head and subsequent exsanguination. Tissues were dissec-

ted out according to the method of Gillespie, (1972); the abdomen was opened along the midline and the intestines removed to one side. The bladder, urethra, vasa deferentia, seminal vesicles and testicles were removed, exposing the descending colon to the point where it passed into the pelvic cavity. The pubic symphysis was split taking care to avoid damaging the ventral bar of the anococcygeus muscle which lies over the colon in this area. The two cut ends of the pubic arch were forced apart exposing the contents of the pubic cavity. The anococcygeus muscles were then seen emerging from under the colon and joining together on it's ventral surface. When the colon was cut at the level of the pubic brim and the pelvic portion pulled ventrally, the full length of the anococcygeus muscles became visible. The connective tissue surrounding the muscles was removed and a thread attached to the tendons at the rostral end. The tendons were then severed and tissues removed by dissecting the anococcygeus away from the colon and cutting through the ventral bar.

In some experiments, the extrinsic nerves innervating the anococcygeus (the genito-femoral and perineal branches of the pudendal nerves) were dissected out and stimulated according to the method of McKirdy & Muir, (1978); the anococcygeus muscles, together with a large area of surrounding connective tissue, were removed and transferred to a petri dish. With the aid of a dissecting microscope, the sheath of connective tissue surrounding the anococcygeus was carefully split and pinned out to reveal the nerve branches innervating the tissue. The genito-femoral and perineal nerves were then each identified and ligated, ready for stimulation.

C. RAT MESENTERIC BED PREPARATION

Age-matched (12-18 weeks) male albino Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats (200-250g) were killed by a blow

to the head and subsequent exsanguination. Tissues were dissected out according to the method of McGregor, (1965); the abdomen was opened and the pancreatico-duodenal, ileo-colic and colic branches of the superior mesenteric artery were tied off. The dorsal aorta was then ligated and cut a few mm posteriorly from it's junction with the superior mesenteric artery. The latter was then isolated by cutting around the intestinal borders of the mesentery. The whole preparation was then removed to a petri dish, cannulated at the junction with the aorta and transferred to a horizontal organ bath.

D. RAT TAIL ARTERY

Rats (SHR and WKY) were killed as previously described. The tail was severed from the body, the cornified epithelium removed and a 3-4 cm length of artery dissected out from the proximal end of the tail (Holman & Surprenant, 1980). Arteries were then transferred to a petri dish, cleared of connective tissue and cannulated with the tip of the cannula pointing away from the body.

E. RABBIT MESENTERIC ARTERY

Male New Zealand rabbits (2-2.5kg) were killed with an overdose of CO_2. Mesenteric bed preparations were dissected out in a similar way to that described for the rat (McGregor, 1965). The abdomen was opened by a midline incision. The superior mesenteric artery was then ligated and severed at it's junction with the aorta. The whole mesenteric bed preparation, comprising the superior mesenteric artery and attached branches, was then removed by cutting around the intestinal border and transferred to a horizontal organ bath. The mesenteric bed was then perfused with Krebs solution by means of a cannula inserted into the superior mesenteric artery. In experiments in which the responses of the superior mesenteric artery alone were investigated, all branches of the artery comprising the mesenteric bed were tied off

then cut. The superior mesenteric artery was then either cannulated to measure mechanical activity, or left uncannulated in experiments where intracellular electrical activity was investigated.

F. RABBIT EAR ARTERY

Rabbits were killed as previously described and the central ear artery dissected out using a method similar to that first described by De la Lande & Rand, (1965). The ears were removed and stripped of their skin and attached fur. The central ear artery was identified at the base of the ear and a ligature placed around it, ready for cannulation. The nerve and vein which run parallel to the artery were separated off. The artery was then cannulated and a 3-4cm long section dissected free from the cartilagenous base of the ear and transferred to a horizontal organ bath.

II. APPARATUS AND TECHNIQUES

A. CONTRACTILE ACTIVITY IN THE MOUSE VAS DEFERENS

To measure contractile responses, both vasa were removed and each mounted in a heated (36 ± 0.5°C) organ bath (5ml) and bubbled with 95% O_2, 5% CO_2 in physiological Krebs solution. The tubing carrying the Krebs and the organ baths were surrounded by a jacket containing water at 42 ± 0.5°C, pumped by a modified Tempette (TE7) pump to maintain the temperature constant at the desired level.

The vas deferens was passed through a pair of chlorided Ag/AgCl ring electrodes (O.D. 2mm). The prostatic end of the muscle was fixed and the epidydimal end attached, via a thread, to a force displacement transducer (Grass FT03C) for monitoring tension. Tissues were stimulated using a Devices isolated stimulator (0.5ms, supramaximal voltage, 5-20Hz) and contractile activity displayed on a Linseis potentiometric

recorder. Contractile responses were recorded also in response to exogenous agonists; noradrenaline (NA) and adenosine-5'-triphosphate (ATP). These drugs were added to the bath in volumes of 0.1-0.3ml from graduated syringes. Mixing occurred rapidly due to the bubbling gas mixture. Agonists were kept in contact with the tissue for 30s. The baths were emptied from below and filled from above by gravity. Drugs were washed out by emptying and filling the bath 3 times and 5min were allowed between addition of each drug. Where one drug was required to be present throughout the experiment, it was added to the reservoir to give the final concentration required.

B. PRESSURE RECORDING IN ARTERIES

Following dissection and cannulation, rat tail artery and mesenteric bed preparations and rabbit ear artery and mesenteric bed preparations were each quickly transferred to a horizontal organ bath (4ml for tail and ear arteries, 10ml for mesenteric bed preparations). Tissues were perfused using a Watson-Marlow pump at a rate of $4mlmin^{-1}$ with oxygenated Krebs solution at 36 ± 0.5°C. Emptying of the bath, via 2 outlets, was carried out by suction. The polythene tubing containing the Krebs solution (O.D. 2mm) was surrounded by an outer tube (O.D. 10mm) containing liquid paraffin at 40 ± 0.5°C, pumped by a modified Tempette (TE7) pump to maintain the temperature constant at the desired level.

Changes in perfusion pressure were measured on a Bell and Howell (4-327-L221) pressure transducer and recorded on a Linseis potentiometric recorder.

Pressure changes were recorded in response to field stimulation (0.5ms, supramaximal voltage, 1-50Hz) using chlorided Ag/AgCl ring electrodes (O.D. 2mm) and bolus injections (0.1-0.3ml) of exogenous agonists, injected through pressure tubing proximal to the cannula.

Antagonists were added to the reservoir to give the final concentration required and were allowed to perfuse the tissues for at least 20min before their effects were investigated.

C. INTRACELLULAR ELECTRICAL (AND SIMULTANEOUS MECHANICAL) RECORDING

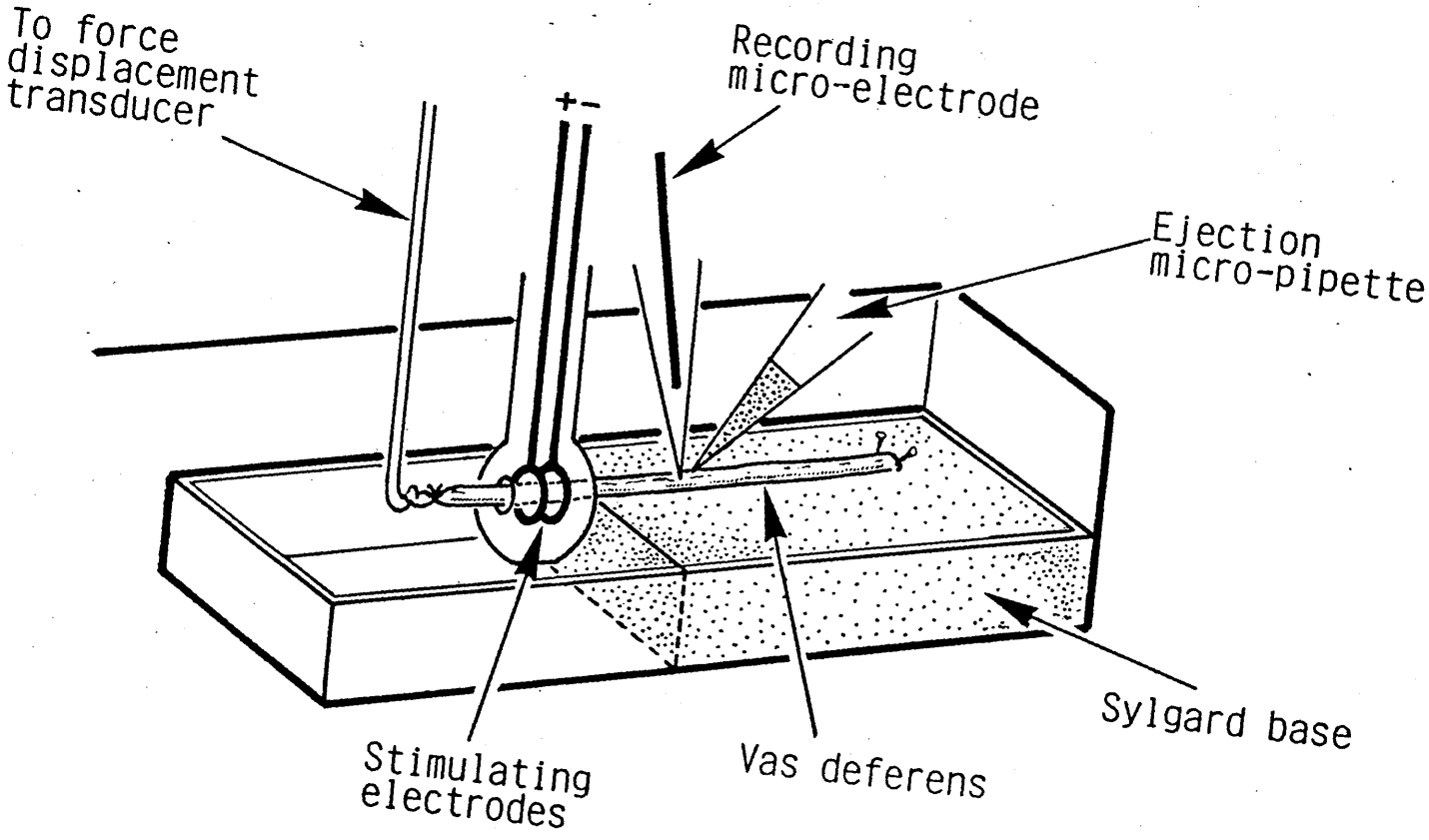
The electrical, and in some cases, simultaneous mechanical responses of the mouse vas deferens, rat anococcygeus muscle, rabbit mesenteric artery and rabbit ear artery to nerve stimulation and to drugs were investigated using conventional microelectrode recording techniques in vitro.

The apparatus (Fig. 1) consisted of an organ bath (5cm x 1cm x 1cm) cut from a perspex block (5cm x 11cm x 2cm), Ag/AgCl ring electrodes (O.D. 2mm), capillary glass micro-electrodes, a Ag/AgCl plated indifferent electrode, D.C. preamplifier, dual beam oscilloscope, voltmeter, transducer, isolated stimulator and gated pulse generator, U.V. oscillograph and tape recorder.

In order to minimise the mechanical vibrations generated in and around the laboratory, the organ bath was bolted to two non-conduction bakelite pillars which were fastened to a steel plate (200kg) on a table mounted on Mufflite (K-150) anti-vibration dampers. The bath was continually perfused ($6 ml min^{-1}$), via 2 inlets, with Krebs solution at 36 ± 0.5°C. Emptying of the bath, via 2 outlets, was carried out by suction. The polythene tubing (O.D. 2mm) containing the Krebs solution was surrounded by an outer tube (O.D. 10mm) containing liquid paraffin at 40 ± 0.5°C, pumped by a modified Tempette (TE7) pump to maintain the temperature constant at the desired level.

To record simultaneous electrical and mechanical activity in the mouse vas deferens and rat anococcygeus muscle, one end of the tissue was attached, via a thread, to an isometric force displacement transducer (Grass FT03C) and the other end, passed through bipolar Ag/AgCl

Fig. 1: Organ bath for combined intracellular electrical and mechanical recordings. The bath comprised a central trough (5cm x 1cm x 1cm) set in a perspex block (5cm x 11cm x 2cm). The block was drilled to accept stainless steel inlet tubes (diameter 2mm) for Krebs solution and outlets for drainage (not shown). One end of the muscle was pinned onto the Sylgard base of the trough and intracellular recordings made from the pinned area. The free end of each tissue was passed through a set of Ag/AgCl ring electrodes (O.D. 2mm) and attached, via a thread, to a force displacement transducer. Membrane potential changes were recorded using conventional glass microelectrodes in response to field stimulation and exogenous agonists, applied locally from a micropipette linked to a pressure-controlled ejection device (Picospritzer II). The bath was perfused with oxygenated pre-heated Krebs solution (36 ± 0.5°C) via 2 inlets by gravity flow. The polythene tubing (diameter 2mm) containing the Krebs solution was surrounded by an outer tube (diameter 10mm) containing liquid paraffin (at 40 ± 0.5°C) pumped from a thermostatically-controlled Tempette pump.


To force
displacement
transducer
+−
Recording
micro-electrode
Ejection
micro-pipette
Stimulating
electrodes
Vas deferens
Sylgard base

ring electrodes (O.D. 2mm and 2mm apart, mounted in Araldite) and pinned to the Sylgard (Dow Corning) base of the bath. Field stimulation was carried out by means of an isolated stimulator (Bell digital stimulator, Mk 3, 0.01-0.5ms, supramaximal voltage).

Intracellular electrical recordings were made with capillary glass micro-electrodes (Clark, GC 150-10; 20-40MΩ) filled with 3M KCl. The micro-electrode was connected to a unity gain high impedance ($10^{10}\Omega$) D.C. preamplifier (W.P.I. M4A) via a Ag\AgCl half-cell attached to a probe, matched and calibrated for the amplifier used. The indifferent Ag/AgCl plated electrode was fixed to the wall of the bath and held in the bath solution. Electrical signals, passed via the preamplifier, were displayed on one channel of a storage oscilloscope (Tektronix 5103N) and monitored on a digital voltmeter (Fairchild M53). The electrical and mechanical signals were recorded permanently on an instrumentation tape recorder (Racal Store 4DE, band width 313-40kHz) and U.V. oscillograph (EMI SE3006).

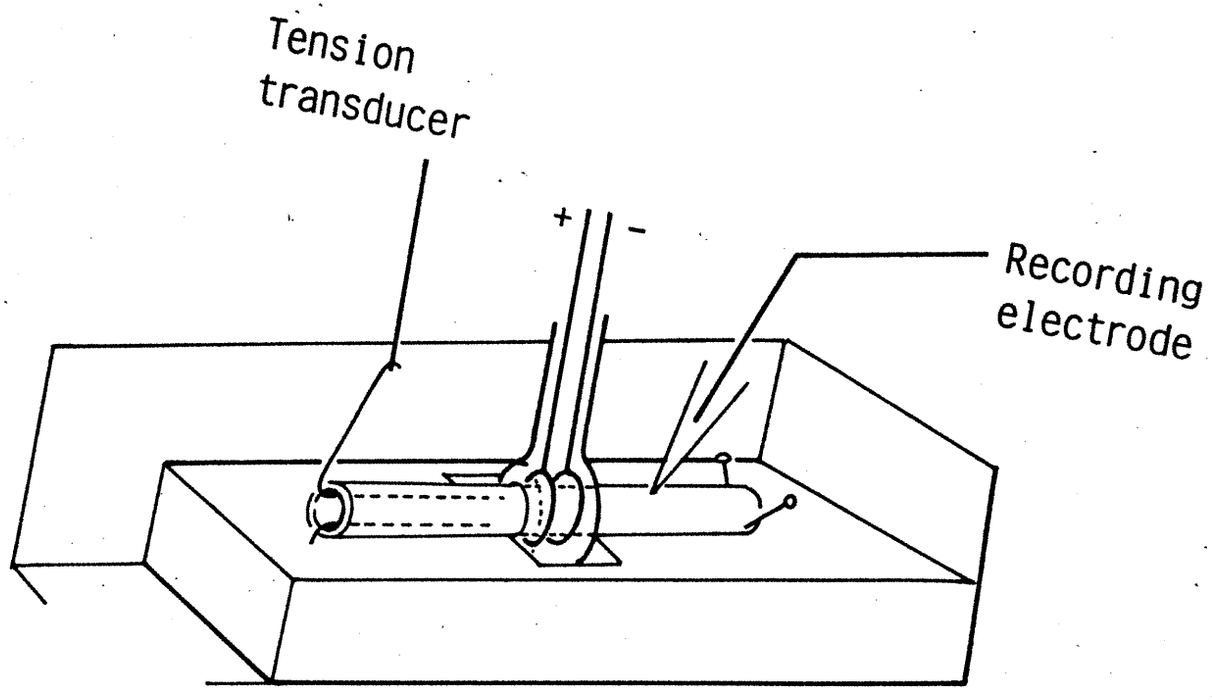
A slightly modified system was used to record simultaneous electrical and mechanical activity in the rabbit superior mesenteric artery (Fig. 2). Two wires were inserted into the lumen of the artery. One was fixed to the Sylgard base of the horizontal organ bath and the other was attached, via a thread, to a force displacement transducer (Grass FT03C). An initial tension of 1g was applied and tissues left to equilibrate for 30min. Intracellular electrical activity was then recorded in the conventional way.

In the rabbit ear artery, electrical activity was recorded alone, the tissues being pinned out firmly to the base of the bath.

D. ELECTRICAL ACTIVITY IN RESPONSE TO LOCALLY APPLIED AGONISTS

In both the mouse vas deferens and rat anococcygeus muscles, membrane potential changes were recorded also in response to exogenous

Fig. 2: A slightly modified arrangement was used to record simultaneous intracellular electrical and mechanical activity in the rabbit superior mesenteric artery. Tissues were pinned out on the Sylgard base of a horizontal organ bath. Stimulation and intracellular electrical recordings were carried out as previously described. To record mechanical activity 2 wires were inserted into the lumen of the artery. One was fixed to the base of the bath, while the other was attached, via a thread, to a force displacement transducer.


Tension
transducer
+
-
Recording
electrode

agonists, applied locally from a pressure-controlled ejection device (Picospritzer II, General Valve Corp. N.J., U.S.A. See fig. 1).

Tissues were dissected out as previously described and pinned out on the Sylgard base of a horizontal organ bath. Cells were impaled using conventional glass micro-electrodes. The drugs, dissolved in Krebs solution (containing, in the case of NA, ascorbic acid, 6 x 10^{-3}M, to prevent oxidation) were applied from ordinary micro-pipettes which had their tips broken back under microscopic control to 1-2μm.

Four factors controlled the amount of drug reaching the recording electrode from the Picospritzer;

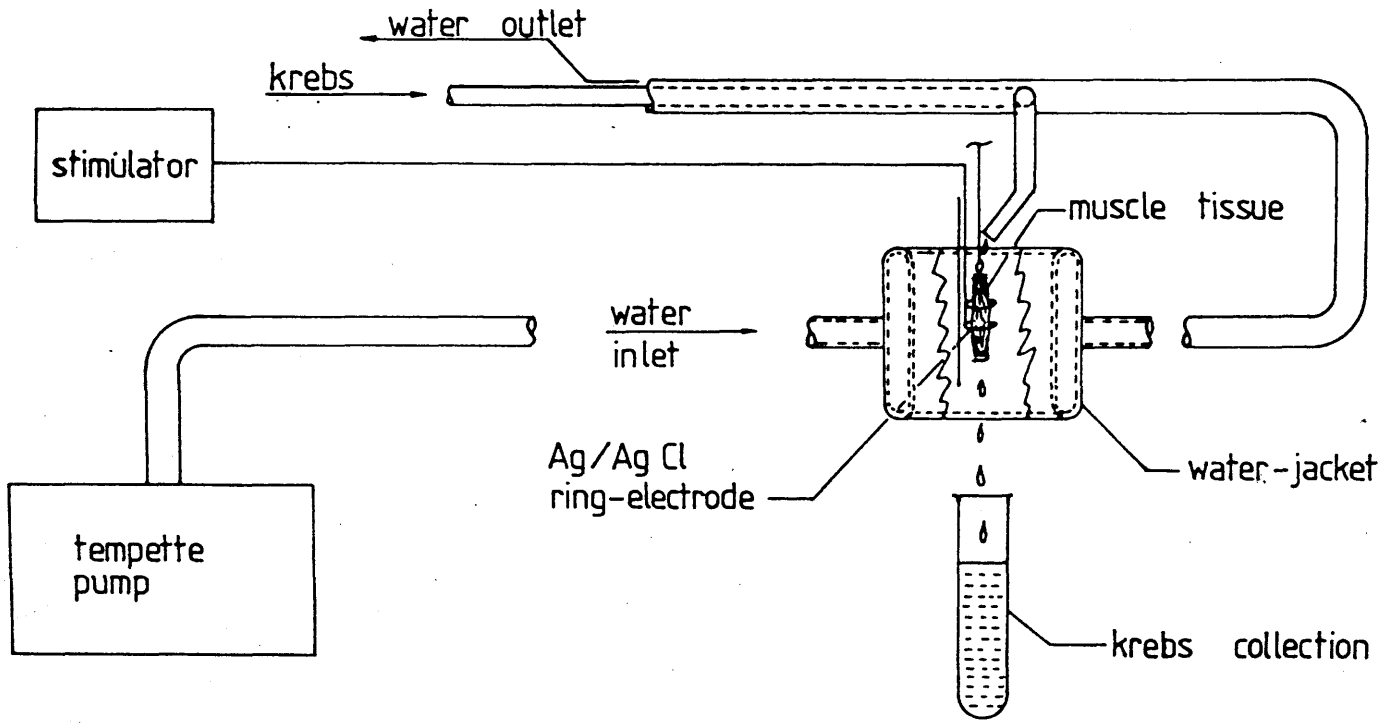
1. The diameter of the pipette tip; to ensure uniformity, the tip was broken back under microscopic control to 1-2μm.
2. The distance of the pipette tip from the recording site; this was kept to within 1mm, as measured with an eyepiece micrometer.
3. The ejection pressure; this was kept to between 40-50p.s.i.
4. The duration of ejection (1-200ms); this was varied as indicated in the text.

E. RADIO-LABELLED TRANSMITTER RELEASE

As an alternative means of measuring transmitter release, the transmitter pool of the nerves of the rat tail artery (SHR and WKY) were labelled radio-chemically and the overflow from each tissue analysed for radio-activity. In this investigation, the term 'overflow' is defined as the amount of neurotransmitter which escaped the uptake processes and was collected from the tissue (Fig. 3).

Tail arteries were dissected out as previously described, then incubated in Krebs solution (37°C) containing (a) [^{3}H]-noradrenaline (20μCiml^{-1}, 2 x 10^{-6}M NA for 60min) or (b) [^{3}H]-adenosine (100μCiml^{-1}, 3 x 10^{-6}M adenosine for 60min). Tissues were oxygenated throughout the loading period.

Fig. 3: Apparatus for measuring [^{3}H] overflow. Each artery, loaded with either [^{3}H]-NA or [^{3}H]-adenosine, was suspended by a thread through a set of bipolar Ag/AgCl ring electrodes. The muscles were then transferred to a heated water jacket (37 ± 0.5°C) and superfused with oxygenated Krebs solution at a constant rate of 2.5mlmin^{-1}. The polythene tubing (diameter 2mm) containing the Krebs solution was surrounded by an outer tube (diameter 10mm) containing water at 42 ± 0.5°C pumped from a thermostatically-controlled Tempette pump. Samples of the perfusate were collected in vials positioned underneath the tissues.


water outlet
krebs
stimulator
muscle tissue
water inlet
Ag/Ag Cl ring-electrode
water-jacket
tempette pump
krebs collection

Following the loading period, tissues were pulled through a pair of bipolar Ag/AgCl ring electrodes (O.D. 2mm) and superfused with oxygenated Krebs solution. The Krebs solution was pumped through polythene tubing (O.D. 2mm) using a Watson-Marlow (Type 22) flow inducer. The tubing was surrounded by an outer jacket (O.D. 10mm) containing water (42 ± 0.5°C) pumped by a Tempette (TE7) pump. The polythene tubing was positioned to allow superfusion of the preparations with Krebs solution at a constant rate of $2.5ml min^{-1}$. 2min samples of superfusate were collected in vials placed under the tissues.

Tail arteries incubated with [^{3}H]-NA ($20\mu Ci ml^{-1}$) were superfused throughout each experiment with Krebs solution containing desmethylimipramine (DMI, $1 \times 10^{-6}M$) and normetanephrine (NMN, $1 \times 10^{-5}M$) to inhibit neuronal and extraneuronal uptake of NA respectively and propranolol ($1 \times 10^{-6}M$) to block any effects of β-adrenoceptors.

It is believed (Westfall et al., 1978), that in the guinea-pig vas deferens, contraction of the muscle per se, contributes to the release of ATP. Accordingly, to prevent muscle contraction, tail arteries incubated with [^{3}H]-adenosine ($100\mu Ci ml^{-1}$) were superfused with Krebs solution containing prazosin ($5 \times 10^{-7}M$) and diltiazem ($3 \times 10^{-6}M$), at concentrations previously determined to virtually abolish all contractile activity.

Following a predetermined washout period of 2h, after which time [^{3}H] release had reached a steady level, tissues incubated in [^{3}H]-NA were stimulated (0.5ms, supramaximal voltage) with 200 pulses at 5, 10 and 20Hz and those incubated with [^{3}H]-adenosine were stimulated (0.5ms, supramaximal voltage) with 500 pulses at 10, 20 and 30Hz.

0.5ml aliquots of the tissue superfusate were added to a scintillation mixture (5ml of Ecoscint, National Diagnostics) containing toluene : triton-X : scintol-2 (12.33 : 6.67 : 1, V : V : V) and the amount of [^{3}H] measured with a scintillation counter (Packard Tri-Carb

2000 CA). The amount of [^{3}H], in counts per min (CPM) was corrected for the efficiency of the counter (approximately 90%) and expressed as the fractional release of [^{3}H], i.e. the overflow evoked by stimulation, as a fraction of the total in the tissue at that time. This was determined after dissolving the tissue at the end of the experiment in Soluene-350 (2ml, United Technologies Packard) and counting an aliquot of the sample.

III. HISTOCHEMICAL TECHNIQUES

The microscopic structure of several tissues was examined using the following techniques:

A. LIGHT MICROSCOPY

Sections of the tail artery and mesenteric bed preparation from both normotensive (WKY) and spontaneously hypertensive (SHR) rats were fixed and processed using the following solutions and methods.

(i) Fixatives

The fixative solutions used were those of Sabitini et al., 1963.

Primary fixative

1M sodium cacodylate	10ml
Distilled water	70ml
25% glutaraldehyde	8ml (E.M. grade)
Sucrose	1.7g
1M calcium chloride	0.1ml

Adjust to pH 7.2, bring to 100ml with distilled water.

Buffer wash

1M sodium cacodylate	10ml
Distilled water	80ml
Sucrose	5.9g

Adjust to pH 7.2, bring to 100ml with distilled water.

Post fixative

1M sodium cacodylate	10ml
Distilled water	50ml
Sucrose	5.1g

Adjust to pH 7.2, bring to 100ml with distilled water.

Buffer	3 parts
4% Osmium tetroxide	1 part

(ii) Method

The artery samples were pinned out on small blocks of Sylgard (Dow Corning) and processed using the following schedule.

1. Primary fixative.	3hr
2. Buffer wash.	3 x 20min
3. Post fixative.	2hr
4. Buffer wash.	Rinse

5. Dehydrate slowly using graded alcohol or acetone.
6. Propylene oxide as intermediate solvent.

7. Propylene oxide\Araldite.	Overnight
8. Fresh unpolymerised resin.	3-4 days

9. Embed in flat embedding trays and polymerize at 60°C.

(iii) Staining

1μm sections were mounted on gelatine\chrome alum subbed slides then stained using the following technique (Ito & Winchester, 1963).

Toluidine blue\Pyronin Y

Sodium borate (Borax)	0.8g
Distilled water	100ml

Dissolve, then add in order;

Toluidine blue	0.8g
Pyronin Y	0.2g

Dissolve, then filter into a stock bottle.

Stain sections at room temperature until the desired depth of staining is obtained. Wash in running tap water, air dry and mount in immersion oil or synthetic mounting medium.

The stained sections were then examined using a Leitz Ortholux microscope and photographed with an Orthomat camera, positioned on top of the microscope.

B. LOCALIZATION OF CATECHOLAMINES BY FALCK HISTOCHEMISTRY

The adrenergic innervation of the mouse vas deferens and rat tail and mesenteric arteries was visualised by the histo-fluorescence technique of Falck et al., 1962. Catecholamines were condensed by exposure to formaldehyde vapour, thereby producing flurophores of isoquinoline which absorb light at 410nm and emit light at 480nm.

Small sections of the tissues were frozen in isopentane, itself cooled by liquid nitrogen. Frozen tissues were transferred to a freeze-drier (Pearse Speedivac) and held at -40°C for 24h under a partial vacume (0.01torr). Tissues were thereafter exposed to paraformaldehyde at 80°C in an oven for 1h, then returned to the freeze drier to be impregnated in wax in vacuo at 56°C. Following wax impregnation, transverse sections were cut and mounted in liquid paraffin for immediate examination.

Sections were examined under ultraviolet light using a Leitz Ortholux microscope fitted with a K530 and a blue-green BG12 narrow band filter. Pictures were taken with an Orthomat camera which was positioned on top of the microscope.

IV. PHYSIOLOGICAL SOLUTIONS : CHANGES IN IONIC COMPOSITION

Krebs solution, with the following composition (mM) was used throughout the investigation;

NaCl; 111.8, $NaHCO_3$; 25.0, $NaHPO_4$; 1.13, KCl; 4.7, $CaCl_2$; 2.7, $MgCl_2$; 1.3, glucose; 11.0, pH 7.4.

When the ionic composition of the Krebs solution was modified, isotonicity was maintained by substituting, or reducing the concentration of another appropriate ion. In K^+-free or low K^+ Krebs, KCl was wholly or partly replaced with NaCl. In solutions containing an increased concentration of K^+, an equivalent reduction in the concentration of NaCl was made. Low Cl^- Krebs was obtained by replacing NaCl with Na benzenesulphonate. Na^+-deficient solutions were prepared by substituting NaCl with choline chloride, the other ions remaining unchanged. Ca^{2+} was removed by replacing $CaCl_2$ with $MgCl_2$. The pH of the Krebs solution was maintained at 7.4 by gassing with a mixture of 95% O_2 and 5% CO_2.

V. CRITERIA FOR CELL PENETRATION

A cell was accepted for electrophysiological investigation provided the following criteria were satisfied;

(a) the penetration was sharp and the membrane potential stable, varying by not more than 2mV.

(b) excitatory junction potentials were observed in response to nerve stimulation.

(c) the voltage measured prior to penetration was restored following withdrawal of the microelectrode.

VI. MEASUREMENT OF SYSTOLIC BLOOD PRESSURE IN RATS

Systolic blood pressure was measured in conscious animals, warmed at 37°C for 15min. Readings were made without anaesthesia by means of

inflation of a tail cuff and a piezo-electric crystal detector connected to a blood pressure recorder (W & W Electronics, Basel, Switzerland).

VII. DRUGS

The following drugs were used:

α,β-methylene adenosine 5'-triphosphate lithium salt (α,βMeATP, Sigma), adenosine hemi-sulphate (Sigma), adenosine 5'-monophosphate (AMP, Sigma), adenosine 5'-diphosphate (ADP, Sigma), adenosine 5'-triphosphate disodium salt (ATP, Sigma), 2,5',8-[^{3}H]-adenosine (Amersham International, 40-60Cimmol^{-1}), (-)adrenaline hydrogen tartrate (BDH), (-)-ascorbic acid (Koch-Light), atropine sulphate (Sigma), bombesin (Sigma), bradykinin triacetate salt (Signa), clonidine hydrochloride (Boehringer), cremophor EL (Sigma), desmethylimipramine hydrochloride (Ciba), diltiazem HCl (Sigma), guanethidine monosulphate (Ciba), 5-hydroxydopamine hydrochloride (Sigma), 6-hydroxydopamine hydrobromide (Sigma), idazoxan hydrochloride (RX781094, Reckitt & Colman), leu-enkephalin (Serva), lidocaine hydrochloride (Sigma), met-enkephalin (Serva), neuropeptide Y (porcine sequence, Sigma), nifedipine (Pfizer), (-)-noradrenaline bitartrate (Koch-Light), (-)-7,8-[^{3}H]-noradrenaline (Amersham International, 8-14Cimmol^{-1}), (±)-normetanephrine hydrochloride (Sigma), phentolamine mesylate (Ciba), (±)-prazosin hydrochloride (Pfizer), (±)-propranolol hydrochloride (Sigma), reserpine (Sigma), somatostatin (Sigma), substance P (Sigma), tetraethylammonium bromide (TEA, Sigma), tetrodotoxin (TTX, Sigma), vasoactive intestinal polypeptide (porcine VIP, Sigma), yohimbine hydrochloride (Sigma).

TTX and reserpine were expressed as the concentration of the base; all other concentrations in the text refer to the salt.

With the following exceptions, drugs were dissolved initially in

0.9% NaCl to give a stock solution, which was then diluted with Krebs to give the desired final concentration. Concentrations in the text refer to those in the bath unless otherwise stated. Nifedipine was dissolved under sodium illumination in the minimum amount of cremophor necessary, then diluted with Krebs. Solutions containing nifedipine were protected from the light. Reserpine was dissolved in glacial acetic acid (0.3ml, 17.5M) and diluted with distilled water. Solutions containing only glacial acetic acid and distilled water served as controls. 6-hydroxydopamine was dissolved by sonication in 0.9% saline containing ascorbic acid (5.7×10^{-3}M), kept at 4°C on ice and bubbled with O_2-free N_2 for at least 30min prior to use. Solutions containing only ascorbic acid served as controls. (-)-7,8-[^{3}H]-noradrenaline supplied in 0.02M acetic acid : ethanol (9 : 1, V : V) was resuspended in distilled water, containing ascorbic acid (5.7×10^{-3}m) to prevent breakdown of catecholamines. 2,5',8[^{3}H]-adenosine, supplied in an aqueous ethanol solution (50%), was resuspended in distilled water.

VIII. ANALYSIS OF RESULTS

Results were expressed as the mean ± standard error of mean (s.e.m.) of a number (n) of observations. Students t-tests were used to test for significance between means. A t-value of $p < 0.05$ was taken as being significant. Three or more tissues were used to investigate each drug

CHAPTER 3: RESULTS

I. MOUSE VAS DEFERENS

A. CONTRACTILE RESPONSES TO NERVE STIMULATION, NA AND ATP

In the mouse vas deferens, field stimulation (0.5ms, supramaximal voltage) with trains (> 25) of pulses at frequencies greater than 5Hz, produced a biphasic contractile response, comprising an initial rapid twitch followed by a slower tonic contraction (Fig. 4). The α-adrenoceptor antagonist phentolamine (1×10^{-6}M) selectively abolished the second phase of the neurogenic response, suggesting that it was mediated by NA, leaving the initial rapid twitch unaffected, or in some cases, as shown in Fig. 4, potentiated. α,βMeATP (1×10^{-6}M), on the other hand, selectively antagonised the initial phase, suggesting that it was mediated by ATP, or a closely related nucleotide. Both drugs, together, abolished all contractile activity to field stimulation.

If the biphasic contractile response in the mouse vas deferens is mediated by the two transmitters NA and ATP, then exogenous application of these substances should mimic, in time course, the respective phases of the neurogenic response. The mean time courses of the contractile responses of the mouse vas deferens to field stimulation (0.3ms, supramaximal voltage, 10Hz for 30s), exogenously-applied ATP (1×10^{-4}M) and NA (1×10^{-5}M) are shown in figures 5 and 6. The frequency of stimulation and doses of agonists used were based on results from preliminary experiments in which complete stimulus-response curves were obtained. The frequency of stimulation used was pre-determined to give a clear biphasic response. Doses of NA and ATP were chosen to be in the middle of the dose-response curve, so that any reduction or enhancement would be obvious. The contraction produced by ATP (1×10^{-4}M, Fig. 6B) was rapid in onset, but declined quickly, even though the drug was still in contact with the tissue and resembled in time course the initial phase of the neurogenic response (Fig. 5). The contraction produced by NA (1

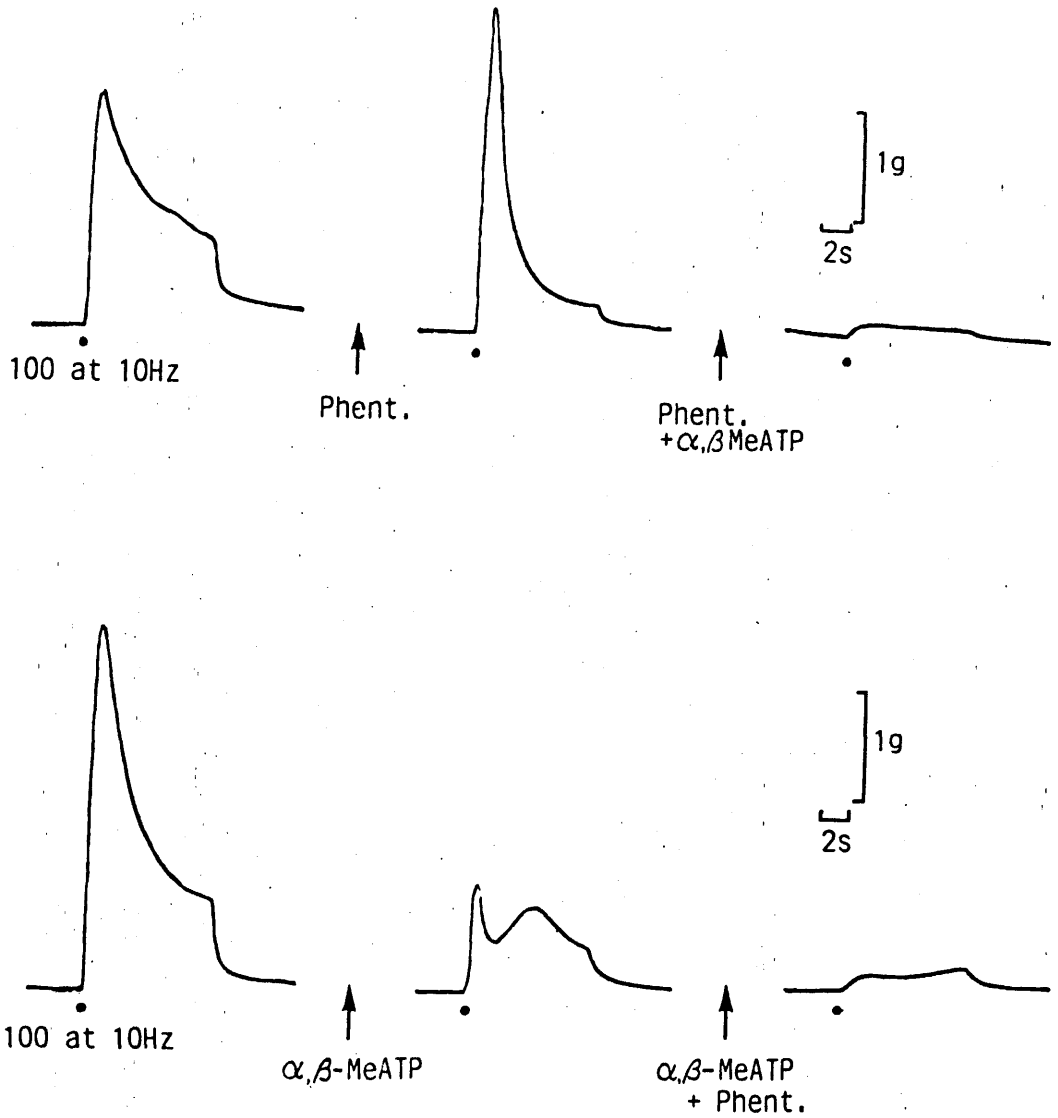


Fig. 4: The contractile responses of the mouse vas deferens to field stimulation (0.5ms, supramaximal voltage, 100 pulses at 10Hz). The response was biphasic, comprising an initial rapid twitch, followed by a slower tonic contraction. The first phase was antagonized by α,βMeATP (1×10^{-6}M), suggesting it was mediated by ATP, or a closely related nucleotide and the second phase, by phentolamine (1×10^{-6}M), suggesting that it was mediated by NA. Both drugs, together, abolished all contractile responses to field stimulation.

Fig. 5: The mean time course of the contractile response (expressed as a % of the maximum in each tissue) of the mouse vas deferens to field stimulation (0.3ms, supramaximal voltage, 10Hz applied for 30s) alone and in the presence of prazosin (1×10^{-7}M) or α,βMeATP (1×10^{-6}M). Each point represents the mean (± s.e.m.) % of the maximum response in a number (n) of tissues, measured 1s after stimulation commenced and at 3s intervals thereafter. In the control situation (left hand graph), the contractile response was biphasic, comprising an initial rapid twitch followed by a slower tonic contraction which lasted throughout the stimulation period. α,βMeATP selectively antagonised the initial twitch, indicating that it was mediated by ATP; prazosin selectively antagonised the second phase, suggesting that it was mediated by NA.

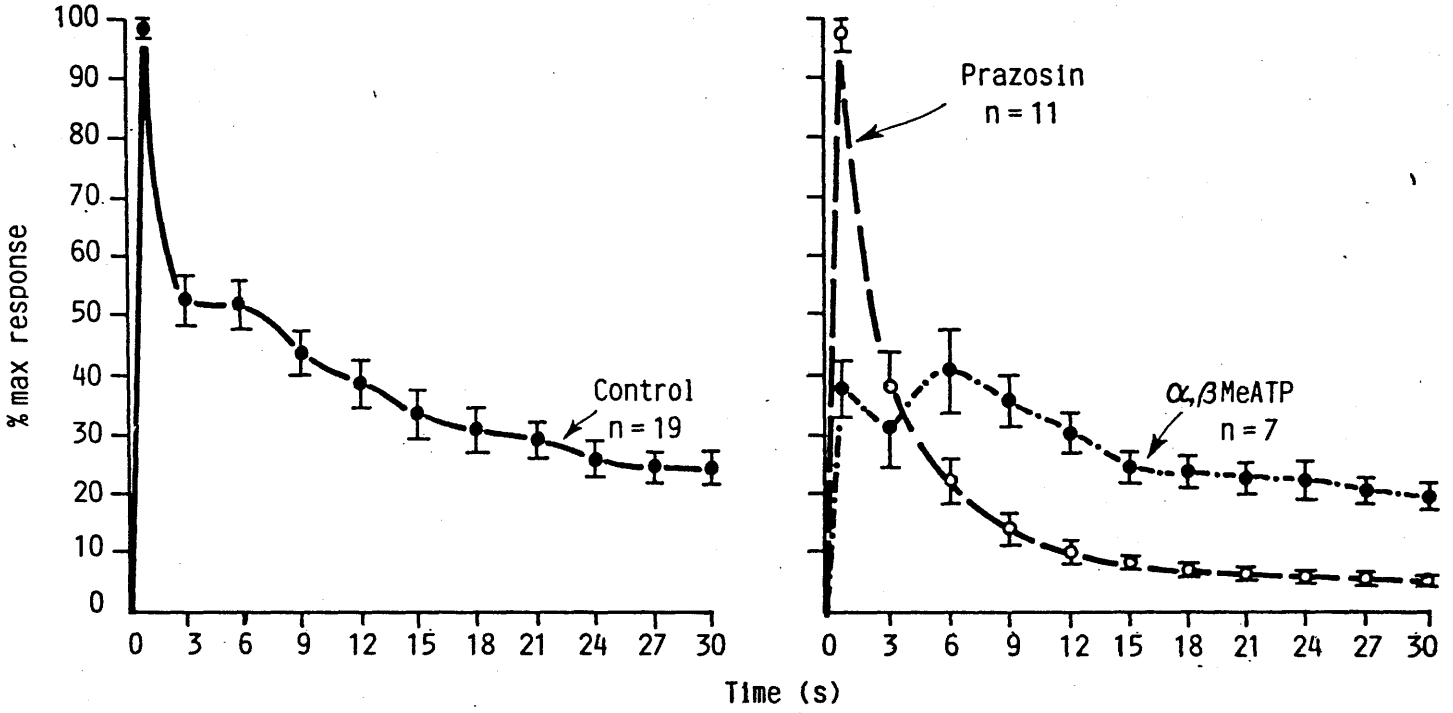
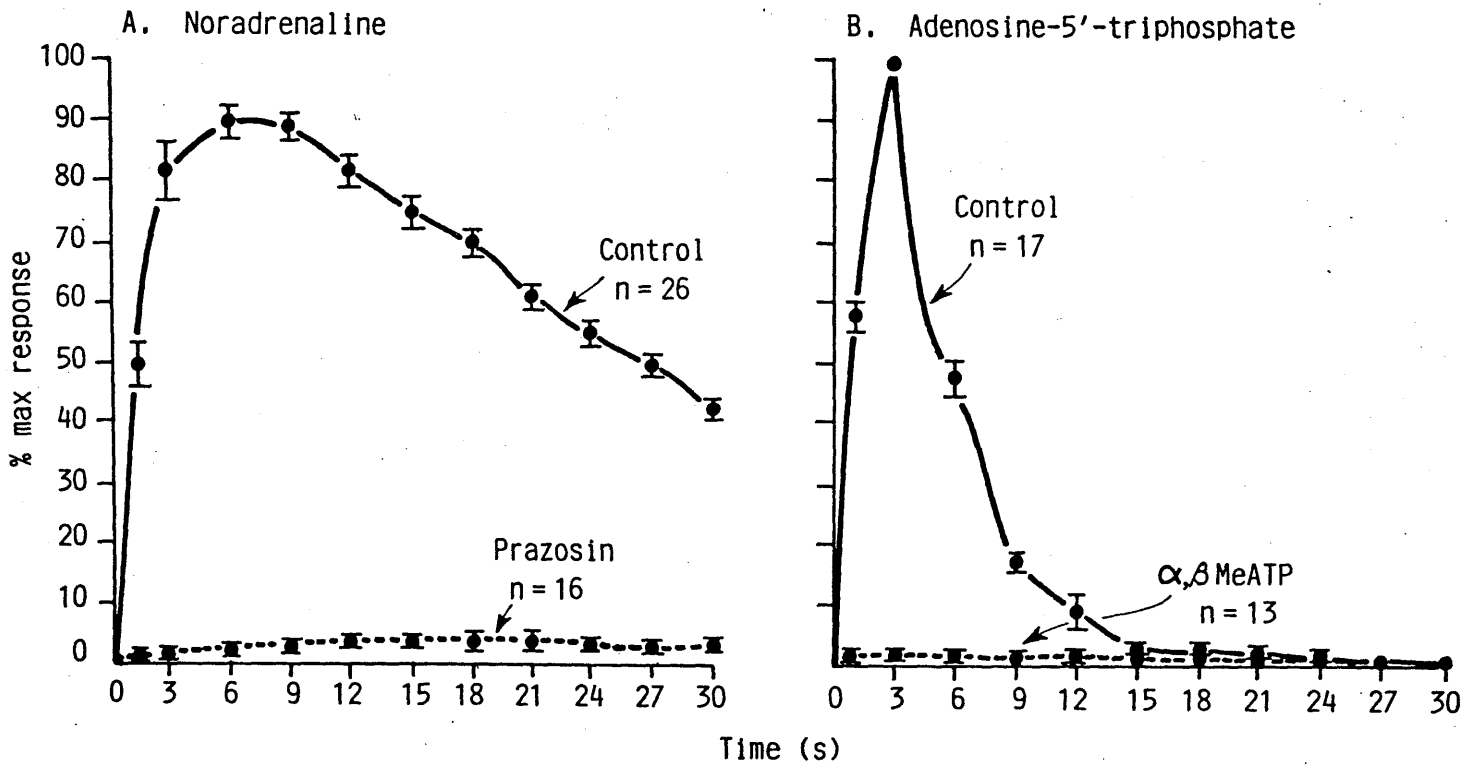

% max response
100
90
80
70
60
50
40
30
20
10
0
0 3 6 9 12 15 18 21 24 27 30
Control
n = 19
Prazosin
n = 11
α,βMeATP
n = 7
Time (s)

Fig. 6: The mean time course of the contractile response (expressed as a % of the maximum in each tissue) of the mouse vas deferens to exogenously added NA (1×10^{-5}M, A.) and ATP (1×10^{-4}M, B.). Both drugs were applied for 30s. Each point represents the mean (± s.e.m.) % of the maximum response in a number (n) of tissues, 1s after addition of the drug to the bath and at 3s intervals thereafter. The rapid transient response to the purine resembled, in time course, the initial phase of the neurogenic response (c.f. Fig. 5), whereas the slower response to the catecholamine more closely resembled the second phase of the neurogenic contraction. Contractile response to NA and ATP were selectively antagonized by prazosin (1×10^{-7}M) and α,βMeATP (1×10^{-6}M) respectively.


A. Noradrenaline
B. Adenosine-5'-triphosphate
% max response
100
90
80
70
60
50
40
30
20
10
0
Control
n = 26
Prazosin
n = 16
Control
n = 17
α,β MeATP
n = 13
0 3 6 9 12 15 18 21 24 27 30
Time (s)

x 10^{-5}M, Fig. 6A), on the other hand, was slower in onset and more prolonged, more closely resembling the second phase of the neurogenic response (Fig. 5). Contractions to exogenously added NA and ATP were selectively abolished by prazosin (1 x 10^{-7}M) and α,βMeATP (1 x 10^{-6}M) respectively (Fig. 6).

B. ELECTRICAL ACTIVITY IN THE MOUSE VAS DEFERENS

(i) Resting membrane characteristics

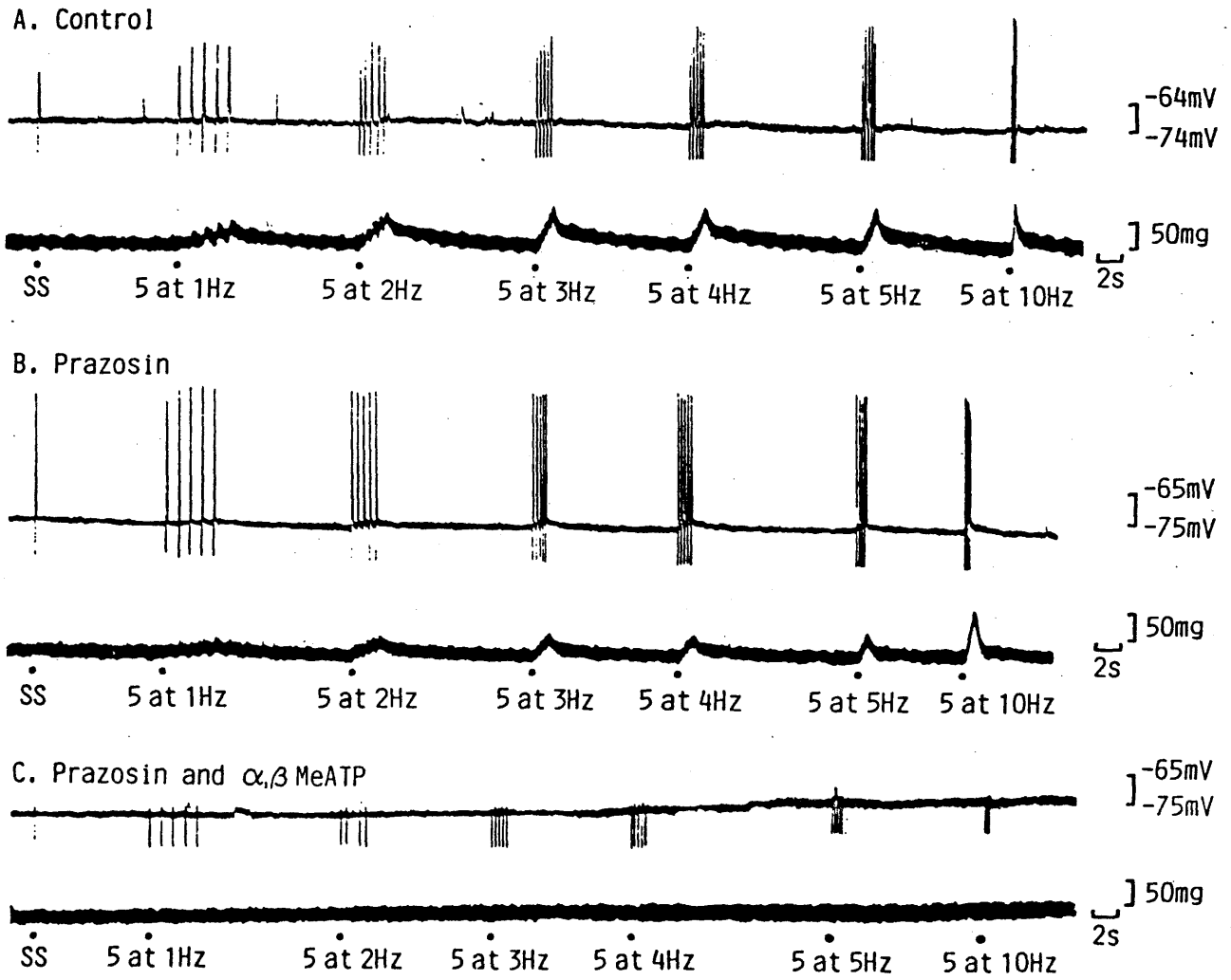
Upon setting up, 1g of stretch was applied to the tissue. The resulting tone decreased slightly over a 30min equilibration period to a value of around 0.7g, which was then maintained throughout the experiment.

The resting membrane potential ranged from -55 to -86mV, (mean -73.6 ± 0.4mV, n=224). Spontaneous excitatory junction potentials (e.j.p.s), with a mean amplitude of 7.3 ± 0.8mV (n=38) were observed in about 90% of all cells impaled and were taken as an indication of a successful penetration. Spontaneous e.j.p.s were unaccompanied by mechanical contractions.

(ii) Evoked membrane activity

Field stimulation (0.01ms, supramaximal voltage, single pulses, ss, or trains of pusles at 1-10Hz) produced e.j.p.s, which readily facilitated to fire action potentials and were accompanied by mechanical contractions (Fig. 7). The mean amplitude of an e.j.p. evoked by a single stimulus was 17.9 ± 0.7mV, n=110 with a duration of around 100ms (mean 91.6 ± 1.8ms, n=103). Figure 8 shows the time course of the electrical response to a single stimulus on an extended time scale. The rate of rise of the e.j.p. was very rapid and even single pulses were sufficient to depolarize the membrane to the threshold required to

Fig. 7: The effects of prazosin alone (1×10^{-7}M, B.) and in the presence of α,βMeATP (1×10^{-6}M, C.), compared with control (A.) on the simultaneous electrical (upper trace in each panel) and mechanical responses of the mouse vas deferens to field stimulation (0.01ms, supramaximal voltage). Prazosin potentiated the e.j.p.s and reduced the mechanical contractions. The additional presence of α,βMeATP abolished all residual electrical and mechanical activity. Electrical recordings were made form the same cell.


A. Control
-64mV
-74mV
50mg
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz
B. Prazosin
-65mV
-75mV
50mg
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz
C. Prazosin and α,β MeATP
-65mV
-75mV
50mg
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz

initiate a propagating action potential.

The α-adrenoceptor antagonist prazosin (1×10^{-7}M) slightly, but significantly ($p < 0.05$) potentiated the e.j.p.s and action potentials and reduced, but did not abolish, the mechanical contractions. The additional presence of α,βMeATP (1×10^{-6}M) abolished the e.j.p.s, action potentials and the residual mechanical activity (Fig. 7). Clearly, field stimulation of the mouse vas deferens released 2 transmitter substances - NA and ATP (or a closely related nucleotide). Both transmitters were involved in the mechanical response, but only ATP appeared to mediate an electrical event.

The mean effects of prazosin (1×10^{-7}M) alone and with α,βMeATP (1×10^{-6}M) on the average amplitude of the summated e.j.p. are shown in figure 9. The potentiating effects of prazosin were more pronounced at low frequencies (< 4Hz) of nerve stimulation, suggesting a presynaptic mechanism of action. α,βMeATP virtually abolished all electrical activity.

The origin of the transmitter substances was investigated using tetrodotoxin (TTX, 1×10^{-6}M, Fig 10) and lignocaine (1×10^{-3}M, Fig. 11) each of which selectively prevent the increase in sodium permeability constituting the rising phase of the action potential and hence block nerve conduction. Both drugs abolished the electrical and mechanical activity in response to field stimulation (0.01ms, supramaximal voltage), suggesting that all responses were mediated by neuronally-released substances. The adrenergic neurone blocking drug guanethidine (1×10^{-5}M, Fig. 12) also abolished the e.j.p.s, action potentials and the mechanical contractions evoked by field stimulation (0.01ms, supramaximal voltage), suggesting that both transmitters (NA and ATP) were released from noradrenergic, rather than from separate noradrenergic and purinergic nerves, confirming the presence of co-transmission in this tissue.

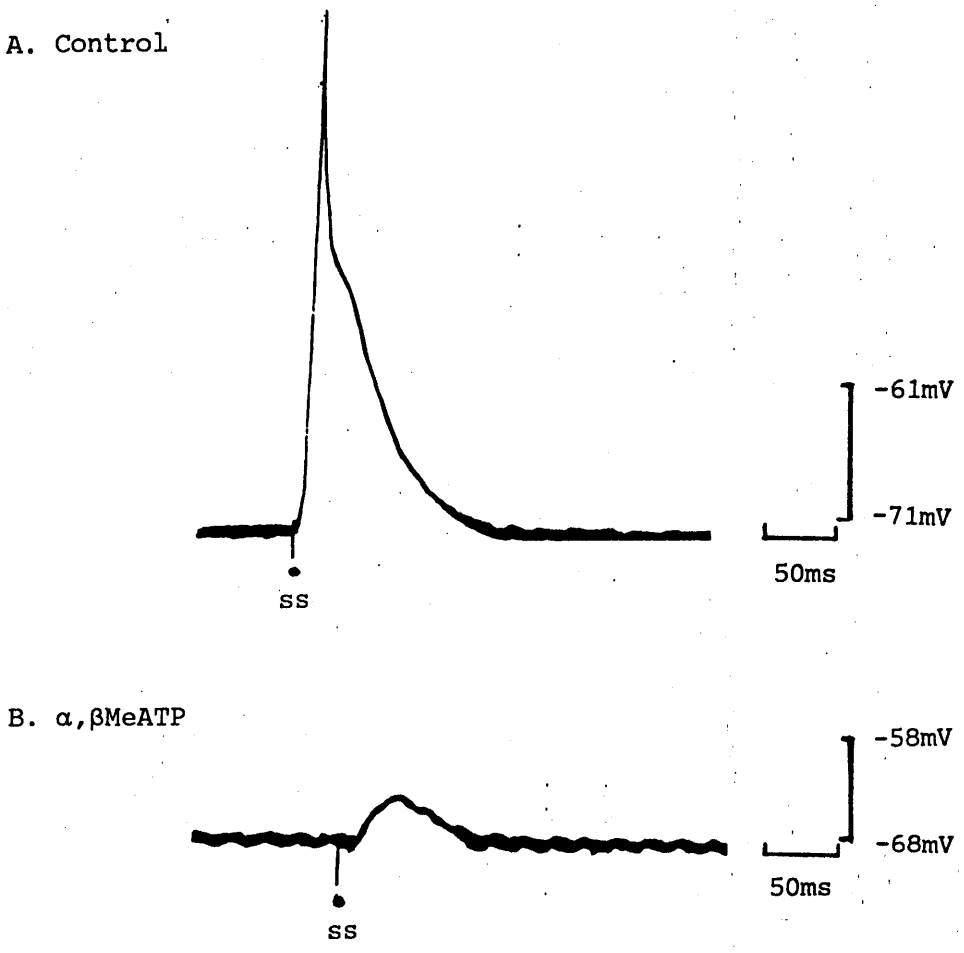


Fig. 8: The effects of α,βMeATP (2×10^{-6}M) on an e.j.p. evoked by a single stimulus (0.01ms, supramaximal voltage) in the mouse vas deferens. Note that even a single pulse was sufficient to generate a propagating action potential, which was abolished by α,βMeATP, suggesting that it was mediated by ATP, or a closely related nucleotide.

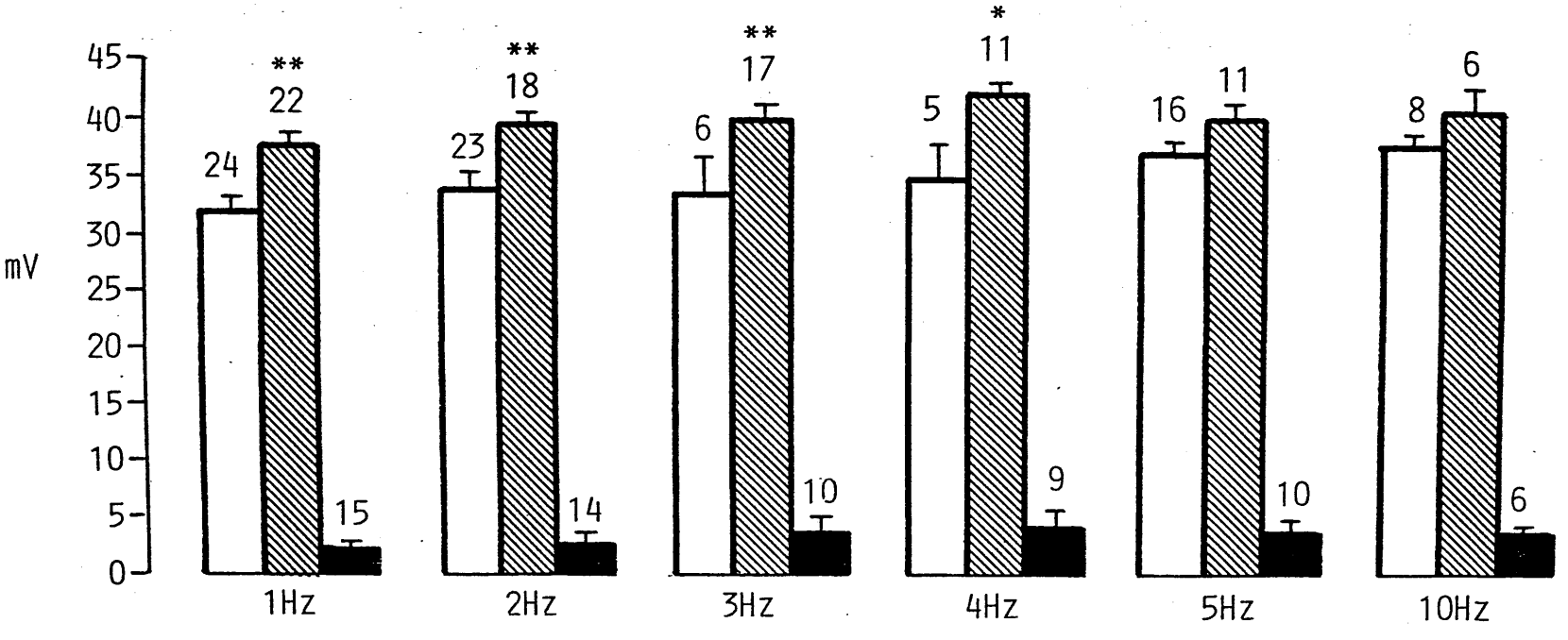


Fig. 9: The effects of prazosin (1×10^{-7}M, ▨) alone and in the presence of α,βMeATP (5×10^{-6}M, ■), compared with control (□), on the mean amplitude of the summated e.j.p. evoked by field stimulation (0.01ms, supramaximal voltage, 5 pulses at 1,2,3,4,5 and 10Hz) in the mouse vas deferens. In each tissue, the mean amplitude was calculated from the 2nd-5th e.j.p. in a train of 5. Each bar graph represents the mean (± s.e.m.) e.j.p. amplitude in a number (n) of tissues. E.j.p.s at lower frequencies (1-4Hz) were significantly (* $p < 0.05$, ** $p < 0.02$) potentiated by prazosin, an effect not seen at higher frequencies (5-10Hz) of stimulation, suggesting a pre-synaptic mechanism of action. All e.j.p.s were [illegible] by α,βMeATP.

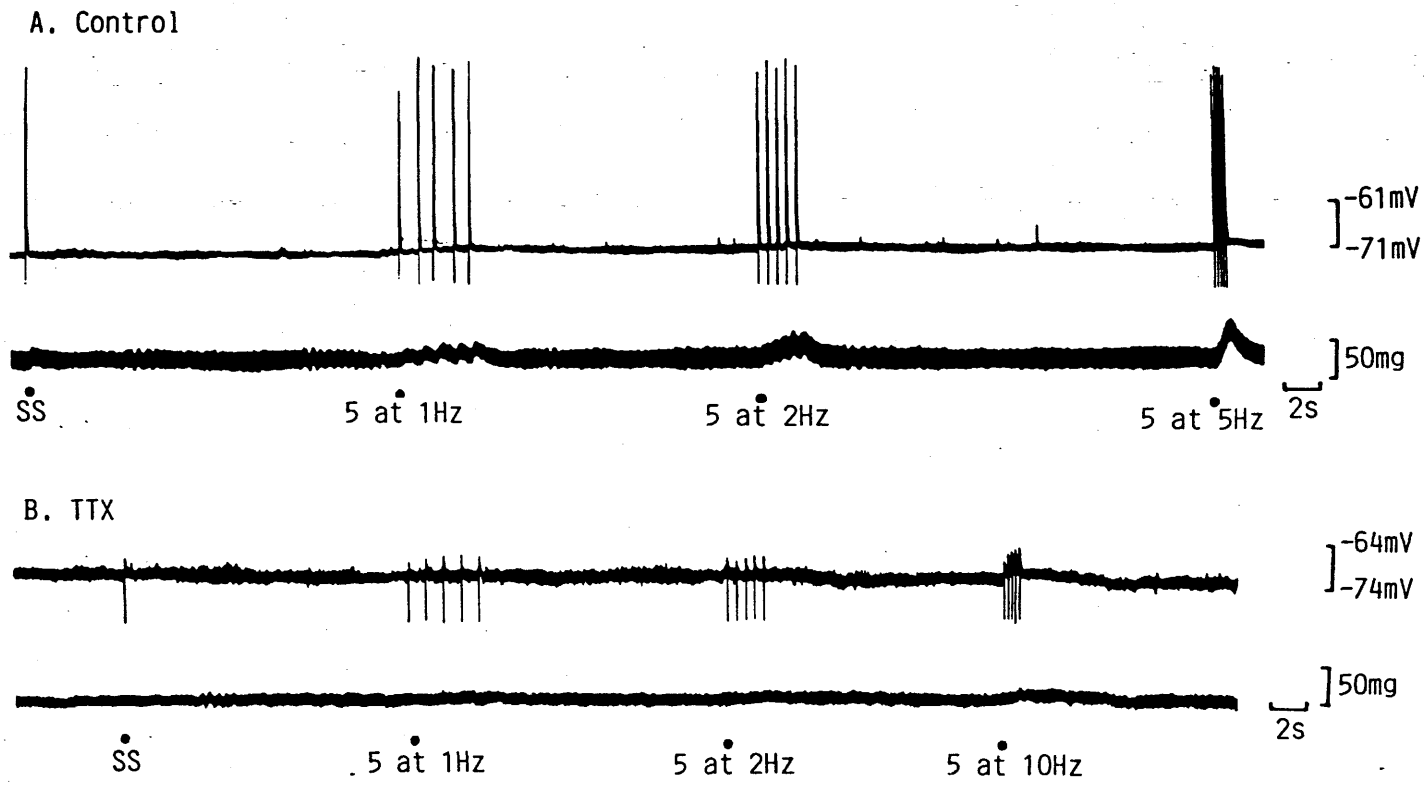
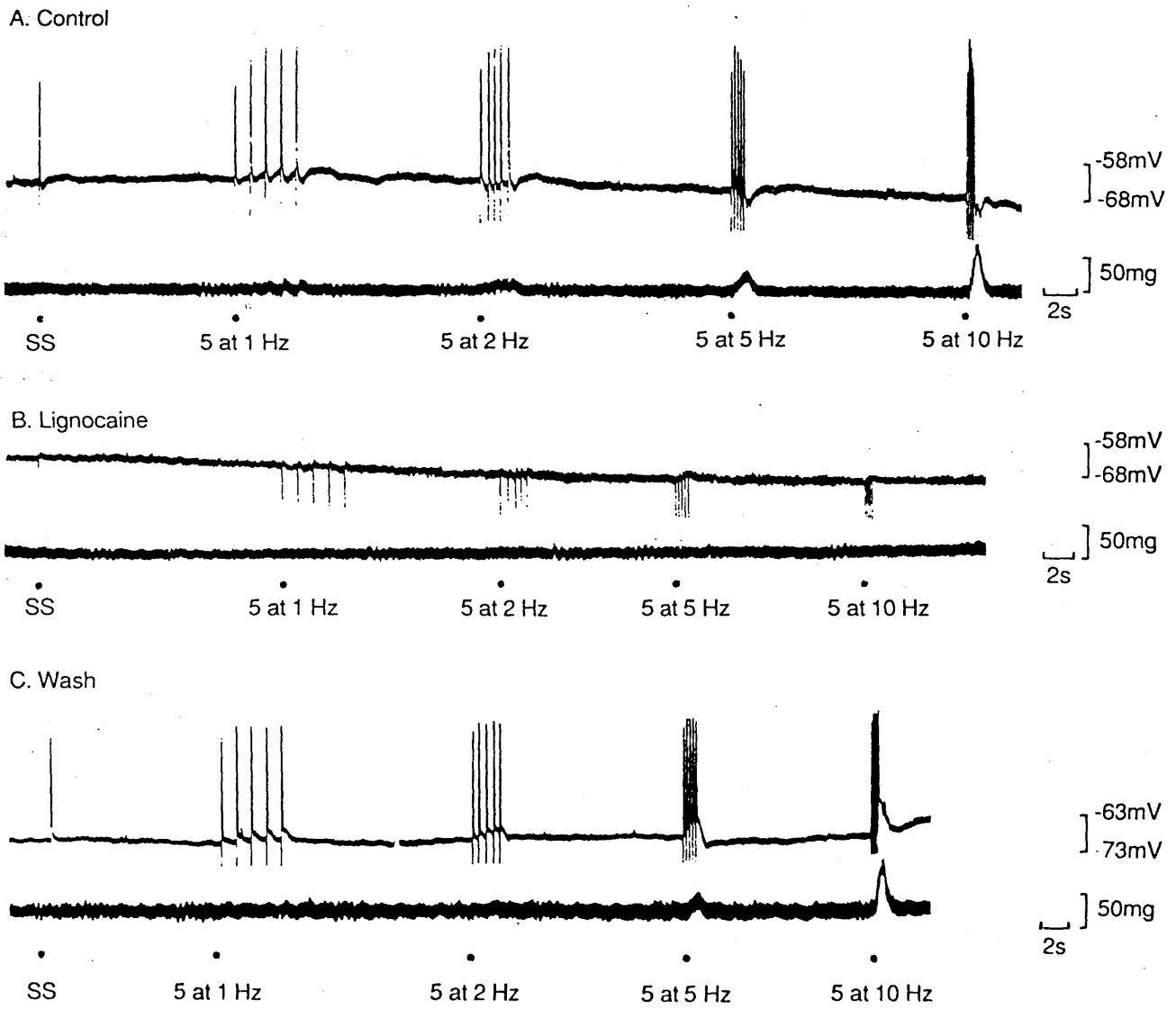


Fig. 10: The effects of tetrodotoxin (TTX, 1 x 10^{-6}M) on the electrical (upper trace in each panel) and mechanical responses of the mouse vas deferens to field stimulation (0.01ms, supramaximal voltage). TTX abolished all electrical and mechanical actity, suggesting that the transmitters were neuronally-released. Electrical recordings were made from the same cell.

Fig. 11: The effects of lignocaine (1×10^{-3}M) on the electrical (upper trace in each panel) and mechanical responses of the mouse vas deferens to field stimulation (0.01ms, supramaximal voltage). The local anaesthetic abolished all electrical and mechanical activity, an effect reversible on washing. Electrical recordings were made from the same cell.


A. Control
-58mV
-68mV
50mg
2s
SS
5 at 1 Hz
5 at 2 Hz
5 at 5 Hz
5 at 10 Hz
B. Lignocaine
-58mV
-68mV
50mg
2s
SS
5 at 1 Hz
5 at 2 Hz
5 at 5 Hz
5 at 10 Hz
C. Wash
-63mV
-73mV
50mg
2s
SS
5 at 1 Hz
5 at 2 Hz
5 at 5 Hz
5 at 10 Hz

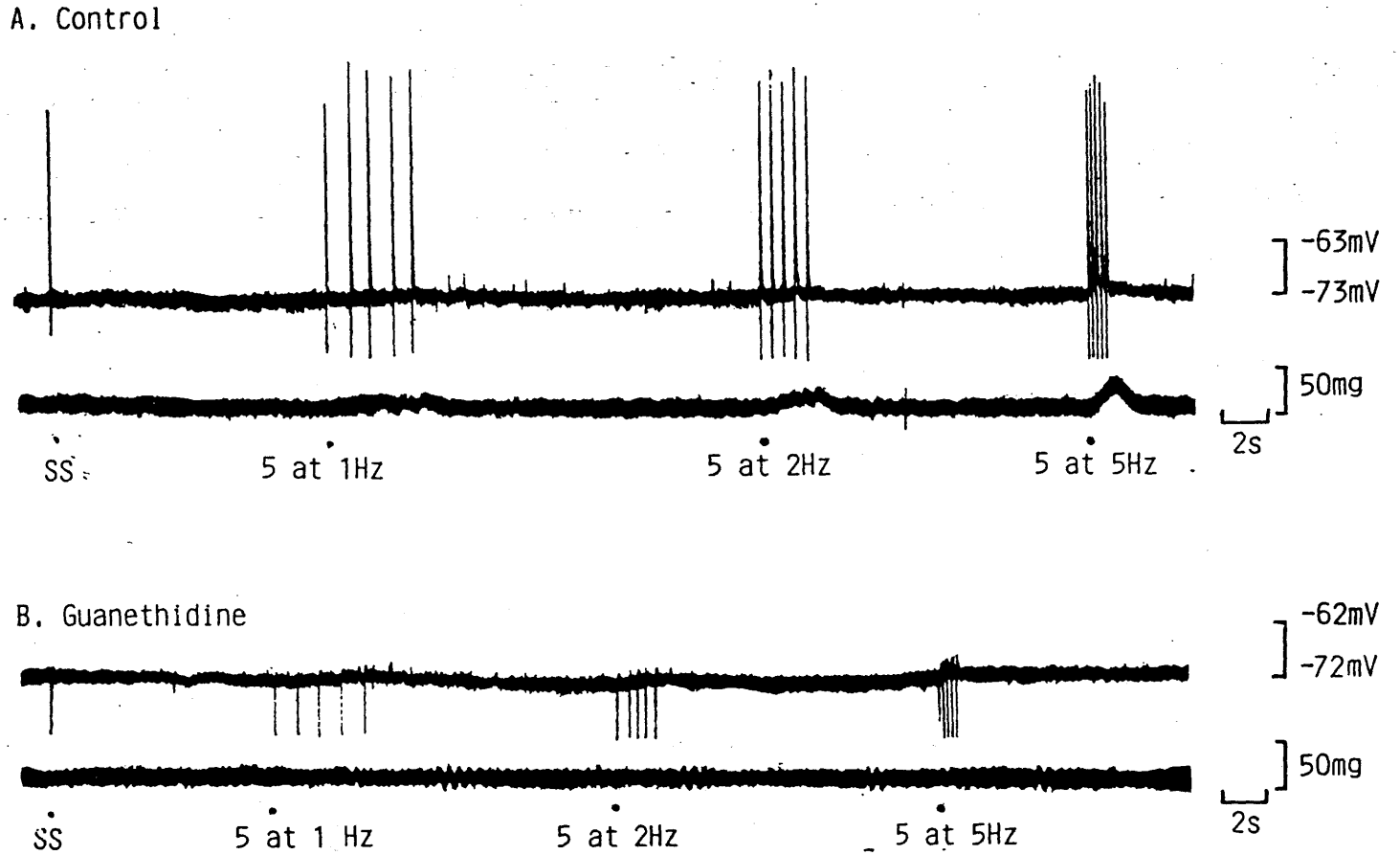


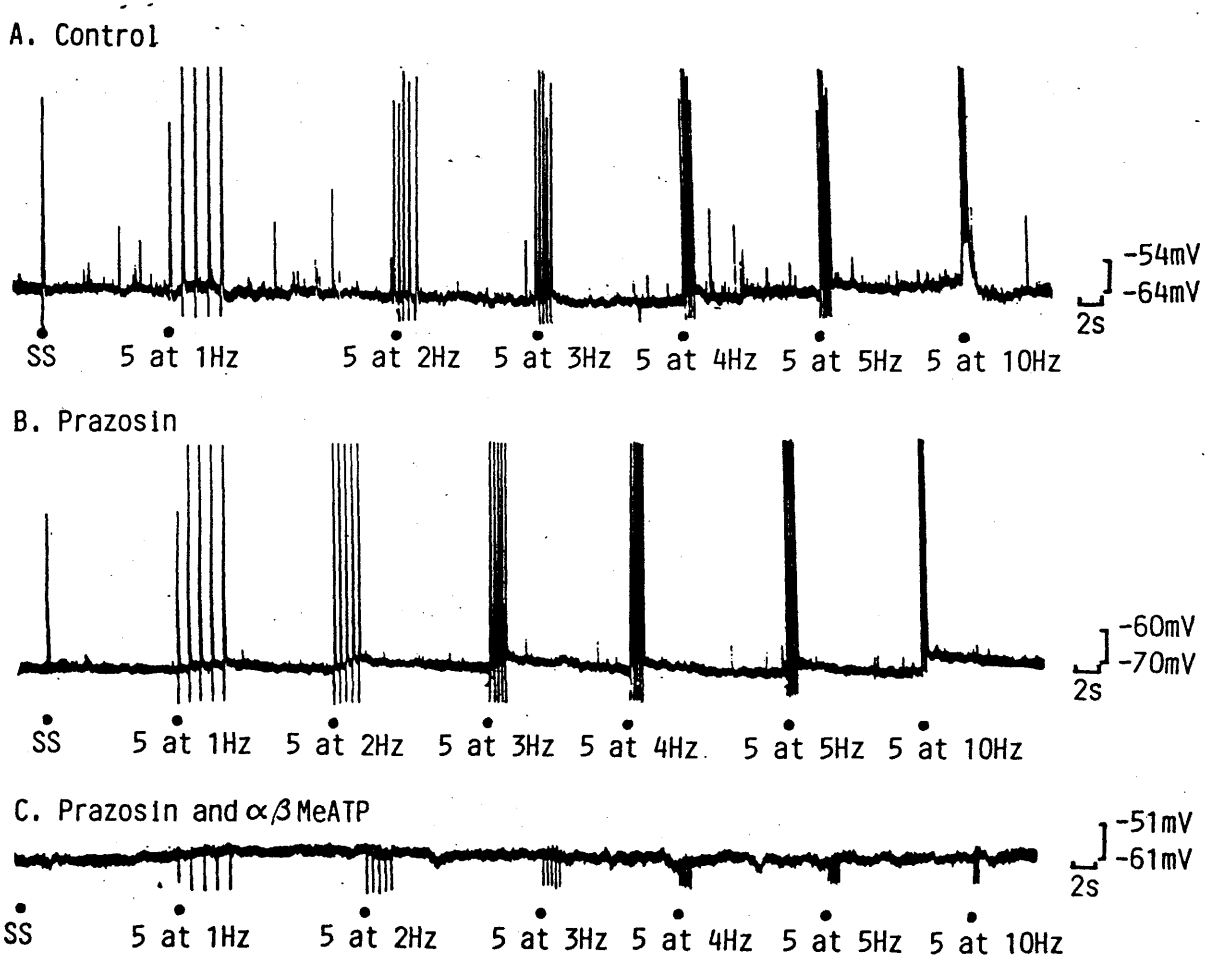
Fig. 12: The effects of guanethidine (1×10^{-5}M) on the electrical (upper trace in each panel) and mechanical responses of the mouse vas deferens to field stimulation (0.01ms, supramaximal voltage). Both the e.j.p.s and the mechanical contractions were abolished by guanethidine, suggesting that both transmitters (NA and ATP) were released from noradrenergic nerves, rather than from separate noradrenergic and purinergic nerves. Electrical recordings were made from the same cell.

The possibility that field stimulation may have been facilitating transmitter release by depolarizing those terminal varicosities already invaded by the action potential (Stjärne, 1977) was investigated using extrinsic nerve stimulation. When the extrinsic hypogastric nerve innervating the mouse vas deferens was dissected out and stimulated (0.1ms, supramaximal voltage) an identical electrical picture emerged (Fig. 13), the e.j.p.s and action potentials being potentiated slightly by prazosin (1×10^{-7}M) and abolished by α,βMeATP (2×10^{-6}M). Thus, field stimulation, at the parameters used, appeared to be specific and did not facilitate transmitter release.

In some tissues, for example the rat tail (Cheung, 1982; 1984 and rabbit ear (Suzuki & Kou, 1983; Suzuki et al., 1984) arteries, where the same co-transmitters are apparently involved, the e.j.p.s were followed by a small, slow noradrenergic depolarization. Attempts to demonstrate such a response in the mouse vas deferens, were it present, by enhancing the amount of NA in the synaptic cleft were made using the noradrenaline uptake blockers normetanephrine (NMN, 1×10^{-5}M) and desemthylimipramine (DMI, 1×10^{-6}M). (Fig. 14). These drugs potentiated the mechanical response to field stimulation (0.01ms, supramaximal voltage), but failed to reveal any noradrenergic component in the electrical response. The mechanical contractions produced by nerve-released NA were therefore mediated by a voltage-independent mechanism.

The ability of the α-adrenoceptor antagonists prazosin (1×10^{-7}M) and phentolamine (1×10^{-6}M) to potentiate both the e.j.p.s (Figs. 7 & 9) and the initial phase of the contractile response (Fig. 4) suggested the presence of pre-synaptic α-adrenoceptors controlling ATP release. This observation was confirmed (Fig. 15) by the ability of the α_2-adrenoceptor agonist clonidine (1×10^{-7}M) to abolish the e.j.p.s, an effect reversed by the additional presence of yohimbine (1

Fig. 13: The effects of prazosin (1×10^{-7}M, B.) alone and in the presence of α,βMeATP (2×10^{-6}M, C.), compared with control (A.), on the electrical responses of the mouse vas deferens to hypogastric nerve stimulation (0.01ms, supramaximal voltage). The e.j.p.s appeared similar to those produced by field stimulation in both their amplitude and response to antagonists. They were potentiated slightly by prazosin and abolished by α,βMeATP. Electrical recordings were made from 3 separate cells.


A. Control
-54mV
-64mV
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz
B. Prazosin
-60mV
-70mV
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz
C. Prazosin and αβMeATP
-51mV
-61mV
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz

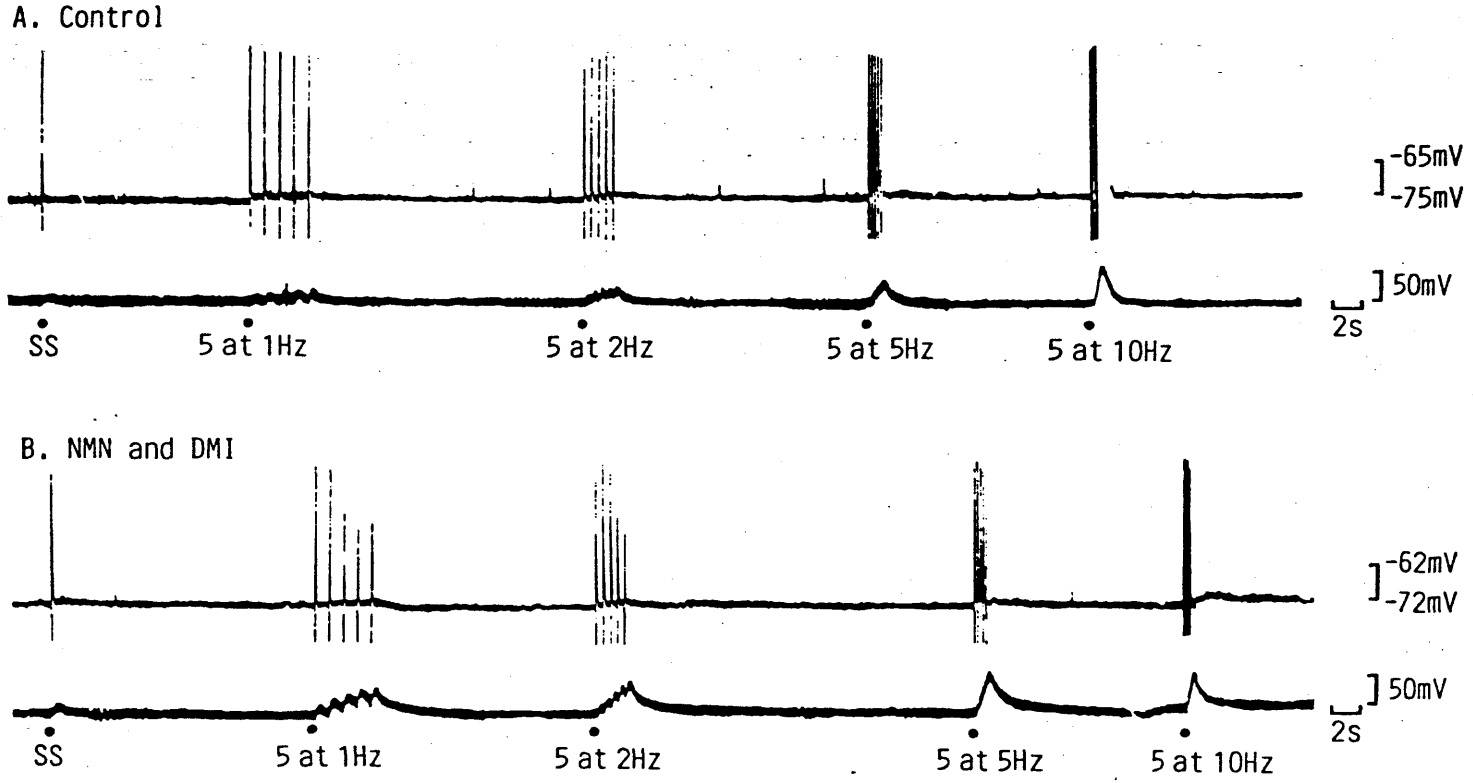
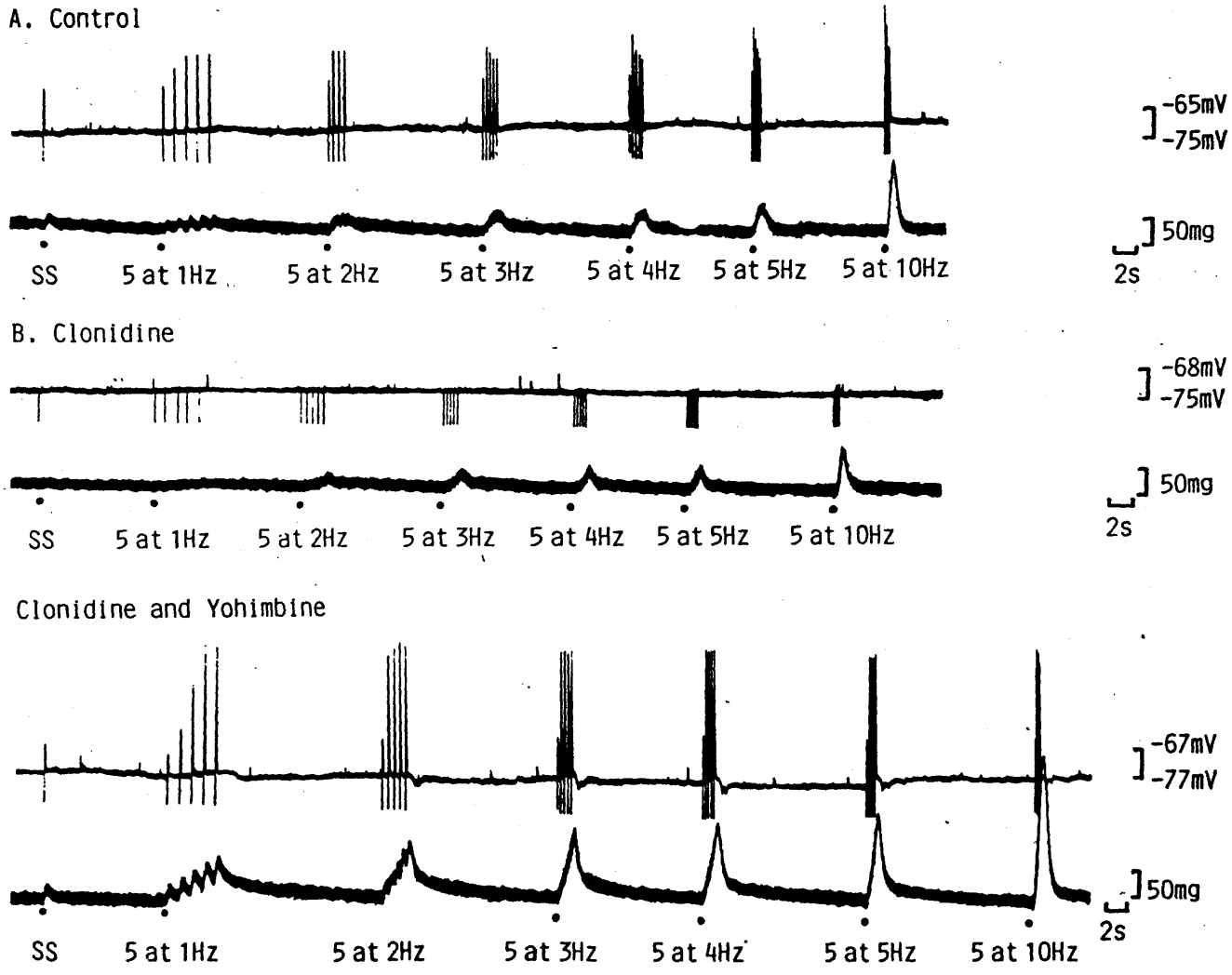


Fig. 14: The effects of normetanephrine (NMN, 1 x 10^{-5}M) and desmethylimipramine (DMI, 1 x 10^{-6}M) together on the electrical (upper trace in each panel) and mechanical responses of the mouse vas deferens to field stimulation (0.01ms, supramaximal voltage). The noradrenaline uptake blockers potentiated the mechanical responses, but failed to reveal any electrical component mediated by NA. Electrical recordings were made from the same cell.

Fig. 15: The effects of clonidine (1×10^{-7}M, B.) alone and in the presence of yohimbine (1×10^{-7}M, C.), compared with control (A.), on the electrical (upper trace in each panel) and mechanical responses of the mouse vas deferens to field stimulation (0.01ms, supramaximal voltage). Clonidine abolished the e.j.p.s, an effect reversed by yohimbine, suggesting the presence of pre-synaptic α_2-adrenoceptors controlling the release of ATP. Mechanical contractions were potentiated by yohimbine, probably due to the blockade of pre-synaptic α_2-adrenoceptors controlling NA release. Electrical recordings were made from 3 separate cells.


A. Control
-65mV
-75mV
50mg
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz
B. Clonidine
-68mV
-75mV
50mg
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz
Clonidine and Yohimbine
-67mV
-77mV
50mg
2s
SS
5 at 1Hz
5 at 2Hz
5 at 3Hz
5 at 4Hz
5 at 5Hz
5 at 10Hz

x 10^{-7}M), an antagonist at these receptors.

(iii) Putative transmitters

If the e.j.p.s are mediated by ATP, then local application of the nucleotide should produce dose-dependent depolarizations.

ATP (1-10 x 10^{-4}M, Fig. 16A), applied exogenously (40p.s.i., tip diameter 1 x 10^{-6}m, 1-50ms) to a small area of the mouse vas deferens, produced a dose-dependent depolarization with a rapid rate of rise and a relatively short duration, closely resembling the neurogenic response. Desensitization occurred rapidly. As with the evoked e.j.p.s, the depolarizations produced by exogenous ATP were abolished by α,βMeATP (1 x 10^{-6}M). NA (1-10 x 10^{-4}M, Fig. 16C) similarly applied produced no such change in membrane potential, though localized mechanical contractions could be seen microscopically.

Neither adenosine (1 x 10^{-3}M), AMP (1 x 10^{-3}M) or ADP (1 x 10^{-3}M) produced any significant change in membrane potential when applied locally from a micropipette (40p.s.i., tip diameters 1-2 x 10^{-6}m, 1-200ms, Fig. 17), suggesting that the effects observed with ATP were due to the nucleotide itself, rather than to one of it's breakdown products. This idea was supported by the observation of dose-dependent depolarizations to local application of the stable analogue of ATP, α,βMeATP (1 x 10^{-6}M, 40p.s.i., tip diameter 1 x 10^{-6}m, 1-10ms, Fig. 17A). α,βMeATP was some 1000 times more potent than ATP at producing membrane depolarizations.

Several biologically-active peptides were also investigated as putative transmitters in the mouse vas deferens. Bradykinin (1-100 x 10^{-7}M), at high doses, produced a small slow membrane hyperpolarization. Vasoactive intestinal polypeptide (VIP, 1-100 x 10^{-7}M), neuropeptide Y (NPY, 1-100 x 10^{-7}M), substance P (1-100 x 10^{-7}M), somatostatin (1-100 x 10^{-7}M), leu-enkephalin (leu-enk, 1-100 x 10^{-7}M), met-

Fig. 16: Intracellularly-recorded membrane potential responses of the mouse vas deferens to micro-application of ATP (1×10^{-3}M, A.) and NA (1×10^{-3}M, C.) for increasing periods of time (5-100ms). Micropipette tip diameters $1\text{-}2 \times 10^{-6}$m and ejection pressure 40p.s.i. Locally-applied ATP produced a dose-dependent rapid depolarization which was abolished by α,βMeATP (1×10^{-6}M, B.). NA similarly applied produced no such change in membrane potential. A. and B. were recorded from the same cell and C. from another cell in the same preparation.

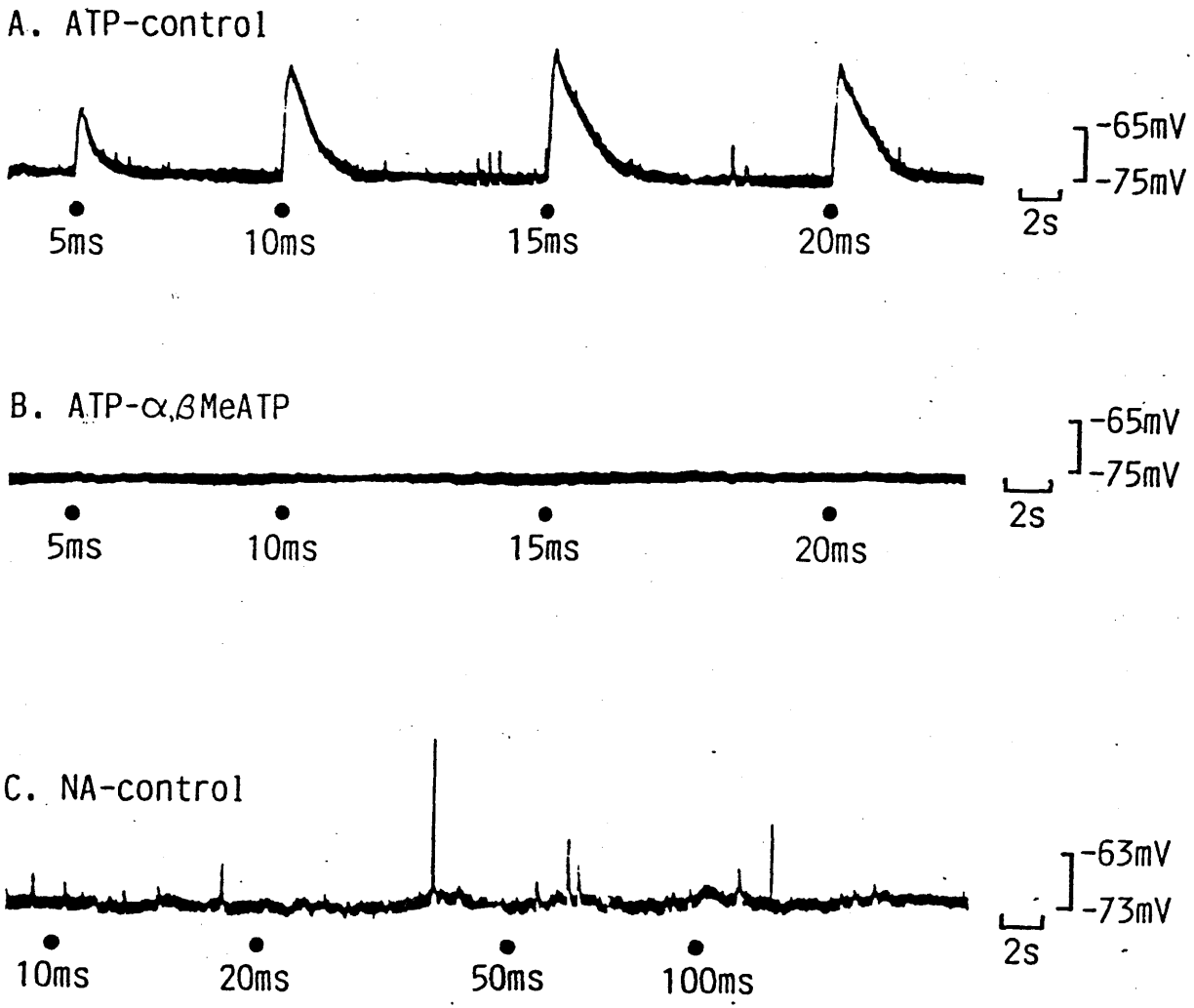
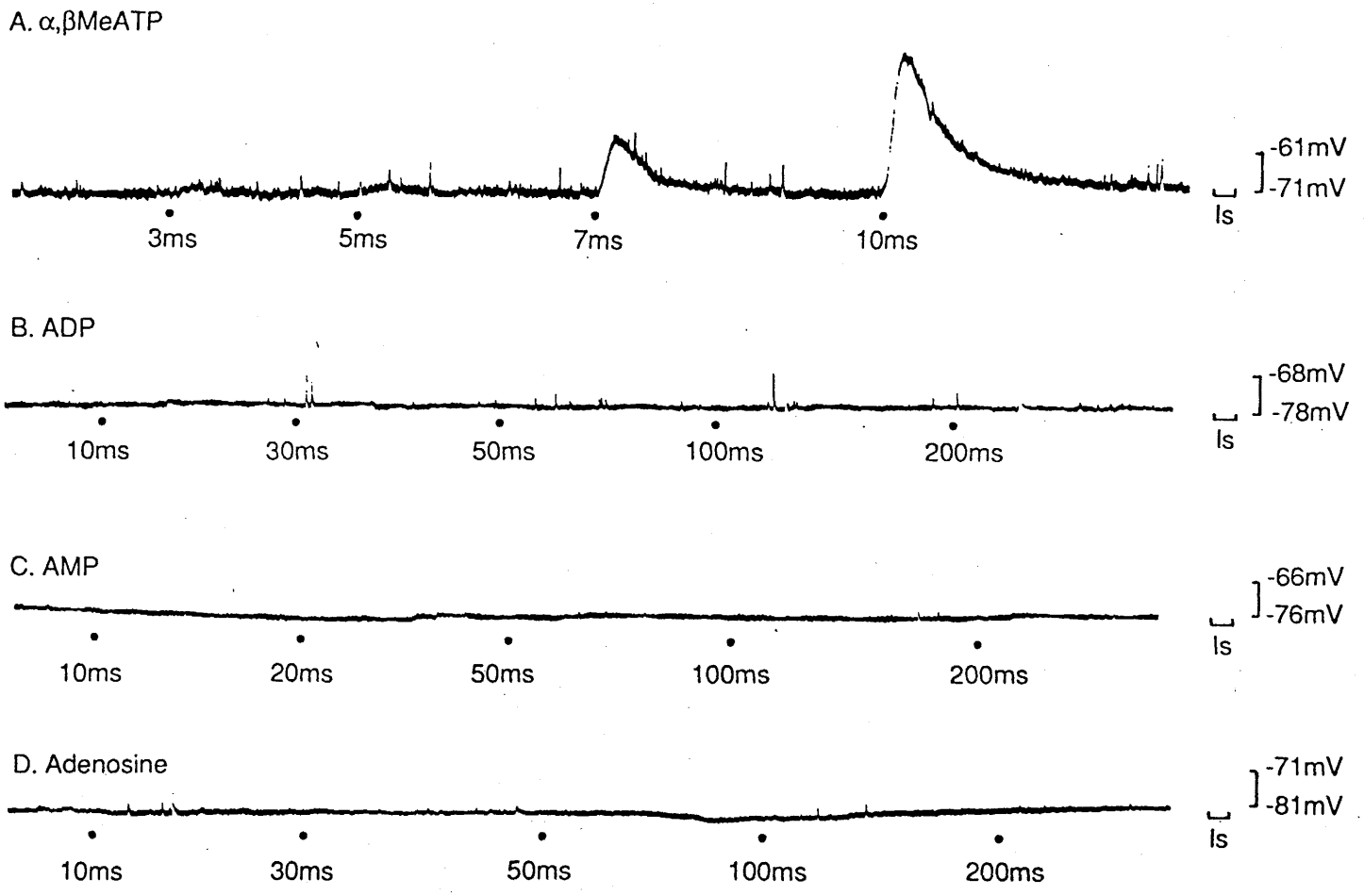

A. ATP-control
-65mV
-75mV
2s
5ms
10ms
15ms
20ms
B. ATP-α,βMeATP
-65mV
-75mV
2s
5ms
10ms
15ms
20ms
C. NA-control
-63mV
-73mV
2s
10ms
20ms
50ms
100ms

Fig. 17: Intracellularly-recorded membrane potential responses of the mouse vas deferens to micro-application of α,βMeATP (1 x 10^{-6}M, A.), adenosine 5'-diphosphate (ADP, 1 x 10^{-3}M, B.), adenosine 5'-monophosphate (AMP, 1 x 10^{-3}M, C.) and adenosine (1 x 10^{-3}M, D.) for increasing periods of time (3-200ms). Micro-pipette tip diameters were 1-2 x 10^{-6}m and ejection pressure 40p.s.i. Only α,βMeATP produced any change in membrane potential, suggesting that the transmitter mediating the e.j.p.s was a purine nucleotide, most likely ATP. Electrical recordings were made from 4 separate cells.


A. α,βMeATP
3ms
5ms
7ms
10ms
-61mV
-71mV
1s
B. ADP
10ms
30ms
50ms
100ms
200ms
-68mV
-78mV
1s
C. AMP
10ms
20ms
50ms
100ms
200ms
-66mV
-76mV
1s
D. Adenosine
10ms
30ms
50ms
100ms
200ms
-71mV
-81mV
1s

enkephalin (met-enk, 1-100 x 10^{-7}M) and bombesin (1-100 x 10^{-7}M) each produced no change in membrane potential when applied locally from a micro-pipette (40p.s.i., 1-200ms, tip diameters 1-2 x 10^{-6}m, Figs. 18 & 19).

(iv) Effects of reserpine and 6-OHDA pre-treatment

The NA content of nerve endings in the guinea-pig vas deferens is reduced by 90% in animals pre-treated with a single dose of reserpine (1mgkg^{-1}, 24h prior to the experiment, Westfall et al., 1975). The effects of reserpine pre-treatment (1mgkg^{-1} for 3 days) on the electrical and mechanical responses of the mouse vas deferens to field stimulation (0.01ms, supramaximal voltage) are shown in figure 20. E.j.p.s and small mechanical contractions were observed. The first few e.j.p.s in a train were smaller than those from untreated animals (perhaps suggesting an excessive dose of reserpine, depleting ATP in addition to NA), but they facilitated to control size (approximately 40mV); as with the e.j.p.s from untreated animals, they were potentiated by prazosin (1 x 10^{-7}M) and abolished by α,βMeATP (3 x 10^{-6}M). Residual mechanical contractions were unaffected by prazosin (1 x 10^{-7}M) but abolished by α,βMeATP (1 x 10^{-6}M). The effectiveness of reserpine pre-treatment was examined using Falck histochemistry (Fig. 21). The virtual absence of fluorescence in treated animals compared with controls suggested that the e.j.p.s and residual mechanical contractions were mediated by a non-adrenergic transmitter, presumably ATP.

When the adrenergic nerves innervating the vas deferens were destroyed by 6-OHDA pre-treatment (150mgkg^{-1} on day 1, 250mgkg^{-1} on day 2, sacrificed on day 3), all electrical and mechanical activity to field stimulation (0.1ms, supramaximal voltage) was abolished (Fig. 22). This suggested that both transmitters were released from noradrenergic nerves, rather than from separate noradrenergic and puriner-

Fig. 18: Intracellularly-recorded membrane potential responses of the mouse vas deferens to micro-application of bradykinin (1 x 10^{-5}M, A.), vasoactive intestinal polypeptide (VIP, 1 x 10^{-5}M, B.), neuropeptide Y (NPY, 1 x 10^{-5}M, C.) and substance P (1 x 10^{-5}M, D.) for increasing periods of time (10-200ms). Micro-pipette tip diameters were 1-2 x 10^{-6}m and ejection pressures 40p.s.i. Bradykinin, at high concentrations (1 x 10^{-5}M), produced small slow hyperpolarizations, while the other peptides were ineffective in producing any change in membrane peotntial. Electrical recordings were made from 4 separate cells.

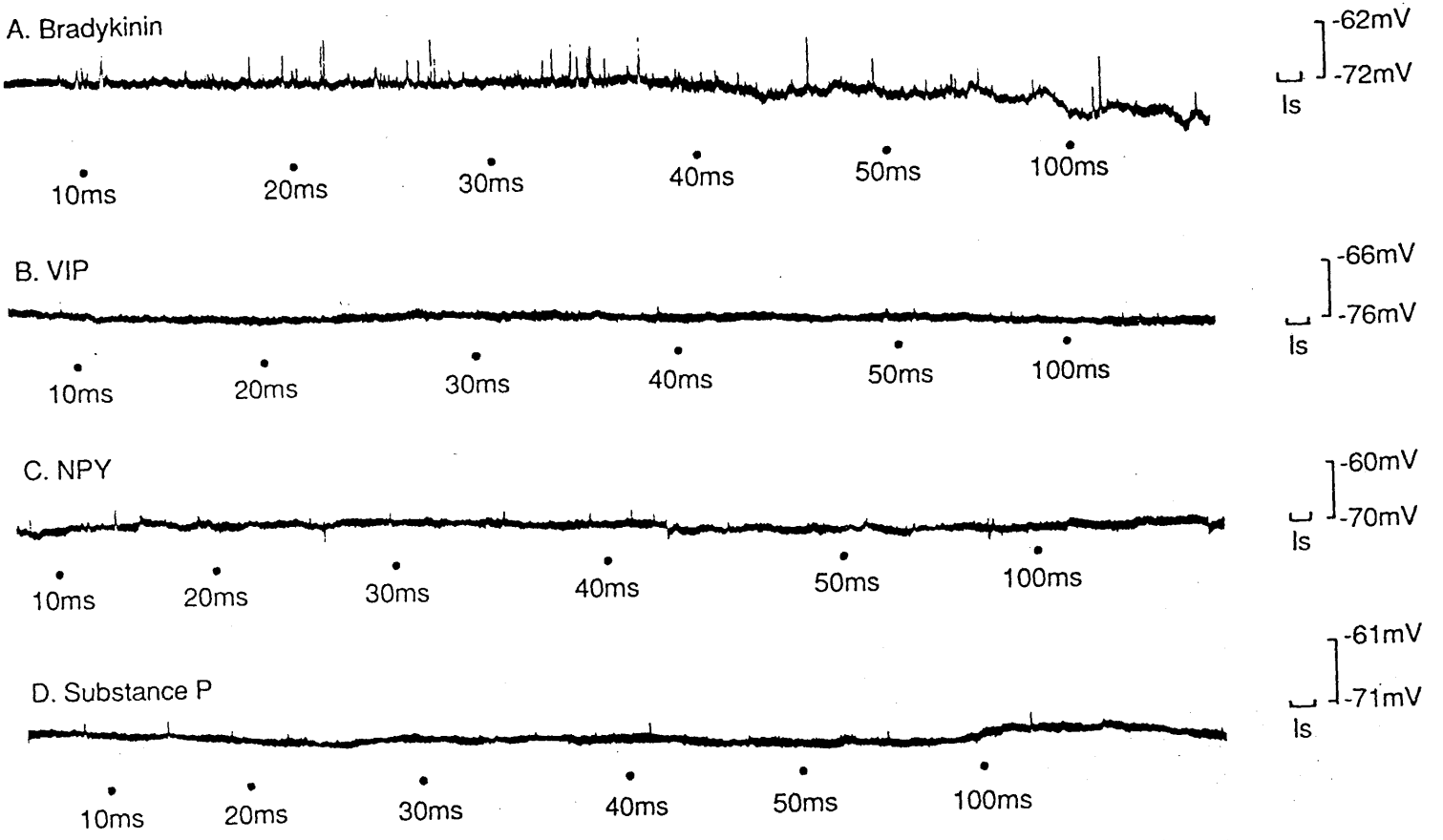

A. Bradykinin
-62mV
-72mV
1s
10ms
20ms
30ms
40ms
50ms
100ms
B. VIP
-66mV
-76mV
1s
10ms
20ms
30ms
40ms
50ms
100ms
C. NPY
-60mV
-70mV
1s
10ms
20ms
30ms
40ms
50ms
100ms
D. Substance P
-61mV
-71mV
1s
10ms
20ms
30ms
40ms
50ms
100ms

Fig. 19: Intracellularly-recorded membrane potential responses of the mouse vas deferens to micro-application of somatostatin (1 x 10^{-5}M, A.), leu-enkephalin (leu-enk, 1 x 10^{-5}M, B.), met-enkephalin (met-enk, 1 x 10^{-5}M, C.) and bombesin (1 x 10^{-5}M, D.) for increasing periods of time (10-200ms). Micro-pipette tip diameters were 1-2 x 10^{-6}m and ejection pressures 40p.s.i. Each peptide was ineffective in producing any change in membrane potential. Electrical recordings were made from 4 separate cells.

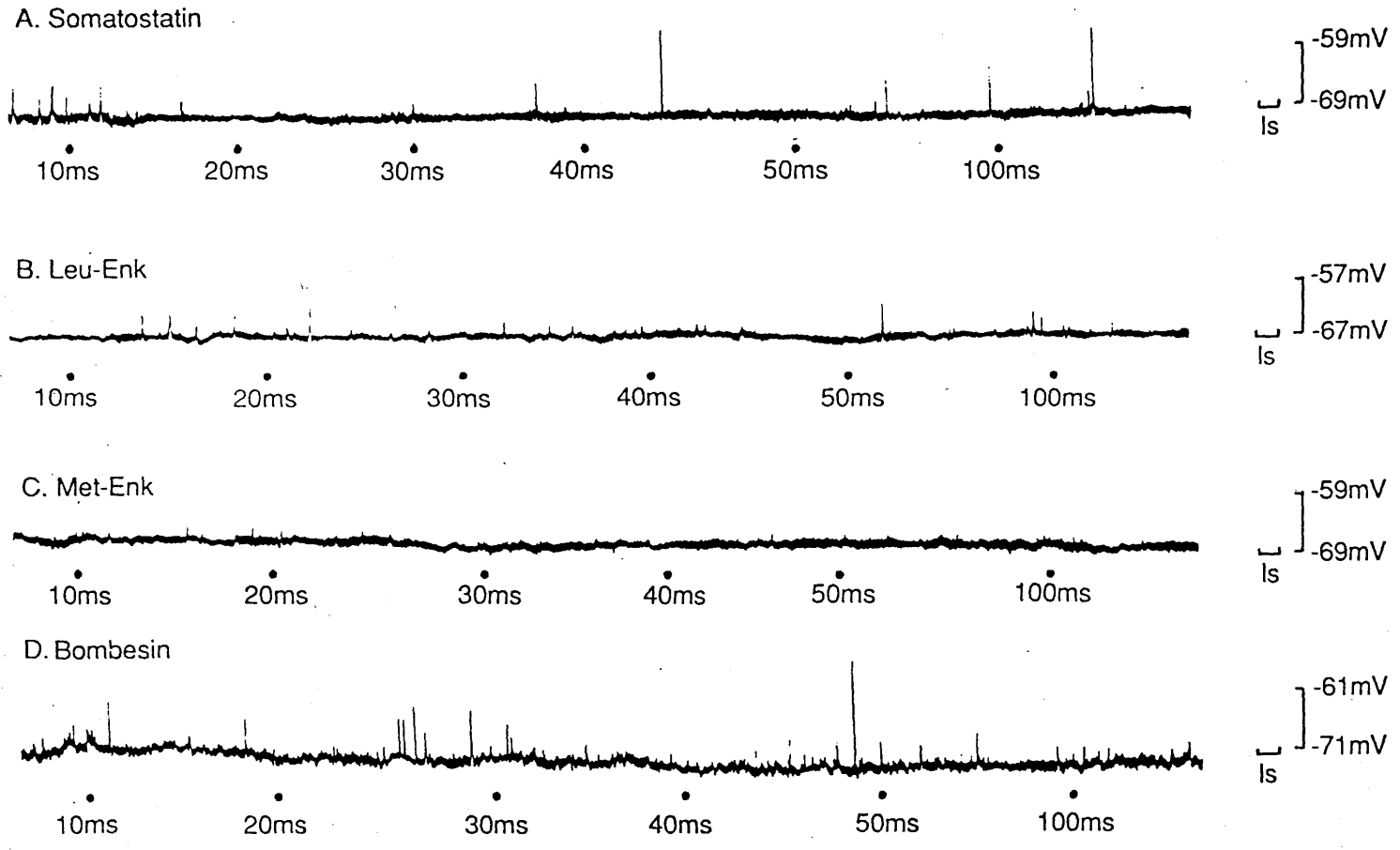
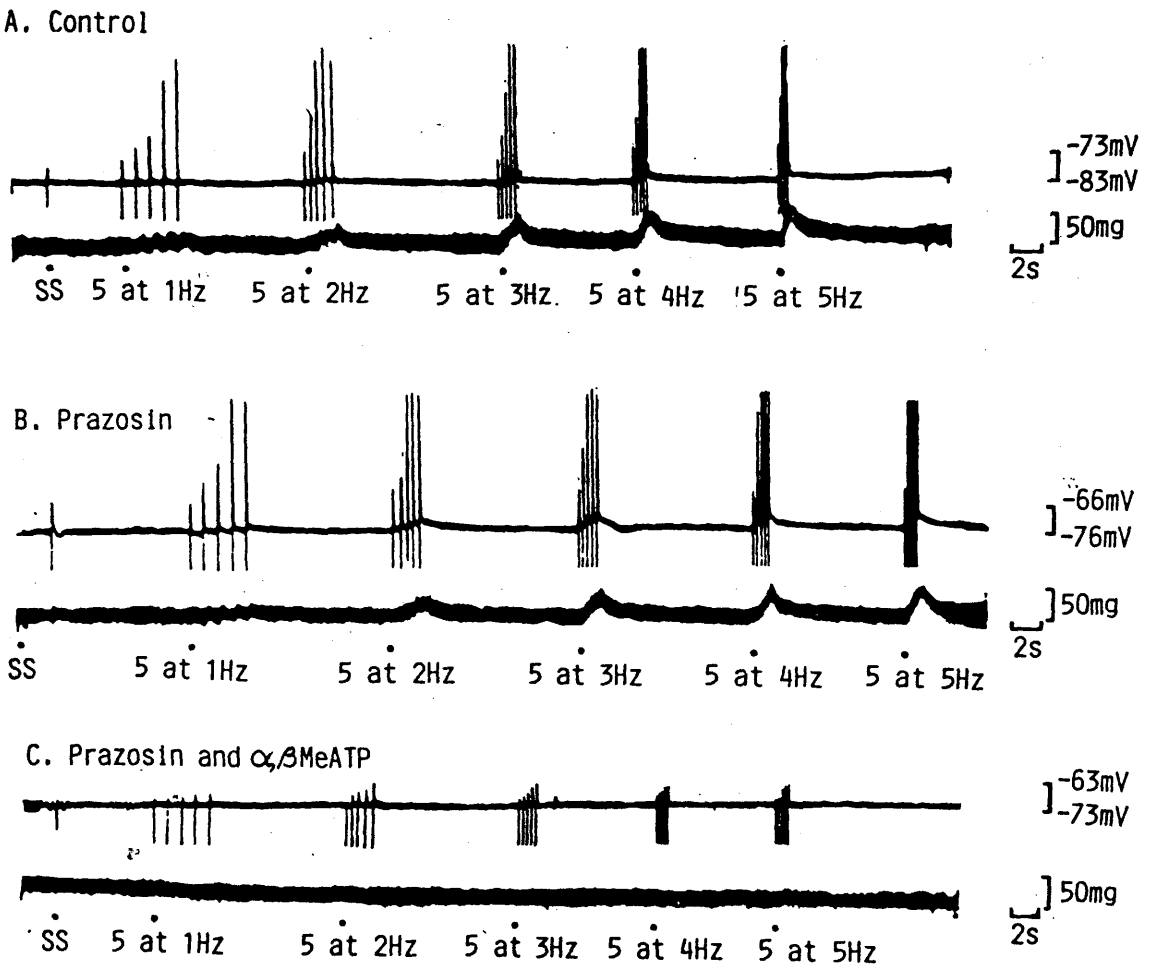
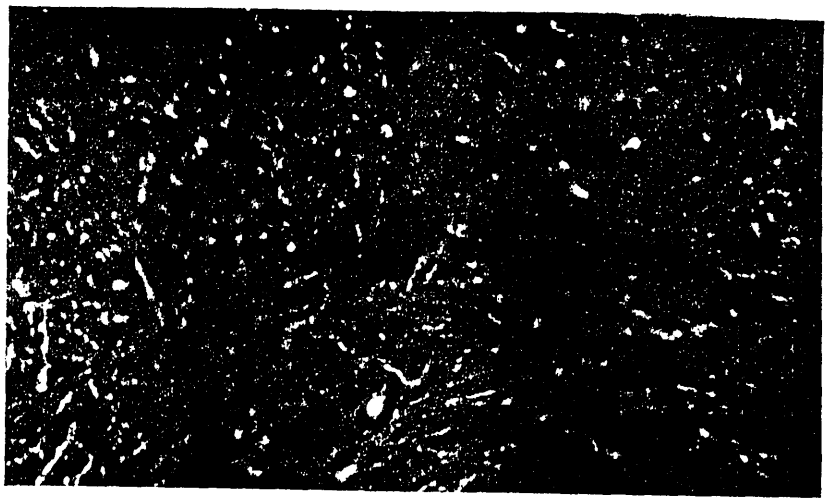

A. Somatostatin
-59mV
-69mV
1s
10ms
20ms
30ms
40ms
50ms
100ms
B. Leu-Enk
-57mV
-67mV
1s
10ms
20ms
30ms
40ms
50ms
100ms
C. Met-Enk
-59mV
-69mV
1s
10ms
20ms
30ms
40ms
50ms
100ms
D. Bombesin
-61mV
-71mV
1s
10ms
20ms
30ms
40ms
50ms
100ms

Fig. 20: The effects of prazosin (1×10^{-7}M, B.) alone and in the presence of α,βMeATP (3×10^{-6}M, C.), compared with control (A.), on the simultaneous electrical (upper trace in each panel) and mechanical responses to field stimulation (0.01ms, supramaximal voltage) of a vas deferens from a mouse pre-treated with reserpine ($1 mg kg^{-1}$ for 3 days). E.j.p.s and small mechanical contractions persisted following reserpine pre-treatment. These electrical and mechanical responses were unaffected by prazosin, but abolished by α,βMeATP, suggesting that they were purinergically mediated. Electrical recordings were made from 3 separate cells.


A. Control
-73mV
-83mV
50mg
2s
SS 5 at 1Hz 5 at 2Hz 5 at 3Hz. 5 at 4Hz 5 at 5Hz
B. Prazosin
-66mV
-76mV
50mg
2s
SS 5 at 1Hz 5 at 2Hz 5 at 3Hz 5 at 4Hz 5 at 5Hz
C. Prazosin and α,βMeATP
-63mV
-73mV
50mg
2s
SS 5 at 1Hz 5 at 2Hz 5 at 3Hz 5 at 4Hz 5 at 5Hz

A.



B.

Fig. 21: The effects of reserpine pre-treatment (1mgkg^{-1} i.p. for 3 days) on the catecholamine content of the mouse vas deferens, determined by histofluorescence (Falck et al., 1962). A. Fluorescence (magnification x 75) of catecholamine-containing neurones in the vas deferens from control mice (pre-treated with the drug vehicle, acetic acid solution). B. The absence of fluorescence (magnification x 75), indicating the depletion of tissue catecholamines by reserpine pre-treatment.

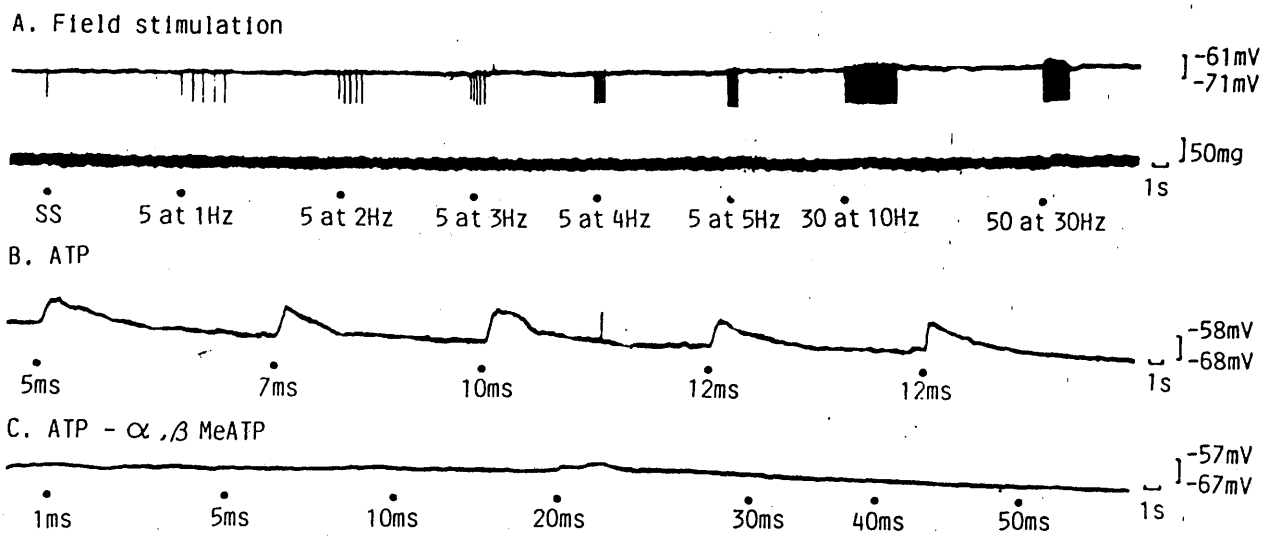


Fig. 22: Intracellularly-recorded electrical (upper trace in A.) and mechanical (A. only) responses of the mouse vas deferens to field stimulation (0.1ms, supramaximal voltage, A.) and exogenously added ATP (1 x 10^{-3}M) in the absence (B.) and presence (C.) of α,βMeATP (1 x 10^{-6}M) following 6-OHDA pre-treatment (150mgkg^{-1} on day 1, 250mgkg^{-1} on day 2, sacrificed on day 3). ATP was applied locally from a micro-pipette by pressure ejection (tip diameter 1 x 10^{-6}m, 40p.s.i., 5-100ms). Following 6-OHDA pre-treatment, field stimulation produced no electrical or mechanical activity, suggesting that both transmitters were released from noradrenergic nerves. Locally-applied ATP still produced membrane depolarizations which were sensitive to α,βMeATP. B. and C. were recorded from the same cell and A. from another cell in the same tissue.

gic nerves - a case of co-transmission.

Furthermore, locally applied ATP (1 x 10^{-3}M) still produced α,βMeATP (1 x 10^{-6}M)-sensitive depolarizations in vasa from 6-OHDA pre-treated mice (Fig. 22), thus ruling out the possibility that exogenously-added ATP was acting pre-synaptically to release NA, which in turn produced the e.j.p.s. The effectiveness of 6-OHDA pre-treatment was again confirmed using Falck histochemistry.

(v) Effects of changing the external ionic environment on the excitatory responses to field stimulation

(a) Potassium

A reduction in $[K^+]_o$, from 4.7 x 10^{-3}M (control) to 2.35 x 10^{-3}M, hyperpolarized the membrane potential from -73.6 ± 0.4mV to -94.2 ± 0.8mV (mean ± s.e.m., n=224 and 17 respectively). E.j.p.s and action potentials were potentiated, but there was little effect on the contractile responses to field stimulation (0.01ms, supramaximal voltage, Fig. 23). Complete removal of $[K^+]_o$ (Fig.24) hyperpolarized and so destabilised the membrane potential (-84.1 ± 1.84mV, n=12), making an accurate evaluation of E_m difficult and induced spontaneous electrical activity. The electrical responses produced by field stimulation (0.01ms, supramaximal voltage) were reduced in amplitude but prolonged in duration, suggesting that K^+ may be involved in the repolarizing phase of the action potential. The accompanying mechanical contractions were greatly reduced.

A doubling in $[K^+]_o$ (to 9.4 x 10^{-3}M) depolarized the membrane potential from -73.6 ± 0.4mV to -61.0 ± 1.6mV (mean ± s.e.m., n=224 and 10 respectively), potentiated the mechanical contractions and reduced the amplitude of the e.j.p.s (Fig. 25).

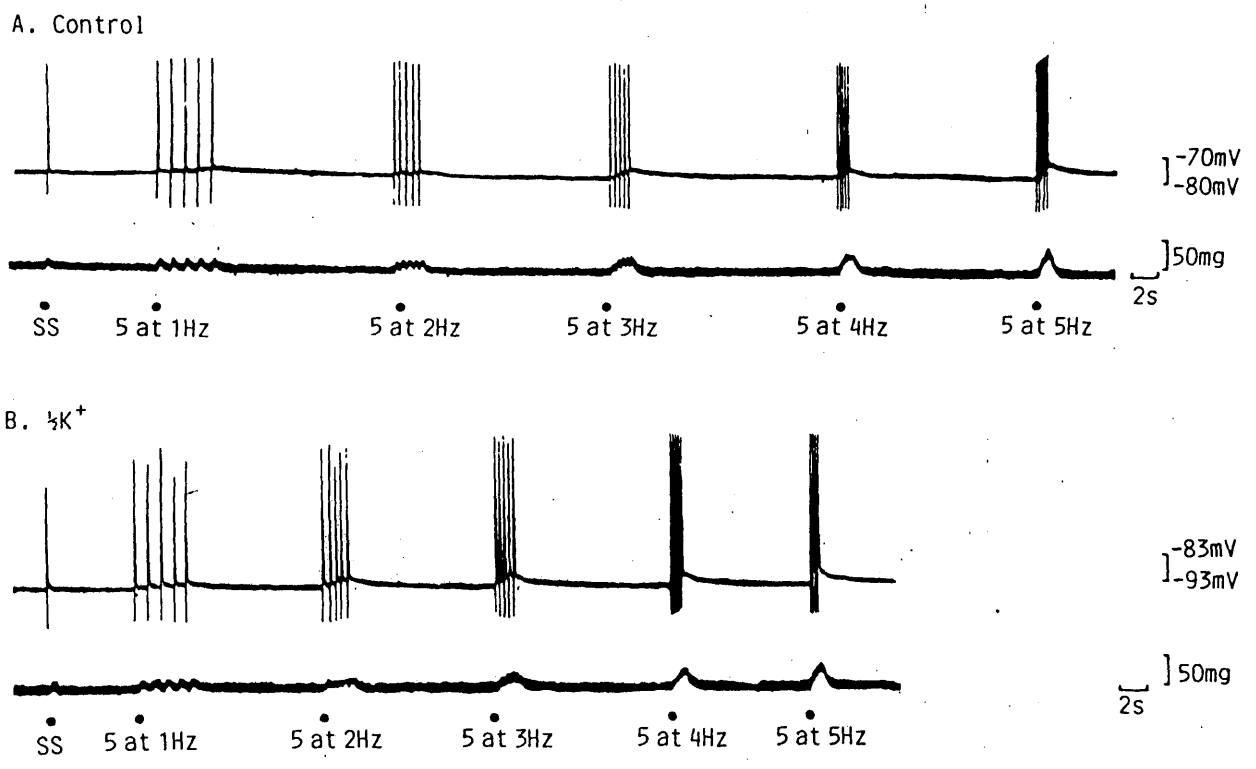


Fig. 23: The effects of halving the $[K^+]_o$ to 2.35 x 10^{-3}M on the electrical (upper trace in each panel) and mechanical activity evoked by field stimulation (0.01ms, supramaximal voltage) in the mouse vas deferens. Halfing the $[K^+]_o$ hyperpolarized the membrane potential, potentiated the amplitude of the e.j.p.s but had little effect on the contractile events. Electrical recordings were made from the same cell.

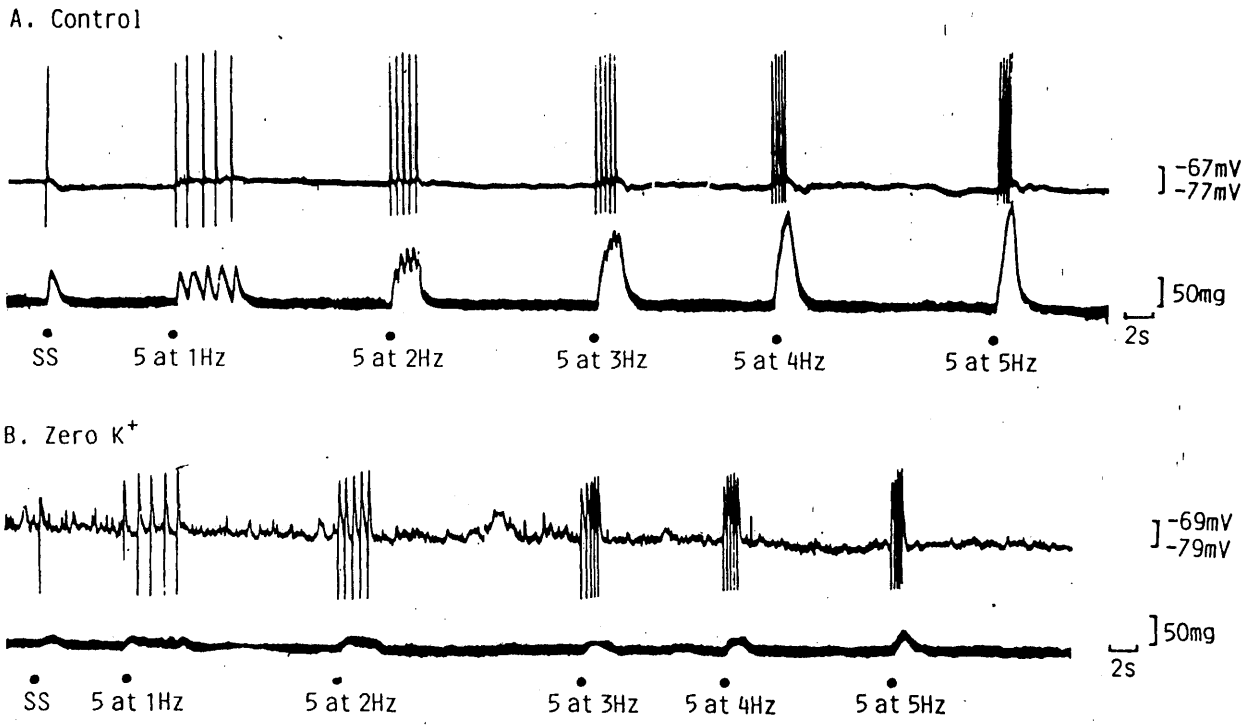


Fig. 24: The effects of complete removal of the $[K^+]_o$ on the electrical (upper trace in each panel) and mechanical activity evoked by field stimulation (0.01ms, supramaximal voltage) in the mouse vas deferens. Complete removal of $[K^+]_o$ hyperpolarized the membrane potential and induced spontaneous electrical activity. The e.j.p.s were reduced in amplitude but prolonged in duration. The accompanying mechanical contractions were greatly reduced. Electrical recordings were made from the same cell.

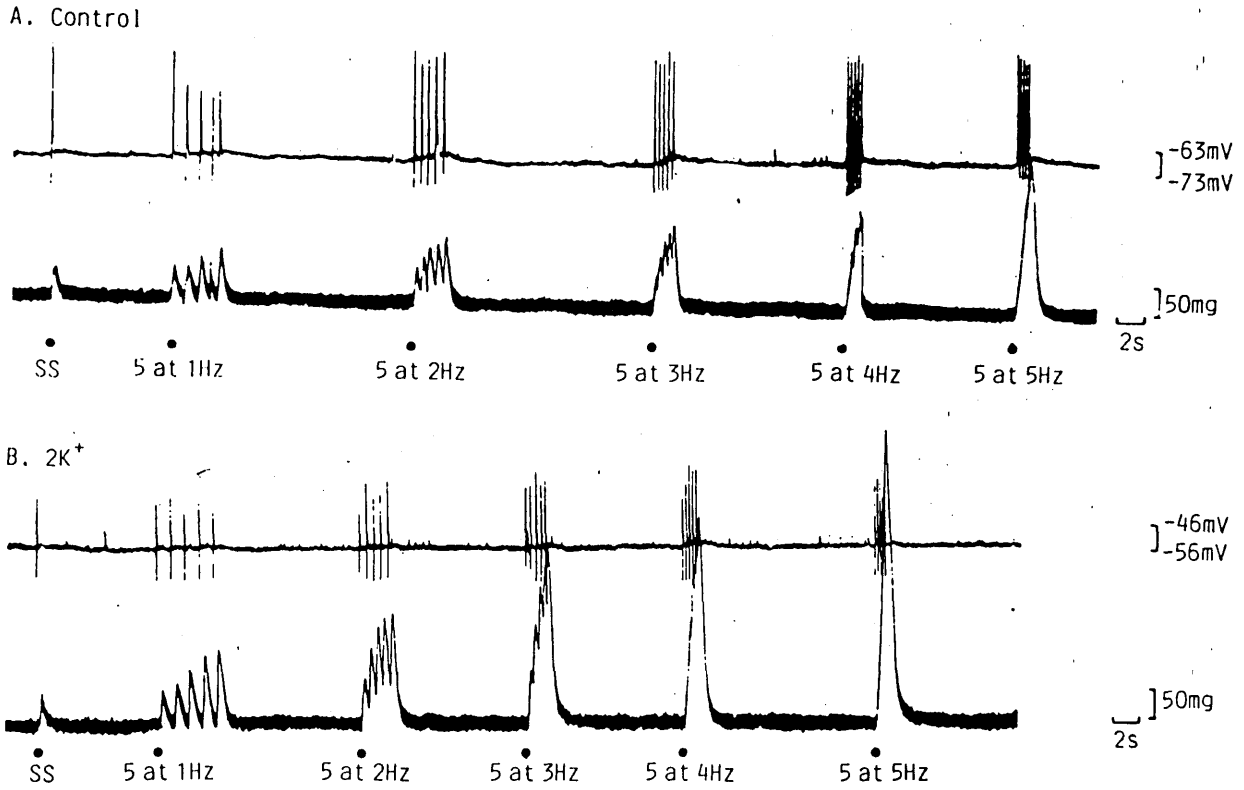


Fig. 25: The effects of doubling the $[K^+]_o$ to 9.4 x 10^{-3}M on the electrical (upper trace in each panel) and mechanical activity evoked by field stimulation (0.01ms, supramaximal voltage) in the mouse vas deferens. Doubling $[K^+]_o$ depolarized the membrane potential, reduced the amplitude of the e.j.p.s and potentiated the mechanical contractions. Electrical recordings were made from the same cell.

(b) Sodium

$[Na^+]_o$-deficient Krebs solution was made by replacing NaCl with choline Cl. A reduction in $[Na^+]_o$ from 144 x 10^{-3}M (control) to 104.5 x 10^{-3}M depolarized the membrane potential from -73.6 ± 0.4mV to -55.1 ± 1.47mV (mean ± s.e.m., n=224 and 18 respectively) and reduced the amplitude of the e.j.p.s and action potentials evoked by field stimulation (0.01ms, supramaximal voltage). A further reduction in $[Na^+]_o$ to 75 x 10^{-3}M (Fig. 26) progressively depolarized the membrane potential (-49.1 ± 1.36mV, n=22) and virtually abolished the e.j.p.s, suggesting the Na^+ was involved in the e.j.p.s and action potentials in this tissue. Mechanical contractions to field stimulation were potentiated by a reduction in the $[Na^+]_o$, probably due to the depolarization of the membrane.

(c) Chloride

Complete removal of $[Cl^-]_o$ slightly depolarized the membrane potential from -73.6 ± 0.4mV to -61.8 ± 1.6mV (n=224 and 14 respectively, Fig. 27). The first e.j.p. in each train was unaffected, but subsequent e.j.p.s were reduced in amplitude, reaching a plateau, but not being abolished. Contractile responses to field stimulation (0.01ms, supramaxamal voltage) were reduced by about 50%.

(d) Calcium

In the absence of $[Ca^{2+}]_o$, both electrical and mechanical responses to field stimulation (0.01ms, supramaximal voltage) were reduced and eventually abolished (after 15-20min) presumably due to the inhibition of transmitter release (Fig. 28).

(e) Effects of tetraethylammonium (TEA)

The effects of TEA, which blocks certain K^+ channels (Imaizumi &

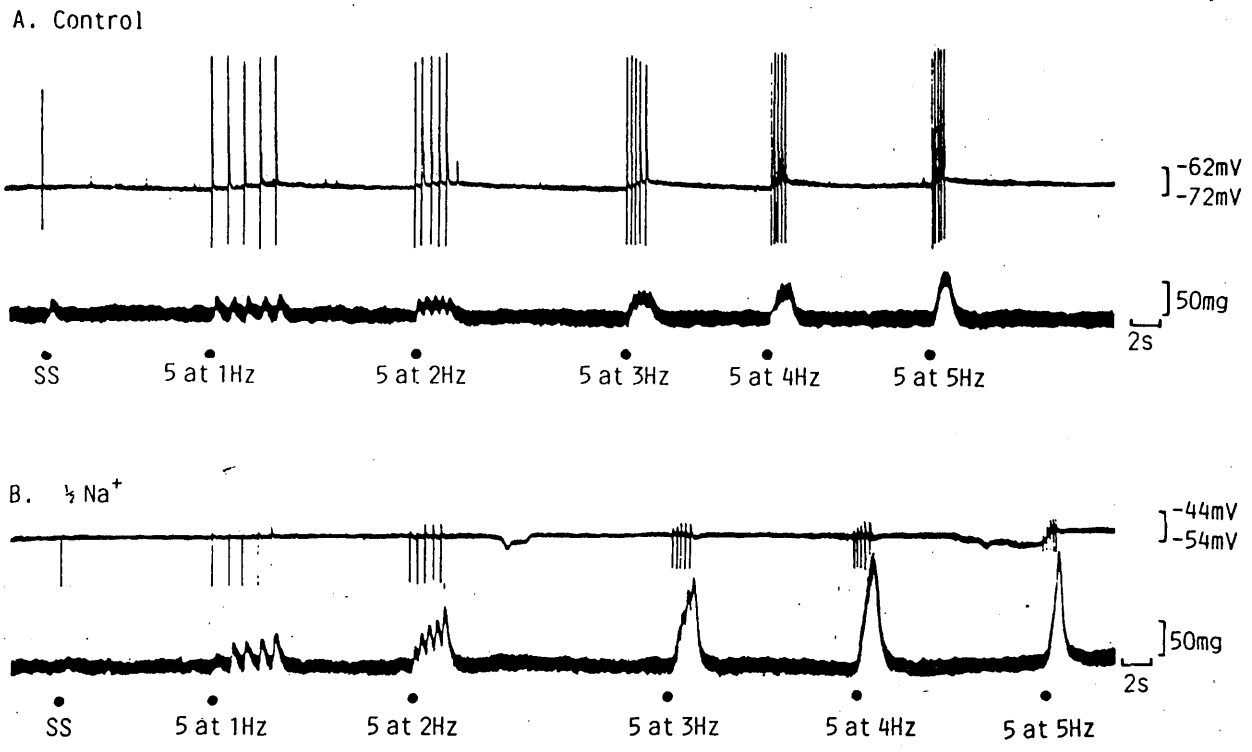


Fig. 26: The effects of reducing the $[Na^+]_o$ to 75 x 10^{-3}M on the electrical (upper trace in each panel) and mechanical activity evoked by field stimulation (0.01ms, supramaximal voltage) in the mouse vas deferens. Halfing the $[Na^+]_o$ depolarized the membrane, virtually abolished the e.j.p.s and potentiated the mechanical contractions Electrical recordings were made from the same cell.

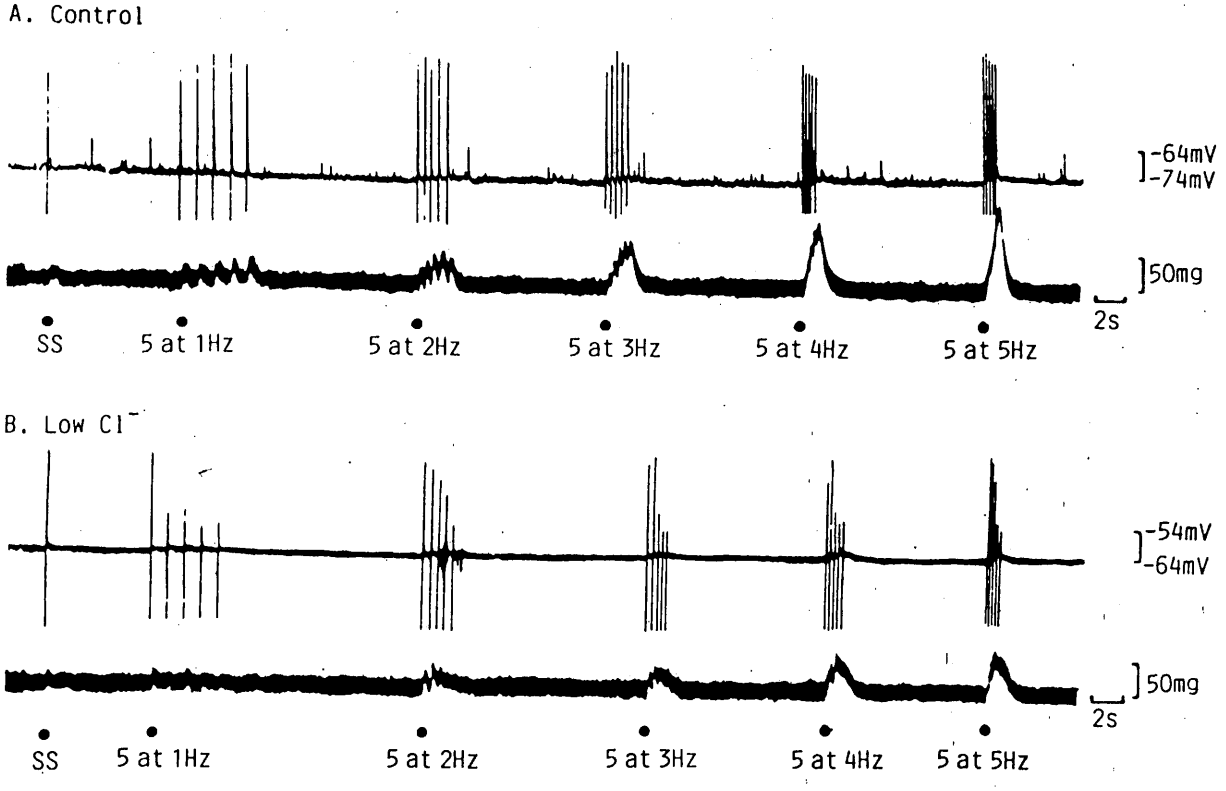


Fig. 27: The effects of low $[Cl^-]_o$ Krebs solution on the electrical (upper trace in each panel) and mechanical activity evoked by field stimulation (0.01ms, supramaximal voltage) in the mouse vas deferens. Cl^- removal depolarized the membrane potential. The first e.j.p. in a train of 5 was unaffected, but subsequent e.j.p.s were reduced in amplitude, as were the mechanical contractions. Electrical recordings were made from the same cell.

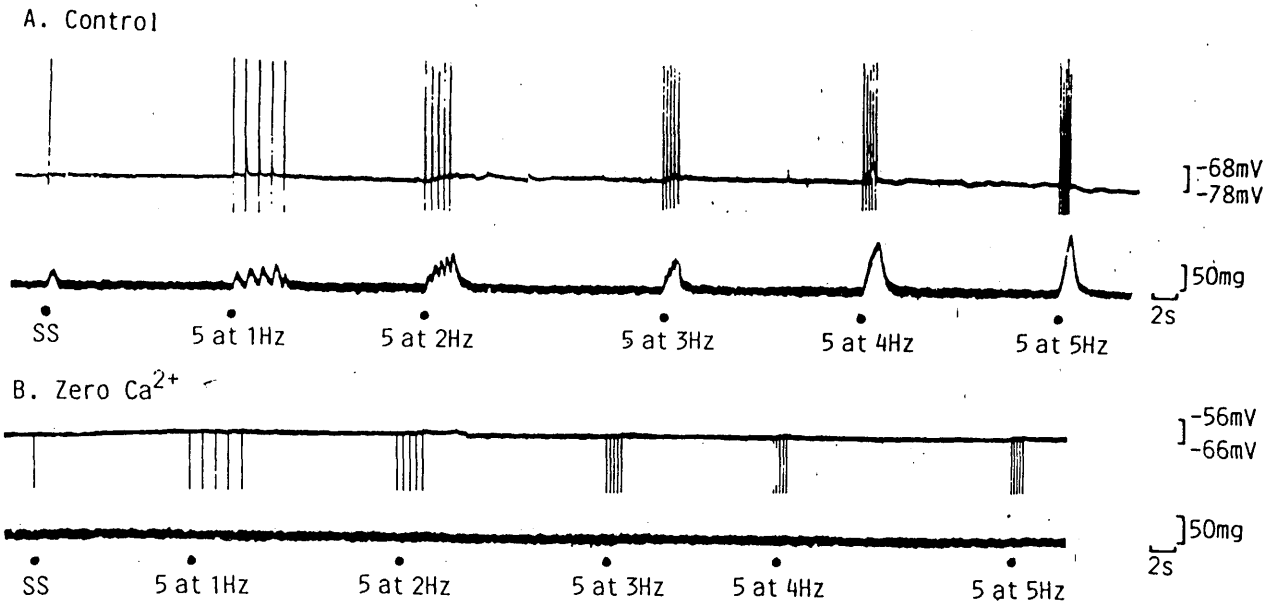


Fig. 28: The effects of $[Ca^{2+}]$ removal (for 20min) on the electrical (upper trace in each panel) and mechanical activity evoked by field stimulation (0.01ms, supramaximal voltage) in the mouse vas deferens. Removal of $[Ca^{2+}]_o$ abolished all electrical and mechanical activity, presumably due to the Ca^{2+}-dependence of transmitter release. Electrical recordings were made form the same cell.

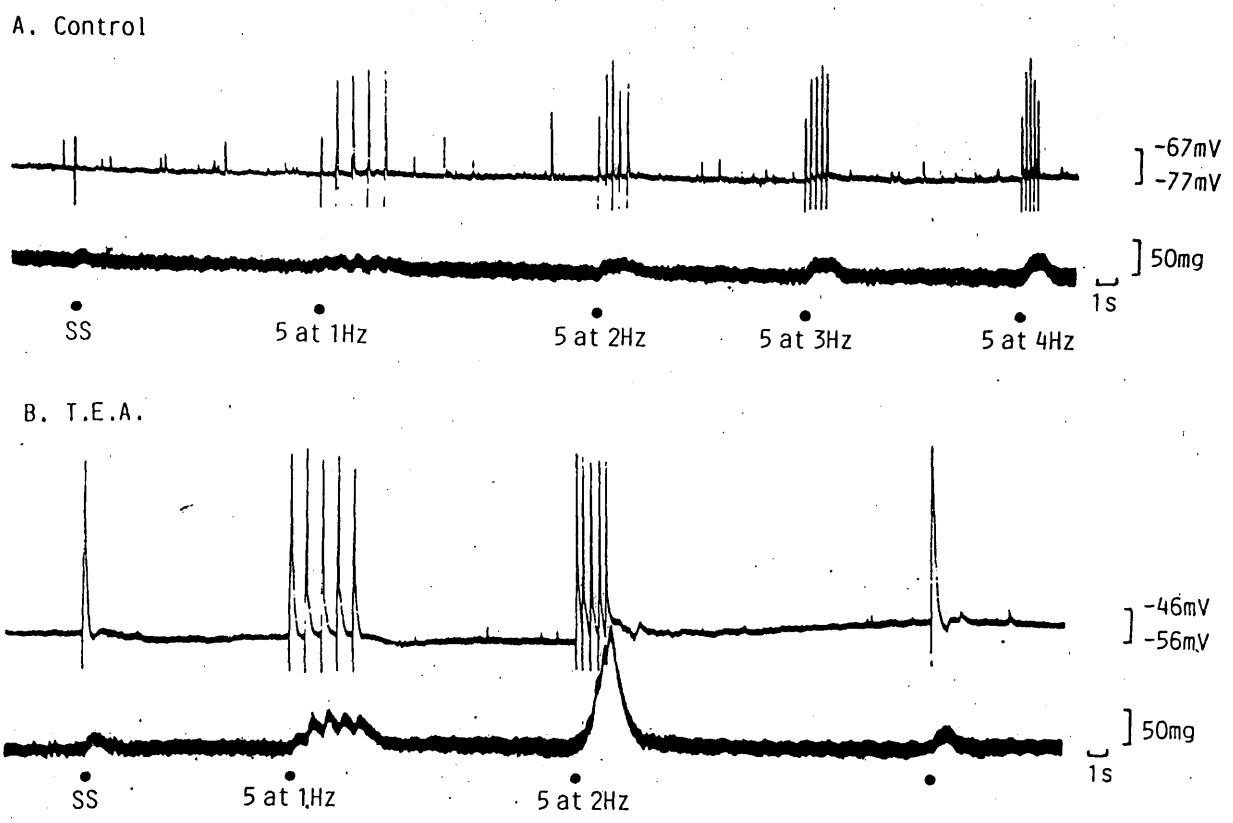


Fig. 29: The effects of tetraethylammonium (TEA, 2 x 10^{-3}M) on the electrical (upper trace in each panel) and mechanical responses to field stimulation (0.01ms, supramaximal voltage) in the mouse vas deferens. TEA depolarised the membrane potential, increased the amplitude and prolonged the duration of the e.j.p.s and potentiated the contractile responses. Electrical recordings were made from the same cell.

Watenabe, 1981), was investigated as a further indication of K^+ involvement in the e.j.p. and action potential in the mouse vas deferens.

TEA (2×10^{-3}M) depolarized the membrane potential from -73.6 ± 0.4mV to -61.3 ± 1.6mV (n=224 and 12 respectively, Fig. 29). The e.j.p.s and action potentials were potentiated in amplitude and prolonged in duration, suggesting that an increase in K^+ permeability underlies the repolarizing phase of the action potential. Contractile responses to field stimulation (0.01ms, supramaximal voltage) were greatly potentiated, presumably due to the depolarizing action of TEA.

II. RABBIT MESENTERIC ARTERY

A. CONTRACTILE RESPONSES TO FIELD STIMULATION, NA AND ATP

The isolated rabbit mesenteric bed preparation was perfused, via the superior mesenteric artery, at a constant rate of $4 ml min^{-1}$. Under these conditions, the basal perfusion pressure, as measured at the end of a 30min equilibration period, was 23.4 ± 0.5mmHg (n=27).

Electrical field stimulation of the superior mesenteric artery (0.5ms, supramaximal voltage, 100 pulses at 1-50Hz, Figs. 30 & 31), NA (0.1-0.3ml bolus injections of 1×10^{-6} -1×10^{-3}M NA, Fig. 32) and ATP (0.1-0.3ml bolus injections of 1×10^{-5} -1×10^{-2}M ATP, Fig. 33) each produced a vasoconstriction. The maximal response to NA exceeded that to either ATP or field stimulation.

The α-adrenoceptor antagonists phentolamine ($1\text{-}10 \times 10^{-6}$M) and prazosin ($1\text{-}10 \times 10^{-7}$M) each depressed the pressor responses to field stimulation by about 80% (Fig. 30), shifted the dose-response curve to NA to the right (Fig. 32), but left that to ATP unaffected (Fig. 33). α,βMeATP ($1\text{-}10 \times 10^{-6}$M), on the other hand, depressed the pressor responses to field stimulation by about 30% (Fig. 31), shifted the dose-response curve to ATP to the right (Fig. 33), but left that to NA

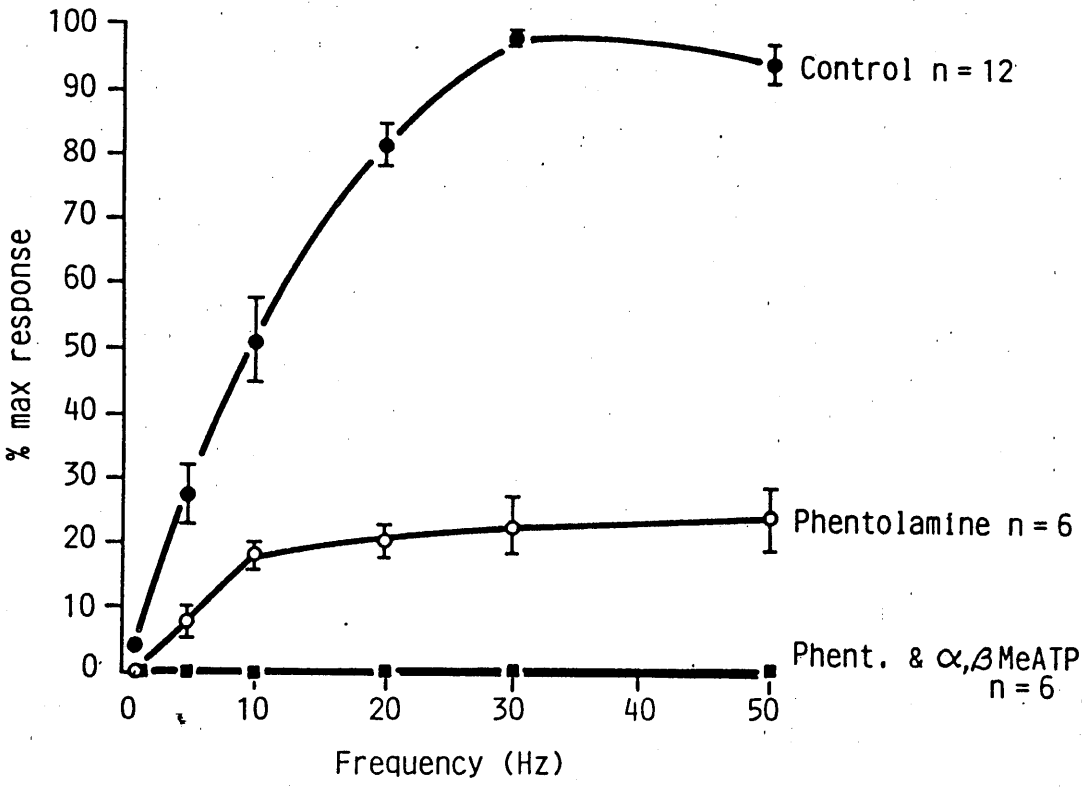


Fig. 30: The effects of increasing frequency on the mean pressor responses, expressed as a % of the maximum control in each tissue, of the perfused isolated rabbit mesenteric bed preparation of field stimulation (0.5ms, supramaximal voltage, 100 pulses at 1-50Hz) via the superior mesenteric artery. Each graph shows the mean (± s.e.m.) of a number (n) of observations. The α-adrenoceptor antagonist phentolamine (5×10^{-6}M) reduced the pressor responses by around 80% and the residual contractions were abolished by the additional presence of α,βMeATP (1×10^{-6}M).

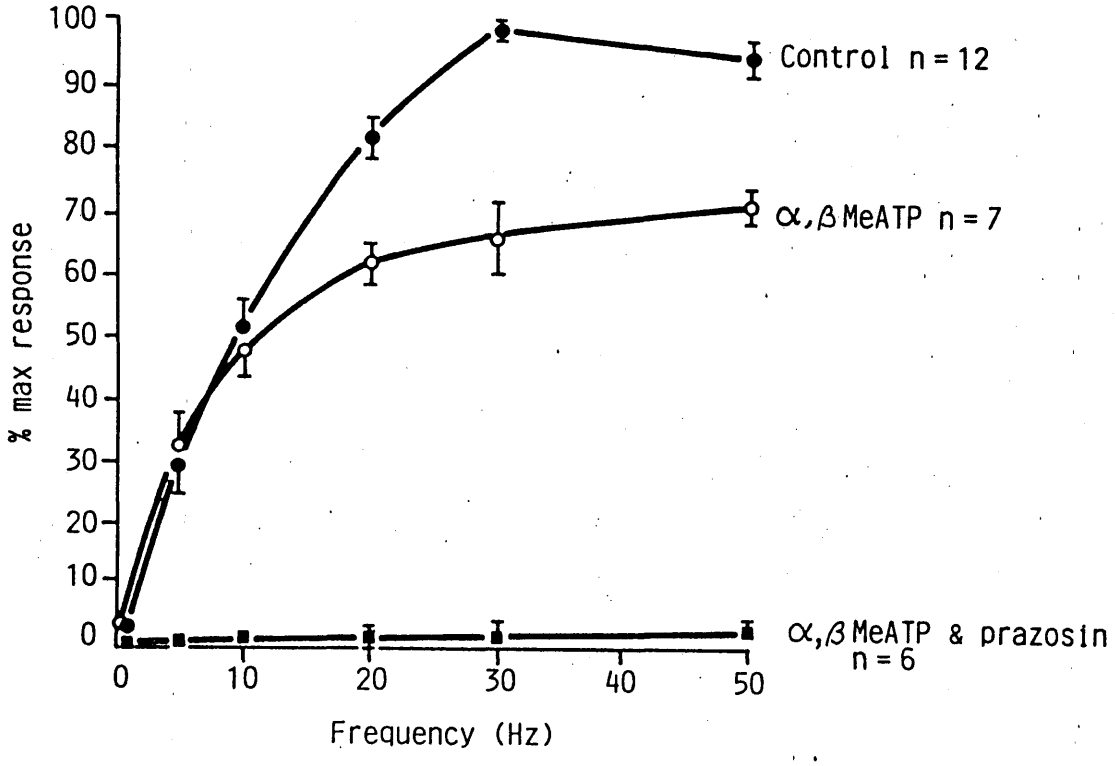


Fig. 31: The effects of increasing frequency on the mean pressor responses, expressed as a % of the maximum control in each tissue, of the perfused isolated rabbit mesenteric bed preparation to field stimulation (0.5ms, supramaximal voltage, 100 pulses at 1-50Hz) via the superior mesenteric artery. Each graph shows the mean (± s.e.m.) of a number (n) of observations. The P_2-receptor antagonist α,βMeATP (1 x 10^{-6}M) reduced the pressor responses by around 30% and the residual contractions were abolished by the additional presence of the α-blocker prazosin (1 x 10^{-7}M).

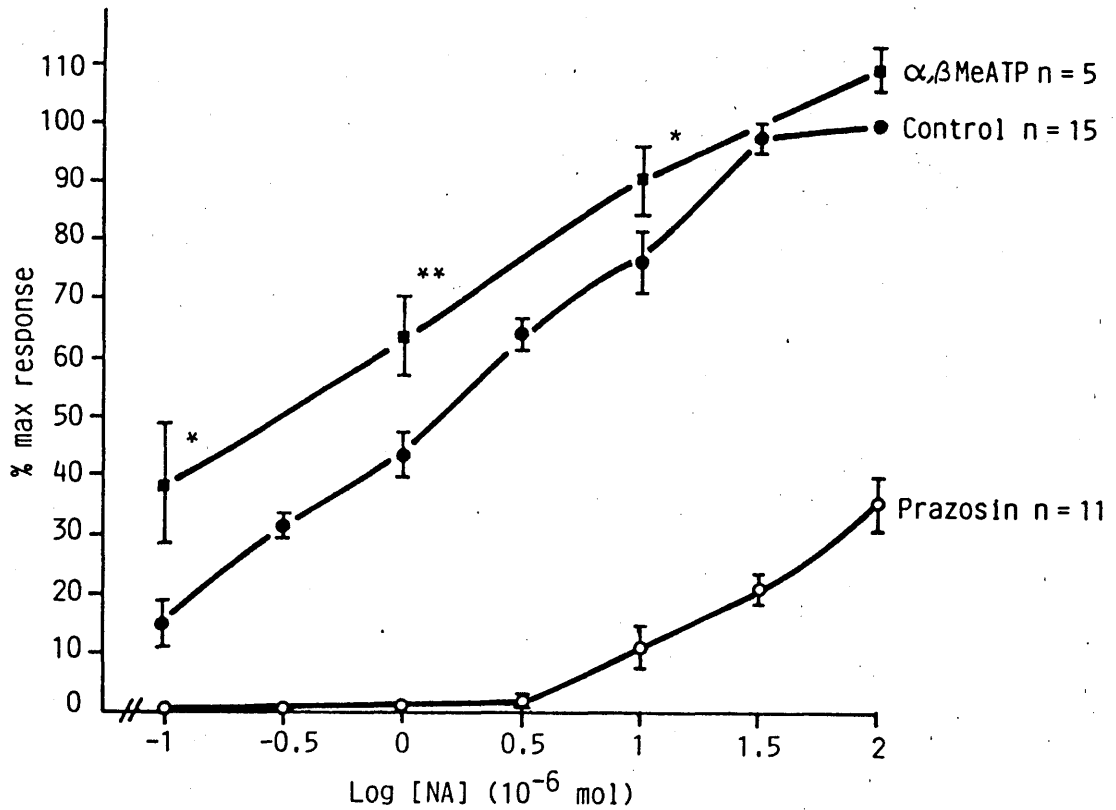


Fig. 32: The mean pressor responses, expressed as a % of the maximum control in each tissue, of the perfused isolated rabbit mesenteric bed preparation to bolus injections (0.1 - 0.3ml) of NA (1×10^{-6} - 1×10^{-3}M), injected via the superior mesenteric artery. Each graph shows the mean (± s.e.m.) of a number (n) of observations. α,βMeATP (1×10^{-6}M) potentiated (* $p < 0.05$, ** $p < 0.02$) the pressor responses, probably due to a depolarizing action of the drug, whereas prazosin (1×10^{-7}M) shifted the dose-response curve to the right.

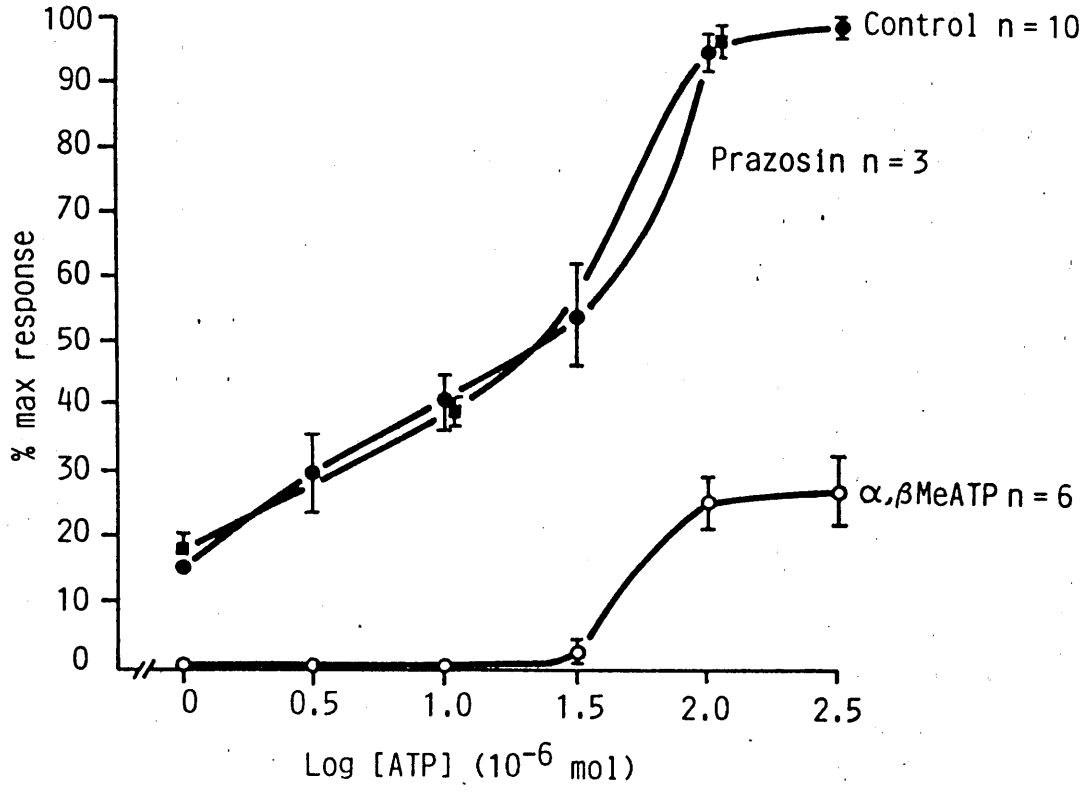


Fig. 33: The mean pressor responses, expressed as a % of the maximum control in each tissue, of the perfused isolated mesenteric bed preparation to bolus injections (0.1 - 0.3ml) of ATP (1×10^{-5} - 1×10^{-3}M), injected via the superior mesenteric artery. Each graph shows the mean (± s.e.m.) of a number (n) of observations. Pressor responses were unaffected by prazosin (1×10^{-7}M), but antagonized by α,βMeATP (1×10^{-6}M).

either unaffected or slightly potentiated (Fig. 32). α,βMeATP (1 x 10^{-6}M) together with either prazosin (1 x 10^{-7}M) or phentolamine (1 x 10^{-6}M) abolished all pressor responses to field stimulation (Figs 30 & 31). From these results it was concluded that both NA and ATP played a role in the contractile response of the rabbit mesenteric bed preparation to field stimulation.

B. ELECTRICAL ACTIVITY IN THE RABBIT SUPERIOR MESENTERIC ARTERY

The mean resting membrane potential of the rabbit superior mesenteric artery, as measured using conventional glass micro-electrodes was -68.7 ± 1.0mV, n=39. Spontaneous e.j.p.s were rarely observed.

Field stimulation (0.1ms, supramaximal voltage, 1-10Hz) of the superior mesenteric artery produced e.j.p.s which facilitated and summated to fire action potentials (Fig. 34).

When intracellular electrical and mechanical activity in response to field stimulation (0.1ms, supramaximal voltage, 5-100Hz) were recorded simultaneously (Figs. 35 & 36), the e.j.p.s and action potentials were seen to be accompanied by contractile events. The α-adrenoceptor antagonist phentolamine (1-10 x 10^{-6}M) had no effect on the electrical activity and reduced, but did not abolish, the mechanical contractions. The e.j.p.s, action potentials and the residual mechanical responses were abolished by the additional presence of α,βMeATP (1 x 10^{-6}M, Fig. 35). When the order of drug addition was reversed (Fig. 36) a similar picture emerged. α,βMeATP (1 x 10^{-6}M) abolished all electrical activity, but only reduced the mechanical contractions. Addition of the α-blocker phentolamine (1 x 10^{-6}M) abolished all residual contractile events.

These results suggested that, in the rabbit mesenteric artery, two transmitters (NA and ATP) played a role in the contractile response

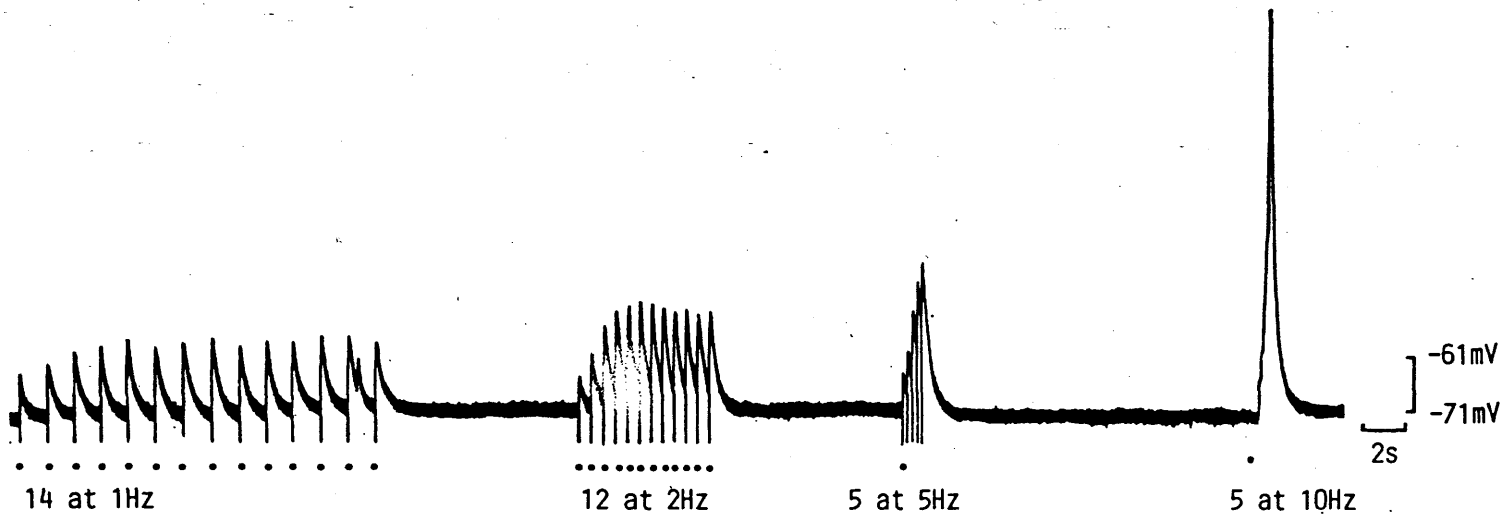


Fig. 34: The intracellularly-recorded electrical responses of the rabbit superior mesenteric artery to field stimulation (0.1ms, supra-maximal voltage, 1-10Hz). The electrical activity consisted of e.j.p.s which facilitated at frequencies of 1Hz and above, facilitated and summated at 2Hz and above and fused at 10Hz to fire action potentials.

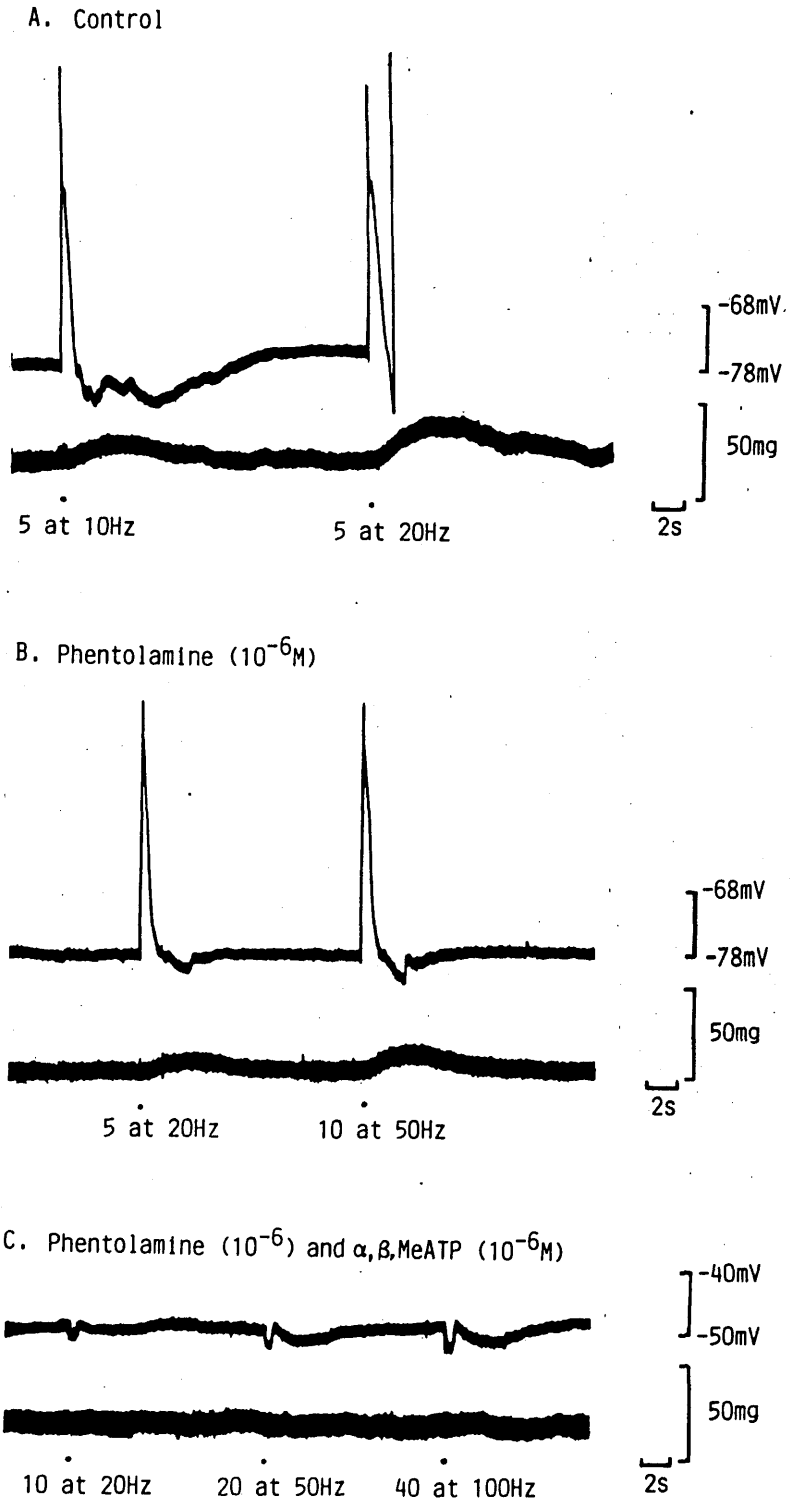
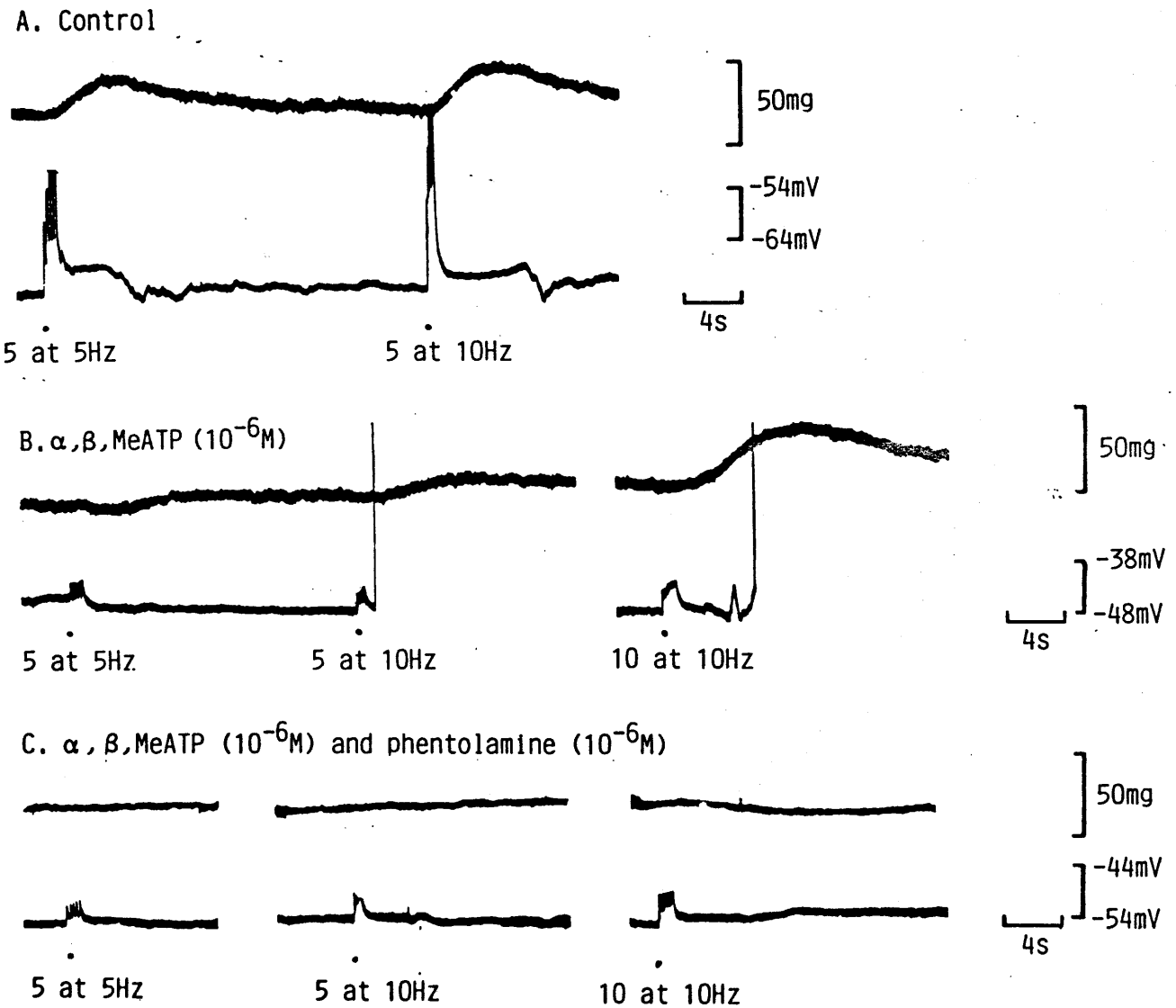


Fig. 35: The intracellular electrical (upper trace in each panel) and mechanical responses of the rabbit superior mesenteric artery to field stimulation (0.1ms, supramaximal voltage, 1-100Hz), In the control situation (A.), field stimulation produced e.j.p.s and action potentials which were accompanied by mechanical contractions. Note impalement of cell lost after 2nd stimulus. The α-adrenoceptor antagonist phentolamine (1 x 10^{-6}M, B.) had no effect on the electrical events, but reduced the mechanical contractions. All residual electrical and mechanical activity was abolished, leaving only stimulus artifacts, by the additional presence of α,βMeATP (1 x 10^{-6}M, C.). Electrical recordings were made from 3 separate cells.

Fig. 36: The intracellular electrical (lower trace in each panel) and mechanical responses of the rabbit superior mesenteric artery to field stimulation (0.1ms, supramaximal voltage, 5-10Hz). In the control situation (A.), field stimulation produced e.j.p.s and action potentials which were accompanied by mechanical contractions. α,βMeATP (1×10^{-6}M, B.) abolished the e.j.p.s and action potentials, leaving only stimulus artifacts, and reduced the mechanical contractions. Note impalement of the cell was lost after the 2nd stimulus, regained, then lost again after the 3rd stimulus. Residual mechanical activity was abolished by the additional presence of the α-blocker phentolamine (1×10^{-6}M, C.). Electrical recordings were made from 3 separate cells.


A. Control
50mg
-54mV
-64mV
4s
5 at 5Hz
5 at 10Hz
B. α,β,MeATP (10^{-6}M)
50mg
-38mV
-48mV
4s
5 at 5Hz
5 at 10Hz
10 at 10Hz
C. α,β,MeATP (10^{-6}M) and phentolamine (10^{-6}M)
50mg
-44mV
-54mV
4s
5 at 5Hz
5 at 10Hz
10 at 10Hz

to field stimulation, but only ATP seemed to mediate the electrical event. Since all electrical activity was abolished by guanethidine (1 x 10^{-6}M) and TTX (1 x 10^{-6}M), it appeared that both transmitters were being released from noradrenergic nerves and that the rabbit mesenteric artery exhibits co-transmission.

III. RABBIT EAR ARTERY

A. CONTRACTILE RESPONSES TO FIELD STIMULATION, NA AND ATP

The isolated rabbit central ear artery was perfused at a constant rate of 4mlmin^{-1}. Under these conditions, the basal perfusion pressure, as measured at the end of a 30min equilibration period, was 16.3 ± 0.4mmHg, n=25.

Electrical field stimulation (0.5ms, supramaximal voltage, 50 pulses at 1-50Hz, Figs. 37 & 38), NA (0.05-0.2ml bolus injections of 1 x 10^{-6} -1 x 10^{-5}M NA, Fig 39) and ATP (0.05-0.4ml bolus injections of 1 x 10^{-3}M ATP, Fig 40) each elicited a vasoconstriction. The maximal response to NA exceeded that to either ATP or field stimulation.

The α-adrenoceptor antagonists phentolamine (1-10 x 10^{-6}M) and prazosin (1-10 x 10^{-7}M) greatly reduced the pressor responses to both field stimulation (Fig. 37) and exogenous NA (Fig. 39), while leaving those to exogenous ATP unaffected. α,βMeATP (1-10 x 10^{-6}M), at a dose which abolished the pressor responses to exogenous ATP (Fig. 40), alone, potentiated the pressor responses to field stimulation (Fig. 38) and in the presence of α-adrenoceptor antagonists (Fig. 37) failed to further reduce the residual vasoconstrictions. Pressor responses resistant to both prazosin (1 x 10^{-7}M) or phentolamine (1 x 10^{-6}M) and α,βMeATP (1 x 10^{-6}M) were abolished by TTX (1 x 10^{-6}M).

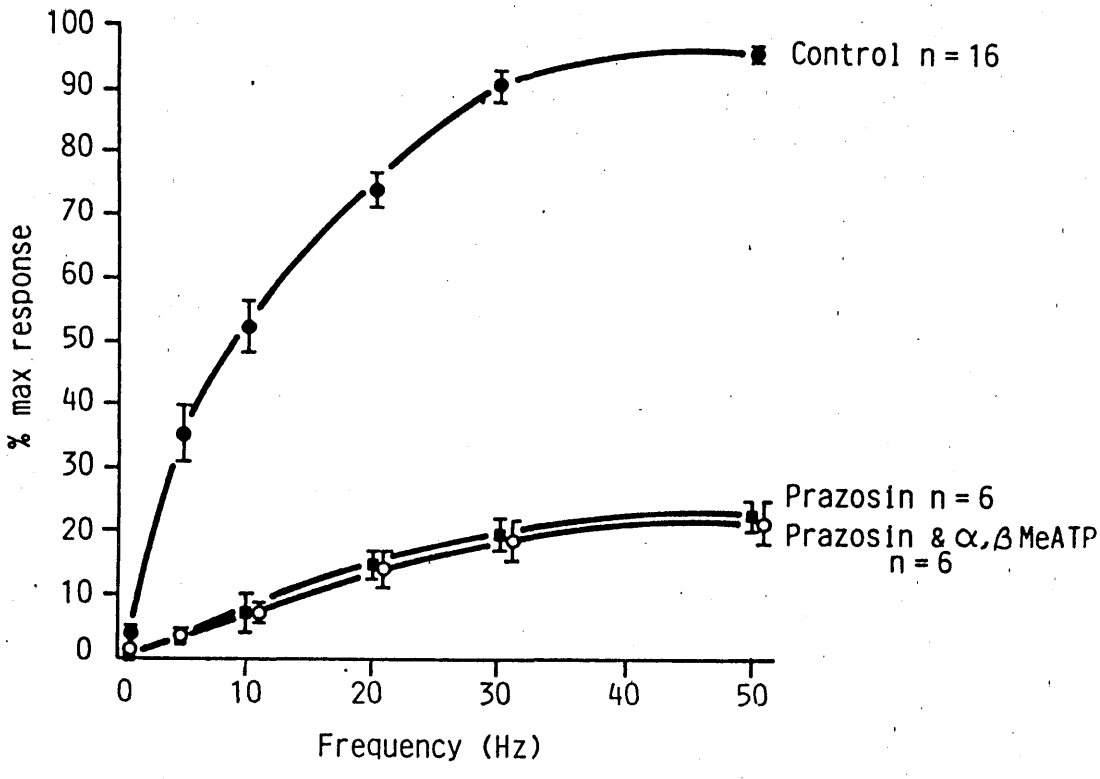


Fig. 37: The effects of increasing frequency on the mean pressor response, expressed as a % of the maximum control in each tissue, of the perfused rabbit central ear artery to field stimulation (0.5ms, supramaximal voltage, 50 pulses at 1-50Hz). Each graph shows the mean (± s.e.m.) of a number, (n), of observations. The α-adrenoceptor antagonist prazosin (1×10^{-7}M) reduced the pressor responses by around 80%, the residual contractions being unaffected by the additional presence of α,βMeATP (1×10^{-6}M).

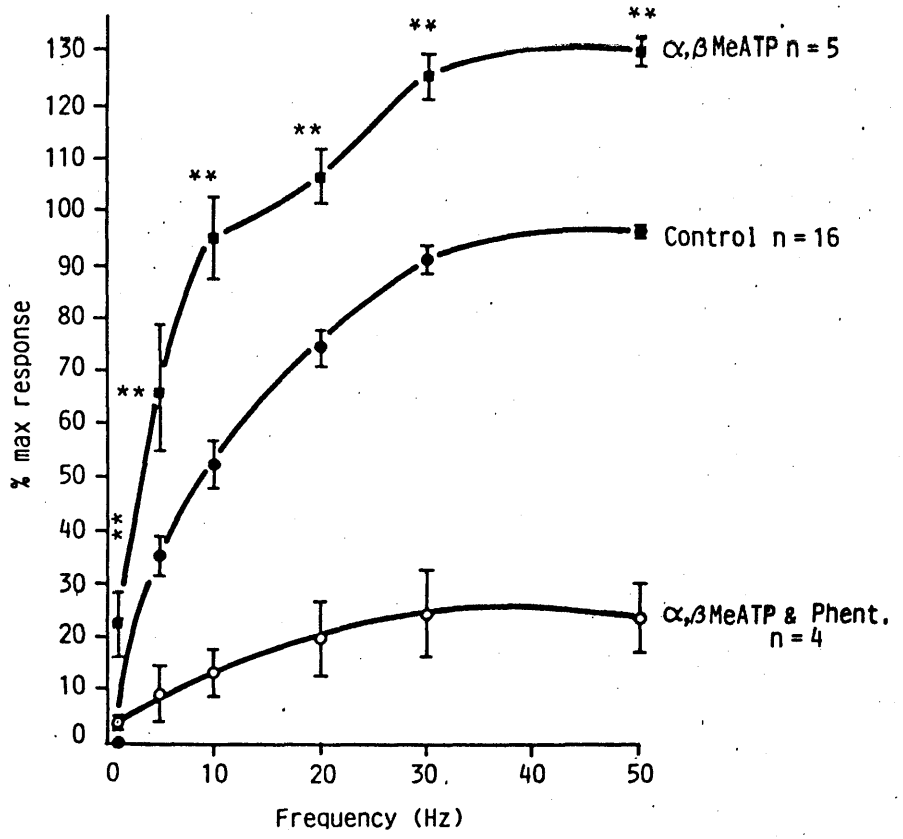


Fig. 38: The effects of increasing frequency on the mean pressor response, expressed as a % of the maximum control in each tissue, of the perfused rabbit central ear artery to field stimulation (0.5ms, supramaximal voltage, 50 pulses at 1-50Hz). Each graph shows the mean (± s.e.m.) of a number, (n), of observations. The pressor responses to field stimulation were potentiated (** $p < 0.02$) by α,βMeATP (1×10^{-6}M), probably due to the depolarizing action of the drug and antagonized by the additional presence of the α-adrenoceptor antagonist phentolamine (5×10^{-6}M).

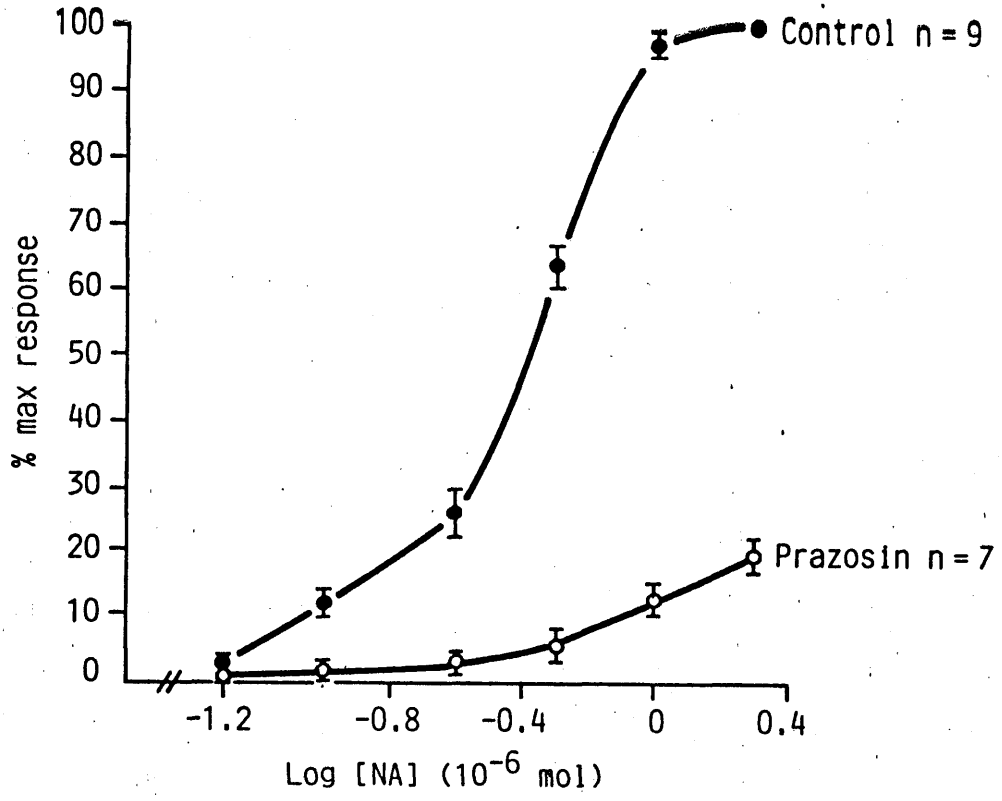


Fig. 39: The mean pressor responses, expressed as a % of the maximum control in each tissue, of the perfused rabbit central ear artery to bolus injections (0.05-0.2ml) of NA (1×10^{-6} - 1×10^{-5}M). Each graph shows the mean (± s.e.m.) of a number, (n), of observations. The dose-response curve to NA was shifted to the right by the α-adrenoceptor antagonist prazosin (1×10^{-7}M).

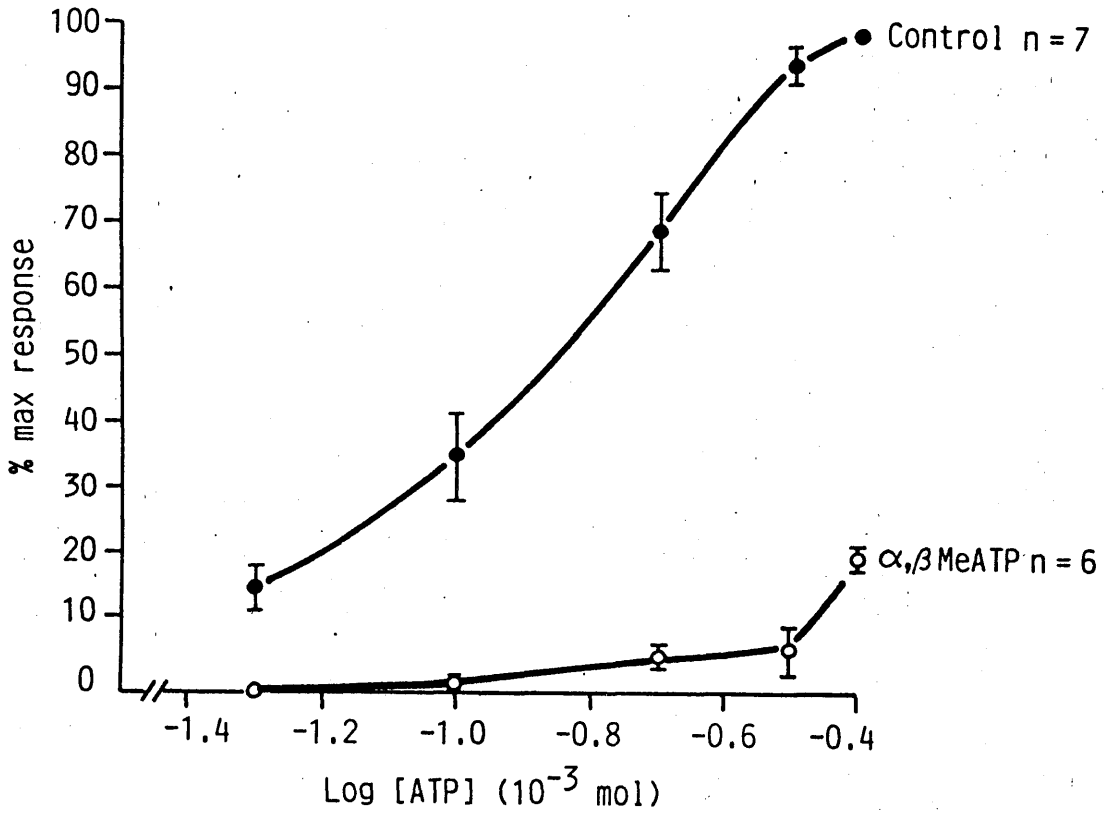


Fig. 40: The mean pressor responses, expressed as a % of the maximum control in each tissue, of the perfused rabbit central ear artery to bolus injections (0.05-0.4ml) of ATP (1 x 10^{-3}M). Each graph shows the mean (± s.e.m.) of a number, (n), of observations. The dose-response curve to ATP was shifted to the right following P_2-purinoceptor depolarization with α,βMeATP (1 x 10^{-6}M).

B. ELECTRICAL ACTIVITY IN THE RABBIT EAR ARTERY

The mean resting membrane potential of the rabbit central ear artery, as measured using conventional glass micro-electrodes was -71.3 ± 0.8mV, n=65. Spontaneous e.j.p.s were absent.

The electrical response to field stimulation (0.01-0.1ms, supra-maximal voltage, 1-10Hz) of the rabbit central ear artery was comprised of e.j.p.s, which facilitated and summated to fire action potentials, followed by a small, slow membrane depolarization lasting some 5-15s (Fig. 41).

The α-adrenoceptor antagonists prazosin (1×10^{-7}M) and phentolamine (1×10^{-6}M) each selectively abolished the slow depolarization, suggesting that it was mediated by NA, but left the e.j.p.s and action potentials unaffected (Fig. 41B). P_2-purinoceptor desensitization with α,βMeATP ($1\text{-}10 \times 10^{-6}$M), on the other hand, abolished the e.j.p.s and action potentials, suggesting that they were mediated by ATP, or a closely related nucleotide, but left the slow depolarization unaffected (Fig. 41C).

Therefore, in the rabbit central ear artery it appeared that both transmitters (NA and ATP) played a role in the electrical response to field stimulation, while only NA seemed to mediate a contractile event. All electrical and mechanical activity was abolished by the adrenergic neurone blocking drug guanethidine (1×10^{-6}M) and by TTX (1×10^{-6}M), suggesting that both transmitters were released from sympathetic nerves, confirming that this tissue exhibits co-transmission.

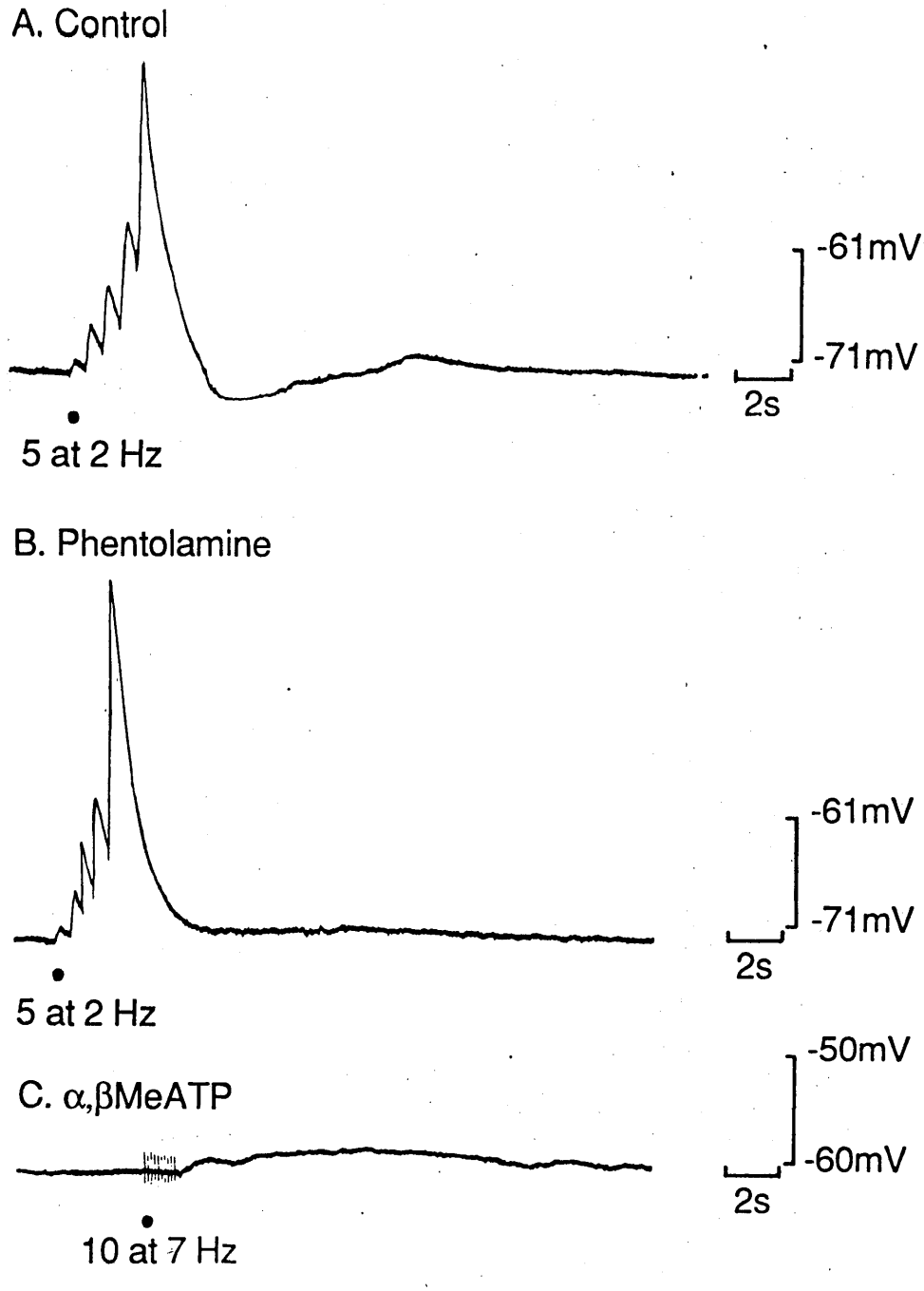


Fig. 41: The intracellular electrical responses of the rabbit central ear artery to field stimulation (0.03ms, supramaximal voltage, 2-7Hz). The control response (A.) was comprised of e.j.p.s, which facilitated to fire action potentials, followed by a small slow membrane depolarization. The α-adrenoceptor antagonist phentolamine (1×10^{-6}M, B.) selectively abolished the slow depolarization, suggesting that it was mediated by NA, whereas α,βMeATP (1×10^{-6}M, C.) selectively abolished the e.j.p.s, suggesting that they were mediated by ATP.

IV. RAT ANOCOCCYGEUS MUSCLE

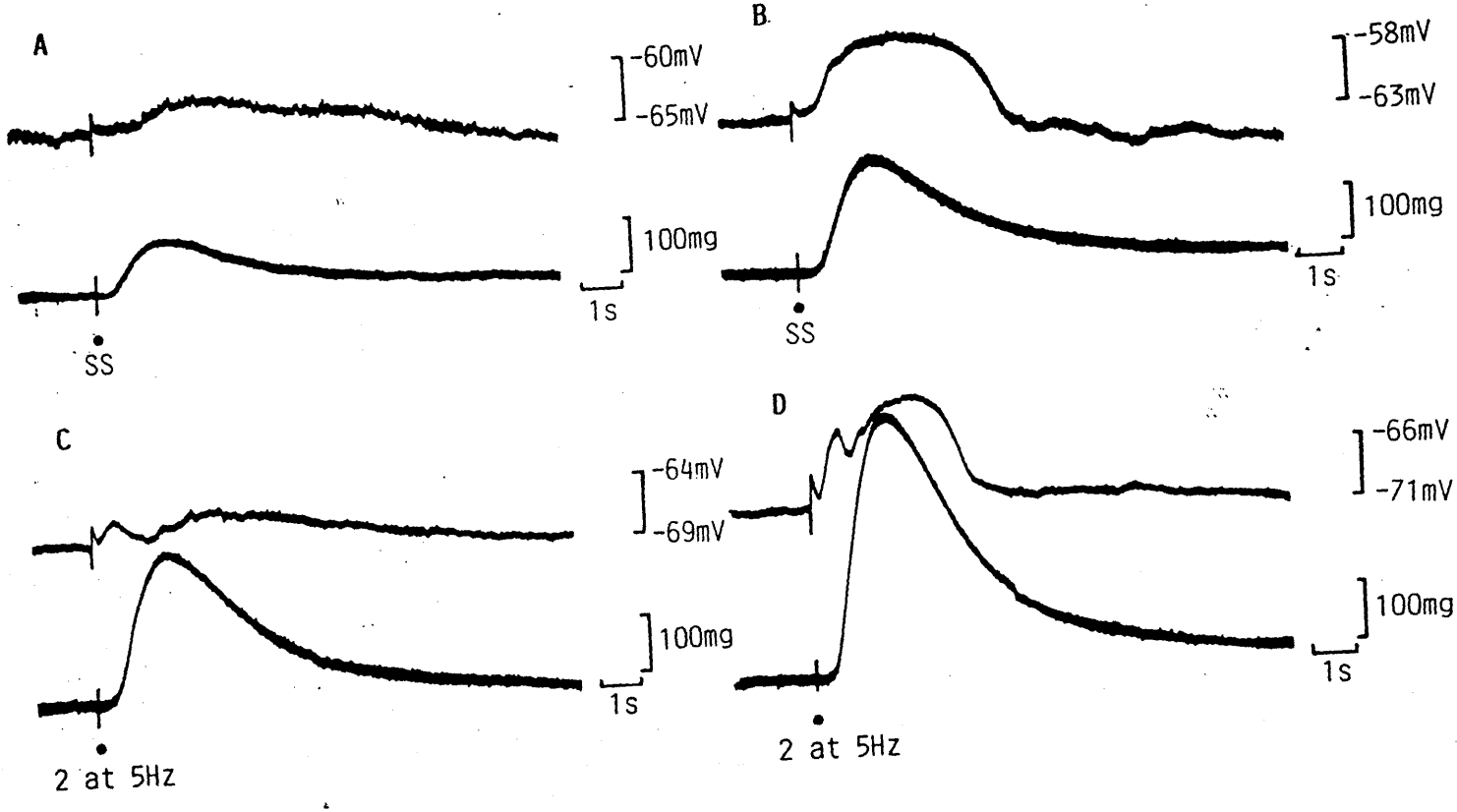
A. SIMULTANEOUS ELECTRICAL AND MECHANICAL RESPONSES TO FIELD AND EXTRINSIC NERVE STIMULATION

Following the setting up of the preparation, the muscle was stretched to a tension of 0.75-1.0g. Tension decayed over a 30min equilibration period, to a lower value (approximately 0.5g) which was maintained throughout the course of the experiment. Under these conditions, the muscle was electrically quiescent. The resting membrane potential ranged from -53 to -76mV, with a mean of -63.7 ± 0.4mV, n=170.

Both field and extrinsic nerve stimulation (0.01-0.5ms, 5-70V) evoked a membrane depolarization and contraction, the amplitude and nature of the depolarization depending on the stimulation parameters used (Fig. 41'). Following single stimuli, by either method, a small slow membrane depolarization, measured as the time between 10 and 90% of the maximum voltage, with a latency of several hundred ms and a duration of several s was always recorded. This event, hereafter termed the 'slow depolarization' frequently appeared to follow the start of the mechanical event. Short trains (2-5) of pulses at 5 or 10Hz, delivered by field or extrinsic nerve stimulation, initially produced a 'fast e.j.p.' (rate of rise 14.9 ± 0.9mVsec^{-1}, n=49) with a latency of less than 100ms and a duration of under 1s, followed by a slow depolarization, similar to that obtained to single pulses (Fig. 41'). The fast e.j.p. always preceded the onset of the contraction. The rates of rise of the slow depolarization and the fast e.j.p.s varied with frequency and stimulus strength. This probably reflected facilitation of transmitter release, a feature characteristic of this muscle (Creed et al., 1975). The mean (± s.e.m.) rate of rise of the slow depolarization measured under identical parameters of stimulation

Fig. 41: The effects of increasing stimulus strength on the simultaneously-recorded electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to field stimulation of sympathetic nerves. Each panel shows, following the stimulus artefact, the responses to submaximal stimuli (A., B., single stimuli, ss, 0.2ms, 20V (left hand side) and 0.3ms, 30V; C., D., two stimuli at 5Hz, 0.1ms, 7V (left hand side) ans 0.1ms, 12V).

The electrical response to single stimuli was a slow depolarization. Following trains of pulses, a biphasic response was obtained. This consisted of an initial fast e.j.p. and a slow depolarization, the amplitude of each depending on stimulus strength. Electrical recordings were obtained from the same cell.


A
-60mV
-65mV
100mg
1s
SS
B
-58mV
-63mV
100mg
1s
SS
C
-64mV
-69mV
100mg
1s
2 at 5Hz
D
-66mV
-71mV
100mg
1s
2 at 5Hz

(1 pulse, 0.2ms, 20V) was 0.6 ± 0.06mVsec^{-1}, n=12.

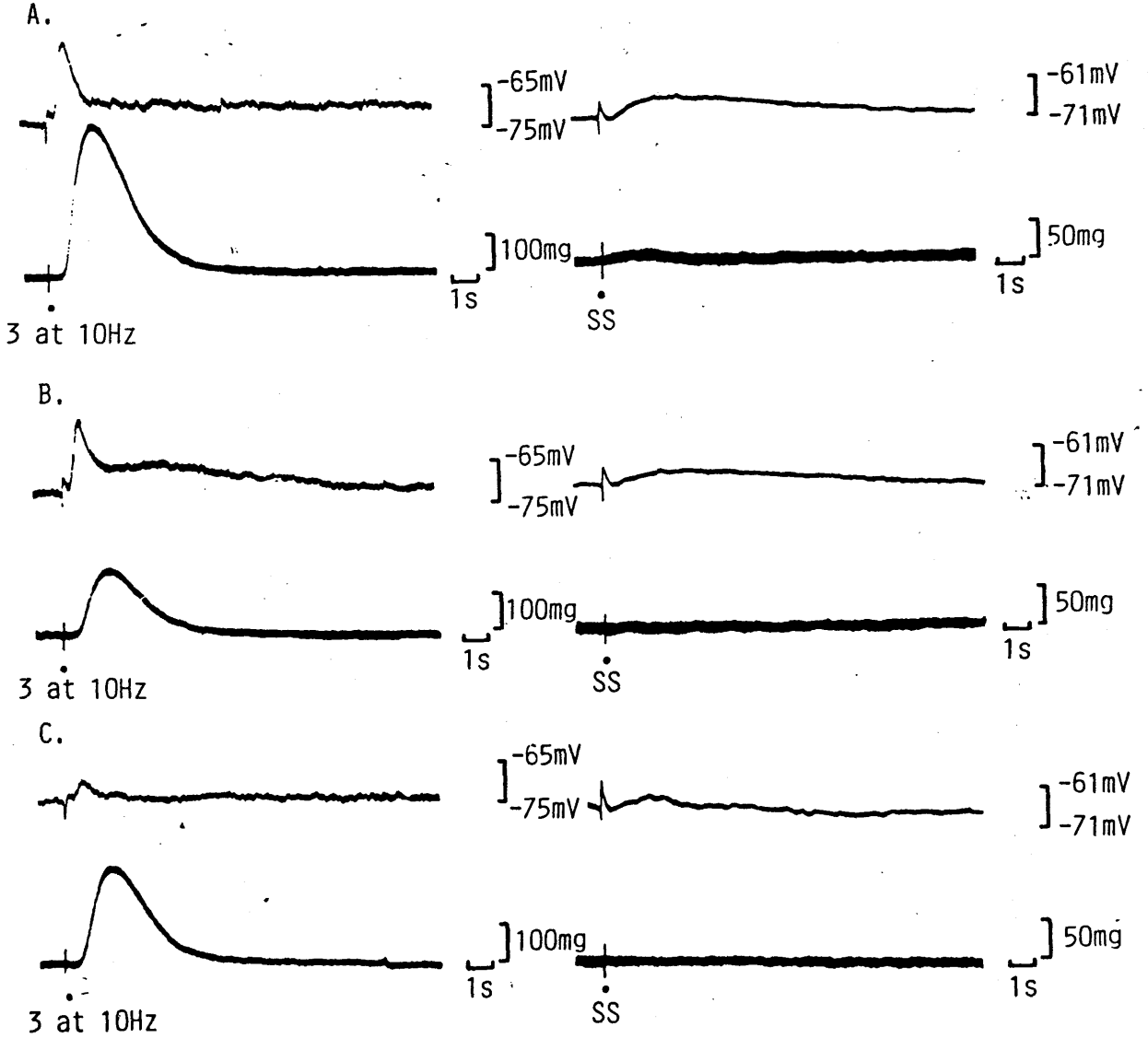
In 3 out of 22 tissues, fast e.j.p.s were absent and only slow depolarizations were evoked by either field or extrinsic nerve stimulation, even with longer trains of pulses (10) at supramaximal voltage (70V). Conversely, in 2 out of 22 tissues, supramaximal field of extrinsic nerve stimulation, using single pulses, produced small (3-4mV) fast e.j.p.s, followed by a slow depolarization.

Both the fast e.j.p.s and the slow depolarizations were recorded in cells throughout the muscle, irrespective of their location, in response to field or extrinsic nerve stimulation. The electrical and mechanical responses to simultaneous stimulation of both the genitofemoral and perineal nerves were larger than those obtained to stimulation of each nerve separately (Fig. 42).

The electrical responses produced by extrinsic nerve stimulation and those to field stimulation were apparently identical. The fast e.j.p.s, slow depolarizations and contractions produced by field (Fig. 43) or extrinsic nerve stimulation (Fig 44) were abolished by the α-adrenoceptor antagonists phentolamine (1 x 10^{-6}M) or prazosin (1 x 10^{-7}M), or by TTX (1 x 10^{-6}M). The stable analogue of ATP, α,βMeATP (1-10 x 10^{-6}M) depolarized the membrane potential, but had no significant inhibitory effect on either the fast e.j.p., the slow depolarization or the contractions produced by either field (Fig. 43) or extrinsic nerve stimulation (Fig. 44). Mechanical contractions were frequently seen to be potentiated by α,βMeATP, probably due to the depolarizing action of the drug.

The absence of a depolarization to nerve stimulation which was resistant to α-adrenoceptor antagonists contrasts with events previously reported in this tissue (Byrne & Large, 1984). To substantiate the present findings, experimental conditions were selected to optimise the possibility of demonstrating such e.j.p.s, were they present (Fig.

Fig. 42: Simultaneously-recorded electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to extrinsic nerve stimulation (left hand column, 3 pulses at 10Hz, 0.3ms, 20V; right hand column, single stimuli, 0.3ms, 30V); A. Perineal and genito-femoral nerves together, B. genito-femoral, and C. perineal nerves alone. As with field stimulation (Fig. 41), trains of pulses produced a biphasic response; an initial fast e.j.p. followed by a slower depolarization whereas single stimuli produced only a slow depolarization. Electrical recordings were obtained from the same cell.


A.
-65mV
-75mV
-61mV
-71mV
100mg
50mg
1s
1s
3 at 10Hz
SS
B.
-65mV
-75mV
-61mV
-71mV
100mg
50mg
1s
1s
3 at 10Hz
SS
C.
-65mV
-75mV
-61mV
-71mV
100mg
50mg
1s
1s
3 at 10Hz
SS

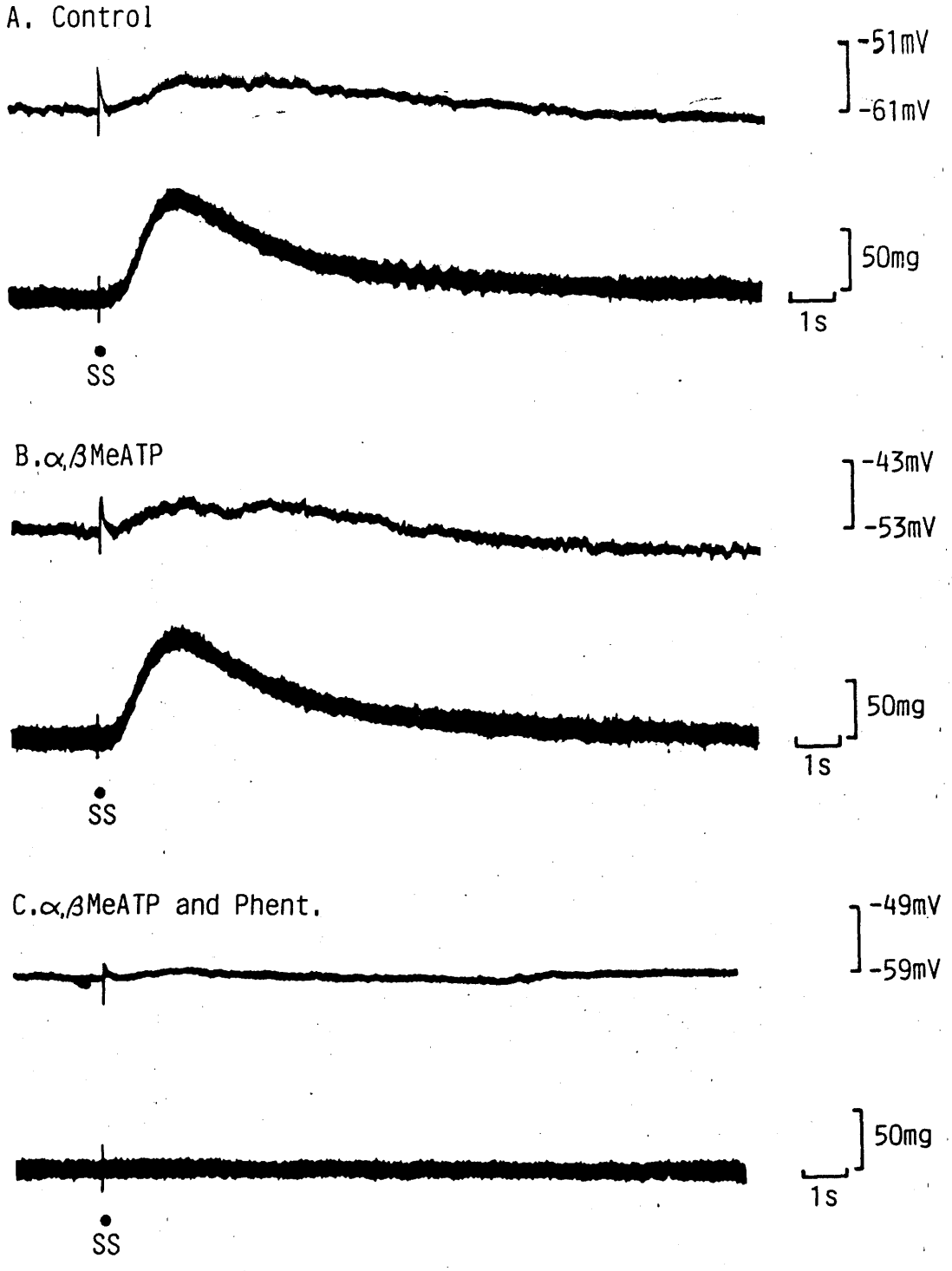


Fig. 43: The effects of α,βMeATP alone (1 x 10^{-6}M, B.) and in the presence of phentolamine (Phent, 1 x 10^{-6}M, C.), compared with control (A.), on the simultaneously-recorded electrical (upper trace in each panel) and mechanical responses of the rat anococcyegus muscle to field stimulation (single stimuli, 0.2ms, 20V). Both the slow depolarization and the mechanical contractions were unaffected by α,βMeATP but were abolished by phentolamine, suggesting that they were noradrenergically mediated. Electrical recordings were made from 3 separate cells in the same tissue.

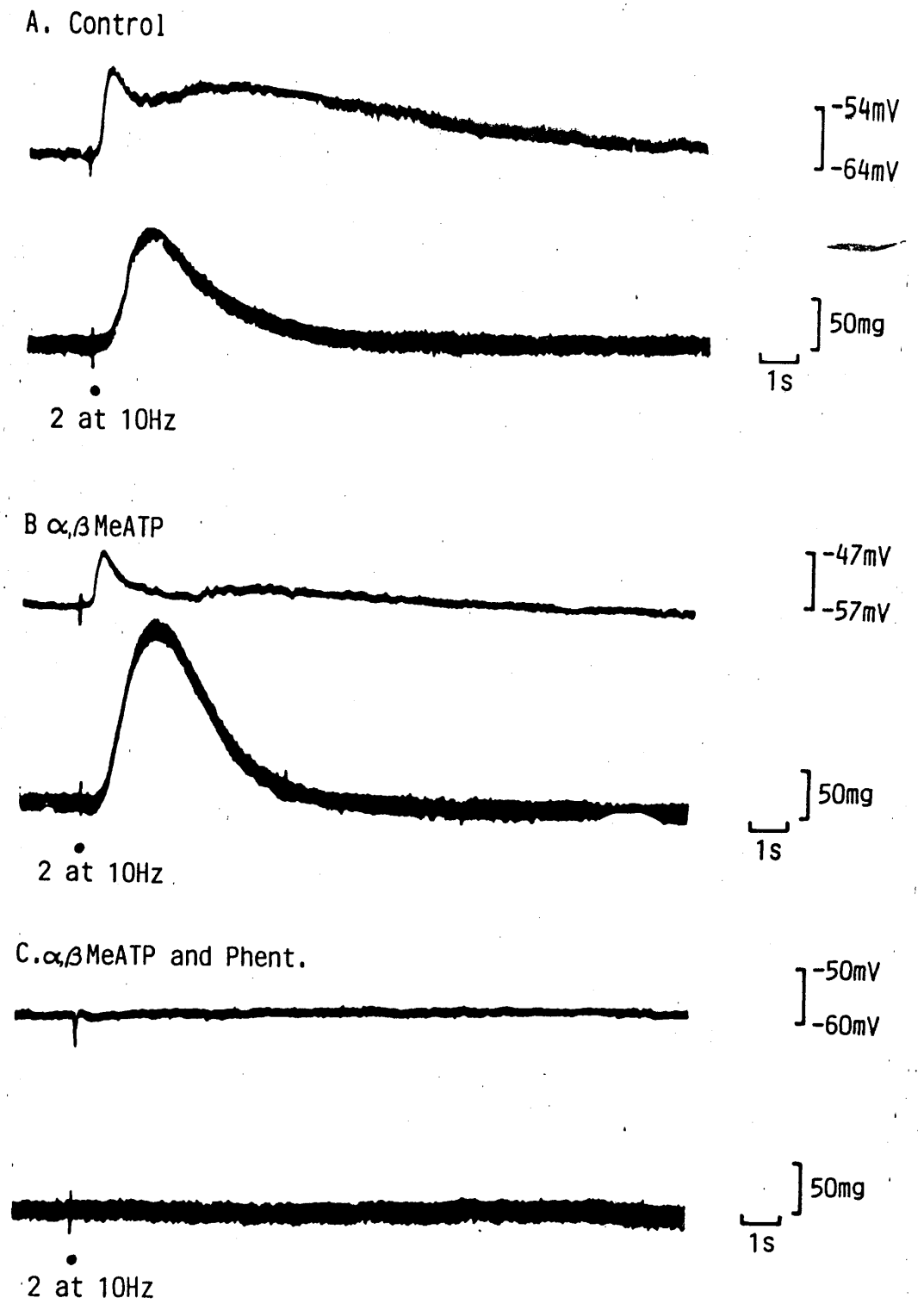


Fig. 44: The effects of α,βMeATP alone (1 x 10^{-6}M, B.) and in the presence of phentolamine (Phent, 1 x 10^{-6}M, C.), compared with control (A.), on the simultaneously-recorded electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to stimulation of the genito-femoral and perineal nerves (2 stimuli at 10Hz, A. 0.3ms, 20V, B. 0.3ms, 15V, C. 0.3m, 50V). The control response to extrinsic nerve stimulation showed a fast e.j.p., a slow depolarization and a mechanical contraction. α,βMeATP enhanced the mechanical response and reduced, but did not abolish, the electrical responses. The enhancement of the mechanical effects were presumably due to the ability of α,βMeATP to depolarize the membrane. The fast e.j.p., the slow depolarization and the contractions were abolished by phentolamine, suggesting that they were noradrenergically mediated. Electrical recordings were made from 3 separate cells in the same preparation.

45).

In addition to the α_1-adrenoceptor antagonist prazosin (1 x 10^{-7}M), idazoxan (1 x 10^{-7}M), which blocks pre-synaptic α_2-adrenoceptors and thus the feedback inhibition on transmitter release, and nifedipine (1 x 10^{-6}M) at a dose which prevented muscle contraction without affecting synaptic potentials (Blakeley et al., 1981) were used. Under these conditions, no post-synaptic depolarization was found, even at high intensity stimulation parameters (5 pulses, 50Hz, 0.01ms, 70V, Fig. 45). These results therefore confirm the absence of non-adrenergic e.j.p.s in the rat anococcygeus muscle.

B. ELECTRICAL RESPONSES TO EXOGENOUS AGONISTS

Pressure application of NA (1 x 10^{-8} -1 x 10^{-6}M, 40p.s.i., 1-50ms) produced a depolarization and a localized contraction which extended some 1-2mm around the point of application. The characteristics of the membrane potential change varied with the amount of NA added (Fig. 46). Low (1 x 10^{-8}-1 x 10^{-7}M) concentrations of NA produced a slow depolarization with a rate of rise of approximately 1mVsec^{-1} (mean 1.45 ± 0.17mVsec^{-1}, n=8) and a duration of several seconds. Higher (1 x 10^{-7}-1 x 10^{-6}M) concentrations produced a more rapid depolarization (mean rate of rise 14.31 ± 1.10mVsec^{-1}, n=12), with a duration of 1-2s. Both the fast and slow depolarizations were abolished by phentolamine (1 x 10^{-6}M) or prazosin (1 x 10^{-7}M, Fig. 47), but were unaffected by α,βMeATP (1-10 x 10^{-6}M).

ATP (1-10 x 10^{-4}M, 40p.s.i., 1-200ms) also produced small membrane depolarization and localized contactions when added exogenously (Fig. 48). The depolarizations were unaffected by phentolamine (1 x 10^{-6}M) or prazosin (1 x 10^{-7}M), but were abolished by α,βMeATP (1 x 10^{-6}M). The rate of rise of the depolarization produced by ATP was graded with the concentration applied (mean 3.8 ± 0.5mVsec^{-1}, n=12);

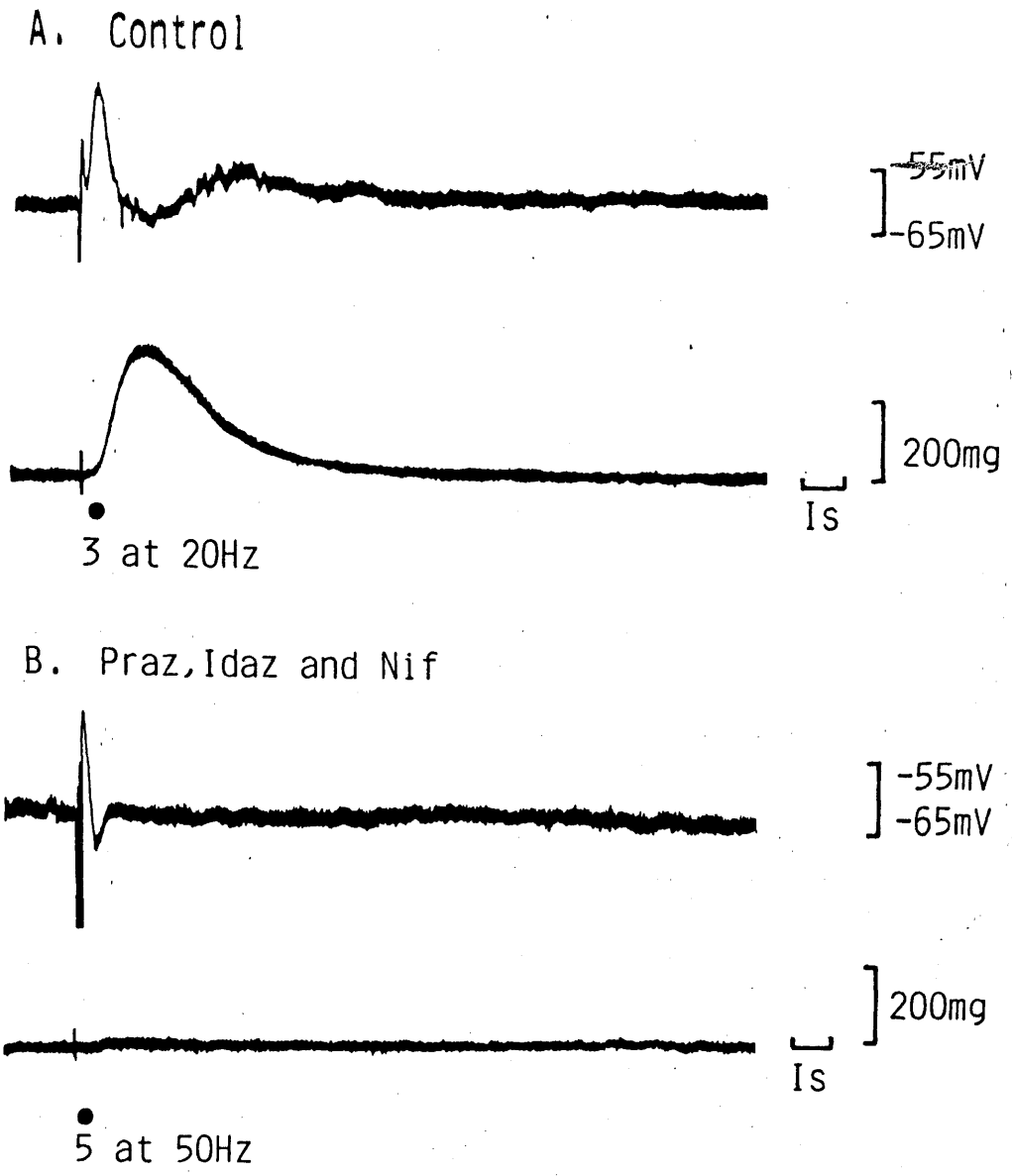


Fig. 45: The effects of prazosin (Praz, 1 x 10^{-7}M), idazoxan (Idaz, 1 x 10^{-7}M) and nifedipine (Nif, 1 x 10^{-6}M) on the electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to field stimulation (A. 3 pulses at 20Hz, 0.01ms, 50V; B. 5 pulses at 50Hz, 0.01ms, 70V). In the control situation (A.), Field stimulation produced a fast e.j.p., a slow depolarizarion and an accompanying mechanical contraction. In the presence of prazosin, idazoxan and nifedipine (B.) the electrical and mechanical responses were abolished, and only a stimulus artefact remained, even when the number of pulses and the stimulus strengths were increased. This confirms the exclusive noradrenergic nature of the evoked e.j.p. Electrical recordings were obtained from the same cell.

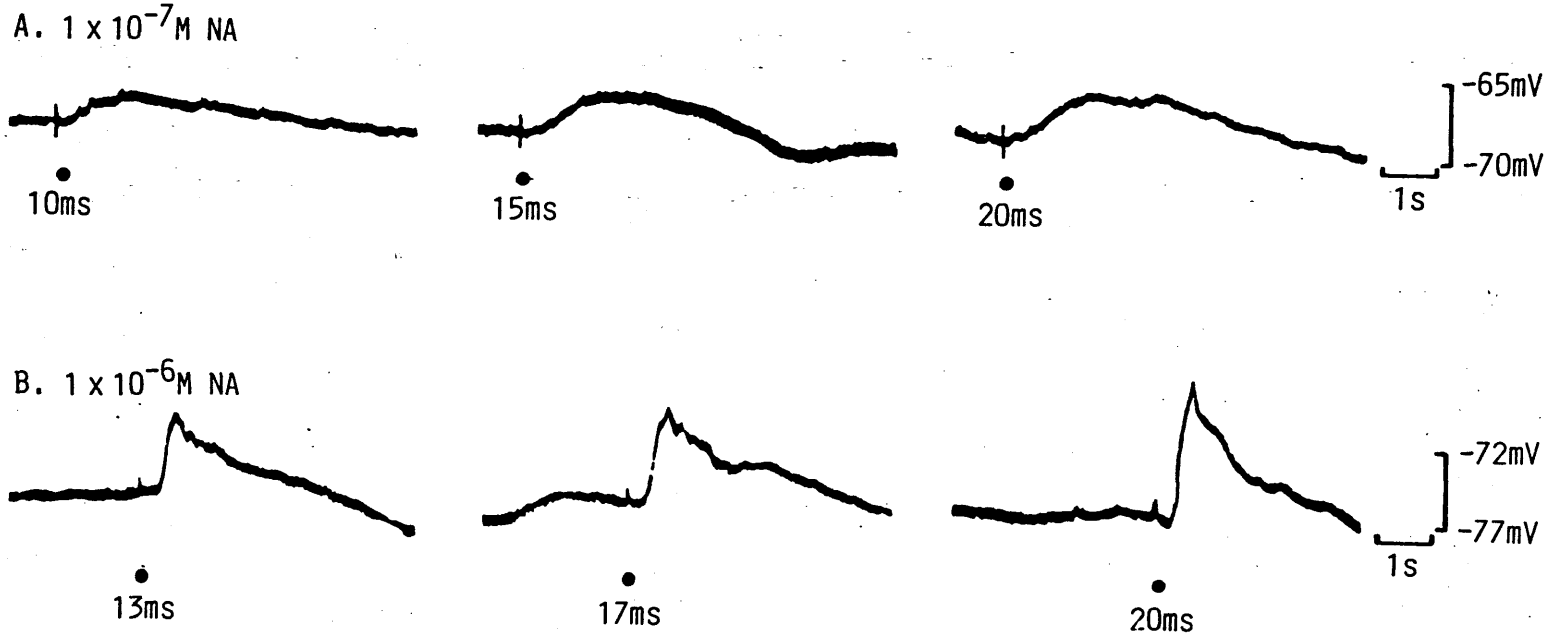


Fig. 46: Intracellularly-recorded membrane potential responses of the rat anococcygeus muscle to micro-application of NA (1×10^{-7}M & 1×10^{-6}M) in two separate cells (A. & B.) in the same preparation for increasing periods of time (10-20ms). Micro-pipette tip diameters were $1\text{-}2 \times 10^{-6}$m and ejection pressures 40p.s.i. When applied locally, close to the recording electrode, NA produced both fast and slow membrane depolarizations, depending on the concentrations applied.

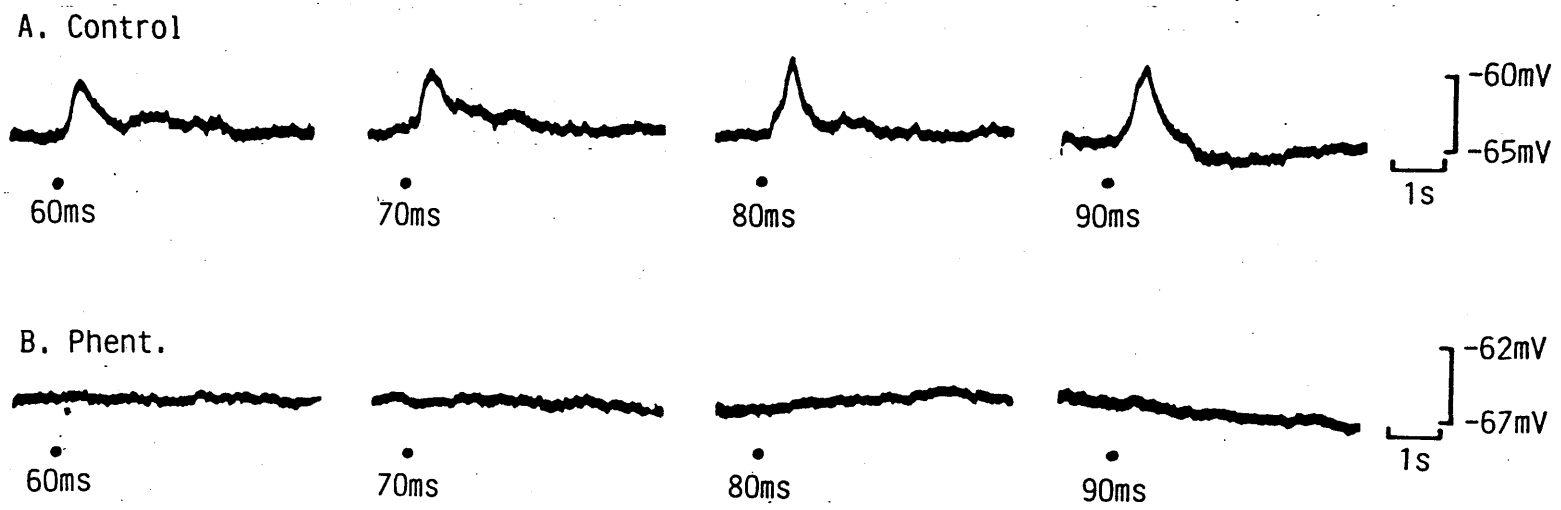


Fig. 47: The effects of phentolamine (Phent, 1 x 10^{-6}M, B.) on the membrane potential changes of the rat anococcygeus muscle to local application of NA (1 x 10^{-7}M, ejection pressure 40p.s.i., micro-pipette tip diameter 1 x 10^{-6}m) for increasing periods of time (ms). Local application of NA for short durations (60-90ms) produced membrane depolarizations which were abolished by the α-adrenoceptor antagonist phentolamine. Electrical recordings were made from the same cell.

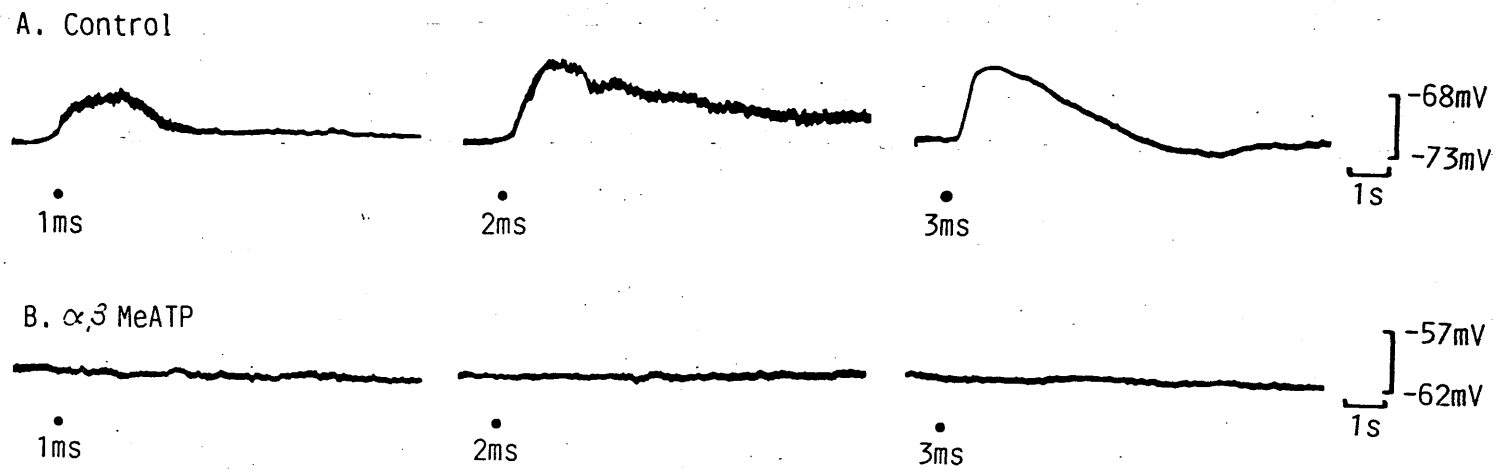


Fig. 48: The effects of α,βMeATP (1×10^{-6}M, B.) on the membrane potential changes of the rat anococcygeus muscle to local application of ATP (1×10^{-3}M, ejection pressure 40p.s.i., micro-pipette tip diameter 2×10^{-6}m) for increasing periods of time (ms). The time interval between successive applications of ATP were sufficient to prevent desensitization to the nucleotide. Local application of ATP produced small membrane depolarizations which were abolished by α,βMeATP. Electrical recordings were obtained from the same cell.

the depolarization appeared uniform and there was no evidence of fast and slow components, as seen with NA.

Several biologically active peptides were also investigated as putative transmitters in the rat anococcygeus muscle. Bradykinin (1-100 x 10^{-7}M), VIP (1-100 x 10^{-7}M), NPY (1-100 x 10^{-7}M), substance P (1-100 x 10^{-7}M), somatostatin (1-100 x 10^{-7}M), leu-enkephalin (1-100 x 10^{-7}M), met-enkephalin (1-100 x 10^{-7}M) and bombesin (1-100 x 10^{-7}M) each produced no change in membrane potential when applied locally form a micro-pipette (40p.s.i., 1-200ms, tip diameter 1-2 x 10^{-6}m). Thus it seems unlikely that any of these peptides act as neurotransmitters by a voltage-dependent mechanism in the rat anococcygeus muscle. It does not, however, rule out the possibility that they act by a voltage-independent mechanism.

V. HYPERTENSION

The possibility that ATP may play a role in the development or maintenance of hypertension (Vidal et al., 1986) was investigated in tail artery and mesenteric bed preparations from age-matched normotensive (WKY) and spontaneously-hypertensive (SHR) rats.

That the animals were hypertensive was confirmed by measuring their systolic blood pressure in vivo by means of an inflated tail cuff (see Materials & Methods). The mean systolic blood pressure in WKY rats was 128 ± 2.5mmHg, n=18, while that in SHR rats was 233 ± 3.3mmHg, n=12.

A. PRESSOR RESPONSES TO FIELD STIMULATION AND EXOGENOUS AGONISTS

At the flow rate used (4mlmin^{-1}), the resting perfusion pressures for both SHR and WKY rat preparations were not significantly different. They ranged from 25-30mmHg for tail arteries and 25-35mmHg for the

mesenteric bed preparations.

In tail artery and mesenteric bed preparations from both WKY and SHR rats, periarterial field stimulation (0.5ms, supramaximal voltage, 200 pulses at 1-50Hz) and injections (0.1-0.3ml) of NA (1×10^{-5} -1×10^{-3}M), ADR (1×10^{-6} -1×10^{-4}M) and ATP (1×10^{-4} -1×10^{-2}M) each produced a vasoconstriction and an increase in perfusion pressure (Figs. 49-52). The order of potency of agonists was ADR > NA > ATP. Pressor responses to either exogenous ADR or NA exceeded those elicited by field stimulation. All pressor responses to periarterial field stimulation were abolished by guanethidine (1×10^{-6}M) and TTX (1×10^{-6}M) and were assumed to be due to transmitters released from sympathetic nerves.

The pressor responses to field stimulation (Figs. 49 & 50), NA (Figs. 51 & 52) and ADR (Figs. 51 & 52) in both tail (Figs 49 & 51) and mesenteric bed preparations (Figs. 50 & 52) from SHR rats were significantly ($p < 0.05$) greater than those from age-matched WKY animals. In contrast, the responses of both tail and mesenteric arteries to exogenous ATP in normo- and hypertensive rats did not differ significantly (Figs. 51 & 52).

In arteries from both WKY and SHR rats, α,βMeATP (1×10^{-6}M) produced a short-lasting pressor response and subsequently inhibited the pressor responses to exogenous ATP (1×10^{-4} -1×10^{-2}M) by about 70%. However, this and higher (< 15×10^{-6}M) concentrations of α,βMeATP had no significant inhibitory effect on the pressor responses to field stimulation of tail arteries (Fig. 53) or mesenteric bed preparations (Fig. 54) in either SHR or WKY rats. Addition of the α-adrenoceptor antagonists phentolamine (2×10^{-6}M) or prazosin (1×10^{-7}M) virtually abolished all pressor responses to both field stimulation (Figs 53 & 54) and exogenously added catecholamines (NA & ADR).

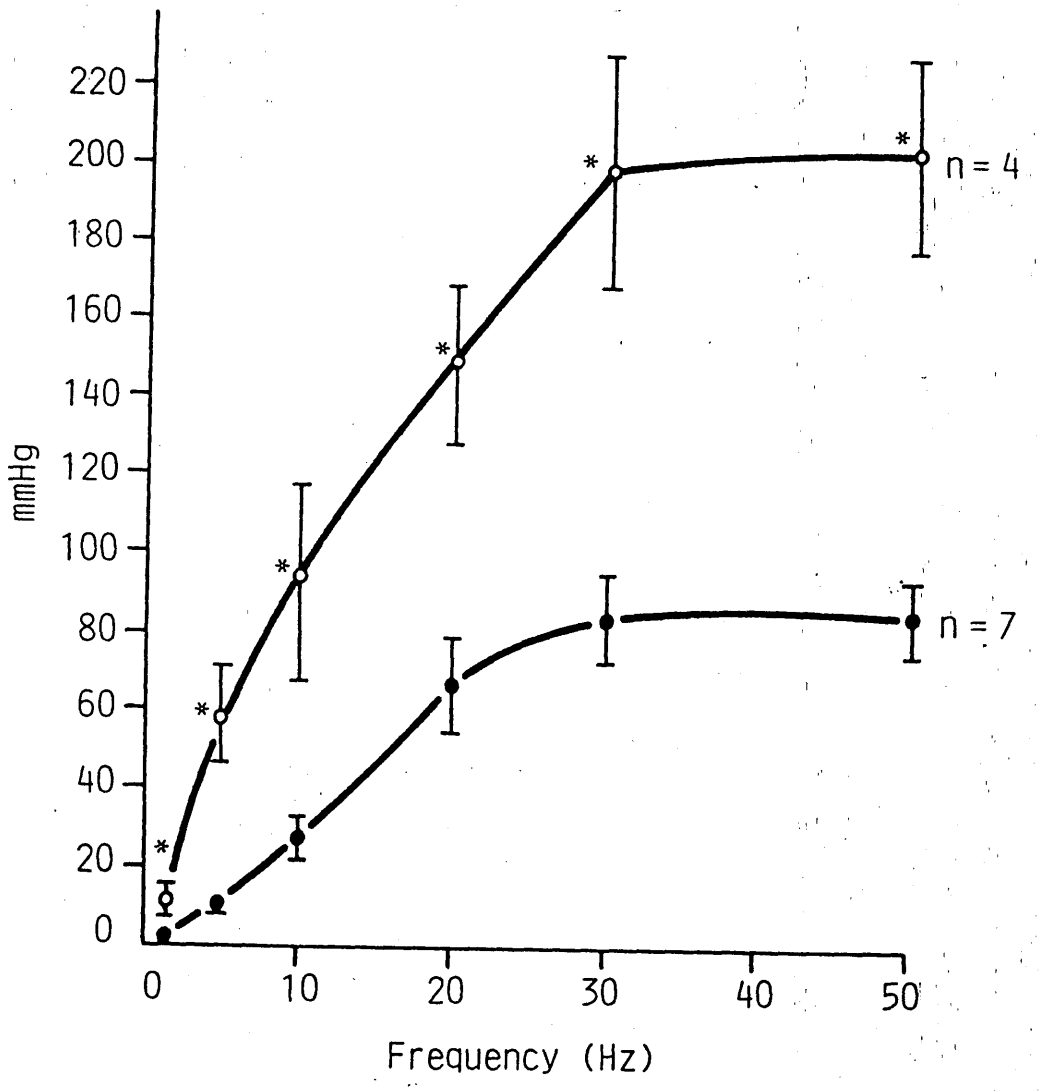


Fig. 49: The effects of increasing frequency on the mean pressor responses (mmHg) of tail arteries from normotensive (WKY, •) and spontaneously-hypertensive (SHR, o) rats to field stimulation (0.5ms, supramaximal voltage, 200 pulses at 1-50Hz). Each graph shows the mean (± s.e.m.) of a number (n) of observations. The pressor responses of SHR arteries were significantly (* $p < 0.05$) greater than those from age-matched WKY animals at each frequency of stimulation.

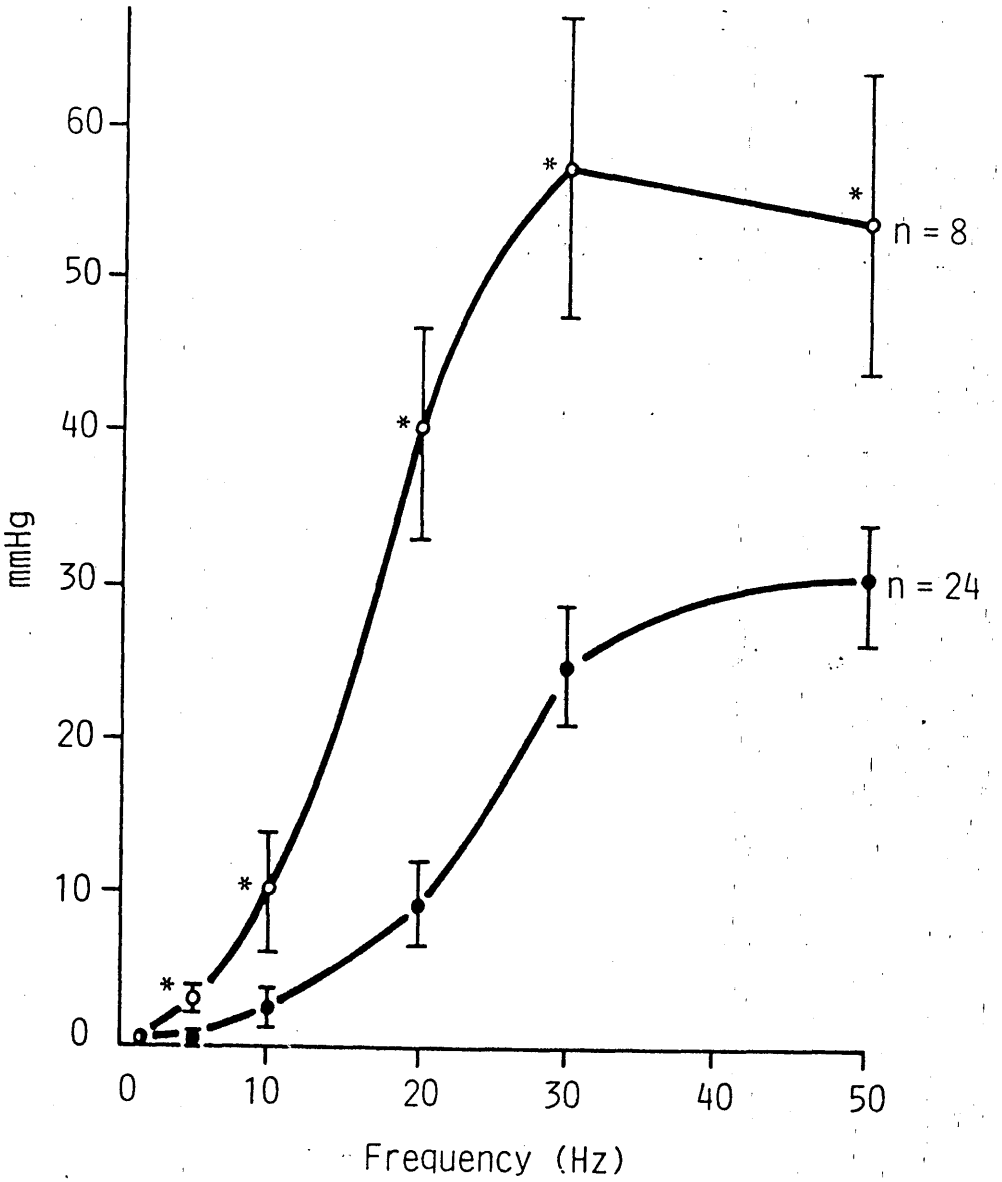


Fig. 50: The effects of increasing frequency on the mean pressor responses (mmHg) of mesenteric bed preparations from normotensive (WKY, ●) and spontaneously-hypertensive (SHR, o) rats to field stimulation (0.5ms, supramaximal voltage, 200 pulses at 1-50Hz). Each graph shows the mean (± s.e.m.) of a number (n) of observations. The pressor responses of SHR arteries were significantly (* $p < 0.05$) greater than those from age-matched WKY animals at all frequencies above 1Hz.

Fig. 51: The mean pressor responses (mmHg) of tail arteries from normotensive (WKY, ●) and spontaneously-hypertensive (SHR, o) rats to injections (0.1 - 0.3ml) of noradrenaline (NA, 1×10^{-6} - 1×10^{-3}M, A.), adrenaline (ADR, 1×10^{-7} - 1×10^{-4}M, B.) and adenosine 5'-triphosphate (ATP, 1×10^{-4} - 1×10^{-2}M, C.). Each graph shows the mean (± s.e.m.) of a number (n) of observations. The pressor responses to NA and ADR, but not those to ATP, were significantly (* $p < 0.05$) greater in arteries from SHR than from WKY animals.

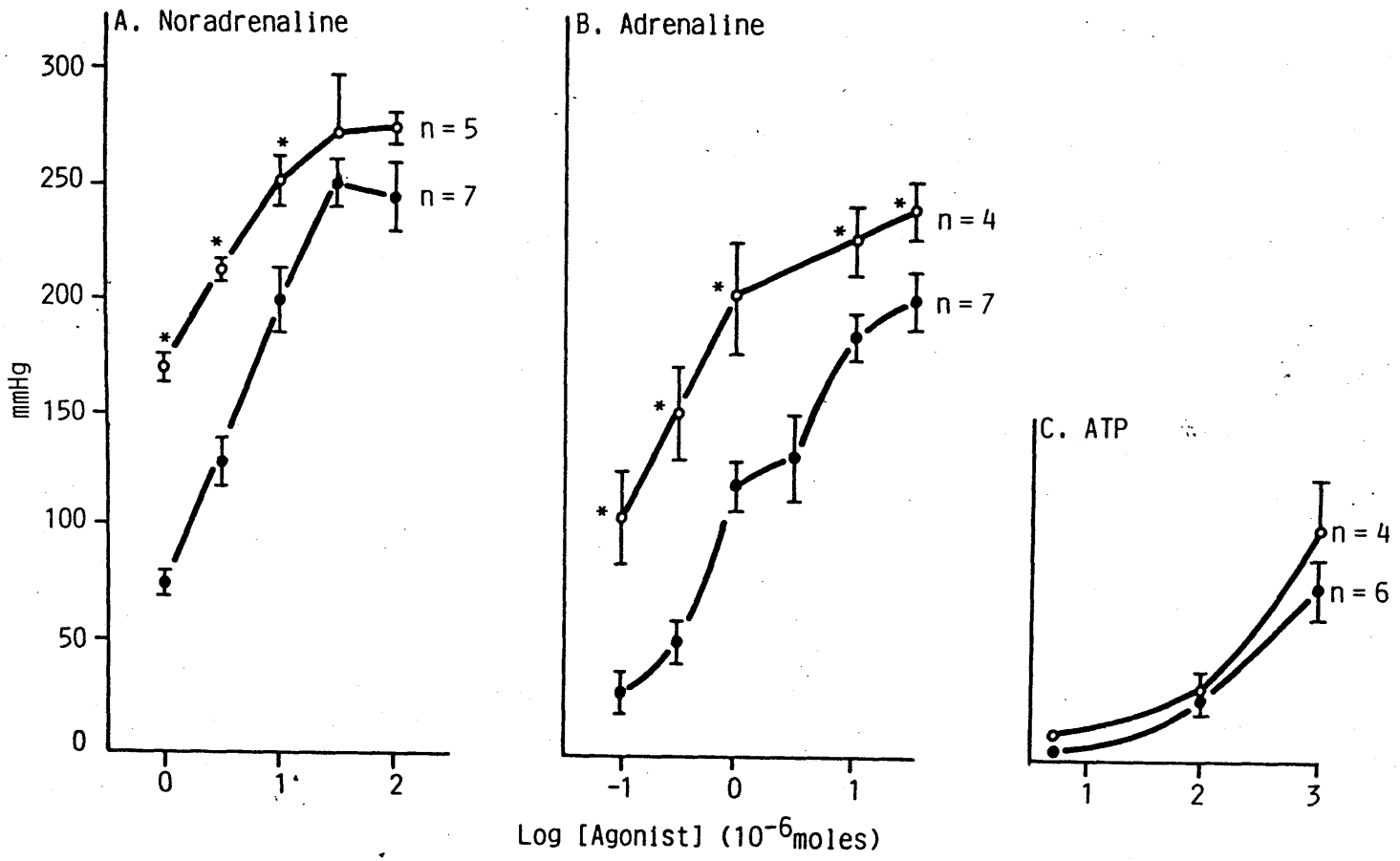

A. Noradrenaline
B. Adrenaline
C. ATP
mmHg
300
250
200
150
100
50
0
n = 5
n = 7
n = 4
n = 7
n = 4
n = 6
0
1
2
-1
0
1
1
2
3
Log [Agonist] (10^{-6}moles)

Fig. 52: The mean pressor responses (mmHg) of mesenteric bed preparations from normotensive (WKY, ●) and spontaneously-hypertensive (SHR, o) rats to injections (0.1 - 0.3ml) of noradrenaline (NA, 1 x 10^{-6} - 1 x 10^{-3}M, A.), adrenaline (ADR, 1 x 10^{-6} - 1 x 10^{-4}M, B.) and adenosine 5'-triphosphate (ATP, 1 x 10^{-4} - 1 x 10^{-2}M, C.). Each graph shows the mean (± s.e.m.) of a number (n) of observations. The pressor responses to NA and ADR, but not those to ATP, were significantly (* $p < 0.05$) greater in mesenteric bed preparations from SHR than from WKY animals.

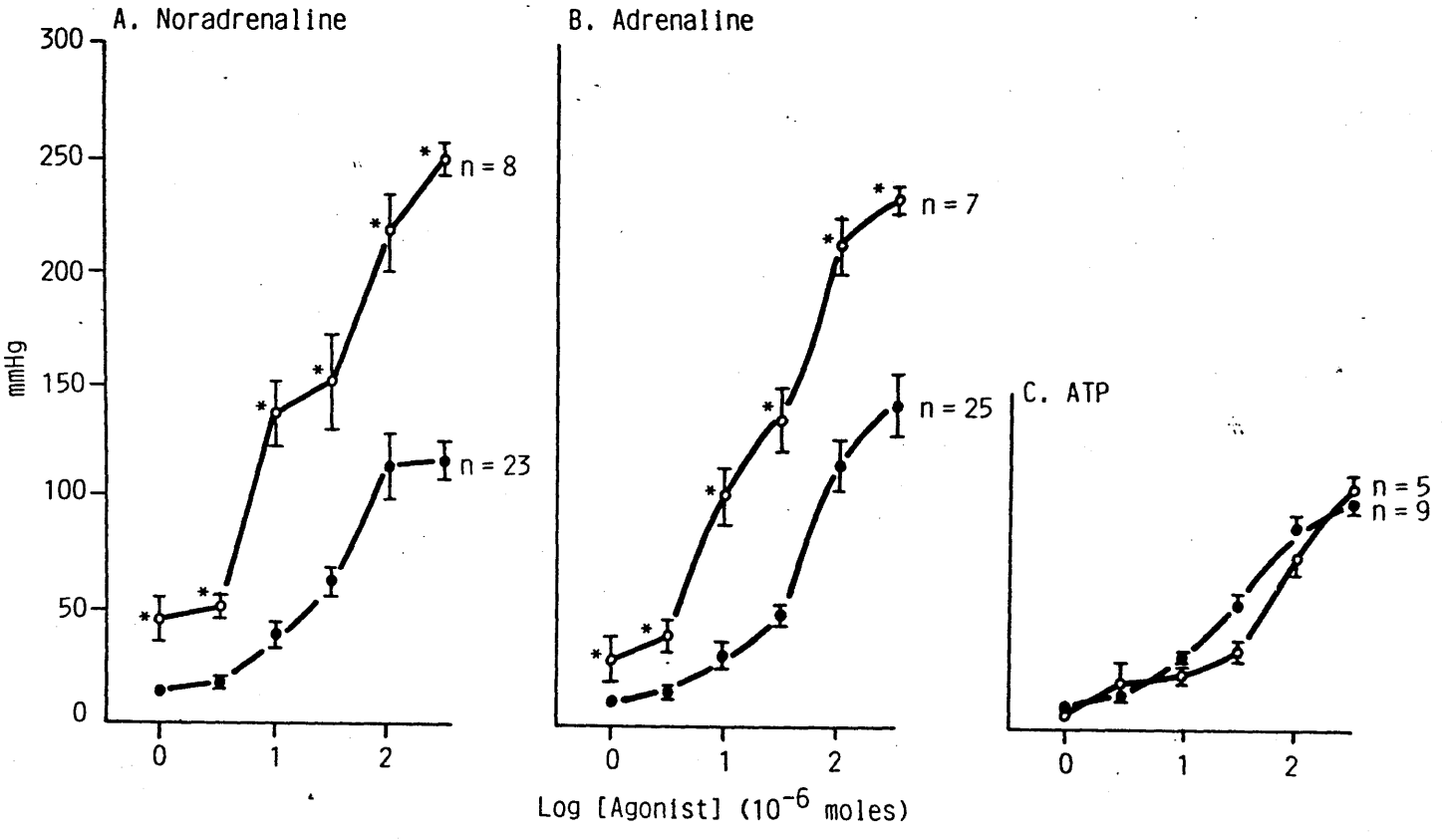

A. Noradrenaline
B. Adrenaline
C. ATP
mmHg
300
250
200
150
100
50
0
n = 8
n = 23
n = 7
n = 25
n = 5
n = 9
0
1
2
Log [Agonist] (10^{-6} moles)

Fig. 53: The effects of α,βMeATP (1×10^{-6}M) alone and in the presence of prazosin (1×10^{-7}M) on the mean pressor responses (mmHg) of tail arteries from normotensive (WKY, ●, B.) and spontaneously-hypertensive (SHR, o, A.) rats to field stimulation (0.5ms, supramaximal voltage, 200 pulses at 1-50Hz). Each graph show the mean (± s.e.m.) of a number (n) of observations. α,βMeATP had no significant inhibitory effect on the pressor activity of arteries from either SHR or WKY rats. The additional presence of prazosin virtually abolished all pressor responses, suggesting that they were mediated by NA.

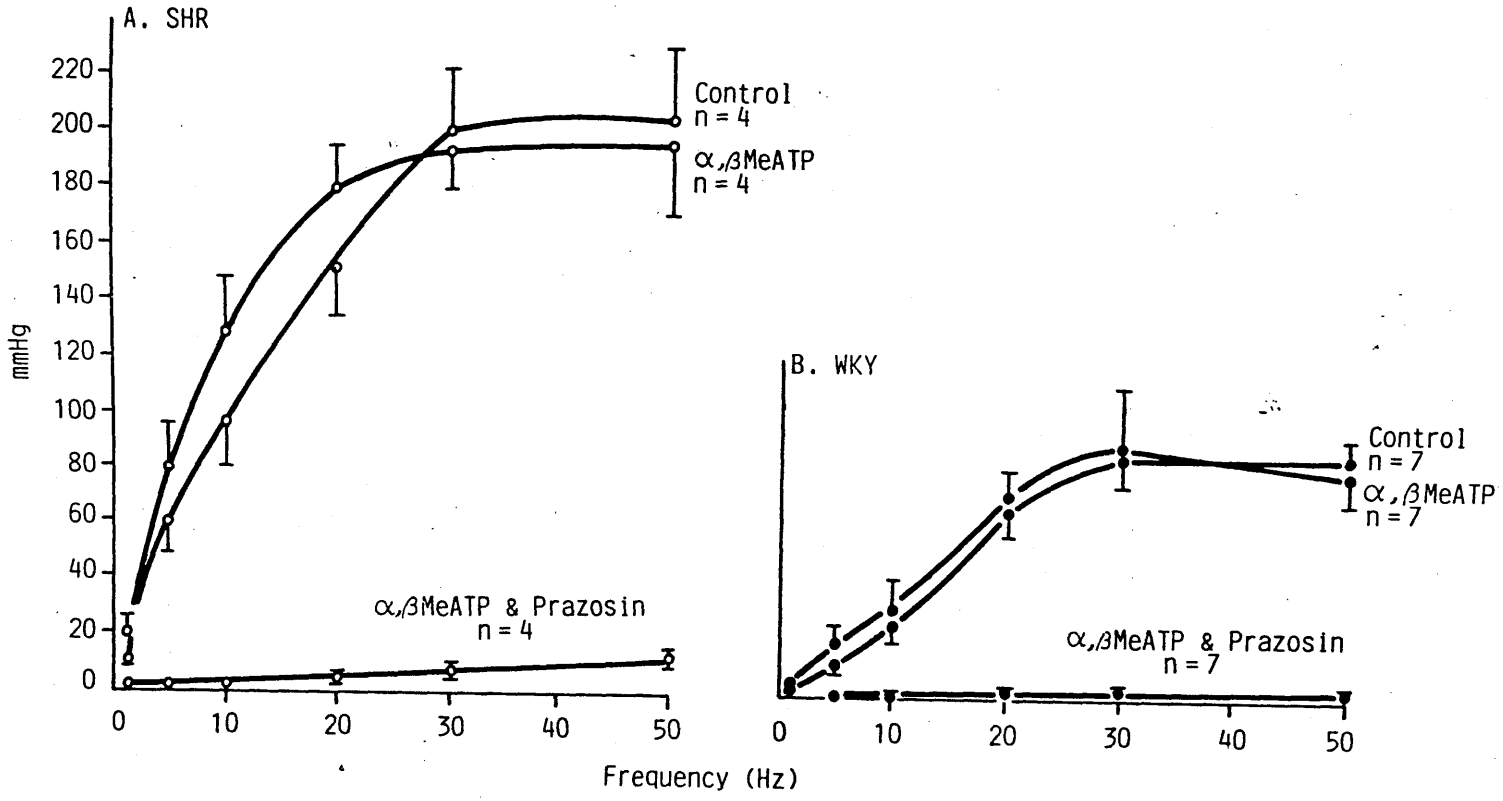
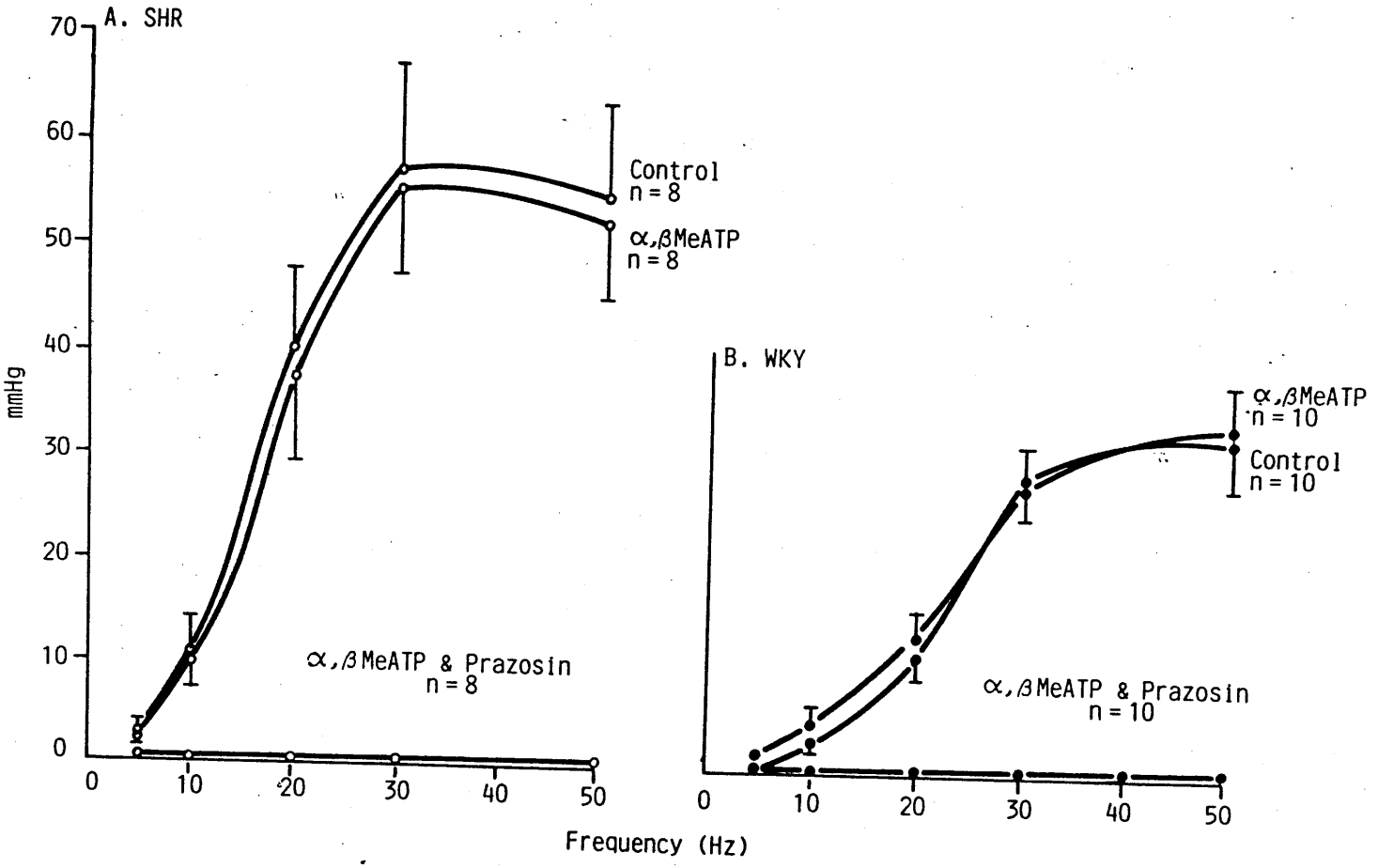

A. SHR
220
200
180
160
140
120
100
80
60
40
20
0
mmHg
Control
n = 4
α,βMeATP
n = 4
α,βMeATP & Prazosin
n = 4
0
10
20
30
40
50
B. WKY
Control
n = 7
α,βMeATP
n = 7
α,βMeATP & Prazosin
n = 7
0
10
20
30
40
50
Frequency (Hz)

Fig. 54: The effects of α,βMeATP (1×10^{-6}M) alone and in the presence of prazosin (1×10^{-7}M) on the mean pressor responses (mmHg) of mesenteric bed preparations from normotensive (WKY, ●, B.) and spontaneously-hypertensive (SHR, o, A.) rats to field stimulation (0.5ms, supramaximal voltage, 200 pulses at 1-50Hz). Each graph show the mean (± s.e.m.) of a number (n) of observations. α,βMeATP had no significant inhibitory effect on the pressor activity of mesenteric bed preparations from either SHR or WKY rats. The additional presence of prazosin virtually abolished all pressor responses, suggesting that they were mediated by NA.


A. SHR
70
60
50
40
30
20
10
0
mmHg
Control
n = 8
α,βMeATP
n = 8
α,βMeATP & Prazosin
n = 8
0
10
20
30
40
50
B. WKY
α,βMeATP
n = 10
Control
n = 10
α,βMeATP & Prazosin
n = 10
0
10
20
30
40
50
Frequency (Hz)

B. [^{3}H] OVERFLOW FOLLOWING PRE-INCUBATION WITH [^{3}H]-NA

In both SHR and WKY tail arteries, the [^{3}H] overflow, i.e. the amount of transmitter which escaped the uptake process and was collected from the tissue, declined exponentially with time, a steady state level being reached after approximately 2h. Subsequent electrical field stimulation (0.5ms, supramaximal voltage, 200 pulses at 5, 10 and 20Hz) produced a TTX (1×10^{-6}M)-sensitive increase in overflow of [^{3}H] into the superfusate. There was no significant difference in the [^{3}H] overflow from SHR and WKY rats (Fig. 55). Exposure to α,βMeATP (3×10^{-6}M for 30min) had no significant inhibitory effect on the stimulation-evoked overflow of [^{3}H] from either group of animals (Fig. 56), so eliminating the possibility that α,βMeATP had any pre-synaptic effects on transmitter release.

C. [^{3}H] OVERFLOW FOLLOWING PRE-INCUBATION WITH [^{3}H]-ADENOSINE

In contrast to the [^{3}H] overflow from tissues pre-incubated with [^{3}H]-NA, which was almost exclusively neuronal in origin, much of the [^{3}H] released from tissues pre-incubated with [^{3}H]-adenosine came directly from the smooth muscle, presumably due to a squeezing effect (Westfall et al., 1978). To abolish this 'squeezing' effect, muscle contractions were abolished by diltiazem (3×10^{-6}M) and prazosin (5×10^{-7}M), which were added to the Krebs solution throughout the experiments.

Following a 2h wash-out period, field stimulation (0.5ms, supramaximal voltage, 500 pulses at 10, 20 and 30Hz) of SHR and WKY tail arteries each produced small, but significant, TTX (1×10^{-6})-sensitive increases in [^{3}H] overflow in tissues pre-incubated with [^{3}H]-adenosine. There was no significant difference, however, in [^{3}H] overflow between SHR and WKY rats (Fig. 57).

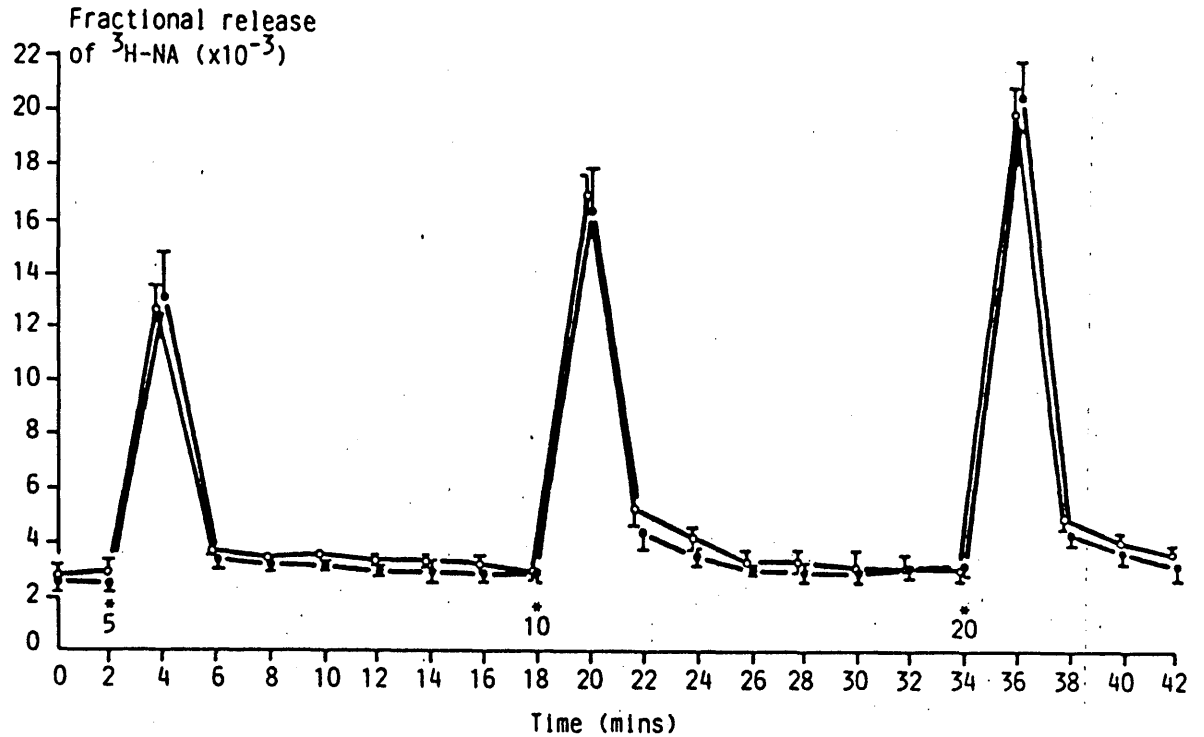
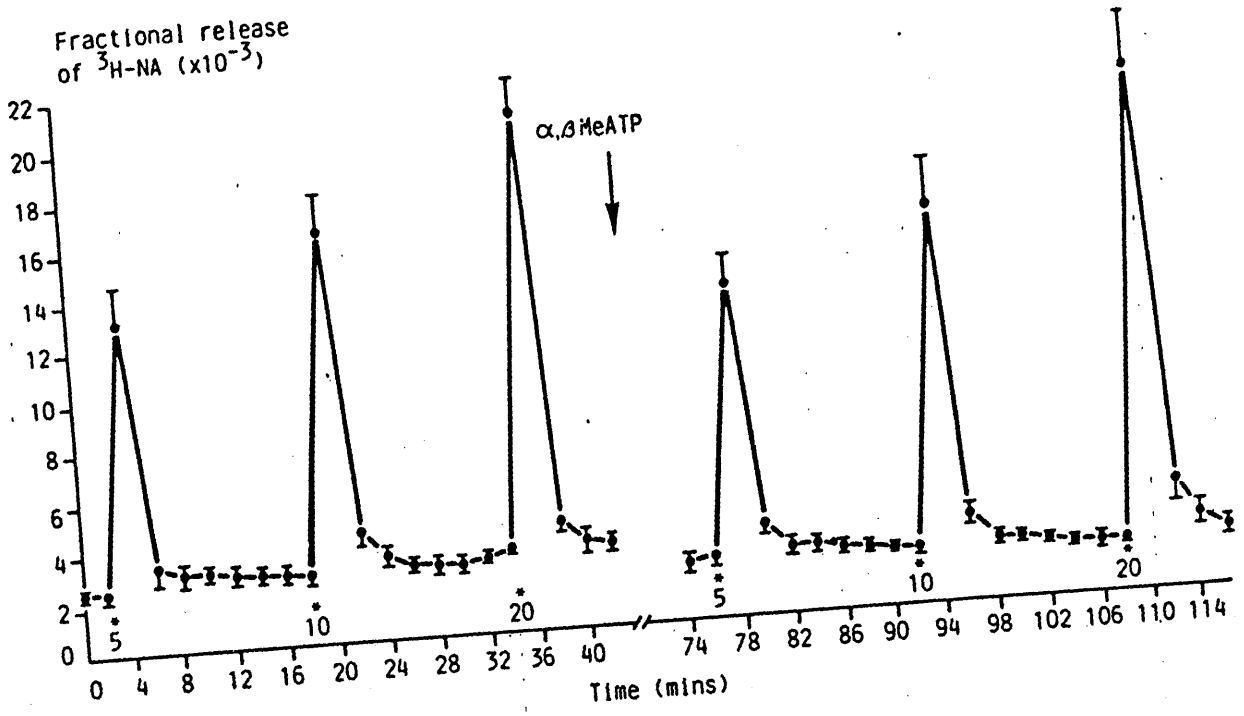
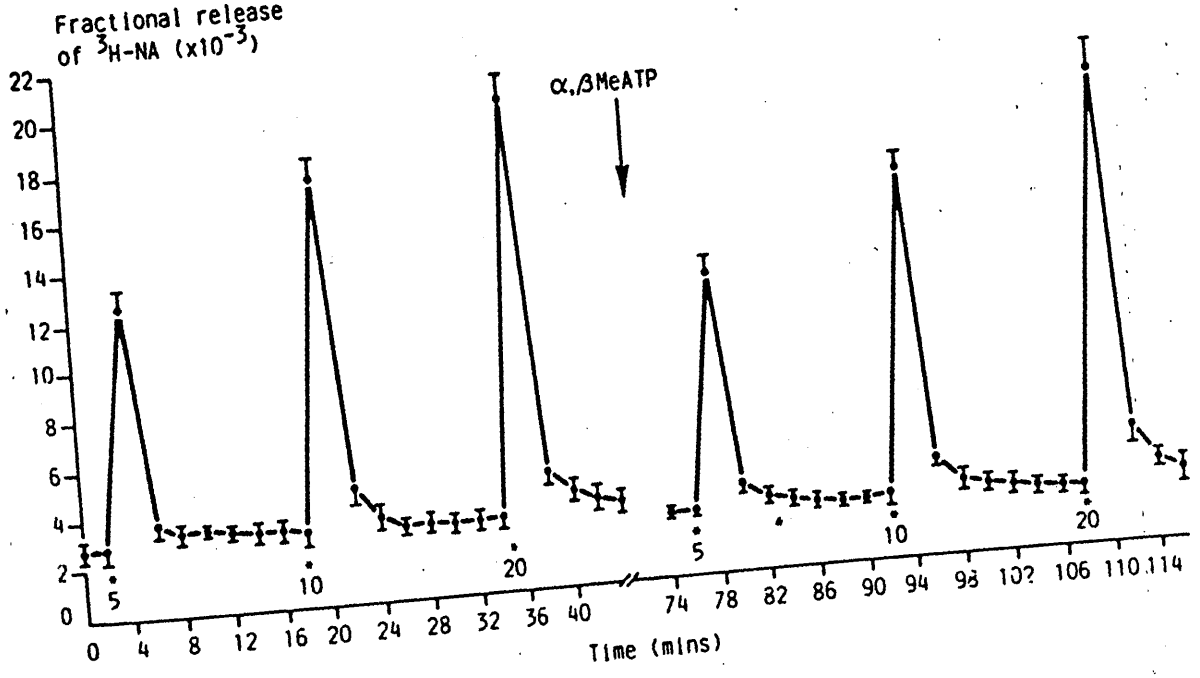


Fig. 55: The mean fractional release of [^{3}H] from tail arteries from normotensive (WKY, •) and spontaneously-hypertensive (SHR, o) rats, pre-incubated with [^{3}H]-NA (25μCiml^{-1}, 2 x 10^{-6}M NA), in response to field stimulation (0.5ms, supramaximal voltage, 200 pulses at 5, 10 and 20Hz, *) of intramural nerves. Each graph shows the mean (± s.e.m.) of a number (n) of observations (n = 6 for WKY, 5 for SHR). NA uptake blockers normetanephrine (NMN, 1 x 10^{-5}M) and desmethylimipramine (DMI, 1 x 10^{-6}M) were present throughout. There was no significant difference between the [^{3}H] overflow following field stimulation in either group of animals.

Fig. 56: The effects of α,βMeATP (3×10^{-6}M, ↓) on the mean fractional release of [^{3}H] from tail arteries from normotensive (WKY, top graph) and spontaneously-hypertensive (SHR) rats, pre-incubated with [^{3}H]-NA (25μCiml^{-1}, 2×10^{-6}M NA) in response to field stimulation (0.5ms, supramaximal voltage, 200 pulses at 5,10 and 20Hz, *) of intramural nerves. Each graph shows the mean (± s.e.m.) of a number (n) of observations (n = 6 for WKY, 5 for SHR). NA uptake blockers normetanephrine (NMN, 1×10^{-5}M) and desmethylimipramine (DMI, 1×10^{-6}M) were present throughout. α,βMeATP had no significant inhibitory effect on the [^{3}H] overflow evoked by field stimulation in either group of animals.


Fractional release
of ^{3}H-NA (x10^{-3})
α,βMeATP
Time (mins)


Fractional release
of ^{3}H-NA (x10^{-3})
α,βMeATP
Time (mins)

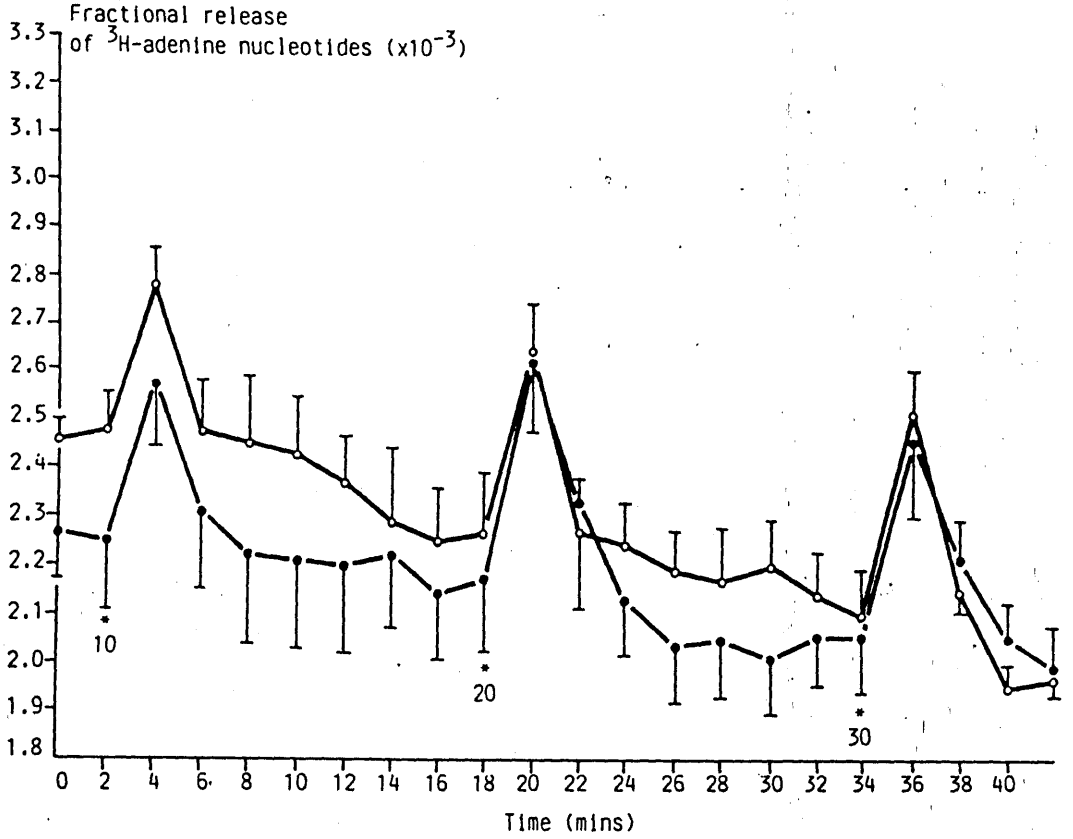


Fig. 57: The mean fractional release of [^{3}H] from tail arteries from normotensive (WKY, ●) and spontaneously-hypertensive (SHR, o) rats, pre-incubated with [^{3}H]-adenosine (100μCiml^{-1}, 1 x 10^{-6}M adenosine), in response to field stimulation (0.5ms, supramaximal voltage, 500 pulses at 10, 20 and 30Hz, *) of intramural nerves. Each graph shows the mean (± s.e.m.) of a number (n) of observations (n = 8 for WKY, 7 for SHR). Muscle contractions were abolished with prazosin (5 x 10^{-7}M) and diltiazem (3 x 10^{-6}M) which were present throughout. Increases in [^{3}H] overflow following field stimulation were small and there was no significant difference between the two groups of animals.

D. LIGHT MICROSCOPY

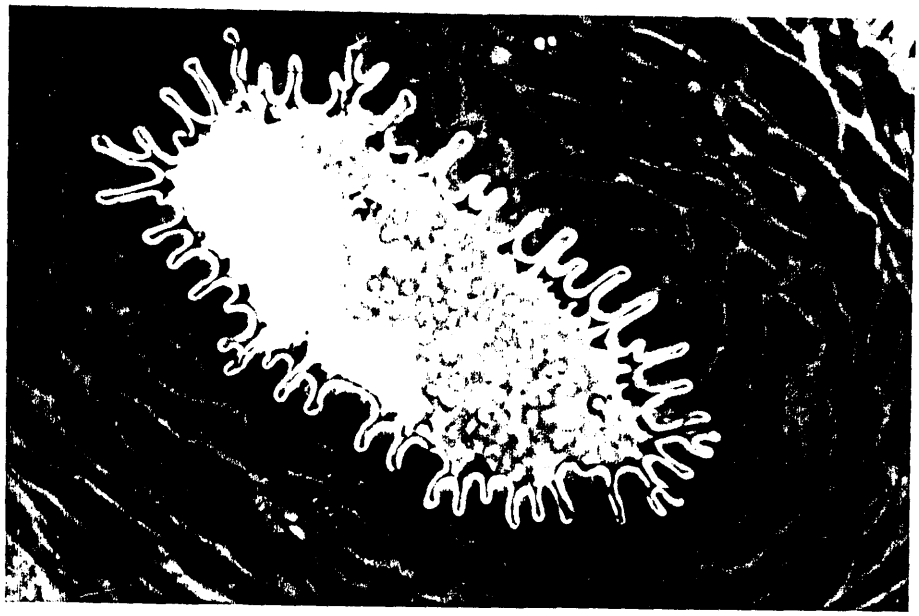
The possibility that structural changes in the vascular smooth muscle could have contributed to the increased vascular reactivity in arteries from spontaneously-hypertensive rats was investigated using light microscopy. Following toluidine blue\pyronin Y staining of freshly-dissected tissues (see Materials & Methods), the tail and superior mesenteric arteries from WKY and SHR rats were examined microscopically and photographed.

The tail artery is a typical example of a 'distributing' or 'muscular' artery. The tunica media in vessels from both normotensive (WKY) and spontaneously-hypertensive (SHR) rats consisted of smooth muscle cells, interspersed with elastin and collagen fibres (Fig. 58). Although the two groups of arteries appeared very similar, close inspection revealed small differences. Compared with those from WKY rats, the smooth muscle cells in the SHR tail artery had a slightly more crenolated appearance, perhaps reflecting the increased total peripheral resistance observed in vivo.

In these arteries, the inner tunica intima layer consisted of a single layer of endothelial cells, resting on a thin internal elastic lamina membrane. This membrane appeared convoluted. The endothelium closely conformed to the irregularities in the internal elastic lamina and sent processes through the fenestrations to establish myoendothelial junctions with the innermost smooth muscle cells on the tunica media. There were no obvious differences in the endothelium from normothesive and hypertensive rat tail arteries.

When the tail arteries were perfused at a constant rate ($4 ml min^{-1}$) with oxygenated physiological saline solution, substantial changes were seen in the preparations (Fig. 59). The lumen of the arteries were greatly enlarged. The convoluted internal elastic lamina was stretched and straightened and some 60-70% of the endothelial cells

A.



B.

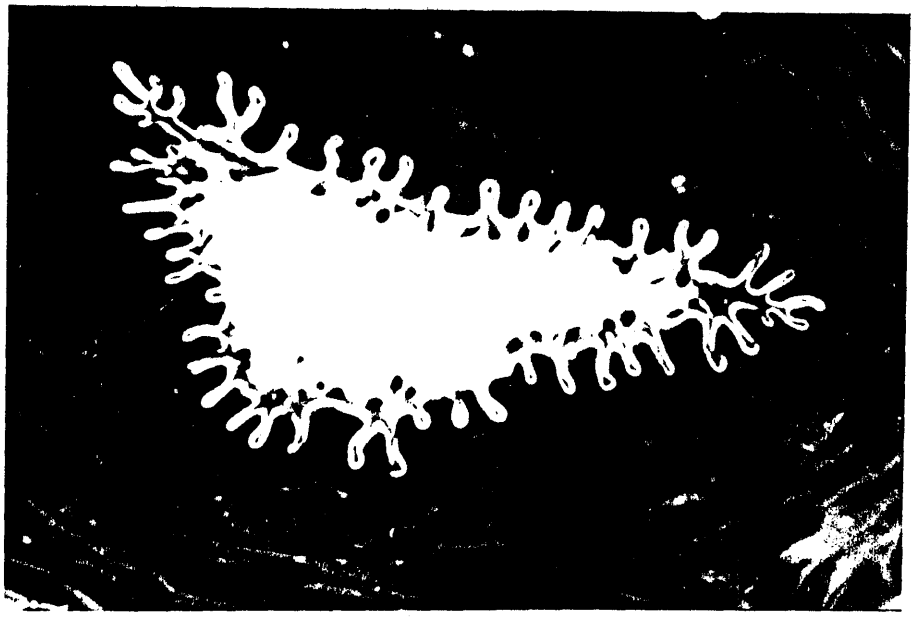
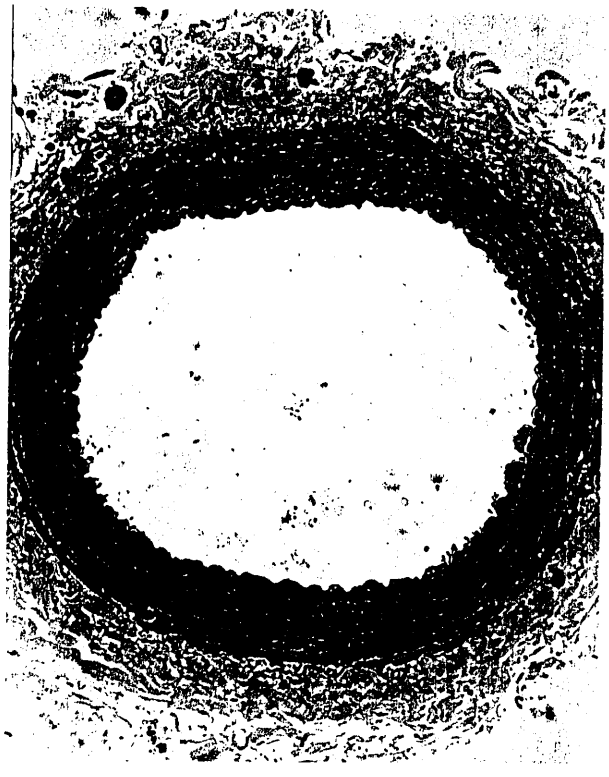


Fig. 58: Transverse sections of the tail artery from a normotensive (WKY, A.) and from a spontaneously-hypertensive (SHR, B.) rat. The tunica media of the vessels consisted mainly of smooth muscle cells, interspersed with elastin and collagen fibres. Both arteries looked very similar, however close inspection revealed that the smooth muscle cells in the SHR artery had a slightly more crenolated appearance. A layer of endothelial cells surrounded the lumen of each artery. Overall magnification x 400.

Fig. 59: Transverse sections of a tail artery from a normotensive (WKY) rat before (A. and C.) and after (B. and D.) perfusion with physiological saline solution ($4 ml min^{-1}$ for 1h). Perfusion greatly stretched the lumen of the artery and removed much of the endothelium. Overall magnification in A. and B. x 100 and in C. and D. x 250.

A.

B.



C.

D.

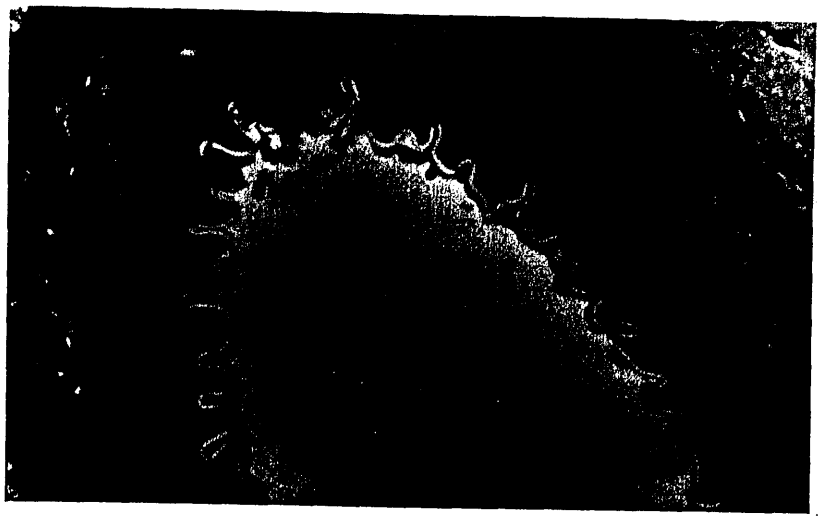
were missing. It appeared, on some occasions, that when α,βMeATP (2 x 10^{-6}M) was added to the perfusing solution, the damage to the artery was greater\more rapid. This may have been due to the vasoconstrictor effects of the drug, with resultant increases in perfusion pressure shedding off more endothelial cells, however more experiments would have had to be carried out before this observation could be confirmed. The degree of damage appeared similar in arteries from normotensive and hypertensive rats. Since all arteries were similarly perfused in the end organ experiments, it seems unlikely that any of the effects observed with field stimulation or exogenous agonists could have been attributed to endothelium-derived factors.

In contrast to the tail artery, the superior mesenteric artery is an 'elastic' or 'conducting' vessel (Fig. 60). The wall-to-lumen ratio was relatively small, with the rounded shape of the vessel being poorly maintained in vitro. Mesenteric arteries from SHR and WKY rats were indistinguishable. The tunica media of these arteries consisted of a thin layer of endothelial cells, separated from the internal elastic lamina by loose connective tissue. The general appearance of the tunica intima was less convoluted than in the muscular (tail) artery and the endothelial layer was less complete. The tunica media of the superior mesenteric artery had a striated appearance, composed of elastic membranes or sheets arranged concentrically, the spaces in between occupied with smooth muscle cells.

E. FALCK HISTOCHEMISTRY

The adrenergic innervation of the tail and superior mesenteric arteries from SHR and WKY rats was examined using Falck histochemistry, to see if the increased vascular reactivity observed in hypertensive animals could be attributed to alterations in the density of the adrenergic nerves.

A.



B.

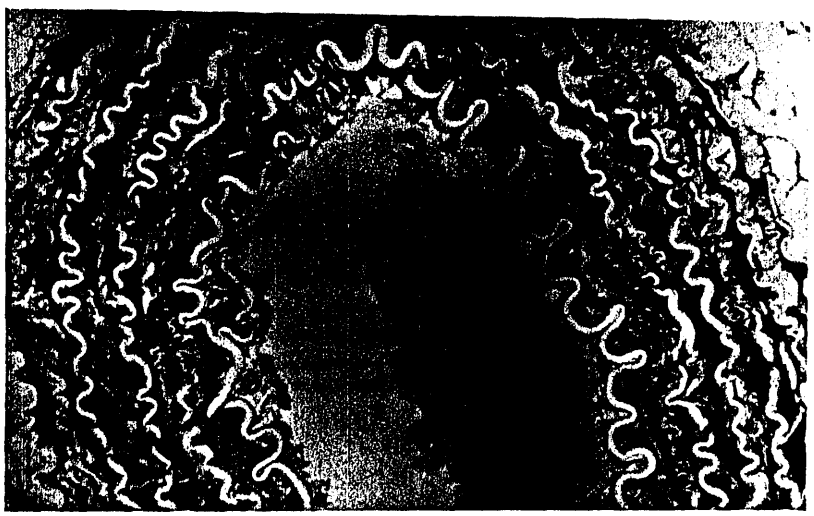
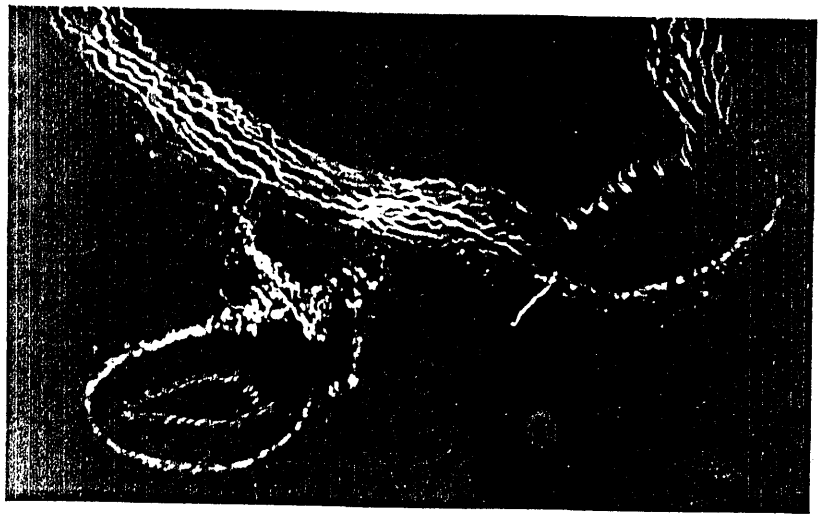


Fig. 60: Transverse sections of the superior mesenteric artery from a normotensive (WKY, A.) and from a spontaneously-hypertensive (SHR, B.) rat. Both arteries appeared similar. The tunica media had a striated appearance, composed of elastic membranes or sheets arranged concentrically, the spaces in between occupied by smooth muscle cells. A layer of endothelial cells surrounded the lumen of each artery. Overall magnification x 250.

Fig. 61 shows superior mesenteric arteries from a normotensive (WKY) and a spontaneously-hypertensive (SHR) rat. Very few adrenergic nerves were seen to innervate the superior mesenteric artery (c.f. tail artery, Fig. 62), an observation reflected in the relatively small pressor responses evoked by field stimulation. There appeared to be no differences in the density of the adrenergic innervation between the two groups of animals. The density of the adrenergic nerves appeared greater in the small branch arteries comprising the mesenteric bed (see Fig. 61A.). The superior mesenteric artery had a striated appearance, with the fluorescent bands representing elastin tissue.

In the rat tail artery from both normotensive (WKY) and spontaneously-hypertensive (SHR) rats, the adrenergic innervation was again restricted to the adventitial-medial border (Fig. 62). General observations from several artery sections showed no difference in the density of the adrenergic innervation between the two groups of animals, however, there may be a suggestion of an increased wall-to-lumen ratio in the hypertensive arteries, although no quantitative measurements were made. Further investigation would be required before this suggestion could be confirmed.

A.



B.

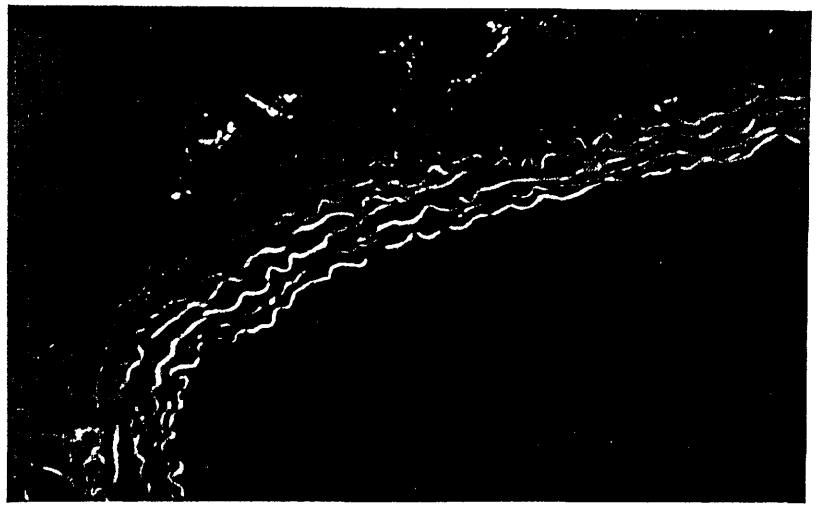
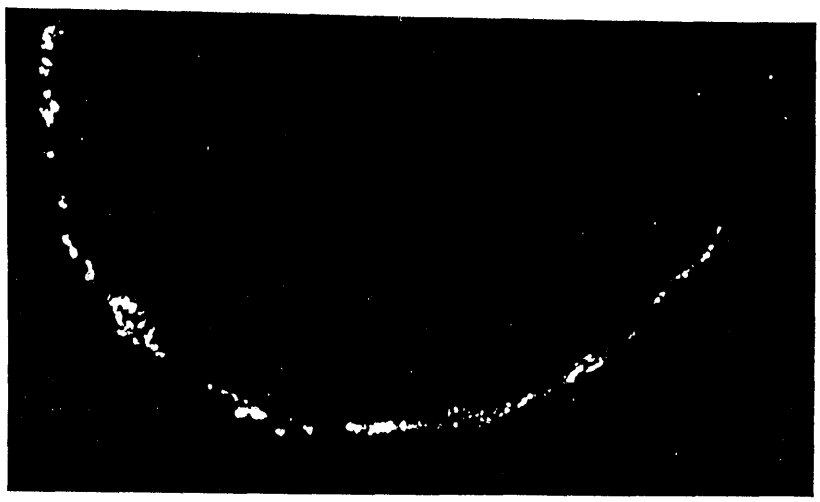


Fig. 61: The catecholamine fluorescence, demonstrated by Falck histochemistry, of the superior mesenteric artery from a normotensive (WKY, A.) and a spontaneously-hypertensive (SHR, B.) rat. The adrenergic innervation was sparse and restricted to the adventitial-medial border, though appeared similar in both groups of animals. The innervation was much more dense in the small branch artery seen in A. The striated bands of fluorescence throughout the wall of the arteries were due to elastin auto-fluorescence. Overall magnification in A. x 50 and in B. x 100.

A.



B.

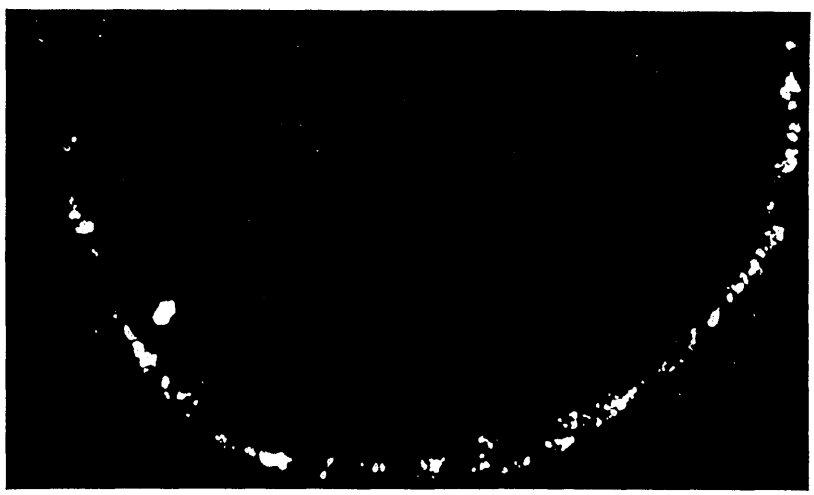


Fig 62: The catecholamine fluorescence, determined by Falck histochemistry, of the tail artery from a normotensive (WKY, A.) and a spontaneously-hypertensive (SHR, B.) rat. The adrenergic innervation was restricted to the adventitial-medial border and appeared similar in both groups of animals. Overall magnification x 100.

CHAPTER 4: DISCUSSION

Compared with those of some 50 years ago, current views on autonomic neurotransmission differ in two quite fundamental ways. First, there has been a virtual explosion in the number of substances proposed as transmitters. In addition to the classical transmitters, NA and ACh, many substances are now believed to be synthesized, stored and released from nerves. These include GABA, 5HT, purines and peptides, and each may have a potential neurotransmitter function. Secondly, in addition to containing different biologically-active compounds, the transmission process itself may, in contrast to the classical one nerve-one transmitter view (Dale, 1935), involve more than one transmitter substance. This latter observation has led directly to the current concept of co-transmission.

The development of the idea of co-transmission, from the advent of histochemical techniques which enabled transmitter substances to be visualized, to the definitive illustration of their presence in the work of Furshpan et al. (1976; 1986) using cell culture techniques, has implied that the genetic information available for producing all transmitter-type molecules is available in every nerve cell and that each neurone may utilize more than one transmitter and thus exhibit co-transmission.

In spite of the widespread reported occurrence of co-transmission, there has been some controversy surrounding the cited evidence. Much of this dispute has centred around the question as to whether co-transmission itself, or merely co-existence or co-release, is involved. This has led to the need for a clearer understanding of what is meant by these terms. It is generally conceded that the term 'co-transmission' refers to the action of two or more transmitters, simultaneously released from the same neurone, on a single target cell. 'Co-existence' and 'co-release', on the other hand, refer to the anatomical co-localization and release, respectively, of 2 or more trans-

mitter substances together from one neurone. Co-existence and co-release are each a necessary, but by themselves an insufficient condition for co-transmission.

Not surprisingly, many of the examples of co-transmitter neurones fail to fulfil these criteria. For example, in the cat submandibular salivary gland, ACh and VIP have been proposed to act as co-transmitters following their simultaneous release from the sphenopalatine nerves (Lundberg et al., 1980). A more recent study, however, (Håkanson et al., 1982) has shown that, under normal conditions, sphenopalatine neurones are normally either VIP-immunoreactive or rich in AChE (indicating the presence of ACh); only after treatment with colchicine did all neurones contain both markers. Thus, since co-storage is a necessary requirement for co-transmission, the cat submandibular salivary gland fails to fulfil the criteria necessary for establishing co-transmission. Furthermore, although NA and NPY are demonstrably co-released from sympathetic nerves innervating the rat vas deferens, they still failed to qualify as co-transmitters since they act on different target cells - NA acts post-synaptically as a true neurotransmitter, whereas NPY mediates its effects pre-synaptically (Lundberg & Stjärne, 1984).

Inherent in the discussion of the foregoing results is the need to establish whether or not co-transmission truly exists. Clearly any experimental contribution to co-transmission must satisfy the criteria of the definition i.e. both substances, following their simultaneous release from a single nerve ending, must act on the same target cell. It was with this problem in mind that intracellular micro-electrode recording was selected as the experimental technique. This allows, not only the measurement of transmitter release, but also of the transmitter effect on target cells.

Among the most interesting facets of proposed co-transmitter

activity is the challenge to examine the electrical and mechanical contribution of the individual transmitters to the overall neurogenic response.

The work outlined in this thesis has provided strong evidence for co-transmission involving NA and ATP (or a closely related nucleotide) in several vascular and non-vascular smooth muscles. The existence of co-transmission is supported by several experimental observations. First, there is little doubt that the origin of each transmitter substance was neuronal, since all electrical and mechanical responses to field stimulation were abolished by TTX and lignocaine, which selectively block the increase in sodium permeability underlying the nerve action potential and by Ca^{2+}-removal from the physiological saline solution, which prevents transmitter release from nerves. Additional confirmation that the substances released were neuronal in origin was the finding that in both the mouse vas deferens and the rat anococcygeus muscle the responses to extrinsic nerve stimulation were indistinguishable from those evoked by field stimulation, thus ruling out the possibility that any of the observed effects following field stimulation were due to artifacts of the method of stimulation employed. Furthermore, there was no evidence to support the claim (Stjärne, 1977; Alberts et al., 1981) that field stimulation facilitated transmitter release, or liberated substances directly from the nerves, other than by action potential propagation in pre-terminal axons.

That these transmitters were contained in the same neurone was implied by the ability of guanethidine and 6-OHDA to each abolish the entire neuronal response. This confirms that both transmitters were co-released from the same nerve ending, rather than from separate noradrenergic and purinergic nerves. In the mouse vas deferens, the observation that both electrical and mechanical responses persisted following reserpine pre-treatment strongly suggested that all of the

responses to sympathetic nerve stimulation could not be attributed to NA and that two different substances were involved in the transmission process.

Results from the foregoing experiments clearly support the idea that neuronally-released substances acted as co-transmitters. Each transmitter substance evoked post-synaptic effects which could be mimicked by exogenous agonists and blocked by antagonists.

The transmitters involved in the neurogenic response were a catecholamine (NA) and a purine nucleotide (most likely ATP). Several observations point towards this conclusion. For example, in the mouse vas deferens, the initial rapid twitch contraction was antagonized by α,βMeATP, suggesting that it was mediated by ATP, whereas the slower tonic contraction was selectively abolished by α-adrenoceptor antagonists, suggesting that it was mediated by NA. Exogenously added NA and ATP each mimicked faithfully the time course of the respective tonic and phasic components of the neurogenic response. This ability of ATP to mimic the nerve-mediated response was also extended to electrical events, where local application of the nucleotide evoked membrane depolarizations with characteristics similar to the e.j.p.s produced by nerve stimulation. These effects of exogenous ATP could be demonstrated in vasa in which the adrenergic nerves had been destroyed with 6-OHDA, thus eliminating the possibility of a pre-synaptic depolarizing action of ATP in transmitter release.

Experimental evidence supporting the idea of ATP as a neurotransmitter has relied heavily on the use of one drug - α,βMeATP. That this drug is specific for purinoceptors is of utmost importance in the evaluation of the experimental results. There have been claims, however, that α,βMeATP may be non-specific. It has been proposed that it could antagonize the e.j.p.s irrespective of whether they are mediated by NA or ATP (Byrne & Large, 1986) or that it may have channel blocking acti-

vity, in a manner similar to apamin (Kotecha & Neild, 1987). No evidence to support these suggestions was obtained from the present investigation. In the rat anococcygeus muscle, α,βMeATP failed to inhibit either the electrical or mechanical responses to neuronally-released or exogenously-added NA. Taken together with the observation that α,βMeATP did not reduce transmitter release in either the rabbit ear (Allcorn et al., 1985) or rat tail artery (this thesis), the present results suggest that this drug is indeed specific for purinoceptors and does not modify post-synaptic effects of NA, thus warranting its use in the study of purinoceptor pharmacology.

While both NA and ATP evoked post-synaptic effects in a variety of autonomically innervated smooth muscles, the contribution of each transmitter to the electrical and mechanical activity varies from one tissue to another.

With regard to the mechanism of action of the co-transmitters, it is clear from the present results that ATP mediates its post-synaptic effects via a voltage-dependent mechanism. The e.j.p.s evoked by sympathetic nerve stimulation were selectively blocked by α,βMeATP, a drug which blocks P_2-purinoceptors by desensitization (Kasakov & Burnstock, 1983) and mimicked by local application of ATP. In both the mouse vas deferens and the rabbit mesenteric artery the functional significance of this purinergic electrical event was clear - the e.j.p.s were part of the mechanism underlying a component of the contractile response to field stimulation. A similar voltage-dependent, purinergically-mediated contractile response has also been reported by other workers in the mesenteric artery of the rabbit (Kügelgen & Starke, 1985) and dog (Muramatsu, 1986) and in the rabbit saphenous (Burnstock & Warland, 1987) and central ear (Kennedy et al., 1986) arteries. In the present study in the rabbit ear artery, no evidence could be found for a purinergic component in the contractile response, although ATP-mediated

e.j.p.s were seen. This result was disappointing; however, it is possible that the purinergic component of the contractile response could have been 'missed' due to the parameters of stimulation used. Thus, it has been reported (Kennedy et al., 1986) that, in the rabbit ear artery, the relative contribution of each co-transmitter (NA and ATP) to the neurogenic contraction is highly dependent of the parameters of stimulation employed, with short (1s) bursts of stimuli at low frequencies (2-5Hz) favouring the prazosin-resistant (purinergic) component of the response.

There is also more direct support for the view that ATP acts as a neurotransmitter from recent electrophysiological experiments. The nature of the ATP-mediated depolarization, in ionic terms, has been mapped following work on dispersed cells using the patch clamp technique. Using this technique on cells from the rabbit ear artery (a tissue in which ATP has been proposed to act as a co-transmitter), Benham et al. (1987) have demonstrated that externally-applied ATP evoked membrane depolarizations which were associated with cationic-selective conductance changes, allowing monovalent and divalent cations to pass across the membrane. Such a selective membrane permeability change is consistent with the idea of a receptor-operated ion channel, activated by ATP.

More recently, such an ion channel has infact been reported in the rabbit ear artery. Benham & Tsien (1987) have proposed a novel receptor-operated Ca^{2+}-permeable channel activated by ATP. This channel is directly operated by ligands, independently of second messengers and its activation may lead to the increased $[Ca^{2+}]_i$ needed to trigger the contractile response. The existence of such an ion channel would strongly favour the suggestion that ATP mediates its effects by a specific receptor-operated mechanism.

Taken together with the present results, it was concluded that,

in many tissues, field stimulation of intramural nerves evokes an ATP mediated, voltage-dependent contractile response -important evidence that the purine nucleotide has a transmitter function.

The nature of the purinergic receptor mediating these effects appeared to be of the P_2 variety. This suggestion was based on several observations. First, all electrical and mechanical responses to exogenously added and neuronally-released ATP were abolished by α,βMeATP, a drug reported to specifically block P_2-purinoceptors, while having no effect on P_1-purinoceptors (Kasakov & Burnstock, 1983). Secondly, in the mouse vas deferens, the rank order of potency of adenine nucleotides in evoking membrane depolarizations was α,βMeATP << ATP << ADP < AMP < adenosine. Such a rank order of potency is consistent with the idea of a P_2-receptor mediated response (Burnstock, 1972; 1979). The observation that the stable analogue of ATP, α,βMeATP, was most potent at producing membrane depolarizations strongly suggests that the observed effects were mediated by ATP itself, rather than one of its breakdown products (e.g. adenosine), and that the receptor involved was of the P_2 variety. No attempts were made to further classify the receptors into the P_{2x} or P_{2y} variety (c.f. Burnstock & Kennedy, 1985) since it was felt that such sub-division would be only speculative until such times as specific receptor antagonists are developed.

In spite of the activity of ATP in the tissues examined, it is clear that the major contractile response to field stimulation is mediated by NA. In each tissue, field stimulation evoked a contractile response in the presence of α,βMeATP, suggesting that NA can evoke contractions independently of the presence of ATP. Unlike the contractions evoked by ATP, however, those evoked by NA appeared to be mediated largely by a voltage-independent mechanism. Only in the rabbit ear artery was there any evidence for an electrical event mediated by NA. This took the form of a small, slow membrane depolarization which

followed the purinergically-mediated e.j.p.s. Such responses, which have previously been reported in several other arteries, including the guinea-pig uterine (Bell, 1969), mesenteric (Spedden, 1964) and the rat tail arteries (Cheung, 1982; 1984) have been proposed as the mechanism underlying the noradrenergic contraction in these tissues. The slow time course, small amplitude and graded nature of the depolarization could best be explained by diffusion of NA, which escapes the uptake processes, to extra-junctional receptors, a notion supported by the finding that a higher stimulation strength was required to elicit the slow depolarization than to obtain the e.j.p. (Cheung, 1982).

It would appear, in fact, (see Bolton & Large, 1986) that, with NA, a wide spectrum of electrical events accompanies the mechanical contraction. This ranges from phentolamine-sensitive, fast e.j.p.s (e.g. in the rat anococcygeus) through slow depolarizations (e.g. in the rabbit ear artery) to completely voltage-independent mechanisms (mouse vas deferens and rabbit mesenteric artery). Where voltage dependent mechanisms operate, tension is most likely generated via the activation of specific voltage-operated Ca^{2+} channels, whereas, where no change in membrane potential is observed, tension is generated by the activation of receptor-operated Ca^{2+} channels.

In the present investigation, no evidence could be found for any electrical accompaniment to the noradrenergic contraction in either the mouse vas deferens or the rabbit mesenteric artery, even in the presence of the NA uptake blockers NMN and DMI. Furthermore, locally applied NA, in contrast to ATP, failed to evoke any change in membrane potential. Although the recording techniques employed may not have been sensitive enough to detect the small changes in membrane potential which might occur, the most likely explanation is that, in the mouse vas deferens and rabbit mesenteric artery, NA mediates its contractile effects via a voltage-independent mechanism. The exact nature of this

mechanism was not investigated; however, in the absence of any alteration in membrane potential, changes in membrane phosphatidylinositol turnover may operate. Interestingly, in the rabbit mesenteric artery, where noradrenergically-mediated vasoconstrictions were unaccompanied by any change in membrane potential, exogenous NA enhanced the breakdown of phosphatidyl inositol 4,5-bisphosphate (PIP_2), an effect inhibited by prazosin (Hashimoto et al., 1986), suggesting that biochemical changes may mediate at least part of the noradrenergic vasoconstriction in this tissue.

It was thus concluded that, with NA, a dual mechanism seems to operate in the generation of tension in smooth muscles, such that voltage-dependent and independent mechanisms co-exist, the relative contribution of each to the overall response varying from one tissue to another.

Although the co-transmission hypothesis is attractive in explaining these results several alternative theories have been proposed. For example, it has been suggested (Von Euler & Hedqvist, 1975) that, in the guinea-pig vas deferens, K^+ ions, released from the adrenergic axons during the recovery phase of the action potential, increase extracellular K^+ concentration and directly depolarize the muscle membrane, thus producing the apparent nonadrenergic component of excitatory transmission in this tissue. K^+ itself, would not satisfy the criteria of a neurotransmitter, since it has no synthesis, no storage vesicles, no breakdown mechanism and no post-synaptic receptors. Furthermore, although this theory cannot be ruled out, it seems unlikely in view of the specific antagonistic effects of α,βMeATP on purinergic responses.

An alternative interpretation of transmission in the mouse vas deferens from that proposed in the present study has been suggested by Stjärne & Astrand (1985). Using radiochemical and mechanical record-

ing, it was proposed that both transmitters, acting in concert, were involved in the neurogenic response, the ''diphasic'' nature of which was an inherent feature of the tissue and could be triggered by NA, ATP or other, different drugs. This view of Stjärne and Astrand does not contradict the co-transmission hypothesis, but it makes the need for a second transmitter unlikely. From the present investigation, there was no evidence to support their suggestion. Here, there was a clear indication that each transmitter (NA and ATP) contributed a single component of the response and there was no evidence to support the idea that the tissue itself determined the ''diphasic'' nature of the contraction.

Nor is it likely that the effects of the nucleotide could be attributed to a relatively non-specific increase in membrane conductance arising from an ATP-induced permeabilization (first demonstrated in mast cells by Dahlquist, 1974; Bennett et al., 1981). The depolarizations evoked by both exogenously-added ATP and neuronally-released transmitter appeared to be receptor-mediated, since they were selectively antagonized by α,βMeATP. In addition, the idea of a non-specific membrane permeabilization by ATP is also questioned by the results from preliminary experiments involving alterations in the external ionic concentration, which strongly suggested the selective involvement of Na^+ and K^+ ions in the membrane depolarization.

The main alternative explanation to the co-transmission hypothesis has come by way of explaining the existence of α-adrenoceptor antagonist resistant responses to sympathetic nerve stimulation on the basis of ɣ-receptors, an idea first proposed by Hirst & Neild (1981). According to this hypothesis, neuronally-released NA acts on a population of junctional receptors on the smooth muscle, called ɣ-receptors, to depolarize the membrane and produce e.j.p.s which are resistant to α-adrenoceptor antagonists. Support for the hypothetical ɣ-adrenocep-

tor was obtained from experiments demonstrating the ability of iontophoretically-applied NA to evoke membrane depolarizations in the guinea-pig mesenteric arterioles (Hirst & Neild, 1981) and rat basilar artery (Hirst et al., 1982; Byrne & Large, 1986). In addition to the γ-adrenoceptor, there are α-adrenoceptors, located extra-junctionally. These are sensitive to α-adrenoceptor antagonists and interaction with NA leads to contractions without any change in the membrane potential.

Several objections have been raised against the γ-receptor hypothesis. For example, the results which form the backbone of the γ-receptor hypothesis have not proved to be easily reproducible. Indeed, only a few groups of workers have been able to provide any evidence for them (Hirst & Neild, 1981; Hirst et al., 1982; Byrne et al., 1985; Byrne & Large, 1986). Furthermore, this evidence has been restricted to a few blood vessels and does not extend to the many other tissues known to produce α-adrenoceptor antagonist-resistant responses.

In the present study no evidence could be found to support the existence of γ-receptors. Two main objections to this theory have been raised. First, in the mouse vas deferens, locally applied NA, in contrast to ATP, produced no significant change in membrane potential. Similar observations have been reported in the guinea-pig vas deferens (Sneddon & Westfall, 1984), rabbit ear artery (Suzuki et al., 1984; Suzuki, 1985) and rabbit and guinea-pig mesenteric arteries (Ishikawa, 1985).

Secondly, the γ-receptor hypothesis assumes that the effects evoked by sympathetic nerve stimulation are attributed to one neurotransmitter acting on two distinct sets of receptors. The most convincing evidence against the idea that only one transmitter substance is involved in the transmission process has come from experiments using reserpine to deplete the tissue content of catecholamines. If the e.j.p.s were mediated by NA acting on γ-receptors, they should be

abolished by reserpine pre-treatment. Such an effect, however, was not observed. When the NA content of the nerves innervating the mouse vas deferens was depleted with reserpine pre-treatment e.j.p.s and small residual contractions persisted. Both were abolished by α,βMeATP, suggesting that they were mediated by ATP.

It may be argued, of course, that locally applied NA did not produce membrane depolarizations because the receptors are located only in the tight junction between the nerve and muscle, whereas ATP can depolarize since its receptors are more widespread at extra-junctional sites. Such an idea, however, seems unlikely since, in the rat anococcygeus muscle, exogenous NA can evoke both slow and fast membrane depolarizations, the latter presumably mediated via junctional receptors. Furthermore, antagonists readily block the neuronally-evoked e.j.p.s, which are mediated by transmitter acting on junctional receptors. Assuming agonists and antagonists alike have similar access to these receptors, it seems unlikely that the inability of NA to evoke a membrane depolarization could be due to an access problem.

Taken together, these results do not preclude the existence of χ-receptors, but make the possibility that neurotransmission involves only one transmitter unlikely.

There is clear evidence that co-transmission, as defined above, occurs in experimental situations. On the other hand, evidence for the phenomenon <u>in vivo</u> is, at present sparse, although there are strong suggestions that it will eventually be shown to occur (see Campbell, 1987). The physiological consequences of co-transmission <u>in vivo</u> are likely to be considerable and already some possibilities as to what they may be have emerged.

One of the more obvious consequences of co-transmission is the increased complexity which it brings to the organization of the nervous system. Before the advent of co-transmission, the nervous system

apparently relied on a relatively few integrative processes, such as spatial or temporal summation, facilitation and fatigue. The existence of many additional transmitters, co-released from a single neurone has added a new order of complexity to the function of the nervous system, with interactions occurring between individual transmitters at both pre-and post-synaptic sites.

As a result of this increased complexity, it has been necessary to re-classify neurones, since it is clear that the existing classical organization is impracticable. Not surprisingly, this up-dated classification has attempted to organize cholinergic and noradrenergic neurones on the basis of the presence of the purine or peptide co-existing with the classical transmitter. For example, the cholinergic neurones of the submucous plexus in guinea-pig ileum can be divided into three subtypes: ACh-substance P; ACh-NPY-SOM-cholecystokinin; and ACh without known peptides (Furness et al., 1984). Attempts at classification on the basis of ancillary transmitters, however, have proved cumbersome and unwieldy.

As an alternative, it has been proposed (Campbell, 1987) that neurones exhibiting co-transmission may be conveniently grouped according to the function they subserve; indeed, natural groupings of transmitters appear to exist. For example, the parasympathetic vasodilator innervation of salivary glands operates by ACh-VIP co-transmission in the cat (Lundberg, 1981), dog (Shimizu & Taira, 1979) and rat (Bloom et al., 1979), but in the rabbit it seems to be purely cholinergic (Morley et al., 1966; Edvinsson et al., 1980). This has led to the interesting suggestion (see Campbell, 1987) that the target organ may influence the co-transmitters released by the nerve. Thus, although a neurone may contain the genetic information required to synthesize many transmitter molecules, it is the target organ which, by determining transmitter function, has overall control on gene expression and hence of the co-

transmitters released. This implies that there will be some situations in which a second transmitter may not be released, simply because the target organ has no function for it. This is a possible explanation for the results obtained in the rat anococcygeus, where all electrical and mechanical activity evoked by field stimulation was abolished by prazosin or phentolamine, suggesting that neurotransmission was purely noradrenergic. No evidence was obtained from the present investigation, to support the suggestion (Byrne & Large, 1984), of a fast e.j.p., resistant to α-adrenoceptor antagonists, and hence the participation of a purine nucleotide in the membrane response to field stimulation in this tissue.

Thus, the present results disagree with those of Byrne and Large (1984). The experimental conditions and, in particular, the temperature at which the work was carried out, may have been responsible. The purinergically-mediated e.j.p. observed by Byrne & Large at room temperature may have been absent from our studies at 36°C due to the rapid hydrolysis of the nucleotide at these temperatures (Cunnane, personal communication). However, the fact that room temperature is an artificial condition for the muscle, raises the possibility that the source of the ATP mediating the e.j.p. may have been non-neuronal and, in fact, an artifact of the experimental conditions.

Although no evidence could be found for co-transmission in the rat anococcygeus muscle, two distinct types of noradrenergically-mediated membrane events were observed in the present investigation. The presence of a two component e.j.p. may indicate some degree of complexity in the transmission process. Alternatively, it may simply reflect differences in the release of the transmitter or its access to receptor sites. It is unlikely that the fast e.j.p. is an enhanced or facilitated slow depolarization, since both the fast and slow membrane changes could be obtained in response to a single stimulus. Except to

establish their common transmitter origin, the function of each component of the depolarization was not investigated. There is, therefore, no evidence to deny the proposal (Byrne & Large, 1984) that the smaller, slow depolarization - the most common response to a single stimulus -arises from the asynchronous release of transmitter, while the larger, faster e.j.p.s are the product of a synchronised release of transmitter following trains of stimuli.

Perhaps the most obvious question which must be addressed following the advent of co-transmission is that of the physiological significance of this process. Several possible roles have been proposed. In many of the tissues examined, both NA and ATP clearly played a physiloical role in the contractile response to nerve stimulation. In the case of, for example, the rabbit ear artery, where ATP, co-released with NA from sympathetic nerves, could be demonstrated to produce only electrical effects post-junctionally, with no accompanying mechanical contractions, the possibility exists that ATP may act as a neuromodulator. Thus, if co-transmission occurs, the 'modulatory' transmitter may have the sole function of regulating the effects of the 'major' transmitter, and have no effect at all if released alone. In the rabbit ear artery, where no functional role for ATP was observed in the contractile response to field stimulation, such a modulatory role may exist. In this tissue, pressor responses to field stimulation were significantly potentiated by α,βMeATP. Such an observation may suggest that ATP reduces the sensitivity of α-adrenoceptors, an effect reversed by α,βMeATP. Such a mechanism may operate <u>in vivo</u>, whereupon ATP down-regulates the sensitivity of the α-adrenoceptors, thus preventing desensitization to NA. Alternatively, the potentiating effects of α,βMeATP may simply reflect the depolarizing actions of the drug, bringing more of the nerve fibres nearer to the threshold for firing.

Evidence suggesting transmission by a major transmitter and a

modulatory substance is already availably in other tissues. The salivation caused by parasympathetic nerve stimulation in cats is fully blocked by atropine (Heidenhain, 1872) and transmission has been regarded as simply cholinergic. However, treatment with VIP antiserum reduced the secretory response, suggesting partial mediation by VIP (Lundberg et al., 1981), but VIP alone does not cause secretion (Bloom & Edwards, 1980). It emerged that VIP increased the secretory response to ACh (Lundberg et al., 1982), perhaps by increasing the affinity of muscarinic receptors for ACh (Lundberg & Hökfelt, 1983). Thus it seems that the role of VIP in the salivary ACh-VIP co-transmission is to facilitate the effects of ACh.

ATP may also act as a co-ordinator of muscle contraction. For example, in blood vessels, the adrenergic innervation is restricted to the adventitial-medial border. ATP, released as a co-transmitter from these nerves, may initiate a propagating action potential, which then spreads throughout the muscle wall, producing a co-ordinated contraction.

Since there are clearly many possible physiological consequences of co-transmission, the likelihood exists that these may also manifest themselves in pathological conditions. Indeed, such an idea has already been proposed (Vidal et al., 1986) according to which ATP, co-released with NA from sympathetic nerves, may play a role in the development and/or maintenance of hypertension.

The pressor response elicited by periarterial nerve stimulation of arteries from hypertensive (SHR) rats exceeded those from age-matched normotensive (WKY) animals (Ekas & Lokhandwala, 1981; Vidal et al., 1986). This increased vascular reactivity in SHR may result from alterations in pre- and/or post-synaptic activity.

Pre-synaptically, increased sympathetic nerve activity originating from the central cardiovascular centres has been implicated in the

development and maintenance of spontaneous hypertension (Juskevich et al., 1978; Saavedra et al., 1978; Takeda & Bunag, 1978). Alternatively, local changes at the level of the post-ganglionic sympathetic neurone, resulting in an increased transmitter release, have also been suggested (for review see Westfall & Meldrum, 1985).

Post-synaptically, increased vascular reactivity has been attributed to an increased receptor sensitivity in SHR arteries (Haeusler & Haefely, 1970; Lais & Brody, 1978; Ekas & Lokhandwala, 1981) as a result of structural changes in the wall-to-lumen ratio and/or factors beyond the vasculature membrane leading to possible changes in the excitation-contraction coupling mechanism.

Results from the present study in both rat tail arteries and mesenteric bed preparations re-affirmed the increased vascular reactivity in hypertensive rats. Since pressor responses to exogenous catecholamines (NA and ADR) were also potentiated in the arteries from SHR animals, alterations in the post-synaptic α-adrenoceptors appeared to be involved in the expression of the hypertensive state. The possibility that an additional pre-synaptic mechanism may exist in the tail arteries seems unlikely, since there was no significant difference in the evoked [^{3}H] overflow in SHR and WKY arteries pre-loaded with [^{3}H]-NA. Thus it seems unlikely that the increased pressor responses evoked by field stimulation in tail arteries from SHR rats was due to an increased NA release.

More recently, it has been suggested (Vidal et al., 1986) that ATP, released as a co-transmitter from sympathetic nerves, may account for the increased neurogenic pressor responses in hypertensive rats. Thus, it was claimed (Vidal et al., 1986), that a component of the contractile response in hypertensive, but not age-matched normotensive, rat tail arteries, was mediated by ATP. Blockade of this component using α,βMeATP reduced the pressor responses of hypertensive arteries

to a level comparable to those from normotensive rats. The present work in both tail arteries and mesenteric bed preparations, however, failed to confirm this suggestion. In each artery, α,βMeATP, at a dose which virtually abolished the pressor responses to exogenous ATP, had no significant inhibitory effect on the pressor response evoked by field stimulation in either SHR or WKY rats. Neurogenic vasoconstrictions were greatly reduced by α-adrenoceptor antagonists, suggesting that in both normotensive and hypertensive rats, they were mediated by NA and that there was no evidence for the involvement of ATP in the hypertensive state.

This lack of involvement of ATP in hypertension is supported by the observation that, while pressor responses to exogenous catecholamines were potentiated in arteries from SHR rats, those to adenine nucleotides were not. Furthermore, the possibility that ATP release was greater in hypertensive arteries also seems unlikely, since there was no significant difference between the evoked [^{3}H] overflow in SHR and WKY rat tail arteries pre-loaded with [^{3}H]-adenosine.

The possibility that the increased neurogenic pressor responses in hypertensive rats may have been due to structural changes in the smooth muscle of the artery wall is also unlikely. In both tail and mesenteric arteries, no obvious differences in either the smooth muscle cells, the endothelium or the density of the adrenergic innervation was observed in arteries from normotensive and hypertensive rats. There was a suggestion, however, of an increased wall-to-lumen ratio in arteries from hypertensive rats, although more accurate studies would have to be carried out for this to be confirmed.

It was concluded, therefore, that the increased vascular reactivity observed in hypertensive rats was mainly due to post-synaptic changes at the level of the α-adrenoceptor, with perhaps an additional contribution of structural changes in the wall of the blood vessel,

resulting in the generation of more smooth muscle. There was no evidence to support the idea (Vidal et al., 1986) that ATP plays a pathological role in the hypertensive state.

The idea, that a neurone acts via one transmitter substance, has dominated our thinking on neurotransmission since its inception by Dale (1935) to the 1970's. This idea may now have to be expanded to embrace the phenomenon of co-transmission, in which two or more transmitter substances, released simultaneously from the same neurone, act on a single target cell to effect transmission. This does not imply any rejection of Dale's principle. It merely reflects the increase in the state of current knowledge. Inherent in Dale's view was the idea that a neurone released the same, single, transmitter from each of its axon terminals. Today, while recognising the value of this concept, for there remain many synapses where neurotransmission involves only one substance, modification is necessary to include the idea of the multiple transmitter neurone. For the physiologist, the concept of co-transmission has added a new order of complexity to the function of the nervous system, with interactions occurring between transmitters at both pre- and post-synaptic sites. For the pharmacologist, the concept of co-transmission is of particular importance for the development of novel strategies in the design of new neuro-pharmacological agents. It is becoming obvious that many of the shortcomings of currently used drugs are due to the actions of previously unknown co-transmitters and that superior therapeutic regimes will soon be developed by the manipulation of co-transmitter systems.

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