CO-TRANSMISSION AND BLOOD VESSEL CONTRACTILITY

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by

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CONTENTS Pg. no.

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PUBLICATIONS

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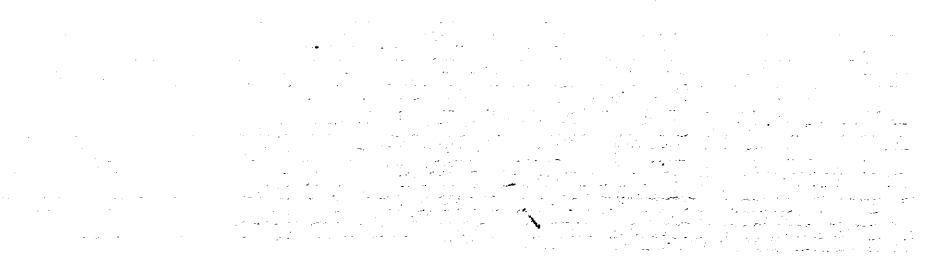


SUMMARY

Co-transmission in vascular smooth muscle was examined with a view to establishing the nature and mechanism of action, of each of the transmitter substances released in response to nerve stimulation.

1. The intracellularly-recorded electrical and simultaneous mechanical responses of the rabbit saphenous artery were examined in vitro in response to field stimulation of intramural sympathetic nerves and to exogenously applied drugs.

2. In the rabbit saphenous artery the resting membrane potential was -69.39 ± 0.26mV (n=250), the space constant (λ) was 0.42 ± 0.12mm (n=12). Field stimulation evoked excitatory junction potentials (e.j.p.s), which facilitated (≥1Hz) and summated (>2Hz). These e.j.p.s were abolished by infusion of α,β-methylene ATP (αβ MeATP 10^{-6}M), but were unaffected by prazosin (10^{-6}M), suggesting that they were mediated by an ATP-like substance. There was no additional electrical event which was associated with NA, implying that NA does not evoke a significant voltage-dependent response in this artery and may act via a voltage-independent mechanism.

3. Exogenous application of ATP (10^{-2}M) or its stable analogue αβ MeATP (10^{-4}M) by close pressure ejection from a micro-pipette, produced dose dependent depolarisations. NA (10^{-2}M) similarly applied was ineffective. Theses results are consistent with the view that the transmitter substances, NA and ATP (or a closely related substance), released from the rabbit saphenous artery produce dissimilar electrical effects.

4. Action potentials were not observed in the absence of drugs in the rabbit saphenous artery. In the presence of tetraethylammonium (TEA, $5x10^{-4}$-10^{-3}M) however, action potentials were observed in response to both single stimuli and trains of pulses. This suggested that action potentials are normally absent in this tissue as a result of a fast rectifying K^+-current, but can be sustained when this conductance is inhibited.

5. Alteration of the external ionic environment by changing $[K^+]_o$, $[Na^+]_o$ and $[Cl^-]_o$ suggested that changes in K^+ and Na^+ conductance may underlie the e.j.p. produced by nerve-released ATP.

6. In the rabbit saphenous artery, approximately 50% of the contraction was abolished by αβ MeATP (10^{-6}M), while the remainder was abolished by prazosin (10^{-6}M). Both NA and ATP therefore contribute to the mechanical event, supporting their co-transmitter role in this tissue.

7. All electrical and mechanical responses were abolished by either tetrodotoxin (TTX, 10^{-6}M) or guanethidine (10^{-6}M), indicating that they were due to transmitters released from the same sympathetic nerves. Idazoxan (10^{-6}M) enhanced both the electrical and mechanical responses, suggesting that α_2-adrenoceptors can pre-junctionally modulate release of both transmitters. This supports the view that both ATP and NA are released from the same nerves.

8. The P_{2x}-purinoceptor desensitising agent αβ MeATP was shown to be a suitable selective compound for analysis of the ATP component of co-transmission in the rabbit saphenous artery. No evidence was obtained from examination of the effect of αβ MeATP (10^{-7}-$4x10^{-6}$M) on membrane conductance by the method of Abe & Tomita (1968) for a non-selective membrane effect of this compound, nor were any differences between the effect of αβ MeATP on the evoked e.j.p.s and on the response to exogenously added ATP observed. αβ MeATP did not alter the contractions evoked by NA(10^{-7}-10^{-5}M), by histamine (10^{-6}-10^{-4}M) or by the post-junctional actions of KCl (1-$1.6x10^{-2}$M).

9. Suramin abolished evoked e.j.p.s and attenuated the contraction evoked by ATP (10^{-5}-10^{-3}M) and αβ MeATP (10^{-7}-10^{-5}M) without affecting the contraction to NA (10^{-7}-10^{-5}M) or the post-junctionally evoked contractions to KCl (10^{-2}-$1.6x10^{-1}$M). It however attenuated the contractions to histamine (10^{-6}-10^{-4}M) and 5-hydroxytryptamine (5-HT, 10^{-6}-10^{-4}M) and thus seemed less selective as an antagonist of P_2-purinoceptors in the rabbit saphenous artery.

10. NA failed, in the rabbit saphenous artery, to evoke hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), as measured by incorporation of <u>myo</u>-[2-^{3}H]-inositol into the water soluble metabolites of PIP_2 hydrolysis - the inositol phosphates. This was the case even in the presence of propranolol ($3x10^{-6}$M) to inhibit any inhibitory effect of β-adrenoceptors on PIP_2 hydrolysis. In the rat tail however, used as a positive control, NA produced a dose dependent

hydrolysis of PIP_2. This implies that PIP_2 hydrolysis is not the basis for the voltage-independent mechanism by which NA produces contraction in the rabbit saphenous artery.

11. Since αβ MeATP (10^{-6}M) and histamine ($3x10^{-6}$M) but not KCl ($8x10^{-2}$M) each evoked hydrolysis of PIP_2 in the rabbit saphenous artery, the mechanism for this hydrolysis exists in this tissue, but was clearly not utilised by NA.

12. The phorbol ester, phorbol 1,2-myristate 1,3-acetate (PMA, 10^{-8}-10^{-7}M) enhanced the contractile responses to exogenous NA (10^{-7}-10^{-5}M) and to field stimulation (0.5ms, supramaximal voltage, 1-32Hz). The post-junctionally evoked contractions to exogenously added KCl (10^{-2}-$1.6x10^{-1}$M) were unaffected. This suggested that protein kinase C (PKC) and the production of diacylglycerol (DAG) were involved in the contraction to NA.

13. Mepacrine ($3x10^{-5}$M) did not alter the contraction to NA (10^{-7}-10^{-4}M), while sodium oleate (NaOl, $2x10^{-6}$M) enhanced and sodium fluoride (NaF, $5x10^{-3}$M)) attenuated the contraction to these doses of NA, suggesting that in the rabbit saphenous artery, phospholipase D (PLD), but not phospholipase A_2 (PLA_2) may be an involved in the contraction to NA.

14. In the rabbit saphenous artery, NA evoked a dose dependent increase in hydrolysis of phosphatidylcholine (PC) as measured by incorporation of ^{3}H-methyl choline chloride into the water soluble

metabolites choline (Cho) and choline phosphate (ChoP). Increased levels of Cho implicated phospholipase D (PLD) in the hydrolysis of PC, while a smaller rise in the levels of ChoP implied that phospholipase C (PLC) was involved to a lesser extent. Levels of a third possible metabolite, glycerophosphocholine (GPC), the presence of which would indicate an action of phospholipase A_2 on PC, were unaffected by NA.

15. αβ MeATP (10^{-7}-10^{-4}M) and histamine (10^{-7}-10^{-4}M) each stimulated hydrolysis of PC resulting in raised levels of Cho and ChoP but not GPC. The increased levels of Cho and ChoP evoked, suggest that αβ MeATP utilises PLC and PLD approximately equally in the hydrolysis of PC while histamine predominantly utilises PLC.

16. A combination of αβ MeATP and NA, (each 10^{-6}-10^{-4}M), evoked no greater a rise in levels of Cho, than did αβ MeATP alone. Increased levels of ChoP produced by the combination of these agonists were however approximately equal to the sum of the individual responses to αβ MeATP and NA. There was no evidence however of synergism. This additive effect suggests that hydrolysis of PC is a level at which ATP and NA interact as co-transmitters in the rabbit saphenous artery.

17. In the <u>in vivo</u> dog preparation, the rank order of potencies for adenosine and adenosine analogues in decreasing hind limb perfusion pressure (HLPP) was 5'-(N-ethylcarboxyamido) adenosine (NECA) > N6-2-phenylamino adenosine (PAA) > adenosine > N6-cyclopentyl adenosine (CPA) = (+)-N6-(2-phenylisopropyl) adenosine (r-PIA), which

is consistent with recognised criteria for an A_2-adenosine receptor and in particular the subtype A_{2a}.

18. The rank order of potencies of adenosine and adenosine analogues in reducing heart rate (HR) in the in vivo dog preparation was NECA> adenosine > CPA > r-PIA > PAA, which more closely resembles an A_2-type adenosine receptor and in particular the A_{2b}-type than the A_1 receptor seen in rabbit and rodent heart.

19. The antagonist PD115199 was selective for the hind limb, while PAA was a selective agonist at this site, suggesting that the receptors in the hind limb differ from those in the heart.

20. PD116948, which has been shown to be relatively A_1-selective in other preparations, was relatively non-selective in the in vivo dog preparation. It produced only a small shift of the dose response curve to adenosine in the canine heart, equivalent to that produced by the A_2-selective PD115199.

21. These results are consistent with the view that the adenosine receptor in the dog hind limb is of the A_{2a}-subtype and of the A_{2b}-subtype in the canine heart.

INTRODUCTION

FOREWORD

When Langley in 1901 noted that adrenal extracts could mimic the response to sympathetic nerve stimulation in supra-renal bodies, it was probably the beginning of our understanding that nerves control the actions of muscles by the release of chemicals, now known as neurotransmitters.

Based primarily on subsequent evidence of the release from nerve endings of either an acetylcholine-like or an adrenaline-like (later found to be noradrenaline, Von Euler, 1946) substance, Dale (1933) coined the terms 'cholinergic' and 'adrenergic' to describe action by these two kinds of chemical transmitters.

Vigorous research over the next 60 years showed that responses to noradrenaline and acetylcholine were initiated, not by interaction with a single receptor type in each case, but by multiple receptor subtypes, mediating a variety of responses.

Cholinergic receptors

The neurotransmitter acetylcholine has been shown to act on two main classes of receptors. These have been distinguished by their different sensitivities to the alkaloid substances muscarine and nicotine (Dale, 1914) and have accordingly been named muscarinic and nicotinic. Muscarinic actions correspond to those of acetylcholine released at post-ganglionic parasympathetic nerve endings, with the exception of sweat secretion evoked by acetylcholine released from cholinergic fibres of the sympathetic nervous system (Rang & Dale, 1987) and the acetylcholine mediated release of endothelium derived relaxing factor

(EDRF, Furchgott, 1981). Muscarinic receptors may also mediate pre-synaptic feedback mechanisms to modify release of transmitter substances from parasympathetic or sympathetic fibres (Rand et al., 1982).

Nicotinic actions correspond to those of acetylcholine released at the ganglionic synapses of the sympathetic and parasympathetic systems, the motor end plate of skeletal muscle and the endings of the splanchnic nerves round the secretory cells of the supra-renal medulla (Rang & Dale, 1987).

Studies in pancreas, submandibular and lacrimal glands (Giraldo et al., 1987; Korc et al., 1987) further subdivided muscarinic receptors into two subtypes - M_1 and M_2 and subsequently, up to five different subtypes of muscarinic receptor have been proposed (Bonner, 1989). Similarly there are considerable pharmacological differences between the nicotinic receptors of striated muscle fibres and those of autonomic ganglia (Paton & Zaimis, 1951), while more recent work has suggested the presence of two more types of nicotinic receptor in the brain (Goldman et al., 1987), with perhaps a different structure for pre-and post-junctional nicotinic receptors.

Adrenoceptors

Based on the relative potencies of sympathomimetics on a variety of tissues, noradrenergic receptors were initially divided into two types - alpha (α) and beta (β) (Ahlquist, 1948). This was later confirmed by the discovery of the selective β adrenoceptor antagonist dichloroisoproteronol (Powell & Slater, 1958), followed by pronethalol (Black & Stephenson, 1962) and propranolol (Black et al., 1964).

β-adrenoceptors are present in many arteries and non-vascular smooth muscle. Interaction with them has been shown in some cases to evoke a hyperpolarisation and relaxation (e.g. rat mesenteric artery, Nilsson et al., 1986). In addition, β-adrenoceptors may exist pre-junctionally and modify evoked transmitter release (see Dahlof, 1981).

Subdivision of α-adrenoceptors occurred on the discovery in rabbit heart (Starke, 1972) and cat nictitating membrane and spleen (Langer, 1973) of pre-synaptic α-receptors which differed from those post-junctionally in effect as well as in location. Langer (1974) proposed that the post-junctional receptors should be termed α_1 and the pre-junctional α_2. Antagonists have since been developed to distinguish between these two sub-types, the most selective of which is the α_1-adrenoceptor antagonist prazosin (Cambridge & Davey, 1979).

It has since been confirmed that α_1-adrenoceptors exist post-junctionally and do not affect the evoked release of NA (Parker et al., 1985; von Kügelgen & Starke, 1985), while pre-junctional α_2-adrenoceptors are widely distributed and are thought to regulate the release of noradrenaline through pre-junctional auto-inhibition (see Starke, 1987). α_2-adrenoceptors also exist post-junctionally and can mediate contraction in a number of vessels (e.g. in hind limb of dog and cat, Gardiner & Peters, 1982; canine femoral bed, Elsner et al., 1984; pithed rat, Flavahan & McGrath, 1980).

Current views on transmission were thus developed from the original work of Dale by detailed questioning and examining. The simplistic view that muscles were controlled throughout the body by only two chemical mechanisms, albeit by interaction with a number of receptor types, failed to explain a number of experimental findings. As far back as 1895, there had been recognition of 'atropine-resistant' responses, both excitatory (e.g. bladder, Langley & Anderson, 1895; Langley, 1898) and inhibitory (e.g. gastrointestinal tract, Langley & Anderson, 1895; Langley, 1898; Bayliss & Starling, 1899), to parasympathetic nerve stimulation. Indeed, even while developing the terms 'cholinergic' and 'adrenergic', Dale was encompassing a number of apparently anomalous results, for example sweat secretion, sympathetic vasodilation and paradoxical contracture of the lips in the dog (Rogowicz, 1885), where effects produced by sympathetic nerves were apparently transmitted by acetylcholine rather than by an adrenaline-type substance.

Despite the proposal by Henderson & Roepke (1934) that some as yet unknown motor transmitter was the major component in the production of the contractile response to nerve stimulation in dog bladder, anomalous observations were generally explained away within the existing cholinergic/adrenergic classification. Atropine resistance was frequently attributed to the release of acetylcholine out with the blockade produced by atropine - the 'barrier' theory (Dale & Gaddum, 1930). Thus if acetylcholine was released outside the atropine 'barrier', its effects were blocked, but if released inside, atropine was ineffective. This theory has largely been disproved by evidence from electron-microscopy that the gaps between autonomic varicosities

where transmitter is released and the smooth muscle where transmitter acts are large and therefore no difficulty in atropine reaching its postsynaptic site of action was evident (see Gillespie, 1982).

Alternatively, anomalous effects were explained away by the so-called 'peripheral mechanism' (McSwiney & Wadge, 1928), in which the resting tone of the tissue was thought to determine the response to nerve stimulation. If tone was high, then either sympathetic or parasympathetic stimulation would produce relaxation. The 'peripheral mechanism' theory has similarly been disproved, since most examples attributed to this mechanism have since been shown to result from mixtures of sympathetic and parasympathetic nerves (e.g. in stomach, Harrison & McSwiney, 1936 and rabbit colon, Gary & Gillespie, 1956).

The use of adrenergic neurone blocking drugs such as guanethidine, has uncovered some anatomically adrenergic nerves, insensitive to block by these drugs, in a manner analogous to those parasympathetic nerves which appeared insensitive to atropine (e.g. guinea-pig taenia coli, Burnstock et al., 1963a). Subsequently, in both the sympathetic and parasympathetic nervous system, examples were found which could not be explained by the classical view of transmission as either cholinergic or adrenergic and indeed many examples were soon found where neither the cholinergic, nor the adrenergic transmitter appeared to be involved. In the guinea-pig taenia coli for example, after blockade of both adrenergic and cholinergic responses, nerve stimulation still produced large transient hyperpolarisations (termed inhibitory junction potentials (i.j.p.s)), accompanied by relaxations (Burnstock et al., 1963; 1964; Bennett et al., 1966a; 1966b) These results led to the idea of their being 'non-adrenergic,

non-cholinergic' (NANC) nerves and by the end of the 1960's, the existence of nerves releasing NANC transmitter substances were postulated in a variety of organs (see Burnstock, 1969; Campbell, 1970), although the nature of the transmitters remained unclear.

NANC NERVES

There is now functional evidence for NANC nerves in a wide variety of tissues. These have been shown to be largely postganglionic, parasympathetic nerves, releasing neither NA nor Ach and may be inhibitory, e.g. guinea-pig gall bladder (Davison et al., 1978), toad lung (Robinson et al., 1971), guinea-pig gastrointestinal tract (see Burnstock, 1979, Gillespie, 1982), guinea-pig trachea (Coburn & Tomita, 1973), rabbit portal vein (Hughes & Van, 1967), bovine retractor penis muscle (Byrne & Muir, 1985) and rat anococcygeus (Gillespie, 1972), or excitatory, e.g. dog urinary bladder (Creed & Tulloch, 1978; Muir & Smart, 1983), guinea-pig ileum (Ambache & Freeman, 1968; Bywater et al., 1981), cat colon (Hulten & Jodal, 1969).

Structural evidence for the existence of NANC nerves has been claimed from electron microscope studies. The predominance of opaque vesicles (LOVs) (Robinson et al., 1971) was observed in tissues, e.g. toad lung, which exhibited NANC responses (Wood & Burnstock, 1967; Campbell, 1971). The distribution of LOVs remained unchanged when adrenergic nerves were abolished by 6-hydroxydopamine (6-OHDA). The morphological similarity of these LOV's to known peptidergic neurosecretory axons led other authors to term them p-type vescicles (Gibbins & Haller, 1979). Similar LOV's were seen in other tissues which showed functional NANC innervation, e.g. avian gizzard (Burnstock, 1972) and bovine penis muscle (Eranko et al., 1976), while in rat anococcygeus muscle (Gibbins & Haller, 1979) a rather smaller, but morphologically similar opaque vesicle was observed, which was

labelled small p-type (sp-type). These observations prompted the proposal that LOV's or sp-type vescicles contained the NANC transmitter in at least some tissues (Burnstock, 1982). While this proposal stimulated much interest in the nature of NANC nerves, such a clear cut ultrastructural distinction between NANC and cholinergic or adrenergic nerves has not always been possible. In a great number of autonomic nerve profiles, e.g. rabbit portal vein, guinea-pig trachea and rabbit urinary bladder, LOV's are no more common in NANC nerves than in cholinergic ones (Gibbins, 1982). The primary transmitter in NANC nerves may therefore be more often stored and released from small clear vescicles and the presence of a particular vesicle profile in nerve endings cannot yet be related to the type of response (Gibbins, 1982). This absence of a unique profile for NANC nerves has proved disappointing in establishing the existence of NANC transmitters in nerves. Attempts to identify the transmitter substances mediating NANC responses have nevertheless continued. Five criteria for the establishment of a substance as a neurotransmitter (Eccles, 1964) have been applied to candidates for this role.

The putative transmitter should:

1) be synthesized and stored in the nerve terminals

2) be released during nerve stimulation

3) produce postjunctional responses on exogenous addition which mimic the responses to nerve stimulation

4) be capable of inactivation by enzymes which are biologically present and/or be removed by an uptake system in the nerve terminals

5) and responses to both exogenous transmitter and nerve stimulation should be antagonised or potentiated by the same drugs.

This last criterion has often proved to be the most difficult to meet, however those NANC substances which have most closely fulfilled the above criteria for transmitter substances can be largely grouped into peptides; released from 'peptidergic nerves' and purines; predominantly ATP, released from 'purinergic nerves' (for reviews see Burnstock, 1979; 1981; 1986; Hökfelt, 1980a) .

Peptides as transmitters

The first realisation that mammalian neurones produced and released peptides followed the characterisation of oxytocin and vasopressin as octapeptides (du Vigneud, 1955). These peptides were released into the blood supply from the posterior pituitary from neurones originating in the supraoptic and paraventricular nuclei of the hypothalamus.

On the basis of their presence in nerves, established by immunohistochemistry, radioimmunoassay and bioassay techniques, over 30 peptides have since been proposed as possible NANC transmitters, in both CNS and periphery. These have included substance P, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), galanin (GAL) and bombesin. (see reviews by Hökfelt et al., 1980a; Iversen, 1983b). Apart from their presence in nerves however, only a few of these come close to fulfilling the rest of the criteria necessary to establish them as transmitters.

Of these, the most promising are VIP and substance P. These peptides are particularly abundant in smooth muscle. For example, the longitudinal muscle of the guinea-pig taenia-coli is innervated by

fibres which are immunoreactive to both VIP and substance P (Jessen et al., 1980). The release of each of these peptides has been demonstrated in vitro following nerve stimulation. For example, VIP was released into the venous outflow when NANC inhibitory nerves supplying cat stomach were stimulated (Fahrenkrug et al., 1978), while substance P was claimed to produce a contraction by its release from intrinsic nerves in the intestine (Franco et al., 1979). In addition, close-arterial infusion of VIP mimicked the nerve evoked relaxation of the cat stomach (Eklund et al., 1979) while substance P mimicked NANC nerve mediated contractions in the guinea-pig ileum (Bauer & Kuriyama, 1982a). The last two of Eccles' criteria, 4 and 5, are however more difficult to apply to these two peptides. Substance P can be rapidly enzymatically degraded (Lee et al., 1981), but no such inactivation mechanism has been demonstrated for VIP, nor has any uptake mechanism been shown for any peptide and only little success has been achieved in the development of peptide antagonists. Some success in blocking the responses to VIP and NANC nerve stimulation have been claimed for active VIP anti-serum in the opossum oesophageal sphincter (Goyal et al., 1980), rabbit internal anal sphincter (Biancani et al., 1983) and cat trachea (Ito & Takeda, 1982), while ($D\text{-pro}^2$, $D\text{-Trp}^{7,9}$)-substance P has been proposed as a substance P antagonist (Leander et al., 1981) which suppressed contraction to NANC nerve stimulation in guinea-pig taenia-coli (see Bauer & Kuriyama, 1982a). These results are however by no means unequivocal.

Despite these problems, the possibility exists that VIP and/or substance P are NANC transmitters in some organs. Given that experimental protocols tend to look at short term effects of

transmitters, while peptides can have actions lasting for an hour or more (e.g. neuropeptide Y (NPY), see Potter, 1985) the effects of the proposed transmitter may therefore have been missed. Other peptides may yet be shown to also act as NANC transmitters.

Purines as transmitters

Apart from the vital role of adenosine nucleotides in cellular metabolism (for review see Gillespie, 1934), the purine nucleotide adenosine 5'-triphosphate (ATP) was also known to stimulate both the peripheral and central nervous systems (see Gillespie, 1934; Buchthal & Kahlson 1944). Indeed, it was proposed almost 40 years ago that ATP might be a transmitter at rabbit sensory nerve endings (Holton & Holton, 1953). Later adenosine or ATP were shown to have potent extracellular actions on excitable membranes which may be involved in physiological regulation of for example, blood flow from the heart (Berne, 1963), skeletal muscle (Scott et al., 1965), kidney (Osswald et al., 1977) and brain (Rubio et al., 1975). Burnstock et al. (1970; 1972) further proposed that a purine nucleotide, probably ATP was more than a modulator and likely to be the NANC transmitter in many smooth muscle preparations, especially those receiving inhibitory NANC nerves of guinea-pig and toad intestine and excitatory nerves of guinea-pig bladder - the 'purinergic nerve' hypothesis.

Application of the five criteria for a neurotransmitter (vide supra, Eccles, 1964), using histological, radiochemical and pharmacological techniques, provided substantial evidence to support the claim that ATP (or a related substance) could be an NANC transmitter. It was proposed (Robinson et al., 1971; Burnstock, 1982), although not universally accepted (Gibbins, 1982), that ATP synthesized in the nerves was stored in the large opaque vesicles (LOVs) previously reported. After its release and its activation of postjunctional receptors, ATP was rapidly broken down by ATPase and 5'-nucleotidase to adenosine. The adenosine so formed was taken up into the nerve terminal by a high affinity uptake system and reconverted into ATP. Exogenous addition of adenine nucleotides, particularly ATP, was shown to closely mimic NANC nerve responses in many smooth muscles. These could be either inhibitory, e.g. guinea-pig taenia-coli (Cocks & Burnstock, Burnstock, 1979) and gall bladder (Davison et al., 1978) or excitatory e.g. guinea-pig urinary bladder (Ambach & Zar, 1970; Muir & Smart, 1983) and ileum (Bauer & Kuriyama, 1982a).

This mimicry extended not only to mechanical events, but to underlying electrical changes. For example, the electrical events associated with the relaxation produced by both ATP and NANC nerve stimulation in the guinea-pig taenia-coli (Axelsson & Holmberg, 1969) and ileum (Bauer & Kuriyama, 1982b) is an increase in K^+ conductance. In those tissues where the electrical response to NANC nerve stimulation is small, e.g. rat anococcygeus (Gillespie, 1982) and bovine retractor penis muscle (Byrne & Muir, 1984), the response to ATP is likewise small.

Rigid application of Eccles last criteria for a neurotransmitter, namely that drugs should antagonise or potentiate both the effects of the transmitter and nerve stimulation, has not always been possible. Furthermore, as with many other peripherally acting neurotransmitters, it now seems likely that there is more than one purinoceptor subtype.

Largely on the basis of the rank order of potencies of agonists, it has been proposed (Burnstock 1978) that purinoceptors be divided into two subtypes - P_1 and P_2. At the P_1-purinoceptor, the order of agonist potency was adenosine > adenosine 5'-monophosphate (AMP) > adenosine 5'-diphosphate (ADP) > adenosine 5'-triphosphate (ATP), while at the P_2-purinoceptor, the opposite order of potency, ATP > ADP > AMP > adenosine, prevailed.

The P_1/P_2 division was further supported by the evidence that P_1-purinoceptors were antagonised by methylxanthines such as theophylline (see Burnstock, 1978) and that their occupation led to changes (either increases or decreases) in intracellular cyclic adenosine monophosphate (cAMP) levels. At the P_2-purinoceptors by comparison, methylxanthines were not antagonists, nor were levels of cAMP altered by occupation of this receptor type.

A variety of actions may be considered for these subtypes of receptor and it has been proposed (Enero & Saidman, 1977; Su, 1978) that purinoceptors may exist both pre and post-junctionally. Thus ATP released from pre-junctional nerve endings may act as a transmitter post-junctionally on P_2-purinoceptors. In addition ATP will be broken down by 5'-nucleotidase and by ATPase to adenosine, which may have further post-junctional actions on P_1-purinoceptors. Adenosine may in

addition act as a neuromodulator on P_1-purinoceptors existing pre-junctionally to modify the release of transmitter substances via a regulatory feedback system.

Since Burnstock's initial (1978) division of purinoceptors into two subtypes, a further subdivision of both P_1 and P_2-purinoceptor types has been proposed.

The P_1-purinoceptor was divided into A_1 and A_2 receptors (van Calker et al., 1979) or alternatively, into R_i and R_a subtypes (Londos et al., 1980). The A_1 receptors appear to be analogous to the R_i and A_2 to R_a and the former nomenclature has been more widely accepted.

Since methylxanthines do not distinguish more than one type of P_1-purinoceptor, further classification of these subtypes has been made biochemically by observing the rank order of potency of analogues in stimulating or inhibiting adenylate cyclase and hence cAMP levels. Pharmacologically, classification has been made by comparison of both the rank order of potency and affinity of adenosine and adenosine analogues as agonists. Thus A_1-receptors display a high affinity for agonists, inhibit adenylate cyclase (Dobson, 1983) and account for the negative chronotropic and inotropic effects of adenosine in rat, guinea-pig and rabbit atria (Collis, 1985; Haleen & Evans, 1985). The rank order of potency of agonists at A_1-receptors is N^6-(2-phenylisopropyl)adenosine (PIA) > adenosine > 5'-(N-ethylcarboxyamido)adenosine (NECA) (Londos et al., 1980), and this receptor exhibits a marked stereo-selectivity for the diastereoisomers of PIA, l-PIA being 50-100 times more potent than

d-PIA.

A_2-receptors on the other hand display a lower affinity for agonists than A_1 receptors, generally stimulate adenylate cyclase (Kukovetz et al., 1978) and are present in bovine coronary arteries (Mustafa & Askar, 1985) aortae (and presumably other blood vessels) and trachea of the guinea-pig (Collis, 1985). The reverse order of potencies exists for A_2-receptors, i. e. NECA > adenosine > PIA and little stereo-selectivity for isomers of PIA exists at this subtype.

While this subdivision of P_1-purinoceptors into A_1 and A_2 has proved valid for a large number of tissues, anomalies have been observed. It has been proposed (Bruns et al., 1986; 1987) that these anomalies may reflect the existence of further subtypes of A_2 receptors - A_{2a} and A_{2b}. At an A_{2a} receptor the order of potencies of adenosine and adenosine analogues is 5'-(N-ethylcarboxyamido) adenosine (NECA) > N^6-2-phenylamino adenosine (PAA) > N^6-cyclohexyl adenosine (CHA) > (+)-N^6-2- phenylisopropyl) adenosine (r-PIA), while at the A_{2b} receptor the order is NECA > CHA ≥ r-PIA > PAA. The selectivity of antagonists for the A_{2a}-subtype is represented by PD115199 > PD116948, while for A_{2b}, PD115199 ≡ PD116948, with both antagonists having a low affinity for this receptor subtype.

In the open chest dog (Kusachi et al., 1983), in the conscious dog and in isolated canine atria (Belloni et al., 1989), the order of agonist potency differed from that accepted for the A_1-receptor subtype in rodent and rabbit heart and in some ways more closely resembled the A_2 subtype of receptor. It was proposed that a novel receptor type existed in the canine heart and this may reflect the presence of one of the subtypes A_{2a} or A_{2b}.

Subtypes of P_2-purinoceptor have likewise been proposed and analysis of the rank order of agonist potency, combined with functional differences, allowed two subtypes, P_{2x} and P_{2y}, to be distinguished (Burnstock & Kennedy, 1985).

The P_{2x} receptor subtype mediates the contractile effects of purines in smooth muscle (e.g. urinary bladder of guinea-pig and rat) and the rank order of potency of ATP and its analogues as agonists is αβ-methylene ATP (αβ MeATP) = βγ-methylene ATP (βγ MeATP) > ATP = 2-methylthioATP. P_{2y} receptors mediate the inhibitory responses evoked by purines (e.g. guinea-pig taenia coli) and the rank order of potency of agonists is 2-methylthio ATP >> ATP > αβ MeATP = βγ MeATP (Burnstock & Kennedy, 1985).

Recently developed structural analogues of ATP which antagonise responses mediated by P_{2x} receptors have made important contributions to the problems of blockade of ATP-mediated responses.

Arylazido aminopropionyl-ATP ($ANAPP_3$), is thought to form covalent bonds with the excitatory P_2-purinoceptor (i.e. P_{2x}) on irradiation with visible light and has been used as an irreversible P_{2x}-purinoceptor antagonist in a number of tissues, e.g. guinea-pig vas deferens (Hogaboom et al., 1980; Fedan et al., 1982), rabbit anococcygeus (Sneddon et al., 1982). It does not antagonise inhibitory responses mediated by P_{2y}-purinoceptors, e. g. guinea-pig taenia coli (Westfall et al., 1982).

Contractions to ATP are also inhibited by repeated administration of the slowly degradable analogue of ATP, αβ methyleneATP (αβ MeATP,

Burnstock & Kennedy 1985). αβ MeATP is a potent agonist producing a transient depolarisation and contraction. Repeated administration causes a prolonged desensitisation of the P_{2x}-purinoceptor, even after the initial depolarisation and contraction have disappeared (Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984b; von Kügelgen & Starke, 1985, Kennedy et al., 1986).

A compound more recently proposed as a suitable antagonist of P_{2x}-purinoceptors is suramin (see methods section for chemical name). In mouse vas-deferens (Dunn and Blakeley, 1988), pithed rat (Schlicker et al., 1989) and rabbit ear artery (Leff et al., 1990), suramin antagonised purine responses evoked by αβ MeATP or ATP, without any significant effect on cholinergic or noradrenergic responses.

Despite the widespread use of αβ MeATP in the study of P_{2x}-purinoceptors, doubts have been cast on the selectivity of this agent.

In the chicken rectum, the initial large depolarisation produced by αβ MeATP did not entirely disappear and this residual depolarisation was accompanied by changes in membrane conductance (Komori et al., 1988). Perhaps as a result of the residual depolarisation caused by this drug, αβ MeATP attenuated the responses evoked by some non-purinergic agonists e.g. KCl and bovine neurotensin (Komori et al., 1988). αβ MeATP also allegedly attenuated depolarisations to NA in the rat basilar artery (Byrne & Large, 1986).

Further, in the chicken rectum, smooth muscle cells regained normal membrane resistance and sensitivity to exogenous addition of ATP on washout of a desensitising block produced by αβ MeATP, more rapidly

than to the evoked e.j.p.s (Komori et al., 1988). This was proposed to be the result of an unknown extra effect of αβ MeATP in addition to its effect on P_2-purinoceptors.

Kotecha & Neild (1987) similarly suggested that αβ MeATP had properties other than desensitisation of purinoceptors and proposed that this compound was a blocker of cation channels in rat tail artery.

While some progress has been made in inhibiting P_{2x} responses, little progress has been made in finding selective antagonists for P_{2y} receptors and this to some extent delayed the acceptance of the 'purinergic nerve' hypothesis.

Apamin, a bee neurotoxin inhibits the relaxation and inhibitory junction potentials in guinea-pig taenia coli (P_{2y}), in the absence of any inhibition of the excitatory (P_{2y}) effects of purines in guinea-pig urinary bladder or rat uterus (Shuba & Vladimirova, 1980). Apamin however acts by blocking certain K^+ channels rather than by interaction with purinergic receptors. Reactive blue 2 has also been used to distinguish between P_{2y} and P_{2x} receptors but this also has a non-specific effect, perhaps acting by interference with biochemical processes regulating the availability of potassium and calcium channels. (Kerr & Krantis, 1979; Manzini et al., 1986). The actions of these drugs have however allowed some distinction between subtypes of P_2-purinoceptor.

Although the hypothesis of purinergic transmission was attractive, it was not universally accepted. The lack of selective antagonists for all receptor subtypes and the criticisms of those existing purinoceptor inhibitors, left this hypothesis not fully proven. The ubiquitousness of ATP and its importance in cell metabolism similarly made it difficult to prove ATP's role as a transmitter. Several authors raised specific doubts about ATP in this role, based on the low potency of ATP in some preparations supplied by NANC nerves (e.g. in producing contraction of rat bladder, Brown et al., 1979). This has since been explained by the proposal that rapid breakdown of ATP to AMP and adenosine accounts for the low potency (Ambache et al., 1977a: 1977b). Other authors found that although tachyphylaxis to ATP in, for example, the rabbit ileum (Burnstock et al., 1970) produced depression of the response to purinergic nerve stimulation, this was not seen in the guinea-pig urinary bladder (Ambache & Zar, 1970). It has since been shown however (Burnstock et al., 1978) that in the presence of indomethacin to inhibit ATP evoked prostaglandin synthesis, the response to nerve stimulation is virtually abolished following desensitisation to ATP.

Accordingly, many of those objections raised to the 'purinergic nerve' hypothesis have been refuted or explained away and there is now good evidence for ATP as a neurotransmitter in some, but not all NANC nerves. Nevertheless, some gaps in the literature still exist and must be filled before ATP's role in transmission is fully accepted.

Other putative NANC transmitters

While purines and peptides are currently the main contenders for the role of NANC transmitters, other classes of compound may also function in this role. 5-hydroxytryptamine (5-HT) or a closely related indolamine, present in enteric neurones, may satisfy the criteria (Eccles, 1964) for a transmitter (e.g. in guinea-pig colon and ileum, Furness & Costa, 1979; guinea-pig small bowel, Wood & Mayer, 1979). Similarly, γ-aminobutyric acid (GABA), which is a major neurotransmitter in the CNS (Iversen, 1972), has been proposed as an NANC transmitter in the gastrointestinal tract (e. g. in guinea-pig taenia coli, Jessen et al., 1979), while dopamine has been proposed for this role in some blood vessels (Bell, 1982). While the evidence for these other substances as NANC transmitters is as yet less convincing, their importance may become apparent as more substantiating facts accrue.

It is clear that control of muscle function by nerves can no longer be seen to be the domain of Ach and NA alone.

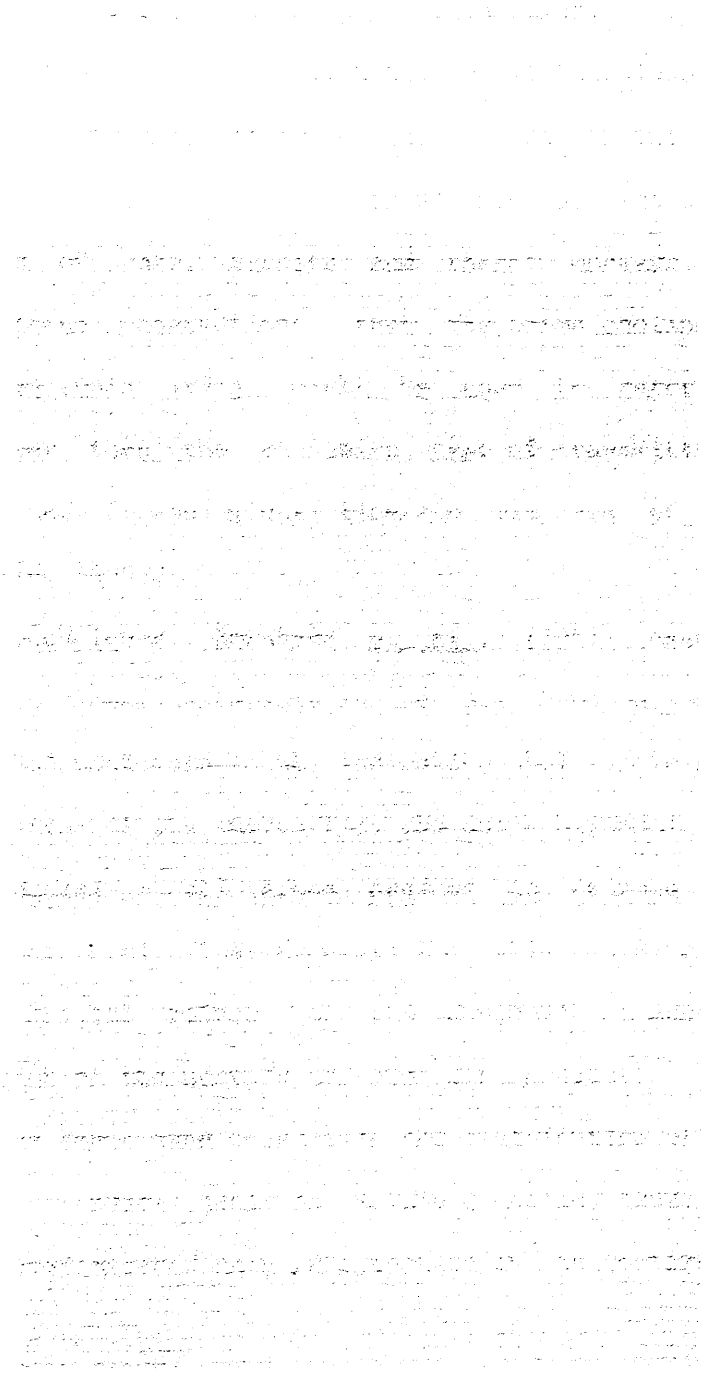
Our understanding of the existence of NANC transmitters was arguably one of the most significant advances in the study of autonomic control since the inception of the concept of chemical transmission. It added to the complexity of our understanding of nervous control and provided

the impetus for a renewal of interest into the nature and function of transmitters. The next major advance was to be concerned with the number of neuronal transmitters involved in the effector response.

The above evidence for NANC transmitters was still encompassed within the concept that each nerve synthesizes and releases only one transmitter (widely known as Dale's Principle), however reexamination of Dale's original views (Dale, 1935) led to the view that while any one class of nerve cell operated at each of its synapses by the same chemical transmission mechanism (Eccles, 1986), this does not exclude the possibility that more than one transmitter could comprise the "chemical transmission mechanism" in each case. (Eccles, 1986). The feasibility of this idea lies in the interpretation of the methods by which transmitters evolve.

Two opposing views have been put forward for the mechanism by which new neurotransmitter systems evolve. One theory proposed that neurones have a common origin and that they differentiate into various types in the course of evolution, due to functional specialisation (see Burnstock, 1969). An alternative view is that there were multiple origins of nerve cells, resulting in a wide diversity of neurotransmitters early in evolution. The eventual reduction in the number of transmitters was the result of selection (Sakharov, 1974). Either way, it seemed conceivable that a gradual evolutionary transmission from one neurotransmitter to another occurred and that this might lead to nerves with more than one transmitter. (see Burnstock, 1981). It was perhaps then not surprising that recent years have seen 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 vescicles, are co-released following stimulation of both parasympathetic and sympathetic nerves and may perhaps act together postjunctionally - the idea of co-transmission.



CO-TRANSMISSION

Early evidence to support this idea came from the observation that an adrenaline-like substance was detected in the perfusate of the cat superior cervical ganglion following pre-ganglionic stimulation (Bülbring, 1944). Subsequently, using histological techniques, Koelle (1955) showed that acetylcholinesterase (AchE) - the enzyme involved in the hydrolysis of acetylcholine (Ach) was present in several adrenergic nerves of cats, rabbits and rhesus monkeys. Koelle concluded from these observations, that the terms cholinergic and adrenergic neurones (Dale, 1933), might be used to refer to the predominant, rather than the exclusive type of transmitter of the nerve fibres and that fibres might liberate mixtures of chemical transmitter (Koelle, 1955).

Using similar techniques, Abrahams et al., (1957) examined the distribution of acetylcholinesterases in the dog hypothalamus. They found that true cholinesterase (AchE, indicating cholinergic neurones) was found in the areas of the supraoptic, the suprachiasmatic and the paraventricular nuclei - all areas assumed to secrete hormones (oxytocin and vasopressin). They proposed that this could reflect the ability of the initial release of the neurone's transmitter to stimulate production of the nerve's own endocrine product.

Histochemistry was again used to examine the distribution of AchE in the cat superior cervical ganglion (Koelle & Koelle, 1959). At this site, functional AchE existed only presynaptically, in contrast to the

neuromuscular junction, where most of the enzyme was located post-synaptically. From this it was inferred that AchE served different primary functions at the two sites. It was proposed (Koelle, 1961), that a dual role existed for Ach, in that released Ach acted initially on pre-synaptic sites to activate the ganglion and cause a secondary release of Ach which acted on post-synaptic sites.

This work suggested that release of even a single transmitter involved a more complex series of events than previously seen. The 'marrying' of the idea of a two phased release of a single transmitter, with the possibility of the release of more than one substance was proposed (Burn & Rand, 1965) to explain the ability of Ach and nicotine to produce effects like those of sympathetic stimulation in many organs. Using post-ganglionic sympathetic nerves, they proposed that the initial response to nerve stimulation was the release of Ach from axonal terminals, which caused an increased influx of calcium ions into the nerve terminal, triggering the release of the actual neuroeffector transmitter, noradrenaline, from the same terminals. This was called the 'cholinergic link' hypothesis (for review see Burn & Rand, 1965) and implied the co-existence and release of both Ach and noradrenaline (NA). While these proposed systems assumed a more complicated picture of transmission than had previously been recognised, neither Koelle, nor Burn & Rand however proposed a postjunctional effect of more than one substance.

Evidence that two transmitter substances may act postjunctionally, came from an electrophysiological study of single isolated sympathetic neurones grown on heart cells (myocytes) previously dissociated from new-born rats. It was shown that some neurones released NA, some Ach

and some released both NA and Ach (Furshpan et al., 1976). Those neurones which released NA evoked myocyte stimulation; those which released Ach produced inhibition; while those which produced both NA and Ach, first inhibited and then stimulated the myocytes.

This then implied that not only was co-existence and co-release of the 'classical' transmitters (i.e. Ach and NA) possible, but that these could in fact operate as co-transmitters.

Co-transmission of the 'classical' transmitters with putative NANC transmitter substances may also exist (see reviews by Hökfelt et al., 1980; Iversen, 1983).

Much of the evidence for co-transmission came from observation of tissues where α-adrenoceptors antagonists were only partly effective in blocking the responses to nerve stimulation. For example, in the rat vas deferens (vide infra, McGrath, 1978), field stimulation evoked an initial rapid contraction, followed by a second, slower and more prolonged contraction. Only the second slow contraction was abolished by α-adrenoceptor antagonists, or mimicked by NA. Furthermore, in the rabbit ear and saphenous artery (Holman & Surprenant, 1980), α-adrenoceptor antagonists depressed, but did not abolish contractions produced by nerve stimulation.

Controversy surrounds the precise definition of co-transmission. It has been defined as simply the pre-junctional release of more than one transmitter (O'Donohue et al., 1985), leading in theory, to four

potential mechanisms by which co-transmitters could interact. First, each transmitter could bind to the same post-junctional receptor, or secondly, to different receptors on different target cells. Thirdly, one transmitter could modulate the action of the other in the synaptic cleft, at the same postjunctional receptor (perhaps by changing the sensitivity of receptors for the other transmitter, see Koketsu, 1984) or at the level of second messengers or ionic channels. Lastly, each transmitter could bind to different receptors on the same cell.

Other authors (see Campbell, 1987) suggest that co-transmission must be defined in terms of the target cell and that co-transmission exists only where two or more transmitters act on the same target cell so that the net result of transmission incorporates interactive effects of the transmitters. For the purposes of this thesis, I have chosen to accept the latter definition.

Not surprisingly perhaps, those substances proposed as transmitters in non-adrenergic, non-cholinergic nerves, were in many cases also the most likely candidates for co-transmitters and while co-transmission with more than one NANC transmitter has been postulated in some cases (e.g., dopamine with cholecystokinin (CCK) in the central nervous system of rat and man (Hökfelt et al., 1980)), the most important examples of co-transmission occur where the classical transmitters -Ach or NA - are combined with one or more NANC putative transmitter substances - particularly peptides or purines.

There is now evidence for co-transmission of purines or peptides together with Ach or NA (see Cuello, 1982; Osborne, 1983; Chan-Palay &

Palay, 1984) in a large number of tissues. The relative importance of these to the concept of co-transmission will be discussed in the following sections.

Co-transmission of 'classical' transmitters with peptides

The possibility that peptides may act as co-transmitters is not perhaps surprising considering the early proposal (Abrahams et al., 1957) that Ach might evoke the release of peptides from dog hypothalamus. Evidence to support the idea of the co-existence of a classical transmitter with a peptide came from work in endocrine cells in gastrointestinal tract (Pearce, 1969), where cells were shown to contain both a biogenic amine, such as 5-HT or histamine and a peptide such as substance P, somatostatin or neurotensin. These cells were part of the APUD (amine content or precursor uptake and decarboxylation) system, the cells of which are 'neuroendocrine -programmed' cells originating from the same ectoblast'. Since neurones have the same embryonic origin as endocrine cells, it was proposed that neurones may also contain both an amine and a peptide (Pearce, 1969). Indeed a population of guinea-pig sympathetic ganglion cells was subsequently found to be somatostatin-immunoreactive (Hökfelt et al., 1977).

Since then, extensive evidence for co-existence of peptides with classical transmitters has come from immunohistochemical techniques, where positive staining for a given peptide has been demonstrated in

autonomic neurones. Iversen (1984) lists 32 peptides that are constituents of neurones and nerve terminals in the central nervous system (CNS) alone.

The importance in transmission of each of these examples is however unlikely to be uniform and while co-existence and indeed co-release of putative transmitters is essential for, it is not sufficient evidence per se, of co-transmission. Not surprisingly then, not all of these substances are themselves transmitters (vide supra, Eccles, 1964) and a variety of functions for these peptides has been suggested, with corresponding names (Dismukes, 1979), such as neurohormones, neuromodulators or neuroregulators. Nevertheless, a number of peptides may fulfil the required criteria for transmission and may be released along with other transmitters during nerve stimulation to act as co-transmitters (for review see Burnstock, 1981).

Evidence that a neurone may contain more than one neurotransmitter has led to investigations concerning the manner in which these putative transmitter may be stored. It has been proposed (Cook & Burnstock, 1976) that in many tissues, the NANC transmitter appears to be stored in discreet vesicles. A separate storage system for each of the putative transmitters would appear to allow differential release of the co-transmitters at, for example, different impulse frequencies.

Such a functional role for co-transmission of a peptide with a 'classical' transmitter and indeed one of the few in vivo examples of co-transmission, was demonstrated with vasoactive intestinal polypeptide (VIP) and acetylcholine (Ach) in the cat submandibular salivary gland (Lundberg & Hökfelt, 1983) . In this case, stimulation of nerves at low frequencies (2Hz) released Ach (as evidenced by

atropine blockade), which produced both salivation and vasodilation. At higher frequencies however (>10Hz), the vasodilation became atropine resistant; an effect attributed to the co-release of VIP which potentiated the Ach-induced salivation by a direct vasodilation. Here co-transmission allowed a response to take place which could be varied physiologically depending on circumstances and which constituted a mechanism by which a demanding situation could be dealt with locally.

Another peptide implicated in co-transmission has been neuropeptide Y (NPY). This is a potent vasoconstrictor in cat pial arteries (Edvinsson et al., 1984), cat salivary glands (Lundberg & Tatemoto, 1982), guinea-pig uterine arteries (Morris et al., 1985) and rat tail arteries (Neild, 1987). At low concentrations, NPY potentiates the contractile responses of each artery to noradrenaline (NA) and to perivascular nerve stimulation. These observations have led to the suggestion that NPY may participate in the physiological control of artery diameter (Nield, 1987) and hence vascular tone.

The finding of peptides, previously associated with NANC transmission in the gut, e.g. VIP (Larsson, 1976), in many areas of the brain, resulted in the view that the CNS, the peripheral autonomic nervous system and the endocrine system have a number of peptides or families of peptides in common (Pearce, 1979). This suggested that co-transmission may also exist in the CNS. A particularly interesting example of this was seen in the occurrence of a CCK-like peptide in a sub-population of dopamine neurones in the meso-limbic system in man.

(Hökfelt et al., 1980a). The meso-limbic dopamine systems have been associated with higher mental functions and disturbances in them may represent one component in the pathogenesis of schizophrenia (Pearce et al., 1977), indeed it has been proposed that an imbalance between peptide and amine could be an aetiologic factor for schizophrenia (Hökfelt et al., 1980c)

Clearly there is evidence for co-transmission of peptides with 'classical' transmitters in both the periphery and the CNS. Much of the evidence rests however, at least in the mean time, on the localization of the peptides in neurones and comparatively little is known about their physiological role. This is partly due to a lack of drugs which can influence peptide-induced events at synapses. Future developments may clarify the importance of co-transmission involving peptides.

Co-transmission of 'classical' transmitters with purines

Evidence that ATP, in addition to being an energy source, could act in its own right, together with other substances, came from the observation 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 (Stevens et al., 1972). It was proposed (Van Dyke et al., 1977) that this ATP acted within the medulla as a 'co-agonist' along with simultaneously released catecholamines. The ATP could act either directly on receptors or indirectly by being transformed into cyclic nucleotides.

Similar results were obtained in the guinea-pig taenia coli, where stimulation of the periarterial adrenergic nerves released [^{3}H] from tissues pre-incubated with [^{3}H]-adenosine (Su et al., 1971). This effect was abolished by the adrenergic neurone blocking agent guanethidine, suggesting that the purine was released from adrenergic rather than purinergic nerves.

In the course of the aforementioned experiments on isolated myocytes, Furshpan et al. (1976) observed a neuronally evoked hyperpolarisation which was atropine resistant. Later work showed this to be antagonised by adenosine receptor blockers (Furshpan et al., 1986) and it was proposed that the hyperpolarisation was mediated by a purine. The authors suggested that in addition to co-releasing NA and Ach, many neurones may also release a purine.

Further evidence that ATP may act together with Ach comes from the observation that both these substances are found together in synaptic vesicles of cholinergic nerves supplying the electric organ of Torpedo (Dowdall et al., 1974). ATP is also released together with Ach from the phrenic nerves in the rat hemi-diaphragm (Silinsky & Hubbard, 1973; Silinsky, 1975). It is however as a putative co-transmitter with NA that ATP has been most often studied and a functional role for ATP as a co-transmitter shown.

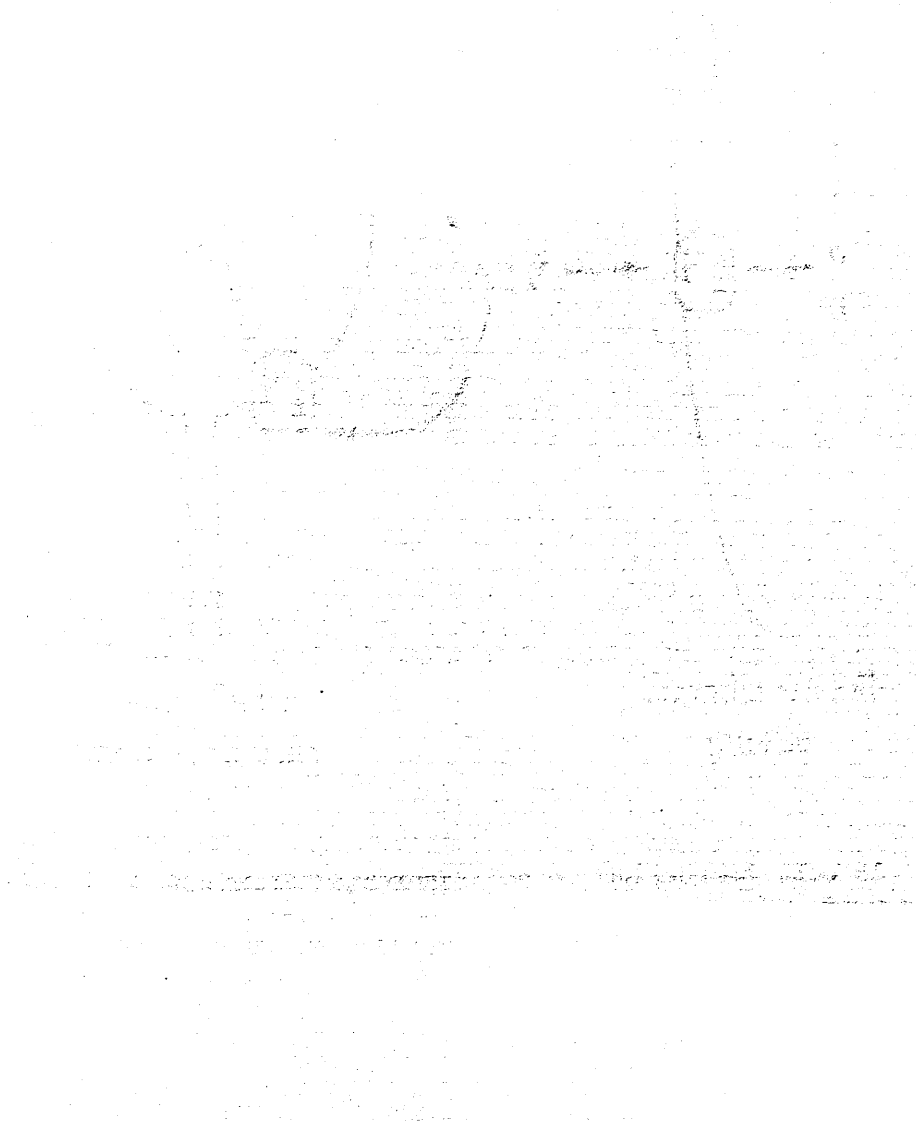
As previously mentioned, work on the rat vas-deferens gave early, relatively clear evidence for ATP and NA as co-transmitters. In this tissue the contractile response to sympathetic nerve stimulation is biphasic (McGrath, 1978). The early rapid twitch contraction was

mimicked by ATP and unaffected by α-adrenoceptor antagonists or by NA depletion with reserpine. The second, slower phase was mimicked by NA and blocked by α-adrenoceptor antagonists. Similarly in the rat tail artery (Cheung, 1982), field stimulation produced e.j.p.s, which were resistant to both phentolamine and yohimbine -respectively α_1 and α_2-adrenoceptor antagonists - and a slow depolarisation which was blocked by phentolamine.

Many studies have since further examined the role of these two putative transmitters in the light of the development of relatively selective antagonists for ATP and have concluded that NA and ATP (or a closely related substance) are co-transmitters in a number of tissues including many vascular tissues, e.g. rat tail artery (Sneddon & Burnstock, 1984a), rabbit ear artery (Suzuki & Kou, 1983; Allcorn et al., 1985), aorta & portal vein (Su, 1978) and dog basilar artery (Muramatsu et al., 1981).

The inclusion of the concept of co-transmission complicates the factors which regulate the nervous control of muscle. Not only must we concerned with the nature of the putative transmitters released, but how as co-transmitters they may act and/or interact, in what ratio and under what conditions of stimulation they are released. While multiple release may permit finer control of muscle by nerves, or may perhaps constitute 'failsafe' mechanisms for the relevant organ, predicting the result of the interaction two or more co-transmitters becomes difficult.

In this thesis, the main points of interest are the post-junctional actions, as co-transmitters, of NA (acting via α_1-adrenoceptors) and ATP (acting via P_{2x}-purinoceptors) in producing contractions in the smooth muscle of blood vessels. As mentioned, both NA and ATP may however also act as co-modulators by stimulating respectively α_2-adrenoceptors and P_1-purinoceptors situated pre-junctionally. A schematic representation is shown in Fig. 1 of some possible interactions between NA and ATP.



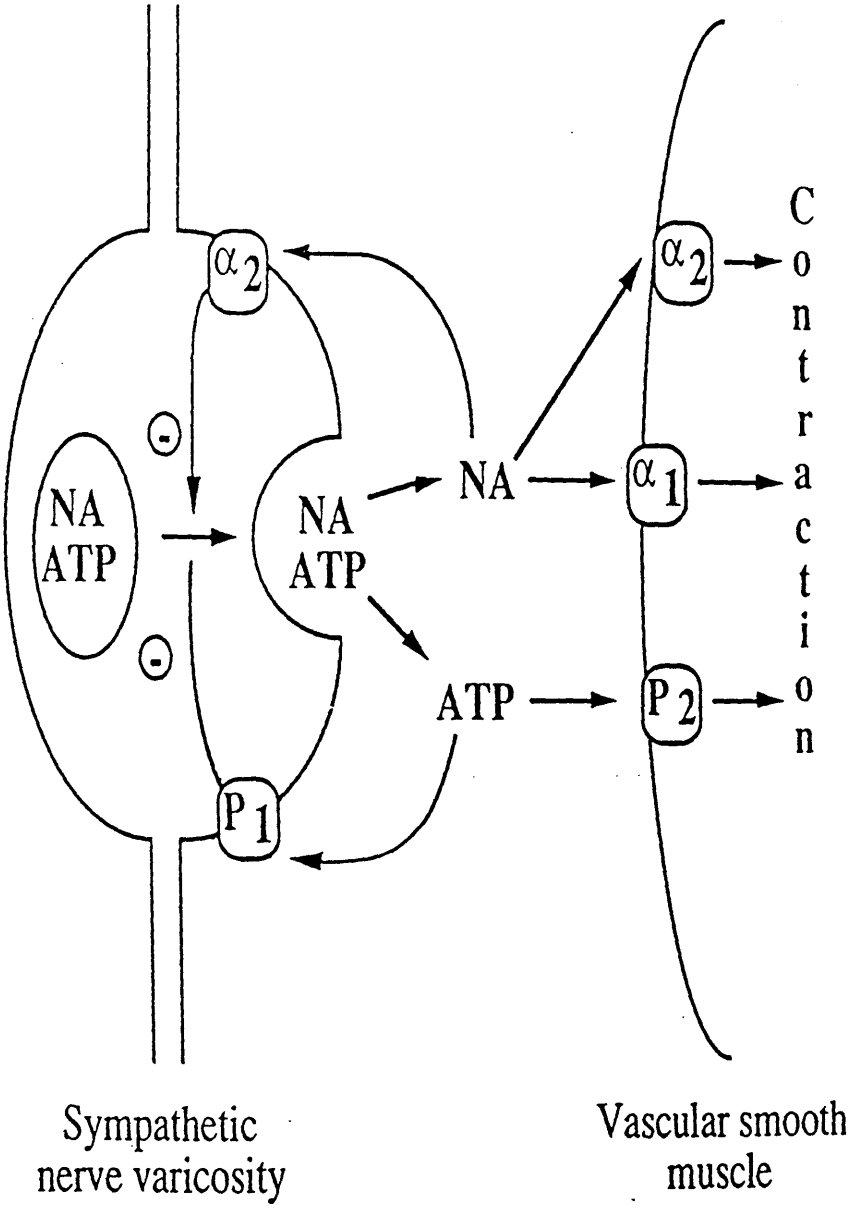


Fig. 1. A schematic representation of the current view of sympathetic neurotransmission by NA and ATP, demonstrating the position of pre-and postsynaptic adrenergic and purinergic receptors.

While many studies have accepted the phenomenon of co-transmission as being a logical and substantiated explanation for those situations where blockade of cholinergic and/or adrenergic transmission unmasked a residual NANC component of the response, an alternative explanation has been proposed in some cases.

For example, a further subtype of adrenoceptor has recently been proposed to explain the inability of α-adrenoceptor antagonists to inhibit the response to field stimulation of intramural nerves in some tissues. Hirst & Neild (1980; 1981), found that responses to iontophoretically applied noradrenaline in guinea-pig arteriolar tissue varied according to the site of application. The contraction evoked by NA added extra-junctionally (i.e. added to regions distant from the sympathetic nerves) was α-adrenoceptor mediated (as evidenced by susceptibility to α-adrenoceptor antagonists) and was unaccompanied by changes in membrane potential. The response to junctionally (i.e. close to the sympathetic nerves) added NA was on the other hand, resistant to α-adrenoceptor blockade and was accompanied by a depolarisation. It was proposed that this latter response was the result of the interaction of NA with a novel adrenoceptor - a 'gamma' (γ) receptor. A γ-adrenoceptor has since been proposed in other tissues, e.g. the guinea-pig mesenteric artery and vein (Hirst & Jobling, 1989), as a low affinity noradrenaline receptor, which may exist in more than one sub-type. In the basilar artery, doses of NA as high as 10^{-3}M failed to produce a contraction, however these high

doses of NA did produce changes in membrane potential which were resistant to α-adrenoceptor blockade (Hirst et al., 1982). It was argued that this reflects the presence of γ-adrenoceptors.

The 'γ' theory has however not received widespread acceptance. Criticisms of this theory have been based on the grounds that the detailed crucial localization experiments have been carried out on only one type of blood vessel, in one species, namely the submucosal arterioles of the guinea-pig. Changes in tone of this tissue were not measured during these experiments, nor was the nature of its innervation or transmitter(s) known. In those experiments in the rat basilar artery, it has been suggested that the presence of a predominance of β-adrenoceptors was overlooked and that the identification of receptors by exclusion in a tissue which does not contract to the physiological agonist seems precarious (Bevan, 1984). Accordingly, while the possibility that γ-adrenoceptors exist has not been excluded, the physiological relevance of these receptors, should they exist, is as yet unclear.

The next stage in the understanding of the relevance of co-transmission has been to evaluate the post-junctional coupling mechanisms by which ATP and NA, the co-transmitters in vascular smooth muscle act and/or interact to produce their physiological response.

MECHANISM OF ACTION OF NA AND ATP

The possibility exists that these putative transmitters may each utilise different receptor-effector linkage mechanisms post-junctionally to produce contraction.

Interaction of a neurotransmitter or an appropriate ligand, with a cell surface receptor triggers a number of receptor-effector linkage mechanisms, which can be grouped into three types.

1) Where the receptor is part of the same macromolecule as an ion channel and directly regulates membrane permeability to ions. This is the fastest type of receptor mediated response and a number of examples have been shown where this occurs, including the L-glutamate receptor in the CNS (Nistri, 1983). The best known example is however the nicotinic acetylcholine receptor (see Bowman, 1980; Blusztajn, 1983). Here Ach acts on the linked receptor/ ion channel complex to transiently increase its permeability to cations, resulting mainly in an increase in sodium and potassium permeability and to a small extent, calcium. This results in a net inward current which depolarises the cell (Skok, 1980) and increases the probability that it will reach the voltage potential required to open voltage-dependent sodium channels and thereby generate an action potential.

2) Where the receptor and ion channel are linked via a G-protein.

A G-protein is a peripheral membrane protein consisting of α, β and γ subunits. There are several subtypes of G-proteins, mediating a variety of effects and distinguishable by differences in the α-subunit of the protein (Bourne, 1986). In general terms however, G-proteins respond when a receptor protein is stimulated by releasing guanosine diphosphate (GDP) and binding guanosine triphosphate (GTP). In its GTP-bound conformation, the G-protein can regulate the function of many effectors. Hydrolysis of bound GTP to GDP terminates the regulatory effect of a G-protein (Bourne, 1986).

Recent studies have reported the possibility that G-proteins may exert direct control over the function of ion channels. It has been shown that muscarinic agonists activated an inward rectifying K^+ channel, which was not prevented by cyclic AMP (cAMP) analogues or by isoprenaline (Pfaffinger et al., 1985; Breitwieser & Szabo, 1985). Further evidence that no second messenger appeared to be involved in activation of this channel came from cell-free patch preparations (Yatani et al., 1987). This channel activation required the presence of a GTP-containing solution and was blocked by pertussis toxin; an agent which catalyses the ADP-ribosylation of some G-proteins (Northrup, 1985). While these experiments do not prove direct interaction between G-protein and channel (see Gilman, 1987), this mechanism nevertheless remains a possible means by which muscarinic agonists act. Similarly, two subtypes of G-protein, G_o and G_i have been suggested as possible mediators of neurotransmitter induced inhibition of voltage-dependent Ca^{2+}-channels (Holz et al., 1986).

3) Where the receptor and ion channel are linked via a G-protein and enzyme system.

There are several mechanisms which can be encompassed within this heading, including activation and inhibition of adenylate cyclase (for review see Gilman, 1987). Within the context of contraction in vascular smooth muscle however, stimulation of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and more recently, stimulation of phosphatidylcholine (PC) hydrolysis are particularly relevant. In each case of this type of receptor-effector linkage system however, G-protein activation leads to alterations in levels of a second messenger within the cell, which subsequently evokes cellular responses. These second messengers may result in changes in the state of phosphorylation of various intracellular proteins whose catalytic activity is thereby regulated (Schulman, 1982), or in changes in levels of calcium (see Putney 1984) .

Stimulation of phosphatidylinositol bisphosphate (PIP_2) hydrolysis.

Hydrolysis of PIP_2 represents a pathway which, by the production of two different second messenger systems, can evoke both types of cellular response mentioned above; phosphorylation of proteins and changes in Ca^{2+} levels.

The binding of a transmitter to a cell surface receptor leads, once again via a G-protein, to the activation of an enzyme, this time a phosphodiesterase, phospholipase C (PLC) and then to the production of the second messengers, inositol 1,4,5-trisphosphate (IP_3) and

diacylglycerol (DAG). PIP_2 hydrolysis is a mechanism which has been shown to occur in response to a wide variety of stimuli (for review see Berridge, 1987) and is relevant to this thesis, since it has been shown to mediate the contraction to agonists in smooth muscle (see later).

In animal cells there are three myoinositol containing phosphatides; phosphatidylinositol [1-(3-sn-phosphatidyl)] -d-myoinositol (PI), phosphatidylinositol -4- phosphate (PIP) and phosphatidylinositol -4,5-bisphosphate (PIP_2) (see Michell, 1975; Downes & Michell, 1982; Berridge, 1984). These phosphatidylinositols (Ptd Ins) are highly metabolically active but comprise less than 10% of the total phospholipid content in animal cells (PI comprising the largest part of this (>90%)).

A chance observation by Hokin & Hokin (1953) that incorporation of [^{32}P]-phosphate into lipids in pigeon pancreas slices was greatly stimulated by acetylcholine (Ach), gave the first evidence that Ptd Ins had a role in cell function. This incorporation of [^{32}P] was later associated with a specific increase in the turnover of Ptd Ins in response to a wide variety of external signals (see Hokin & Hokin, 1964; Michell, 1975; Putney, 1981; Berridge, 1984).

Degradation of the predominant inositol containing lipid - PI -was initially believed to be the primary consequence of receptor occupancy and accordingly the term phosphatidylinositol or 'PI' response was used to describe this phenomenon. Later however the G-protein mediated hydrolysis of PIP_2 by phospholipase C (PLC), with the subsequent

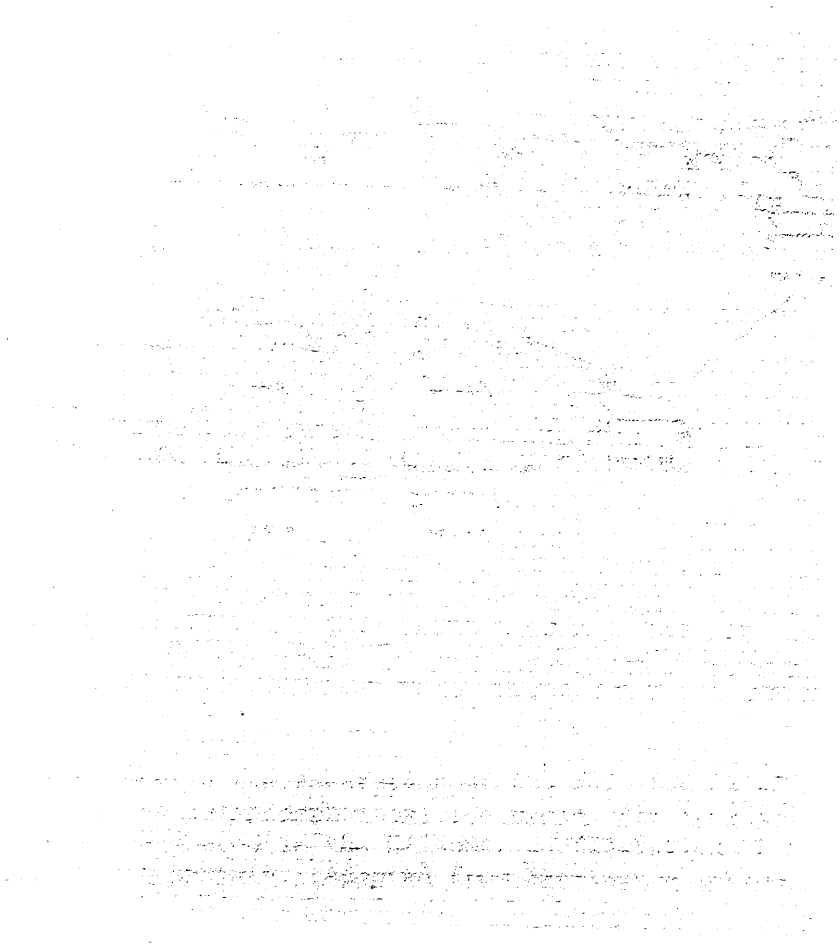
formation of diacylglycerol (DAG) and inositol (1,4,5) triphosphate (IP_3) (Michell et al., 1981; Berridge, 1983) was shown to be the initial response. That the inositol lipid cycle might in some way serve as a biochemical coupling mechanism linked to receptor activation and cellular Ca^{2+} mobilisation had already been proposed (Michell, 1975) and the means by which receptor occupation was linked to changes in Ca^{2+} was presumed to be via a second messenger. It was Berridge (1983) who suggested that inositol (1,4,5) triphosphate ($I(1,4,5)P_3$) was a likely candidate for a second messenger, it being rapidly formed in response to receptor occupation and able to release Ca^{2+} from the endoplasmic reticulum (Berridge, 1983; Berridge & Irvine, 1984; Putney, 1986).

Receptor mediated changes in intracellular Ca^{2+} have now been shown to occur as a result of both intracellular release by IP_3, probably from a Ca^{2+} pool in the endoplasmic reticulum (Burgess et al., 1983; Streb et al., 1984) and sequential or concomitant slow entry of extracellular Ca^{2+} (Putney, 1979; Putney et al., 1981).

Since then the metabolic pathway for the production and degradation of $I(1,4,5)P_3$ has been extensively studied and this has led to the discovery of a number of inositol phosphates, including 1,3,4,5-tetrakisphosphate in rat cerebral cortical slices (IP_4)(Batty et al., 1985; Irvine et al., 1986). The rapid metabolism of IP_4, as well as its rapid formation after receptor stimulation has led to debate over its possible role as a second messenger. The role of IP_4 is as yet unclear, however many agonists can promote a slow influx of external Ca^{2+} in addition to mobilising internal Ca^{2+} and both IP_4 and IP_3 have been implicated in controlling this. In some tissues IP_4 may

control the transfer of Ca^{2+} between intracellular pools (Irvine & Moore, 1986; Berridge, 1987) and in so doing, alter the influx of Ca^{2+} into the endoplasmic reticulum from the extracellular space by an as yet unknown mechanism, to replace stores.

Fig. 2 shows a simplified diagram of some of the general concepts of the hydrolysis of PIP_2.



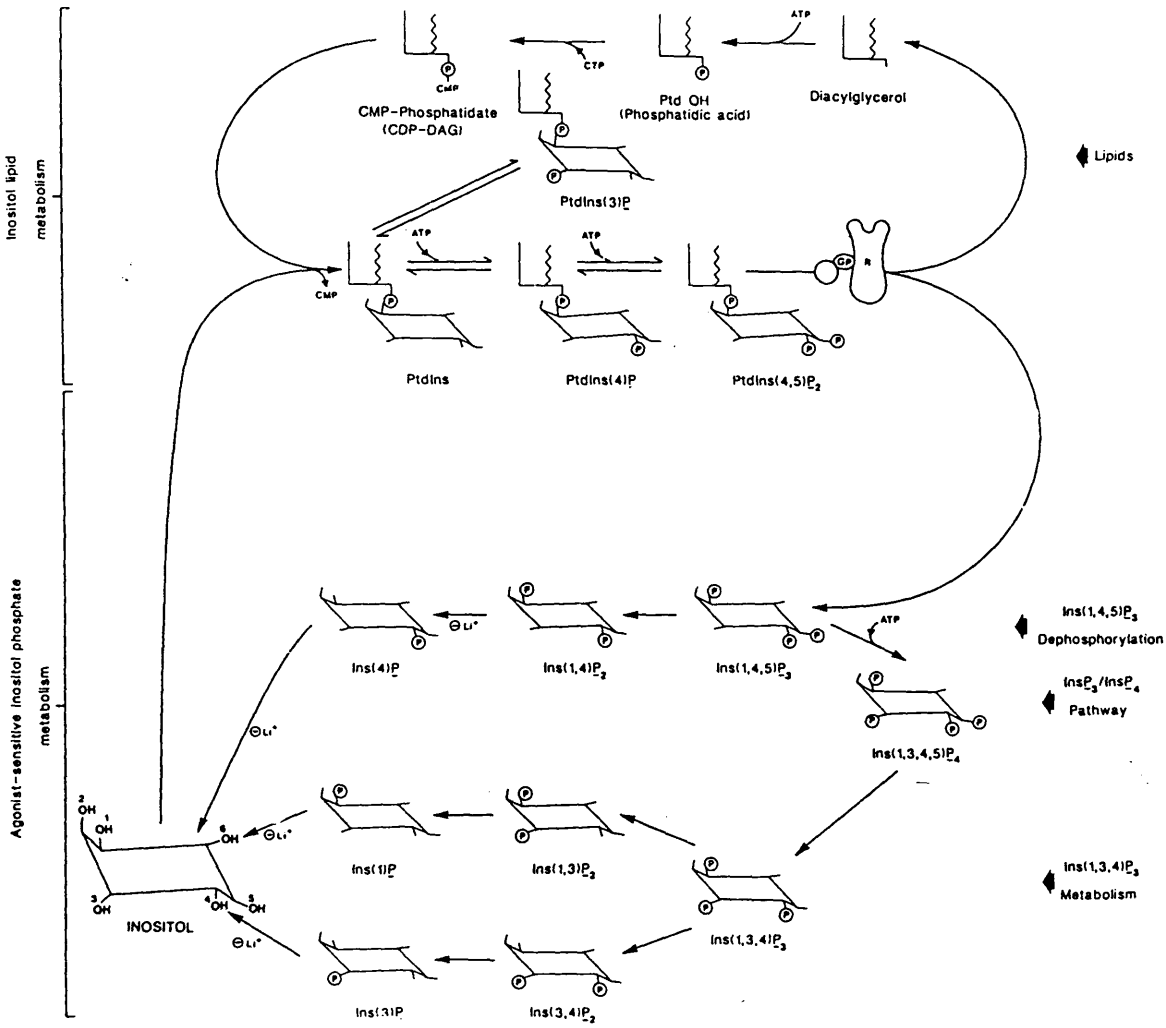


Fig. 2. A Schematic representation of the main routes of metabolism in the polyphosphoinositide cycle. All inositol phosphates are numbered in the D-isomer configuration. R = receptor; Gp = G-protein. (Adapted from Berridge & Irvine, 1989).

Diacylglycerol and protein kinase C

The other primary product of phospholipase C (PLC) stimulated hydrolysis of PIP_2 is diacylglycerol (DAG) and there is now strong evidence that DAG also functions as a second messenger (Nishizuka, 1983).

Under normal conditions, DAG is almost absent from membranes and it appears only transiently on receptor stimulation. This is due to its rapid phosphorylation to phosphatidic acid (PA) by diacylglycerol kinase and thence conversion back into inositol phospholipids and/or its further degradation into monoacylglycerol and arachidonic acid by diacylglycerol lipase (see Nishizuka, 1986). The diacylglycerol produced in response to receptor occupation initiates the action of a protein kinase C, so that information from extracellular signals can be translated directly across the membrane to protein phosphorylation (Nishizuka, 1983). The protein kinase C identified for this process requires calcium, acidic phospholipids (especially phosphatidylserine, however phosphatidylcholine exerts a negative effect) and DAG for maximum activity (Nishizuka, 1984). DAG greatly increases the affinity of the protein kinase for Ca^{2+}, so that in the presence of DAG, maximum activity of the kinase occurs at basal cytosolic concentrations of Ca^{2+}.

Protein kinase C is found in various tissues in mammals and other eukaryotic organisms (Kuo et al., 1980). In unstimulated cells it exists in the cytosol, however on activation of the cell receptors,

* Phosphorylation of myosin light chain may lead to conformational changes of myosin light chain, similar to those evoked by myosin light chain kinase (MLCK) and hence bring about contraction directly (Kamm et al., 1989). Phosphorylation of MLCK by PKC, may alter the sensitivity of MLCK to calcium, evoking the activation of MLCK at resting cell calcium levels, similarly leading to a contraction as a result of PKC activation (Singer et al., 1989).

production of DAG allows PKC to bind to phosphatidylserine at the plasma membrane and it is this that shifts the calcium dependence of PKC from high (10^{-5}-10^{-4}M) to low (<10^{-6}M) concentrations, thereby activating the kinase.

Protein kinase C can also be activated by synthetic diacylglycerols or tumor-promoting phorbol esters such as phorbol 1,2-myristate 1,3-acetate (PMA), which possess a DAG-like structure (Castagna et al., 1982). The action of phorbol esters lasts however for hours, while that of DAG is transient.

Once activated, PKC phosphorylates a large number of proteins including smooth muscle myosin light chain (Naka et al., 1983; Nishikawa et al., 1983) and myosin light chain kinase (Nishikawa et al., 1984; Ikebe et al., 1985). PKC may also modulate ion conductance by phosphorylating ion channels, pumps and ion exchange proteins (Nishizuka, 1986). It may also have a role in extrusion of calcium immediately after its mobilization into the cytosol (e.g. via a voltage-dependent Ca^{2+} channel or by the actions of IP_3, Drummond, 1985; Albert & Tashjian, 1985) and the Ca^{2+} transport ATPase is also a possible target of this protein kinase (Limas, 1980; Iwas & Hosey, 1984).

Alternative sources of 1,2-diacylglycerol

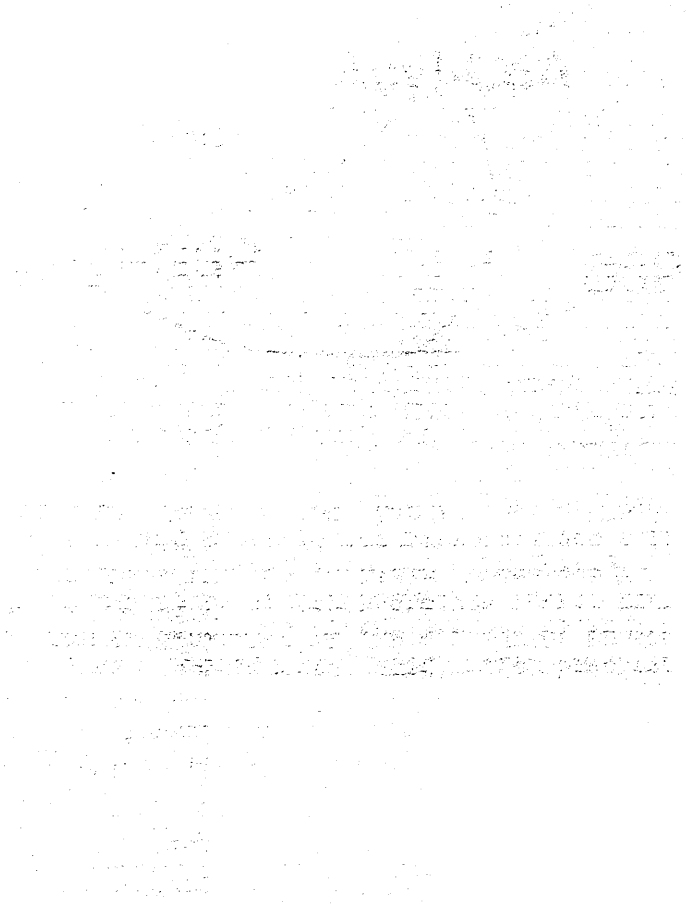
While production of DAG from hydrolysis of PIP_2 is now well documented, it has become apparent that the phosphatidylinositols are not the only source of DAG.

Following the suggestion by Bocckino et al. (1985) that hormonally induced-DAG was partially derived from sources other than inositol phospholipids, Exton (1988) concluded that the generation of DAG from phosphatidylcholine exceeded that from PIP_2 hydrolysis in isolated hepatocytes. Phosphatidylcholine is found almost exclusively in eukaryotic cell membrane and forms the largest part of the cell membrane. Evidence for the role of phosphatidylcholine (PC) in DAG production is now fairly conclusive in isolated cells preparations. The evidence in favour of a receptor-regulated choline phospholipid hydrolysis is based on the following observations. Activation of various receptors has been shown to enhance the release of choline, phosphocholine and glycerophosphocholine - each metabolites of PC (Besterman et al., 1986; Cabot et al., 1988a; 1988b; Welsh et al., 1988). The fatty acid composition of the evoked DAG and phosphatidic acid (PA) was closer to that of phosphatidylcholine than of phosphatidylinositol The amount of DAG or PA formed in response to stimuli often exceeded the release of inositol phosphates (Bocckino et al., 1985). The only phospholipid that declined was in many cases phosphatidylcholine (Bocckino et al., 1987) and while PI-phospholipase C is blocked in zero external calcium (Uhing et al., 1986), PA

accumulation due to PC hydrolysis was not (Bocckino et al., 1987).

The pathways proposed for the hydrolysis and synthesis of phosphatidylcholine (PC) are shown in fig. 3. There are three phospholipases by which DAG may be produced from PC, phospholipase C (PLC), phospholipase D (PLD) and phospholipase A_2 (PLA_2).

In contrast to the phospholipase C catalyzed hydrolysis of PIP_2, the hydrolysis of PC produces DAG without altering cytoplasmic levels of Ca^{2+}. As previously discussed however, DAG produced from PC can still propagate a signal by enhancing the affinity of PKC for available Ca^{2+} and thereby activating the kinase.



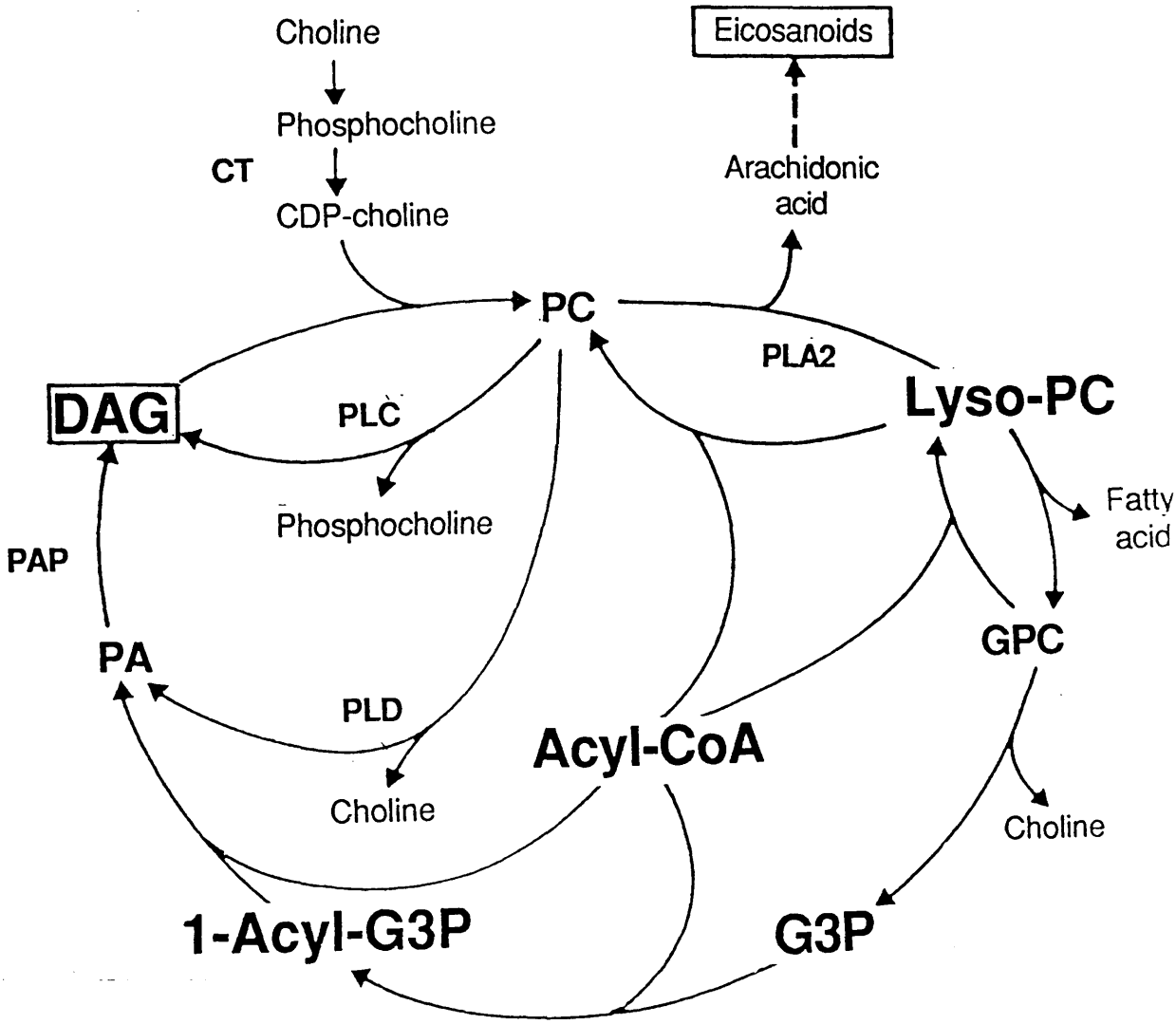


Fig. 3. Phosphatidylcholine cycle for generation of diacylglycerol (DAG). DAG can be generated directly via phospholipase C (PLC), or by the action of phospholipase D (PLD) to yield phosphatidic acid (PA) which is cleaved to DAG by PA phosphohydrolase (PAP). Alternatively, DAG may be formed from PA generated by the actions of phospholipase A_2 (PLA_2), in which case, arachidonic acid, the precursor or the eicosanoids is also produced. DAG can react with CDP-choline, formed by the action of phosphocholine cytidylyltransferase (CT) on phosphocholine, to complete the PC turnover cycle. (Reproduced from Pelech & Vance, 1989)

The mechanisms of action of the two transmitters NA and ATP may involve at least two of the above mentioned types of transduction mechanisms, 1), 2) and 3).

Some indication of the importance of the first above mentioned receptor-effector mechanism has been obtained by examining the effects of ATP and NA on the resting membrane potential of smooth muscle cells and the associated contraction.

Stimulation of sympathetic nerves supplying smooth muscle may, for example in rabbit saphenous artery (Holman & Surprenant, 1979), evoke junction potentials (e.j.p.s), which represent a rapid shift in membrane potential, such that the entry of calcium into the cell through voltage-sensitive calcium channels is likely to be increased (Bolton & Large, 1986), leading, in many cases, to action potential and contraction. In some cases, the fast e.j.p.s are followed by a slow depolarisation (e.g. rat tail artery, Cheung, 1982) which is easier to observe after a train of stimuli (Bolton & Large, 1986) and may similarly be associated with a contraction.

Use of antagonists has allowed separation of the components of the electrical and mechanical responses to NA and ATP released during nerve stimulation and it has become apparent that the degree to which each of these transmitter utilises voltage-dependent mechanisms, varies among different tissues.

Thus in the rabbit ear artery (Allcorn et al., 1985), field stimulation of intramural nerves evokes e.j.p.s followed by a slow

depolarisation and a contraction. The evoked e.j.p.s are mediated by ATP, as evidenced by the use of the desensitising agent αβ MeATP. Electrically, NA produces only the slow depolarisation yet is responsible for the entire mechanical response, which is abolished by prazosin. In the rabbit mesenteric artery (Lim et al., 1986), nerve released NA does not alter the membrane potential, while ATP produces e.j.p.s which summate to produce action potentials. The contraction is mediated by both ATP and NA. Similarly, there are many examples (e.g. rabbit ear artery, Droogmans et al., 1977; rabbit saphenous artery, Holman & Surprenant, 1979) where addition of exogenous ATP produces a depolarisation whilst exogenous addition of NA does not. By contrast, in the rat mesenteric artery (Mulvaney et al., 1982), contractions to exogenously added NA are accompanied by membrane depolarisations.

It seems likely therefore that in many smooth muscles, the co-transmitter ATP acts via a voltage-dependent mechanisms to produce contraction. There is however evidence (Benham & Tsein, 1987) that in the rabbit ear artery, ATP mediates its effect, not only via voltage dependent Ca^{2+} channels, but also via voltage-independent channels which are operated directly by ligands, in the absence of any change in second messengers. This may constitute an example of the second type of mechanism listed above.

It has been proposed that in some arteries e.g. the rabbit mesenteric artery (Nelson et al., 1988) that NA similarly acts via voltage-dependent Ca^{2+} channels. In many cases however, NA produces only small electrical changes which appear insufficiently large to be underwritten by substantial ion changes. The contractile basis for NA

must, to a large degree, involve those mechanisms where cellular events do not rely on extracellular ion movements, but which are mainly concerned with providing calcium from intracellular stores, or with altering the sensitivity of the cell to resting calcium. NA is more likely therefore to utilise the last of the three mechanisms described above, i.e. where the receptor and ion channel are linked via a G-protein and enzyme system. Indeed, the PIP_2 cycle has now been shown to be activated by NA in a number of vascular tissues, e. g. rabbit mesenteric artery (Hashimoto et al., 1986), rat tail artery (Fox et al., 1985) rabbit aorta (Campbell et al., 1985) and rat aorta (Legan et al., 1985). Stimulation of PC hydrolysis has not yet been shown for noradrenaline.

The possibility that, in addition to acting via voltage-operated ion channels, ATP may also stimulate PIP_2 hydrolysis has been less often studied. There is some evidence however, that in rat tail artery (Jenkinson, 1990) in addition to producing e.j.p.s, ATP may also stimulate PIP_2 hydrolysis. ATP has also been shown to stimulate PIP^2 hydrolysis in a number of cell lines, including hepatocytes (Charest et al, 1985) and Ehrlich ascites tumor cells (Dubyak, 1986). ATP has similarly been implicated in the receptor mediated hydrolysis of PC in isolated hepatocytes (Boccking et al., 1987). The question of whether the ATP stimulated PIP_2 or PC hydrolysis precedes, or is a consequence of the electrical changes in the smooth muscle cell has yet however to be answered.

Within the mechanisms of signal transduction described, synergism and/or reciprocation is possible on several levels. First, the possibility exists of a multiple effect of the second messengers released as a result of PIP_2 hydrolysis. IP_3 so formed will mediate release of calcium from the endoplasmic reticulum, as well as controlling - possibly along with IP_4 - the influx of extracellular Ca^{2+}. DAG will stimulate protein kinase C by enhancing its sensitivity to Ca^{2+}. This may further evoke changes in Ca^{2+} levels by the PKC-mediated phosphorylation of channels, or by kinase effects on Ca^{2+} sequestration, as discussed above.

Secondly, there exists the possibility of an interaction between elevated calcium levels, resulting from a voltage-dependent mechanism (e.g. e.j.p.s produced by ATP) and DAG mediated activation of PKC (produced by a voltage-independent mechanism, such as is proposed to explain the action of NA in many tissues). Evidence for interactions of these types is already available, in a variety of isolated cells, from the concomitant use of calcium ionophores such as A23187 to raise calcium levels and phorbol esters to stimulate PKC e.g. in platelets (Kaibuchi et al., 1983), lymphocytes (Rink et al., 1983) and hepatocytes (Mastro & Smith, 1983). In most of these studies, neither component alone produces the maximal physiological response, but do so only when they are together. Thus voltage-independent mechanisms may reinforce or synergize with the voltage-dependent mechanisms inherent in the e.j.p.s - commonly produced by ATP - in a co-transmission system, providing a basis for a 'dual-control' mechanism.

AIMS OF THE THESIS

The emergence of the concept of co-transmission in both the parasympathetic and sympathetic nervous system has provided a potential degree of complexity in autonomic control of muscle, hitherto not encountered.

Co-transmission therefore provides a challenge to pharmacologists to unravel the means by which the transmitters concerned operate to achieve the physiological response. Just as drug control of cholinergic and noradrenergic responses has had clinical uses, the possibility exists that once the mechanisms of action of co-transmitters are fully understood, manipulation of multiple transmitters will have similar clinical ramifications.

Much work has been done to establish the means by which co-transmitters operate in producing a physiological response. This thesis represents an attempt to do this by studying transmitter mediated events in a vascular site - the rabbit saphenous artery -where co-transmission is believed to operate.

Much of the information on the action of co-transmitters has been gained from mechanical recordings with the accompanying limitations on the interpretation of the underlying mechanisms producing the contraction. Accordingly in this thesis, mechanical recordings were accompanied by intracellular micro-electrode and biochemical recording techniques, to investigate the mechanisms underlying the contractions evoked by the released transmitters. In particular, this thesis aimed to investigate whether both transmitters evoked similar, or different types of cellular change on interaction with their separate receptors

and if different cellular changes were evoked by each transmitter, at what level these transmitter substances interact.

In addition, studies in the canine heart and femoral vascular bed were undertaken, to establish the nature of the purine receptor subtypes for adenosine, mediating respectively, bradycardia and vasodilation in the dog.

MATERIALS AND METHODS

Tissues for mechanical, intracellular or biochemical recording were removed from animals as quickly as possible, as described below. Tissues were left for a minimum of 30 min after being set up before the start of each experiment. Sodium oleate and antagonists, with the exception of staurosporine, were added to tissues 15 min before their effects were investigated. Staurosporine and phorbol 1,2-myristate 1,3-acetate (PMA) were each added 1h before investigation.

I. DISSECTION OF TISSUES

A. RABBIT SAPHENOUS ARTERY

Male New Zealand White rabbits 1.3-2.5Kg, were killed either by CO_2 overdose or by a blow to the head. In each case, exsanguination followed. Saphenous arteries were removed by a method similar to that of Holman & Surprenant (1979). The leg was stripped of skin and attached fur and a 3-5cm length of artery dissected from above the ankle to below the knee. The artery was then transferred to an oxygenated, Krebs-filled petri dish the base of which was coated with Sylgard (Dow-Corning) and, at room temperature under a dissecting microscope, cleaned of fat and connective tissue .

B. RAT TAIL ARTERY.

Male albino Wistar rats (200-400g) were killed by a blow to the head followed by exsanguination. The tail was severed approximately 1cm from the body and removed to an oxygenated, Krebs-filled petri dish, the base of which was coated with Sylgard. The cornified epithelium was removed and a 3-5cm length of artery dissected out from the proximal end of the tail (Holman & Surprenant, 1979). Under a dissection microscope, the artery was then cleaned of fat and connective tissue.

II. APPARATUS & TECHNIQUES

A. MEASUREMENT OF CONTRACTILE ACTIVITY.

To measure contractile responses from in vitro preparations, 0.5-1cm lengths of rabbit saphenous artery were mounted horizontally in a heated (37 ± 0.5°C) organ bath (10ml) and bubbled with 95% O_2 & 5% CO_2 in a modified physiological Krebs solution. The tubing carrying the Krebs solution and the organ bath were surrounded by a jacket containing water at 42 ± 0.5°C, pumped by a tempette (TE7) pump, to maintain the Krebs in the organ bath at 37 ± 0.5°C.

Segments of arteries were mounted under 1g tension by placing two platinum wires into the lumen. One was attached to a force displacement transducer (Grass FT03C) to monitor tension, while the other was fixed. Ag/AgCl electrodes were placed parallel to the artery segment and stimulation of intramural nerves carried out using an

isolated square wave stimulator (Grass SD9, 0.5mS, supramaximal voltage, 1-64Hz). The parameters used ensured that no direct stimulation of muscle occurred. Contractile activity was displayed on a heat sensitive linear chart recorder (Electromed MX216).

Drugs were added to the organ bath in volumes of 0.1 - 0.3ml from graduated syringes, or using a Gilson pipetman of suitable size. Agonists were kept in contact with the tissue for 1min. The organ baths were emptied from below and filled from above by gravity. Where appropriate, drugs were washed out by emptying and filling the bath three times and 5 min were allowed between each addition. For continuous or long term infusion, drugs were added to the reservoir to give the final concentration required.

B. SIMULTANEOUS INTRACELLULAR ELECTRICAL AND MECHANICAL RECORDING

The electrical and simultaneous mechanical responses of the rabbit saphenous artery to field stimulation of intra-mural nerves and to drugs, were investigated using intracellular microelectrode and conventional mechanical recording techniques in vitro

The apparatus (Fig 4) consisted of a horizontal organ bath (5cm x 1cm x 1cm) cut from a perspex block, the base of which was coated with Sylgard, two Ag/AgCl ring electrodes, an indifferent Ag/AgCl electrode, capillary glass microelectrodes, D.C. preamplifier, dual beam oscilloscope, voltmeter, transducer, isolated stimulator and gated pulse generator, U.V. oscillograph and a tape recorder.

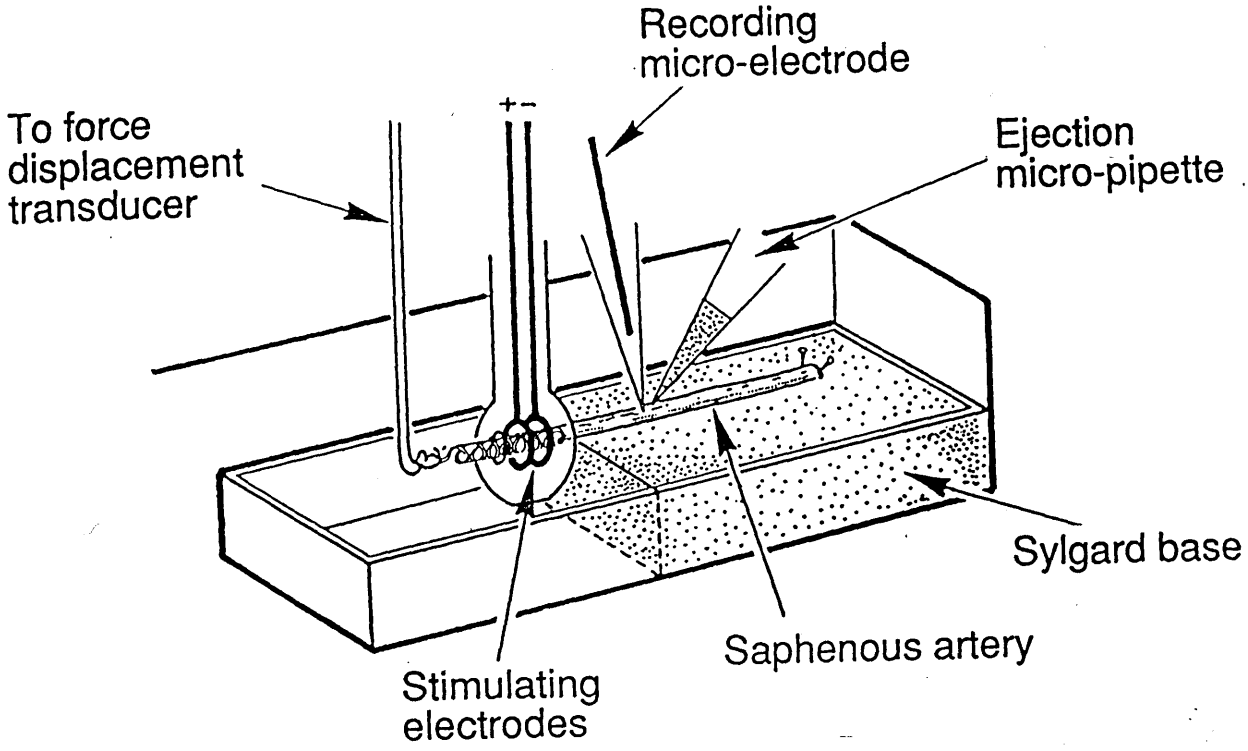


Fig. 4 Organ bath for simultaneous mechanical and intracellular electrical recordings of the rabbit saphenous artery. A trough (5cm x 1cm x 1cm) was cut from a perspex block, which was drilled to accept inlet and outlet tubes (0.2cm diameter) for continual perfusion of Krebs solution. Tissues were passed through Ag/AgCl (V^+-V^-) ring electrodes attached to a square wave isolated stimulator. One end was cut open, flattened and pinned onto Sylgard, while the other was cut into a spiral and attached to a force displacement transducer under 1g tension, for measurement of contractions. Membrane potential changes were recorded using conventional glass microelectrodes in response to field stimulation and exogenous agonists, applied locally from a micro-pipette linked to a pressure-controlled ejection device (Picospritzer II). The bath was perfused with oxygenated, heated (37 ± 0.5°C) Krebs solution, pumped by a tempette (TE7) pump.

Lengths of artery (2cm) were cleaned of fat and connective tissue as described. The artery was cut into a spiral along half its length, while the other half was opened lengthwise. The artery was then placed in the organ bath and constantly perfused (5ml/min) via two inlets, with Krebs solution at 37 ± 0.5 °C, pre-bubbled with 95% O_2 & 5% CO_2. Emptying of the bath was by suction applied from a water pump. Polythene tubing (O.D. 2mm) containing Krebs solution was surrounded by an outer tube (O.D. 10mm) containing liquid paraffin at 42 ± 0.5°C, pumped by a Tempette (TE7) pump to maintain Krebs at the constant required temperature.

To minimise mechanical vibrations, the organ bath was bolted to two non-conducting Bakelite pillars fastened to a steel plate (200Kg) on a table mounted on Mufflite (K-150) anti-vibration dampeners.

The spiralled end of the artery was attached to a force displacement transducer (Grass FT03C) and the other end passed through the bipolar Ag/AgCl ring electrodes (O.D. 2mm, 2mm apart, mounted in epoxy resin (Araldite)) and pinned flat on the Sylgard, serosa uppermost. Field stimulation was carried out via an isolated square wave stimulator (Grass SD9, 0.5mSec, supramaximal voltage, 0.2-64Hz).

Intracellular recordings were made from cells impaled using glass capillary microelectrodes (Clark, GC 150-10, 40 - 80 MΩ) filled with filtered KCl (3M). Signals were passed to a unity gain high impedance ($10^{10}\Omega$) DC preamplifier (Neurolog NL 102) via a Ag/AgCl half-cell attached to a probe, matched and calibrated for the amplifier used.

The indifferent Ag/AgCl electrode was fixed to the wall of the bath and held in the bath solution. Mechanical signals were amplified via a low pass filter/amplifier (made by Mr J.Sinclair, Institute of physiology, University of Glasgow). Signals were displayed on a storage oscilloscope (Telequipment DM63) and displayed on a digital voltmeter (Fairchild M53). The electrical and mechanical signals were stored permanently on an instrumentation tape recorder (Raccal store 4DE, band width 313-40kHz) and U.V. Oscillograph (EMI SE3006) Drug reached the tissue following;

1) Addition of small bolus quantities by graduated micro-syringe (Hamilton), directly to the organ bath

2) Addition as perfusate to give the final concentration required.

3) Local addition by a hydrostatic pressure controlled ejection device (Picospritzer II, Gen. Valve Corp., N.J., U.S.A.). Drugs for application were filled into ordinary glass micropipettes the tips of which had been broken back under microscopic control to 1-2 μm and ejected close to the cell from which intracellular recording was being made. This technique minimises the possibility of desensitisation of receptors by addition of excess drug, while increasing greatly the likelihood of obtaining drug responses from the impaled cell.

The amount of drug reaching the recording electrode from the picospritzer depended upon the diameter of the tip (1-2μm), the distance of the tip from the recording site (kept to <1mm as measured with an eyepiece micrometer), the ejection pressure (held constant at 40-50 p.s.i.) and the duration of ejection (1-640mS, varied as indicated in the text).

C. MEASUREMENT OF CHANGES IN CONDUCTANCE AND RESISTANCE.

Passive membrane properties were measured and changes in membrane conductance and resistance under the influence of drugs observed, in vitro using the partition method of Abe & Tomita (1968).

The apparatus (Fig 5) comprised an organ bath (5cm x 1cm x 1cm) with a sylgard base, cut from a perspex block. The bath was divided into two main compartments - for stimulating and recording respectively -by means of two silver plate electrodes (50μm thickness x 1cm x 1cm, 1cm apart). The surface exposed to the recording chamber was coated with epoxy resin (Araldite) to act as an insulating partition. The electrodes were connected to an isolated square wave stimulator (Devices type 2533) through a resistance (30kΩ) to obtain constant current. There was a small hole (0.3mm) in each of the plates through which the tissue was passed, to be pinned flat in the recording chamber.

Relative current intensity was measured from the voltage gradient of the solution in the stimulating chamber via two Ag/AgCl recording electrodes 2mm apart in the chamber wall. Field stimulation of intramural nerves was carried out by two Ag/AgCl ring electrodes in the recording chamber, attached to an isolated square wave stimulator (Grass SD9, 0.5mS, supramaximal voltage, 1Hz) and intracellular microelectrode recordings were obtained from within the recording chamber, using glass capillary microelectrodes (Clark, GC 150F-10, 40-80MΩ). Both chambers were independently perfused with Krebs solution pre-bubbled with 95% O_2, 5% CO_2, pre-heated to 37 ± 0.5°C. The polythene tubing

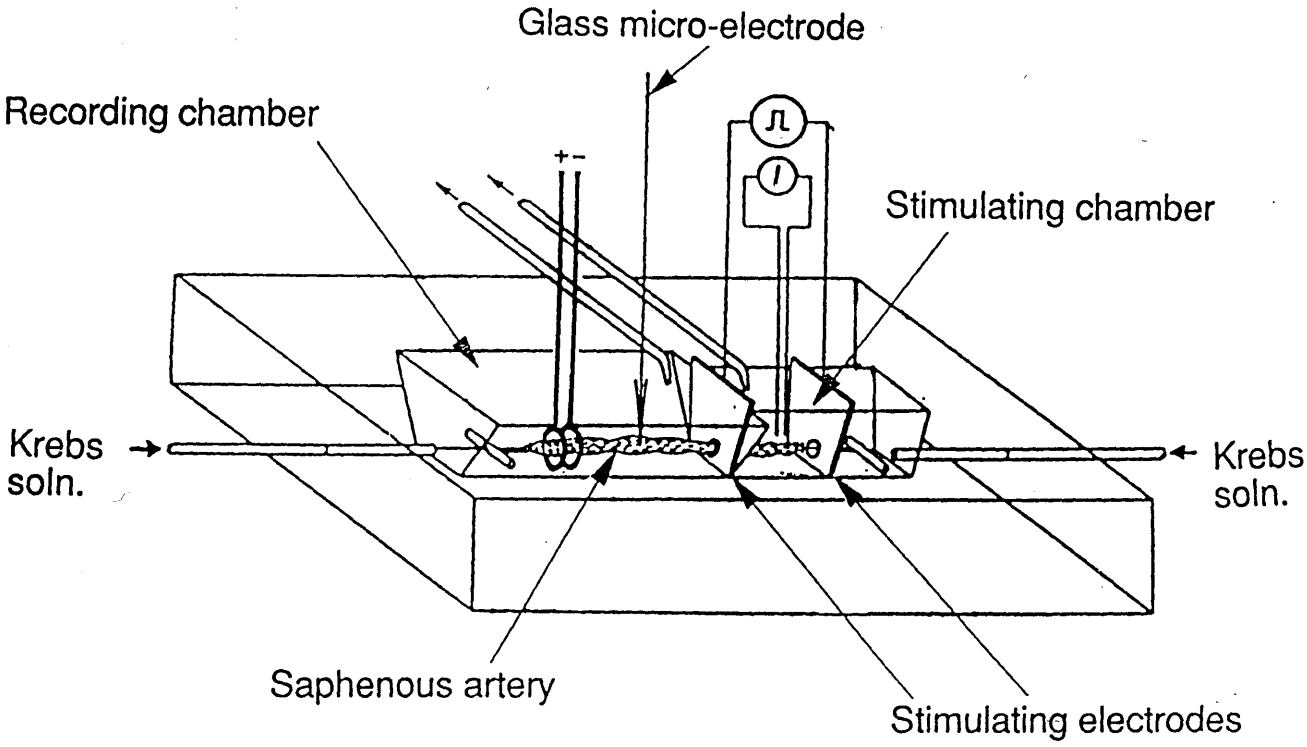


Fig. 5 Modified Abe & Tomita (1968) partition bath used in the investigation of membrane conduction, resistance and passive electrical properties in the rabbit saphenous artery.

Two Ag plate electrodes divided an organ bath (5cm x 1cm x 1cm) into both a recording and a stimulating chamber. The tissue was anchored at one end, then passed through a small hole in each of the plate electrodes and pinned onto Sylgard in the recording chamber, where changes in membrane potential were measured intracellularly using glass microelectrodes. Current was applied to the muscle via the Ag plate electrodes which were connected to an isolated square wave stimulator. The relative current intensity was measured from the voltage gradient of the Krebs solution in the stimulating chamber via two Ag/AgCl electrodes. Field stimulation of intramural nerves was via two Ag/AgCl ring electrodes situated in the recording chamber. Each chamber was separately irrigated with oxygenated, heated (37 ± 0.5°C) Krebs solution pumped by a tempette (TE7) pump.

(O.D. 2mm) carrying the Krebs solution was surrounded by outer tubing (O.D. 10mm) containing liquid paraffin at 42 ± 0.5°C to maintain the required temperature.

Drugs were added to the Krebs in the perfusion reservoir.

D. MEASUREMENT OF THE PRODUCTS OF PHOSPHATIDYLINOSITOL -4,5 BISPHOSPHATE (PIP_2) HYDROLYSIS)

The involvement of PIP_2 hydrolysis in the mechanical response to agonists in vitro was assessed by radiochemical measurement of inositol phosphates released from segments of rabbit saphenous and rat tail arteries incubated with myo-[2-^{3}H]-inositol.

Ion exchange resin (Dowex 1 x 8-400, Chloride form) was prepared by washing with the following solutions; distilled water (1l), NaOH (2M, 2l), distilled water (2l), formic acid (2M, 0.2l), followed by distilled water until pH 4.5 - 5 was obtained. Aliquots (1ml) of the formate form of resin so produced were added to Pasteur pipettes (5ml), serving as chromatography columns, the tips of which were plugged with glass wool. Columns were prepared by washing with ammonium formate (2.4M, 15ml), followed by distilled water (15-29ml) until pH 4.5 - 5 was once again obtained.

The products of PIP_2 hydrolysis were examined using a modified version of the method of Akhtar & Abdel Latif (1986)

Segments (1-2mm) of arteries were incubated in glass vials (3ml), for 3h in Krebs solution containing 8μCi/ml myo-[2-^{3}H] inositol at 37 ± 0.5°C and bubbled with 95% O_2, 5% CO_2. Constant temperature was maintained by immersion of the glass vials in a bath containing water at 40 ± 0.5°C, pumped by a tempette (TE7) pump. Tissues were then further incubated in Krebs solution containing the same quantity of myo-[2-^{3}H] inositol, but with the substitution of 10mM NaCl, with 10mM LiCl, to inhibit inositol phosphate phosphatase (IP phosphatase) and hence prevent inositol phosphate being broken down to inositol and subsequent cycling of the phosphatidylinositol (PI) cycle (Hallcher & Sherman, 1980). Tissues were then washed twice in fresh Li^{+} substituted Krebs solution and drugs added, by graduated syringe, direct to the vial.

Agonists were left in contact with the tissue for 25min and antagonists were added 15 min prior to agonists. The reaction was then stopped by plunging the tissue into ice-cold trichloroacetic acid (TCA, 1ml, 10%) in a glass test tube (5ml). Tissues were homogenised in the TCA, using a hand held glass pestle homogeniser, then centrifuged at 2,000g for 15 min at 5°C. The supernatant, which now contained all soluble compounds, was decanted off for analysis and the pellet discarded. Any residual TCA was removed by extraction 3 times with water saturated diethyl ether (3ml) and excess diethyl ether driven off by placing the tube in a heated water bath at 100°C for 5min. The remaining supernatant was neutralised with 0.1M NaOH and the neutralised extract was then applied to the previously prepared columns for separation of the constituents of the samples by a method similar to that of Downes et al., (1986).

The columns were washed with 10ml each; distilled water to elute [^{3}H]-inositol, Sodium borate (5mM) plus sodium formate (60mM) to elute glycerylphoryl inositols, ammonium formate (0.2M) plus formic acid (0.1M) to elute [^{3}H]-Inositol phosphate (IP), ammonium formate (0.4M) plus formic acid (0.1M), to elute [^{3}H]-inositol bisphosphate (IP_2), and finally ammonium formate (1.2M) plus formic acid (0.1M), to elute [^{3}H]-inositol trisphosphate (IP_3) and [^{3}H]-inositol tetrakisphosphate (IP_4) together since separation of these two components was not possible. In each experiment, columns were calibrated using standard samples of inositol, IP, IP_2, IP_3 and IP_4 to confirm the elution profile.

A sample (1ml) of each eluate was added to scintillation fluid (10ml, Ecoscint) and total radioactivity determined by liquid scintillation counting (Hewlett-Packard 2000CA Tri-carb). Total inositol phosphate levels (tIP) were taken as the sum of the amounts of IP, IP_2, IP_3 and IP_4 obtained. Results were adjusted for the whole sample and translated to degradations per minute (d.p.m.) per mg of tissue (wet weight).

E. EXTRACTION AND ANALYSIS OF THE PRODUCTS OF PHOSPHATIDYLCHOLINE (PC) HYDROLYSIS

The involvement of PC hydrolysis in the mechanical response to agonists in vitro was assessed in the rabbit saphenous artery by measurement of the breakdown products released from tissues incubated with [^{3}H]-methyl choline.

Columns were prepared by washing ion exchange resin (Dowex, 50-WH$^+$) extensively with HCl (1M, 4l) followed by distilled water (4l), until pH 5.5 was obtained. Aliquots (1ml) of the prepared resin were added to glass wool-plugged Pasteur pipettes (5ml).

The products of hydrolysis of PC were examined using a modification of the method of Cook & Wakelam (1989).

Lengths of rabbit saphenous artery (4cm) were incubated in Krebs containing 2µci/ml [^{3}H]-methyl choline for 18 hours in glass vials (3ml); this being the time which allowed the tissue to come closest to isotopic equilibrium without significantly impairing the contractile response. After incubation, tissues were washed twice in fresh Krebs solution and drugs added. The reaction was stopped after the required time, by immersing the tissue in ice cold methanol (1ml). Tissues were left for 1h in methanol, before chloroform and distilled water were added to give a final ratio of methanol : chloroform : water of 1:1:0.9. Tissues were homogenised in a hand held glass pestle homogeniser and centrifuged at 2,000g for 15 min at 5°C. The upper

aqueous phase was taken for analysis. Samples were diluted (to 5ml) and loaded onto the prepared columns. Samples were then further eluted with a distilled water (4ml), which was added to the initial run off, followed by HCl (10ml of 0.01M and finally 20ml of 1M) to elute respectively, glycerophosphocholine (GPC), phosphocholine (ChoP) and choline (Cho). In each experiment, standards were loaded onto separate columns to check the elution profiles.

F. IN VIVO MEASUREMENT OF HEART RATE (HR) AND HIND LIMB PERFUSION PRESSURE (HLPP)

Female Beagle dogs (12-15Kg) were anaesthetized with sodium pentobarbitone (45-60mg/Kg i.v.) to induce deep anaesthesia, and the animal was ventilated with air delivered by a respirator pump (Palmer ideal, s.v.; 170-230ml, rate; 24/min). The following blood vessels were catheterised; right jugular vein for administration of maintenance anaesthesia (pentobarbitone 5mg/ml delivered at 22ml/h); the right brachial vein for administration of antagonists; the right brachial artery for measurement of systemic blood pressure using a pressure transducer (Bell & Howell 4/327/L211) and for blood sampling; the left carotid artery for administration of agonists. The catheter to the left carotid artery was introduced into the left ventricle to administer agonists directly to the heart.

The right hind limb was perfused from the right iliac artery, via a perfusion pump (Watson Marlow, 520s) back to the right femoral artery, at a constant flow rate (30-40ml/min) and the right leg was tied off

just above the ankle to isolate the vascular bed of the perfused limb. Perfusion pressure was measured by a (Bell & Howell 4/327/L211) pressure transducer.

Both Vagi, the right femoral and the right sciatic nerves were ligated and severed and ECG recording electrodes were placed around the chest wall. Heart rate measurements were taken from the ECG signal.

Each animal was given a bolus injection of the anticoagulant heparin (1250u) to prevent blood clotting and the β_1- partial agonist xamoterol hemi-fumarate (1mg/Kg) to maintain a stable high heart rate and so facilitate measurement of drug induced responses. The adenosine uptake blocker s-(p-nitrobenzyl)-6-thioinosine (NBTI, 0.5mg/Kg) was given to prevent purine uptake and hence maximise the effects of adenosine.

Samples of blood were taken for blood gas analysis (Ciba-Corning 278 blood gas system), any blood acidosis corrected by administration of sodium bicarbonate (8.4%w/v). Blood CO_2 and O_2 levels were maintained by alteration of the tidal volume and animals were allowed to acclimatise for one hour before the start of experiments.

Except where otherwise stated, drug addition was by bolus injection into the relevant catheter.

At the end of experiments, dogs were sacrificed by intravenous injection of a saturated solution (25ml) of KCl.

III. 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 field stimulation of intramural nerves

(c) The resting membrane potential was more negative than -60mV

(d) following withdrawal of the microelectrode, the membrane potential was restored to the levels observed before penetration.

IV. PHYSIOLOGICAL SOLUTIONS AND CHANGES IN IONIC COMPOSITION

Except where stated, Krebs solution of the following composition (mM) was used throughout the investigation. NaCl, 118.4, $NaHCO_3$, 25, NaH_2PO_4, 1.13, KCl, 4.7, $MgCl_2$, 1.3, $CaCl_2$, 2.7 and glucose, 11.

Where 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 by NaCl. In solution containing an increased concentration of K^+, an equivalent reduction in NaCl was made. Na^+ deficient solutions were made by substituting NaCl with choline chloride. Cl^- free solutions were made by substituting NaCl

with Na benzenesulphonate, KCl with K_2SO_4, $MgCl_2$ with $MgSO_4$ and $CaCl_2$ with $CaSO_4$. Ca^{2+} free solutions were made by substituting $CaCl_2$ with $MgCl_2$. Where Li^+ substituted Krebs was required, an equivalent amount of NaCl was withdrawn.

The pH of the Krebs solution in each case was maintained at 7.4 by gassing with a mixture of 95% O_2 and 5% CO_2.

V. DRUGS

The following drugs were used:

αβ Methylene adenosine 5'-triphosphate sodium (αβ MeATP, Sigma), adenosine sodium (Sigma), adenosine 5'-triphosphate sodium (ATP, Sigma), atropine sulphate (Sigma), 8,8'-[carbonylbis [imino-3,1-phenylene carbonylimino (4-methyl-3,1-phenylene) carbonylimino]]bis -1,3,5-napthalene trisulfonic acid hexasodium (Suramin, I.C.I.), N^6-cyclopentyl adenosine (CPA, Research Biochemicals Inc.), desmethylimipramine hydrochloride (DMI, Ciba), diltiazem hydrochloride (Sigma), N-[2-(dimethylamino) ethyl]-N-methyl-4-(2,3,6,7-tetrahydro -2,6-dioxo-1,3-dipropyl -1H-purin-8-yl) benzenesulphonate (PD115199, synthesized by I.C.I.), 1,3-dipropyl 8-cyclopentyl xanthine (PD 116948, synthesized by I.C.I.), 5'-(N-ethylcarboxyamido) adenosine (NECA, Sigma), guanethidine monosulphate (Ciba), heparin sodium B.P.(Evans), histamine diphosphate (Sigma), 5-hydroxytryptamine hydrochloride (5-HT, Sigma), 6-hydroxydopamine hydrobromide (6-OHDA, Sigma), 2-(2-(1,4 benzodioxanyl)) 2-imidazoline hydrochloride (Idazoxan,

Reckitt & Coleman), [^{3}H] inositol phosphate marker set (Amersham Int.), [^{3}H]-methyl choline chloride (Amersham Int.), myo-[2-^{3}H]-inositol (Amersham Int.), s-(p-nitrobenzyl)-6-thioinosine (NBTI, Sigma), (-) noradrenaline bitartrate (NA, Sigma), (±) normetanephrine hydrochloride (NMN, Sigma), N^6-2-phenylamino adenosine (PAA, Research Biochemicals Inc.), (+)-N^6-(2-phenylisopropyl) adenosine, (r-PIA, Research Biochemicals Inc.), 8-phenyltheophylline (Research products Inc.), phorbol 1,2-myristate 1,3-acetate (PMA, Sigma), potassium chloride (KCl, Koch-light), (±) prazosin (Pfizer), (±) propranolol hydrochloride (Sigma), quinacrine dihydrochloride (mepacrine, Sigma), sodium fluoride (NaF, Hopkins & Williams), sodium oleate (NaOl, Sigma), sodium pentobarbitone (Sagatal, May & Baker), staurosporine (Sigma), tetraethylammonium bromide (TEA, Sigma), tetrodotoxin (TTX, Sigma), theophylline (Sigma), trichloroacteic acid (TCA, B.D.H. Ltd.). xamoterol hemi-fumarate (Corwin, I.C.I.),

Concentrations in the bath refer to the salts, except TTX theophylline and staurosporine, which are expressed as concentration of the base.

With the following exceptions, drugs were dissolved in distilled water, prior to their dilution in saline (0.9%) to give the required final concentration. PD116948, PD115199 and 8-phenyltheophylline were dissolved in polyethylene glycol 400/ sodium hydroxide (0.1M, 1:1, v/v); NBTI was dissolved in polyethylene glycol 400/saline 0.9% (1:1 v/v); PMA was dissolved in 95% ethanol; PIA and staurosporine in 95% ethanol/distilled water (1:1 v/v). Dilutions were made in saline (0.9%) as before. In each case, controls used vehicle alone.

VI. ANALYSIS OF RESULTS

Results were expressed as mean ± standard error of the mean (s.e.m.), n = number of observations. Students t-test or one way analysis of variance were used to test for significance between means. A t - value of $p<0.05$ was taken as being significant.

Characteristic, individual examples from electrophysiological experiments, have been chosen to present most significant findings. Three or more tissues were used to investigate each drug or parmeter and each tissue provided several cells. The 'n' values given represent the total number of cells from which recordings were made.

In most electrophysiological studies undertaken in the present work, the experimental arrangement permits simultaneous electrical recording from one cell and mecahnical recording from the entire tissue, however in experiments using a Picospritzer, mechanical recordings cannnot be obtained, due to the very small number of cells stimulated by this method.

RESULTS

I. RESTING MEMBRANE CHARACTERISTICS

The resting membrane potential of the rabbit saphenous artery ranged from -59mV to -86mV, with a mean value of -69.39 ± 0.26mV (n=250). The resting membrane potentials formed a population with normal distribution, giving evidence of only one population of cells (Fig. 6)

Spontaneous excitatory junction potentials (s.e.j.p.s) 2-6mV amplitude (mean 3.5 ± 0.6mV, n=45) were observed in over 95% of cells examined. They occurred singly, infrequently (every 3 - 10 min) and at irregular intervals.

II. PASSIVE ELECTRICAL PROPERTIES

The passive membrane properties of the smooth muscle cells of the rabbit saphenous artery were investigated using the techniques of Abe & Tomita (1968). Using the apparatus previously described, rectangular pulses of constant inward (hyperpolarising) or outward (depolarising) current were applied (for 5sec) to the tissue. Electrotonic potentials produced in response to this stimulation, were recorded intracellularly from the outer layer of the artery, via glass microelectrodes in the recording chamber, held at several distances (0.5-3mm) from the stimulating plate electrodes. For both depolarising and hyperpolarising stimuli, the amplitude of the electrotonic potential was linearly proportional to the stimulus intensity and rectification was not apparent over the ranges studied (Fig. 7).

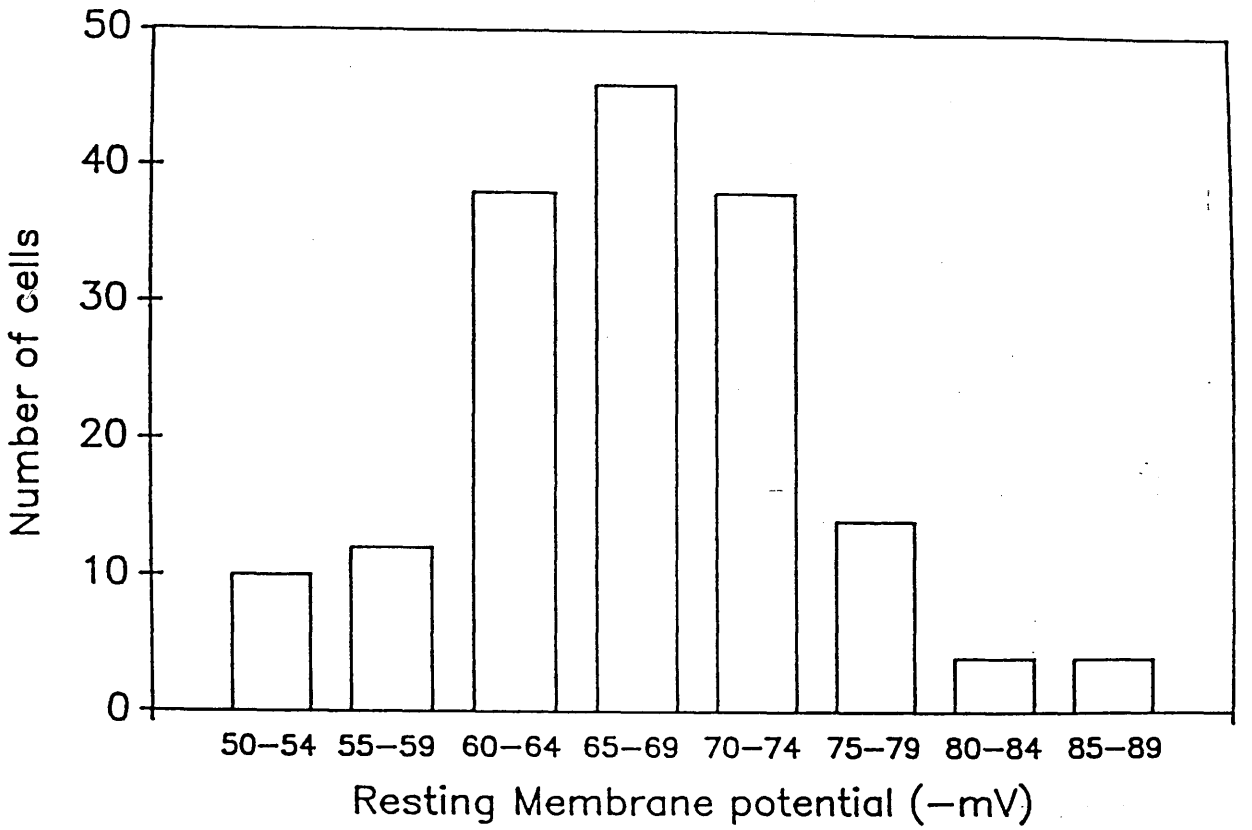


Fig. 6. Resting membrane potential plotted against the number of cells shows a normal distribution about the mean (69.3 ± 0.26mV), with no evidence of more than one cell type.

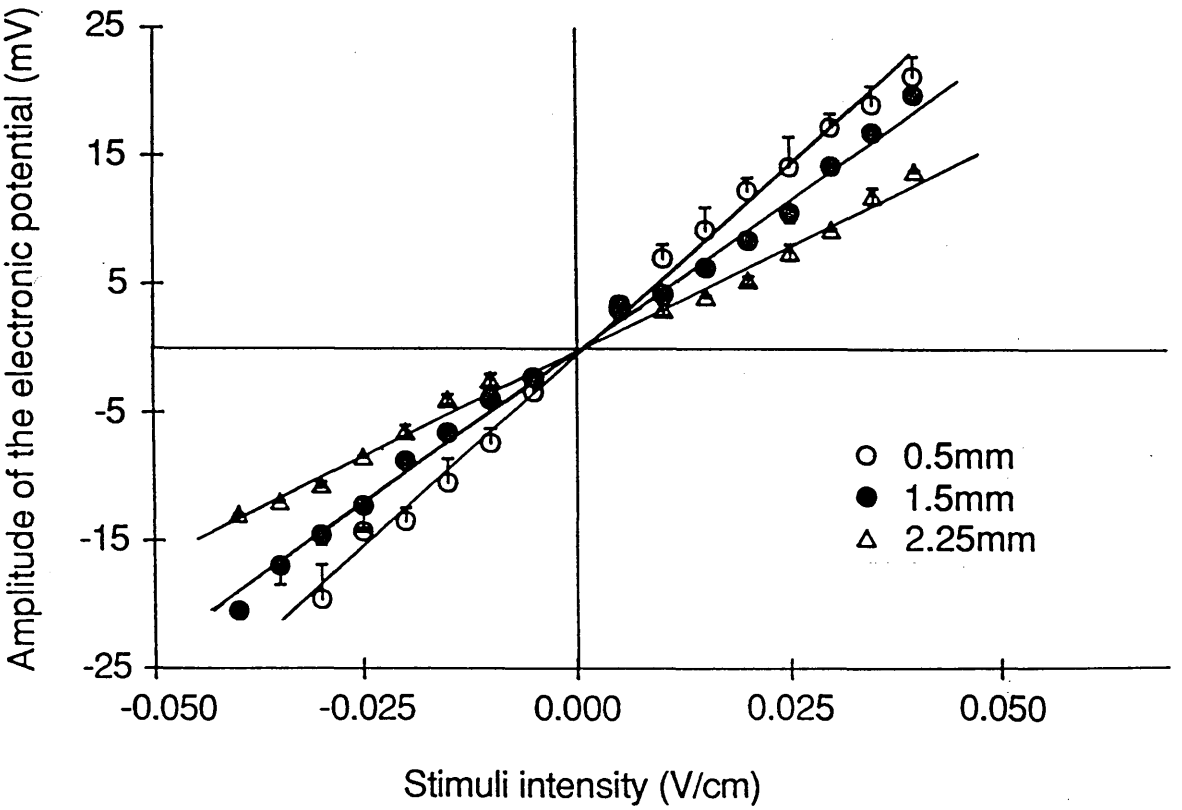


Fig. 7. Current (V/cm)/ voltage (mV) relationship in rabbit saphenous artery measured using the Abe & Tomita (1968) partition bath technique. Recordings of electrotonic potentials evoked by graded stimuli (square wave pulses, 5s duration), were taken intracellularly at 0.5, 1.5 and 2.25mm from the stimulating plate electrodes. No rectification was observed in response to inward (depolarising) or outward (hyperpolarising) currents over the range used.

Electrotonic potentials could be measured up to 4mm from the stimulating plate electrode. The amplitude of the transmembrane electrotonic potential to a uniform hyperpolarising or depolarising stimulus, decayed exponentially with increasing distances of recording site from the stimulating plate electrode. From the slope of semi-log plots of these two parameters (amplitude against distance), the mean space constant (λ = 0.42 ± 0.12mm, n=12) was calculated, Fig. 8).

III. RESPONSE TO FIELD STIMULATION

(A) EVOKED MEMBRANE ACTIVITY

Field stimulation (0.5msec, supramaximal voltage, single pulse or trains of pulses at 1-64Hz) of the intramural nerves of the rabbit saphenous artery evoked, in the absence of drugs, an electrical response which consisted solely of excitatory junction potentials (e.j.p.s). Single pulses evoked e.j.p.s ranging from 2-18mV in amplitude (mean 6.7 ± 0.3mV, n=115), with duration of around 500msec (mean 489 ± 17msec, n=35). Trains of pulses evoked e.j.p.s which facilitated (at or above 0.5Hz) and summated (above 2Hz) to reach a maximum of 20mV. These e.j.p.s often had an uncommonly sharp or 'peaky shape (see Holman & Surprenant, 1979) (Fig. 9). Prolonged stimulation (4Hz for 1min) failed to uncover any additional electrical event. There was no accompanying late slow membrane depolarisation, nor were action potentials observed in untreated tissues with any stimulation parameter.

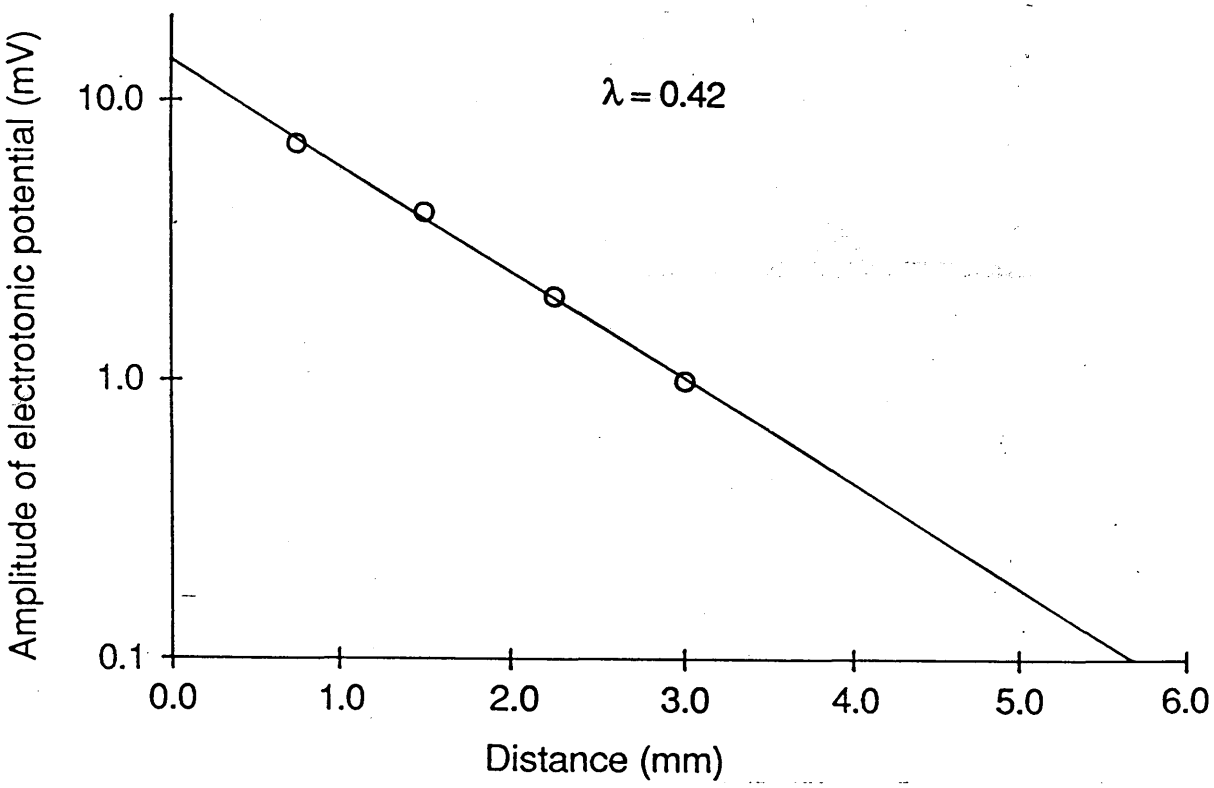


Fig. 8. Estimation of the length constant (λ) for the rabbit saphenous artery was obtained from the slope of a semi-log plot of the amplitude of electrotonic potential (ordinate mV) evoked by a uniform depolarising or hyperpolarising pulse, against distance from the stimulating electrode (λ = 0.42 ± 0.21, n=12)

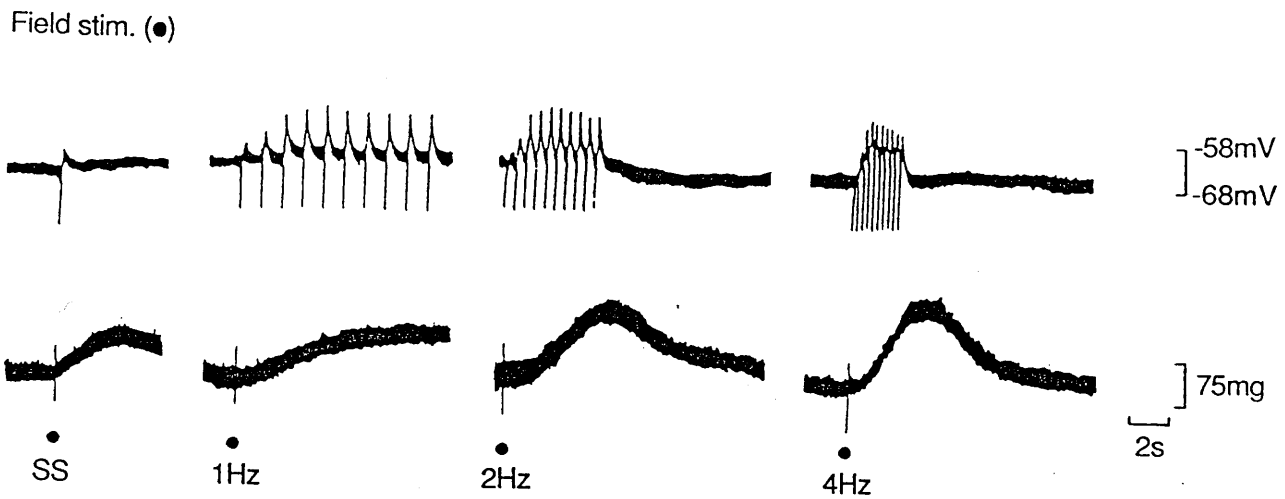


Fig. 9. Electrical (upper trace in each case) and simultaneously-recorded mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, single stimulus and trains of pulses at 1, 2 and 4Hz) of the rabbit saphenous artery. Single stimulus evoked e.j.p.s and, on occasion, contractions. Trains of pulses evoked discrete e.j.p.s which facilitated (≥1Hz), summated (>2Hz) and were accompanied by monophasic contractions. Intracellular microelectrode recordings were made from the same cell.

Infusion of TEA ($5x10^{-4}$-10^{-3}M, Fig. 10), a substance which blocks certain potassium channels (Imaizumi & Watenabe, 1981), produced no significant change in the resting membrane potential of the rabbit saphenous artery but increased the amplitude of evoked e.j.p.s to a mean value of 15.2 ± 0.8mV, (n=15, range 10-25mV). In the presence of this drug, action potentials (32-40mV) were seen superimposed on depolarisations of 12mV or more in response to single pulses or trains of stimuli.

The action potentials thus produced were abolished by the calcium channel blocking drug, diltiazem (10^{-5}M). Diltiazem failed however to alter the amplitude of the evoked e.j.p.s. in the presence of TEA.

(B) EVOKED MECHANICAL ACTIVITY

Trains of pulses (0.5msec, supramaximal voltage, 1-64Hz) and on occasion single stimuli, (0.5msec, supramaximal voltage), produced fast monophasic contractions (Fig. 9). In the presence of TEA, the evoked contraction was greatly increased (Fig. 10).

IV. EFFECTS OF ALTERATION OF EXTERNAL ENVIRONMENT

The underlying ionic basis for the electrical response to intramural nerve stimulation was examined by changing the external ionic environment.

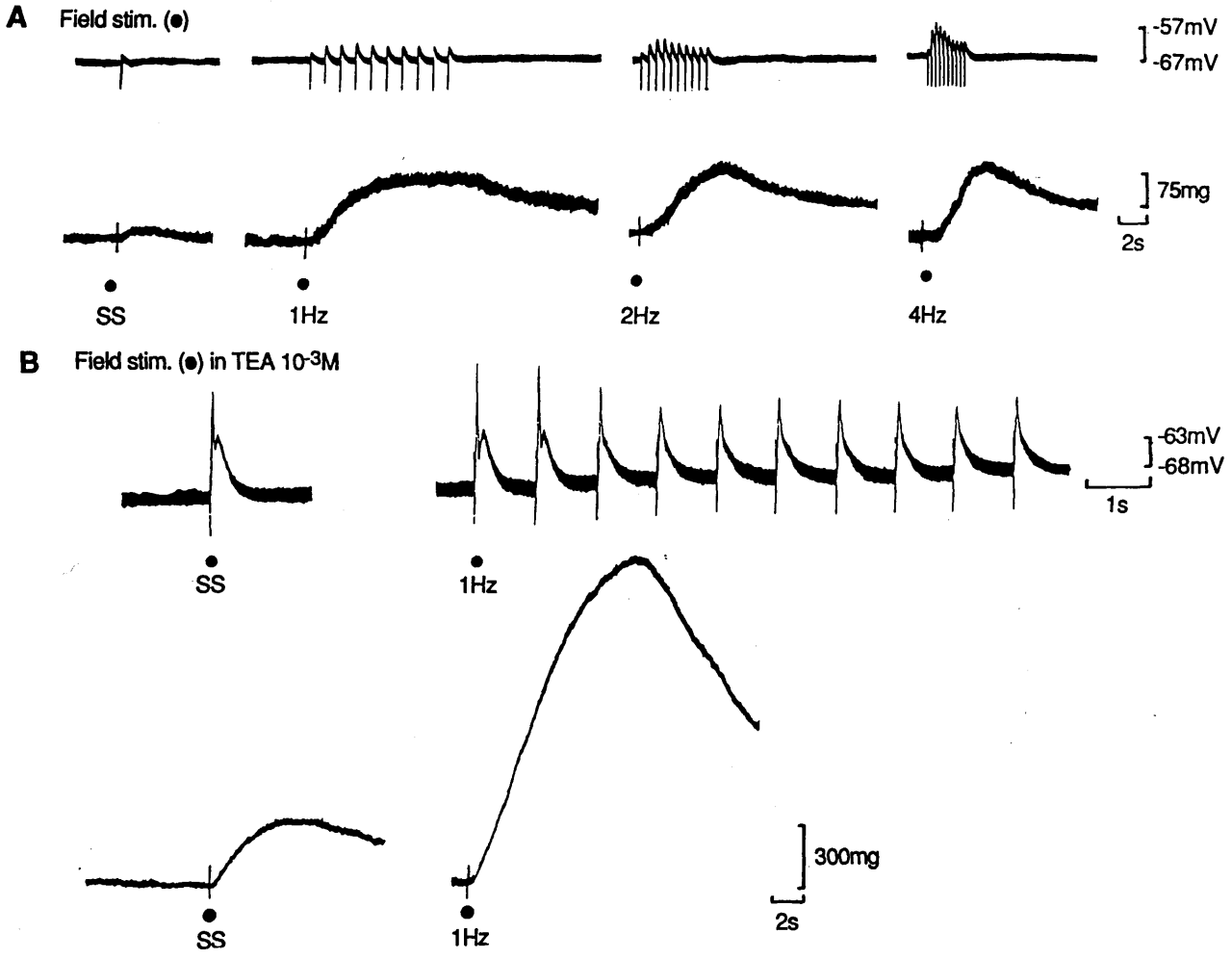


Fig. 10. Electrical (upper trace in each case) and simultaneously recorded mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, single stimulus and trains of pulses at 1, 2 and 4Hz) in the absence (A) and in the presence (B) of TEA (10^{-3}M). The amplitude of the evoked e.j.p.s and the mechanical response were enhanced in the presence of TEA. Action potentials were seen superimposed on depolarisations of 12mV or more in the presence of TEA. Electrical recordings were made from the same cell. Overall the mean increase in e.j.p. amplitude and contraction was 226±11.4% and 1154±12% respectively ($p<0.001$, $n=15$)

(A) POTASSIUM

Removal of external potassium ($[K^+]_o$, control 4.7mM) produced no significant change in membrane potential (n = 9). During an initial period of approximately 30 min, e.j.p.s were unaffected, thereafter e.j.p.s were progressively suppressed to approximately 5% of control. In a small number of cells, a greatly increased level of frequency and amplitude of spontaneous e.j.p.s was observed in the minutes following introduction of K^+ free Krebs solution, which subsided after 20-30 min.

The mechanical response to field stimulation (0.5msec, supramaximal voltage, 1-4Hz) was initially enhanced in K^+ free Krebs solution but as the e.j.p.s started to decline after 30min, a large mechanical oscillation was observed which made measurement of the contraction to field stimulation difficult (Fig. 11).

These results suggest that K^+ may be involved in production of the evoked e.j.p..

An increase in $[K^+]_o$ from 4.7mM (control) to 9.4mM, significantly depolarised the membrane ($p<0.001$) from -68.2 ± 0.73mV (n=59) to -59.4 ± 0.55mV (n=62). The amplitude of the evoked e.j.p.s was significantly reduced ($p<0.005$), while the mechanical response to field stimulation (0.5msec, supramaximal voltage, 1-4Hz) was potentiated, possibly as a result of the depolarisation (Fig. 12).

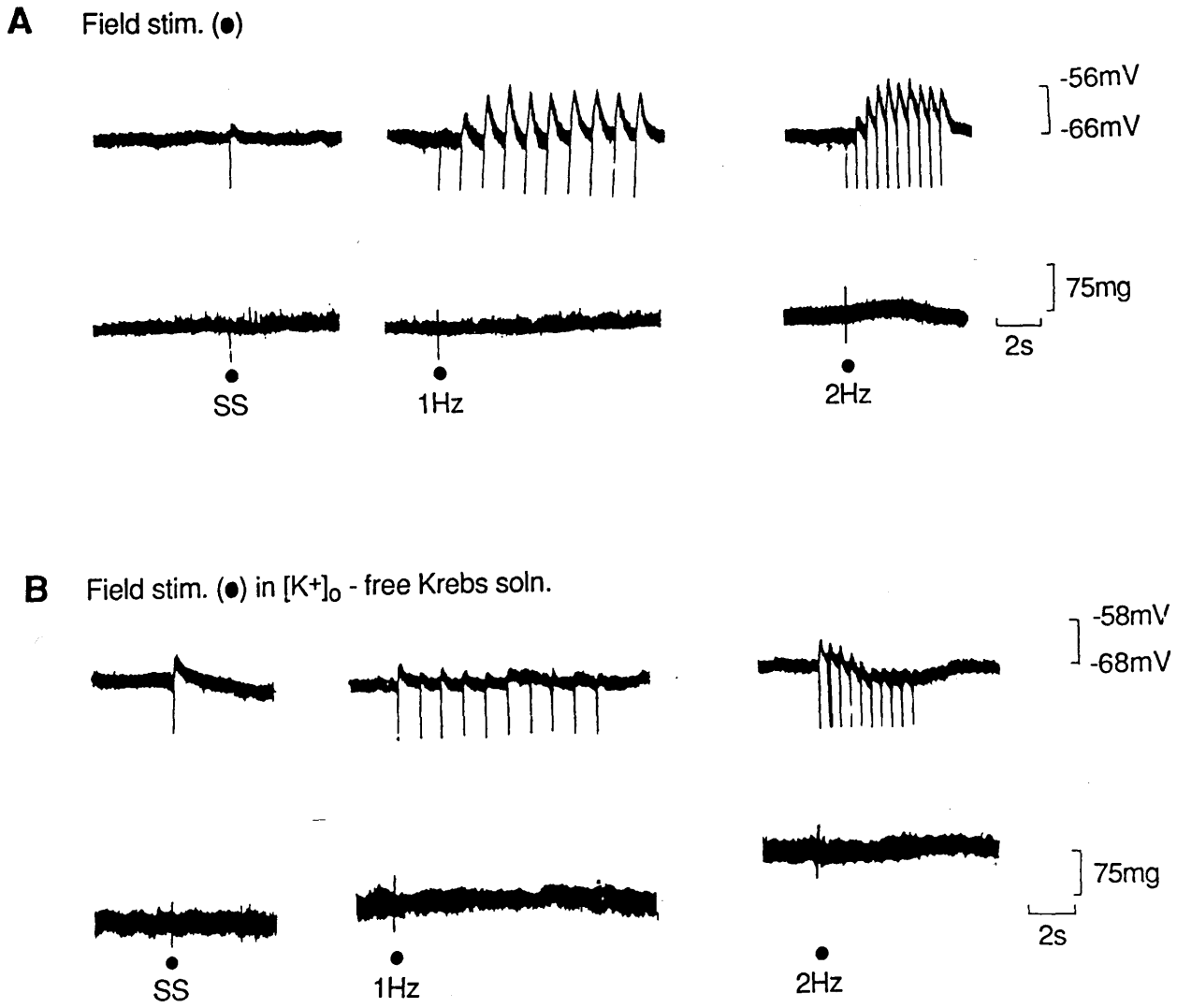


Fig. 11 The effect on the intracellularly-recorded electrical (upper trace in each case) and simultaneously recorded mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, single stimuli and trains of pulses at 1 and 2Hz), of removal of $[K^+]_o$ (control = 4.7 x 10^{-3}M). After approximately 30 mins, the evoked e.j.p.s were greatly attenuated and a large mechanical oscillation in tone of up to 55mg was observed. Intracellular microelectrode recordings were made from the same cell. Overall the mean decrease in e.j.p. amplitude was 95±3.2% ($p<0.001$, $n=15$)

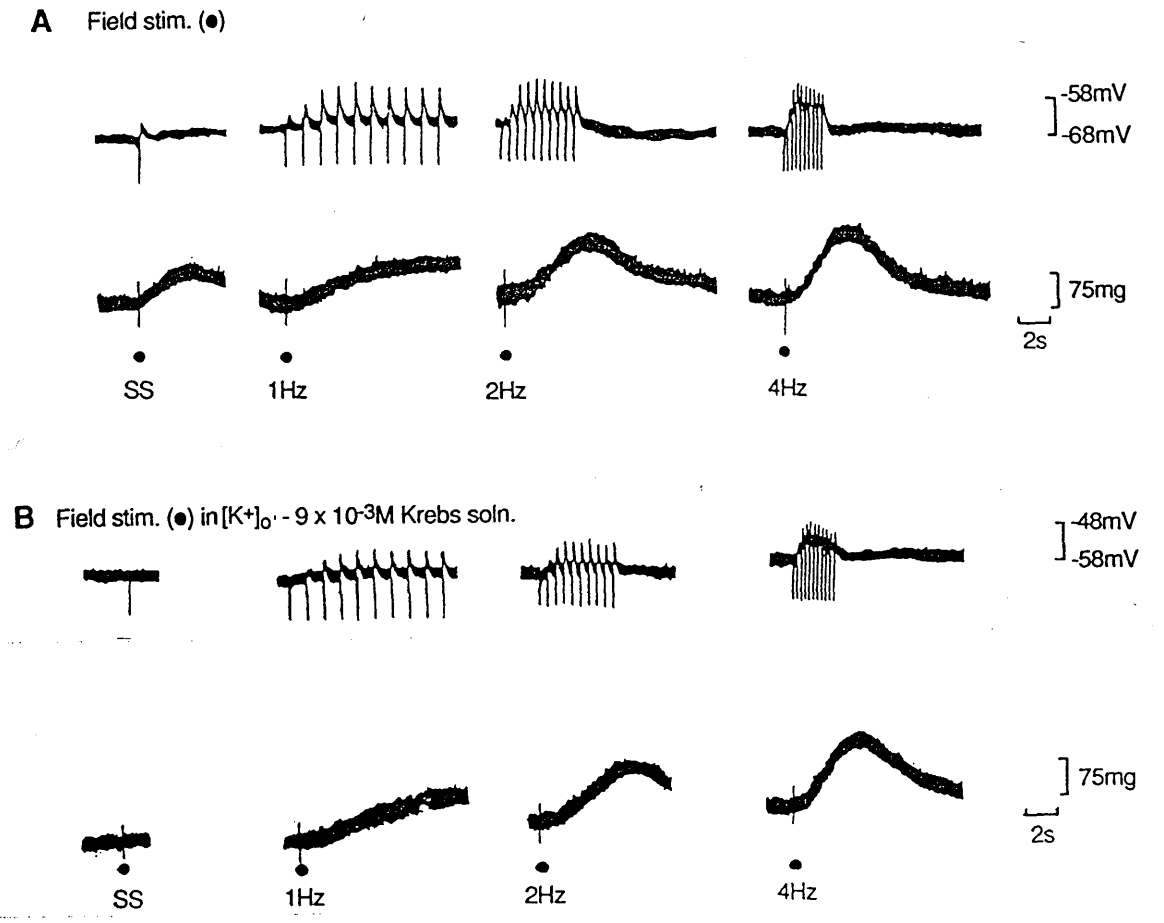


Fig. 12. The effect of doubling $[K^+]_O$ from (A) control (4.7×10^{-3}M), to (B) 9.4×10^{-3}M on the electrical (upper panel in each case) and mechanical responses to field stimulation (•, 0.5msec, supramaximal voltage, single stimulus and trains of pulses at 1, 2 and 4Hz). Doubling $[K^+]_O$ depolarised the membrane, reduced the amplitude of the e.j.p.s and potentiated the contractions. Intracellular microelectrode recordings were made from the same cell. Overall, the mean reduction in e.j.p amplitude and contraction was, respectively 32±8.4% ($p<0.01$) and 24±5.1% ($p<0.05$), n=14.

(B) SODIUM

A reduction in external sodium ($[Na^+]_o$) from 144.5mM (control) to 108.3mM, depolarised the membrane from -69.3 ± 0.68mV (n=40) to -58.6 ± 1.13mV (n=18). Subsequent evoked e.j.p.s (0.5msec, supramaximal voltage, 1-4Hz) were greatly attenuated ($p<0.005$). A large transient contraction was observed immediately on introduction of the low sodium Krebs, which subsided after 5-8min. Thereafter mechanical responses to field stimulation were enhanced.

A further reduction in $[Na^+]_o$ to 72.2mM depolarised the membrane further, to -54.2 ± 1.73mV (n=9) and abolished evoked e.j.p.s. Again a large transient contraction was observed, after which the contraction to field stimulation (0.5msec, supramaximal voltage, 1-4Hz) was abolished (Fig. 13).

These results suggest that sodium is also involved in the evoked e.j.p.

(C) CHLORIDE

Removal of extracellular chloride ($[Cl^-]_o$), significantly depolarised ($p<0.05$) the membrane to -58.7 ± 1.73mV (n=15) and substantially reduced (>90%) or abolished, the evoked e.j.p.s. Contractions to field stimulation (0.5msec, supramaximal voltage, 1-4Hz) were reduced by some 50% (Fig. 14).

(D) CALCIUM

Removal of extracellular calcium ($[Ca^{2+}]_o$) did not alter the resting membrane potential but progressively reduced both the electrical and mechanical responses to field stimulation (0.5msec, supramaximal

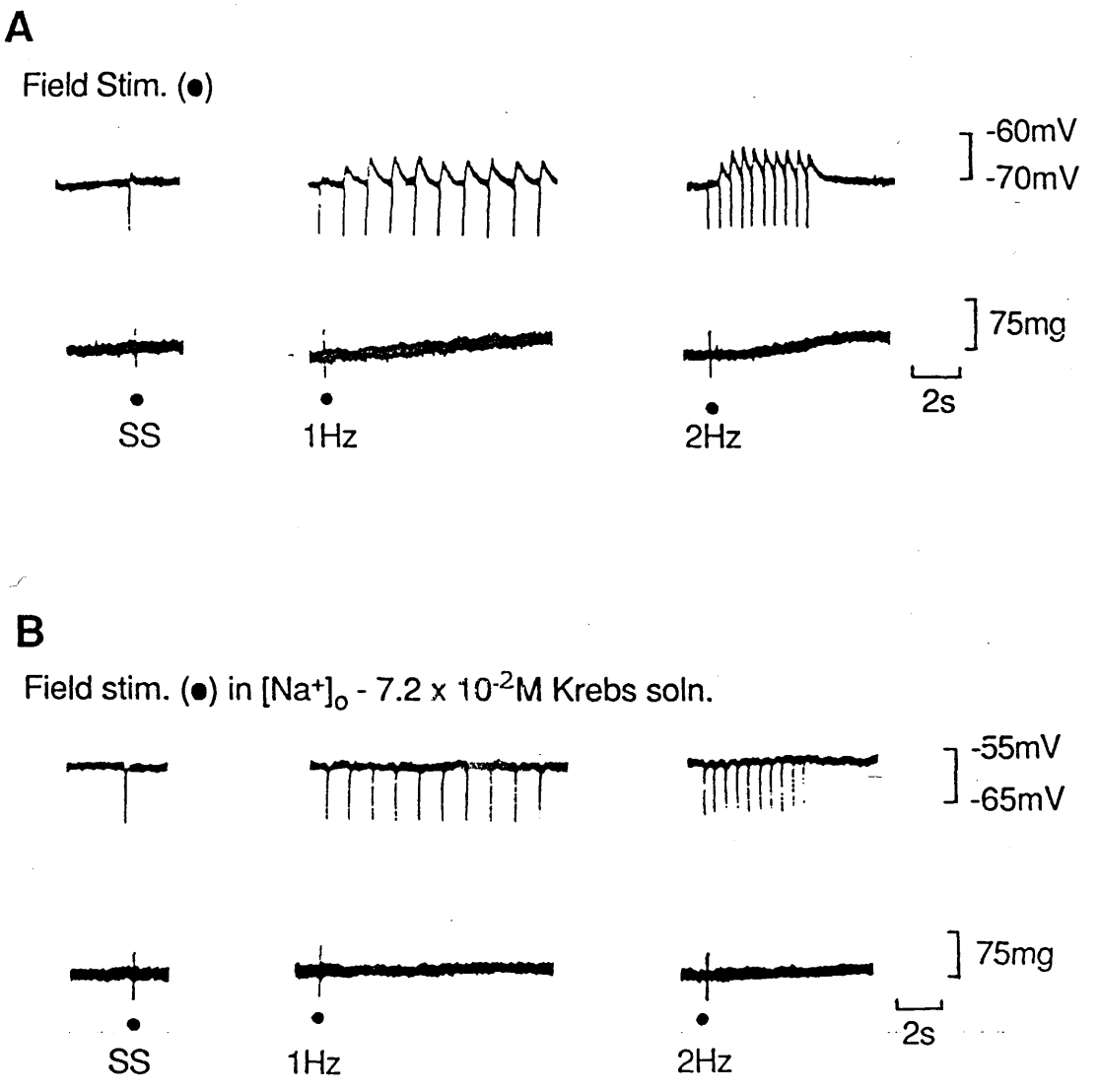


Fig. 13. The effect of reducing $[Na^+]_o$ from (A) control (14.4×10^{-4}M) to (B) 7.2×10^{-4}M on the electrical (upper trace in each case) and mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, single stimulus and trains of pulses at 1 and 2Hz). Reduction of $[Na^+]_o$ to 50% of control, depolarised the membrane and abolished both the evoked e.j.p.s and mechanical response. Intracellular microelectrode recordings were made from the same cell. Total number of cells from which recordings were made (n)= 15.

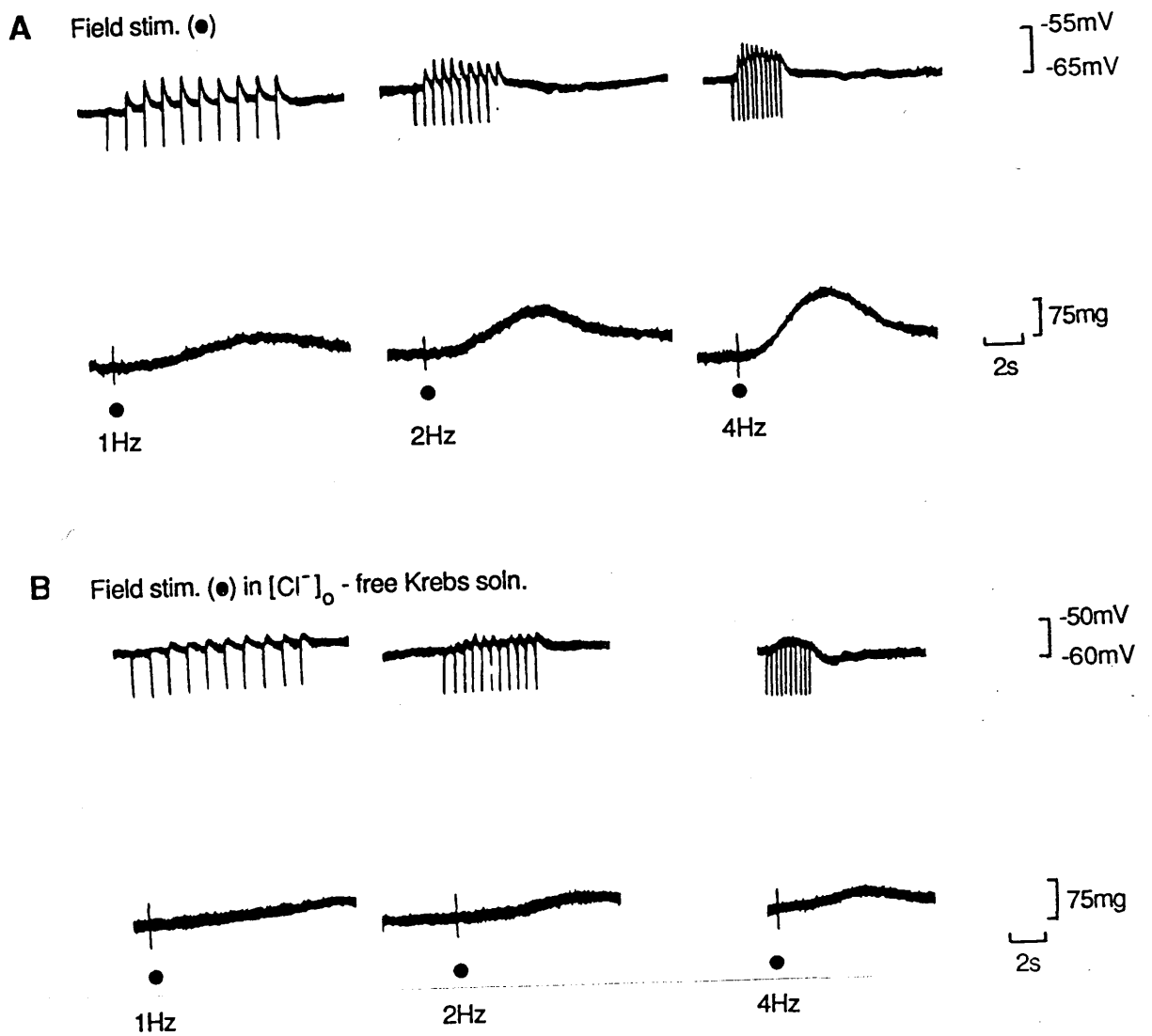


Fig. 14. The effect of removal of $[Cl^-]_o$ on the electrical (upper trace in each case) and mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, trains of pulses at 1, 2 and 4Hz) in the rabbit saphenous artery. The membrane was depolarised and the evoked e.j.p.s attenuated or abolished. Contractions were approximately halved. Intracellular microelectrode recordings were taken from the same cell. Overall, the mean reduction in e.j.p. amplitude and contraction was 98.4±0.6% and 54.7±11.1% respectively ($p<0.001$, $n=14$).

voltage, 1-16Hz), until after 10-15min they were abolished (Fig. 15), presumably resulting from an inhibition of transmitter release.

V. THE EFFECT OF DRUGS ON THE EXCITATORY RESPONSE TO FIELD STIMULATION

The contractile effect of putative transmitter substances released following nerve stimulation in the rabbit saphenous artery have been identified as NA and ATP (Burnstock & Warland, 1987). Their electrical and simultaneously recorded mechanical effects were investigated using agents which antagonise the actions of these two substances.

(A) PRAZOSIN and αβ MeATP.

Perfusion with the α_1-adrenoceptor antagonist prazosin (10^{-6}M) had no effect on either the resting membrane potential, or the evoked e.j.p.s but reduced the mechanical response to field stimulation (0.5msec, supramaximal voltage, 1-16Hz), by 30-50%. Perfusion of the P_2-purinoceptor desensitising agent αβ MeATP (10^{-6}M), evoked an initial depolarisation from -69.3 ± 0.26mV (n=257) to -53.4 ± 1.02mV (n=28). The membrane potential however returned to control values within 3-5min and recordings in all cases were taken after the initial depolarisation had subsided, at which time the tissue was insensitive to further bolus addition of αβ MeATP (10^{-5}M) and the P_2-purinoceptors were considered desensitised. Subsequent references to desensitisation with αβ MeATP refer to this process. αβ MeATP abolished the evoked e.j.p.s as well as reducing by 50-70%, the mechanical response to

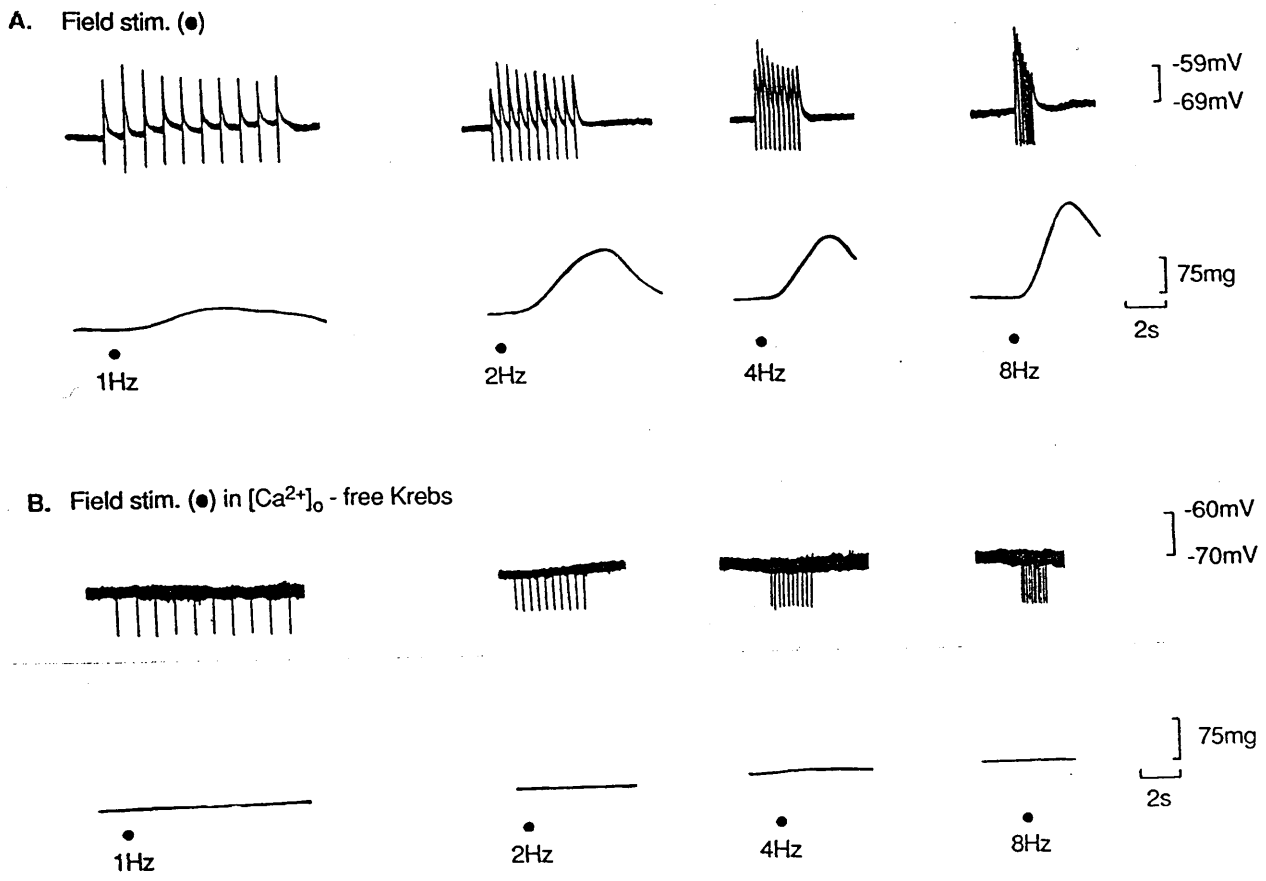


Fig. 15. The effects of removal of $[Ca^{2+}]_o$ on the electrical (upper trace in each case) and mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, trains of pulses at 1, 2, 4 and 8Hz). Removal of $[Ca^{2+}]_o$ abolished all electrical and mechanical activity. Intracellular microelectrode recordings were made from the same cell. Total number of cells from which recordings were made (n) = 15.

field stimulation (0.5msec, supramaximal voltage, 1-16Hz).

A combination of prazosin and αβ MeATP (each 10^{-6}M) abolished both the electrical (e.j.p.s) and the mechanical response (Fig. 16).

This suggests that in the rabbit saphenous artery, NA and ATP (or a closely related substance) are released following nerve stimulation, and mediate effects post-junctionally. The non-adrenergic component alone mediates the electrical response via P_2-purinoceptors, while both NA acting via α_1-adrenoceptors and ATP (or a closely related compound) mediate the contraction.

The lack of electrical response to NA, as evidenced by the results from experiments with prazosin, was at odds with results found in other vascular tissues, such as the rabbit ear artery (Suzuki & Kou, 1983, Allcorn et al., 1985), where the same substances have been proposed as co-transmitters. In that tissue, the e.j.p.s which were non-adrenergic were followed by a slow depolarisation attributable to NA. Attempts were therefore made to maximise any NA-mediated responses in the rabbit saphenous artery.

(B) NORMETANEPHRINE (NMN) and DESMETHYLIMIPRAMINE (DMI)

To maximise any nerve released NA in the synaptic cleft, NMN (10^{-6}M) and DMI (10^{-6}M) which respectively block NA uptake into non-neuronal and neuronal sites were added to the perfusate. In the presence of a combination of these drugs, the mechanical response to field stimulation (0.5msec, supramaximal voltage, 1-16Hz) was greatly enhanced, however e.j.p.s were unaffected and no additional electrical

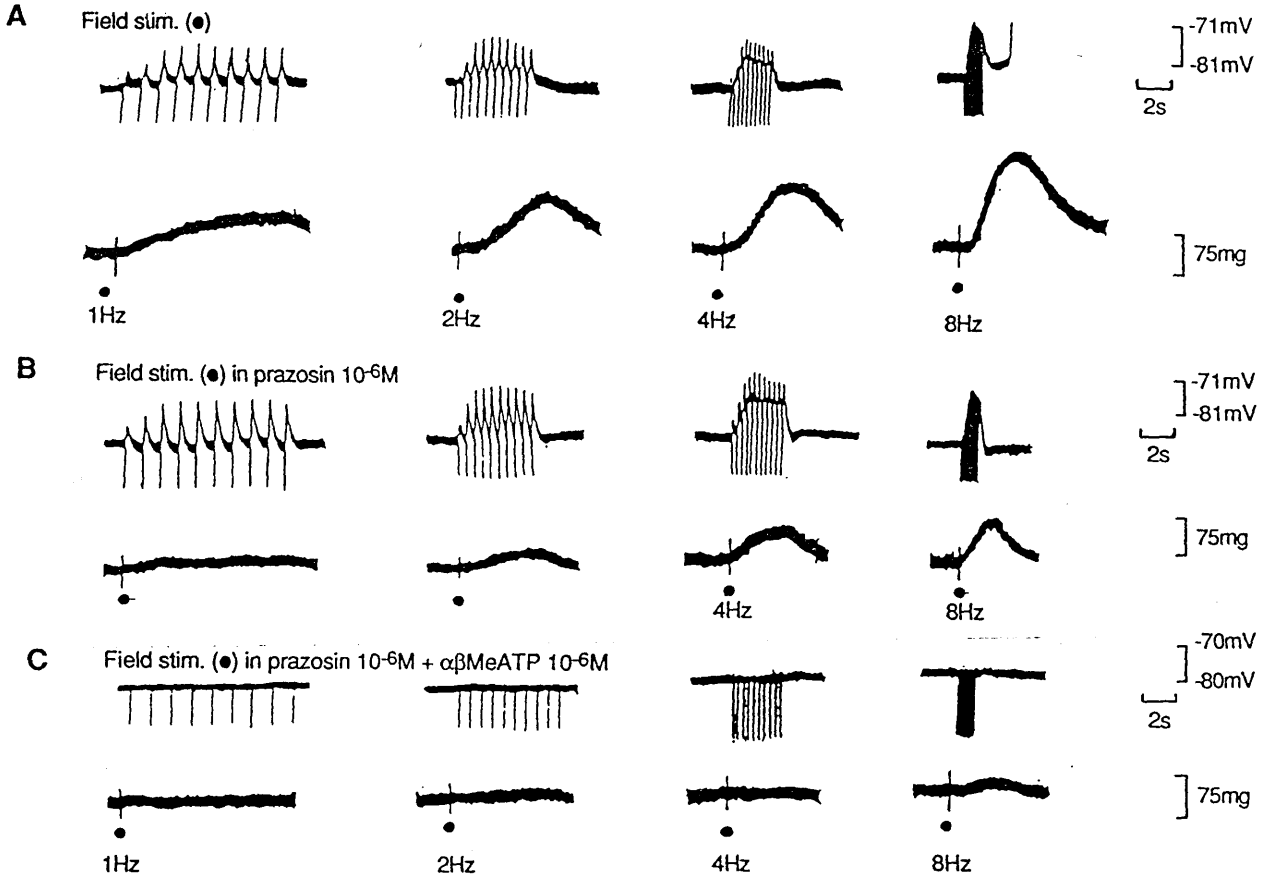


Fig. 16. The effect in the rabbit saphenous artery on the electrical (upper trace in each case) and mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, trains of pulses at 1, 2, 4 and 8Hz), of prazosin (10^{-6}M, B) alone and in combination with αβ MeATP (10^{-6}M, C), compared to control (A). Prazosin had no significant effect on the e.j.p.s but reduced the contraction overall by 57±8.2% (n=15). Prazosin and αβ MeATP together abolished both the electrical and mechanical responses. Intracellular microelectrode recordings shown were taken from two different cells. Total number of cells from which recordings were made (n) = 30.

effects were observed (Fig. 17).

In addition, long periods of stimulation also failed to demonstrate any electrical NA response. The role of other receptors in modulating the response to the putative transmitters was next examined.

(C) PROPRANOLOL

The presence of (±) propranolol (10^{-6}-10^{-5}M) to antagonise β-adrenoceptors, did not alter the resting membrane potential, the e.j.p.s or the contraction to field stimulation (0.5msec, supramaximal voltage, 1-16Hz), indicating an absence of β-adrenoceptor control of the neuronal response.

(D) ATROPINE

Atropine (10^{-6}M) likewise had no effect on the resting membrane potential, or on mechanical or electrical responses to field stimulation (0.5msec, supramaximal voltage, 0.2-16Hz) indicating that cholinergic receptors do not modify the neuronal response in this tissue.

VI. The origin of the putative transmitter substances ATP and NA

(A) TETRODOTOXIN (TTX)

TTX selectively prevents the increase in sodium permeability

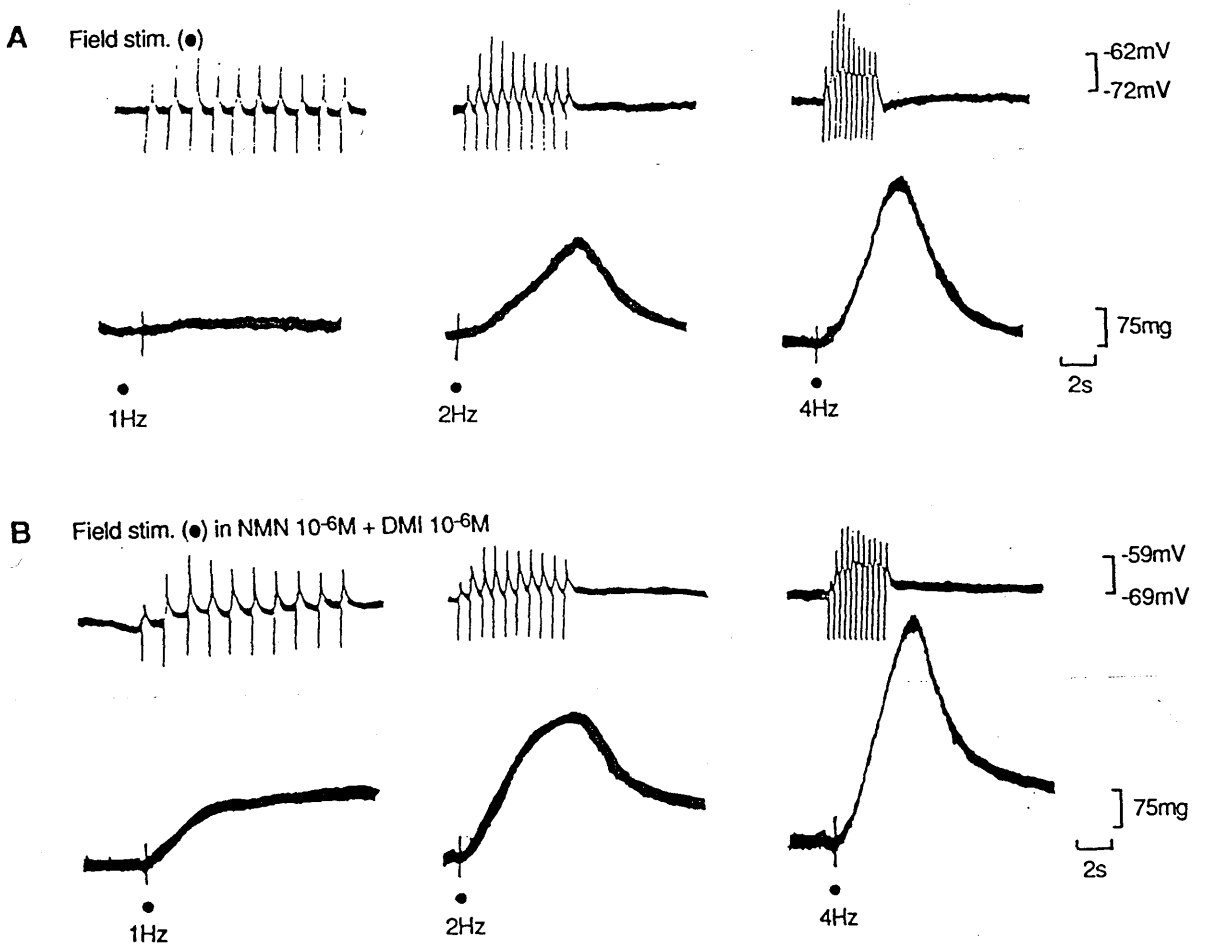


Fig. 17. Electrical (upper trace in each case) and mechanical responses to field stimulation (•, 0.5msec, supramaximal voltage, trains of stimuli at 1, 2 and 4Hz) in the absence (A) and the presence (B) of a combination of the NA uptake blockers normetanephrine (NMN, 10^{-6}M) and desmethylimipramine (DMI, 10^{-6}M). E.j.p.s were not significantly affected by the presence of NMN and DMI, while the mechanical response was greatly enhanced (overall increase 72.6±9.4%, $p<0.001$, n=15). NMN and DMI together failed to uncover any secondary electrical event associated with NA. Intracellular microelectrode recordings were made from the same cell.

responsible for the rising phase of the action potential and hence blocks nerve conduction and subsequent pre-junctional release of excitatory substances. TTX (10^{-6}M) abolished both the electrical and mechanical responses to field stimulation (0.5msec, supramaximal voltage, 1-32Hz, Fig. 18), in the rabbit saphenous artery, confirming the neuronal origin of the putative transmitter substances.

(B) GUANETHIDINE

The adrenergic neurone blocking agent guanethidine (10^{-6}M) also abolished both the electrical and mechanical responses to field stimulation (0.5msec, supramaximal voltage, 1-32Hz, Fig. 19), suggesting that both ATP and NA were released from the same nerve in the rabbit saphenous artery.

(C) IDAZOXAN

The α_2-adrenoceptor antagonist idazoxan (10^{-6}M) produced significant increases in both the mean amplitude of the evoked e.j.p.s ($p<0.05$) and the mean mechanical response ($p<0.001$) to field stimulation (0.5msec, supramaximal voltage, 1-8Hz, Fig. 20). 50% of the increased mechanical response was blocked by addition of prazosin (10^{-6}M), while a combination of prazosin (10^{-6}M) and $\alpha\beta$ MeATP (10^{-6}M) abolished both the enhanced e.j.p.s and mechanical response. This implies that α_2-adrenoceptors on the pre-junctional nerve terminal modulate both NA and ATP release, suggesting once again, that NA and ATP are co-released from the same nerve.

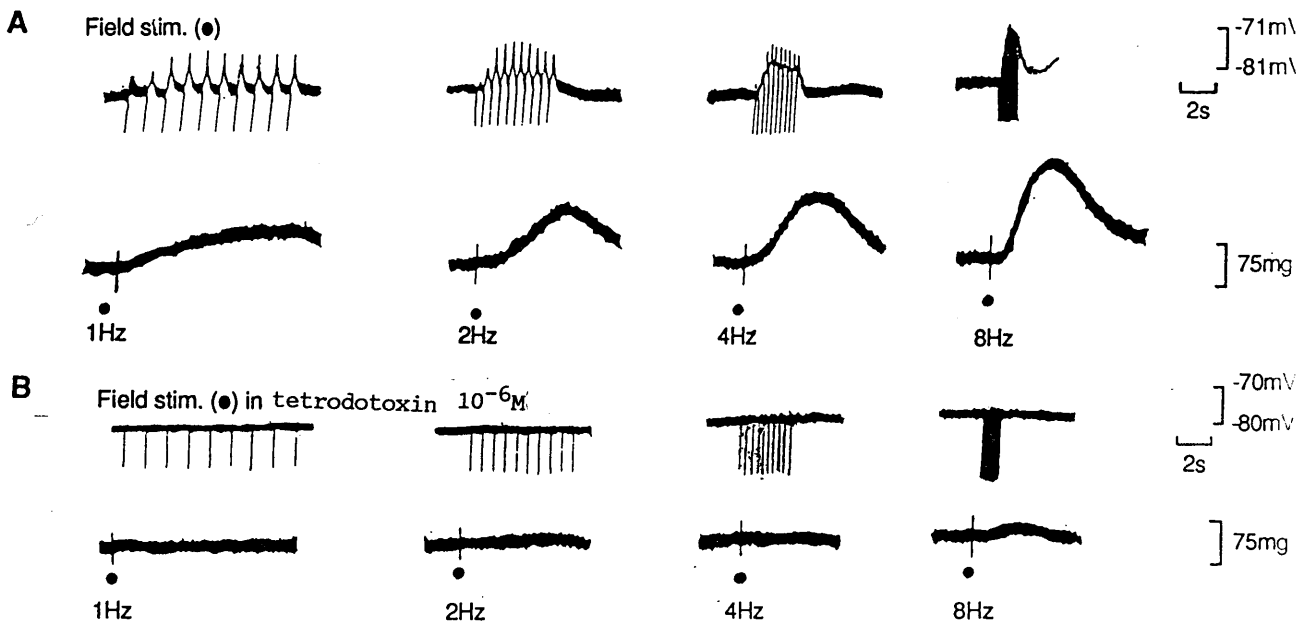


Fig. 18. The effect in the rabbit saphenous artery of tetrodotoxin (TTX, 10^{-6}M, B) on the electrical (upper trace) and mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, 1-8Hz). All electrical responses were abolished, suggesting a neuronal origin for the transmitter substances. Intracellular microelectrode recordings were made from the same cell. Total number of cells from which recordings were made (n) = 15.

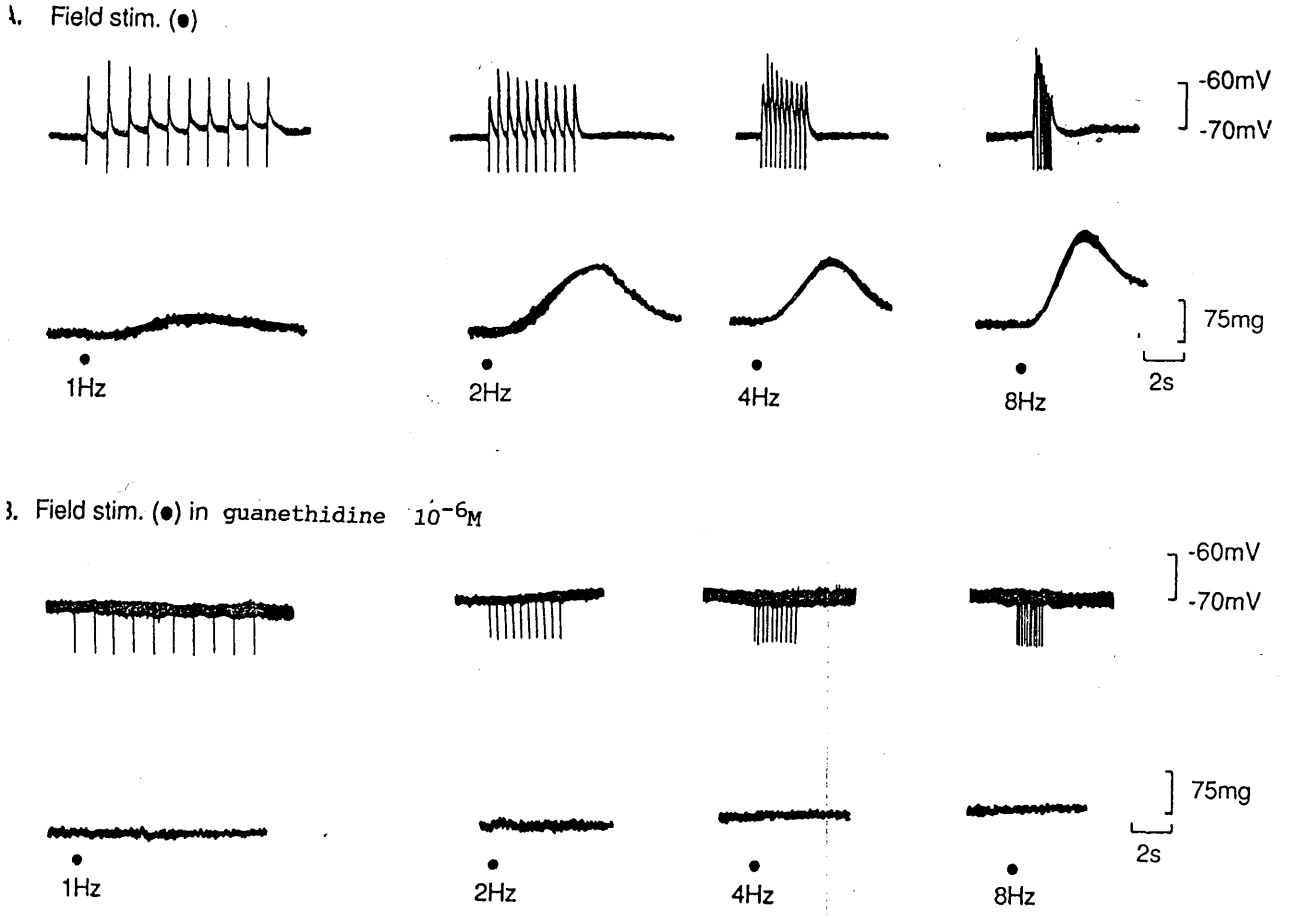


Fig. 19. The effect in the rabbit saphenous artery of the adrenergic neurone blocking agent guanethidine (10^{-6}M, B) on the electrical (upper trace in each case) and mechanical responses to field stimulation (●, 0.5msec, supramaximal voltage, 1-8Hz). Guanethidine abolished both the electrical and mechanical responses, suggesting that both the putative transmitters (NA and ATP) were released from adrenergic nerves. Intracellular microelectrode recordings were made from the same cell. Total number of cells from which recordings were made (n) = 15.

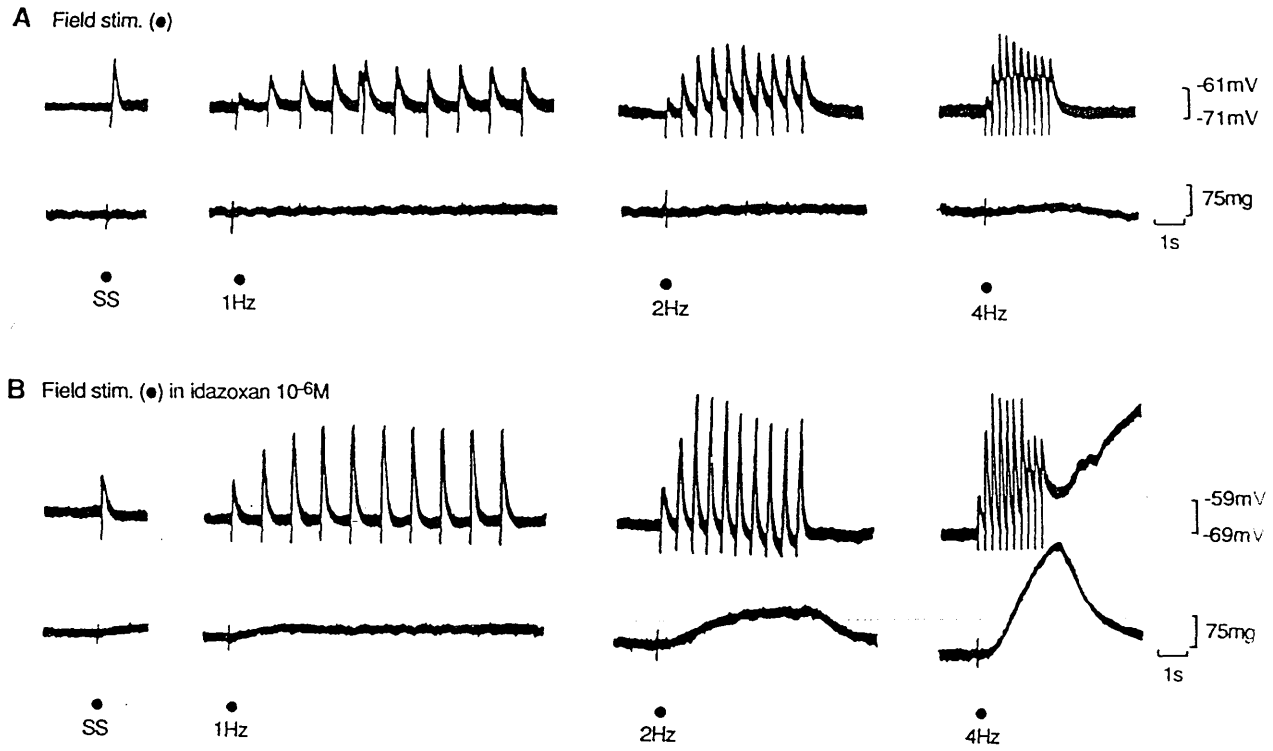


Fig. 20. Electrical (upper trace in each case) and mechanical responses of the rabbit saphenous artery to field stimulation (●, 0.5msec, supramaximal voltage, trains of stimuli at 1, 2 and 4Hz) in the absence (A) and the presence (B) of the α_2-adrenoceptor antagonist idazoxan (10^{-6}M). Idazoxan increased both the mechanical and electrical responses, suggesting that α_2-adrenoceptors control the pre-junctional release of both putative transmitters. Intracellular microelectrode recordings were made from the same cell. Overall, the mean increase in e.j.p. amplitude and contraction was 86.2±5% and 500±18% respectively ($p<0.001$, n=9)

VIII. THE EFFECT OF EXOGENOUS ADDITION OF THE PUTATIVE TRANSMITTER SUBSTANCES

If NA and ATP are indeed co-transmitters in the rabbit saphenous artery (see Burnstock & Warland, 1987), then their exogenous addition to the artery should mimic the response obtained to field stimulation of intramural nerves. Thus if e.j.p.s are mediated by ATP, then exogenous application of the nucleotide should produce a comparable depolarisation. This should be absent when NA is added exogenously.

Application of ATP (10^{-2}M) by Picospritzer (40 p.s.i., 5-640msec, tip diameter $1x10^{-6}$m) produced a dose-dependent depolarisation of up to 15mV, which resembled in shape and size, that produced in response to nerve stimulation. This depolarisation was blocked by a desensitising infusion of αβ MeATP (10^{-6}M), as were the evoked e.j.p.s (Fig. 21).

Responses to αβ MeATP (10^{-3}M) applied by Picospritzer (40 p.s.i., 5-160msec, tip diameter $1x10^{-6}$), do not produce desensitisation provided the time between individual applications of the drug exceeds 10min. Applied in this manner, αβ MeATP evokes dose-dependent depolarisations, similar to those produced by ATP (Fig. 22). This suggests that the effects observed with ATP are due to the nucleotide itself rather than one of its breakdown products. αβ MeATP was some 200 times more potent than ATP in depolarising the cell membrane.

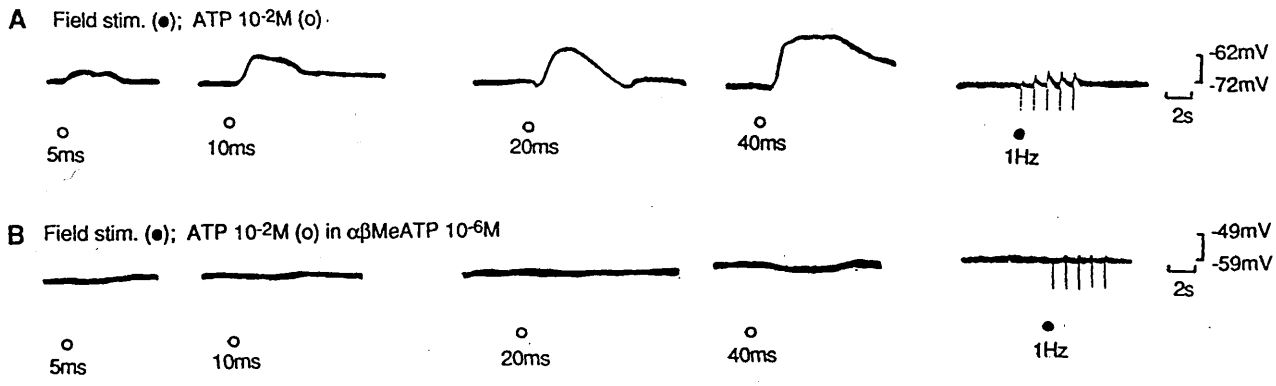


Fig. 21. Intracellularly-recorded electrical response to field stimulation (●, 0.5msec, supramaximal voltage, trains of stimuli at 1Hz) and to ATP applied by close pressure ejection (o, 10^{-2}M, 5-40ms) in the absence (A) and the presence (B) of a continuous desensitising infusion of αβ MeATP (10^{-6}M). Both the e.j.p.s evoked by field stimulation and the depolarisation evoked by ATP were abolished by infusion of αβ MeATP. Intracellular microelectrode recordings were made from the same cell. Total number of cells from which recordings were made (n) =12.

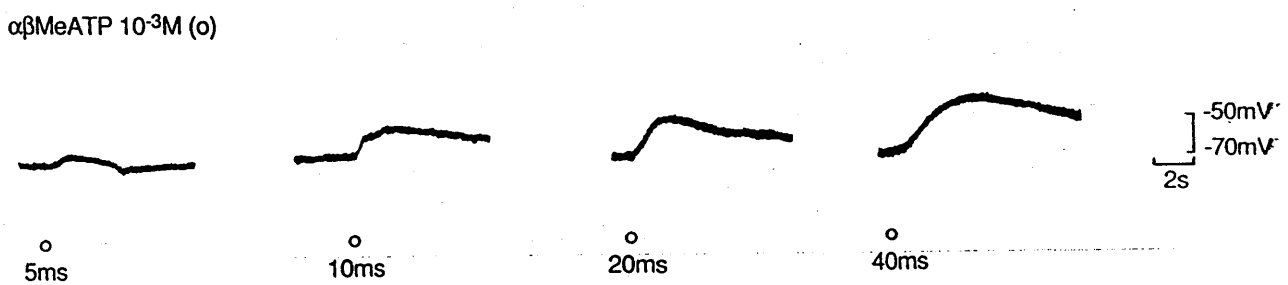


Fig. 22. Intracellularly-recorded electrical responses to doses of αβ MeATP, applied by pressure ejection (o, 10^{-3}M, 5-40ms). αβ MeATP produced a dose dependent depolarisation. Intracellular microelectrode recordings were taken from the same cell. Total number of cells from which recordings were made (n) = 9.

Addition of NA (10^{-2}M)by picospritzer (40 p.s.i., 5-640msec, tip diameter $1x10^{-6}$m) failed to alter the membrane potential, even in the presence of the NA uptake blocking agents NMN (10^{-6}M) and DMI (10^{-6}M) in combination (Fig. 23), confirming the absence of any noradrenergically mediated electrical event.

VIII. SUITABILITY OF ANTAGONISTS

(A) αβ MeATP

Where purinergic substances have been proposed as co-transmitters, αβ MeATP has been widely used to desensitise P_{2x}-purinoceptors and so antagonise contractile responses mediated by ATP. Recent doubts raised however regarding its selectivity for these receptors led to a re-examination of the actions of αβ MeATP.

This was done in three series of experiments.

(1) The effect of αβ MeATP on the contractile responses to a variety of agonists.

To establish the selectivity of αβ MeATP at P_2-purinoceptors, the effect of αβ MeATP on a number of purinergic and non-purinergic agonists was examined.

Contractile responses to ATP (10^{-5}-10^{-3}M) and αβ MeATP

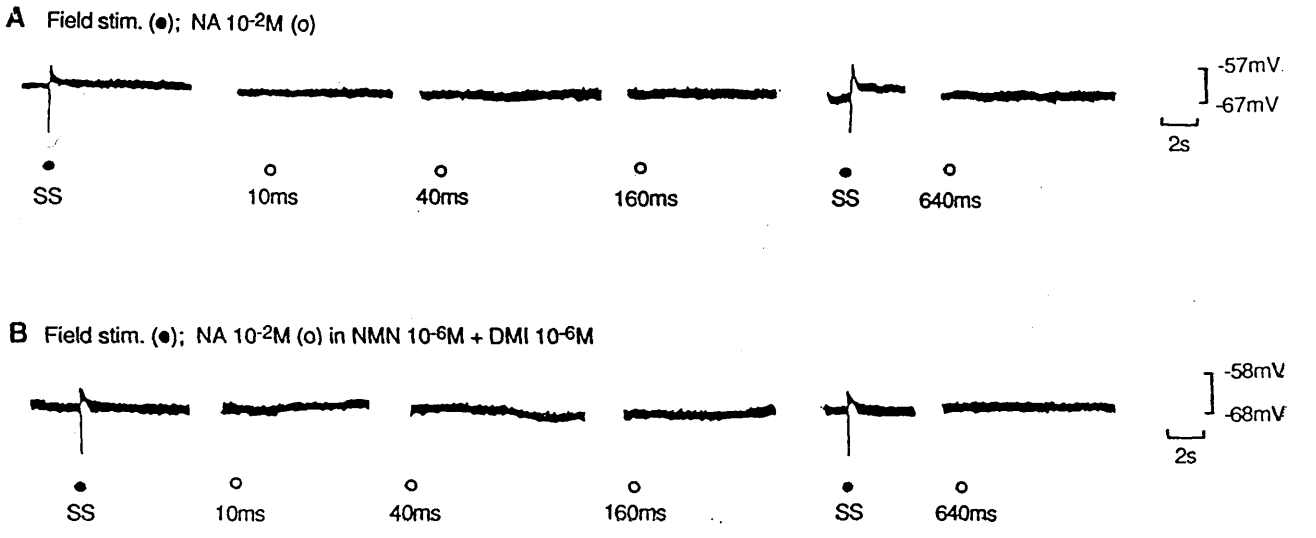


Fig. 23. Intracellularly-recorded electrical response to field stimulation (•, 0.5msec, supramaximal voltage, single stimuli) and to NA applied by close pressure ejection (o, 10^{-2}M, 10-640ms) in the absence (A) and the presence (B) of normetanephrine (NMN, 10^{-6}M) and desmethylimipramine (DMI, 10^{-6}M). NA produced no change in the membrane potential even in the presence of the NA uptake blocking agents NMN and DMI. The presence of evoked e.j.p.s was used to confirm cell penetration. Intracellular microelectrode recordings were taken from the same cell. Total number of cells from which recordings were made (n) = 9.

(10^{-6}-10^{-4}M) were each inhibited by a desensitising infusion of αβ MeATP (10^{-6}M, Fig. 24A). Contractile responses to NA (10^{-7}-10^{-5}M, Fig. 24B), histamine (10^{-6}-10^{-4}M, Fig. 24C) and 5-HT (10^{-6}-10^{-4}M) were each however unaffected by infusion of αβ MeATP (10^{-6}M).

Contractile responses evoked by the depolarising agent KCl (1-16x 10^{-2}M) presumably resulting from activation of voltage dependent calcium channels, were attenuated by constant desensitising infusion of αβMeATP (10^{-6}M). KCl however also evokes pre-junctional release of excitatory substances, in addition to its post-junctional actions. To prevent this pre-junctional release of transmitters by KCl, adrenergic nerves were destroyed by pre-treatment in vitro of the tissues with 6-OHDA ($5x10^{-4}$M, 2h) until no contraction was evoked by field stimulation (0.5msec, supramaximal voltage, 8Hz). Subsequently, contractions evoked by KCl (1-16x10^{-2}M) and presumably due to the post-junctional effects of this compound, were unaffected by αβ MeATP (10^{-6}M, Fig. 24D).

(2) The effect of αβ MeATP on the membrane depolarisation produced by KCl.

Since KCl acts to produce a contraction by a voltage dependent mechanism, any effect of αβ MeATP on the associated depolarisation produced by KCl would be seen to be a non-receptor effect. Tissues were pre-treated with 6-OHDA ($5x10^{-4}$M) as before.

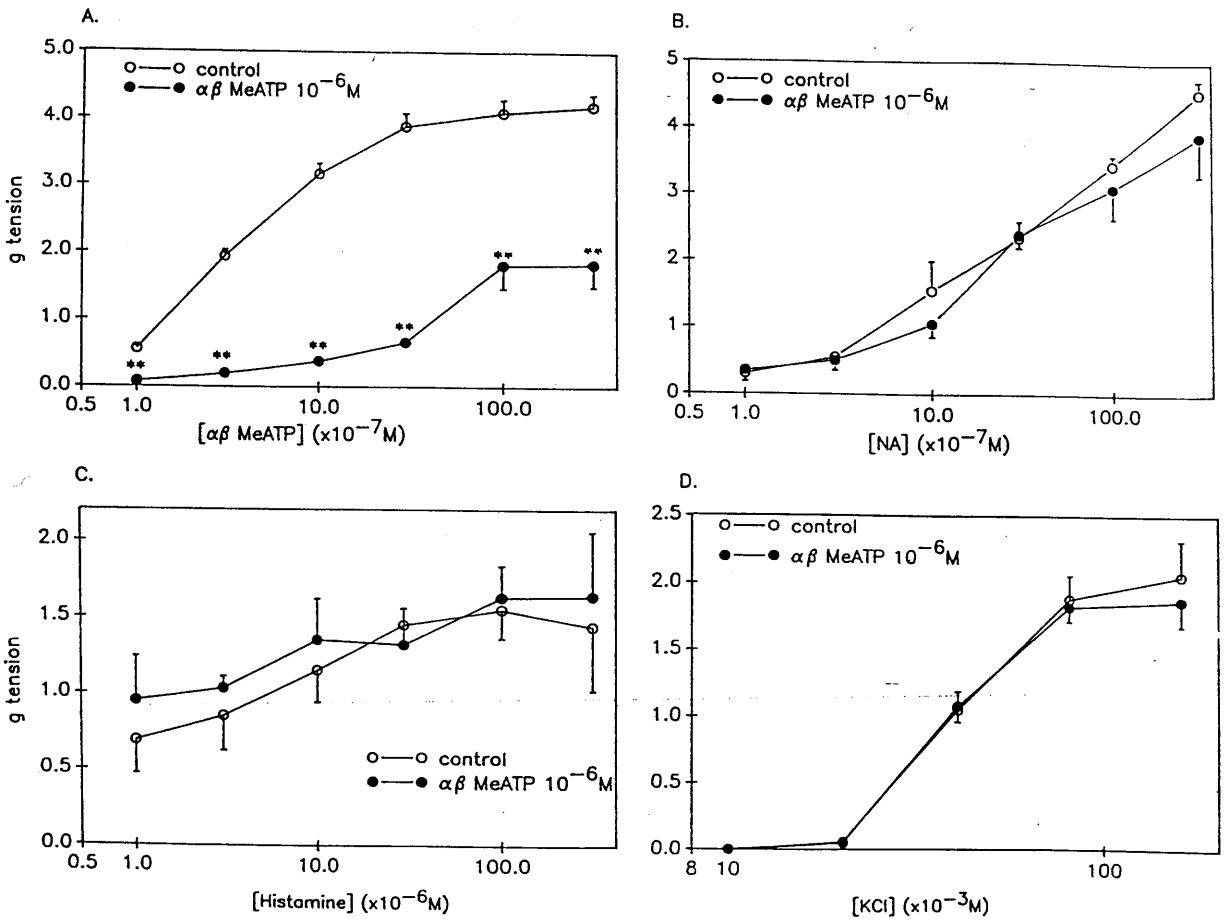


Fig. 24. The effect of continuous desensitising infusion of αβ MeATP (●-●, 10^{-6}M) on the mechanical responses to αβ MeATP (10^{-7}-$3x10^{-5}$M, A), NA (10^{-7}-$3x10^{-5}$M, B) and histamine (10^{-6}-$3x10^{-4}$M, C). The effect of infusion of αβ MeATP on the contractions to KCl (1-$16x10^{-2}$M, D) were examined after pre-treatment of the tissue with 6-OHDA in vitro for 2h to abolish any effect of pre-junctional release of transmitter. Each graph shows the mean (± s.e.m.); number of observations > 5. αβ MeATP infusion antagonised the contractions to bolus addition of αβ MeATP compared with control (** $p<0.001$) but not those to NA, to histamine, or KCl.

Addition of KCl (10^{-1}M) by picospritzer (40 p.s.i., 5-80msec, tip diameter $1x10^{-6}$m), produced a dose dependent depolarisation of the membrane potential. No change in the depolarisation evoked by KCl was seen during infusion of αβ MeATP (10^{-6}M) sufficient to produce desensitisation of P_2-purinoceptors as previously described (Fig. 25). These results suggest the absence of any non-receptor mediated post-synaptic effect of αβ MeATP and strengthens the view that it is specific for P_{2x}-purinoceptors.

(3) The effect of αβ MeATP on membrane potentials, electrotonic potentials and evoked e.j.p.s

Electrotonic potentials (2-45mV) in response to hyperpolarising currents (5-60V/cm), applied (for 5s) through extracellular plate electrodes, were recorded intracellularly in the recording partition of an Abe & Tomita (1968) bath, as previously described. E.j.p.s evoked by field stimulation (0.5msec, supramaximal voltage, 1Hz) of intramural nerves of the rabbit saphenous artery, were likewise recorded intracellularly and the effect of desensitising infusions of αβ MeATP (10^{-7}-$4x10^{-6}$M) on these parameters was measured.

There was no alteration in the amplitude of the induced electrotonic potential and therefore, by implication no alteration in membrane conductance and resistance in the presence of these doses of αβ MeATP, while e.j.p.s evoked by trains of pulses were abolished (Fig. 26). This indicated a lack of non-selective membrane effect of αβ MeATP in this tissue.

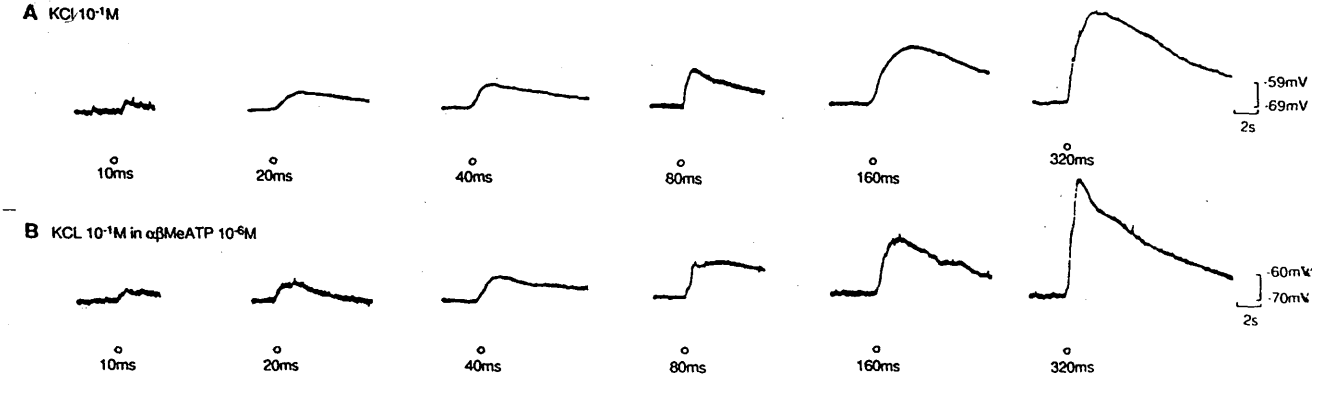


Fig. 25. The effect of a desensitising infusion of αβ MeATP (10^{-6}M) on the intracellularly-recorded electrical response to micro-application of KCl (o, 10^{-1}M, 10-320msec (ms)), where any pre-junctional effects of KCl had been removed using <u>in vitro</u> pre-treatment with 6-OHDA ($5x10^{-4}$M). KCl produced a dose dependent depolarisation, which was unaffected by αβ MeATP. Imtracellular microelectrode recordings were med from the same cell. Total numberof cells from which recordings were made (n) =10.

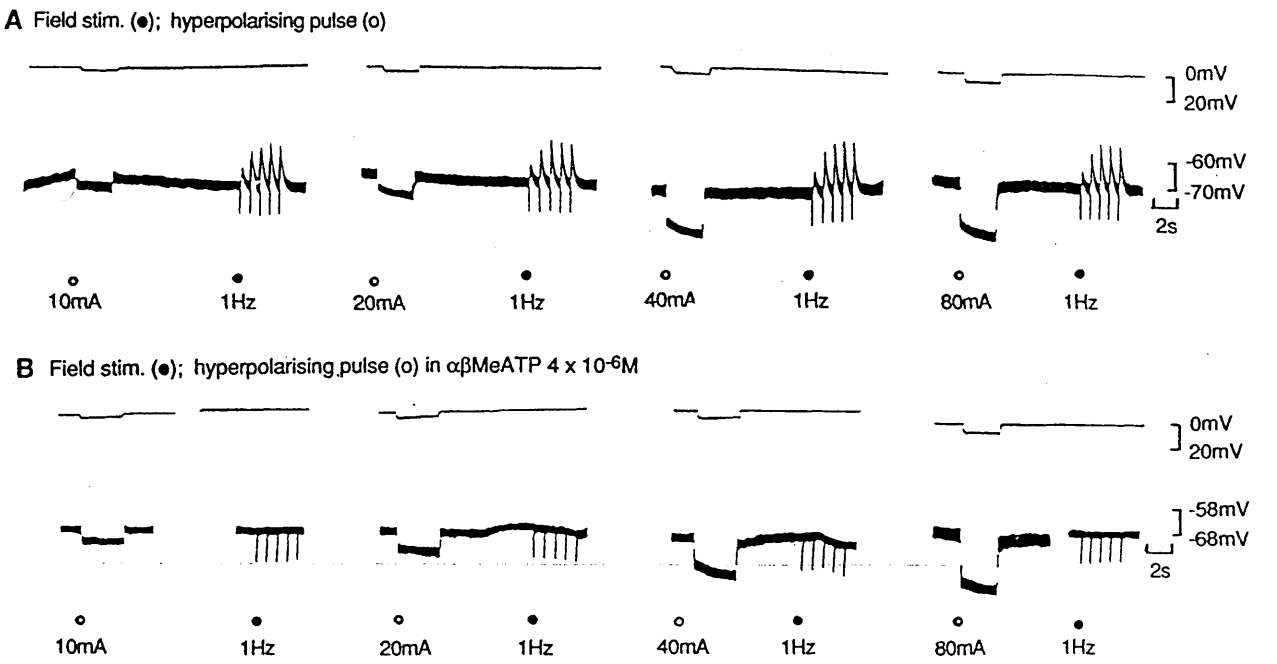


Fig. 26. Membrane potential changes in response to hyperpolarising pulses (o, 10-80mA) and to field stimulation (•, 0.5msec, supramaximal voltage, trains of stimuli at 1Hz) in the absence (A) and the presence (B) of a continuous infusion of αβ MeATP ($4x10^{-6}$M). The evoked electrotonic potentials were unaffected by αβ MeATP, while evoked e.j.p.s were abolished. Intracellular microelectrode recordings were taken from the same cell. Total number of cells from which recordings were made (n) = 9.

(4) The recovery rate of evoked responses to field stimulation and ATP, on washout of αβ MeATP.

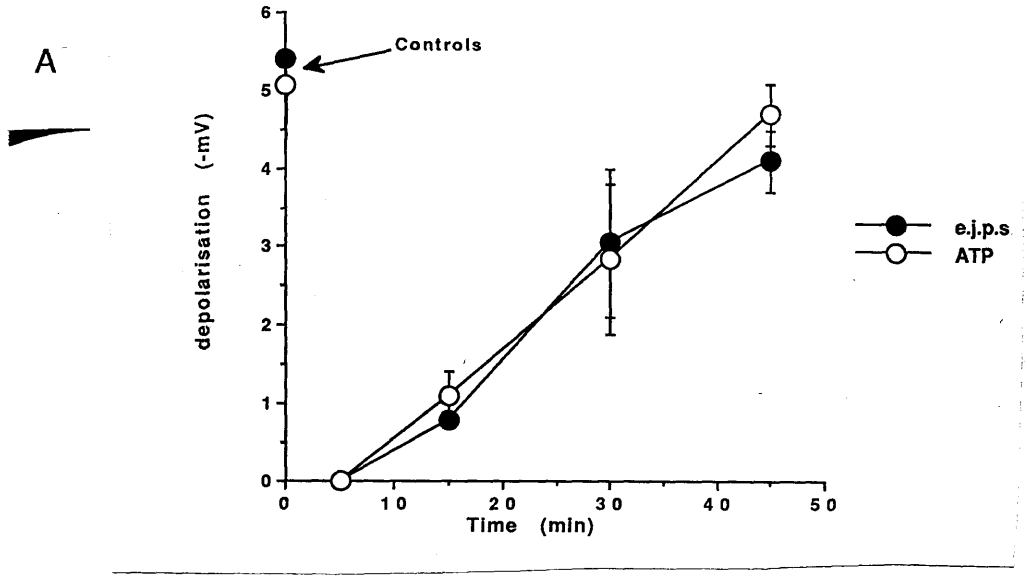
To establish that αβ MeATP abolished both evoked e.j.p.s and the responses to exogenously added ATP, by acting on the same purinergic receptor, intracellular microelectrode recordings were used. The responses to these two types of stimulation in the absence, presence and on wash out of a desensitising infusion of αβ MeATP were then compared.

ATP applied by Picospritzer (40 p.s.i., 20msec, tip diameter $1x10^{-6}M$) depolarised the artery, while field stimulation (0.5msec, supramaximal voltage, 1Hz), evoked e.j.p.s. Both responses were abolished in the presence of a desensitising infusion of αβ MeATP ($10^{-6}M$). The time course of recovery of the responses to both field stimulation and ATP, on wash out of αβ MeATP was comparable in all cases examined (Fig. 27), suggesting that αβ MeATP had a similar effect on both neuronally released non-adrenergic transmitter and on exogenously added ATP and that these are likely to be acting on the same receptor.

αβ MeATP does not show signs of any action, other than its ability to desensitise P_2-purinoceptors in the rabbit saphenous artery.

(B) SURAMIN

Dunn & Blakeley (1988), proposed that suramin also inhibited P_2-purinoceptors without antagonising noradrenergic receptors. The opportunity was therefore taken to examine the suitability of this drug in the rabbit saphenous artery as a purinergic antagonist.



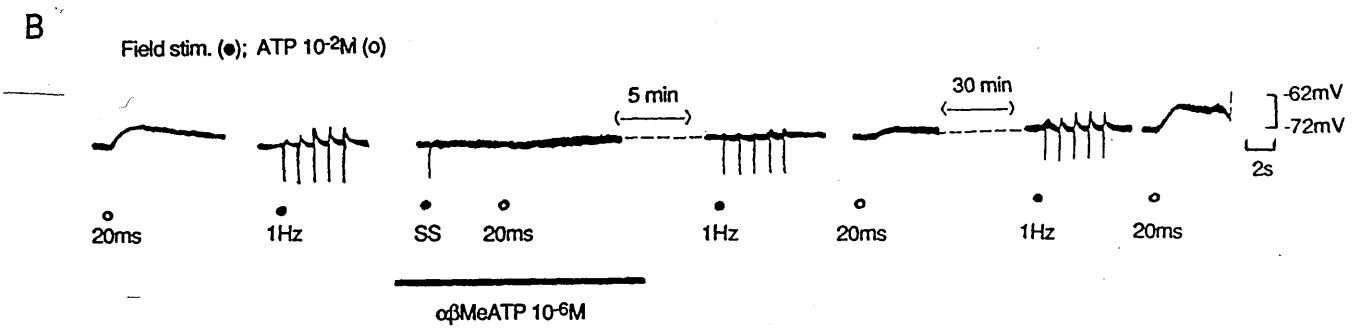


Fig. 27. (A) The intracellularly-recorded electrical response to field stimulation (•, 0.5msec, supramaximal voltage, trains of stimuli at 1Hz) and to ATP applied by pressure ejection (o, 10^{-2}M, 20msec (ms)) compared to controls, during recovery from a desensitising infusion of αβ MeATP (10^{-6}M) plotted against time (min). (B) A characteristic and typical intracellular recording of the response to the aforementioned stimuli during and on washout of αβ MeATP. Both the evoked e.j.p.s and the depolarisation to ATP were abolished by αβ MeATP. The recovery rate of these two parameters on washout of αβ MeATP was not significantly different. Intracellular microelectrode recordings were taken from the same cell. Total number of cells from which recordings were made (n) =9.

Suramin alone produced no change in membrane potential, nor any contraction.

Contractions to αβ MeATP (10^{-7}-10^{-5}M, Fig. 28A), ATP (10^{-5}-10^{-3}M) and field stimulation (0.5msec, supramaximal voltage, 1-16Hz) recorded from ring preparations were antagonised by constant infusion of suramin (10^{-3}M). Contractions to NA (10^{-7}-10^{-5}M, Fig. 28B), were unaffected, as were the post-junctionally evoked contractions to KCl(1-16x10^{-2}M, Fig. 28D). Any pre-junctional release of transmitter substances by KCl, was abolished by pre-treatment <u>in vitro</u> with 6-OHDA (5x10^{-4}M) to destroy adrenergic nerves, as previously described. Contractions to histamine (10^{-6}-10^{-4}M, Fig. 28C) and to 5-HT (10^{-6}M-10^{-5}M), were however attenuated by suramin (10^{-3}M).

Intracellular electrical and simultaneous mechanical recordings showed that suramin (10^{-3}M) abolished the evoked e.j.p.s and reduced by approximately 50%, the mechanical response to field stimulation (0.5msec, supramaximal voltage, 1-16Hz, Fig. 29). The residual mechanical response was abolished by prazosin (10^{-7}M).

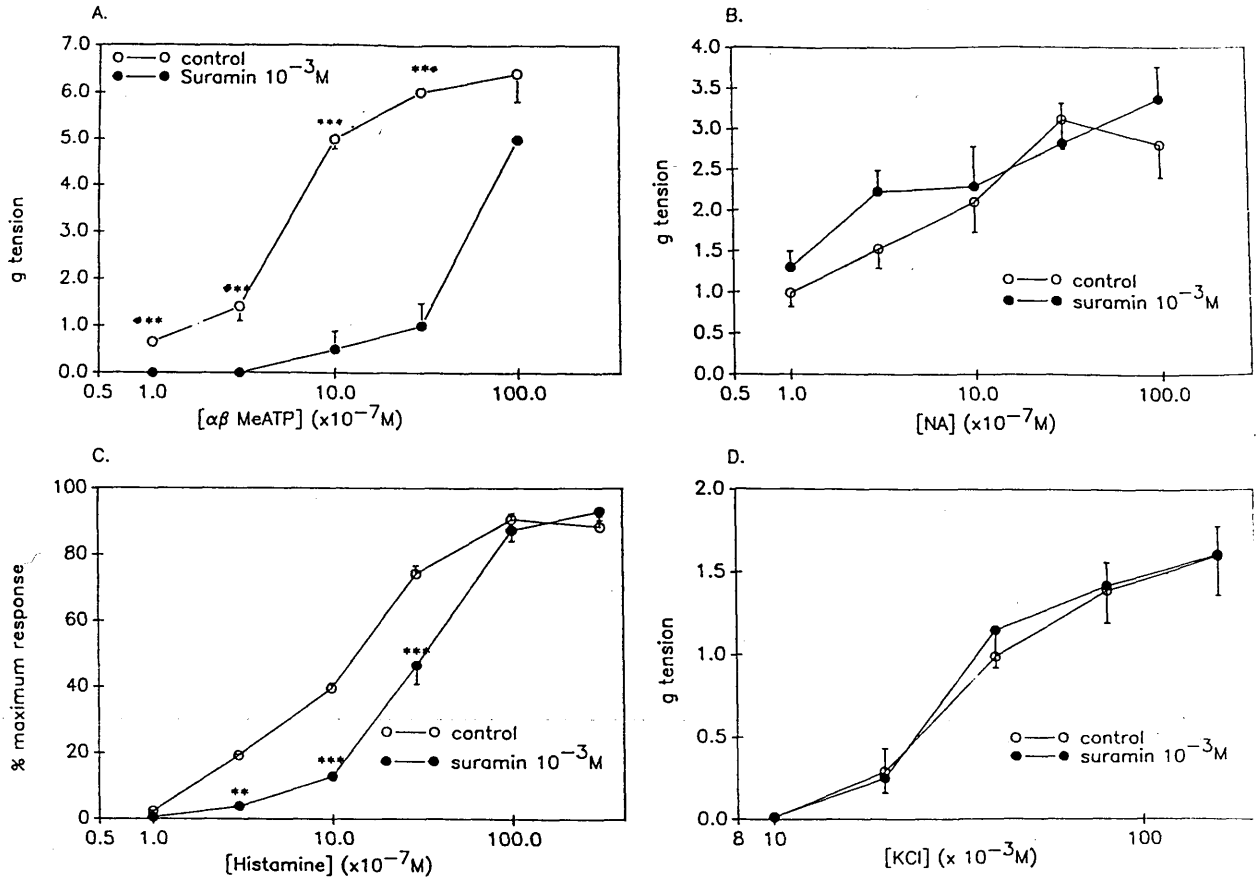


Fig. 28. The effect in the rabbit saphenous artery of infusion of suramin (10^{-3}M) on the contractile responses to αβ MeATP (10^{-7}-10^{-5}M, A), NA (10^{-7}-10^{-5}M, C) and histamine (10^{-7}-$3x10^{-5}$M), (each plotted as g tension). The effect of suramin on contractions evoked by KCl ($1\text{-}16x10^{-2}$M, D, plotted as percentage maximum response), were examined after pre-junctional release of transmitter had been prevented by in vitro treatment of the tissue with 6-OHDA ($5x10^{-4}$M). Each graph shows the mean (± s.e.m.); number of observations > 5. Suramin attenuated the responses to both αβ MeATP and histamine (** P<0.05, *** P<0.001), but did not alter the contractions to NA or to the post-junctional effects of KCl.

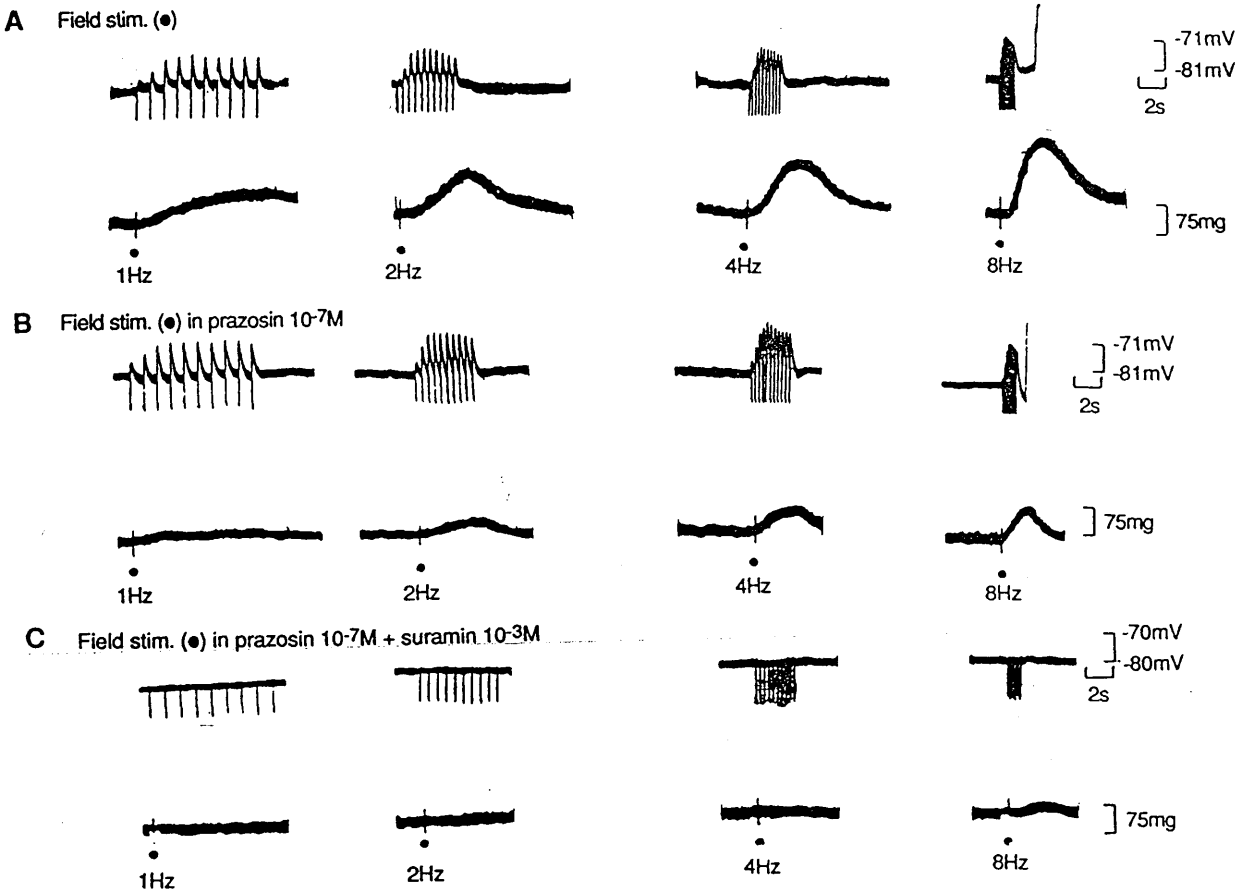


Fig. 29. Intracellularly recorded electrical (upper trace in each case) and mechanical responses to field stimulation (•, 0.5msec, supramaximal voltage, trains of stimuli at 1, 2, 4 and 8Hz) in the presence of prazosin (10^{-7}M, B) alone and together with suramin (10^{-3}M, C), compared with control (A). Suramin abolished the electrical response and any residual mechanical response left by treatment with prazosin alone. Intracellular microelectrode recordings were taken from three different cells. Total number of cells from which recordings were made (n) = 15.

IX. THE BIOCHEMICAL BASIS FOR THE CONTRACTION TO AGONISTS.

(A) NORADRENALINE

(1) Phosphatidylinositol bisphosphate (PIP_2) hydrolysis

Hydrolysis of PIP_2 has been associated with contraction in a number of vascular tissues (see Introduction). Its importance in the rabbit saphenous artery and rat tail artery was assessed by measurement of incorporation of [^{3}H]inositol into total inositol phosphates (tIPs), in response to NA.

In the rabbit saphenous artery, NA (10^{-7}-10^{-4}M) failed to raise the levels of tIPs, even in the presence of propranolol ($3x10^{-6}$M), to inhibit any negative effect of NA on PIP_2 hydrolysis via β-adrenoceptors (see Hall & Hill, 1988).

In the rat tail artery, here used as a positive control, NA (10^{-7}-10^{-4}M) increased in a dose-dependent manner, levels of tIPs produced by hydrolysis of PIP_2. The maximum increase was at 10^{-4}M NA and was of the order of a ten fold increase over control levels (Fig. 30). The increase in tIPs produced by NA was abolished by prazosin (10^{-6}M), indicating that NA acts in the rat tail artery but not in the rabbit saphenous artery, to produce PIP_2 hydrolysis via α_1-adrenoceptors.

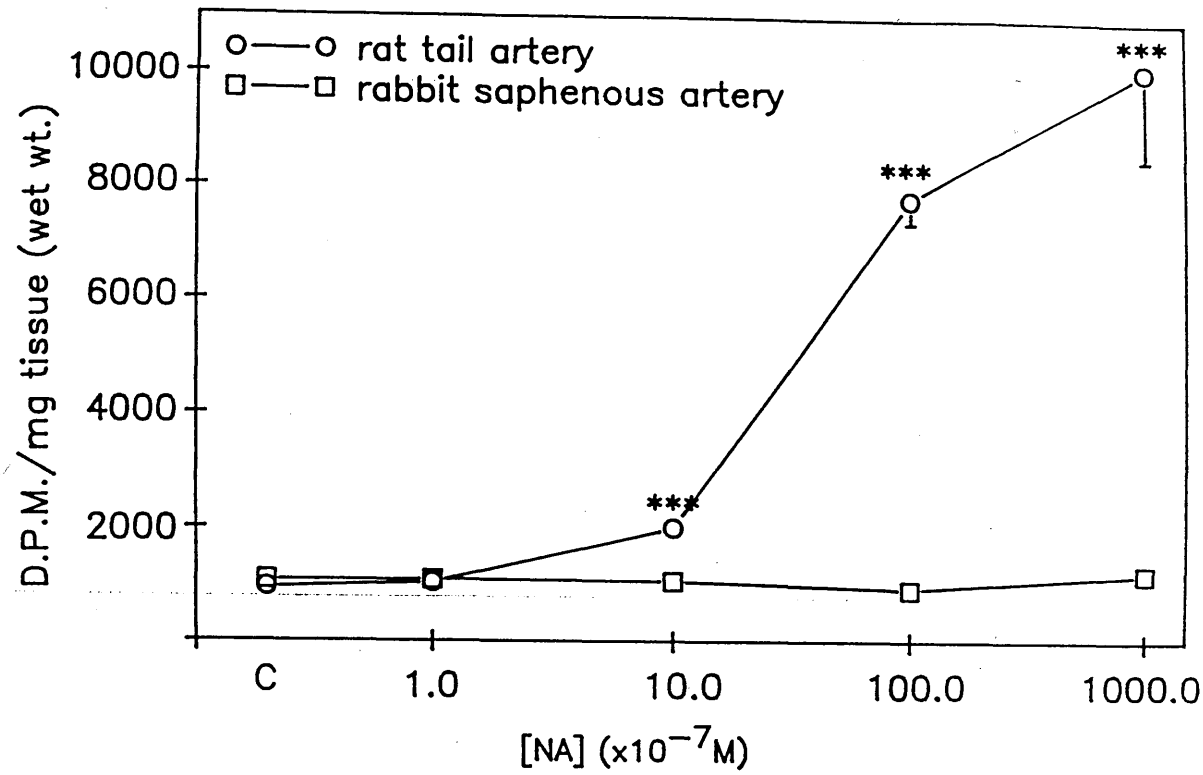


Fig 30. The effect of increasing doses of noradrenaline (NA) (10^{-7}-10^{-4}M) on accumulation of total [^{3}H]-inositol phosphates (tIP), measured as disintegrations per minute (d.p.m.) per mg of tissue (wet weight), in rat tail (o) and rabbit saphenous (□) arteries pre-incubated with myo-[^{3}H]-inositol, compared with drug free controls. Number of observations > 9 in each case. NA evoked a dose dependent rise in tIP in the rat tail (*** P<0.001) but not in the rabbit saphenous arteries.

(2) The effect of PMA.

The phorbol ester PMA (10^{-8}-10^{-7}M), is reported to stimulate protein kinase C by a mechanism analogous to that of diacylglycerol (Nishizuka, 1983).

In ring segments of rabbit saphenous artery, PMA increased the contractile response to field stimulation (0.5msec, supramaximal voltage, 1-32Hz, Fig. 31A) and NA (10^{-7}-10^{-5}M, Fig. 31B). The contractile response to KCl (10^{-2}-$1.6x10^{-1}$M, Fig. 31C), where pre-treatment of the artery in vitro with 6-OHDA had been carried out as previously described, was unaffected by pre-treatment with PMA.

This indicated that protein kinase C activation potentiated the receptor-mediated contractile response to NA in the rabbit saphenous artery.

(3) The effect of staurosporine.

The antibiotic staurosporine (10^{-9}-10^{-7}M), which inhibits a number of protein kinases, including protein kinase C (Rüegg et al., 1989), did not attenuate the contractile responses of the rabbit saphenous artery to NA (10^{-7}-10^{-5}M, Fig. 32A), nor did it alter contractions to KCl (10^{-2}-$1.6x10^{-1}$M, Fig. 32B), in tissues which had been pre-treated with 6-OHDA as before.

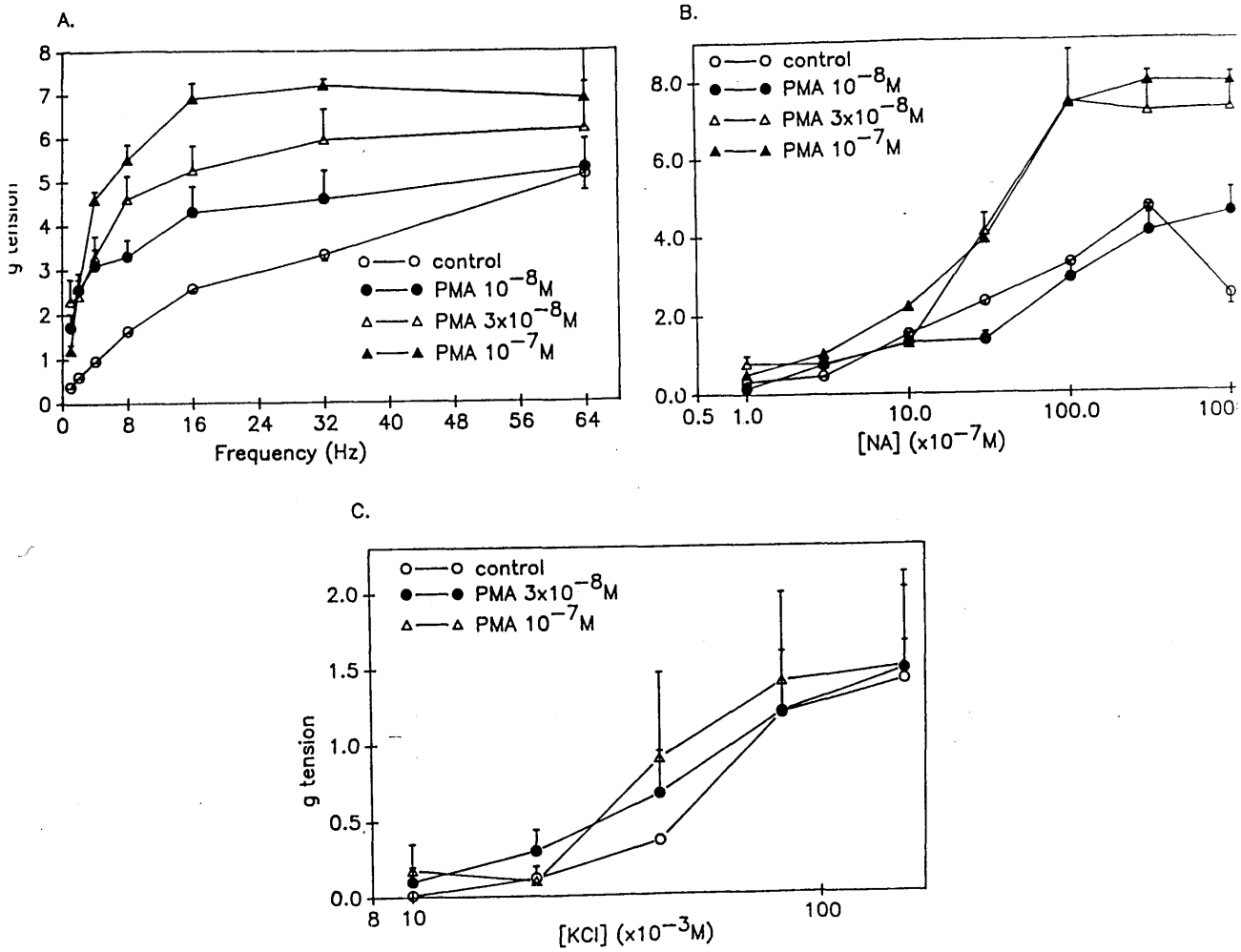


Fig. 31. The effect of the phorbol ester, phorbol 1,2-myristate 1,3-acetate (PMA, 10^{-7}-10^{-8}M) on the contractions of the rabbit saphenous artery to field stimulation (0.5msec, supramaximal voltage, 1-64Hz, A) and NA (10^{-7}-10^{-4}M, B). The effect of PMA on KCl (1-16x10^{-2}M, C) was investigated after pre-treatment with 6-OHDA (5x10^{-4}M) <u>in vitro</u> had been carried out as before. Each graph shows the mean (± s.e.m.); number of observations > 5. The responses to both NA (>10^{-6}M) and field stimulation (≥ 1Hz) were significantly enhanced by the presence of PMA ($P<0.001$). The contractions to KCl were however unaffected, suggesting that PKC potentiates the receptor-mediated contraction to NA.

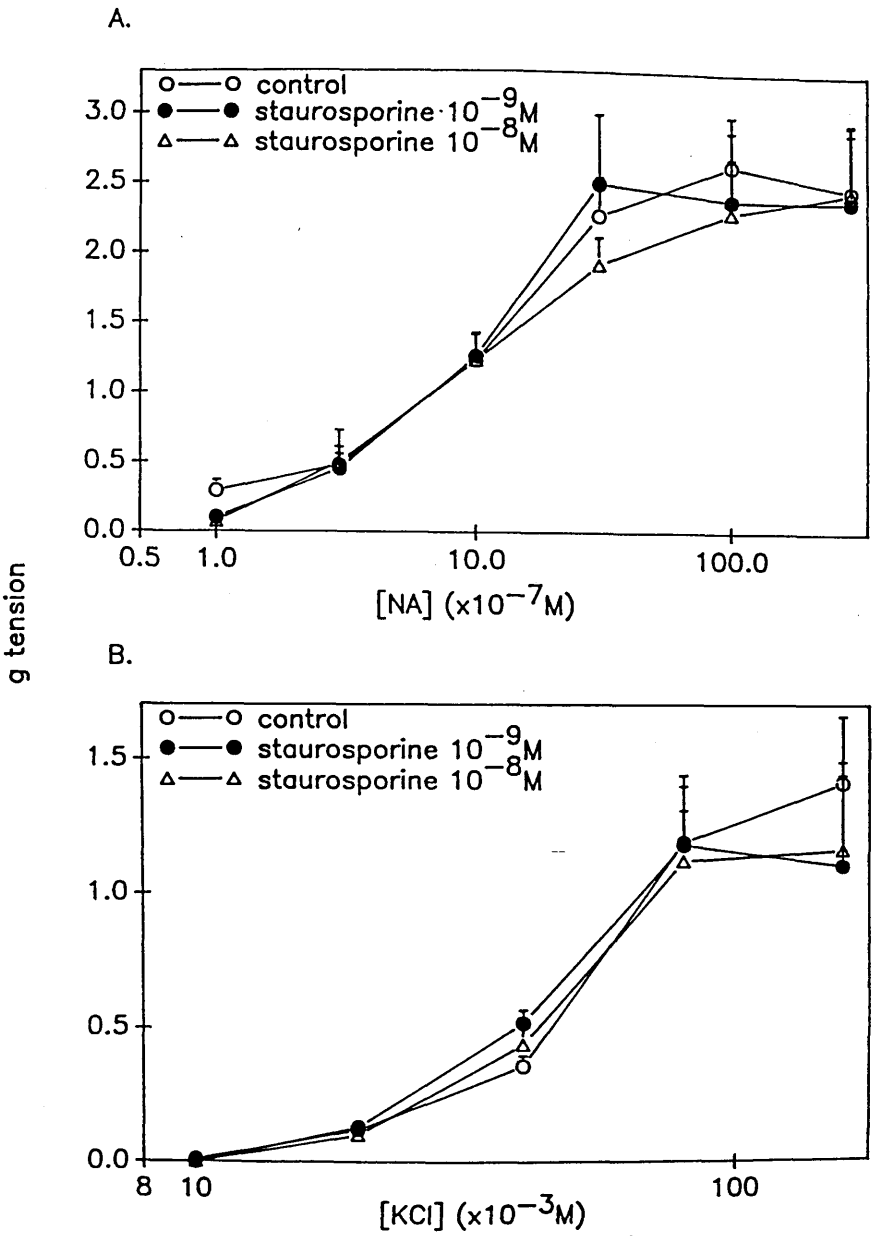


Fig. 32 The effect of staurosporine (10^{-9}-10^{-8}M) on the contractions of the rabbit saphenous artery to NA (10^{-7}-10^{-4}M, A) and KCl (1-16x10^{-2}M, B). In the case of KCl pre-treatment with 6-OHDA (5x10^{-4}M) in vitro had been carried out to abolish any pre-junctional release of transmitter substances. Each graph shows the mean (± s.e.m.); number of observations > 5. Neither the contractions to NA or to KCl were altered by the presence of staurosporine.

(4) The effect of mepacrine.

The involvement of phospholipase A_2 (PLA_2) in the mechanical response to NA was assessed using mepacrine, which is known to inhibit this phospholipase. (Lindmar et al., 1986)

Mepacrine ($3x10^{-5}M$) did not alter the response of ring segments of rabbit saphenous artery to NA (10^{-7}-$10^{-4}M$, Fig. 33), implying that PLA_2 is not an enzyme involved in the contraction to NA in this tissue.

(5) The effect of sodium oleate (NaOl) and sodium fluoride (NaF).

While these drugs may have additional actions, NaOl and NaF can respectively stimulate (Hattori & Kanfer, 1984) and inhibit (Bocckino et al., 1987) phospholipase D (PLD). They were therefore used as an indicator of the possible involvement of PLD in the contraction to NA in the rabbit saphenous artery.

In ring segments of artery, NaOl ($2x10^{-6}M$) enhanced (Fig. 34A) and NaF ($5x10^{-3}M$) inhibited (Fig. 34B) the mechanical response to NA (10^{-7}-$10^{-4}M$), giving some indication that PLD may indeed be involved in the mechanical response to NA in this tissue.

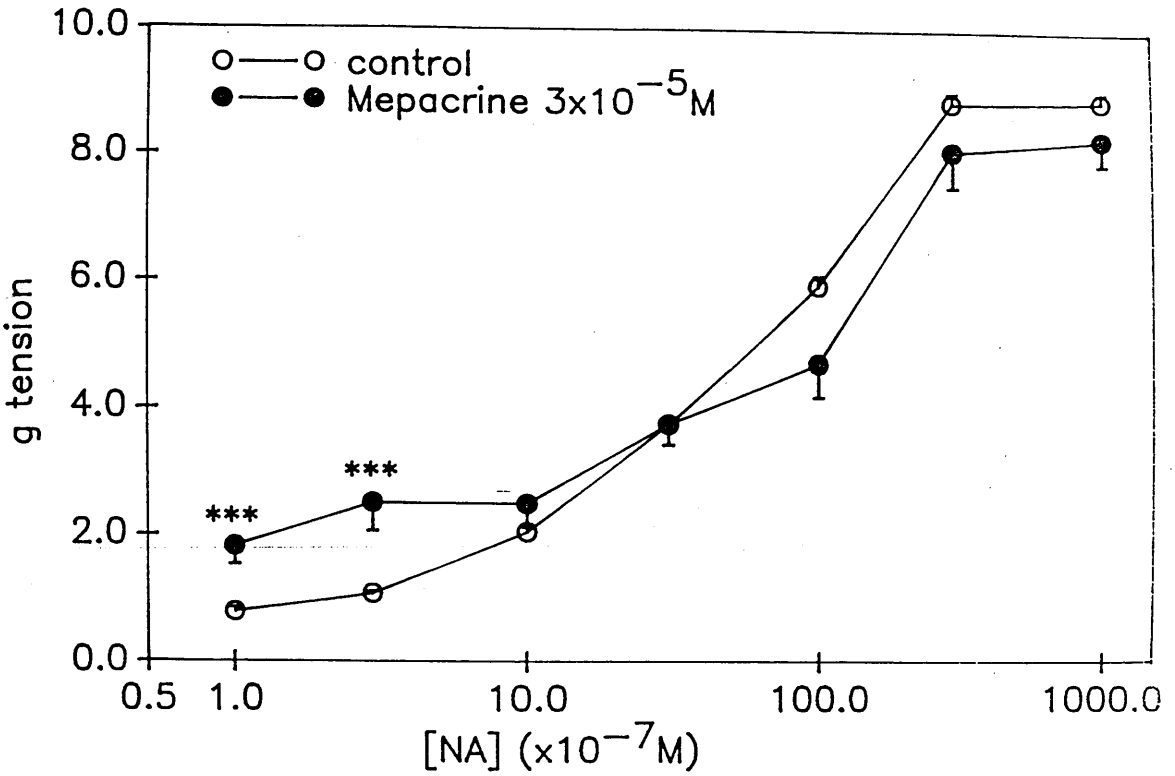


Fig. 33. The failure of mepacrine ($3x10^{-5}M$), which inhibits phospholipase A_2, to attenuate the contractions of the rabbit saphenous artery to NA (10^{-7}-$10^{-4}M$). Each graph shows the mean (± s.e.m.); number of observations > 5; *** $p< 0.001$.

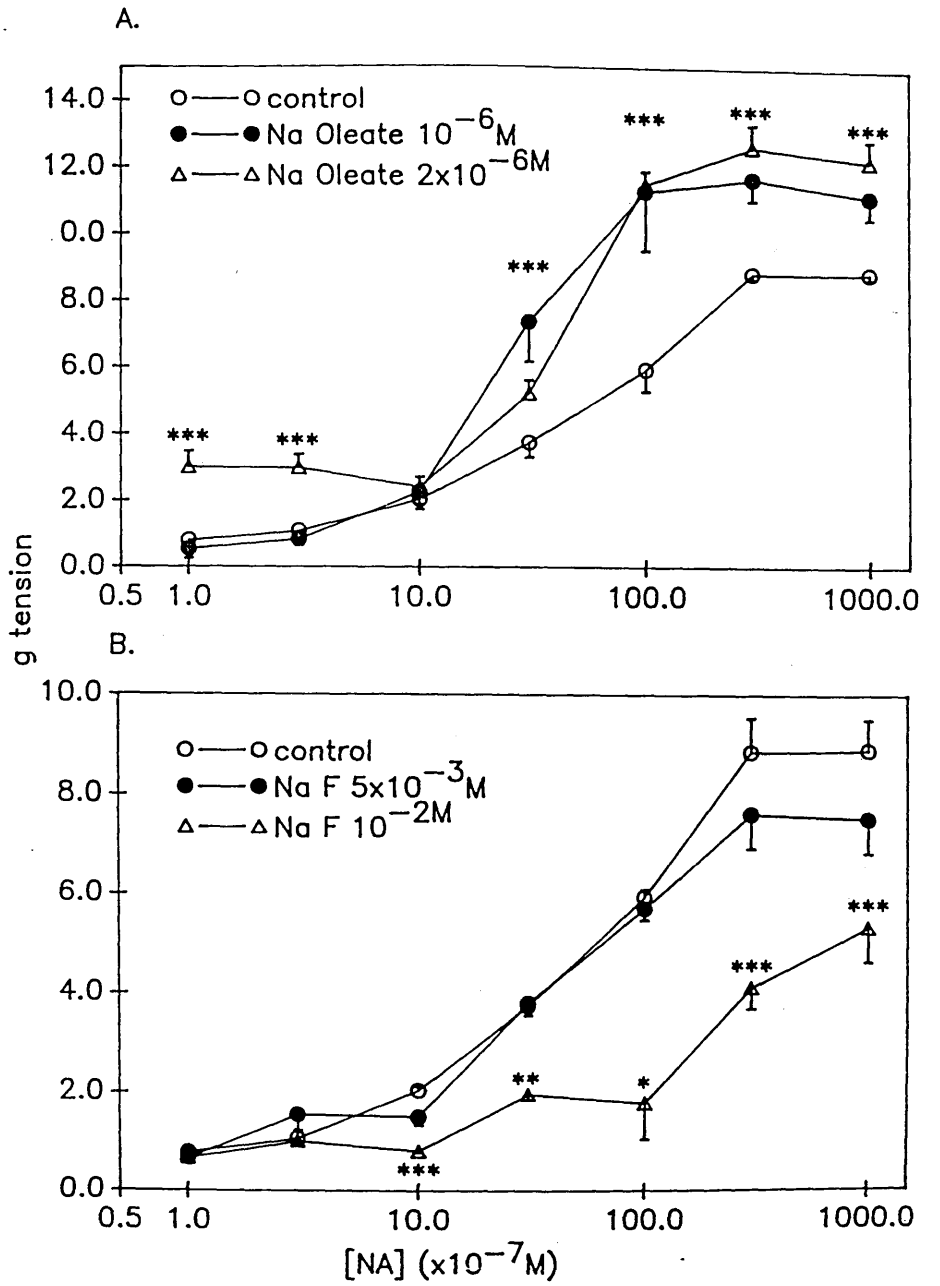


Fig. 34. Contraction of the rabbit saphenous artery to NA (10^{-7}-10^{-4}M) in the presence of sodium oleate (NaOl, 10^{-6}-$2x10^{-6}$M, A) and sodium fluoride (NaF, $5x10^{-3}$-10^{-2}M, B), each alone, which may respectively enhance and inhibit, phospholipase D. Each graph shows the mean (± s.e.m.); number of observations > 5. NaOl increased (*** $p<0.001$)and NaF reduced (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) the contractions evoked by NA in this tissue.

(6) Phosphatidylcholine (PC) hydrolysis.

PC hydrolysis leads via different phospholipases (see introduction), to glycerophosphocholine (GPC, via PLA_2), choline (Cho, via PLD) and to phosphocholine (ChoP, via PLC). Measurement of changes in the levels of these metabolites can be used to give an estimation of the involvement of PC hydrolysis in the contractile response to agonists.

Stimulation of the rabbit saphenous artery with NA (10^{-6}-10^{-4}M) evoked a rapid, dose dependent rise in levels of Cho and ChoP but not of GPC (measured 30s after addition, Fig. 35). The absence of any change in the levels of GPC suggest that PLA_2 is not involved in this response, confirming the previously observed inability of mepacrine to alter the contraction evoked by NA in ring segments of artery.

Analysis in the time course for changes in Cho and ChoP levels (Fig. 36) in response to NA (10^{-5}m) showed an large initial fast rise (after 15-30s) in Cho, accompanied by a smaller rise in ChoP, consistent with the onset of the contractile response to NA seen in ring segments of artery. This suggests that PLD and, to a lesser extent, PLC are acting to catalyse the hydrolysis of PC in this tissue. The suggestion that PLD is a part of the response is in keeping with the results previously obtained using NaOl and NaF.

Subsequent to the initial rise, Cho levels were not maintained, but returned to basal levels within 1-2min. By contrast the initial rise in ChoP were followed by a larger, later rise (at 2-4min). This may result from phosphorylation of the earlier

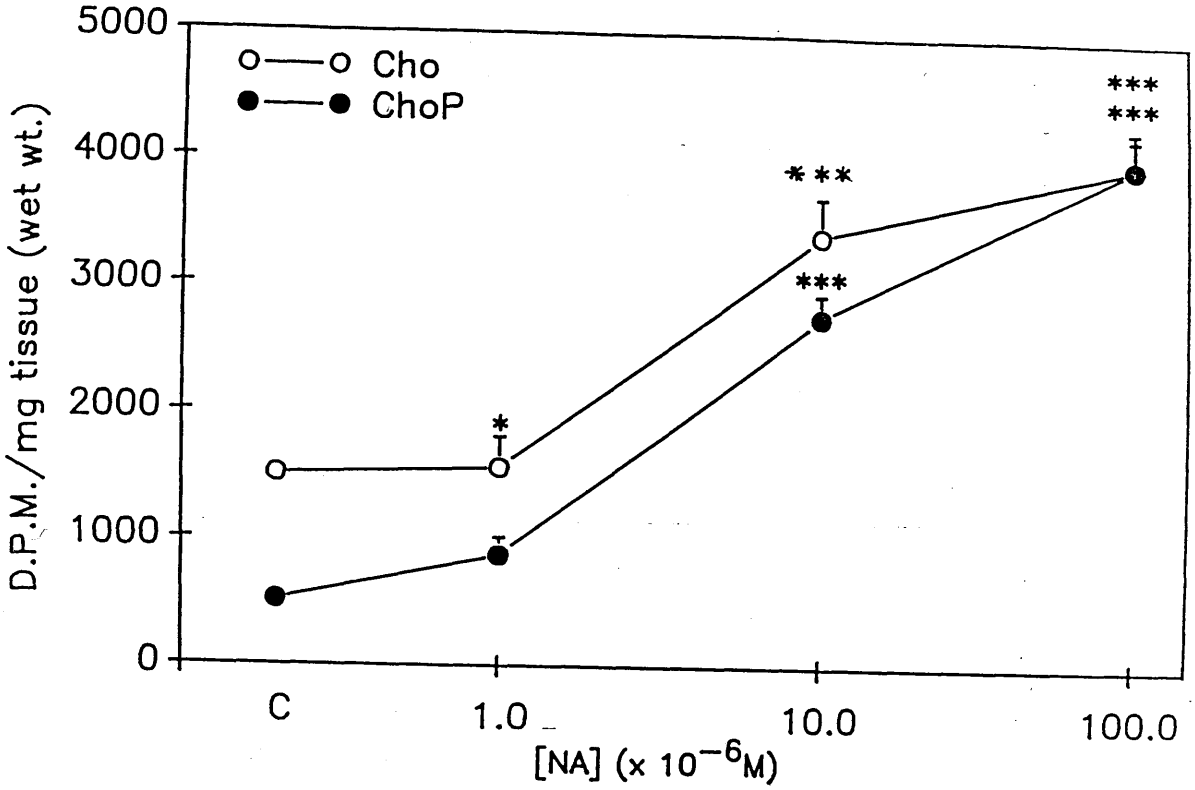
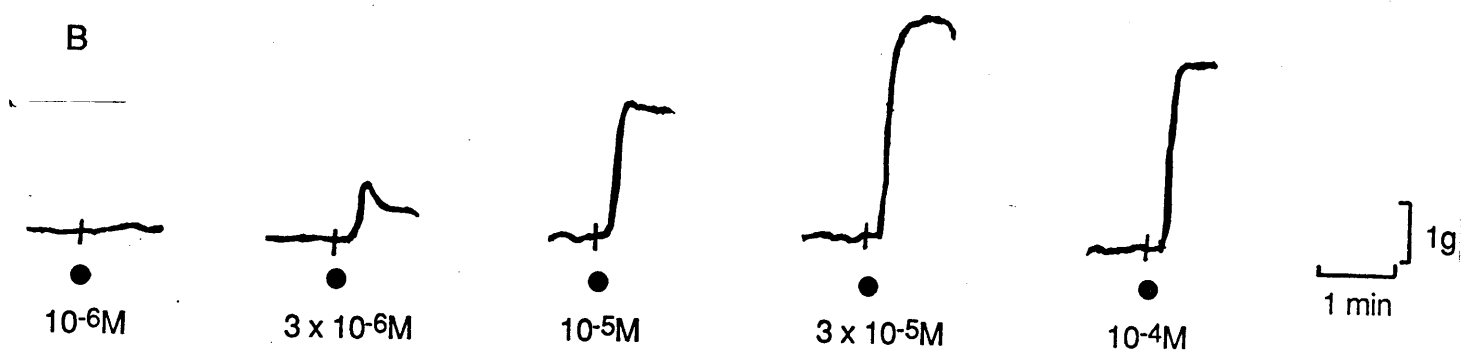


Fig. 35. The effect, measured after 30s, of increasing doses of NA (10^{-6}-10^{-4}M) on the release of [^{3}H]-choline (Cho) and [^{3}H]-choline phosphate (ChoP) in rabbit saphenous arteries, pre incubated with [^{3}H]-choline chloride. NA evoked a dose dependent rise in the levels of both Cho and ChoP, (*$p<0.05$, *** $p<0.001$) measured as disintegrations per minute (d.p.m.) per mg tissue (wet weight).


B
10-6M
3 x 10-6M
10-5M
3 x 10-5M
10-4M
1g
1 min

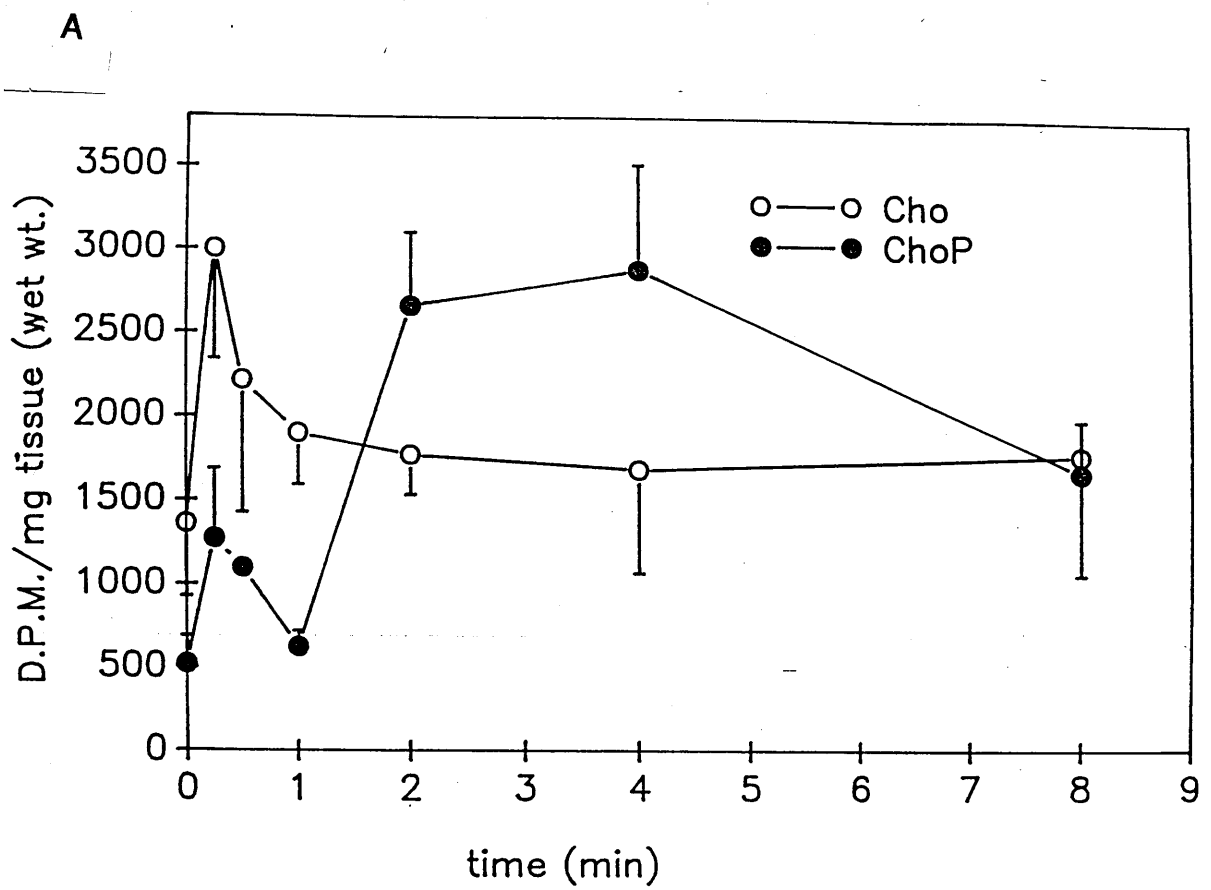


Fig. 36. (A) Time dependent changes in the levels of [^{3}H]-choline (Cho) and [^{3}H]-choline phosphate (ChoP) measured as disintegrations per minute (d.p.m.) per mg tissue (wet weight), in response to NA (10^{-5}M, number of observation (n) > 9). There was an initial (15-30s) large rise in Cho, accompanied during the same period, by a smaller rise in ChoP. A larger, much later rise was seen in ChoP at 2-4min. (B) The effect of increasing doses of NA on changes in tension (g wt.) the time to reach maximum contraction corresponds with the initial rise in Cho and ChoP observed in Fig 36 (A).

evoked Cho by choline kinase, as has been postulated for the recycling of phosphatidyl choline, or from further actions of a PLC. Both the evoked rise in Cho and ChoP was abolished by pre-incubation of the artery with prazosin (10^{-7}M).

The ability of NA to stimulate a rise in levels of Cho and ChoP suggest that hydrolysis of phosphatidylcholine by PLD and PLC but not PLA_2 may be a mechanism by which NA, acting on α-adrenoceptors, mediates contraction in the rabbit saphenous artery.

(B) NON-ADRENERGIC AGONISTS

(1) PIP_2 hydrolysis

The effects of αβ MeATP, histamine and KCl on incorporation of [^{3}H] inositol into total inositol phosphates (tIPs) was examine, to establish whether the inability of NA to evoke hydrolysis of PIP^2 was unique to the noradrenergic co-transmitter, or in fact indicated a general absence, in the rabbit saphenous artery, of the mechanism for this hydrolysis.

The slowly hydrolysable compound αβ MeATP was used to indicate the responses evoked by ATP; the putative co-transmitter in this artery. Histamine was used as an alternative example of a compound which evokes a receptor-operated contraction and KCl as a mediator of a voltage-operated, receptor-independent contraction.

In contrast to noradrenaline's apparent inability to hydrolyse PIP_2 and hence evoke a rise in total inositol phosphate levels, both αβ MeATP (10^{-6}M; ED80 for contraction) and histamine ($3x10^{-6}$M; ED80 for contraction) significantly raised tIP content of the rabbit saphenous artery (Fig. 37). This suggests that the mechanism for receptor activated hydrolysis of PIP^2 exists in this artery and can be utilised by agonists other than NA, including the putative co-transmitter ATP.

By contrast, addition to tissues treated <u>in vitro</u> with 6-OHDA in the manner previously described, of KCl ($8x10^{-2}$M; ED80 for contraction), a compound which evokes a depolarisation and contraction, presumably by activation of voltage-dependent calcium channels, did not elicit any alteration in levels of tIPs (Fig. 37). This suggests that the hydrolysis of PIP_2 evoked by, for example αβ MeATP, is not a direct consequence of depolarisation, or of raised calcium levels, but that PIP_2 hydrolysis is likely to result from receptor occupation by some agonists.

<u>(2) PC hydrolysis</u>

The ability of other agonist, including the putative co-transmitter ATP, to evoke PC hydrolysis was examined in the rabbit saphenous artery. As before, αβ MeATP was used to indicate responses evoked by ATP.

In a manner analogous to that seen with NA, αβ MeATP (10^{-7}-10^{-4}M) evoked a dose dependent rise in levels of both Cho and ChoP in

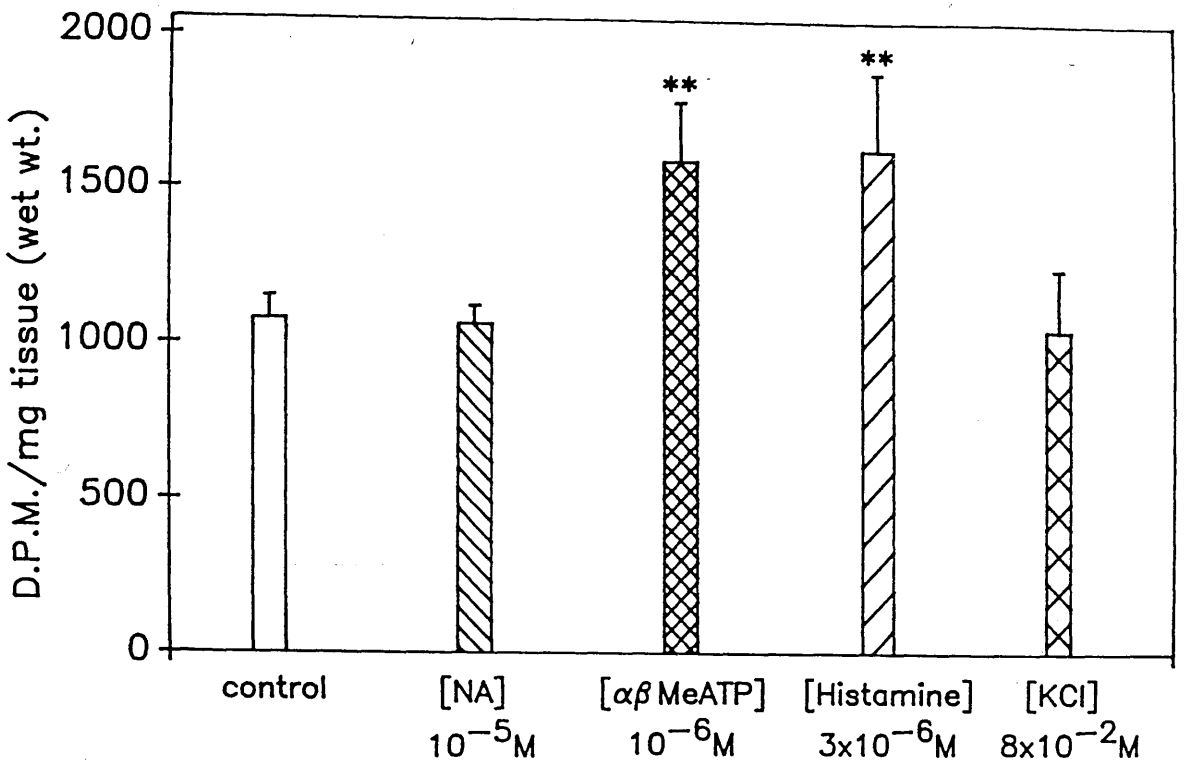


Fig. 37. The effect of NA (10^{-5}M), αβ MeATP (10^{-6}M), histamine ($3x10^{-6}$M) and KCl ($8x10^{-2}$M) on the accumulation of total [^{3}H]-inositol phosphates (tIP, number of observations (n) > 9 in each case), measured as disintegrations per minute (d.p.m.) per mg of tissue (wet weight), in rabbit saphenous arteries pre-incubated with myo-[^{3}H]-inositol. While NA and KCl were each ineffective, αβ MeATP and histamine each evoked a significant rise in tIP levels (** $p < 0.005$).

the rabbit saphenous artery (Fig. 38A). Levels of GPC were not altered at any dose of αβ MeATP used.

Histamine (10^{-7}-10^{-4}M) similarly evoked a dose dependent increase in both Cho and ChoP, in the absence of any change in GPC (Fig. 38B), suggesting that histamine likewise stimulated PC hydrolysis.

The analysis of the time dependency of generation of Cho and ChoP in response to αβ MeATP (10^{-5}M, Fig 39A) and histamine (10^{-5}M, Fig 39B) showed that, as with NA induced stimulation, early rises in both Cho and ChoP were evoked by each of these agonists. ChoP levels showed a biphasic pattern, similar to that seen with NA, however Cho levels did not significantly decline over the period observed.

The initially evoked rise in ChoP in response to αβ MeATP was approximately equivalent to that of Cho, suggesting that αβ MeATP can stimulate hydrolysis of PC, with equal dependence on both PLD and PLC.

In contrast, levels of ChoP evoked by histamine (10^{-5}M, Fig 39B). were substantially higher than the rise in levels of Cho, suggesting the predominant involvement of PLC in the response to histamine in the rabbit saphenous artery. The rise in levels of both Cho and ChoP evoked by histamine were well maintained throughout the period examined, perhaps reflecting the more prolonged contraction observed in response to this agonist, compared to NA.

These results suggest that a number of agonists evoke contraction in the rabbit saphenous artery by stimulating the hydrolysis of

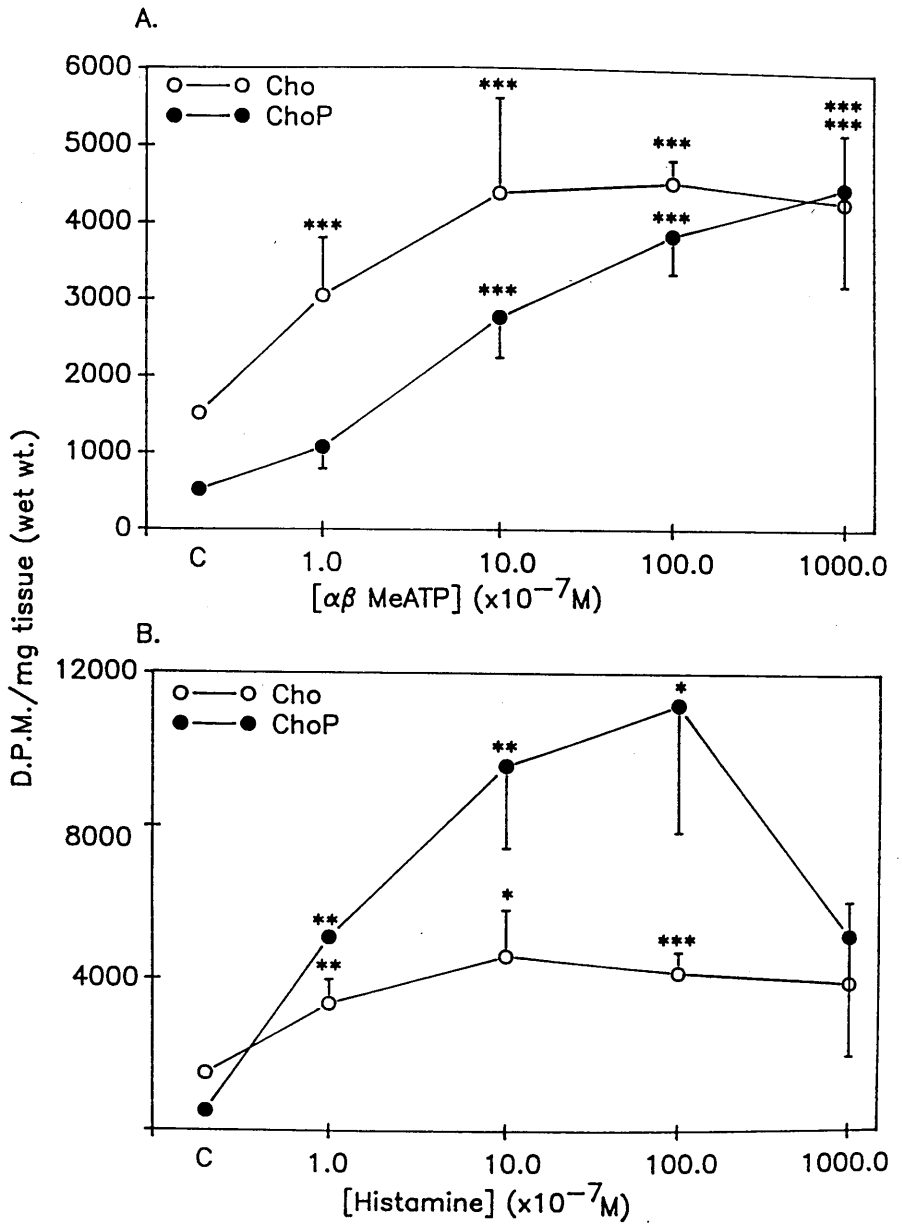


Fig. 38. The effect, measured after 30s, of increasing doses of αβ MeATP (10^{-7}-10^{-4}M, A) and histamine (10^{-7}-10^{-4}M, B) on the release of [^{3}H]-choline (Cho) and [^{3}H]-choline phosphate (ChoP) in rabbit saphenous arteries, pre-incubated with [^{3}H]-methyl choline chloride (number of observations (n) > 9). Histamine and αβ MeATP each evoked a rise in levels of both Cho and ChoP, (* $p<0.05$, ** $p<0.005$, *** $p<0.001$) measured as degradations per minute (d.p.m.) per mg tissue (wet weight).

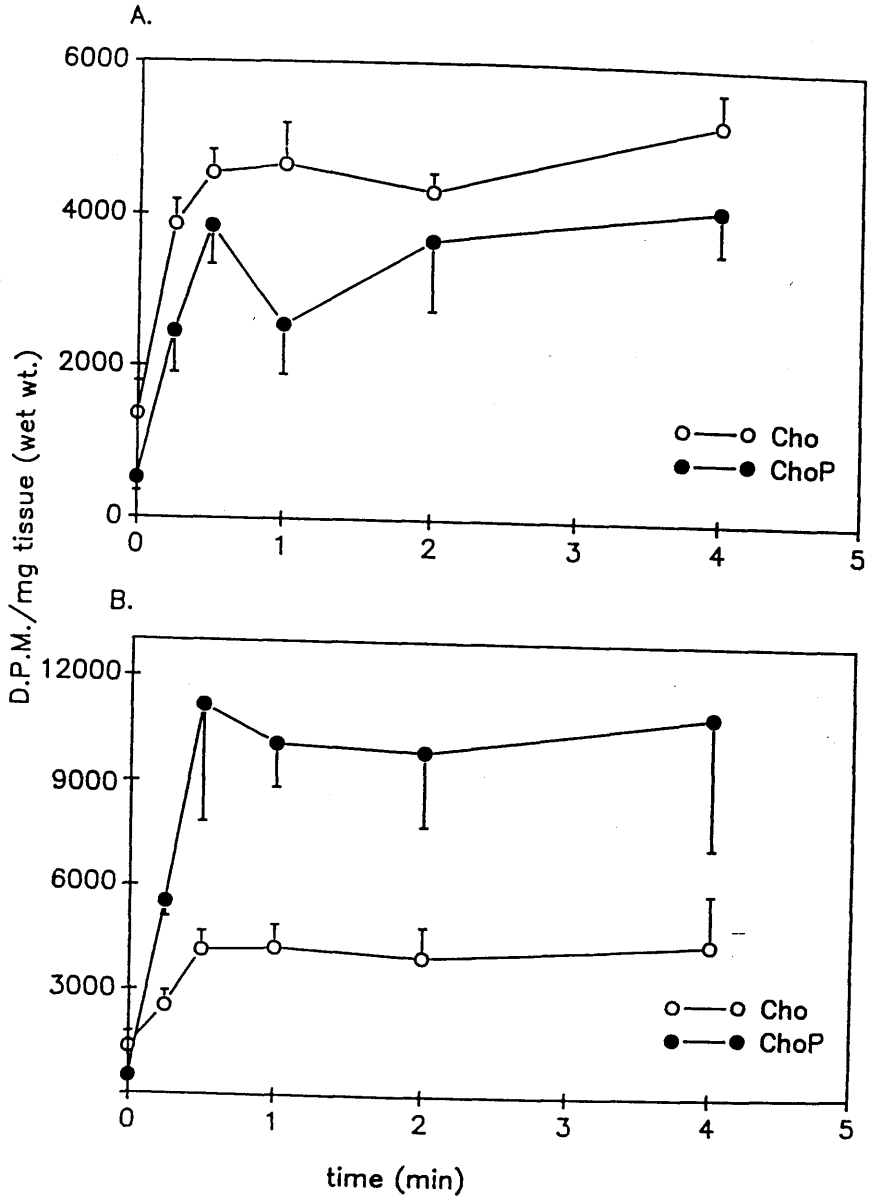


Fig. 39. Time dependent changes in the levels of [^{3}H]-choline (Cho) and [^{3}H]-choline phosphate (ChoP) measured as disintegrations per minute (d.p.m.) per mg tissue (wet weight), in response to αβ MeATP (10^{-5}M, A) and histamine (10^{-5}M, B) (number of observations (n) > 9). αβ MeATP evoked an initial rise at 15-30s in levels of both Cho and ChoP, with the rise in ChoP being proportionally slightly greater. A larger, later rise in ChoP was observed at 2-4min. Histamine evoked a large rise in ChoP, accompanied by a smaller rise in Cho. The increased levels of both Cho and ChoP evoked by histamine were maintained during the period investigated.

PC. The extent however, to which PLD and PLC are utilised in the hydrolysis of this phospholipid, varies. In addition, while NA stimulates the hydrolysis of PC in the absence of any action on PIP_2, other agonists, including the putative co-transmitter ATP, may act to produce contraction by stimulation of a combination of these mechanisms.

(C) THE EFFECT OF CO-TRANSMISSION ON THE HYDROLYSIS OF PC

The effects on hydrolysis of phosphatidylcholine (PC) of NA and αβ MeATP added singly and in combination were examined to investigate any synergism that may exist between the putative co-transmitters at this level.

Addition of NA (10^{-6}-10^{-4}M) and αβMeATP (10^{-6}-10^{-4}M) alone and in combination (10^{-6}-10^{-4}M using matched concentrations in each case), evoked dose dependent increases in Cho (Fig. 40A) and ChoP (Fig. 40B). Comparison of equimolar doses of these agonists showed that αβ MeATP was more potent than NA evoking hydrolysis of PC.

The increase in Cho levels evoked by αβ MeATP in conjunction with NA was not significantly greater than the response to αβ MeATP alone. The increase in levels of ChoP evoked by a combination of αβ MeATP and NA was however significantly larger than the response to either of the individual compounds. Within experimental limits this increase was approximately equal to the arithmetic combination of the individual response to NA and αβ MeATP. There was no evidence of synergism.

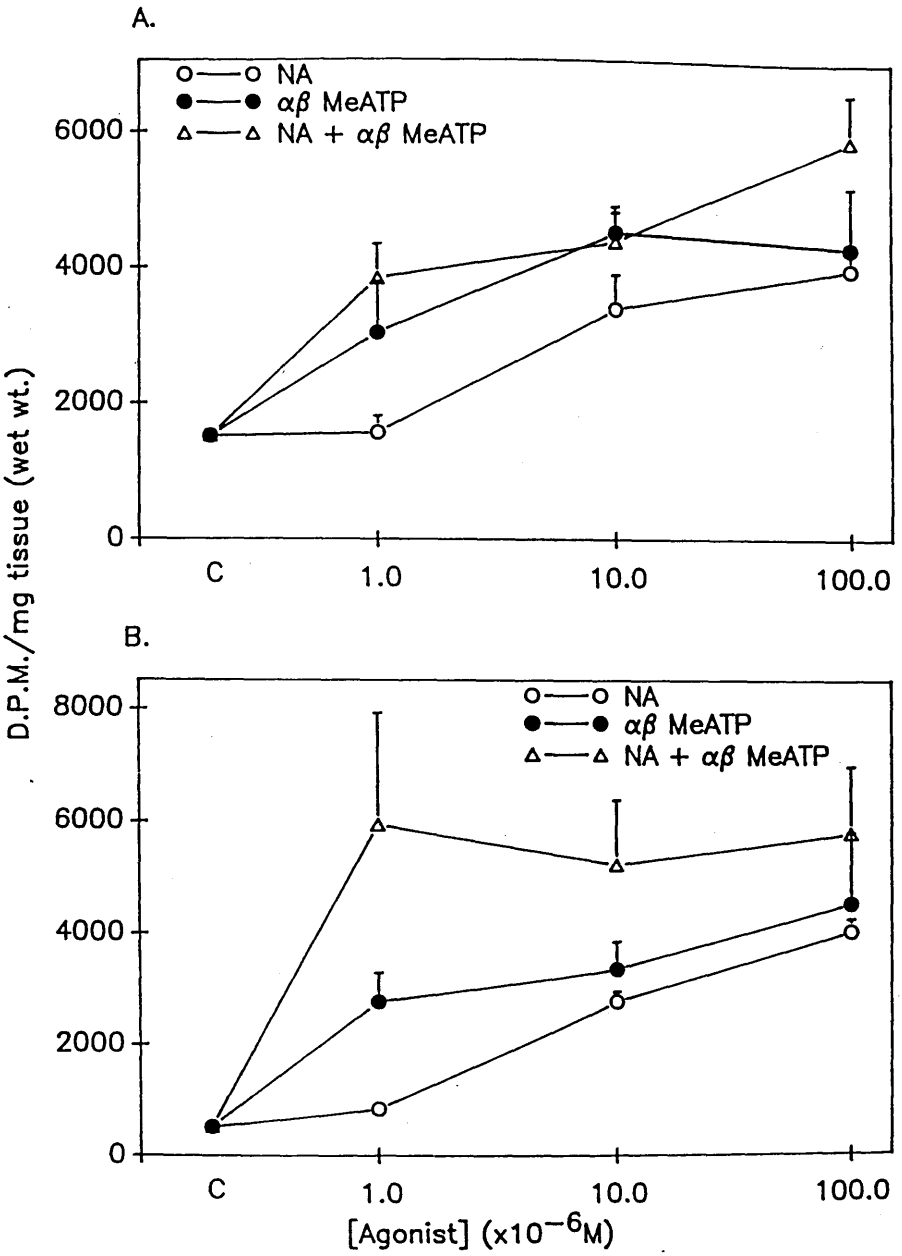


Fig. 40. The effect, measured after 30s, of increasing doses of NA (10^{-6}-10^{-4}M), αβ MeATP (10^{-6}-10^{-4}M) and a combination of matched doses of NA (10^{-6}-10^{-4}M) and αβ MeATP (10^{-6}-10^{-4}M) on the release of [^{3}H]-choline (Cho, A) and [^{3}H]-choline phosphate (ChoP, B) in rabbit saphenous arteries, pre incubated with [^{3}H]-choline chloride. The combination of NA and αβ MeATP evoked a dose dependent rise in ChoP, which was significantly ($p<0.05$) greater than the rise evoked by either agonist alone, being approximately equivalent to the arithmetical sum of the individual responses but showed no evidence of synergism. The rise in Cho evoked by a combination of NA and αβ MeATP was no greater than that seen with αβ MeATP alone. Results were measured as disintegrations per minute (d.p.m.) per mg tissue (wet weight). Number of observations > 6.

THE EFFECT OF ADENOSINE AND ADENOSINE ANALOGUES ON HEART RATE (HR) AND HIND LIMB PERFUSION PRESSURE (HLPP) IN THE DOG.

(A) POTENCY OF AGONISTS

To establish the subtypes of adenosine receptor mediating bradycardia and vasodilation in the dog, a potency order for adenosine analogues was first established.

Concentration-response curves for adenosine (0.001-0.3mg/Kg) and adenosine analogues N^6-cyclopentyl adenosine (CPA, 0.001-0.3mg/Kg), r-N^6-phenylisopropyl adenosine (r-PIA, 0.001-0.3mg/Kg), 2-phenylamino adenosine (PAA, 0.001-0.3mg/Kg) and 5'-N-ethylcarboxamide adenosine (NECA, 0.0001-0.1mg/Kg) in canine heart are shown in Fig. 41A. With the exception of PAA, all agonists produced a dose dependent decrease in heart rate (HR, measured as beats per minute). PAA evoked a slight tachycardia. The order of potencies for decrease in heart rate was NECA > adenosine > CPA > r-PIA > PAA.

Concentration-response curves were also obtained for these agonists in the hind limb of the dog. Adenosine and all the adenosine analogues examined, produced a decrease in hind limb perfusion pressure (HLPP, measured as mmHg, Fig. 41B). The order of potencies for this, calculated from the ED_{50} values (shown in parenthesis) from the sigmoidal curve fits shown in Fig. 41B, was NECA, (0.001)> PAA, (0.0033)> adenosine, (0.025)> r-PIA, (0.05) = CPA, (0.055).

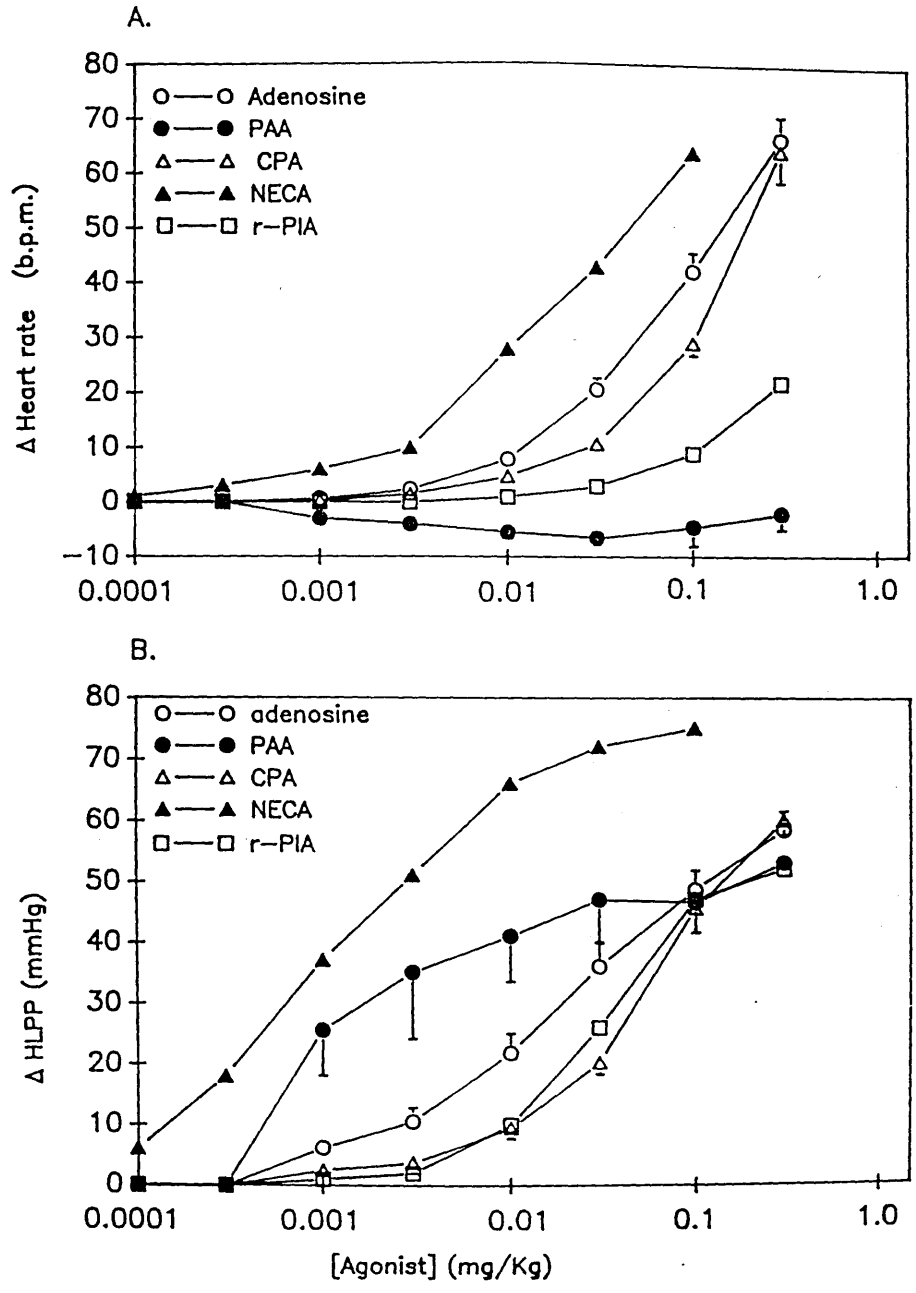


Fig. 41. The effect of adenosine (0.001-0.3mg/Kg, number of observations (n) = 19), CPA (0.001-0.3mg/Kg, n=4), r-PIA (0.001-0.3mg/Kg, n=1), PAA (0.001-0.3mg/Kg, n=2) and NECA (0.0001-0.1mg/Kg, n=1) on (A) heart rate (HR), measured as beats per minute (b.p.m.) and (B) hind limb perfusion pressure (HLPP) measured as mmHg, in the unconscious in vivo dog. The order of potencies derived for heart rate was NECA > adenosine > CPA > r-PIA > PAA and for hind limb perfusion pressure, was NECA > PAA > adenosine > r-PIA = CPA>

(B) THE EFFECT OF ANTAGONISTS

Antagonist potency was assessed by the dose required to produce a two-fold rightward shift of the adenosine dose response curve (DR2).

(1) Theophylline.

Dose-response curves to adenosine for both HR (Fig. 42A) and HLPP (Fig. 42B) were shifted to the right in a dose dependent manner by theophylline (1-10mg/Kg). The slope of the curve was not significantly altered in each case, suggesting a competitive antagonism. Theophylline was relatively non-selective however; the DR2 for heart rate was 3.46mg/Kg, while for HLPP the DR2 was 0.73mg/Kg, indicating only a small degree of selectivity for the adenosine receptor in the femoral arterial bed.

(2) PD116948

Where adenosine was added as a bolus injection into the left ventricle, the induced decrease in heart rate was inhibited in a dose dependent manner by the A_1-selective antagonist PD116948 (0.3-3mg/Kg), with a DR2 of 0.4mg/Kg (Fig. 43A), while the reduction in HLPP (Fig. 43B) produced by adenosine was antagonised by PD116948 with a DR2 of 0.9mg/Kg. This suggests that, rather surprisingly, PD116948 is relatively non-selective for the adenosine receptors mediating bradycardia in the dog heart and femoral

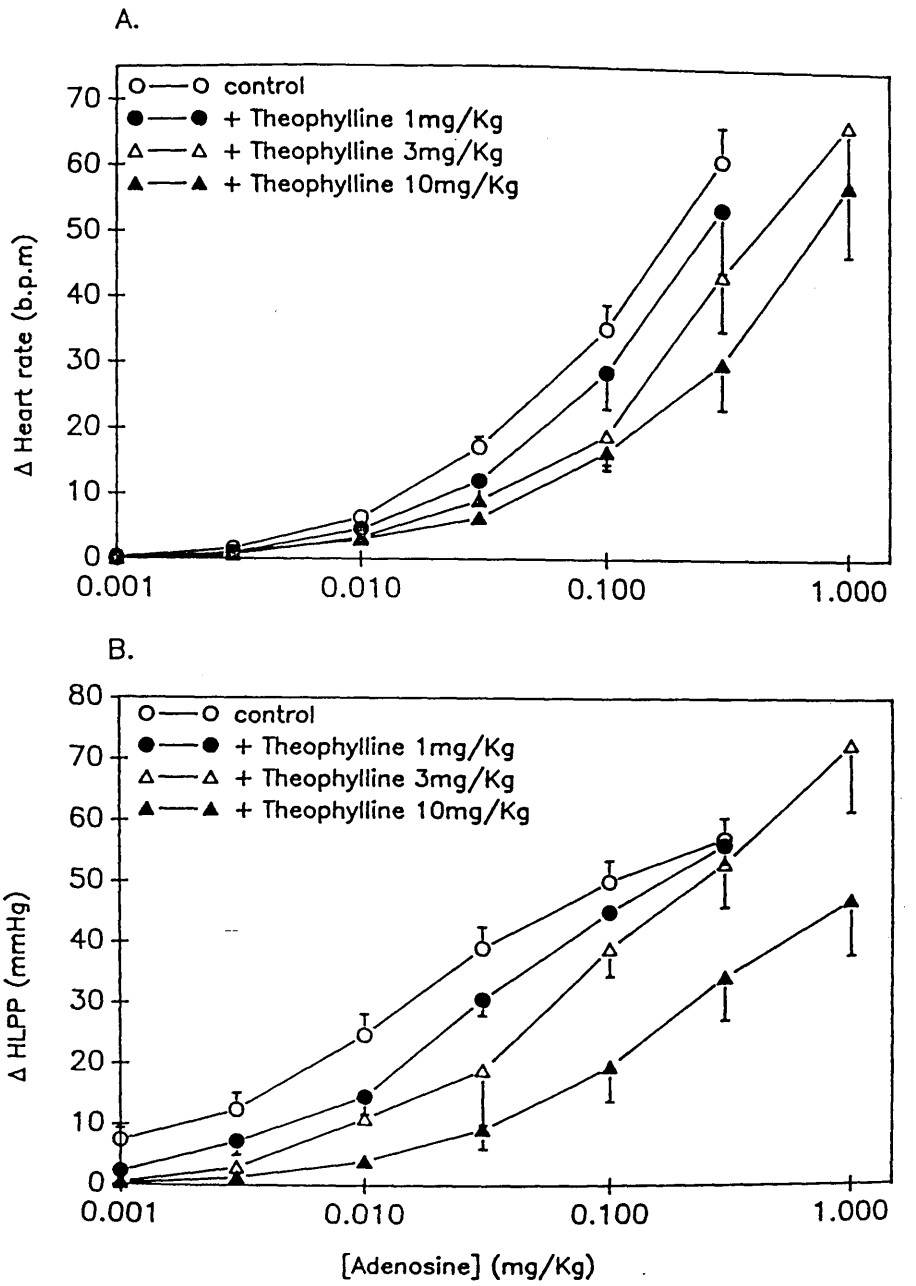


Fig. 42. The effect of theophylline (1-10mg/Kg) on the changes evoked by adenosine (0.001-1mg/Kg) of (A) heart rate (HR) measured as beats per minute (b.p.m.) and (B) hind limb perfusion pressure (HLPP) measured as mmHg, in the unconscious in vivo dog. Number of observations (n) = 3. The dose response curves for both HR and HLPP to adenosine, were shifted to the right in a relatively non-selective, dose dependent manner by theophylline.

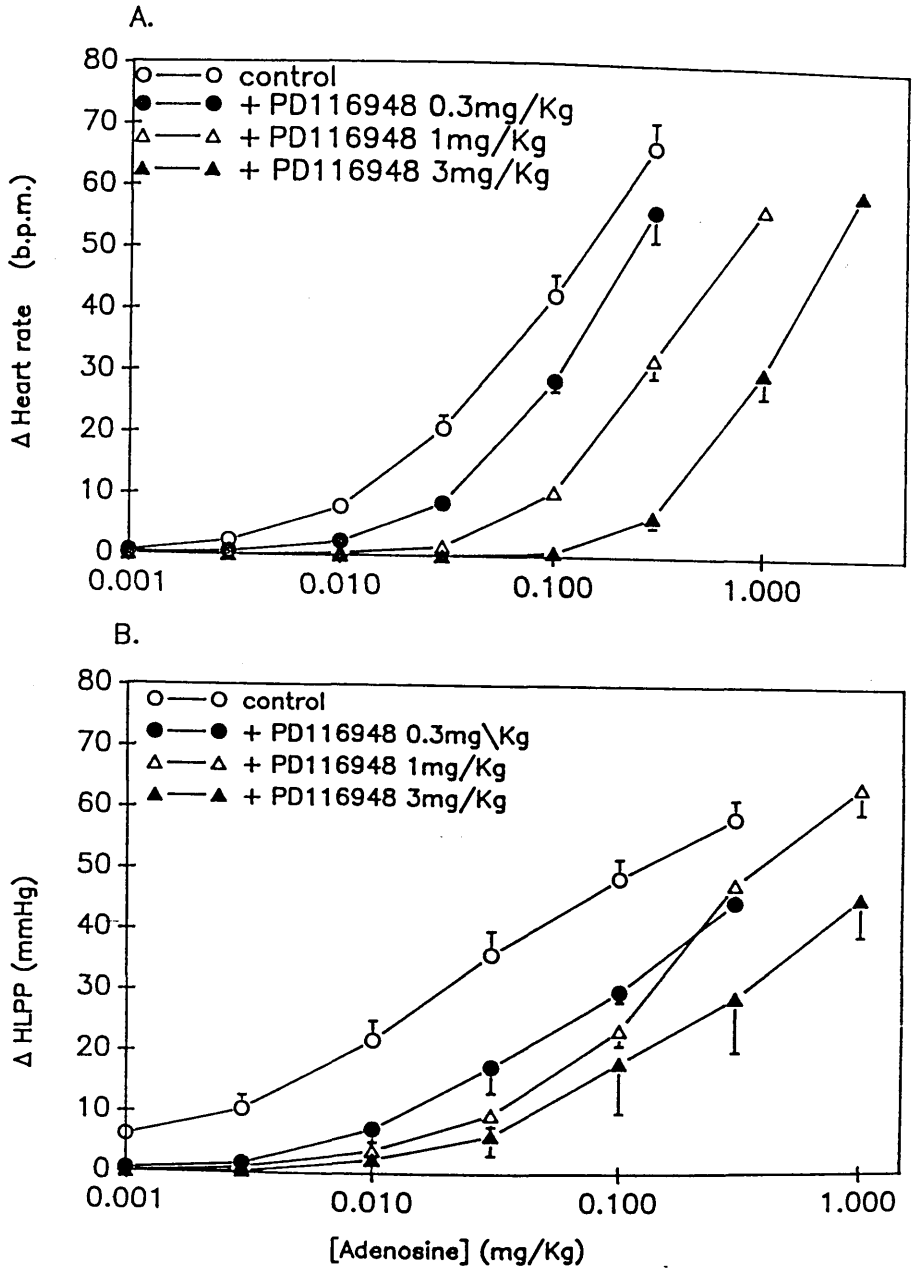


Fig 43. The effect of PD116948 (0.3-3mg/Kg) on the dose response curves to bolus addition of adenosine (0.001-1mg/Kg) for (A) heart rate (HR) measured as beats per minute (b.p.m.) and (B) hind limb perfusion pressure (HLPP) measured as mmHg, in the unconscious in vivo dog. Number of observation (n) = 3. The adenosine dose response curves for both HR and HLPP, were shifted to the right in a relatively non-selective, dose dependent manner by PD116948.

arterial bed.

In a single experiment, adenosine was added by infusion into the left ventricle (Fig. 44). On this occasion, PD116948 produced an average inhibition 3.1 fold greater than that seen with the same dose under conditions of bolus addition. HLPP could not be measured in this experiment since infusion of adenosine produced marked desensitisation in the hind limb.

The possibility exists that lack of full receptor occupation may explain the apparent lack of selectivity of this antagonist in the dog. A greater sample number would be required however before any conclusions could be properly drawn from this result.

(3) PD115199.

The adenosine induced changes in HR (Fig. 45A) and HLPP (Fig. 45B), were both attenuated in a dose dependent manner by the selective A_2 antagonist PD115199 (0.3-3mg/Kg). The DR2 for HLPP was 0.034mg/Kg, compared with 1.37mg/Kg for HR, indicating that PD115199 was some 40 fold selective as an antagonist of HLPP compared to HR.

The effects of PD116948 and PD115199 each alone, on the response to the relatively A_1 selective agonist CPA were investigated.

PD116948(1mg/Kg) produced very little shift of the dose response curve to CPA in either the heart (Fig. 46A) or the hind limb (Fig.

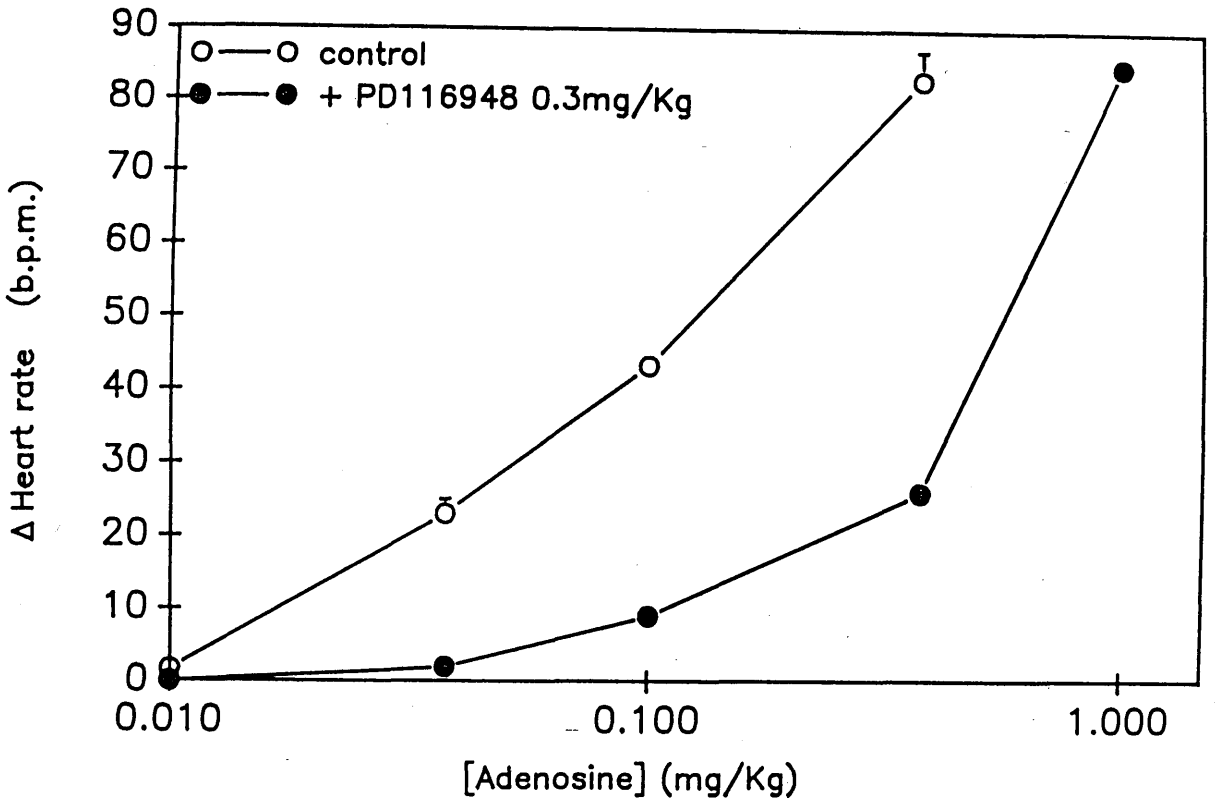


Fig. 44. The effect of PD116948 (0.3mg/Kg) on the dose response curves to addition of adenosine (0.01-1mg/Kg) by infusion into the left ventricle for heart rate (HR), measured as beats per minute (b.p.m.) in the unconscious in vivo dog. Number of observations (n) = 1. PD116948 produced an average inhibition of the response to adenosine, which was more than 3 fold greater than that observed with bolus addition.

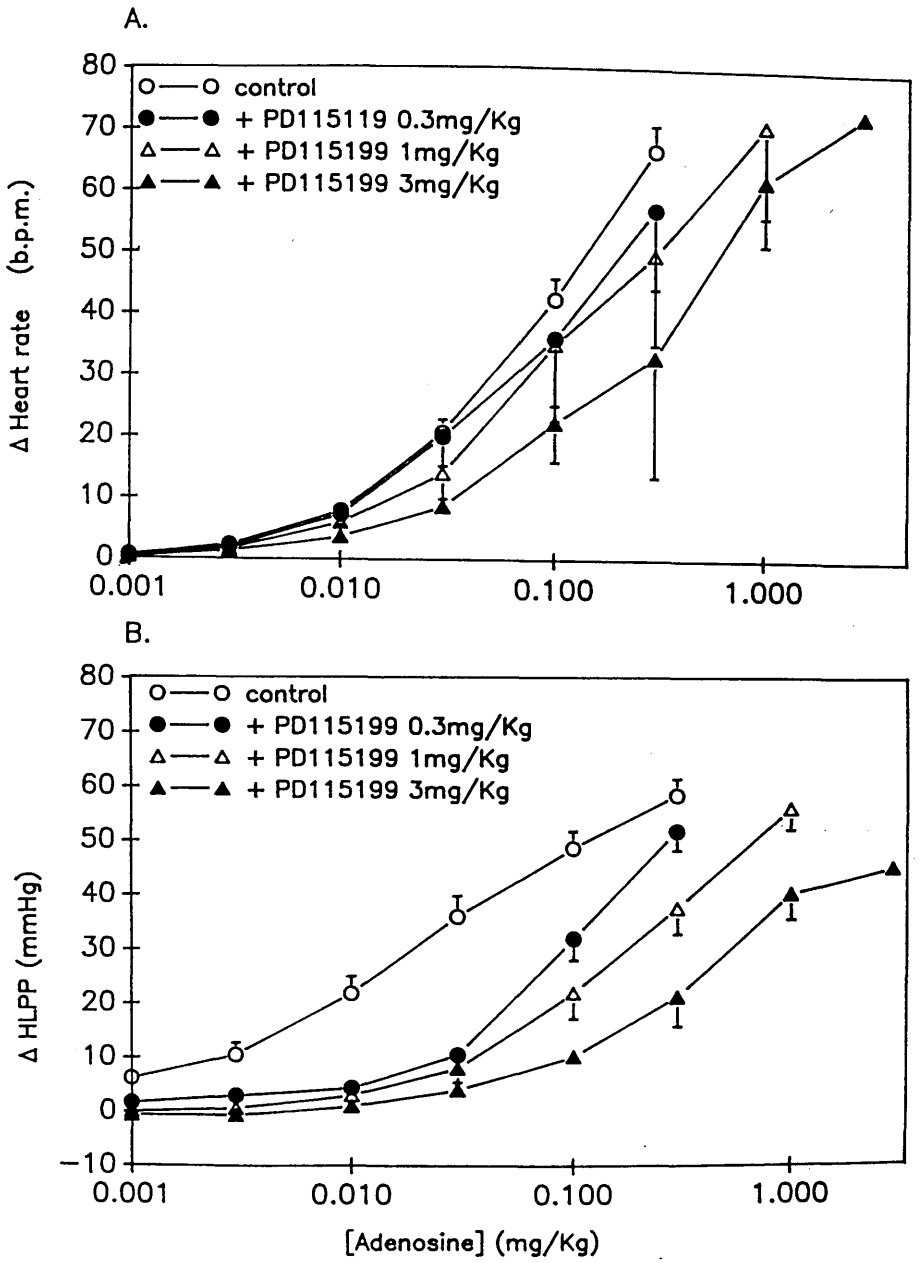


Fig. 45. The effect of PD115199 (0.3-3mg/Kg) on the dose response curves to bolus addition of adenosine (0.001-1mg/Kg) for (A) heart rate (HR) measured as beats per minute (b.p.m.) and (B) hind limb perfusion pressure (HLPP) measured as mmHg, in the unconscious in vivo dog. Number of observations (n) = 3. The adenosine dose response curves for both HR and HLPP, were shifted to the right by PD115199. The DR2 for HLPP was 0.034mg/Kg, compared with a DR2 of 1.37mg/Kg, indicating a selectivity of 40:1 for HLPP : HR.

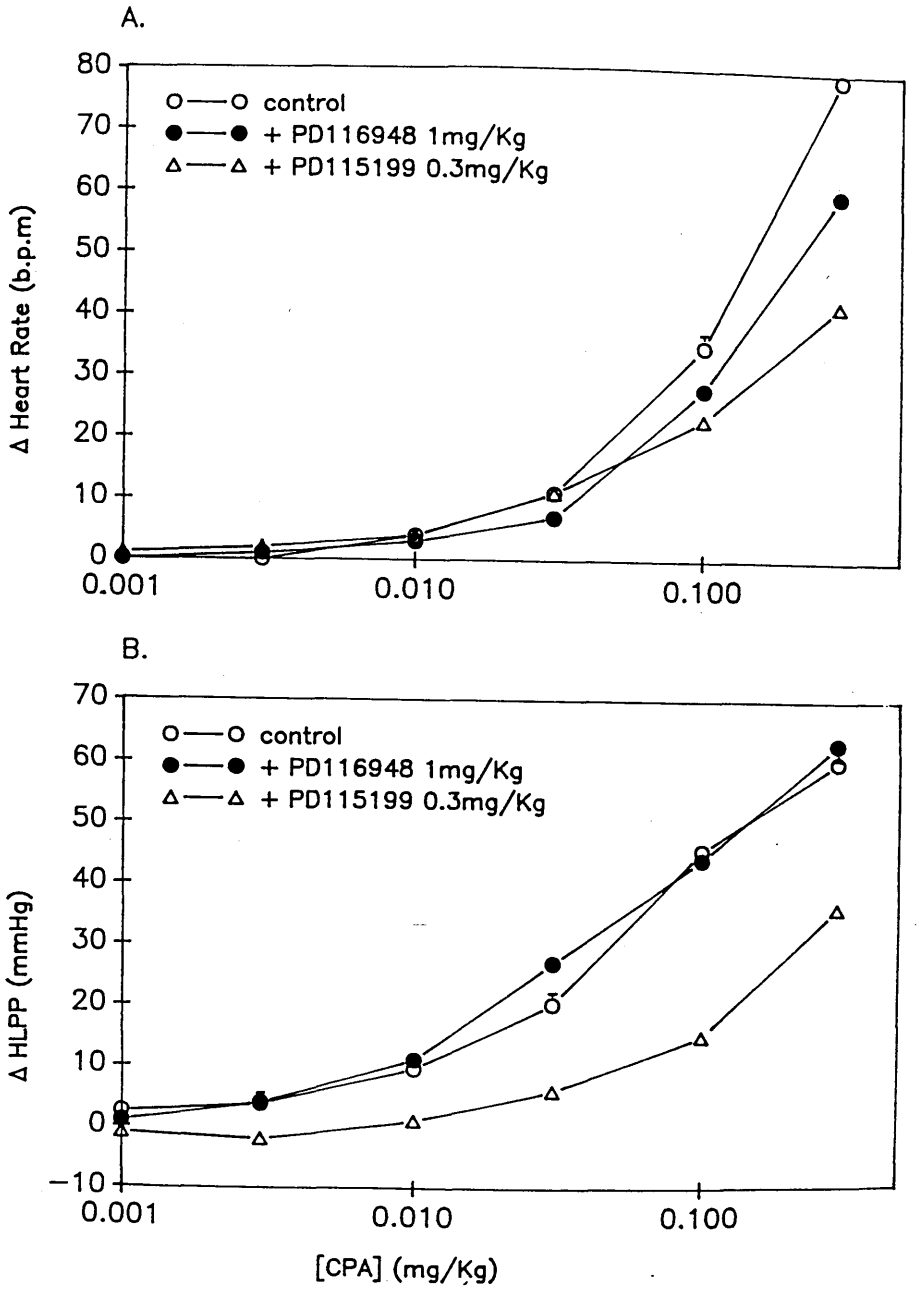


Fig. 46. The effect of PD116948 (1mg/Kg) and PD115199 (0.3mg/Kg) each alone, on the dose response curves to CPA (0.001-1mg/Kg) for (A) heart rate (HR) measured as beats per minute (b.p.m.) and (B) hind limb perfusion pressure (HLPP) measured as mmHg. Number of observation (n) = 2. PD116948 showed little selectivity between the two parameters measured. PD115199 however produced a significant attenuation of the hind limb response in the absence of any effect on the heart rate response to CPA.

46B). PD115199 (0.3mg/Kg) likewise did not alter the response to CPA in the heart (Fig. 46A), but produced a significant attenuation of the HLPP (Fig. 46B). These results suggest that any lack of selectivity exhibited by PD116948 was unlikely to be the result of a mixed receptor population in the heart.

DISCUSSION

CO-TRANSMISSION IN ARTERIAL SMOOTH MUSCLE

In response to the substantial amount of evidence presented over the last 50 years, our understanding of nerve transmission has been greatly altered and widened, to incorporate co-existence, co-release and co-transmission of a variety of biologically active compounds in the fine control of muscle by nerves.

Co-existence - the anatomical co-localization of more than one transmitter substance in peripheral and central neurones, is a widespread phenomenon. Co-release has been demonstrated in a large number of examples, but perhaps the most important control mechanism for cells is that of co-transmission.

Understanding the mechanisms by which multiple co-transmitters act or interact in a single target cell in vitro is important if the role of co-transmitters in the whole animal is to be understood. Previous studies have commonly measured only the contractile response as a means by which to study co-transmitters, with the inherent problems of interpretation that this method involves. This study therefore sought to examine in greater detail, the mechanisms underlying the contraction. The results outlined in this thesis can accordingly be discussed under three categories;

1) Those arising from investigation of the electrical properties of the transmitter by the use of intracellular microelectrode and simultaneous mechanical recording techniques. These techniques allow for a degree of separation of the component parts of co-transmission

as well as giving an indication of the degree of importance of voltage-dependent mechanisms in the actions of each putative transmitter.

2) Those obtained using biochemical measurements, intended to determine the receptor mediated transduction systems utilised by the transmitters.

3) Those obtained from an in vivo investigation of purine (adenosine) receptors in the dog heart and hind limb, obtained from experiments carried out in ICI laboratories.

Electrical and simultaneous mechanical recordings.

The rabbit saphenous artery was chosen for this study, it being a well innervated vascular preparation, which had been previously shown, by mechanical means, to exhibit co-transmission by NA and ATP (Burnstock & Warland, 1987) or a related compound. This artery was easily accessible for dissection, gave reproducible, maintained results and by spiralling one end of the artery for attachment to a force displacement transducer, allowed simultaneous mechanical and electrical recordings to be made. Intracellular microelectrode studies showed the mean resting membrane potential of the rabbit saphenous artery (-69mV) to be similar to that seen in other vascular smooth muscles. e.g. guinea-pig mesenteric artery (-69mV, Ito et al, 1983) and rabbit ear artery (-67mV, Surprenant, 1980). On the basis that the resting membrane potentials were represented by a uniform population, there was no evidence of there being more than one population of cells in this study. The space constant (λ) was relatively short (0.42mm),

comparable with that seen in, for example, rat tail artery (0.41mm, Cassell et al., 1988, see also Holman & Surprenant, 1979) and consistent with the circular orientation of the smooth muscle (Holman & Surprenant, 1979) bundles.

The electrical and simultaneous mechanical results provide strong evidence for co-transmission involving NA and ATP, or a related substance, in the rabbit saphenous artery. There is little doubt that the origin of the transmitters producing these responses was neuronal, since electrical and mechanical events were abolished in the presence of tetrodotoxin (TTX), which abolishes the increase in sodium permeability underlying the nerve action potential. Similarly, all electrical and mechanical events were abolished by removal of Ca^{2+} from the Krebs solution; calcium being required for transmitter release from nerves. In addition, the transmitter substances released were likely to have originated from the same adrenergic nerves, rather than from separate adrenergic and purinergic nerves, since the adrenergic neurone blocking agent guanethidine abolished all electrical and mechanical responses. In addition, the α_2-adrenoceptor antagonist idazoxan potentiated both the electrical and mechanical response, suggesting the presence of α_2-adrenoceptors which control the release of the two transmitters..

Pharmacological evidence confirmed that the transmitters involved in the response to field stimulation in the rabbit saphenous artery were noradrenaline (NA) and ATP, or a related substance. This conclusion was supported by the observation that the mechanical and electrical responses to each component could be separated by the use of antagonists. Thus NA appeared to mediate a part of the contraction, but did not evoke any electrical event, as shown by the ability of the α_1-adrenoceptor antagonist prazosin to abolish some 50% of the contraction. This antagonism occurred in the absence of any effect on membrane potential or on the evoked e.j.p.s, even in the presence of normetanephrine and desmethylimipramine to block both neuronal and non-neuronal uptake of NA. ATP (or a closely related substance) on the other hand was responsible for the entire electrical event (e.j.p.s) as well as the remainder of the contraction, as evidenced by the actions of the P_{2x}-purinoceptor desensitising agent $\alpha\beta$ MeATP. This pattern of co-transmitter responses was similar to that seen in the rabbit mesenteric artery (Lim et al., 1986). The suggestion that ATP mediated the evoked e.j.p.s was further strengthened by the ability of both $\alpha\beta$ MeATP and ATP, applied by close pressure ejection, to mimic the electrical response to nerve stimulation, while, even in the presence of normetanephrine and desmethylimipramine, NA failed to evoke a depolarisation when applied in this manner.

The e.j.p.s mediated by the co-transmitter ATP, were unusually 'peaky' in shape (c/f Holman & Surprenant, 1979) and were associated with contractions. E.j.p.s did not however summate to produce action potentials, in the absence of the potassium channel blocking agent tetraethylammonium (TEA). This is in contrast with other arterial smooth muscles where summation of e.j.p.s reaches the threshold required for action potentials. e.g. rat tail artery (Cassell et al., 1988), rabbit ear and guinea-pig mesenteric arteries (Surprenant, 1980). The lack of action potentials in the absence of TEA resembles the situation in the rat anococcygeus (Creed et al., 1975) and bovine retractor penis (Byrne & Muir, 1984) muscles, where field stimulation likewise produced e.j.p.s associated with contractions while action potentials were initiated in the presence of TEA.

In the rabbit saphenous artery, it seems likely that the upstroke of the action potential evoked in the presence of TEA is carried by calcium current, since these action potentials were abolished by diltiazem which inhibits voltage dependent calcium channels (Beattie et al., 1986).

The question remains as to whether, in the rabbit saphenous artery, TEA unmasks a calcium current which is normally present, but cannot be observed under control conditions, or whether this calcium current is normally absent. The unusual shape of the e.j.p.s would perhaps indicate the presence of 'truncated' action potentials, suggesting that the former explanation is more plausible. Further studies, perhaps using patch clamp techniques would be necessary to confirm this. It seems unlikely however that the e.j.p.s alone account for the contraction, since a calcium component of the e.j.p.s was not unmasked

by the presence of the calcium antagonist diltiazem. This lack of calcium current assosciated with e.j.p.s was in contrast with the situation seen in other arteries, such as the rabbit ear artery (Benham & Tsien, 1987), where ATP activates channels with a degree of selectivity for Ca^{2+} over Na^{+}.

The results with TEA suggest that there is a rectifying potassium current which under control conditions 'aborts' the action potential upstroke. Where however the method of Abe & Tomita (1968) was used to assess the passive membrane properties of the rabbit saphenous artery, the amplitude of the electrotonic potential evoked in this technique was linearly proportional to the stimulus intensity. Thus no rectification was observed over the stimulation parameters used. This may however reflect an inability to sufficiently depolarise the cell membrane using this technique and hence demonstrate this phenomenon (see Holman & Surprenant 1979).

The reduction in the amplitude of the e.j.p.s following reduction or removal of Na^{+} or K^{+}, together with the evidence from the use of TEA, suggests that each of these ions may contribute to the evoked response in this artery. Doubling $[K^{+}]_{o}$ produced a membrane depolarisation as expected. This may explain the reduced amplitude of the e.j.p.s and the potentiated mechanical response. The absence of any significant change in the membrane potential when the tissue is exposed to K^{+} free Krebs was however unexpected. This may reflect a similar mechanism to that found in the guinea-pig taenia coli, where it was proposed (Casteels, 1970) that although K^{+} free Krebs solution increased K^{+} permeability, resulting in a decrease in intracellular K^{+}

* The unexpected depolarisation in response to a decrease in $[Na^+]_o$ may have resulted from a number of factors. Since Cl^- uptake into smooth muscle may be driven by the Na^+ gradient (Brading, 1979; 1980), any reduction in Na^+ might evoke a decrease in $[Cl^-]_i$, resulting in depolarisation. The possibility however that Choline chloride, used to substitute for NaCl, produces a membrane effect cannot be discounted (Lim, 1985).

concentration, this was offset by an accompanying increase in intracellular Na^+ ions, so negating any hyperpolarisation.*

The ability of Cl^- withdrawal to reduce or abolish e.j.p.s may be due to its effect on Na^+ conductance and the Na^+/K^+ ATPase pump.

The experimental evidence supporting both the role of ATP as the transmitter responsible for the e.j.p.s and the conclusion that ATP is acting via P_{2x}-purinoceptors, relies heavily on the use of the ATP analogue α,β-Methylene ATP (αβ MeATP) to selectively desensitise P_{2x}-purinoceptors. As previously described however, there have been a number of criticisms of this compound.

The foregoing results suggest however that αβ MeATP is a suitable selective agent for the study of ATP as a co-transmitter in the rabbit saphenous artery.

In support, αβ MeATP did not alter the responses, either mechanical or electrical to the non-purinergic agonists used, irrespective of whether these responses were mediated via receptors, e.g. 5-hydroxytryptamine (5-HT), histamine and noradrenaline (NA) or via KCl operated, voltage-dependent channels. These results support the view that αβ MeATP was selective for purinoceptors.

In contrast to the situation seen in the chicken rectum (Komori et al., 1988), constant infusion of αβ MeATP in the rabbit saphenous artery resulted in an initial depolarisation which quickly declined, leaving no residual depolarisation. Subsequently, changes in membrane conductance and resistance evoked by square wave hyperpolarising currents were unaffected by the presence of this drug. This implies

the lack of a non-selective membrane effect of αβ MeATP. Similarly, there was no difference in the rate at which the smooth muscle of the rabbit saphenous artery regained responsiveness to exogenously added ATP, or to neuronally released transmitter (e.j.p.s), on washout of αβ MeATP. This suggests that this drug is acting similarly on those areas utilised by both the neuronal transmitter and exogenous ATP, i.e. purinoceptors.

These results demonstrate that αβ MeATP is a suitable agent for blocking the actions of ATP in this tissue.

The alternative antagonist examined in the present study was suramin. This was found to be less suitable than αβ MeATP as an antagonist of P_{2x}-purinoceptors in the rabbit saphenous artery.

Purinoceptors were antagonised by suramin in the absence of an effect on adrenoceptors. There did not appear to be any non-selective, non-receptor mediated effect of this compound, since the post-junctionally mediated contractions to KCl were likewise unaffected by suramin. In contrast to αβ MeATP however, suramin attenuated the responses to some non-purinergic agonists, namely 5-HT and histamine, which may reflect suramin's ability to bind to a large number of proteins (Wills & Wormall, 1950). This relative non-selectivity of suramin for purinoceptors, makes it unsuitable for further use in the study of ATP as a co-transmitter in the rabbit saphenous artery. Since it shows some affinity for purinoceptors, suramin may however provide a starting point for the development of more selective P_2-purinoceptor antagonists. Suramin has also been shown to inhibit the ectonucleotidases responsible for breakdown of

ATP (Hourani & Chown 1989) and this may be reflected in the large doses (10^{-3}M) required to block purinoceptors. It is therefore possible that any use suramin may have in the study of co-transmission will lie in the development of analogues which potentiate the effects of ATP by inhibiting its breakdown.

The apparent inability of NA to produce an electrical event either when released during field stimulation, or on exogenous addition is in contrast to the situation in a number of other vascular tissues. Thus for example, NA released by neural stimulation produces e.j.p.s and action potentials in the guinea-pig pulmonary artery (Suzuki, 1983) and guinea-pig submucosal arteries (Hirst, 1977). In other tissues, e.g. rabbit ear artery (Allcorn et al., 1985), nerve released NA is responsible for only the small slow depolarisation following the main electrical event (e.j.p.). This latter event may be mediated by another putative transmitter. In addition, exogenously added NA can produce depolarisation in a large number of tissues. e.g. rat mesenteric (Mulvany et al., 1982) and guinea-pig ear artery (Kajiwara et al., 1981). In the rabbit saphenous artery, as in the rabbit pulmonary artery (Casteels et al., 1977), NA acting via α-adrenoceptors, does not significantly alter the membrane potential and may act to produce a contraction via voltage-independent mechanisms.

The possibility cannot however be discounted that voltage changes occur in response to NA in the rabbit saphenous artery, but that they cannot be measured by the current technique. Using patch clamp techniques, Amédéé et al. (1990) showed that in the rabbit ear artery,

NA elicited mixed inward and outward currents. The possibility cannot be excluded that in the rabbit saphenous artery, NA evokes equivalent inward and outward currents which result in a net absence of change in membrane potential. This remains to be investigated in this tissue.

There is some controversy regarding the ability of NA to act via voltage-independent mechanisms. Notwithstanding direct evidence for a stimulatory action of NA on phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis (see introduction), some authors have proposed that NA contracts arteries solely by activating voltage-dependent Ca^{2+} channels. e.g. in rabbit mesenteric artery (Nelson et al., 1988). Other authors have however proposed an action of NA on receptor operated Ca^{2+} channels (Bolton & Large, 1986). It seems likely, in view of the conflicting evidence, that NA can act via a variety of mechanisms, depending on the tissue involved and the stimulation parameters.

From examination of the effects of ATP and NA on the membrane potential of the rabbit saphenous artery, it would appear that these co-transmitters act via different mechanisms to evoke contractions. NA appears to act solely via voltage-independent mechanisms in this tissue, while ATP clearly evokes voltage-dependent mechanisms to produce e.j.p.s associated with a contraction.

The fact that ATP produces a depolarisation in this tissue, does not however exclude the possibility that ATP also evokes second-messenger mediated transduction mechanisms, in the production of contraction. Indeed ATP has been shown to stimulate phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis in the rat tail artery (Jenkinson,

1990), in addition to producing e.j.p.s.

The foregoing results, obtained by biochemical measurements, give evidence of the mechanism by which NA may act in the rabbit saphenous artery and suggest that both NA and ATP can evoke production of second-messengers in this tissue.

Biochemical Recordings.

The rabbit saphenous artery was found to be suitable for the study of biochemical pathways, by reason of its robust nature; necessary for the long incubations required to reach isotopic equilibrium during some of these experiments. In addition, this artery was available in suitable quantities for examination. The rabbit saphenous artery is a resistance vessel, likely to be representative of others involved in vascular tone.

Since a number of vascular smooth muscle preparations (see introduction) had previously been shown to act via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), it seemed natural to examine the involvement of this pathway in the production of contraction in the rabbit saphenous artery.

It was clear however from the results obtained that, in the rabbit saphenous artery, NA did not stimulate PIP_2 hydrolysis, as measured by incorporation of myo[^{3}H]-inositol into the water soluble metabolites

of this hydrolysis - the inositol phosphates. This is in contrast to the situation in, for example, the rabbit mesenteric artery - an artery which exhibits a similar pattern of co-transmission in terms of electrical and mechanical events, i.e. the electrical response is mediated by the non-adrenergic component alone, while the contraction is mediated by NA and ATP (Lim et Al., 1986). In the rabbit mesenteric artery, exogenously added NA enhances the breakdown of PIP_2 (Hashimoto et al., 1986).

This result was therefore rather surprising and a number of possible explanations were sought.

The lack of ability of NA to evoke PIP_2 hydrolysis in the rabbit saphenous artery was shown not to be the result of β_2-adrenoceptor stimulation mediating a negative effect on PIP_2 hydrolysis, since pre-incubation with propranolol neither enhanced the contraction to NA nor evoked hydrolysis of PIP_2.

The possibility that the methodology used was in some way deficient was next considered, but no support for this was evident. The rat tail artery, in which exogenous NA has been clearly shown to stimulate hydrolysis of PIP_2 (Fox et al., 1985) was used as a positive control. By comparison with the rabbit saphenous artery, the rat tail evoked a dose dependent rise in inositol phosphates levels, confirming the validity of the method used.

A third possibility was that the lack of PIP_2 hydrolysis evoked by NA was the result of the characteristics of the particular ligand. The lack of hydrolysis of PIP_2 in the rabbit saphenous artery was not due to the absence of the mechanism for this hydrolysis since histamine

evoked a rise in inositol phosphates in this tissue, confirming similar observations in a number of tissues and cell lines (e.g. bovine tracheal smooth muscle, Hall & Hill, 1988).

More significantly, αβ MeATP also evoked a rise in levels of inositol phosphates in the rabbit saphenous artery. αβMeATP was used as a stable analogue of the putative transmitter ATP, since ATP is rapidly degraded to adenosine by 5'-nucleotidase and ATPase. Hence, of the two co-transmitters in the rabbit saphenous artery, NA does not produce a contraction via hydrolysis of PIP_2, while this hydrolysis may be a mechanisms by which ATP acts, in addition to activating voltage-operated channels (VOCs).

It is unlikely that the ATP stimulated PIP_2 hydrolysis is simply consequential to Ca^{2+} entry, since KCl, which elevates $[Ca^{2+}]_i$ by opening voltage-dependent Ca^{2+} channels, did not stimulate PIP_2 hydrolysis in the rabbit saphenous artery.

With the widespread acceptance of PIP_2 hydrolysis as a mechanism mediating contraction in a number of tissues, the ability of histamine and αβMeATP to stimulate this hydrolysis is not perhaps unusual. PIP_2 hydrolysis has indeed been shown to be evoked by a large number of neurotransmitters and hormones (see Berridge, 1984). What is unusual is that NA, which has been shown to stimulate PIP_2 hydrolysis via α-adrenoceptors in a number of tissues (see introduction), including other rabbit tissues, does not do so in the rabbit saphenous artery. The functional reasons for this are not yet known. Clearly however, PIP_2 hydrolysis does not represent a mechanism shared by NA and ATP.

The search for the voltage-independent mechanism underlying the contraction to NA, led to my investigating its effect on other membrane phospholipids in the rabbit saphenous artery.

Evidence that diacylglycerol (DAG) production, albeit not from PIP_2 hydrolysis and hence protein kinase C (PKC) activation was involved in the contractile response, came from the use of the phorbol ester, phorbol 1,2-myristate 1,3-acetate (PMA). PMA enhanced the contractile response to NA. It seemed likely that this was an effect mediated via α-adrenoceptors rather than an indirect effect on contraction, since contractions to KCl were unaffected by PMA. The ability of phorbol esters to modulate α-adrenoceptor responsiveness, by their actions on protein kinase C has previously been shown (Cotecchia et al., 1985). Staurosporine, which has been proposed to act by inhibiting protein kinase C (Tamaoki et al., 1986) did not attenuate the responses to either NA or KCl. Since however subsequent studies have suggested that staurosporine inhibits several protein kinases (Rüegg et al., 1989),

no firm conclusions can be drawn from this result.

An alternative source of DAG (see introduction) is from the hydrolysis of phosphatidylcholine (PC). This hydrolysis may be catalysed by number of phospholipases; by phospholipase C (PLC), leading to phosphocholine and diacylglycerol (DAG); by phospholipase D (PLD), producing choline and phosphatidic acid which is then metabolised to DAG; or by phospholipase A_2 (PLA_2), producing a number of metabolites, including glycerophosphocholine (GPC) and ultimately DAG. The foregoing evidence suggests that in the rabbit saphenous artery, this hydrolysis is implicated in the contraction evoked by a

number of agonists.

PC hydrolysis was estimated from changes in levels of the water soluble metabolites of the hydrolysis, choline (Cho), choline phosphate (ChoP) and glycerophosphocholine (GPC). NA evoked dose dependent rises in both Cho and ChoP, suggesting that NA evoked production of DAG by hydrolysis of this phospholipid and that this may be a mechanism by which NA evoked contraction in the rabbit saphenous artery.

This is the first time, to the best of my knowledge, that PC hydrolysis has been implicated in the response to an agonist in smooth muscle. Furthermore, although in cell lines, a number of agonists have been shown to act via PC hydrolysis, the role of NA - an important neurotransmitter throughout the body - in the hydrolysis of PC has not before been established.

In the majority of examples so far studied, where PC hydrolysis in response to hormones and neurotransmitters exists, this has mostly occurred in conjunction with PIP_2 hydrolysis. This led to much debate as to whether PC hydrolysis occurred as a consequence of PIP_2 hydrolysis. Recently some cell lines have been demonstrated to exhibit PC hydrolysis in the absence of a 'PI response' (e.g. T lymphocytes, Rosoff et al., 1988), thus clearly separating these two phenomena. NA hydrolysis of PC in the rabbit saphenous artery would likewise appear to occur independently of PIP_2 hydrolysis, confirming that these phenomenon are not necessarily linked. This mechanism of action of NA, via hydrolysis of PC in the absence of PIP_2 hydrolysis, is very calcium sparing in that it does not require Ca^{2+} mobilisation either from intracellular or extracellular pools. Utilisation of PC

hydrolysis as a source of DAG was shown not to be unique to NA in this tissue, since histamine and αβ MeATP could similarly stimulate PC hydrolysis, thus both histamine and αβMeATP could each evoke hydrolysis of both PC and PIP_2.

Based on the observation that NA, histamine and αβ MeATP each raised levels of Cho and ChoP but not GPC, it seemed likely that phospholipase D (PLD) and phospholipase C (PLC) but not phospholipase A_2 (PLA_2) were implicated in the hydrolysis of PC in the rabbit saphenous artery (see Pelech & Vance, 1989). The inability of mepacrine, which inhibits PLA_2, to attenuate the mechanical response appeared to confirm the lack of activity of PLA_2 in the contraction. The ability of sodium fluoride (NaF) to inhibit and sodium oleate (NaOl) to enhance the contraction to NA does not give unequivocal evidence of the involvement of PLD, however it perhaps strengthens the evidence that PLD is involved in the hydrolysis of PC in this tissue. The above evidence notwithstanding, the true nature of the phospholipases involved were better indicated by studying the time course of the hydrolysis.

NA does indeed evoke the activity of PLD, as evidenced by an initial large rise in Cho. In addition, PLC activity was also evoked by NA, since the rise in Cho was accompanied by a rise in ChoP. These rises correspond in time to the maximum contractile response to exogenously added NA. The rise in Cho was much greater than the rise in ChoP. This may reflect an ability of the smooth muscle of the rabbit saphenous artery to utilise PLD to a greater extent than PLC in producing contraction. The larger secondary rise in ChoP at around 2-4min, being well after the time for maximum contraction may result from a

secondary activation of PLC, due perhaps to the actions of activated protein kinase C, but is perhaps more likely to be due to phosphorylation of the initially liberated choline. Phosphorylation of Cho by choline kinase, to ChoP has been proposed as part of the cyclical resynthesis of phosphatidylcholine (see Pelech & Vance, 1989). In the absence of a selective inhibitor of choline kinase, this study was unable to further investigate this secondary rise.

Histamine similarly evoked early rises in both Cho and ChoP, however these were more sustained than those seen with NA, perhaps reflecting the slower time course seen for contraction to histamine and the ratio of these metabolites differed. In the presence of histamine, the rise in ChoP was very much larger than the rise in Cho. This implies that in producing a contraction, histamine utilises predominantly PLC, with a smaller contribution from PLD.

A slightly different pattern again was seen with αβ MeATP, which similarly evoked a rise in both Cho and ChoP. Although the maximum level of Cho obtained with αβ MeATP exceeded the maximum level of ChoP, the rise in ChoP was proportionally similar, perhaps indicating an approximately equal reliance on the actions of both PLD and PLC.

Thus in the rabbit saphenous artery, PC hydrolysis would seem to be evoked by a number of agonists, including both putative co-transmitters. The possibility that this represented a level at which these co-transmitters interacted was addressed by examining the effect of a combination of NA and αβ MeATP on levels of Cho and ChoP. The foregoing results show that, while ATP and NA evoke an additive effect, at least in terms of levels of ChoP evoked by a combination of

these drugs, there was no evidence of synergism, the rise in ChoP being approximately equal to the sum of the response to each agonist alone. The picture is less clear with Cho levels, however there is some evidence of an additive effect of these agonists at some doses used.

Although PC hydrolysis and hence presumably DAG production and protein kinase activation, is a level at which some interaction between NA and ATP can be shown, it is unlikely that this alone accounts for the additive effect of neuronally-evoked ATP and NA in producing a contraction in the rabbit saphenous artery. It would appear that NA evokes only one mechanism in the production of contraction, i.e. a rise in PC hydrolysis, while ATP uses a combination of voltage-dependent and voltage-independent post-junctional mechanisms. ATP may open voltage-dependent Ca^{2+} channels and initiate PI-phospholipase C mediated hydrolysis of PIP_2 -both mechanisms for increasing $[Ca^{2+}]_i$ - as well as evoking both phospholipase D and PC-phospholipase C mediated hydrolysis of PC. This, along with PIP_2 hydrolysis, will produce DAG to stimulate protein kinase C and increase the sensitivity of the cell to existing levels of Ca^{2+}.

These mechanisms may act synergistically to produce the total response to ATP, however the possibility also exists that one or more of these mechanism may predominate during different levels of stimulation, perhaps as mechanisms to deal with stressful situations. Similarly, rather than producing independent components of the contraction, ATP and NA may interact in a manner similar to that seen in hepatocytes and other cell lines (see introduction), where

elevation of $[Ca^{2+}]_i$ acts in addition to activation of protein kinase C to produce a maximum response.

In conclusion, the foregoing results suggest that in the rabbit saphenous NA evokes a voltage-independent contraction, mediated via the second messenger DAG. ATP on the other hand produces a contraction associated with e.j.p.s, which may evoke the subsequent opening of voltage-dependent Ca^{2+} channels. In addition, a component of the contraction evoked by ATP may be mediated by voltage-independent mechanisms. These mechanisms are likely to include both hydrolysis of PIP_2 (leading to formation of the second messengers IP_3 and DAG) and, in common with NA, hydrolysis of PC.

The question remains as to the functional significance of co-transmission in neurotransmission. In the rabbit saphenous artery, both transmitters play an approximately equal role in the production of contraction <u>in vitro</u>. This may simply reflect the gradual evolutionary change within neurones, from transmission with one transmitter, to transmission with another, conveniently observed in the rabbit saphenous artery at a midpoint in the transition. Alternatively, there may be a reason why two transmitters are advantageous to the functioning of this artery <u>in vivo</u> and perhaps the answer to this lies in the possibility of synergism between those mechanisms which directly raise intracellular Ca^{2+}. and those which raise the sensitivity of protein kinase C to existing Ca^{2+} levels.

Whatever the likely answer, physiologists and pharmacologists can no longer countenance the simplistic one neurone, one transmitter view of nerve transmission, but instead must view nervous control of muscle as a complex interaction of several transmitter substances, included among which are NA and ATP. In studying contraction in arterial smooth muscle, the degree of complexity of the system goes however beyond the number of transmitters released to encompass a complex arrangement of pre-and post-junctional receptors, coupled to a multiplicity of post-junctional mechanisms by which contraction is mediated. The possible requirements for the large number of pathways proposed, may lie in 'failsafe' mechanisms to cope with stressful conditions. How these interacting mechanisms, are altered in disease states, e.g. hypertension, has yet to be fully established and the role of PC hydrolysis in disorders of this nature has yet to be addressed. This may constitute interesting future research. Only by advances in our knowledge of the events occurring during normal nervous control of muscle will analysis of the nature of abnormalities occurring in disease states become more likely and the physiological role of co-transmitters better understood.

Once fully analysed, the multiple actions of co-transmitters may allow pharmacologists great scope for the development of new means to modify nerve control of muscle and to develop therapeutic regimes with a full understanding of the actions and targets of agents in the manipulation of co-transmitter systems.

AN INVESTIGATION OF THE ADENOSINE RECEPTORS IN HEART AND VASCULATURE OF THE DOG

The foregoing results describe a research project undertaken in ICI, Alderley Edge, England, as part of the SERC CASE award requirement for industrial experience.

The complexity of our understanding of the actions of purines often lies in the number of subtypes of receptors which may exist to mediate drug effects. This work in the canine cardiovascular system, allowed examination of subtypes of the adenosine receptor and suggested that an atypical adenosine receptor may mediate bradycardia in the canine heart.

Hind limb perfusion pressure (HLPP) was used as a measure of vasodilator activity in resistance vessels. In a previous study (Belloni et al, 1988), vasodilation was measured in the coronary vasculature. Clearly however, coronary output would effect measurements of this sort. Accordingly, in the present study, vasodilation in the hind limb was measured under conditions of constant flow; this being unaffected by changes in cardiac output.

By measuring the ability of adenosine and adenosine analogues to reduce HLPP, a rank order of potencies was obtained (NECA > PAA > adenosine > CPA = r-PIA, for abbreviations see methods), which was

consistent with recognised criteria for an A_2 receptor subtype, in particular the A_{2a} and was comparable with the results obtained for other vascular tissues, e.g. bovine coronary arteries (Mustafa & Askar, 1985) and guinea-pig aorta (Collis, 1985). Examination of the canine cardiac receptor mediating bradycardia, revealed however an order of potencies (NECA > adenosine > CPA > r-PIA > PAA), more in keeping with an A_{2b} subtype, than the A_1 type seen in other preparations, such as rat, guinea-pig and rabbit heart (Collis, 1985; Haleen & Evans, 1985). Thus, with the exception of the response to PAA, there was a similar order of potencies observed for adenosine analogues in reducing heart rate (HR) as in producing vasodilation. In addition, PD116948, which has been shown in other preparations to be A_1 selective, (see Collis et al, 1989) has less selectivity for adenosine receptors in the in vivo dog preparation, than the relatively non-selective adenosine antagonist theophylline, where adenosine is added by bolus injection. PD116948 had however, a much greater antagonist effect against adenosine added by infusion, implying that adenosine receptors are not saturated when a bolus addition is used. This however was examined during only one experiment and needs to examined further before strong conclusions can be made.

The possibility existed that the unusual order of potencies and the lack of selectivity of PD116948 was a result of a mixed A_1/A_2 population in canine heart, especially since the A_2 selective agonist PAA could produce a positive chronotropic effect in heart. PD116948 however produced only a small rightward shift of the dose response curve to the relatively A_1-selective agonist CPA, which was not greater than the shift of the adenosine dose response curve produced

by this antagonist. This implied that a mixed receptor population does not mediate the atypical responses to adenosine analogues in the canine heart.

The ability of both PAA and the A_2 selective antagonist PD115199 to distinguish clearly between the receptors in the hind limb, and in the heart, suggests that these receptors differ. The receptor in canine heart does however appear to be atypical and while the greater effect of PD116948 on the bradycardia evoked by adenosine infusion cannot be ignored, it appears nevertheless, that the adenosine receptor in the canine heart displays some of the characteristics of an A_2 receptor, as evidenced by the order of potencies of adenosine and adenosine analogues. It is however distinguishable from a A_{2a} receptor on the basis of evidence from selective A_2 agonists and antagonists. The adenosine receptor in canine heart closely resembles the A_{2b} subtype proposed by Bruns et al. (1986;1987).

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