THE EFFECTS OF DRUGS ON CONDUCTION AND TRANSMISSION IN AUTONOMICALLY-INNERVATED SMOOTH MUSCLE

A thesis presented for the degree of Doctor of Philosophy in the University of Glasgow

by

DAVID THOMAS BEATTIE

Department of Pharmacology,
University of Glasgow.

February 1987

CONTENTS

ACKNOWLEDGEMENTS

I am grateful to Professor J.S. Gillespie, not only for the opportunity to carry out this project in his department, but also for his interest in my work and academic welfare.

To Dr T.C. Muir, I express my sincere appreciation. His supervision throughout this project was constant and his guidance and instruction, so readily given, invaluable. I will always value his friendship.

My sincere thanks to Dr J.R.C. Baird for his supervision during my period of research at Pfizer Central Research. The financial assistance he secured for me and his interest in my work are also appreciated.

I am grateful to Dr T.C. Cunnane for his instruction in the first year of this project.

To Dr Siew Peng Lim, I express special words of thanks, not only for sharing her expertise in microelectrode recording and collaborating in some of the radioisotope experiments, but also for her inexhaustible enthusiasm for research, which was a constant stimulus to me. Her words of comfort during the writing of this thesis are much appreciated. I value her friendship very highly.

I also acknowledge the following, who were, at various times during the last three years, my colleagues: Archibald McNicol and Mark Bushfield, who explained the complexities of the 'PI Cycle' to me, Anita MacDonald, Adriano Rossi, Linda MacMillan and Kay Wardle. To Jane Paterson, Kathy Bevan and Maria Hawkins, I express my gratitude for their help during my period with Pfizer.

I am grateful for the assistance provided by members of the technical staff , in particular Mr John Craig and Mr Trevor Clark. Thanks to Mr Jim Sinclair for repairing any electrical apparatus which I managed to break and to the staff of the Medical Illustration Unit for their preparation of the figures.

My sincere thanks to Mrs Marjory Wright for her valuable library assistance and to Mrs Lorna Peedle for her skilful typing of this thesis.

Finally, I acknowledge the unfailing support of my parents. Their assistance in the preparation of this thesis is also appreciated.

PUBLICATIONS

Several aspects of the work described in this thesis have been published.

Beattie, D.T., Cunnane, T.C. & Muir, T.C. (1984). Effects of Ca^{2+}-entry blockers on conduction in preterminal nerves in the guinea-pig vas deferens. In: Proceedings 9th IUPHAR Congress of Pharmacology, 1643P.

Beattie, D.T., Cunnane, T.C. & Muir, T.C. (1985). Effects of Ca^{2+}entry blockers on spontaneous and electrically-evoked release of transmitter from guinea-pig vas deferens nerves. Br. J. Pharmac., 84, 29P.

Beattie, D.T., Lim, S.P. & Muir, T.C. (1985). The accumulation and release of $[^3H]$ -adenine nucleotides by nerves in the guinea-pig internal anal sphincter. Digest. Dis. Sci., 30, 759P.

Beattie, D.T., Clark, K.L., Lim, S.P. & Muir, T.C. (1986). Action potential conduction and transmitter release from non-adrenergic, non-cholinergic nerves: effects of Ca^{2+}-antagonists. Br. J. Pharmac., 88, 252P.

Beattie, D.T., Cunnane, T.C. & Muir, T.C. (1986). Effects of calcium channel antagonists on action potential conduction and transmitter release in the guinea-pig vas deferens. Br. J. Pharmac., 89, 235-244.

Baird, J.R.C., Beattie, D.T. & Muir, T.C. (1986). Effects of Ca^{2+} channel antagonists on action potential conduction in the guinea-pig hypogastric nerve *in vivo*. In: Proceedings Br. Pharmac. Soc. December, P108.

SUMMARY

1. The prejunctional actions of a number of Ca^{2+} channel antagonists were investigated. Their effects were assessed on both nerve action potential (AP) conduction and neuromuscular transmission in the vas deferens and internal anal sphincter (i.a.s.) of the guinea-pig and in the rat anococcygeus. Nerve APs were recorded extracellularly with a suction electrode and transmitter release measured by intracellular electrical recording of smooth muscle junction potentials and analysis of the overflow of radiolabelled transmitter.

2. In high concentrations, verapamil ($0.5 - 2 \times 10^{-4}$M), diltiazem ($1 - 8 \times 10^{-4}$M), amlodipine ($0.5 - 2 \times 10^{-4}$M), nicardipine ($0.5 - 1 \times 10^{-3}$M) and cobalt ($4 - 6 \times 10^{-2}$M), each inhibited stimulation (0.5 Hz, 0.05 - 0.5 ms, supramaximal voltage) -evoked APs in the hypogastric nerve fibres running to the guinea-pig vas deferens *in vitro*. Nifedipine ($1 - 5 \times 10^{-3}$M) was ineffective. It is possible that at these high concentrations, inhibition of APs was achieved by a local anaesthetic action, involving Na^{+}, rather than Ca^{2+} channel blockade. The APs were abolished by tetrodotoxin (TTX, 1×10^{-6}M) and lignocaine ($0.5 - 2 \times 10^{-3}$M). However, AP amplitude was reduced following Ca^{2+}-withdrawal from the perfusing Krebs solution, suggesting that the antagonists may block a Ca^{2+} component in the AP.

3. APs, in response to stimulation (single pulses, 0.1 - 0.5 ms, supramaximal voltage), were inhibited *in vitro* by verapamil ($0.5 - 2 \times 10^{-4}$M), nifedipine ($0.5 - 1 \times 10^{-4}$M) and cobalt ($0.5 - 1.5 \times 10^{-2}$M) in the genito-femoral nerve fibres running

to the rat anococcygeus. As in the hypogastric nerve, the antagonists may have inhibited AP conduction by blocking Na^+ and/or Ca^{2+} channels.

4. The significance of the ability to block AP conduction *in vitro* was examined by studying *in vivo*, in the anaesthetised guinea-pig, the effects of the organic compounds on AP conduction compared with those on the cardiovascular system. Verapamil (0.01 - 0.3 $mgkg^{-1}$), diltiazem (0.01 - 0.3 $mgkg^{-1}$) and amlodipine (0.1 - 1.6 $mgkg^{-1}$), when given i.v., each failed to affect stimulation (0.2 Hz, 0.1 - 0.5 ms, supramaximal voltage)-evoked APs, recorded from the hypogastric nerve fibres running to the vas deferens, at a time when blood pressure and heart rate were significantly depressed. Verapamil (1 - 3 $mgkg^{-1}$), injected close-arterially, however, inhibited the APs. The inhibition produced by verapamil was probably not due to an action on conduction itself, but rather to the prevention of preganglionic transmitter release. This was suggested by the ability of acetylcholine (0.01 mg i.a.) to produce a spontaneous postganglionic AP discharge during blockade by verapamil (3 $mgkg^{-1}$ i.a.).

5. Postganglionic transmitter release, as measured *in vitro* by intracellularly-recorded junction potentials, and mechanical responses, were inhibited in each tissue by the Ca^{2+} channel antagonists.

The excitatory junction potentials (e.j.ps) and contractions in the guinea-pig vas deferens, evoked by field stimulation (5-20 pulses at 1 and 2 Hz, 0.1 - 0.5 ms, supramaximal voltage),

were inhibited by verapamil (1 - 2 x 10^{-4}M), diltiazem (1 - 5 x 10^{-4}M), amlodipine (0.5 - 1 x 10^{-4}M) and cobalt (1 x 10^{-3}M). Nifedipine (5 x 10^{-4}M) failed to affect the e.j.ps, but inhibited the contractions.

The e.j.ps and contractions in the rat anococcygeus, in response to extrinsic nerve stimulation (5 pulses at 5-20 Hz, 0.5 ms, supramaximal voltage), were inhibited by verapamil (0.1 - 5 x 10^{-5}M), nifedipine (0.01 - 1 x 10^{-4}M) and cobalt (0.5 - 2 x 10^{-3}M).

The inhibitory junction potentials (i.j.ps) produced by field stimulation (single pulse and 5 pulses at 5 and 10 Hz, 0.5 ms, supramaximal voltage), the tone of the guinea-pig i.a.s. and the spontaneous spike discharge were each inhibited by verapamil (0.01 - 1 x 10^{-4}M), diltiazem (0.05 - 5 x 10^{-4}M), nifedipine (0.01 - 5 x 10^{-4}M) and cobalt (1 x 10^{-3}M).

The concentrations of the organic agents required to inhibit the junction potentials in each tissue were similar to those which blocked nerve AP conduction, suggesting that this action was responsible for the inhibition of release. Cobalt, on the other hand, inhibited release at a concentration which failed to affect AP conduction. Thus, cobalt, unlike the organic antagonists, appeared to prevent release by acting at or near the nerve terminals. Each organic agent blocked, postjunctionally, the responses of the tissues in preference to those prejunctionally; the mechanical responses of each preparation and the spontaneous spike discharge in the i.a.s. were inhibited when release was little affected. Cobalt was

equally effective pre- and postjunctionally.

6. The effects of the antagonists on transmitter release were also measured directly by radiochemical analysis in the vas deferens and i.a.s. of the guinea-pig.

Stimulation (400-2000 pulses at 20 Hz, 0.5 ms, supramaximal voltage)-evoked ^{3}H overflow from the nerves of the vas deferens, incubated with $[^{3}H]$ -adenosine, was inhibited by the antagonists at similar concentrations to those which blocked the junction potentials. Superfusion with either diltiazem ($5 \times 10^{-4}M$) or cobalt ($2 \times 10^{-3}M$) prevented ^{3}H overflow. Nifedipine ($5 \times 10^{-4}M$), ineffective on nerve AP conduction and e.j.ps, also failed to affect ^{3}H overflow.

In the vas deferens incubated with $[^{3}H]$ -noradrenaline, the stimulation (60 pulses at 1-20 Hz, 0.5 ms, supramaximal voltage) - evoked ^{3}H overflow was inhibited by cobalt ($2 \times 10^{-3}M$). Superfusion with either verapamil ($0.1 - 1 \times 10^{-4}M$), diltiazem ($0.5 - 1 \times 10^{-4}M$), amlodipine ($0.05 - 1 \times 10^{-4}M$) or nifedipine ($1 - 5 \times 10^{-4}M$) increased markedly the resting overflow of ^{3}H, such that any effect of stimulation could not be easily determined. This drug-induced release of ^{3}H consisted mainly of the noradrenaline metabolite, 3,4-dihydroxyphenylglycol (DOPEG) and originated from the sympathetic nerves independently of extracellular Ca^{2+}. The effect may have been due to either a reserpine- or tyramine-like action.

Although the identity of the non-adrenergic, non-cholinergic (NANC) transmitter in the guinea-pig i.a.s. is unknown, results from electrical studies have suggested that adenosine 5'-triphosphate (ATP) is a strong possibility (Lim & Muir, 1986).

Following incubation of the i.a.s. with $[^3H]$ -adenosine, field stimulation (900 pulses at 2-20 Hz, 0.5 ms, supramaximal voltage) of the NANC nerves produced an overflow of ^3H. The overflow had a neural origin; it was prevented by TTX (1×10^{-6}M) and Ca^{2+}-withdrawal from the superfusing medium, but unaffected by paralysis of the muscle with verapamil (1×10^{-6}M). Furthermore, sodium nitroprusside (1×10^{-4}M), a directly-acting muscle relaxant, failed to affect the resting ^3H overflow. Superfusion with verapamil (1×10^{-4}M) blocked the overflow of ^3H in response to stimulation.

7. The possibility that ATP is indeed the NANC transmitter in the guinea-pig i.a.s. was investigated. Chromatographic separation of the $[^3H]$ -compounds in the stimulation (2400 pulses at 20 Hz, 0.5 ms, supramaximal voltage)-evoked overflow from the i.a.s., incubated with $[^3H]$ -adenosine, however, showed that only hypoxanthine, adenosine 5'-monophosphate (AMP) and adenosine 3',5'-cyclic monophosphate (cAMP) were present. The existence of these compounds may have been the result of ATP metabolism. The luciferase-luciferin assay failed to detect any release of intact ATP from the i.a.s., following field stimulation (100-1000 pulses at 10 and 20 Hz, 0.2 ms, supramaximal voltage). In the vas deferens, ATP release was detected following field stimulation (200-2000 pulses at 10 and 20 Hz, 0.2 ms, supramaximal voltage), but was associated with the contraction of the muscle <u>per se</u>; it was inhibited when the muscle was paralysed selectively with sucrose (0.37M).

The failure to detect neuronally-released ATP in the vas deferens and the i.a.s. possibly indicated that it was being metabolised, if indeed this substance is the NANC transmitter in both tissues.

8. It is concluded that organic Ca^{2+} channel antagonists act prejunctionally, albeit in high concentrations, to inhibit both nerve AP conduction and transmitter release in autonomically-innervated tissues. It is likely that release is inhibited by failure of the AP to reach the release sites. A local anaesthetic action may be involved. The organic agents block selectively the postjunctional responses in smooth muscle, implying that the Ca^{2+} channels at this site differ from those prejunctionally. Cobalt, in contrast to the organic antagonists, blocks release at the same time as it prevents the postjunctional responses, but before affecting AP conduction.

CHAPTER 1: INTRODUCTION

THE ROLE OF Ca^{2+}

Ca^{2+} is of fundamental importance in the regulation of many biological processes, including contraction of cardiac, smooth and skeletal muscle (Bolton, 1986; Ebashi & Endo, 1968; Herzig, 1985), secretion of transmitters and hormones (Katz & Miledi, 1965; Rubin, 1970), generation and conduction of nerve and muscle action potentials (Bolton, 1986; Hagiwara, 1973; Hagiwara & Byerly, 1981; Tomita, 1981) and activation of enzymes (Cohen, 1982). It is not surprising, therefore, that drugs which interfere with the Ca^{2+} economy of cells should have profound biological effects. This thesis is concerned with one such group of drugs, the Ca^{2+} channel antagonists. Of particular interest is the actions of these agents on autonomic neuromuscular transmission. To appreciate why the antagonists should affect this process, our understanding of the precise role of Ca^{2+} in autonomic neuromuscular transmission should be defined. For convenience, the involvement of Ca^{2+} will be discussed, first in terms of its prejunctional neuronal actions and then with respect to its postjunctional effects in smooth and cardiac muscle.

AT PREJUNCTIONAL SITES

(a) In the nerve action potential

Elucidation of the role of sodium (Na^+) and potassium (K^+) ions as the predominant carriers of the current underlying the nerve action potential (AP, Hodgkin & Huxley, 1952) was soon followed by evidence for an involvement of Ca^{2+} (for reviews

see Hagiwara, 1973; Hagiwara & Byerly, 1981; Kostyuk, 1981). It is now clear that in some nerves, Ca^{2+}, usually in conjunction with Na^{+}, can carry part of the inward current of the AP, although to varying extents in different species. A basic requirement for the involvement of Ca^{2+} is that channels in the neuronal membrane are activated and allow its entry into the cell.

A role for Ca^{2+} in the nerve AP, as in other cell functions, is biophysically 'plausible' due to its large electrochemical gradient across the plasma membrane. The extracellular concentration of Ca^{2+} ($[Ca^{2+}]_o$) is high (in the millimolar range, Blaustein, 1974), while that, free (i.e. unbound) within the cell ($[Ca^{2+}]_i$), is low (approximately 10^{-7}M, Schweitzer & Blaustein, 1980), due to Ca^{2+} uptake into organelles (mainly the mitochondria and endoplasmic reticulum) and into systems which bind divalent cations (Baker, 1972, 1976).

The importance of Ca^{2+} in the nerve AP varies among different regions of the neurone, possibly reflecting the relative densities of Ca^{2+} and Na^{+} channels at each site. In the nerve cell body, for example, Ca^{2+} contributes significantly to the generation of the AP of a variety of species, including the frog (Koketsu et al., 1959), chick (Dichter & Fischbach, 1977), mollusc (Oomura et al., 1961) and pigeon (Llinás & Hess, 1976). Ca^{2+} involvement has also been identified in mammalian nerve cell bodies, e.g. mouse dorsal root ganglion (Matsuda et al.

1976, 1978; Ransom & Holz, 1977) and neuroblastoma cells (Spector et al., 1973; Moolenaar & Spector, 1979) and Auerbach's plexus of the guinea-pig small intestine (Hirst & Spence, 1973; North, 1973). The precise function of these Ca^{2+} currents in the nerve cell body is unclear. They may participate, probably together with Na^{+}, in initiating the propagating AP in the axon and also in metabolic processes within the cell (Kostyuk, 1981), such as cyclic nucleotide synthesis (Rasmussen & Goodman, 1977; Schultz et al., 1973) and protein kinase activity (Greengard, 1979; Schulman & Greengard, 1978).

Ca^{2+} currents also exist in the non-terminal portion of the nerve axon, although, unlike those in the cell body, generally do not carry a significant amount of charge in comparison to that carried by Na^{+} (Hodgkin & Keynes, 1957). Ca^{2+} involvement has been identified in the axons of several species, e.g. the squid (Hodgkin & Keynes, 1957), snail (Eckert & Lux, 1977), barnacle (Edgington & Stuart, 1979) and Helix (Akaike et al., 1978), as well as in the postganglionic neurones of the rat superior cervical ganglion (McAfee & Yarowsky, 1979; Horn & McAfee, 1980).

Powerful Ca^{2+} currents have been demonstrated, however, in the terminal regions of nerve axons in the frog (Ito & Komatsu, 1979), lobster (Kawai & Niwa, 1980) and chick (Dunlap & Fischbach, 1978). It has been proposed, on the basis of the inhibitory action of removal of $\left[Ca^{2+}\right]_o$ and addition of the inorganic Ca^{2+} channel antagonist, manganese, that Ca^{2+} may also be involved in the APs of the guinea-pig hypogastric nerve

fibres (Cunnane & Stjärne, 1984). Ca^{2+} currents are stronger in the terminals than in the axon, presumably reflecting the role of Ca^{2+} in transmitter release (see next section), in addition to its ability to carry the charge of the propagating AP at this site (Hagiwara & Byerly, 1981; Kostyuk, 1981).

From the large number of experiments claiming to have identified neuronal Ca^{2+} currents, several criteria have been proposed which require to be satisfied to establish a role for Ca^{2+} in the AP (Hagiwara, 1973; Hagiwara & Byerly, 1981). Thus, the AP should persist in the presence of tetrodotoxin (TTX), which selectively inhibits Na^{+} influx and following substitution of $[Ca^{2+}]_o$ with barium or strontium, but should be inhibited by inorganic Ca^{2+} channel antagonists such as manganese, cobalt and lanthanum. The overshoot or maximum rate of rise of the AP should be unaffected by replacing Na^{+} with an organic cation such as $tris^{+}$, but vary directly with $[Ca^{2+}]_o$ and the amount of Ca^{2+} entering should be measurably sufficient (e.g. by radiochemical analysis) to charge the membrane to the potential reached by the spike.

(b) <u>In transmitter release</u>

Ca^{2+} is vital in transmitter release (Blaustein, 1979; Douglas, 1974; Rubin, 1970). An influx of Ca^{2+} into the nerve terminal, following arrival of the AP, provides the signal for the release of transmitter into the synaptic cleft (Rubin, 1970).

Studies supporting a role for Ca^{2+}, based primarily on

the effects of altering $[Ca^{2+}]_o$, have involved both direct measurement of release by the assay of tissue effluent (radiochemically or spectrofluorimetrically) following nerve stimulation, and an indirect measure of effector responses. Thus, it has been demonstrated that Ca^{2+} causes release of transmitter from nerves in all species, both primitive and developed. Of particular interest is the role of Ca^{2+} in transmission from sympathetic and parasympathetic nerves, where the release of noradrenaline, acetylcholine or the non-adrenergic, non-cholinergic (NANC) transmitter (or transmitters) is produced.

$[Ca^{2+}]_o$-dependent noradrenaline release has been demonstrated directly in the rabbit heart (Hukovič & Muscholl, 1962; Löffelholz, 1967), cat spleen (Kirpekar & Misu, 1967; Kirpekar & Wakade, 1968), guinea-pig vas deferens (Nakazado & Onada, 1980) and rat atria (Keenan & Callanan, 1980). Acetylcholine release in the cat superior cervical ganglion (Douglas et al., 1961; Harvey & MacIntosh, 1940), guinea-pig intestine (Gerhards et al., 1964) and rabbit sinoatrial node (Vincenzi & West, 1965) has also been shown to be dependent on $[Ca^{2+}]_o$.

Postsynaptic potentials, recorded electrophysiologically, provide an indirect measure of transmitter release (Bennett, 1973; Bennett & McLachlan, 1972; Elmquist & Quastel, 1965; Holman & Weinrich, 1975). These have been used to show a $[Ca^{2+}]_o$-dependent release in sympathetic nerves of the vas deferens of the guinea-pig (Kuriyama, 1964) and mouse (Einstein & Lavidis, 1984) and in parasympathetic terminals of the chick ciliary ganglion (Martin & Pilar, 1964) and squid stellate

ganglion (Charlton et al., 1982). The demonstration that removal of $[Ca^{2+}]_o$ reduces the mechanical responses of the rabbit ileum (Burn & Gibbons, 1964), atria (Burn & Gibbons, 1965) and ear artery (Farmer & Campbell, 1967) to nerve stimulation but not to agonist addition has been suggested to provide further, indirect, evidence for Ca^{2+} involvement in release. A requirement for Ca^{2+} has also been demonstrated in NANC transmitter release in the guinea-pig small intestine (Holman & Weinrich, 1975), urinary bladder (Burnstock et al., 1978) and internal anal sphincter (Lim, 1985).

Notwithstanding its importance, the precise role of Ca^{2+} in release is unclear. Ca^{2+} conductance in the nerve terminal increases when depolarization, produced by the AP or K^+ addition, reaches a critical level (Wakade & Wakade, 1982). 'Voltage-operated' channels (V.O.Cs), so-called because they are opened by depolarization, allow Ca^{2+} influx. Ca^{2+}, entering the terminal down its electrochemical gradient (McGraw et al., 1982), triggers a burst of transmitter release (Llinás & Nicholson, 1975; Miledi, 1973; Miller, 1985) by exocytosis of the vesicle contents (Douglas, 1974). The release is transient and declines within 1-2 ms (McGraw et al., 1982), probably due to an abrupt fall in $[Ca^{2+}]_i$ (Katz & Miledi, 1968) by buffering or sequestratio (McGraw et al., 1982). The link, however, between Ca^{2+} entry and transmitter release remains obscure. Ca^{2+} may trigger exocytosis by neutralizing opposing electrostatic forces between the vesicle and cell membranes (Blioch et al., 1968), or by facilitating fusion of the granule with lipids in the

plasma membrane (Blaschko, 1967). It is unlikely, however, that Ca^{2+} acts directly on the vesicle (Rubin, 1970), having little effect on isolated secretory granules (Banks, 1966; Hillarp, 1958). An intermediate energy-requiring step, possibly involving the splitting of ATP may be responsible (Kirpekar *et al.*, 1970; Rubin, 1970). ATPase is located in vesicle fractions (Germain & Proulx, 1965) and ATP together with Ca^{2+} produces a more consistent release from isolated vesicles (Poisner & Trifaró, 1967). Theories of an interaction of Ca^{2+} with calmodulin (De Lorenzo, 1981), synapsin I (Miller, 1985), adenosine 3',5'-cyclic monophosphate (cAMP, Geschwind, 1969), prostaglandins (Ramwell *et al.*, 1966) and lysolecithin (Blaschko, 1967) have been introduced as the step linking Ca^{2+} entry to release. Elucidation of the mechanism involved requires further work.

AT POSTJUNCTIONAL SITES

The involvement of Ca^{2+} in postjunctional cellular activity was first suggested by Ringer (1883), who demonstrated that the ion was required to maintain the beating of the frog heart *in vitro*. In subsequent experiments, Ca^{2+}, in addition to its involvement in muscle contraction was shown to 'stabilize' the membranes of excitable cells. Addition of Ca^{2+} to a bathing saline (NaCl) solution reduced evoked contractions of the frog sartorius muscle (Mines, 1908). This action of Ca^{2+} was not restricted to skeletal muscle; egg membranes disrupted by needle puncture were repaired only in the presence of $[Ca^{2+}]_o$ (Heilbrunn, 1943), while sheep and calf Purkinje fibres were

inactivated when $[Ca^{2+}]_o$ was increased four-fold (to 10.4×10^{-3}M, Weidmann, 1955).

The term 'stabilization', used to define these effects of Ca^{2+}, was, of necessity, vague; the mechanisms involved were imprecisely understood. Stabilization suggested a resistance to stimulation on the part of the biological membrane being investigated. Membranes which had been stabilized had the following characteristics:-

(a) stronger currents were required for their stimulation,

(b) spontaneous activity in them was slowed or suppressed and

(c) conduction of impulses in them, blocked (Weidmann, 1955).

While the presence of Ca^{2+} stabilized biological membranes, removal of the cation produced an increase in excitability (or 'labilization'). Thus, spontaneous fibrillations were observed following withdrawal of $[Ca^{2+}]_o$ in both frog sartorius muscle (Adrian & Gelfan, 1933; Kuffler, 1944) and in sheep and calf Purkinje fibres (Weidmann, 1955).

Modern technology and the growth in knowledge have led to the replacement of the terms 'stabilizer' and 'labilizer' by more precise descriptions of the actions of Ca^{2+}. Current views on the biological activity of the cation postjunctionally have emphasised, rather than diminished, its importance. They have been obtained largely from two types of investigation :- (a) those which have examined, electrophysiologically, its role as a mediator of membrane conductance, and (b) those in which the availability of Ca^{2+} has been linked to biochemical membrane transduction mechanisms. Together, the results obtained from

each type of investigation provide the basis of our understanding of how Ca^{2+} is involved in many cellular functions. Thus, in muscle contraction, hormone secretion and enzyme activation, a rise in $[Ca^{2+}]_i$, which provides the ultimate signal for many processes, is achieved by a trans-membrane Ca^{2+} current, acting alone or in conjunction with a biochemical mobilization of Ca^{2+} from within the cell. It is likely that the relative importance of these electrophysiological and biochemical components varies in different tissues and in different types of cellular responses. Attempts to measure, simultaneously, their individual contribution to responses in the same cell, have been largely unsuccessful to date. Until this is achieved, it is reasonable to discuss them separately.

(a) Ca^{2+} as a mediator of membrane conductance

An influx of Ca^{2+} is largely responsible for providing the current which underlies or controls the excitability of both smooth and cardiac muscle (Bolton, 1986; Hagiwara & Byerly, 1981; Holman & Neild, 1979; Hurwitz, 1986; Reuter, 1973; Trautwein, 1973). Ca^{2+} currents are involved in most types of electrical activity observed. This applies to the resting membrane potential and to the action potentials (APs), whether spontaneous or evoked, and 'slow wave' depolarizations which may contribute to contractile responses of smooth muscle (Bolton, 1986; Casteels, 1981; Reuter, 1973, 1985; Tomita, 1981; Trautwein, 1973).

For example, the presence of Ca^{2+} in the extracellular fluid makes an important contribution to the maintenance of

the resting membrane potential of cells (Casteels, 1981). An increase in $[Ca^{2+}]_o$ hyperpolarizes and a decrease depolarizes the membrane potential in, for example, the guinea-pig vas deferens (Bennett, 1967) and taenia coli (Brading et al., 1969) and the rabbit main pulmonary artery (Casteels et al., 1977). These effects do not depend directly on the Na^+-K^+ pump; the hyperpolarization with increased $[Ca^{2+}]_o$ occurs in the presence of ouabain (Casteels, 1981). However, Na^+ is involved; the depolarization following reduction of $[Ca^{2+}]_o$ depends on its presence in the extracellular fluid (Brading et al., 1969). An interference with a Na^+-Ca^{2+} exchange process (Bolton, 1986), which allows the transportation of these cations across the plasma membrane in either direction, may be responsible for the alterations in membrane potential produced by changing $[Ca^{2+}]_o$.

Ca^{2+} also contributes significantly to the active electrical responses of tissues. Inward Ca^{2+} currents, which raise $[Ca^{2+}]_i$, are associated with contraction in both smooth and cardiac muscle and the secretion of hormones from glands. In smooth muscle, Ca^{2+} currents are responsible for the generation of APs, whether spontaneous or evoked, in tissues as diverse in function as the taenia coli, portal vein, vas deferens, stomach, myometrium and ureter (Bennett, 1967; Bülbring & Tomita, 1970; Holman, 1957; Kuriyama, 1970; Kuriyama et al., 1971; Kuriyama & Tomita, 1970; Tomita, 1975). The current underlying the 'slow wave' depolarizations, characteristic of some (particularly gastrointestinal) smooth muscles, is also carried by Ca^{2+} in,

for example, the cat, dog and guinea-pig stomach (Tomita, 1981). Ca^{2+} is not the only ion involved, however, in 'slow waves'; Na^{+} together with Ca^{2+} carries the current underlying the depolarizations in the stomach of the cat and dog (El-Sharkawy et al., 1978; Papasova et al., 1968) and the jejunum of the rabbit (El-Sharkawy & Daniel, 1975).

Ca^{2+} also carries the current underlying the depolarization of the plateau component of the cardiac AP. The other, sharp spike-like component, which precedes the plateau, is the result of an inward movement of Na^{+}, rather than Ca^{2+} (Reuter, 1973, 1985; Trautwein, 1973; Weidmann, 1974). Ca^{2+} currents are also responsible for excitation at the sinoatrial and atrioventricular nodes (Noma et al., 1980a,b) and for the conduction, rhythmicity and excitability of Purkinje fibres (Reuter, 1967).

It has been proposed that Ca^{2+} carries inward current into the cell by passing through V.O.Cs. The influx of Ca^{2+} through these channels is sensitive to the Ca^{2+} gradient across the membrane, unaffected by TTX and inhibited by inorganic Ca^{2+} channel antagonists, such as manganese and cobalt. Barium and strontium can replace Ca^{2+} as carriers of the inward current (Bülbring & Tomita, 1970; Hotta & Tsukui, 1968; Kohlhardt et al., 1973; Nayler & Grinwald, 1981; Nonomura et al., 1966). It is also apparent that in cardiac muscle the V.O.Cs open and close slowly, relative to the fast Na^{+} channels and are operational over a different voltage range (between -45 and -25 mV for Ca^{2+} and -80 and -50 mV for Na^{+}).

V.O.Cs are opened by membrane depolarization. This may be achieved by the pacemaker activity characteristic of some tissues, e.g. the guinea-pig taenia coli (Brading et al., 1969) and internal anal sphincter (Lim, 1985; Lim & Muir, 1985). In general, however, the depolarization which opens the V.O.Cs to allow Ca^{2+} influx, arises from a different population of channels. In cardiac muscle, it is the depolarization created by the initial Na^{+} influx which is responsible, while in many smooth muscles and hormone-secreting glands, so-called 'receptor-operated' channels (R.O.Cs) may be involved. These R.O.Cs are opened by agonist/receptor combination independently of membrane potential (Bolton, 1979, 1986) and, together with Ca^{2+}, may admit other small ions (Bolton, 1979). The R.O.Cs are presumably responsible for generation of the e.j.ps observed in some smooth muscles, e.g. in the guinea-pig vas deferens (Burnstock & Holman, 1961). If the e.j.ps reach a sufficient level of depolarization, the V.O.Cs are opened, Ca^{2+} enters the cell and in so doing, carries the current underlying the AP (Bolton, 1986). Even in tissues where APs are normally absent, e.g. in the rat anococcygeus (Creed et al., 1975) and the sheep carotid and rabbit ear arteries (Droogmans et al., 1977; Keatinge, 1964), a significant amount of Ca^{2+} may enter the cell through the V.O.Cs, opened by individual or summated e.j.ps (Bolton, 1986).

Despite the importance of membrane depolarization, it is now clear that the responses in some tissues occur independently of changes in membrane potential and, by inference, without an influx of Ca^{2+} through V.O.Cs (Bolton & Large, 1986). Thus, contractions, unaccompanied by membrane depolarization, are

evident in the rabbit and the canine main pulmonary arteries and aortae, in response to either serotonin, noradrenaline or histamine (Somlyo & Somlyo, 1968) and in the guinea-pig ileum to acetylcholine, histamine and substance P (Glossmann et al., 1982). Also, some depolarized tissues are capable of contracting when no further depolarization is possible, e.g. in response to carbachol in the guinea-pig taenia coli (Durbin & Jenkinson, 1961a,b) and to acetylcholine and serotonin in the chick amnion, rat uterus, guinea-pig ileum and cat intestine (Evans et al., 1958).

This voltage-independent mechanism of tension generation, termed 'pharmacomechanical'-, as distinct from 'electromechanical'-coupling, is thought to involve mobilization of Ca^{2+} from within the cell (Berridge, 1985a; Somlyo & Somlyo, 1968). Indeed, the importance of the release of intracellularly-stored Ca^{2+} is now recognised (Berridge, 1985a,b; Bolton, 1986; Wollheim, 1985). Upon reduction or removal of $[Ca^{2+}]_o$, some smooth muscles are still capable of contracting, e.g. in response to K^+ addition in the guinea-pig taenia coli (Imai & Takeda, 1967) and circular muscle of the stomach antrum (Kuriyama et al., 1975). Responses to acetylcholine in the rabbit mesenteric artery (Kuriyama & Suzuki, 1978) and to noradrenaline in the pulmonary artery (Ito et al., 1977) also remain following $[Ca^{2+}]_o$-removal.

Ca^{2+} mobilization is not restricted to contraction. It is also involved in many other biological processes, including secretion of insulin from the β-cells of the pancreas in response to glucose and to cholinergic agonists (Wollheim, 1985),

glycogen breakdown in the liver, secretion of histamine from mast cells and prolactin from the pituitary gland, fertilization of sea urchin oocytes, DNA synthesis in lymphocytes and platelet aggregation (Berridge, 1985a,b; Berridge & Irvine, 1984).

Although the importance of intracellular mobilization of Ca^{2+} has been recognised for some time, the underlying mechanisms have only recently been uncovered. It is now clear that transduction pathways, located in the cell membrane and activated by agonist/receptor combination, regulate, principally through Ca^{2+}, cell function. These will now be described.

(b) Ca^{2+} in transduction mechanisms

In molecular terms, the transduction pathways which have been identified involve a series of proteins within the cell membrane, each of which transmits information by inducing a conformational change in adjacent proteins (Fig. 1). Thus, agonist/receptor combination changes the shape of a transducer 'G' protein, so-called because of its dependence on guanosine 5'-triphosphate (GTP) for its activity, which, in turn, activates an effector molecule located at the inner surface of the cell membrane. This results in the activation of small molecules or ions in the cytoplasm - the 'second messengers', which diffuse throughout the cell and interact with intracellular 'acceptor' proteins. These proteins change in shape and trigger the cellular response.

Two major transduction pathways are now recognised (Fig. 2); one employs the second messengers, Ca^{2+}, inositol 1,4,5-trisphosphate and diacylglycerol, and the other, cAMP. Guanosine 3',5'-cyclic monophosphate (cGMP) also has a second messenger

Fig. 1: The transduction of an extracellular signal (i.e. an agonist) into an intracellularly-mediated response. Following agonist/receptor combination, a transducer 'G' protein, dependent on GTP for its activity, changes its conformation. This stimulates an effector - an enzyme, which, following its activation catalyses the production of a second messenger. The second messenger combines with an acceptor protein to trigger the cellular response.

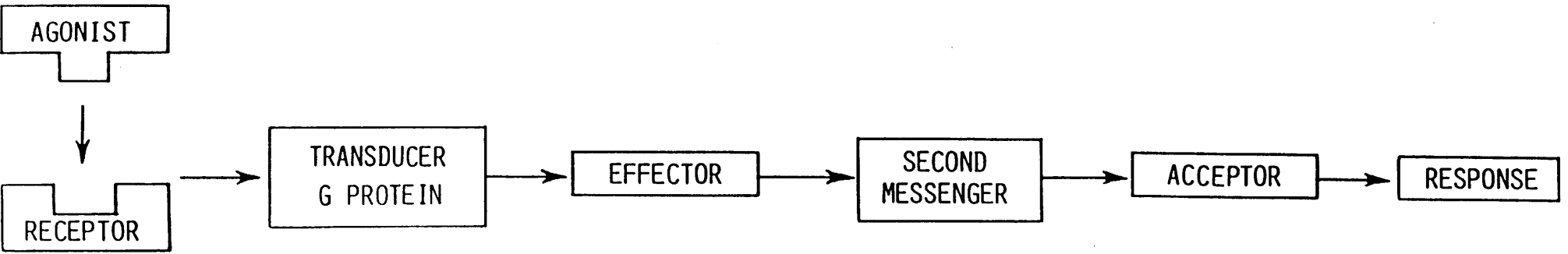
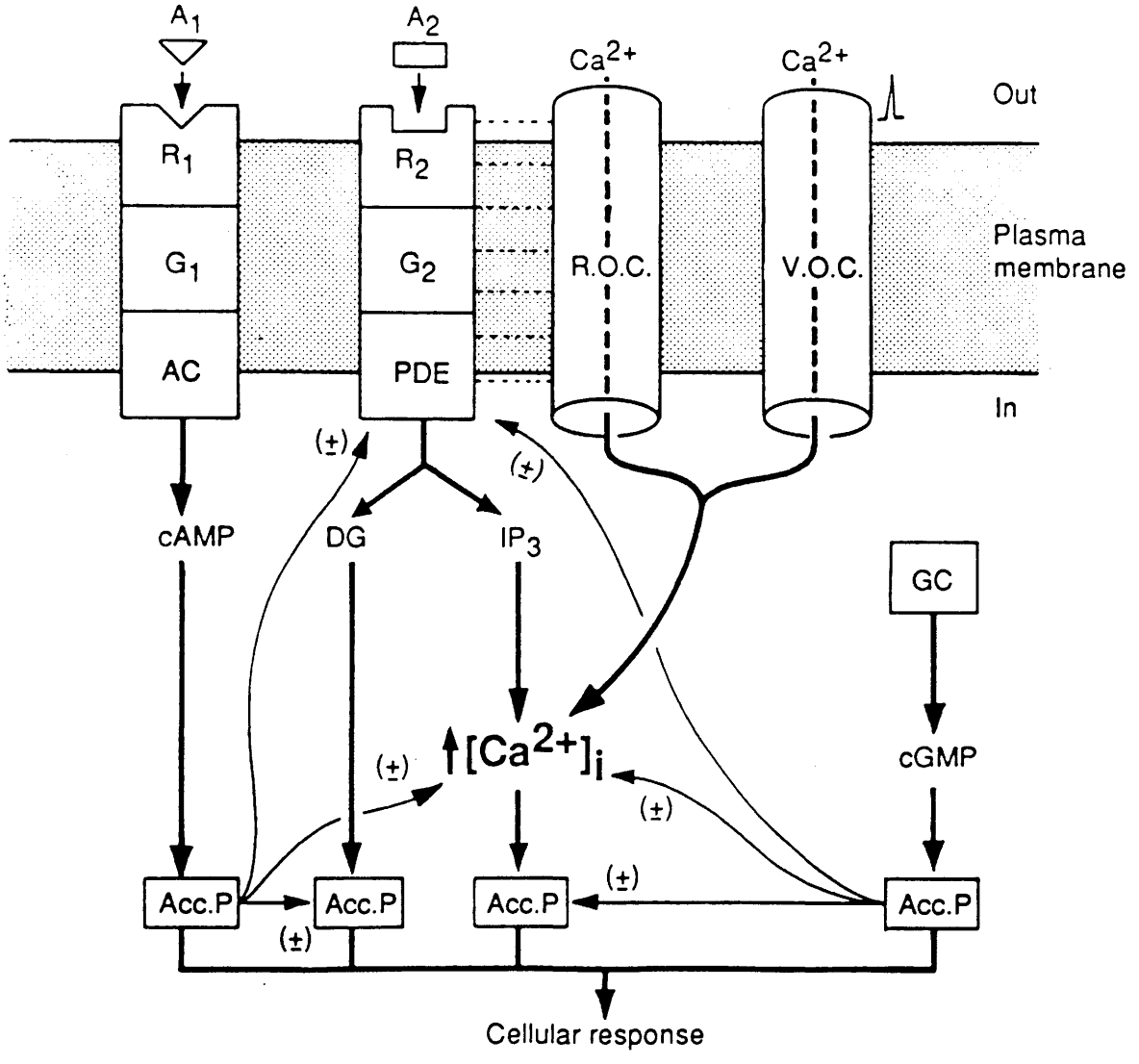

AGONIST
RECEPTOR
TRANSDUCER
G PROTEIN
EFFECTOR
SECOND
MESSENGER
ACCEPTOR
RESPONSE

Fig. 2: Diagrammatic representation of the two major transduction pathways identified in the cell and their influence on the availability or activity of Ca^{2+}. In the first, an agonist (A_1) interacts with its receptor (R_1) at the plasma membrane, changing the conformation of a 'G' protein (G_1), which results in activation of adenylate cyclase (AC). Adenylate cyclase catalyses the conversion of ATP to cAMP. cAMP binds to an acceptor protein (Acc.P) in the cytoplasm to initiate a cellular response, either directly, or by interacting with the second transduction pathway. In this second pathway, an agonist (A_2) interacts with its receptor (R_2), stimulating, firstly a 'G' protein (G_2) and then, in turn, a phosphodiesterase (PDE), phospholipase C. The phosphodiesterase catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). Inositol 1,4,5-trisphosphate mobilises intracellularly-stored Ca^{2+}, which then binds to its acceptor protein and triggers the cellular response. Guanylate cyclase (GC), in the cytoplasm, converts GTP to cGMP. Following interaction of cGMP with its acceptor protein, the availability and/or activity of Ca^{2+} may be influenced. In addition, $\left[Ca^{2+}\right]_i$ may be raised by influx through either 'voltage-operated' channels (V.O.Cs), opened by membrane depolarization, or 'receptor-operated' channels (R.O.Cs), opened by agonist/receptor combination.


A1
A2
Ca2+
Ca2+
Out
R1
R2
G1
G2
AC
PDE
R.O.C.
V.O.C.
Plasma membrane
In
(±)
(±)
cAMP
DG
IP3
GC
[Ca2+]i
(±)
(±)
cGMP
Acc.P
Acc.P
Acc.P
Acc.P
(±)
(±)
Cellular response

role, although does not appear to be associated with a transduction mechanism in the cell membrane. An enzyme, guanylate cyclase, converts GTP, in the cytoplasm, into cGMP. Of all these second messengers, Ca^{2+} has a pivotal role. In regulating cell function, Ca^{2+} is not only a second messenger in its own right, but is also influenced by the other compounds. Thus, inositol 1,4,5-trisphosphate is involved in the mobilization of Ca^{2+}, while cAMP and cGMP may influence both the availability of Ca^{2+} and its activity.

Our present understanding of the precise mechanisms involved in the mobilization of Ca^{2+} stems from the observation (Hokin & Hokin, 1953) that, in the pigeon pancreas, acetylcholine increased the incorporation of radioactive phosphate into phosphatidylinositol, one of the phospholipid constituents of the cell membrane. Michell (1975) suggested that the change in phosphatidylinositol lipid turnover was responsible for generating the increase in $\left[Ca^{2+}\right]_i$ underlying the responses to many agonists. It is now clear that the key event, following agonist/receptor interaction and activation of a G protein, is the hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phosphodiesterase, phospholipase C, to give diacylglycerol and inositol 1,4,5-trisphosphate (Berridge, 1985b). Inositol 1,4,5-trisphosphate diffuses into the cytoplasm and releases Ca^{2+} from the endoplasmic reticulum (Biden et al., 1984). Its ability to do this was first recognised in rat pancreatic cells (Streb et al., 1983) and has since been confirmed in many other tissues, e.g. in the smooth muscle of the porcine coronary artery (Suematsu et al., 1984), in canine cardiac

muscle (Hirata et al., 1984) and in frog skeletal muscle (Vergara et al., 1985), as well as in hepatocytes (Burgess et al., 1984) and platelets (O'Rourke et al., 1985). Mobilization of Ca^{2+} by inositol 1,4,5-trisphosphate may contribute to such diverse cellular responses as smooth muscle contraction (Ochs, 1986), the secretion of insulin from the β-cells of the pancreas (Wollheim, 1985), the light response in Limulus photoreceptors, fertilization in sea urchin and starfish oocytes and initiation of a chloride current in Xenopus oocytes (Berridge, 1985a,b; Berridge & Irvine, 1984). A particularly interesting possibility is that inositol 1,4,5-trisphosphate might be important in mobilizing Ca^{2+} for excitation-contraction coupling in skeletal muscle (Ochs, 1986; Vergara et al., 1985). Depolarization of the T-tubule membrane may release inositol 1,4,5-trisphosphate, which could then diffuse to the sarcoplasmic reticulum to release Ca^{2+} (Berridge, 1985a). Ca^{2+} entry through R.O.Cs, following agonist/receptor interaction, may also be dependent on inositol 1,4,5-trisphosphate, although this remains to be established.

Elucidation of the role of cAMP as a second messenger began with its discovery in 1957 (Sutherland & Rall). The transduction pathway, resulting in cAMP formation, has since been elucidated (see Gilman, 1984). The key event in the cell membrane, following receptor and then G protein activation, is the conversion of ATP to cAMP by adenylate cyclase (Fig. 2). It is now established that in many processes, cAMP provides the 'fine tuning' in the cell by regulating the economy of Ca^{2+}. cAMP, following its combination with a protein

kinase, modulates the availability of Ca^{2+}, governing such responses as heart rate and force of contraction, glycogen breakdown in skeletal muscle and liver, platelet activation and secretion of saliva (Berridge, 1985b; Bushfield et al., 1985). In cardiac muscle, cAMP stimulates the Ca^{2+} pump of the sarcoplasmic reticulum, which accelerates relaxation and allows the organelle to discharge a larger pulse of Ca^{2+} on the next beat (Berridge, 1985a). cAMP also reduces $[Ca^{2+}]_i$ to relax smooth muscle, following β-adrenoceptor stimulation (Bolton, 1986): there is an increased storage of Ca^{2+} in skinned smooth muscle (Itoh et al., 1982) and in microsomes containing endoplasmic reticulum (Fitzpatrick & Szentivanyi, 1977; Webb & Bhalla, 1976). Ca^{2+} entry through V.O.Cs may also be inhibited by cAMP (Meisheri & van Breemen, 1982). In human platelets too, cAMP antagonises the mobilization of Ca^{2+} and stimulates its sequestration and/or extrusion (Bushfield et al., 1985; Zavoico & Feinstein, 1984). The mechanism responsible may involve an interference by cAMP with the activity of phospholipase C (Lapetina et al., 1977) and in consequence, production of inositol 1,4,5-trisphosphate (Watson et al., 1984) and Ca^{2+} mobilization.

Although the precise role of cGMP in the cell is not well understood, it is clear that it too, can interact with Ca^{2+} (Fig. 2). Thus, in platelets, cGMP inhibits elevation of $[Ca^{2+}]_i$, possibly by interacting with phospholipase C activity (Nakashima et al., 1986) and also stimulates sequestration or efflux of Ca^{2+} (MacIntyre et al., 1985). cGMP may interact with Ca^{2+}

in the process of visual excitation, although this has yet to be established (Waloga, 1983).

Following the increase in $[Ca^{2+}]_i$, by whichever mechanism or combination of mechanisms, and its possible interaction with other second messengers, the functional response of the cell is initiated. The means by which Ca^{2+} achieves this will now be discussed, with respect to three important processes; muscle responses, enzyme activation and hormone secretion.

(i) Muscle responses

The precise role of Ca^{2+} in the contraction of smooth and cardiac muscle differs. In smooth muscle, Ca^{2+} acts directly as an activator to trigger contraction, while in cardiac muscle it behaves as a derepressor, removing an inhibition to allow the contractile machinery to operate.

In smooth muscle, Ca^{2+} triggers contraction by binding to calmodulin, an important Ca^{2+}-binding protein (Berridge, 1985a; Bolton, 1986; Herzig, 1984). Ca^{2+}-calmodulin activates myosin light chain kinase, an enzyme which phosphorylates a thick protein filament composed of myosin, allowing the formation of cross-bridges with a thin filamentous protein, actin (Bagshaw, 1982; Bolton, 1986; Herzig, 1984). As in skeletal muscle ('sliding filament theory'; Huxley & Hanson, 1954), actin and myosin are believed to slide over each other by cross-bridge cycling, thus generating tension. Ca^{2+}, in addition to activating myosin light chain kinase, is also required by myosin ATPase to enhance the splitting of ATP, the energy source for cross-bridge cycling (Herzig, 1985).

There may be other, less important mechanisms by which Ca^{2+} regulates smooth muscle contraction (Bolton, 1986). Some of these involve actin; purified actin binds Ca^{2+} and activates myosin ATPase (Marston, 1982; Marston et al., 1980). In some smooth muscles, Ca^{2+} regulates actin-myosin interaction independently of phosphorylation; the rate of ATP splitting and muscle shortening (and hence cross-bridge cycling) declines with little change in myosin phosphorylation (Butler & Siegman, 1983; Siegman et al., 1984). The reason for this may be that Ca^{2+} binds to leiotonin, a protein which triggers actin-myosin interaction without myosin phosphorylation (Ebashi et al., 1982).

In cardiac muscle, Ca^{2+} binds to troponin C, a regulatory protein, which is attached to actin filaments. Following the binding of Ca^{2+}, troponin C changes shape, moving another protein, tropomyosin out of its inhibitory position on the actin molecule, thereby allowing interaction of actin with myosin (Herzig, 1984, 1985). Cross-bridges are formed between actin and myosin and cross-bridge cycling, as in smooth muscle, generates tension. Some Ca^{2+} also binds to calmodulin, but in cardiac muscle, unlike in smooth muscle, the Ca^{2+}-calmodulin complex seems to have only a modulatory function, affecting the velocity of cross-bridge cycling and hence of muscle shortening (Rüegg et al., 1983).

A fall in the free $\left[Ca^{2+}\right]_i$ initiates relaxation in both smooth and cardiac muscle. In smooth muscle, this reduction results in the dissociation of Ca^{2+}-calmodulin, which deactivates myosin light chain kinase causing dephosphorylation of myosin

(Bolton, 1986). In cardiac muscle, Ca^{2+}-troponin C dissociates and tropomyosin returns to its inhibitory position on the actin filament (Herzig, 1984, 1985). $[Ca^{2+}]_i$ may be depleted by sequestration in the sarcoplasmic reticulum or other intracellular organelles (Itoh et al., 1982), by extrusion (Bülbring & den Hertog, 1980) or by a reduction in Ca^{2+} influx through V.O.Cs (Meisheri & van Breemen, 1982).

(ii) Enzyme activity

The multitude of enzymes dependent on Ca^{2+} for their activity include myosin light chain kinase, Ca^{2+} and Mg^{2+}-ATPase, phospholamban kinase, glycogen synthase kinase, cyclic nucleotide phosphodiesterase and tyrosine and tryptophan hydroxylase (Adelstein & Elsenberg, 1980; Imahori, 1982; Le Peuch et al., 1979; Payne & Soderling, 1980; Teo & Wang, 1973; Yamauchi et al., 1981). Although the cellular responses to these, and other Ca^{2+}-dependent enzymes are diverse, including, for example, muscle contraction, Ca^{2+} sequestration and glycogen and neurotransmitter synthesis, the regulatory role of Ca^{2+}, in the main, follows its combination with the same molecule - calmodulin (Cohen, 1982). It is believed that four Ca^{2+} binding sites on calmodulin must be occupied for activation of cyclic nucleotide phosphodiesterase and myosin light chain kinase (Adelstein, 1981), although this number may be different for other enzymes (Cohen, 1982). The interaction changes the molecular conformation of calmodulin and this, in turn, stimulates enzyme combination and activation. Although the importance of this Ca^{2+}-calmodulin interaction is firmly established,

it is not yet clear if one molecule of Ca^{2+}-calmodulin activates several different enzymes simultaneously or if each is stimulated by a different $[Ca^{2+}]_i$ (Cohen, 1982; Kuznicki et al., 1981).

It is now recognised that although Ca^{2+}-calmodulin interaction is required for the activity of most Ca^{2+}-dependent enzymes, it is not essential for some. Protein kinase C, for example, is activated by Ca^{2+} without the involvement of calmodulin or any related Ca^{2+} binding protein. Interaction of Ca^{2+} with diacylglycerol may be involved (Cohen, 1982).

(iii) Hormone secretion

The many hormones dependent on a rise in $[Ca^{2+}]_i$ for their secretion include adrenaline, insulin, corticosteroids, vasopressin, oxytocin, prolactin, calcitonin and thyrotrophin (Rubin, 1970; Trifaró, 1977; Wollheim, 1985; Wollheim & Sharp, 1981). The mechanisms proposed to explain how Ca^{2+} induces hormone secretion are the same (with the exception of that applying to corticosteroid release) as those applied to neurotransmitter release (vide infra). Ca^{2+}, by some, as yet unknown mechanism, but probably involving an intermediate energy step (e.g. ATP splitting), triggers exocytosis of hormones (Trifaró, 1977). The dependence on Ca^{2+} for corticosteroid secretion is due to its requirement for the synthesis of the compounds, rather than for release per se (Rubin, 1970). In the adrenal cortex, there are no intracellular organelles containing corticosteroids for release and it is likely that following

adrenocorticotrophin stimulation, corticosteroids are synthesised de novo and then immediately secreted (Rubin, 1970). The site of action may be within the mitochondrion, where many of the steps of the biosynthetic pathway from cholesterol to corticosterone are carried out (Peron & McCarthy, 1968).

Ca^{2+} CHANNEL ANTAGONISTS

The comprehensive role of Ca^{2+} in cellular activity implies that any drug which interferes with the availability of this ion will, most likely, have profound effects on the cell. Not surprisingly, one group of drugs, the principal action of which is to prevent Ca^{2+} influx into the cell, has been used, both to study the involvement of Ca^{2+} in many cellular processes and clinically to treat disorders, such as angina pectoris, hypertension and cardiac dysrrhythmias (Snyder & Reynolds, 1985). The terminology of these drugs is confusing. Originally designated 'Ca^{2+} antagonists', several synonyms, such as 'slow-channel blockers', 'Ca^{2+} channel antagonists', 'Ca^{2+}-entry blockers' and 'Ca^{2+}-blockers' have been introduced to describe them (Fleckenstein, 1983; Janis & Scriabine, 1983; Vanhoutte, 1981). The term, 'Ca^{2+} channel antagonists', used throughout this thesis, was recommended by the Nomenclature Working Party of the British Pharmacological Society.

HISTORY

The first Ca^{2+} channel antagonists were inorganic compounds. In 1869, magnesium (Mg^{2+}, as the sulphate) was noted by Jolyet and Cahours to produce respiratory paralysis in dogs (see Aikawa, 1963; Engbaek, 1948). In the presence of Mg^{2+}, stimulation, directly, but not indirectly via the sciatic nerve, resulted in muscle contraction; an effect attributed, at this time, to a 'curare-like' action. The precise mechanism of the Mg^{2+}-induced antagonism remained unknown for almost a century, when it was shown that the release of acetylcholine at the frog sartorius neuromuscular junction was related directly to $\left[Ca^{2+}\right]_o$ and inversely to $\left[Mg^{2+}\right]_o$ (del Castillo & Engbaek, 1954). Ca^{2+} and Mg^{2+}, it was claimed, in the rat phrenic nerve/diaphragm preparation, competed for the same neuronal release sites (Hubbard, 1961).

Interaction with Ca^{2+} was evident with other alkaline earth metals. When substituted for Ca^{2+}, strontium and barium each maintained the resting membrane potential and the excitability of nerve axons (Blaustein & Goldman, 1968; Guttman, 1940). On the other hand, some cations were predominantly antagonistic - the so-called 'inorganic Ca^{2+} channel antagonists'. Ca^{2+} spikes in crayfish muscle were inhibited by manganese (Fatt & Ginsborg, 1958) and in a variety of tissues, including the cat ventricle (Kohlhardt *et al.*, 1973), the rods of the toad retina (Fain *et al.*, 1980) and the muscle fibres of the barnacle (Hagiwara & Takahashi, 1967), by cobalt, nickel, cadmium and lanthanum.

The widespread interest in Ca^{2+} channel antagonists, arising from their clinical importance in hypertension and associated disorders, is not, however, due to the availability of inorganic agents, but stems, rather, from the emergence of new, chemically-unrelated, organic compounds. Like several other clinically-important drugs, e.g. chlorpromazine, the sulphonamides, penicillin and warfarin, the pharmacological activity of these compounds was discovered entirely by chance (Fleckenstein, 1983). The first members of this class were synthesised in 1963 by the German pharmaceutical companies, Knoll and Hoechst, in the search for clinically-useful coronary vasodilators. Two such compounds produced both a vasodilatation and a hitherto unexplained cardiodepressant effect. Both were given to Professor Albrecht Fleckenstein in Freiburg for further investigation. He demonstrated that each mimicked the cardiac effects of $\left[Ca^{2+}\right]_o$-withdrawal, inhibiting excitation-contraction coupling and reducing Ca^{2+}-dependent high-energy phosphate utilization and oxygen consumption (Fleckenstein, 1983). The inhibitory effects were reversed by measures which increased the supply of Ca^{2+} to the contractile system, i.e. addition of either β-adrenoceptor agonists, cardiac glycosides or Ca^{2+} itself. These drugs, later named 'verapamil' and 'prenylamine', were thought to interfere with the mediator function of Ca^{2+} in excitation-contraction coupling in the heart.

Interest in this new pharmacodynamic principle, soon to be called 'Ca^{2+} channel antagonism', and the success of verapamil

and prenylamine in the treatment of angina and coronary heart disease in Germany, led to the search for other, similarly-acting agents. In 1968, Fleckenstein was approached by Dengel, the chief chemist of the Knoll company, to examine the effects of the 600th compound he had synthesised, 'D 600', a methoxy derivative of verapamil. It had similar actions to verapamil, but was more potent on both the myocardium and on smooth muscle. One year later, 'Bay a 1040' and 'Bay a 7168', synthesised by the Bayer Company, were noted to inhibit contractile activity in the myocardium. Fleckenstein confirmed that these compounds acted in a similar manner to verapamil and D 600. Bay a 1040 and Bay a 7168, later named 'nifedipine' (Fleckenstein et al., 1972) and 'niludipine' (Fleckenstein et al., 1979) respectively, currently enjoy a prominent position in medicine.

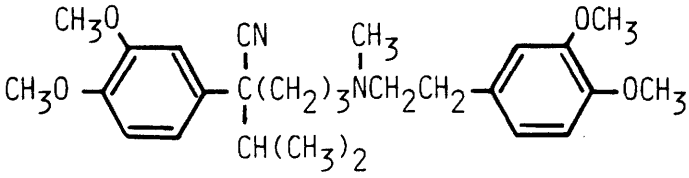
The 1970s were noted in the field of drug development by a world-wide acceptance of the Ca^{2+} channel antagonistic principle and by a rapid numerical growth of the Ca^{2+} channel antagonist family. To provide a degree of order and to assist the clinician's choice of agent from the many available, some means of classification was required; several have been attempted.

First, on the basis of their different potencies and selectivities in blocking electrophysiologically-measured Ca^{2+} and Na^{+} currents in the myocardium, organic Ca^{2+} channel antagonists have been classified into two groups (Fleckenstein, 1983; Fleckenstein & Reuter, 1985). The members of the first (e.g. verapamil, nifedipine and diltiazem) inhibited by 90-100%

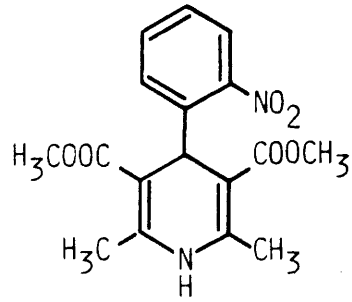
and the second (e.g. prenylamine and fendiline) by 50-70%, the Ca^{2+} current, without affecting that mediated by Na^{+}.

A second classification, based on results from radioligand binding studies, recognised that three different binding sites existed in the plasma membrane of a number of tissues, such as the brain and skeletal and cardiac muscle (Glossmann et al., 1982, 1985). Compounds designated 'Class 1' (i.e. the dihydropyridines such as nifedipine) were bound at one high-affinity site, 'Class 2' (i.e. verapamil and its derivatives) at another and 'Class 3' (i.e. diltiazem), yet another. That different binding sites should be involved is not surprising, considering the diversity in the chemical structures of the organic antagonists (Fig. 3). The binding sites communicate allosterically; combination at one site influences interaction at another (Glossmann et al., 1985). Thus, the compounds in Class 2 inhibit, non-competitively, the binding of those in Class 1 by acting at their own site. Interestingly, Class 3 compounds, following their combination, stimulate rather than inhibit binding of 'Class 1' agents.

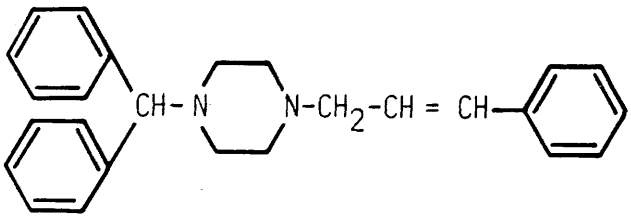
A third classification was based on the effectiveness of BAY K 8644, a dihydropyridine Ca^{2+} agonist (Schramm & Towart, 1985), to reverse the inhibitory effects of the antagonists on Ca^{2+}-evoked contractions in the depolarized guinea-pig taenia coli (Spedding & Berg, 1984). Compounds in Class 1 (e.g. nifedipine) were those producing an inhibition which was reversed competitively by BAY K 8644. The inhibitory effects of Class 2 compounds (e.g. verapamil and diltiazem), on the



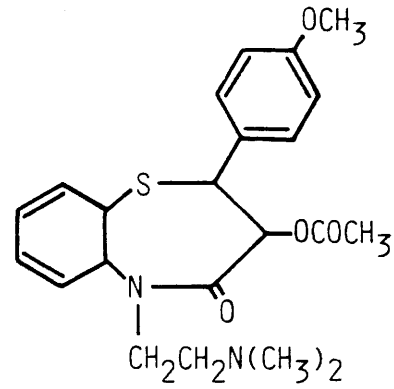
Verapamil



Nifedipine



Flunarizine



Diltiazem

Fig. 3: The structural formulae of the organic Ca^{2+} channel antagonists, verapamil (a phenylalkylamine), nifedipine (a dihydropyridine), flunarizine (a diphenylalkylamine) and diltiazem (a benzothiazepine). As a consequence of their diverse chemical structures, it is unlikely that these agents have a common molecular site of action.

other hand, were reversed by BAY K 8644 in a non-competitive manner, while those of Class 3 (e.g. flunarizine) were unaffected.

These classifications have proved valuable, providing some degree of order among an increasing number of compounds. The demonstration that the compounds vary in potency and selectivity, possess different binding sites and produce characteristically-different functional responses has proved expedient, both pharmacologically and clinically. The classifications enable their dissimilarities to be described, if not necessarily explained and may be related clinically with the variable effectiveness of the compounds which has been demonstrated in the treatment of a number of disorders. Thus, although all the antagonists which are currently used are efficacious in alleviating hypertension, verapamil and diltiazem are undoubtedly superior anti-dysrrhythmic agents than nifedipine (Henry, 1980; Snyder & Reynolds, 1985). However, it is clear that there are anomalies among the classifications. Thus, although the inhibitions by verapamil and diltiazem are each prevented by BAY K 8644, these compounds have opposite effects on dihydropyridine binding (Glossmann et al., 1982; Spedding & Berg, 1984). However, stimulation of BAY K 8644 binding by diltiazem may explain why inhibition with this agent is reversed more effectively than that with verapamil.

EFFECTS

Ca^{2+} channel antagonists act both pre- and postjunctionally to influence autonomic neuromuscular transmission. Their postjunctional effects are particularly well characterised and, in the case of the organic compounds, important clinically. It is the postjunctional actions which underlie their effectiveness in the treatment of disorders, such as angina pectoris, hypertension, cardiac dysrrhythmias and cerebral vasospasm (Braunwald, 1980; Halperin & Cubeddu, 1986; Henry, 1980; Snyder & Reynolds, 1985). These postjunctional actions will be described first.

(a) Effects at postjunctional sites

The compounds inhibit, selectively, Ca^{2+}-dependent electrical and mechanical responses, whether spontaneous or evoked, in cardiac and in both vascular and non-vascular smooth muscle (Fleckenstein, 1983; French & Scott, 1981; Golenhofen, 1981). In each tissue investigated, responses mediated by an influx of Ca^{2+} across the cell membrane, from the extracellular fluid, are prevented more effectively than those which involve the mobilization of the ion from intracellular storage sites (van Nueten & Vanhoutte, 1981).

The effects of Ca^{2+} channel antagonists are particularly marked on the cardiovascular system. In the isolated heart, they produce a negative inotropic response by inhibiting the inward Ca^{2+} current of the AP. This reduces the amount of Ca^{2+} available to the intracellular contractile proteins (Millard et al., 1982; Nayler & Grinwald, 1981). The rate of both

sinoatrial node ('pacemaker') discharge and conduction at the atrioventricular node - themselves mediated by an influx of Ca^{2+} - are also reduced, resulting in a negative chronotropic response (Fleckenstein, 1983; Fleckenstein-Grün et al., 1984). In consequence, the antagonists reduce ATP utilization in the beating ventricles (Fleckenstein, 1983). This, in turn, lowers the cardiac oxygen requirement and thus, the demand on the heart (Fleckenstein-Grün et al., 1984).

The vascular component of the cardiovascular system is also affected, both in vitro and in vivo. The compounds reduce the resting, Ca^{2+}-dependent tone of vascular smooth muscle, including the coronary vasculature; the resulting vasodilatation lowers blood pressure (Halperin & Cubeddu, 1986; Hof, 1985). Ca^{2+} channel antagonists also oppose agonist-induced vasoconstriction. Thus, K^{+}-, Ca^{2+}- and noradrenaline-evoked responses are prevented in a variety of isolated blood vessels, including the human, rabbit and rat mesenteric arteries, the rat and rabbit aortae and the pig coronary artery (Fleckenstein, 1983; Godfraind & Dieu, 1981; Godfraind & Kaba, 1969; van Breemen et al., 1981).

In vivo, their effects on the blood vessels activate homeostatic mechanisms (Hof, 1985). For example, the vasodilatation produced by the compounds reflexly increases both heart rate and contractility. The extent to which the reflex overcomes the direct cardiac depressant effects of the compounds varies with each antagonist, reflecting, presumably, their relative potencies on cardiac and vascular smooth muscle.

The response produced by each compound also varies among species and between conscious and anaesthetised animals. In conscious dogs, for example, nifedipine increases, verapamil decreases and diltiazem fails to affect the force of myocardial contraction, although each accelerates heart rate (Millard et al., 1982). In anaesthetised animals, heart rate is reduced by each (Millard et al., 1982). In anaesthetised cats, verapamil and diltiazem, but not nifedipine, each reduces heart rate (Hof, 1985). In conscious humans, verapamil increases and diltiazem decreases heart rate; neither normally affects the force of myocardial contraction (Henry, 1980). Such differences may be important clinically. A reflex tachycardia would, for example, be undesirable if cardiac dysrrhythmias were already present.

In non-vascular smooth muscle also, Ca^{2+} channel antagonists inhibit spontaneous electrical activity, whether manifested as an AP discharge, as in the guinea-pig taenia coli (Tomita, 1981) and internal anal sphincter (Lim & Muir, 1985), or as a slow membrane depolarization, as in the rat uterus (Reiner & Marshall, 1975) and the guinea-pig trachea (Ahmed et al., 1985). Accompanying spontaneous mechanical activity is also inhibited. Interestingly, in tissues where biphasic mechanical responses are evident, the effectiveness of individual antagonists against the phasic and the tonic component differs. Thus, in a variety of tissues in the guinea-pig, verapamil, D 600 and nifedipine each inhibit the phasic component of the contractions selectively (Golenhofen, 1981). The tonic component may be resistant as a result of its relying on the mobilization of intracellular stores of Ca^{2+}, rather than on trans-membrane

flux, against which, the antagonists, in the main, are relatively ineffective (van Nueten & Vanhoutte, 1981).

Evoked contractions, in response to a number of stimuli, are also inhibited in non-vascular smooth muscle by Ca^{2+} channel antagonists, again to varying extents. Responses to either K^+ addition or electrical stimulation are blocked readily in a variety of tissues, such as the rodent vas deferens (Blakeley et al., 1981) and the guinea-pig ileum (Frankish, 1983), while those to receptor agonists are little affected in most preparations, e.g. in the rat anococcygeus to noradrenaline (Oriowo, 1984) and in the guinea-pig trachea to either acetylcholine, histamine or serotonin (Advenier et al., 1984). This may be due to the ability of K^+-depolarization and receptor agonists to utilize different channels - V.O.Cs and R.O.Cs respectively (Bolton, 1979, 1986). This does not explain, however, why the antagonists should more effectively inhibit responses mediated by nerve stimulation, which presumably utilizes R.O.Cs, than by receptor agonists. Exogenously-added agonists could, possibly, prolong the open time of the R.O.Cs or, alternatively, may rely to a greater extent on intracellularly-bound Ca^{2+}. Such suggestions are plausible; confirmation awaits experimental investigation.

(b) Effects at prejunctional sites

By comparison with their postjunctional activity, little attention has been paid to the prejunctional effects of Ca^{2+} channel antagonists. Their postjunctional activity, giving rise to their clinical success, has held the stage of experimental

endeavour. The lack of interest in their prejunctional actions is, however, surprising in the light of Ca^{2+} involvement in both transmitter release and in the AP of some nerves (vide infra). The few prejunctional studies which have been carried out have confirmed that the antagonists affect both AP conduction and transmitter release (Andersson et al., 1983; Cunnane & Stjärne, 1984; Takata & Kato, 1984). The organic compounds appear to be less active pre- than postjunctionally, inhibiting contractile responses in concentrations too small to affect release (Andersson et al., 1983; Haeusler, 1972; Högestätt et al., 1982; Kajiwara & Casteels, 1983).

Both organic (i.e. D 600) and inorganic (i.e. manganese and lanthanum) antagonists inhibit AP conduction in small preterminal sympathetic nerve fibres running to the guinea-pig vas deferens (Cunnane & Stjärne, 1984). This action is not restricted to autonomic nerves; verapamil and D 600 also inhibit APs in the somatic fibres of the rat phrenic nerve (Hay & Wadsworth, 1982).

The effects of the compounds on transmitter release appear to vary in different tissues. In most, they produce an inhibition. Thus, release, measured radiochemically, in response to either electrical stimulation or K^{+}-depolarization, is reduced in the rabbit urethra (Andersson et al., 1983) and heart (Göthert et al., 1979), the canine saphenous vein (Takata & Kato, 1984) and guinea-pig portal vein (O'Connor, 1982). The reduction in inhibitory junction potential (i.j.p.) amplitude in the guinea-pig ileum may also be due to an

interference with release (Bornstein et al., 1985).

In contrast, the organic compounds may, in some cases, potentiate transmitter release. Stimulation-evoked $[^3H]$ - noradrenaline release, for example, is increased in the rat tail artery (Wolchinsky & Zsotér, 1985) and rabbit ear artery (Steinsland et al., 1985), while i.j.p. amplitude is enhanced in the guinea-pig ileum at a lower concentration than that producing an inhibition (Bornstein et al., 1985). The underlying mechanism of this potentiation is unclear. It may be mediated intracellularly independent of Ca^{2+} (Wolchinsky & Zsotér, 1985) or, at least in some cases, could involve a prejunctional inhibition of α_2-adrenoceptors (Galzin & Langer, 1983), thus removing a negative feedback control on release.

It is clear, however, that in some tissues, the organic antagonists (particularly nifedipine) are inactive. Thus, stimulation-evoked noradrenaline release in the guinea-pig portal vein (O'Connor, 1982), rabbit urethra (Andersson et al., 1983) and ear artery (Steinsland et al., 1985) and e.j.ps in the guinea-pig vas deferens (Blakeley et al., 1981) are unaffected by either nifedipine or nitrendipine.

MECHANISM OF ACTION

Ca^{2+} channel antagonists could, theoretically, interfere with Ca^{2+}-dependent responses at pre- and postjunctional sites, either by inhibiting the influx of Ca^{2+} from the extracellular fluid, the mobilization of the ion from intracellular stores, or by stimulating its efflux from, or sequestration within the cell. That Ca^{2+} channel antagonists produce their effects principally by inhibiting Ca^{2+} influx is now established (Fleckenstein, 1977, 1983; Janis & Scriabine, 1983; Kohlhardt et al., 1973; Reuter, 1983; Schramm & Towart, 1985). However, inhibition of both the mobilization of Ca^{2+} and its activity within the cell has also been demonstrated (Morgan et al., 1983; Rahwan, 1985).

Evidence suggesting that the antagonists inhibit Ca^{2+} influx across the plasma membrane has arisen from both direct and indirect investigation in a variety of tissues. The inward, Ca^{2+}-mediated current, measured electrophysiologically, is inhibited in both cardiac and smooth muscle cells (Ehara & Kaufmann, 1978; Lee & Tsien, 1983; Osterrieder et al., 1981; Reuter, 1983). Also, $^{45}Ca^{2+}$ uptake is prevented in a variety of tissues, including the rabbit aorta (van Breemen et al., 1981) and the rat aorta and mesenteric artery (Godfraind & Dieu, 1981).

It is clear, however, that Ca^{2+} influx through V.O.Cs and R.O.Cs is prevented by organic and inorganic antagonists to varying extents. The V.O.Cs are blocked readily by most compounds. Thus, responses to K^+ addition, which opens V.O.Cs to allow Ca^{2+} influx (Godfraind, 1981), are inhibited in the

human mesenteric artery and vein (Mikkelsen et al., 1979), the rabbit aorta (van Breemen et al., 1981) and the guinea-pig trachea (Advenier et al., 1984). Ca^{2+} influx through R.O.Cs, on the other hand, although prevented by the inorganic antagonists, is relatively resistant to the organic compounds (Flaim et al., 1985; Oriowo, 1984). The inorganic agents are thought to simply 'plug' Ca^{2+} channels in a competitive manner (Schramm & Towart, 1985). They prevent Ca^{2+} influx, not only through V.O.Cs and R.O.Cs, but also through channels formed by Ca^{2+}-ionophores (Snyder & Reynolds, 1985). In contrast, the organic antagonists have a more complex mechanism of action. Ca^{2+} channel blockade through V.O.Cs by either verapamil, D 600 or nifedipine increases with increasing depolarization and frequency of stimulation. They do not, however, slow activation of the channels (Lee & Tsien, 1983; Trautwein, 1985). The V.O.Cs may exist in different forms; resting (closed but available for activation), open (Ca^{2+}-conducting) and inactive (closed and unavailable for activation) states. Organic antagonists block either open or inactive states (Janis & Scriabine, 1983; Lee & Tsien, 1983).

The site and mechanism of action of organic Ca^{2+} channel antagonists has also been clarified by radioligand binding studies. These have emphasised the existence of binding sites associated with Ca^{2+} channels. The binding sites are located in the plasma membranes of, for example, the heart (Glossmann et al., 1982), ileum (Bolger et al., 1983) and aorta (Godfraind, 1981). They are, most likely, proteins associated with phospholipids; they are denatured by heat and sensitive to

the proteolytic enzyme, trypsin and to phospholipases A and C (Glossmann et al., 1982). It is now clear that several different binding sites exist for the organic Ca^{2+} channel antagonists (Schramm & Towart, 1985). Thus, for example, verapamil and its derivatives bind at a site at the 'inner mouth' of the Ca^{2+} channel; quaternary compounds are effective only on the inside of the cell (Hescheler et al., 1982). On the other hand, dihydropyridines are thought to bind at a site at the 'outer mouth' of the channel (Glossmann et al., 1982). For each organic compound, the binding is consistent with an action at a specific site; it is saturable, rapid, reversible and stereoselective (Janis & Scriabine, 1983).

In addition to Ca^{2+} channel blockade, the antagonists have an affinity for blocking both, other channels and receptors at the plasma membrane. These include Na^{+} (Bayer et al., 1975) and K^{+} channels (Kass, 1982), together with α_2-adrenergic (Galzin & Langer, 1983), muscarinic (Fairhurst et al., 1980) and serotoninergic receptors (Affolter et al., 1985). The extent of these actions varies with each compound, but, in the main, only occurs with high concentrations, at which presumably, the drugs lose their selectivity for the Ca^{2+} channels

The antagonists may also have actions within the cell. Indeed, one group of compounds, the methylenedioxyindenes, appear to exert their effects primarily by inhibiting Ca^{2+} mobilization from the endoplasmic reticulum with little contribution being made by Ca^{2+} channel blockade (Rahwan, 1985). For the majority of agents, however, intracellular mechanisms

appear to be less important. Generally, higher concentrations are required than those at which Ca^{2+} influx is inhibited. Notwithstanding, however, it is evident that the organic compounds can interact with the contractile proteins. Thus, in cardiac muscle, experiments with aequorin, a protein, which on combination with Ca^{2+} emits light (the 'Ca^{2+} signal'), have shown that the degree of tension for the same amount of 'Ca^{2+} signal' is smaller in the presence of the antagonists, than in their absence (Morgan et al., 1983). Also, Ca^{2+}-induced contractions of the guinea-pig taenia coli, treated with detergents to disrupt the plasma membrane, are inhibited by cinnarizine, fendiline, flunarizine and pimozide (Spedding, 1983). An inhibitory interaction with calmodulin may be involved (Boström et al., 1981; Johnson, 1983). Indeed, calmodulin-dependent phosphodiesterase activity is inhibited by both verapamil and nifedipine (Epstein et al., 1982).

AIMS OF THE THESIS

Interest in Ca^{2+} channel antagonists has stemmed largely from their ability to prevent, postjunctionally, Ca^{2+} influx into smooth and cardiac muscle and from their resulting benefits in the treatment of cardiac dysrrhythmias, hypertension and angina pectoris. There is, however, no *a priori* reason why interest should be restricted to their activity at postjunctional sites, since a physiological role for Ca^{2+} prejunctionally in transmitter release and in the AP of some nerves, is now established. Indeed, that Ca^{2+} entry is required for the release of all neurotransmitters, suggests that Ca^{2+} channel antagonists could provide an important pharmacological and, in consequence, clinically-useful means of controlling neuromuscular transmission in both smooth and cardiac muscle.

To date, the few investigations of such prejunctional activity, which have been carried out, have been fragmentary and the resulting effects attributed to the compounds, equivocal. The opportunity to contribute to this potentially important aspect of the activity of the antagonists was sufficiently challenging to prompt the present work. This thesis, therefore, represents attempts made to examine, systematically and in detail, the prejunctional effects of a number of organic and inorganic Ca^{2+} channel antagonists. To do this, the release, of both excitatory and inhibitory neurotransmitters in tissues receiving different types of innervation, was measured *in vitro*, both directly, by collecting the overflow of radiolabelled transmitter and indirectly, by recording, with intracellular

microelectrodes, junction potentials in smooth muscle. As interference with nerve AP conduction influences transmitter release, neuronal activity was also measured. This was achieved extracellularly with a suction electrode. The relevance of any prejunctional actions produced by the compounds was assessed by evaluating, in parallel, their effects, postjunctionally and *in vivo*.

CHAPTER 2: METHODS

The methods were designed to allow investigation of the actions, especially at prejunctional sites, of a number of Ca^{2+} channel antagonists. Prejunctional activity was examined by measurement of:-

(a) AP conduction along autonomic nerves. Interference with this process would have profound effects on both transmitter release and effector activity.

(b) neurotransmitter release from autonomic nerve terminals. This was done by studying drug-induced changes on the overflow of radiolabelled transmitter and on evoked junction potentials in smooth muscle (Burnstock & Holman, 1961), which represent the initial membrane response of the muscle to transmitter.

The choice of preparation was governed by several factors. In studying prejunctional activity, a preparation with a readily accessible extrinsic nerve, suitable for AP conduction measurement, was required. It was also desirable that the neurally-released transmitter(s) in the preparation should have been identified and their responses, characterised.

Several such preparations were considered, e.g. the guinea-pig, mouse and rat vas deferens (for review, see Sjöstrand, 1981), guinea-pig urinary bladder (Langley & Anderson, 1895), cat spleen (Gillespie & Kirpekar, 1965) and rat anococcygeus (Gillespie, 1972). Of these, two were chosen: the guinea-pig vas deferens and the rat anococcygeus. Each preparation was appropriate for the measurement of AP conduction, receiving a dense innervation by readily accessible extrinsic nerves. The guinea-pig vas deferens is innervated

by excitatory sympathetic nerves. Although termed 'short adrenergic neurones' (Sjöstrand, 1965), because of the close proximity of their cell bodies to the muscle, these nerves are sufficiently long to allow measurement of AP conduction in response to both pre- and postganglionic stimulation (Ferry, 1967). Indeed the suitability of this tissue for AP conduction measurement has been demonstrated both *in vitro* and *in vivo* (Ferry, 1967).

The rat anococcygeus is innervated by separate extrinsic excitatory sympathetic and inhibitory non-adrenergic, non-cholinergic (NANC) nerves (Gibson & Gillespie, 1973), which, being relatively long, facilitate measurement of AP conduction. The dual innervation in this tissue suggested that an investigation of drug effects on AP conduction in each type of nerve was feasible.

The suitability of the guinea-pig vas deferens and rat anococcygeus for the measurement of transmitter release has also been established. The sympathetic nerves to the guinea-pig vas deferens are thought to release two transmitters - noradrenaline and adenosine 5'-triphosphate (ATP, Burnstock & Sneddon, 1984; Sneddon & Westfall, 1984) and the tissue is suitable for analysing, radiochemically, the release of both substances (Alberts *et al.*, 1981; Westfall *et al.*, 1978). Transmitter release can also be measured by recording the e.j.ps in the smooth muscle of the guinea-pig vas deferens (Burnstock & Holman, 1961). In the rat anococcygeus, noradrenaline, released from the sympathetic nerves, is measured

readily by analysis of the large evoked e.j.ps in this tissue (Creed et al., 1975). However, the rat anococcygeus is not so suitable for studying the effects of drugs on the release of the other, as yet unidentified, NANC transmitter, because the evoked i.j.ps are small and even insignificant (Creed et al., 1975). To overcome this problem, a preparation was sought, in which NANC-mediated i.j.ps were large and measurable. The guinea-pig internal anal sphincter (i.a.s.), previously shown in this laboratory to exhibit large i.j.ps in response to NANC nerve stimulation (Lim & Muir, 1985), was chosen. The NANC transmitter in the guinea-pig i.a.s. has been suggested (Lim & Muir, 1986), on the basis of results from intracellular electrical studies, to be ATP or a related nucleotide. It was hoped that this could be determined by radiochemical analysis. A disadvantage in using the guinea-pig i.a.s. was that the intrinsic nature of its NANC innervation (Costa & Furness, 1973) would not allow measurement of AP conduction.

The postjunctional actions of the Ca^{2+} channel antagonists were also analysed by isometric monitoring of the mechanical activity of the tissues under investigation. This allowed a direct comparison to be made of the pre- and postjunctional actions of Ca^{2+} channel antagonists.

MORPHOLOGY AND INNERVATION OF TISSUES USED

(i) Guinea-pig vas deferens

The vas deferens is responsible for the transport of spermatozoa and seminal fluid from the epididymis of the testis to the seminal vesicle duct near the prostate gland. It comprises three smooth muscle layers: an outer and inner longitudinal, which surround a circular layer. The smooth muscle cells are surrounded by connective tissue and overlap in an interweaving fashion (Merrillees, 1968).

The innervation of the guinea-pig vas deferens has been described extensively (Merrillees, 1968; Sjöstrand, 1965, 1981). The tissue receives principally an excitatory sympathetic innervation by the hypogastric nerves, which originate at the T9-T10 level of the spinal cord and run to the inferior mesenteric ganglion. The hypogastric nerves, containing mainly preganglionic sympathetic fibres, pass from the inferior mesenteric ganglion to a plexus of ganglia located within 1 cm of the prostatic end of the vas deferens. From this plexus, postganglionic, mainly non-myelinated sympathetic nerve fibres, run first to the prostatic and then towards the epididymal end, sending off radial branches into the muscle.

The guinea-pig vas deferens also receives a parasympathetic cholinergic innervation (Sjöstrand, 1981). Most parasympathetic axons innervate the circular muscle layer and a few, the external longitudinal layer. The function of the parasympathetic nerves is unclear. They may provide an excitatory innervation or modify the sympathetic neurotransmission (Sjöstrand, 1981).

(ii) Rat anococcygeus

The anococcygeus is a paired smooth muscle, suggested, as a consequence of its close proximity to the terminal colon, to be involved in the defaecation reflex (McGrath, 1973). The tissue consists of parallel bundles of longitudinal smooth muscle fibres, arising from a tendinous origin attached to coccygeal vertebrae 1 and 2. Each muscle runs caudally, first behind the colon and then passes ventrally around either side of the terminal colon to fuse and so form a 'ventral bar' (Gillespie, 1972).

The rat anococcygeus receives both an excitatory (from postganglionic sympathetic adrenergic nerves, Gillespie, 1972) and an inhibitory (NANC) innervation, the transmitter of which is unknown. The sympathetic and NANC nerve fibres are believed to be separate (Gibson & Gillespie, 1973), and have a different spinal origin (T10 to L3 and L5 to S2 respectively). Two extrinsic nerve bundles, containing both excitatory and inhibitory fibres and originating from the posterior scrotal division of the perineal nerve and the external spermatic division of the genito-femoral nerve (Gillespie & Lüllmann-Rauch, 1974), run towards the anal end of the anococcygeus. The nerves divide as they approach the muscle to form a nerve network on its lateral surface (McKirdy & Muir, 1978).

(iii) Guinea-pig internal anal sphincter

The internal anal sphincter (i.a.s.), which is involved in defaecation, coincides in the guinea-pig with a thickening (approximately 3 mm long and 5 mm broad) of the rectum wall

(Fig. 4a). The sphincter consists of a band of circular smooth muscle fibres, more densely arranged than elsewhere in the rectum wall (Fig. 4b).

Of the two muscle layers of the rectum, the outer, longitudinal muscle extends over the inner, circular, sphincteric area. Each layer terminates 5-10 mm short of the anal margin and is separated from the skin by a band of largely connective tissue with a few circular smooth muscle fibres. The term 'internal anal sphincter' (i.a.s.) in this thesis denotes the circular muscle free from the longitudinal layer.

The i.a.s. is innervated by discrete excitatory sympathetic adrenergic and inhibitory NANC nerves (Costa & Furness, 1973). The sympathetic and NANC nerves enter the sphincteric muscle from the pelvic plexuses within the rectum wall. Extrinsic sympathetic nerves also enter the i.a.s. in the pudendal nerves which originate at the L6 and S1 level of the spinal cord. The longitudinal muscle, extending over the sphincteric area, receives a parasympathetic excitatory cholinergic innervation, the nerves of which also originate in the pelvic plexuses.

DISSECTION OF TISSUES

Adult male Duncan-Hartley guinea-pigs (300-1000g) and Wistar rats (150-350g) were killed by cervical dislocation or stunning and then bled. Following a midline incision in the abdomen, the peritoneal cavity and its contents were exposed and the gastrointestinal tract displaced. During each

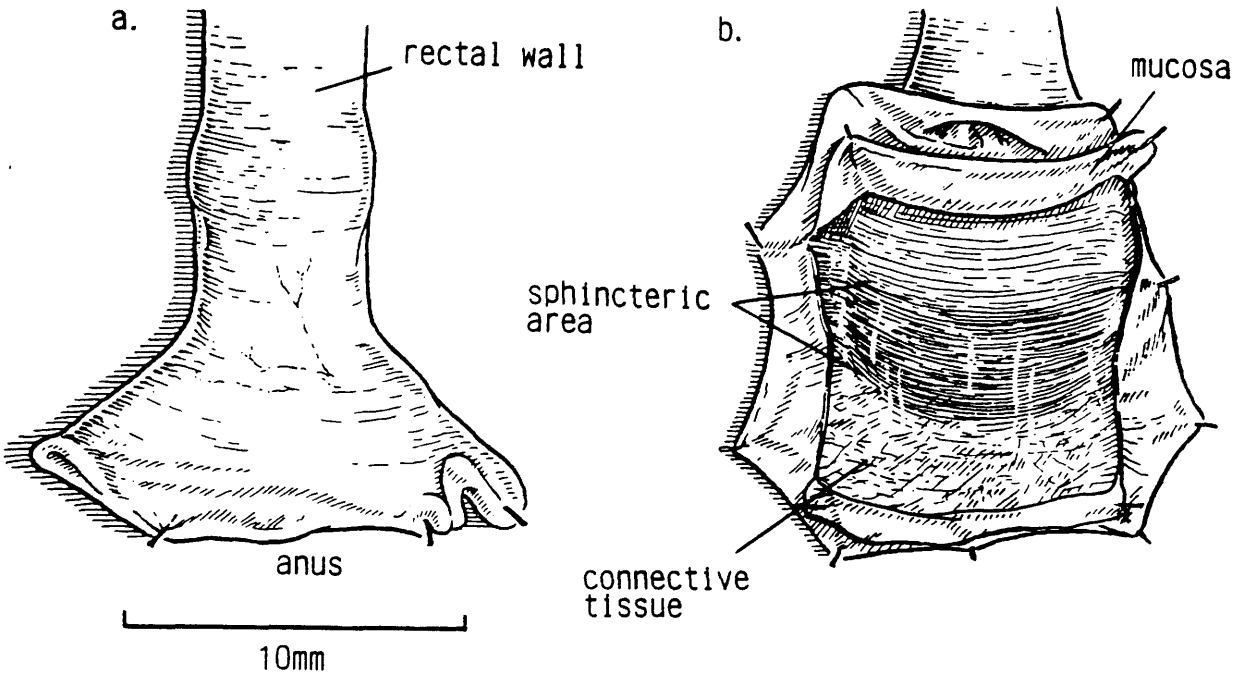


Fig. 4: Diagrammatic indication of the position of the guinea-pig internal anal sphincter (magnification x 5). In this stretched preparation, the approximate location of the sphincter is indicated by a slight swelling of the rectum wall (a). In b., a longitudinal incision in the rectum has been made and the mucosa and submucosa removed. The sphincter can be identified as a band of circular smooth muscle in which the fibres are more densely arranged than elsewhere in the rectum wall. The sphincter is easily differentiated from the connective tissue adjacent to the anus.

dissection, tissues were kept moist with Krebs solution (see later for formula), continuously bubbled with 95% O_2 and 5% CO_2 (pH - 7.4).

(i) Guinea-pig vas deferens

The testes were pushed into the peritoneal cavity and one vas deferens was freed at the prostatic end. Following separation from the testis, the vas deferens, together with its attached connective tissue, was transferred to a beaker of Krebs solution, bubbled with 95% O_2 and 5% CO_2 at room temperature, until used as described below.

(ii) Rat anococcygeus

Following removal of vasa deferentia and seminal vesicles, the pubic symphysis was split and the bladder and urethra, discarded. The colon, ligated and cut at the level of the pelvic brim, was pulled forward and the connective tissue behind it, removed until the two anococcygeus muscles were visible. Both muscles were ligated at their tendinous points of attachment to the coccygeal vertebrae and at the 'ventral bar'. Each anococcygeus, together with the surrounding connective tissue which contained the extrinsic nerves, was then transferred to a beaker of oxygenated Krebs, until used.

(iii) Guinea-pig internal anal sphincter

Following splitting of the pubic symphysis, a segment of the rectum, including the anal region, was transferred to a Sylgard (Dow Corning)-coated petri dish containing Krebs solution, bubbled with 95% O_2 and 5% CO_2 (pH - 7.4). The rectum was pinned out and the skeletal muscle and connective tissue forming the external anal sphincter, removed. A

longitudinal incision in the ventral wall of the rectum (approximately 2 cm in length) was made cranially from the anus. The mucosa and submucosa were then removed and the i.a.s. identified under a dissection microscope as a dense band of circular muscle (Fig. 4b). A horizontal strip (1 cm long and 0.2 cm broad, unstretched) of sphincter with attached longitudinal layer was dissected out. The longitudinal muscle layer was then carefully removed under the microscope.

MEASUREMENT OF ACTION POTENTIAL CONDUCTION IN AUTONOMIC NERVES

APPARATUS

(i) Organ bath

This was the same as that used for intracellular electrical studies (Fig. 9). It consisted of a trough (4 cm x 1.5 cm x 1 cm) cut from a perspex block (10.5 cm x 6.5 cm x 2 cm). To minimize mechanical vibrations, the bath was bolted to two non-conducting pillars of Bakelite, which were fastened to a steel plate (200 kg) on a table mounted on Muffelite (K-150) anti-vibration dampers. The bath (4 ml capacity) was perfused continuously (6 ml min^{-1}) via two inlets (0.2 cm internal diameter) with Krebs solution (36 $\pm$ 0.5°C), held in small diameter (0.15 cm internal diameter) polythene tubing. This tubing was surrounded, in turn, by an outer polythene tube of larger diameter (1 cm internal diameter) containing liquid paraffin at 40 $\pm$ 0.1°C, pumped from a Tempette (TE7) pump.

Drugs were either perfused with the Krebs solution or added directly to the bath by syringe.

(ii) Suction electrode

Nerve activity was recorded extracellularly by a suction electrode (Figs. 5 and 6). This consisted of a glass microelectrode, filled with Krebs solution, the shaft of which was connected by polythene tubing (0.15 cm internal diameter) to the needle of a glass syringe (1 ml), filled with liquid paraffin. Changes in pressure at the microelectrode tip were induced when the plunger of the syringe, surrounded by a steel spring, was moved by rotation of a screw, thus allowing a nerve bundle to be sucked into the microelectrode tip. A silver wire inside the microelectrode served as the recording electrode and a Ag/AgCl pellet in the organ bath, as the indifferent electrode. Electrical signals were passed from the silver wire in the suction electrode to an A.C. preamplifier (Neurolog NL103), filtered (low and high frequency cutoffs - 100 and 1000 Hz respectively (Neurolog NL 115)) and displayed on a storage oscilloscope (Tektronix 510 3N), from which signals were recorded on an F.M. tape recorder (Racal 4DS).

RECORDING FROM GUINEA-PIG HYPOGASTRIC NERVES IN VITRO

A pair of bipolar Ag/AgCl ring electrodes (0.3 cm diameter, 0.2 cm separation), placed midway along the tissue (Fig. 7), allowed postganglionic stimulation (0.5 Hz, 0.05 - 0.5 ms, supra- or submaximal voltage) of the hypogastric nerves to be carried out. Both ends of the preparation were pinned, at

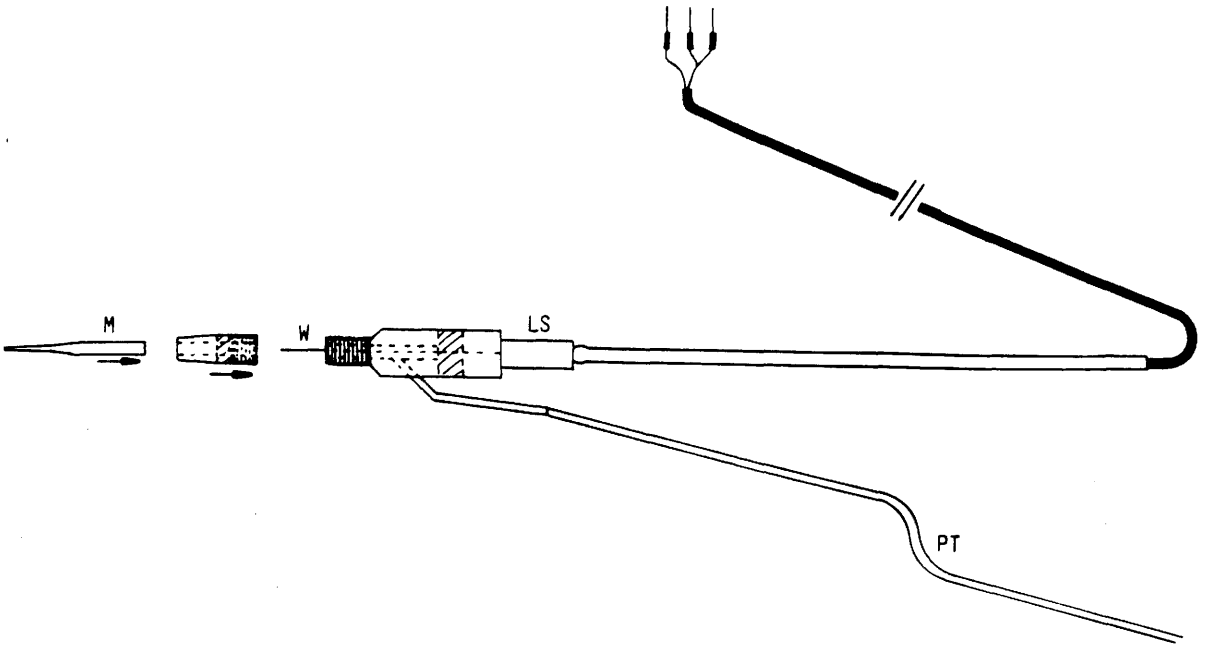


Fig. 5: Suction electrode for extracellular measurement of nerve AP conduction. A silver wire (W) was connected electrically via a Limo socket (LS), embedded in perspex, to the recording circuitry (Fig. 7). A glass microelectrode (M) was placed over the silver wire and connected via polythene tubing (PT), filled with liquid paraffin, to the remainder of the apparatus (Fig. 6).

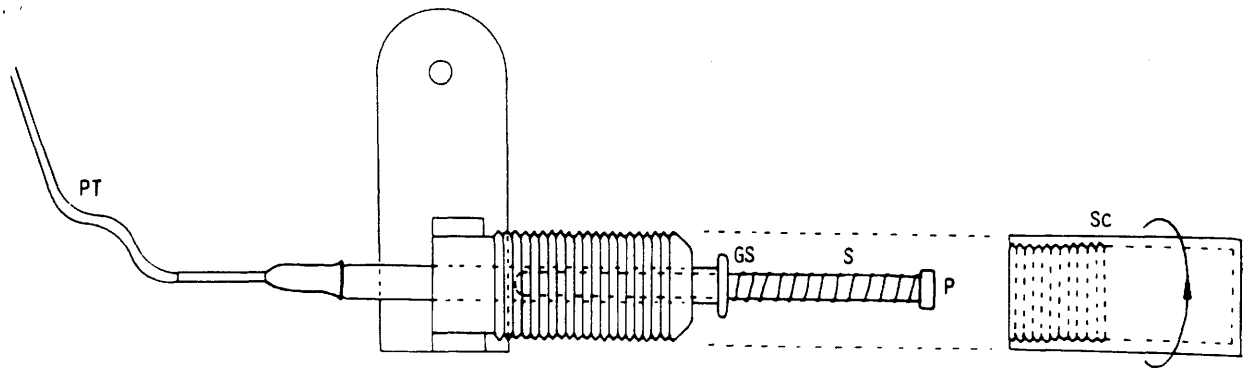


Fig. 6: Suction electrode for extracellular measurement of nerve AP conduction. Changes in pressure at the microelectrode tip (Fig. 5) were induced by movement of the plunger (P) of a glass syringe (GS), in either direction, by means of a perspex screw (Sc) and a steel spring (S) placed around the plunger. The syringe, filled with liquid paraffin, was connected to the remainder of the suction electrode (Fig. 5) by polythene tubing (PT).

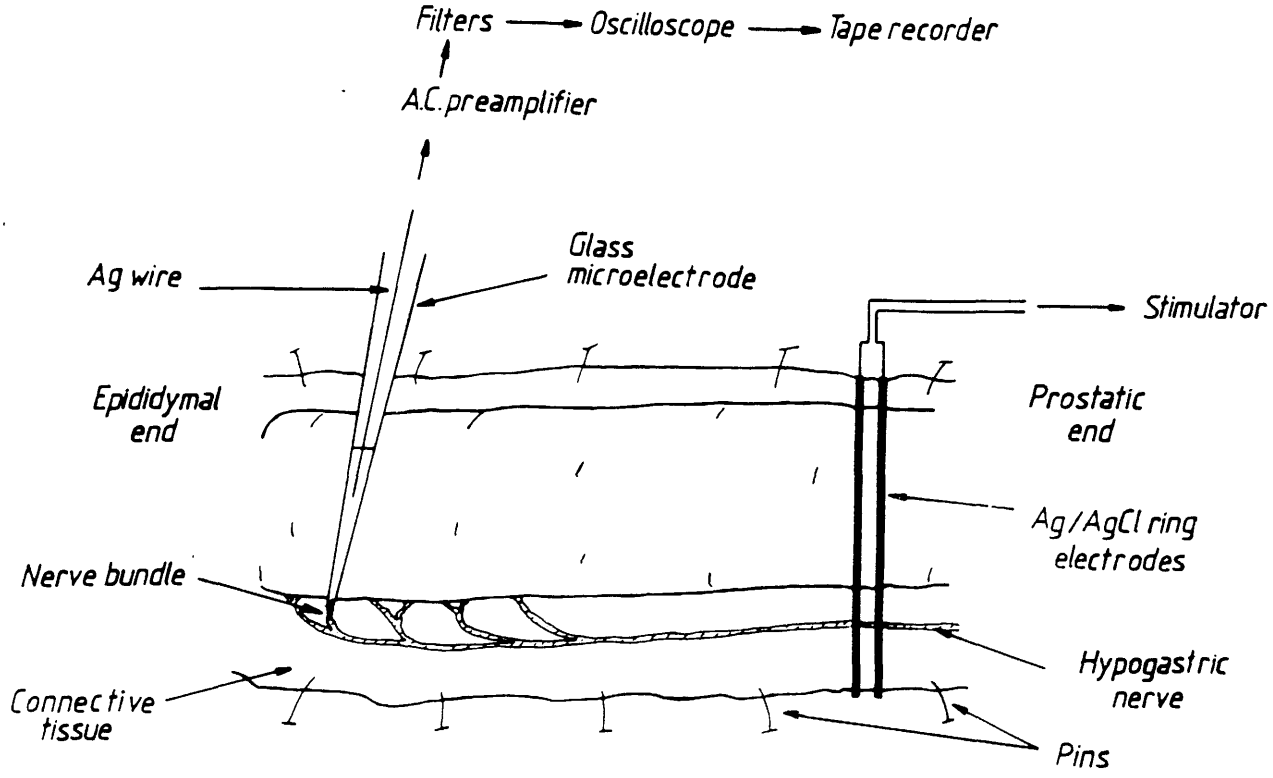


Fig. 7: Extracellular recording of AP conduction in vitro, in a small bundle of guinea-pig hypogastric nerve fibres, using a suction electrode. The central cut end of a small nerve branch at the epididymal end of the vas deferens was sucked into the broken tip of a glass microelectrode, filled with Krebs solution. APs were recorded following stimulation of the main nerve trunk with a pair of Ag/AgCl ring electrodes placed midway along the tissue. Electrical signals from the silver recording electrode were amplified, filtered and displayed on an oscilloscope, from which they were recorded on tape.

resting length, to the Sylgard base of the bath. The electrodes were connected to a square pulse stimulator (Devices Mk.III), which was triggered by a pulse generator (Devices 2521), gated in turn by a Digitimer.

To gain access to the small hypogastric nerves, a longitudinal incision was made through the connective tissue overlying the muscle. The surrounding connective tissue was pinned back and laterally stretched to allow the nerves to be visualized microscopically. A small hypogastric nerve bundle (30-100 μm in diameter) was cut near its point of entry into the epididymal end of the tissue. The glass microelectrode tip was broken under microscopic control and its diameter matched approximately to that of an available nerve bundle. The central cut end of the nerve bundle was drawn into the microelectrode tip by applying suction, to allow measurement of stimulation-evoked APs to be made.

RECORDING FROM GUINEA-PIG HYPOGASTRIC NERVES IN VIVO

The ready accessibility of the vas deferens and its extrinsic nerves made this investigation feasible (Ferry, 1967). Adult male Duncan-Hartley guinea-pigs (400-800g), anaesthetised with urethane (1.7 g kg^{-1} i.p.), were placed, ventral side uppermost, on a thermostatically-controlled heated (37 $\pm$ 0.1°C) blanket (C.F. Palmer 8185). The trachea was cannulated to allow artificial respiration, the right jugular vein for i.v. administration of drugs and the left carotid artery for measurement of blood pressure and heart rate. The arterial

cannula, containing heparinized saline (90 I.U. ml^{-1} of 0.9% NaCl in distilled water) to prevent coagulation, was connected via a blood pressure transducer (Gould P23ID), to a pen recorder (Harvard Universal Oscillograph).

The abdomen was opened by a midline incision and the overlying skin sutured to a metal ring (4.6 cm internal diameter), clamped above the abdomen (Fig. 8). The gastrointestinal tract was displaced and the peritoneal cavity filled with Krebs solution. The temperature of the animal was monitored rectally by a probe connected to the heated blanket and maintained at $37 \pm 1^{o}C$.

On occasion, drugs were added by close-arterial injection via the left external iliac artery, cannulated retrogradely, peripheral to its junction with the internal iliac artery. The patency of this route was confirmed by injection of indian ink.

For convenience, pre- rather than postganglionic stimulation (0.2 Hz, 0.1 - 0.5 ms, supramaximal voltage) of the hypogastric nerves, approximately 2 cm from the vas deferens, was carried out using a pair of platinum hook electrodes. The electrodes were connected to an isolated stimulator (Digitimer DS2) and this, in turn, to a frequency generator (Scientific and Research Instruments 6030). The left vas deferens was then pinned on to a piece of silicone rubber, placed inside the peritoneal cavity. The subsequent dissection and recording of APs were carried out in a manner similar to that described previously for the in vitro investigation. APs were passed to a preamplifier and filtered (low and high frequency cutoffs - 80 and 250 Hz respectively (Tektronix RM 122)). Signals

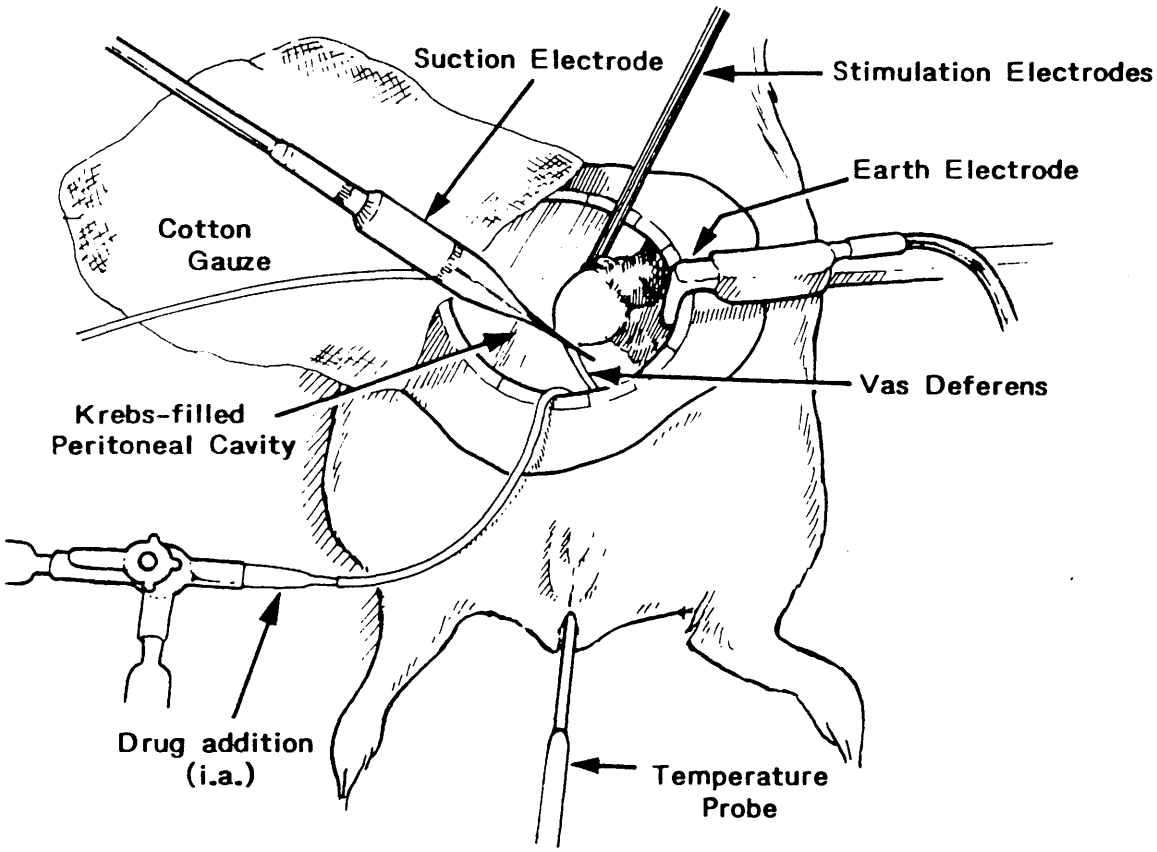


Fig. 8: Extracellular recording of AP conduction in vivo in a small bundle of hypogastric nerve fibres in the anaesthetised guinea-pig (1.7 gkg^{-1} urethane i.p.). Following a midline incision, the abdominal wall was sutured to a metal ring clamped above the animal, the gastrointestinal tract placed under moistened cotton gauze and the peritoneal cavity filled with Krebs solution. The temperature of the animal was monitored rectally with a probe and maintained at 37 $\pm$ 1°C. A pair of platinum hook electrodes was placed under both hypogastric nerve trunks, some 2 cm from the vas deferens, to provide preganglionic stimulation. The vas deferens was pinned to a piece of silicone rubber and APs recorded from the small central cut end of a nerve bundle at the epididymal end of the tissue, using a suction electrode. The earth electrode consisted of a metal plate placed inside the peritoneal cavity. Cannulation of the external iliac artery allowed close-arterial drug addition to the vas deferens. Drugs were also given i.v. by cannulation of the jugular vein (not shown).

were displayed on a storage oscilloscope (Telequipment DM64) and recorded permanently on tape (Racal 4DS). An image analyser and computer (Hewlett Packard) were used to determine the latency, duration and area of the AP discharges.

RECORDING FROM RAT ANOCOCCYGEUS NERVES IN VITRO

The anococcygeus was pinned to the Sylgard base of the organ bath and the attached genito-femoral and perineal branches of the pudendal nerve gently pulled through a separate pair of bipolar Ag/AgCl ring electrodes (0.2 cm diameter, 0.2 cm separation) for stimulation (single pulses, 0.1 - 0.5 ms, supramaximal voltage). The electrodes were connected to a stimulator (Devices Mk III), triggered by a pulse generator (Devices 2521) and Digitimer. Stimulation-evoked APs were recorded as already described.

In order to record APs from the NANC nerves of the anococcygeus, the sympathetic nerves were destroyed by pre-treating rats with reserpine (1 mg kg^{-1} i.p. daily for 3 days) and 6-hydroxydopamine (150 and 250 mg kg^{-1} i.p. for the following 2 days). The effectiveness of the pretreatment was assessed by the Falck histofluorescence technique (modified by Gillespie & Kirpekar, 1966). In this technique, catecholamines in sympathetic nerves are condensed by exposure to formaldehyde vapour, producing fluorophores of isoquinoline which absorb light at 410 nm and emit light at 480 nm. Tissues were frozen in isopentane, itself cooled by liquid nitrogen, to remove any moisture and so avoid tissue damage on freezing,

and then transferred to a freeze-drier (Pearse Speedivac) under a partial vacuum (0.01 torr) at -40°C for 24 h. Muscles were exposed thereafter to paraformaldehyde at 80°C in an oven for 1 h. Transverse sections (6 μ thick) were cut, mounted in liquid paraffin and examined under u.v. light using a Leitz Orthoplan microscope with a BG 12 narrow band filter for excitation and a Zeiss No.47 barrier filter. Photographs were taken with a Leitz Orthomat camera.

INTRACELLULAR RECORDING OF SMOOTH MUSCLE ACTIVITY

In these experiments, simultaneous electrical and mechanical recordings were made from the guinea-pig vas deferens and i.a.s. and the rat anococcygeus. In each tissue, evoked junction potentials were used to assess the effects of Ca^{2+} channel antagonists on transmitter release.

Each tissue was pinned to the Sylgard base of a horizontal perspex organ bath (Fig. 9), the characteristics of which have been previously described. In the case of the vas deferens and i.a.s., field stimulation (single pulses and 5-20 pulses at 1-20 Hz, 0.1 - 0.5 ms, supramaximal voltage) was provided by a pair of bipolar Ag/AgCl ring electrodes (0.3 cm diameter, 0.2 cm separation), placed midway along each tissue. The genito-femoral and perineal branches of the pudendal nerve, which innervate the anococcygeus, were stimulated (5 pulses at 5-20 Hz, 0.1 - 0.5 ms, supramaximal voltage) simultaneously by means of two pairs of bipolar Ag/AgCl ring electrodes (0.2 cm diameter, 0.2 cm separation). The anococcygeus was stimulated via its extrinsic innervation, rather than by field

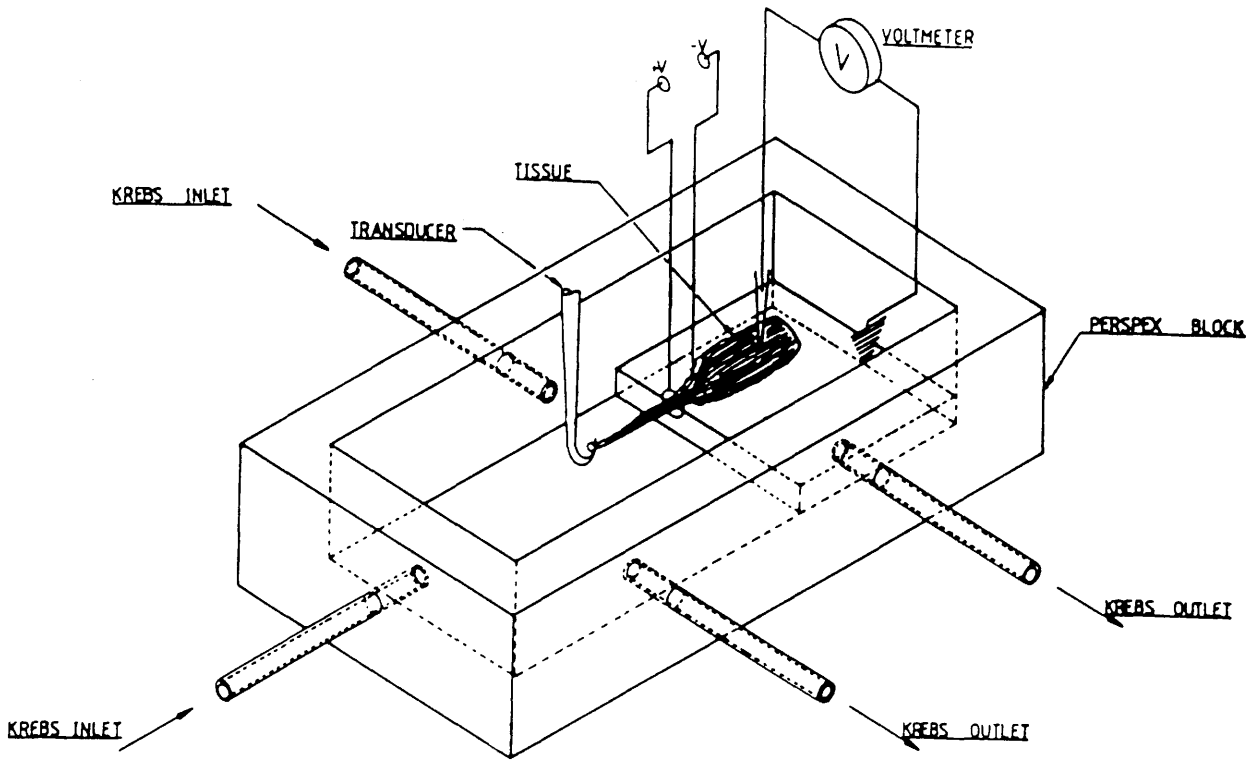


Fig. 9: Organ bath for combined intracellular electrical and mechanical recording. The bath comprised a central trough (4 cm x 1.5 cm x 1 cm) cut from a perspex block. The block was drilled to accept stainless steel inlet tubes (0.2 cm internal diameter) for Krebs solution and outlets for drainage. The body of the muscle was pinned to the Sylgard base of the trough and intracellular recordings made from the pinned area. One free end was tied by thread to an isometric transducer. Field stimulation by an isolated stimulator was effected via Ag/AgCl ring electrodes (+V and -V). The bath was perfused with oxygenated pre-heated Krebs solution (36 $\pm$ 0.5^{o}C), supplied via two inlets by gravity flow. The polythene tubing (0.2 cm internal diameter) containing the Krebs solution was surrounded by an outer tube (1.0 cm internal diameter) containing liquid paraffin (at 40 $\pm$ 0.1^{o}C), pumped from a thermostatically-controlled Tempette pump.

stimulation, to allow simultaneous measurement of nerve AP conduction (by suction electrode), e.j.ps and mechanical responses to be made. The electrodes were connected to a square wave pulse stimulator (Devices Mk III) which was triggered by a pulse generator (Devices 2521) and gated by a Digitimer. The mechanical activity of each tissue was monitored: the i.a.s., vas deferens (prostatic end) and anococcygeus (tendinous end) were each connected by thread to an isometric force-displacement transducer (Grass FT03C) under tension (1g).

Changes in membrane potential were recorded intracellularly using capillary glass microelectrodes (15-40 MΩ), filled with 3M KCl. Electrical measurements from the vas deferens and i.a.s. were made up to 0.4 cm from the stimulating electrodes. The microelectrode was connected to a unity gain high impedance (10^{10} Ω) D.C. preamplifier (WPI M44A) via a Ag/AgCl half cell, attached to a probe, matched and calibrated for the amplifier used. The indifferent electrode was a Ag/AgCl pellet in the organ bath. Electrical signals, passed via the preamplifier, were displayed on one channel of a storage oscilloscope (Tektronix 5103N) and on a digital voltmeter (Fairchild M 53). The mechanical responses measured by the transducer were displayed on the other channel of the oscilloscope. Electrical and mechanical measurements were amplified and recorded on a u.v. recorder (SE Oscillograph 3006 and 6150) and stored on tape (Racal 4DS).

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 2 mV.

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

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

RADIOLABELLED TRANSMITTER RELEASE

As an alternative means of measuring transmitter release, the transmitter pools of the nerves to the guinea-pig vas deferens and i.a.s. were labelled radiochemically and the overflow from each tissue analysed for radioactivity. In this investigation, the term 'overflow' is defined as the amount of neurotransmitter which escaped the uptake processes and was collected from the tissue.

Tissues were incubated in Krebs solution (37°C) containing either (a) $[^3H]$ -noradrenaline (25 μCi ml^{-1}, 2 x 10^{-6}M noradrenaline for 30 min) or (b) $[^3H]$ -adenosine (50 and 150 μCi ml^{-1}, 1 and 3 x 10^{-6}M adenosine for 1 h) and then rinsed with Krebs solution. Tissues were pulled gently through a pair of bipolar Ag/AgCl ring electrodes (0.3 cm diameter, 0.2 cm separation) located inside a heated (37 $\pm$ 0.5°C) water jacket, and attached at one end to a hook on the electrode assembly (Fig. 10). The other end of each tissue (the

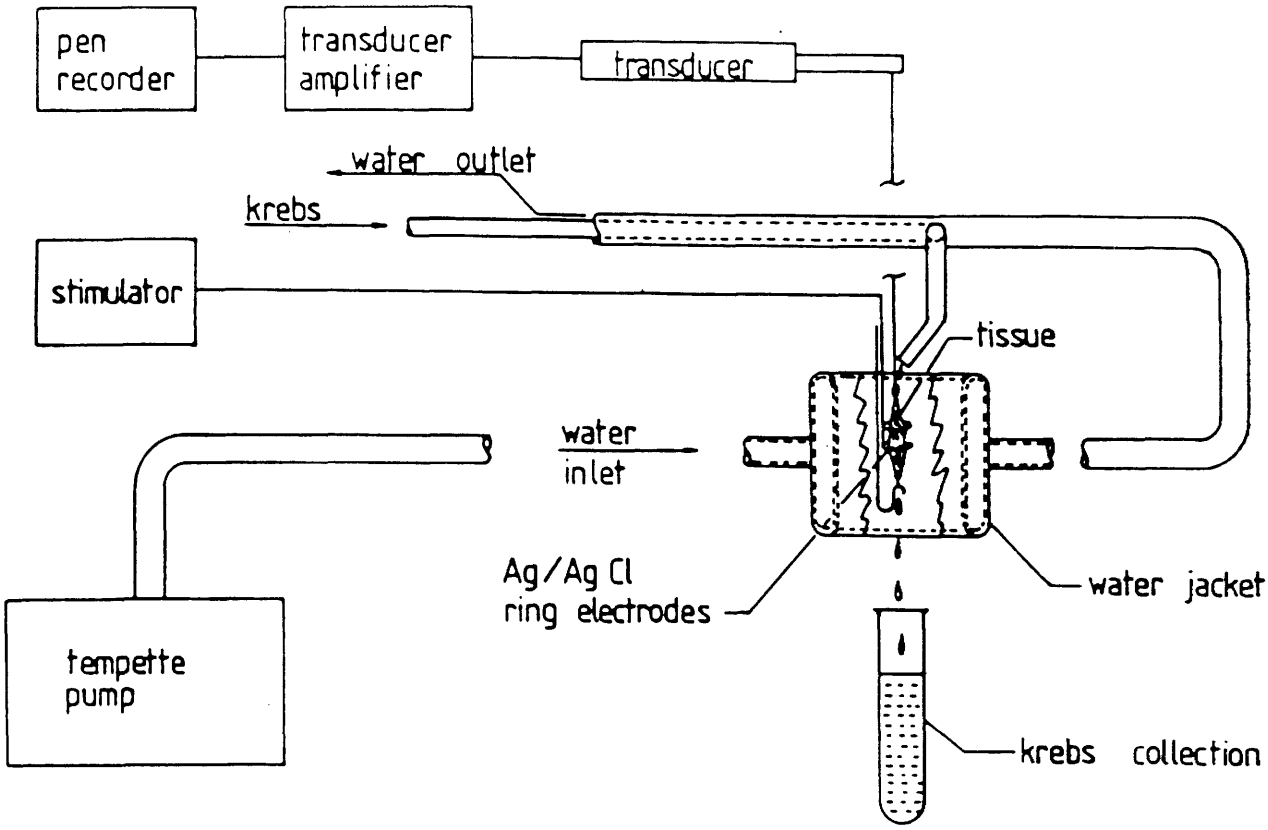


Fig. 10: Apparatus for measuring ^{3}H overflow. Each tissue, loaded with either $[^{3}H]$ -noradrenaline or $[^{3}H]$ -adenosine, was attached by thread to an isometric force-displacement transducer at one end and the other end passed through bipolar Ag/AgCl ring electrodes and securely anchored by thread to a hook on the electrode assembly. The tissue was located inside a heated glass water jacket ($37 \pm 0.5^{\circ}C$) and superfused (1 $mlmin^{-1}$) with oxygenated Krebs solution. The polythene tubing (0.2 cm internal diameter) containing the Krebs solution was surrounded by an outer tube (1.0 cm internal diameter) containing water at $42 \pm 0.5^{\circ}C$, circulated by a thermostatically-controlled pump.

epididymal end, in the case of the vas deferens) was connected, under tension (1g), by thread to an isometric force-displacement transducer (Grass FT03C). The ring electrodes were connected to a stimulator (Grass SD9) for field stimulation. Drugs were added to the Krebs solution, which was pumped through polythene tubing (0.2 cm internal diameter) using a Watson-Marlow (Type 22) flow inducer. The tubing was surrounded by an outer jacket (1 cm internal diameter) containing water ($42 \pm 0.5^{\circ}C$) pumped by a Tempette (TE7) pump. The polythene tubing was positioned to allow superfusion of the preparation with Krebs solution (1 ml min^{-1}, $37^{\circ}C$). 2 min samples of superfusate were collected in vials placed under the tissue.

Vasa incubated with [^{3}H] -noradrenaline (25 μCi ml^{-1}) were superfused throughout each experiment with Krebs solution containing ascorbic acid (1.1×10^{-4}M) to inhibit catecholamine oxidation, desipramine (6×10^{-7}M) and normetanephrine (1×10^{-5}M) to inhibit neuronal and extraneuronal catecholamine uptake respectively and atropine (2.6×10^{-6}M) to block the cholinergic transmission which exists in this tissue (Sjöstrand, 1981). Under these conditions, approximately 90% of the catecholamine in the tissue is believed to be noradrenaline (Alberts et al., 1981). The effects of drugs on stimulation (60 pulses at 1-20 Hz, 0.5 ms, supramaximal voltage)-evoked ^{3}H overflow were examined 15 min after commencing drug superfusion.

It is believed (Westfall et al., 1978) that in the guinea-pig vas deferens, contraction of the muscle per se, contributes

to the release of ATP. Accordingly, to prevent muscle contractions, vasa incubated with $[^3H]$ -adenosine (150 μCi ml^{-1}) were superfused with a hypertonic Krebs solution containing sucrose (0.37M). Additionally, α, β methylene adenosine 5'-triphosphate (α, β meATP) and prazosin (each 5 x 10^{-6}M) were added to prevent the postjunctional effects of ATP and noradrenaline respectively, which are released from the sympathetic nerves (Burnstock & Sneddon, 1984). One tissue from each animal was superfused with the drug being studied, while the contralateral tissue served as control and both were stimulated simultaneously (400-2000 pulses at 20 Hz, 0.5 ms, supramaximal voltage), 15 min after commencing drug superfusion.

The guinea-pig i.a.s. incubated with $[^3H]$ -adenosine (50 μCi ml^{-1}) was superfused with Krebs solution containing atropine (1 x 10^{-6}M) to inhibit cholinergic responses of any residual longitudinal muscle and phentolamine and guanethidine (each 1 x 10^{-6}M) to inhibit responses to, and release of, noradrenaline respectively. Drug effects on stimulation (200-900 pulses at 2-20 Hz, 0.5 ms, supramaximal voltage) - evoked ^3H overflow were examined 15 min after commencing superfusion.

0.5 ml aliquots of the tissue superfusate were added to a scintillation mixture (10 ml) containing toluene: triton-X: scintol-2 (12.33: 6.67: 1, v:v:v) and the amount of ^3H measured with a scintillation counter (Packard 3390). The amount of ^3H, in counts per minute (CPM), was corrected for the efficiency of the counter (approximately 30%) and expressed as disintegrations per minute (DPM), or as the fractional release of

^{3}H, i.e. the overflow evoked by stimulation, as a fraction of the total ^{3}H in the tissue at that time. This was determined after dissolving the tissue at the end of the experiment in potassium hydroxide (0.5M) and counting an aliquot of the sample.

CHROMATOGRAPHIC IDENTIFICATION OF $[^{3}H]$ -COMPOUNDS

The radioisotope experiments per se, measured the total ^{3}H and did not allow identification of the compounds into which the ^{3}H was incorporated.

To do this, chromatographic techniques were used and the major metabolites of both noradrenaline and ATP (Norman et al., 1974; Pull & McIlwain, 1972; Tarlov & Langer, 1971), separated and identified. Identification of the $[^{3}H]$ -compounds in the overflow allowed a further analysis of drug and nerve-induced effects on release to be made.

The guinea-pig vas deferens and i.a.s. were incubated with $[^{3}H]$ -noradrenaline and $[^{3}H]$ -adenosine respectively, as before, and samples of superfusate collected for chromatography.

(i) Separation and identification of $[^{3}H]$ -noradrenaline and its metabolites

The ^{3}H in the superfusate from vasa incubated with $[^{3}H]$ -noradrenaline was separated into noradrenaline and its principal metabolites (Tarlov & Langer, 1971), 3,4-dihydroxyphenylglycol (DOPEG), 4-hydroxy 3-methoxymandelic acid (VMA) and 4-hydroxy 3-methoxyphenylglycol (MOPEG), 3,4-dihydroxymandelic acid (DOMA) and normetanephrine (NMN). The method of separation (Fig. 11) was that of Graefe et al. (1973), Nicol (1975) and Tillman (1977).

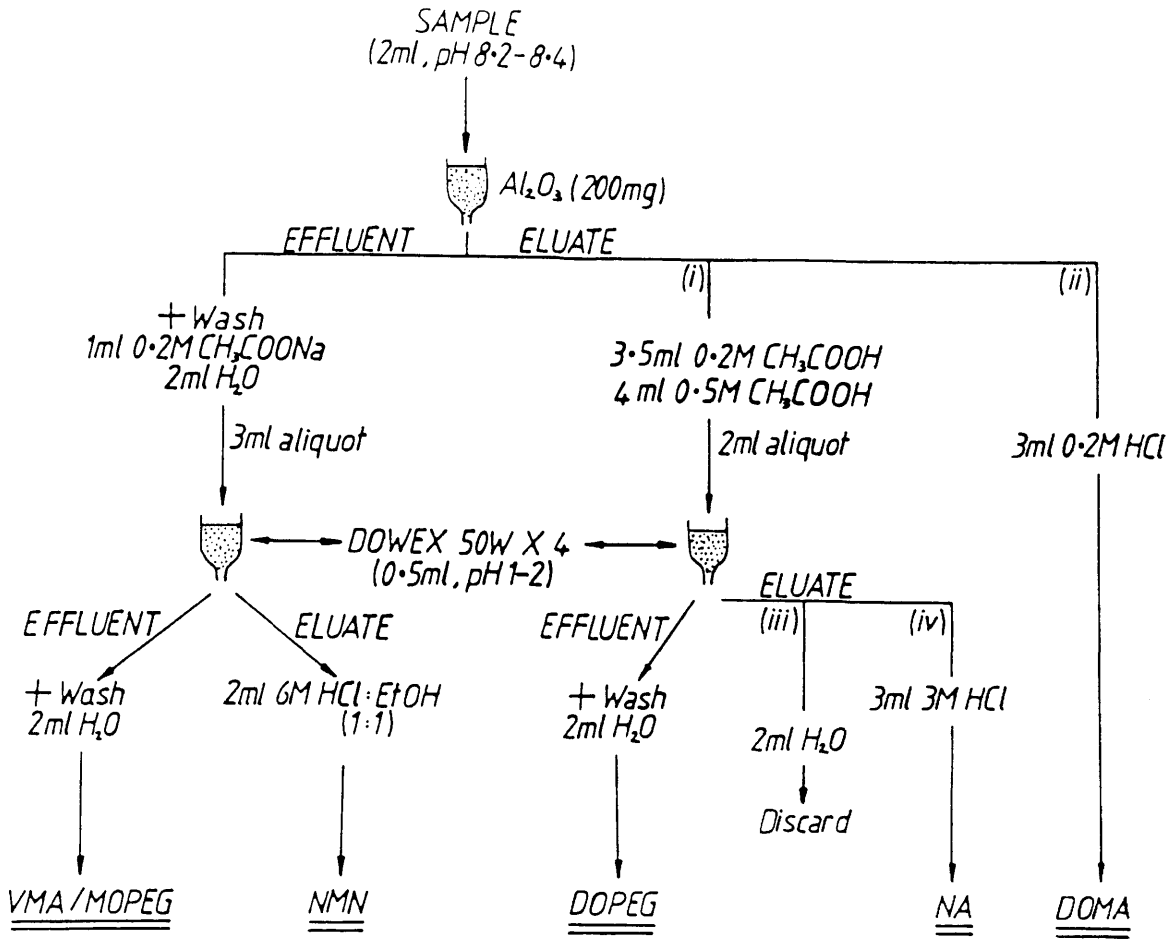


Fig. 11: Flow diagram of the chromatographic separation of $[^3H]$ -noradrenaline from its metabolites. 2 ml samples (pH 8.2 - 8.4) were applied to a column containing alumina (Al_2O_3). The compounds in the effluent and washings from this column were separated by another column, containing Dowex 50W x 4 resin. 4-Hydroxy 3-methoxymandelic acid (VMA) and 4-hydroxy 3-methoxyphenylglycol (MOPEG) were present in the effluent and washing and normetanephrine (NMN) in the eluate, following addition of HCl:EtOH. Noradrenaline (NA) and 3,4-dihydroxy-phenylglycol (DOPEG) were eluted from the alumina-containing column with CH_3COOH (i) and 3,4-dihydroxymandelic acid (DOMA), with HCl (ii). The NA/DOPEG fraction was separated by another column, filled with Dowex 50W x 4 resin, the effluent and washing from which contained DOPEG. Following further washing with H_2O (iii), NA was eluted with HCl (iv).

The chromatographic procedure involved passing the superfusate from the guinea-pig vas deferens through three columns; one containing alumina (Sigma) and two, Dowex 50W x 4 (200-400 mesh, Sigma), a strong cation exchange resin. Each column consisted of a shortened glass Pasteur pipette stoppered with cotton wool (length 10 cm, diameter 0.5 cm to 0.2 cm at the tip). The alumina and Dowex resin were prepared prior to use. Alumina (0.2 g), prepared by the method of Crout (1961), was added dry to columns and washed with sodium acetate (5 ml, 0.2M, pH 8.2). Dowex resin was washed several times with NaOH (2M) containing ethylenediamine tetra-acetic acid (EDTA, 0.03M), at 50°C, until the supernatant was clear and then with distilled water, HCl (2M), distilled water again and finally stored in HCl (0.01M). Dowex resin was added to columns as a slurry (0.5 ml) and then washed with distilled water.

Samples of superfusate (2 ml) were collected prior to, and following field stimulation (300 pulses at 20 Hz, 0.5 ms, supramaximal voltage), in vials placed on ice. Each sample was adjusted to pH 1-2 with perchloric acid (0.4M), which contained EDTA (3×10^{-3}M) and sodium metabisulphite (5.3×10^{-3}M) to precipitate protein and minimize catecholamine breakdown. Unlabelled noradrenaline, DOPEG, VMA, MOPEG, DOMA and NMN (each 10 µl, 1 mg ml^{-1}) were then added as carriers for the much smaller amounts of labelled compounds.

The pH of each sample of superfusate was adjusted to 8.2 - 8.4 with Tris buffer (0.5M) and added to the column containing alumina. The column was then washed with sodium

acetate (1 ml, 0.2M) and distilled water (2 ml). VMA, MOPEG and NMN, which are not retained by the alumina, were collected in the effluent and washing. To separate VMA and MOPEG from NMN, an aliquot (3 ml) of the effluent and washing from the alumina and distilled water (2 ml) were added to the first of the two columns filled with Dowex resin. VMA and MOPEG passed through the column, while NMN, retained by the Dowex resin, was eluted with HCl-ethanol (2 ml, 6M, 1:1 v/v).

The compounds retained by the alumina were eluted; noradrenaline and DOPEG with acetic acid (3.5 ml, 0.2M and 4 ml, 0.5M) and DOMA with HCl (3 ml, 0.2M). Separation of noradrenaline from DOPEG was achieved using the remaining column filled with Dowex resin. An aliquot (2 ml) of the combined noradrenaline and DOPEG fraction, followed by distilled water (2 ml), was added to the column. DOPEG, which is not retained by the Dowex resin, passed straight through the column. After further washing with distilled water (2 ml), to ensure complete removal of DOPEG, noradrenaline, bound to the Dowex resin, was eluted with HCl (3 ml, 3M).

Each separated fraction (0.5 ml) was added to scintillant (10 ml, composition as before) and the ^{3}H counted. The amount of radioactivity in each of the metabolite samples, compared to that in the sample before separation, gave an indication of the recovery and efficiency of each column. The mean recovery for the column filled with alumina was 94.8 $\pm$ 2.5% (n = 45) and for the two columns with Dowex resin, 97.1 $\pm$ 3.3% and 86.4 $\pm$ 2.3% (n = 45).

(ii) Separation and identification of $[^3H]$ -ATP and its metabolites

The ^3H in the superfusate from the i.a.s., together with the total amount retained by nerves and muscle, following incubation with $[^3H]$ -adenosine, was separated into ATP and its major breakdown products (Norman et al., 1974), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), hypoxanthine, cAMP and adenosine and inosine.

Samples of superfusate (5 ml) were collected prior to, and following field stimulation (2400 pulses at 20 Hz, 0.5 ms, supramaximal voltage) in vials placed on ice. The i.a.s. was then homogenised in perchloric acid (5 ml, 0.25M) to disrupt the cells and prevent enzyme activity. The cellular debris and proteins were then sedimented by centrifugation at 0^oC (15500 rpm for 15 min) and the supernatant transferred to a vial placed on ice.

The technique used to separate and identify the $[^3H]$ -compounds was thin layer chromatography (TLC), as described by Norman et al. (1974) and Pull & McIlwain (1972). Identification of compounds by TLC necessitates the use of a small sample volume. Preliminary studies, however, with a 50 µl aliquot of the superfusate from the i.a.s. revealed that the amount of ^3H was insufficient to enable an accurate detection to be made. Accordingly, the radioactivity in each sample was concentrated prior to TLC separation (Tsuboi & Price, 1959). The $[^3H]$ -compounds in each sample of superfusate or tissue supernatant were adsorbed on to charcoal (5 mg) in perchloric

acid (1.25 ml, 1M, 4°C). After centrifugation (2000 rpm for 5 min), to sediment the charcoal and adsorbed material, the supernatant of each sample was discarded and the ^{3}H eluted by addition of pyridine (0.5 ml, 1.26M), with occasional mixing for 2 h. The pyridine-charcoal mixture was then centrifuged and the resulting supernatant, which contained the $[^{3}H]$ -compounds, transferred to a small vial for the next step. A gentle stream of O_2-free N_2 was passed over the supernatant to evaporate the pyridine and leave a concentrated aqueous sample.

In preparation for TLC separation, methyl orange (2 µl, 0.25M) was added to each concentrated sample. Perchloric acid (1M) was then added to adjust the pH to 3-4, at which value, methyl orange changed colour to red. Each sample (50 µl), together with standard solutions of ATP, ADP, AMP, hypoxanthine, cAMP, adenosine and inosine (10 µl, 1 mg ml^{-1}), were spotted on to two silica gel-coated TLC glass plates (Merck $60F_{254}$). A different solvent system was used for each plate, allowing separation and identification of most of the $[^{3}H]$ -compounds present. cAMP, hypoxanthine and adenosine and inosine (R_f values 0.23, 0.31 and 0.39 respectively), but not ATP, ADP or AMP, which remained at or near the origin, were separated with butan-1-ol:ethyl acetate:methanol:ammonia (7:4:3:4, by vol.). ATP, ADP and AMP (R_f values 0.30, 0.39 and 0.53 respectively), but not the remaining compounds (R_f value 0.65), were separated using isobutylalcohol:amylalcohol:ethoxyethanol:ammonia:water (15:10:30:15:25, by vol.). Using both these solvent systems,

adenosine and inosine had the same R_F value and therefore could not be separated. Spots produced by the standards were located under u.v. light, outlined in pencil and the silica gel scraped and transferred to scintillation vials for counting.

RELEASE OF ATP IN THE GUINEA-PIG INTERNAL ANAL SPHINCTER

In addition to the radioisotope experiments, two further attempts were undertaken to determine if ATP is the NANC transmitter in the guinea-pig i.a.s. (Lim, 1985; Lim & Muir, 1986).

The i.a.s. was superfused as before (Fig. 10) and samples of superfusate collected prior to, and following field stimulation (100-1000 pulses at 10 and 20 Hz, 0.1 - 0.5 ms, supramaximal voltage). The Krebs solution contained atropine (1×10^{-6}M) to inhibit cholinergic responses of residual longitudinal muscle and phentolamine and guanethidine (each 1×10^{-6}M) to inhibit responses to, and release of, noradrenaline respectively. Sodium vanadate (1×10^{-4}M), an ATPase inhibitor (Cantley *et al.*, 1977), was added to prevent the breakdown of ATP. Samples of superfusate were subjected to the following procedures.

(i) Analysis by thin layer chromatography

Trichloroacetic acid (5 ml, 1.2M) was added to each sample of superfusate (10 ml) to denature any enzymes present. Following mixing and centrifugation (4000 rpm for 15 min), 4 volumes of water-saturated diethyl ether were added to the

supernatant and the organic phase, containing the trichloroacetic acid, discarded. The aqueous phase, containing ATP and its metabolites, was then freeze-dried (Edwards Modulyo) and resuspended in distilled water (1 ml). An aliquot (0.1 ml) of each sample was spotted on a polyethyleneimine-cellulose-coated TLC plastic sheet (Merck 5579). The solvent used was lithium chloride (1M). Spots were detected by u.v. light and identified by comparison to those produced by standard solutions of ATP and its metabolites (10 µl, 1 mg ml^{-1}).

(ii) Luciferase-luciferin luminescence

The luciferase-luciferin luminescence technique is a sensitive means of assaying ATP (Strehler & Totter, 1952). The principle behind this method is the interaction of ATP with synthetic luciferin and with luciferase extracted from firefly tails. This interaction emits luminescence which can be detected.

Luciferase-luciferin (0.1 ml, 40 mg ml^{-1}) was added to an aliquot (0.5 ml) of each sample of superfusate and the luminescence detected with a luminescence aggregometer (Chronolog Corporation), connected to a pen recorder (Linseis). The luminescence from samples of superfusate was compared to that produced by known concentrations of ATP, dissolved in Krebs solution.

ANALYSIS OF RESULTS

Where appropriate, results were expressed as the mean ± the standard error of mean (s.e. mean); n = number of observations. Student's t-tests were used to test for significance ($p < 0.05$) between means.

PHYSIOLOGICAL SOLUTIONS

The ionic composition of the Krebs physiological saline solution (Krebs & Henseleit, 1932), used throughout the investigation, was (x 10^{-3}M): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.7, NaH_2PO_4 1.1, $MgCl_2$ 1.3, $NaHCO_3$, 25.0 and (+)-glucose 11.0. To prevent precipitation of insoluble cobalt salts when used in high concentrations (above 2 x 10^{-3}M), and to maintain isotonicity, tris buffer (2.5 x 10^{-3}M) and choline chloride (23.6 x 10^{-3}M) replaced NaH_2PO_4 and $NaHCO_3$ in the Krebs solution. The modified Krebs solution itself was inactive, having no effect on nerve AP conduction in either the guinea-pig vas deferens or rat anococcygeus.

DRUGS

The following drugs were used: adenosine (Sigma), 2,5',8-$[^3H]$-adenosine (Amersham International, 40-60 Cimmol^{-1}), adenosine 3',5'-cyclic monophosphate (cAMP, Sigma), adenosine 5'-monophosphate (AMP, Sigma), adenosine 5'-diphosphate (ADP, Sigma), adenosine 5'-triphosphate (ATP, Sigma), amlodipine maleate (Pfizer), (-)-ascorbic acid (Koch-Light), atropine sulphate (Sigma), choline chloride (Hopkin and Williams), cobaltous chloride (Koch-Light), cremophor EL (Sigma),

desipramine hydrochloride (Ciba), (±)-3,4-dihydroxymandelic acid (DOMA, Sigma), (±)-3,4-dihydroxyphenylglycol (DOPEG, Sigma), diltiazem hydrochloride (Sigma), dipyridamole (Sigma), ethylene diamine tetra-acetic acid (EDTA, Sigma), guanethidine sulphate (Ciba), heparin sodium (Evans), 6-hydroxydopamine hydrobromide (Sigma), (±)-4-hydroxy 3-methoxymandelic acid (VMA, Sigma), 4-hydroxy 3-methoxyphenylglycol (MOPEG, Sigma), hypoxanthine (Sigma), inosine (Sigma), lignocaine hydrochloride (Sigma), luciferase-luciferin (Sigma), α,β methylene adenosine 5'-triphosphate (α,β meATP, Sigma), methyl orange (Sigma), nicardipine hydrochloride (Sigma), nifedipine (Pfizer), (-)-7,8-$[^3H]$-noradrenaline (Amersham International, 8-14 Cimmol^{-1}), (-)-noradrenaline bitartrate (Koch-Light), (±)-normetanephrine hydrochloride (NMN, Sigma), paraformaldehyde (Hopkin and Williams), pentolinium tartrate (May & Baker), perchloric acid (Hopkin and Williams), phentolamine mesylate (Ciba), potassium hydroxide (Hopkin and Williams), (±)-prazosin hydrochloride (Pfizer), reserpine (Sigma), sodium metabisulphite (BDH), sodium nitroprusside (BDH), sodium vanadate (Riedel-De Haën AG Seelze-Hannover), sucrose (Formachem), tetrodotoxin (TTX, Sigma), trichloroacetic acid (Koch-Light), tris (hydroxymethyl) aminomethane (Sigma), tris (hydroxymethyl) aminomethane hydrochloride (Sigma), urethane (Sigma), (±)-verapamil hydrochloride (Sigma). TTX and reserpine were expressed as the concentration of the base; other concentrations in the text refer to the salts. The solvents used, were all of analytical grade or better.

With the following exceptions, drugs were dissolved in 0.9% NaCl in distilled water. Nifedipine was dissolved under Na^+ illumination in the minimum amount of cremophor necessary and diluted in Krebs. Solutions containing nifedipine were protected from the light. Reserpine was dissolved in glacial acetic acid (0.3 ml, 17.5M) and diluted with distilled water. Solutions containing only glacial acetic acid and distilled water served as controls. Luciferase-luciferin, supplied as a solid in glycine buffer, was dissolved in distilled water and the resulting solution protected from the light and kept at 4°C. (-)-7,8- $[^3H]$ -noradrenaline, supplied in 0.02M acetic acid:ethanol (9:1, v:v) was resuspended in distilled water, containing ascorbic acid (5.7×10^{-3}M) to prevent breakdown of the catecholamine. 2,5',8- $[^3H]$ - adenosine, supplied in an aqueous ethanol solution (50%), was resuspended in distilled water. 6-Hydroxydopamine was dissolved by sonication in 0.9% saline containing ascorbic acid (5.7×10^{-3}M), kept at 4°C on ice and bubbled with O_2-free N_2 for at least 30 min prior to use. Solutions containing only ascorbic acid served as controls.

CHAPTER 3: RESULTS

ACTIONS OF Ca^{2+} CHANNEL ANTAGONISTS ON THE GUINEA-PIG VAS DEFERENS

(i) Nerve AP conduction in vitro

APs were recorded, routinely, from a small hypogastric nerve bundle, in response to stimulation (0.5 Hz, 0.05-0.5 ms). Supramaximal stimulation (0.5 Hz, 0.5 ms) produced a discharge of several APs (Fig. 12a). These had a mean amplitude of 0.2 ± 0.02 mV (n = 66) and a latency of 26.9 ± 2.0 ms (n = 60). The amplitude and latency of each AP varied, presumably reflecting their different diameters and conduction velocities. The mean duration of the AP discharges, in response to a single pulse (0.5 ms, supramaximal voltage), was 28.7 ± 2.8 ms (n = 30). The APs were postganglionic; hexamethonium (1×10^{-4}M) was ineffective.

The pattern of AP discharge was related to current strength; single biphasic APs were obtained by reducing the stimulation voltage and/or pulse width (Fig. 12b). These had a mean amplitude of 0.3 ± 0.02 mV (n = 30) and a duration of 2.7 ± 0.1 ms (n = 30).

Perfusion of the vas deferens with either verapamil ($0.5 - 2 \times 10^{-4}$M), diltiazem ($1 - 8 \times 10^{-4}$M), amlodipine ($0.5 - 2 \times 10^{-4}$M), nicardipine ($0.5 - 1 \times 10^{-3}$M) or cobalt ($4 - 6 \times 10^{-2}$M), inhibited both the amplitude and number of APs in a dose-dependent manner (Figs. 13 to 15). The order of potency of the organic Ca^{2+} channel antagonists was amlodipine > verapamil > diltiazem > nicardipine.

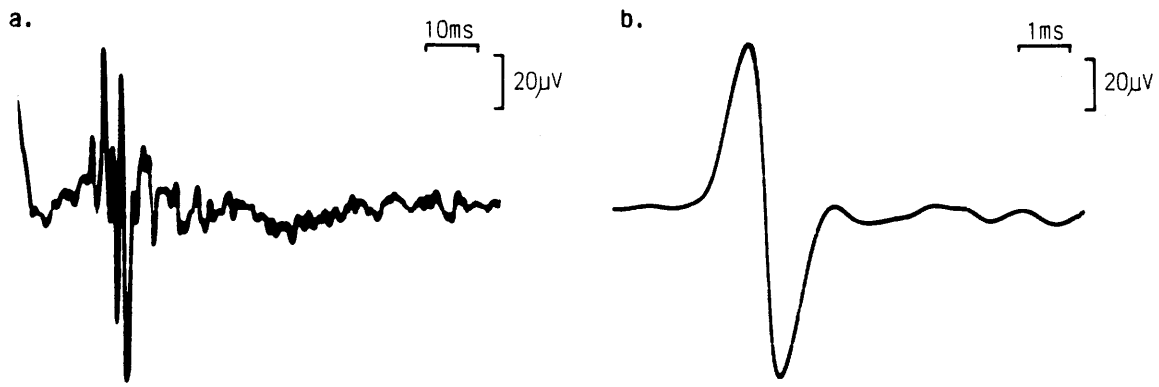


Fig. 12: APs, recorded extracellularly in vitro, from a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens, in response to a. supramaximal (0.5 Hz, 0.5 ms) and b. submaximal stimulation (0.5 Hz, 0.1 ms, 10V). Supramaximal stimulation produced an AP discharge (mean duration of 28.7 $\pm$ 2.8 ms, n = 30). The APs had a mean amplitude of 0.2 $\pm$ 0.02 mV (n = 66) and latency of 26.9 $\pm$ 2.0 ms (n = 60). Submaximal stimulation produced single biphasic APs. These had a mean amplitude of 0.3 $\pm$ 0.02 mV (n = 30) and duration of 2.7 $\pm$ 0.1 ms (n = 30).

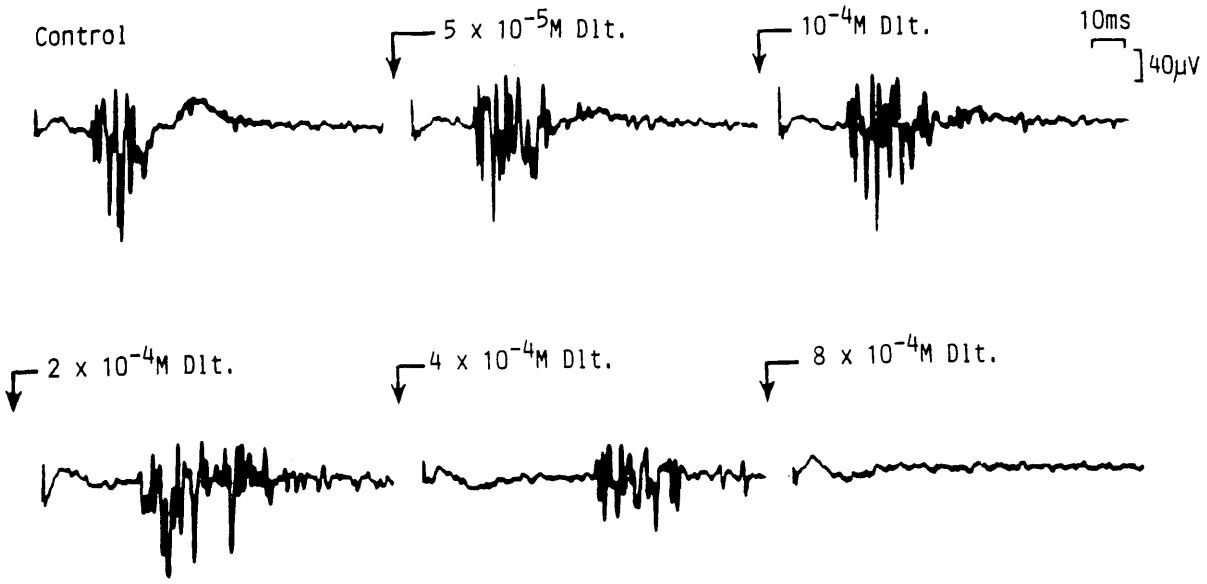


Fig. 13: The dose-dependent inhibitory effect of diltiazem (Dlt., 5×10^{-5} - 8×10^{-4}M) on AP conduction in vitro, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens, evoked by stimulation (0.5 Hz, 0.2 ms, supramaximal voltage). The time between each panel was 15 min.

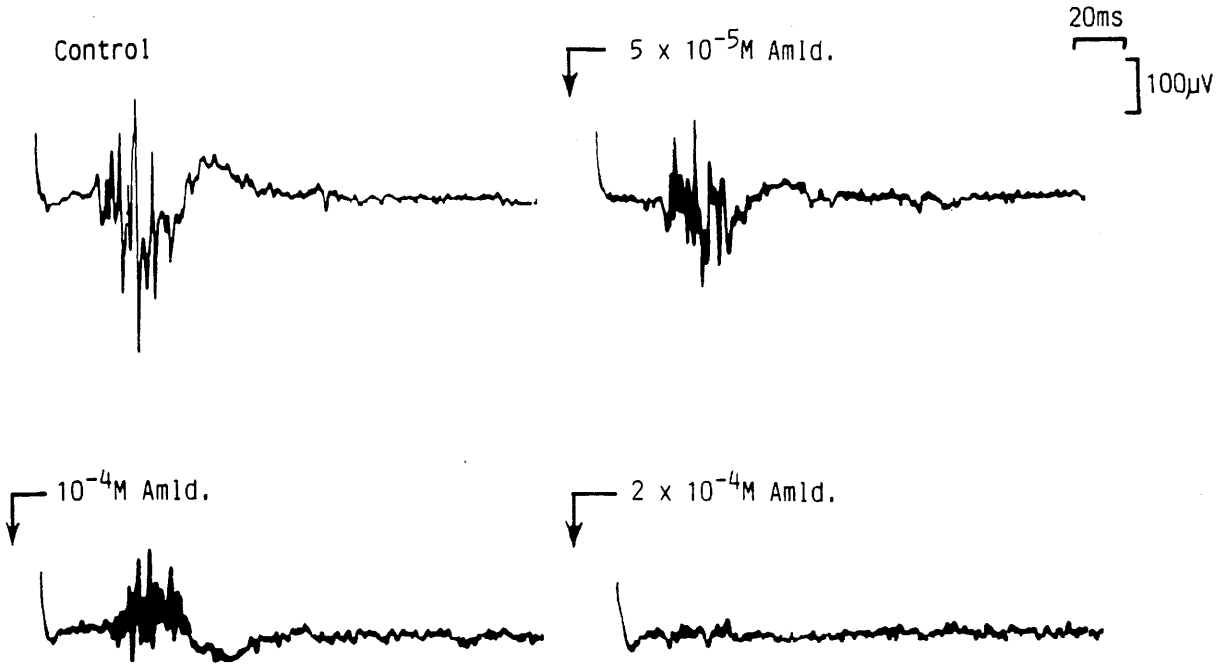


Fig. 14: The dose-dependent inhibitory effect of amlodipine (Amld., 5×10^{-5} - 2×10^{-4}M) on AP conduction in vitro, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens, evoked by stimulation (0.5 Hz, 0.2 ms, supramaximal voltage). The time between each panel was 15 min.

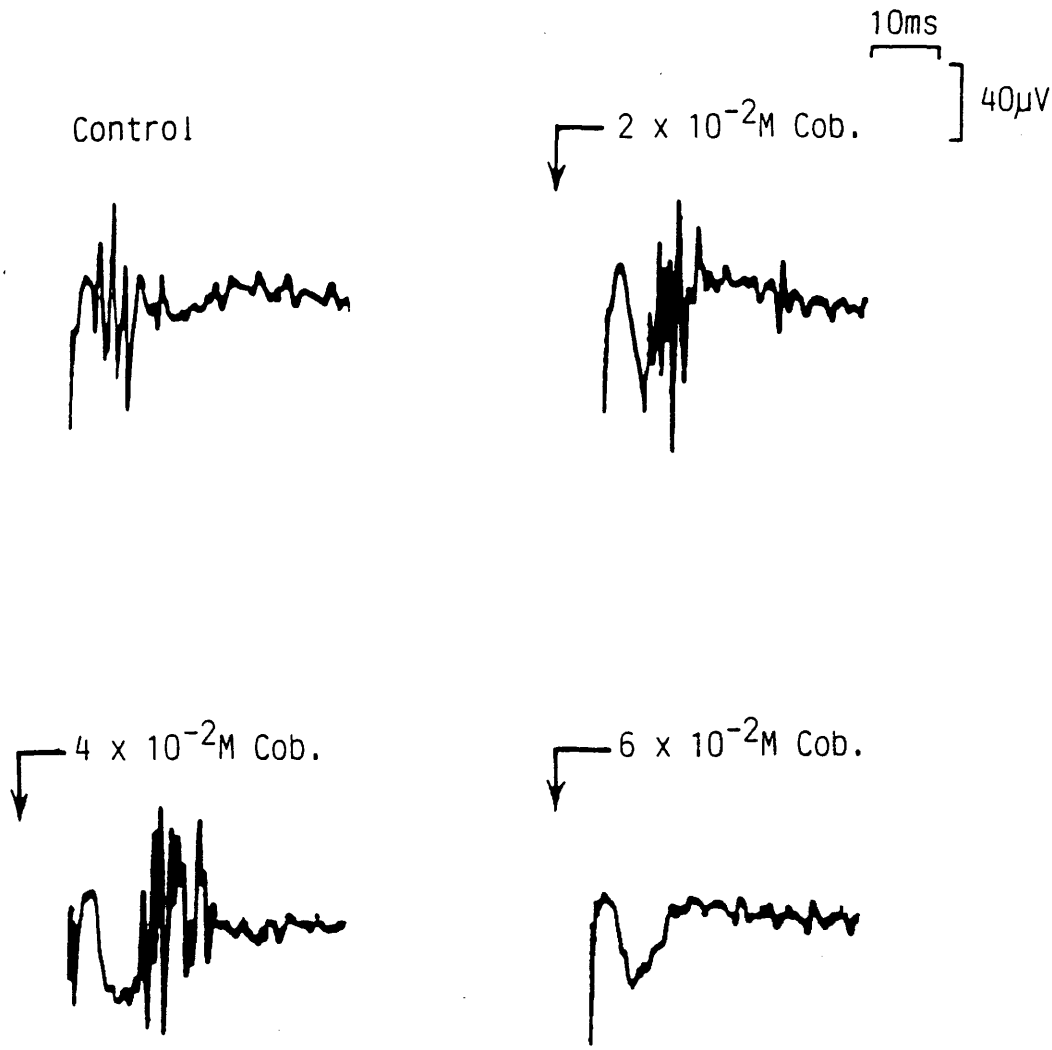


Fig. 15: The inhibitory effect of cobalt (Cob., 2 - 6 x 10^{-2}M) on AP conduction in vitro, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens, evoked by stimulation (0.5 Hz, 0.5 ms, supramaximal voltage). Higher concentrations of cobalt, compared to the organic agents diltiazem and amlodipine (previous 2 figures), were required to inhibit the APs. The time between each panel was 15 min.

The immediate effects of these antagonists were seen more clearly following bolus injection, than after perfusion. Lower concentrations were effective when added by injection, presumably because APs were blocked prior to the equilibration of drugs with the Krebs bathing solution. The initial effect of each antagonist was to inhibit, progressively, the latency and then to reduce the amplitude of the AP in either a graded or an 'all-or-none' manner, depending upon concentration (Fig. 16).

In contrast to the other antagonists, nifedipine, even at very high concentrations (up to 5×10^{-3}M), had no significant effect on AP conduction (Fig. 17).

The ionic basis of the APs was examined to determine the mechanism underlying the inhibitory effects of the Ca^{2+} channel antagonists. Removal of Ca^{2+} from the perfusing Krebs solution, without ionic compensation, reduced the amplitude of single biphasic APs; an effect reversed by readmission of Ca^{2+} (Fig. 18). TTX (1×10^{-6}M) and the local anaesthetic, lignocaine ($0.5 - 2 \times 10^{-3}$M), which block Na^{+} channels, each abolished APs (as shown for lignocaine in Fig. 19), suggesting that Na^{+} rather than Ca^{2+} carried the inward current of the AP.

(ii) Nerve AP conduction in vivo

APs were recorded in the anaesthetised guinea-pig from a small postganglionic bundle of hypogastric nerve fibres, in response to preganglionic stimulation (0.2 Hz, 0.1 - 0.5 ms, supramaximal voltage). Systemic blood pressure and heart rate were recorded simultaneously.

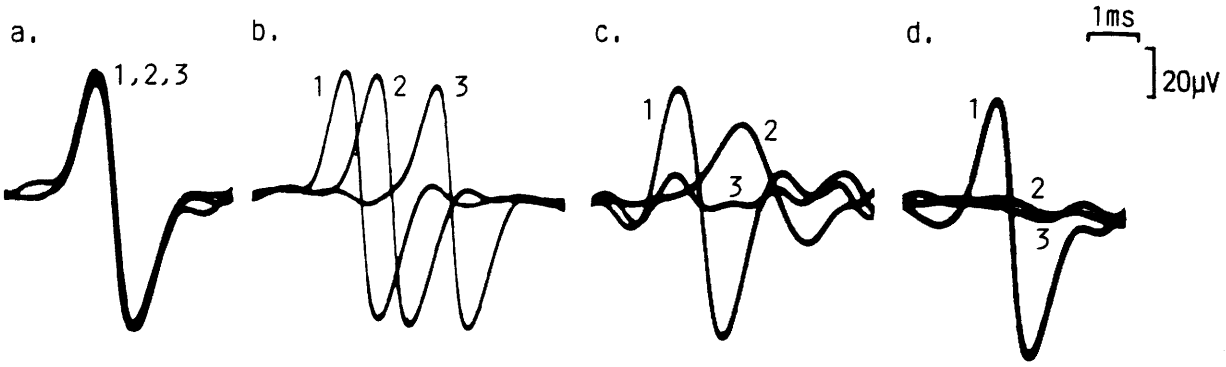


Fig. 16: The effect of verapamil (2.5×10^{-6}M (b), 3.75×10^{-6}M (c) and 1.25×10^{-5}M (d)), compared with a saline control (a), on AP conduction in vitro. APs were recorded from a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens, in response to submaximal stimulation (3 pulses at 0.5 Hz, 0.2 ms, 10V). In each trace the numbers refer to responses to 3 consecutive stimuli: 1 taken immediately prior to and 2 and 3, respectively, 2 and 4s after addition of verapamil (b-d) or saline (a). AP latency was increased initially by verapamil (2.5×10^{-6}M) and the amplitude then reduced in a graded (at 3.75×10^{-6}M) or 'all-or-none' manner (at 1.25×10^{-5}M). Verapamil was added by bolus injection to the bath rather than by perfusion.

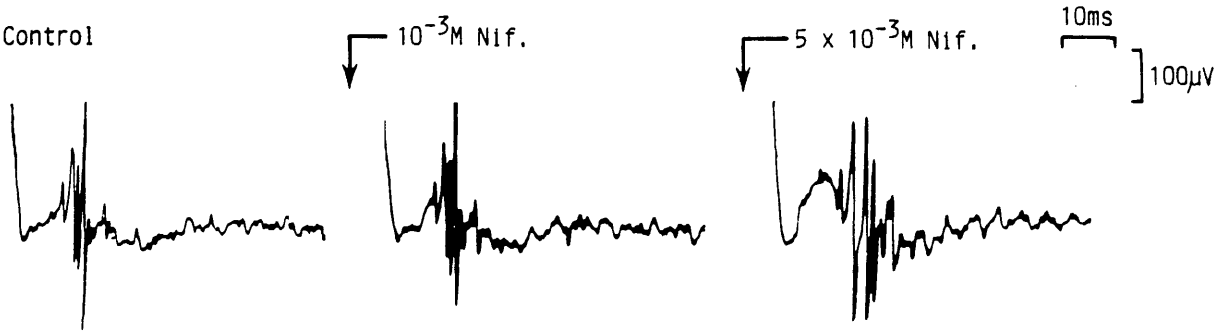
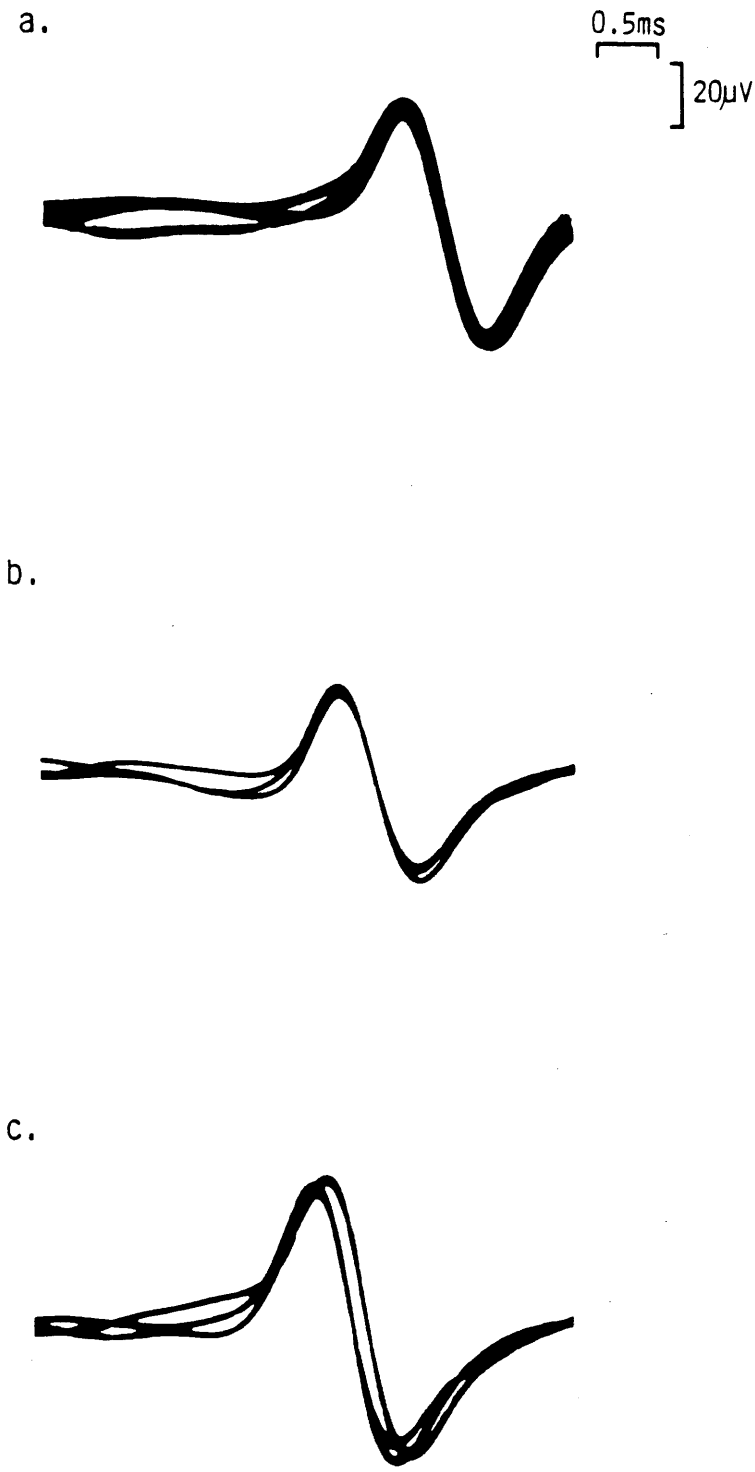


Fig. 17: The lack of effect of nifedipine (Nif., 1 and 5 x 10^{-3}M) on AP conduction in vitro, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens. APs were evoked by stimulation (0.5 Hz, 0.5 ms, supramaximal voltage) of the hypogastric nerve trunk. Nifedipine, in contrast to the other Ca^{2+} channel antagonists, was ineffective. The time between each panel was 15 min.

Fig. 18: The effect of Ca^{2+}-withdrawal from the perfusing Krebs solution, on AP conduction in vitro, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens. APs were evoked by submaximal stimulation (3 pulses at 0.5 Hz, 0.1 ms, 5V) of the hypogastric nerve trunk. Compared to the control (2.7×10^{-3}M $CaCl_2$ (a)), Ca^{2+}-withdrawal produced a small reduction in AP amplitude (b), which was reversed upon readmission of $CaCl_2$ (2.7×10^{-3}M) to the perfusing Krebs solution (c). The time between each panel was 15 min.


a.
0.5ms
20μV
b.
c.

APs were discharged following stimulation; their mean latency (28.3 ± 1.6 ms, n = 60) and the duration of each discharge (35.1 ± 2.3 ms, n = 20), evoked by single pulses (0.5 ms, supramaximal voltage), was similar to that found *in vitro*, although the amplitude (0.1 ± 0.01 mV, n = 60) was significantly smaller. Connective tissue and fascia surrounding the hypogastric nerves *in vivo* may have reduced the current density of the stimulus and consequently AP amplitude. Ganglia were present in the nerve pathway; pentolinium (0.1 - 0.6 mg kg^{-1} i.v.) inhibited the APs (Fig. 20).

The mean heart rate was 275 ± 8 beats min^{-1} (n = 34) and the diastolic and systolic blood pressures, 49.3 ± 2.5 and 75.8 ± 3.5 mm Hg (n = 34), respectively.

I.v. administration of either verapamil (0.01 - 0.3 mg kg^{-1}), diltiazem (0.01 - 0.3 mg kg^{-1}) or amlodipine (0.1 - 1.6 mg kg^{-1}), reduced both blood pressure and, except for amlodipine which was ineffective, heart rate (Fig. 21). AP conduction was unaffected by each agent at these doses; the latency, area and duration of the discharge, as analysed by computer, were not significantly different from controls (Fig. 22). In larger doses, verapamil, diltiazem and amlodipine (1, 1 and 3.2 mg kg^{-1} i.v. respectively), each produced a complete cardiovascular collapse.

Verapamil was then injected i.a. via the external iliac artery. When added in this way, higher doses of verapamil could be given than were possible i.v.; the compound was tolerated better by the guinea-pig, presumably due to its

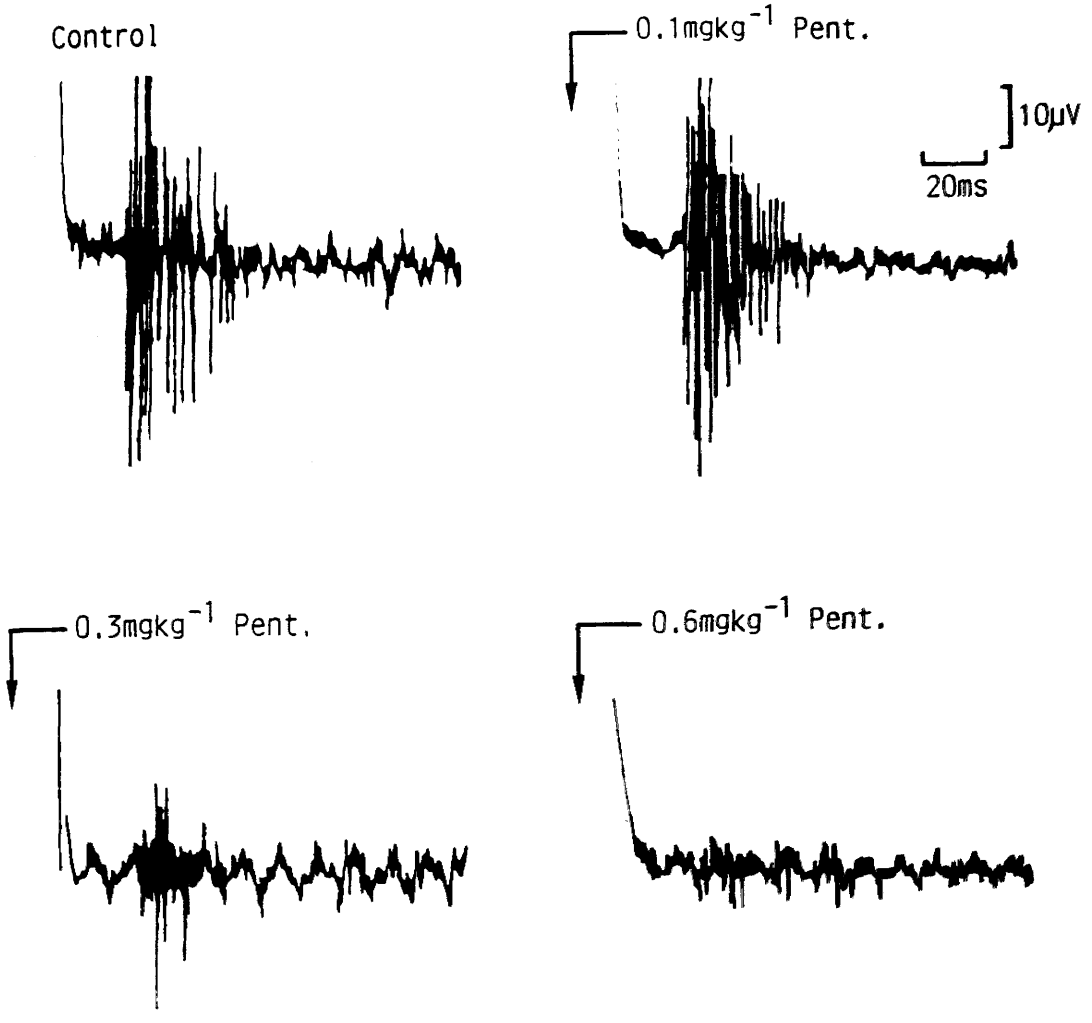


Fig. 20: The dose-dependent inhibitory effect of pentolinium (Pent., 0.1 - 0.6 mgkg^{-1} i.v.) on AP conduction *in vivo*, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens. APs were evoked by stimulation (0.2 Hz, 0.5 ms, supramaximal voltage) of the hypogastric nerve trunk. The time between each panel was 15 min.

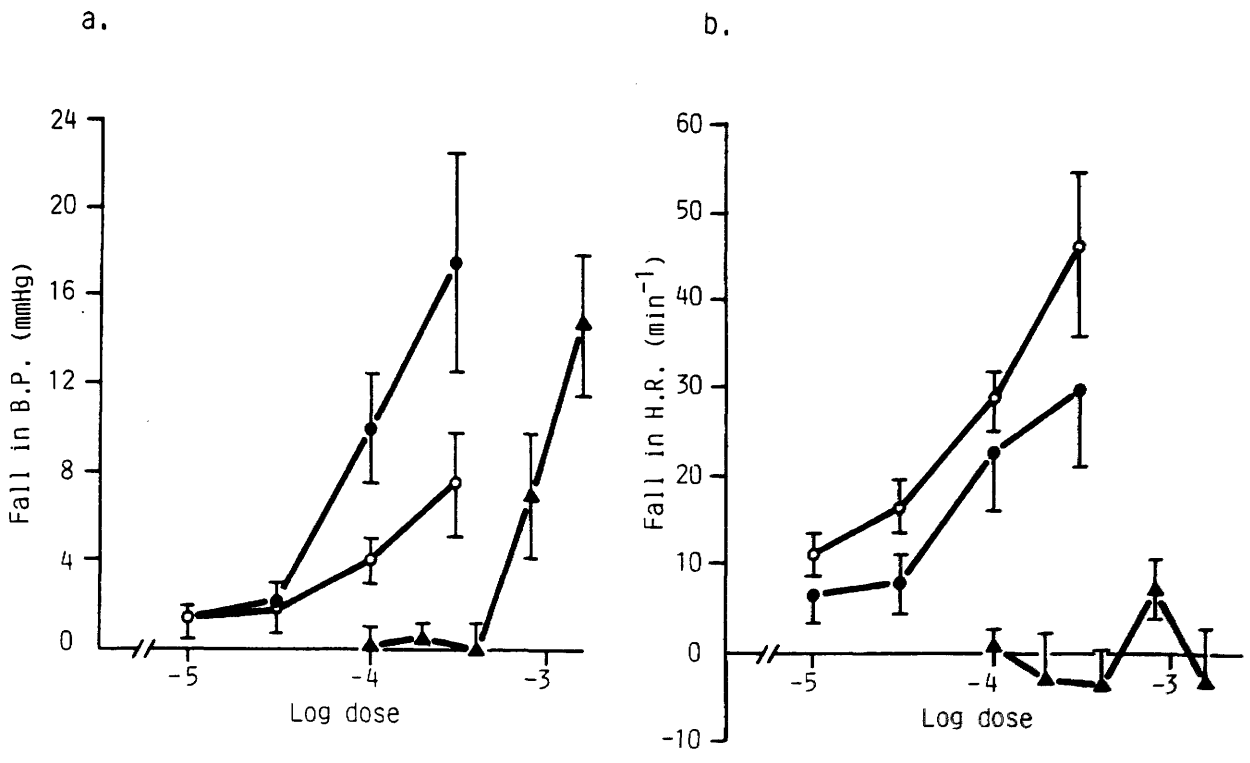
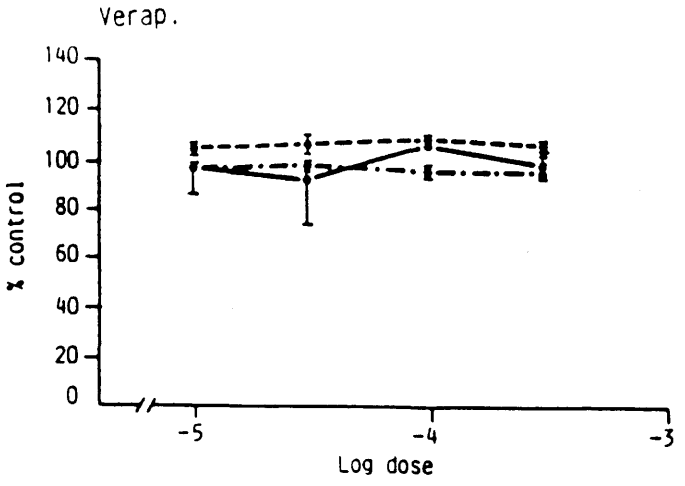
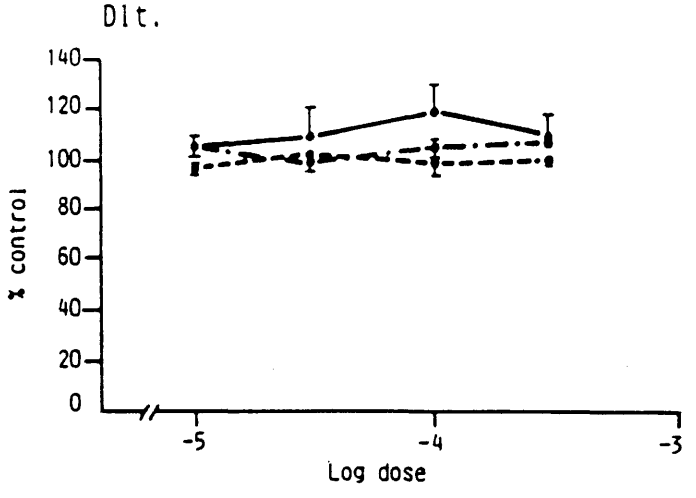
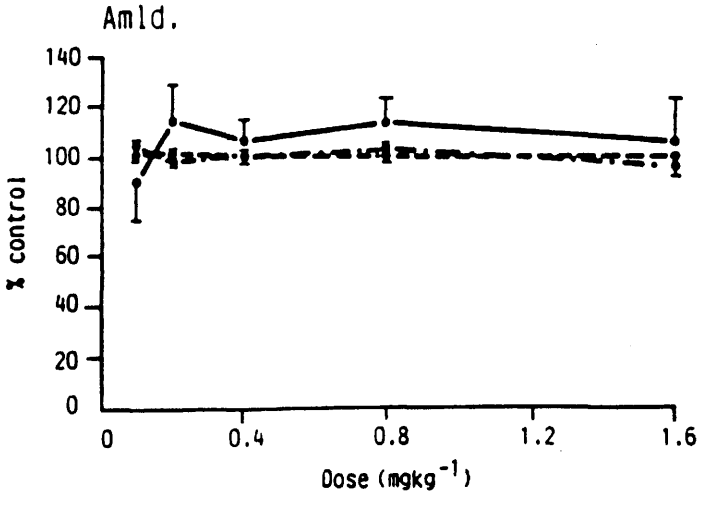


Fig. 21: The effect (± s.e. mean, n = 6-8) of verapamil (0.01 - 0.3 mgkg^{-1}, ●-●), diltiazem (0.01 - 0.3 mgkg^{-1}, o-o) and amlodipine (0.1 - 1.6 mgkg^{-1}, ▲—▲), administered i.v., on a. the blood pressure (mm Hg) and b. the heart rate (min^{-1}) of the anaesthetised guinea-pig. Verapamil, diltiazem and amlodipine, in descending order of potency, each produced a dose-dependent fall in blood pressure. Heart rate was reduced to a similar extent by verapamil and diltiazem, while amlodipine was ineffective.

Fig. 22: The lack of effect of verapamil (Verap., 0.01 - 0.3 $mgkg^{-1}$, $\pm$ s.e. mean, n = 3), diltiazem (Dlt., 0.01 - 0.3 $mgkg^{-1}$, $\pm$ s.e. mean, n = 4), and amlodipine (Amld., 0.1 - 1.6 $mgkg^{-1}$, $\pm$ s.e. mean, n = 4), administered i.v., on AP conduction *in vivo*, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens. APs were evoked by stimulation (0.2 Hz, 0.5 ms, supra-maximal voltage) of the hypogastric nerve trunk. The results, determined by computer analysis, are expressed as a percentage of the control AP discharge, with respect to the latency (●--●), area (●—●) and duration (●-·-●).


Verap.
140
120
100
80
60
40
20
0
% control
-5
-4
-3
Log dose


Dlt.
140
120
100
80
60
40
20
0
% control
-5
-4
-3
Log dose


Amld.
140
120
100
80
60
40
20
0
% control
0
0.4
0.8
1.2
1.6
Dose (mgkg^{-1})

delayed passage to the heart. Verapamil (1 - 3 mg kg^{-1} i.a.) inhibited the APs; the duration and the area of the discharge were reduced and the latency increased (Figs. 23 and 24). The blood pressure and heart rate were also reduced in a dose-dependent fashion (Fig. 25).

Verapamil may have inhibited the APs by ganglion blockade, as presumably occurred with pentolinium, or by interfering with either pre- or postganglionic AP conduction. To determine which was the case, acetylcholine (0.01 mg) was added i.a., when evoked APs had been blocked with either verapamil (3 mg kg^{-1} i.a.) or pentolinium (0.6 mg kg^{-1} i.v.). Activation of ganglionic nicotinic receptors by acetylcholine would discharge APs, only if its receptors and the postganglionic fibres were unaffected by either compound. During blockade of evoked APs by verapamil, but not by pentolinium, acetylcholine produced a spontaneous discharge of APs (Fig. 26). Thus, the ganglionic nicotinic receptors, blocked by pentolinium, and the postganglionic nerve fibres, were unaffected by verapamil. As a consequence of its lack of effect on postganglionic AP conduction, it is likely that preganglionic conduction was similarly unaffected and probable that verapamil blocked the evoked APs by inhibiting preganglionic transmitter release.

(iii) Excitatory junction potentials

The actions of Ca^{2+} channel antagonists on postganglionic transmitter release were next examined. E.j.ps were used as one measure of release.

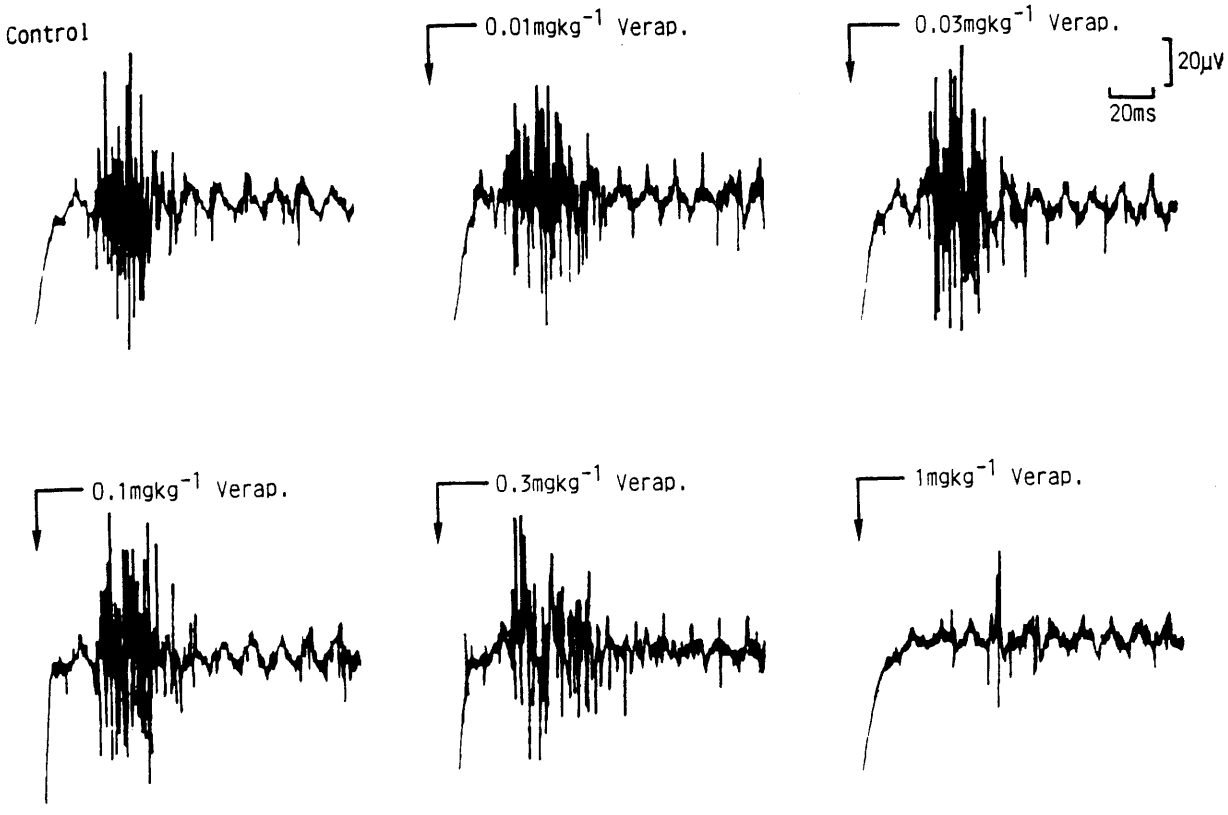


Fig. 23: The dose-dependent inhibitory effect of verapamil (Verap., 0.01 - 1 mgkg^{-1} i.a.) on AP conduction *in vivo*, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens. APs were evoked by stimulation (0.2 Hz, 0.5 ms, supramaximal voltage) of the hypogastric nerve trunk. The time between each panel was 15 min.

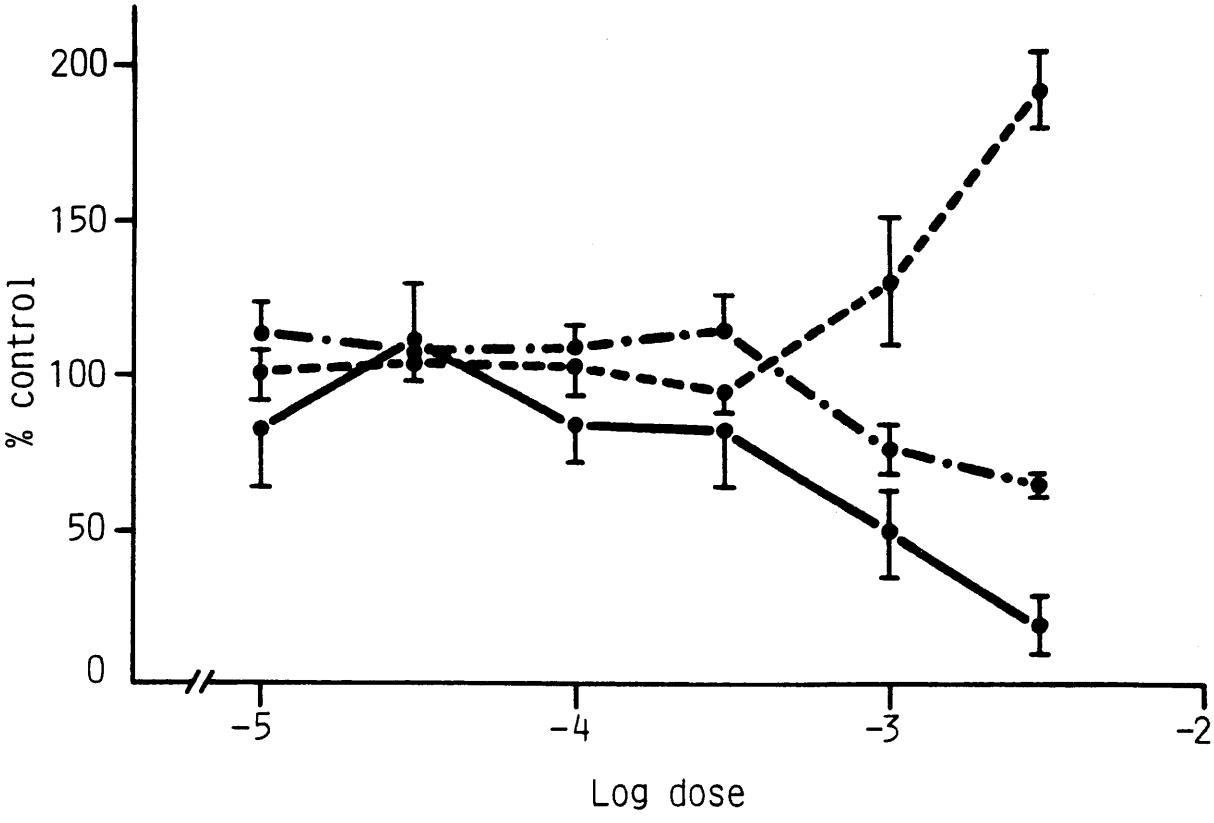


Fig. 24: The dose-dependent inhibitory effect (± s.e. mean, n = 3) of verapamil (0.01 - 3 $mgkg^{-1}$ i.a.) on AP conduction in vivo, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens. APs were evoked by stimulation (0.2 Hz, 0.5 ms, supramaximal voltage) of the hypogastric nerve trunk. The results, determined by computer analysis, are expressed as a percentage of the control AP discharge, with respect to the latency (●- - ●), area (●—●) and duration (●-·-●).

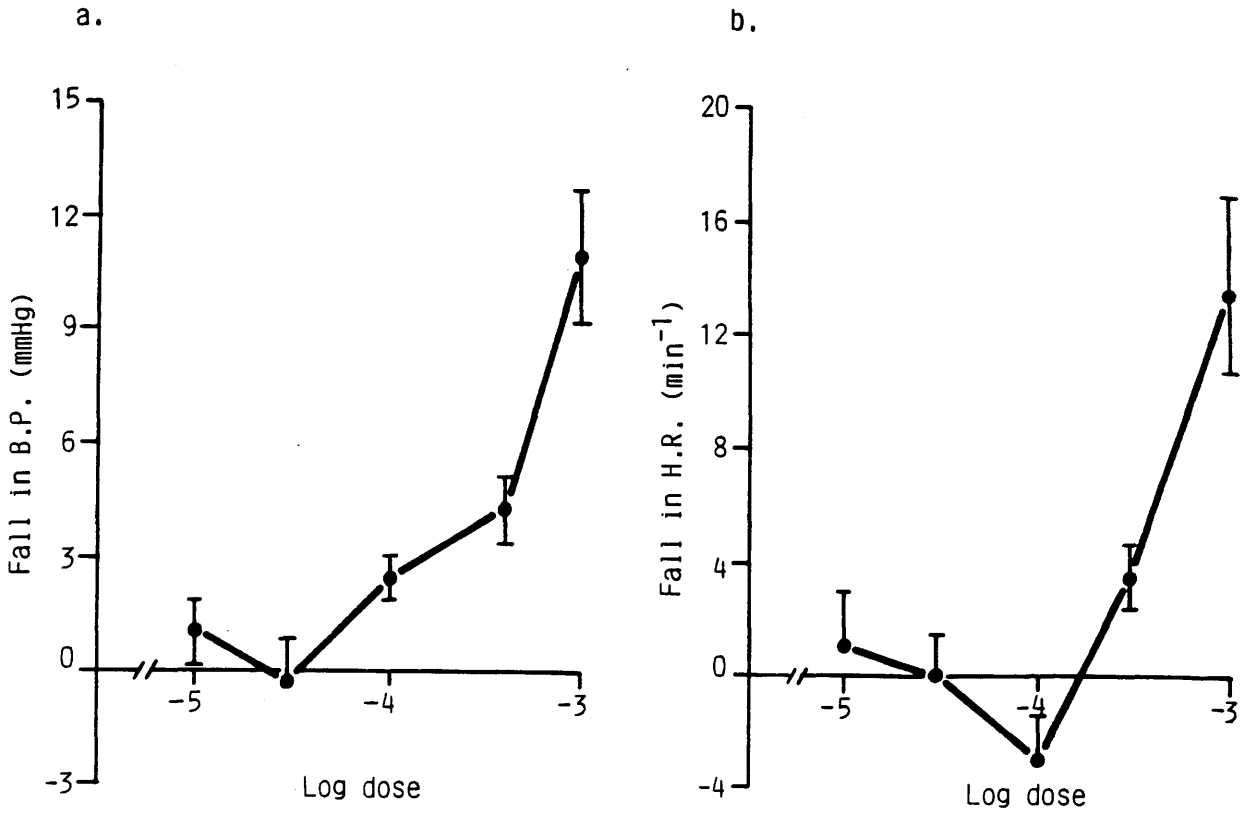
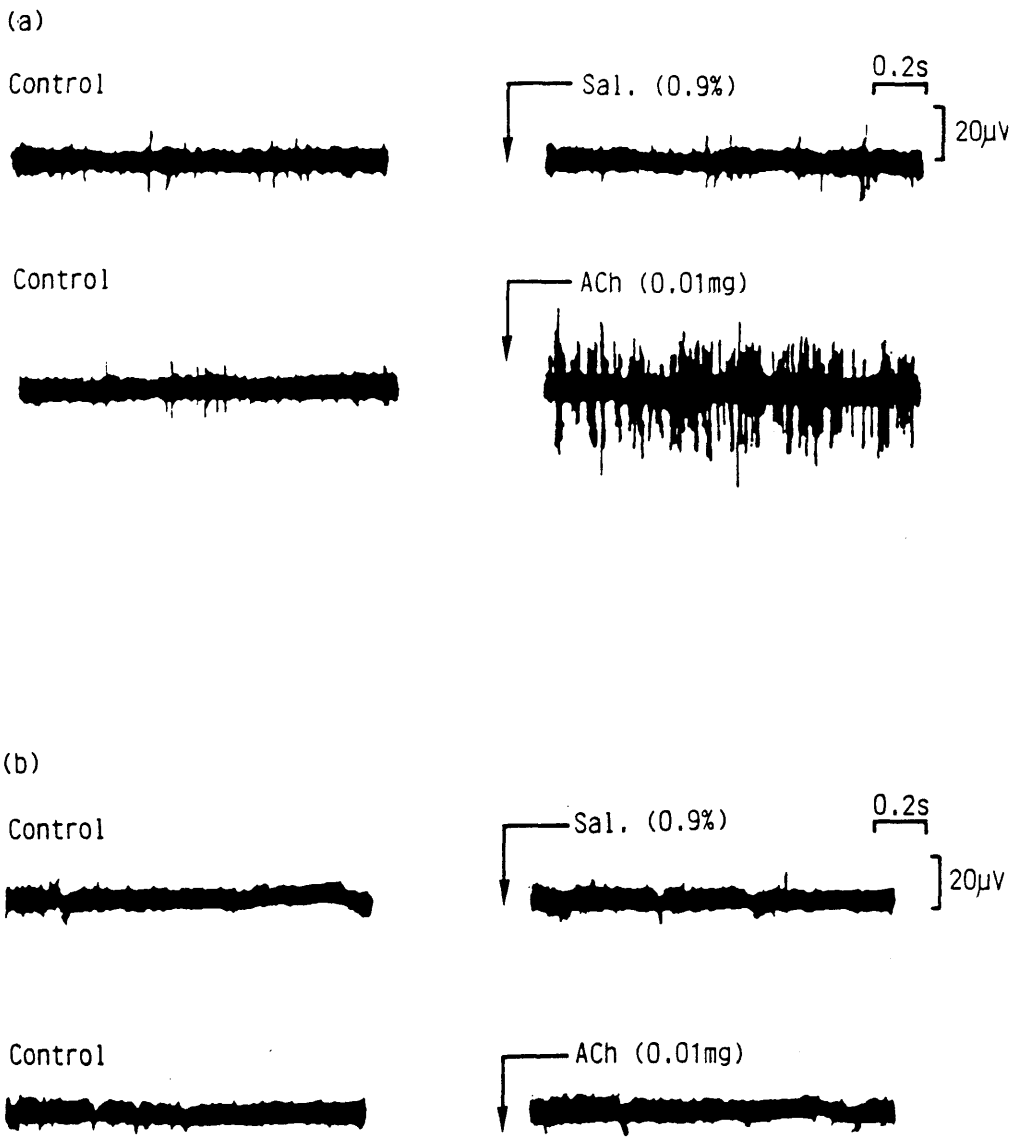


Fig. 25: The effect ($\pm$ s.e. mean, n = 3,4) of verapamil (0.01 - 1 mgkg^{-1} i.a.) on a. the blood pressure (mm Hg) and b. the heart rate (min^{-1}) of the anaesthetised guinea-pig. Verapamil produced a dose-dependent fall in both parameters.

Fig. 26: The effect of acetylcholine (ACh, 0.01 mg i.a.), compared to its vehicle, saline (Sal., 0.9% NaCl in distilled water, i.a.), on the resting electrical activity *in vivo*, in a small bundle of guinea-pig hypogastric nerve fibres running to the vas deferens. Stimulation (0.2 Hz, 0.5 ms, supramaximal voltage)-evoked APs were blocked with either (a) verapamil (3 $mgkg^{-1}$ i.a.) or (b) pentolinium (0.6 $mgkg^{-1}$ i.v.). Acetylcholine produced a spontaneous discharge following blockade of stimulation-evoked APs by verapamil, but not by pentolinium. Saline was ineffective.


(a)
Control
Sal. (0.9%)
0.2s
20μV
Control
ACh (0.01mg)
(b)
Control
Sal. (0.9%)
0.2s
20μV
Control
ACh (0.01mg)

When first set up, the vas deferens had no tone. Each preparation was then gently stretched to a tension of 1g, which was well maintained throughout experiments. The smooth muscle of the vas deferens had a mean resting membrane potential of -55.5 ± 0.4 mV (n = 113 cells) and frequently showed spontaneous e.j.ps with a mean amplitude of 5.0 ± 0.4 mV (n = 50), a duration of 85.9 ± 4.3 ms (n = 50) and time to peak of 20.0 ± 1.3 ms (n = 50). These were unaccompanied by any increase in tone.

Field stimulation (5-20 pulses at 1 and 2 Hz, 0.1 - 0.5 ms, supramaximal voltage) evoked e.j.ps, which at 1 Hz had a mean amplitude of 6.8 ± 0.4 mV (n = 50), a latency of 13.1 ± 1.0 ms (n = 50), a duration of 724.7 ± 22.8 ms (n = 50) and a time to peak of 56.8 ± 3.4 ms (n = 50). These values were comparable to those found in other investigations (Burnstock & Holman, 1961; Cunnane, 1979). The e.j.ps facilitated in amplitude at 1 Hz and summated at 2 Hz, successive e.j.ps arising during the falling phase of the preceding depolarization. They were inhibited by TTX (1×10^{-6}M), indicating their neural origin (Fig. 27). E.j.ps summated to produce APs and a contraction. However, contractions were also recorded in the absence of APs in an impaled cell, presumably as a result of electrical activity (APs) in other cells.

Perfusion of the vas deferens with either verapamil ($1 - 2 \times 10^{-4}$M), diltiazem ($1 - 5 \times 10^{-4}$M), amlodipine ($0.5 - 1 \times 10^{-4}$M) or cobalt (1×10^{-3}M), inhibited e.j.ps and muscle contractions in a dose-dependent manner (Figs. 28

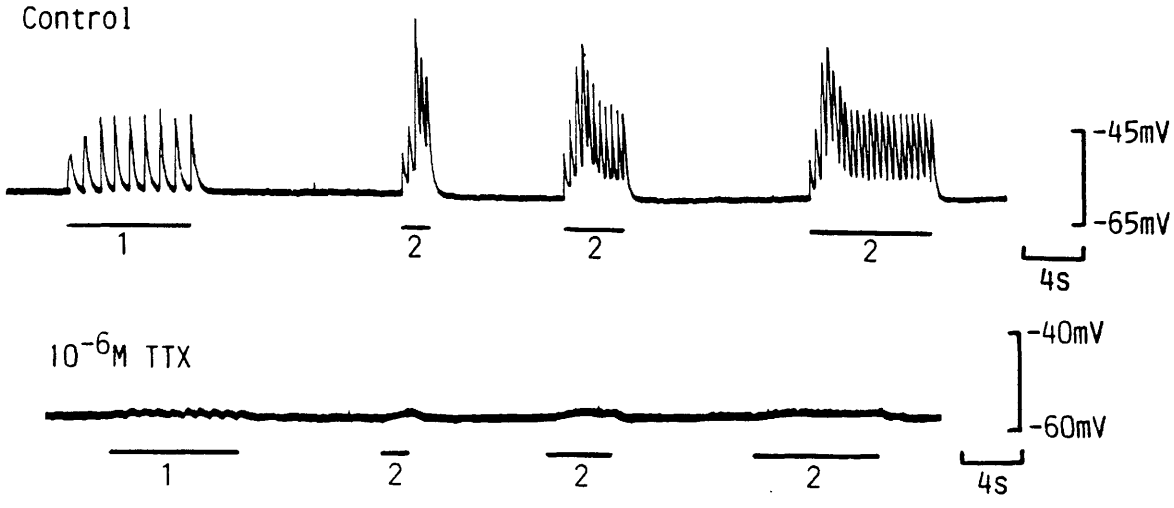


Fig. 27: Intracellularly-recorded e.j.ps in response to field stimulation of the guinea-pig vas deferens (10 pulses at 1 Hz and 5, 10 and 20 pulses at 2 Hz, 0.1 ms, supramaximal voltage) in the absence (upper trace) and presence of TTX (1×10^{-6}M). E.j.ps, which facilitated at 1 Hz and summated at 2 Hz, were abolished by TTX, indicating their mediation by nerves. The duration of stimulation is indicated by the horizontal bars and the frequency by the numbers underneath. The time between each trace was 15 min.

to 30). The organic agents were each more potent in blocking muscle contractions than e.j.ps, while cobalt was equally effective. Nifedipine (5×10^{-4}M), unlike the other antagonists, had no effect on the e.j.ps, but inhibited both muscle APs and contractions (Fig. 31).

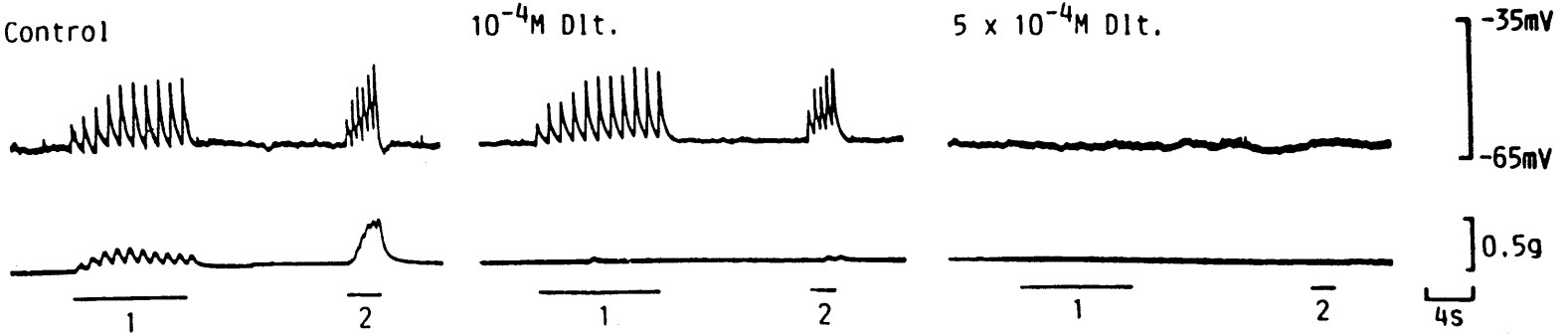
(iv) Radiolabelled transmitter release

The effects of Ca^{2+} channel antagonists on transmitter release were measured directly. To do this, the sympathetic nerves were treated with radiolabelled transmitter material and the effect of the antagonists on the overflow measured.

(a) $[^3H]$-noradrenaline incubation

After commencing superfusion, the overflow of ^3H from the vas deferens declined exponentially, in the absence of nerve stimulation (Fig. 32), reaching a steady value by 3 h, at which time stimulation was carried out. Field stimulation (60 pulses at 1-20 Hz, 0.5 ms, supramaximal voltage) in the presence of ascorbic acid (1.1×10^{-4}M) to prevent oxidation of catecholamines, desipramine (6×10^{-7}M) and normetanephrine (1×10^{-5}M) to inhibit uptake-1 and uptake-2 processes respectively and atropine (2.6×10^{-6}M) to block any cholinergic influence on release, increased ^3H overflow (Fig. 33). The amount of evoked ^3H overflow increased with increasing frequency of stimulation (maximum at 10-20 Hz, Fig. 34) and was abolished by TTX (1×10^{-6}M), indicating its mediation by nerves.

Fig. 28: The effect of diltiazem (Dlt., 1 and 5 x 10^{-4}M) on intracellularly-recorded e.j.ps (upper trace of each set) and accompanying mechanical activity in the guinea-pig vas deferens, in response to field stimulation (10 pulses at 1 Hz and 5 pulses at 2 Hz, 0.2 ms, supramaximal voltage). Diltiazem inhibited e.j.ps and contractions in a dose-dependent manner. Contractions were blocked by diltiazem at a time when e.j.ps were present. The duration of stimulation is indicated by the horizontal bars and the frequency by the numbers underneath. The time between each set was approximately 15 min.


Control
10⁻⁴M Dlt.
5 x 10⁻⁴M Dlt.
-35mV
-65mV
0.5g
1
2
1
2
1
2
4s

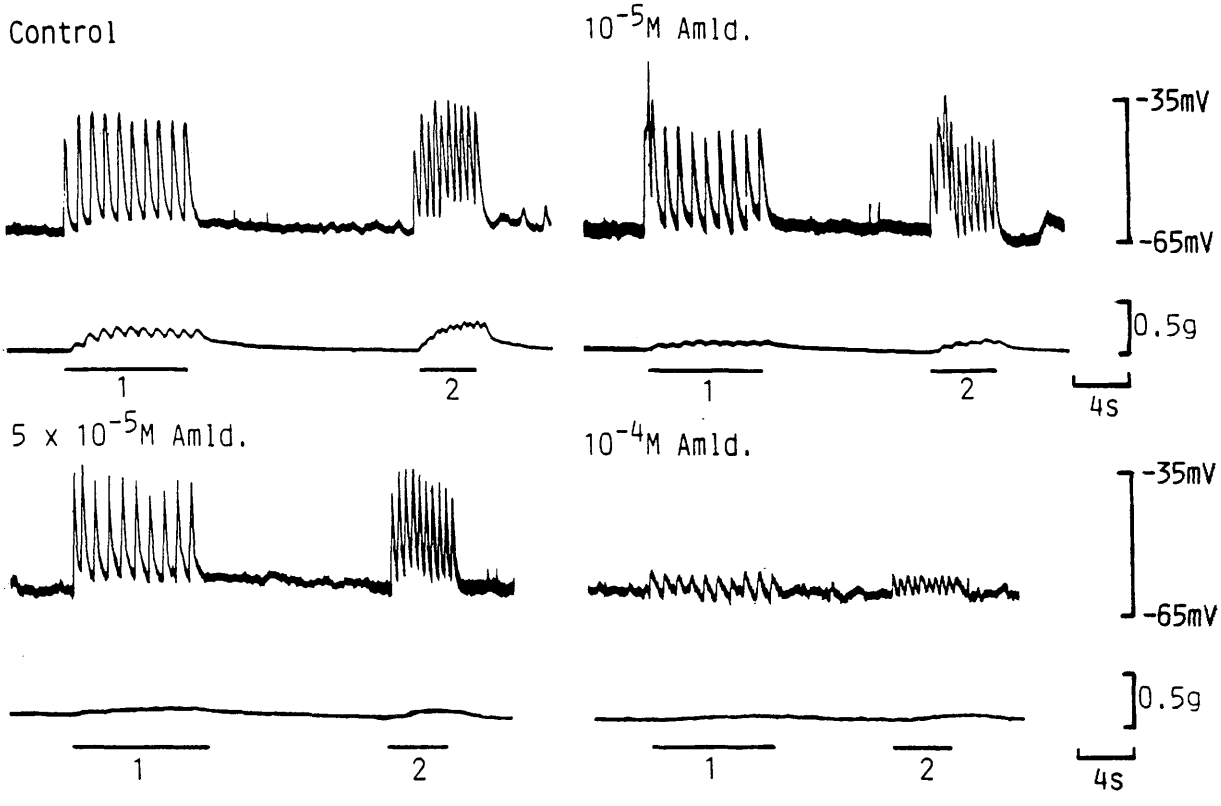


Fig. 29: The effect of amlodipine (Amld., 1 x 10^{-5} - 1 x 10^{-4}M) on intracellularly-recorded e.j.ps (upper trace of each set) and accompanying mechanical activity in the guinea-pig vas deferens, in response to field stimulation (10 pulses at 1 and 2 Hz, 0.5 ms, supramaximal voltage). Amlodipine inhibited e.j.ps and contractions in a dose-dependent manner. The contractions were blocked at a lower concentration than the e.j.ps. The duration of stimulation is indicated by the horizontal bars and the frequency by the numbers underneath. The time between each set was approximately 15 min.

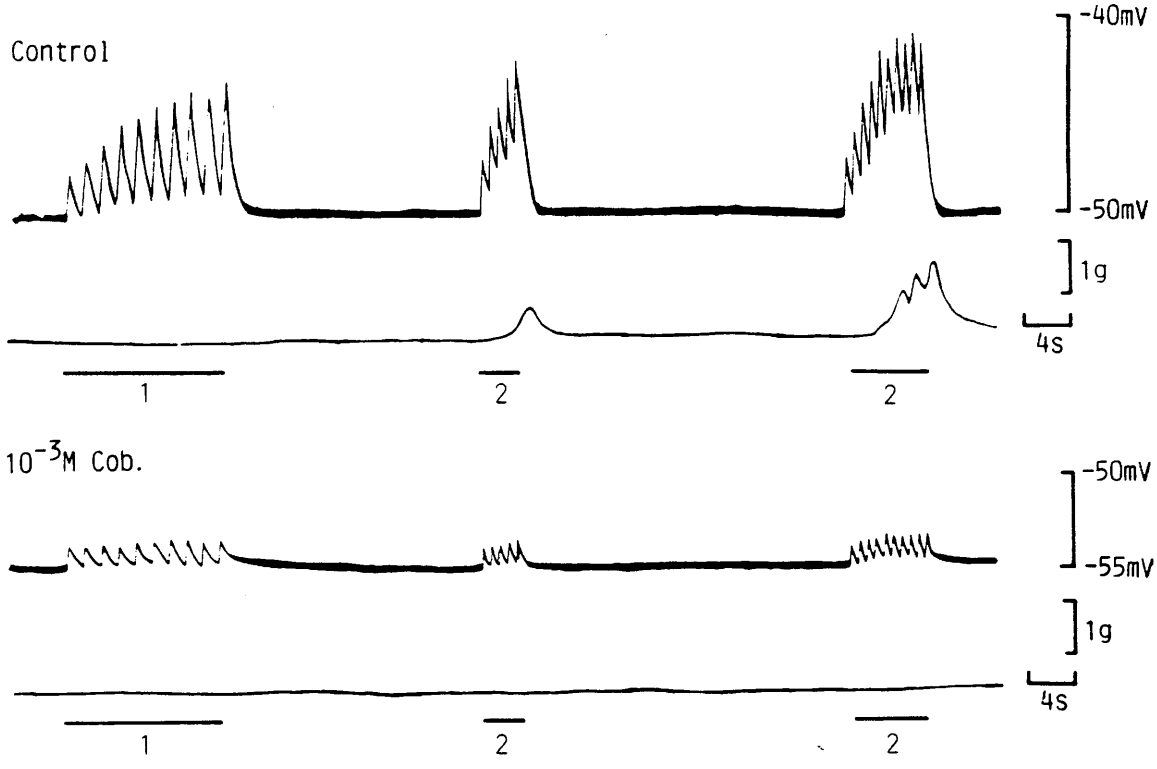


Fig. 30: The effect of cobalt (Cob., 1 x 10^{-3}M) on intracellularly-recorded e.j.ps (upper trace of each set) and accompanying mechanical activity in the guinea-pig vas deferens, in response to field stimulation (10 pulses at 1 Hz and 5 and 10 pulses at 2 Hz, 0.5 ms, supramaximal voltage). Cobalt abolished contractions and produced an almost complete blockade of e.j.ps. The duration of stimulation is indicated by the horizontal bars and the frequency by the numbers underneath. The time between the two sets was 25 min.

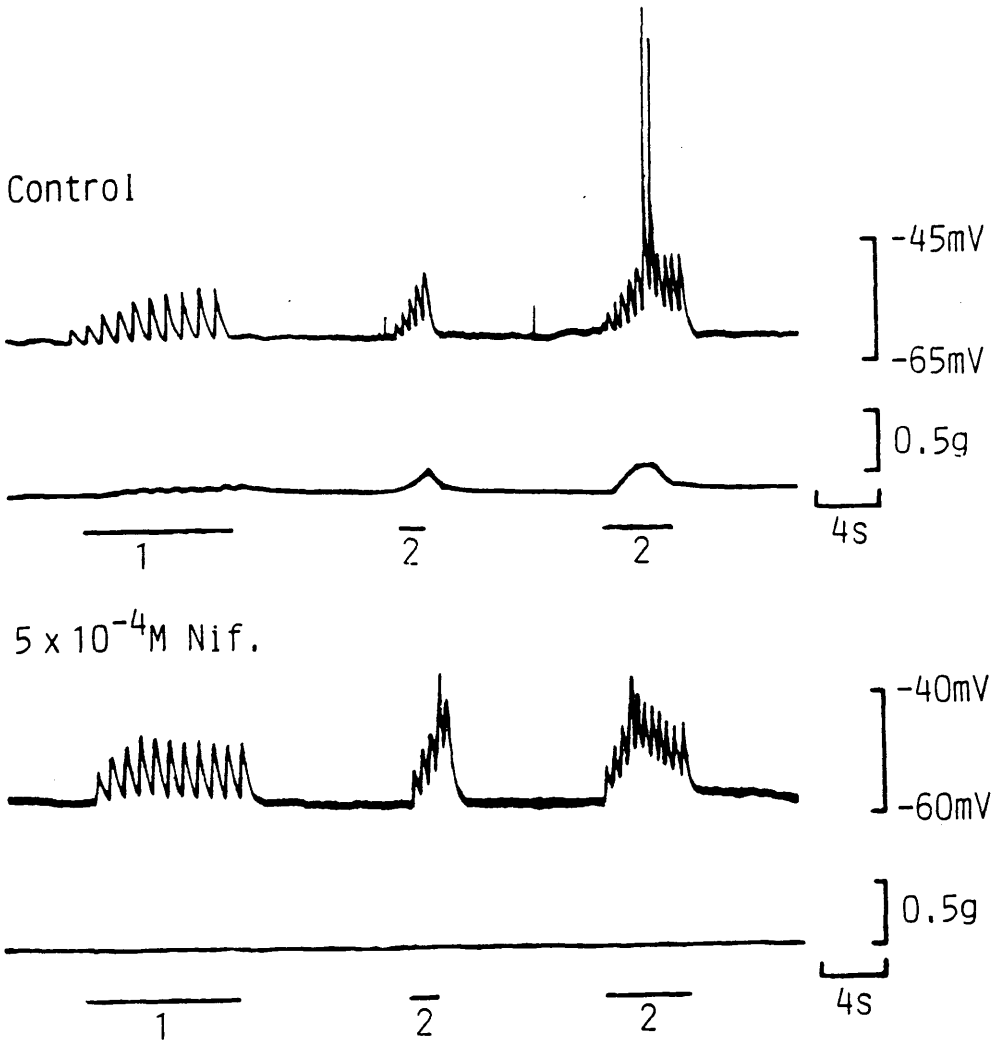


Fig. 31: The effect of nifedipine (Nif., 5 x 10^{-4}M) on intracellularly-recorded e.j.ps (upper trace of each set) and accompanying mechanical activity in the guinea-pig vas deferens, in response to field stimulation (10 pulses at 1 Hz and 5 and 10 pulses at 2 Hz, 0.5 ms, supramaximal voltage). Nifedipine, in contrast to the other Ca^{2+} channel antagonists examined, had no effect on the e.j.ps but inhibited both muscle APs and mechanical contractions. The duration of stimulation is indicated by the horizontal bars and the frequency by the numbers underneath. The time between the two sets was 20 min.

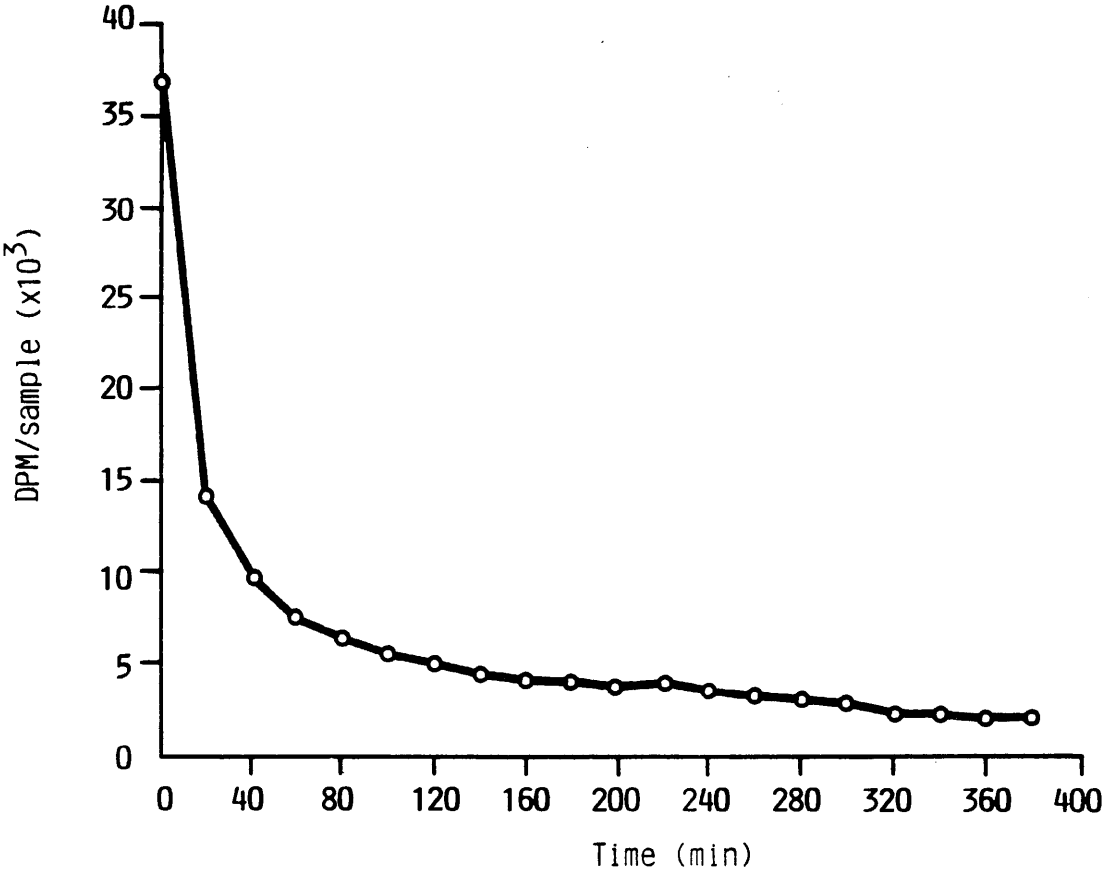


Fig. 32: The exponential decline with time in the resting ^3H overflow, expressed as DPM/sample (x 10^3), from the guinea-pig vas deferens incubated with [^3H] -noradrenaline. By 3 h, approximately, ^3H overflow had reached a relatively steady value, enabling an accurate assessment to be made of the effects of field stimulation and of drugs.

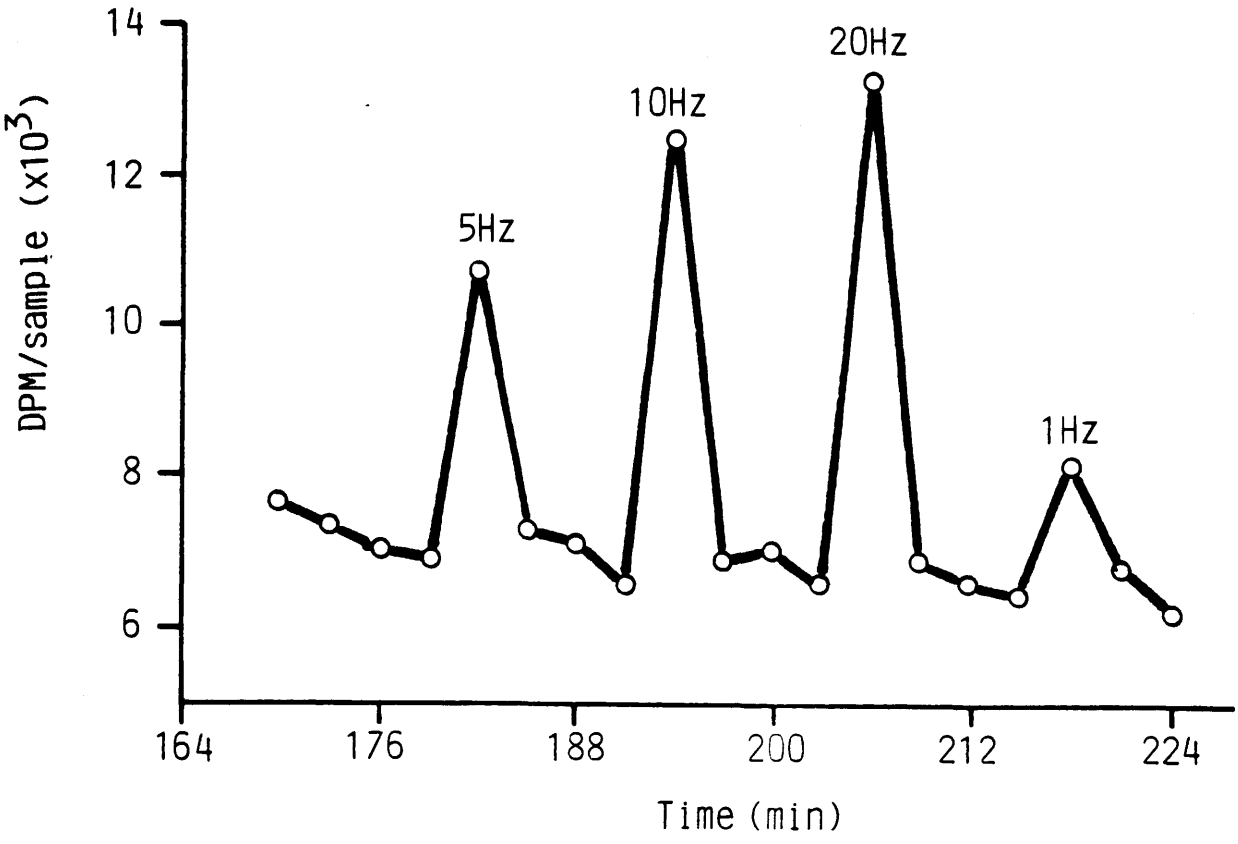


Fig. 33: The effect of field stimulation (60 pulses at 1, 5, 10 and 20 Hz, 0.5 ms, supramaximal voltage) on the resting ^3H overflow, expressed as DPM/sample (x 10^3), from the guinea-pig vas deferens incubated with [^3H] -noradrenaline. Stimulation produced a frequency-dependent increase in ^3H overflow. The following drugs were present throughout: ascorbic acid (1.1 x 10^{-4}M) to prevent oxidation of catecholamines, desipramine (6 x 10^{-7}M) and normetanephrine (1 x 10^{-5}M) to inhibit, respectively, uptake-1 and uptake-2 processes and atropine to block any cholinergic influence on release.

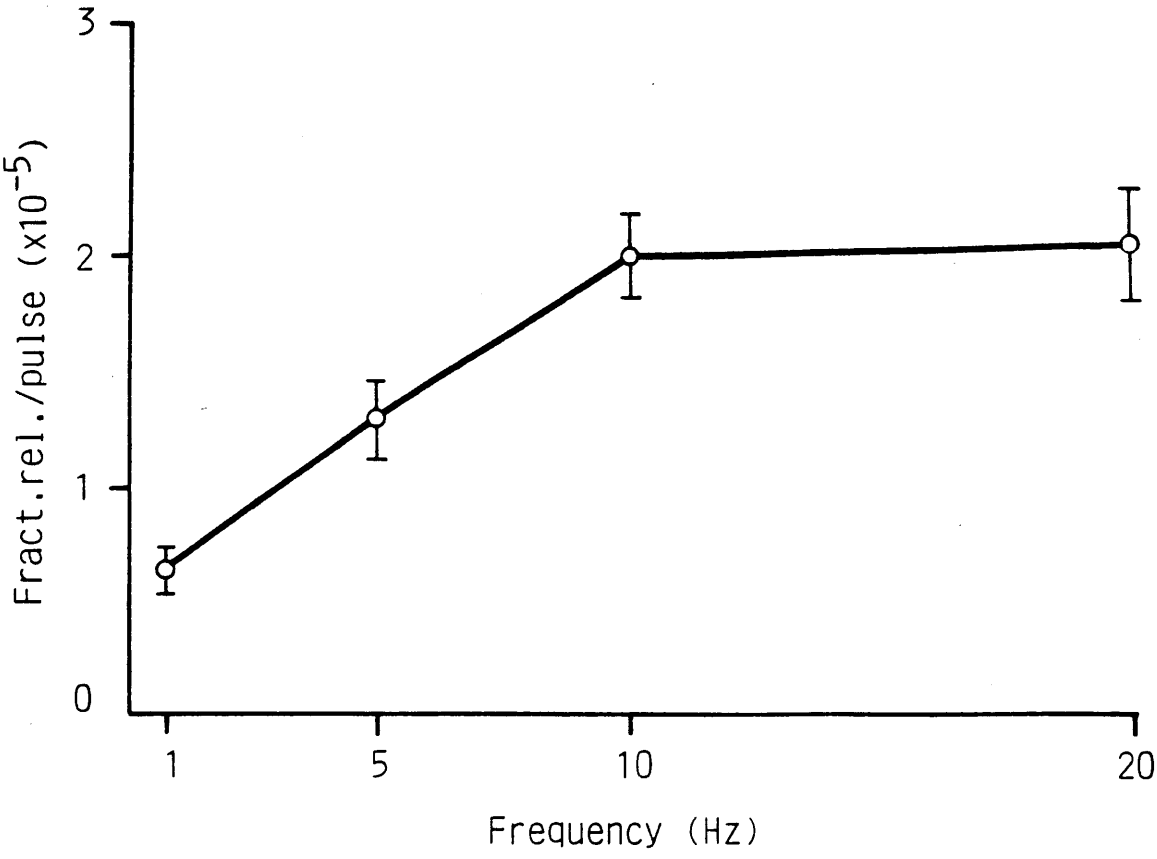


Fig. 34: ^{3}H overflow, expressed as the fractional release per pulse of stimulation (± s.e. mean, n = 12), evoked by field stimulation (60 pulses at 1, 5, 10 and 20 Hz, 0.5 ms, supramaximal voltage) of the guinea-pig vas deferens incubated with [^{3}H] -noradrenaline. ^{3}H overflow increased with the frequency of stimulation, reaching a maximum between 10 and 20 Hz.

The major constituents in the resting ^{3}H overflow, as revealed by chromatography, were 4-hydroxy 3-methoxymandelic acid (VMA) and 4-hydroxy 3-methoxyphenylglycol (MOPEG), 3,4-dihydroxyphenylglycol (DOPEG) and noradrenaline (Fig. 35a). Field stimulation (300 pulses at 20 Hz, 0.5 ms, supramaximal voltage) released mainly noradrenaline (Fig. 35b).

Cobalt ($2 \times 10^{-3}M$), while having little effect on the resting ^{3}H level, significantly inhibited stimulation (60 pulses at 1-20 Hz, 0.5 ms, supramaximal voltage)-evoked overflow of ^{3}H at each frequency (Fig. 36).

Verapamil ($0.1 - 1 \times 10^{-4}M$), diltiazem ($0.5 - 1 \times 10^{-4}M$), amlodipine ($0.05 - 1 \times 10^{-4}M$) and nifedipine ($1 - 5 \times 10^{-4}M$), each markedly increased the resting ^{3}H overflow in a dose-dependent manner (Figs. 37 to 39). This effect obscured any increase in ^{3}H overflow which may have been produced by nerve stimulation. In contrast to that produced by field stimulation, the principal constituent released by either amlodipine ($5 \times 10^{-6}M$, Fig. 40) or verapamil ($1 \times 10^{-5}M$, Fig. 41) was DOPEG. The amounts of VMA and MOPEG, DOMA, NMN or noradrenaline itself, were not affected significantly by either compound.

The source of the increased resting ^{3}H overflow was examined in vasa of guinea-pigs pretreated with 6-hydroxydopamine (150 $mgkg^{-1}$ on day 1 and 250 $mgkg^{-1}$ on day 2; experiments on day 3) to destroy the sympathetic innervation. Destruction was confirmed by the absence of fluorescence (Fig. 42), using a modification of the Falck histofluorescence technique (Gillespie & Kirpekar, 1966). Following 6-hydroxydopamine

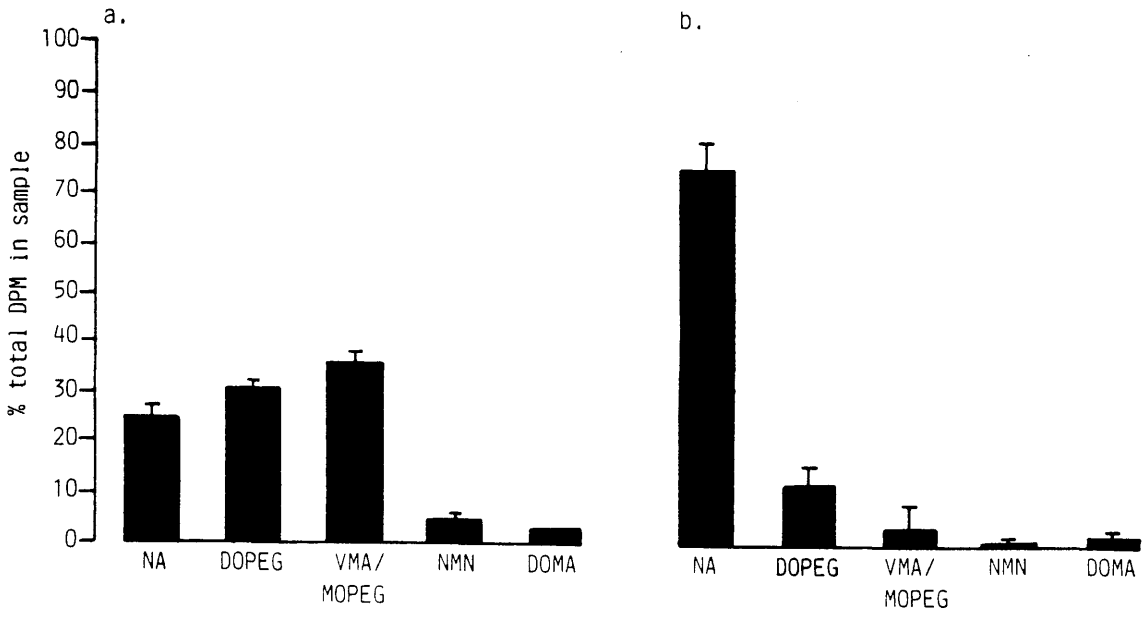


Fig. 35: The relative amounts (± s.e. mean, n = 12) of [^{3}H] -noradrenaline and its metabolites, expressed as a percentage of the total disintegrations per minute (DPM), in a sample of the ^{3}H overflow, a. at rest and b. following field stimulation (300 pulses at 20 Hz, 0.5 ms, supramaximal voltage) of the guinea-pig vas deferens. At rest, [^{3}H] -labelled 4-hydroxy 3-methoxymandelic acid (VMA) and 4-hydroxy 3-methoxyphenylglycol (MOPEG), 3,4-dihydroxyphenylglycol (DOPEG) and noradrenaline (NA) were the major constituents. Only small amounts of [^{3}H] -normetanephrine (NMN) and 3,4-dihydroxymandelic acid (DOMA) were found. Following field stimulation [^{3}H] -noradrenaline was the principal product.

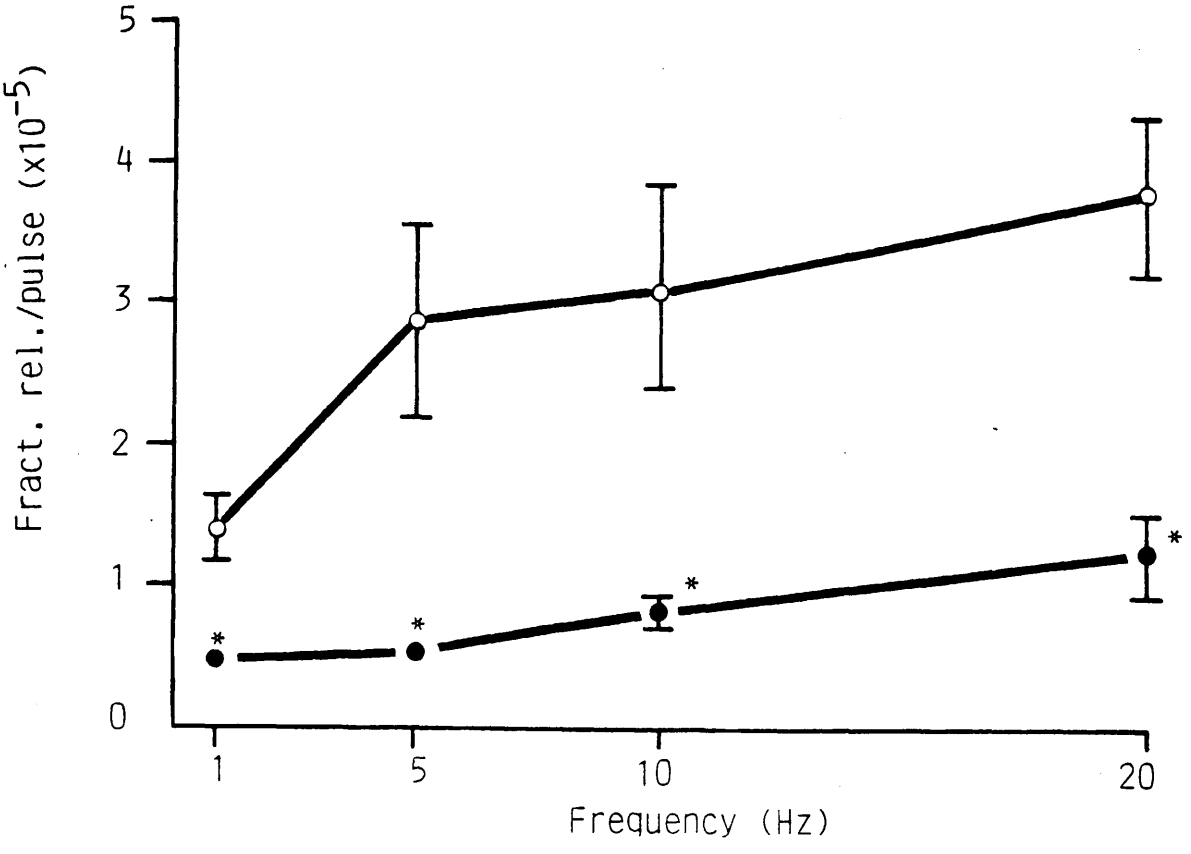


Fig. 36: ^{3}H overflow, expressed as the fractional release per pulse of stimulation ($\pm$ s.e. mean, n = 3), evoked by field stimulation (60 pulses at 1, 5, 10 and 20 Hz, 0.5 ms, supramaximal voltage) of the guinea-pig vas deferens incubated with $[^{3}H]$ -noradrenaline, in the absence (o-o) and presence (●-●) of cobalt (2 x 10^{-3}M). ^{3}H overflow was significantly (*, $p < 0.05$) inhibited by cobalt at each frequency. Ascorbic acid (1.1 x 10^{-4}M), desipramine (6 x 10^{-7}M), normetanephrine (1 x 10^{-5}M) and atropine (2.6 x 10^{-6}M) were present throughout (see legend for Fig. 33).

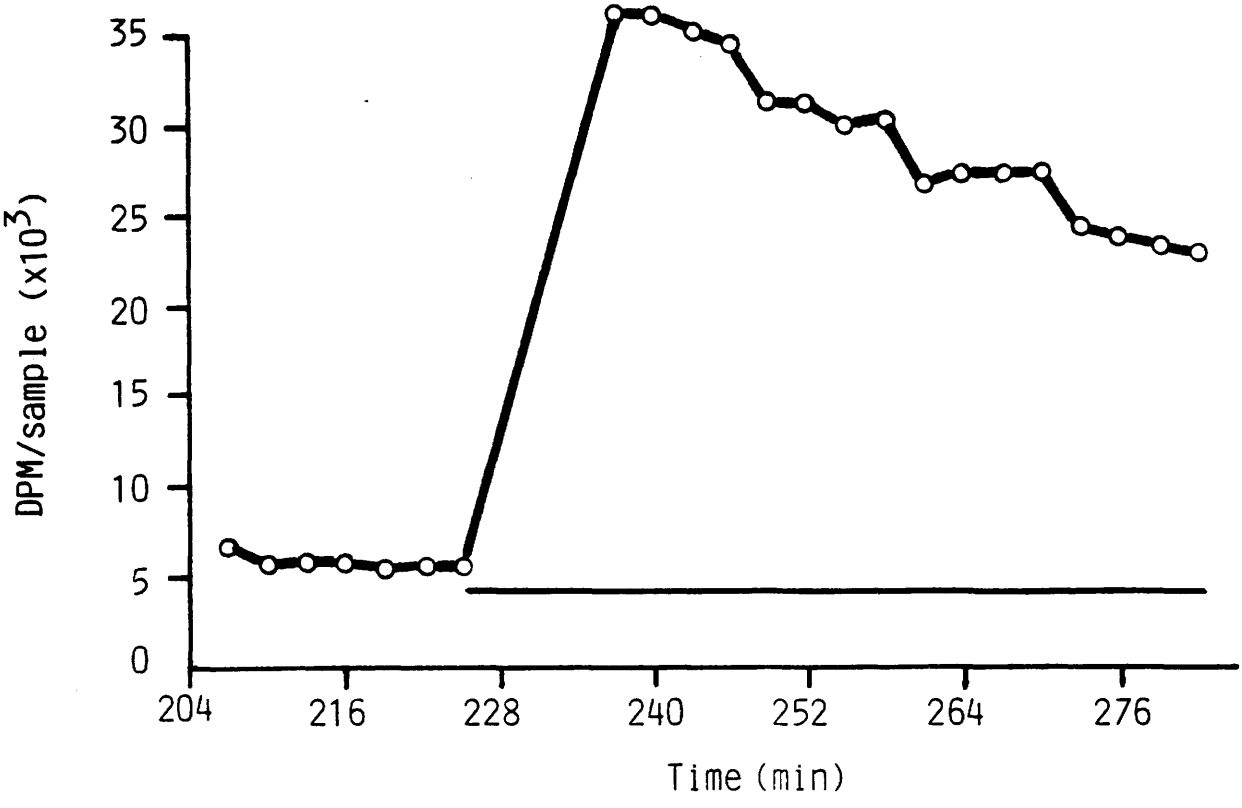


Fig. 37: The effect of verapamil (1 x 10^{-4}M for the period indicated by the horizontal bar) on the resting ^{3}H overflow, expressed as DPM/sample (x 10^3), from the guinea-pig vas deferens incubated with [^{3}H] -noradrenaline. Verapamil markedly increased the resting ^{3}H overflow. Ascorbic acid (1.1 x 10^{-4}M), desipramine (6 x 10^{-7}M), normetanephrine (1 x 10^{-5}M) and atropine (2.6 x 10^{-6}M) were present throughout (see legend for Fig. 33).

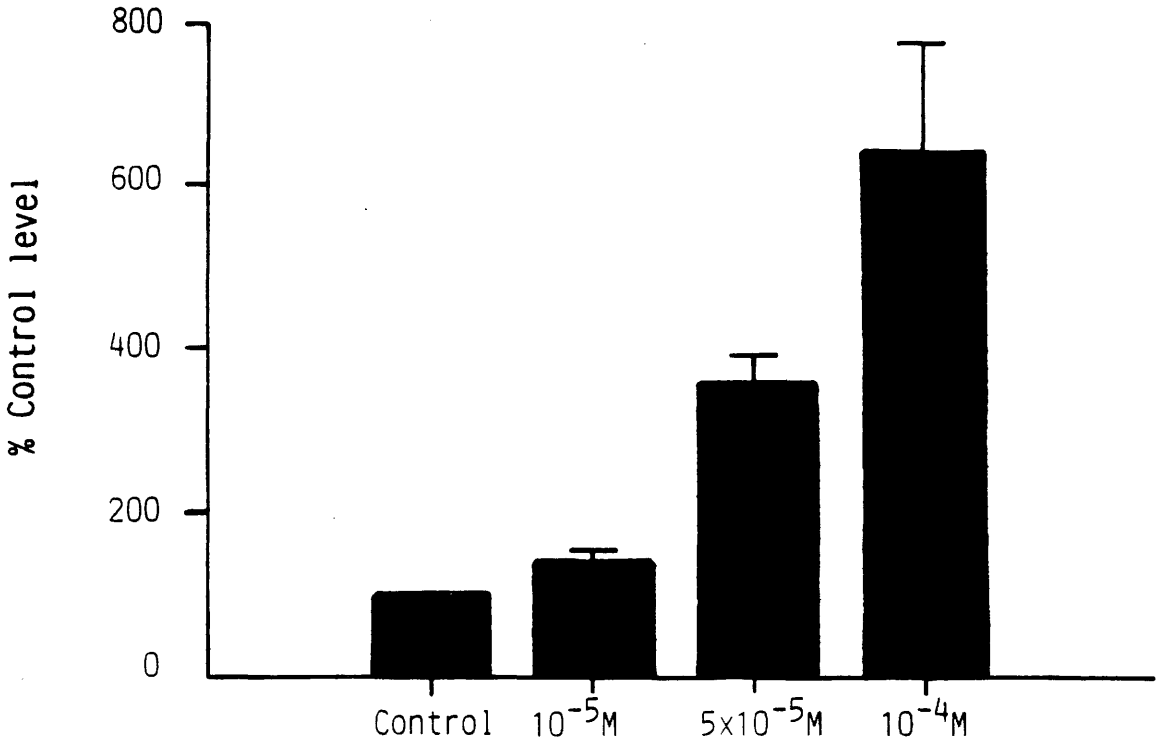


Fig. 38: The effect (± s.e. mean, n = 3) of verapamil (1×10^{-5} - 1×10^{-4}M), expressed as a percentage of the control (100%), on the resting ^{3}H overflow from the guinea-pig vas deferens incubated with $[^{3}H]$ -noradrenaline. Verapamil produced a dose-dependent increase in the resting ^{3}H overflow.

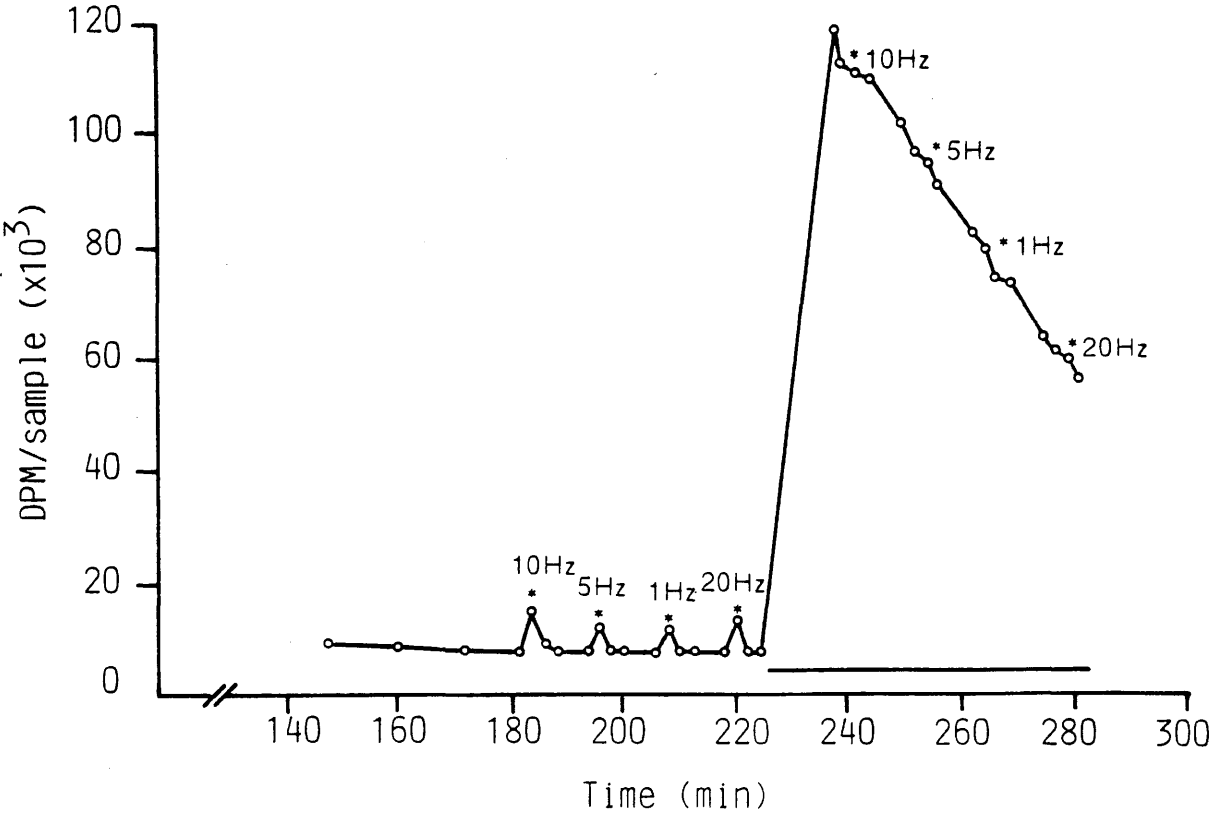


Fig. 39: The effect of amlodipine (5×10^{-5}M for the period indicated by the horizontal bar) on ^{3}H overflow, expressed as DPM/sample ($\times 10^{3}$), evoked by field stimulation (60 pulses at 1, 5, 10 and 20 Hz, 0.5 ms supramaximal voltage) of the guinea-pig vas deferens incubated with $[^{3}H]$ -noradrenaline. Amlodipine increased the resting ^{3}H overflow sufficiently to mask the effect of field stimulation at each frequency. Ascorbic acid (1.1×10^{-4}M), desipramine (6×10^{-7}M), normetanephrine (1×10^{-5}M) and atropine (2.6×10^{-6}M) were present throughout (see legend for Fig. 33).

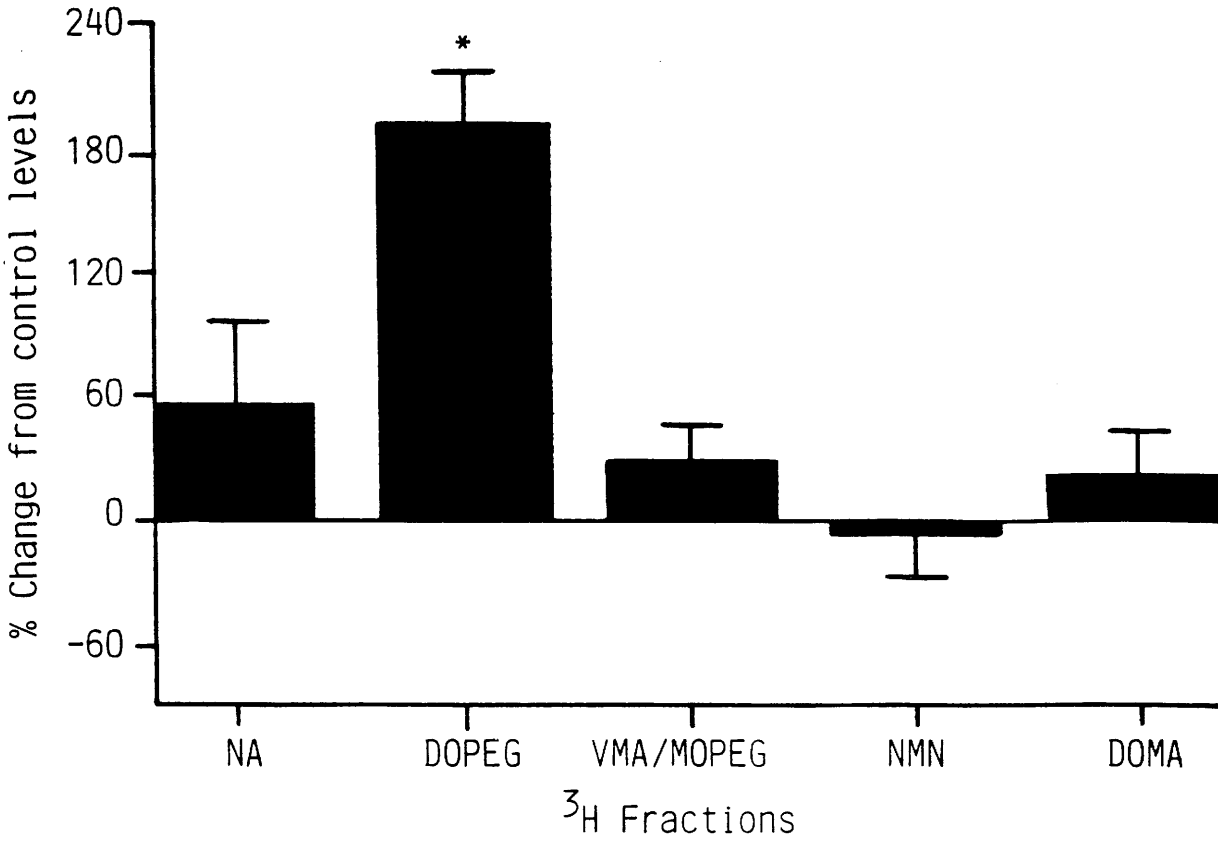


Fig. 40: The effect ($\pm$ s.e. mean, n = 3) of amlodipine (5×10^{-6}M), expressed as a percentage change from the resting, control, overflow levels of $[^3H]$ -noradrenaline and its metabolites in the guinea-pig vas deferens. Amlodipine significantly (*, $p < 0.05$) increased the level of $[^3H]$ -3,4-dihydroxyphenylglycol (DOPEG), but had no significant effect on the levels of $[^3H]$ -4-hydroxy 3-methoxymandelic acid (VMA) and 4-hydroxy 3-methoxyphenylglycol (MOPEG), normetanephrine (NMN), 3,4-dihydroxymandelic acid (DOMA) or noradrenaline (NA).

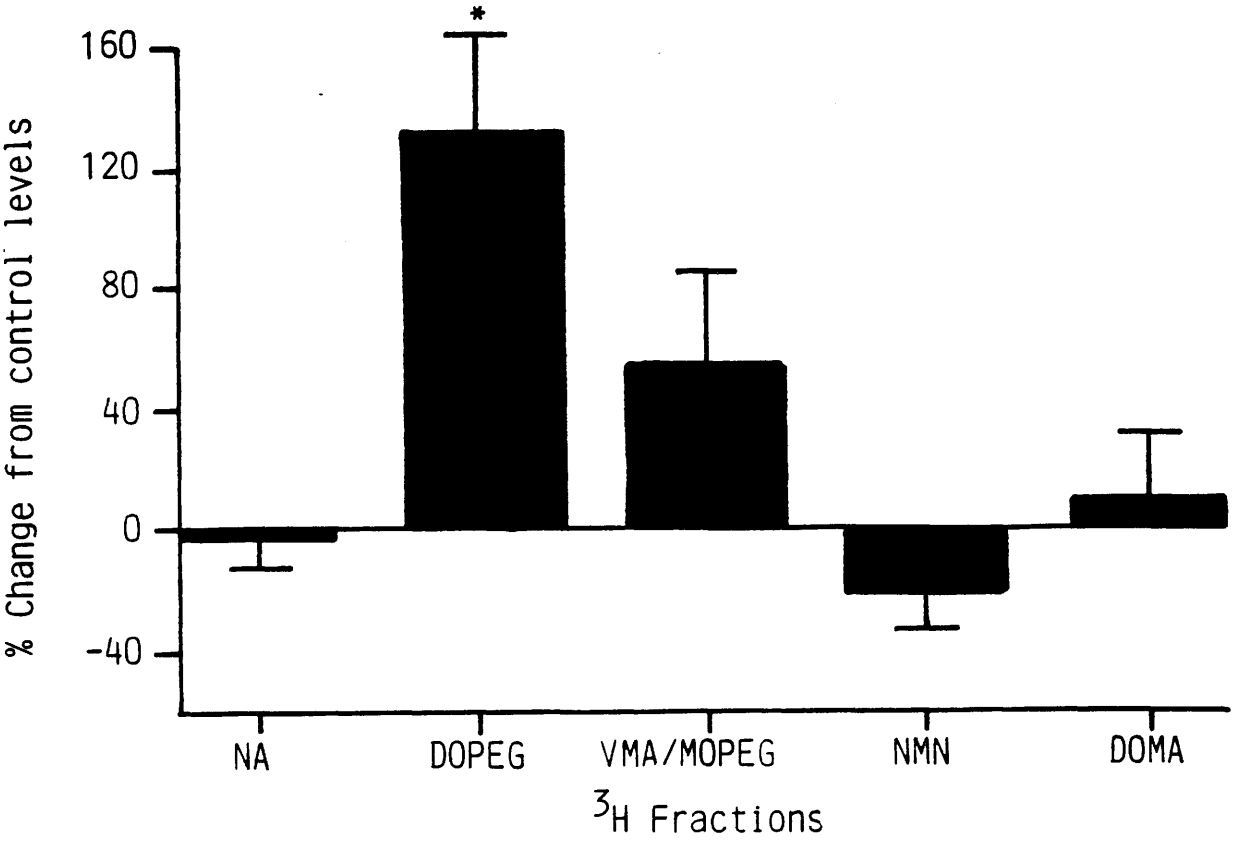
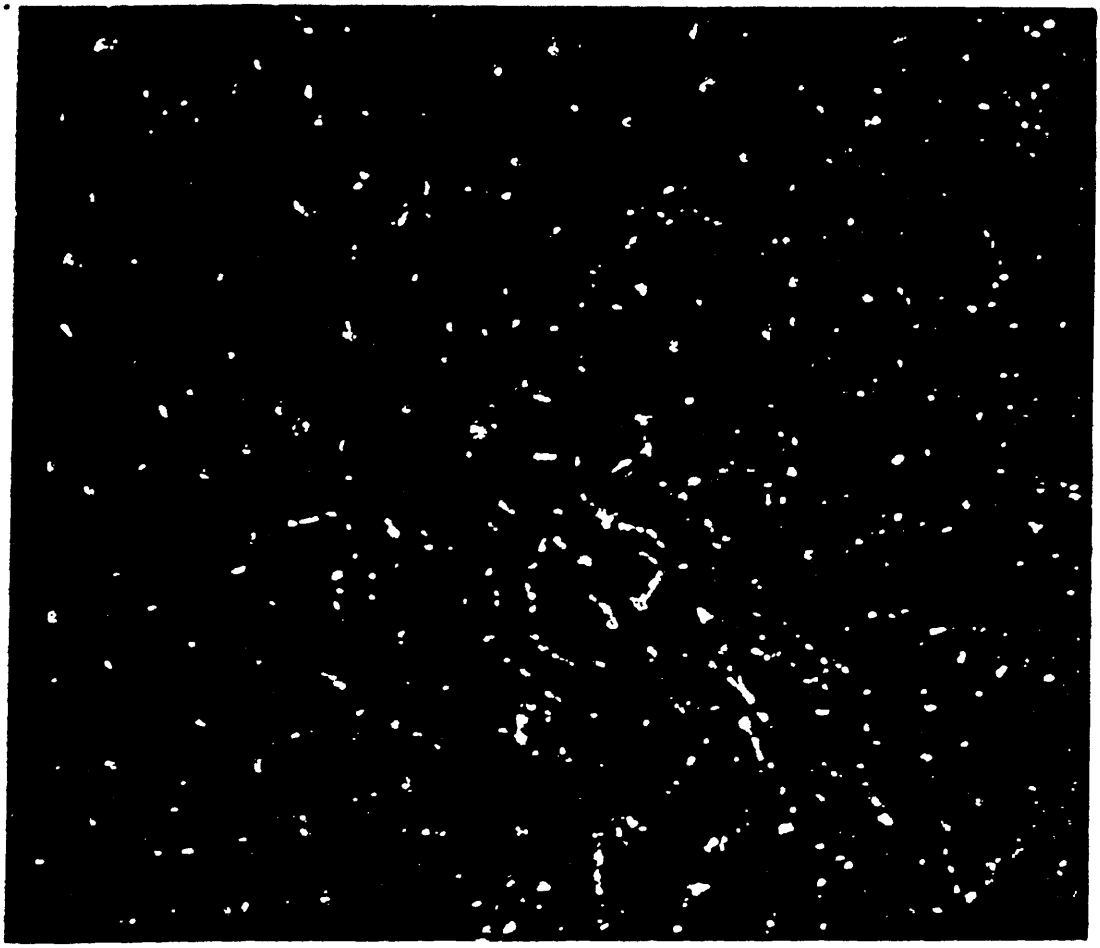


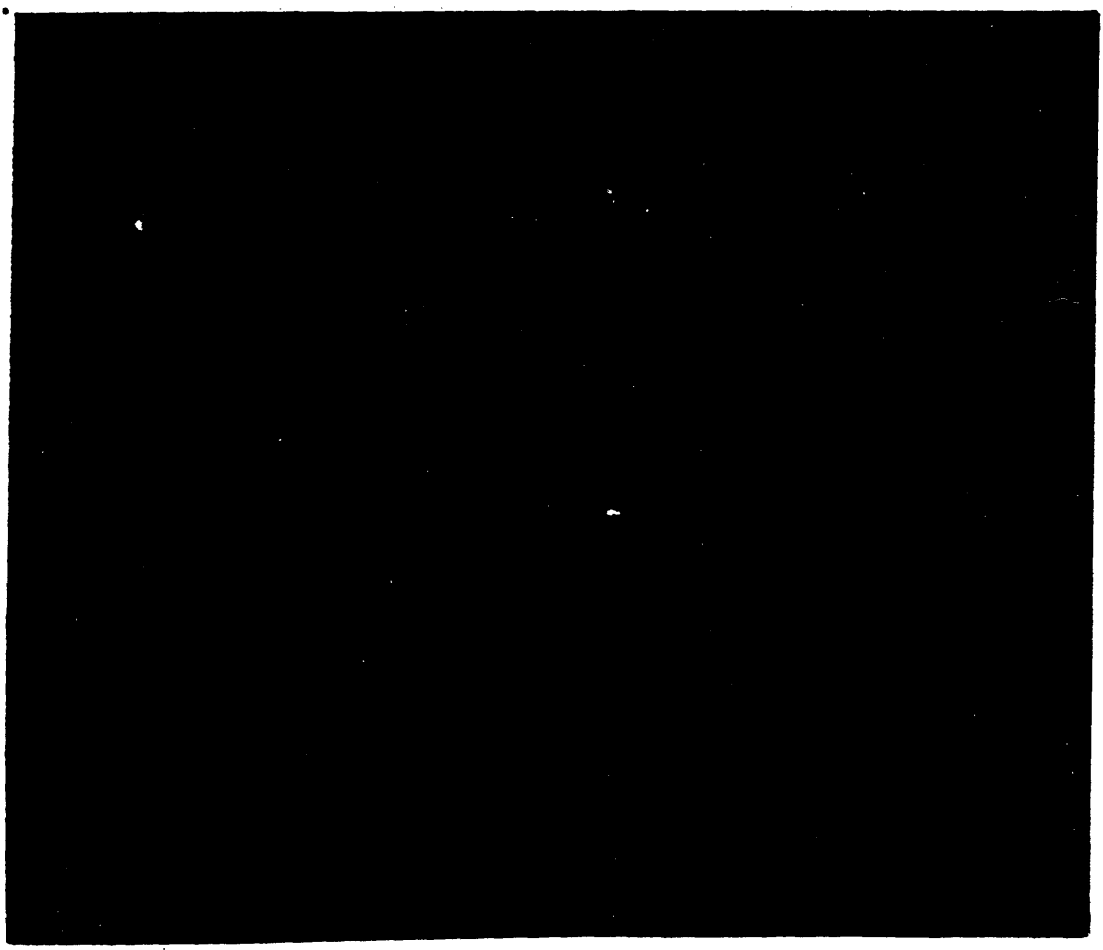
Fig. 41: The effect (± s.e. mean, n = 4) of verapamil (1 x 10^{-5}M), expressed as a percentage change from the resting, control, overflow levels of [^{3}H] -noradrenaline and its metabolites in the guinea-pig vas deferens. Verapamil significantly (*, $p < 0.05$) increased the level of [^{3}H] - 3,4-dihydroxyphenylglycol (DOPEG), but had no significant effect on the levels of [^{3}H] -4-hydroxy 3-methoxymandelic acid (VMA) and 4-hydroxy 3-methoxyphenylglycol (MOPEG), normetanephrine (NMN), 3,4-dihydroxymandelic acid (DOMA) or noradrenaline (NA).

Fig. 42: The effect of 6-hydroxydopamine pretreatment (150 $mgkg^{-1}$ i.p. on day 1 and 250 $mgkg^{-1}$ on day 2; experiments on day 3) on the catecholamine content of the guinea-pig vas deferens, determined by histofluorescence (Gillespie & Kirpekar, 1966).
a) Fluorescence (magnification x 25) of catecholamine-containing neurones in the vas deferens from control guinea-pigs (pretreated with the drug vehicle, ascorbic acid).
b) The absence of fluorescence (magnification x 25), indicating destruction of catecholamine-containing neurones by 6-hydroxydopamine pretreatment.

a.



b.



pretreatment, the effect of verapamil (1×10^{-4}M) on the resting overflow of ^{3}H, compared to that in untreated vasa, was greatly reduced (Fig. 43). Thus, the origin of the increased ^{3}H overflow appeared to be the sympathetic nerves. The effect of verapamil (1×10^{-4}M) on the resting ^{3}H overflow was unaffected by removal of Ca^{2+} from the superfusing Krebs solution. This suggested that inhibition of Ca^{2+} influx was not involved in the action of verapamil.

(b) $[^{3}H]$ -adenosine incubation

The resting overflow of ^{3}H from the vas deferens declined exponentially. The amount of ^{3}H released by field stimulation, measured 128 min after commencing superfusion, when ^{3}H overflow had reached a steady value, was poor compared to that following incubation with $[^{3}H]$ -noradrenaline. Accordingly, the conditions of field stimulation adopted (400-2000 pulses at 20 Hz, 0.5 ms, supramaximal voltage) were more severe. Moreover, to inhibit the muscle contractions, which contribute to the overflow of ^{3}H (Fredholm et al., 1982; Westfall et al., 1978), a hypertonic Krebs solution containing sucrose (0.37M) was superfused. α, β meATP and prazosin (both 5×10^{-6}M) were also added to prevent the postjunctional effects of ATP and noradrenaline respectively (Sneddon & Westfall, 1984). Under these conditions, field stimulation produced an increase in ^{3}H overflow, which declined in magnitude after 3 stimulation periods. The same tissue could not be used to measure the evoked ^{3}H overflow in both the absence and presence of drugs.

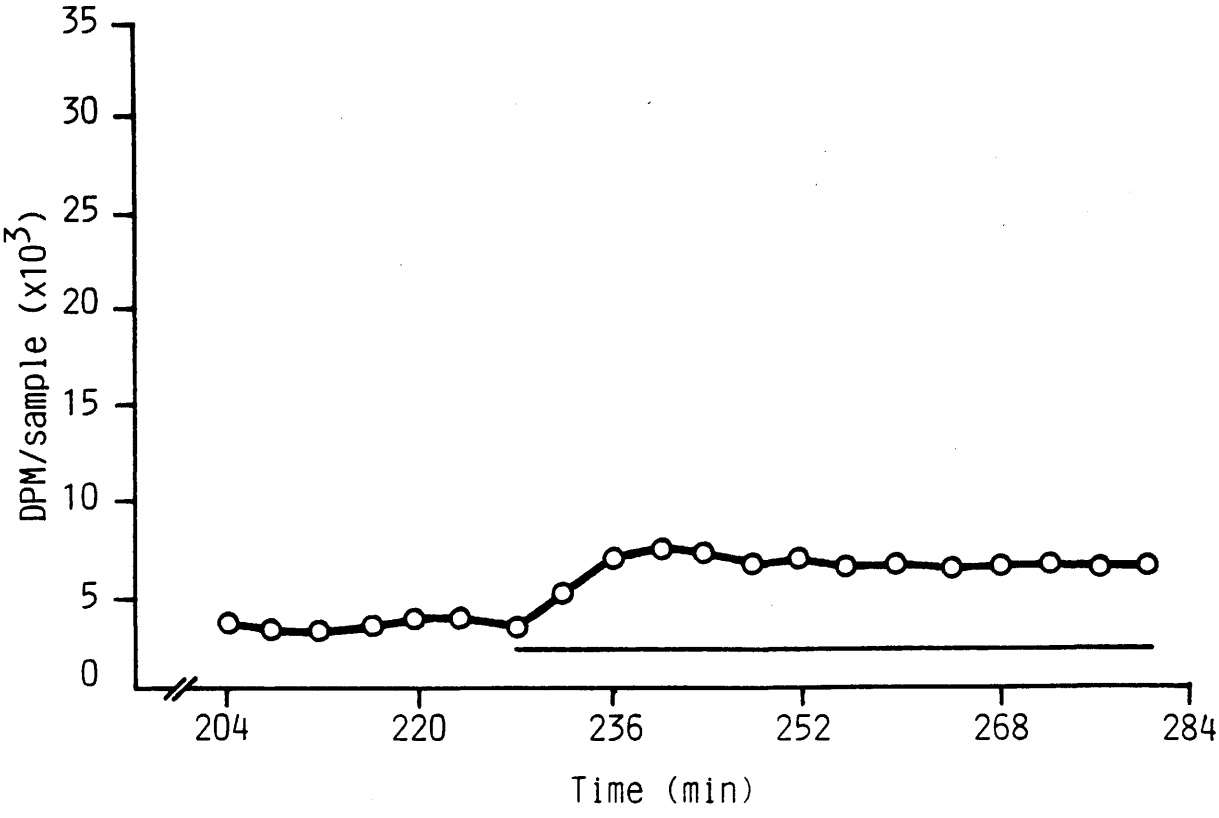


Fig. 43: The effect of verapamil (1×10^{-4}M for the period indicated by the horizontal bar) on the resting ^{3}H overflow, expressed as DPM/sample ($\times 10^{3}$), in the guinea-pig vas deferens incubated with $[^{3}H]$ -noradrenaline. The animal was pretreated with 6-hydroxydopamine (150 $mgkg^{-1}$ i.p. on day 1 and 250 $mgkg^{-1}$ on day 2; experiments on day 3) to destroy the sympathetic innervation. Verapamil increased the resting overflow of ^{3}H, but to a lesser extent than in tissues from untreated animals (Fig. 37). Ascorbic acid (1.1×10^{-4}M), desipramine (6×10^{-7}M), normetanephrine (1×10^{-5}M) and atropine (2.6×10^{-6}M) were present throughout (see legend for Fig. 33).

Accordingly, one vas from each guinea-pig was used for drug treatment and the contralateral tissue for the control. The amount of ^{3}H in the resting overflow from each tissue varied greatly (e.g. Fig. 46), even between control preparations. The cause of this variation is unknown.

The stimulation-evoked increase in ^{3}H overflow originated from nerves; it was prevented by TTX (2×10^{-6}M, Fig. 44). Verapamil (1×10^{-4}M, Fig. 45) had little effect on the resting overflow of ^{3}H when $[^{3}H]$ -adenosine was used. Stimulation-evoked overflow was inhibited by diltiazem (5×10^{-4}M) and cobalt (2×10^{-3}M, Fig. 46), but was unaffected by nifedipine (5×10^{-4}M, Fig. 47).

ACTIONS OF Ca^{2+} CHANNEL ANTAGONISTS ON THE RAT ANOCOCCYGEUS

(i) Nerve AP conduction in vitro

A discharge of APs was recorded from a small genito-femoral nerve bundle in response to stimulation (single pulses, 0.1 - 0.5 ms, supramaximal voltage). The APs had a mean amplitude of 0.1 $\pm$ 0.1 mV (n = 74) and a latency of 10.5 $\pm$ 0.8 ms (n = 77). The AP discharges in response to a single pulse lasted 18.8 $\pm$ 1.0 ms (n = 20). There were no ganglia in the nerve pathway; hexamethonium (1×10^{-4}M) was ineffective. Na^{+} involvement in the APs was indicated by the inhibitory action of TTX (2×10^{-6}M).

Perfusion with either verapamil ($0.1 - 5 \times 10^{-5}$M), nifedipine ($0.01 - 1 \times 10^{-4}$M) or cobalt ($0.05 - 1 \times 10^{-2}$M), inhibited the APs in a dose-dependent manner (Figs. 48 to 50).

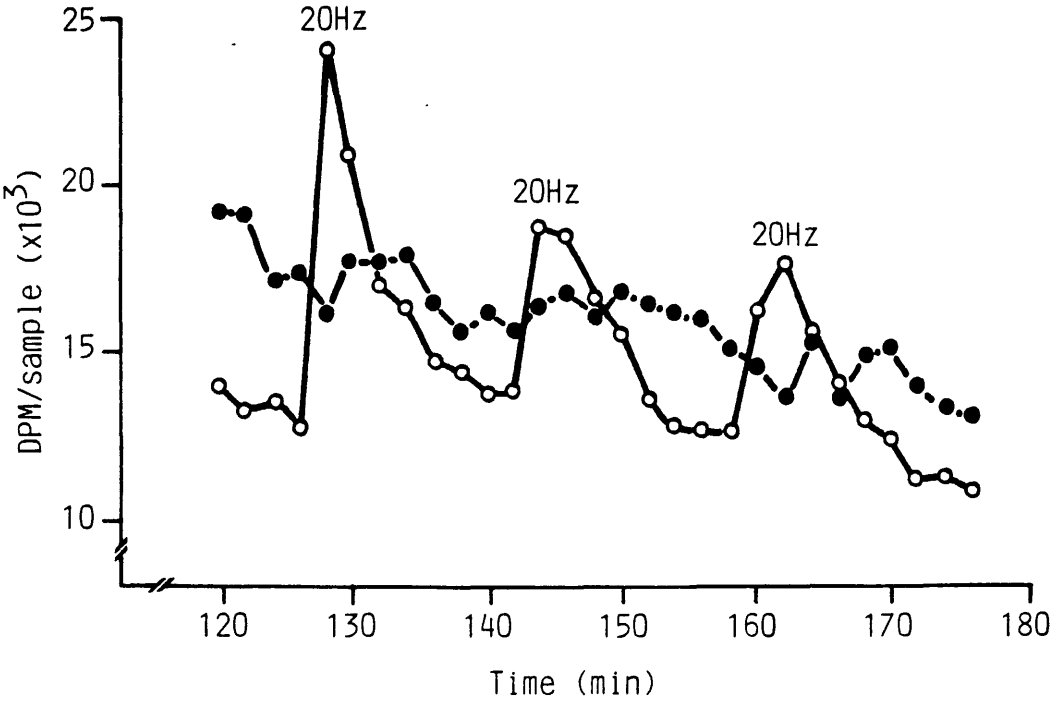


Fig. 44: ^{3}H overflow, expressed as DPM/sample (x 10^{3}), from guinea-pig vasa incubated with $[^{3}H]$ -adenosine, in the absence (o-o) and presence (●-●) of TTX (2 x 10^{-6}M). TTX prevented ^{3}H overflow in response to field stimulation (2000 pulses at 20 Hz, 0.5 ms, supramaximal voltage), indicating that the ^{3}H originated from nerves. Sucrose (0.37M), α, β meATP and prazosin (both 5 x 10^{-6}M) were present throughout, to inhibit contractions which contribute to the release of ATP (Westfall et al., 1978). Vasa from the same animal were superfused separately, but stimulated simultaneously.

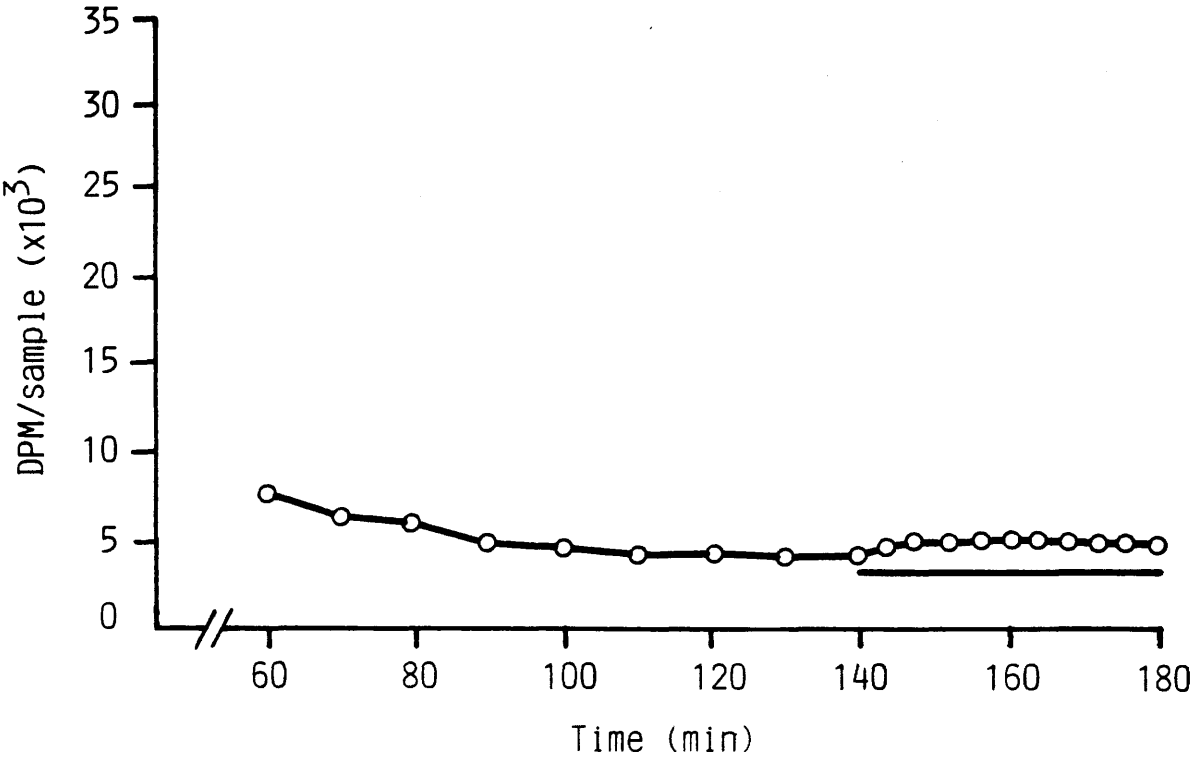


Fig. 45: The effect of verapamil (1 x 10^{-4}M for the period indicated by the horizontal bar) on the resting ^{3}H overflow, expressed as DPM/sample (x 10^3), from the guinea-pig vas deferens incubated with [^{3}H] -adenosine. Verapamil had little effect on the resting ^{3}H overflow compared with its effect on vasa incubated with [^{3}H] -noradrenaline (Fig. 37).

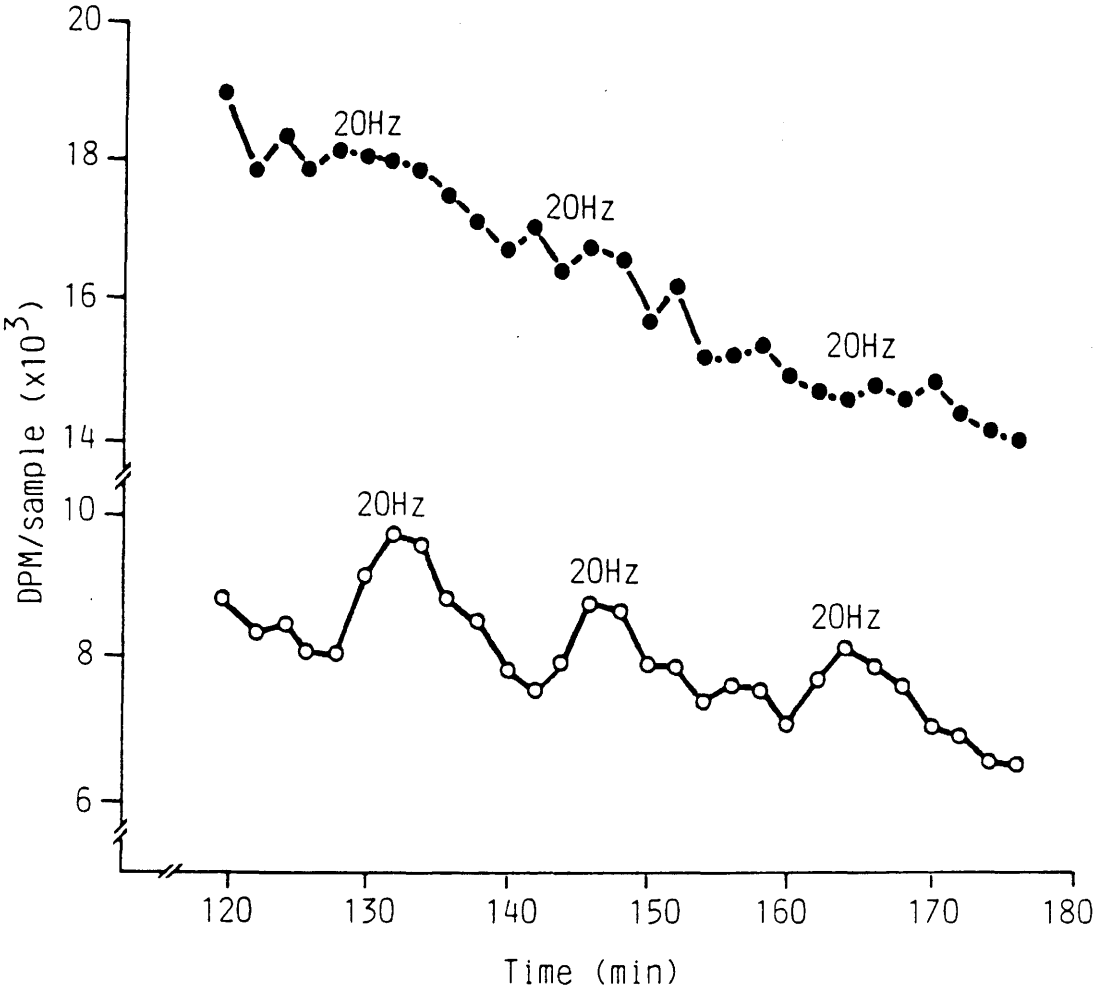


Fig. 46: ^{3}H overflow, expressed as DPM/sample (x 10^3), from guinea-pig vasa incubated with [^{3}H] -adenosine, in the absence (o-o) and presence (●-●) of cobalt (2 x 10^{-3}M). Cobalt prevented ^{3}H overflow in response to field stimulation (2000 pulses at 20 Hz, 0.5 ms, supramaximal voltage). Sucrose (0.37M), α, β meATP and prazosin (both 5 x 10^{-6}M) were present throughout, to inhibit contractions. Vasa from the same animal were superfused separately, but stimulated simultaneously.

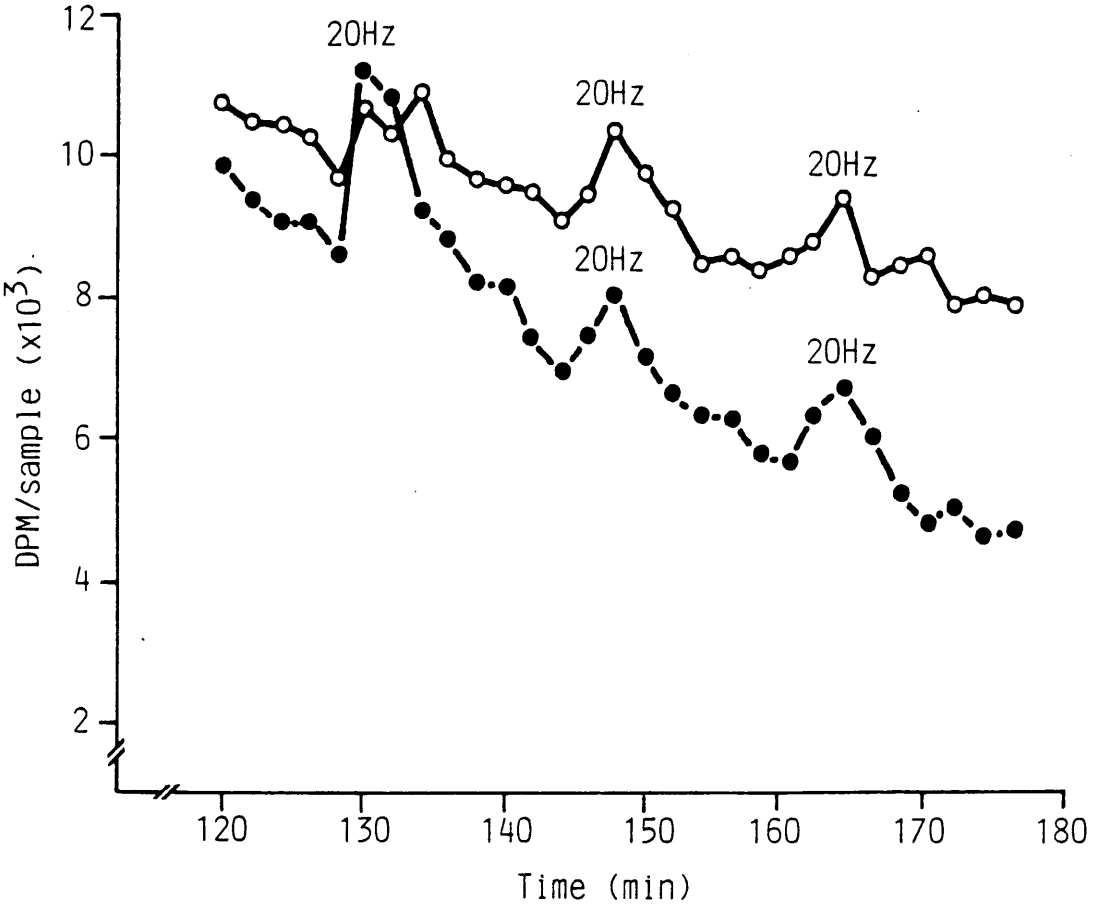


Fig. 47: ^{3}H overflow, expressed as DPM/sample (x 10^{3}), from guinea-pig vasa incubated with $[^{3}H]$ -adenosine, in the absence (0-0) and presence (●-●) of nifedipine (5 x 10^{-4}M). Nifedipine had no effect on ^{3}H overflow in response to field stimulation (2000 pulses at 20 Hz, 0.5 ms, supramaximal voltage). Sucrose (0.37M), α, β meATP and prazosin (both 5 x 10^{-6}M) were present throughout, to inhibit contractions. Vasa from the same animal were superfused separately, but stimulated simultaneously.

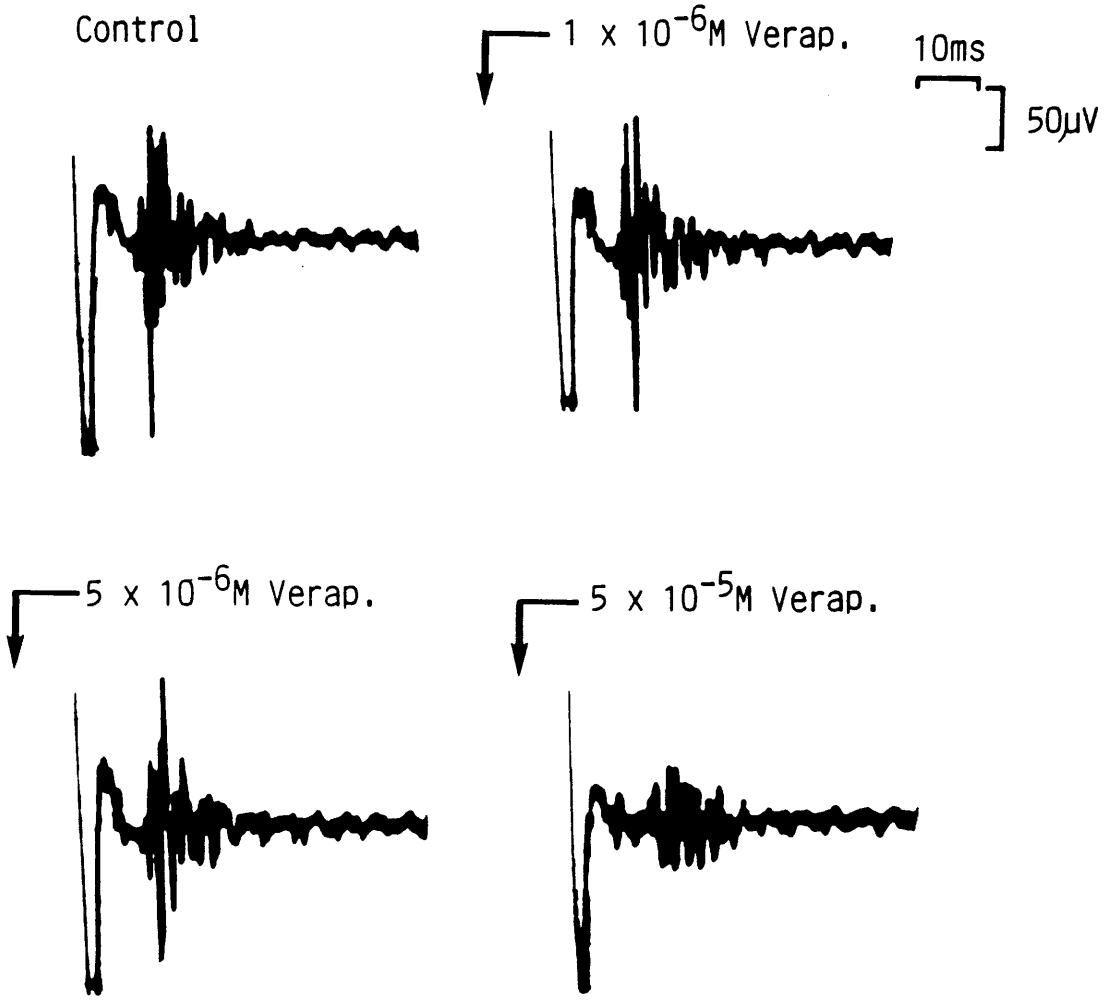


Fig. 48: The effect of verapamil (Verap., 1 x 10^{-6} - 5 x 10^{-5}M) on AP conduction in vitro, in a small bundle of genito-femoral nerve fibres running to the rat anococcygeus. Verapamil inhibited the APs evoked by stimulation (single pulses, 0.5 ms, supramaximal voltage) of the genito-femoral nerve trunk, in a dose-dependent manner. Both the number and amplitude of APs were reduced. The time between each panel was 15 min.

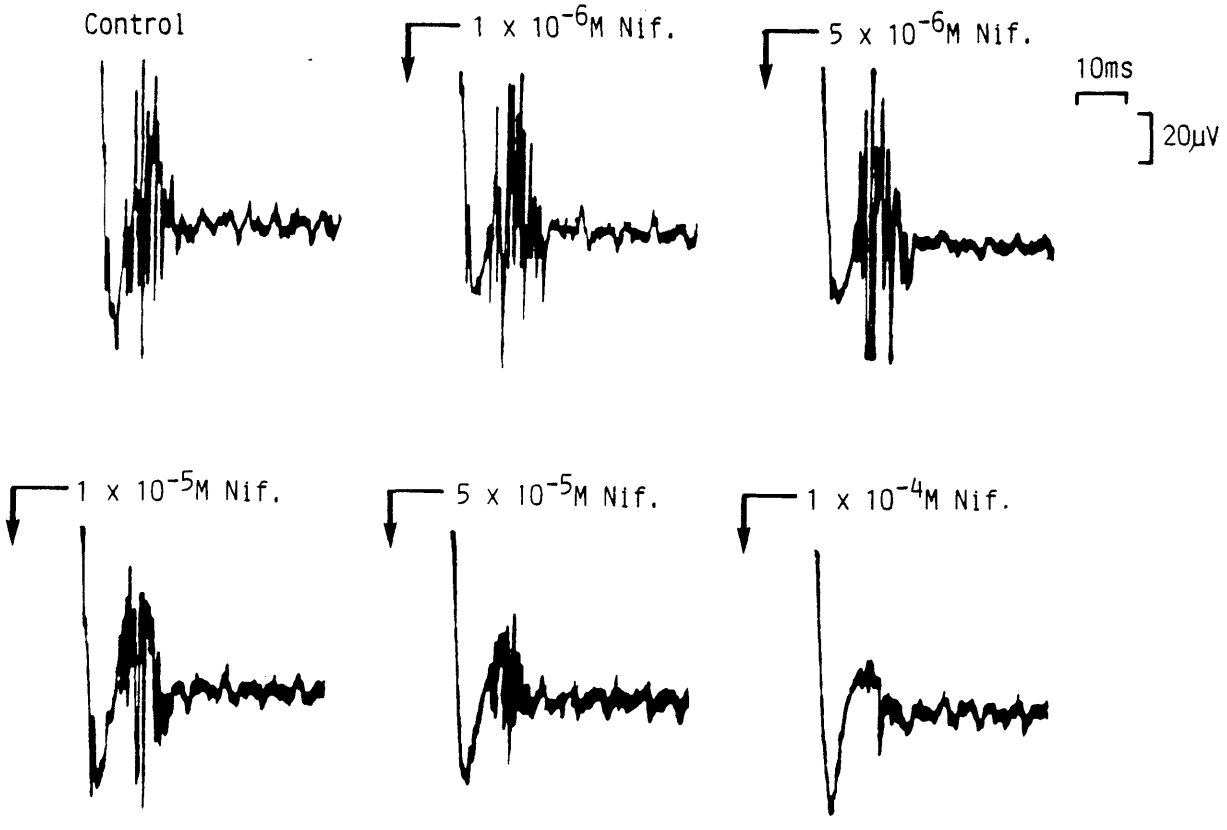


Fig. 49: The effect of nifedipine (Nif., 1 x 10^{-6} - 1 x 10^{-4}M) on AP conduction *in vitro*, in a small bundle of genito-femoral nerve fibres running to the rat anococcygeus. Nifedipine inhibited the APs evoked by stimulation (single pulses, 0.5 ms, supramaximal voltage) of the genito-femoral nerve trunk, in a dose-dependent manner. The time between each panel was 15 min.

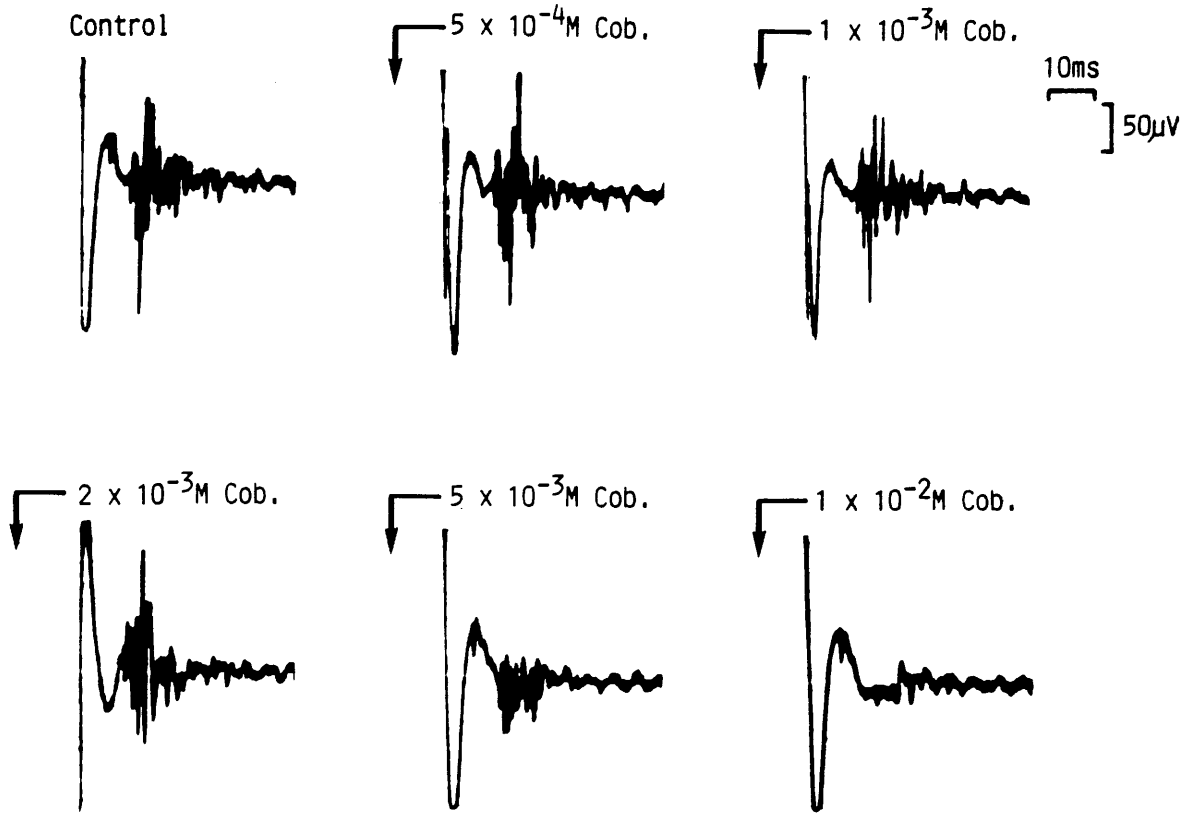


Fig. 50: The effect of cobalt (Cob., 5×10^{-4} - 1×10^{-2}M) on AP conduction in vitro, in a small bundle of genito-femoral nerve fibres running to the rat anococcygeus. Cobalt inhibited, dose-dependently, the APs evoked by stimulation (single pulses, 0.5 ms, supramaximal voltage) of the genito-femoral nerve trunk. The time between each panel was 15 min.

It was intended to study the effects of these agents on AP conduction in the NANC nerve fibres running to the rat anococcygeus. This appeared feasible since NANC and sympathetic nerve fibres are separate in this tissue and inhibitory responses present in 6-hydroxydopamine-treated animals (Gibson & Gillespie, 1973). To destroy selectively the sympathetic innervation, rats were pretreated with reserpine (1 $mgkg^{-1}$ i.p. daily for the first 3 days) and 6-hydroxydopamine (150 $mgkg^{-1}$ i.p. on day 4 and 250 $mgkg^{-1}$ on day 5; experiments on day 6). Destruction was achieved; there were no catecholamine-containing nerve fibres, as indicated by the absence of fluorescence (Gillespie & Kirpekar, 1966; Fig. 51), nor were contractions observed in pretreated tissues following extrinsic nerve stimulation. APs and NANC-mediated relaxations, recorded in separate experiments, were, however, evident following pretreatment. The APs were similar to those recorded from untreated tissues, with respect to their amplitude and latency. Perfusion with either verapamil ($0.05 - 2 \times 10^{-4}$M) or cobalt ($0.1 - 1.5 \times 10^{-2}$M), at similar concentrations to those effective in untreated nerves, inhibited the APs (Figs. 52 and 53).

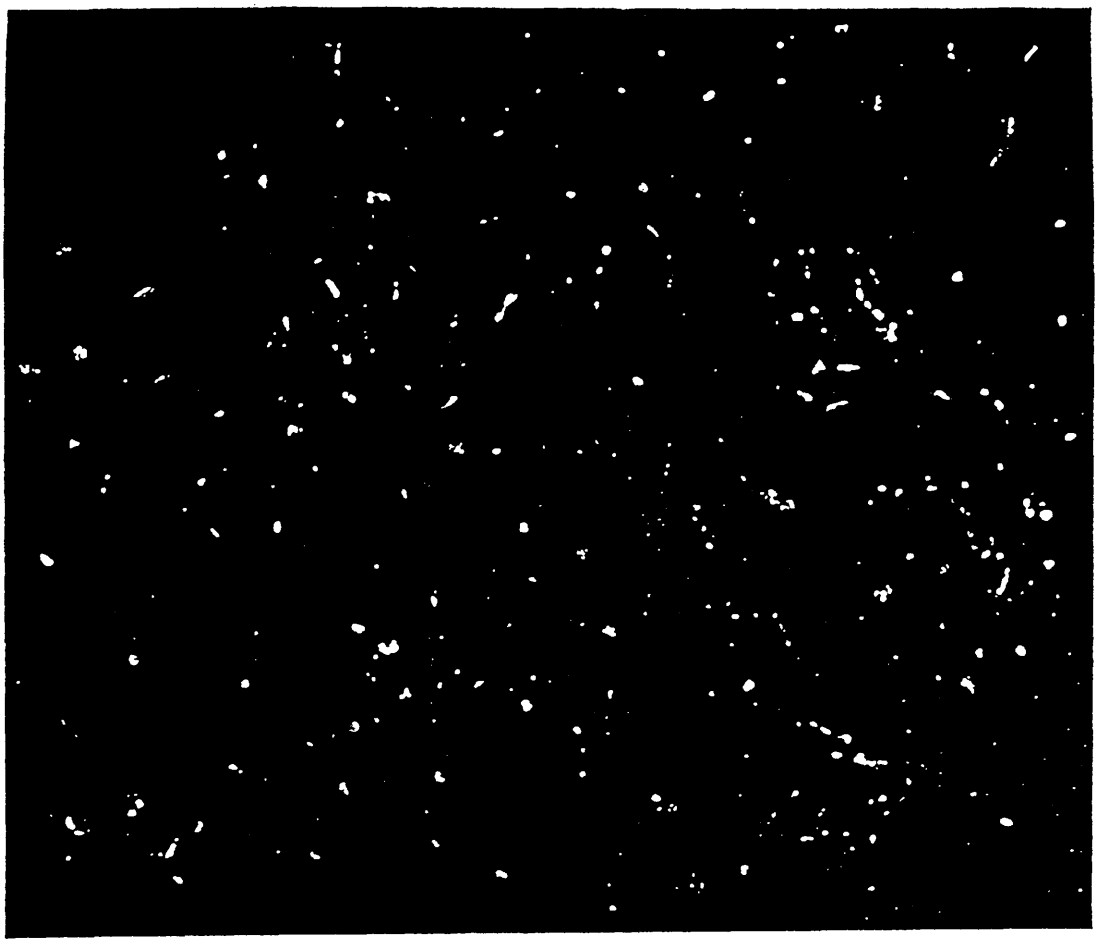
Together, the results of these observations are at best equivocal. It is likely that pretreatment with reserpine and 6-hydroxydopamine destroyed some sympathetic nerve fibres. There is, however, no evidence that the fibres from which APs were subsequently recorded, were exclusively NANC, although it was clear that mechanical responses to NANC (but not sympathetic) nerve stimulation persisted following pretreatment.

Fig. 51: The effect of reserpine (1 $mgkg^{-1}$ i.p. daily for the first 3 days) and 6-hydroxydopamine (150 $mgkg^{-1}$ i.p. on day 4 and 250 $mgkg^{-1}$ on day 5; experiments on day 6) on the catecholamine content of the rat vas deferens, determined by histofluorescence (Gillespie & Kirpekar, 1966).

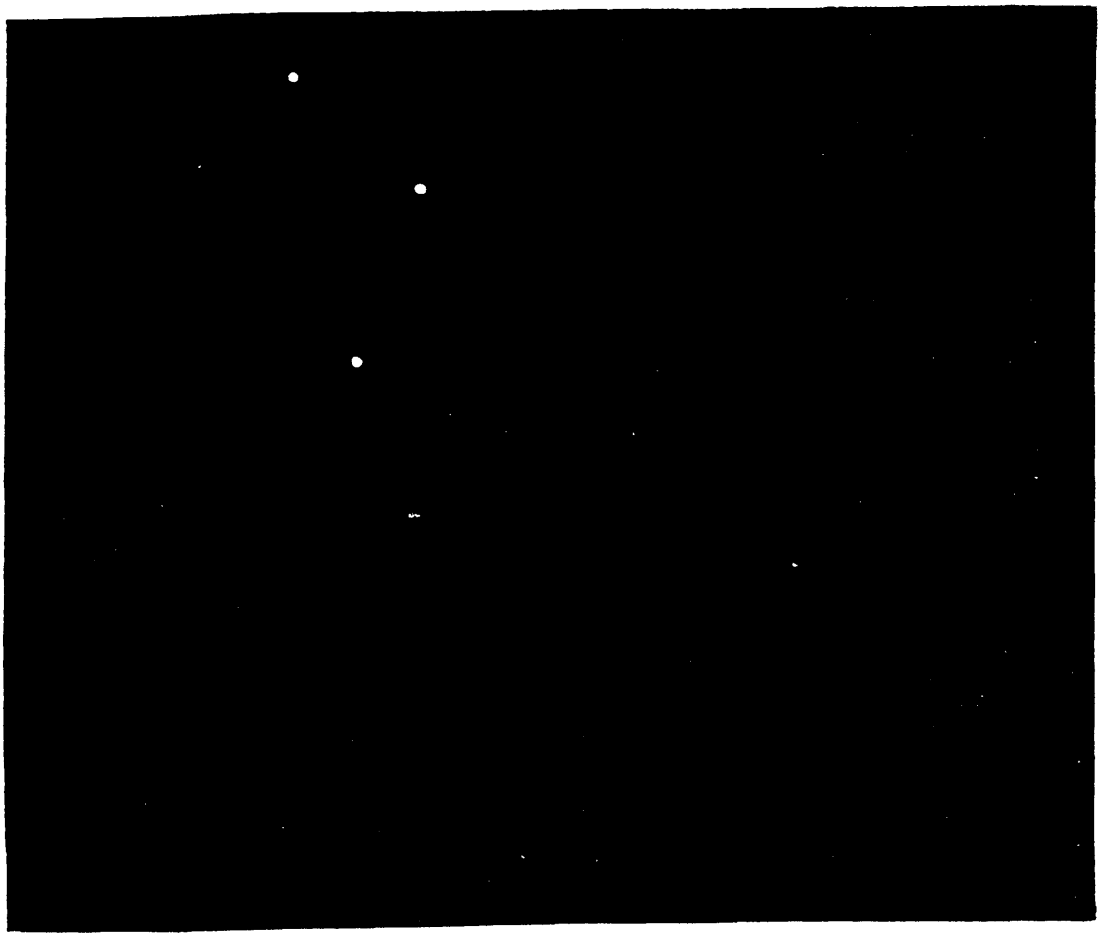
a) Fluorescence (magnification x 25) of catecholamine-containing neurones in the vas deferens from control rats (pretreated with the drug vehicles, glacial acetic acid and ascorbic acid).

b) The absence of fluorescence (magnification x 25), indicating destruction of catecholamine-containing neurones by reserpine and 6-hydroxydopamine pretreatment.

a.



b.



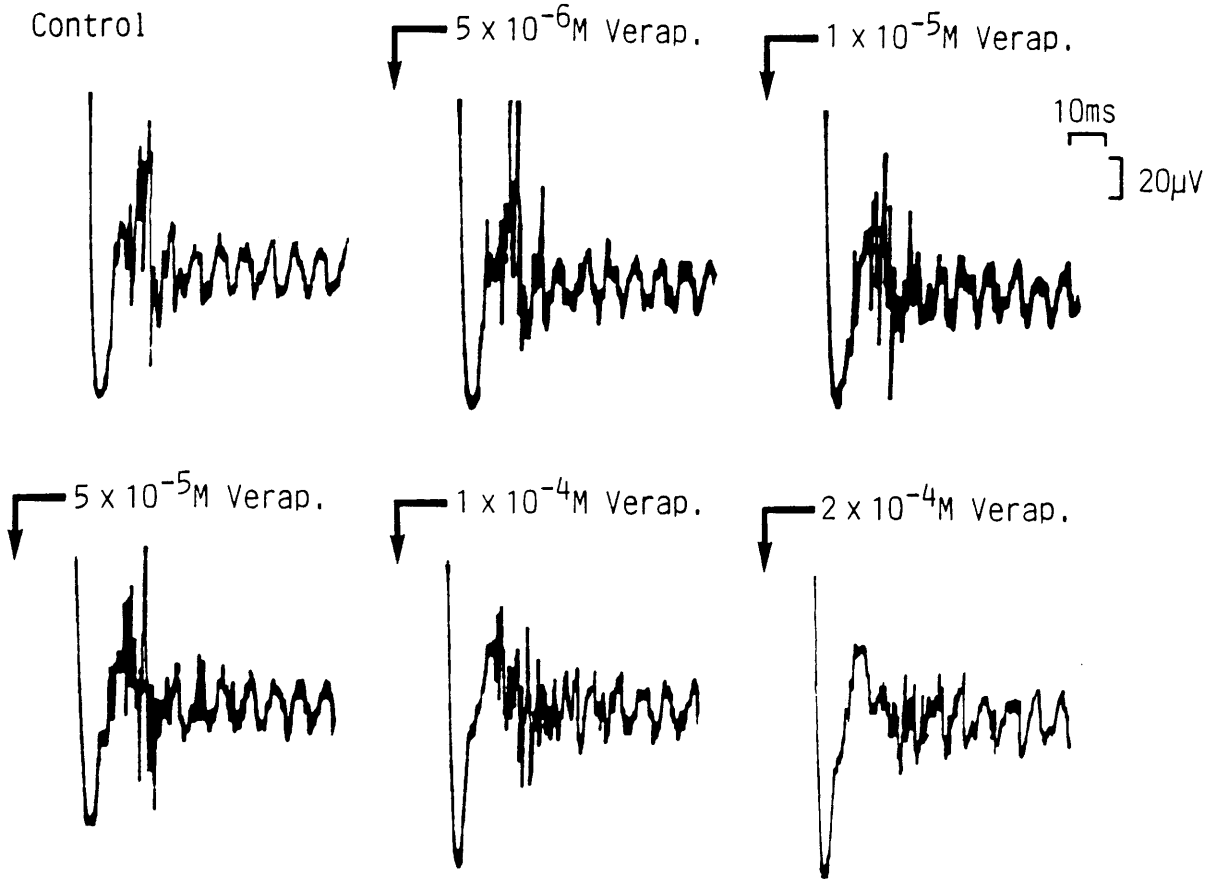


Fig. 52: The effect of verapamil (Verap., 5×10^{-6} - 2×10^{-4}M) on AP conduction *in vitro*, in a small bundle of genito-femoral nerve fibres running to the rat anococcygeus. The animal was pretreated with reserpine (1 $mgkg^{-1}$ i.p. daily for the first 3 days) and 6-hydroxydopamine (150 $mgkg^{-1}$ i.p. on day 4 and 250 $mgkg^{-1}$ on day 5; experiments on day 6) to destroy the sympathetic innervation. Verapamil inhibited the APs in response to stimulation (single pulses, 0.5 ms, supramaximal voltage) of the genito-femoral nerve trunk, in a dose-dependent manner. These APs may have originated from NANC nerve fibres (see text). The time between each panel was 15 min.

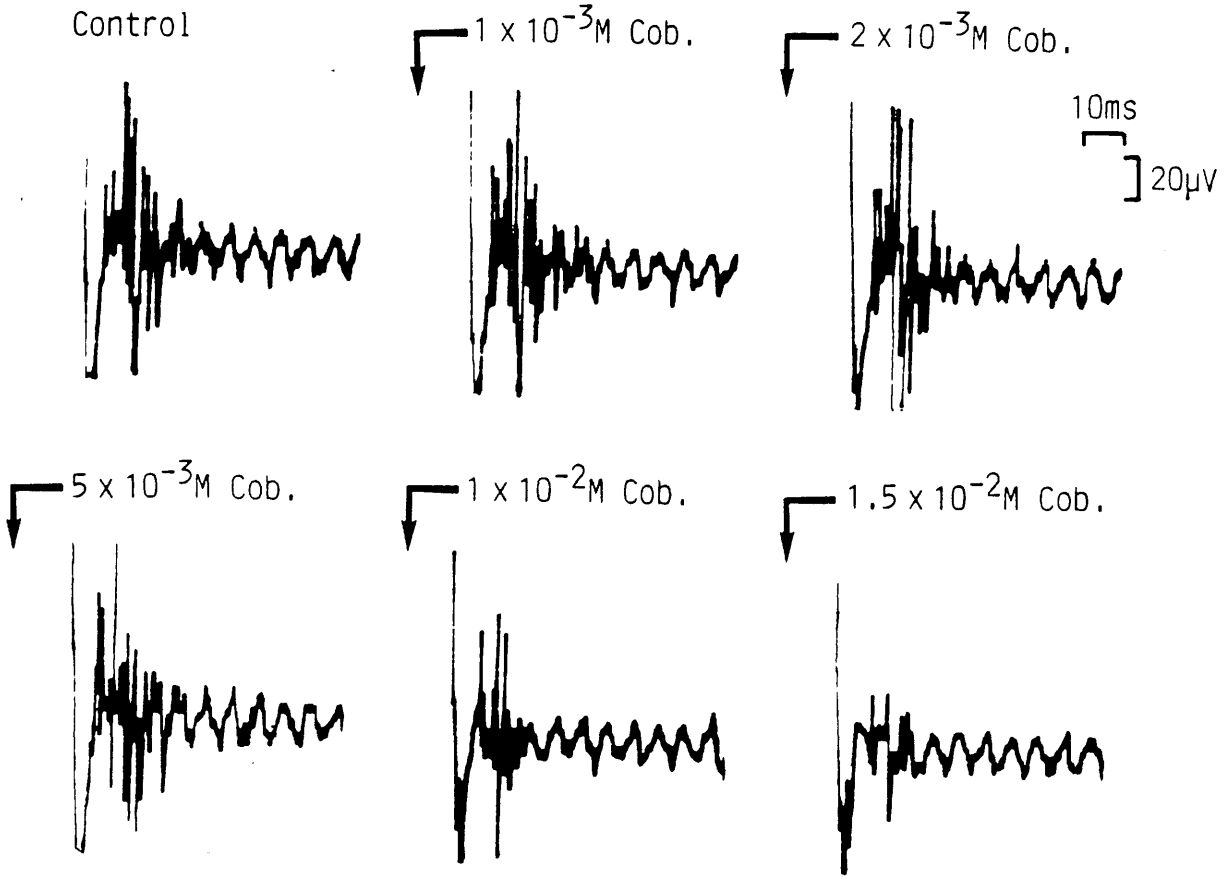


Fig. 53: The effect of cobalt (Cob., 1 x 10^{-3} - 1.5 x 10^{-2}M) on AP conduction *in vitro*, in a small bundle of genito-femoral nerve fibres running to the rat anococcygeus. The animal was pretreated with reserpine (1 $mgkg^{-1}$ i.p. daily for the first 3 days) and 6-hydroxydopamine (150 $mgkg^{-1}$ i.p. on day 4 and 250 $mgkg^{-1}$ on day 5; experiments on day 6) to destroy the sympathetic innervation. Cobalt inhibited, dose-dependently, the APs in response to stimulation (single pulses, 0.5 ms, supramaximal voltage) of the genito-femoral nerve trunk. These APs may have originated from NANC nerve fibres (see text). The time between each panel was 15 min.

(ii) Excitatory junction potentials

To assess the effects of the antagonists on transmitter release in the anococcygeus, e.j.ps, in response to extrinsic nerve stimulation, were measured intracellularly using glass microelectrodes.

When first set up, the anococcygeus had no tone. Each preparation was then gently stretched to a tension of approximately 1g, which was maintained throughout each experiment. The smooth muscle of the anococcygeus had a mean resting membrane potential of -70.1 $\pm$ 0.5 mV (n = 140 cells). No spontaneous electrical activity was present.

Stimulation (5 pulses at 5-20 Hz, 0.1 - 0.5 ms, supra-maximal voltage) of the genito-femoral, together with the perineal branches of the pudendal nerve running to the ano-coccygeus, evoked an e.j.p. and contraction. Two types of e.j.ps, possessing different rates of rise, were evident. In 80% of cells (Fig. 54a), stimulation with 5 pulses at 10 and 20 Hz evoked e.j.ps with a short time to peak. These 'fast' e.j.ps, up to 40 mV in amplitude with a mean duration of 1.0 $\pm$ 0.03 s (n = 35), were followed by a 'slow' e.j.p. which lasted several seconds (4.7 $\pm$ 0.3s, n = 31) and had a mean amplitude of 4.8 $\pm$ 0.4 mV (n = 36). In these cells, stimulation with 5 pulses at 5 Hz generally produced only a 'slow' e.j.p. In the remainder of cells, only 'fast' e.j.ps were observed (Fig. 54b). TTX (1×10^{-6}M) inhibited both e.j.ps and contractions, indicating their mediation by nerves.

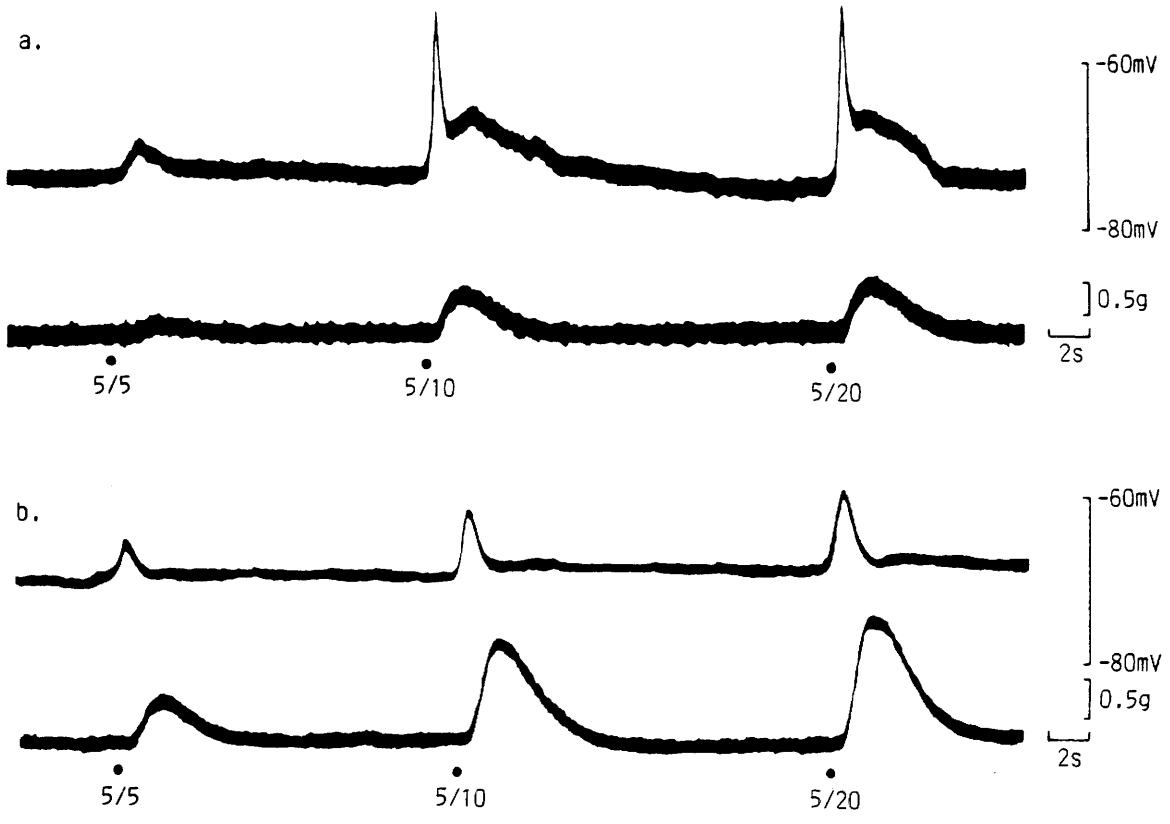


Fig. 54: Intracellular electrical (upper trace) and accompanying contractile activity in the rat anococcygeus, evoked by stimulation (5 pulses at 5, 10 and 20 Hz, 0.5 ms, supramaximal voltage) of both the genito-femoral and perineal branches of the pudendal nerve. a. E.j.ps evoked by stimulation (5 pulses) at 10 and 20 Hz, in some 80% of cells, consisted of a 'fast' and 'slow' component and were accompanied by mechanical contractions. Stimulation (5 pulses) at 5 Hz normally produced only a 'slow' e.j.p. b. 'Fast' e.j.ps only, again accompanied by a contraction, were observed in approximately 20% of cells.

Verapamil (0.1 - 5 x 10^{-5}M), nifedipine (0.01 - 1 x 10^{-4}M) and cobalt (0.5 - 2 x 10^{-3}M), each inhibited, in a dose-dependent manner, both evoked (5 pulses at 5-20 Hz, 0.5 ms, supramaximal voltage) 'fast' and 'slow' e.j.ps and contractions (Figs. 55 to 57). The organic agents were each more potent in blocking muscle contractions than in reducing the e.j.ps, while cobalt was equally effective against both parameters.

ACTIONS OF Ca^{2+} CHANNEL ANTAGONISTS ON THE GUINEA-PIG INTERNAL ANAL SPHINCTER

(i) Inhibitory junction potentials

I.j.ps were used as a measure of NANC transmitter release.

The i.a.s., when first set up, had no tone. Following gentle stretch (1g), however, some 2-3g developed spontaneously and persisted for several hours, accompanied frequently by oscillations in tone. Tone was myogenic; it was unaffected by either cholinoceptor (e.g. atropine, 1 x 10^{-6}M) or adrenoceptor (e.g. phentolamine, 1 x 10^{-6}M) antagonists, or by TTX (1 x 10^{-6}M).

There was a spontaneous spike discharge in the i.a.s., two different patterns of which were evident (Fig. 58). The first, observed in 40% of cells, consisted of large (up to 60 mV) spikes, discharging at 1-2 Hz. Each spike was preceded by a slow membrane depolarization, characteristic of pacemaker activity (Bennett, 1972) and followed by a hyperpolarization of some 5-7 mV. The second type consisted of smaller spikes (2-3 Hz, 30-40 mV in amplitude), which could be either continuous or intermittent, often superimposed on a membrane depolarization. The mean resting membrane potential, measured

Fig. 55: The dose-dependent inhibitory effect of verapamil (Verap., 1 x 10^{-6} - 5 x 10^{-5}M) on intracellularly-recorded e.j.ps (upper trace of each set) and accompanying contractions in the rat anococcygeus, in response to extrinsic nerve stimulation (5 pulses at 5, 10 and 20 Hz, 0.5 ms, supramaximal voltage). The contractions were inhibited before the e.j.ps. The time between each set was approximately 15 min.

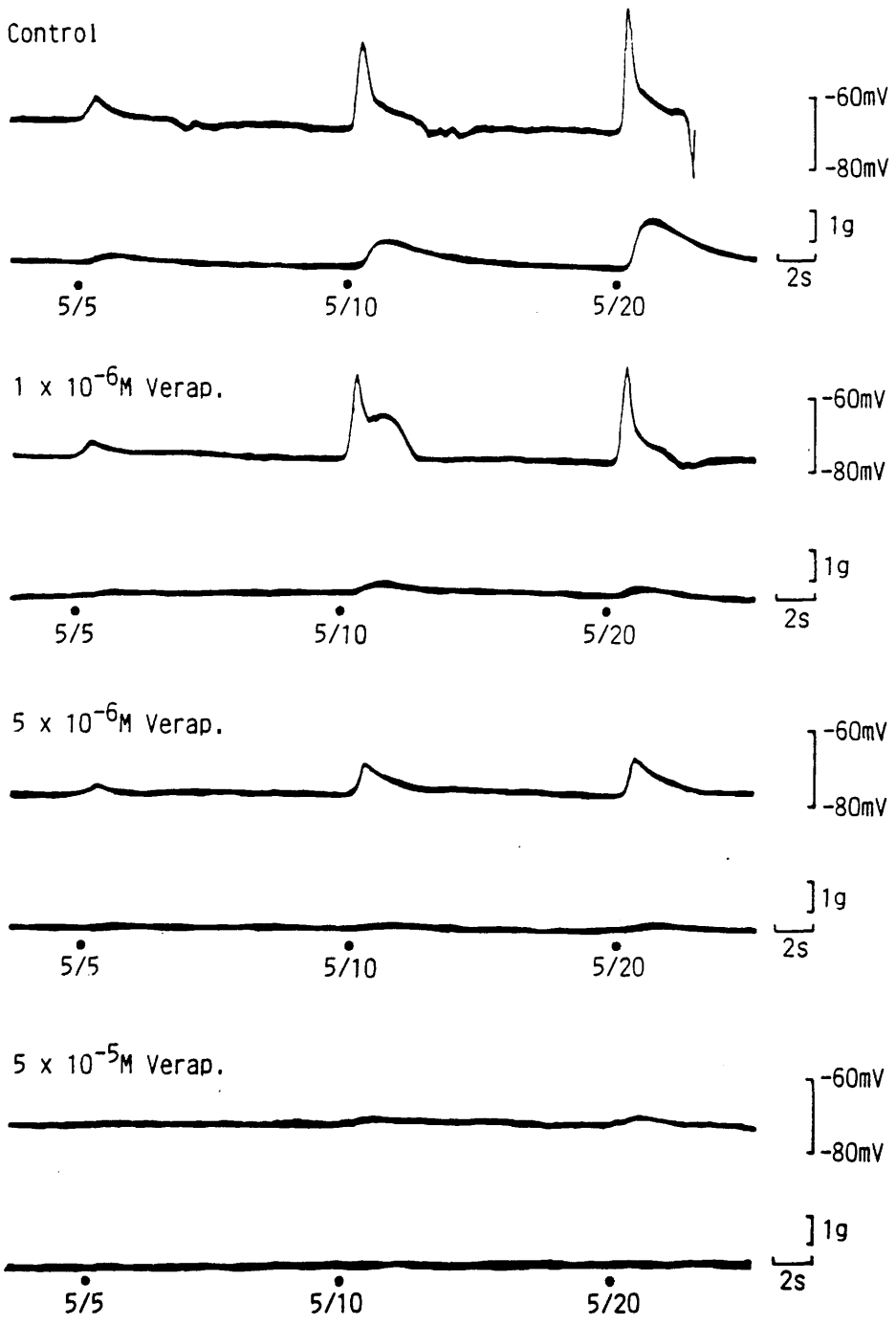

Control
-60mV
-80mV
1g
2s
5/5
5/10
5/20
1 x 10-6M Verap.
-60mV
-80mV
1g
2s
5/5
5/10
5/20
5 x 10-6M Verap.
-60mV
-80mV
1g
2s
5/5
5/10
5/20
5 x 10-5M Verap.
-60mV
-80mV
1g
2s
5/5
5/10
5/20

Fig. 56: The dose-dependent inhibitory effect of nifedipine (Nif., 1 x 10^{-6} - 1 x 10^{-4}M) on intracellularly-recorded e.j.ps (upper trace of each set) and accompanying contractions, evoked by extrinsic nerve stimulation (5 pulses at 5, 10 and 20 Hz, 0.5 ms, supramaximal voltage), in the rat anococcygeus. As with verapamil (Fig. 55), nifedipine inhibited the contractions before the e.j.ps. In this experiment, the effects of nifedipine on nerve AP conduction (Fig. 49) were studied simultaneously with its actions on release. The time between each set was approximately 15 min.

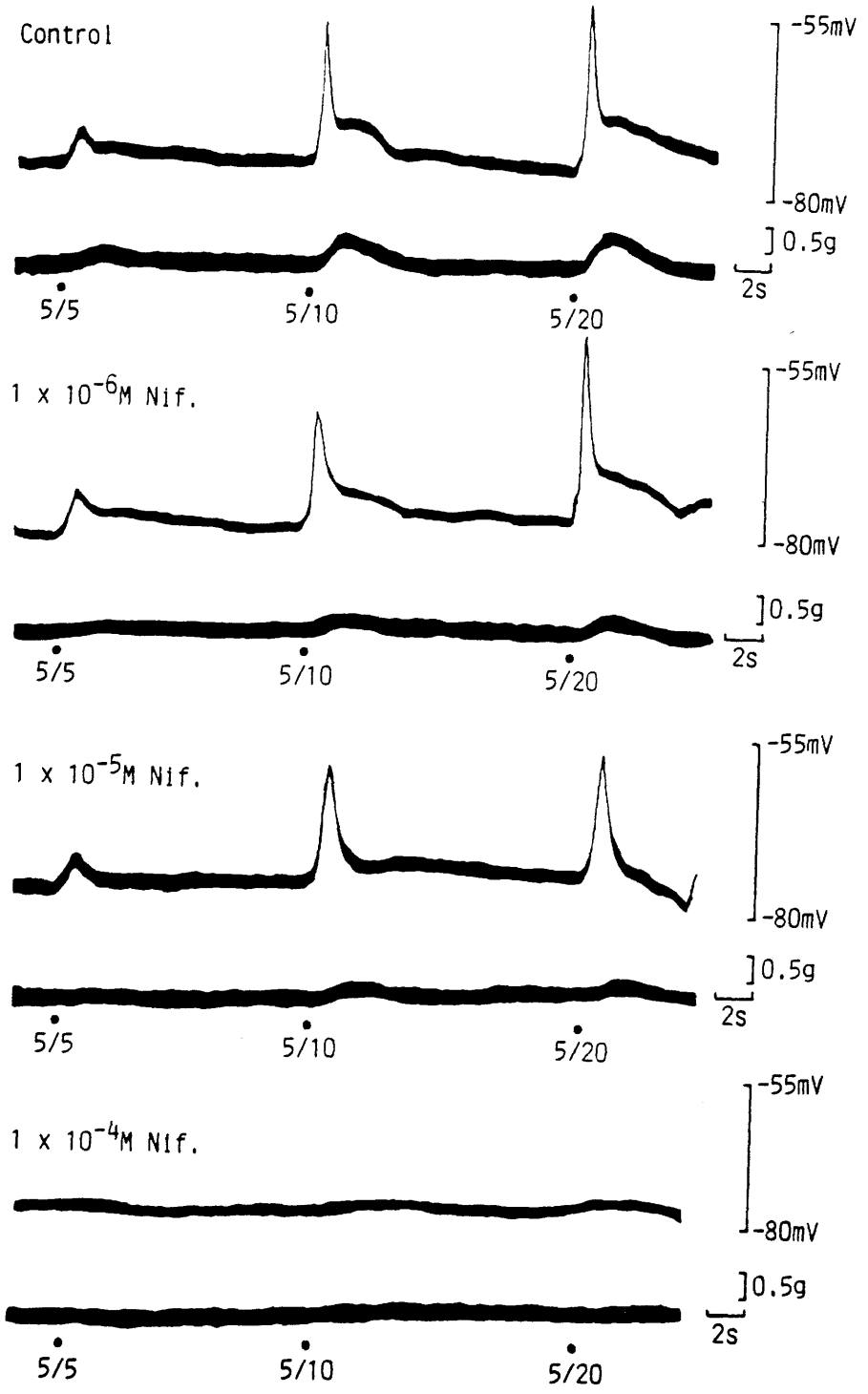
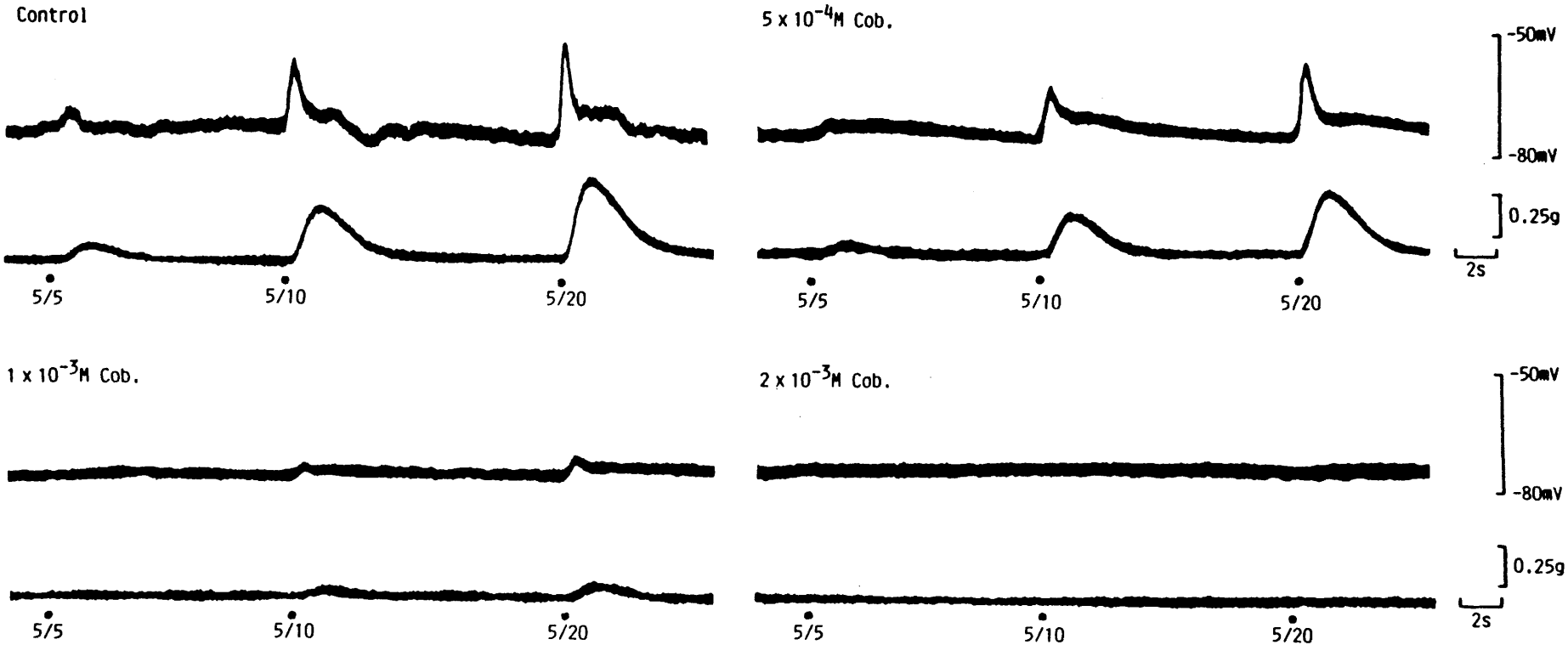

Control
-55mV
-80mV
0.5g
2s
5/5
5/10
5/20
1 x 10-6M Nif.
-55mV
-80mV
0.5g
2s
5/5
5/10
5/20
1 x 10-5M Nif.
-55mV
-80mV
0.5g
2s
5/5
5/10
5/20
1 x 10-4M Nif.
-55mV
-80mV
0.5g
2s
5/5
5/10
5/20

Fig. 57: The effect of cobalt (Cob., 5×10^{-4} - 2×10^{-3}M) on intracellularly-recorded e.j.ps (upper trace of each set) and accompanying contractions in the rat anococcygeus, in response to extrinsic nerve stimulation (5 pulses at 5, 10 and 20 Hz, 0.5 ms, supramaximal voltage). Cobalt was equally effective at inhibiting both e.j.ps and contractions. The time between each set was approximately 15 min.


Control
5 x 10⁻⁴M Cob.
-50mV
-80mV
0.25g
2s
5/5
5/10
5/20
1 x 10⁻³M Cob.
2 x 10⁻³M Cob.
-50mV
-80mV
0.25g
2s
5/5
5/10
5/20

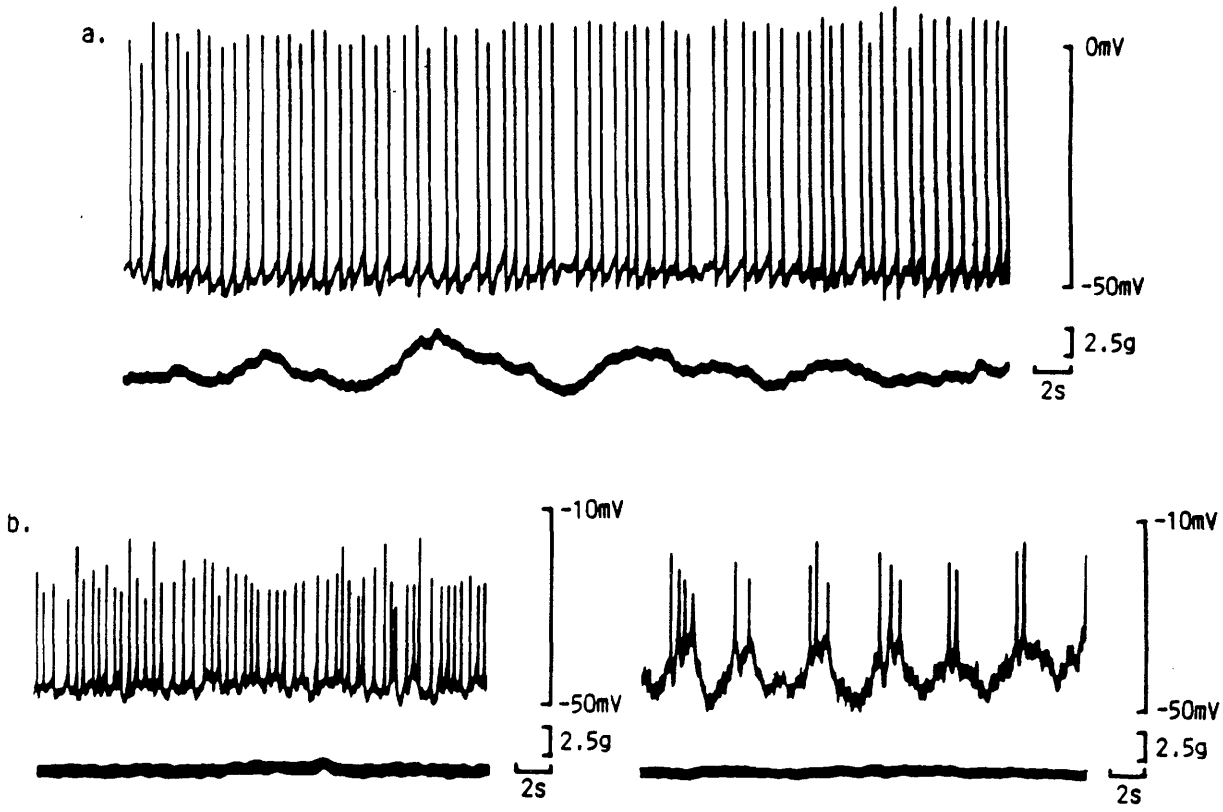


Fig. 58: Spontaneous electrical (upper trace of each set) and accompanying mechanical activity in the guinea-pig i.a.s. a. Large spikes (seen in 40% of cells) of some 60 mV in amplitude preceded by a slow depolarization and accompanied by an after-hyperpolarization and oscillations in tone. b. Smaller spikes (seen in 60% of cells), either continuous (left hand trace) or intermittent, of 30-40 mV in amplitude, often superimposed on a membrane depolarization.

between spontaneous spikes, was -43.5 ± 0.3 mV (n = 135 cells).

In the presence of tone, field stimulation (single pulses and trains of 5 pulses at 5 and 10 Hz, 0.5 ms, supramaximal voltage) inhibited spike discharge, hyperpolarized the membrane producing an inhibitory junction potential (i.j.p.) and relaxed tone (Fig. 59). Single stimuli produced i.j.ps of up to 30 mV in amplitude (16.4 ± 0.8 mV, n = 50) with a mean latency of 112.0 ± 5.1 ms (n = 20), a duration of 815.0 ± 40.2 ms (n = 20) and a time to peak of 231.2 ± 12.7 ms (n = 20). Upon stimulation with 5 pulses at 5 and 10 Hz (0.5 ms, supramaximal voltage), the i.j.ps summated. A post-stimulus increase in spike frequency and a rebound contraction, common characteristics of the responses to NANC nerve stimulation (Bennett et al., 1966; Furness, 1969), were often observed. I.j.ps were not significantly affected by hexamethonium (1 x 10^{-6}M), but were abolished by TTX (1 x 10^{-6}M), confirming their postganglionic neural origin. The i.j.ps were mediated by NANC nerves; they were unaffected by atropine, phentolamine and guanethidine (each 1 x 10^{-6}M), perfused in the Krebs solution throughout each experiment.

Perfusion of the i.a.s. with either verapamil (0.01 - 1 x 10^{-4}M), diltiazem (0.05 - 5 x 10^{-4}M), nifedipine (0.01 - 5 x 10^{-4}M) or cobalt (1 x 10^{-3}M), inhibited both spontaneous and evoked electrical and mechanical responses. The organic agents, in low doses (1 - 5 x 10^{-6}M), each blocked both the spontaneous spikes and tone, but had no inhibitory effect on the i.j.ps, except at higher concentrations (1 - 5 x 10^{-4}M,

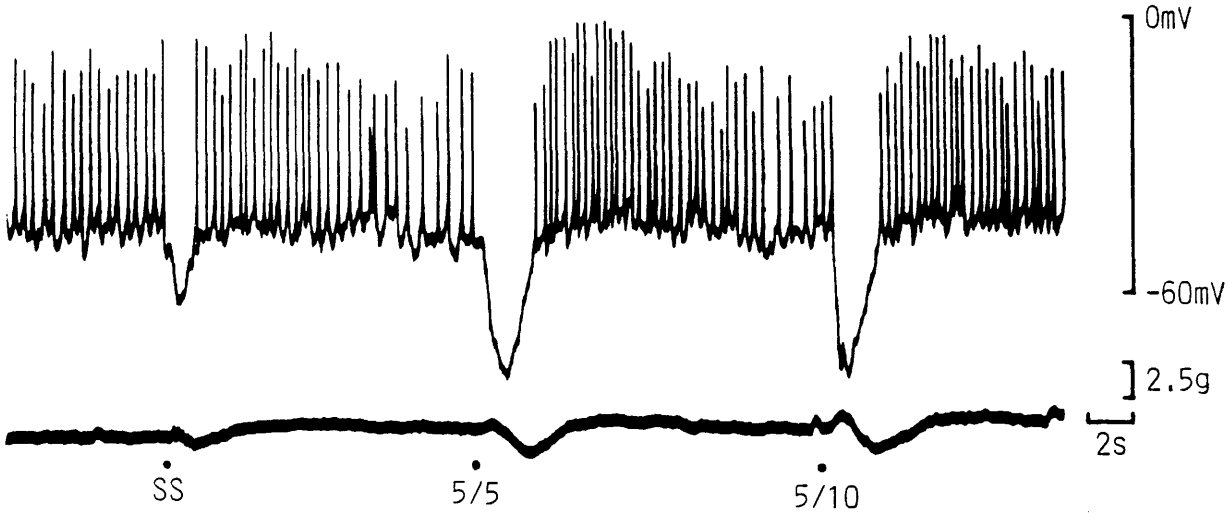


Fig. 59: Intracellular electrical (upper trace) and accompanying mechanical responses in the guinea-pig i.a.s., evoked by field stimulation (single pulse(ss) and 5 pulses at 5 and 10 Hz, 0.5 ms, supramaximal voltage) of NANC inhibitory nerves. Atropine, phentolamine and guanethidine (each 1 x 10^{-6}M) were present throughout. Field stimulation inhibited the spontaneous spikes, hyperpolarized the membrane to give an i.j.p. and relaxed the tone.

Figs. 60 and 61). Cobalt (1×10^{-3}M) inhibited, with equal effectiveness, both electrical and mechanical responses in the i.a.s. (Fig. 62).

(ii) Radiolabelled transmitter release

The i.a.s. was incubated with [^{3}H] -adenosine and the effects of the drugs on the overflow determined. If, as has been suggested (Lim, 1985; Lim & Muir, 1986), ATP is the NANC transmitter in this tissue, the nerves should accumulate and release this compound or its metabolites (Su et al., 1971).

In the absence of stimulation, the overflow of ^{3}H from the i.a.s. declined exponentially. Field stimulation (200-900 pulses at 2-20 Hz, 0.5 ms, supramaximal voltage) and drug additions were carried out 86 min after commencing superfusion, when ^{3}H overflow had reached a steady level. Field stimulation, in the presence of atropine, phentolamine and guanethidine (each 1×10^{-6}M), increased ^{3}H overflow from the i.a.s. and produced a relaxation. TTX (1×10^{-6}M) inhibited these responses, indicating their mediation by nerves (Fig. 63).

Two additional experiments were carried out to confirm that the stimulation-evoked overflow of ^{3}H arose from the NANC nerves, rather than from the relaxation of the muscle per se. First, Ca^{2+}, necessary for transmitter release (Katz & Miledi, 1965), was withdrawn from the superfusing Krebs solution, without compensation. This increased the resting ^{3}H overflow substantially and abolished tone (Fig. 64). Field stimulation (900 pulses at 2 and 5 Hz, 0.5 ms, supramaximal voltage) now

Fig. 60: The effect of verapamil (1×10^{-6} and 1×10^{-4}M) on the intracellular electrical (upper trace in each set) and accompanying mechanical responses in the guinea-pig i.a.s., evoked by field stimulation (single pulse (ss) and 5 pulses at 5 and 10 Hz, 0.5 ms, supramaximal voltage) of NANC inhibitory nerves. Atropine, phentolamine and guanethidine (each 1×10^{-6}M) were present throughout. Verapamil (1×10^{-6}M) inhibited the spontaneous spike discharge and reduced tone (as indicated by the arrow), but had no effect on the i.j.ps. When the concentration was increased to 1×10^{-4}M, the i.j.ps were abolished. The time between each set was approximately 15 min.

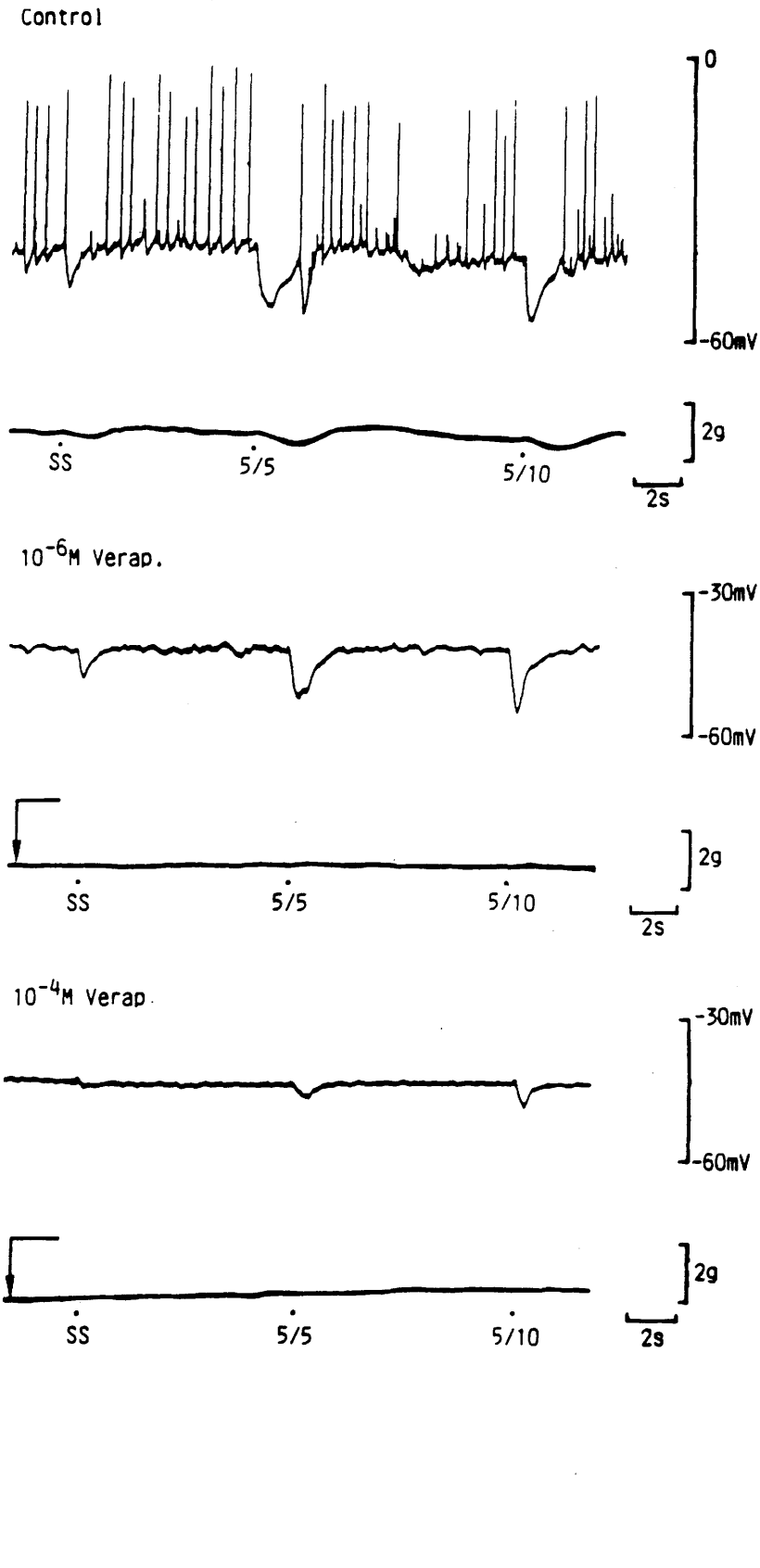
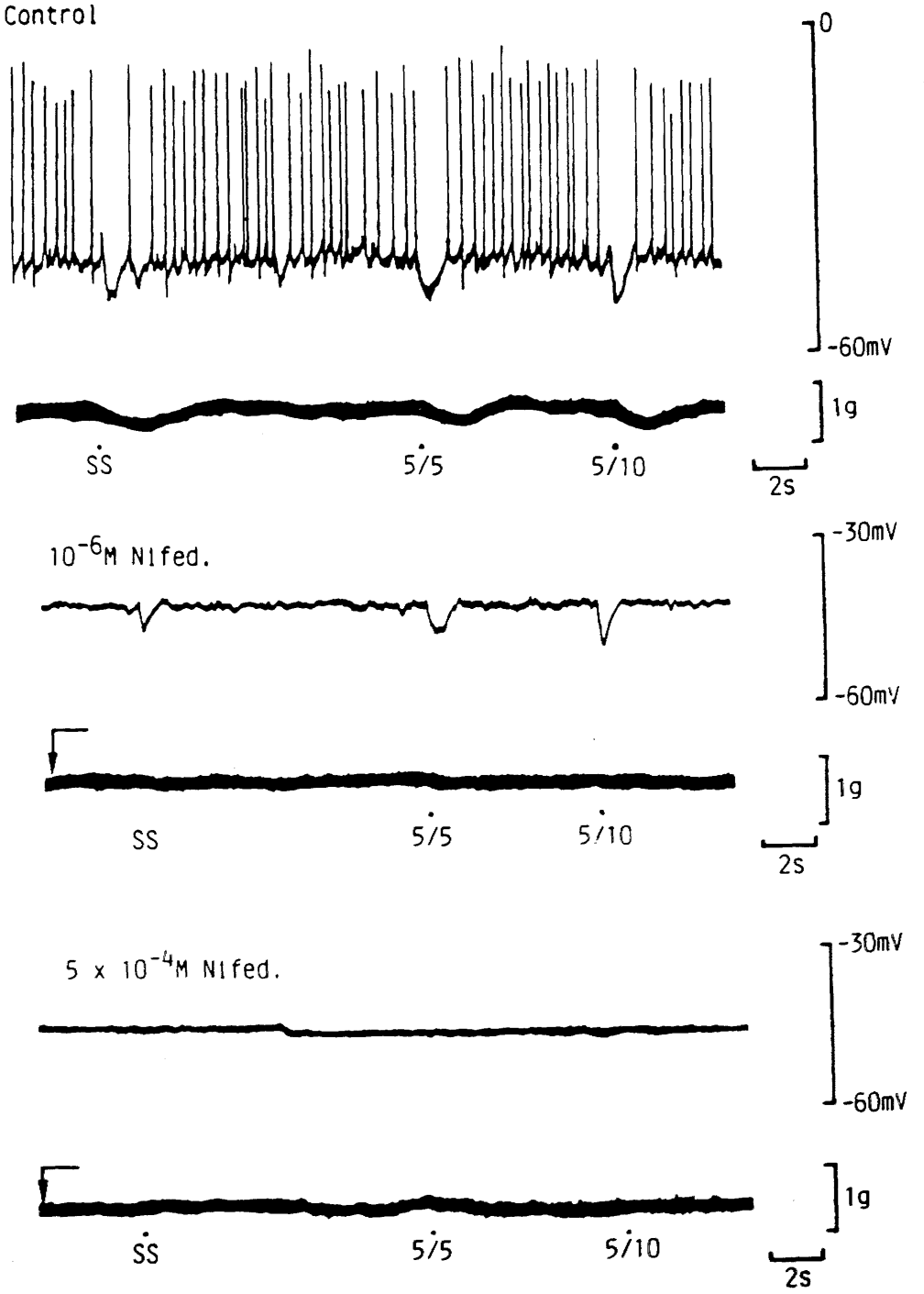

Control
0
-60mV
2g
SS
5/5
5/10
2s
10-6M Verap.
-30mV
-60mV
2g
SS
5/5
5/10
2s
10-4M Verap.
-30mV
-60mV
2g
SS
5/5
5/10
2s

Fig. 61: The effect of nifedipine (1×10^{-6} and 5×10^{-4}M) on the intracellular electrical (upper trace in each set) and accompanying mechanical responses in the guinea-pig i.a.s., evoked by field stimulation (single pulse (ss) and 5 pulses at 5 and 10 Hz, 0.5 ms, supramaximal voltage) of NANC inhibitory nerves. Atropine, phentolamine and guanethidine (each 1×10^{-6}M) were present throughout. Nifedipine (1×10^{-6}M) inhibited the spontaneous spike discharge and reduced tone (as indicated by the arrow) but had no effect on the i.j.ps. When the concentration was increased to 5×10^{-4}M, the i.j.ps were abolished. The time between each set was approximately 15 min.


Control
0
-60mV
1g
SS
5/5
5/10
2s
10^{-6}M Nifed.
-30mV
-60mV
1g
SS
5/5
5/10
2s
5 x 10^{-4}M Nifed.
-30mV
-60mV
1g
SS
5/5
5/10
2s

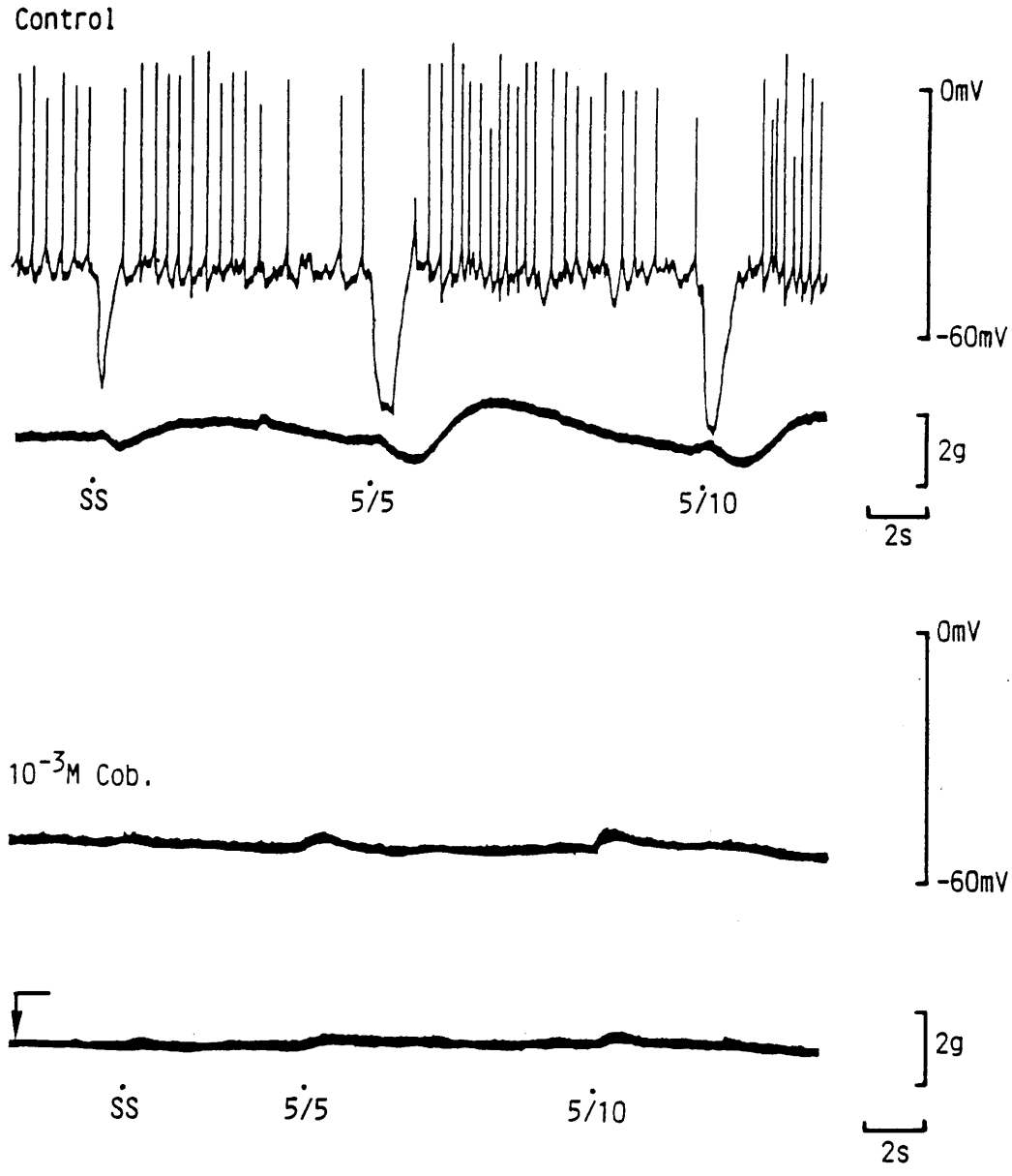


Fig. 62: The effect of cobalt (1 x 10^{-3}M) on the intracellular electrical (upper trace of each set) and accompanying mechanical responses of the guinea-pig i.a.s., evoked by field stimulation (single pulse (ss) and 5 pulses at 5 and 10 Hz, 0.5 ms, supramaximal voltage) of NANC inhibitory nerves. Atropine, phentolamine and guanethidine (each 1 x 10^{-6}M) were present throughout. Cobalt inhibited the spontaneous spike discharge and abolished both the i.j.ps and tone. The time between the two sets was 15 min.

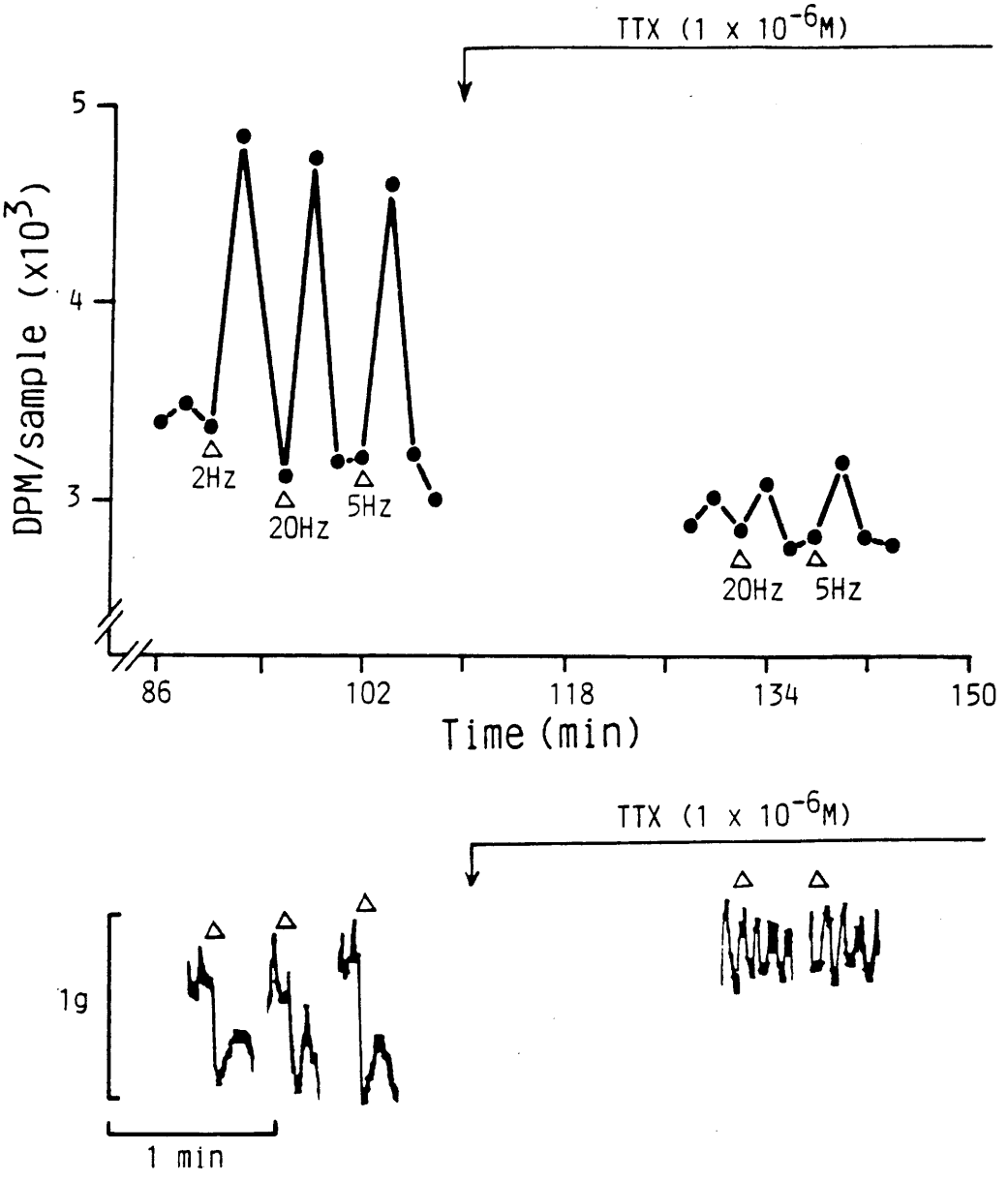
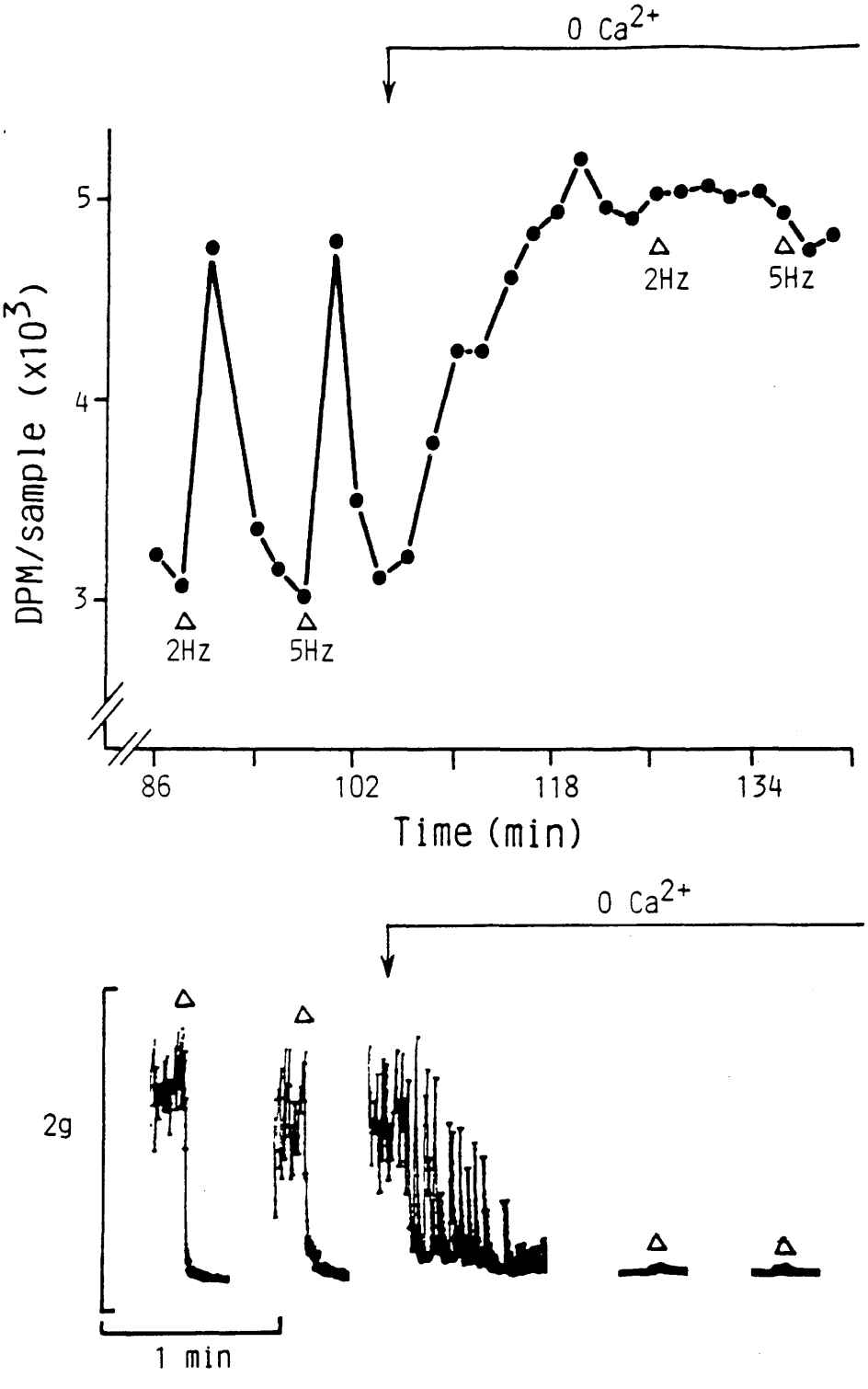


Fig. 63: ^{3}H overflow (upper graph) and simultaneously-recorded mechanical relaxations (lower trace) of the guinea-pig i.a.s., evoked by field stimulation (900 pulses at 2, 5 and 20 Hz, 0.5 ms, supramaximal voltage), in the absence and presence of TTX (1 x 10^{-6}M for the period indicated by the horizontal bar). The mechanical relaxations produced by field stimulation were accompanied by an increase in ^{3}H overflow. Both were inhibited by TTX indicating their mediation by nerves. Phentolamine, guanethidine and atropine (each 1 x 10^{-6}M) were present throughout.

Fig. 64: The effect of Ca^{2+}-withdrawal from the superfusing Krebs solution (for the period indicated by the horizontal bar) on the ^{3}H overflow (upper graph) and simultaneously-recorded mechanical relaxations (lower trace) of the guinea-pig i.a.s., evoked by field stimulation (900 pulses at 2 and 5 Hz, 0.5 ms, supramaximal voltage). Removal of Ca^{2+} increased the resting ^{3}H overflow and abolished tone. Field stimulation now had no effect on either ^{3}H overflow or mechanical activity. Phentolamine, guanethidine and atropine (each 1 x 10^{-6}M) were present throughout.


0 Ca2+
DPM/sample (x10^3)
5
4
3
2Hz
5Hz
2Hz
5Hz
86
102
118
134
Time (min)
0 Ca2+
2g
1 min

produced neither an increase in ^{3}H overflow nor a relaxation. The rise in resting ^{3}H overflow has also been seen following Ca^{2+}-withdrawal from other autonomically-innervated tissues (e.g. canine saphenous vein; Takata & Kato, 1984) and attributed to autonomic nerves (Boullin, 1967). It may be due to a decrease in stability of the neuronal membrane or to an inhibition of the NANC transmitter uptake-1 mechanism.

Secondly, sodium nitroprusside (SNP, 1 x 10^{-4}M), which relaxes smooth muscle independently of nerve activity, probably via an increase in cGMP (Bowman & Drummond, 1984), reduced the tone of the i.a.s. without an accompanying increase in ^{3}H overflow (Fig. 65).

As a consequence of the inhibitory effects of TTX (1 x 10^{-6}M) and Ca^{2+}-withdrawal on the stimulation-evoked increase in ^{3}H overflow and the failure of direct muscle relaxation by SNP (1 x 10^{-4}M) to affect the resting overflow, it is likely that the ^{3}H arose from nerves. These nerves were NANC; the influence of the other, sympathetic, innervation in the i.a.s. was prevented by guanethidine (1 x 10^{-6}M).

Verapamil was added to study the effects of Ca^{2+} channel antagonists on ^{3}H overflow. Verapamil (1 x 10^{-6}M) abolished tone, but had no effect on the evoked ^{3}H overflow (Fig. 66). At a higher concentration (1 x 10^{-4}M), the resting ^{3}H overflow was unaffected, while that evoked by stimulation was inhibited (Fig. 67). Thus, verapamil preferentially blocked the muscle response in comparison to transmitter release, as it did in the other tissues.

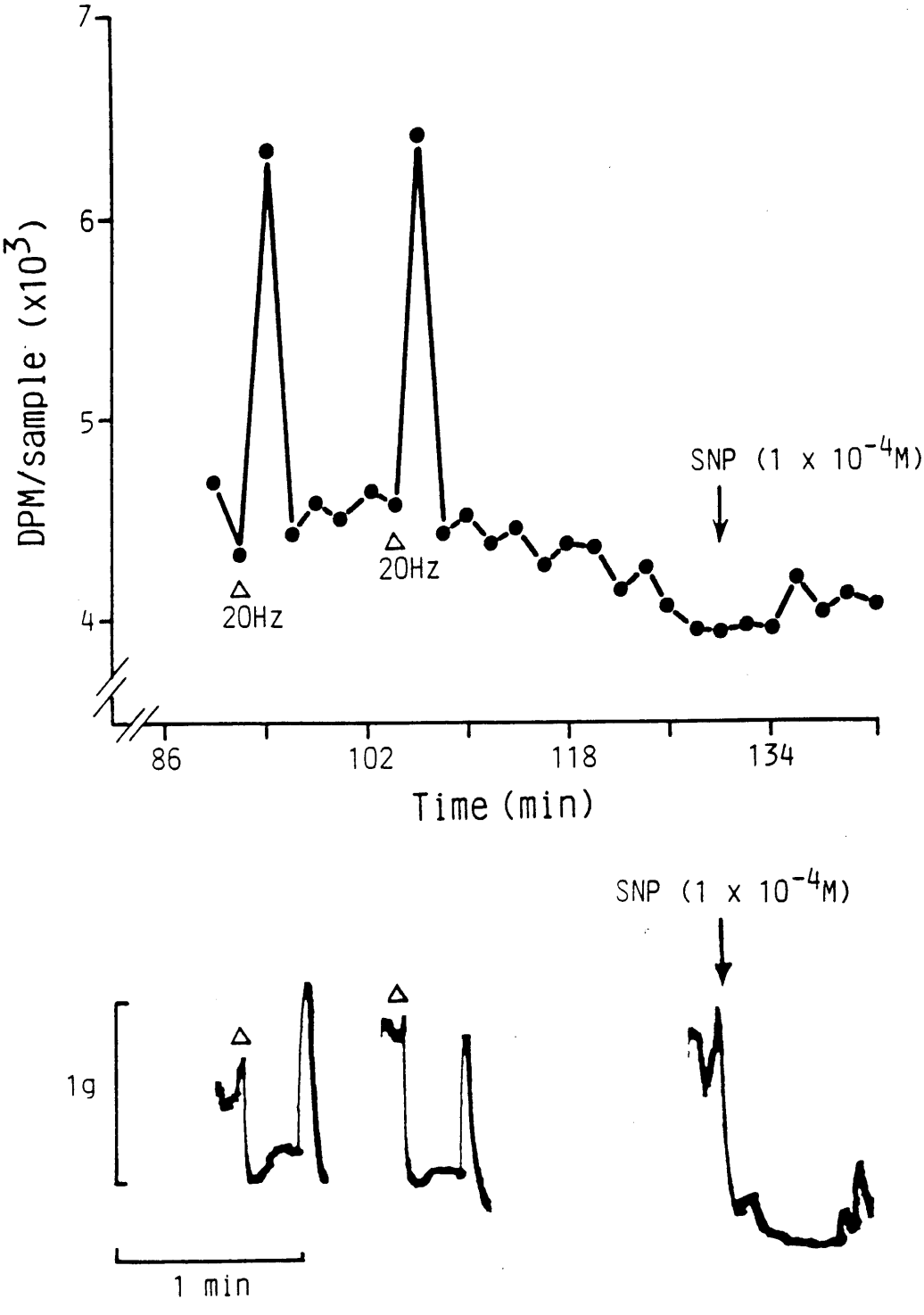


Fig. 65: ^{3}H overflow (upper graph) and simultaneously-recorded mechanical relaxations (lower trace) of the guinea-pig i.a.s., in response to field stimulation (900 pulses at 20 Hz, 0.5 ms, supramaximal voltage) and to addition of sodium nitroprusside (SNP, 1 x 10^{-4}M). Sodium nitroprusside, unlike field stimulation, relaxed the tissue without affecting ^{3}H overflow. Phentolamine, guanethidine and atropine (each 1 x 10^{-6}M) were present throughout.

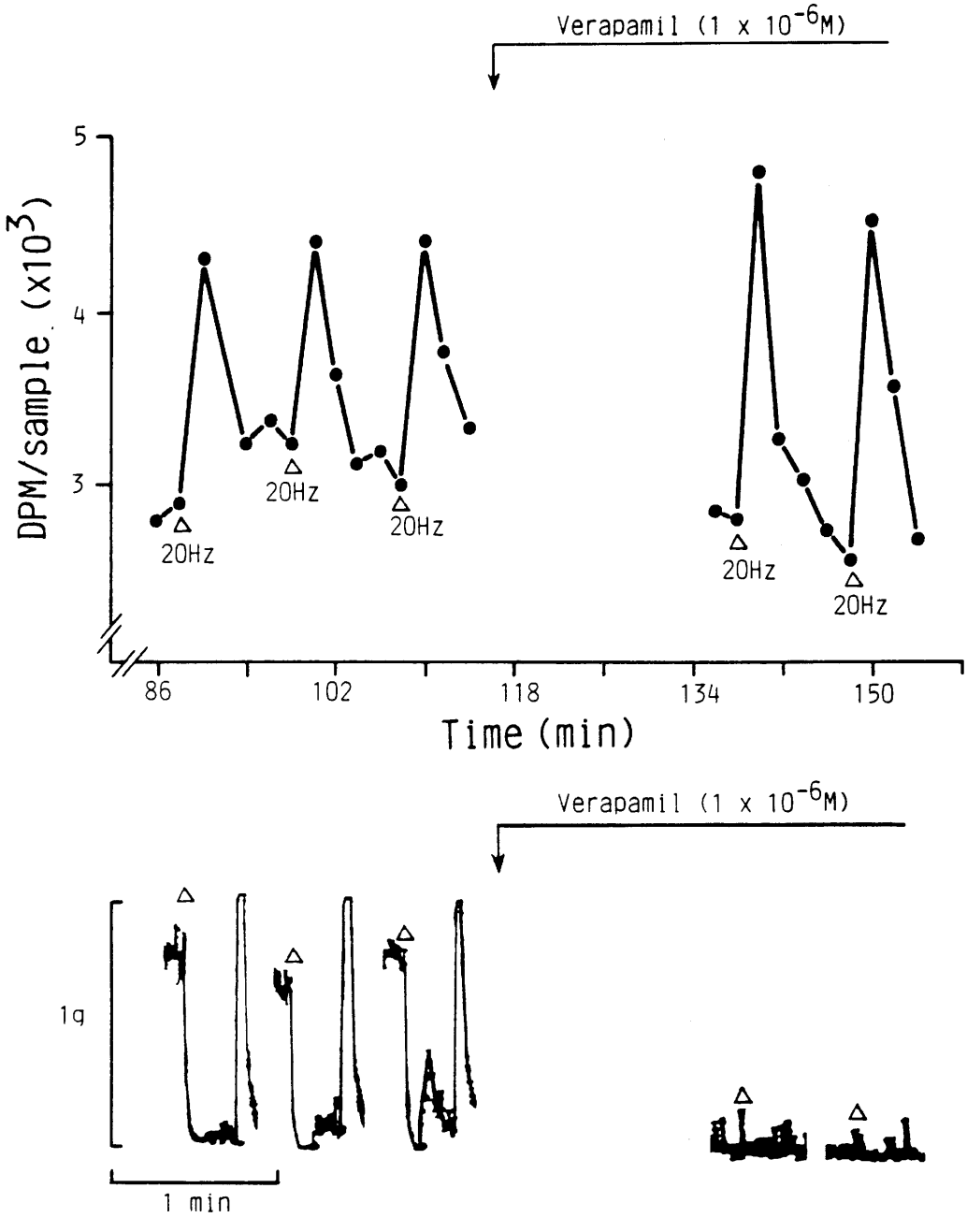
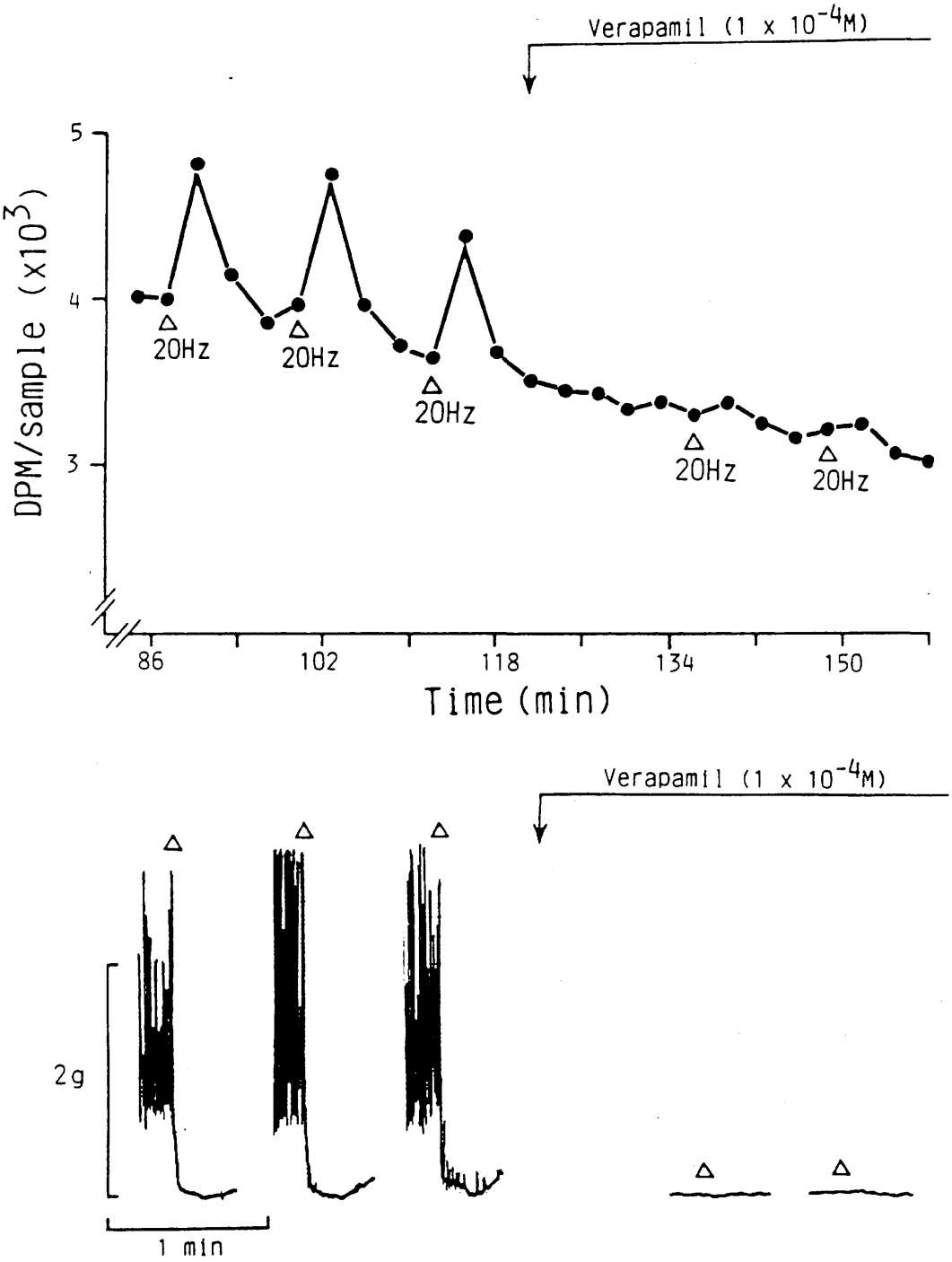


Fig. 66: The effect of verapamil (1 x 10^{-6}M for the period indicated by the horizontal bar) on the ^{3}H overflow (upper graph) and simultaneously-recorded mechanical relaxations (lower trace) of the guinea-pig i.a.s., in response to field stimulation (900 pulses at 20 Hz, 0.5 ms, supramaximal voltage). Verapamil abolished the tone of the i.a.s., but had no inhibitory effect on the ^{3}H overflow evoked by field stimulation. Phentolamine, guanethidine and atropine (each 1 x 10^{-6}M) were present throughout.

Fig. 67: The effect of verapamil (1×10^{-4}M) for the period indicated by the horizontal bar) on the ^{3}H overflow (upper graph) and simultaneously-recorded mechanical relaxations (lower trace) of the guinea-pig i.a.s., in response to field stimulation (900 pulses at 20 Hz, 0.5 ms, supramaximal voltage). Verapamil abolished the tone of the i.a.s. and inhibited the ^{3}H overflow evoked by field stimulation. The resting ^{3}H overflow was unaffected by verapamil. Phentolamine, guanethidine and atropine (each 1×10^{-6}M) were present throughout.


Verapamil (1 x 10^{-4}M)
DPM/sample (x10^3)
5
4
3
20Hz
20Hz
20Hz
20Hz
20Hz
86
102
118
134
150
Time (min)
Verapamil (1 x 10^{-4}M)
2g
1 min

The problem of the nature of the substance(s) released by stimulation remained. To separate and identify the $[^3H]$ - compounds from the i.a.s., chromatography was used. The major constituents in the resting overflow were breakdown products of ATP; hypoxanthine, cAMP, AMP and adenosine and inosine (Fig. 68a). Field stimulation (2400 pulses at 20 Hz, 0.5 ms, supramaximal voltage) released mainly hypoxanthine, AMP and cAMP (Fig. 68b), while most of the ^3H in the tissue, itself, was incorporated into the nucleotides, ATP and ADP (Fig. 68c).

ATP AS A CANDIDATE FOR THE NANC TRANSMITTER IN THE GUINEA-PIG INTERNAL ANAL SPHINCTER

The presence of breakdown products of ATP in the overflow following field stimulation, was consistent with the view (Lim & Muir, 1986), that in the i.a.s., ATP is the NANC transmitter. In an attempt to detect ATP release from the NANC nerves, two further investigations were carried out.

(i) Analysis by thin layer chromatography

Even in the presence of the ATPase inhibitor, sodium vanadate (1×10^{-4}M), thin layer chromatography failed to detect any ATP or its metabolites in samples of the superfused i.a.s., collected either at rest or following field stimulation (1000 pulses at 10 Hz, 0.1 - 0.5 ms, supramaximal voltage). It is likely that the sensitivity of this procedure was too low; the standards, ATP, ADP, AMP, adenosine and inosine were only detected at concentrations above 1×10^{-9}M.

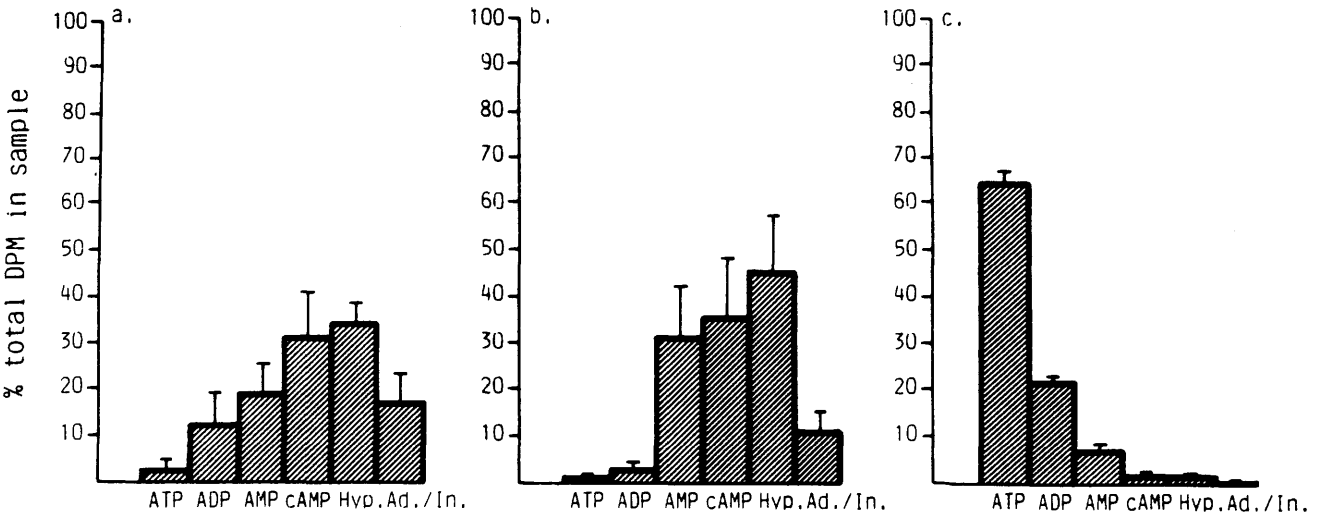


Fig. 68: The relative amounts (± s.e. mean, n = 4) of $[^3H]$ - adenosine 5'-triphosphate ($[^3H]$ -ATP) and its metabolites, each expressed as a percentage of the total DPM/sample, of a. the ^3H overflow at rest, b. following field stimulation (2400 pulses at 20 Hz, 0.5 ms, supramaximal voltage) and c. the ^3H content of the guinea-pig i.a.s. following homogenisation. At rest, the major constituents were hypoxanthine (Hyp.), adenosine 3',5'-cyclic monophosphate (cAMP), adenosine 5'-monophosphate (AMP) and adenosine and inosine (Ad./In.). Field stimulation released mainly hypoxanthine (Hyp.), AMP and cAMP, while the ^3H content of the i.a.s. consisted principally of ATP and adenosine 5'-diphosphate (ADP).

(ii) Luciferase-luciferin luminescence

This technique was a sensitive assay for ATP; as little as 6×10^{-19} moles of ATP could be detected (Fig. 69). Despite this sensitivity, NANC nerve stimulation (100 - 1000 pulses at 10 and 20 Hz, 0.2 ms, supramaximal voltage), while relaxing the i.a.s., failed to release a measurable amount of ATP. Addition of sodium vanadate (1×10^{-4}M) had no effect (Fig. 70).

This failure to detect ATP may have been due to either a lack of sensitivity of the technique, the metabolism of released ATP or, alternatively, may indicate that ATP is not the NANC transmitter. To investigate these alternatives, the luciferase-luciferin technique was applied to the guinea-pig vas deferens, as in this tissue, ATP is likely to be a transmitter (Burnstock & Sneddon, 1984; Sneddon & Westfall, 1984).

Field stimulation (200 pulses at 10 Hz, 0.2 ms, supramaximal voltage) of the guinea-pig vas deferens produced a TTX (2×10^{-6}M)-sensitive contraction and an accompanying increase in ATP release (Fig. 71). To inhibit contractions of the vas deferens, which contribute to the release of ATP (Westfall et al., 1978), a hypertonic Krebs solution containing sucrose (0.37M) was superfused. Field stimulation (200 pulses at 10 Hz, 0.2 ms, supramaximal voltage) in the presence of sucrose, when there were no contractions, failed to release any ATP (Fig. 72). It is likely, therefore, that in the absence of sucrose, ATP was released as a consequence of the muscle contraction rather than from nerves. In an attempt to detect neuronally-released ATP in the vas deferens, the ATPase inhibitor, sodium vanadate

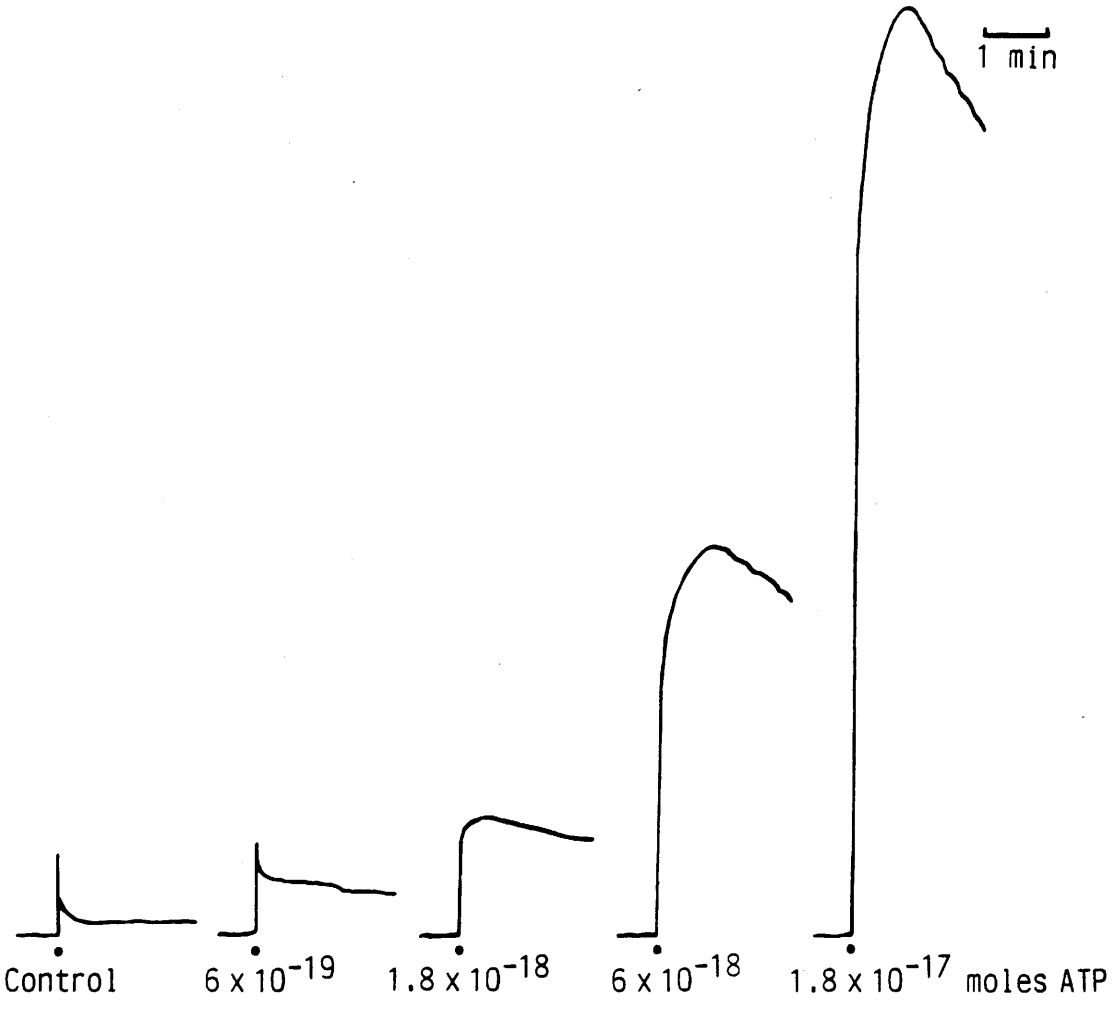


Fig. 69: The degree of luciferase-luciferin luminescence detected in samples (0.5 ml) of ATP (6×10^{-19} - 1.8×10^{-17} moles, dissolved in Krebs solution). Compared to the control (0.5 ml Krebs), ATP increased the amount of luminescence in a concentration-dependent manner.

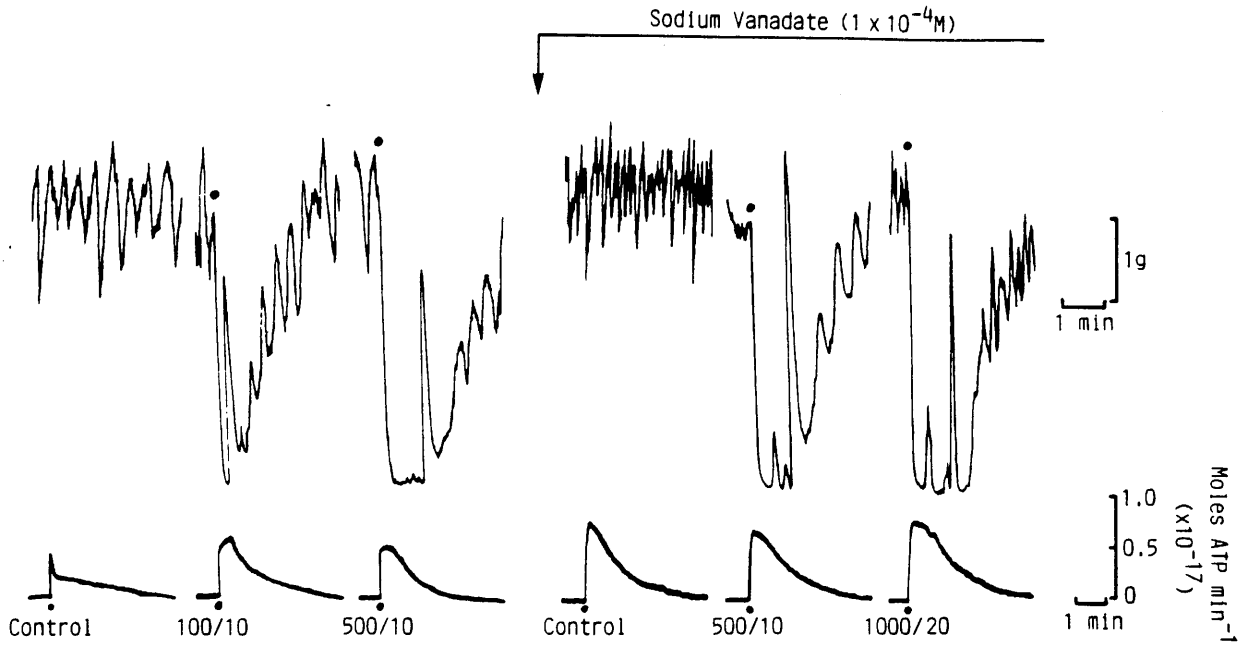


Fig. 70: The effect of field stimulation (100 and 500 pulses at 10 Hz and 1000 pulses at 20 Hz, 0.2 ms, supramaximal voltage) of the superfused guinea-pig i.a.s., on the mechanical activity (upper trace) and accompanying ATP release, in the absence and presence of the ATPase inhibitor, sodium vanadate (1 x 10^{-4}M for the period indicated by the horizontal bar). Field stimulation, before and during addition of sodium vanadate, relaxed the i.a.s., but failed to produce any release of ATP. Samples from the superfused i.a.s. (0.5 ml), collected prior to stimulation, served as controls.

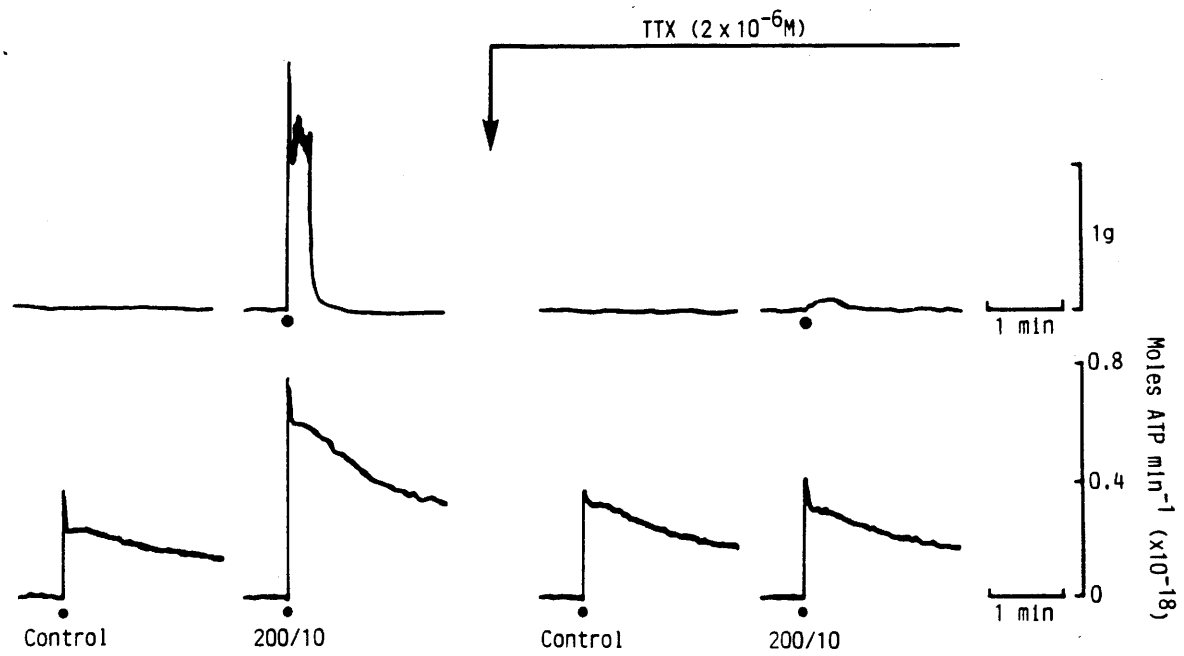


Fig. 71: The effect of field stimulation (200 pulses at 10 Hz, 0.2 ms, supramaximal voltage) of the superfused guinea-pig vas deferens, on the mechanical activity (upper trace) and accompanying ATP release, in the absence and presence of TTX (2 x 10^{-6}M for the period indicated by the horizontal bar). Field stimulation produced a contraction and a release of ATP, both of which were inhibited by TTX. Samples from the superfused vas deferens (0.5 ml), collected prior to stimulation, served as controls.

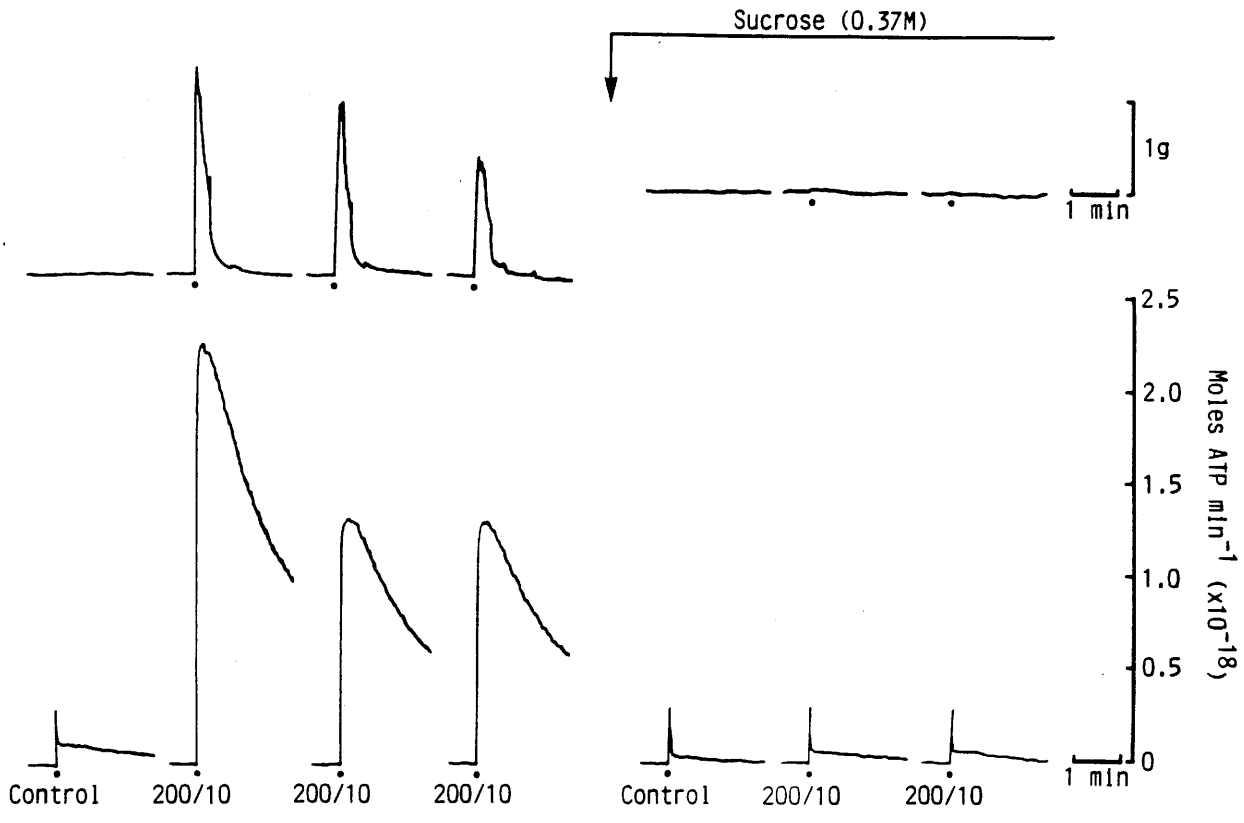


Fig. 72: The effect of field stimulation (200 pulses at 10 Hz, 0.2 ms, supramaximal voltage) of the superfused guinea-pig vas deferens, on the mechanical activity (upper trace) and accompanying ATP release, in the absence and presence of sucrose (0.37M for the period indicated by the horizontal bar) to inhibit, selectively, the contractions. In the absence of sucrose, field stimulation contracted the vas deferens and released ATP. In the presence of sucrose, muscle tone increased, while contractions and ATP release, in response to field stimulation, were prevented. Samples from the superfused vas deferens (0.5 ml), collected prior to stimulation, served as controls.

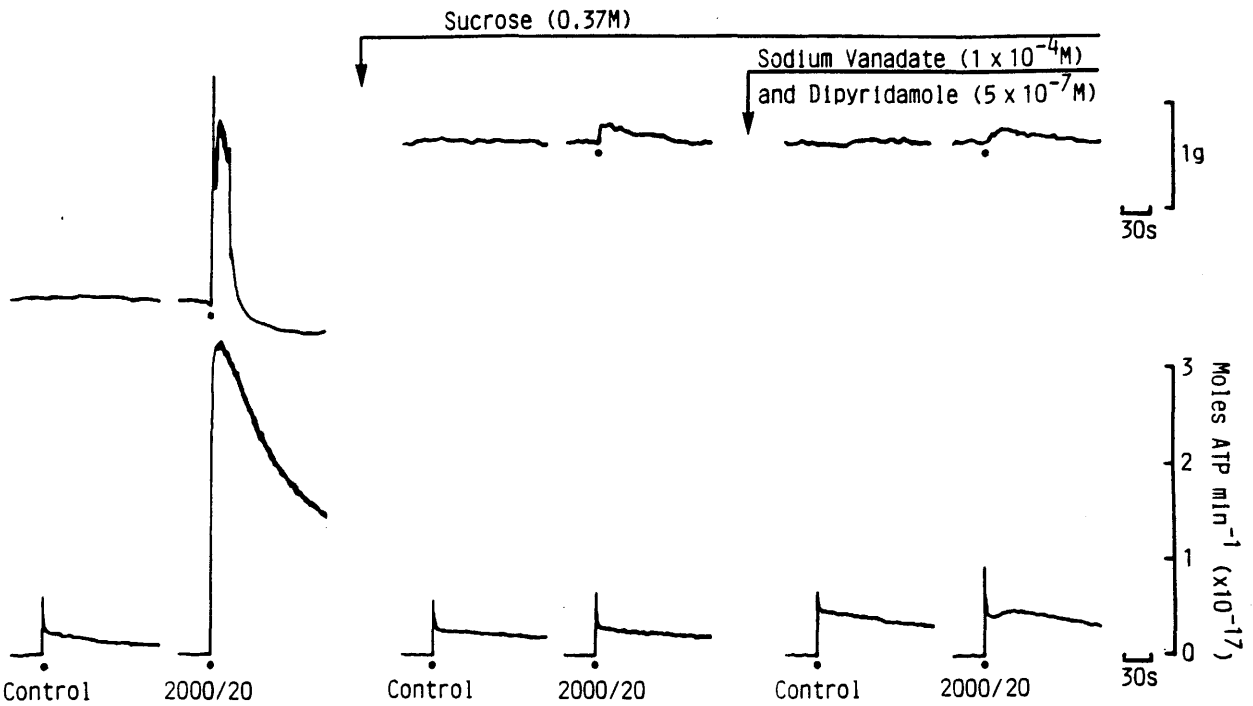


Fig. 73: The effect of field stimulation (2000 pulses at 20 Hz, 0.2 ms, supramaximal voltage) of the superfused guinea-pig vas deferens, on the mechanical activity (upper trace) and accompanying ATP release. Sucrose (0.37M) was added to block contractions, sodium vanadate (1×10^{-4}M) to inhibit ATPase and dipyridamole (5×10^{-7}M) to prevent adenosine uptake. In the absence of drugs, field stimulation contracted the vas deferens and released ATP. In the presence of sucrose, either alone, or together with sodium vanadate and dipyridamole, muscle tone increased, while contractions and ATP release, in response to field stimulation, were prevented. Samples from the superfused vas deferens (0.5 ml), collected prior to stimulation, served as controls.

(1×10^{-4}M) and adenosine uptake blocker, dipyridamole (5×10^{-7}M), were superfused in the presence of sucrose (0.37M). It was hoped that these compounds, by preventing the disappearance of ATP, might allow its detection. Field stimulation (2000 pulses at 20 Hz, 0.2 ms, supramaximal voltage), however, in the presence of sodium vanadate, dipyridamole and sucrose failed to produce a detectable release of ATP (Fig. 73).

CHAPTER 4: DISCUSSION

The methods used in this investigation have clearly demonstrated that Ca^{2+} channel antagonists possess prejunctional activity in autonomically-innervated smooth muscle. The use of nerve AP conduction, radiolabelled transmitter overflow and junction potentials in smooth muscle are accepted measures of prejunctional activity (Andersson et al., 1983; Bennett, 1973; Cunnane & Stjärne, 1984), although, as with all experimental methods, each has its limitations. In this study, APs were unavoidably measured from preterminal, rather than terminal regions of nerves, due to the latter's inaccessibility. The radiolabelled transmitter measured, may have included both neuronally- and extraneuronally-derived material, consisting of metabolites, as well as the transmitter itself. Measures were taken to overcome these problems. Sucrose, for example, was used to paralyse selectively the muscle response in radioisotope experiments (Westfall et al., 1978), thereby minimizing its contribution to the overflow. TTX was used to inhibit and thus confirm the extent of neuronal involvement. Inhibitors of metabolism and uptake were used to reduce transmitter loss, and chromatographic techniques to identify the compound(s) collected.

The use of junction potentials as a measure of transmitter release is complicated by the contribution of postjunctional events. Clearly, any change in membrane potential of the smooth muscle will affect the amplitude of evoked potentials, as, for example, in the case of isoprenaline-induced enhancement of spontaneous m.e.p.ps at the skeletal neuromuscular junction

(Kuba, 1970). In the present study, significant postjunctional involvement was unlikely. During blockade of either e.j.ps or i.j.ps by the organic Ca^{2+} channel antagonists, the ability of the postjunctional membrane to respond, electrically, to either noradrenaline or ATP was unimpaired.

The present investigation has clarified the principal prejunctional sites at which Ca^{2+} channel antagonists act. The organic compounds probably inhibited transmitter release by preventing invasion of the nerve terminals by the AP, rather than by interfering with the secretory process itself. In support of this view, similar concentrations of the drugs were required to prevent transmitter release and AP conduction in each tissue, while compounds ineffective on release, also failed to inhibit the APs, e.g. nifedipine in the vas deferens.

AP blockade may have arisen from an inhibition of Na^{+} and/or Ca^{2+} conductance. The ability of the antagonists to inhibit, at high concentrations, AP conduction was reminiscent of local anaesthetics, with which they have been compared (Bayer et al., 1975; Hay & Wadsworth, 1982; Nagao et al., 1972). Not surprisingly, therefore, the AP in the hypogastric nerve was blocked by TTX, but not by nifedipine, which lacks local anaesthetic activity (Hay & Wadsworth, 1982). Moreover, cobalt, at the high concentrations required to inhibit APs in the hypogastric nerve, antagonises Na^{+}, in addition to Ca^{2+} influx (Edwards, 1982).

A Ca^{2+}, as well as Na^{+} component, sensitive to the antagonists may, however, also be present in the AP. $[Ca^{2+}]_o$-withdrawal in the hypogastric nerve reduced, reversibly, AP

amplitude (seen also by Cunnane & Stjärne, 1984). Despite their sensitivity to TTX, the APs in the genito-femoral nerve were inhibited, not only by nifedipine, which has no local anaesthetic activity (Hay & Wadsworth, 1982), but also by concentrations of cobalt close to those proposed to block Ca^{2+} channels selectively (Edwards, 1982). The existence of Na^{+} and Ca^{2+} components in the AP occurs in, for example, the postganglionic neurones of the rat superior cervical ganglion (McAfee & Yarowsky, 1979) and the axons of the Helix (Akaike et al., 1978) and squid (Hagiwara, 1973). In these tissues, conductance mediated by Na^{+} and Ca^{2+} involves different channels; a Ca^{2+}-, but not Na^{+}- mediated current remains following TTX addition. However, Ca^{2+} channels may allow the influx of Na^{+} (Hurwitz, 1986; Reuter & Scholz, 1977). In consequence, the possibility exists that in this study the Ca^{2+} channel antagonists inhibited AP conduction by preventing both Na^{+} and Ca^{2+} influx through the same channel.

The ineffectiveness of the organic Ca^{2+} channel antagonists at the release site was surprising, since Ca^{2+} influx, which is required for the secretory process (Katz & Miledi, 1965; Rubin, 1970) and occurs through V.O.Cs (Wakade & Wakade, 1982), might have been expected to be sensitive. Difficulty of access to the Ca^{2+} channels at the nerve terminal could have been responsible, but is an unlikely explanation. More likely, the antagonists, themselves, may have had little affinity for the channels. There is no doubt that Ca^{2+} is involved. This was underlined by the ability of cobalt, in contrast to the organic agents, to inhibit transmitter release when preterminal

nerve AP conduction was unaffected. Its site of action in these circumstances is, presumably, the terminal, rather than preterminal nerve regions.

Results from *in vivo* experiments, designed to investigate the potential clinical value of the prejunctional actions of the organic antagonists, in the anaesthetised guinea-pig, were disappointing. It is unlikely that the prejunctional activity of the compounds investigated will be of clinical value. When given i.v. to block nerve AP conduction, each compound produced cardiovascular collapse. This investigation did, however, produce some interesting results. When AP conduction was inhibited, following close-arterial injection of verapamil, preganglionic transmitter release, rather than conduction itself, was prevented. Thus, acetylcholine (i.a.), added during blockade by verapamil, caused AP discharge. Clearly, the effectiveness of verapamil at the preganglionic nerve terminal contrasts with its ineffectiveness at the postganglionic release site. Although in agreement with previous studies which have investigated the effects of the organic agents at either site (Göthert *et al.*, 1979; Ito & Nishi, 1982; O'Connor, 1982; Reinish *et al.*, 1986), the reason why this should be so, is unclear.

Notwithstanding, however, the ability of the organic antagonists to act prejunctionally *in vivo* and *in vitro* is clearly secondary to their affinity for postjunctional sites. Cobalt, in contrast, was equally effective at either site. Thus, the organic compounds, but not cobalt, prevented evoked

contractions, spontaneous muscle spikes and tone in the tissues investigated at doses lower than those required to block nerve AP conduction and transmitter release. This selectivity of the organic Ca^{2+} channel antagonists is well known (Haeusler, 1972; Högestätt et al., 1982; Kajiwara & Casteels, 1983) and suggests that the Ca^{2+} channels in smooth muscle differ from those at the nerve terminal. The nature of this difference is unclear. The proteins surrounding or lying within the channel - the organic antagonist binding sites (Glossmann et al., 1982, 1985) - may be arranged differently at pre- and postjunctional locations. Presumably, the effectiveness of cobalt at each site reflects its ability, not shared by the organic agents, to compete directly with Ca^{2+} for entry through the channel itself (Schramm & Towart, 1985). This apparent heterogeneity in configuration is not restricted to Ca^{2+} channels; the different affinities of neuronal and muscular Na^{+} channels for TTX may, it has been proposed, be due to their structural differences (Baer et al., 1976). Differences in channel structure, moreover, may be analogous to those already accepted to exist between different receptors. Thus, the proteins which comprise the β-adrenergic and M_1 and M_2 muscarinic receptors, despite possessing similar amino acid sequences, are thought to be arranged in different configurations (Kubo et al., 1986). This presumably explains the ability of drugs to act selectively at a particular receptor, e.g. prazosin and yohimbine at α_1- and α_2-adrenoceptors respectively

(Cambridge et al., 1977; Langer, 1977). In consequence, the discovery of an organic compound blocking selectively prejunctional Ca^{2+} channels is a possibility. The therapeutic value of such an antagonist is not in question. It could, by providing a potent means of controlling neuromuscular transmission, benefit the treatment of a variety of disorders, including angina pectoris and hypertension. Although problems restricting drug action to a particular tissue can be foreseen, the clinical promise of a prejunctionally-acting Ca^{2+} channel antagonist should, in itself, be sufficient stimulus for future research. Investigation utilizing compounds chemically-related to the currently available drugs and employing appropriate measures of prejunctional activity, such as electrical and radiochemical analysis of transmitter release, may be the most rewarding.

Apparently quite unrelated to their inhibitory activity, organic Ca^{2+} channel antagonists stimulated catecholamine release from autonomic nerves. Such an action has been reported previously (Takata & Kato, 1984; Wolchinsky & Zsotér, 1985). It may be relevant clinically; elevation of noradrenaline levels could have several, potentially important, consequences. First, noradrenaline could stimulate vascular smooth muscle, attenuating the vasodilator and hypotensive effects of the antagonists. This could be achieved through R.O.Cs, which, unlike the V.O.Cs, are resistant to the organic antagonists (Flaim et al., 1985; Oriowo, 1984). Secondly, noradrenaline could augment the reflex tachycardia which follows the hypotensive effects of some of the drugs (Hof,

1985). In addition, as a consequence of depleting noradrenaline-containing stores, especially in the long-term, Ca^{2+} channel antagonists could detrimentally affect maintenance of sympathetic tone and influence cardiovascular performance. Significantly, elevation of plasma noradrenaline concentration occurs clinically, following administration of nifedipine and nitrendipine (Professor John Reid, personal communication; Ventura et al., 1983). While, in the present investigation, the metabolite DOPEG was released, rather than noradrenaline itself, this compound could have been derived from noradrenaline, giving credence to these possibilities.

The mechanism responsible for the catecholamine release is unclear. The site of action was clearly neuronal; the effect of verapamil was reduced markedly following chemical sympathectomy by 6-hydroxydopamine. A non-specific reduction in the stability of the nerve terminals, with an accompanying liberation of transmitter, although a possibility, fails to explain why ^{3}H was released following $[^{3}H]$ -noradrenaline, but not after $[^{3}H]$ -adenosine incubation. Two more likely explanations of what appears to be a selective, prejunctionally-mediated, mechanism are a tyramine- or reserpine-like action. Reminiscent of a tyramine action was the fast onset of ^{3}H release and its independence of $[Ca^{2+}]_o$. However, the presence of $[^{3}H]$ -DOPEG and absence of $[^{3}H]$ -noradrenaline in the overflow was inconsistent with this and more in keeping with a reserpine-like action (Kopin & Gordon, 1962).

Spontaneous release was not produced by the Ca^{2+} channel antagonists following incubation with $[^3H]$ -adenosine, unlike the situation following labelling with noradrenaline. This allowed stimulation-evoked overflow in the vas deferens and i.a.s., incubated with $[^3H]$ -adenosine, to be measured. The amount of ^3H released by nerve stimulation in each tissue was lower following labelling with adenosine than with noradrenaline. This may reflect the small quantity of ATP in the synaptic vesicles (in the vas deferens, estimated at 1/50th of that of noradrenaline; Stjärne & Åstrand, 1984). Alternatively, $[^3H]$ -adenosine may penetrate the nerve terminal poorly. The possibility cannot be ignored, however, that ATP may not have a transmitter role in either tissue (see later). At any rate, the present evidence confirmed the release of ^3H from nerves. In the vas deferens, when sucrose was added to paralyse the muscle (and so eliminate this as a source of ATP (Westfall et al., 1978) and TTX to remove the neuronal contribution, no ^3H was released by stimulation. A similar situation occurred in the i.a.s., following muscle paralysis with verapamil and inhibition of neuronal conduction by TTX.

Together, the results in both the vas deferens and i.a.s. demonstrated neuronal release of ATP metabolites, rather than the nucleotide itself. The putative role of ATP as the NANC transmitter in each tissue (Burnstock & Sneddon, 1984; Lim & Muir, 1986) would be supported by detecting the release of intact ATP following stimulation. It was hoped that the highly sensitive luciferase-luciferin luminescence technique

might achieve this. The results were, however, disappointing. No neuronally-released ATP in either the i.a.s. or vas deferens could be measured, even in the presence of the ATPase inhibitor, sodium vanadate (Cantley et al., 1977). This failure may be explained in at least one of three ways.

First, ATP may not be the transmitter in either tissue. Evidence to the contrary is, however, considerable. In the guinea-pig vas deferens, ATP mimics and α, β meATP, a compound which antagonises purinoceptors, blocks the depolarization and initial twitch component produced by nerve stimulation (Burnstock & Sneddon, 1984; Sneddon & Westfall, 1984). In the guinea-pig i.a.s., both ATP and NANC nerve stimulation hyperpolarize and relax the muscle by a Ca^{2+}-dependent increase in K^+ conductance (Lim, 1985; Lim & Muir, 1986).

Alternatively, the amount of neuronally-released ATP may have been insufficient for detection by the luciferase-luciferin assay. However, considering that the ATP: noradrenaline ratio in the secretory vesicles of the vas deferens is claimed to be 1:50 (Stjärne & Åstrand, 1984) and that each stimulus should have released approximately 1×10^{-13} moles of noradrenaline (Macrae, 1983), this seems unlikely; 1×10^{-18} moles of ATP could be detected easily by the assay.

The third and perhaps most likely explanation is that the ATP was metabolised following its release from nerves. This is consistent with the recovery of $[^3H]$ -ATP metabolites and the absence of the nucleotide itself, from the i.a.s. following NANC nerve stimulation. Failure of sodium vanadate

to protect ATP from destruction may reflect the complex nature of ATP metabolism. The nucleotide is rapidly broken down at the plasma membrane by at least three enzymes - Ca^{2+} and Mg^{2+}-ATPase, 5'-nucleotidase and Na^{+} and K^{+}-ATPase (Manery & Dryden, 1979). The principal enzyme, Ca^{2+} and Mg^{2+}-ATPase, has been identified in sympathetic nerves in the guinea-pig vas deferens (Burnstock, 1972) and non-myelinated nerves of the rat intestine (Rostgaard & Barrnett, 1964) and retina (Marchesi et al., 1964). The importance of this enzyme in ATP breakdown may explain the inability of sodium vanadate, which blocks selectively Na^{+} and K^{+}-ATPase (Cantley et al., 1977), to allow accumulation of sufficient ATP.

Conclusions

The project was undertaken to investigate the influence of Ca^{2+} channel antagonists on autonomic neuromuscular transmission. The results showed that the compounds, albeit in high concentrations, inhibited both nerve AP conduction and transmitter release in the guinea-pig vas deferens and i.a.s. and the rat anococcygeus. The organic agents probably inhibited release by preventing invasion of the nerve terminals by the AP. They may have done so, by blocking Na^{+} and/or Ca^{2+} involvement in the AP. In contrast, cobalt, it is likely, acted at or near the terminal to prevent Ca^{2+} influx and, in consequence, transmitter release. In each tissue, the organic agents, but not cobalt, acted postjunctionally at lower doses than those required to inhibit nerve APs and transmitter

release. The possibility exists that in the future an organic Ca^{2+} channel antagonist might be developed which would act selectively at prejunctional sites.

In the course of the investigation, the ability of the antagonists to stimulate spontaneous catecholamine liberation, in a manner analogous to that of tyramine or reserpine, was recognised. These experiments also allowed investigation of the nature of the transmitters in the guinea-pig vas deferens and i.a.s. Noradrenaline was released from the sympathetic nerves of the vas deferens. Also, the presence of a neuronally-mediated release of ^{3}H, following incubation with $[^{3}H]$ - adenosine, suggested that in both the vas deferens and i.a.s. this compound may have a transmitter role. Attempts to detect intact ATP from the nerves in either tissue were, however, disappointing, probably due to its metabolism. Unequivocal evidence that ATP is the NANC transmitter in the vas deferens or i.a.s., therefore, remains elusive.

REFERENCES

ADELSTEIN, R.S. (1981).
Calcium and calmodulin in Kyoto.
Nature, 294, 693-694.

ADELSTEIN, R.S. & ELSENBERG, E.A. (1980).
Regulation and kinetics of the actin-myosin-ATP interaction.
A. Rev. Biochem., 49, 921-956.

ADRIAN, E.D. & GELFAN, S. (1933).
Rhythmic activity in skeletal muscle fibres.
J. Physiol., 78, 271-281.

ADVENIER, C., CERRINA, J., DURROUX, P., FLOCH, A. & RENIER, A. (1984).
Effects of five different organic calcium antagonists on guinea-pig isolated trachea.
Br. J. Pharmac., 82, 727-733.

AFFOLTER, H., BURKARD, W.P. & PLETSCHER, A. (1985).
Verapamil, an antagonist at 5-hydroxytryptamine receptors of human blood platelets.
Eur. J. Pharmac., 108, 157-162.

AHMED, F., FOSTER, R.W. & SMALL, R.C. (1985).
Some effects of nifedipine in guinea-pig isolated trachealis.
Br. J. Pharmac., 84, 861-869.

AIKAWA, J.K. (1963).
In: The role of magnesium in biologic processes.
Springfield and Illinois: Charles C. Thomas.

AKAIKE, N., LEE, K.S. & BROWN, A.M. (1978).
The calcium current of Helix neuron.
J. gen. Physiol., 71, 509-531.

ALBERTS, P., BARTFAI, T. & STJÄRNE, L. (1981).
Site(s) and ionic basis of α-autoinhibition and facilitation of [^{3}H]noradrenaline secretion in guinea-pig vas deferens.
J. Physiol., 312, 297-334.

ANDERSSON, K.E., HÖGESTÄTT, E.D., LARSSON, B. & MATTIASSON, A. (1983).
Effects of three Ca^{2+} entry blockers on contraction, noradrenaline release and α adrenoceptor binding in female rabbit urethra.
Br. J. Pharmac., 80, 614P.

BAER, M., BEST, P.M. & REUTER, H. (1976).
Voltage-dependent action of tetrodotoxin in mammalian cardiac muscle.
Nature, 263, 344-345.

BAGSHAW, C.R. (1982).
In: Muscle Contraction, eds. Brammar, W.J. and Edidin, M. London and New York: Chapman and Hall.

BAKER, P.F. (1972).
Transport and metabolism of calcium ions in nerve.
Prog. Biophys. Mol. Biol., 24, 177-223.

BAKER, P.F. (1976).
Regulation of intracellular Ca and Mg in squid axons.
Fed. Proc., 35, 2589-2595.

BANKS, P. (1966).
An interaction between chromaffin granules and calcium ions.
Biochem. J., 101, 18-20.

BAYER, R., KALUSCHE, D., KAUFMANN, R. & MANNHOLD, R. (1975).
Inotropic and electrophysiological actions of verapamil and D600 in mammalian myocardium.
Naunyn-Schmiedebergs Arch. Pharmac., 290, 81-97.

BENNETT, M.R. (1967).
The effect of cations on the electrical properties of the smooth muscle cells of the guinea-pig vas deferens.
J. Physiol., 190, 465-479.

BENNETT, M.R. (1972).
In: Autonomic Neuromuscular Transmission. London: Cambridge University Press.

BENNETT, M.R. (1973).
An electrophysiological analysis of the storage and release of noradrenaline at sympathetic nerve terminals.
J. Physiol., 229, 515-531.

BENNETT, M.R., BURNSTOCK, G. & HOLMAN, M.E. (1966).
Transmission from nerves to the smooth muscle of the guinea-pig taenia coli.
J. Physiol., 182, 541-558.

BENNETT, M.R. & McLACHLAN, E.M. (1972).
An electrophysiological analysis of the storage of acetylcholine in preganglionic nerve terminals.
J. Physiol., 221, 657-668.

BERRIDGE, M.J. (1985a).
Calcium: a universal second messenger.
Triangle, 24, 79-90.

BERRIDGE, M.J. (1985b).
The molecular basis of communication within the cell.
Sci. Amer., 253, 142-152.

BERRIDGE, M.J. & IRVINE, R.F. (1984).
Inositol trisphosphate, a novel second messenger in cellular transduction.
Nature, 312, 315-321.

BIDEN, T.J., PRENTKI, M., IRVINE, R.F., BERRIDGE, M.J. & WOLLHEIM, C.B. (1984).
Inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+} from permeabilized insulin-secreting cells.
Biochem. J., 223, 467-473.

BLAKELEY, A.G.H., BROWN, D.A., CUNNANE, T.C., FRENCH, A.M., McGRATH, J.C. & SCOTT, N.C. (1981).
Effects of nifedipine on electrical and mechanical responses of rat and guinea-pig vas deferens.
Nature, 294, 759-761.

BLASCHKO, H. (1967).
Biochemical studies on chromaffin cells.
Naunyn-Schmiedebergs Arch. Pharmac., 257, 143-147.

BLAUSTEIN, M.P. (1974).
The interrelationship between sodium and calcium fluxes across cell membranes.
Rev. Physiol., Biochem. Pharmac., 70, 33-82.

BLAUSTEIN, M.P. (1979).
The role of calcium in catecholamine release from adrenergic nerve terminals. In: The Release of Catecholamines from Adrenergic Neurons, ed. Paton, D.M., pp.39-58. Oxford: Pergamon Press.

BLAUSTEIN, M.P. & GOLDMAN, D.E. (1968).
The action of certain polyvalent cations on the voltage-clamped lobster axon.
J. gen. Physiol., 51, 279-291.

BLIOCH, Z.L., GLAGOLEVA, I.M., LIBERMAN, E.A. & NENASHEV, V.A. (1968).
A study of the mechanism of quantal transmitter release at a chemical synapse.
J. Physiol., 199, 11-35.

BOLGER, G.T., GENGO, P., KLOCKOWSKI, E., LUCLOWSKI, E., SIEGEL, H., JANIS, R.A., TRIGGLE, A.M. & TRIGGLE, D.J. (1983).
Characterization of binding of the Ca^{2+} channel antagonist (^{3}H) nitrendipine to guinea-pig ileal smooth muscle.
J. Pharmac. exp. Ther., 225, 291-309.

BOLTON, T.B. (1979).
Mechanisms of action of transmitters and other substances on smooth muscle.
Physiol. Rev., 59, 606-692.

BOLTON, T.B. (1986).
Calcium metabolism in vascular smooth muscle.
Br. Med. Bull., 42, 421-429.

BOLTON, T.B. & LARGE, W.A. (1986).
Are junction potentials essential? Dual mechanism of smooth cell activation by transmitter released from autonomic nerves.
Quart. J. exp. Physiol., 71, 1-28.

BORNSTEIN, J.C., LEES, G.M. & McBAIN, C.J. (1985).
Comparative effects of calcium antagonists on inhibitory junction potentials of circular muscle of guinea-pig ileum.
Br. J. Pharmac., 86, 724P.

BOSTRÖM, S., LJUNG, B., MÅRDH, S., FORSEN, S. & THULIN, E. (1981).
Interaction of the antihypertensive drug felodipine with calmodulin.
Nature, 292, 777-778.

BOULLIN, D.J. (1967).
The action of extracellular cations on the release of the sympathetic transmitter from peripheral nerves.
J. Physiol., 189, 85-99.

BOWMAN, A. & DRUMMOND, A.H. (1984).
Cyclic GMP mediates neurogenic relaxation in the bovine retractor penis muscle.
Br. J. Pharmac., 81, 665-674.

BRADING, A.F., BÜLBRING, E. & TOMITA, T. (1969).
The effect of sodium and calcium on the action potential of the smooth muscle of the guinea-pig taenia coli.
J. Physiol., 200, 637-654.

BRAUNWALD, E. (1980).
Calcium channel blockers.
Am. J. Cardiol., 46, 1045-1046.

BÜLBRING, E. & DEN HERTOG, A. (1980).
The action of isoprenaline on the smooth muscle of the guinea-pig taenia coli.
J. Physiol., 304, 277-296.

BÜLBRING, E. & TOMITA, T. (1970).
Effects of Ca removal on the smooth muscle of the guinea-pig taenia coli.
J. Physiol., 210, 217-232.

BURGESS, G.M., IRVINE, R.F., BERRIDGE, M.J., McKINNEY, J.S. & PUTNEY, J.W. (1984).
Actions of inositol phosphates on Ca^{2+} pools in guinea-pig hepatocytes.
Biochem. J., 224, 741-746.

BURN, J.H. & GIBBONS, W.R. (1964).
The part played by calcium in determining the response to stimulation of sympathetic postganglionic fibres.
Br. J. Pharmac., 22, 540-548.

BURN, J.H. & GIBBONS, W.R. (1965).
The release of noradrenaline from sympathetic fibres in relation to calcium concentration.
J. Physiol., 181, 214-223.

BURNSTOCK, G. (1972).
Purinergic nerves.
Pharmac. Rev., 24, 509-581.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978).
Purinergic innervation of the guinea-pig urinary bladder.
Br. J. Pharmac., 63, 125-138.

BURNSTOCK, G. & HOLMAN, M.E. (1961).
The transmission of excitation from autonomic nerve to smooth muscle.
J. Physiol., 155, 115-133.

BURNSTOCK, G. & SNEDDON, P. (1984).
Evidence that excitatory junction potentials in response to sympathetic nerve stimulation of some smooth muscles are mediated by ATP.
J. Physiol., 351, 28P.

BUSHFIELD, M., McNICOL, A. & MacINTYRE, D.E. (1985).
Inhibition of platelet-activating-factor-induced human platelet activation by prostaglandin D_2.
Biochem. J., 232, 267-271.

BUTLER, T.M. & SIEGMAN, M.J. (1983).
Chemical energy usage and myosin light chain phosphorylation in mammalian smooth muscle.
Fed. Proc., 42, 57-61.

CAMBRIDGE, D., DAVEY, M.J. & MASSINGHAM, R. (1977).
Prazosin, a selective antagonist of post-synaptic α-adrenoceptors.
Br. J. Pharmac., 59, 514-515P.

CANTLEY, L.C., JOSEPHSON, L., WARNER, R., YANAGISAWA, M., LECHENE, C. & GUIDOTTI, G. (1977).
Vanadate is a potent (Na, K)-ATPase inhibitor found in ATP derived from muscle.
J. biol. Chem., 252, 7421-7423.

CASTEELS, R. (1981).
Membrane potential in smooth muscle cells.
In: Smooth Muscle: an assessment of current knowledge, eds. Bülbring, E., Brading, A.F., Jones, A.W. and Tomita, T., pp.105-126. London: Edward Arnold.

CASTEELS, R., KITAMURA, K., KURIYAMA, H. & SUZUKI, H. (1977).
Excitation-contraction coupling in the smooth muscle cells of the rabbit main pulmonary artery.
J. Physiol., 271, 63-79.

CHARLTON, M.P., SMITH, S.J. & ZUCKER, R.S. (1982).
Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse.
J. Physiol., 323, 173-193.

COHEN, P. (1982).
The role of protein phosphorylation in neural and hormonal control of cellular activity.
Nature, 296, 613-620.

COSTA, M. & FURNESS, J.B. (1973).
The innervation of the internal anal sphincter of the guinea-pig. In: Proceedings of the IVth International Symposium on Gastrointestinal Motility, ed. Daniel, E.E., pp.681-690. Vancouver: Mitchell Press.

CREED, K.E., GILLESPIE, J.S. & MUIR, T.C. (1975).
The electrical basis of excitation and inhibition in the rat anococcygeus muscle.
J. Physiol., 245, 33-47.

CROUT, J.R. (1961).
Catecholamines in urine. In: Standard Methods of Clinical Chemistry, ed. Seligson, D., Vol. 3, pp.62-80. New York and London: Academic Press.

CUNNANE, T.C. (1979).
In: Some Mechanisms of Neuromuscular Transmission. Ph.D. Thesis, University of Glasgow.

CUNNANE, T.C. and STJÄRNE, L. (1984).
Frequency dependent intermittency and ionic basis of impulse conduction in postganglionic sympathetic fibres of guinea-pig vas deferens.
Neurosci., 11, 211-229.

DEL CASTILLO, J. & ENGBAEK, L. (1954).
The nature of the neuromuscular block produced by magnesium.
J. Physiol., 124, 370-384.

DE LORENZO, R.J. (1981).
The calmodulin hypothesis of neurotransmission.
Cell Calcium, 2, 365-385.

DICHTER, M.A. & FISCHBACH, G.D. (1977).
The action potential of chick dorsal root ganglion neurones maintained in cell culture.
J. Physiol., 267, 281-298.

DOUGLAS, W.W. (1974).
Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence.
Biochem. Soc. Symp., 39, 1-28.

DOUGLAS, W.W., LYWOOD, D.W. & STRAUB, R.W. (1961).
The stimulant effect of barium on the release of acetylcholine from the superior cervical ganglion.
J. Physiol., 156, 515-522.

DROOGMANS, G., RAEYMAEKERS, L. & CASTEELS, R. (1977).
Electro- and pharmacomechanical coupling in the smooth muscle cells of the rabbit ear artery.
J. gen. Physiol., 70, 129-148.

DUNLAP, K. & FISCHBACH, G.D. (1978).
Neurotransmitters decrease the calcium component of sensory neurone action potentials.
Nature, 276, 837-839.

DURBIN, R.P. & JENKINSON, D.H. (1961a).
The effect of carbachol on the permeability of depolarized smooth muscle to inorganic ions.
J. Physiol., 157, 74-89.

DURBIN, R.P. & JENKINSON, D.H. (1961b).
The calcium dependence of tension development in depolarized smooth muscle.
J. Physiol., 157, 90-96.

EBASHI, S. & ENDO, M. (1968).
Calcium ion and muscle contraction.
Prog. Biophys. Mol. Biol., 18, 123-183.

EBASHI, S., NONOMURA, Y., NAKAMURA, S., NAKASONE, H. & KOHAMA, K. (1982).
Regulatory mechanism in smooth muscle: actin-linked regulation.
Fed. Proc., 41, 2863-2867.

ECKERT, R. & LUX, H.D. (1977).
Calcium-dependent depression of a late outward current in snail neurons.
Science, 197, 472-475.

EDGINGTON, D.R. & STUART, A.E. (1979).
Calcium channels in the high resistivity axonal membrane of photoreceptors of the giant barnacle.
J. Physiol., 294, 433-445.

EDWARDS, C. (1982).
The selectivity of ion channels in nerve and muscle.
Neurosci., 7, 1335-1366.

EHARA, T. & KAUFMANN, R. (1978).
The voltage- and time-dependent effects of (-)-verapamil on the slow inward current in isolated cat ventricular myocardium.
J. Pharmac., exp. Ther., 207, 49-55.

EINSTEIN, R. & LAVIDIS, N.A. (1984).
The dependence of excitatory junction potential amplitude on the external calcium concentration in narcotic tolerant mouse vas deferens.
Br. J. Pharmac., 83, 853-861.

ELMQUIST, D. & QUASTEL, D.M.J. (1965).
A quantitative study of end-plate potentials in isolated human muscle.
J. Physiol., 178, 505-529.

EL-SHARKAWY, T.Y. & DANIEL, E.E. (1975).
Ionic mechanisms of intestinal electrical control activity.
Am. J. Physiol., 229, 1287-1298.

EL-SHARKAWY, T.Y., MORGAN, K.G. & SZURSZEWSKI, J.H. (1978).
Intracellular electrical activity of canine and human gastric smooth muscle.
J. Physiol., 279, 291-307.

ENGBAEK, L. (1948).
Investigations on the course and localisation of magnesium anesthesia.
Acta Pharmac. Toxic., 4, Suppl. 1, 1-189.

EPSTEIN, P.M., FISS, K., HACHISU, R. & ADRENYAK, D.M. (1982).
Interaction of calcium antagonists with cyclic AMP phosphodiesterases and calmodulin.
Biochem. Biophys. Res. Commun., 105, 1142-1149.

EVANS, D.H.L., SCHILD, H.O. & THESLEFF, S. (1958).
Effects of drugs on depolarized plain muscle.
J. Physiol., 143, 474-485.

FAIN, G.L., GERSCHENFELD, H.M. & QUANDT, F.N. (1980).
Calcium spikes in toad rods.
J. Physiol., 303, 495-513.

FAIRHURST, A.S., WHITTAKER, M. & EHLERT, F.J. (1980).
Interactions of D600 (methoxyverapamil) and local anaesthetics with rat brain adrenergic and muscarinic receptors.
Biochem. Pharmac., 29, 155-162.

FARMER, J.B. & CAMPBELL, I.K. (1967).
Calcium and magnesium ions: influence on the response of an isolated artery to sympathetic nerve stimulation, noradrenaline and tyramine.
Br. J. Pharmac., 29, 319-328.

FATT, P. & GINSBORG, B.L. (1958).
The ionic requirements for the production of action potentials in crustacean muscle fibres.
J. Physiol., 142, 516-543.

FERRY, C.B. (1967).
The innervation of the vas deferens of the guinea-pig.
J. Physiol., 192, 463-478.

FITZPATRICK, D.F. & SZENTIVANYI, A. (1977).
Stimulation of calcium uptake into aortic microsomes by cyclic AMP and cyclic AMP-dependent protein kinase.
Naunyn-Schmiedebergs Arch. Pharmac., 298, 255-257.

FLAIM, S.F., RATZ, P.H., SWIGART, S.C. & GLEASON, M.M. (1985).
Bepridil hydrochloride alters potential-dependent and receptor-operated calcium channels in vascular smooth muscle of rabbit aorta.
J. Pharmac. exp. Ther., 234, 63-71.

FLECKENSTEIN, A. (1977).
Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. A. Rev. Pharmac. Toxicol., 17, 149-166.

FLECKENSTEIN, A. (1983).
History of calcium antagonists.
Circ. Res., 52, Suppl. I, 3-16.

FLECKENSTEIN, A., FLECKENSTEIN-GRÜN, G., BYON, Y.K., HAASTERT, H.P. & SPAH, F. (1979).
Vergleichende untersuchungen über die Ca^{++}-antagonistischen grundwirkungen von niludipin (Bay a 7168) und nifedipin (Bay a 1040) auf myokard, myometrium und glatte gefaßmuskulatur.
Arzneimittelforsch., 29, 230-246.

FLECKENSTEIN, A. & REUTER, H. (1985).
Calcium antagonism in heart and vascular smooth muscle.
Med. Res. Rev., 5, 395-425.

FLECKENSTEIN, A., TRITTHART, H., DÖRING, H.J. & BYON, Y.K. (1972).
Bay a 1040-ein hochaktiver Ca^{++}-antagonistischer inhibitor der elektromechanischen koppelungsprozesse im warmblüter-myokard.
Arzneimittelforsch., 22, 22-33.

FLECKENSTEIN-GRÜN, G., FREY, M. & FLECKENSTEIN, A. (1984).
Calcium antagonists: mechanisms and therapeutic uses.
Trends Pharmac. Sci., 5, 283-286.

FRANKISH, N.H. (1983).
Verapamil and bepridil on potassium-evoked contractures of guinea-pig ileum.
Br. J. Pharmac., 80, 610P.

FREDHOLM. B.B., FRIED, G. & HEDQVIST, P. (1982).
Origin of adenosine released from rat vas deferens by nerve stimulation.
Eur. J. Pharmac., 79, 233-243.

FRENCH, A.M. & SCOTT, N.C. (1981).
A comparison of the effects of nifedipine and verapamil on rat vas deferens.
Br. J. Pharmac., 73, 321-323.

FURNESS, J.B. (1969).
An electrophysiological study of the innervation of the smooth muscle of the colon.
J. Physiol., 205, 549-562.

GALZIN, A.M. & LANGER, S.Z. (1983).
Presynaptic α_2-adrenoceptor antagonism by verapamil but not by diltiazem in rabbit hypothalamic slices.
Br. J. Pharmac., 78, 571-577.

GERHARDS, K.P., RÖTTCHER, M. & STRAUB, R.W. (1964).
Wirkungen von Ca und Mg auf freisetzung und synthese von acetylcholin am ruhiggestellten darm.
Pflügers Arch., 279, 251-264.

GERMAIN, M. & PROULX, P. (1965).
Adenosine triphosphatase activity in synaptic vesicles of rat brain.
Biochem. Pharmac., 14, 1815-1819.

GESCHWIND, I.I. (1969).
Mechanism of action of releasing factors.
In: Frontiers in Neuroendocrinology, eds. Ganong, W.F. and Martini, L., pp.389-431. New York, London and Toronto: Oxford University Press.

GIBSON, A. & GILLESPIE, J.S. (1973).
The effect of immunosympathectomy and of 6-hydroxydopamine on the responses of the rat anococcygeus to nerve stimulation and to some drugs.
Br. J. Pharmac., 47, 261-267.

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

GILLESPIE, J.S. & KIRPEKAR, S.M. (1965).
The inactivation of infused noradrenaline by the cat spleen.
J. Physiol., 176, 205-227.

GILLESPIE, J.S. & KIRPEKAR, S.M. (1966).
The histological localization of noradrenaline in the cat spleen.
J. Physiol., 187, 69-79.

GILLESPIE, J.S. & LÜLLMANN-RAUCH, R. (1974).
On the ultrastructure of the rat anococcygeus muscle.
Cell Tiss. Res., 149, 91-104.

GILMAN, A.G. (1984).
G proteins and dual control of adenylate cyclase.
Cell, 36, 577-579.

GLOSSMANN, H., FERRY, D.R., GOLL, A., STRIESSNIG, J. & SCHOBER, M. (1985).
Calcium channels: basic properties as revealed by radioligand binding studies.
J. Cardiovasc. Pharmac., 7, S20-S30.

GLOSSMANN, H., FERRY, D.R., LÜBBECKE, F., MEWES, R. & HOFMANN, R. (1982).
Calcium channels: direct identification with radioligand binding studies.
Trends Pharmac. Sci., 3, 431-437.

GODFRAIND, T. (1981).
Mechanisms of action of calcium entry blockers.
Fed. Proc. 40, 2866-2871.

GODFRAIND, T. & DIEU , D. (1981).
The inhibition by flunarizine of the norepinephrine-evoked contraction and calcium influx in rat aorta and mesenteric arteries.
J. Pharmac. exp. Ther., 217, 510-515.

GODFRAIND, T. & KABA, A. (1969).
Blockade or reversal of the contraction induced by calcium and adrenaline in depolarized arterial smooth muscle.
Br. J. Pharmac., 36, 549-560.

GOLENHOFEN, K. (1981).
Differentiation of calcium activation processes in smooth muscle using selective antagonists.
In: Smooth Muscle: an assessment of current knowledge, eds. Bülbring, E., Brading, A.F., Jones, A.W. and Tomita, T., pp.157-170. London: Edward Arnold.

GÖTHERT, M., NAWROTH, P. & NEUMEYER, H. (1979).
Inhibitory effects of verapamil, prenylamine and D-600 on Ca^{2+}-dependent NA release from the isolated rabbit hearts.
Naunyn-Schmiedebergs Arch. Pharmac., 310, 11-20.

GRAEFE, K.H., STEFANO, J.E. & LANGER, S.Z. (1973).
Preferential metabolism of (-)-^{3}H-noradrenaline through the deaminated glycol in the rat vas deferens.
Biochem. Pharmac., 22, 1147-1160.

GREENGARD, P. (1979).
Cyclic nucleotides, phosphorylated proteins, and the nervous system.
Fed. Proc., 38, 2208-2217.

GUTTMAN, R. (1940).
Stabilization of spider crab nerve membranes by alkaline earths, as manifested in resting potential measurements.
J. gen. Physiol., 23, 343-364.

HAEUSLER, G. (1972).
Differential effect of verapamil on excitation-contraction coupling in smooth muscle and on excitation-secretion coupling in adrenergic nerve terminals.
J. Pharmac. exp. Ther., 180, 672-682.

HAGIWARA, S. (1973).
Ca spike.
Adv. Biophys., 4, 71-102.

HAGIWARA, S. & BYERLY, L. (1981).
Calcium channel.
A. Rev. Neurosci., 4, 69-125.

HAGIWARA, S. & TAKAHASHI, K. (1967).
Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane.
J. gen. Physiol., 50, 583-601.

HALPERIN, A.K. & CUBEDDU, L.X. (1986).
The role of calcium channel blockers in the treatment of hypertension.
Am. Heart J., 111, 363-382.

HARVEY, A.M. & MacINTOSH, F.C. (1940).
Calcium and synaptic transmission in a sympathetic ganglion.
J. Physiol., 97, 408-416.

HAY, D.W.P. & WADSWORTH, R.M. (1982).
Local anaesthetic activity of organic calcium antagonists: relevance to their actions on smooth muscle.
Eur. J. Pharmac., 77, 221-228.

HEILBRUNN, L.V. (1943).
In: An Outline of General Physiology. 2nd edition.
Philadelphia: W.B. Saunders.

HENRY, P.D. (1980).
Comparative pharmacology of Ca^{2+} antagonists: nifedipine, verapamil and diltiazem.
Am. J. Cardiol., 46, 1047-1058.

HERZIG, J.W. (1984).
Contractile proteins: possible targets for drug action.
Trends Pharmac. Sci., 5, 296-300.

HERZIG, J.W. (1985).
Calcium ions and the electrochemical coupling of the heart.
Triangle, 24, 115-129.

HESCHELER, J., PELZER, D., TRUBE, G. & TRAUTWEIN, W. (1982).
Do organic calcium channel blockers act from inside or outside of the cardiac cell membrane?
Pflügers Arch., 393, 287-291.

HILLARP, N.A. (1958).
The release of catecholamine from the amine containing granules of the adrenal medulla.
Acta Physiol. Scand., 43, 292-302.

HIRATA, M., SUEMATSU, E., HASHIMOTO, T., HAMACHI, T. & KOGA, T. (1984).
Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate.
Biochem. J., 223, 229-236.

HIRST, G.D.S. & SPENCE, I. (1973).
Calcium action potentials in mammalian peripheral neurones.
Nature New Biol., 243, 54-56.

HODGKIN, A.L. & HUXLEY, A.F. (1952).
Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo.
J. Physiol., 116, 449-472.

HODGKIN, A.L. & KEYNES, R.D. (1957).
Movements of labelled calcium in squid giant axons.
J. Physiol., 138, 253-281.

HOF, R.P. (1985).
Pharmacological characterization of calcium antagonists: What they do and where they act.
Triangle, 24, 143-156.

HÖGESTÄTT, E.D., ANDERSSON, K.E. & EDVINSSON, L. (1982).
Effects of nifedipine on potassium-induced contraction and noradrenaline release in cerebral and extracranial arteries from rabbit.
Acta Physiol. Scand., 114, 283-296.

HOKIN, M.R. & HOKIN, L.E. (1953).
Enzyme secretion and the incorporation of P^{32} into phospholipides of pancreas slices.
J. biol. Chem., 203, 967-977.

HOLMAN, M.E. (1957).
The effect of changes in sodium chloride concentration on the smooth muscle of the guinea-pig's taenia coli.
J. Physiol., 136, 569-584.

HOLMAN, M.E. & NEILD, T.O. (1979).
Membrane properties.
Br. Med. Bull., 35, 235-241.

HOLMAN, M.E. & WEINRICH, J.P. (1975).
The effects of calcium and magnesium on inhibitory junctional transmission in smooth muscle of guinea-pig small intestine.
Pflügers Arch., 360, 109-119.

HORN, J.P. & McAFEE, D.A. (1980).
Alpha-adrenergic inhibition of calcium-dependent potentials in rat sympathetic neurones.
J. Physiol., 301, 191-204.

HOTTA, Y. & TSUKUI, R. (1968).
Effect on the guinea-pig taenia coli of the substitution of strontium or barium ions for calcium ions.
Nature, 217, 867-869.

HUBBARD, J.I. (1961).
The effect of calcium and magnesium on the spontaneous release of transmitter from mammalian motor nerve endings.
J. Physiol., 159, 507-517.

HUKOVIĆ, S. & MUSCHOLL, E. (1962).
Die noradrenalin-abgabe aus dem isolierten kaninchenherzen bei sympathischer nervenreizung und ihre pharmakologische beeinflussung.
Naunyn-Schmiedebergs Arch. Pharmac., 244, 81-96.

HURWITZ, L. (1986).
Pharmacology of calcium channels and smooth muscle.
A. Rev. Pharmac. Toxicol., 26, 225-258.

HUXLEY, H.E. & HANSON, J. (1954).
Changes in cross-striations of muscle during contraction and stretch and their structural interpretation.
Nature, 173, 973-976.

IMAHORI, K. (1982).
Calcium-dependent neutral protease: its characterization and regulation.
In: Calcium and Cell Function, ed. Cheung, W.Y., Vol. 3, pp.473-485. New York, London, Paris, San Diego, San Francisco, Sao Paulo, Sydney, Tokyo and Toronto: Academic Press.

IMAI, S. & TAKEDA, K. (1967).
Calcium and contraction of heart and smooth muscle.
Nature, 213, 1044-1045.

ITO, F. & KOMATSU, Y. (1979).
Calcium-dependent regenerative responses in the afferent nerve terminal of the frog muscle spindle.
Brain Res., 175, 160-164.

ITO, H. & NISHI, K. (1982).
Frequency-dependent depression of ganglionic transmission by propranolol and diltiazem in the superior cervical ganglion of the guinea-pig.
Br. J. Pharmac., 77, 359-362.

ITO, Y., SUZUKI, H. & KURIYAMA, H. (1977).
On the roles of calcium ion during potassium induced contracture in the smooth muscle cells of the rabbit pulmonary artery.
Jap. J. Physiol., 27, 755-770.

ITOH, T., IZUMI, H. & KURIYAMA, H. (1982).
Mechanisms of relaxation induced by activation of β-adrenoceptors in smooth muscle cells of the guinea-pig mesenteric artery.
J. Physiol., 326, 475-493.

JANIS, R.A. & SCRIABINE, A. (1983).
Sites of action of Ca^{2+} channel inhibitors.
Biochem. Pharmac., 32, 3499-3507.

JOHNSON, D. (1983).
Interaction of hydrophobic inhibitory ligands with calmodulin.
Biophys. J., 41, 306P.

KAJIWARA, M. & CASTEELS, R. (1983).
Effects of Ca-antagonists on neuromuscular transmission in the rabbit ear artery.
Pflügers Arch., 396, 1-7.

KASS, R.S. (1982).
Nisoldipine: a new, more selective calcium current blocker in cardiac purkinje fibers.
J. Pharmac. exp. Ther., 223, 446-456.

KATZ, B. & MILEDI, R. (1965).
The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction.
Proc. Roy. Soc. Bull., 161, 483-495.

KATZ, B. & MILEDI, R. (1968).
The role of calcium in neuromuscular facilitation.
J. Physiol., 195, 481-492.

KAWAI, N. & NIWA, A. (1980).
Neuromuscular transmission without sodium activation of the presynaptic nerve terminal in the lobster.
J. Physiol., 305, 73-85.

KEATINGE, W.R. (1964).
Mechanism of adrenergic stimulation of mammalian arteries and its failure at low temperatures.
J. Physiol., 174, 184-205.

KEENAN, A.K. & CALLANAN, K. (1980).
The calcium dependence of evoked ^{3}H-NA release from rat atria: A study using dantrolene and D600.
Irish J. Med. Sci., 149, 447P.

KIRPEKAR, S.M. & MISU, Y. (1967).
Release of noradrenaline by splenic nerve stimulation and its dependence on calcium.
J. Physiol., 188, 219-234.

KIRPEKAR, S.M., PRAT, J.C. & YAMAMOTO, H. (1970).
Effects of metabolic inhibitors on norepinephrine release from the perfused spleen of the cat.
J. Pharmac. exp. Ther., 172, 342-350.

KIRPEKAR, S.M. & WAKADE, A.R. (1968).
Release of noradrenaline from the cat spleen by potassium.
J. Physiol., 194, 595-608.

KOHLHARDT, M., BAUER, B., KRAUSE, H. & FLECKENSTEIN, A. (1973).
Selective inhibition of the transmembrane Ca conductivity in mammalian myocardial fibers by Ni, Co and Mn ions.
Pflügers Arch., 338, 115-123.

KOKETSU, K., CERF, J.A. & NISHI, S. (1959).
Further observations of the electrical activity of frog spinal ganglion cells in sodium-free solutions.
J. Neurophysiol., 22, 693-703.

KOPIN, I.J. & GORDON, E.K. (1962).
Metabolism of norepinephrine-H^3 released by tyramine and reserpine.
J. Pharmac. exp. Ther., 138, 351-359.

KOSTYUK, P.G. (1981).
Calcium channels in the neuronal membrane.
Biochim. Biophys. Acta, 650, 128-150.

KREBS, H.A. & HENSELEIT, K. (1932).
Unter suchungen über die harnstoffbildung im tierkörper.
Hoppe-Seylers Z. Physiol. Chem., 210, 33-66.

KUBA, K. (1970).
Effects of catecholamines on the neuromuscular junction in the rat diaphragm.
J. Physiol., 211, 551-570.

KUBO, T., FUKUDA, K., MIKAMI, A., MAEDA, A., TAKAHASHI, H., MISHINA, M., HAGA, T., HAGA, K., ICHIYAMA, A., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. (1986).
Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor.
Nature, 323, 411-416.

KUFFLER, S.W. (1944).
The effect of calcium on the neuromuscular junction.
J. Neurophysiol., 7, 17-26.

KURIYAMA, H. (1964).
Effect of calcium and magnesium on neuromuscular transmission in the hypogastric nerve - vas deferens preparation of the guinea-pig.
J. Physiol., 175, 211-230.

KURIYAMA, H. (1970).
Effects of ions and drugs on the electrical activity of smooth muscle. In: Smooth Muscle, eds. Bülbring, E., Brading, A.F., Jones, A.W. and Tomita, T., pp.366-395.
London: Edward Arnold.

KURIYAMA, H., MISHIMA, K. & SUZUKI, H. (1975).
Some differences in contractile responses of isolated longitudinal and circular muscle from the guinea-pig stomach.
J. Physiol., 251, 317-331.

KURIYAMA, H., OHSHIMA, K. & SAKAMOTO, Y. (1971).
The membrane properties of the smooth muscle of the guinea-pig portal vein in isotonic and hypertonic solutions.
J. Physiol., 217, 179-199.

KURIYAMA, H. & SUZUKI, H. (1978).
The effects of acetylcholine on the membrane and contractile properties of smooth muscle cells of the rabbit superior mesenteric artery.
Br. J. Pharmac., 64, 493-501.

KURIYAMA, H. & TOMITA, T. (1970).
The action potential in the smooth muscle of the guinea-pig taenia coli and ureter studied by the double sucrose-gap method.
J. gen. Physiol., 55, 147-162.

KUZNICKI, J., GRABAREK, Z., BRZESKA, H. & DRABIKOWSKI, W. (1981).
Stimulation of enzyme activities by fragments of calmodulin.
F.E.B.S. Lett., 130, 141-145.

LANGER, S.Z. (1977).
Presynaptic receptors and their role in the regulation of transmitter release.
Br. J. Pharmac., 60, 481-497.

LANGLEY, J.N. & ANDERSON, H.K. (1895).
The innervation of the pelvic and adjoining viscera. Part II. The bladder.
J. Physiol., 19, 71-139.

LAPETINA, E.G., SCHMITGES, C.J., CHANDRABOSE, K. & CUATRECASAS, P. (1977).
Cyclic adenosine 3',5'-monophosphate and prostacyclin inhibit membrane phospholipase activity in platelets.
Biochem. Biophys. Res. Comm., 76, 828-835.

LEE, K.S. & TSIEN, R.W. (1983).
Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells.
Nature, 302, 790-794.

LE PEUCH, C.J., HAIECH, J. & DEMAILLE, J.G. (1979).
Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate dependent and calcium-calmodulin-dependent phosphorylations.
Biochem., 18, 5150-5157.

LIM, S.P. (1985).
In: Electrical basis for inhibition and excitation in non-propulsive autonomically innervated smooth muscle.
Ph.D. Thesis, University of Glasgow.

LIM, S.P. & MUIR, T.C. (1985).
Mechanisms underlying the electrical and mechanical responses of the guinea-pig internal anal sphincter to field stimulation and to drugs.
Br. J. Pharmac., 86, 427-437.

LIM, S.P. & MUIR, T.C. (1986).
Neuroeffector transmission in the guinea-pig internal anal sphincter: an electrical and mechanical study.
Eur. J. Pharmac., 128, 17-24.

LLINÁS, R. & HESS, R. (1976).
Tetrodotoxin-resistant dendritic spikes in avian Purkinje cells.
Proc. Natl. Acad. Sci. U.S.A., 73, 2520-2523.

LLINÁS, R. & NICHOLSON, C. (1975).
Calcium role in depolarization-secretion coupling: An aequorin study in giant synapse.
Proc. Natl. Acad. Sci. U.S.A., 72, 187-190.

LÖFFELHOLZ, K. (1967).
Untersuchungen über die noradrenalin-freisetzung durch acetylcholin am perfundierten kaninchenherzen.
Naunyn-Schmiedebergs Arch. Pharmac., 258, 108-122.

MacINTYRE, D.E., BUSHFIELD, M. & SHAW, A.M. (1985).
Regulation of platelet cytosolic free calcium by cyclic nucleotides and protein kinase C.
F.E.B.S. Lett., 188, 383-388.

MACRAE, I.M. (1983).
In: The release of catecholamines by nerve stimulation. Ph.D. Thesis, University of Glasgow.

MANERY, J.F. & DRYDEN, E.E. (1979).
Ecto-enzymes concerned with nucleotide metabolism. In: Physiological and regulatory functions of adenosine and adenine nucleotides, eds. Baer, H.P. and Drummond, G.I., pp.323-339. New York: Raven Press.

MARCHESI, V.T., SEARS, M.L. & BARRNETT, J.R. (1964).
Electron microscopic studies of nucleoside phosphatase activity in blood vessels and glia of the retina. Invest. Opthalmol., 3, 1-21.

MARSTON, S.B. (1982).
The regulation of smooth muscle contractile proteins. Prog. Biophys. Mol. Biol., 41, 1-41.

MARSTON, S.B., TREVETT, R.M. & WALTERS, M. (1980).
Calcium ion-regulated thin filaments from vascular smooth muscle.
Biochem. J., 185, 355-365.

MARTIN, A.R. & PILAR, G. (1964).
Quantal components of the synaptic potential in the ciliary ganglion of the chick.
J. Physiol., 175, 1-16.

MATSUDA, Y., YOSHIDA, S. & YONEZAWA, T. (1976).
A Ca-dependent regenerative response in rodent dorsal root ganglion cells cultured *in vitro*.
Brain Res., 115, 334-338.

MATSUDA, Y., YOSHIDA, S. & YONEZAWA, T. (1978).
Tetrodotoxin sensitivity and Ca component of action potentials of mouse dorsal root ganglion cells cultured *in vitro*.
Brain Res., 154, 69-82.

McAFEE, D.A. & YAROWSKY, P.J. (1979).
Calcium-dependent potentials in the mammalian sympathetic neurone.
J. Physiol., 290, 507-523.

McGRATH, J.C. (1973).
In: The inhibitory and motor innervation of the anococcygeus muscle.
Ph.D. Thesis, University of Glasgow.

McGRAW, C.F., NACHSHEN, D.A. & BLAUSTEIN, M.P. (1982).
Calcium movement and regulation in presynaptic nerve terminals. In: Calcium and Cell Function, ed. Cheung, W.Y. Vol. 2, pp.81-110. New York, London, Paris. San Diego, San Francisco, Sao Paulo, Sydney, Tokyo and Toronto: Academic Press.

McKIRDY, H.C. & MUIR, T.C. (1978).
An investigation of the role of ganglia in the innervation of the rat anococcygeus muscle: an electrical and mechanical study.
Br. J. Pharmac., 64, 173-184.

MEISHERI, K.D. & VAN BREEMEN, C. (1982).
Effects of β-adrenergic stimulation on calcium movements in rabbit aortic smooth muscle: relationship with cyclic AMP.
J. Physiol., 331, 429-441.

MERRILLEES, N.C.R. (1968).
The nervous environment of individual smooth muscle cells of the guinea-pig vas deferens.
J. Cell Biol., 37, 794-817.

MICHELL, R.H. (1975).
Inositol phospholipids and cell surface receptor function.
Biochim. Biophys. Acta, 415, 81-147.

MIKKELSEN, E., ANDERSSON, K.E. & PEDERSEN, O.L. (1979).
Verapamil and nifedipine inhibition of contractions induced by potassium and noradrenaline in human mesenteric arteries and veins.
Acta Pharmac. Toxicol., 44, 110-119.

MILEDI, R. (1973).
Transmitter release induced by the injection of calcium ions into nerve terminals.
Proc. R. Soc. Lond. B, 183, 421-425.

MILLARD, R.W., LATHROP, D.A., GRUPP, G., ASHRAF, M., GRUPP, I.L. & SCHWARTZ, A. (1982).
Differential cardiovascular effects of calcium channel blocking agents: potential mechanisms.
Am. J. Cardiol., 49, 499-506.

MILLER, R.J. (1985).
Second messengers, phosphorylation and neurotransmitter release.
Trends Neurosci., 8, 463-465.

MINES, G.R. (1908).
On the spontaneous movements of amphibian skeletal muscle in saline solutions with observations on the influence of potassium and calcium chlorides on muscular excitability.
J. Physiol., 37, 408-444.

MOOLENAAR, W.H. & SPECTOR, I. (1979).
The calcium current and the activation of a slow potassium conductance in voltage-clamped mouse neuroblastoma cells.
J. Physiol., 292, 307-323.

MORGAN, J.P., WEIR, W.G., HESS, P. & BLINKS, J.R. (1983).
Influence of Ca^{++}-channel blocking agents on calcium transients and tension development in isolated mammalian heart muscle.
Circ. Res., 52, I-47-I-52.

NAGAO, T., SATO, M., IWASAWA, Y., TAKEDA, T., ISHIDA, R., NAKAJIMA, H. & KIYOMOTO, A. (1972).
Studies on a new 1,5-benzothiazepine derivative (CRD-401). III. Effects of optical isomers of CRD-401 on smooth muscle and other pharmacological properties.
Jap. J. Pharmac., 22, 467-478.

NAKASHIMA, S., TOHMATSU, T., HATTORI, H., OKANO, Y. & NOZAWA, Y. (1986).
Inhibitory action of cyclic GMP on secretion, polyphosphoinositide hydrolysis and calcium mobilization in thrombin-stimulated human platelets.
Biochem. Biophys. Res. Comm., 135, 1099-1104.

NAKAZADO, Y. & ONADA, Y. (1980).
Barium and strontium can substitute for calcium in noradrenaline output, induced by excess potassium in the guinea-pig.
J. Physiol., 305, 59-71.

NAYLER, W.G. & GRINWALD, P. (1981).
Calcium entry blockers and myocardial function.
Fed. Proc., 40, 2855-2861.

NICOL, C.J.M. (1975).
In: Amine accumulation by rabbit erythrocytes.
Ph.D. Thesis, University of Glasgow.

NOMA, A., IRISAWA, H., KOKOBUN, S., KOTAKE, H., NISHIMURA, M. & WATANABE, Y. (1980a).
Slow current systems in the A-V node of the rabbit heart.
Nature, 285, 228-229.

NOMA, A., KOTAKE, H. & IRISAWA, H. (1980b).
Slow inward current and its role mediating the chronotropic effect of epinephrine in the rabbit sinoatrial node.
Pflügers Arch., 388, 1-9.

NONOMURA, Y., HOTTA, Y. & OHASHI, H. (1966).
Tetrodotoxin and manganese ions: effects on electrical activity and tension in taenia coli of guinea-pig.
Science, 152, 97-99.

NORMAN, G.A., FOLLETT, M.J. & HECTOR, D.A. (1974).
Quantitative thin-layer chromatography of ATP and the products of its degradation in meat tissue.
J. Chromatog., 90, 105-111.

NORTH, R.A. (1973).
The calcium-dependent slow after-hyperpolarization in myenteric plexus neurones with tetrodotoxin-resistant action potentials.
Br. J. Pharmac., 49, 709-711.

OCHS, R.S. (1986).
Inositol trisphosphate and muscle.
Trends Biol. Sci., 11, 388-389.

O'CONNOR, P.C. (1982).
Influence of calcium antagonists on ^{3}H-noradrenaline release from sympathetic nerve terminals.
Irish J. Med. Sci., 151, 401P.

OOMURA, Y., OZAKI, S. & MAENO, T. (1961).
Electrical activity of a giant nerve cell under abnormal conditions.
Nature, 191, 1265-1267.

ORIOWO, M.A. (1984).
Effect of calcium-entry blockers and divalent cations on noradrenaline-induced contractions of the rat anococcygeus muscle.
Arch. Internat. Pharmacodyn. Therap., 271, 45-52.

O'ROURKE, F.A., HALENDA, S.P., ZAVOICO, G.B. & FEINSTEIN, M.B. (1985).
Inositol 1,4,5-trisphosphate releases Ca^{2+} from a Ca^{2+}-transporting membrane vesicle fraction derived from human platelets.
J. biol. Chem., 260, 956-962.

OSTERRIEDER, W., PELZER, D., YANG, Q.F. & TRAUTWEIN, W. (1981).
The electrophysiological basis of the bradycardiac action of AQA 39 on the sinoatrial node.
Naunyn-Schmiedebergs Arch. Pharmac., 317, 233-237.

PAPASOVA, M.P., NAGAI, T. & PROSSER, C.L. (1968).
Two-component slow waves in smooth muscle of cat stomach.
Am. J. Physiol., 214, 695-702.

PAYNE, M.E. & SODERLING, T.R. (1980).
Calmodulin-dependent glycogen synthase kinase.
J. biol. Chem., 255, 8054-8056.

PERON, F.G. & McCARTHY, J.L. (1968).
Corticosteroidogenesis in the rat adrenal gland.
In: Functions of the Adrenal Cortex, ed. McKerns, K.W.
Vol. I, pp.261-337. New York: Appleton-Century-Croft.

POISNER, A.M. & TRIFARÓ, J.M. (1967).
The role of ATP and ATPase in the release of catecholamines from the adrenal medulla. I. ATP-evoked release of catecholamines, ATP and protein from isolated chromaffin granules.
Mol. Pharmac., 3, 561-571.

PULL, I. & McILWAIN, H. (1972).
Metabolism of [^{14}C] adenine and derivatives by cerebral tissues, superfused and electrically stimulated.
Biochem. J., 126, 965-973.

RAHWAN, R.G. (1985).
The methylenedioxyindene calcium antagonists.
Life Sci., 37, 687-692.

RAMWELL, P.W., SHAW, J.E., DOUGLAS, W.W. & POISNER, A.M. (1966).
Efflux of prostaglandin from adrenal glands stimulated with acetylcholine.
Nature, 210, 273-274.

RANSOM, B.R. & HOLZ, R.W. (1977).
Ionic determinants of excitability in cultured mouse dorsal root ganglion and spinal cord cells.
Brain Res., 136, 445-453.

RASMUSSEN, H. & GOODMAN, D.B.P. (1977).
Relationships between calcium and cyclic nucleotides in cell activation.
Physiol. Rev., 57, 421-509.

REINER, O. & MARSHALL, J.M. (1975).
Action of D-600 on spontaneous and electrically stimulated activity of the parturient rat uterus.
Naunyn-Schmiedebergs Arch. Pharmac., 290, 21-28.

REINISH, L.W., LJUNG, B. & TUTTLE, R.S. (1986).
Separation of antihypertensive from ganglioplegic effects of felodipine in the cat.
J. Cardiovasc. Pharmac., 8, 367-375.

REUTER, H. (1967).
The dependence of slow inward current in Purkinje fibres on the extracellular calcium-concentration.
J. Physiol., 192, 479-492.

REUTER, H. (1973).
Divalent cations as charge carriers in excitable membranes.
Prog. Biophys. Mol. Biol., 26, 3-43.

REUTER, H. (1983).
Calcium channel modulation by neurotransmitters, enzymes and drugs.
Nature, 301, 569-574.

REUTER, H. (1985).
Calcium movements through cardiac cell membranes.
Med. Res. Rev., 5, 427-440.

REUTER, H. & SCHOLZ, H. (1977).
A study of the ion selectivity and the kinetic properties of the calcium dependent slow inward current in mammalian cardiac muscle.
J. Physiol., 264, 17-47.

RINGER, S. (1883).
A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart.
J. Physiol., 4, 29-42.

ROSTGAARD, J. & BARRNETT, R.J. (1964).
Fine structural localization of nucleoside phosphatases in relation to smooth muscle cells and unmyelinated nerves in the small intestine of the rat.
J. Ultrastruct. Res., 11, 193-206.

RUBIN, R.P. (1970).
The role of calcium in the release of neurotransmitter substances and hormones.
Pharmac. Rev., 22, 389-428.

RÜEGG, J.C., KUHN, H.J., GRÜTH, K., PFITZER, G. & HOFFMANN, F. (1983).
In: Cross bridge mechanism in muscle contraction, eds. Sugi, H. and Pollack, G.J. New York: Plenum Press.

SCHRAMM, M. & TOWART, R. (1985).
Modulation of calcium channel function by drugs.
Life Sci., 37, 1843-1860.

SCHULMAN, H. & GREENGARD, P. (1978).
Ca^{2+}-dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium-dependent regulator".
Proc. Natl. Acad. Sci. U.S.A., 75, 5432-5436.

SCHULTZ, G., HARDMAN, J.G., SCHULTZ, K., BAIRD, C.E. & SUTHERLAND, E.W. (1973).
The importance of calcium ions for the regulation of guanosine 3':5'-cyclic monophosphate levels.
Proc. Natl. Acad. Sci. U.S.A., 70, 3889-3893.

SCHWEITZER, E. & BLAUSTEIN, M.P. (1980).
Calcium buffering in presynaptic nerve terminals: Free calcium levels measured with arsenaro III.
Biochem. Biophys. Acta, 600, 912-921.

SIEGMAN, M.J., BUTLER, T.M., MOOERS, S.U. & MICHALEK, A. (1984).
Ca^{2+} can affect Vmax without changes in myosin light chain phosphorylation in smooth muscle.
Pflügers Arch., 401, 385-390.

SJÖSTRAND, N.O. (1965).
The adrenergic innervation of the vas deferens and the accessory male genital organs.
Acta Physiol. Scand., 65, suppl. 257, 1-82.

SJÖSTRAND, N.O. (1981).
Smooth muscles of vas deferens and other organs in the male reproductive tract. In: Smooth Muscle: an assessment of current knowledge, eds. Bülbring, E., Brading, A.F., Jones, A.W. and Tomita, T., pp.367-376. London: Edward Arnold.

SNEDDON, P. & WESTFALL, D.P. (1984).
Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens.
J. Physiol., 347, 561-580.

SNYDER, S.H. & REYNOLDS, I.J. (1985).
Calcium-antagonistic drugs: receptor interactions that clarify therapeutic effects.
New Eng. J. Med., 313, 995-1002.

SOMLYO, A.V. & SOMLYO, A.P. (1968).
Electromechanical and pharmacomechanical coupling in vascular smooth muscle.
J. Pharmac. exp. Ther., 159, 129-145.

SPECTOR, I., KIMHI, Y. & NELSON, P.G. (1973).
Tetrodotoxin and cobalt blockade of neuroblastoma action potentials.
Nature New Biol., 246, 124-126.

SPEDDING, M. (1983).
Direct inhibitory effects of some 'calcium antagonists' and trifluoperazine on the contractile proteins in smooth muscle.
Br. J. Pharmac., 79, 225-231.

SPEDDING, M. & BERG, C. (1984).
Interactions between "calcium channel agonist", Bay K 8644, and calcium antagonists differentiate calcium antagonist subgroups in K^+-depolarized smooth muscle.
Naunyn-Schmiedebergs Arch. Pharmac., 328, 69-75.

STEINSLAND, O.S., JOHNSON, C.E. & SCRIABINE, A. (1985).
Comparative effects of nitrendipine and verapamil on neuroeffector transmission in the rabbit ear artery.
J. Cardiovasc. Pharmac., 7, 990-995.

STJÄRNE, L. & ÅSTRAND, P. (1984).
Discrete events measure single quanta of ATP secreted from sympathetic nerves of guinea-pig and mouse vas deferens.
Neurosci., 13, 21-28.

STREB, H., IRVINE, R.F., BERRIDGE, M.J. & SCHULZ, I. (1983).
Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol - 1,4,5-trisphosphate.
Nature, 306, 67-69.

STREHLER, B.L. & TOTTER, J.R. (1952).
Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination.
Arch. Biochem. Biophys., 40, 28-41.

SU, C., BEVAN, J.A. & BURNSTOCK, G. (1971).
[^{3}H] Adenosine triphosphate: Release during stimulation of enteric nerves.
Science, 173, 336-338.

SUEMATSU, E., HIRATA, M., HASHIMOTO, T. & KURIYAMA, H. (1984).
Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery.
Biochem. Biophys. Res. Commun., 120, 481-485.

SUTHERLAND, E.W. & RALL, T.W. (1957).
The properties of an adenine ribonucleotide produced with cellular particles, ATP, Mg^{++}, and epinephrine or glucagon.
J. Am. Chem. Soc., 79, 3608P.

TAKATA, Y. & KATO, H. (1984).
Effects of Ca antagonists on the norepinephrine release and contractile responses of isolated saphenous veins to transmural nerve stimulation.
Jap. J. Pharmac., 34, 397-409.

TARLOV, S.R. & LANGER, S.Z. (1971).
The fate of ^{3}H-norepinephrine released from isolated atria and vas deferens: effect of field stimulation.
J. Pharmac. exp. Ther., 179, 186-197.

TEO, T.S. & WANG, J.H. (1973).
Mechanism of activation of a cyclic adenosine 3':5'-monophosphate phosphodiesterase from bovine heart by calcium ions.
J. biol. Chem., 248, 5950-5955.

TILLMAN, J. (1977).
In: Transmitter Release and Drug Responses in the Cat Spleen.
Ph.D. Thesis, University of Glasgow.

TOMITA, T. (1975).
Electrophysiology of mammalian smooth muscle.
Prog. Biophys. Mol. Biol., 30, 185-203.

TOMITA, T. (1981).
Electrical activity (spikes and slow waves) in gastrointestinal smooth muscles.
In: Smooth Muscle: an assessment of current knowledge, eds. Bülbring, E., Brading, A.F., Jones, A.W. and Tomita, T., pp.127-156. London: Edward Arnold.

TRAUTWEIN, W. (1973).
Membrane currents in cardiac muscle fibers.
Physiol. Rev., 53, 793-835.

TRAUTWEIN, W. (1985).
Calcium channels in the membrane of the cardiac myocyte: Physiological and pharmacological control.
Triangle, 24, 101-114.

TRIFARÓ, J.M. (1977).
Common mechanisms of hormone secretion.
A. Rev. Pharmac. Toxicol., 17, 27-47.

TSUBOI, K.K. & PRICE, T.D. (1959).
Isolation, detection and measure of microgram quantities of labeled tissue nucleotides.
Arch. Biochem. Biophys., 81, 223-237.

VAN BREEMEN, C., HWANG, O. & MEISHERI, K.D. (1981).
The mechanism of inhibitory action of diltiazem on vascular smooth muscle contractility.
J. Pharmac. exp. Ther., 218, 459-463.

VANHOUTTE, P.M. (1981).
Calcium entry blockers and the cardiovascular system.
Introduction: Why calcium blockers?
Fed. Proc., 40, 2851.

VAN NUETEN, J.M. & VANHOUTTE, P.M. (1981).
Calcium entry blockers and vascular smooth muscle heterogeneity.
Fed. Proc., 40, 2862-2865.

VENTURA, H.O., MESSERLI, F.H., OIGMAN, W., DUNN, F.G., REISEN, E. & FROHLICH, E.D. (1983).
Immediate hemodynamic effects of a new calcium-channel blocking agent (nitrendipine) in essential hypertension.
Am. J. Cardiol., 51, 783-786.

VERGARA, J., TSIEN, R.Y. & DELAY, M. (1985).
Inositol 1,4,5-triphosphate: a possible chemical link in excitation-contraction coupling in muscle.
Proc. Natl. Acad. Sci. U.S.A., 82, 6352-6356.

VINCENZI, F.F. & WEST, T.C. (1965).
Modification by calcium of the release of autonomic mediators on the isolated sino-atrial node.
J. Pharmac. exp. Ther., 150, 349-360.

WAKADE, A.R. & WAKADE, T.D. (1982).
Relationship between membrane depolarization, calcium influx and norepinephrine release in sympathetic neurons maintained in culture.
J. Pharmac. exp. Ther., 223, 125-129.

WALOGA, G. (1983).
Effects of calcium and guanosine-3':5'-cyclic-monophosphoric acid on receptor potentials of toad rods.
J. Physiol., 341, 341-357.

WATSON, S.P., McCONNELL, R.T. & LAPETINA, E.G. (1984).
The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin.
J. biol. Chem., 259, 13199-13203.

WEBB, R.C. & BHALLA, R.C. (1976).
Calcium sequestration by subcellular fractions isolated from vascular smooth muscle: effect of cyclic nucleotides and prostaglandins.
J. Mol. Cell. Cardiol., 8, 145-157.

WEIDMANN, S. (1955).
Effects of calcium ions and local anaesthetics on electrical properties of Purkinje fibres.
J. Physiol., 129, 568-582.

WEIDMANN, S. (1974).
Heart: electrophysiology.
A. Rev. Physiol., 36, 155-169.

WESTFALL, D.P., STITZEL, R.E. & ROWE, J.N. (1978).
The postjunctional effects and neural release of purine compounds in the guinea-pig vas deferens.
Eur. J. Pharmac., 50, 27-38.

WOLCHINSKY, C. & ZSOTÉR, T.T. (1985).
The effect of diltiazem on noradrenaline release.
Br. J. Pharmac., 85, 387-393.

WOLLHEIM, C.B. (1985).
The mechanism of insulin release, an example of the role of calcium in stimulus-secretion coupling in endocrine cells.
Triangle, 24, 131-141.

WOLLHEIM, C.B. & SHARP, G.W.G. (1981).
Regulation of insulin release by calcium.
Physiol. Rev., 61, 914-973.

YAMAUCHI, T., NAKATA, H. & FUJISAWA, H. (1981).
A new activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca^{2+}-, calmodulin-dependent protein kinase.
J. biol. Chem., 256, 5404-5409.

ZAVOICO, G.B. & FEINSTEIN, M.B. (1984).
Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: antagonism between stimulators and inhibitors of adenylate cyclase.
Biochem. Biophys. Res. Commun., 120, 579-585.