INHIBITION AND EXCITATION IN NON-PROPULSIVE MAMMALIAN SMOOTH MUSCLE

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

by

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October, 1990

C O N T E N T S

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I would like to express my thanks to:

Professor J.S. Gillespie and subsequently Professor T.W. Stone for allowing me to carry out this project in the Department of Pharmacology at the University of Glasgow.

Dr. T.C. Muir, for suggesting the initial idea of this project and for his continued interest, enthusiasm and encouragement in his supervision of my work.

Dr. J.R.C. Baird for providing me with the opportunity to carry out research in the industrial environment of Pfizer Central Research. I am also grateful for his help and advice during my stay with Pfizer.

Dr. S.B. Guild who helped me understand the world of biochemistry a little better and how to carry out radioimmunoassays.

Josie Odber whose friendship I have valued for many years and who always had time to listen to even my most trivial problems.

Jane Nally for providing her stereo-cassette, bad jokes and chatter. We shared the laboratory and equipment, and I am especially grateful to her for making the period of my project a more enjoyable one.

My fellow research students whose paths I crossed, and chatted with, along the way. They are Kay Wardle, Christine Newlands, Leslie Berry, Claire Allen, Shonna Moodie and Kevin Buchan.

John Craig for technical expertise and Trevor Clarke for his help in making and altering organ baths.

Mrs. Marjorie Wright for valuable library assistance.

The staff of Medical Illustration for providing me with slides and posters.

My parents who provided me with never ceasing support and encouragement and especially my Mum for coping with my dreadful handwriting and typing of this thesis.

Finally, my husband, Iain, who helped proof-read and collate this thesis, but most of all for his patience and tolerance when I need them.

PUBLICATIONS

PUBLICATIONS

Several aspects of the work described in the thesis have been published and listed here chronologically.

Muir, T.C. & Stirrat, A.A. (1987). Putative inhibitory transmitters in the guinea-pig internal anal sphincter. Dig. Dis. Sci., 32, 922.

Muir, T.C. & Stirrat, A.A. (1988). Smooth muscle relaxation and membrane hyperpolarisation in guinea-pig internal anal sphincter. Br. J. Pharmacol., 95, 623P.

Baird, A.A. & Muir, T.C. (1989). Cyclic nucleotides and relaxation of guinea-pig internal anal sphincter. J.Gastrointest. Motil., 1, 58.

Baird, A.A. & Muir, T.C. (1990). Membrane hyperpolarisation, cyclic nucleotide levels and relaxation in the guinea-pig internal anal sphincter. Br. J. Pharmacol., 100, 329-335.

SUMMARY

1 Mechanisms underlying relaxation in response to inhibitory NANC nerve stimulation and putative neurotransmitters of these nerves have been examined in the guinea-pig internal anal sphincter (IAS) and compared with those in the bovine retractor penis muscle (BRP) and guinea-pig taenia caeci.

2 Two types of techniques were employed. One which measured the effects of nerve stimulation and drugs on electrical membrane properties where intracellular microelectrode and simultaneous mechanical recording techniques were used. Drugs, for example ATP or cromakalim were applied by perfusion in the Krebs' solution, microinjection into the bath, or by hydrostatic pressure ejection. A second method assessed the underlying biochemical changes accompanying relaxation by measuring alterations in second messenger systems, for example cyclic AMP and cyclic GMP using radioimmunoassay techniques.

3 Electrical events were clearly an important accompaniment to mechanical inhibition in the IAS. Field stimulation (single pulse and 5 pulses at 5,10 & 20Hz; 0.5ms; supramaximal voltage) produced large inhibitory junction potentials of up to 15mV in amplitude which accompanied relaxation of 80% of muscle tone. Indeed, hyperpolarising electrotonic current passed into the IAS produced relaxation.

The neurotransmitter which is released by field stimulation of the inhibitory nerves is probably ATP since exogenous application of purine by hydrostatic pressure ejection ($5.8x10^{-4}$M; 10-55ms) produced a dose-dependent hyperpolarisation. The membrane potential change was similar in

size, rate of decline and duration to the ijp. Neither hyperpolarisation nor relaxation could be achieved with the P_{2x}-purinergic agonist, αβMeATP (10^{-5}-10^{-3}M) or the P_1-purinergic agonist adenosine (10^{-3}M) thus ATP was acting on the P_{2y}-purinergic receptor. Inhibitory NANC neurotransmission was not peptidergic since VIP (10^{-7}-10^{-5}M), bradykinin (10^{-3}M), neuropeptide Y (10^{-5}M), bombesin (10^{-5}M), leu-enkephalin ($1.8x10^{-4}$M), met-enkephalin ($1.8x10^{-5}$M), somatostatin (10^{-6}-10^{-3}M) and substance P ($7.6x10^{-6}$ - $7.6x10^{-4}$M) each had no effect on the membrane potential of the IAS.

There is also evidence that stimulation of β-adrenoceptors by isoprenaline (10^{-9} - 10^{-5}M) produced relaxation which was accompanied by hyperpolarisation of the IAS.

In all cases where hyperpolarisation and relaxation are associated in the IAS, the mechanism underlying the electrical change appeared to be an increase in K^+ conductance. Apamin (4.5×10^{-6}M) which blocks certain Ca^{2+}-mediated K^+ channels, antagonised the electrical and mechanical responses produced by field stimulation and ATP. Similarly, TEA ($8x10^{-2}$M), which blocks most K^+ channels, antagonised the hyperpolarisations and relaxations produced by field stimulation, ATP and isoprenaline.

Indeed, the K^+ channel activator cromakalim (10^{-9}-10^{-5}M) produced hyperpolarisation and relaxation of the IAS suggesting that an increase in K^+ conductance is important in the mediation of mechanical inhibition of the IAS.

4 Relaxation of the IAS was also produced without a significant change in membrane potential by altering the levels of cyclic nucleotides within the smooth muscle cells of the IAS. Forskolin (10^{-9}-10^{-5}M), which activates adenylate cyclase with a subsequent increase in cyclic AMP, relaxed the IAS. Similarly, sodium nitroprusside (10^{-9}-10^{-5}M) - a guanylate cyclase activator, M&B22948 (10^{-9}-10^{-4}M) - a cyclic GMP phosphodiesterase inhibitor, and 8-bromo-cyclic GMP (10^{-4}M) each increased cyclic GMP and produced relaxation of the IAS.

Direct measurement of cyclic nucleotide levels of the IAS showed that field stimulation (80 pulses at $8H_Z$; 0.5ms; supramaximal voltage) and ATP (10^{-4}) elevated the cyclic AMP and cyclic GMP contents of the IAS. All other stimuli which produced slow, prolonged electrical and mechanical changes increased the level of only one cyclic nucleotide. Isoprenaline (10^{-4}M), cromakalim (10^{-5}M) and forskolin (10^{-5}M) increased cyclic AMP content while sodium nitroprusside (10^{-5}M) increased the cyclic GMP content.

Further investigation of other second messenger systems involved in relaxation of the IAS showed that increase in inositol phosphate turnover was not associated with stimulation of inhibitory P_{2y}-purinoceptors by ATP (10^{-2}M) in the IAS. However, an increase in inositol phosphate accumulation was produced by noradrenaline (10^{-4}M) and associated with contraction.

5 A method was devised to measure the intraluminal pressure

changes of the internal anal sphincter in the anaesthetised guinea-pig using a Millar pressure transducer. Using this method the *in vitro* results were largely confirmed by this *in vivo* study. Basal intraluminal sphincter pressure was increased by noradrenaline acting on α-adrenoceptors and decreased by isoprenaline acting on β-adrenoceptors, ATP on P_{2y}-purinoceptors and 2-chloroadenosine on P_1-purinoceptors

CHAPTER I

INTRODUCTION

1. PREFACE

Smooth muscle is diverse in structure and function. However, despite structural and functional dissimilarities contraction and relaxation are fundamental common concerns of all smooth muscles. The understanding of the mechanisms underlying relaxation is important from several points of view. Clinically, it is important in trying to obtain relief from a myriad of pathophysiological disorders such as hypertension, asthma, achalasia, intestinal colic, and biliary spasm. In these conditions the smooth muscle has become contracted for a variety of different reasons and has lost its ability to relax and therefore to perform its physiological role. In many instances this inability to relax can become life threatening. Clearly an understanding of the mechanisms underlying the control of smooth muscle relaxation is a prerequisite to being able to successfully treat smooth muscle dysfunction.

Relaxation of smooth muscle can be induced by the activity of inhibitory nerves, drugs or hormones. Inhibitory nerves through the activity of the neurotransmitters they release, modify and regulate smooth muscle tone. The precise mechanisms by which inhibitory stimuli relax smooth muscle have been a subject of interest for nearly a century. This interest has been maintained with the development of ever more sophisticated techniques to study individual mechanisms of smooth muscle relaxation, for example patch clamp analysis of ion channel activity. Furthermore, in the last few years the importance of smooth muscle relaxation has gained fresh impetus with the establishment of new methods by which smooth muscle tone can be relaxed. For example, it is now accepted that a number of agents thought to act primarily in smooth muscle

cells produce their effects by acting initially on vascular endothelial cells which release a secondary relaxant factor (nitric oxide) to inhibit smooth muscle tone.

In spite of the ability of presynaptic mechanisms to modulate the activity of neurotransmitters, hormones and drugs, the principal site of action of these substances remains post-synaptic. To measure these events a variety of biological techniques has been employed which fall roughly into two categories. First, post-junctional events can be measured electrophysiologically using extracellular, intracellular and patch clamp analysis to ascertain if changes in electrical excitabilty of the cell membrane are responsible for an alteration in muscle tone. Additionally and complementarily, biochemical techniques measure how external signals, which are detected by receptors, are translated into an intracellular effect by investigating signal transduction mechanisms, that is second messenger systems. The best known second messenger involved in smooth muscle relaxation is the cyclic nucleotide, cyclic AMP, although the other cyclic nucleotide, cyclic GMP, is also becoming associated with relaxation as a second messenger. Ideally, both electrical and biochemical techniques should be used in conjunction with one another to ascertain a complete picture of smooth muscle relaxation. In some cases this is not possible, but where it can be done, it can provide a most useful approach to the problem of how relaxation is achieved.

The contribution of both electrical and biochemical measurements will be discussed in this investigation. To date, however, studies of smooth muscle function by relating electrophysiological and second messenger-mediated events carried out in the same laboratory are few. The evidence for the post-synaptic events implicated in relaxation has

been built up from a variety of sources using different types of smooth muscle and experimental techniques. This thesis represents an attempt to eludicate how relaxation occurs in smooth muscle using both electrophysiological and biochemical techniques.

2 CONTROL OF SMOOTH MUSCLE RELAXATION

Mechanical activity in smooth muscle is controlled predominantly by autonomic nerves. The autonomic pathways from the CNS were first traced by Gaskell (1866) who later described them as the cranial, thoraco-lumbar and sacral outflows (see Gaskell, 1916). These outflows were collectively termed the autonomic nervous system (Langley, 1898). Within the autonomic nervous system, two systems were distinguished on the basis of their anatomical location: sympathetic (thoraco-lumbar) and parasympathetic (cranial, sacral).

The sympathetic nervous system has short pre-ganglionic fibres which synapse within ganglia in the paravertebral chains and long post-ganglionic fibres which emanate from these ganglia to their effector organs. The parasympathetic system, on the other hand, has long pre-ganglionic fibres which synapse with ganglia in, or near, the effector organ, and short post-ganglionic fibres which leave these ganglia. The two systems were thought to differ in more than an anatomical way: parasympathetic activity was antagonised by atropine whereas the sympathetic system was not (see Mitchell, 1953; Campbell, 1970). When stimulated the sympathetic and parasympathetic nerves produced apparently antagonistic effects on many of the organs which they supplied.

The concept of neurochemical transmission arose from a study of the

autonomic nervous system. Historically the idea of chemical transmission was first proposed by Elliot in 1905 who suggested that post-ganglionic sympathetic nerves acted by releasing "adrenalin". In 1921, Loewi showed that vagal stimulation of a perfused frog's heart released a substance which slowed the rate of a second frog's heart. He also showed that stimulation of sympathetic nerves to the first heart increased the rate of beating of the second heart. Loewi called the substances 'Vagusstoff' and 'Acceleranstoff', respectively. These observations established that autonomic nerves released specific neurotransmitters which acted on the effector organ (see Bacq, 1975). Nerves were, therefore, classified from this point onwards in terms of the neurotransmitter which they released. During the 1930s Dale and his colleagues demonstrated that in the autonomic nervous system all pre-ganglionic fibres and post-ganglionic parasympathetic fibres released acetylcholine (ACh). Cannon showed that post-ganglionic sympathetic fibres released an adrenaline-like substance he called 'sympathin' - later found to be noradrenaline (Von Euler, 1946). Dale (1933) introduced the terms 'adrenergic' and 'cholinergic' to define the two parts of the autonomic nervous system - 'adrenergic' implying the release of adrenaline (later noradrenaline) and 'cholinergic' implying ACh.

(a) Classical Neurotransmitters

(i) Noradrenergic nerves

Smooth muscle inhibition may occur following stimulation of sympathetic nerves or by application of catecholamines acting on either α- or β-adrenoceptors. That catecholamines acted on two types of membrane adrenoceptors was originally proposed by Ahlquist (1948) and since then

has been amply confirmed and extended by the use of agonists and antagonists. α-Adrenoceptor activation is normally evoked by catecholamines with a potency order of adrenaline ≥ noradrenaline >> isoprenaline and antagonised by drugs including the ergot alkaloids and phentolamine, whilst β-adrenoceptor activation has a potency order of isoprenaline ≥ adrenaline >> noradrenaline and is antagonised by drugs like propranolol.

In general, gastrointestinal smooth muscles, e.g. guinea-pig taenia caecum, contain α-adrenoceptors which mediate relaxation. The response produced by activation of α-adrenoceptors is rapid in onset, short in duration and fast in recovery. (Bolton, 1979; Bülbring et al., 1981).

The mechanical inhibition produced by adrenaline in the guinea-pig taenia caecum is associated with the abolition of spontaneous spike discharge, membrane hyperpolarisation (Bülbring, 1954; 1957) and a reduction in membrane resistance (Shuba, 1961). An increase in membrane permeability was also produced by α-adrenoceptor activation in this tissue (Jenkinson & Morton, 1967a, b) as shown by an increased influx and efflux of K^+. This was abolished by α-adrenoceptor antagonists but not β-adrenoceptor antagonists.

In stomach muscles, catecholamines cause relaxation, contraction or both. The response can be differentiated into α-adrenoceptor and β-adrenoceptor mediated components in the stomach of the guinea-pig, (Bailey, 1971; Yamaguchi & Tomita, 1974), rabbit (Haffner, 1971) and rat (Ogle & Wong, 1971). In the guinea-pig, relaxation of the circular muscle of the stomach is mediated predominatly by α-adrenoceptors; in the longitudinal muscle by β-adrenoceptors (Yamaguchi & Tomita, 1974). In the guinea-pig stomach noradrenaline (NA) caused hyperpolarisation and a

reduction in membrane resistance. The slow waves measured in the stomach remained unaffected by low concentrations and were abolished by high concentrations of NA. In the dog stomach, on the other hand, high concentrations of noradrenaline, largely mediated by α-adrenoceptor activation, decreased the amplitude and duration of the plateau component and increased the frequency of slow waves. NA rarely hyperpolarised the membrane but induced a transient after-hyperpolarisation following each slow wave (El-Sharkawy & Szurszewski, 1978). It would appear that α-adrenoceptor activation produces mechanical inhibition by volage-dependent mechanisms. β-adrenoceptor activation also produces relaxation of smooth muscle and has therefore been studied predominantly on spontaneously active smooth muscles, e.g. myometrium, portal vein and muscles of the gastrointestinal tract. Relaxation mediated by β-adrenoceptors is slow in onset and recovery, in contrast to that mediated by α-adrenoceptors which is fast and transient (Bolton, 1979; Bülbring et al., 1981).

β-Adrenoceptor activation interferes with tension generation; isoprenaline reduced the size of contractions associated with spike potential discharge in strips of smooth muscle (Bülbring & Den Hertog, 1977; Bülbring & Tomita, 1969b). The relaxation produced by β-adrenoceptor activation may (Bülbring & Den Hertog 1980; Diamond & Marshall, 1969a,b; Kawarabayashi & Osa, 1976; Kroeger & Marshall, 1974; Magaribuchi & Osa, 1971; Marshall & Kroeger, 1973; Szurszewski, 1973) or may not (Bülbring & Tomita, 1969b) be associated with membrane hyperpolarisation. Where hyperpolarisation is found, a small increase in the K^+ permeability is probably involved although increases in Ca^{2+} permeability have also been detected (Kroeger & Marshall, 1973; Marshall,

1977). The situation seems very similar to that seen in liver cells (Haylett & Jenkinson, 1972a, b).

Despite the slight hyperpolarisation, the main inhibitory actions of β-adrenoceptor activation seem to be on the complex calcium economy of the cell (Bülbring et al., 1981). Therefore, the contribution which cyclic AMP makes to any β-adrenoceptor mediated response has to be discussed. As in other tissues (Robison et al., 1971), activation of β-adrenoceptors in smooth muscle seems to involve an adenylate cyclase system which generates cyclic 3',5'-adenosine monophosphate (cyclic AMP). The involvement of the cyclic nucleotide in the regulation of smooth muscle has been investigated and widely debated (for review see Hardman, 1981). The relaxation mediated by β-adrenoceptors in rabbit colon was preceded by, and correlated with, an increase in cyclic AMP content (Andersson & Nilsson, 1972). Isoprenaline significantly increased the intracellular level of cyclic AMP in guinea-pig taenia coli (Inatomi et al., 1974). In the same preparation the action of isoprenaline was enhanced and prolonged in the presence of theophylline - a cyclic AMP phosphodiesterase inhibitor (Bülbring & Kuriyama, 1973). In rat uterus, isoprenaline increased tissue cyclic AMP levels, and the increase in the concentration of cyclic AMP followed the same time course as that for relaxation at 37°C and 10°C (Marshall & Kroeger, 1973).

In spite of the many correlations which have been claimed among β-adrenoceptor activation, cyclic AMP and relaxation, evidence exists which prohibits the formation of causal relationship. For example in the rat uterus, adrenaline acting on β-adrenoceptors caused relaxation and increased the level of cyclic AMP and these effects were blocked by propranolol. Yet, in the same tissue, prostaglandins which are excitatory

also caused an increase in the level of cyclic AMP which was not antagonised by propranolol (Vesin & Harbon, 1974; Harbon et al., 1976; 1984; Do Khac et al., 1986) and in guinea-pig taeni caeci, Honda and colleagues (1977) found that relaxation produced by isoprenaline preceded the rise in tissue cyclic AMP. These experimental findings reiterate that, to date, cyclic AMP may not be the exclusive link between β-adrenoceptor activation and smooth muscle relaxation. This evidence suggests that relaxation in response to β-adrenoceptor activation is mediated by cyclic AMP - dependent and - independent means.

(ii) Cholinergic nerves

In the early part of the twentieth century (Dale, 1937) it was known that acetylcholine (ACh) had two main types of action on post-synpatic membranes : 1. a muscarinic, and 2. a nicotinic action. The muscarinic actions are typically those exerted by ACh released from post-ganglionic parasympathetic nerve terminals in the heart, smooth muscle and exocrine glands and are antagonised by atropine. The nicotinic actions of the ACh, on the other hand, are those produced on autonomic ganglia and skeletal muscle and are antagonised by hexamethonium or tubocurarine. However, the muscarinic actions of ACh on smooth muscle are the main concern of this section.

Until 1980 it was assumed that ACh was a potent vascular relaxant in vivo, however it is now well accepted that the vasodilator actions of ACh are dependent on the presence of endothelial cells (for review see Furchgott, 1984). The main effect of ACh on muscarinic receptors on smooth muscle is contraction, with the exception of the rabbit and cat anococcygeus muscles which relax in response to ACh (Creed & Gillespie,

1976; Creed et al., 1977: Gillespie & McGrath, 1974). Thus, ACh has very few inhibitory effects on smooth muscle cells themselves.

(b) Non-Adrenergic, Non-Cholinergic (NANC) Neurotransmission

Indications of a divergence from the "classical" view of peripheral autonomic neurotranmission based on two transmitters, noradrenaline and acetylcholine came to light first of all with the availability of the powerful cholinergic blocking agent, atropine. It was shown that excitation of the urinary bladder in response to pelvic nerve stimulation was not antagonised by atropine (Langley & Anderson, 1895).

Such observations were not restricted to contractile responses. Langley (1898) also showed atropine resistant inhibition of the rabbit stomach in response to nerve stimulation. In dog small intestine vagal stimulation caused relaxation followed by a powerful contraction and neither component was abolished by atropine. This atropine resistance was rationalised within the existing concepts of classical cholinergic and adrenergic neurotransmission at the time, by suggesting the existence of a "peripheral mechanism" (McSwiney & Wadge, 1928) in which the effector itself could determine the response to nerve stimulation, irrespective of the division of the autonomic nervous system being stimulated. Thus, the response to either type of stimulation in low tone would be contraction and in high tone relaxation. Atropine-resistance was also attributed to stimulation of sympathetic nerves within the vagus (Harrison & McSwiney, 1936), and to the inaccessibility of ACh receptors to atropine in the neuroeffector junction (Dale & Gaddum, 1930).

However, it was not until selective, potent adrenergic neurone blocking agents, e.g. guanethidine, were introduced that conclusive

evidence for NANC neurones was discovered. Their availability, along with that of the cholinergic antagonist atropine, allowed both divisions of the autonomic nervous system to be blocked and any remaining neurogenic response to be studied. Such neurone blocking drugs were additionally advantageous because they acted on pre-junctional sites to inhibit transmitter release and were unaffected by problems of access encountered with atropine (Dale & Gaddum, 1930). Adrenergic neurone blocking drugs were used in the investigation of the response of the stomach to nerve stimulation. Relaxation of the cat stomach following vagal stimulation in the presence of atropine was unaffected by adrenergic neurone blocking agents (Martinson & Muren, 1963; Martinson, 1965). Thus the inhibitory responses to vagal stimulation persisted while those to sympathetic nerve stimulation were blocked. The first observation that such responses were produced by non-adrenergic inhibitory neurones, came from the powerful inhibitory response of the guinea-pig taenia coli to transmural nerve stimulation which was not antagonised by bretylium (Burnstock et al, 1963).

Following the initial work by Burnstock et al., (1963) NANC responses were further studied and characterised throughout the 1960s. Field stimulation in the guinea-pig taenia caeci produced inhibitory junction potentials (ijps), which were resistant to adrenergic and cholinergic blocking agents but sensitive to tetrodotoxin (TTX) (Burnstock et al., 1963; 1964; Bennett et al., 1966a; Kuriyama et al., 1967). These responses were quite different from those produced by perivascular stimulation of the sympathetic nerves which innervate the guinea-pig taenia caeci. Stimulation of sympathetic nerves with single pulses, produced neither a mechanical nor an electrical response.

Repetitive stimulation at low frequencies (1-5Hz) reduced spontaneous spike discharge and contractions but did not alter the membrane potential. Small hyperpolarisations became evident only with stimulation at frequencies greater than 10Hz (Gillespie, 1962; Bennett et al., 1966a). On the other hand, single pulses of stimulation to the intramural NANC nerves produced a large membrane hyperpolarisation (up to 25mV) and relaxation. Repetitive stimulation also produced hyperpolarisations which summated to give values of up to 40mV (Bennett et al., 1966b). The maximal electrical and mechanical responses to NANC nerve stimulation were achieved at much lower frequencies (10Hz) than those required for the maximum sympathetically-mediated response (Bennett et al., 1966a,b). The mechanical response to NANC nerve stimulation was more rapid though less well sustained than that produced by sympathetic nerve stimulation (Burnstock et al., 1966). When NANC nerve stimulation ceased, the relaxation was followed by a rebound excitation which could trigger a burst of action potentials and a contraction (Bennett 1966; Campbell 1966; Daniel et al., 1983). This rebound phenomenon may be mediated by a secondary release of prostaglandins (Burnstock et al., 1975) since the after-contraction was abolished by indomethacin, or by run-down of the store of inhibitory transmitter (Holman & Weinrich, 1975) or by the activation of slow excitatory substance P receptors during trains of stimulation (Neil et al., 1983). More recently, a fourth explanation of "rebound" has emerged which suggests that hyperpolarisation activates a cation - specific inward rectifying current (Benham et al., 1986).

NANC-inhibitory responses have been demonstrated throughout the mammalian gastrointestinal tract and include guinea-pig taenia coli

(Kuriyama et al., 1967; Hidaka & Kuriyama, 1969) and the guinea-pig and rabbit colon (Furness, 1969a, b). They are not exclusive to the alimentary canal however, and were found in the trachea (Coburn & Tomita, 1973), lung (Robinson et al., 1971), blood vessels (Hughes & Vane, 1967), gall bladder (Davison et al., 1978) and the accessory muscles of the reproductive tract, including the bovine retractor penis muscle (BRP), (Klinge & Sjöstrand, 1974; Byrne & Muir, 1985) anococcygeus (Gillespie, 1972; Creed et al., 1975) and rectococcygeus (King & Muir, 1981).

Following descriptions of the electrical and mechanical effects subsequent to nerve stimulation, attempts were also made to distinguish NANC nerves by electron microscopical evidence. This had some degree of success and three types of nerve profiles were detected in Auerbach's plexus. Small agranular vesicles (35-60nm) were identified as containing ACh and small dense cored vesicles (40-80nm) were degraded by pretreatment with 6-hydroxydopamine (6-OHDA) and identified as adrenergic. The third type of nerve profile contained large dense cored vesicles (80-110nm) and was termed p-type profiles since they resembled the known peptide-containing vesicles found in the hypothalamus (Baumgarten et al., 1970). Similar results were also reported by Burnstock (1972) and named large opaque vesicles (LOVs). Such nerve profiles (LOVs) had also been noted in a variety of preparations which were known to exhibit NANC reponses, for example toad lung (Robinson et al., 1971), guinea-pig myenteric plexus (Gabella, 1972), avian gizzard (Burnstock, 1972), BRP muscle (Eranko et al., 1976) and rat anococcygeus (Gibbins & Haller, 1979).

Despite the interest shown in the ultrastructure of NANC nerve fibres, the p-type profile as a discrete characteristic has not been

extensively accepted because many discrepancies emerged. For example, in the circular muscle of the rabbit jejunum ijps of up to 25mv resistant to atropine and guanethidine were recorded although few p-type profiles were found (Daniel et al., 1977). Furthermore, detailed comparisons of the nerve profiles from a variety of tissues showed no significant correlation between p-type profiles and NANC responses. Indeed, the profile of NANC nerves in rabbit anococcygeus muscle, hepatic portal vein and toad lung were no different from those of the cholinergic nerves in the atria of the rabbit, guinea-pig and toad (Gibbins, 1982).

Although electron microscopical studies proved ambiguous, other evidence from pharmacological, electrical and histochemical studies have substantiated that a group of nerve fibres exist within the autonomic nervous system which are neither adrenergic nor cholinergic. Arising from and forming an integral part of these subdivisions has been the search to establish the identity of the transmitter(s) involved in the NANC neurones. For any substance to be regarded as a neurotransmitter five criteria must be satisfied (Eccles, 1964). These criteria for a neurotransmitter are:

(1) the substance must be synthesised and stored in nerve terminals; (2) it should be released by a Ca^{2+}- dependent process during nerve stimulation; (3) post-junctional responses following exogenous application of the substance should mimic those produced by nerve stimulation; (4) an inactivation process by enzymes and/or an uptake system for the transmitter and its metabolites should be present; (5) drugs should produce parallel antagonism or potentiation of the responses produced by nerve stimulation and exogenous application of the transmitter. Within this framework of criteria, two main types of

substances have been proposed as inhibitory NANC neurotransmitters; a) purines, e.g. ATP (Burnstock, 1972), which gave rise to the term 'purinergic' nerves and b) peptides, e.g. VIP, substance P, somatostatin, leu-enkephalin, neurotensin (Bloom & Polak, 1978; Humphrey & Fischer, 1978), hence "peptidergic" nerves. It is about the chemical identity of the transmitter that dispute has been greatest and is a topic which has been widely reviewed (Burnstock, 1979, 1981, 1986; Hökfelt et al., 1977; 1980a,b). Application of the criteria for a substance to have a transmitter role is necessary in view of their importance in smooth muscle relaxation. The evidence for both purines and peptides will be considered.

(i) Purinergic Nerves

A wealth of evidence supports the existence of purinergic nerves. The first suggestion that adenosine 5'-triphosphate (ATP) was involved in chemical transmission came from a study of sensory nerve endings (Holton & Holton, 1953; 1954) in which a role in capillary dilation for the purine was proposed. This proposal was supported by the demonstration that ATP was released from the perfused rabbit ear artery preparation in response to antidromic stimulation of the sensory nerves (Holton, 1958). An extensive case has since been put forward for ATP as the NANC neurotransmitter mainly by Burnstock and his colleagues (for reviews see Burnstock, 1979; 1981; 1986).

(1) Presence in nerve fibres

Obviously no substance can be considered as a neurotransmitter if it is not synthesised within the nerve fibres concerned. However, ATP,

because it is vital in energy metabolism, is present and synthesised in all living cells. Furthermore, it is associated with storage vesicles in nerve fibres which are not NANC in nature. Thus its presence in nerves need not imply a neurotransmitter function. Nevertheless, accumulation of [^{3}H]-adenosine occurred in the guinea-pig taenia caeci and the nucleoside was rapidly converted to [^{3}H] -ATP in the region of Auerbach's plexus and therefore presumably in nerves (Su et al.,1971). Conversion of [^{3}H]-adenosine to [^{3}H]-ATP by nerves has been shown in many other tissues which produce NANC responses, for example in the guinea-pig vas deferens (Westfall et al., 1978), thoracic aorta, ear artery and portal vein of the rabbit (Su, 1975).

Histochemistry, involving the use of quinacrine which binds to ATP to produce a fluorescent complex (Irvin & Irvin, 1954), has shown the presence of ATP in nerves. The nucleotide was shown in both cell bodies and varicose fibres in Auerbach's plexus (Olson et al., 1976), urinary bladder (Burnstock et al., 1978b) and anococcygeus muscle (Burnstock et al., 1978a). Moreover, the fluorescence produced by the quinacrine - ATP complex was reduced by depolarisation (Alund & Olson, 1979). These findings have been used to support the view that quinacrine binds to a compound, presumably ATP, which is released by nerve stimulation.

(2) Release by nerve stimulation

Studies on the perfused stomach of the guinea-pig and toad gave the first indications that ATP was released by nerve stimulation. Stimulation of the vagus nerve to Auerbach's plexus in the tissues (Burnstock, 1970) produced the ATP breakdown products adenosine and inosine in the perfusion fluid. That ATP itself had been released from

nerve endings was merely inference. Radioactivity was released in response to nerve stimulation from guinea-pig taenia caeci pre-incubated with radiolabelled adenosine and this release was abolished by TTX (Su et al., 1971). The luciferin - luciferase luminescence technique has more recently made it possible to measure ATP directly (Strehler & Totter, 1952). This method relies on the interaction of ATP with synthetic luciferin and with luciferase extracted from firefly tails to produce luminescence. Using this technique, detection of ATP following field stimulation was claimed in guinea-pig taenia caeci, urinary bladder, rat anococcygeus and rabbit rectococcygeus (Burnstock et al., 1978 a, b & c; Cocks et al., 1979). Furthermore, lowering the external calcium concentration reduced release and TTX abolished it completely in the guinea-pig taenia caeci (Burnstock, 1978).

The possibility remained that, because of its universal occurrence and the myriad of functions in which it is involved, the ATP released may not have been located within the nerve terminals themselves, but may have arisen from another source. The nerve membrane is a potential source of ATP during the propagation of an action potential, for example (Burnstock et al., 1978c). However, the amount of nucleotide collected during stimulation of NANC nerves was estimated to be some 1000-fold greater than that released as a direct result of activation of the axon membrane during impulse propagation. ATP could also have been released secondarily from the muscle rather than the nerve terminals. This problem has been addressed and resolved. It was shown that while a 2-6 fold increase in ATP release from the guinea-pig taenia caeci and urinary bladder occurred subsequent to activation of NANC nerves, no significant rise in ATP release occurred in response to direct muscle stimulation (Burnstock et

al., 1978c).

More recently, release of endogenous ATP was shown in response to transmural electrical stimulation of sympathetic nerves in the guinea-pig vas deferens (Lew & White, 1987) in the presence of 4-aminopyridine to enhance the response by the luciferin-luciferase technique. The release of ATP was not due to contraction since the measured response persisted in the presence of prazosin and αβmethylene ATP (αβMeATP) which blocked the contraction, although it was TTX-sensitive. This evidence suggested that ATP was released presynaptically giving further credibility to the work carried out by Burnstock and his colleagues in the 1970s on the release of ATP presynaptically from nerves. Furthermore, release of ATP has been measured not only during the contractile response in which the nucleotide very often acts as a co-transmitter released from sympathetic nerves (Lew & White, 1987), but also in the inhibitory response. ATP has been measured by the luciferin-luciferase technique following transmural stimulation of NANC nerves in the guinea-pig internal anal sphincter (Beattie, 1986). Thus release of ATP from nerves has been shown in both excitatory and inhibitory responses.

(3) Mimicry of nerve stimulation

The exogenous application of a substance proposed as a neurotransmitter should produce a response similar to that produced by stimulation of the nerves themselves.

Adenine nucleotides, particularly ATP, closely mimic both NANC nerve-mediated inhibitory and excitatory responses (Ambache & Zar, 1970; Burnstock et al., 1970; 1972; Burnstock, 1972; 1979, 1981). In the case of the inhibitory response, the latency and duration of the relaxation

produced by ATP and nerve stimulation is similar. This is particularly well documented in gastrointestinal smooth muscle, for example the stomach, colon and ileum of the guinea-pig and rabbit, the rat gastric fundus, duodenum and colon, the mouse duodenum and colon (Burnstock et al., 1970; Okwuasaba et al., 1977; McKenzie et al., 1977; Huizinga & Den Hertog, 1980) and the guinea-pig internal anal sphincter (Crema et al., 1983; Lim & Muir, 1986). The post-synaptic 'rebound' excitation is also a feature of the response produced by exogenous ATP, previously noted as a characteristic of the NANC response.

The inhibitory NANC nerve-mediated responses can be mimicked by ATP in vascular smooth muscle as well as the smooth muscles of the alimentary canal. Vasodilatation is observed with nerve stimulation and ATP in guinea-pig uterine artery (Bell, 1976), rabbit portal vein (Hughes & Vane, 1967) and bull penile artery (Klinge & Sjöstrand, 1974).

Indeed, ATP not only mimics the relaxation produced by NANC nerve activation but also the accompanying electrical effects. This similarity has been shown in the guinea-pig taenia coli (Axelsson & Holmberg, 1969; Tomita and Watanabe, 1973; Jager & Schevers, 1980) ileum (Bauer & Kuriyama, 1982b), stomach (Vladimirova & Shuba, 1978), internal anal sphincter (Lim & Muir, 1986) and rabbit caecum (Small, 1974). In all these cases NANC nerve stimulation produced powerful, rapid and brief inhibitory junction potentials which immediately preceded the relaxation. Similarly, exogenously applied ATP also produced large, rapid and brief hyperpolarisations which preceded the inhibition of tone in the guinea-pig internal anal sphincter (Lim & Muir, 1986).

(4) **Blockage of the responses to NANC nerve stimulation and ATP**

Any substance which antagonises the effects of a putative neurotransmitter should also block the effects produced by NANC nerve stimulation itself. However, selective antagonists have, as yet, been very difficult to find and their absence is therefore a major obstacle in the acceptance of the 'purinergic' nerve hypothesis.

As with many other peripherally acting neurotransmitters, it seems that there is more than one type of antagonist. Methylxanthines are claimed to be competitive antagonists of adenosine in both vascular and non-vascular smooth muscle (see Burnstock, 1978). Both the vasodilation in the brain (Oberdörster et al., 1975) and the vasoconstriction in the kidney (Osswald, 1975) produced by the nucleoside were antagonised by theophylline, for example. In non-vascular smooth muscle a similar picture is observed. The relaxation of the trachea (Coleman, 1976) and ileum (Ally & Nakatsu, 1976) produced by adenosine were blocked by aminophylline and theophylline respectively.

The ability of those components to block the responses to adenosine however, was greater than their ability to antagonise those to NANC nerve stimulation. This was shown clearly in the rabbit duodenum, where theophylline inhibited the responses to adenosine but not to NANC nerve stimulation (Huizinga & Den Hertog, 1980). Such evidence weakens the case for the use of such compounds in the investigation of the NANC neurotransmitter.

Specific ATP antagonists have been more difficult to obtain and although quinidine, 2-substituted imadazolines and 2'2-pyridylisatogen tosylate have been used they are non-specific (Burnstock, 1978). For example, 2'2 pyridylisatogen tosylate (PIT) blocked ACh and histamine

receptors at similar concentrations to those which inhibited ATP responses (Burnstock et al., 1978a). In the guinea-pig taenia coli this drug attentuated the inhibitory effect produced by exogenous ATP but not that of NANC nerve stimulation (Spedding et al., 1975). Phentolamine and imadazoline, in a concentration exceeding that required to block α-adrenoceptors, also abolished the inhibitory response of the guinea-pig taenia coli to exogenous ATP without antagonising that to field stimulation (Ambache et al., 1977a). From this evidence it seemed there was a lack of specificity of these compounds as antagonists for either the neuronally-released transmitter(s) or ATP.

More recently however, major contributions to this field have been made by the availability of two substances. First, arylazido aminopropionyl ATP ($ANAPP_3$; Hogaboom et al., 1980) was claimed to be a specific ATP antagonist. Indeed, $ANAPP_3$ abolished the contractile response to ATP and NANC nerve stimulation in the urinary bladder of the guinea-pig (Westfall et al., 1983) and cat (Theobald, 1982) and in the guinea-pig vas deferens (Sneddon & Westfall, 1984). However, this antagonist failed to inhibit the relaxant effects produced by ATP and NANC nerve stimulation in guinea-pig stomach (Frew & Lundy, 1982) and rabbit rectococcygeus (Sneddon et al., 1982). This inability to block inhibitory but not excitatory responses to ATP was also demonstrated with the second substance, αβmethylene ATP (αβMeATP; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984; Allcorn et al., 1985) a stable analogue of ATP, which acts by desensitisation (Kasakov & Burnstock, 1983). However, doubt was cast on αβMeATP as a specific antagonist of the excitatory responses produced by ATP. In the rat basilar artery αβMeATP inhibited not only the depolarisation produced by ATP but also that

produced by noradrenaline (Byrne & Large, 1984). Furthermore it was suggested that αβMeATP acted not by desensitisation as previously described (Kasakov & Burnstock, 1983) but by blocking ion channels (Kotecha & Neild, 1987). Nevertheless, from the work using these compounds there are differences between the receptors mediating contraction and those mediating relaxation which have been identified.

Apamin, a bee-venom peptide, irreversibly blocked the hyperpolarisation produced by ATP and NANC neurotransmission in the guinea-pig taenia coli (Vladimirova & Shuba, 1978). This led to the belief that apamin was a specific antagonist of ATP and gave strong support for ATP as the inhibitory NANC neurotransmitter. However, it soon became clear that apamin also blocked the hyperpolarisation produced in response to α-adrenoceptor agonists and the ijps in response to perivascular stimulation of the guinea-pig taenia coli. Thus it appeared that apamin acted not as a purine nucleotide blocking drug but rather it blocked particular Ca^{2+}- mediated K^{+} channels (Banks et al., 1979; Maas & Den Hertog, 1979; Maas et al., 1980).

The existence of a class of ATP antagonists, the anthraquinane-sulphonic acid derivatives, including reactive blue 2 were described by Kerr & Krantis (1979). The ATP - but not the adenosine - induced relaxations of the guinea-pig internal anal sphincter were antagonised by reactive blue 2 (Crema et al., 1983). Similarly, relaxation in the rat duodenum produced by ATP and low frequency (0.1Hz) field stimulation was antagonised by the compound (Manzini et al., 1985). Further studies in the rat caecum showed that reactive blue 2 antagonised ijps produced by field stimulation and αβMeATP-induced hyperpolarisations (Manzini et al., 1986). These workers suggested that reactive blue 2 acted on

post-junctional P_2-purinoceptors and/or interfered with biochemical processes regulating the availability of K^+ or Ca^{2+} channels, underlying spike potential generation or the ijp. Thus, reactive blue 2 and other anthraquinane-sulphonic acid derivatives cannot, as yet, be described as specific P_2-purinoceptor antagonists.

Recently it was found that the anti-trypanosomal agent suramin, which inhibited intracellular oxidative enzymes (Fairlamb & Bowman, 1977) and calcium transport processes (Layton & Azzi, 1974), also inhibited the contraction produced by ATP in the mouse vas deferens (Dunn & Blakely, 1988). The hyperpolarisation and relaxation produced by ATP in the guinea-pig taenia caeci were also suppressed by this agent without affecting the α-adrenoceptor response (Den Hertog et al., 1989). However, as with other purine antagonists, the selectivity of this compound has been questioned. In the rabbit saphenous artery suramin inhibited the contractile responses produced not only by ATP but also by 5-hydroxy-tryptamine (5-HT) and histamine (Nally & Muir, personal communication).

Until selective antagonists for purines are developed, one of the main criteria for the establishment of a substance as a neurotransmitter remains unfulfilled.

(5) Inactivation

Both the excitatory and inhibitory responses produced by NANC nerve stimulation not only developed rapidly but also recovered rapidly, which suggested the presence of a quick and effective inactivation process for the transmitter. For ATP this does not present a problem, since the nucleotide can be dephosphorylated by ecto-ATPases and a 5'-nucleotidase to adenosine (see Maguire & Satchell, 1979). The adenosine is then

inactivated by either deamination to inosine, or uptake into smooth muscle or neurones. Burnstock (1972; 1979) has built up a complete picture of inactivation from these observations and proposed the 'purinergic' hypothesis. After release and the activation of purinergic receptors, ATP is rapidly broken down by extracellular enzymes (Mg^{2+}-activated ATPase and 5-nucleotidase) to adenosine and then taken back into nerve endings by a high affinity uptake system for resynthesis into ATP within the large dense-cored vesicles, where it is available for release. Any adenosine which is not taken up is further metabolised to inosine by adenosine deaminase and is removed in the circulation, since inosine is pharmacologically inactive and cannot be taken up into nerves.

Summary of how purines meet the criteria

In spite of the wealth of evidence which has been obtained in support of the idea that a purine, like ATP, can act as a neurotransmitter it is still viewed with scepticism throughout the scientific community. The main reservations are undoubtedly because of the ubiquitous nature of ATP as the cofactor or provider of energy on which many enzyme systems rely. However, dubiety has also arisen partly because some groups had difficulty repeating certain results proposed by proponents of the hypothesis and partly because of the lack of specificity of antagonists claimed to be selective for ATP. Indeed, the antagonists which were used in the 'purinergic' hypothesis (Burnstock 1972) including theophylline and 2,2'-pyridylisatogen tosylate, were subsequently shown to be non-specific (Small & Weston, 1979; Spedding et al., 1975).

Objections have also been raised because of the high concentration of ATP required to mimic the NANC nerve response. One explanation for the use of these high concentrations has come from work carried out on the urinary bladder of the rat (Brown et al., 1979). This suggested that there was a rapid breakdown of ATP (which is contractile) to adenosine (which is relaxant) and so the breakdown product would be antagonistic and inhibit the ATP response. In support of this, the stable analogue of ATP, αβMeATP which is not degraded to adenosine is more potent than ATP itself in producing contractile responses.

Furthermore, while many of the preparations used by Burnstock and his colleagues (1970) responded to ATP in a similar manner to NANC nerve stimulation, many more including the pig stomach (Ohga & Taneika, 1977), opossum lower oesophageal sphincter (Daniel et al., 1979) and rat anococcygeus (Gillespie, 1972) did not. Moreover, in the guinea-pig ileum (Weston, 1973a), taenia caeci (Ambache et al., 1977a), urinary bladder (Ambache & Zar, 1970; Ambache et al 1977b), rabbit duodenum (Weston, 1973b) and pig stomach (Ohga & Taneika, 1977) it was demonstrated that all were desensitised to ATP while simultaneously retaining their response to nerve stimulation. In the anococcygeus muscles of the cat, rabbit and rat which have an inhibitory NANC innervation (Creed & Gillespie, 1977; Creed et al., 1977; Gillespie & McGrath, 1974; Gillespie & McKnight, 1978) ATP was shown to mimic nerve stimulation in the cat and rabbit. However, it was argued that the purine could not be the neurotransmitter because it produced contraction of the rat anococcygeus and the transmitter was likely to be the same substance in the three species. This paradox was given an explanation since in the presence of indomethacin, a prostaglandin synthesis inhibitor, the contraction

produced by ATP in the rat anococcygeus was converted to a relaxation (Burnstock et al., 1978a). This suggested that the inhibitory action of ATP was masked by the potent contractile action of secondary prostaglandins. Thus ATP could be the inhibitory NANC neurotransmitter in the anococcygeus muscles of the three species, rat, cat and rabbit.

In the final analysis, the evidence weighs heavily on the side of ATP as an NANC neurotransmitter although it need not be the universal NANC neurotransmitter. Many gaps in the literature still exist and must be filled before the 'purinergic' hypothesis receives widespread acceptance.

(i) Purinergic Receptors

Given that a purine could be a neurotransmitter in NANC nerves it seems that, as with many other peripherally acting neurotransmitters, there is more than one type of purinoceptor (Burnstock, 1978). This conclusion was based on the rank order of potency of agonists which demonstrated two main categories; P_1-purinoceptors which were more sensitive to adenosine and adenosine 5'-monophosphate (AMP) than to ATP, and P_2-purinoceptors, which were more sensitive to ATP and adenosine 5'-diphosphate (ADP). Furthermore P_1-purinoceptor occupation was suggested to lead to changes in intracellular cyclic AMP levels whereas P_2-purinoceptor occupation did not, although in some cases the latter evoked prostaglandin synthesis.

Biochemical, pharmacological and receptor binding studies led to a proposed subdivision of P_1-purinoceptors into A_1 and A_2 receptors (Van Calker et al., 1979) or R_i and R_a receptors (Londos et al, 1980). The R_i and A_1 receptors appear to be analogous and their occupation

leads to the activation of adenylate cyclase (see Kennedy & Burnstock, 1984). The R_a or A_2 receptor is also more susceptible to 5'-carboxamide analogues of adenosine such as 5'-N-ethylcarboxamide adenosine (NECA) and less responsive to N^6-substituted analogues such as L-N^6-phenylisopropyl adenosine (L-PIA), whereas the reverse is true for R_i or A_1 receptors (Bruns et al., 1980).

Like the P_1-purinoceptors, and many other receptor populations, P_2-purinoceptors do not form a homogeneous group. A proposed subdivision of P_2-purinoceptors was based on the actions of apamin (Shuba & Vladimirova, 1980) into those mediating contraction and those mediating relaxation and also on anatomical location (Su, 1981). However, support for a subclassification of P_2-purinoceptors from studies of rank order of potency of structural analogues of ATP (see Burnstock & Kennedy, 1985) is perhaps more substantive. P_2-purinoceptors which showed a potency order of αβMeATP, βγMeATP > ATP = 2-methylthio ATP have been designated P_{2x}-purinoceptors. On the other hand, those which showed a potency order of 2-methylthio ATP >> ATP > αβMeATP, βγMeATP have been designated P_{2y}-purinoceptors. More basic differences exist between the subdivision of P_2-purinoceptors as suggested previously from the evidence on apamin (Shuba & Vladimirova, 1980). The P_{2x}-purinoceptor mediates contraction, for example in the rat vas deferens and urinary bladder of the guinea-pig (Kasakov & Burnstock, 1983; Burnstock et al., 1983; Fedan et al., 1982; Meldrum & Burnstock, 1983; Burnstock et al., 1985; Brown et al., 1979; Dahlen & Hedquist, 1980; Taylor et al., 1983) while the P_{2y}-purinoceptor mediates relaxation seen in the guinea-pig taenia coli and internal anal sphincter (Burnstock et al., 1983; Gough et al., 1973; Satchell & Maguire, 1975; Lim & Muir, 1986).

P_{2x}-purinoceptors were antagonised by $ANAPP_3$ and selectively desensitised by αβMeATP. In contrast P_{2y}-purinoceptors were only partially antagonised by these drugs although the action of ATP mediated by P_{2y}-purinoceptors could be blocked by apamin.

More recently a third subclassification of the purinoceptor has come to light. This third type of receptor was shown to be presynaptic and controlled the release of NA from sympathetic nerve endings in the rat caudal artery (Westfall et al., 1989). This unique receptor was termed P_3-purinoceptor and both P_1-purinoceptor and P_2-purinoceptor agonists were shown to interact with it. Furthermore xanthines antagonised responses mediated by P_3-purinoceptors as did αβMeATP.

(ii) Peptidergic Nerves

Peptides have also been proposed as NANC neurotransmitters especially in the gastro-intestinal tract. The term 'peptidergic neurone' was initially proposed to describe those neurones in the hypothalamus involved in the secretion of hormones (Bargmann et al., 1967). Since then, over thirty peptides including substance P, neuropeptide Y, somatostatin, vasoactive intestinal polypeptide (VIP) and bombesin have been proposed as neurotransmitters in the peripheral and central nervous systems (for reviews see Otsuka & Takahashi, 1977; Hökfelt et al., 1980a,b; Iversen, 1983).

Uncertainty that a peptide could act as a neurotransmitter has arisen partly because 'classical' transmitters have always been small molecules (molecular weight ~200) whereas some of the peptides are much larger and consist of up to 30 amino acids (molecular weight ~3000) and

partly because many peptides were previously thought of as peripheral hormones rather than neuoronally-released transmitters. However, the idea of a compound being both a hormone and a neurotransmitter is not unusual, after all NA and adrenaline have long been accepted in such a dual role. More importantly, however, the method of replenishment of peptide neurotransmitters in the nerve endings differs fundamentally from that of classical neurotransmitters. Intraneuronal NA levels are maintained by enzymatic synthesis in nerve endings, re-uptake from extraneuronal space and by supply of amine in storage vesicles from the cell body by axonal transport (for review see Hökfelt et al., 1980a). Peptides could be released intermittently rather than tonically and such intermittent release would be compensated for by a long duration of action. Furthermore, peptides may be more potent than 'classical' neurotransmitters, hence the amount of peptide released would be small which would compensate for an inefficient replacement of released transmitter (for reviews see Hökfelt et al., 1980a, b; Iversen 1983).

As with purines, the peptides which have been suggested as neurotransmitters must fulfill the criteria proposed by Eccles (1964) for a neurotransmitter, however, few have been successful to date.

(1) Presence in nerves

Immunohistochemistry, radioimmunoassay and bioassay techniques have been important means of showing the presence of peptides in autonomic nerves.

In the mid 1970s important findings suggested that all NANC nerves were unlikely to belong to a homogeneous population served by one neurotransmitter. Systematic electron microscopic studies (Cook &

Burnstock, 1976) revealed up to nine morphologically distinguishable types of neurone in the enteric plexi, including some nerve profiles containing a complex mixture of vesicles. This suggested that the profiles might contain more than one transmitter. Immunohistochemistry methods have localised biologically active polypeptides in autonomic nerves such as enkephalin, substance P, VIP, neurotensin, somatostatin, gastrin releasing peptide (GRP), neuropeptide Y (NPY)/pancreatic peptide (PP), bombesin (BN), cholecystokinin (CCK), calcitonin gene-related peptide (CGRP), and most recently galanin (GAL) (Hökfelt et al., 1980a, b; Furness & Costa, 1981; Melander et al., 1985). VIP and substance P immunoreactive fibres were the most abundant, particularly in autonomic ganglia and Auerbach's plexus. In the colon, VIP was localised in p-type (allegedly NANC) fibres in the lamina propria and submucous plexus (Larrson, 1977). The taenia coli was innervated by both VIP and substance P immunoreactive nerves (Jessen et al., 1980), suggesting possible roles in the inhibitory and "rebound" responses, respectively, produced by NANC nerve stimulation. Nerve cell bodies containing VIP in the myenteric plexus, project in an anal direction to supply the circular coat of the intestinal wall (Furness & Costa, 1980) and these may mediate the descending inhibition which is involved in peristalsis (Hirst & McKirdy, 1974; Costa & Furness, 1976).

(2) Release by nerve stimulation

Vasoactive inhibitory peptide levels rose in the portal and systemic circulation in the pig and cat during stimulation of the vagus or pelvic nerves to the colon. The release was maximal at 8Hz and did not fatigue readily (Fahrenkrug, et al., 1978 a, b). VIP release was also observed

from other gastrointestinal smooth muscles with a known NANC innervation including lower oesphageal sphincter (Goyal & Cobb, 1981), taenia coli (Fahrenkrug et al., 1978a) and small intestine (Fahrenkrug et al., 1978b), as well as from enteric vasodilator nerves (Fahrenkrug et al., 1978b). These results suggested that VIP could be the neurotransmitter released by NANC inhibitory nerves in these tissues.

(3) Mimicry of nerve stimulation

VIP is a powerful inhibitor of many smooth muscles including those of the gastrointestinal (Furness & Costa, 1981), respiratory (Kitamura, et al., 1980) and urinogenital (Sjöstrand et al., 1981) tracts.

VIP relaxed the lower oesophageal sphincter (Rattan et al., 1977; Uddmann et al., 1978; Behar et al., 1979; Siegel et al., 1979), the cat stomach (Fahrenkrug et al., 1978b; Eklund et al., 1979), the small intestine (Kachelhaffer et al., 1976) and the guinea-pig taenia coli (Cocks & Burnstock, 1979). Close-arterial infusion of VIP relaxed the stomach (Eklund et al., 1979) and, along with evidence that VIP is released from the corpus fundus of the stomach during stimulation of the vagus (Fahrenkrug et al., 1978 a, b), strongly supported a transmitter role for VIP. Furthermore, in the lower oesophageal sphincter of the opossum VIP mimicked the relaxation produced by stimulation of the NANC nerves. However, there is a clear discrepancy between the response produced by VIP and that by NANC nerve stimulation in the guinea-pig taenia coli. The relaxation to VIP was slow in onset, slow to develop and persistent whereas that to NANC nerve stimulation had a short latent period, developed rapidly and was poorly maintained (Cocks & Burnstock, 1979). Thus it would seem that in the guinea-pig taenia coli at least, another

neurotransmitter is involved although VIP can elicit relaxation.

Low concentrations of VIP (10^{-12} - 10^{-10}M) relaxed the smooth muscle of the cat trachea with no accompanying electrical change. However, at higher concentrations (10^{-8}M) VIP produced both hyperpolarisation and relaxation (Ito & Takeda, 1982).

There is evidence which suggests that VIP may be one of the inhibitory NANC neurotransmitters and in some cases VIP mimics NANC nerve-mediated responses, however the picture is as yet not complete.

(4) Blockade of response to NANC nerve stimulation and peptides

The lack of any synthetic receptor blocking drug has hindered pharmacological analysis of the role of peptides as neurotransmitters. Advantage has therefore been taken of the ability of peptides to produce antisera which can act as selective blocking agents by antagonising and desensitising the relevant receptors to the particular peptide. VIP-antiserum reduced the relaxation of opossum lower oesophageal sphincter (Goyal et al., 1980), rabbit internal anal sphincter (Biancani et al., 1983) and cat trachea (Ito & Takeda, 1982) in response to both exogenous VIP and electrical or chemical stimulation of NANC nerves. In the opossum lower oesophageal sphincter, the effects appeared to be selective because the relaxation produced by isoprenaline was not blocked (Goyal et al., 1980; Rattan et al., 1982). However, this work could not be repeated in human oesophageal and gastric muscles (de Carle & Pye, 1982) with VIP-antiserum.

Desensitisation to the inhibitory effect of VIP on the cat trachea was accompanied by a reduction in the response to inhibitory nerve stimulation (Ito & Takeda, 1982), suggesting that receptors occupied by

exogenous VIP and neuronally-released transmitter were similar. However, in the guinea-pig taenia coli chymotrypsin which digests certain peptides abolished the inhibitory effect of exogenously added VIP but had no effect on the inhibitory reponse produced by the NANC nerve-mediated response. Conversely, apamin blocked the NANC nerve-mediated response but failed to antagonise the actions of VIP (MacKenzie & Burnstock, 1980; Hills et al, 1983). Thus, VIP may be the neurotransmitter in some cases but there is clear evidence that it is not the universal NANC neurotransmitter.

(5) Inactivation

The rapid recovery following cessation of NANC nerve stimulation suggests an efficient inactivation mechanism exists for the neurotransmitter(s). This is generally achieved for other neurotransmitters by enzymatic breakdown and/or reuptake into nerve endings or at post-junctional sites. No evidence for such a mechanism for peptides has been reported to date (Hökfelt et al., 1980a).

VIP has a half-life of between 4 and 15 minutes (Fahrenkrug, 1979) which suggested a slow destruction by a peptidase. This is consistent with the prolonged inhibition produced by VIP (Cocks & Burnstock, 1979) and is not encouraging for its role as a transmitter involved in the mediation of rapid and brief inhibitions of smooth muscle.

Summary of how peptides meet the criteria

There is a great deal of evidence for the presence of biologically-active peptides in the central and peripheral nervous systems. However in spite of this, few, if any, fulfill all of the

criteria necessary to establish a substance as a neurotransmitter. The major stumbling blocks lie with the lack of specific antagonists and the lack of rapid inactivation mechanisms.

In the gastrointestinal tract, despite its slow and prolonged actions, VIP is the most likely candidate as a neurotransmitter. However, these observations, together with VIP's ability to circulate in the local blood supply with a prolonged half-life, are more in keeping with a role associated with a neurohumoral agent rather than a neurotransmitter. Hence VIP might better be described as part of the neuroendocrine system.

VIP has been implicated not only in NANC neurotransmission but also in co-transmission. Discovery of the co-existence of more than one neuroactive substance in the same neurone was the result of histological work. One of the first of these studies was carried out by Koelle (1955) who showed the distribution of acetylcholinesterase (AChE) in the cells of ciliary and stellate ganglia from cats, rabbits and rhesus monkeys. High concentrations of AChE were localised, not surprisingly, in the cholinergic neurones of all species but variable concentrations were present also in several adrenergic and sensory neurones. More recently, a growing body of evidence has shown that more than one substance can be released in response to nerve stimulation (Burnstock, 1976; 1982; 1985; 1986; Cuello, 1982; Osborne, 1983).

In the majority of cases where co-transmission has been shown the mechanical effector responses are contractile (Neild, 1987; Langer & Pinto, 1976; Westfall et al., 1978; Su, 1975; Head et al., 1977; McGrath, 1978). One example where an inhibitory effect has been observed with co-transmission is in the submandibular salivary gland of the cat

(Lundberg & Hökfelt, 1983). Here, parasympathetic nerves innervate both the blood vessels and the exocrine acinar cells (Garrett, 1974) and, with nerve stimulation, produce salivary secretion and an increased local blood flow. Immunohistochemical analysis (Lundberg, 1981) suggested that, in addition to ACh, these parasympathetic nerves contained a VIP-like peptide. Furthermore, electrical stimulation increased the overflow of both ACh and VIP into the venous effluent of the gland (Lundberg et al., 1982) confirming the earlier immunohistochemical reports. At low frequencies of nerve stimulation (2Hz) the induced secretion and vasodilation was potentiated by eserine and abolished by atropine which suggested that ACh mediated both salivation and the increased blood flow at these frequencies. At higher frequencies (>10Hz) however, the vasodilation was atropine-resistant and there was a particularly large release of immunoreactive VIP (Lundberg et al., 1981; 1982). The effect then at the higher frequencies was attributed to VIP.

(iii)Nitric Oxide

Endothelium-derived relaxant factor (EDRF) is released from vascular endothelial cells in response to a number of substances including acetylcholine and bradykinin (for review see Furchgott, 1984). The existence of EDRF was first described by Furchgott and Zawadzki (1980) to account for the discrepancy between the depressor response of ACh in vivo and its pressor response in vitro. With the discovery of this labile humoral substance, speculation concerning its chemical nature developed, and over the last few years it has been identified as nitric oxide (NO; Palmer et al., 1987; Hutchinson et al., 1987; Ignarro et al., 1987a,b; 1988; Radomski et al., 1987) or a closely related substance. NO is

synthesised from the terminal guanido nitrogen atom(s) of the amino acid L-arginine (Palmer et al., 1988a). This biosynthetic reaction was shown to be specific because the D-isomer and other analogues of the amino acid were not able to act as substrates for the synthesis of NO. Indeed, L-N^9-monomethyl arginine inhibited the reaction in endothelial cells (Palmer et al., 1988b). Furthermore, on its on own L-N^9-monomethyl arginine produced endothelial-dependent contractions. This suggests that tonic production of NO exists which can be regulated by manipulating the substrate for the reaction.

Both EDRF and NO relaxed not only vascular smooth muscle but also a variety of non-vascular smooth muscles, for example BRP muscle, bovine trachea, rat anococcygeus and stomach fundus, guinea-pig trachea and rabbit taenia coli (Gillespie & Sheng, 1988b; Buga et al., 1989) all of which contain inhibitory NANC innervation. This evidence may have helped to elucidate the nature of a naturally occurring neuronally-released relaxant which had been isolated from the BRP muscle (Ambache et al., 1975; Gillespie & Martin, 1980) and which mimicked inhibitory NANC nerve stimulation in this tissue. The response produced by the extract was insensitive to TTX which implied it had a direct effect on the smooth muscle. Furthermore, the response produced by neither NANC nerve stimulation nor the extract was antagonised by muscarinic, adrenergic, or histamine blocking agents. The substance was not a purine since adenine nucleotides could be removed from the extract by adsorption onto alumina without losing its inhibitory action (Bowman et al., 1979). Nor could the inhibitory activity be attributed to a peptide because it was unaffected by trypsin, subtillisin or pepsin or a fatty acid derivative (Gillespie & Martin, 1980; Gillespie et al., 1981).

Other gastrointestinal smooth muscles with an inhibitory NANC innervation were also relaxed by the extract. In the guinea-pig taenia caeci the extract produced rapid brief inhibitions of tone similar to those produced by NANC nerve stimulation. Similarly, relaxations were produced in the stomach fundal strip and colon, although the extract was less active in the ileum (Crossley & Gillespie, 1983). Indeed, in the BRP muscle the extracted inhibitory factor mimicked the relaxation produced by NANC nerve stimulation and the underlying electrical mechanisms were similar in each case (Byrne & Muir, 1985). These observations suggest that the "inhibitory factor" may be a candidate for the role of an NANC neurotransmitter.

The "inhibitory factor", NO and EDRF have a great deal in common, beside their relaxant effects. They have extremely short half-lives, in the order of 4-5s (Moncada et al., 1988a; Gillespie et al., 1981) and their actions are abolished by haemoglobin (Moncada et al., 1988b; Bowman et al., 1982). In the BRP muscle the inhibition produced by NANC nerve stimulation and the extract were both antagonised by oxyhaemoglobin (Bowman & Gillespie, 1982; Bowman et al., 1982; Byrne & Muir, 1984), whereas in the guinea-pig taenia caeci relaxation to the extract was abolished but that to NANC nerve stimulation was unaffected. Apamin, however, was effective against the actions of NANC nerve stimulation but not against those of the extract in the guinea-pig taenia coli (Bowman & Gillespie, 1981). Therefore, the BRP muscle and guinea-pig taenia caeci may have different NANC neurotransmitters but the action of haemoglobin on the extract is similar regardless of the tissue.

Haemoglobin had previously been shown to antagonise vascular smooth muscle relaxation produced by NO (Gruetter et al.,1979; 1980) and more

recently in non-vascular smooth muscle (Gillespie & Sheng, 1988b; Buga et al., 1989). This is because haemoglogin has a greater affinity for NO than O_2 and therefore binds the NO molecule before it diffuses into the smooth muscle cell (see Moncada et al., 1988a). The actions of EDRF are also antagonised by haemoglobin in a similar fashion (Martin et al., 1986).

It has now been accepted that EDRF is in fact NO (see Moncada et al., 1988a) and the accumulating evidence suggests that the inhibitory factor is also NO (Martin et al., 1988; Gillespie & Sheng, 1988b). Thus NO could be the putative NANC inhibitory neurotransmitter in the BRP muscle and anococcygous muscles (Gillespie & Sheng, 1988b; Gibson & Mirzazadeh, 1989). However, like the purines and peptides, the NO story is incomplete. It was found that haemoglobin was an effective antagonist only after acid-activation of the inhibitory factor. The relaxant action of inactive inhibitory factor was not abolished by haemoglogin (Gillespie & Sheng, 1988b). It was suggested that NO is liberated from inhibitory factor by acid activation of nitrate (Martin et al., 1988) and the released NO is then stabilized by an unidentified component of the semipurified extract.

Unlike the other putative neurotransmitters, NO does not act on a membrane bound receptor but rather, because of its lypophilic properties, is able to pass through the plasma membrane easily. Thus NO acts directly on the cytosolic guanylate cyclase enzyme. Soluble guanylate cyclase therefore is the receptor for NO, with cyclic GMP as the second messenger (Moncada et al., 1988b). The actions of cyclic GMP will be discussed later.

It seems that NANC nerves may liberate different biologically active

substances; that is, the NANC neurotransmitter does not form a single population but varies from tissue to tissue. The transmitter may be selected by the receptors on and the sensitivity of the effector cells which the nerves serve (Hökfelt et al., 1980b; Burnstock, 1986). Thus it may be that peptidergic, purinergic and NO-releasing nerves will co-exist within the autonomic nervous system along with adrenergic and cholinergic to provide "fine" and "course" control of smooth muscles at any one time.

3 MECHANISMS OF SMOOTH MUSCLE RELAXATION

The control of smooth muscle relaxation has been discussed so far in terms of the activities of chemical messengers liberated from neurones to smooth cells. On the arrival at the external surface of a responsive smooth muscle cell, most intracellular and drug-mediated messages are read and interpreted by receptors - specific proteins in the plasma membrane or, as in the case of NO, in the cytosol. To permit the cell to express its response to these stimuli, mechanisms must exist to translate them into a language understandable within the cell. The activation of receptors which produce relaxation can be viewed as involving either electrically-mediated mechanisms, i.e. voltage-dependent or biochemically-mediated mechanisms, i.e. voltage-independent (see Bolton, 1979). Generally, however, this type of classification tends to reflect the method by which the relaxation process has been studied rather than a description of two mutually exclusive events involved in the inhibition of smooth muscle tension.

a) Voltage-Dependent Mechanisms

From the early 1950s the electrical characteristics of smooth muscle

have been studied extensively and characterised by the use of microelectrodes. The resting membrane potential and action potentials have been recorded in a variety of different smooth muscles including taenia coli and other intestinal and intestinally-related smooth muscles, including vas deferens, uterus and a variety of blood vessels (for reviews see Bennett, 1972; Bolton, 1979; Bülbring et al., 1981; Bolton & Large, 1986). The range in complexity among those muscles showing electrical activity is broad. The BRP muscle which is electrically quiescent at rest and shows very little change in membrane potential during field stimulation of NANC nerves (Byrne & Muir, 1984), is at one end of the spectrum and the guinea-pig taenia coli and internal anal sphincter which show spontaneous action potential discharge at rest and large inhibitory junction potentials upon field stimulation of NANC nerves at the other. The first smooth muscle to be well documented electrically was the guinea-pig taenia coli where the resting tone was shown to be accompanied by spontaneous action potential discharge. Augmentation of tone was associated with an increase, and a reduction in tone with a decrease in action potential discharge (Bülbring, 1955). This work provided evidence that electrical, i.e. voltage-dependent mechanisms existed which were associated with the mechanical properties of smooth muscle and led the way to a vast expansion in our knowlege of how voltage changes in smooth muscle cell membranes produced changes in mechanical tone.

Subsequent to the work of Bülbring in the 1950's, it was shown that many of the substances which inhibited smooth muscle tone also induced membrane hyperpolarisation. For example, substances which activated α-adrenoceptors and ATP on the guinea-pig taenia coli (Bennett et al., 1966a; Bülbring et al., 1981; Tomita & Watanabe, 1973; Jager, 1974;

Bauer, 1982) or on coronory artery (Takata & Kuriyama, 1980) or on the mouse uterus (Nimomiya & Suzuki, 1983) produced membrane hyperpolarisation at concentrations which exerted a strong relaxation. More importantly, stimulation of NANC inhibitory nerve fibres in the muscles of the alimentary canal including stomach (Beani et al., 1971), small intestine (Lang 1979a,b; Bauer & Kuriyama, 1982b) and large intestine (Bennett et al., 1966b; Bülbring & Tomita, 1967; Furness, 1969a,b; Ito & Kuriyama, 1973) produced large transient hyperpolarisations along with muscle relaxation. As mentioned earlier, these NANC inhibitory responses may be mediated by stimulation of 'purinergic' (Burnstock 1972; 1979; 1986) or 'peptidergic' (Hökfelt et al., 1980a,b; Iversen, 1983) nerves, but it seems that in many cases, even where the nature of the neurotransmitter is disputed, the post-junctional events leading to relaxation are voltage-dependent and involve inhibitory junction potentials (ijps).

In those muscles where changes in membrane potential were an integral part of the post-synaptic events subsequent to stimulation of smooth muscles by neurotransmitters or drugs leading to relaxation, interest was turned to the ionic basis underlying these electrical changes. In the guinea-pig taenia coli (Bennett et al., 1963; Tomita, 1972; Den Hertog & Jager, 1975) and jejunum (Hidaka & Kuriyama, 1969) the ionic basis of the ijp was demonstrated as a selective increase in K^+ conductance. The amplitude of the ijp was increased by reduction or removal $[K^+]_o$ (Bennett et al., 1963; Hidaka & Kuriyama, 1969; Tomita, 1972) and decreased by raising $[K^+]_o$. Furthermore, during passive hyperpolarisation of the membrane produced by passage of electrotonic current pulses, the ijps elicited by field stimulation of inhibitory nerves were attenuated and subsequently abolished at a membrane potential

of -80 to -90mV the K^+ equilibrium potential in smooth muscle (Tomita, 1972; Creed & Gillespie, 1977; Bauer & Kuriyama, 1982a). During further membrane hyperpolarisation beyond -90mV, evoked responses became depolarisations. This evidence strongly supported the proposal that K^+ is the ion underlying the ijp. In addition, it has been demonstrated that there is an increase in membrane conductance and the rate of $^{42}K^+$ efflux during field stimulation of guinea-pig taenia caeci preloaded with $^{42}K^+$ (Den Hertog & Jager, 1975). This work suggested that relaxation is mediated by an increase in membrane permeability of K^+ which produces hyperpolarisation.

From the work carried out to identify the ionic changes underlying the hyperpolarisation associated with smooth muscle relaxation very little information could be gleaned concerning how K^+ left the smooth muscle cell with the techniques used. Indeed, intracellular microelectrode studies and those observing ion fluxes allowed only a broad overview of how smooth muscle cell membranes under resting and stimulated conditions reacted electrically. However, to advance knowledge of the mechanisms underlying the potential changes, ionic currents crossing the membrane were analysed to the extent of investigating the measurements of charged 'gating' molecules in the cell membrane believed to regulate ionic channels admitting different ions (see Armstrong, 1974). The voltage-clamp technique and more importantly its successor, the extracellular patch clamp technique, allowed currents in single ionic channels to be observed (Neher & Sakmann, 1976). This latter technique involves pressing a heat polished glass pipette against the cell membrane to form an electrical seal with a very high resistance (Neher et al., 1978) which ensures that any currents emanating from the small patch of

membrane flow into the pipette. The patch clamp technique has advanced so far that electrical seals with gigaohm resistances can be obtained (Hamill et al., 1981) which allow a higher current resolution, direct membrane patch potential control and physical isolation of membrane patches. It is therefore a very useful experimental tool for the electrophysiologist to study the molecular events in the cell membrane which are responsible for the observed potential changes. Indeed, it has prompted a paper entitled "The patch clamp is much more useful than anyone had expected" by Sigworth (1984).

Smooth muscle studied with patch clamp methods showed that their excitability is determined by the relative contribution of inward and outward currents flowing through the plasma membrane. The inward current is carried mainly by Ca^{2+} ions and the outward by K^{+} ions (Bolton, 1979; Tomita, 1981; 1982). The inward current carried by Ca^{2+} is responsible for the action potential and flows through a voltage-sensitive Ca^{2+} channel (for a review see Tomita, 1988). There are also receptor-operated Ca^{2+} channels which are involved in smooth muscle contraction (for example, Benham & Tsein, 1987). However, it is the outward K^{+} current which is more interesting where smooth muscle relaxation is concerned. Before the application of the patch clamp technique to single smooth muscle cells, two sorts of K^{+}-conductance were known to exist: a voltage-dependent conductance responsible for repolarisation of the action potential and one responsible for the resting membrane potential . With single channel studies a plethora of K^{+} channels have since been identified (for review see Cook, 1988; Tomita, 1988). The best characterised is a Ca^{2+}-activated K^{+} channel in the rabbit jejunum and guinea-pig mesenteric artery (Benham et al., 1985;

1986) which was shown to be sensitive to potential and intracellular Ca^{2+} concentrations. A similar K^+ channel was found in the canine trachea (McCann & Welsh, 1986) and a further three Ca^{2+} -activated K^+ channels in the rabbit portal vein (Inoue et al., 1985; 1986) identified according to conductance and sensitivity to tetraethylammonium (TEA). One type has a large conductance (K_l) and was sensitive to externally applied TEA whilst a second had a small conductance (K_s) and was insensitive to TEA (Inoue et al., 1985). The third had an intermediary conductance (K_m) and was more sensitive to internally than externally applied TEA.

Knowledge of K^+ channels and their physiological role has progressed by the contribution which the use of many animal toxins has made. Similar to the crucial role which tetrodotoxin played in understanding Na^+ channels, toxins such as apamin (Banks et al., 1979) the dendrotoxins (Harvey & Anderson, 1985) and charybdotoxin (Anderson et al., 1988; MacKinnon & Miller, 1988) have helped to elucidate the physiological roles of K^+ channels. Dendrotoxins block the voltage-sensitive K^+ channels which prolong interspike interval bursts whilst charybdotoxin blocks Ca^{2+}-activated K^+ channels involved in repolarisation of action potentials (see Cook, 1988). Apamin blocks the K_s channel (Blatz & Magleby, 1986) and had previously been shown to inhibit the relaxation and hyperpolarisation produced by NANC nerve stimulation and exogenous ATP in the guinea-pig taenia coli (Vladimirova & Shuba, 1978; Maas & Den Hertog, 1979; Müller & Baer, 1980). This evidence suggested that K^+ efflux and, by implication, hyperpolarisation are important mediators of relaxation produced by certain agonists.

In the last few years the K^+ channel has become a site of

therapeutic interest following in the footsteps of both Na^+ and Ca^{2+} channels. Blocking K^+ channels promoted secretion of insulin and suppressed dysrhythmias of the heart and the converse of opening K^+ channels to produce hyperpolarisation and relaxation was investigated for the alleviation of hypertension and asthma. Nicorandil was the first compound shown to possess the ability to hyperpolarise smooth muscle membranes and produce coronary vasodilation (Furukawa et al., 1981). The hyperpolarisation produced by nicorandil was examined and attributed to an increase in K^+ conductance (Furukawa et al., 1981; Karashima et al., 1982). Confirmation of these results was demonstrated by the efflux of $^{86}Rb^+$ from preloaded tissues in response to nicorandil (Weir & Weston, 1986a,b). However, the relaxant actions produced by nicorandil may not have been due solely to its ability to open K^+ channels; the nitro moiety which it possesses could have contributed to its inhibitory effects by stimulating guanylate cyclase (Itoh et al., 1981; Holzmann, 1983). Furthermore the vasodilator action of nicorandil on rabbit aortic rings was significantly attenuated by methylene blue - a guanylate cyclase inhibitor (Lefer & Lefer, 1988). Thus, it appeared that nicorandil has some properties in common with organic nitrates in addition to K^+ channel activators.

Membrane hyperpolarisation associated with potent relaxant actions is not a property attributed to nicorandil alone; many other compounds have been identified which produce membrane hyperpolarisation and relaxation including cromakalim, pinacidil, diazoxide and minoxidil sulphate (see Cook, 1988). Cromakalim produced relaxation of guinea-pig taenia caeci (Weir & Weston, 1986a), isolated trachealis (Allen et al, 1986c), portal vein (Quast, 1987), rat portal vein (Hamilton et al., 1986)

and rabbit blood vessels (Weir & Weston, 1986b) by opening K^+ channels. This was supported by evidence which demonstrated that a concentration-dependent membrane hyperpolarisation, which took the membrane potential close to the theoretical K^+ equilibrium potential, was produced by cromakalim (Hamilton et al., 1986; Kreye et al., 1987a,b; Southerton et al., 1987b). Subsequent work showed that pinacidil produced similar electrical and mechanical effects to cromakalim in rat aorta and portal vein (Bray et al., 1987; Southerton et al., 1987a). Furthermore, in rat aorta, portal vein, rabbit aorta, pulmonary, ear and mesenteric arteries and guinea-pig trachea (Hamilton et al., 1986; Weir & Weston, 1986a,b; Bray et al., 1987; Coldwell & Howlett, 1986; 1987; Cook et al., 1988; Kreye et al., 1987a,b; Quast, 1987; Southerton et al., 1987a,b; Allen et al., 1986 a,b,c) preloaded with the potassium marker $^{86}Rb^+$, or $^{42}K^+$ itself, cromakalim and pinacidil produced a significant increase in K^+ efflux. However, in the guinea-pig detrusor muscle preloaded with $^{86}Rb^+$ or $^{43}K^+$, cromakalim appeared to produce a selective efflux of $^{43}K^+$ over $^{86}Rb^+$ (Foster et al., 1989), whilst in rat uterine muscle cromakalim did not modify the efflux of either $^{86}Rb^+$ or $^{43}K^+$. This suggested that the K^+ channel population on which cromakalim acted was not a homogeneous one.

The electrical and mechanical effects produced by cromakalim and nicorandil were not inhibited by apamin in the guinea-pig taenia caeci (Weir & Weston, 1986a), suggesting that the small conductance Ca^{2+}-activated K^+ channels were not involved (Banks et al., 1979; Blatz & Magleby, 1986). Further single channel analysis suggested that cromakalim opened the large-conductance Ca^{2+}-activated K^+ channel (Quast & Cook, 1989). However, charybdotoxin, a potent blocker of these

channels, did not attenuate the relaxation and $^{86}Rb^{+}$ efflux produced by K^{+} channel activators (see Quast & Cook, 1989). K^{+} channel blocking agents such as TEA, procaine and aminopyridines antagonised the effects produced by cromakalim and pinacidil (Coldwell & Howlett, 1987; Southerton & Weston, 1987;Wilson, 1987). But, the millimolar range at which TEA showed effective blockage of the hyperpolarisation and relaxation produced by K^{+} channel activators is inconsistent with the view that large conductance Ca^{2+}-activated K^{+} channels are opened, since these channels are blocked at submillimolar concentrations of the blocking agent. More recently, evidence has come to light proposing that the K^{+} channel activators open an ATP-sensitive K^{+} channel (K_{ATP}). K_{ATP} channels were first identified in cardiac muscle and have subsequently been found in pancreatic β-cells, skeletal muscle, cortical neurones and arterial smooth muscle (Ashcroft, 1988; Standen, et al., 1989; Ashford, et al., 1988). The potent and selective blocker of K_{ATP} channels, glibenclamide was shown to inhibit both the vasodilation and the increase in $^{86}Rb^{+}$ efflux in vitro and the vasodilation in vivo produced by cromakalim (Quast & Cook, 1989). This evidence implied that K^{+} channel activators act on K_{ATP} channels. Support for this hypothesis has come from single channel analysis of dispersed cells from rabbit and rat mesenteric arteries (Standen et al., 1989). Channel activity recorded from these cells was abolished by ATP and subsequently restored in the presence of cromakalim and this was inhibited by glibenclamide. This work provided direct electrophysiological evidence that K_{ATP} channels existed in vascular smooth muscle and cromakalim acted on them. Hence, the mechanical inhibition produced by the K^{+} channel activators may be due to their opening of K_{ATP} channels which in

resting conditions in normal smooth muscle remain closed (Quast & Cook, 1989).

This evidence gained from the use of these drugs suggested that voltage - dependent mechanisms are an important facet of smooth muscle relaxation. This is strengthened by work which demonstrated that neither cromakalim nor pinacidil increased the tissue content of cyclic GMP and cyclic AMP (Kauffman et al., 1986; Coldwell & Howlett, 1987; Taylor et al., 1988; Gillespie & Sheng, 1988a).

It seems that in many cases hyperpolarisation is a pre-requisite of relaxation and that the change in membrane potential is mediated via a Ca^{2+} - activated K^+ channel in the case of inhibitory nerve stimulation or via an ATP - sensitive K^+ channel in the case of drugs. Other ions, such as Cl^- may also be involved in hyperpolarisation, especially in those muscles where the ijp associated with relaxation produced by inhibitory NANC nerve stimulation is small.

b) Voltage-Independent Mechanisms

In many smooth muscles, especially those which do not exhibit spontaneous action potential discharge, for example cat and bovine trachea (Ito & Takeda, 1982; Cameron et al., 1983), rat anococcygeus (Creed et al., 1975, Creed & Gillespie, 1977; Byrne & Large, 1984), rabbit rectococcygeus (Blakely et al., 1979; King & Muir, 1981) and BRP muscle (Byrne & Muir, 1984, 1985) stimulation of inhibitory NANC nerves produced relaxation accompanied by little or no hyperpolarisation. It seemed unlikely that the powerful relaxation in these cases was mediated by voltage-dependent mechanisms when the change in membrane potential was so small. Indeed, in the BRP muscle the hyperpolarisation produced in

response to inhibitory NANC nerve stimulation was abolished in the presence of TEA, although the relaxation remained unaffected (Byrne et al., 1984). This suggested that other voltage-independent mechanisms were more important in the mediation of relaxation. A similar observation had been made previously concerning contraction where calcium mobilization with subsequent contraction was not mediated by depolarisation (Somlyo & Somlyo, 1968). This process was termed 'pharmacomechanical coupling'. It is now clear that many biochemical signal transduction mechanisms exist which regulate cell function independent of a change in membrane potential.

In general, agonists bind to receptors to produce effects in one of four fundamentally different ways. The first three all involve opening or closing ion channels, where the receptor is the channel itself, for example the nicotinic receptor (Noda et al., 1983), where information is transduced from the receptor to a channel by a GTP-binding protein (Yatani et al., 1987), or where binding of the agonist to the receptor produces alteration in the activity of an enzyme and hence the level of the latter's product which subsequently alters the open/closed state of ion channels (see Christie & North, 1988). The fourth involves the production of second messengers generated within the cell as a result of the agonist-receptor combination. These second messengers then act to alter cellular enzymes, membranes or contractile proteins to trigger the cells' overall response. (Figure 1). Of the known second messengers, Ca^{2+}, inositol phosphates, diacylglycerol and the cyclic nucleotides, cyclic AMP and cyclic GMP, are the best understood. (Figure 2).

One of the first observations that biochemical processes alone were involved in receptor mechanisms can be traced to work which showed that

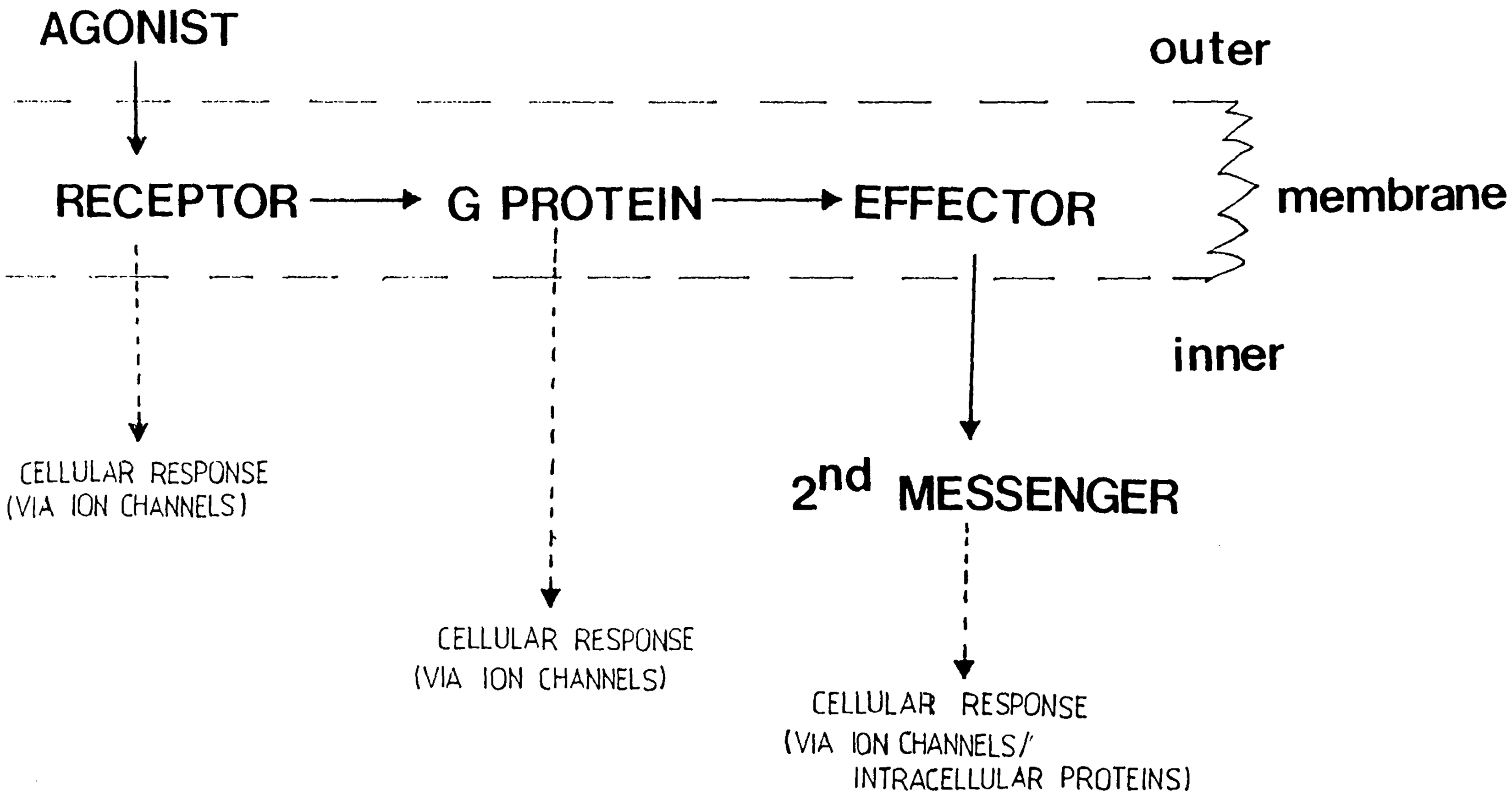
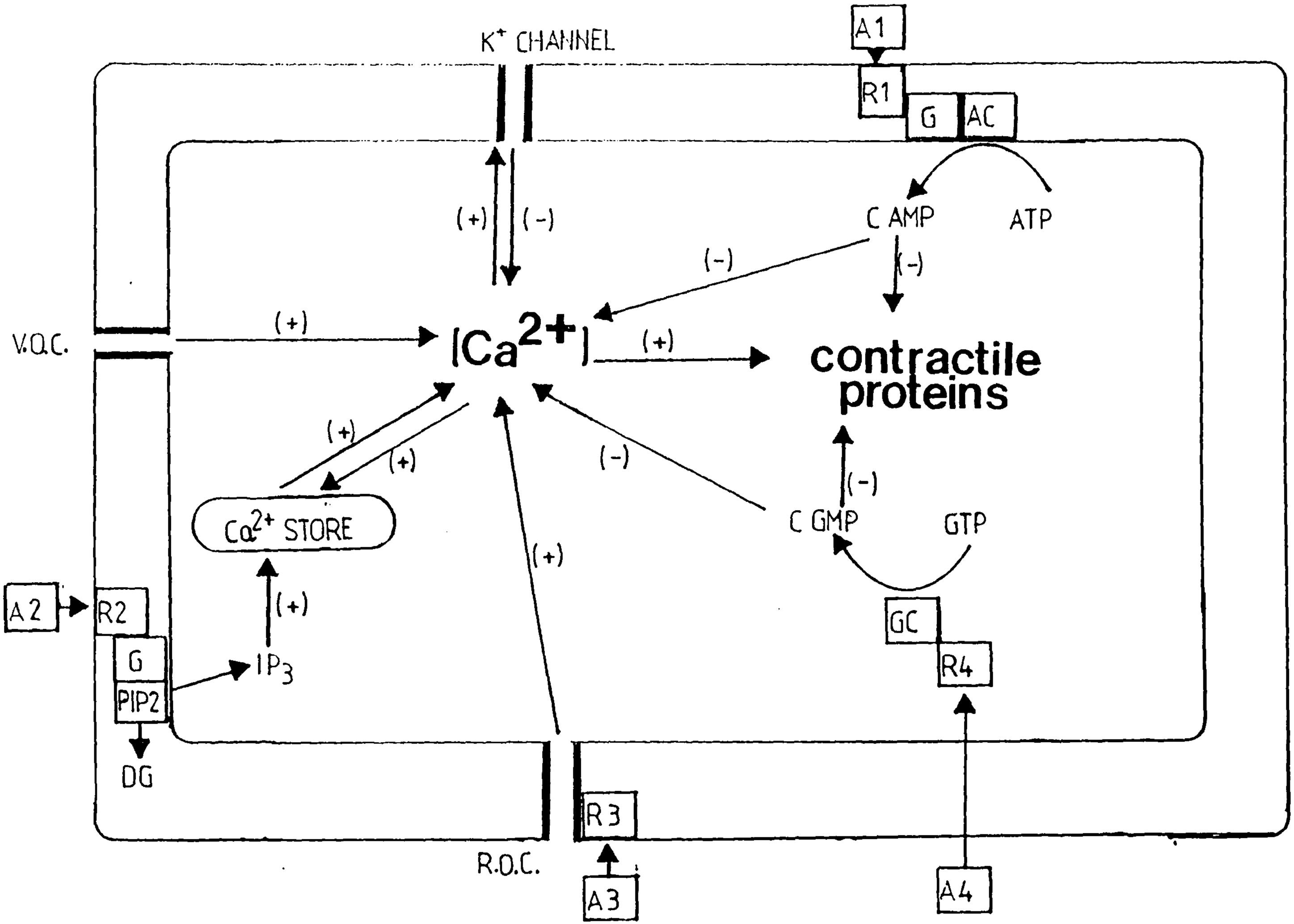


FIGURE 1
Diagrammatic representation of signal cascade involved in agonist-receptor interaction.

FIGURE 2

Diagrammatic representation of a smooth muscle cell and some of the signal transduction pathways which are known. Abbreviations used: A_{1-4}*, agonist;* R_{1-4}*, receptor; G, G-protein; cAMP, cyclic 3':5' adenosine monophosphate;* ^{1}AC*, adenylate cyclase; cGMP, cyclic 3':5' guanosine monophosphate; GC, guanylate cyclase;* PIP_2*, phosphatidylinositol 4,5 bisphosphate;* IP_3*, inositol 1,4,5-triphosphate; V.O.C., voltage operated channel; R.O.C.* ^{3}*, receptor operated channel; + , stimulatory effect; - , inhibitory effect.*


K+ CHANNEL
A1
R1
G
AC
(+)
(-)
C AMP
ATP
(-)
(-)
V.O.C.
(+)
[Ca2+]
(+)
contractile proteins
(+)
(+)
(-)
(-)
Ca2+ STORE
(+)
(+)
C GMP
GTP
A2
R2
G
PIP2
IP3
DG
GC
R4
R3
R.O.C.
A3
A4

activation of muscarinic cholinergic receptors in the exocrine pancreas provoked an increase in ^{32}P incorporation into phosphatidylinositol and its precursor phosphatidic acid (Hokin & Hokin, 1953). However it was not until the mid 1970s that the role of polyphosphoinositides in receptor function was fully recognised (see Michell, 1975). In his review Michell (1975) pointed out that: (1) whenever receptors were associated with phosphoinositide (PI turnover), these same receptors activated Ca^{2+} mobilization, and (2) activation of phosphoinositide turnover was not a consequence of Ca^{2+} mobilization. It was therefore concluded that Ca^{2+} mobilization and phosphoinositides were linked. Due to an imprecise knowledge of the biochemical pathways involved it was nearly a decade later before evidence came to light which showed that the minor inositide, phosphatidylinositol 4,5-bisphosphate (PIP_2) was initially broken down in PI turnover (Kirk et al., 1981). It is now clear that PIP_2 hydrolysis by phospholipase C (a phosphodiesterase) initiated by agonist/receptor binding yields two second messengers, inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (see Berridge, 1985). The IP_3 molecule was recognised as a likely second messenger to activate release of Ca^{2+} from intracellular stores (Berridge, 1983) and was shown to release Ca^{2+} from a non-mitochondrial store in permeabilized pancreatic acinar cells (Streb et al., 1983). In smooth muscle, IP_3 acts as a second messenger in the mediation mainly of contraction (Ochs, 1986).

Interestingly, reports have emerged which implicate phospholipase C - catalysed inositol phosphate formation subsequent to P_{2y}-purinergic receptor activation in cultured endothelial cells (Pirotton et al., 1987) and turkey erythrocytes (Boyer et al., 1989). The P_{2y}-purinoceptor has been linked with relaxation in smooth muscle (see Burnstock & Kennedy,

1985), although inositol phosphate formation and relaxation have not been associated as yet.

Where smooth muscle relaxation is concerned, the second messengers most commonly involved are the cyclic nucleotides, cyclic AMP and cyclic GMP. Their role in the regulation of smooth muscle tone has been investigated and debated since the late 1950s (for reviews see Hardman, 1981; Waldman & Murad, 1987). These second messengers will now be discussed.

(i) Cyclic AMP

Subsequent to its discovery in 1958 by Sutherland and Rall, cyclic AMP has been of major importance in helping to understand many of the mechanisms underlying the effects of neurotransmitters, hormones and drugs on their target tissues. Cyclic AMP is synthesised within a cell by the action of the enzyme adenylate cyclase on ATP following agonist-receptor interaction (see R_1 in Figure 2). However, because receptors lie on the outer surface of the membrane, a G-protein which lies within the membrane is necessary to link the receptor to adenylate cyclase which faces inside the cell. The cyclic nucleotide acts within the cell on cyclic AMP-dependent protein kinases and is degraded by phosphodiesterases. The purpose of cyclic AMP is probably best described as an aid in the management of the Ca^{2+} economy within the cell. The amount of free Ca^{2+} within the cell is very important in determining smooth muscle tone. The ion enters cells by voltage-operated channels and receptor operated channels and can be stored in intracellular sites such as the endoplasmic reticulum, sarcoplasmic reticulum or mitochondria. Elevation of cyclic AMP produces its effect by four different methods:

(1) it promotes Ca^{2+} extrusion from the cell by activating pumps in the membrane,

(2) it promotes uptake of Ca^{2+} into intracellular stores,

(3) it decreases the sensitivity of myosin light chain kinase to Ca^{2+}, and

(4) it reduces Ca^{2+} entry into the cell by inactivating voltage-operated channels.

Many smooth muscle relaxants produce their effects by stimulating adenylate cyclase activity with a subsequent increase in the level of cyclic AMP and a reduction in $[Ca^{2+}]_i$. These agents include adenosine (see Burnstock & Kennedy, 1985), VIP (Frandsen et al., 1978), histamine (Mitznegg et al., 1975), prostaglandins (see Bolton, 1979), and forskolin (Seamon & Daly, 1986) as well as the β-adrenergic agents such as isoprenaline (see Bolton, 1979; Hardman, 1981).

One of the actions then of cyclic AMP to reduce free Ca^{2+} is by promoting extrusion mechanisms as seen in the isoprenaline-induced relaxation of smooth muscle (Marshall & Kroeger, 1973). This Ca^{2+} extrusion could be due to activation of Na^{+}-Ca^{2+} exchange resulting from a cyclic AMP-dependent increase in Na^{+}-K^{+}-ATPase (Scheid et al., 1979). Alternatively an electrogenic Ca^{2+} extrusion may have been responsible, presumably due to modulation by calmodulin (Caroni & Carafoli, 1981, Lamers et al., 1981) which increases the affinity of the pump for Ca^{2+} (see Berridge, 1985).

Relaxation of smooth muscle by reduction in free $[Ca^{2+}]_i$ can be achieved not only by Ca^{2+} extrusion from the cell but also by uptake of Ca^{2+} into intracellular stores. Cyclic AMP along with cyclic AMP-dependent protein kinase has been implicated in the accumulation of

Ca^{2+} in storage sites in smooth muscle, mainly the "sarcoplasmic reticulum". Isoprenaline-induced relaxation of guinea-pig mesenteric artery via β-adrenoceptor activation, which is associated with an increase in intracellular cyclic AMP, was proposed to be mediated by Ca^{2+} extrusion from the cell and accumulation of Ca^{2+} in internal storage sites (Itoh et al., 1982). This was demonstrated in studies using saponin-skinned guinea-pig mesenteric artery where Ca^{2+}-accumulation in the storage sites was increased in the presence of high concentrations of Ca^{2+}, cyclic AMP and cyclic AMP-dependent protein kinase. This is presumably by the activation of the Ca^{2+} pump on the sarcoplasmic reticulum enabling it to sequester a greater share of Ca^{2+} than under resting conditions (see Tada & Katz, 1982).

Application of cyclic AMP itself suppressed Ca^{2+}-induced contractions (Rüegg et al., 1981). Similarly, high concentrations of catalytic subunits of cyclic AMP-dependent protein kinase inhibited Ca^{2+}-induced contractions of chemically skinned guinea-pig taenia caeci (Rüegg et al., 1981; Sparrow et al., 1981). Because the latter set of experiments was carried out in chemically-skinned preparations, Ca^{2+}-extrusion from the cell was not the method by which relaxation was induced and it was unlikely that sequestration of Ca^{2+} was the sole method by which intracellular free Ca^{2+} was reduced. Therefore a third method by which cyclic AMP produced relaxation must have existed. This third method involves the suppression of Ca^{2+} binding by the contractile proteins, actin and myosin. Cyclic AMP is known to activate the free catalytic subunits of cyclic AMP-dependent protein kinase. This activtion results in phosphorylation of the myosin light chain kinase (MLCK) and the binding between calmodulin, the Ca^{2+} receptor of the

contractile protein, and the MLCK is weakened. This results in impaired MLCK activity which decreases the amount of myosin which can be phosphorylated and relaxation ensues (Conti & Adelstein, 1980; Adelstein et al., 1980; Adelstein & Eisenberg, 1980).

More recently, a fourth method by which the second messenger cyclic AMP can induce relaxation has been noted. Cyclic AMP can modulate ion channels and is known to inhibit Ca^{2+} entry through voltage-operated ion channels (Meisheri & van Breemen, 1982)

Relaxation mediated by cyclic AMP is produced by many signal pathways which all involve reduction in Ca^{2+} within the cell.

(ii) Cyclic GMP

Cyclic GMP has with some justification, until recently, been very much the poor relative of cyclic AMP. It was discovered accidentally in a study originally designed to identify organic phosphates in urine (Ashman et al., 1963) and the subsequent investigation of the role of this nucleotide has progressed very slowly. There are superficial similarities between cyclic AMP and cyclic GMP which suggest they may have analogous functions, i.e. as second messengers. These similarities are: (1) cyclic GMP is also formed by the action of an enzyme, in this case guanylate cyclase, on the guanine nucleotide, guanosine triphosphate (GTP) subsequent to receptor occupation (see R_4, in Figure 2) and (2) once formed, cyclic GMP activates cyclic GMP-dependent protein kinase and is subsequently degraded by cyclic GMP-specific phosphodiesterase. Although guanylate cyclase has been described in virtually all cell types it exists, unlike adenylate cyclase, as a polymorphic protein with a cytosolic (soluble) and a membrane-bound (particulate) form. The

co-existence of these isoenzymes in the same cell is largely responsible for the difficulties encountered in the study and understanding of guanylate cyclase and the role of cyclic GMP as a second messenger. The actions of cyclic GMP are much less clear than those of cyclic AMP, although cyclic GMP is known to promote relaxation by impairing MLCK activity and accelerating Ca^{2+} extrusion from the cells. However, the synthesis and function of cyclic GMP has been studied and extensively reviewed by Waldman & Murad (1987).

A relationship between raised levels of cyclic GMP and relaxation in response to drugs and inhibitory NANC nerve stimulation has been noted in many smooth muscles. For example, in the BRP muscle (Bowman & Drummond, 1984) a rise in cyclic GMP immediately preceded relaxation in a frequency-dependent fashion in response to field stimulation. Similarly, a rise in cyclic GMP accompanied relaxation produced by field stimulation in the lower oesophageal sphincter of the opossum (Torphy et al., 1986), although it is not known if this rise preceded relaxation. Rises in cyclic GMP also accompany relaxation produced by a number of drugs in a variety of vascular and non-vascular smooth muscles (Katsuki et al., 1977a,b; Rapoport et al., 1983b; Katsuki & Murad, 1977; Schultz et al., 1977).

A group of drugs, collectively termed nitrovasodilators, has been helpful in elucidating the mechanisms by which cyclic GMP may be involved in smooth muscle relaxation. These drugs have been used since the nineteenth century following the synthesis of amylnitrite (Balard, 1844). Since then this group has grown to include nitroprusside, nitroglycerin, hydroxylamine and sodium nitrate. However, the mechanisms of action of these compounds was not well understood until around 15 years

ago. In 1975 it was noted that sodium nitroprusside which produced relaxation of rat myometrium and canine femoral artery also elevated cyclic GMP concentrations within the cells (Diamond & Holmes, 1975; Diamond & Blisard, 1976). Subsequent reports indicated that other nitrovasodilators, including sodium azide, hydroxylamine and sodium nitrate also raised cyclic GMP levels in both vascular and non-vascular smooth muscle (see Waldman & Murad, 1987). The correlation was thus made that the action of nitrovasodilators was mediated by activation of guanylate cyclase and raised levels of cyclic GMP (Schultz et al., 1977; Katsuki et al., 1977b). A direct correlation between smooth muscle relaxation and cyclic GMP by these agents has however been questioned. Sodium nitroprusside failed to relax rat vas deferens, although cyclic GMP levels were raised 15-fold, whilst verapamil and hydralazine relaxed these preparations but failed to elevate cyclic GMP concentrations (Janis & Diamond, 1979). Additionally, sodium nitroprusside produced a greater elevation of cyclic GMP in rat myometrium and vas deferens than nitroglycerin, although relaxation was produced by nitroglycerin alone (Diamond, 1983).

However, in spite of these anomalies the proposal that nitrovasodilators exert their effects by mechanisms involving cyclic GMP has gained support. This support has come from the knowledge that nitrovasodilators generate nitric oxide either spontaneously or enzymatically and that this free radical is the proximal activator of guanylate cyclase (Murad & Aurbach, 1977; Murad et al., 1981; Katsuki et al., 1977a; Arnold et al., 1977; Murad et al., 1978). That cyclic GMP is the second messenger produced by nitric oxide to induce cellular activation has gained credibility recently with work involving EDRF. It

has been shown that EDRF and probably inhibitory factor released from some inhibitory NANC nerves (Moncada et al., 1988b; Gillespie & Sheng, 1988b; Martin et al., 1988) are nitric oxide or a closely related substance and that EDRF and inhibitory factor produce increased levels of cyclic GMP in their target organs. This suggests that actions of nitric oxide are indeed mediated by cyclic GMP.

Furthermore, agents which inhibit guanylate cyclase activity and hence the production of cyclic GMP, including methylene blue, cyanide and ferricyanide also inhibit relaxation induced by nitrovasodilators (Katsuki et al., 1977 a, c; Gruetter et al., 1979; 1980; 1981 a,b; Holzmann, 1983; Murad et al., 1978; Kruszynar et al., 1982). Similarly, in rat aorta substances which directly interfere with the guanylate cyclase enzyme by interacting with sulfhydryl groups, for example cystamine, also block relaxation and the elevated cyclic GMP concentrations produced by nitrovasodilators (Brandwein et al., 1981; Katsuki et al., 1977a; Rapoport et al., 1981). Therefore it seems that the evidence weighs heavily on the side of cyclic GMP as a mediator of relaxation.

While there is support for a correlation between cyclic GMP and relaxation, it is still not clear, if they are related, how cyclic GMP functions to produce relaxation. The effects of cyclic GMP were recently proposed to be mediated via cyclic nucleotide kinases with subsequent protein phosphorylation. Sodium nitroprusside which is known to increase cyclic GMP levels and produce relaxation (Schultz et al., 1977) also activated cyclic GMP-dependent protein kinase in a dose- and time-dependent manner in rabbit and canine arteries (Fosterman et al., 1986). Furthermore, sodium nitroprusside and the stable cyclic GMP analogue, 8-bromo-cyclic GMP, produced a concentration-dependent decrease

in the phosphorylation of cellular proteins in rat aorta prelabelled with ^{32}P (Rapoport et al., 1982). These results have been extended to include the effects of other endothelium-dependent agents on rat aorta which raise the level of cyclic GMP and induce relaxation (Rapoport et al., 1983 a,b; Rapoport & Murad, 1983; Murad, 1986). In these cases the amount of phosphorlyated myosin light chain decreased, which is known to correlate with smooth muscle relaxation (Draznin et al., 1983; 1986). Thus, these studies proposed that activation of guanylate cyclase caused accumulation of cyclic GMP which in turn activated cyclic GMP-dependent protein kinase. The activation of cyclic GMP-dependent protein kinase then results in an accumulation of unphosphorylated myosin light chain and relaxation.

Accumulation of unphosphorylated myosin light chain is not the sole method by which cyclic GMP has been proposed to have its effects. Recent evidence from both skinned and intact muscle of rabbit mesenteric artery showed that cyclic GMP reduced the amount of free Ca^{2+} within the cells (Itoh et al., 1985) by extrusion. From this work it was deduced that cyclic GMP, together with cyclic GMP-dependent protein kinase, accelerated Ca^{2+} extrusion thereby reducing the amount of stored and free Ca^{2+} in the myoplasm and hence promoting relaxation (Itoh et al., 1985).

Cyclic GMP mediates the actions of nitrovasodilators, EDRF and inhibitory factor released from inhibitory NANC nerves. The cyclic nucleotide, like its cousin cyclic AMP, acts via more than one pathway which results in relaxation. However the management of Ca^{2+} within the smooth muscle cells seems to be its major effect.

4 AIMS OF THESIS

It is clear that relaxation of smooth muscle may involve both changes in membrane potential and second messenger systems. Ideally, both types of measurement should be considered in any attempt to investigate mechanisms of relaxation. While such a goal remains an ideal rather than a reality, it is nonetheless necessary to attempt to consider both aspects in a study of relaxation. This thesis had as its aim a study of the mechanisms by which sphincteric smooth muscle relaxation was brought about. It attempts to correlate the outcome of experiments using intracellular electrical recording techniques, which indicate membrane events, with those biochemical measurements which identify ways in which post-synaptic membrane changes are translated into cellular events.

In this thesis, the work described is a study mainly of spontaneously active, non-propulsive smooth muscle, namely the circular muscle of the guinea-pig internal anal sphincter (IAS). Initially, experiments were carried out to elucidate the nature of the neurotransmitter involved in the IAS, which contains a well developed NANC inhibitory innervation, using electrophysiological techniques. Having established, as far as possible, the neurotransmitter involved, electromechanical coupling was then investigated.

Generally, there are two types of electromechanical relationships. One where hyperpolarisation is a predominant feature of the relaxant response, for example the guinea-pig taenia caeci (Bülbring, 1954), and the other is found, for example, in the rat anococcygeus (Creed et al., 1975) where hyperpolarisation is an unimportant part of the inhibitory response. Thus, in this thesis electrophysiological techniques were used

further to assess: 1) if, in the IAS, relaxation produced by a variety of known smooth muscle relaxants, including inhibitory NANC nerve stimulation, was always accompanied by membrane hyperpolarisation; 2) if, in those cases where relaxation was accompanied by hyperpolarisation in the IAS, the membrane potential change was integral to the mechanical response; and 3) if pharmacologically-induced hyperpolarisation could produce relaxation in a smooth muscle, the BRP muscle which is known not to require a membrane potential change for relaxation. (Byrne et al., 1984).

The second part of the investigation was an attempt to characterise the second messenger systems involved in relaxation of the IAS under similar conditions of stimulation to those used in the electrophysiological study. This was assessed by measuring cyclic nucleotide levels by radioimmunoassay and inositol polyphosphate production by radiochemical detection during relaxation. Subsequently, correlations among electrical, biochemical and mechanical changes in the IAS produced by different relaxants could be made to establish some of the mechanisms underlying smooth muscle relaxation.

Subsequent to in vitro studies which examined some of the precise details underlying smooth muscle relaxation, an opportunity was then taken to investigate the activity of smooth relaxants in vivo. Clearly for any drug to be useful clinically, it must show a similar mode of action in vivo as in vitro. Therefore the last part of the thesis is concerned with experiments carried out in the anaesthetised guinea-pig. Initially these experiments were conducted to determine whether or not anal sphincter pressure could be monitored in this small animal. Then having measured anal sphincter pressure under resting conditions, drugs with

known effects on the IAS *in vitro* were administered intravenously to the animal to investigate if similar effects could be seen on *in vivo* anal sphincter pressure levels.

CHAPTER II

METHODS

Three non-vascular smooth muscle preparations were examined, anatomically related to, or an integral part of, the alimentary canal.

1 ANATOMY

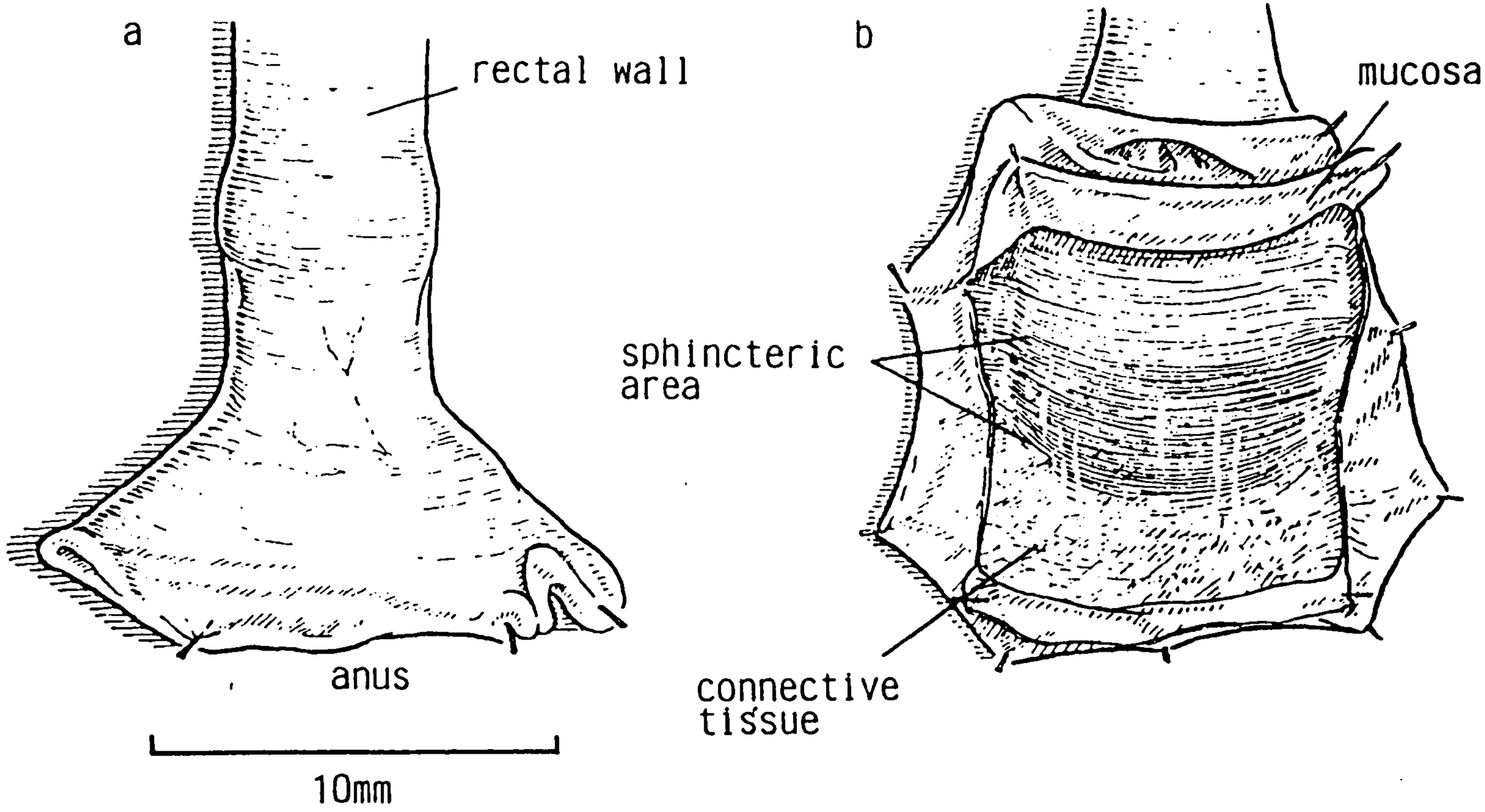
a) Guinea-Pig internal anal sphincter

The internal anal sphincter (IAS) in this species appears as a thickening of the rectal wall (3 x 5mm), and consists of a band of circular fibres more densely arranged than elsewhere in the anorectal region. The rectum consists of two muscle layers, an outer longitudinal covering which extends over an inner circular band known as the IAS. Each muscle layer ends some 5 - 10mm short of the anal margin and is separated from the skin by a band of mainly connective tissue and a few circular muscle fibres. (Figure 3).

The IAS is innervated by three types of efferent nerves. Firstly, postganglionic noradrenergic excitatory axons which reach the IAS via the pudendal nerves and from the pelvic plexi in the rectal wall. Secondly, cholinergic excitatory nerves enter the IAS from the pelvic plexi. The third type of efferent neurone is parasympathetic with cell bodies located in the sacral region of the spinal cord. Axons of this third type leave by the sacral ventral roots and run in the pelvic nerves to synapse with postganglionic non-adrenergic, non-cholinergic (NANC) intramural neurones in the pelvic plexi which then enter the IAS (Costa & Furness, 1973). Recent evidence suggests the inhibitory motor neurones to the IAS are Type 3 rectal neurones which emanate from the myenteric plexus and are activated to an excitable state only during rectal distension (Tamura, et al., 1989).

FIGURE 3:

Diagrammatic indication of the position of the guinea-pig internal anal sphincter (x5). In this stretched preparation (a), the approximate location of the sphincter is indicated by a slight swelling of the rectal wall. In (b), a longitudinal incision in the rectum has been made and the mucosa and sumucosa 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 rectal wall. The sphincter is thus easily differentiated from the connective tissue, which is adjacent to the anus.


a
rectal wall
sphincteric area
anus
10mm
b
mucosa
connective tissue

The longitudinal muscle which extends over the specialised sphincteric area receives parasympathetic excitatory cholinergic nerve fibres which also arise in the pelvic plexi (Costa & Furness, 1973). (Figure 4).

b) Guinea-pig taenia caeci

The large intestine consists of caecum, transverse colon and descending colon, and the musculature and lamination remains the same here as elsewhere throughout the alimentary canal. Circular muscle is arranged in accordance with other parts of the gastrointestinal tract. However, the longitudinal layer of the large intestine is not distributed evenly throughout the wall, but is collected into three distinct bundles termed taenia coli which can be seen through the serous coat. The taenia coli cause puckering in the gut wall called haustra. In the guinea-pig the longitudinal coat of the caecum, but not the colon, is arranged in taeniae hence, the more correct term of the preparation described in this thesis is taenia caeci.

The taenia caeci receives both excitatory parasympathetic, cholinergic nerves and a sparse inhibitory sympathetic innervation. In addition, like many other parts of the gastrointestinal tract, the taenia caeci receives intramural inhibitory NANC nerve fibres with their cell bodies in Auerbach's plexus (Bennett & Rogers, 1967).

c) Bovine retractor penis muscle

The BRP muscle (BRP) is a paired smooth muscle originating from the first two coccygeal vertebrae and passes along the ventral surface of the bulbocavernosus muscle to insert into the distal part of the penis.

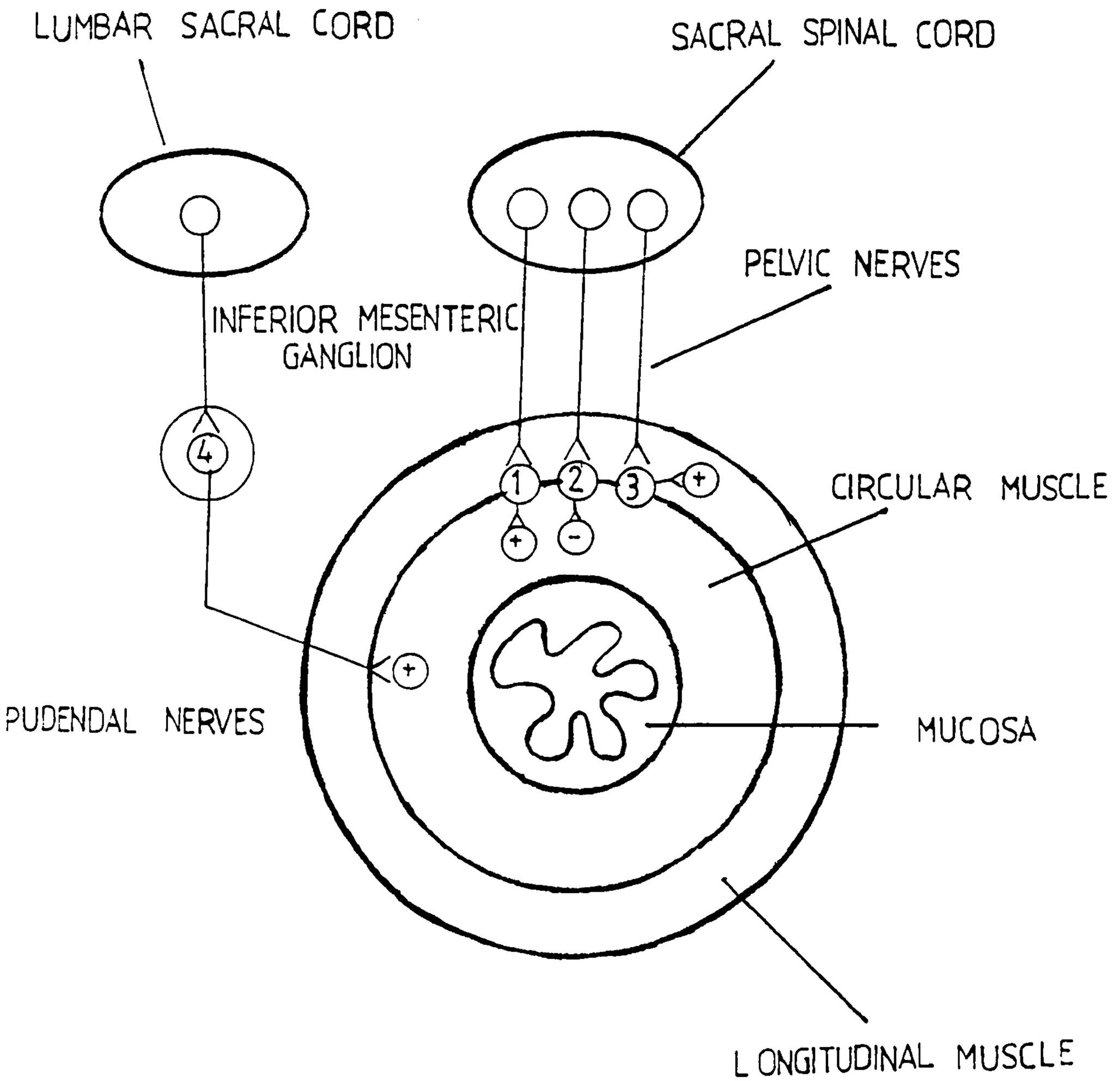


FIGURE 4:

Schematic representation of the innervation of the internal anal sphincter. 1 & 3 are cholinergic intramural neurones; 2 is the intramural inhibitory NANC neurone; and 4 is the sympathetic excitatory neurone. (Based on a diagram in Gonella et al., 1987).

When contracted, the BRP muscles keep the penis withdrawn in the characteristic sigmoid flexure. (Figure 5).

The muscles have a dense excitatory adrenergic innervation which reaches the muscle mainly by the pudic nerve, although a small proportion of fibres run in the hypogastric or pelvic nerves. There is also an inhibitory innervation which is non-adrenergic, non-cholinergic in nature and which consists of postganglionic, parasympathetic fibres (Klinge & Sjöstrand, 1974).

2 DISSECTION OF TISSUES

Tissues were removed as quickly as possible and transferred to petri dishes containing oxygenated Krebs' solution. Connective tissue, fat and blood vessels were removed under a dissecting microscope and tissues prepared as described below for mechanical, intracellular electrical, radio-chemical recording or radioimmunoassay. All tissues were left to equilibrate for at least 30min before starting each experiment.

a) Guinea-pig internal anal sphincter

Adult guinea pigs of either sex (300 - 500g) were killed by cervical dislocation with subsequent exsanguination. The abdominal cavity was opened by a midline incision and the intestines removed to one side. The remaining viscera were removed to expose the descending colon to the point where it passed into the pelvic cavity. The pubic symphisis was then split to reveal the descending colon, rectum and anus. A segment of rectum down to and including the anus, together with a small portion of

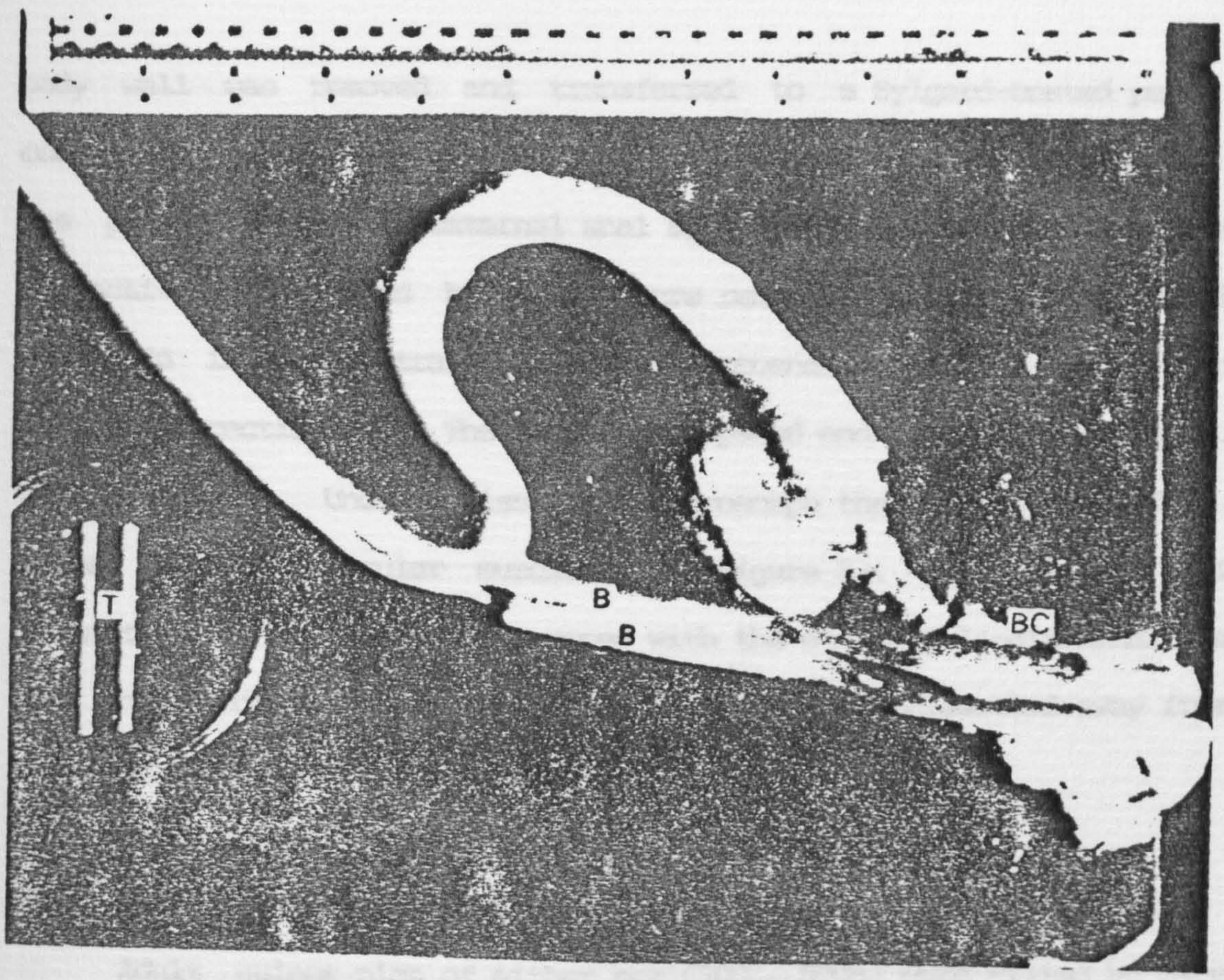


FIGURE 5:

The bovine retractor penis in its characteristic sigmoid flexure and the attached retractor penis muscles (B) extending to the bulbocavernosus (BC). Thin strips of tissue (T) taken from the midportion of the retractor penis were used for simultaneous electrical and mechanical recording. The size of the bovine retractor penis is indicated by the 30cm ruler in the background.

body wall was removed and transferred to a Sylgard-coated petri dish containing oxygenated Krebs' solution. This segment of rectum and anus was pinned out and the external anal sphincter comprising skeletal muscle, connective tissue and body wall were removed. A longitudinal incision was made in the ventral wall of the rectoanal canal from the anus in the cranial direction. The canal was opened and the mucosa and submucosa were removed. Under a dissecting microscope the IAS was identified as a dense band of circular muscle. (Figure 3). A horizontal strip of sphincter (10 x 3mm) was removed with the attached longitudinal muscle. The longitudinal muscle layer was then carefully dissected away from the circular muscle under the microscope.

b) Guinea-pig taenia caeci

Adult guinea-pigs of either sex (300 - 500g) were killed by cervical dislocation and subsequently exsanguinated. The abdominal cavity was opened by a midline incision to reveal the intestines. The taenia caeci was identified as a thin strip of longitudinal smooth muscle running the length of the caecum. It was ligated and a length dissected free from the caecum (1 x 70mm) by severing the connective tissue between the muscle layer and the wall of the caecum. It was placed in a Sylgard-coated petri dish, pinned out and cut into equal potions (1 x 15mm).

c) Bovine retractor penis muscle

Bovine retractor penis was obtained fresh from the abattoir on the day of each experiment. After slaughter, a cut was made through the midline at the level of the pelvic region to expose the penis. The penis was then severed at the level of the ischiocavernosus and the

bulbocavernosus muscles and, together with the retractor muscles, removed by cutting it free from connective tissue and fat. Specimens were transported to the laboratory. (Figure 5).

In the laboratory, the retractor muscles were dissected free from the penis and attached connective tissue and fat, and then transferred to a Sylgard-coated petri dish containing oxygenated Krebs' solution. Thin strips of muscle (3 - 4mm broad) 15 - 20mm long were dissected from the middle of the BRP by cutting along the lines of cleavage separating the smooth muscle bundles.

3 APPARATUS AND TECHNIQUES - <u>IN VITRO</u>

a) Intracellular and simultaneous mechanical recording

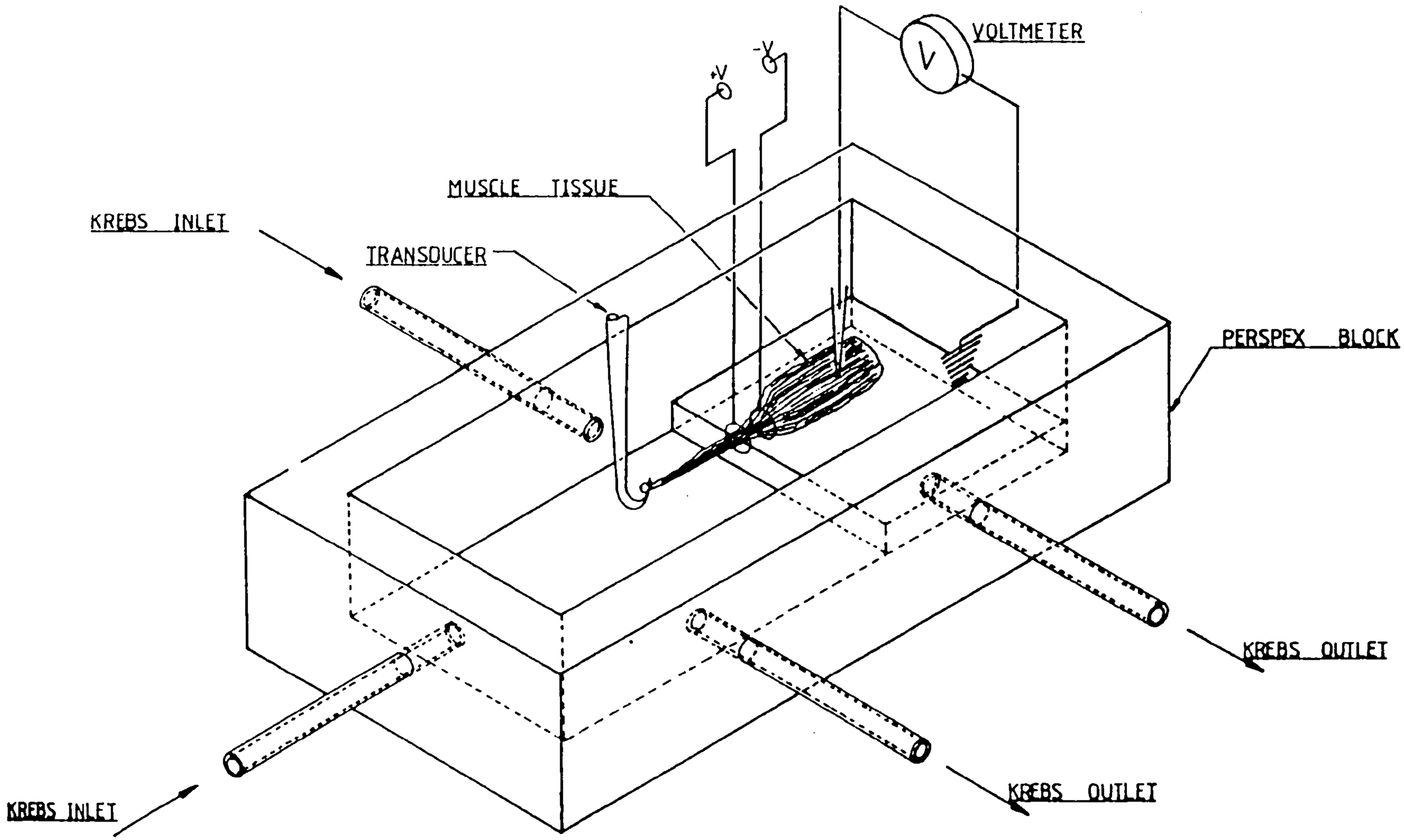
These experiments involved simultaneous measurement of electrical and mechanical events from each of the IAS and BRP. In each tissue, the effects of relaxant stimuli, including intramural NANC nerve stimulation, were used to assess the relationship between the electrical and mechanical events.

The apparatus (Figure 6) consisted of a horizontal organ bath (50 x 10 x 10mm) cut from a perspex block (110 x 80 x 20mm), Ag/AgCl ring electrodes (O.D. 2mm), capillary glass microelectrodes, a Ag/AgCl plated indifferent electrode, D.C. preamplifier, dual beam oscilliscope, voltmeter and gated pulse generator, U.V. oscillograph, and tape recorder.

In order to minimise the mechanical vibrations generated in and around the laboratory, the organ bath was bolted to two non-conducting Bakelite pillars which were fastened to a steel plate (200kg) on a table

FIGURE 6:

Organ bath for the combined intracellular electrical and mechanical recordings. The bath comprised a central trough (50 x 10 x 10mm) cut from a perspex block (110 x 80 x 20mm). The block was drilled to accept stainless steel inlet tubes (diameter, 2mm) for the Krebs' solution and two outlets for drainage. The body of the muscle was pinned on to the Sylgard base of the trough and intracellular recordings were made from the pinned area. One free end was tied by a thread to an isometric tension transducer as shown. Field stimulation by an isolated stimulator was effected via Ag/AgCl ring electrodes (V^+-V^-). The bath was perfused by gravity flow with oxygenated pre-heated Krebs' solution (36 ± 0.5°C) supplied via two inlets. The polythene tubing (diameter 2mm) containing Krebs' solution was surrounded by an outer tubing (diameter, 10mm) containing liquid paraffin (at 40 ± 0.5°C) pumped by a thermostatically controlled Tempette pump.


VOLTMETER
V
+V
-V
MUSCLE TISSUE
KREBS INLET
TRANSDUCER
PERSPEX BLOCK
KREBS OUTLET
KREBS OUTLET
KREBS INLET

mounted on Muffelite (K-150) antivibration dampers. The bath was continually perfused (6mlmin^{-1}) via two inlets with Krebs' solution at 36 ± 0.5°C. Emptying of the bath, via two outlets, was carried out by suction. The polythene tubing (O.D. 2mm) containing Krebs' solution was surrounded by an outer tube (O.D. 10mm) containing liquid paraffin at 40 ± 0.5°C, pumped by a modified Tempette (TE7) pump to maintain the temperature constant (± 0.5°C) at the desired level.

To record simultaneous electrical and mechanical activity in the IAS and BRP muscle, one end of each tissue was attached, via a thread, to an isometric force displacement transducer (Grass FT03C) and the other end passed through bipolar Ag/AgCl ring electrodes (O.D. 2mm and 2mm apart, mounted in Araldite) and pinned to the Sylgard (Dow-Corning)-coated base of the bath. Field stimulation was carried out by means of an isolated square wave stimulator (Devices type 2521) triggered from a Devices Digitimer (0.5ms; supramaximal voltage). Drugs were added in two ways:

1. by addition to the perfusate with a 15min equilibration period to measure dose-response relationships, or 2. from a microsyringe directly into the organ bath close to the recording site, when drugs with a short half-life were used or to assess the amplitude, rate of decline and duration of the induced-electrical response. The latter method was chosen to measure these characteristics of the electrical responses because the rate of decline and duration of the response were more accurately assessed by addition of a single discrete dose rather than a continuous dose. However the known concentration of drug applied by this method is much less precise than that of continuous perfusion. In all cases, an interval of 15min between doses was maintained throughout.

Intracellular electrical recordings were made with capillary glass

microelectrodes (Clark, GC150-10; 20 - 40MΩ) filled with 3M KCl. The microelectrode was connected to a unity gain high impedance ($10^{10}\Omega$) D.C preamplifier (W.P.I. M4A) via a Ag/AgCl half-cell attached to a probe, matched and calibrated for the preamplifier used. The indifferent Ag/AgCl-plated electrode was fixed to the wall of the bath and held in the bathing solution. Electrical signals, passed via the preamplifier, were displayed on one channel of a storage oscilliscope (Tektronix 5103N) and monitored on a digital voltmeter (Fairchild M53). The electrical and mechanical signals were stored permanently on an instrumentation tape recorder (Racal 4DS, band width 313 - 400KHz) and a U.V. oscillograph. (Figure 7).

b) Electrical activity in response to locally applied agonists

Membrane potential changes were recorded in response to exogenous agonists applied locally from a pressure controlled ejection device (Picospritzer II, General Valve Corporation, N.J., U.S.A.). This technique allowed a more accurate assessment of the electrical effects of substances, including those proposed as NANC neurotransmitters on smooth muscle membrane. The small amount of drug applied directly to the recording site more closely mimics the extrusion of neurotrasmitter into the synaptic cleft from a nerve ending than microinjection into the organ bath. In addition, the substances applied are less susceptible to enzymatic degradation or destruction before reaching the post-synaptic membrane and less liable to produce desensitisation because of the small amount applied. Mechanical responses could not be recorded when drugs were applied in this manner, since only a few muscle bundles at the intracellular recording site were activated, which is insufficient to

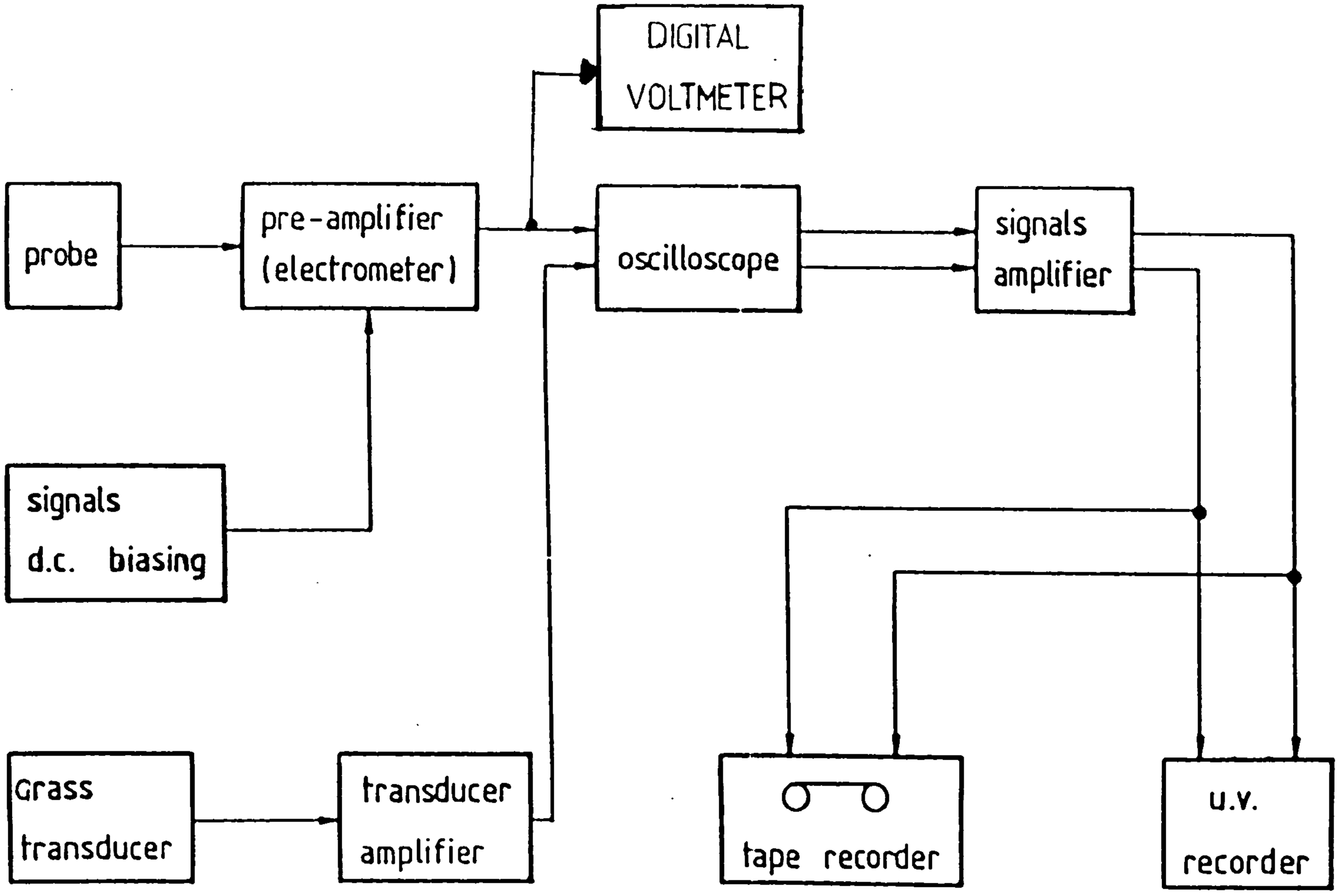


FIGURE 7:
Diagrammatic arrangement of the apparatus used to record intracellular electrical changes.

exert a change in tension on the transducer.

IAS muscles were dissected out and pinned on the Sylgard base of a horizontal organ bath, as previously described. Cells were impaled using conventional glass microelectrodes (20 - 40MΩ) containing 3M KCl to measure intracellular electrical changes. The drugs, dissolved in Krebs' solution, were applied from ordinary micropipettes which had their tips broken back under microscopic control to 2 - 10μm diameter. (Figure 8). Four factors controlled the amount of drug reaching the recording electrode from the Picospritzer:

1 the diameter of the pipette tip; to ensure uniformity the tip was broken back under microscopic control to 2 - 10μm;

2 the distance of the pipette tip from the recording site; this was kept to within 1mm, as measured by an eyepiece micrometer;

3 the ejection pressure; this was kept to within 40 - 50 psi;

4 the duration of ejection (1 - 300ms); this was varied as indicated in the text.

c) **Mechanical response following displacement of the membrane potential**

The partition method of Abe & Tomita (1968) was used to pass current directly into the muscle to investigate the effect of membrane potential displacement on the mechanical tone of the IAS. In these experiments the bath arrangement ensured that the stimulating current from a constant current source was applied by large external electrodes to a discrete area of tissue. Large external electrodes are necessary to pass enough current to overcome membrane and junctional resistance of smooth muscle

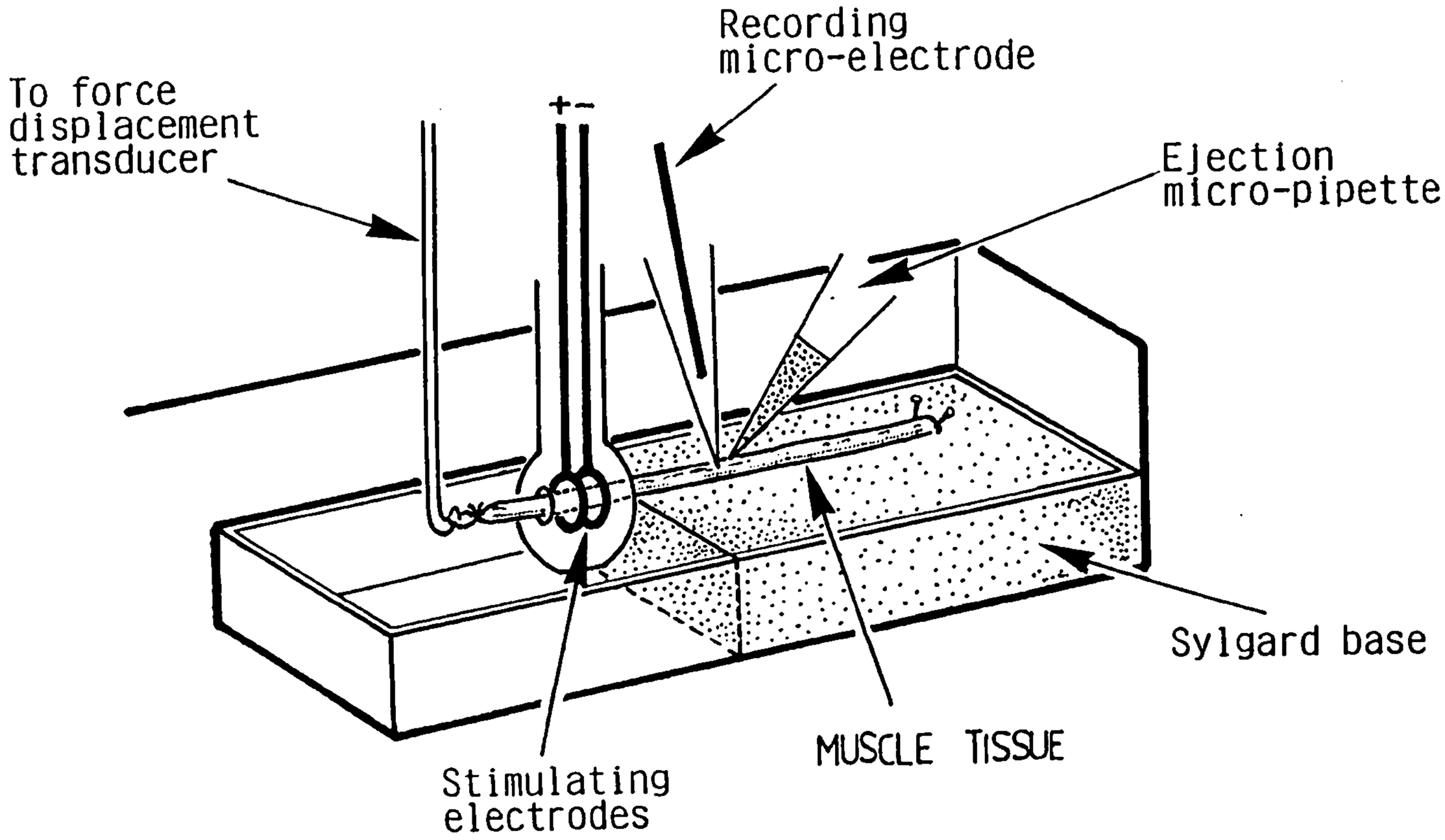


FIGURE 8:

Organ bath for the measurement of intracellular electrical changes recorded using conventional glass microelectrodes in response to field stimulation and exogenous agonists, applied locally from a micropipette, linked to a pressure-controlled ejection device (Picospritzer II). The organ bath was perfused by gravity flow with oxygenated pre-heated Krebs' solution (36 ± 0.5°C) via two inlets.

(Tomita, 1966; 1967) and avoid the dissipation of current which occurs in smooth muscle following focal stimulation. This procedure has already been used to show that current passed into the IAS by this method altered the membrane potential in a manner predicted by cable properties (Lim, 1985).

Two Ag/AgCl plates (10 x 10 x 5mm) each breached with a hole (3mm diameter) and 10mm apart, divided the organ bath (previously described) into three compartments: one for recording mechanical activity, a second for passing and monitoring current, and a third for anchoring the tissue. (Figure 9). The recording compartment contained a force displacement transducer (Grass FT03C) and the current-passing chamber, a pair of Ag/AgCl wires 2mm apart, used to monitor current. The surfaces of the plates facing the recording and anchoring compartments were coated with Araldite to insulate both chambers from the current-passing chamber.

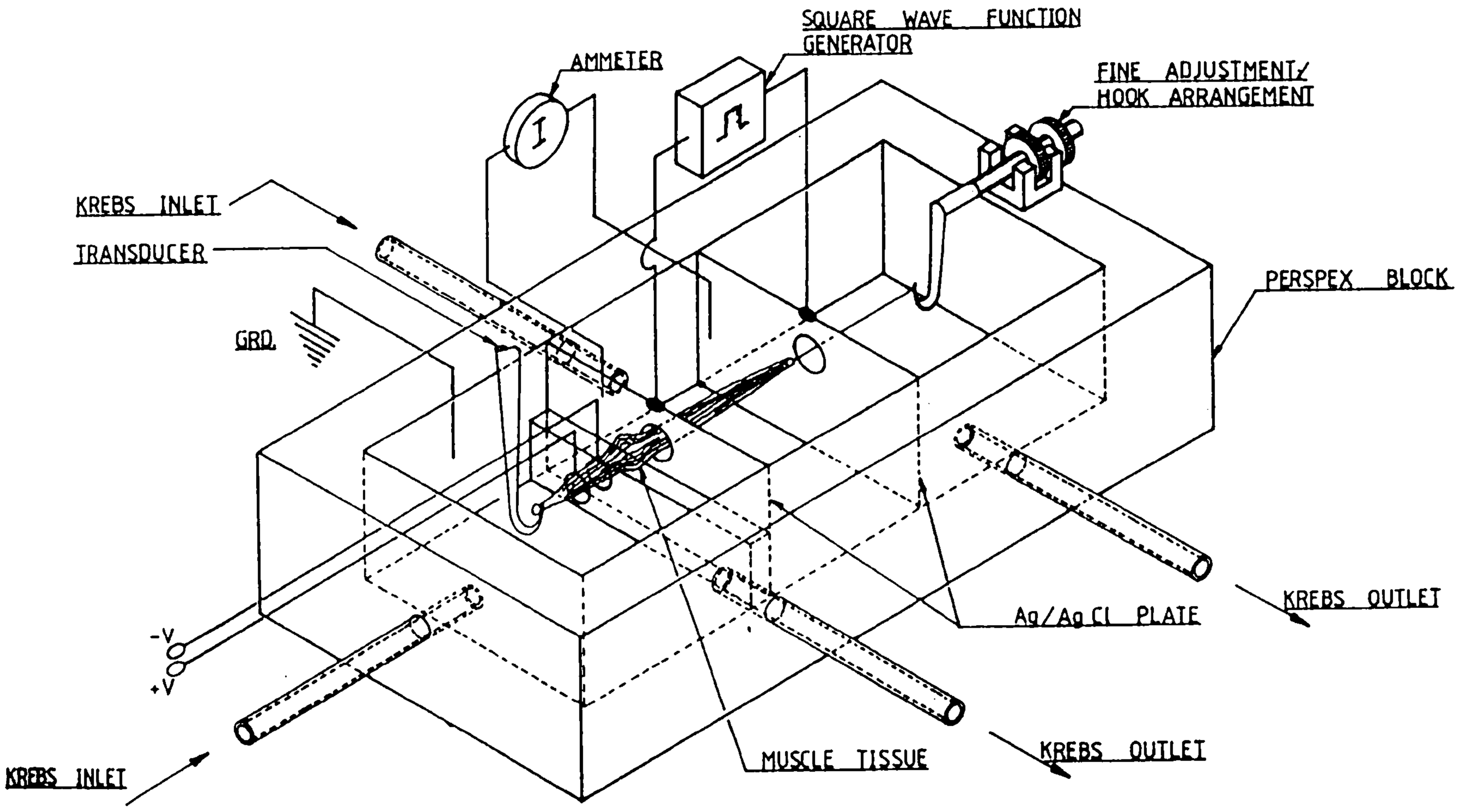
One end of the sphincter was attached via a thread to the transducer, passed through the hole in one plate and anchored via a second thread to a stainless steel metal hook in the third compartment. The length of tissue extending into the current-passing chamber was 4-6mm. Current pulses of 5s duration were delivered to the plates by a constant current source triggered manually.

d) Measurement of cyclic nucleotides

The levels of cyclic AMP and cyclic GMP were measured in the IAS and guinea-pig taenia caeci each by radioimmunoassay and the effects of field stimulation or of drug application on these tissues assessed by measurement of changes in the levels of these cyclic nucleotides. Radioimmunoassay (Steiner, et al., 1972) allowed absolute quantification

FIGURE 9:

Organ bath modified to allow displacement of the membrane potential by the technique of Abe & Tomita (1968). The bath comprised a central trough (50 x 10 x 10mm) cut from a perspex block (110 x 80 x 20mm). The trough was divided by two Ag/AgCl plates into three compartments. Changes in muscle tone were recorded in the recording compartment (r). Current was applied to the muscle via the Ag/AgCl plates which enclosed the current-passing chamber (C). The relative current intensity (I) was measured via two Ag/AgCl wires dipped into the current-passing chamber and were connected differentially to one channel of the oscilloscope. The recording compartment contained bipolar Ag/AgCl ring electrodes for field stimulation. The bath was perfused with oxygenated pre-heated Krebs' solution (36 ± 0.5°C) supplied via two inlets (diameter, 25mm) by gravity flow. The polythene tubing containing the Krebs' solution was surrounded by an outer tube (diameter, 10mm) containing liquid paraffin (at 40 ± 0.5°C) pumped from a thermostatically controlled modified Tempette pump.


SQUARE WAVE FUNCTION GENERATOR
AMMETER
FINE ADJUSTMENT/ HOOK ARRANGEMENT
KREBS INLET
TRANSDUCER
PERSPEX BLOCK
GRD.
-V
+V
Ag/AgCl PLATE
KREBS OUTLET
MUSCLE TISSUE
KREBS OUTLET
KREBS INLET

of basal cyclic AMP and cyclic GMP levels because it is more sensitive to small amounts (fmol) of cyclic nucleotides than protein binding assays for cyclic nucleotide measurement. Furthermore unlike the incorporation of ^{32}P into cellular proteins which are affected by cyclic nucleotide-dependent protein kinases and where drug-induced responses have to be compared to similar responses produced by the analogues of the cyclic nucleotides, radioimmunoassay directly measured the amounts of cyclic AMP and cyclic GMP present in samples after field stimulation or drug application.

(i) Sample collection

Tissues were dissected out as previously described, weighed and mounted in a heated vertical organ bath (10ml) containing Krebs' solution at 37 ± 0.5°C bubbled with 95%O_2 and 5%CO_2. The organ bath was surrounded by an outer jacket containing water at 40 ± 0.5°C maintained by a modified Tempette pump.

Each tissue was passed through a pair of Ag/AgCl ring electrodes (O.D. 2mm). One end was fixed, via a loop, onto a hook on the electrode and the other was attached, via a thread, to a force displacement transducer (Grass FT03C) to monitor tension. Tissues were stimulated using a Grass SD9 isolated stimulator (0.5ms; supramaximal voltage) and mechanical activity displayed on a Becks pen recorder. Changes in mechanical tone were also recorded in response to exogenous agonists. Drugs were added directly to the organ bath in small volumes from graduated syringes. Mixing occurred rapidly due to the bubbling gas. At the point of peak relaxation tissues were removed rapidly (less than 10s) from the organ bath and frozen by immersion in isopentane cooled in

liquid nitrogen.

(ii) Preparation of Samples

Frozen samples were thawed in trichloroacetic acid (1ml; 10% w/v) and homogenised using a ground glass homogeniser. Precipitated proteins were removed by centrifugation (3000g; 15min; 4°C). Portions of the acid-soluble fraction (the supernatant) were removed and extracted with water-saturated diethyl ether (4 times; 4 volumes), the traces of which were driven off by placing sample tubes in a heated water bath (2min at 70°C), At this point samples could be frozen until required for assay.

(iii) Radioimmunoassay for cyclic AMP and cyclic GMP

Frozen samples were thawed and an aliquot (10µl) of each was made up to 100µl with sodium acetate buffer (50mM, pH6.2). Cyclic AMP and cyclic GMP levels were each measured by radioimmunoassay using the acetylation method of Harper & Brooker (1975). The assay involves the competition between radioactive and non-radioactive antigens for a fixed number of antibody binding sites (Figure 10) and a method to separate antibody-bound from -free material. Unlabelled antigens, in this case cyclic AMP or cyclic GMP from samples or standards, together with a constant amount of radioactively-labelled cyclic AMP or cyclic GMP are allowed to react with a fixed amount of antibody raised to the appropriate cyclic nucleotide. If the sample contains a high amount of the cyclic nucleotide then the amount of radioactive cyclic nucleotide bound to the antibody decreases. Thus, an inverse relationship exists between the amount of bound radioactivity and the amount of cyclic AMP or cyclic GMP present in the sample. Efficient separation of the bound from the free

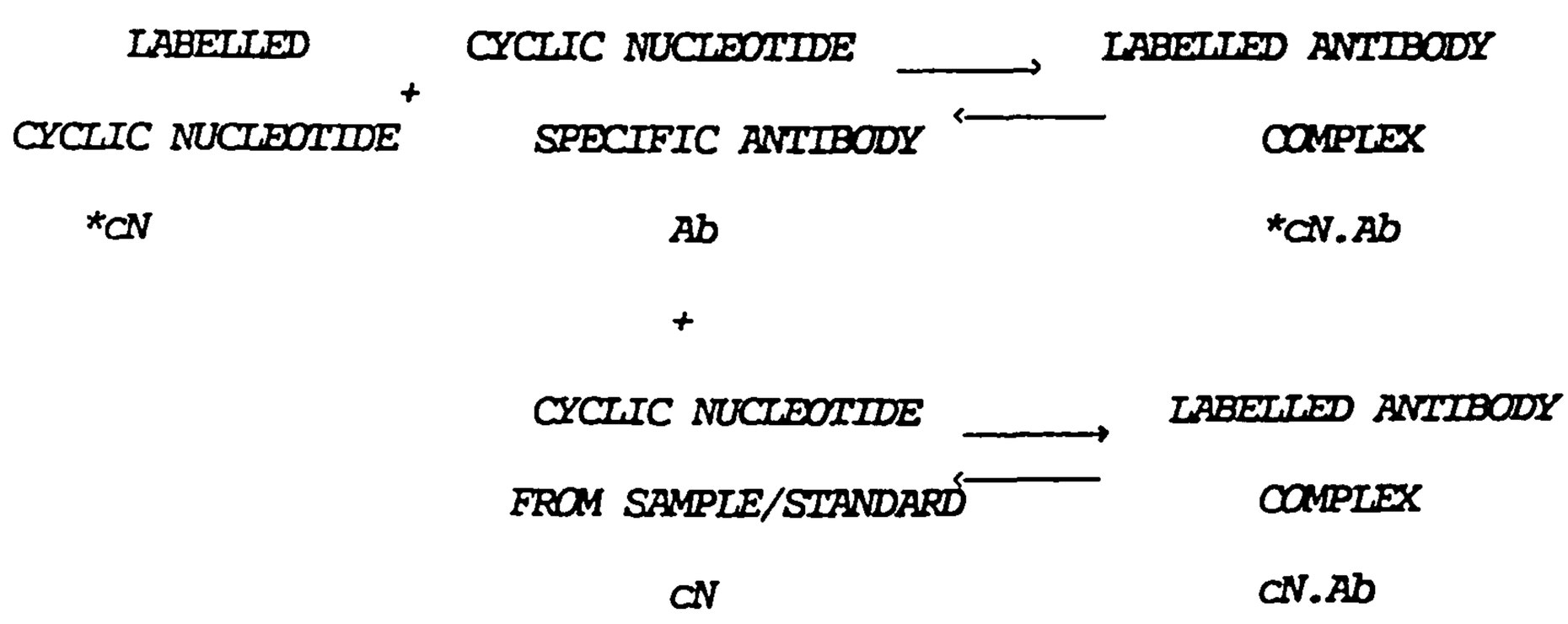


FIGURE 10:

*Schematic representation of the competition between radiolabelled (*cN) and unlabelled (cN) cyclic nucleotides for antibody-binding sites in a radioimmunoassay. This forms the basis for the measurement of the amounts of cyclic AMP and cyclic GMP present in both guinea-pig IAS and taenia caeci.*

radioactivity in the assay mixture is necessary before the final measurement. This separation can be achieved by addition of activated charcoal or finely divided silicates to extract the unbound radioactively-labelled cyclic nucleotide. However, the double antibody method was used here which precipitates the bound radioactivity by addition of a second antibody raised to the cyclic nucleotide in a different species and therefore does not require a separation step. Acetylation was used since it markedly increases the sensitivity of the assay by increasing the affinity of the cyclic nucleotide for its antibody. The samples were acetylated at room temperature by adding 5µl of the acetylating reagent: a mixture of acetic anhydride and triethylamine (1:2, v/v), directly into the solution, and vortexed rapidly. Under these conditions more than 25000 pmol of either cyclic AMP or cyclic GMP can be acetylated. A solution of bovine serum albumin (0.1%, w/v) plus either succinyl cyclic AMP tyrosine methyl ester-[^{125}I] or succinyl cyclic GMP tyrosine methyl ester-[^{125}I], diluted to give at least 6000cpm in sodium acetate buffer was added (100µl) to each sample. Subsequently, either cyclic AMP antiserum complex or cyclic GMP antiserum complex, containing pre-reacted first and second antibodies, was then added (100µl). The samples were mixed using a vortex mixer and incubated overnight (at 4°C).

After incubation, samples were centrifuged (3000g; 15min; 4°C) to precipitate out the labelled-cyclic nucleotide-antibody complex. To facilitate this process n-propanol (500µl) was added to each sample before centrifugation. The supernatant was decanted off and the pellet counted for the amount of radioactivity contained therein either by an EMI Gamma Counter or a Cobra Packard Gamma Counter.

A standard curve was constructed for each assay (eg. Figure 11 and Figure 12); this consisted of a duplicate sample of each of the following concentrations of cyclic AMP (expressed as $pmol\,mg^{-1}$):

0, 0.1, 0.25, 0.5, 1, 2, 4

or concentrations of cyclic GMP (expressed as $fmol\,mg^{-1}$):

0, 1, 2.5, 5, 10, 25, 50, 100, 250, 500.

The standards were assayed as described above. The standard curve was used to obtain the unknown cyclic AMP or cyclic GMP content (respectively) present in each sample.

e) Measurement of inositol phosphates

The involvement of inositol phospholipids in receptor mechanisms was first shown by Hokin & Hokin (1953). These workers assessed phosphoinositide turnover by measuring the incorporation of ^{32}P into phosphatidic acid, which is a by-product of phosphatidylinositol 4-5-bisphosphate (PIP_2) and subsequent diacylglycerol breakdown. The method chosen here is similar to that used much later by Downes & Michell (1981) which employs the integration of [^{3}H]-myoinositol into the PI cycle from the free inositol pool present in cells. This technique, rather than that used by Hokin & Hokin (1953), allows direct measurement of the labelled products of the cycle, including inositol 1,4,5 -triphosphate (IP_3), the proposed second messenger, by anion exchange chromatography on Dowex columns.

Sphincteric strips (10 x 3mm) were dissected out as previously described, then incubated in a small volume (2ml) of Krebs' solution, at 37°C containing [^{3}H]-inositol ($8\mu Ci\,ml^{-1}$) for 3h. Tissues were gassed throughout the loading period with 95%O_2 and 5%CO_2.

FIGURE 11:

Typical standard curve for the radioimmunoassay of cyclic AMP. The concentration of cyclic AMP (pmolmg^{-1}) is plotted against the radioactivity of the antibody - labelled antigen complex (cmp). There is an inversely proportional relationship between the concentration of cyclic AMP and the bound radioactivity.

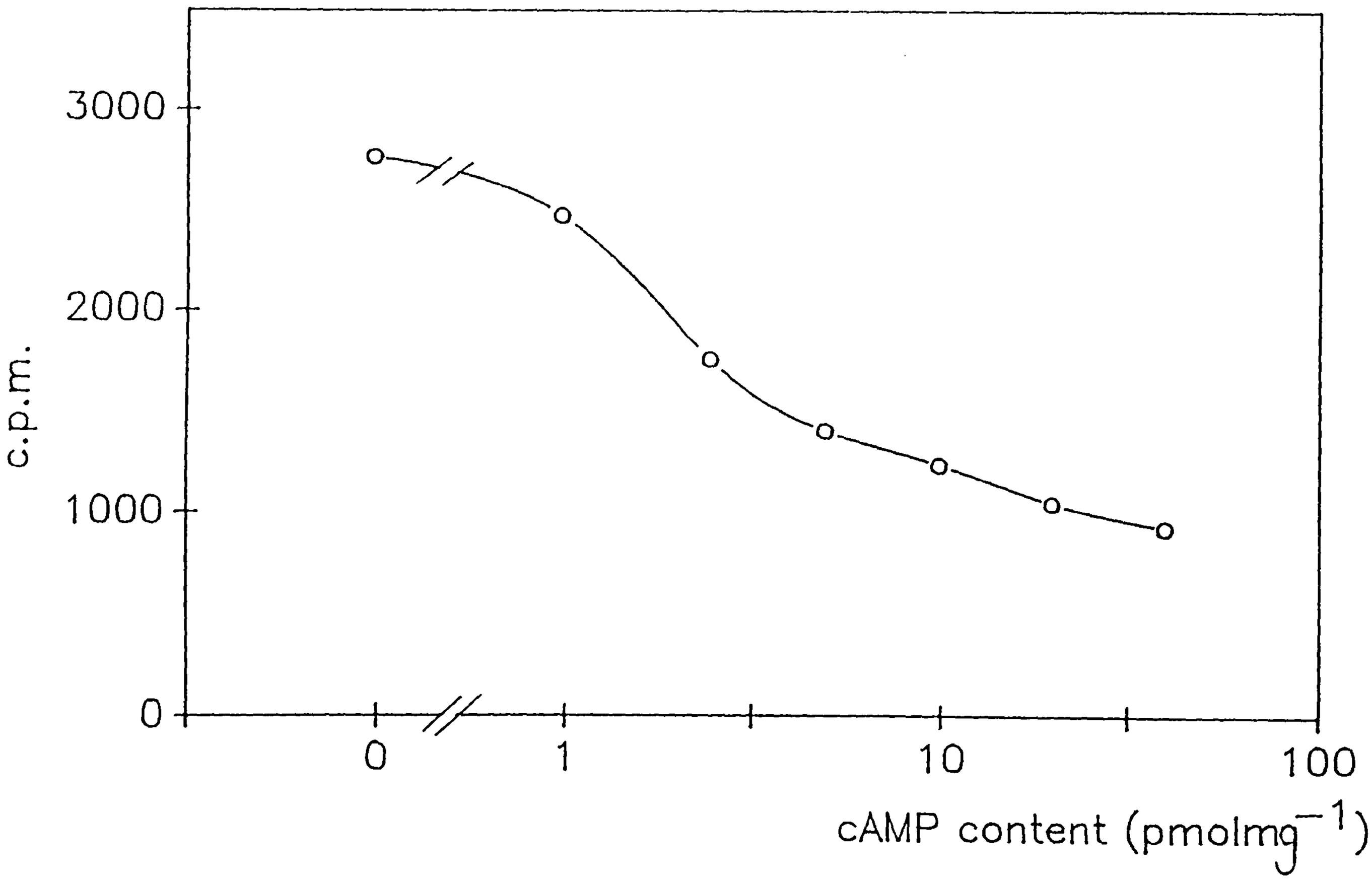
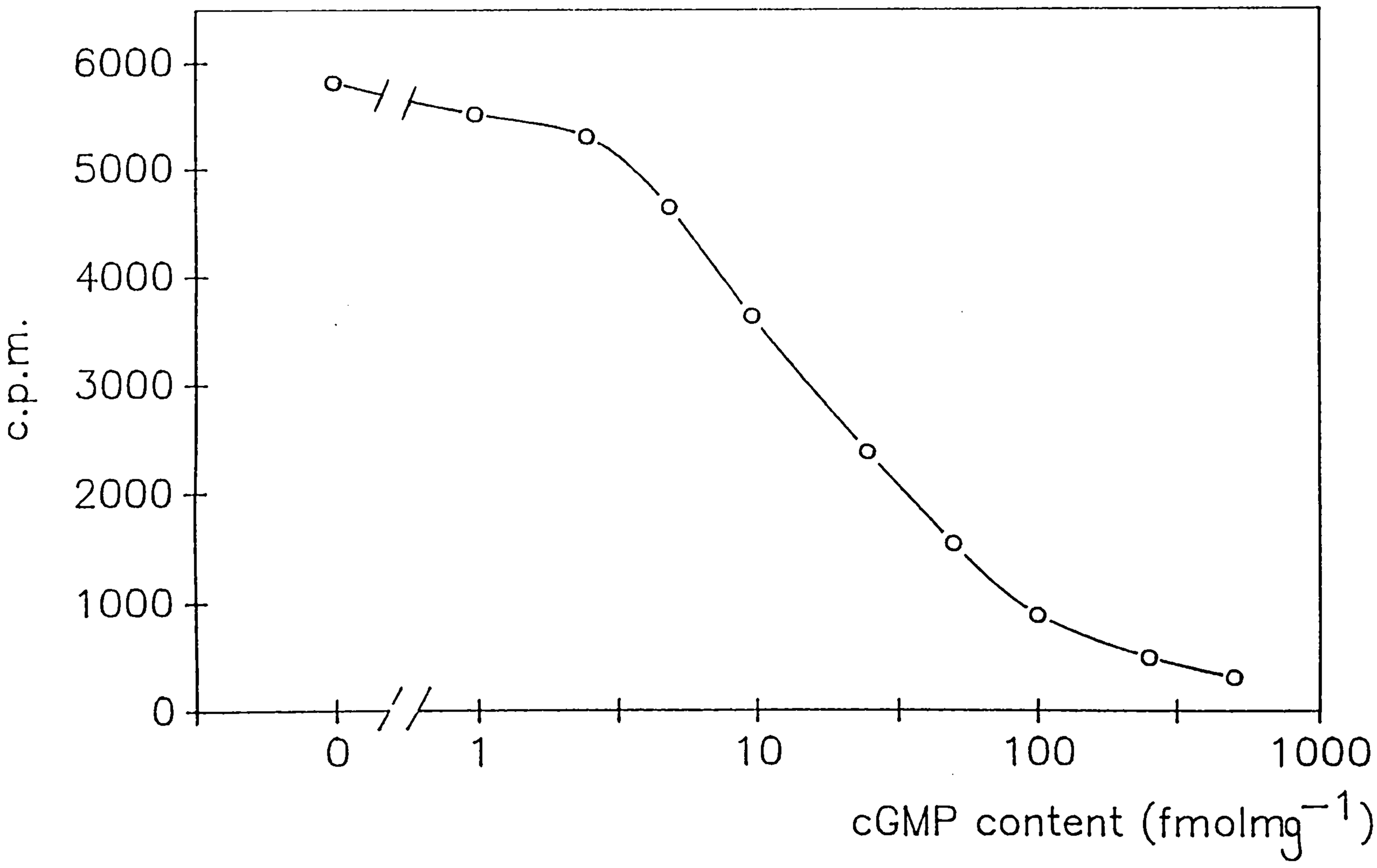

3000
2000
1000
0
c.p.m.
0
1
10
100
cAMP content (pmolmg^{-1})

FIGURE 12:

Typical standard curve for the radioimmunoassay of cyclic GMP. The concentration of cyclic GMP (fmolmg^{-1}) is plotted against the radioactivity of the antibody - labelled antigen complex (cmp). There is an inversely proportional relationship between the concentration of cyclic GMP and the bound radioactivity.


6000
5000
4000
3000
2000
1000
0
c.p.m.
0
1
10
100
1000
cGMP content (fmolmg^{-1})

Following this loading period, tissues were transferred to fresh incubation media containing 10mM Li^+-substituted Krebs' solution and [^{3}H]-inositol (8μCiml^{-1}), bubbled with 95%$_2$ and 5%CO_2. Li^+ was present from this point onwards in the experimental procedure since the cation inhibits the enzyme inositol 1-phosphatase which converts IP to free inositol. In effect, this increases the amount of [^{3}H]-inositol which is taken up into the cycle and greatly amplifies any response to drug application. After this period, tissues not designated as controls were removed and placed in an incubation medium containing the desired agonist together with Li^+-substituted Krebs' solution (as above) and [^{3}H]-inositol (as above) bubbled with 95%O_2 and 5%CO_2 for up to 30min.

Tissues were then removed from either the control medium or the agonist-containing medium and each immersed in ice-cold trichloroacetic acid (1ml, 10%w/v) to stop inositol phospholipid turnover. Samples were homogenised with a ground glass homogeniser and the precipitated proteins removed by centrifugation (1500g; 15min; 4°C). The supernatant (acid-soluble fraction) was removed and stored frozen until required for radioactive determination.

Acid-soluble fractions of the samples were thawed and neutralised by extraction with water-saturated diethyl ether (4 times ; 4 volumes). Residual ether was driven off in a boiling water bath and the extract allowed to cool to room temperature. Subsequently, if the pH of the sample was not >5, a few drops of NaOH (0.1M) were added to this end. Extracts were placed onto columns of Dowex (1-8, 100-200 mesh, Cl^- form) anion exchange resin which had been washed with ammonium formate (2.4M) to change the resin to the formate form. The inositol phosphates were

separated by elution, after free [^{3}H]-inositol had been washed out of the column with distilled water (10ml) and the [^{3}H]-glycerol phosphates with 60mM sodium formate/5mM borax (10ml). [^{3}H]-inositol phosphate was eluted with 0.1M formic acid/0.2M ammonium formate (10ml), [^{3}H]-inositol bisphosphate with 0.1M formic acid/0.4M ammonium formate (10ml) and [^{3}H]-inositol trisphosphate together with [^{3}H]-inositol tetrakisphosphate with 0.1M formic acid/1.2M ammonium formate (10ml). Columns were calibrated with [^{3}H]-inositol and [^{3}H]-inositol phosphate standards.

Aliquots (1ml) of separated [^{3}H]-inositol phosphates were quantified by liquid scintillation spectrometry (Packard Model 2000CA). The scintillation fluid used was Ecoscint (National Diagnostics, Alyesbury) 10 volumes/volume sample.

4 APPARATUS AND TECHNIQUES -IN VIVO

Intraluminal pressure recordings were made from the anal sphincter of the guinea-pig in vivo in response to intravenous administration of drugs. Adult Duncan-Hartley guinea-pigs (250 - 400g) of either sex were anaesthetised with urethane (1.7gkg^{-1}, i.p.) and placed ventral side uppermost on an operating table. The trachea was cannulated to allow artificial respiration to be applied if necessary. The left jugular vein was cannulated for the administration of drugs and the right carotid artery for the measurement of blood pressure and heart rate. The arterial cannula contained heparinised saline (90 I.U.ml^{-1} of 0.9% saline) to prevent coagulation and was connected via a blood pressure transducer (Gould Pressure Transducer) and tachograph (Grass Preamplifier

7P44B) to a pen recorder (Grass Model 7D Polygraph, 7 DWU).

Manometric changes in the rectum and anal canal have been recorded until now by means of a balloon catheter (Schuster, et al., 1963; Kerremans & Penninckx, 1970). However, this method of measurement was precluded in the guinea-pig because of the small diameter of the anorectal canal (approx. 3mm). Thus, the intraluminal pressure of the anal canal was monitored by a Millar pressure transducer (O.D. 2mm, Millar Mikro-tip^{R} Catheter Pressure Transducer, Model PC-340, size 4F) placed in the rectoanal canal and connected via a Millar control box to a pen recorder (as above). This pressure transducer is more routinely used to monitor left ventricular pressure in the heart in vivo and has a smaller outer diameter than the balloon of a balloon catheter. It is therefore particularly suited to the measurement of small movements in pressure from small diameter vessels. The sphincter area is characterised by a region of endoluminal pressure which is higher than that observed in the rectum (Duthie & Bennett, 1963; Hill et al., 1960). Consequently, the optimum position of the transducer in the anal canal for the measurement of sphincteric pressures was determined by the area of highest pressure measured as the transducer was inserted in the anorectal canal. The IAS is known to contract in response to noradrenaline (Lim & Muir, 1985) but other non-sphincteric gastrointestinal muscles (e.g. taenia caeci, Bülbring, 1954) are known to relax in vitro. Therefore confirmation that the pressure transducer was placed in the sphincteric region was obtained by administration of noradrenaline ($8\mu g kg^{-1}$, i.v.) - an increase in intraluminal pressure denoted the position of the sphincter.

5 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 that 2mV, over an initial period of 10s,

b) inhibitory junction potentials were observed in response to field stimulation,

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

6 PHYSIOLOGICAL SALT SOLUTIONS

Krebs' solution with the following composition (mM) was used throughout the investigation:

NaCl, 118.4; $NaHCO_3$, 25.0; NaH_2PO_4, 1.13; KCl, 4.7; $CaCl_2$, 2.7; $MgCl_2$, 1.3; glucose 11.0; pH 7.4.

Where ionic composition of the Krebs' solution was modified, isotonicity was maintained by substituting or reducing the concentration of another appropriate ion.

7 DRUGS

The following drugs were used:

adenosine 5'-triphosphate disodium (ATP, Sigma), apamin (Sigma), atropine sulphate (Sigma), bombesin (Sigma), bradykinin acetate (Sigma), 8-bromoguanosine 3':5' cyclic monophosphate sodium (8-Br-cGMP,Sigma), carbachol chloride (Sigma), 2-chloroadenosine (Sigma), cromakalim

(Beecham), forskolin (Sigma), heparin sodium (Evans), isoprenaline sulphate (Aldrich), leu-enkephalin acetate (Sigma), met-enkephalin acetate (Sigma), αβmethylene adenosine 5'-triphosphate lithium (Sigma), βγ adenosine 5'-triphosphate sodium (Sigma), myo-[2-^{3}H]-inositol (Amersham), neuropeptide Y (porcine, Sigma), (-)noradrenaline bitartrate (Koch-Light), phentolamine mesylate (Ciba), 8-phenyltheophylline (Research Products Inc.), dl-propranolol hydrochloride (Sigma), 2-O-propoxyphenyl-8-azapurin-6-one (M&B 22948, May & Baker), sodium nitroprusside (Sigma), sodium pentobarbitone (May & Baker), somatostatin (Sigma), substance P acetate (Sigma), tetraethylammonium bromide (TEA, Sigma), tetrodotoxin (TTX, Sigma), urethane (Aldrich), vasoactive intestinal polypeptide (porcine, VIP, Sigma). Concentrations in the bath refer to the salts except TTX, apamin, vasoactive intestinal polypeptide, somatostatin and neuropeptide Y, which are expressed as concentrations of the base.

With the following exceptions, drugs were dissolved in saline (0.9%) prior to their addition to the organ bath, by the desired method of application. Cromakalim and forskolin, each were dissolved in 95% ethanol/0.1M sodium hydroxide (3:1, v/v). Noradrenaline was dissolved in saline (0.9%) containing ascorbic acid (2×10^{-4}M) and ethyl diamine tetra-acetic acid (3×10^{-5}M) to prevent oxidation of the catecholamine.

8 ANALYSIS OF RESULTS

Where appropriate, results were expressed as the mean ± standard deviation (S.D.), of n (number of observations). Student's t-test or one-way analysis of variance followed by the Tukey test, was used to test

for significance between means. A t-value of $p<0.05$ was taken as being significant. Three or more tissues were used to investigate each drug. Significance values are shown as asterisks, where $p<0.05$ is denoted by *, $p<0.01$ by ** and $p<0.001$ by *** throughout the thesis.

CHAPTER III

RESULTS

1 ELECTRICAL AND MECHANICAL

a) Guinea-pig Internal Sphincter

(1) Resting properties

When initially set up in the organ bath in Krebs' solution at 36 ± 0.5°C, the IAS had no tone. Noradrenaline (NA, 10^{-5}M) was applied directly to the bath to test that the preparation was indeed the IAS and not part of the rectum. This test procedure was carried out at the start of each experiment; gastrointestinal sphincters contract in response to α-adrenoceptor stimulation, non-sphincteric muscles relax. After washout of NA and gentle stretch (1g), a further 2 - 3g tone developed within 15 - 30min, and persisted for several hours throughout each experiment. The development of tone depended on the maintenance of spontaneous action potential (spike potential) discharge and was unaffected by cholinoceptor (atropine, 10^{-6}M) or adrenoceptor (phentolamine, 10^{-6}M) blockade or TTX (10^{-6}M) confirming its non-neuronal origin or control. In the absence of tone spike potentials were not observed; resting tone and membrane electrical activity are closely related.

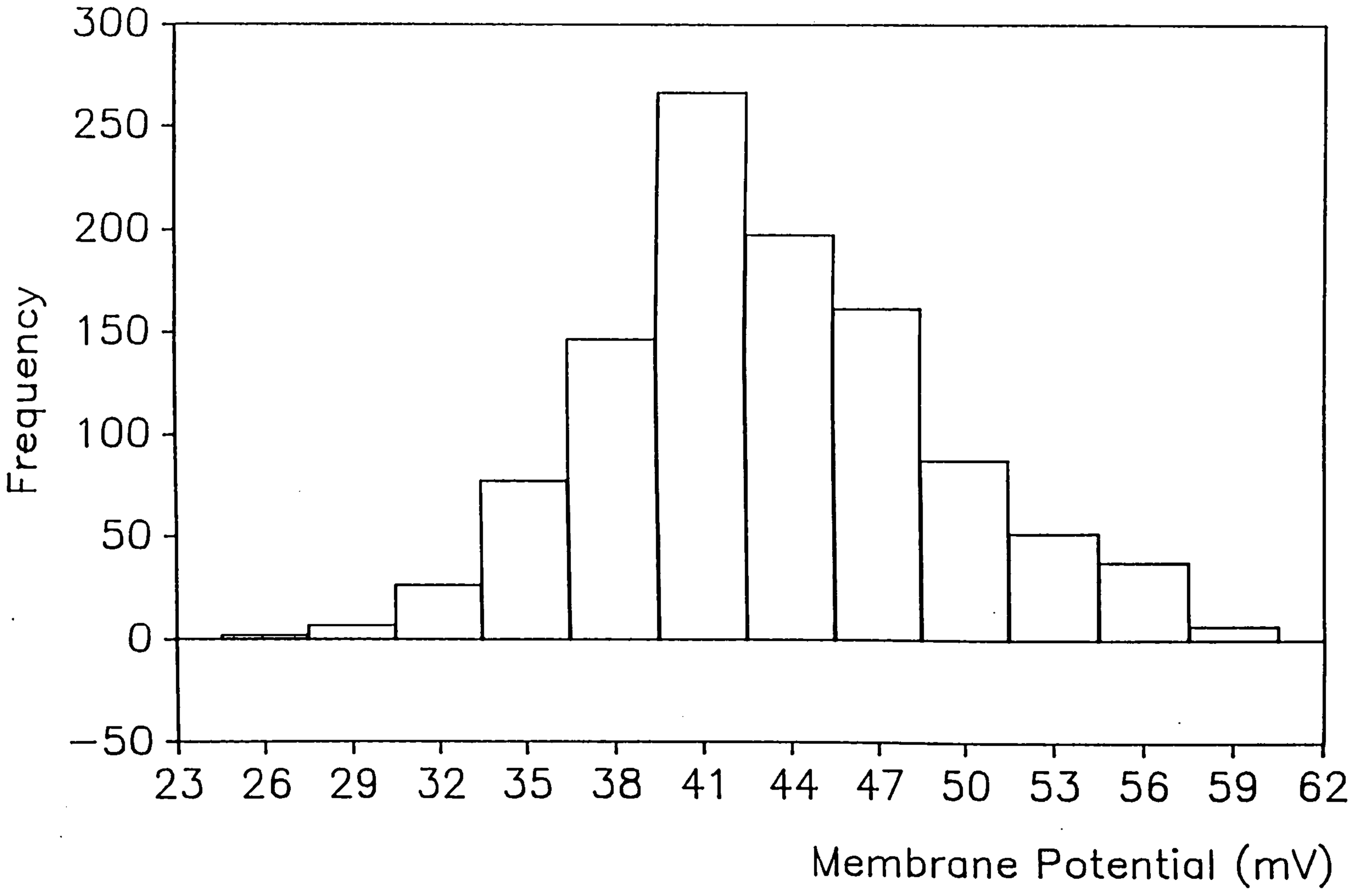
The resting membrane potential was distributed normally around a mean of -42±6mV (n=257). (Figure 13). Spike potentials reached 50mV in amplitude and were discharged a rate of 1 - 2Hz. Accompanying oscillations in tone were observed. Spontaneous ejps and ijps were not seen.

(ii) Response to field stimulation

When the tone had developed, field stimulation of intramural inhibitory nerves (single pulse, 5 pulses at 5, 10 & 20Hz; 0.5ms;

FIGURE 13:

Graphical representation of the population distribution of the resting membrane potential of cells impaled for intracellular electrical recording in the IAS.


300
250
200
150
100
50
0
−50
Frequency
23
26
29
32
35
38
41
44
47
50
53
56
59
62
Membrane Potential (mV)

supramaximal voltage) inhibited spike discharge and hyperpolarised the membrane (giving an ijp). These were immediately followed by relaxation. (Figure 14). The electrical and mechanical responses to field stimulation were each frequency-dependent (Figure 15) and unaffected by atropine and phentolamine (each 10^{-6}M) but blocked by TTX (10^{-6}M) confirming that they were produced by NANC inhibitory nerve stimulation. The optimum frequency of stimulation was 10Hz and at this frequency the ijp had a mean amplitude of 16 ± 9mV (n=69), a rapid rate of decline to the lowest point (41 ± 20mVs^{-1}, n=69) and a short duration (2.7 ± 0.8s, n=69) to recovery at the resting membrane potential. A post-stimulus increase in the frequency of spike discharge and a "rebound" contraction were often observed. These rebound effects are common characteristics of NANC inhibitory nerve stimulation (Bennett 1966). In some cases these "rebound" effects can be abolished by indomethacin suggesting a secondary release of prostaglandins (Burnstock et al., 1975). They have also in part been explained by the run down of inhibitory transmitter (Holman & Weinrich, 1975) or by the activation of slow excitatory substance P receptors (Neil et al., 1983). More recently, the rebound depolarisation has been explained by an inward rectification activated upon hyperpolarisation and represents the activation of a cation-specific conductance change (Benham et al., 1987). In the IAS there is no evidence available to support any of these suggestions.

It was proposed that responses produced by NANC inhibitory nerve stimulation were mediated by an increase in K^+ conductance (Lim & Muir, 1985). TEA (8 x 10^{-2}M) which non-selectively blocks K^+ channels abolished the ijp and the relaxation.(Figure 16 A & C). Furthermore, the bee venom apamin which blocks certain Ca^{2+}-dependent K^+ channels

FIGURE 14:

The simultaneously recorded electrical (upper trace) and mechanical responses of the IAS to field stimulation (single pulse, ss; 5 pulses at 5, 10Hz; 0.5ms; supramaximal voltage). Atropine and phentolamine were present throughout (each 10^{-6}M). Field stimulation produced spike inhibition, ijps and relaxed the muscle.

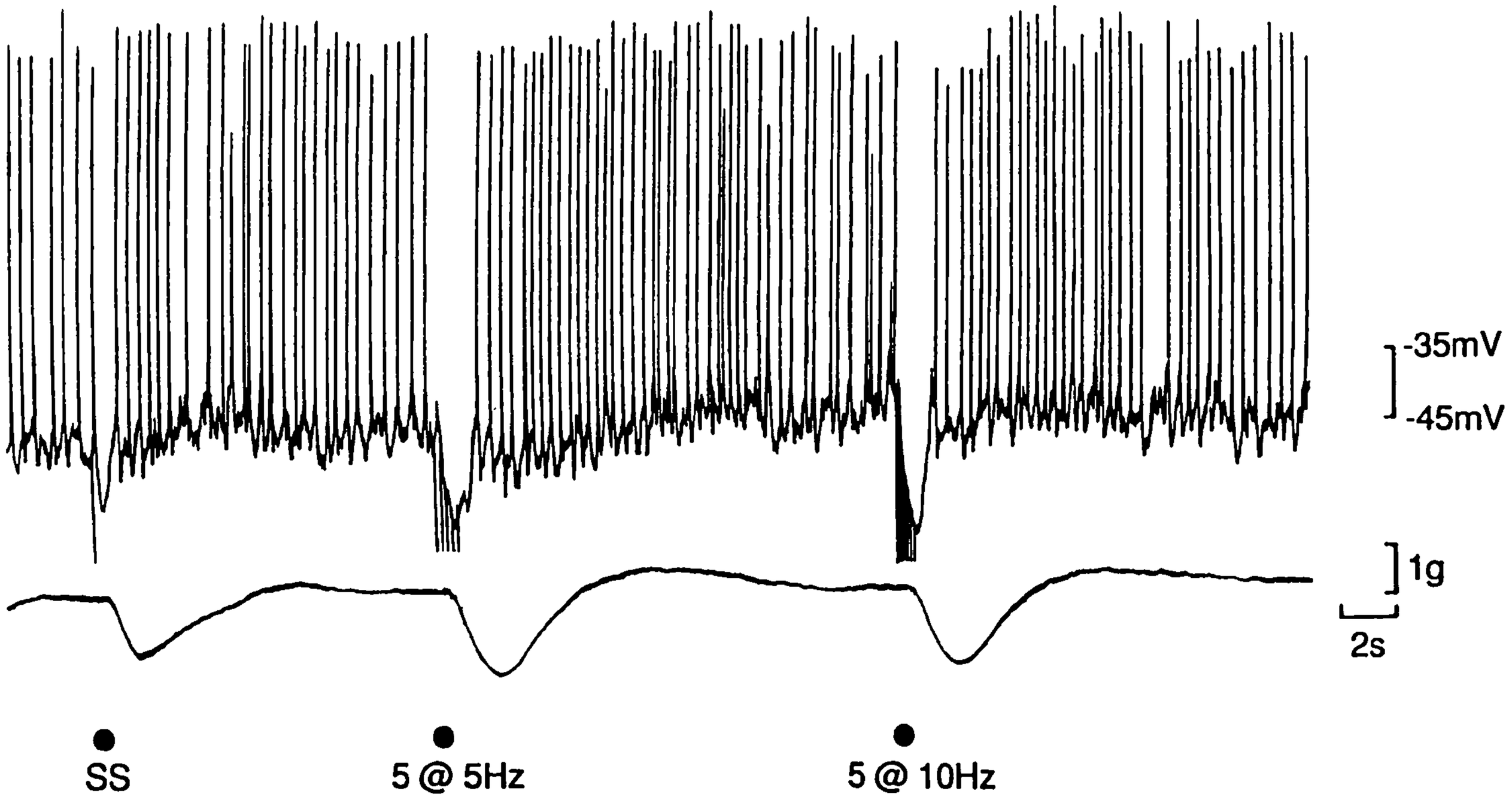
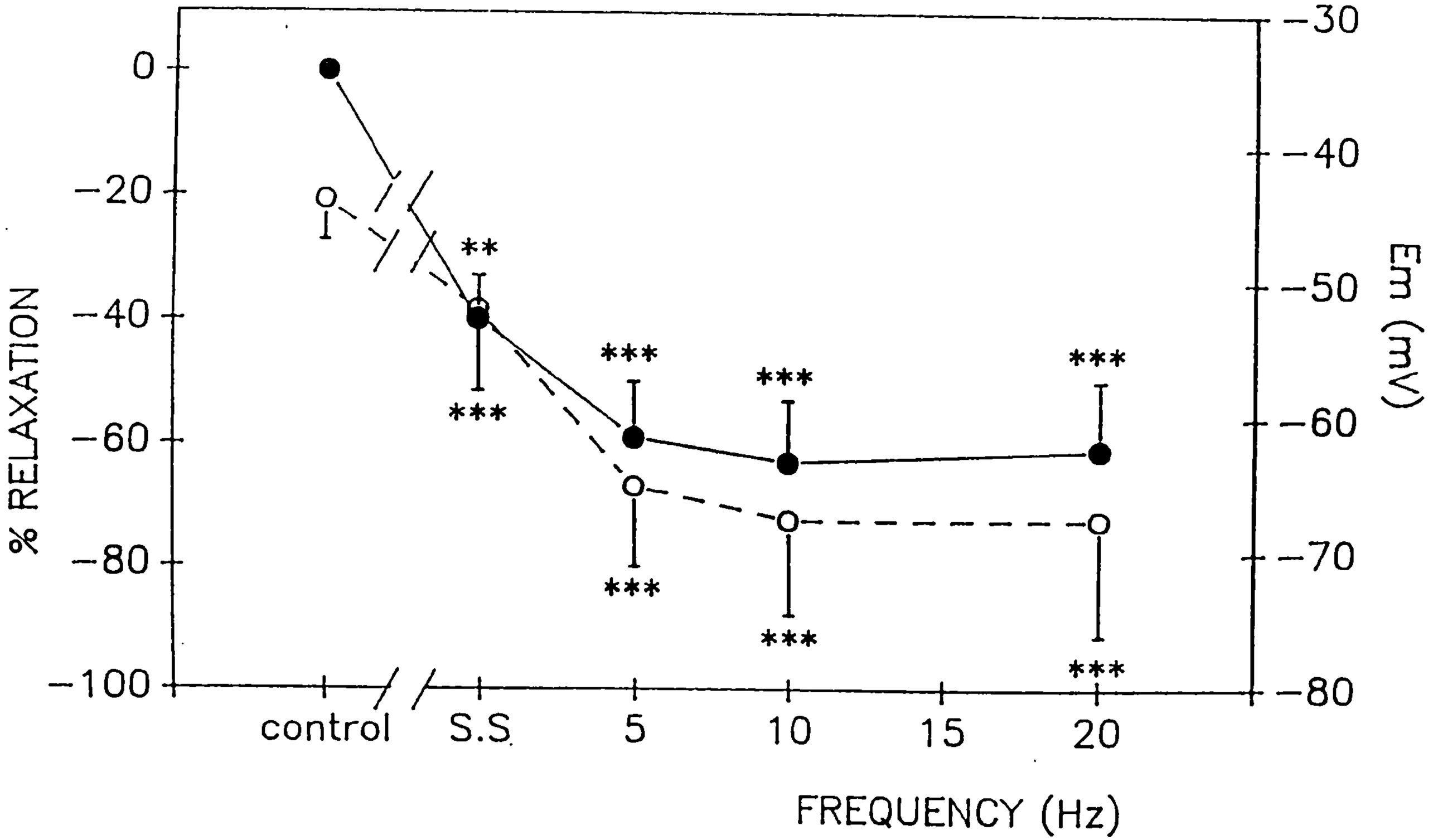

-35mV
-45mV
1g
2s
SS
5 @ 5Hz
5 @ 10Hz

FIGURE 15:

The effects of increasing the frequency of field stimulation (single pulse ss, 5 pulses at 5, 10 and 20 Hz; 0.5 ms; supramaximal voltage) on the electrical (Em, o-----o) and mechanical (% relaxation, ●——●) responses of the IAS. Each point represents the mean ± S.D. of at least 10 observations. Atropine and phentolamine (each 10^{-6} M) were present throughout. The graph shows a frequency-dependent hyperpolaristion and accompanying relaxation in response to field stimulation of inhibitory nerves.


0
−20
−40
−60
−80
−100
% RELAXATION
−30
−40
−50
−60
−70
−80
Em (mV)
control
S.S
5
10
15
20
FREQUENCY (Hz)
**
***

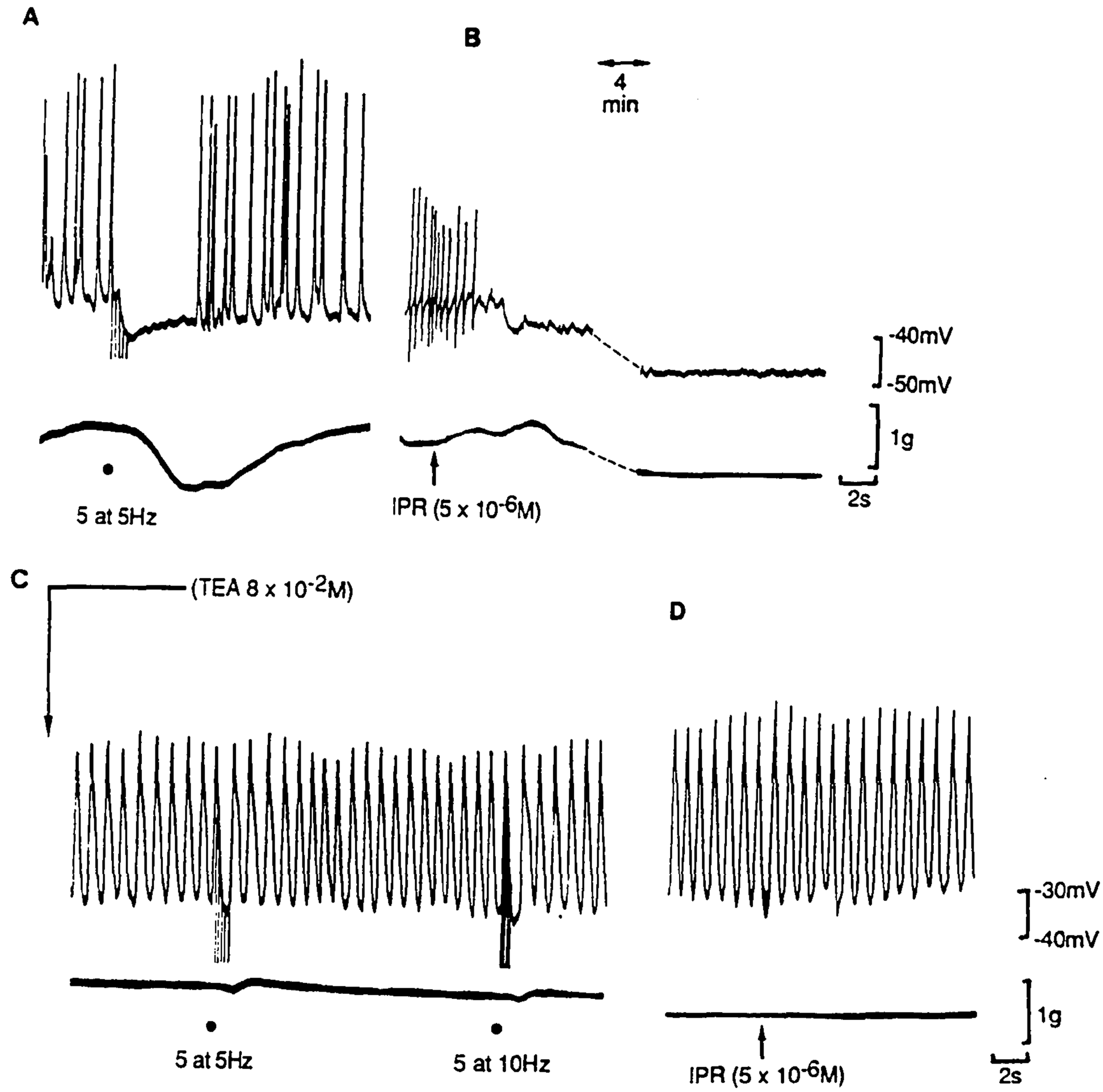


FIGURE 16:

The effect of field stimulation (5 pulses at 5 & 10 Hz; 0.5ms; supramaximal voltage) and of isoprenaline (IPR, 5 x 10^{-6}M) alone (A and B, respectively) and in the presence of tetraethylammonium (TEA, 5 x 10^{-2}M) (C & D respectively) added at the arrow for the duration of the experiment on the simultaneously-recorded electrical (upper trace) and mechanical responses of the IAS. Atropine and phentolamine (each 10^{-6}M) were present throughout. The ijps and relaxation produced by field stimulation and the hyperpolarisation to IPR were abolished by TEA.

A & B were from the same cell; C & D were from the same cell. Time between C & D was approximately 5 min.

(Banks et al., 1979) abolished the electrical and mechanical responses produced by NANC inhibitory nerve stimulation. (Figure 17 A & C). The results suggest that the response produced by inhibitory nerve stimulation is mediated by an increase in K^+ conductance and emphasises the importance of hyperpolarisation in the relaxant response; when hyperpolarisation is blocked, then relaxation is inhibited.

(iii) Effect of electrical current pulses

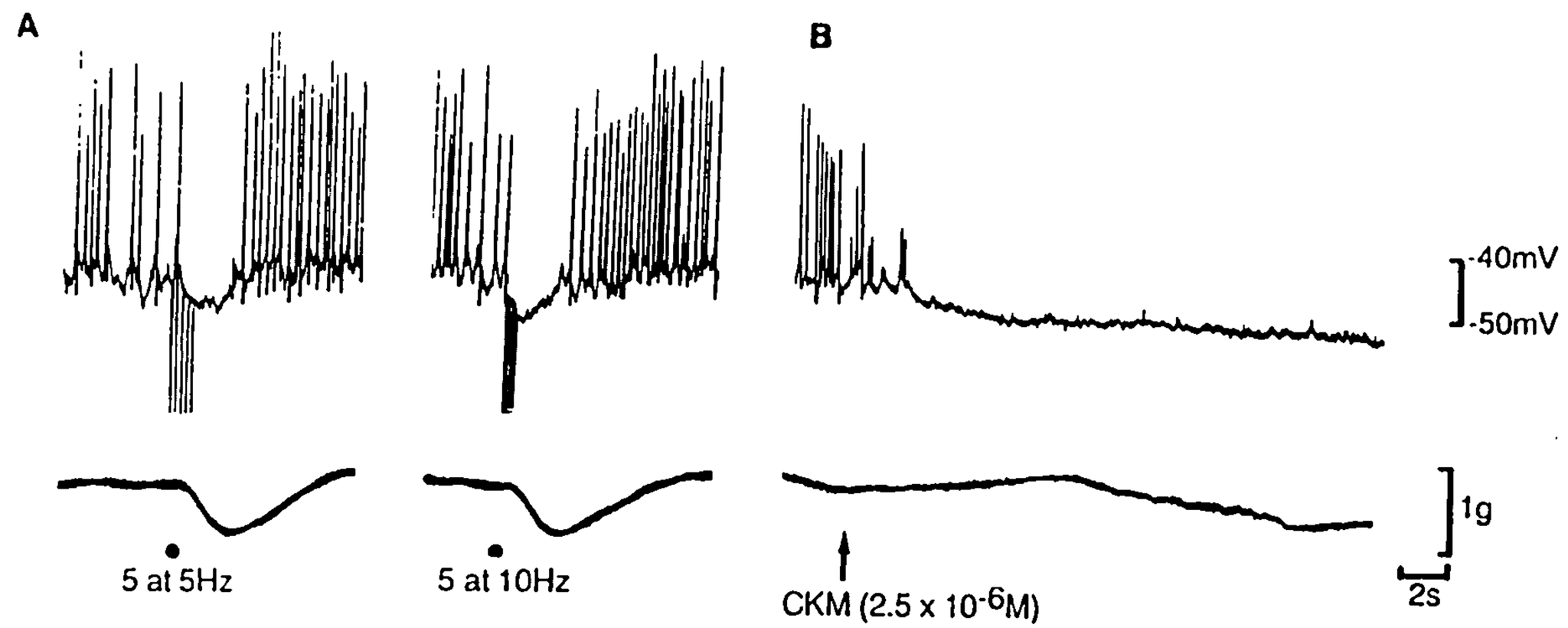
The effect of membrane hyperpolarisation on mechanical tone was studied by passing constant electrotonic current pulses applied for 5s directly into the muscle cells in an Abe & Tomita (1968) partition bath. Application of large inward currents (20mA) produced small reductions in tone (up to 17.5% inhibition). (Figure 18). Large outward currents, on the other hand, produced small contractions (20% excitation). From these experiments it seems that the tone of the IAS is dependent on the membrane potential of the muscle cells directly. The use of large external electrodes ensures current is passed directly into the muscle cells to alter the membrane potential (Abe & Tomita, 1968), rather than affecting the intramural nerves resulting in neurotransmitter release. The release of neurotransmitter could alter not just the membrane potential but also a variety of voltage-independent second messenger systems. From these experiments then, it seems that the tone of the IAS is dependent on the membrane potential directly.

(iv) Putative transmitters

Since the inhibitory intramural nerves of the IAS are NANC, a variety of putative transmitters was applied to investigate if the

FIGURE 17:

The effect of field stimulation (5 pulses each at 5 & 10 Hz: 0.5 ms; supramaximal voltage) and of cromakalim (CKM, 2.5 x 10^{-6}M) alone (A and B, respectively) and in the presence of apamin (4.5 x 10^{-6}M) (C and D, respectively) added at the arrow for the duration of the experiment on the simultaneously-recorded electrical (upper trace) and mechanical responses of the IAS. Atropine and phentolamine (each 10^{-6}M) were present throughout. Field stimulation produced ijps and relaxation which were abolished by apamin, however, the hyperpolarisation produced by cromakalim was unaffected. In the presence of apamin, tone declined and with it the ability to demonstrate mechanical inhibition (C & D). A & B were from the same cell, C & D were from the same cell; time between panels joined by the dotted line was 1 min.


A
B
-40mV
-50mV
5 at 5Hz
5 at 10Hz
CKM (2.5 x 10-6M)
1g
2s
C
Apamin (4.5 x 10-6M)
D
-25mV
-35mV
5 at 5Hz
5 at 10Hz
CKM (2.5 x 10-6M)
1g
2s

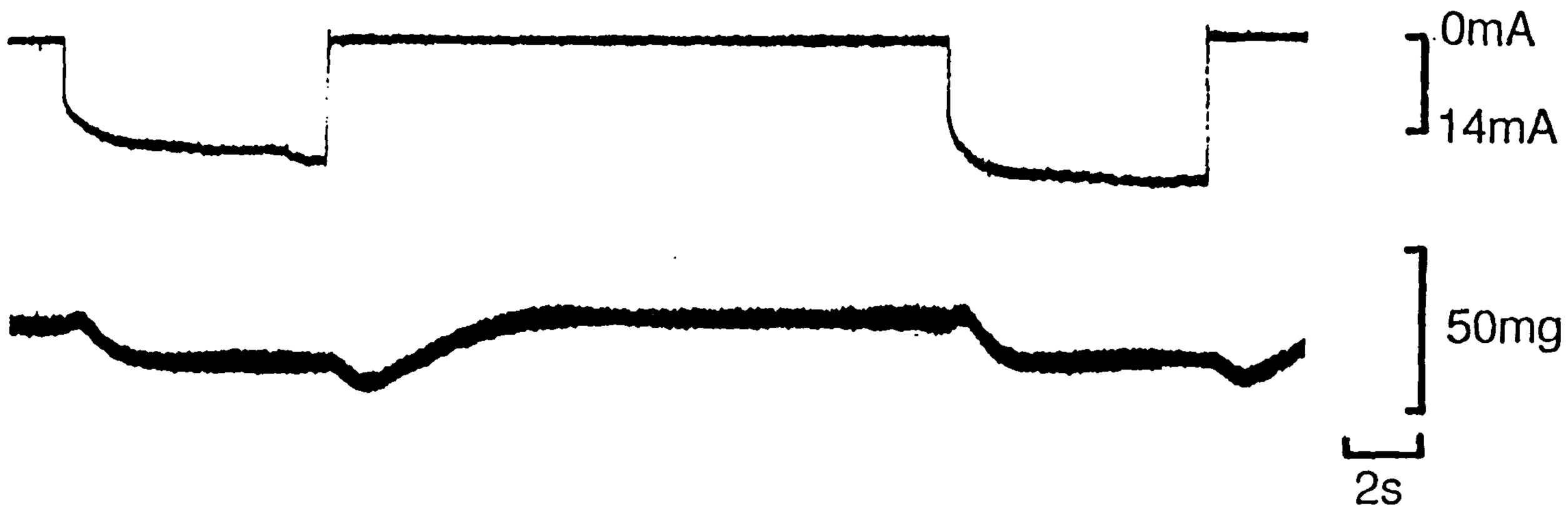


FIGURE 18:
The effect of constant hyperpolarising electronic current pulses (upper trace; 14mA; 5s) on the mechanical tone of the IAS. These constant current pulses produced relaxation of the sphincter. Atropine and phentolamine were present throughout (each 10^{-6}M).

neuronal response could be mimicked. To minimise loss and ensure their discrete localisation to the electrical recording site, the compounds were applied by hydrostatic pressure ejection (Picospritzer^{R}, General Valve Corporation, N.J., U.S.A.) from a broken off micropipette (O.D. 2 - 10μm) and the membrane changes studied. Because of the small volume of drug applied to the tissue, mechanical effects were restricted to a small number of muscle bundles and could not be monitored. To measure accompanying mechanical responses, a separate series of experiments were conducted in which drugs were added directly into the organ bath by microsyringe (10 - 100μl) as close to the recording site as possible.

Purines

Previous reports (Lim, 1985; Lim & Muir, 1986) proposed that the purine, adenosine 5'-triphosphate (ATP) was the inhibitory neurotransmitter in the IAS. ATP (5.8×10^{-4}M) applied by hydrostatic pressure ejection (10 - 55ms; 40p.s.i.) immediately produced a spike inhibition and a dose-dependent hyperpolarisation. (Figure 19). The amplitude of the membrane potential change (ATP, 5.8×10^{-4}M) was 14 ± 6mV (n=12). It had a rapid decline (18 ± 24mVs^{-1}, n=12) and a short duration (14 ± 9s, n=12). In these respects the membrane hyperpolarisations resembled the ijps observed in response to field stimulation.

ATP did not produce hyperpolarisation alone. When applied by microsyringe, ATP (10^{-7} - 10^{-3}M) produced hyperpolarisations followed by relaxations which were dose-dependent. (Figure 20). Like those to field stimulation, the electrical and mechanical responses produced by ATP were virtually abolished by TEA (8×10^{-2}M) (Figure 21A) or apamin (5 x

FIGURE 19:

A. The electrical responses of the IAS to ATP (5.8×10^{-4}) applied locally from a micropipette by pressure ejection (tip diameter 2-10μm; 40p.s.i.; 10-40ms). B. The electrical responses to field stimulation (5 pulses at 5 Hz; 0.5 ms; supramaximal voltage) and to exogenously applied ATP (5.8×10^{-4}M) in the same cell. Atropine and phentolamine were present throughout (each 10^{-6}M). ATP hyperpolarised the membrane to a comparable extent to the ijp produced by field stimulation. ATP and field stimulation each inhibited spike discharge and hyperpolarised the membrane. The amplitude, rate of onset and duration of the membrane change to each stimulus were comparable. Time between A & B was 5 min.

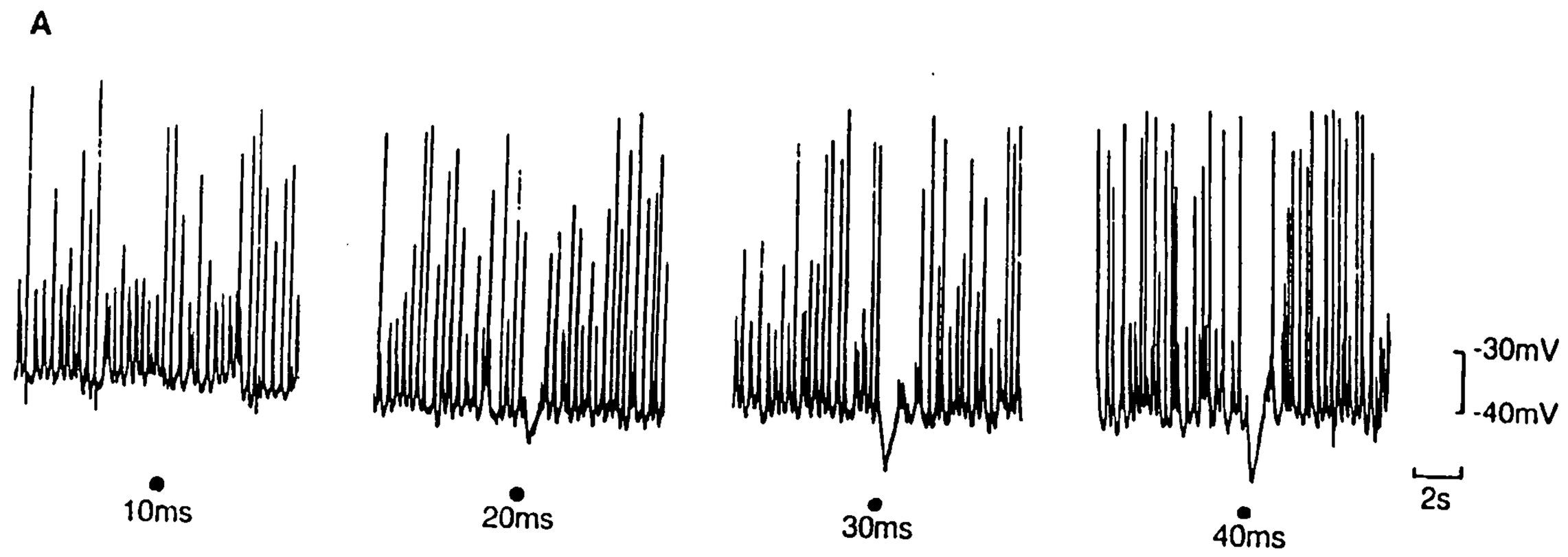

A
10ms
20ms
30ms
40ms
-30mV
-40mV
2s
B
45ms
5 at 5Hz
47ms
-30mV
-40mV
2s

FIGURE 20:

The effect of increasing concentrations of ATP (10^{-7} - 10^{-3}M) on the electrical (Em, o-----o) and mechanical (% relaxation, ●——●) responses of the IAS. Each point represents the mean ± S.D. of at least 9 observations. Atropine and phentolamine (each 10^{-6}M) were present throughout. ATP produced a dose-dependent hyperpolarisation followed by relaxation of the IAS.

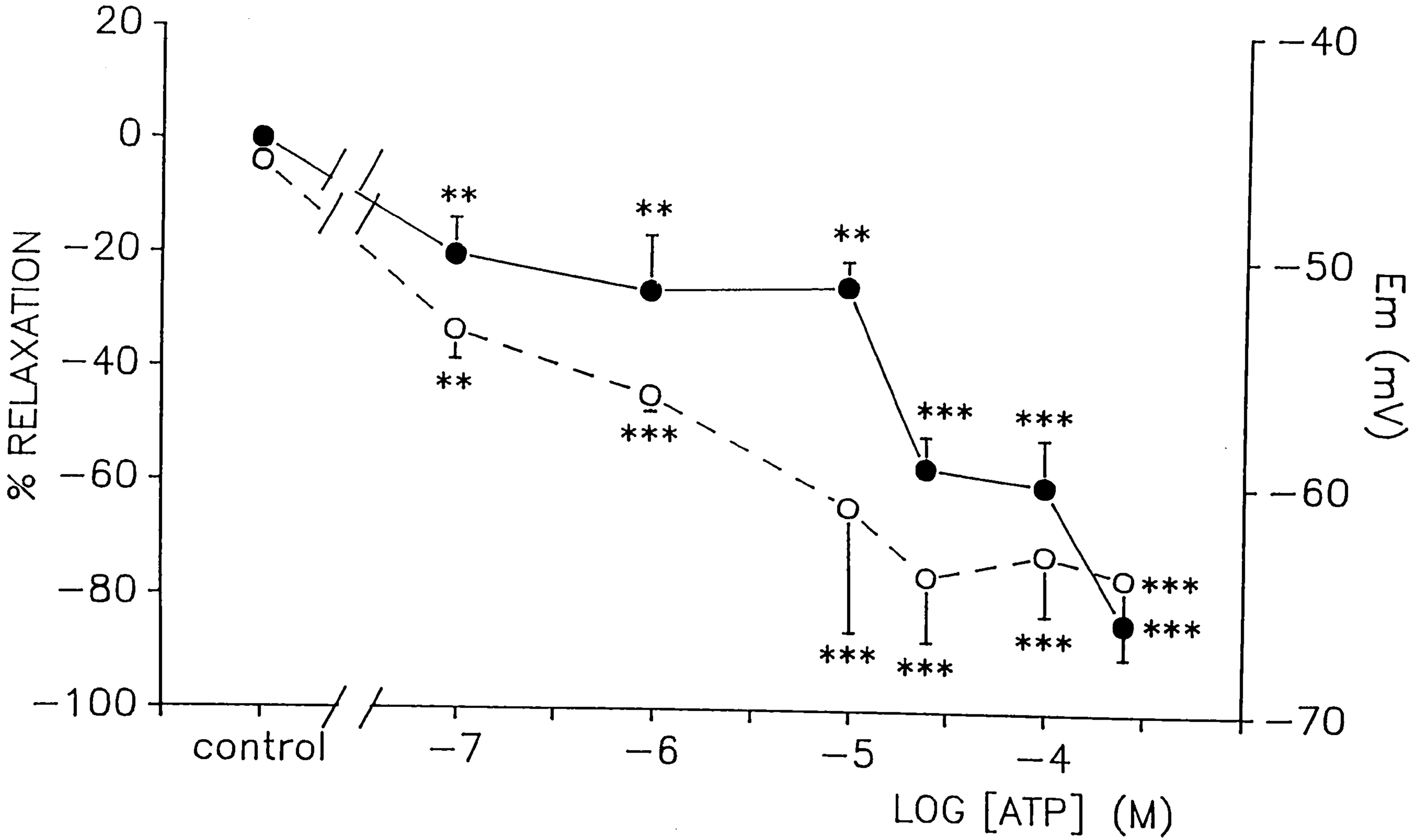
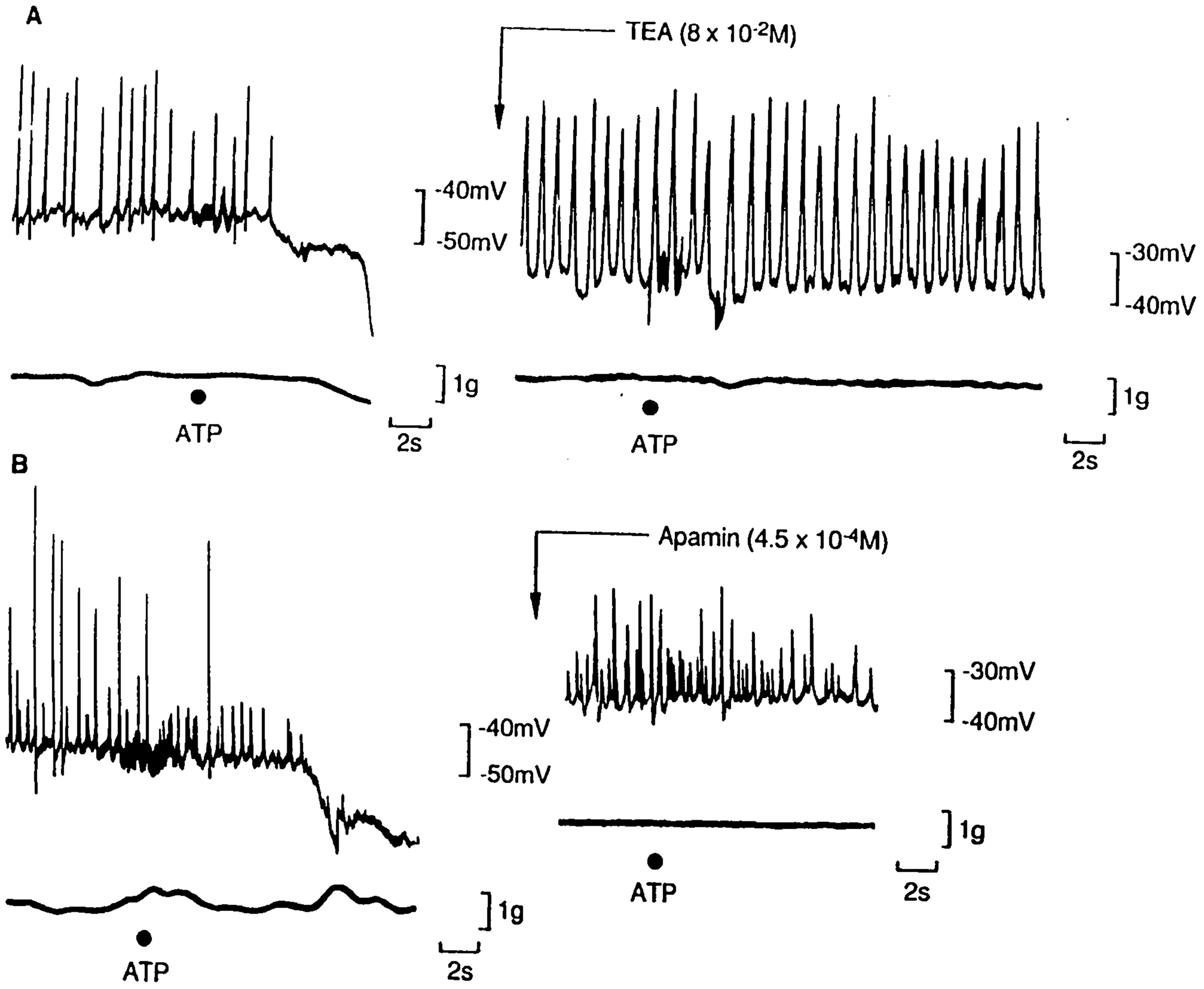

20
0
−20
−40
−60
−80
−100
% RELAXATION
−40
−50
−60
−70
Em (mV)
control
−7
−6
−5
−4
LOG [ATP] (M)
**
***

FIGURE 21:

A. The effect of tetraethylammonium (TEA, 8 X 10^{-2}M) applied at the arrow for the duration of the experiment on the simultaneously recorded intracellular electrical (upper trace) and mechanical responses of the IAS to exogenous application of ATP (10^{-4}M) from a microsyringe. Atropine and phentolamine were present throughout (each 10^{-6}M). TEA abolished the hyperpolarisation and relaxation produced by ATP.

B. The effect of apamin (4.5 x 10^{-6}M) on the simultaneously recorded electrical (upper trace) and mechanical responses of the IAS to exogenous application of ATP (10^{-4}M) from a microsyringe. Atropine and phentolamine (each 10^{-6}M) were present throughout. Apamin abolished the hyperpolarisation and relaxation produced by ATP.

Recordings A & B were taken from separate tissues.


A
TEA (8 x 10-2M)
-40mV
-50mV
-30mV
-40mV
1g
ATP
2s
1g
ATP
2s
B
Apamin (4.5 x 10-4M)
-40mV
-50mV
-30mV
-40mV
1g
ATP
2s
1g
ATP
2s

10^{-6}M) (Figure 21B).

In contrast to the results produced by ATP, spike discharge and membrane potential remained unaffected by either αβMeATP (10^{-5} - 10^{-3}M; 5-200ms; 40p.s.i.) - the P_{2x}-purinoceptor agonist (Figure 22) or adenosine (10^{-3}M; 5-480ms; 40p.s.i.) - the P_1-purinoceptor agonist (Figure 23).

The evidence presented here from the local application of ATP supports the proposal that ATP is the inhibitory NANC neurotransmitter acting on P_{2y}-purinoceptors (Burnstock & Kennedy, 1985) in the IAS. These results also highlight the interdependence of the electrical and mechanical responses since neither hyperpolarisation nor relaxation exists without the other.

Peptides

NANC neurotransmission, it has been proposed, may be mediated via peptides in the IAS - in particular vasoactive intestinal polypeptide (Biancani et al., 1983). A variety of bioactive peptides were studied by discrete localised ejection from a broken-off micropipette (O.D. 2 - 10μm).

Bradykinin (10^{-6}M; 15-55ms; 40p.s.i) had no effect on spike potential discharge or membrane potential. However, at 10^{-3}M (2-70ms; 40p.s.i.) spike inhibition and membrane hyperpolarisation were evident. The amplitude of the hyperpolarisation was 3 ± 2.5mV (n=10) with a slow rate of decline (0.8 ± 1.1mVs^{-1}, n=10) and prolonged duration (13 ± 6s, n=10) (Figure 24) some 8s after application.

Vasoactive intestinal polypeptide (VIP, 10^{-7} & 10^{-5}M; 5-75ms; 40p.s.i.) was usually ineffective, although 10^{-7}M (35ms; 40p.s.i.) on

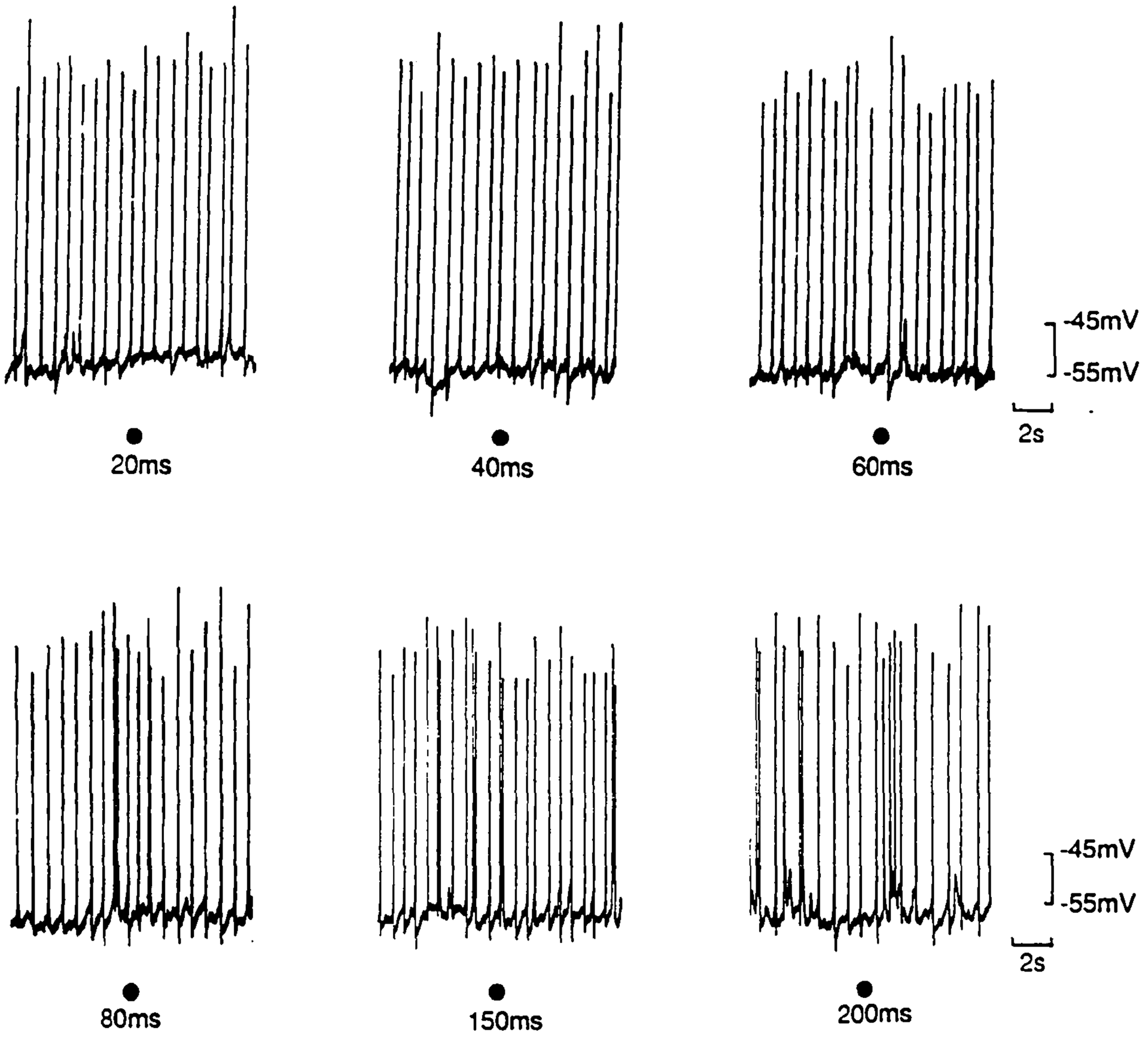


FIGURE 22:

Intracellular electrical responses of the IAS to addition of αβmethyleneATP (αβMeATP, 10^{-5}M) applied locally from a micropipette by pressure ejection (tip diameter 2μm; 40p.s.i; 20-200ms). Atropine and phentolamine were present throughout (each 10^{-6}M). αβMeATP produced no change in either spike potential discharge or membrane potential.

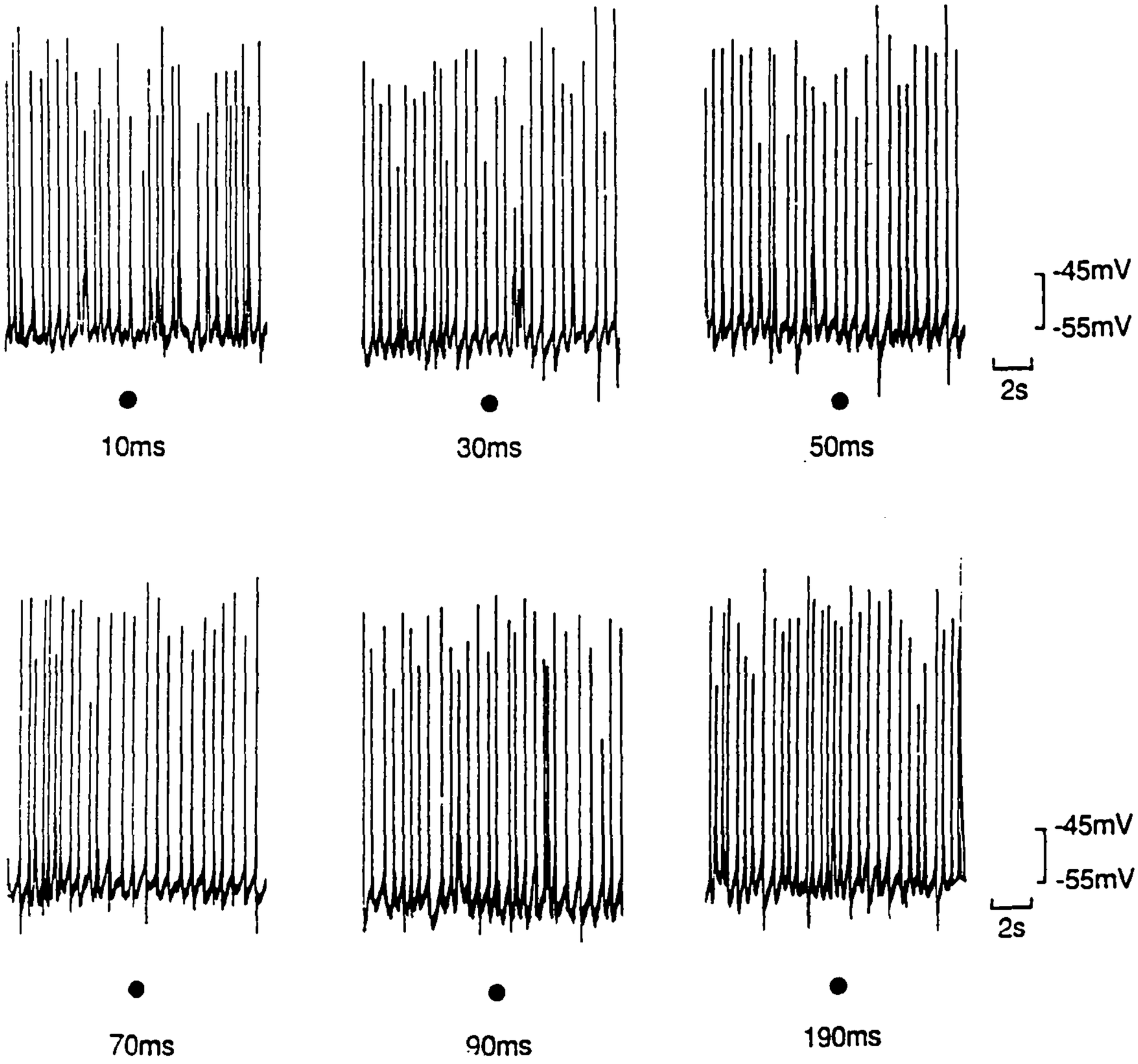


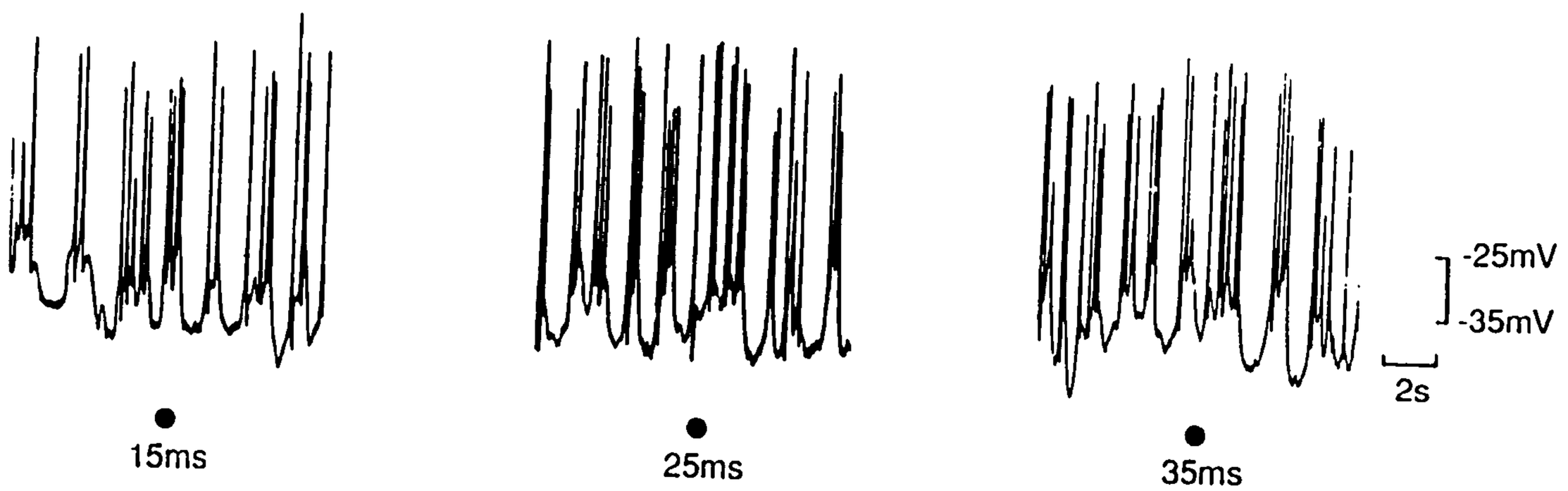
FIGURE 23:

Intracellular electrical responses of the IAS to addition of adenosine (10^{-3}M) applied locally from a micropipette by pressure ejection (tip diameter 2-10μm; 40p.s.i; 10-190ms). Atropine and phentolamine were present throughout (each 10^{-6}M). Adenosine produced no change in either spike potential discharge or membrane potential.

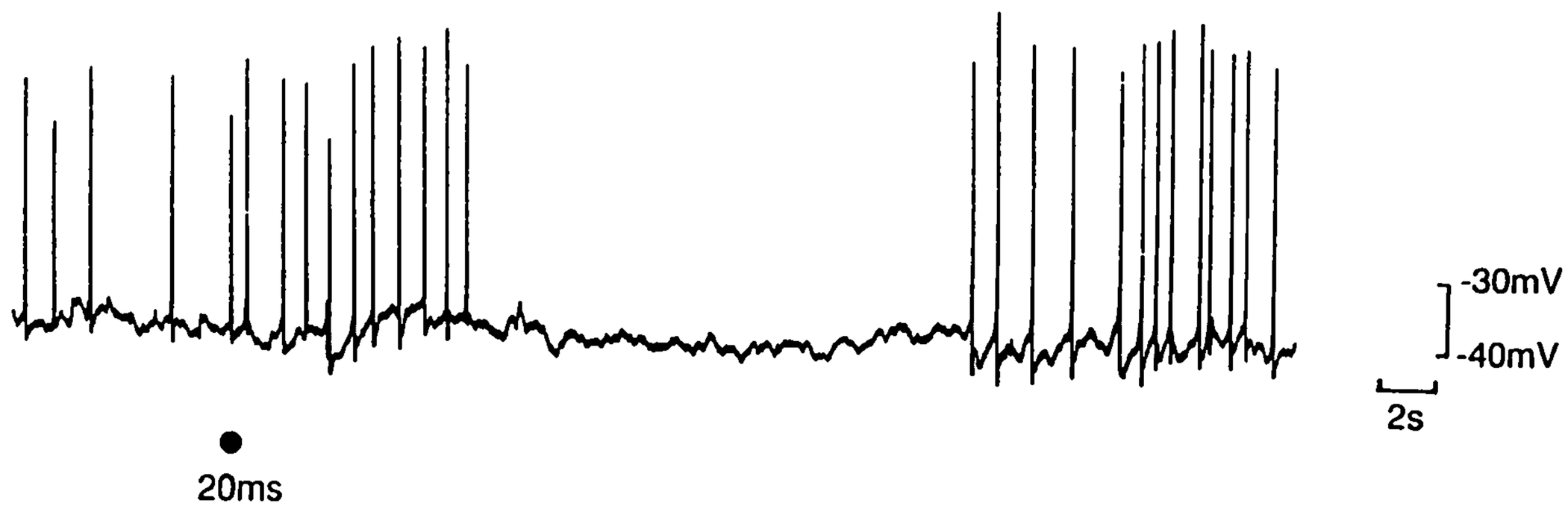
FIGURE 24:

The effect of bradykinin (10^{-6}M in A and 10^{-3}M in B) on the intracellular electrical responses of IAS applied by hydrostatic pressure ejection in two different cells (tip diameter 2-10μm; 40p.s.i; 15-55ms in A and 20ms in B). Atropine and phentolamine were present throughout (each 10^{-6}M). At a higher concentration (10^{-3}M) bradykinin inhibited spike potential discharge and produced a small, slow hyperpolarisation.

A. Bradykinin (● 10^{-7}M)



B. Bradykinin (● 10^{-3}M)



occasion produced brief, irregular hyperpolarisations some 7s after application. (Figure 25).

Despite the effects of bradykinin (10^{-3}M) and VIP (10^{-7}M) neither peptide produced membrane potential changes comparable with those observed to field stimulation. The responses produced by these peptides were not immediate on application. With bradykinin the hyperpolarisation was small, slow and prolonged and with VIP the hyperpolarisations were brief and irregular.

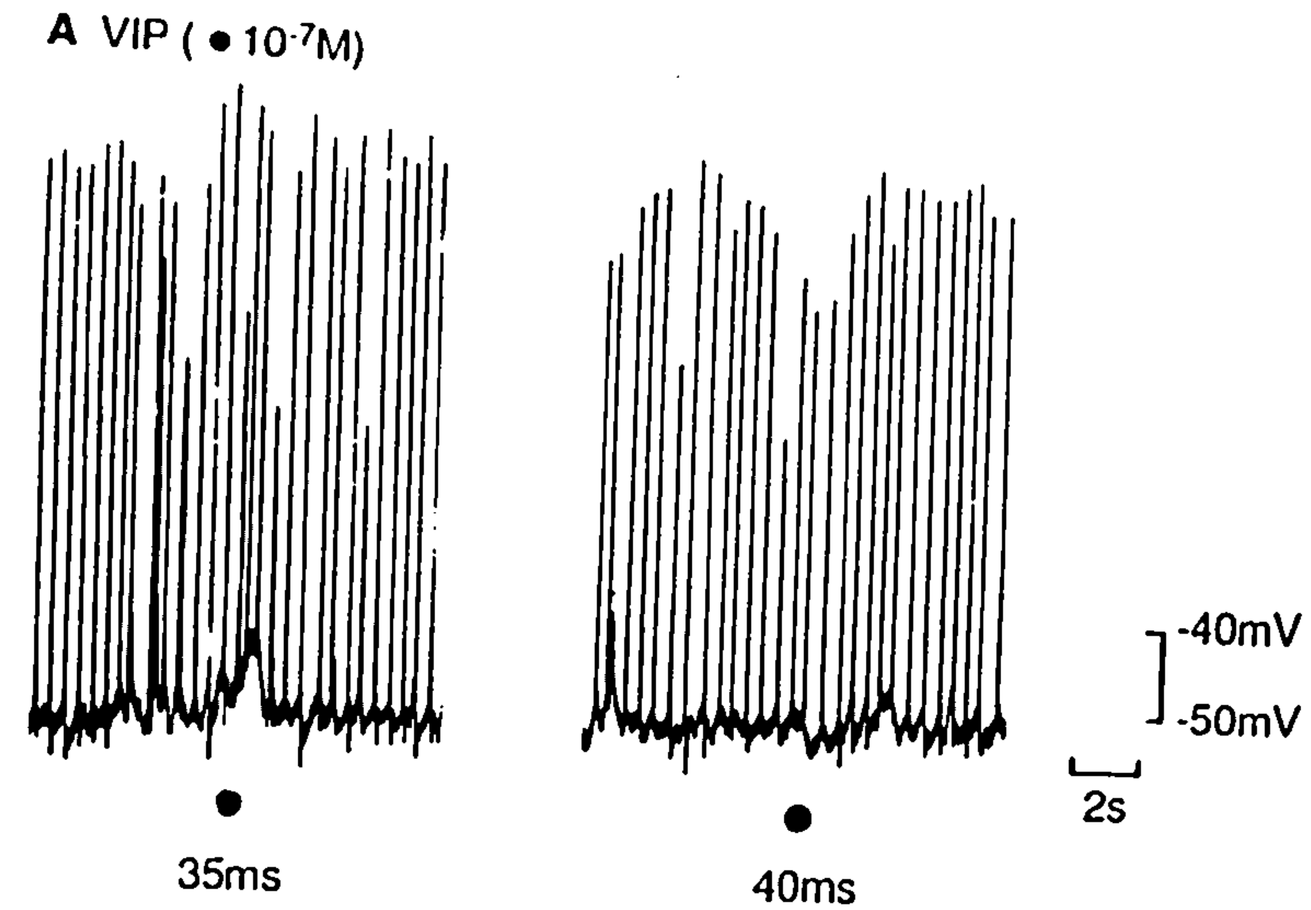
Like saline (0.9%; 5-175ms; 40p.s.i.) (Figure 26B), other peptides had no effect on either spike discharge or the resting membrane potential. These peptides were: bombesin (10^{-5}M; 5-300ms; 40p.s.i.) (Figure 27), leu-enkephalin ($1.8x10^{-4}$M; 5-400ms; 40p.s.i.), met-enkephalin ($1.8x10^{-5}$M; 10-150ms; 40p.s.i.) (Figure 28), neuropeptide Y (10^{-5}M; 10-200ms; 40p.s.i.), somatostatin 10^{-6}- 10^{-3}M; 10-100ms; 40p.s.i.) (Figure 29) and substance P (7.4×10^{-6}- 7.4×10^{-4}M; 10-100ms; 40p.s.i.) (Figure 26). Therefore it is evident from the results presented here that none of these peptides is a candidate for the putative inhibitory neurotransmitter in the IAS.

(v) Effects of β-adrenergic stimulation

Relaxation can be induced in vascular and non-vascular smooth muscle by catecholamines activating β-adrenoceptors (Bülbring & Den Hertog, 1980; Magaribuchi & Osa, 1971; Itoh et al., 1982). Isoprenaline inhibited spike discharge and produced membrane hyperpolarisation which was followed by relaxation in the IAS. The hyperpolarisation and mechanical response (isoprenaline 10^{-4}M) were smaller in amplitude than those produced by field stimulation (7.5 ± 3mv, $n=6$, $p< 0.01$ and 20%

FIGURE 25:

The effect of vasoactive intestinal polypeptide (VIP 10^{-7}M, in A & B) on the intracellular electrical responses of two different cells in the same preparation of the IAS applied by hydrostatic pressure ejection (tip diameter 2-10μm; 40p.s.i; 35-40ms in A & 35ms in B). Atropine and phentolamine were present throughout (each 10^{-6}M). VIP was for the most part ineffective, however, occasionally irregular membrane hyperpolarisations were observed.


A VIP (• 10-7M)
-40mV
-50mV
2s
35ms
40ms

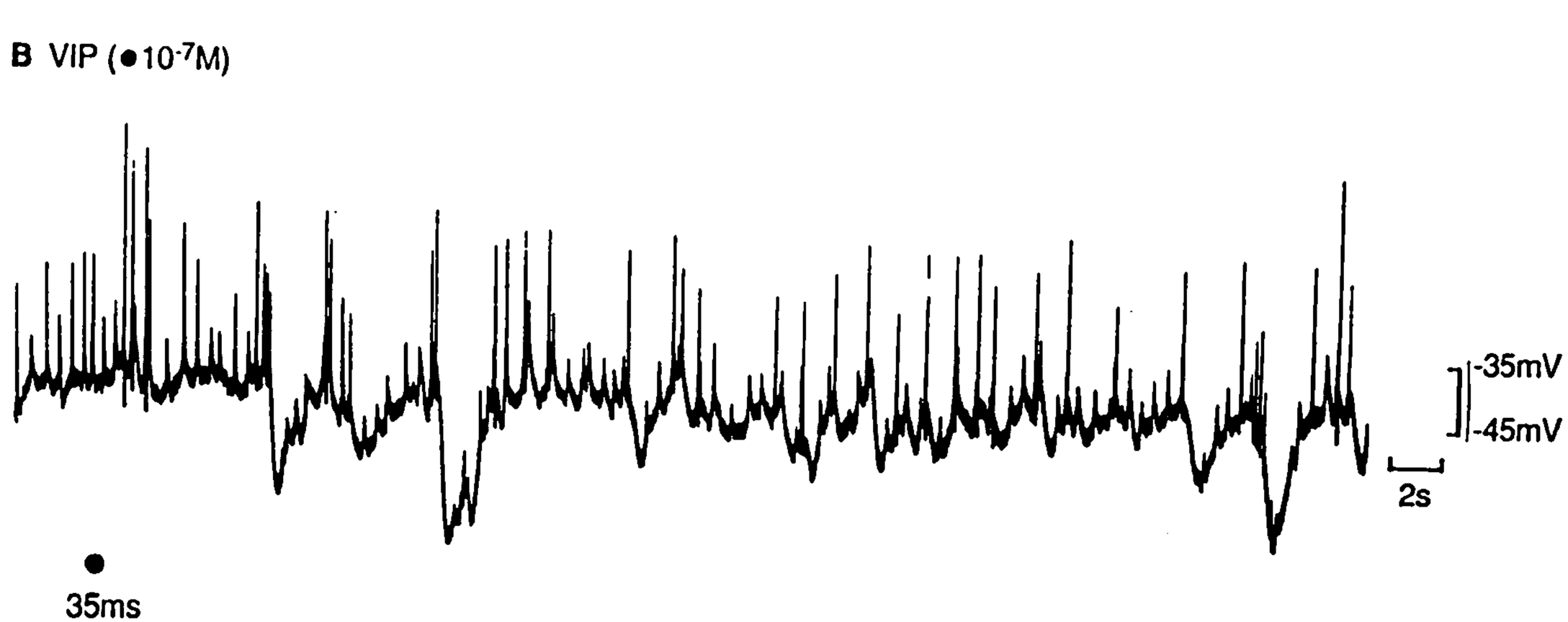
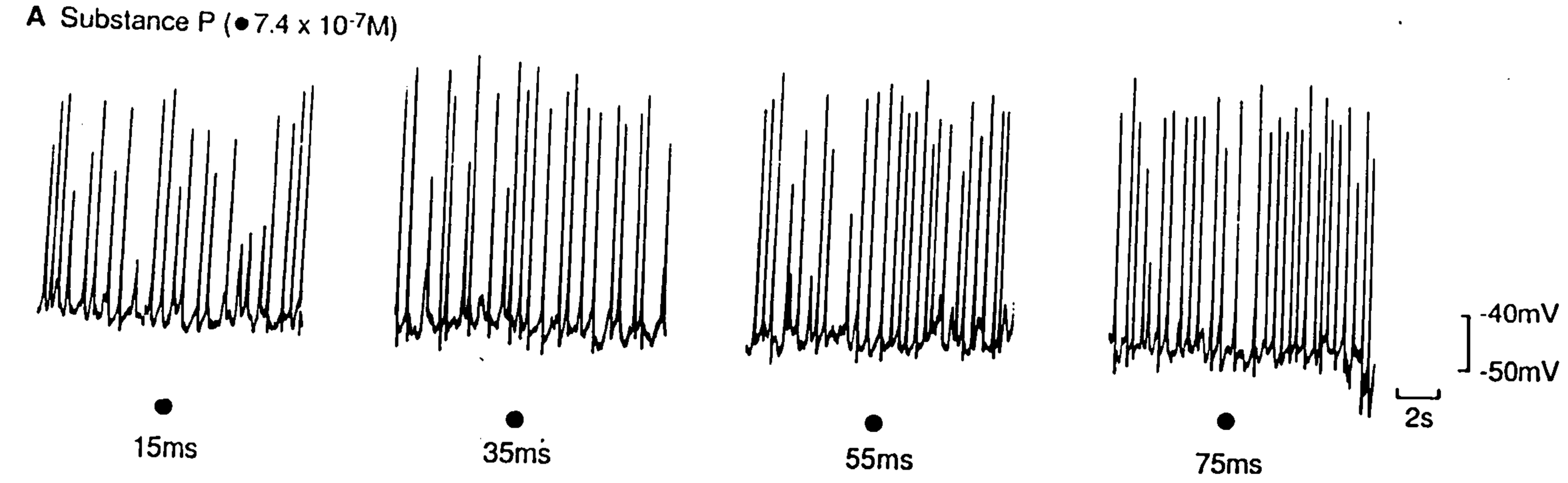

B VIP (•10-7M)
-35mV
-45mV
2s
35ms

FIGURE 26:

Intracellular electrical responses from two different cells in the same preparation of the IAS to application of substance P ($7.4x10^{-7}$ M, in A) and saline (0.9%, in B) from a micropipette by hydrostatic pressure ejection (tip diameter 2-10μm, 40p.s.i.; 15-75ms in A, 15-155ms in B). Atropine and phentolamine were present throughout (each 10^{-6} M). Neither substance P nor saline had any effect on either spike potential discharge or the resting membrane potential.


A Substance P (● 7.4 x 10-7M)
-40mV
-50mV
2s
15ms
35ms
55ms
75ms
B. Saline (● 0.9%)
-40mV
-50mV
2s
15ms
35ms
65ms
155ms

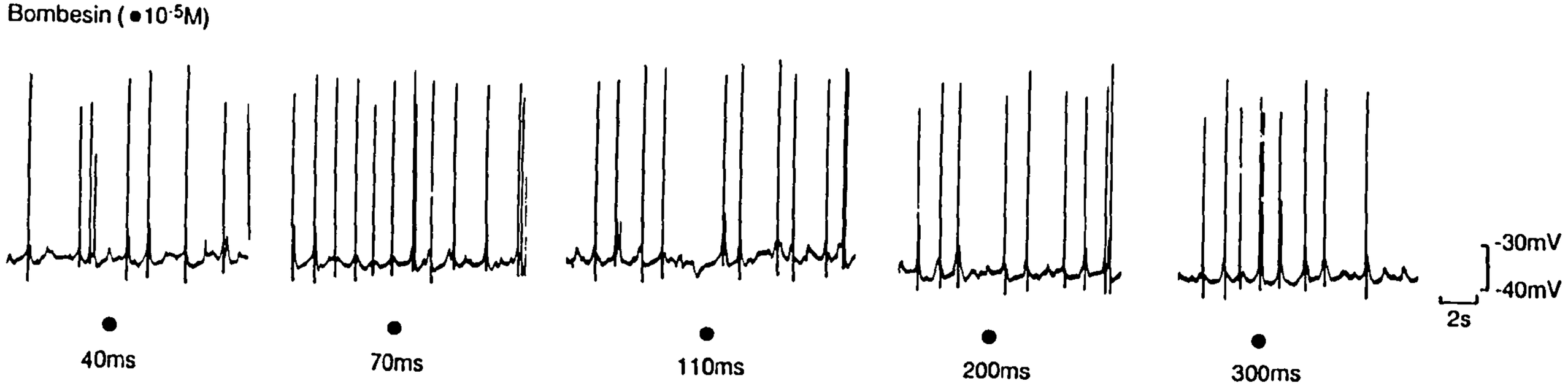


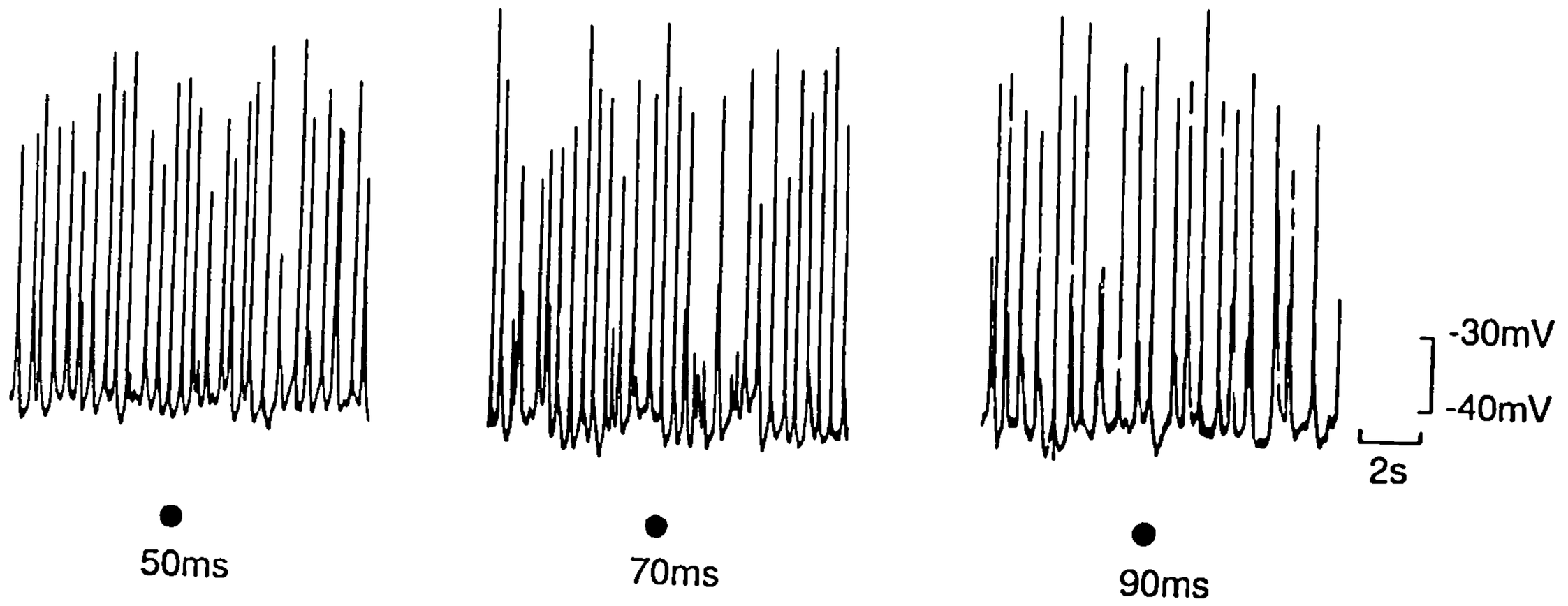
FIGURE 27:

Intracellular electrical responses from the same cell in the IAS to local application of bombesin (10^{-5}M) from a micropipette by hydrostatic pressure ejection (tip diameter 2-10μm; 40p.s.i.; 40-300ms). Atropine and phentolamine were present throughout (each 10^{-6}M). Bombesin had no effect on either spike potential discharge or the resting membrane potential.

FIGURE 28:

The intracellular electrical responses of two cells from the same preparation of the IAS to local application of leu-enkephalin ($1.8x10^{-4}$M, in A) and met-enkephalin ($1.8x10^{-5}$M, in B) from a micropipette by hydrostatic pressure ejection (tip diameter 2-10μm; 40p.s.i.; 50-90ms in A, 10-70ms in B). Atropine and phentolamine were present throughout (each 10^{-6}M). Neither leu-enkephalin nor met-enkephalin had any effect on spike potential discharge or the resting membrane potential.

A Leu-Enkephalin (●1.8 x 10^{-4}M)



B Met-Enkephalin (●1.8 x 10^{-5}M)

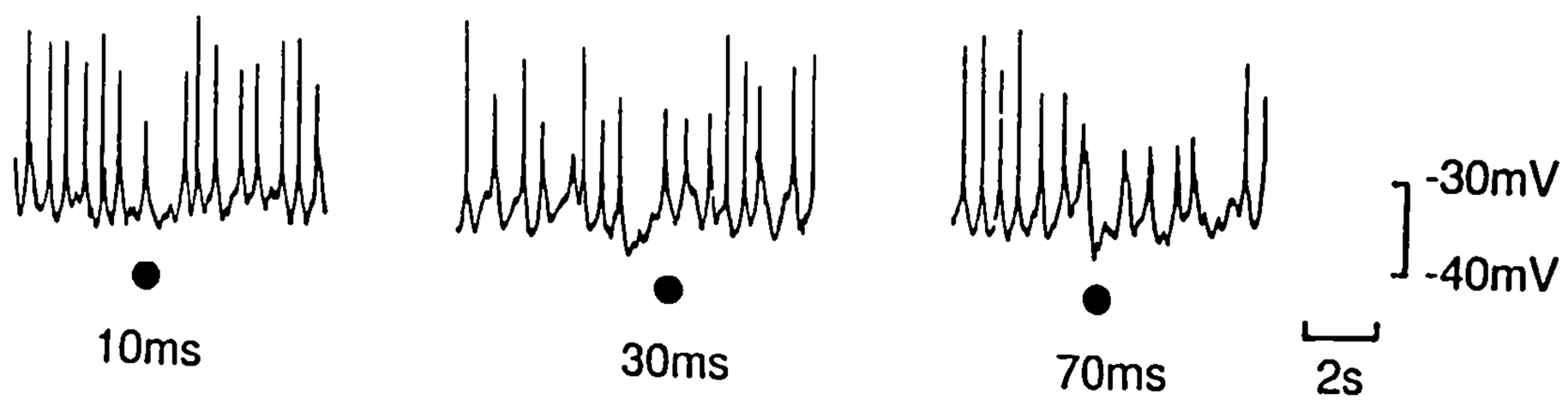
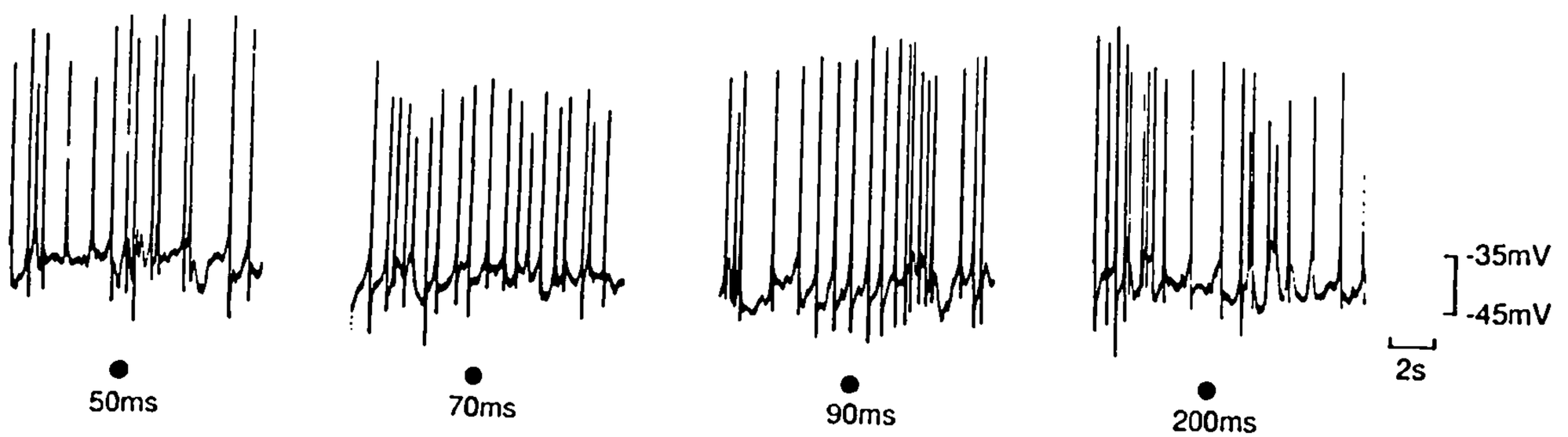


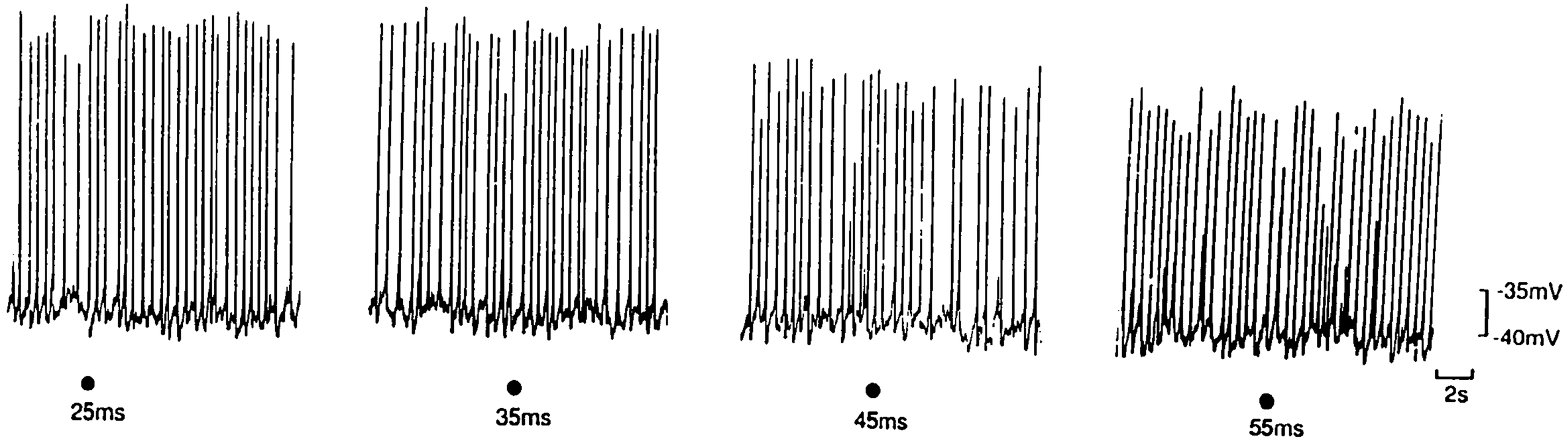
FIGURE 29:

The intracellular electrical responses of two cells in the same preparation of the IAS to local application of neuropeptide Y(10^{-5} in A) and somatostatin (10^{-6}M in B) by hydrostatic pressure ejection (tip diameter 2-10μm; 40p.s.i.; 50-200ms in A, 25-55ms in B). Atropine and phentolamine were present throughout (each 10^{-6}M). Neither neuropeptide Y nor somatostatin produced any effect on spike potential discharge or the resting membrane potential.

A. Neuropeptide Y (● 10^{-5}M)



B. Somatostatin (● 10^{-6}M)



reduction in tone,respectively). (Figure 16B). The membrane potential change was slow (0.6 ± 0.5mVs^{-1}, n=6) and prolonged (40 ± 4 s, n=6). Both the electrical and mechanical responses produced by isoprenaline (10^{-9} - 10^{-4}M) were dose-dependent (Figure 30A) and any dose which produced hyperpolarisation produced relaxation.

Propranolol (10^{-6}M), a β-adrenoceptor antagonist (Figure 31) and TEA ($8x10^{-2}$M) (Figure 16B&D) each abolished the electrical and mechanical responses produced by isoprenaline (10^{-4}M). In contrast, apamin ($4.5x10^{-6}$M) was ineffective against either the electrical or the mechanical responses to isoprenaline (10^{-4}M). From the use of these antagonists it seems that isoprenaline is activating β-adrenoceptors and the response is mediated by an increase in K^+ conductance, although the Ca^{2+}-mediated K^+ conductance affected by apamin is not involved.

The responses produced by this catecholamine serve to highlight the close association between membrane hyperpolarisation and relaxation.

(vi) Effect of cromakalim

A group of drugs which has been implicated in smooth muscle relaxation, over recent years, are the K^+ channel activators. These include pinacidil (Bray et al., 1987), nicorandil (Weir & Weston, 1986 a,b; Allen et al., 1986b) and cromakalim (Weir & Weston, 1986 a,b; Hollingsworth et al., 1987). Cromakalim (10^{-5} - 10^{-3}M; 5-110ms; 40p.s.i.) applied by Picospritzer^R produced no effect on either spike discharge or resting membrane potential. (Figure 32). On the other hand, when cromakalim (10^{-9} - 10^{-5}M) was applied by microsyringe to the bath or in the perfusion fluid, spike inhibition and membrane hyperpolarisation followed by relaxation of the sphincter were observed.

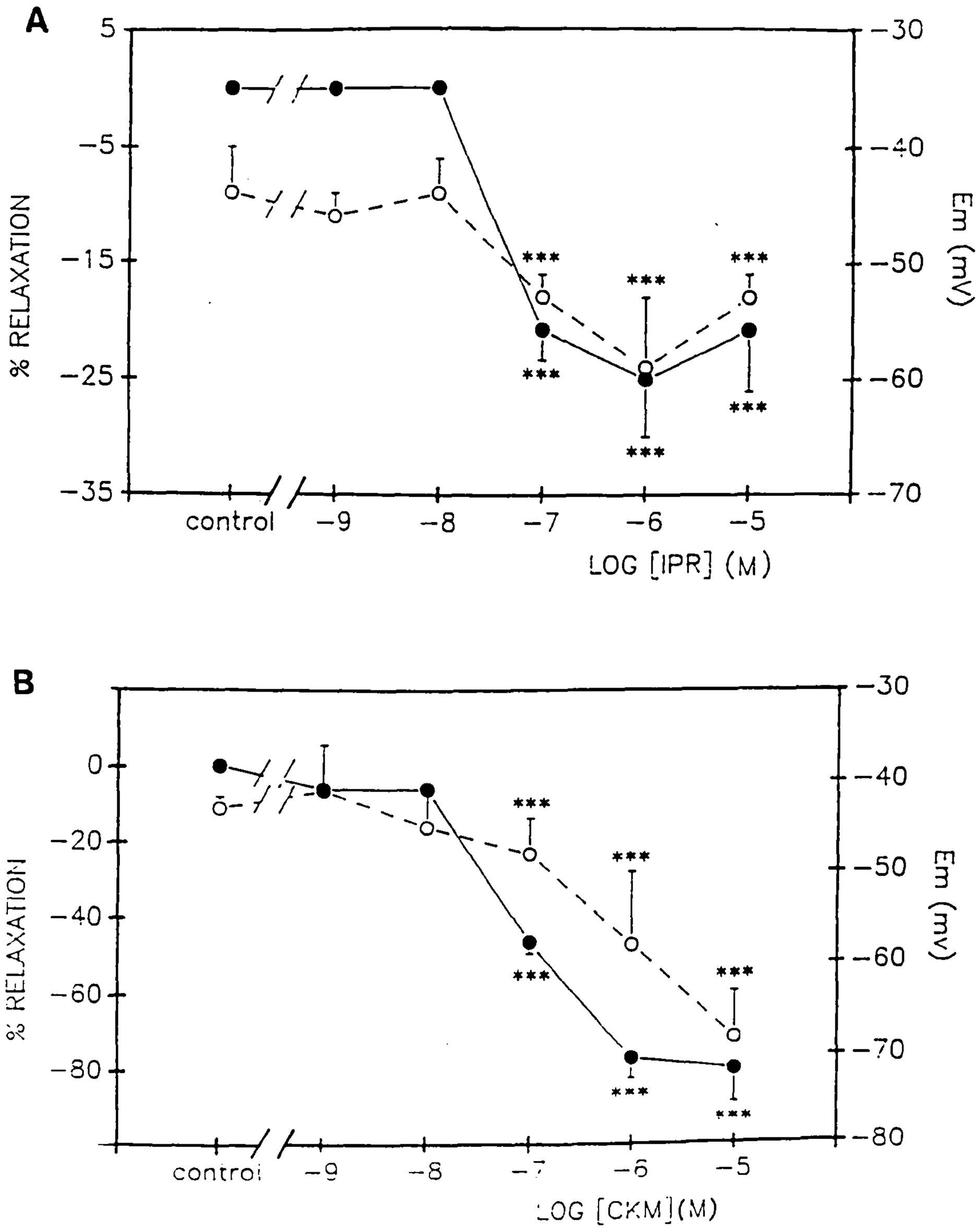
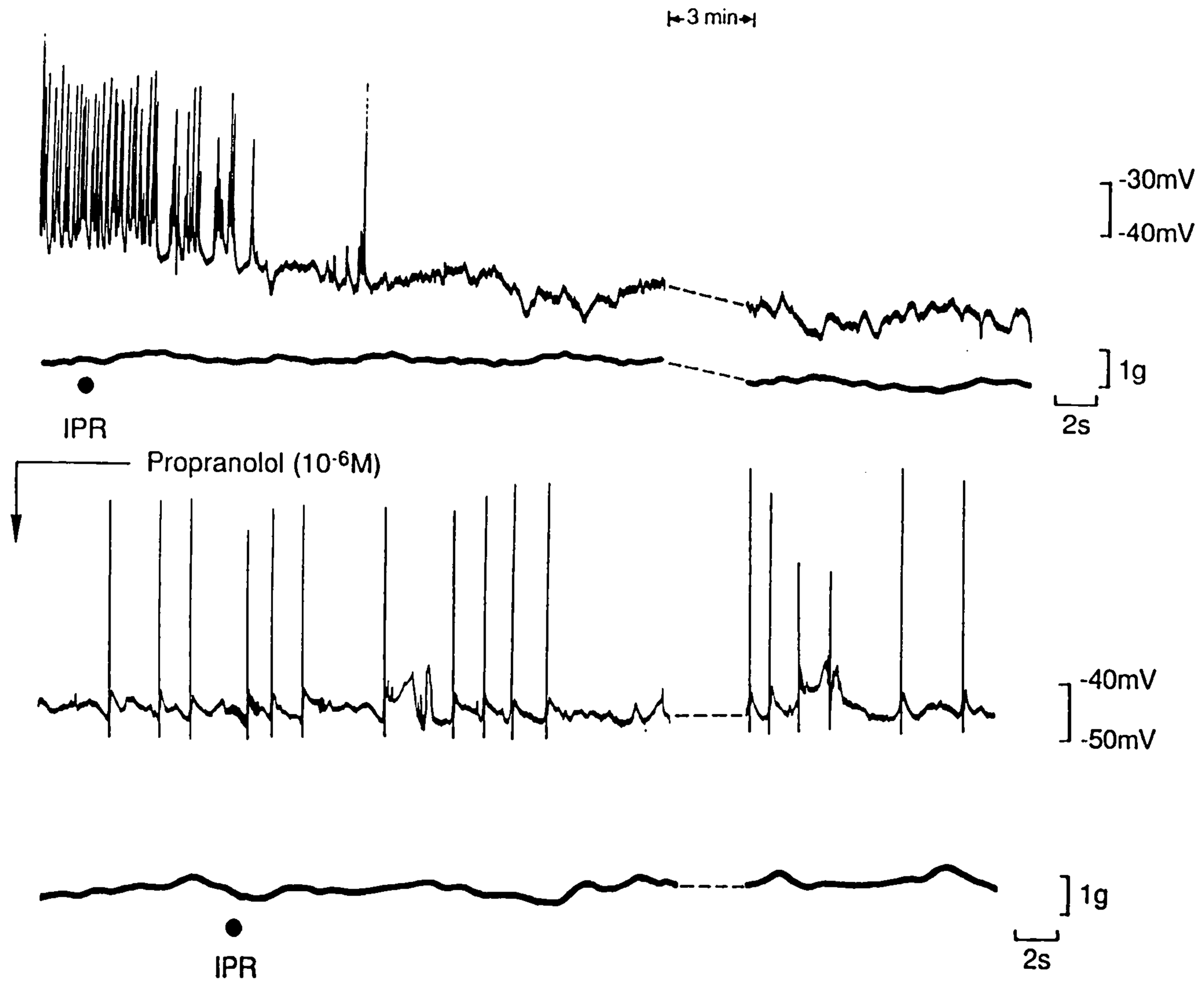


FIGURE 30:

A. The effects of increasing concentrations of isoprenaline (IPR) on the electrical (E_m, o-----o) and mechanical (% relaxation, ●——●) responses of the TAS. Each point represents the mean ± S.D. of at least 9 observations. Isoprenaline produced a concentration-dependent membrane hyperpolarisation and reduction of tone. B. The effects of increasing the concentration of cromakalim (CKM, 10^{-9}-10^{-5}M) on the electrical (E_m, o----o) and mechanical (% relaxation, ●——●) responses of the gpTAS. Each point represents the mean ±.S.D. of at least 9 observations. Cromakalim produced a concentration dependent membrane hyperpolarisation and accompanying relaxation. Atropine and phentolamine were present throughout (each 10^{-6}M).

FIGURE 31:

The effect of propranolol (10^{-6}M) added at the arrow for the duration of the experiment on the electrical (upper trace) and mechanical responses of the IAS to isoprenaline (IPR, 10^{-4}M). Atropine and phentolamine were present throughout (each 10^{-6}M). IPR reversibly inhibited spike discharge, hyperpolarised the membrane and lowered tone (upper panel). Propranolol abolished the hyperpolarisation and relaxation produced by isoprenaline.


3 min
-30mV
-40mV
1g
2s
IPR
Propranolol (10^{-6}M)
-40mV
-50mV
1g
2s
IPR

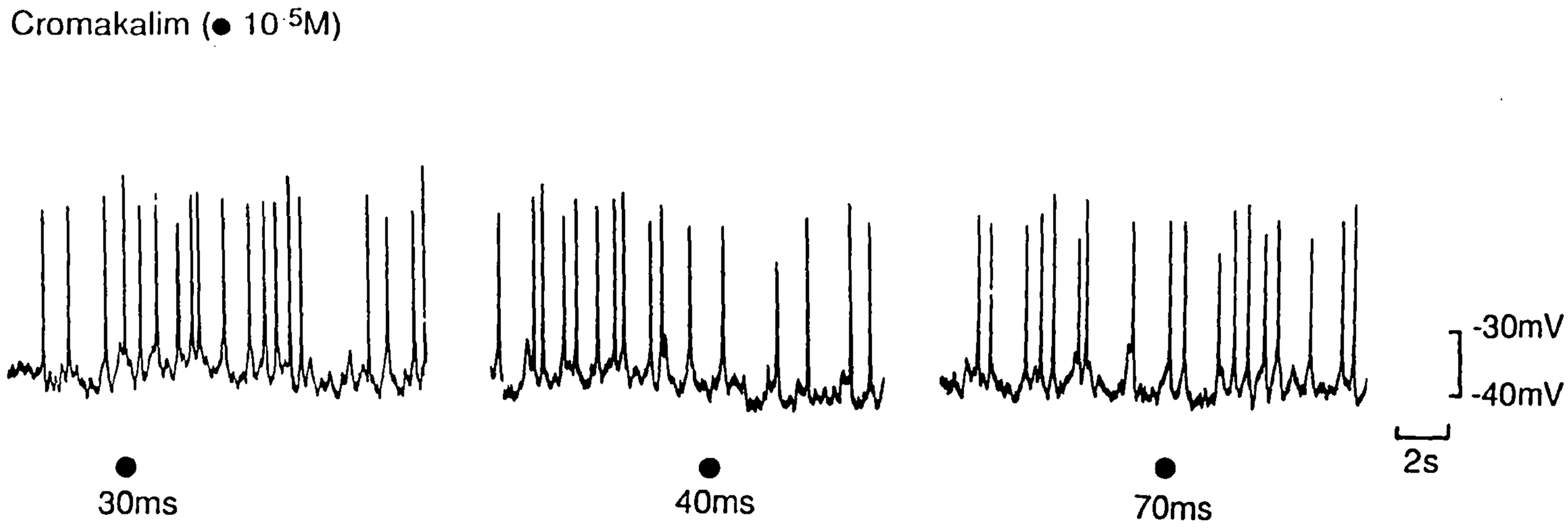


FIGURE 32:
The effect of cromakalim (10^{-5}M) applied locally from a micropipette by hydrostatic pressure ejection (tip diameter 2-10μm; 40p.s.i.; 30-70ms) on the intracellular electrical responses from the same cell in the IAS. Atropine and phentolamine were present throughout (each 10^{-6}M). Cromakalim had no effect on either spike potential discharge or the resting membrane potential by this method of application.

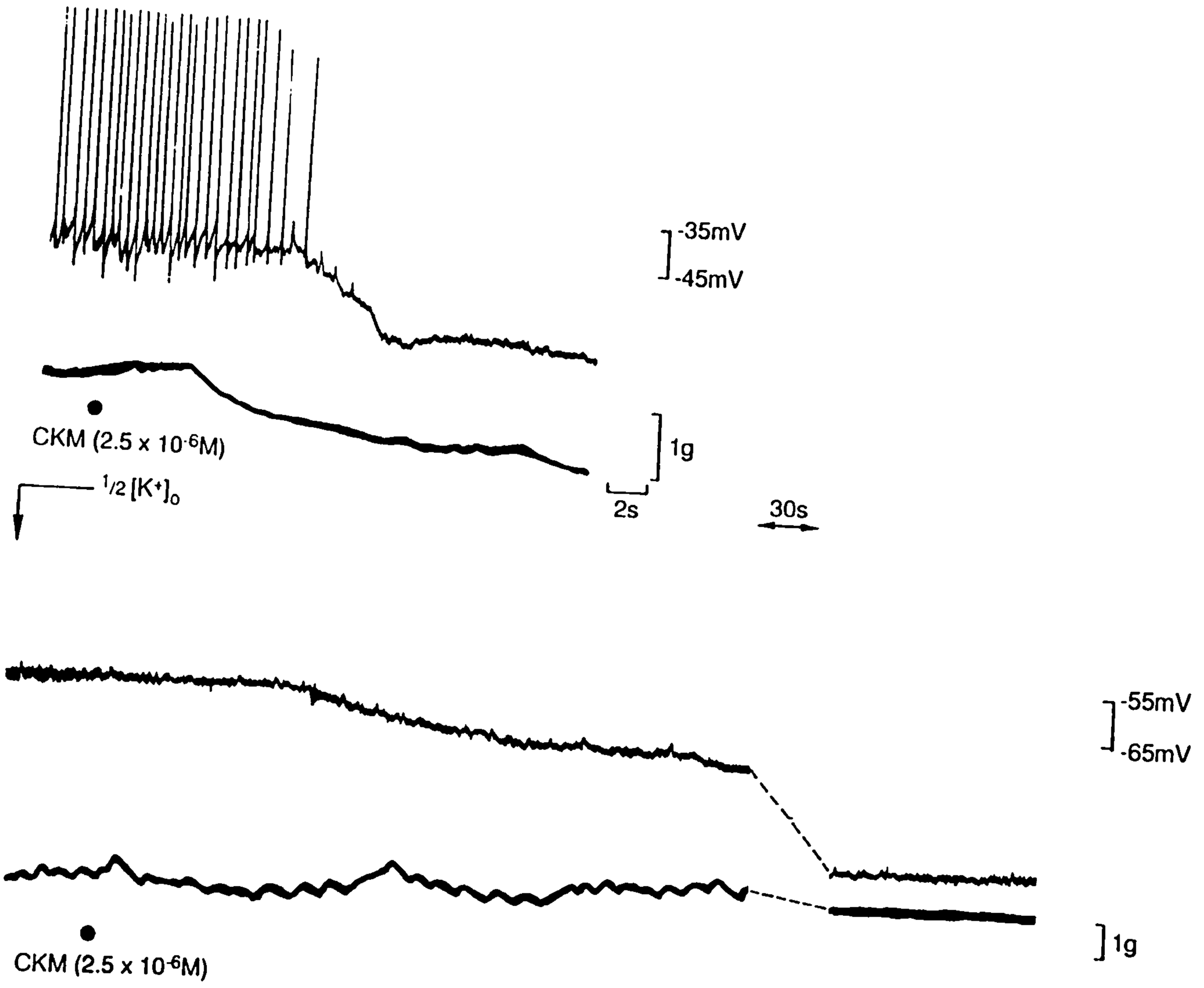
(Figure 30B). Both the electrical and mechanical responses to cromakalim (10^{-5}M) resembled in amplitude those produced by field stimulation (19 ± 6mV, n=12, and 80% reduction in tone, respectively). (Figure 17B). The hyperpolarisation, however, was much slower in decline (0.6 ± 0.2mVs^{-1}, n=12) and more prolonged (596 ± 238s, n=12) than the ijp.

The electrical change produced by cromakalim (10^{-5}M) was further investigated by altering the ionic composition of the Krebs' solution surrounding the muscle and thereby changing the equilibrium potential of the chosen ion. Halving $[K^+]_o$ from 4.7mM to 2.35mM hyperpolarised the resting membrane potential to -56 ± 7mV (n=5) and increased the amplitude of the electrical change produced by cromakalim (10^{-5}M) to 26 ± 6mV (n=4). (Figure 33). Doubling $[K^+]_o$ to 9.4mM depolarised the resting membrane potential to -34 ± 2mV (n=6) and decreased the amplitude of the drug-induced hyperpolarisation to 11 ± 1.4mV (n=3). While quadrupling $[K^+]_o$ to 18.8mM further depolarised the membrane potential to -30 ± 1.2mV (n=7) and attenuated the hyperpolarisation in response to cromakalim (10^{-5}M) further to 1.5 ± 1.5mV (n=3). (Figure 34). These observations underline the proposal that cromakalim has its actions via K^+ efflux which moves the resting membrane potential towards the K^+ equilibrium potential.

TEA (8 x 10^{-2}M) abolished the membrane potential change and reduction in tone produced by cromakalim (10^{-5}M) (Figure 35), although these responses were insensitive to apamin (4.5 x 10^{-6}M) (Figure 17 B&D). These results support the previous evidence that responses produced by cromakalim are mediated by an increase in K^+ conductance, however, this is not via the apamin-sensitive Ca^{2+}- dependent K^+ channel. The importance of the role of hyperpolarisation in relaxation

FIGURE 33:

The effect of reducing $[K^+]_o$ by half to 2.35mM on the electrical (upper trace) and mechanical responses of the IAS to cromakalim (CKM, $2.5x10^{-6}$M). Atropine and phentolamine were present throughout (each 10^{-6}M). In the presence of $\frac{1}{2}[K^+]_o$ spike potential discharge was abolished, the resting membrane potential hyperpolarised and the muscle relaxed. The ijp produced by field stimulation remained the same as that in normal $[K^+]_o$ but the hyperpolarisation produced by cromakalim was increased. The mechanical responses to field stimulation and cromakalim were each smaller in $\frac{1}{2}[K^+]_o$ presumably due to the lack of tone.


-35mV
-45mV
CKM (2.5 x 10-6M)
1/2 [K+]o
1g
2s
30s
-55mV
-65mV
CKM (2.5 x 10-6M)
1g

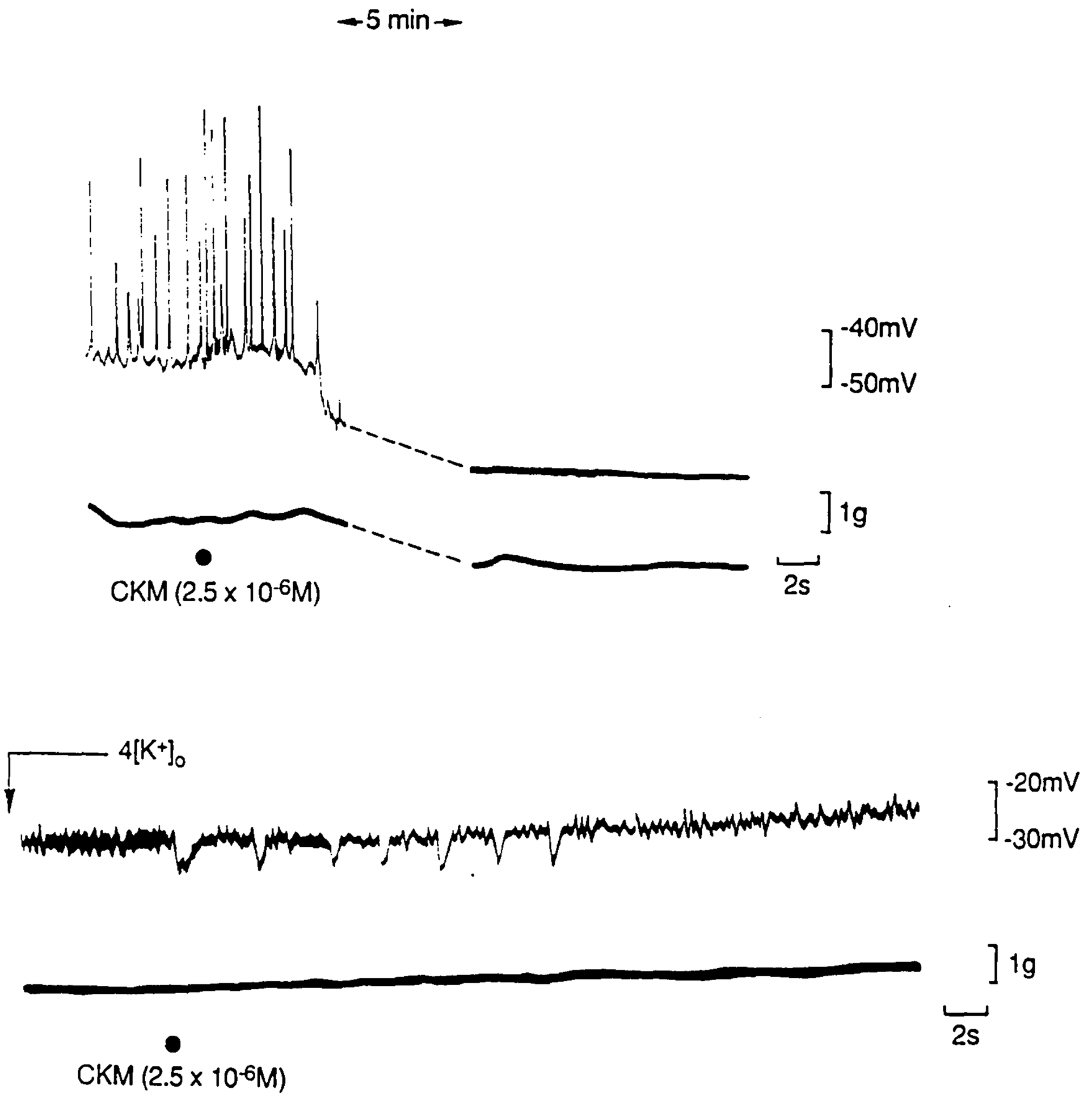


FIGURE 34:

The effect of increasing $[K^+]_o$ by 4-fold to 18.8mM on the electrical (upper trace) and mechanical responses of the IAS to cromakalim (CKM, 2.5×10^{-6}M). Atropine and phentolamine were present throughout (each 10^{-6}M). The resting membrane potential became depolarised, spike potential discharge was inhibited and tone was increased in the presence of $4[K^+]_o$. The electrical and mechanical responses to cromakalim were attenuated. The lack of mechanical response was presumably due to the induced muscle contraction.

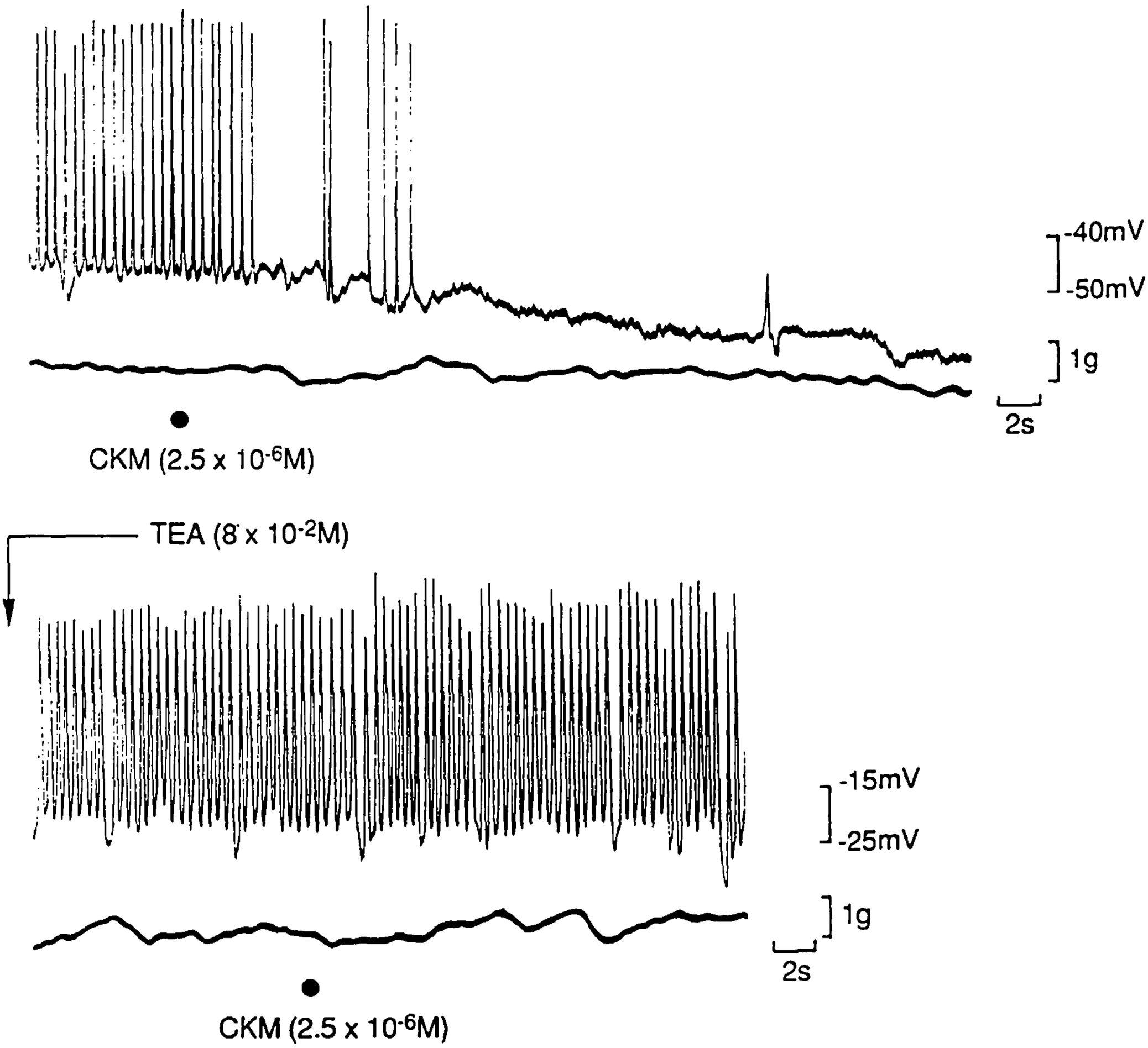


FIGURE 35:
The effect of tetraethylammonium (TEA, $8x10^{-2}M$) added at the arrow for the duration of the experiment on the electrical (upper trace) and mechanical responses from two cells in the same preparation of the IAS to cromakalim (CKM, $2.5x10^{-6}M$). Atropine and phentolamine were present throughout (each $10^{-6}M$). TEA abolished the hyperpolarisation and relaxation produced by cromakalim indicating its mediation by K^{+} conductance.

is also emphasised since relaxation cannot be produced without hyperpolarisation using cromakalim.

(vii) Effects of guanylate cyclase activation

Previous reports have suggested that levels of cyclic GMP are raised during smooth muscle relaxation in response to NANC inhibitory nerve stimulation and a variety of drugs, including the nitrovasodilators (Bowman & Drummond, 1984; Torphy et al., 1986). Compounds which affected the level of the cyclic nucleotides were examined to see if the relaxation they produced was accompanied by a membrane hyperpolarisation.

Sodium nitroprusside

A dose-dependent reduction in muscle tone without a change in either the amplitude and frequency of the spikes or the membrane potential was observed with sodium nitroprusside (10^{-9} - 10^{-6}M). (Figure 36). At a higher concentration, which virtually abolished tone, sodium nitroprusside (10^{-5}M) inhibited spike potential discharge but hyperpolarised the membrane only to a small extent (10mV). (Figure 37A). During administration of sodium nitroprusside (10^{-5}M), field stimulation (5 pulses at 5, 10 & 20Hz; 0.5ms; supramaximal voltage) produced ijps superimposed on the drug-induced membrane hyperpolarisation. (Figure 38). These ijps were of the same order of magnitude as control ijps suggesting that the drug-induced membrane hyperpolarisation is mediated via a different mechanism to the ijp.

The effects of a number of potential antagonists on the electrical changes and accompanying reduction on tone produced by sodium nitroprusside (10^{-5}M) were studied. Three drugs were selected, two to

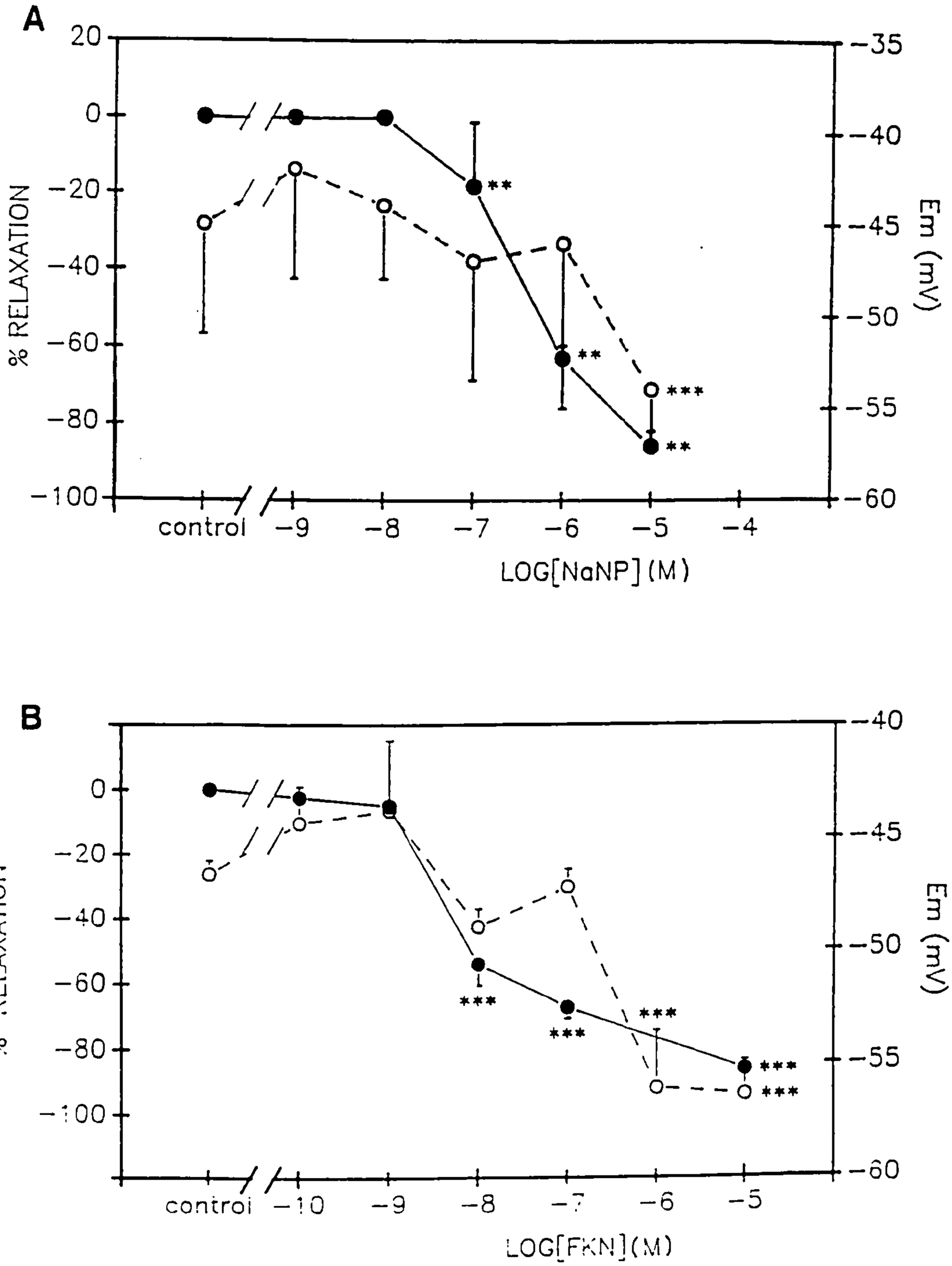


FIGURE 36:

The effects of increasing concentrations of sodium nitroprusside (NaNP in A) and forskolin (FKN in B) on the electrical (E_m o----o) and mechanical (% relaxation, ●——●) responses of the IAS. Atropine and phentolamine (each 10^{-6}M) were present throughout. Each point represents the mean ± S.D. of at least 10 observations. NaNP and FKN each relaxed tone initially without affecting the membrane potential though higher concentrations also hyperpolarised the membrane.

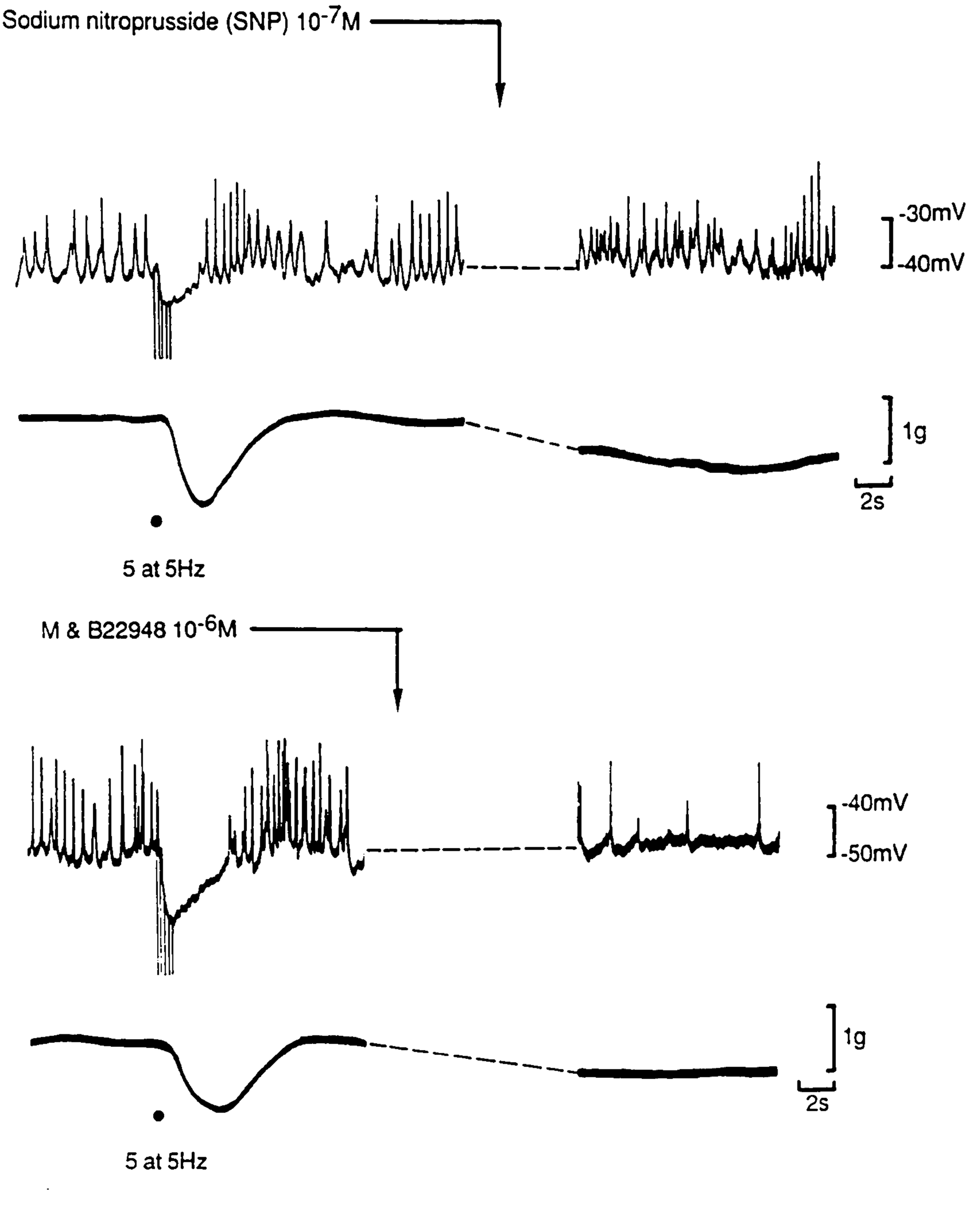
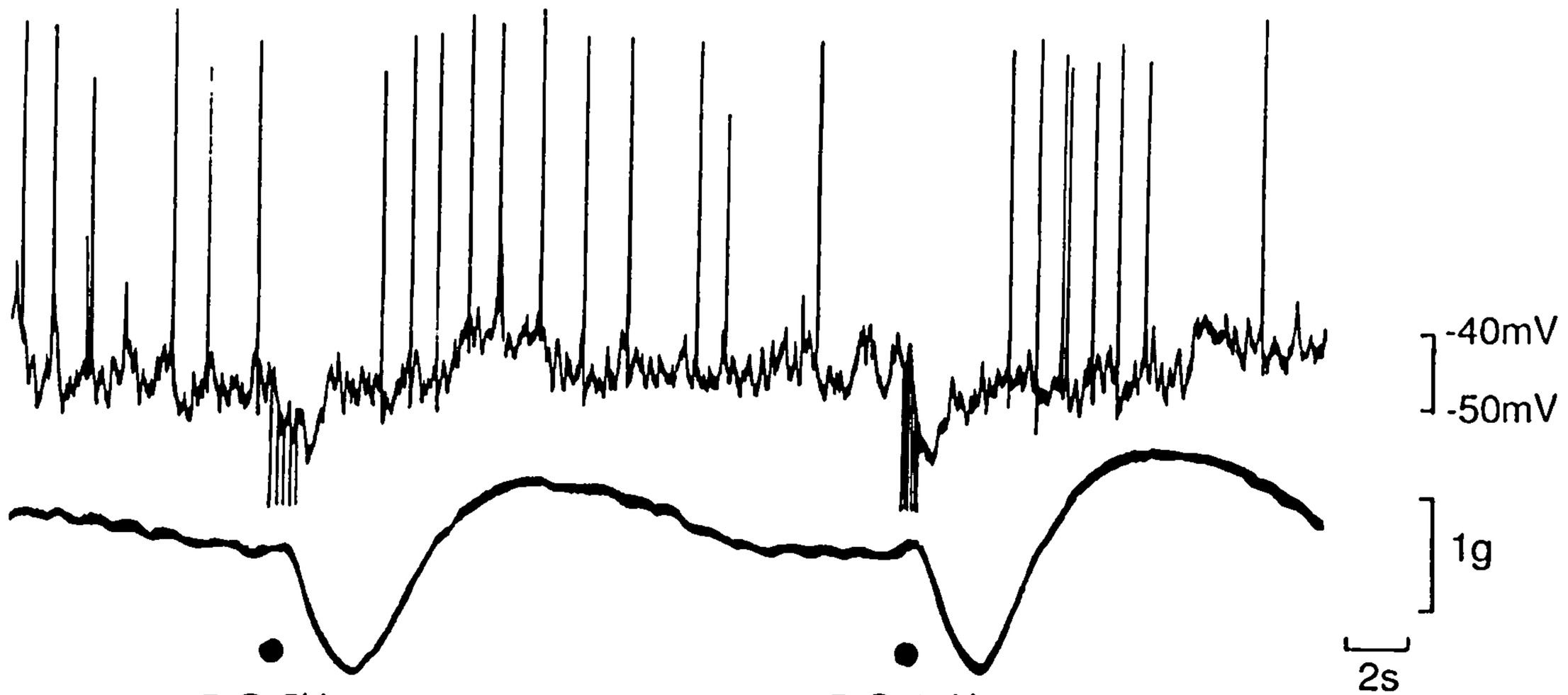
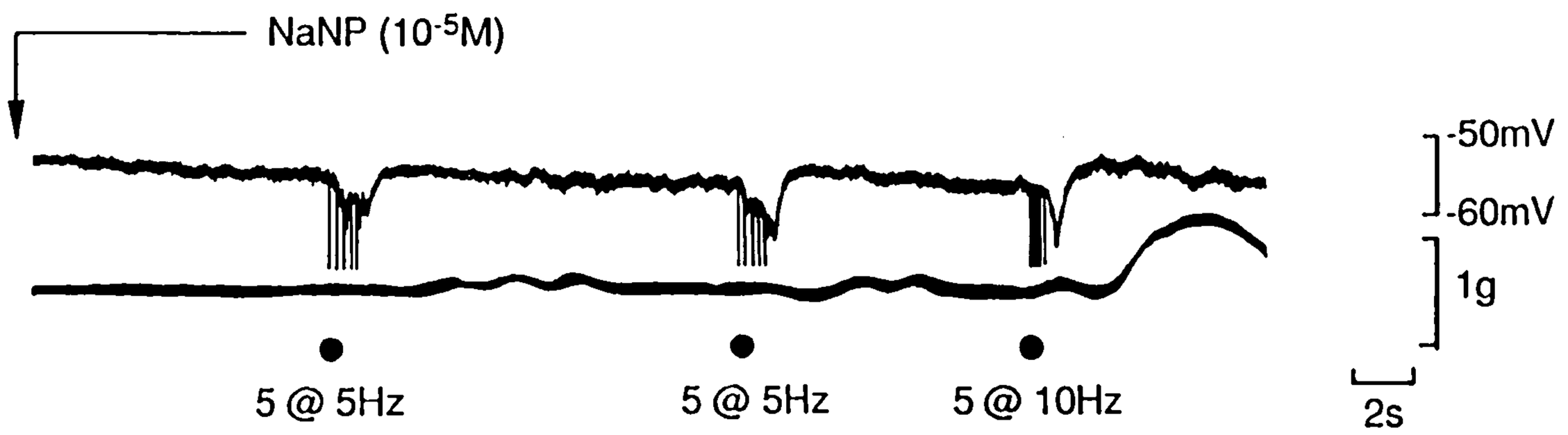


FIGURE 37:
The effect of sodium nitroprusside (NaNP, 10^{-7}M, in A) and M&B22948 (10^{-6}M, in B) added by perfusion at the arrow on the electrical (upper trace) and mechanical responses of two cells from different preparations of the IAS. Sodium nitroprusside and M&B22948 each produced relaxation without a change in membrane potential or spike potential discharge. Atropine and phentolamine (each 10^{-6}M) were present throughout.

FIGURE 38:

The effect of sodium nitroprusside (NaNP, 10^{-5}M) added at the arrow by perfusion, on the electrical (upper trace) and mechanical responses of the IAS to field stimulation (5 pulses at 5 & 10Hz; 0.5ms; supramaximal voltage). Atropine and pentolamine were present throughout (each 10^{-6}M). Sodium nitroprusside abolished spike potential discharge, hyperpolarised the membrane and relaxed tone by up to 80%. The ijps produced by field stimulation were unaffected although no relaxation was observed due to the decrease in tone produced by sodium nitroprusside. Under these conditions the contraction in the last panel was probably a rebound contraction.


-40mV
-50mV
1g
2s
5 @ 5Hz
5 @ 10Hz


NaNP (10-5M)
-50mV
-60mV
1g
2s
5 @ 5Hz
5 @ 5Hz
5 @ 10Hz

establish if the hyperpolarisation was mediated by an increase in K^+ conductance similar to that of NANC neurotransmission and a third to investigate the effect of blocking β-adrenoceptors on the response.

TEA (8×10^{-2}M) abolished the spike inhibition and relaxation although the hyperpolarisation persisted. (Figure 39). On the other hand, neither apamin (4.5×10^{-6}M) nor propranolol (10^{-6}M) (Figure 40) were effective. Therefore an increase in K^+ conductance mediates the relaxation produced by sodium nitroprusside (10^{-5}M) but not the hyperpolarisation. Again this shows that sodium nitroprusside allows the dissociation of the electrical and mechanical responses of the IAS.

M&B 22948

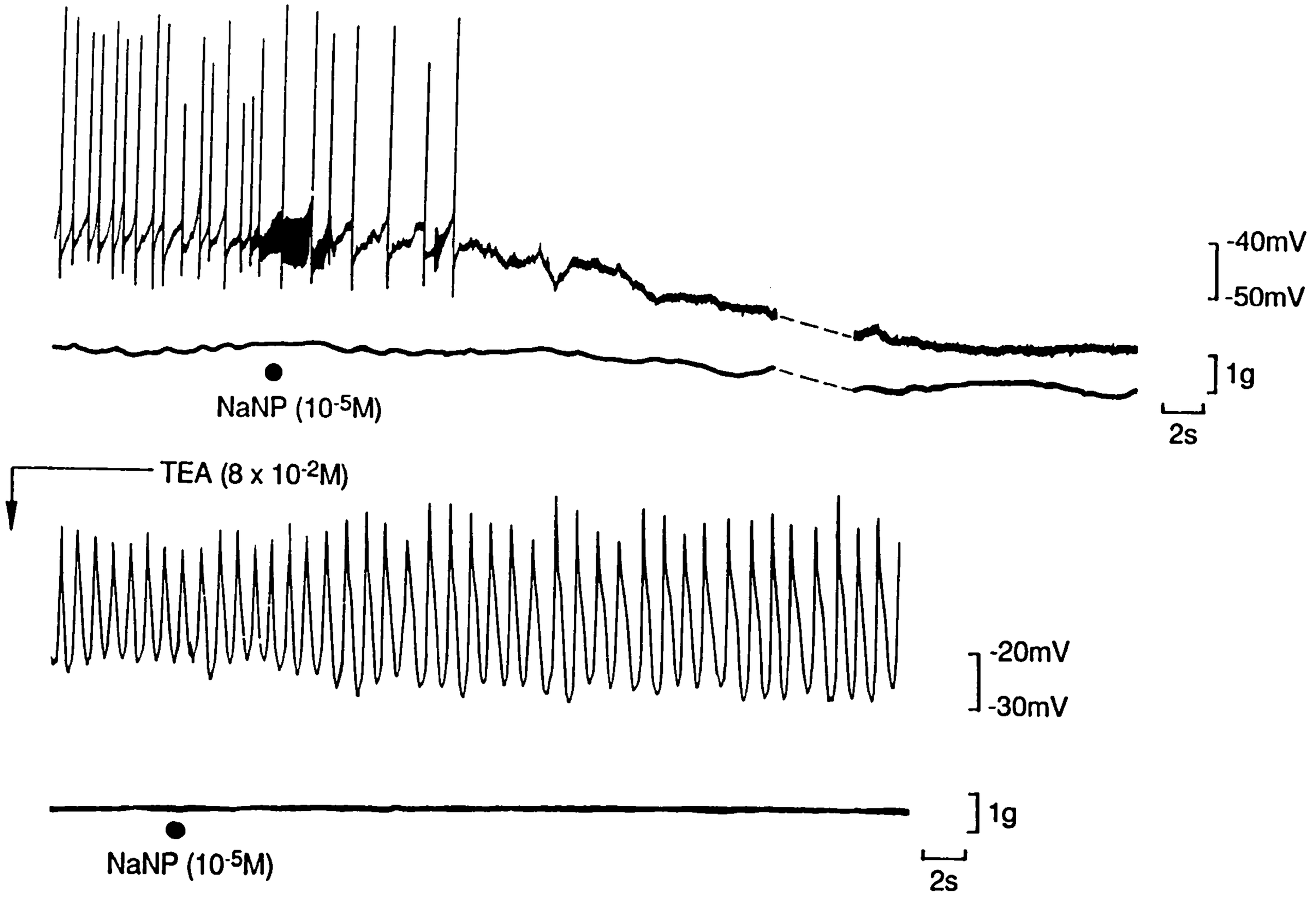
This cyclic GMP-specific phosphodiesterase inhibitor (10^{-9} - 10^{-5}M) produced a dose-dependent relaxation with no effect on either spike discharge or resting membrane potential. (Figures 37B & 41). A small (<10mV) membrane hyperpolarisation and spike inhibition were observed at a higher concentration (10^{-4}M). Like sodium nitroprusside, M&B 22948 (10^{-4}M) virtually abolished tone. In addition, field stimulation (5 pulses at 5, 10 & 20HZ; 0.5ms; supramaximal voltage) produced ijps superimposed on the induced membrane hyperpolarisation. These were not different from control ijps.

8-Bromo-cyclic GMP

To propose that a cyclic nucleotide is acting as a second messenger in the mediation of the action of a drug, hormone or neurotransmitter certain criteria must be fulfilled. One such criterion is that the action of the drug, hormone or neurotransmitter should be

FIGURE 39:

The effect of tetraethylammonium (TEA, $8x10^{-2}$M) added at the arrow for the duration of the experiment, on the electrical (upper trace) and mechanical responses of the IAS produced by sodium nitroprusside (NaNP, 10^{-5}M). TEA abolished the inhibition of spike potential discharge and the relaxation produced by sodium nitroprusside, although some hyperpolarisation was still produced by NaNP. Atropine and phentolamine were present throughout (each 10^{-6}M). The time between panels was 10min.


-40mV
-50mV
1g
2s
NaNP (10-5M)
TEA (8 x 10-2M)
-20mV
-30mV
1g
2s
NaNP (10-5M)

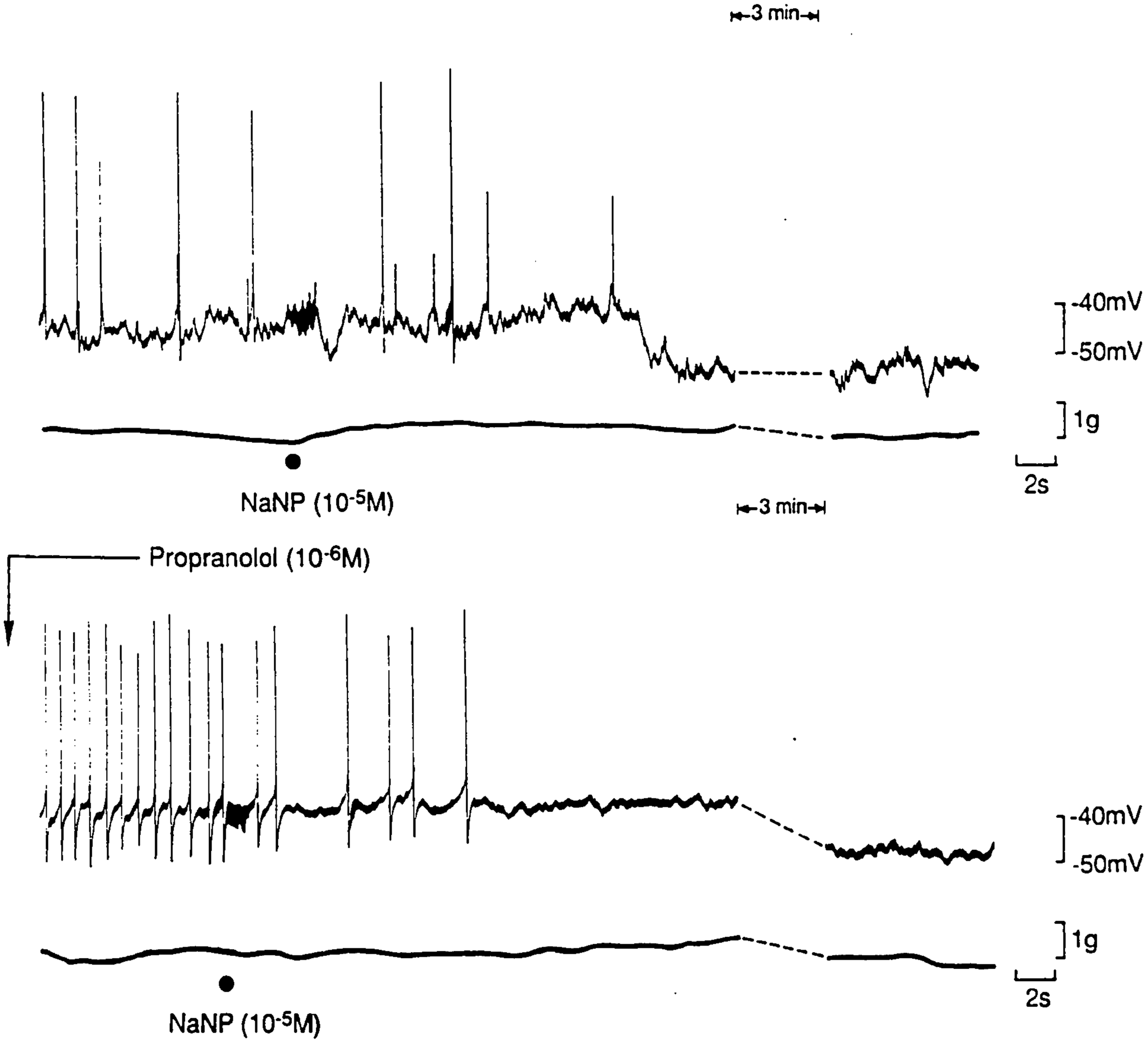
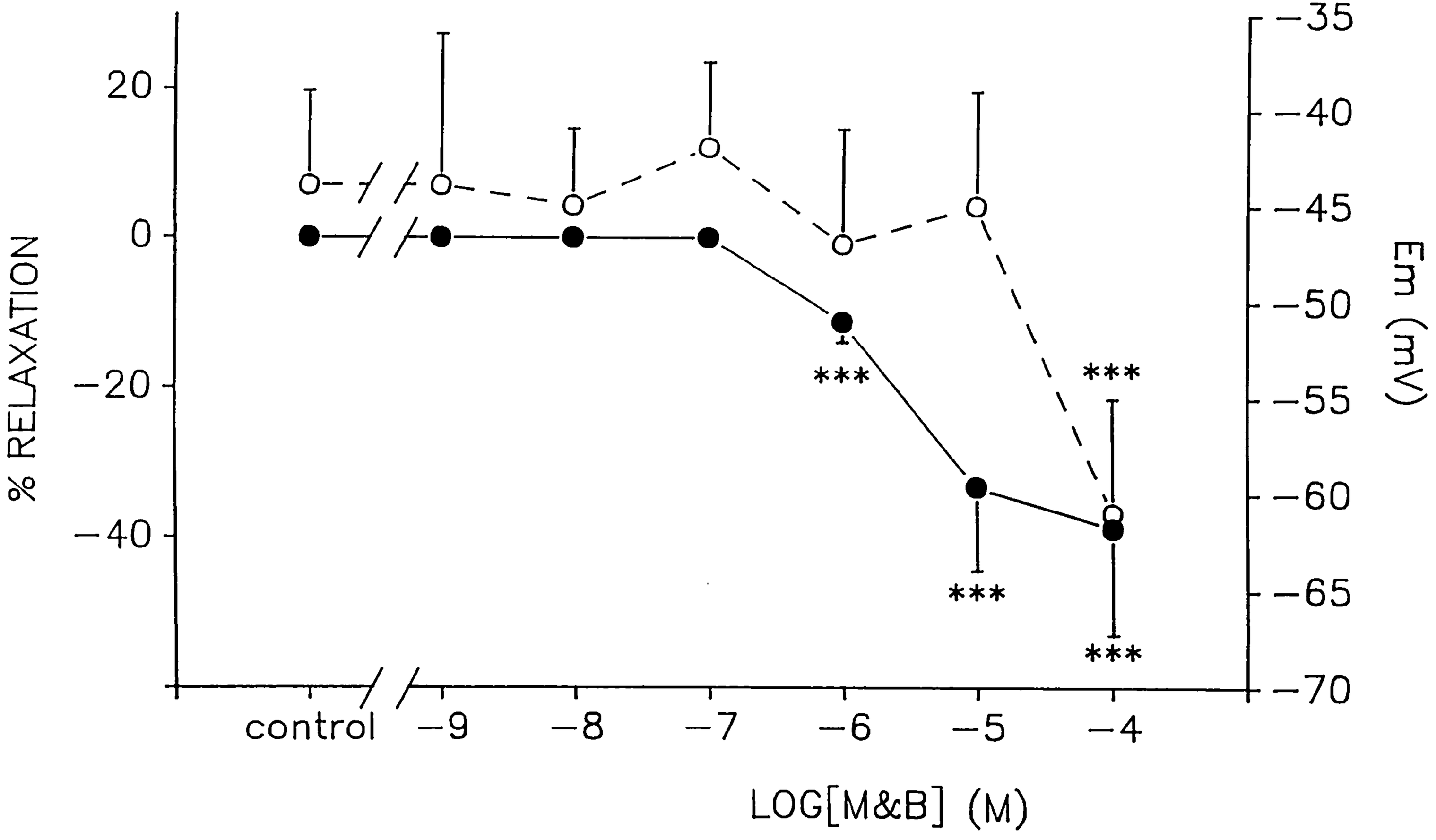


FIGURE 40:

The effect of propranolol (10^{-6}M), added at the arrow for the duration of the experiment, on the electrical (upper trace) and mechanical responses of the IAS produced by sodium nitroprusside (NaNP, 10^{-5}M). Propranolol had no effect on the relaxation, inhibition of spike potential discharge or membrane hyperpolarisation produced by sodium nitroprusside. This indicates that β-adrenoceptors were not involved in the response to sodium nitroprusside. Atropine and phentolamine were present throughout (each 10^{-6}M).

FIGURE 41:

The effect of increasing the concentration of M&B22948 (10^{-9}-10^{-4}M) on the electrical (E_m, o-----o) and mechanical (% relaxation, ●——●) responses of the IAS. M&B22948 produced a dose-dependent relaxation without a change in membrane potential. Only at maximum inhibition of tone (M&B22948, 10^{-4}M) was a small hyperpolarisation observed. Atropine and phentolamine were present throughout (each 10^{-6}M).


20
0
−20
−40
% RELAXATION
−35
−40
−45
−50
−55
−60
−65
−70
Em (mV)
***
***
***
***
control
−9
−8
−7
−6
−5
−4
LOG[M&B] (M)

mimicked by exogenous application of the appropriate cyclic nucleotide or its stable analogue (Sutherland et al., 1968). In this case, 8-bromo-cyclic GMP (10^{-4}M) was employed. It is 2 - 5 times more active than cyclic GMP as an activator of cyclic GMP-dependent protein kinase (Kuo et al., 1974; 1976) and it is resistant to degradation by phosphodiesterase (Revanker & Robins, 1982). It reduced tone by some 80%, inhibited spike discharge and hyperpolarised the membrane to a small extent (<10mV). (Figure 42).

As previously shown with sodium nitroprusside and M&B 22948, field stimulation (5 pulses at 5, 10 & 20Hz; 0.5ms; supramaximal voltage) during administration of the analogue (10^{-4}M) produced ijps superimposed on the induced electrical change. (Figure 42).

(viii) Effects of adenylate cyclase activation

It is already well accepted that cyclic AMP is important in smooth muscle relaxation. However the question arose as to whether relaxation of the IAS itself was regulated by cyclic AMP and if increases in the level of the cyclic nucleotide produced hyperpolarisation.

Activation of adenylate cyclase by perfusion of forskolin (10^{-10} - 10^{-7}M) produced a dose-dependent muscle relaxation without a change in either spike discharge or membrane potential. (Figure 36B). Only at concentrations of forskolin (10^{-6} & 10^{-5}M) which virtually abolished sphincter tone were spike potentials inhibited and membrane hyperpolarisation evident (<10mV). (Figure 43). Furthermore, ijps produced by field stimulation (5 pulses at 5, 10 & 20Hz; 0.5ms; supramaximal voltage) were added to the membrane hyperpolarisation resulting from forskolin (10^{-5}M). (Figure 43). This suggests that the

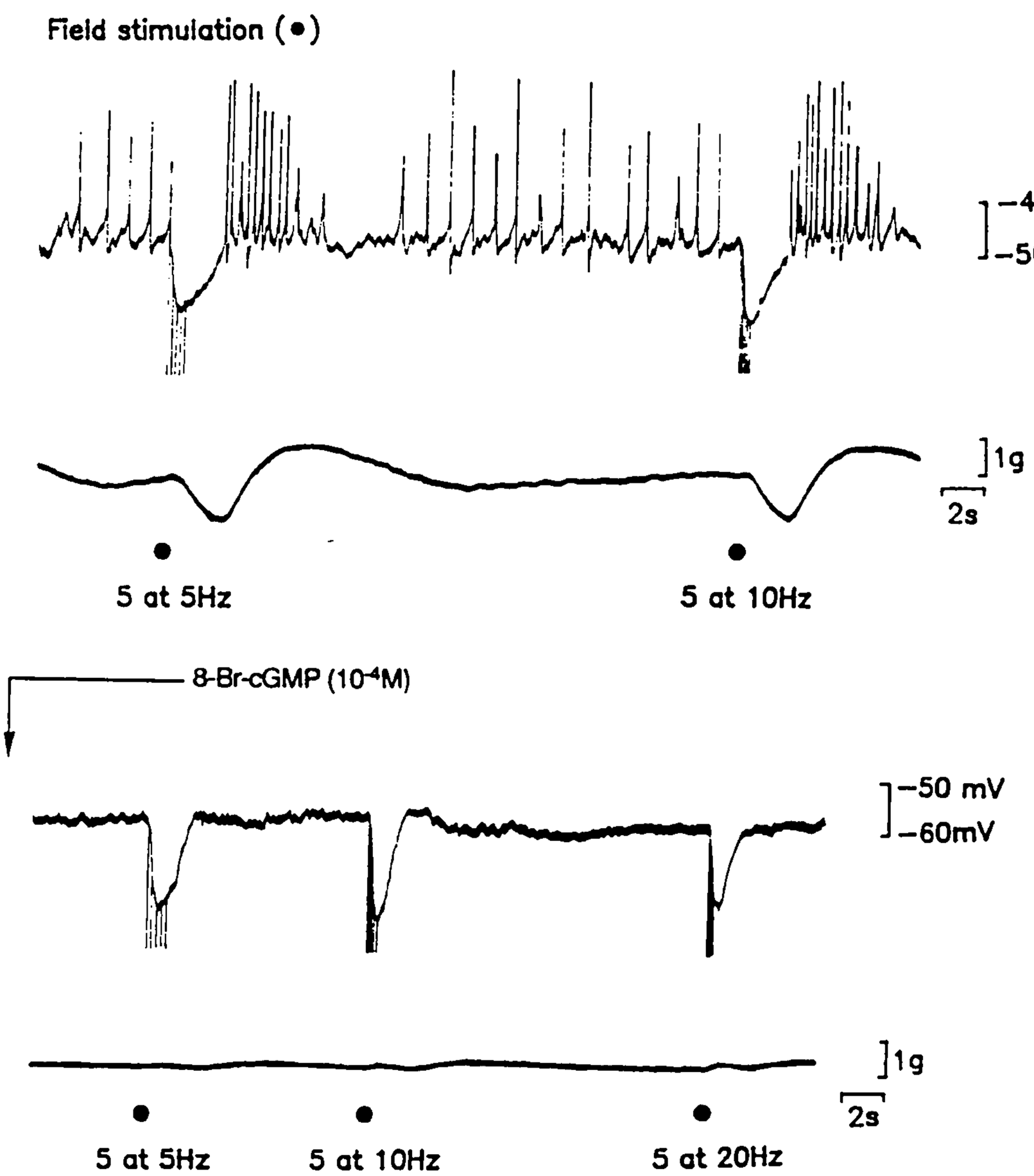
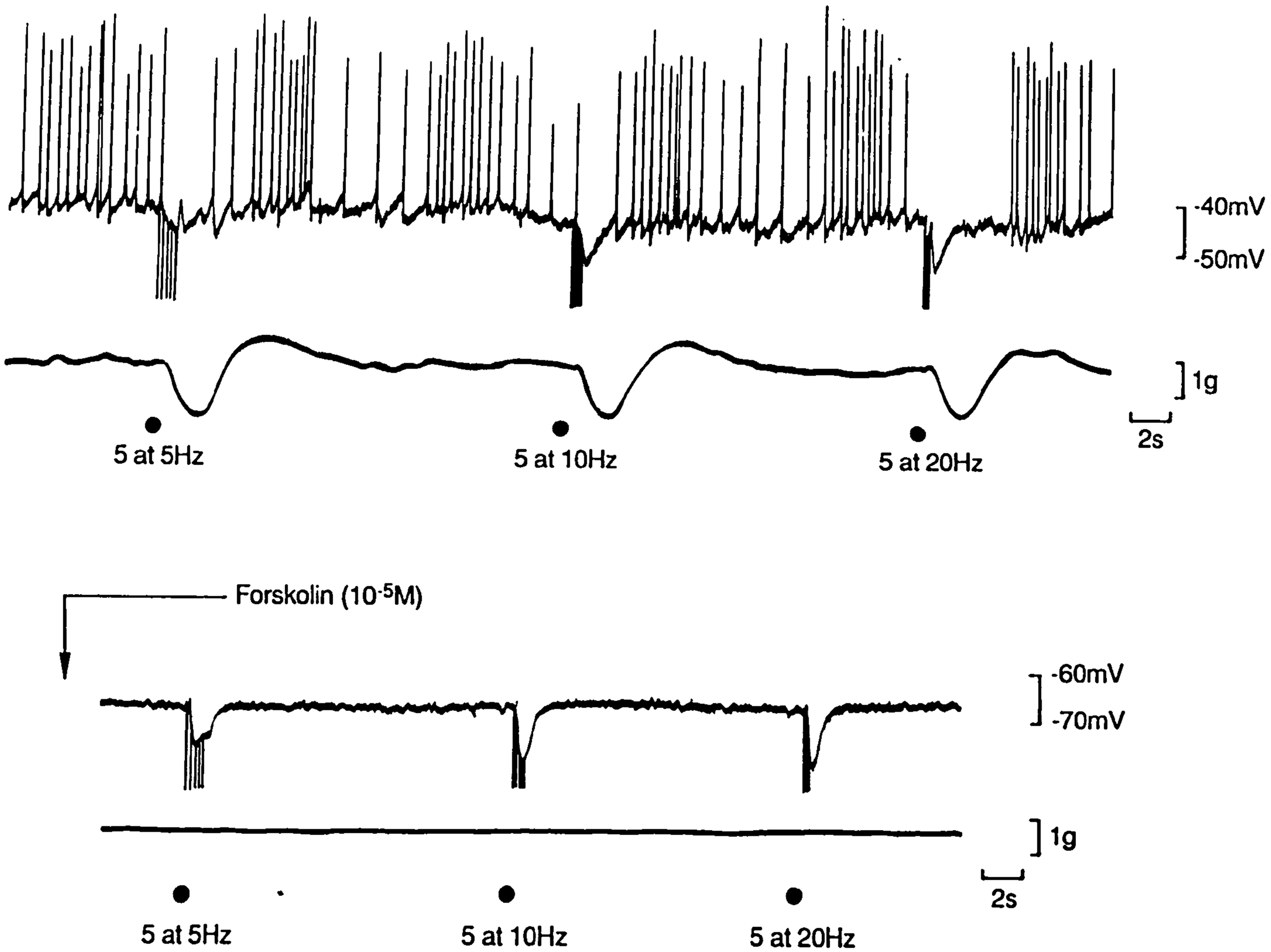


FIGURE 42:

The effects of 8-bromo-cGMP (8-Br-cyclic GMP, 10^{-4}M) added by perfusion in the Krebs' on the simultaneously recorded electrical (upper trace) and mechanical responses of the IAS in two cells from the same preparation to field stimulation (5 pulses at 5 & 10 Hz; 0.5 ms; supramaximal voltage). Atropine and phentolamine (each 10^{-6}M) were present throughout. 8-Br-cGMP hyperpolarised the membrane abolished spike discharge and muscle tone so that further relaxation could not be observed. However, ijps to field stimulation were not diminished during the hyperpolarisation produced by the cyclic nucleotide. Time between top and bottom panels was 20 min.

FIGURE 43:
The effect of forskolin (10^{-5}M) added by perfusion in the Krebs' solution, on the electrical (upper trace) and mechanical responses of the IAS to field stimulation (5 pulses at 5, 10 & 20Hz; 0.5ms; supramaximal voltage). Atropine and phentolamine were present throughout (each 10^{-6}M). Forskolin inhibited spike potential discharge, hyperpolarised the membrane and relaxed the muscle. The ijps produced by field stimulation remained in the presence of forskolin, however, further relaxation was not observed presumably due to lack of tone.


-40mV
-50mV
1g
2s
5 at 5Hz
5 at 10Hz
5 at 20Hz
Forskolin (10^{-5}M)
-60mV
-70mV
1g
2s
5 at 5Hz
5 at 10Hz
5 at 20Hz

mechanism involved in the ijp is different to that of the forskolin-induced hyperpolarisation.

b) Bovine Retractor Penis Muscle

(i) Resting properties

Initially, the BRP muscle had no resting tone with a stable membrane potential of -51 ± 6mV (n=16). As with the IAS, subsequent to gentle stretch (1g) of the preparation a further 4 - 5g of tone developed within a period of 1 - 2h. Guanethidine (5×10^{-6}M) was added to the perfusion fluid for this purpose if necessary. With the development of tone, the membrane potential depolarised to -40 ± 3mV (n = 75; $p<0.05$) but the tissue remained quiescent. These results confirm the previous findings of Byrne & Muir (1984).

(ii) Effect of intramural field stimulation

In the absence of tone field stimulation (single pulse and 3 - 10 pulses at 10Hz; 0.5ms; supramaximal voltage) produced ejps accompanied by contractions of the muscle. (Figure 44A). In the presence of tone, field stimulation (single pulse and 3 - 8 pulses at 5 & 10Hz; 0.5ms; supramaximal voltage) produced ijps and relaxation of the muscle. (Figure 44B). The electrical and mechanical responses of the BRP muscle to field stimulation are much slower than those of the IAS.

(iii) Effects of cromakalim

The hyperpolarisation produced in response to field stimulation in the BRP muscle is not a prerequisite for relaxation, Byrne et al., (1984) since it was demonstrated that in the presence of TEA the ijp was abolished

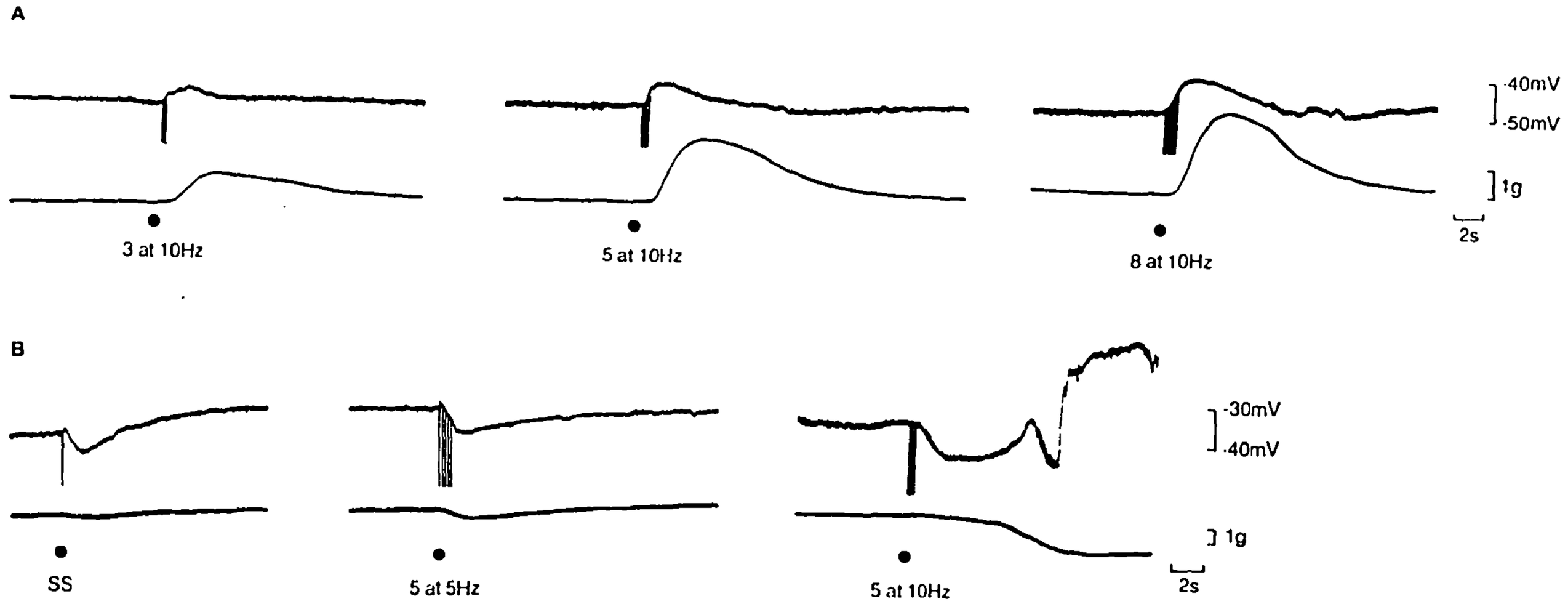


FIGURE 44:

The simultaneously recorded electrical (upper trace) and mechanical responses of the BRP muscle to field stimulation (5 & 8 pulses at 10Hz, in A and single pulse ss, & 5 pulses at 5 & 10Hz in B; 0.5ms; supramaximal voltage) before (A) and after (B) the development of tone in two different cells from the same preparation. Field stimulation produced ejps and contractions in the relaxed BRP muscle but ijps and relaxations in the contracted state. Impalement was lost in the last panel. Time between A and B was 60min.

but the relaxation in response to field stimulation was unaffected. Thus, relaxation could be achieved without hyperpolarisation. Byrne & Muir (1985) also showed that K^+ was not the ion involved in the response of the BRP muscle to field stimulation of inhibitory NANC nerves, unlike that in the IAS (Lim & Muir, 1985). The set of experiments presented here investigates firstly whether or not the BRP muscle could be relaxed by K^+ channel activation and secondly whether or not the relaxation was accompanied by hyperpolarisation.

Cromakalim (5×10^{-6} - 2.5×10^{-5}M) produced a dose-dependent membrane hyperpolarisation and accompanying relaxation. (Figure 45). Both the electrical and mechanical responses were prolonged (up to 25min) at lower doses, whilst at higher doses the actions of cromakalim were difficult to reverse. The induced hyperpolarisation (cromakalim 2.5×10^{-5}M) was much greater than the ijp produced by field stimulation and as the membrane potential change increased then the reduction in tone also increased. These results suggest that the electrical and mechanical responses of the BRP muscle to cromakalim were associated with one another unlike those to field stimulation. The difference may be due to the ionic conductances involved in the responses produced by cromakalim - a K^+ conductance - and field stimulation - a Na^+ and K^+ conductance (Byrne & Muir, 1985).

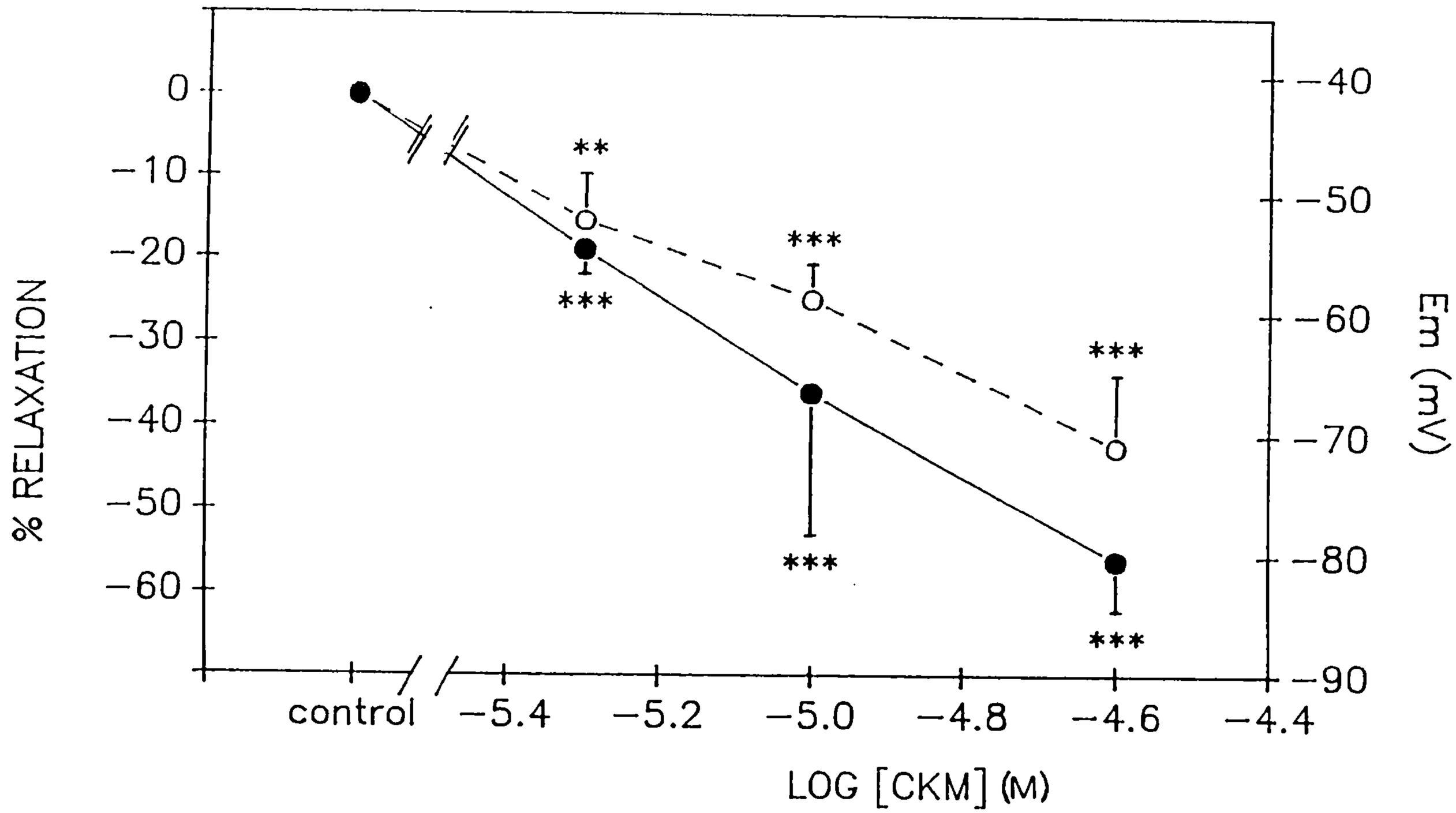


FIGURE 45:

The effect of increasing concentrations of cromakalim (CKM) on the electrical (E_m, o-----o) and simultaneously recorded mechanical (●——●) responses of the BRP. Cromakalim hyperpolarised the membrane and relaxed the muscle in a dose-dependent fashion. Each point represents the mean ± S.D. of 9 observations.

2 CYCLIC NUCLEOTIDE CONTENT

a) Guinea-pig Internal Anal Sphincter

(i) Effect of field stimulation

Both the cyclic AMP and the cyclic GMP contents of the IAS were increased significantly from control values of 1.84 ± 1.63pmolmg^{-1} (n=11) to 4.28 ± 2.27pmolmg^{-1} (n=10) and from 4.2 ± 4.6fmolmg^{-1} (n=11) to 15.7 ± 9.5fmolmg^{-1} (n=10), respectively, in response to field stimulation (80 pulses at 8Hz; 0.5ms; supramaximal voltage) of inhibitory NANC nerves. These increases were prevented by TTX (10^{-6}M) confirming the neurogenic origin of the rise in both cyclic nucleotides. Apamin (5 x 10^{-6}M), already shown to antagonise both the electrical and mechanical responses of the IAS to field stimulation, inhibited the increase in cyclic AMP levels but not that in cyclic GMP. (Figure 46).

(ii) The putative inhibitory neurotransmitter

The putative inhibitory neurotransmitter ATP (10^{-4}M) produced quantitatively similar increases in both cyclic nucleotides to those produced by field stimulation (4.1 ± 2.5pmolmg^{-1},n=11 and 19.8 ± 15.4fmolmg^{-1}, n=11, cyclic AMP and cyclic GMP respectively). (Figure 46). This further emphasises the similarity between the effects produced by the adenosine nucleotide and the inhibitory NANC neurotransmitter.

(iii) Effect of other non-neuronal stimuli

Cromakalim (10^{-5}M) and isoprenaline (10^{-4}M) each raised the level of cyclic AMP significantly from control values (Figure 47), while the cyclic GMP content remained unaffected. The response observed here

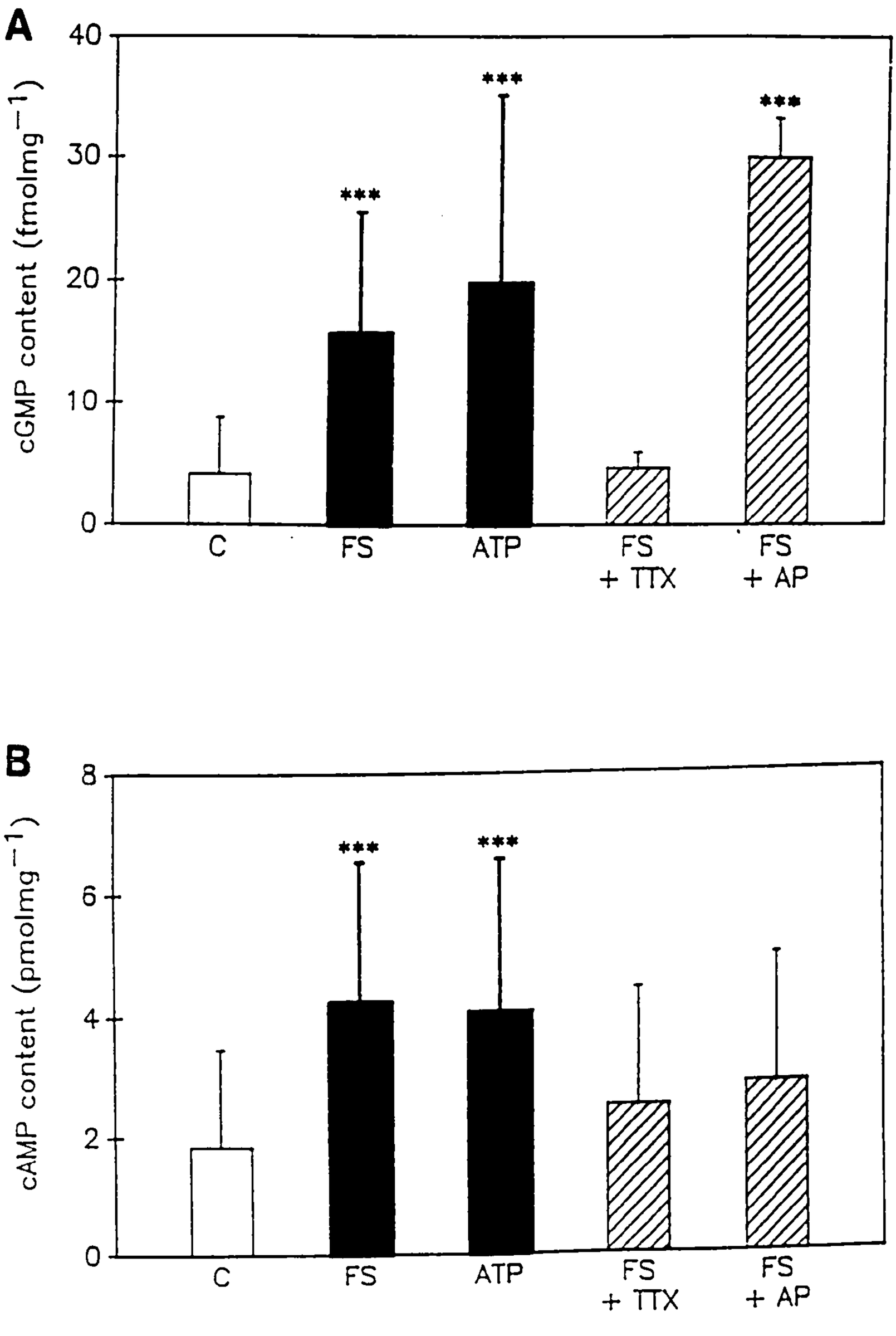


FIGURE 46:

The effects of ATP (10^{-4}M) and field stimulation (FS; 80 pulses at 8 Hz; supramaximal voltage; 0.5 ms) alone and in the presence of TTX (10^{-6}M) (FS + TTX) or apamin (5×10^{-6}M) (FS + AP) on the content of cyclic GMP (fmol mg^{-1}) (A) and cyclic AMP (pmol mg^{-1}) (B) in the IAS. Each bar represents the mean ± S.D. of at least 10 observations. Atropine and phentolamine were present (each 10^{-6}M) in the Krebs' solution. Field stimulation and ATP raised the level of each nucleotide significantly compared with controls (c). TTX inhibited the effects of FS, and apamin the ability of FS to enhance the cyclic AMP but not the cyclic GMP content of this tissue.

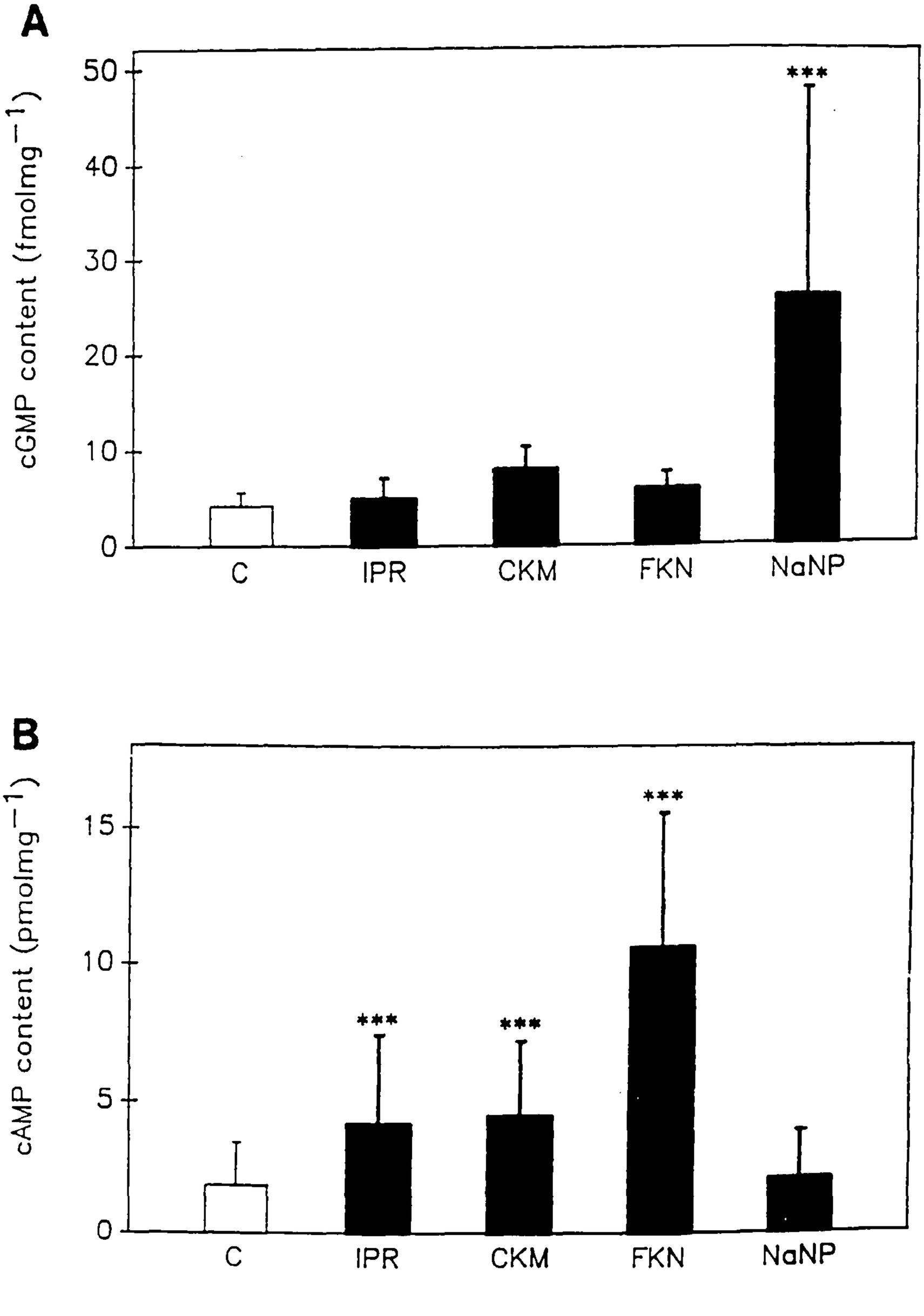


FIGURE 47:

The effects of isoprenaline (IPR 10^{-4}M), cromakalim (CKM 10^{-5}M), forskolin (FKN 10^{-5}M) and sodium nitroprusside (NaNP 10^{-5}M) on the cyclic GMP (fmolmg^{-1}) (A) and cyclic AMP contents (pmolmg^{-1}) (B) of the IAS. Each bar represents the mean ± S.D. of at least 10 observations. Atropine and phentolamine were present (each 10^{-6}M) in the Krebs' solution. NaNP alone failed to raise the cyclic AMP content of this tissue significantly and only NaNP raised the levels of cyclic GMP compared with controls (c).

to cromakalim contrasts with other findings (Coldwell & Howlett, 1987; Gillespie & Sheng, 1988a) where cyclic nucleotides were not raised during the inhibitory response to this drug. These results suggest that in the IAS the effects of cromakalim did not involve K^+ channel activation alone, but also an increase in adenylate cyclase activity.

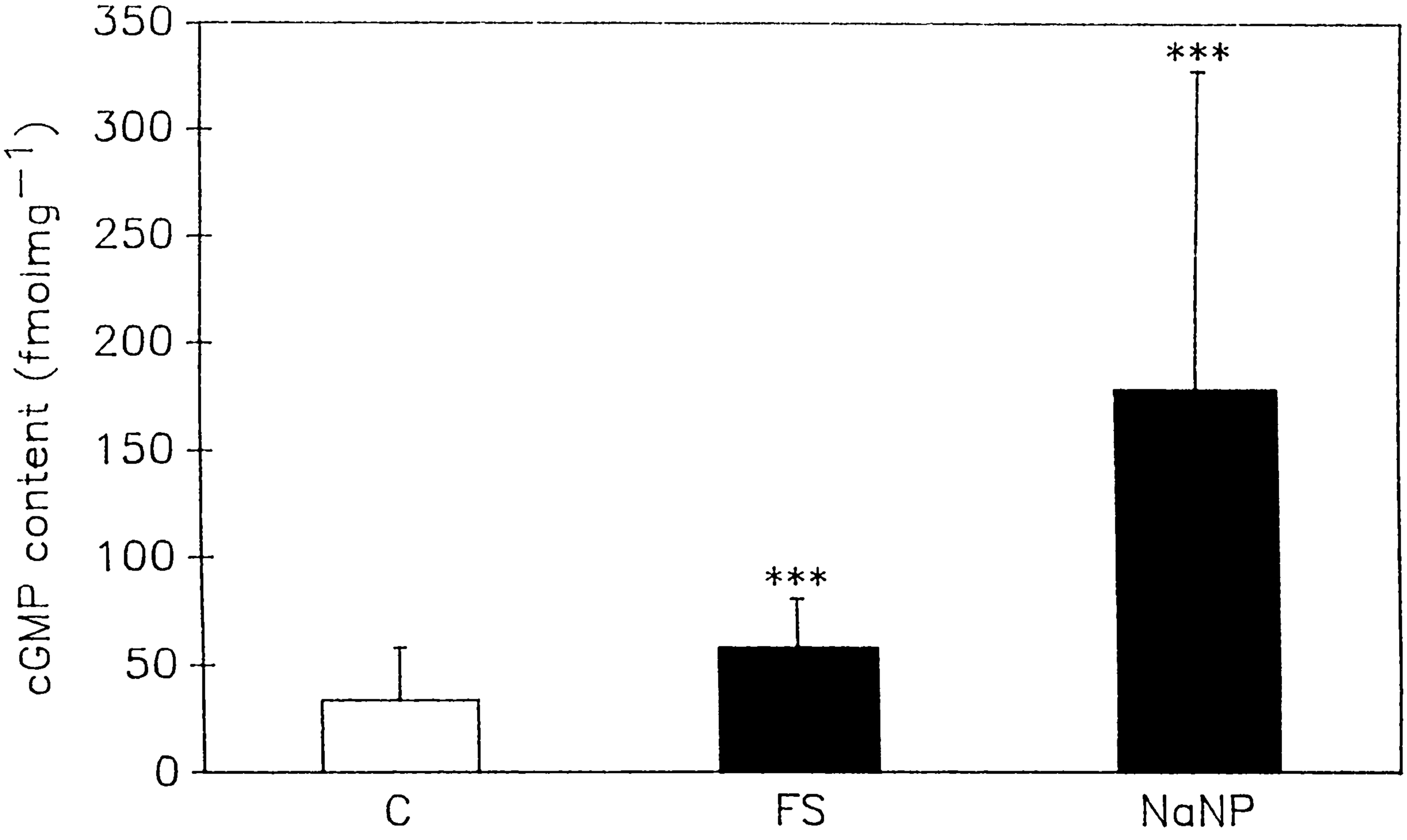
Sodium nitroprusside (10^{-5}M) raised the cyclic GMP content approximately 6-fold to 26.1 ± 21.9fmolmg^{-1} (n=19), a rise which was the same order of magnitude as that observed in the response of the BRP muscle to this drug (Bowman & Drummond, 1984). (Figure 47). Forskolin (10^{-5}M) raised only cyclic AMP levels approximately 6-fold, again a similar rise to that noted in the BRP muscle to this drug (Bowman & Drummond, 1984). (Figure 47).

b) Guinea-pig Taenia Caeci

Field stimulation (80 pulses at 8Hz; 0.5ms; supramaximal voltage) of the intramural NANC nerves raised the cyclic GMP content of the muscle cells significantly from 33.7 ± 24.7fmolmg^{-1} (n=12) to 58.8 ± 22.6fmolmg^{-1} (n=13). Sodium nitroprusside (10^{-5}M) produced a 6-fold rise in the amount of cyclic GMP present (170.0 ± 149.3fmolmg^{-1}, n=16). (Figure 48).

FIGURE 48:

The cyclic GMP content (fmolmg^{-1}) of the guinea-pig taenia caeci alone and in response to field stimulation (FS, 80 pulses at 8Hz; 0.5ms; supramaximal voltage) and sodium nitroprusside (NaNP, 10^{-5} M). Each bar represents the mean ± S.D. of at least 12 observations. Field stimulation and sodium nitroprusside each significantly increased the cyclic GMP content.


350
300
250
200
150
100
50
0
cGMP content (fmolmg^{-1})
***
***
C
FS
NaNP

3 INOSITOL PHOSPHATE TURNOVER IN THE GUINEA-PIG INTERNAL ANAL SPHINCTER

a) Effect of Adenosine 5'-Triphosphate

ATP (10^{-3} & 10^{-2}M) did not stimulate formation of [^{3}H]-inositol phosphates in the IAS prelabelled with [^{3}H]-inositol. These results are contrary to those reported by Pirotton et al., (1987) who stated that ATP (10^{-6} - 10^{-4}M) produced increased formation of inositol phosphates in bovine aortic endothelial cells.

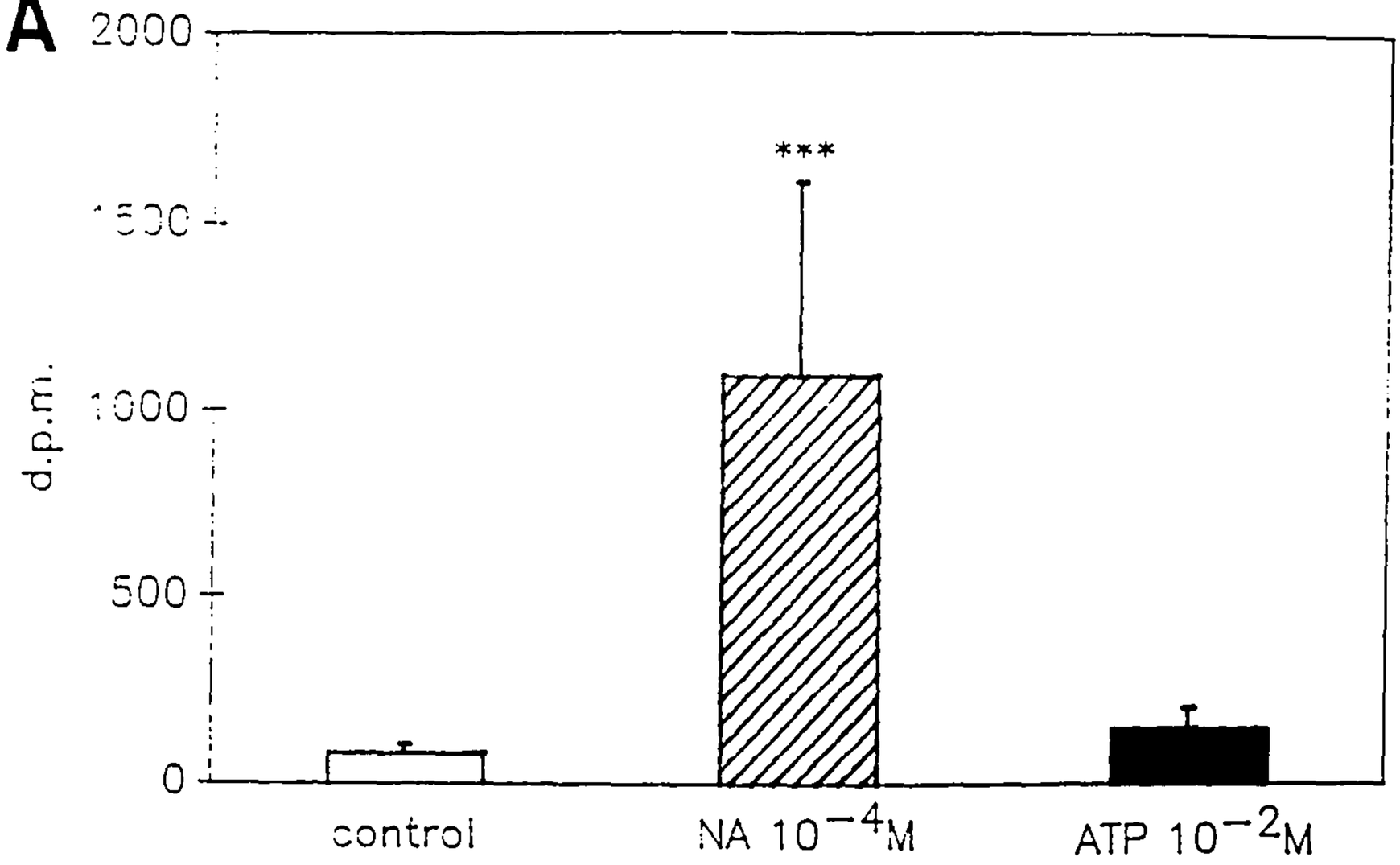
b) Effect of Noradrenaline

Noradrenaline (10^{-4}M), unlike ATP, significantly increased the formation of [^{3}H]-inositol phosphates in the IAS from control values by 1266% (IP), 1220% (IP_2), 955% (IP_3 & IP_4). IP_3 and IP_4 could not be separated independently on the the Dowex columns which were used and therefore were eluted together. These increases produced by NA (10^{-4}M) were also significantly greater than those produced by ATP (10^{-2}M) by 596% (IP), 636% (IP_2) and 542% (IP_3 & IP_4) as analysed by one-way analysis of variance followed by the Tukey test. (Figure 49A,B & C).

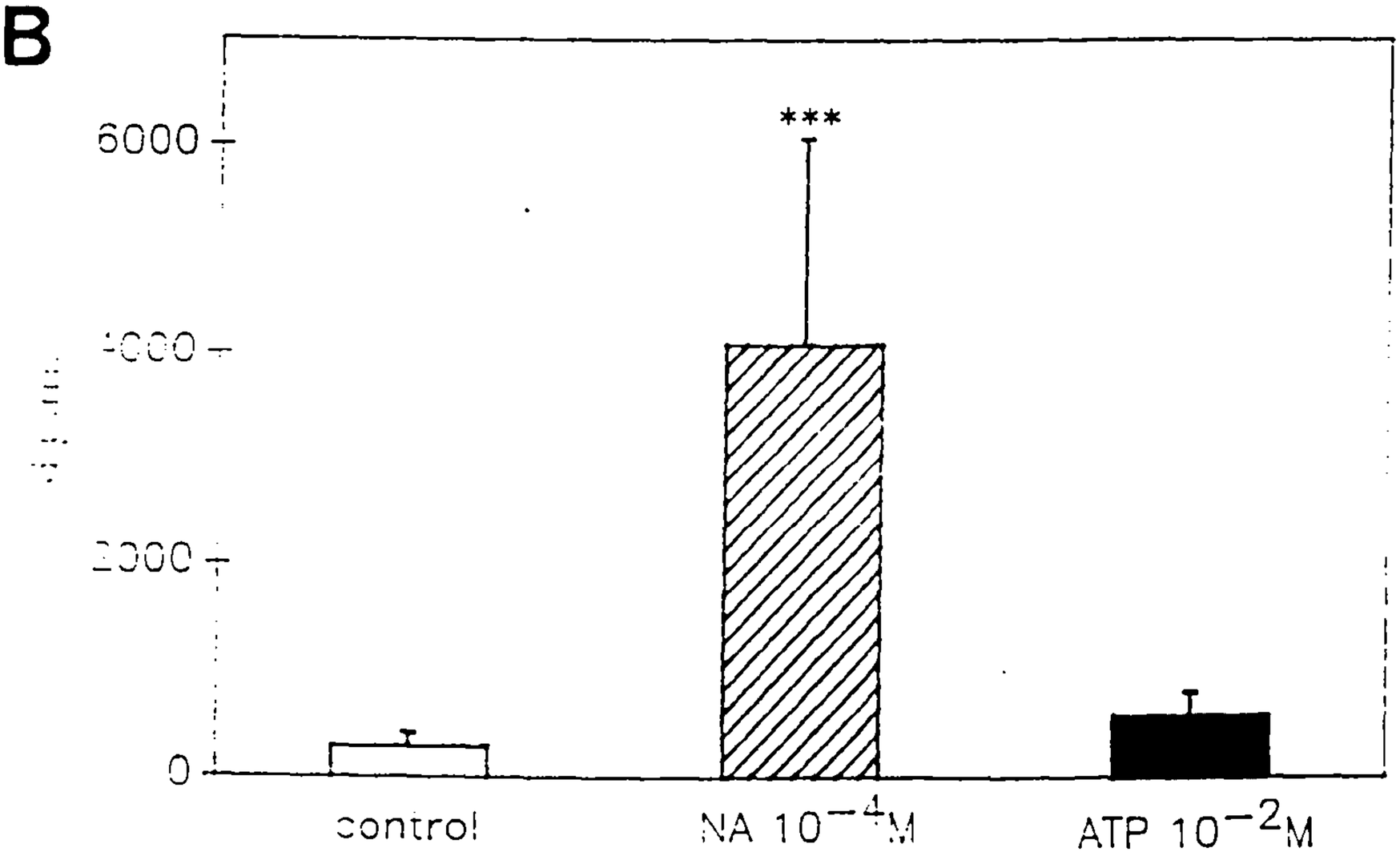
FIGURE 49:

The effect of noradrenaline (NA, 10^{-4}M) and ATP (10^{-2}M) on the resting [^{3}H] content of inositol 1-phosphate (IP, A), inositol 1,4-bisphosphate (IP_2, B) and inositol 1,4,5-trisphosphate (IP_3, C) in the IAS incubated in [^{3}H]-inositol. Each bar represents the mean ± S.D. of n=8 observations. Noradrenaline significantly increased the accumulation of [^{3}H]-IP, [^{3}H]-IP_2 and [^{3}H]-IP_3 from control values, whilst ATP had no significant effect.

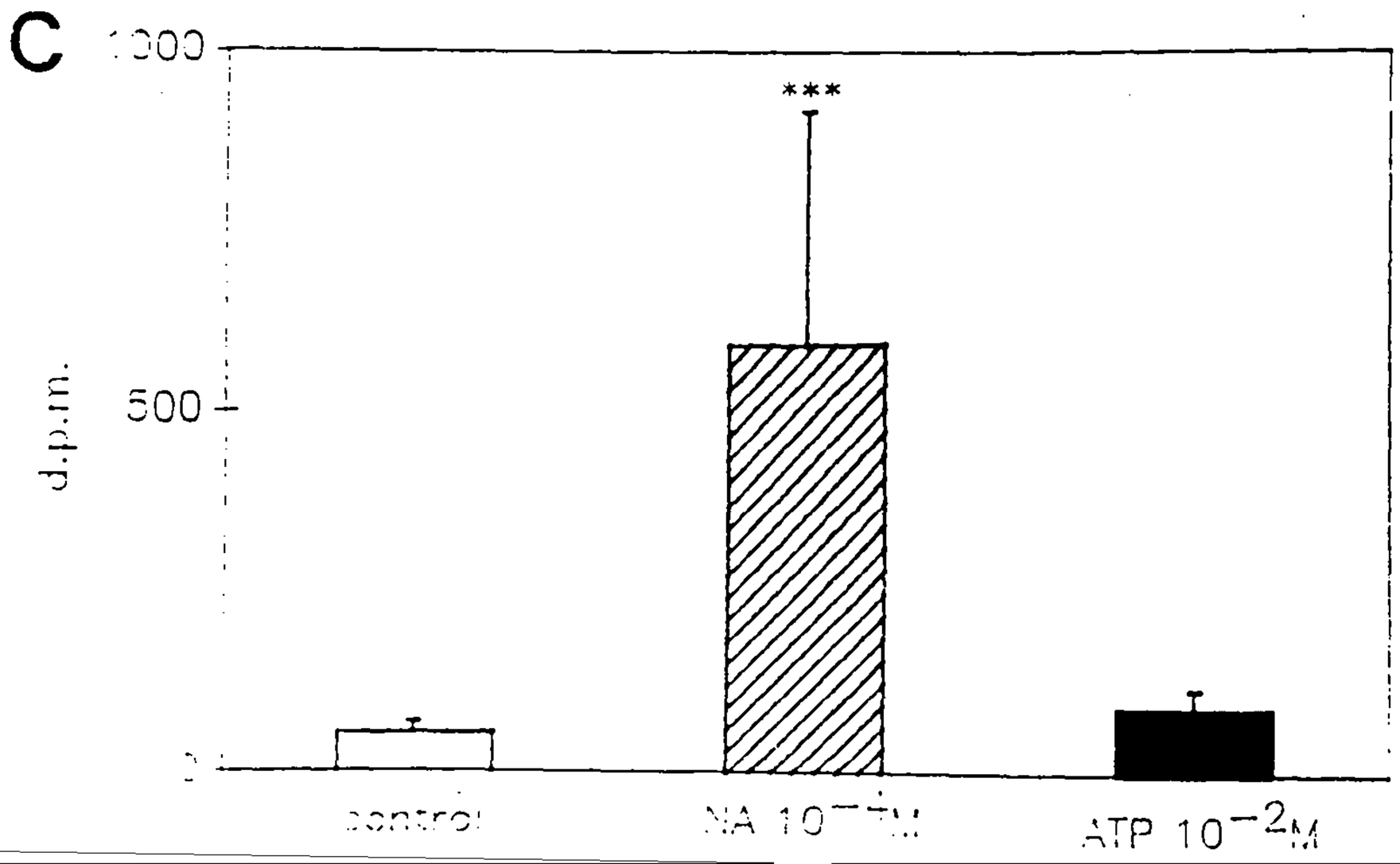
A


2000
1500
1000
500
0
d.p.m.
***
control
NA 10^{-4}M
ATP 10^{-2}M

B


6000
4000
2000
0
***
control
NA 10^{-4}M
ATP 10^{-2}M

C


1000
500
d.p.m.
***
control
NA 10^{-4}M
ATP 10^{-2}M

4 INTRALUMINAL PRESSURE IN THE GUINEA-PIG INTERNAL ANAL SPHINCTER IN VIVO

a) Characteristics of the Preparation

Animals were given 30min to equilibrate following anaesthesia with urethane ($1.7gkg^{-1}$, i.p.) and subsequent cannulation of the trachea, left jugular vein (for drug administration) and right carotid artery (to monitor blood pressure and heart rate). At the end of this equilibration period the mean diastolic and systolic blood pressures were 41 ± 12mmHg (n=42) and 77 ± 17mmHg (n=42), respectively, and the mean heart rate was 276 ± 76 $beatsmin^{-1}$ (n=42). The resting intraluminal pressure of the internal anal sphincter was 8.8 ± 7.0mmHg (n=39) measured from a baseline control level which was obtained at the end of each experiment after administration of a fatal dose of pentobarbitone ($60mgkg^{-1}$). The baseline was taken as the lowest pressure measured from the anal canal after death, since at this point all muscular tone affecting the intraluminal pressure had been lost and only the effects of body weight on the pressure transducer were evident. During measurement, oscillations were noted superimposed on the resting levels of the anal sphincter pressure; small rapid changes observed were attributed to respiratory movements and larger slower changes to the mixing and propulsive movements of the large intestine and rectum. Because of these changes, the resting anal pressure was measured where possible between these oscillations. This value comprised at least three components; the pressure exerted by the circular smooth muscle, the longitudinal smooth muscle and the skeletal muscle of the external anal sphincter and will be referred to as the "anal sphincter region" *in vivo*. This situation is unlike that

in vitro where the circular muscle of the sphincteric region was isolated.

The responses of the cardiovascular system to drugs were measured as absolute changes in the mean arterial blood pressure (mmHg) and heart rate ($beatsmin^{-1}$). However, the responses to drugs in the anal sphincter region were measured from the lowest pressure immediately prior to drug administration and taken as a percentage increase or decrease from that point. Concentration-dependent changes in blood pressure were produced by all drugs used. Only in the upper range of the blood pressure concentration response curves were changes in anal sphincter pressure evident, and because of the maximal effect of these drugs on the cariovascular system, concentration-dependent responses in the anal sphincter region were not studied. Saline (0.9%; $0.17mlkg^{-1}$; i.v.) had no effect on anal sphincter pressure, blood pressure or heart rate . (Figure 50).

b) **Effect of Sympathomimetics**

(i) **Noradrenaline**

In the cardiovascular system NA (0.2 - $8\mu gkg^{-1}$, i.v.) produced a dose-dependent increase in blood pressure (Figure 51), whilst the direction of change in heart rate was dependent on the dose administered; $3\mu gkg^{-1}$ (i.v.) produced a decrease and $8\mu gkg^{-1}$ (i.v.) produced an increase. Propranolol ($100\mu gkg^{-1}$, i.v.) reduced the resting blood pressure and slowed the heart rate (Figure 52B), however the pressor responses produced by the catecholamine ($8\mu gkg^{-1}$, i.v.) remained unaffected. (Figure 53). Phentolamine ($100\mu gkg^{-1}$, i.v.) also lowered the blood pressure and raised the heart rate (Figure 52A) and profoundly reduced the pressor responses produced by NA ($8\mu gkg^{-1}$, i.v.) on the

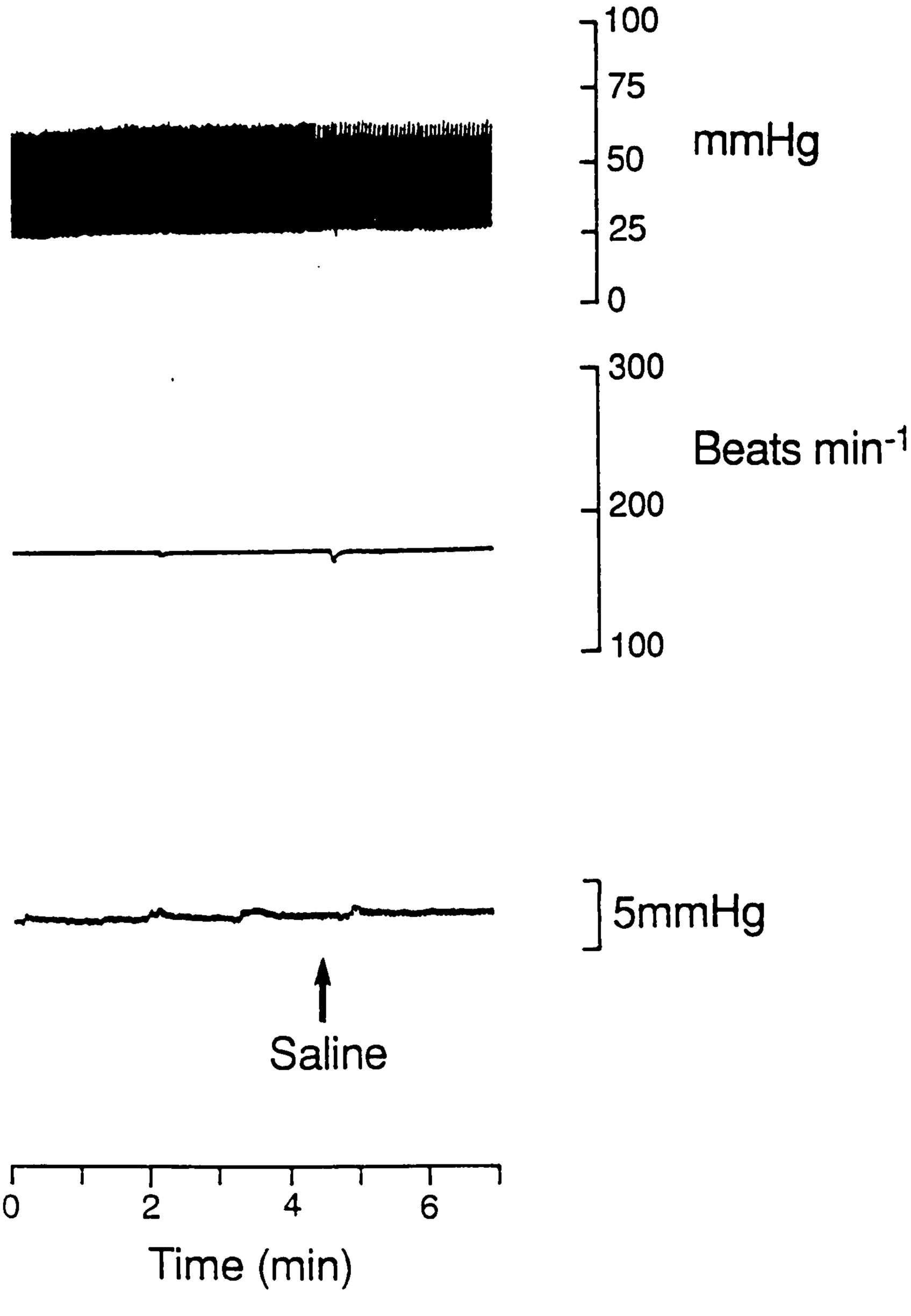


FIGURE 50:
The lack of effect of saline (0.2mlkg^{-1}, i.v.) on the blood pressure (mmHg, top panel), heart rate (beatsmin^{-1}, middle panel) and anal sphincter pressure (mmHg, bottom panel) of the anaesthetised guinea-pig <u>in vivo</u>.

FIGURE 51:
The dose-dependent effect of noradrenaline (NA, 0.3-8μgkg^{-1}, i.v.) on the blood pressure (mmHg) of the anaesthetised guinea-pig. Each point represents the mean ± S.D. of at least 4 observations.

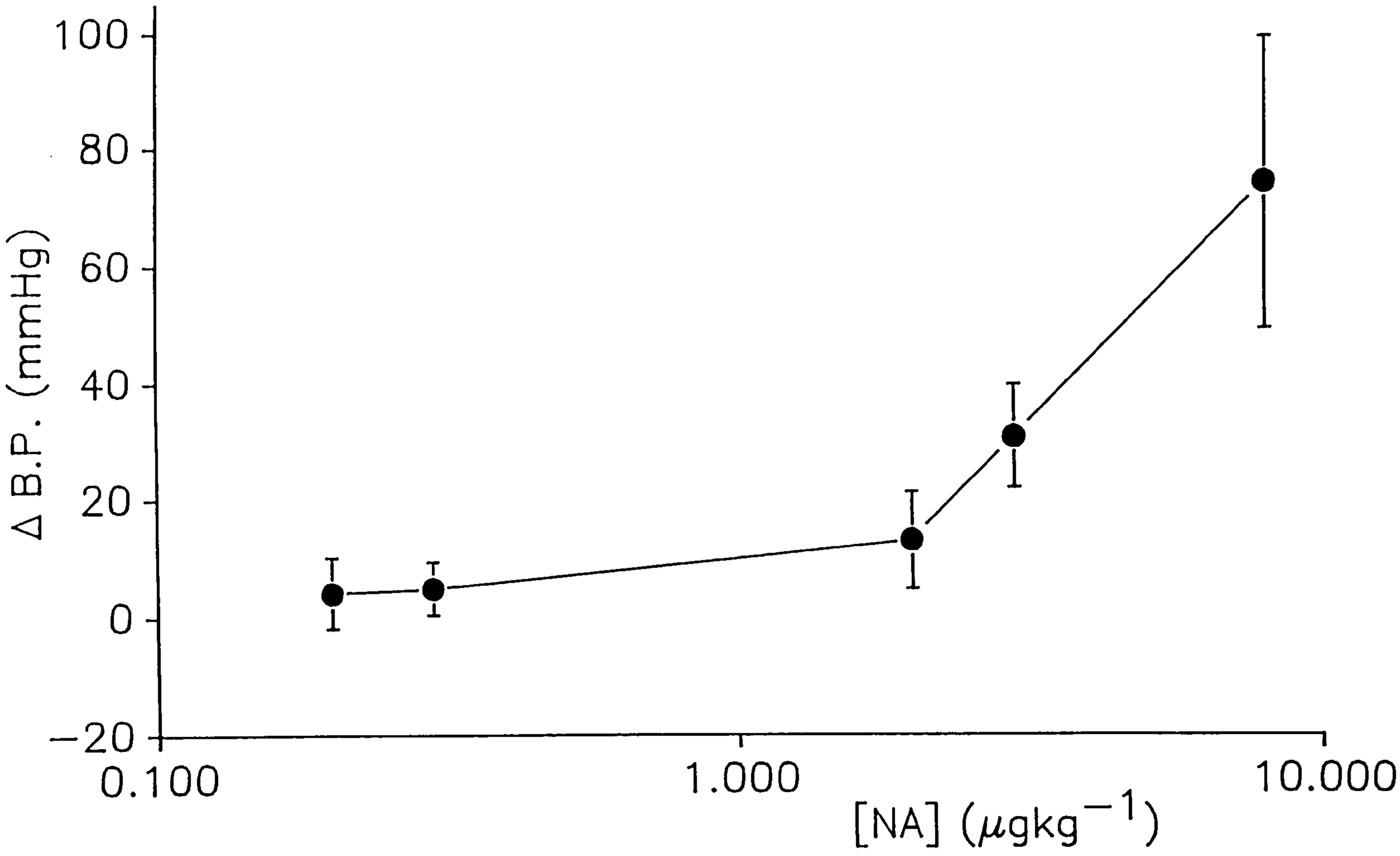

100
80
60
40
20
0
−20
Δ B.P. (mmHg)
0.100
1.000
10.000
[NA] (μgkg⁻¹)

FIGURE 52:

The effect of phentolamine (100μgkg^{-1},i.v.) (A) and propranolol (100μgkg^{-1},i.v.)(B) on the blood pressure (mmHg, top panels), heart rate (beatsmin^{-1}, middle panels) and anal sphincter pressure (mmHg, bottom panels) of the anaesthetised guinea-pig <u>in vivo</u>. Both propranolol and phentolamine produced a decrease in blood pressure, and anal sphincter pressure.

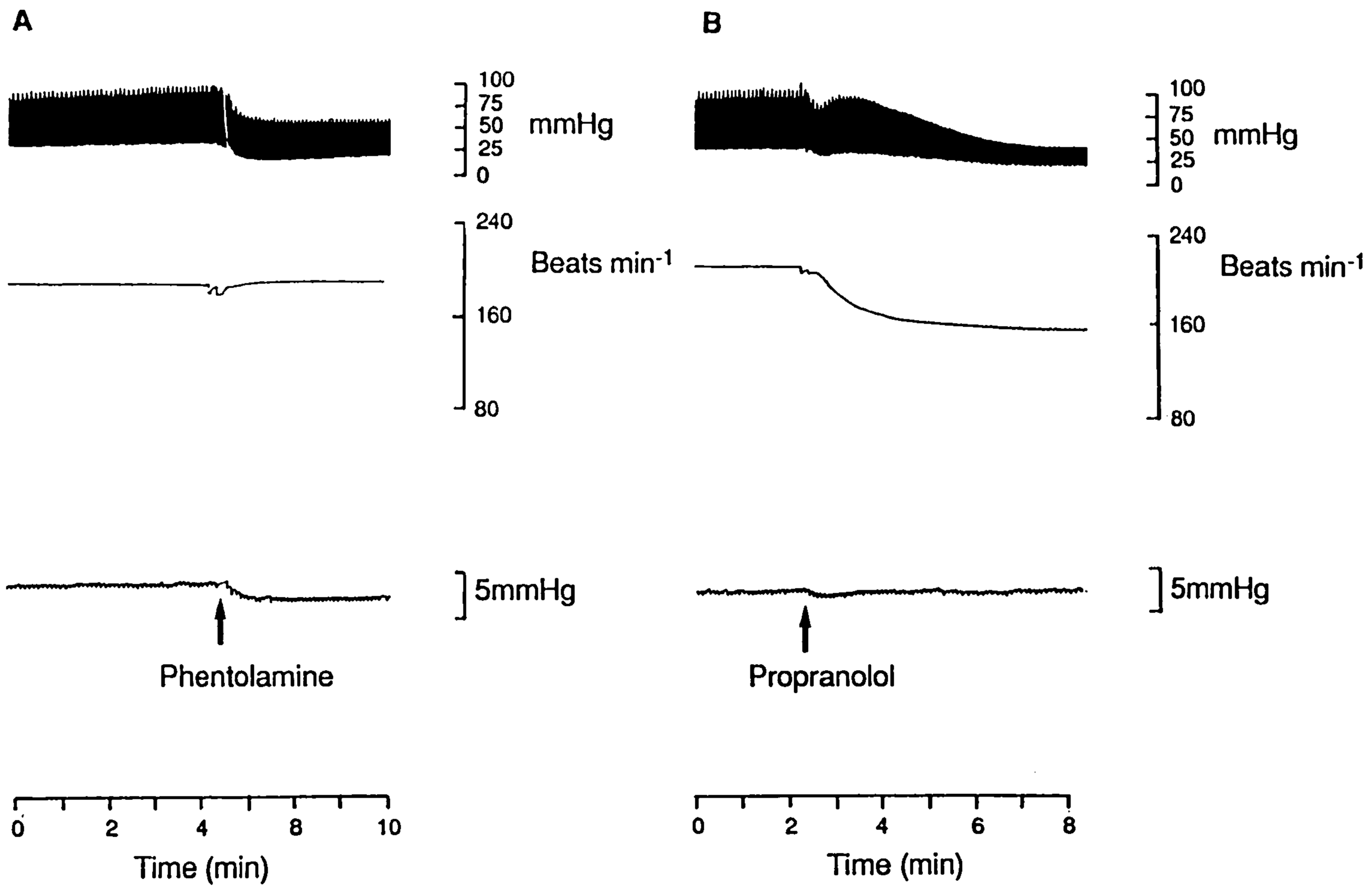
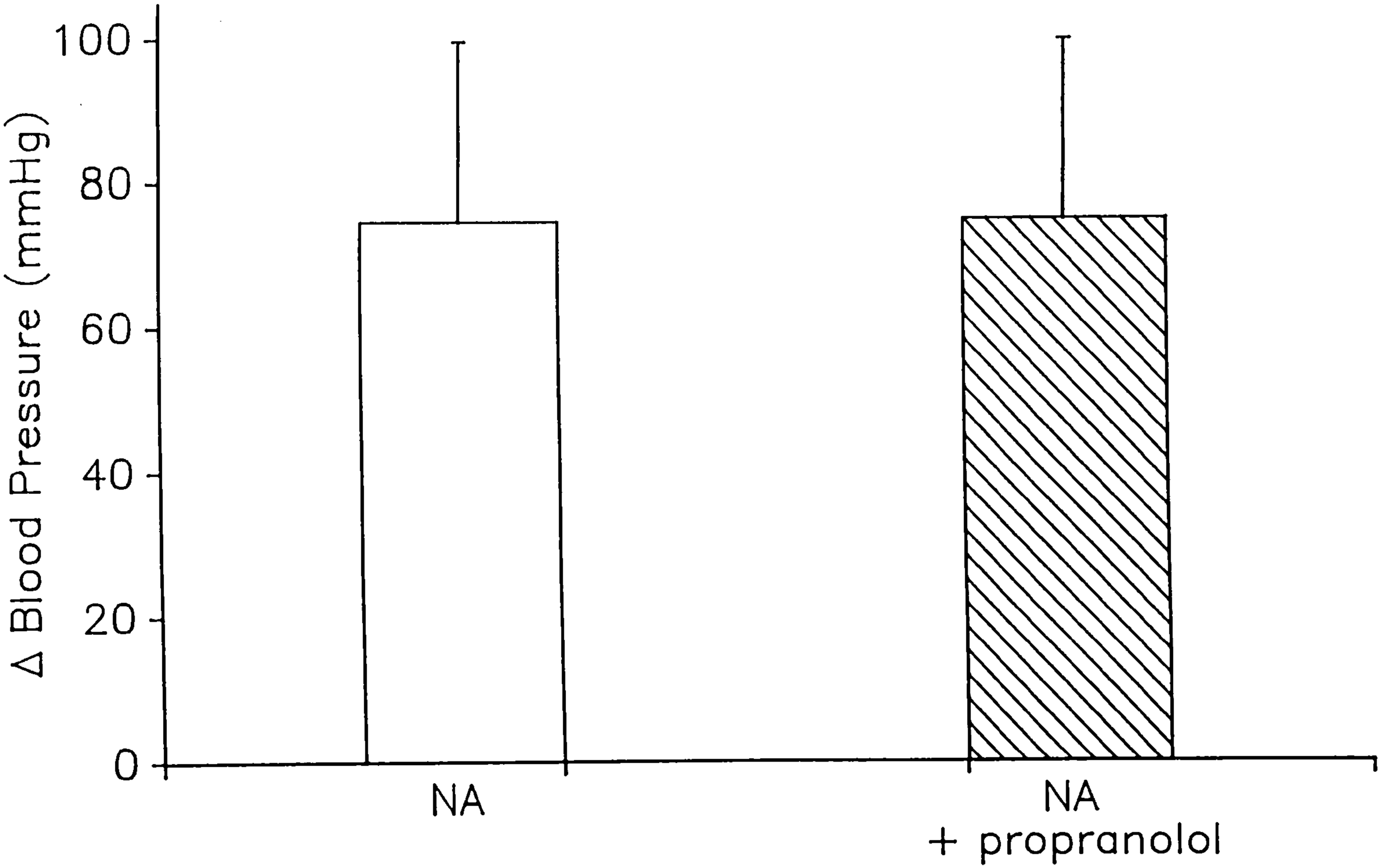

A
B
100
75
50
25
0
mmHg
240
160
80
Beats min-1
5mmHg
Phentolamine
Propranolol
0
2
4
8
10
6
Time (min)

FIGURE 53:
The lack of effect of propranolol (100μgkg^{-1},i.v.) on the blood pressure (mmHg) response produced by noradrenaline (NA; 8μgkg^{-1},i.v.) in the anaesthetised guinea-pig in vivo. Each bar represents the mean ± S.D. of at least 8 observations.


100
80
60
40
20
0
Δ Blood Pressure (mmHg)
NA
NA
+ propranolol

cardiovascular system. (Figure 54).

NA ($8\mu gkg^{-1}$, i.v.) increased anal sphincter pressure by 62.4 ± 48.3% (n=29) (Figure 55). The excitatory response in the IAS *in vivo* was characteristic of that produced at all gastrointestinal sphincters and mirrored that of the IAS *in vitro* to the catecholamine (Lim, 1985). In the presence of the β-adrenoceptor antagonist propranolol ($100\mu gkg^{-1}$, i.v.), the basal intraluminal pressure of the anal sphincter region was decreased by 22.6 ± 33.6% (n=10) (Figure 52B), although the increase in pressure produced by NA ($8\mu gkg^{-1}$, i.v.) remained unaffected. (Figure 56). Phentolamine ($100\mu gkg^{-1}$, i.v.), the α-adrenergic antagonist, reduced the basal level of anal sphincter pressure by 22.8 ± 22.6% (n=10) (Figure 52A) and abolished the rise in anal sphincter pressure produced by NA ($8\mu gkg^{-1}$, i.v.). (Figure 54).

In the anal sphincter region *in vivo*, as *in vitro*, α-adrenoceptors are present which mediate the excitatory response produced by NA. *In vivo* there appears to be a degree of adrenergic control which can be reduced by propranolol and phentolamine. These results seem to be contradictory to those obtained from experiments *in vitro* where propranolol ($10^{-6}M$) and phentolamine ($10^{-6}M$) are shown to have no effect on the resting tone of the IAS (Lim, 1985). This anomoly probably arises because *in vitro* the circular muscle of the anal sphincter is isolated from other surrounding muscles and from any extrinsic autonomic control and is therefore able to show myogenic control of tone alone.

(ii) Isoprenaline

Isoprenaline ($3\mu gkg^{-1}$, i.v.) lowered blood pressure and raised heart rate (Figure 57), however in the presence of propranolol ($100\mu gkg^{-1}$,

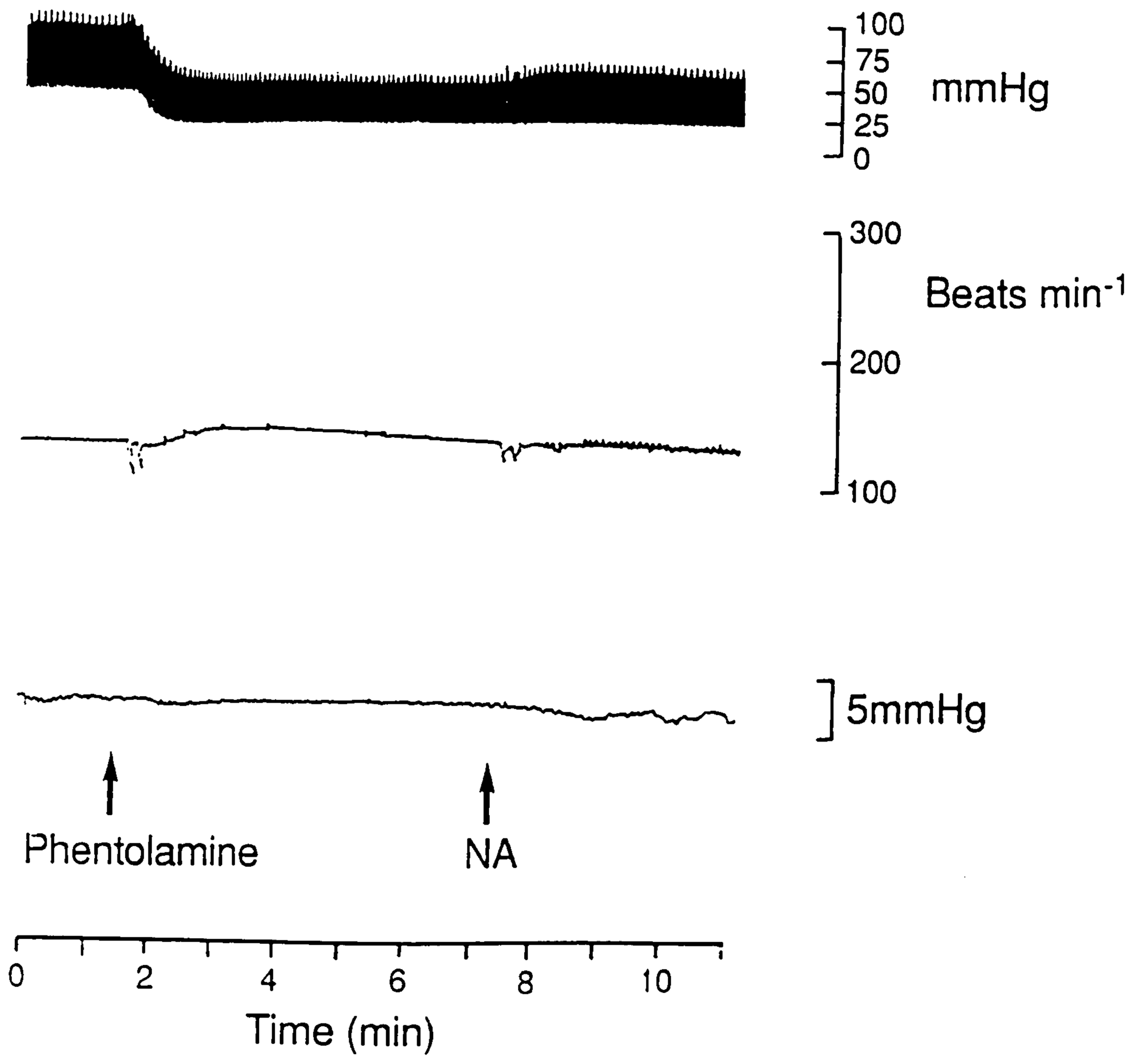


FIGURE 54:

The effect of phentolamine (100μgkg^{-1}, i.v.) on the blood pressure (mmHg, top panel), heart rate (beatsmin^{-1}, middle panel) and anal sphincter pressure (mmHg, bottom panel) responses produced by noradrenaline (NA; 8μgkg^{-1}, i.v.) in the anaesthetised guinea-pig in vivo. Phentolamine abolished the pressor responses in the cardiovascular system and the anal sphincter.

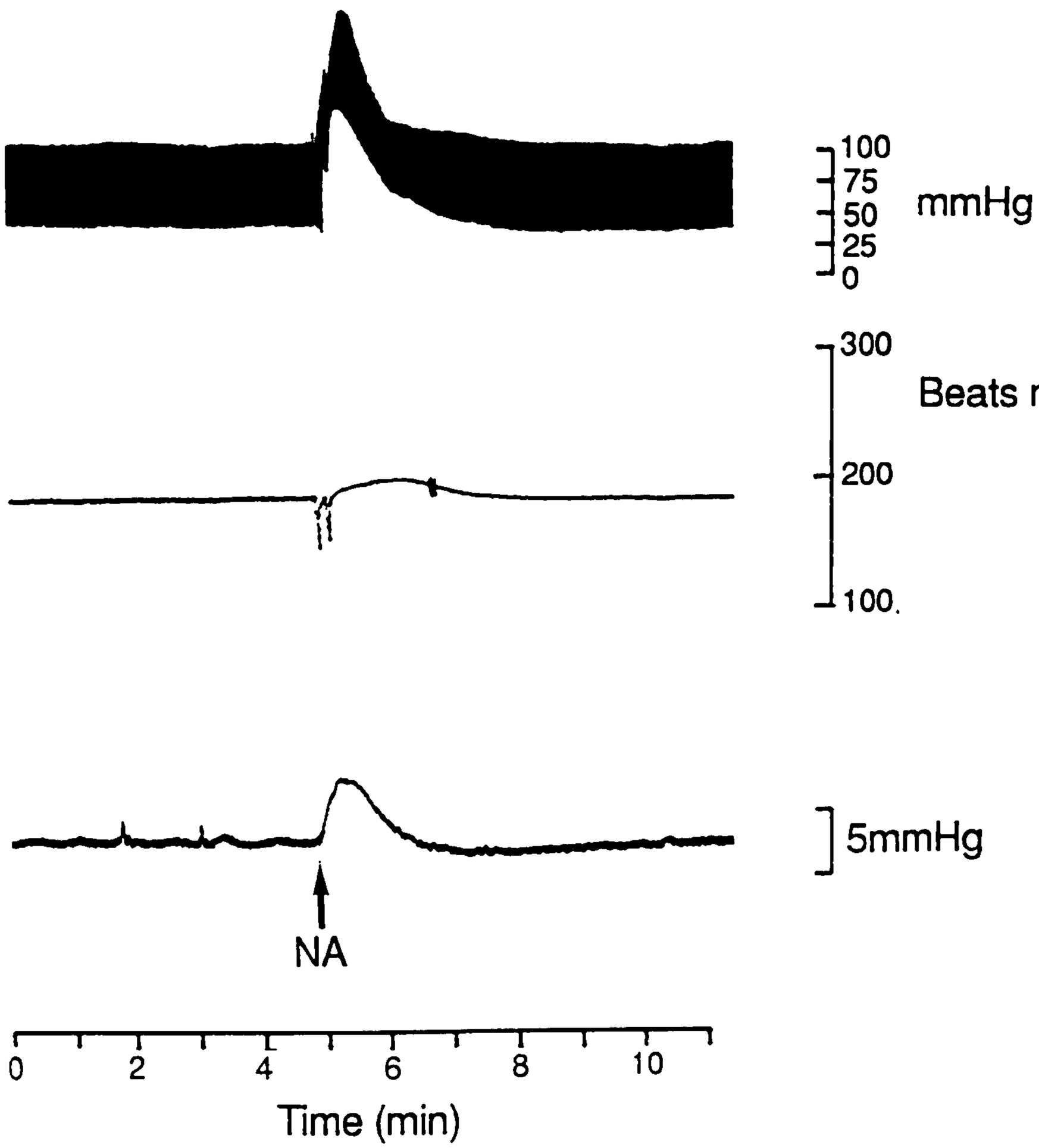
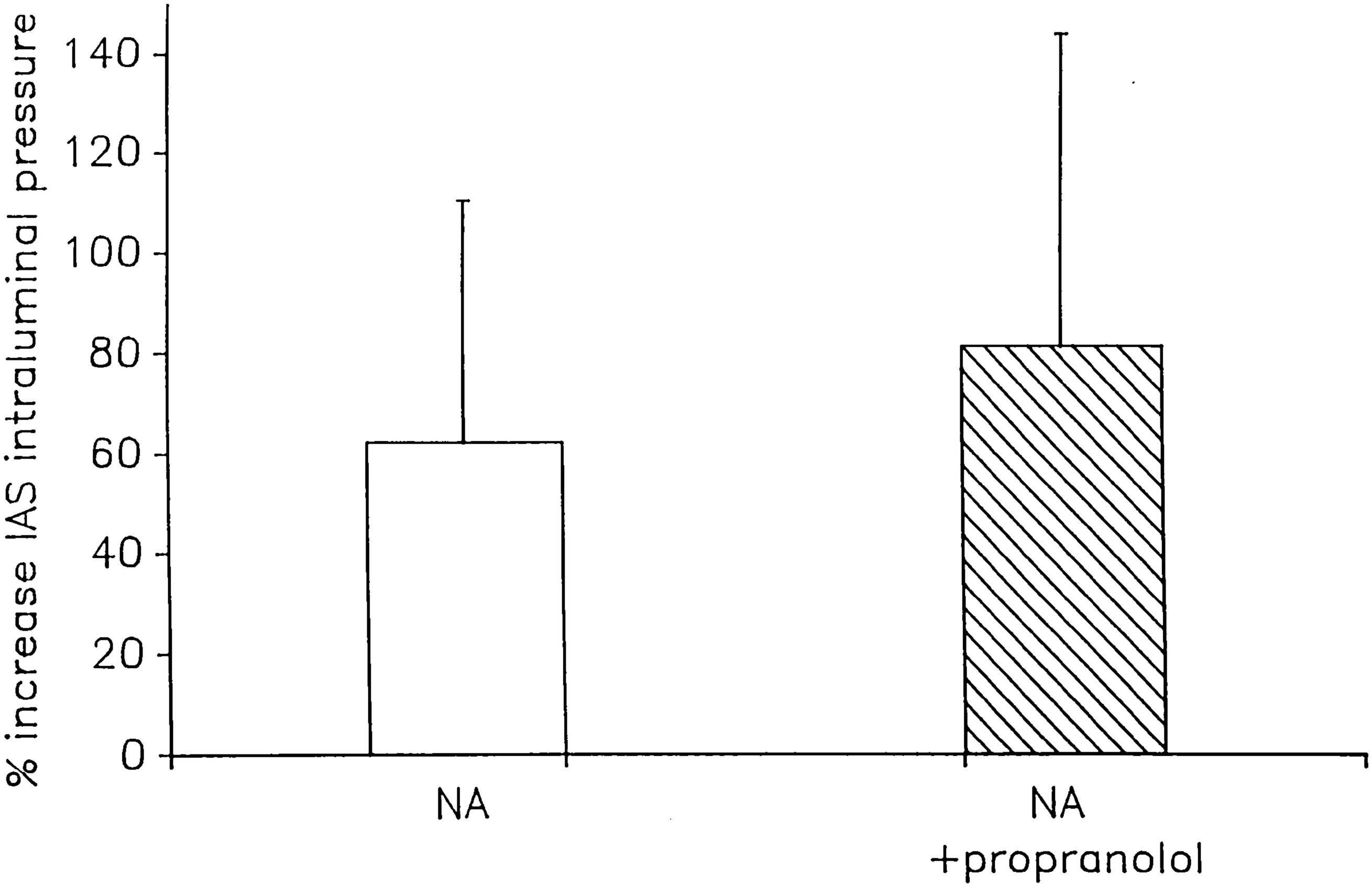


FIGURE 55:

The effect of noradrenaline (NA; 8μgkg^{-1},$_{-1}$i.v.) on the blood pressure (mmHg, top panel), heart rate (beatsmin^{-1}, middle panel) and anal sphincter pressure (mmHg, bottom panel) in the anaesthetised guinea-pig in vivo. NA produced an increase in blood pressure and anal sphincter pressure and a tachycardia..

FIGURE 56:

The lack of effect of propranolol (100μgkg^{-1}, i.v.) on the increase in anal sphincter pressure (% increase IAS intraluminal pressure) produced by noradrenaline (NA; 8μgkg^{-1}, i.v.) in the anaesthetised guinea-pig in vivo. Each bar represents the mean ± S.D. of at least 8 observations.


% increase IAS intraluminal pressure
140
120
100
80
60
40
20
0
NA
NA
+propranolol

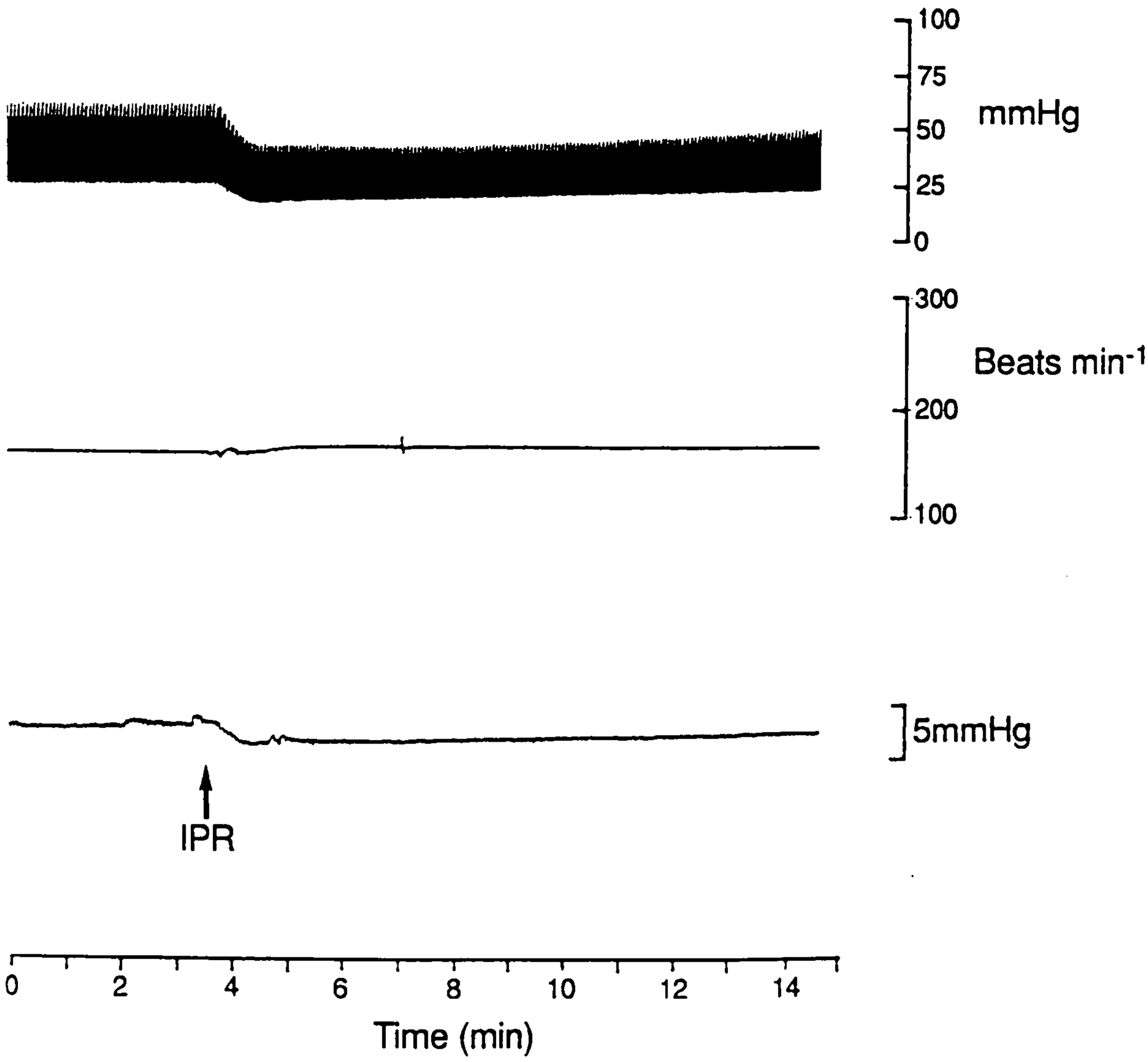


FIGURE 57:
The effect of isoprenaline (3μgkg^{-1}, i.v.) on the blood pressure (mmHg, top panel), heart rate (beatsmin^{-1}, middle panel) and anal sphincter pressure (mmHg, bottom panel) of the anaesthetised guinea-pig in vivo. Isoprenaline produced a decrease in blood pressure, and anal sphincter pressure.

i.v.) the depressor response was converted to a small pressor response. (Figure 58). This could presumably have been mediated by the weak action of α-adrenoceptors in the cardiovascular system.

The basal intraluminal pressure of the anal sphincter region was reduced by 21.0 ± 5.0% (n=10) by isoprenaline (3μgkg^{-1}, i.v.). (Figure 57). Propranolol (100μgkg^{-1}, i.v.) attenuated this reduction to 14.3 ± 5.0% (n=4) inhibition of basal tone. (Figure 59). These results suggest that the effects of isoprenaline <u>in vivo</u> in the anal sphincter region are, on the whole, mediated by β-adrenoceptors. The effect of isoprenaline on the IAS <u>in vitro</u> then is mirrored <u>in vivo</u>. The remaining reduction in anal sphincter pressure produced by isoprenaline (3μgkg^{-1}, i.v.) in the presence of propranolol (100μgkg^{-1}, i.v.), was abolished by further administration of phentolamine (100μgkg^{-1}, i.v.). This suggests that either β-adrenoceptor blockade was not achieved with the dose of propranolol used or isoprenaline acted on inhibitory α-adrenoceptors unmasked by β-adrenoceptor blockade in the anal sphincter region which could be antagonised by phentolamine.

c) Effect of Purines

(i) 2-Chloroadenosine

2-Chloroadenosine produced a dose-dependent (0.04 - 0.8μgkg^{-1}, i.v.) reduction in the blood pressure and a slowing of the heart rate. (Figure 60). The depressor responses produced by 2-chloroadenosine (0.8μgkg^{-1}, i.v.) and the bradycardia were attentuated by apamin (30μgkg^{-1}, i.v.) and virtually abolished by 8-phenyltheophylline (100μgkg^{-1}, i.v.). (Figure 61).

FIGURE 58:

The effect of propranolol (100μgkg^{-1}, i.v.) on the fall in blood pressure (mmHg) of the anaesthetised guinea-pig <u>in vivo</u> produced by isoprenaline (IPR; 3μgkg^{-1}.i.v.). Propranolol converted the depressor response to a pressor effect produced by isoprenaline. Each bar represents the mean ± S.D. of at least 4 observations.

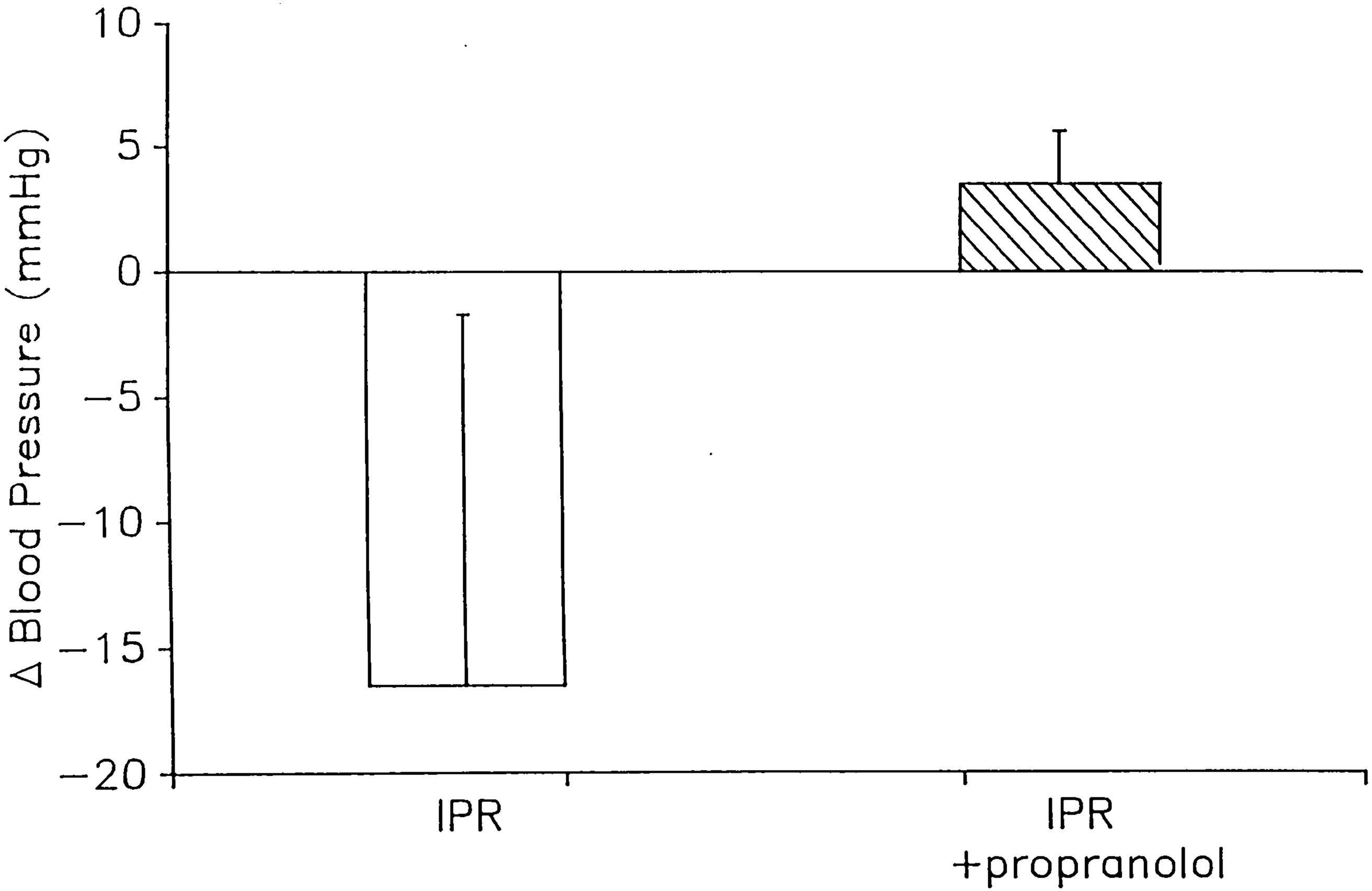

10
5
0
−5
−10
−15
−20
Δ Blood Pressure (mmHg)
IPR
IPR
+propranolol

FIGURE 59:

The effect of propranolol (100μgkg^{-1}, i.v.) on the anal sphincter pressure response (% decrease IAS intraluminal pressure) produced by isoprenaline (3μgkg^{-1}, i.v.) in the anaesthetised guinea-pig <u>in vivo</u>. Propranolol significantly ($p<0.05$) reduced the decrease in anal sphincter pressure produced by isoprenaline. Each bar represents the mean ± S.D. of at least 4 observations.

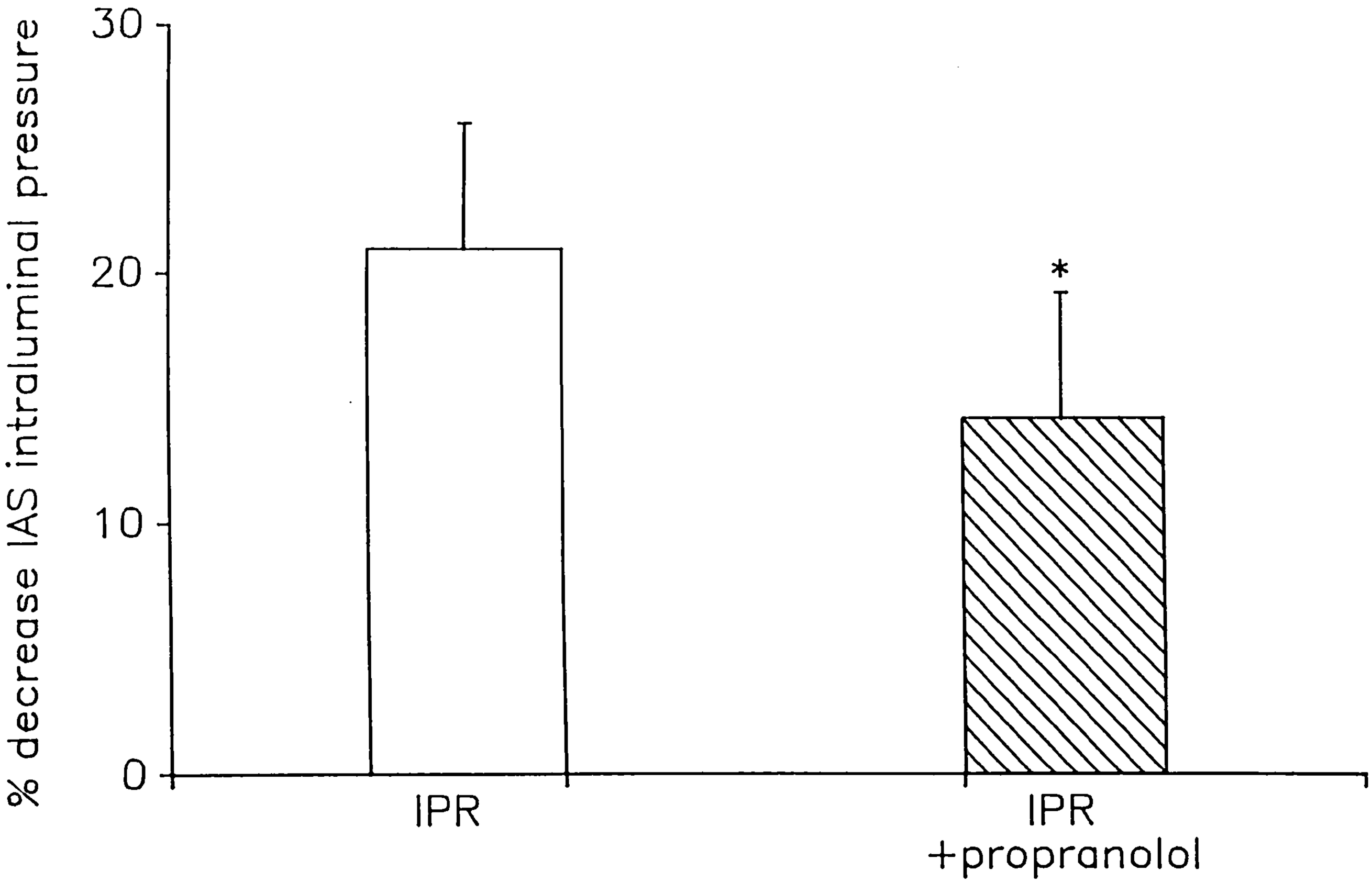

% decrease IAS intraluminal pressure
30
20
10
0
*
IPR
IPR
+propranolol

FIGURE 60:

The effect of increasing the concentration of 2-chloroadenosine (2-ClAd; 0.04-0.8μgkg^{-1}, i.v.) on the blood pressure (mmHg) of the anaesthetised guinea-pig in vivo. The first three points represent the average of 2 observations while the latter two points are from the mean ± S.D. of at least 6 observations. 2-Chloroadenosine produced a dose-dependent decrease in blood pressure.

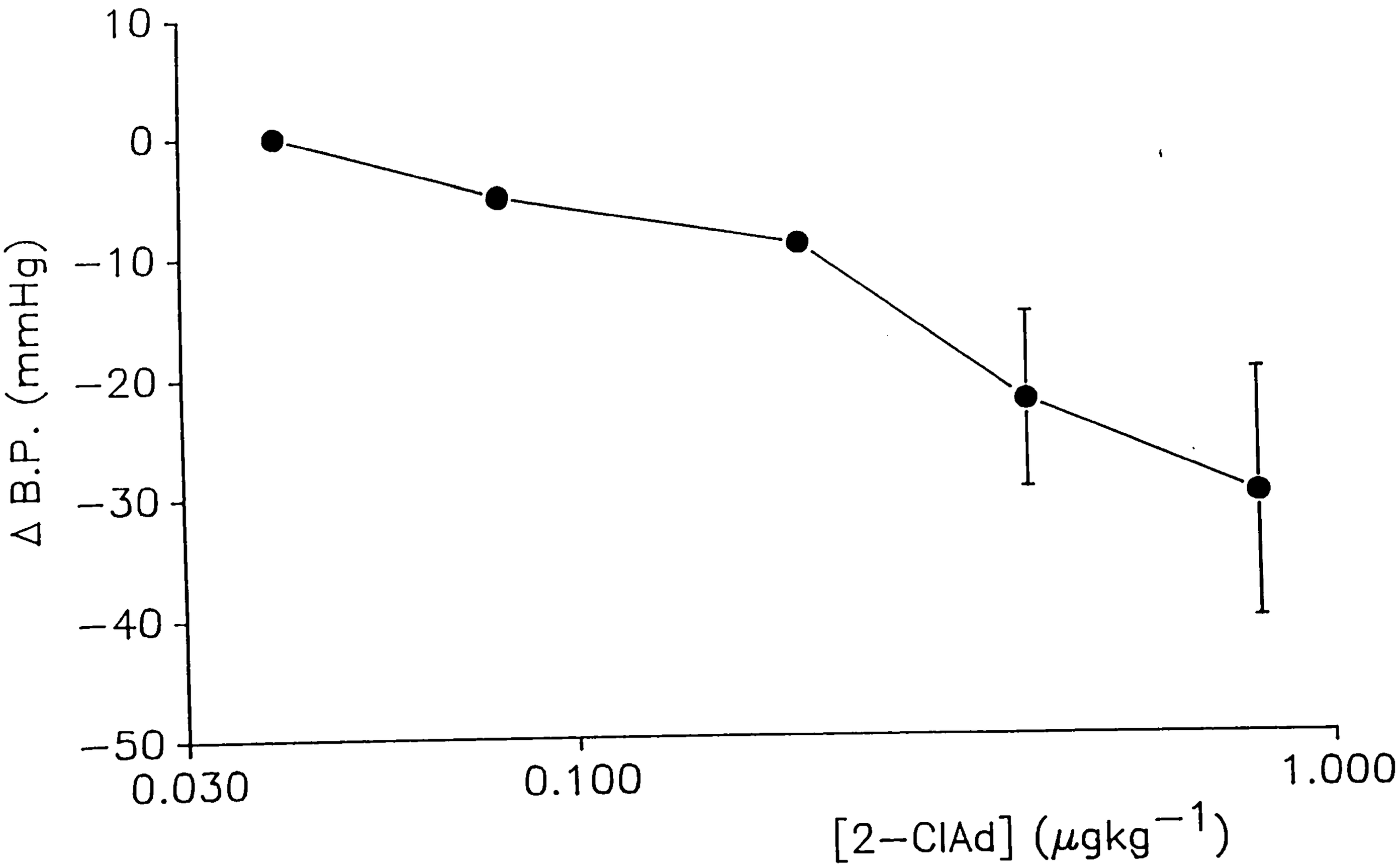
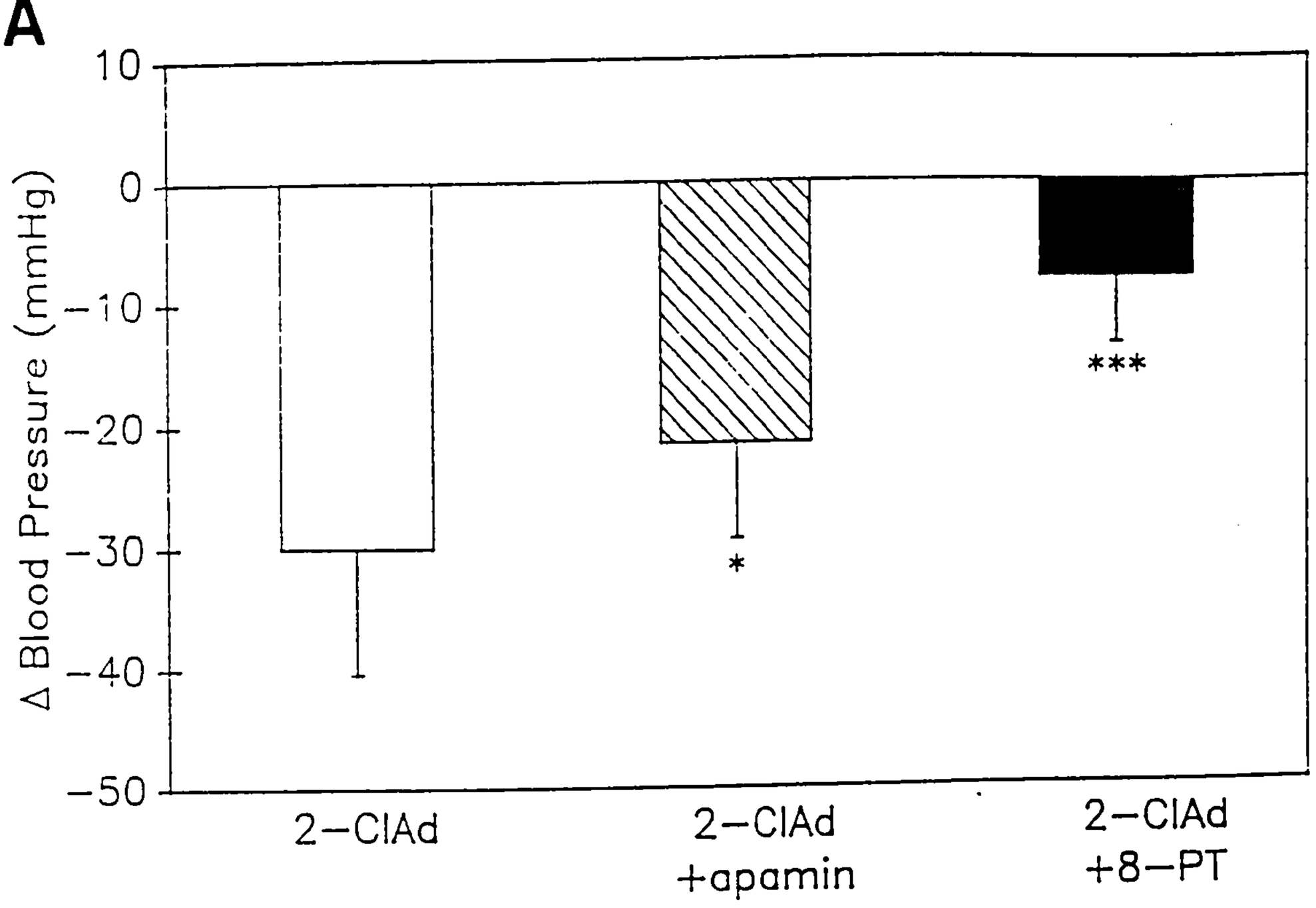

10
0
−10
−20
−30
−40
−50
Δ B.P. (mmHg)
0.030
0.100
1.000
[2−ClAd] (μgkg⁻¹)

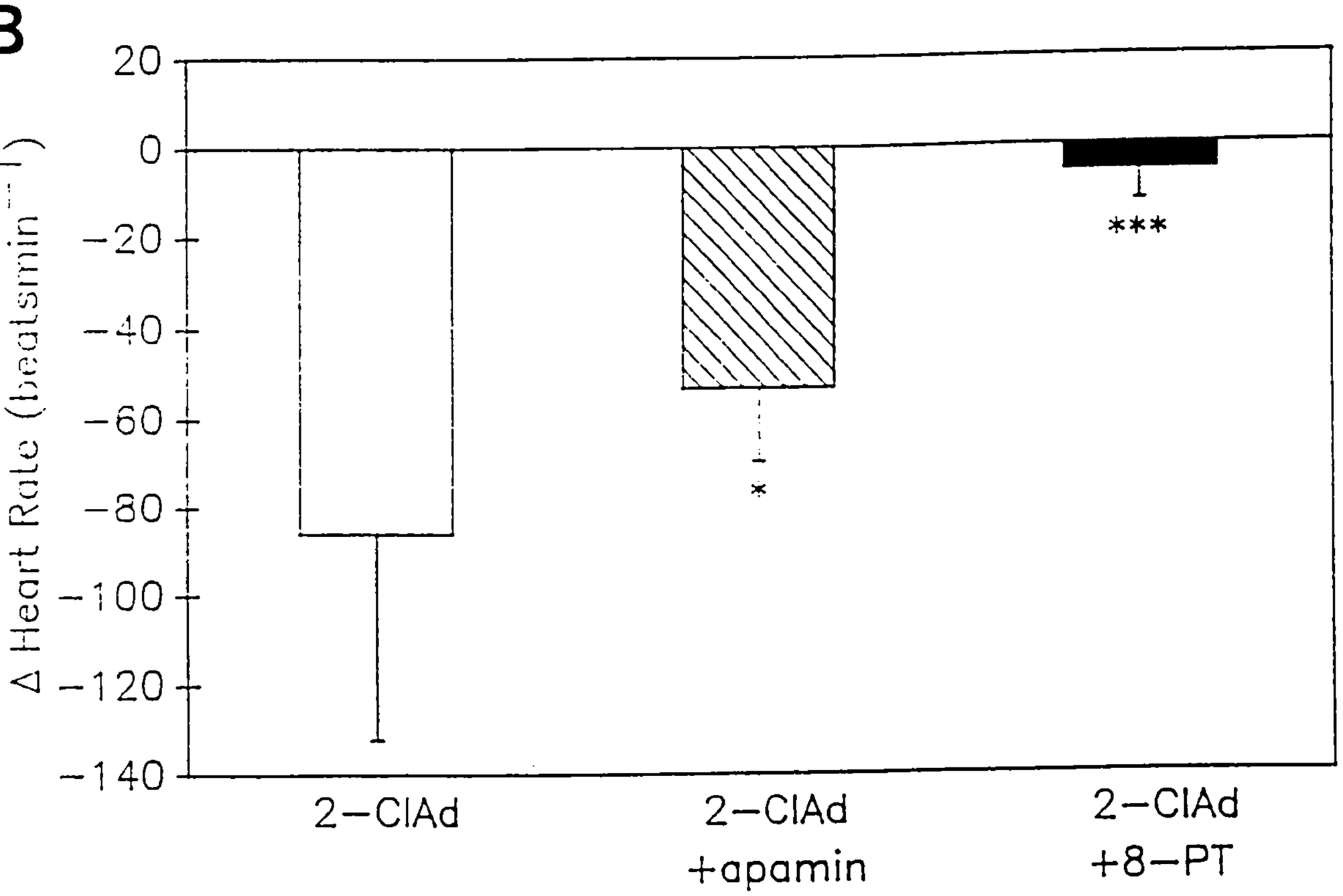
FIGURE 61:

The effect of apamin ($30\mu gkg^{-1}$, i.v.) and 8-phenyltheophylline (8-PT; $100\mu gkg^{-1}$, i.v.) on the blood pressure (mmHg) (A) and heart rate ($beats\,min^{-1}$) (B) responses of the anaesthetised guinea-pig <u>in vivo</u> produced by 2-chloroadenosine (2-ClAd; $0.8\mu gkg^{-1}$, i.v.). Each bar represents the mean ± S.D. of at least 5 observations. Apamin ($p<0.05$) and 8-phenyltheophylline ($p<0.01$) each reduced the inhibitory responses produced by 2-chloroadenosine on blood pressure and heart rate.

A


10
0
−10
−20
−30
−40
−50
Δ Blood Pressure (mmHg)
*
***
2−ClAd
2−ClAd
+apamin
2−ClAd
+8−PT

B


20
0
−20
−40
−60
−80
−100
−120
−140
Δ Heart Rate (beatsmin⁻¹)
*
***
2−ClAd
2−ClAd
+apamin
2−ClAd
+8−PT

2-Chloroadenosine ($0.8\mu gkg^{-1}$, i.v.) decreased the resting pressure of the anal sphincter by 30 ± 13.7% (n=22). Apamin ($30\mu gkg^{-1}$, i.v.), which is proposed to inhibit the action of P_{2y}-purinoceptors (Burnstock & Kennedy, 1985), had no effect on either the resting anal pressure or in the reduction in pressure produced by 2-chloroadenosine ($0.8\mu gkg^{-1}$, i.v.). 8-Phenyltheophylline ($100\mu gkg^{-1}$, i.v.), the P_1-purinoceptor antagonist had no effect on the resting anal sphincter pressure, although, in contrast to apamin, it abolished the decrease in pressure produced by 2-chloroadenosine ($0.8\mu gkg^{-1}$, i.v.) in the anal canal. (Figure 62). From these results it seems that effects produced by 2-chloroadenosine in the anal canal in vivo are mediated by P_1-purinoceptors. This is in contrast to results obtained in vitro, where reduction of muscle tone is attributed to P_{2y}-purinoceptors alone (Lim & Muir, 1986). This difference may be due to the isolation of the IAS in vitro from the many systems which will affect it in vivo, for example other smooth muscles or extrinsic nervous control.

(ii) Adenosine 5'-triphosphate (ATP)

Blood pressure was lowered by ATP ($80\mu gkg^{-1}$,i.v.) and accompanied by a brief asystole of the heart. Apamin ($30\mu gkg^{-1}$,i.v.) reduced the bradycardia produced by ATP but the decrease in blood pressure remained unaffected. 8-Phenyltheophylline ($100\mu gkg^{-1}$,i.v.) reduced the depressor responses produced by ATP ($80\mu gkg^{-1}$,i.v.) on the blood pressure and heart rate, while TEA ($1mgkg^{-1}$,i.v.) attenuated the depressor effect ($80\mu gkg^{-1}$,i.v.) on the blood pressure but not the heart rate. (Figure 63).

FIGURE 62:

The effect of apamin (30μgkg^{-1}, i.v.) on the anal sphincter pressure (% decrease IAS intraluminal pressure) produced by 2-chloroadenosine (2-ClAd; 0.8μgkg^{-1}, i.v.) in the anaesthetised guinea-pig <u>in vivo</u>. Each bar represents the mean ± S.D. of at least 5 observations. Apamin produced no effect on the inhibitory response to 2-chloroadenosine.

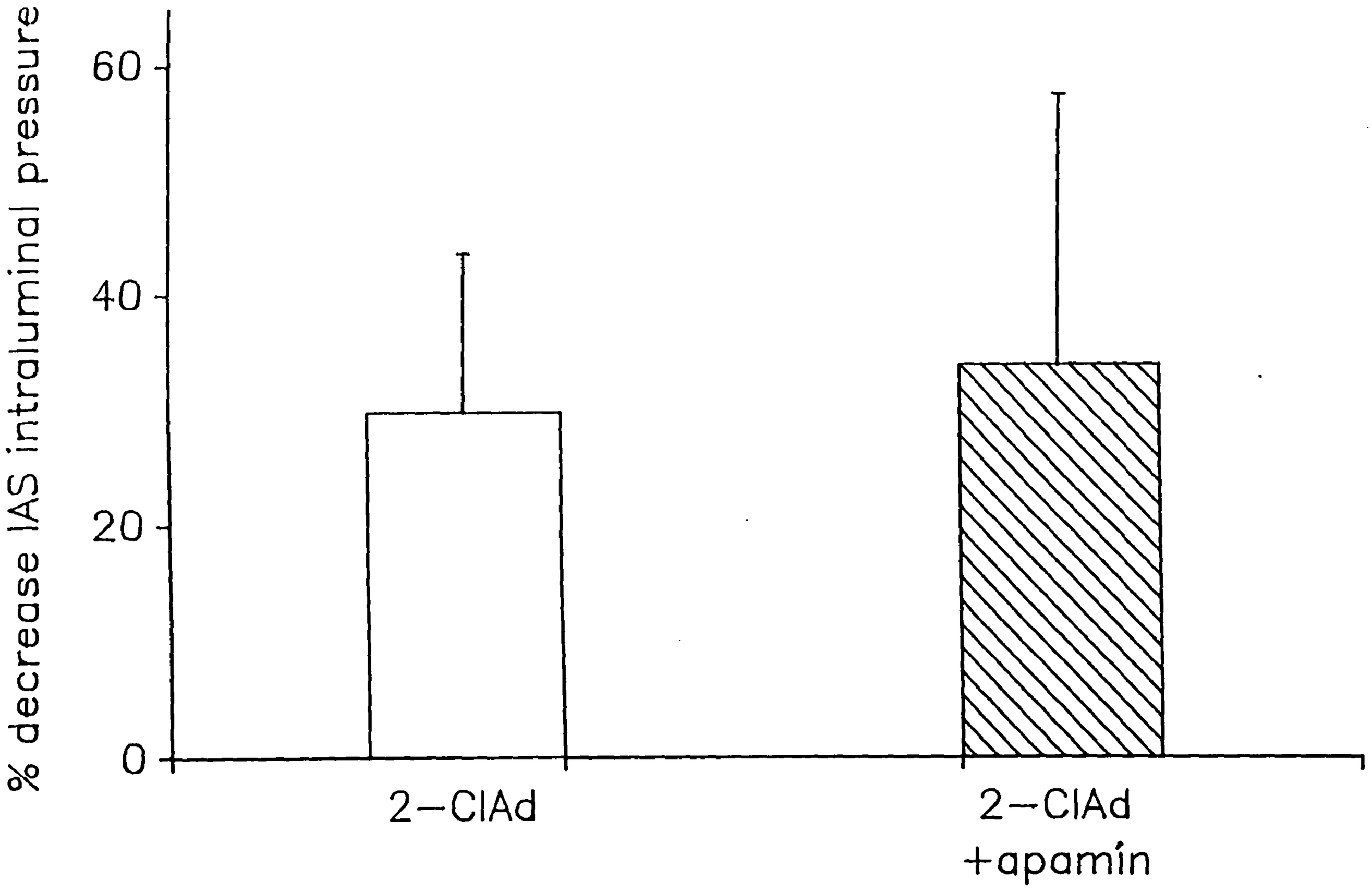
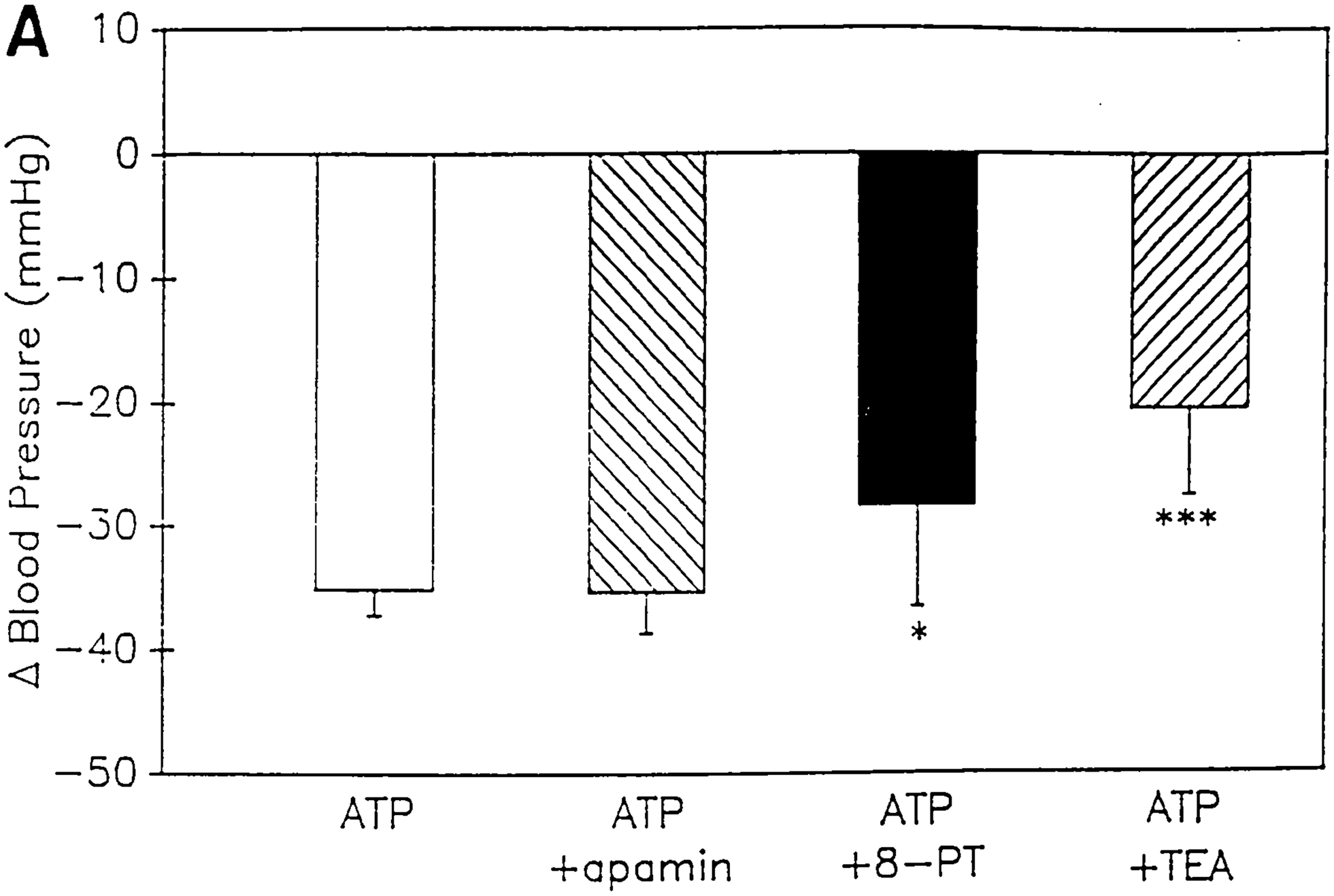
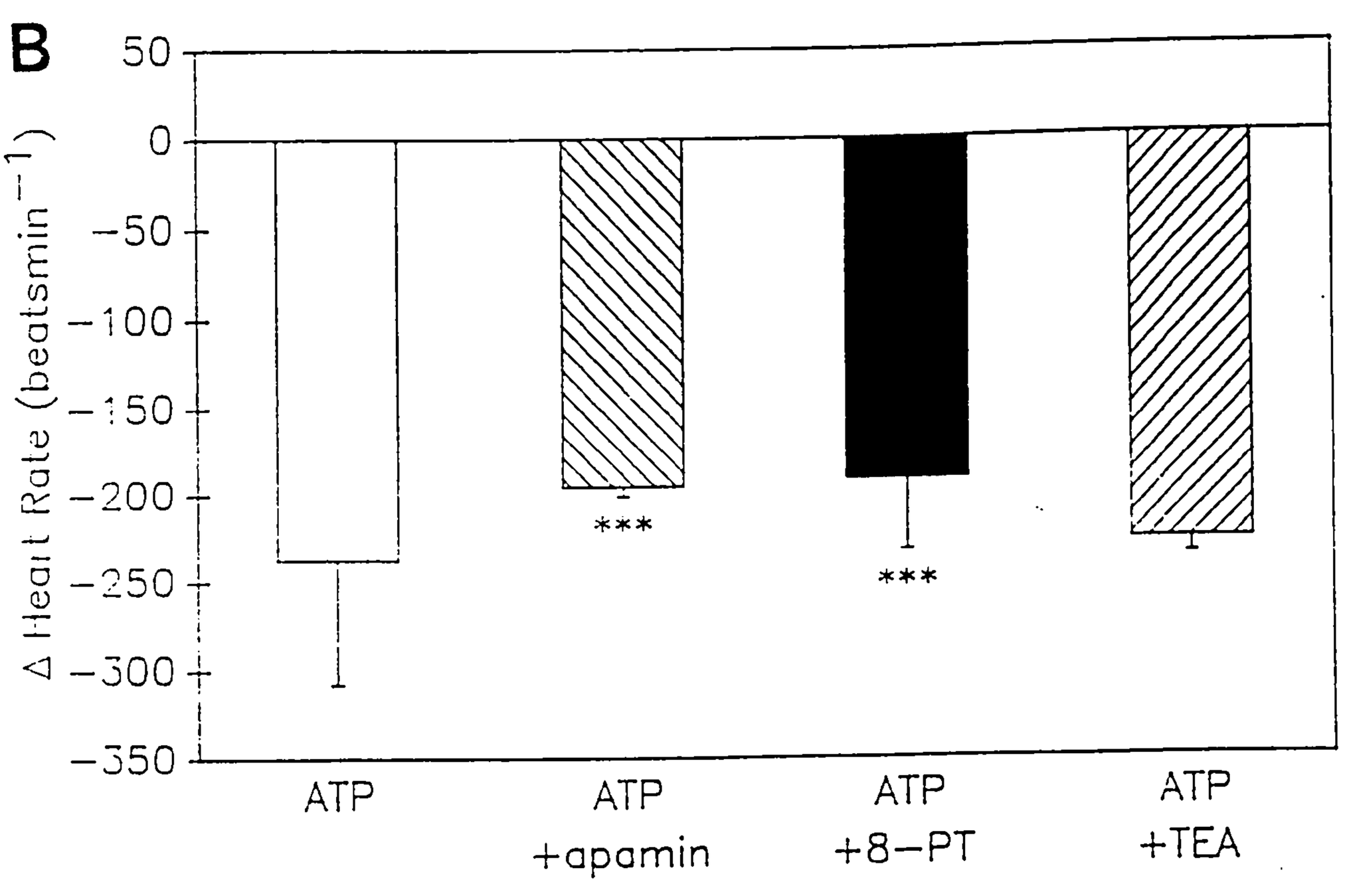

% decrease IAS intraluminal pressure
60
40
20
0
2–ClAd
2–ClAd
+apamin

FIGURE 63:

The effect of apamin ($30\mu gkg^{-1}$, i.v.), 8-phenyltheophylline (8-PT; $100\ \mu gkg^{-1}$, i.v.) and tetraethylammonium (TEA; $1mgkg^{-1}$, i.v.) each, on the reduction in blood pressure (mmHg) (A) and heart rate ($beatsmin^{-1}$) (B) in the anaesthetised guinea-pig <u>in vivo</u> produced by ATP ($80\mu gkg^{-1}$, i.v.). Each bar represents the mean ± S.D. of at least 4 observations. 8-Phenyltheophylline ($p<0.05$) and TEA ($p<0.01$) each attenuated the decrease in blood pressure produced by ATP and apamin had no effect. The bradycardia produced by ATP was attenuated by apamin ($p<0.01$) and 8-phenyltheophylline ($p<0.01$), but was unaffected by TEA.


A
10
0
−10
−20
−30
−40
−50
Δ Blood Pressure (mmHg)
***
*
***
ATP
ATP
+apamin
ATP
+8−PT
ATP
+TEA


B
50
0
−50
−100
−150
−200
−250
−300
−350
Δ Heart Rate (beatsmin^{-1})
***
***
ATP
ATP
+apamin
ATP
+8−PT
ATP
+TEA

ATP ($80\mu gkg^{-1}$,i.v.) inhibited anal sphincter intraluminal pressure by 30.8 ± 20.6% (n=13). This reduction was unaffected by 8-phenyltheophylline ($100\mu gkg^{-1}$,i.v.) (Figure 64). Apamin ($80\mu gkg^{-1}$,i.v.) showed inconsistent effects; in two out of four cases the toxin abolished the reduction in pressure produced by ATP, whilst in the other two it had no effect. TEA ($1mgkg^{-1}$,i.v.), which non-specifically blocks K^+-channels, alone decreased the basal pressure of the anal sphincter and consistently abolished the inhibition produced by ATP ($80\mu gkg^{-1}$,i.v.). These in vivo responses mirrored those obtained in vitro where ATP inhibited muscle tone by an interaction at P_{2y}-purinocepters and presumably mediated by an increase in K^+-conductance.

(iii) αβMethylene adenosine 5'-triphosphate (αβMeATP)

In the cardiovascular system, αβMeATP ($3\mu gkg^{-1}$,i.v.) produced an increase in blood pressure and slowed the heart rate. These results are similar to those seen in the pithed rat with αβMeATP (Flavahan et al., 1985; Bulloch & McGrath, 1988). The effect of antagonists were not as consistent in the cardiovascular system as with the anal sphincter. Phentolamine ($100\mu gkg^{-1}$,i.v.) attenuated the rise in blood pressure and bradycardia produced by αβMeATP ($3\mu gkg^{-1}$,i.v.) as did 8-phenyltheophylline ($100\mu gkg^{-1}$,i.v.).

TEA ($1mgkg^{-1}$,i.v.), on the other hand, had no effect on the pressor response but augmented the bradycardia. (Figure 65).

An increase in the intraluminal pressure of the anal sphincter region of 62.8 ± 41.9% (n=20) was observed with αβMeATP ($3\mu gkg^{-1}$,i.v.). This rise in anal sphincter pressure was

FIGURE 64:

The lack of effect of 8-phenyltheophylline (8-PT; 100μgkg^{-1}, i.v.) on the decrease in anal sphincter pressure$_{-1}$ (% decrease IAS intraluminal pressure) produced by ATP (80μgkg^{-1}, i.v.) in the anaesthetised guinea-pig <u>in vivo</u>. Each bar represents the mean ± S.D. of at least 4 observations.

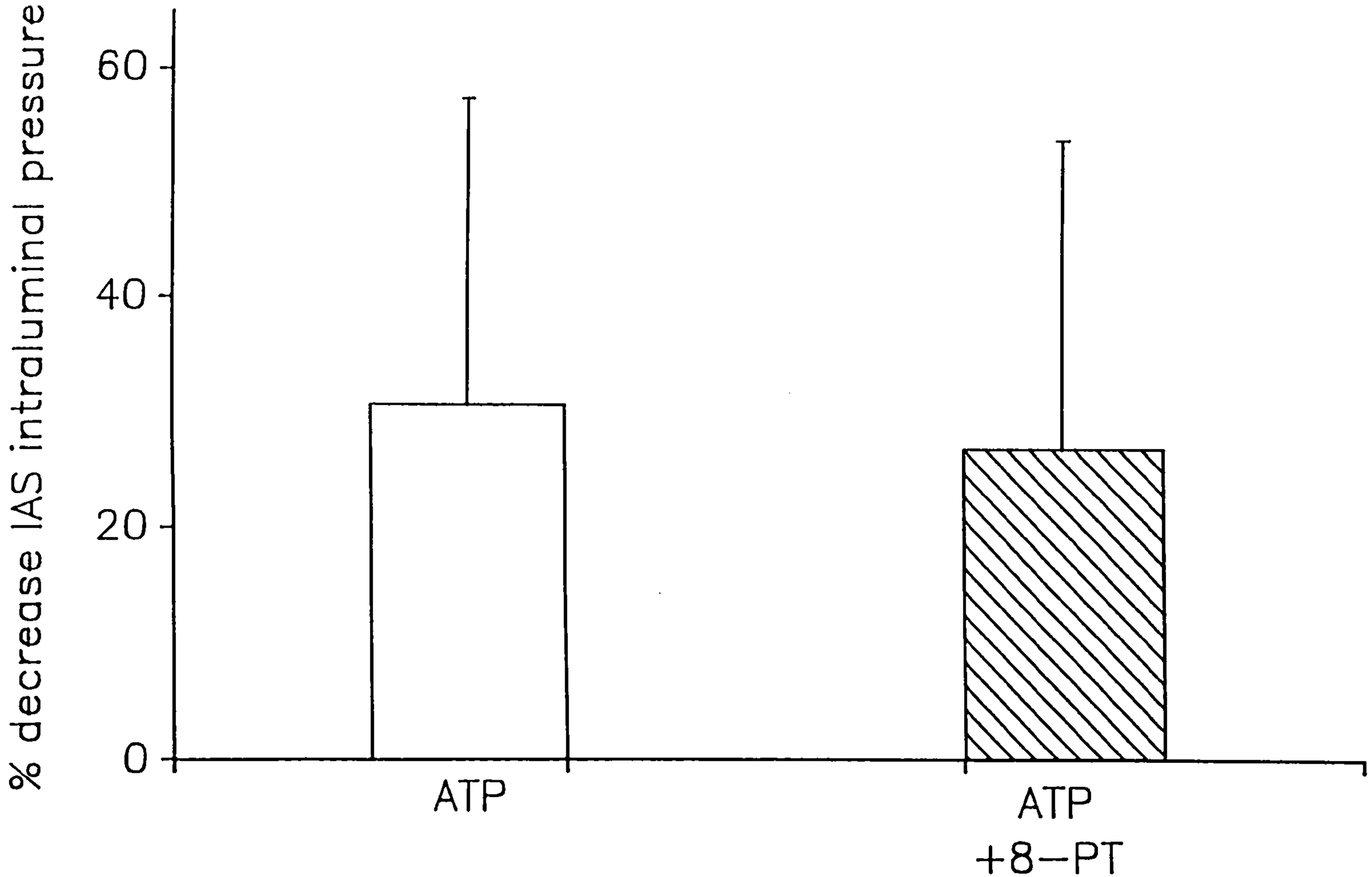
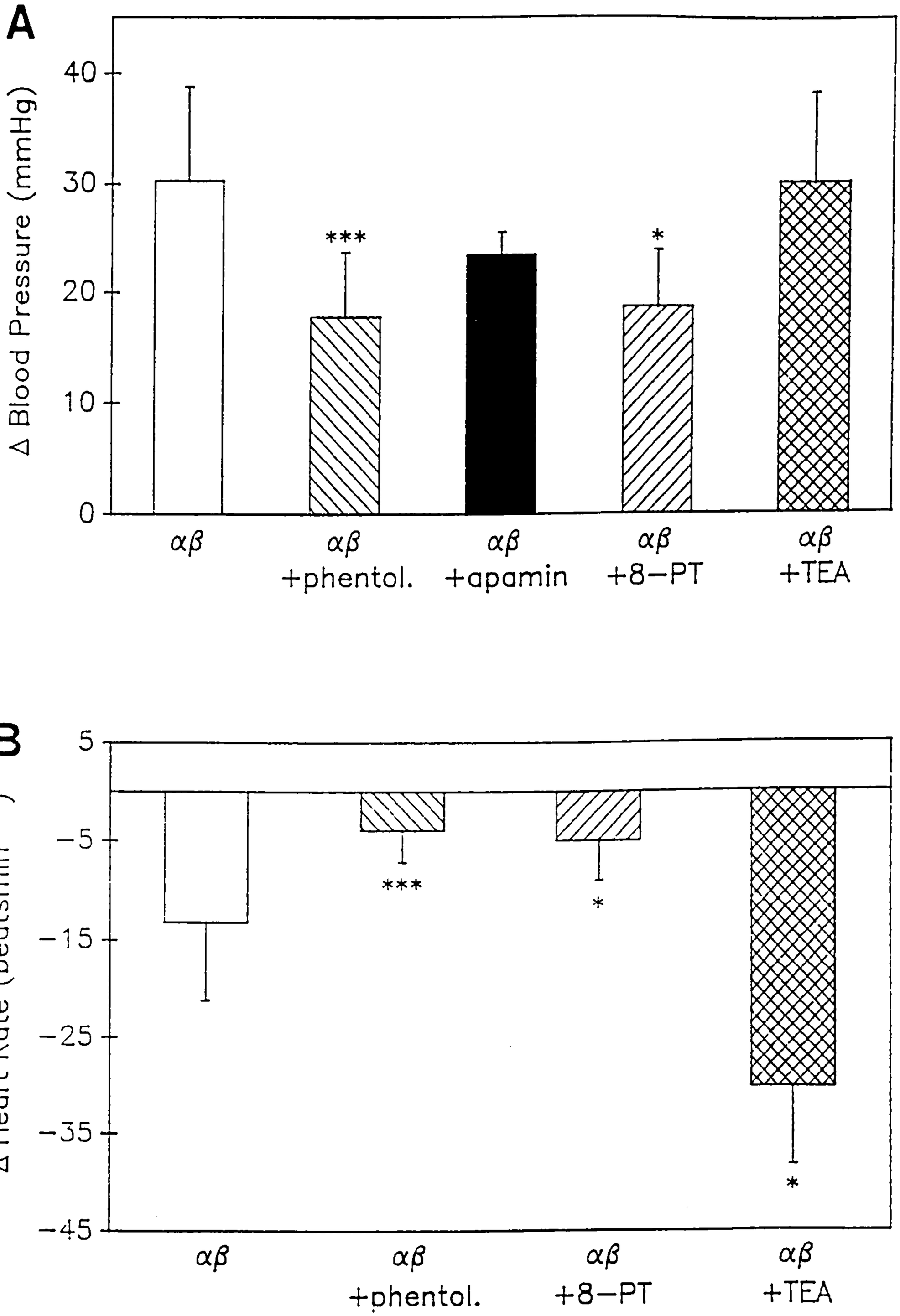

% decrease IAS intraluminal pressure
60
40
20
0
ATP
ATP
+8–PT

FIGURE 65:

The effect of phentolamine ($100\mu gkg^{-1}$, i.v.) (A & B), apamin ($30\mu gkg^{-1}$, i.v.) (A alone), 8-phenyltheophylline (8-PT; $100\mu gkg^{-1}$, i.v.) (A & B) and tetraethylammonium (TEA; $1mgkg^{-1}$, i.v.) (A & B) each on the increase in blood pressure (mmHg) (A) and decrease in heart rate ($beatsmin^{-1}$) (B) produced by αβmethylene ATP (αβ; $3\mu gkg^{-1}$, i.v.) in the anaesthetised guinea-pig <u>in vivo</u>. Each bar represents the mean ± S.D. of at least 6 observations. The pressor response in blood pressure produced by αβmethylene ATP was reduced by phentolamine ($p<0.01$) and 8-phenyltheophylline ($p<0.05$) but was unaffected by either apamin or TEA. The bradycardia produced by αβmethylene ATP was attenuated by phentolamine ($p<0.01$) and 8-phenyltheophylline ($p<0.05$) and augmented by TEA ($p<0.05$).


A
Δ Blood Pressure (mmHg)
40
30
20
10
0
***
*
αβ
αβ +phentol.
αβ +apamin
αβ +8–PT
αβ +TEA
B
Δ Heart Rate (beatsmin⁻¹)
5
−5
−15
−25
−35
−45
***
*
*
αβ
αβ +phentol.
αβ +8–PT
αβ +TEA

consistently augmented by each of phentolamine (100μgkg^{-1},i.v.), 8-phenyltheophylline (100μgkg^{-1},i.v.), apamin (30μgkg^{-1},i.v.) and TEA (1mgkg^{-1},i.v.). (Figure 66).

The response of this ATP analogue is quite different *in vivo* to that *in vitro*, where the compound has no effect on the IAS. This suggests that there are no P_{2x}-purinoceptors on the isolated IAS muscle. However, *in vivo* αβMeATP may act on P_{2x}-purinoceptors on sites other than the circular muscle of the IAS, but which nevertheless contribute to the total intraluminal pressure of the anal canal. The enhancement of the pressor response to αβMeATP *in vivo* by phentolamine may be due to the latter's presynaptic actions which may increase the amount of circulating NA to augment any contractile responses in the anal sphincter region. The enhancement of contractile response by phentolamine has already been noted in the vas deferens (Barnett *et al.*, 1968; Jurkiewicz & Jurkiewicz 1976) and in the urinary bladder (Muir & Smart, 1983). 8-Phenyltheophylline may also augment the pressor response in the anal canal by blocking the inhibitory action of adenosine, a by-product of any muscle contraction. Apamin and TEA most likely have their potentiating effects by blocking K^+- conductances, thus inhibiting the repolarisation of cells which have been depolarised by the agonist, hence allowing more Ca^{2+} into the depolarised cells.

(iv)βγMethylene adenosine 5'-triphosphate (βγMeATP)

A rise in blood pressure with a decrease in heart rate was produced by βγMeATP (30μgkg^{-1},i.v.). These were unaffected by phentolamine (100μgkg^{-1},i.v.), 8-phenyltheophylline (100μgkg^{-1},i.v.), or propranolol (100μgkg^{-1},i.v.). (Figure 67).

FIGURE 66:

The augmenting effect of phentolamine (100μgkg^{-1}, i.v.), 8-phenyltheophylline (8-PT; 100μgkg^{-1}, i.v.), apamin (30μgkg^{-1}, i.v.) and tetraethylammonium (TEA; 1mgkg^{-1}, i.v.) on the pressor response of the anal sphincter pressure (% increase IAS intraluminal pressure) produced by αβmethylene ATP (αβ; 3μgkg^{-1}, i.v.) in the anaesthetised guinea-pig in vivo. Each bar represents the mean ± S.D. of at least 3 observations. (= p<0.05 whilst *** = p<0.01).*

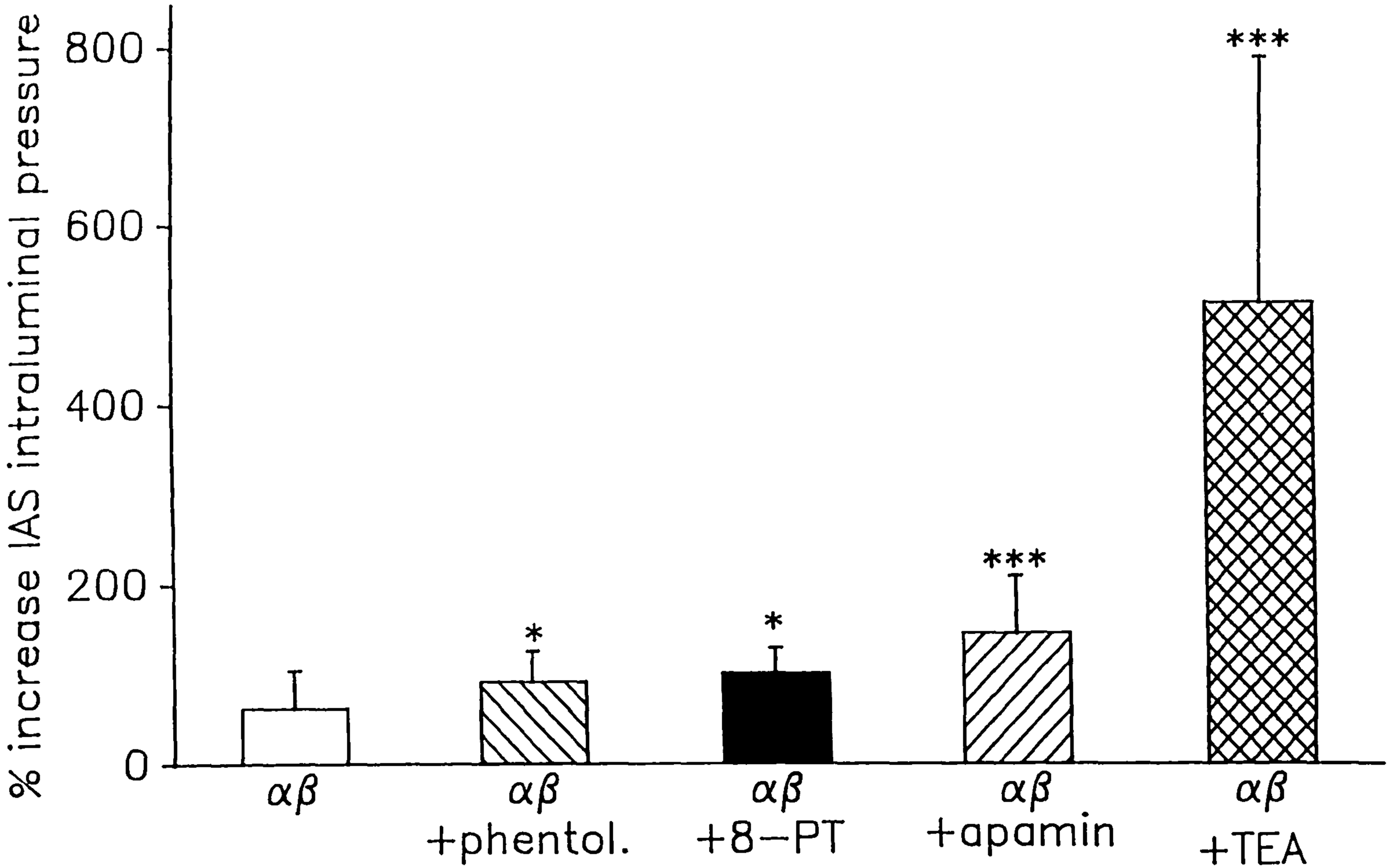
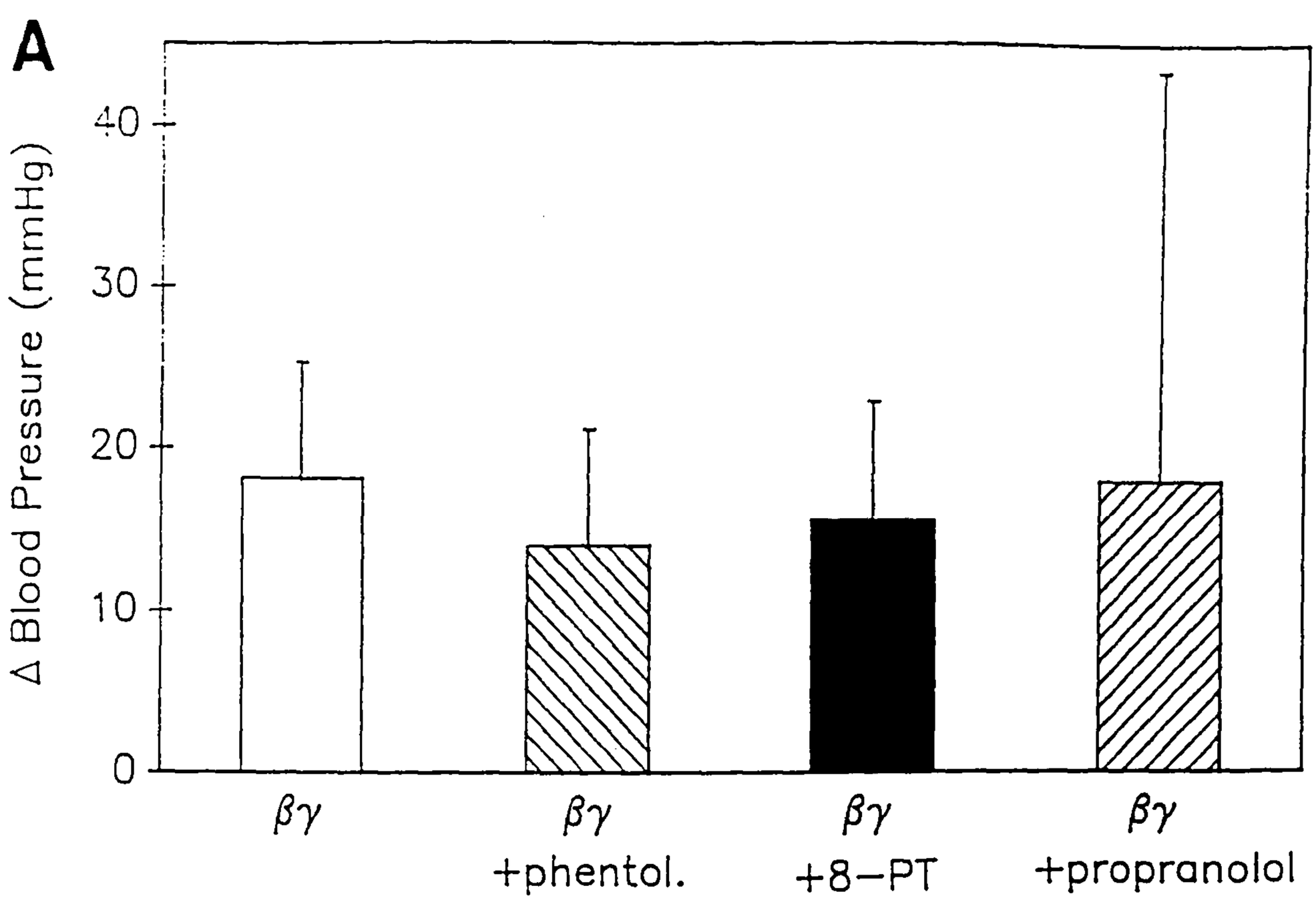

% increase IAS intraluminal pressure
800
600
400
200
0
***
*
*
***
αβ
αβ +phentol.
αβ +8−PT
αβ +apamin
αβ +TEA

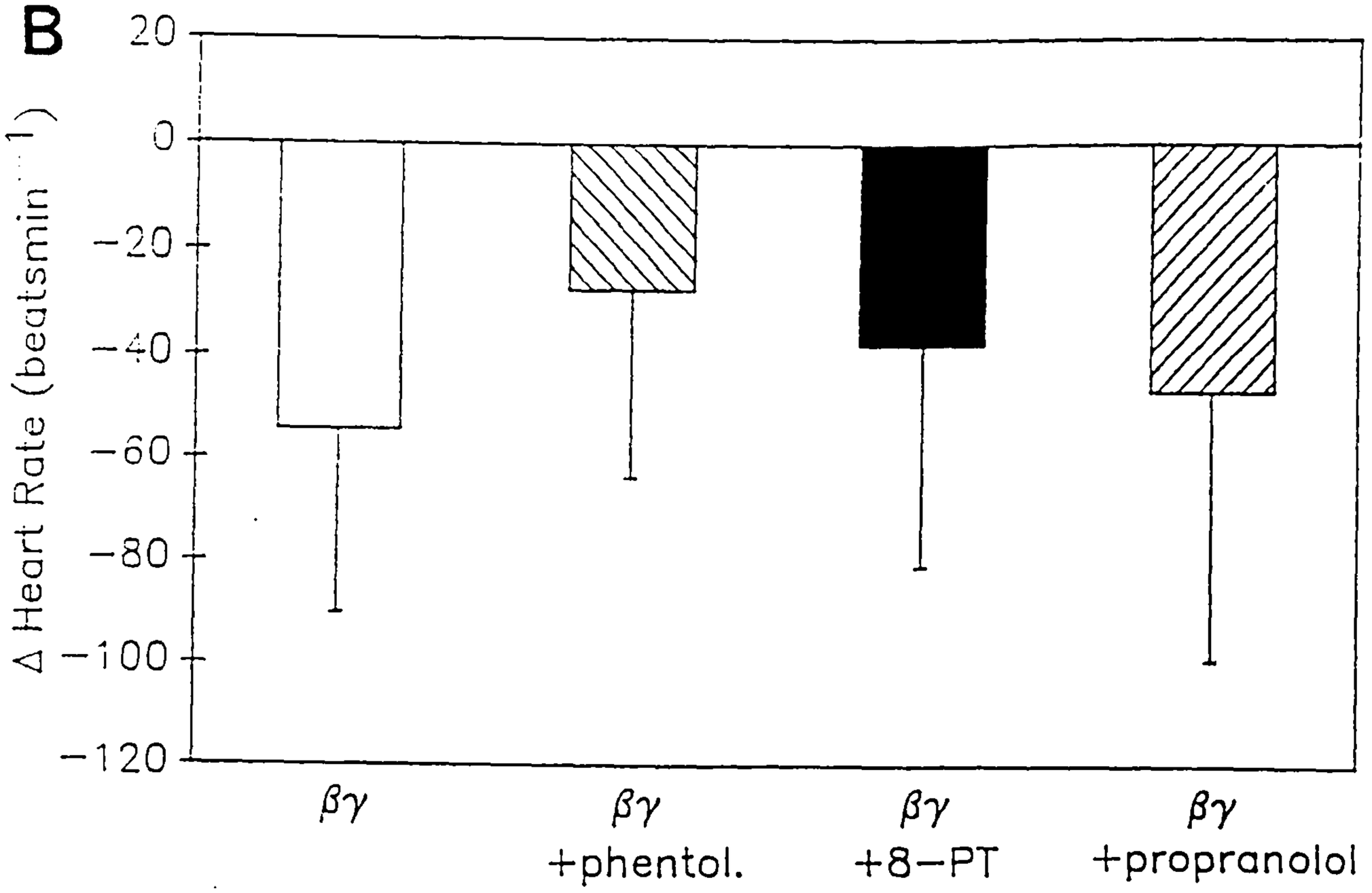
FIGURE 67:

The effect of phentolamine ($100\mu gkg^{-1}$, i.v.), 8-phenyltheophylline (8-PT; $100\mu gkg^{-1}$, i.v.) and propranolol ($100\mu gkg^{-1}$ i.v.) each on the blood pressure (mmHg) (A) and heart rate ($beatsmin^{-1}$) (B) responses produced by $\beta\gamma$ methylene ATP ($\beta\gamma$; $30\mu gkg^{-1}$, i.v.) in the anaesthetised guinea-pig in vivo. Each bar represents the mean ± S.D. of at least 5 observations. Neither the increase in blood pressure nor the bradycardia produced by $\beta\gamma$ methylene ATP was affected by phentolamine, 8-phenyltheophylline or propranolol.

A


Δ Blood Pressure (mmHg)
40
30
20
10
0
βγ
βγ +phentol.
βγ +8–PT
βγ +propranolol

B


Δ Heart Rate (beatsmin⁻¹)
20
0
−20
−40
−60
−80
−100
−120
βγ
βγ +phentol.
βγ +8–PT
βγ +propranolol

Like αβMeATP, β_γMeATP($30\mu gkg^{-1}$) produced a rise in the intraluminal pressure of the sphincter area of 146.2 ± 151.1% (n=15). This pressor effect was unaffected by phentolamine ($100\mu gkg^{-1}$,i.v.), 8-pherylltheophylline ($100gkg^{-1}$,i.v.) or propranolol ($100\mu gkg^{-1}$, i.v.). (Figure 68). This is presumably because the rise in intraluminal pressure produced by αβMeATP is maximal and cannot be potentiated.

d) **Effect of Sodium Nitroprusside**

Sodium nitroprusside (0.02 - $30\mu gkg^{-1}$,i.v.) produced no effect on intraluminal pressure of the anal sphincter region or heart rate but produced a dose-dependent decrease in blood pressure (Figure 69). The lack of effect in the anal canal presumably could have been due to the short life of the drug <u>in vivo</u>, since sodium nitroprusside had profound relaxant effects on the IAS <u>in vitro</u>.

e) **Effect of Carbachol**

Carbachol ($0.2\mu gkg^{-1}$,i.v.) lowered the blood pressure and slowed the heart rate. In the presence of atropine ($100\mu gkg^{-1}$,i.v.) the heart rate was reduced by 15.0± 26.0 $beatsmin^{-1}$ (n=3) and the depressor effects produced by carbachol ($0.2\mu gkg^{-1}$,i.v.) on the blood pressure and heart rate were profoundly reduced. (Figure 70).

Of the four experiments carried out, carbachol ($0.2\mu gkg^{-1}$,i.v.) had no effect on the basal intraluminal pressure of the sphincter area in two cases, but in the other two cases a decrease of 37.9±35.8% (n=5) of basal pressure was produced. Where carbachol ($0.2\mu gkg^{-1}$,i.v.) lowered the anal sphincter pressure, the response was abolished by atropine ($100\mu gkg^{-1}$,i.v.). Atropine

FIGURE 68:
The lack of effect of phentolamine ($100\mu gkg^{-1}$,i.v.), 8-phenyltheophylline (8-PT, $100\mu gkg^{-1}$,i.v.) and propranolol ($100\mu gkg^{-1}$,i.v.) on the pressor response of the anal sphincter (% increase IAS intraluminal pressure) produced by $\beta\gamma$ methylene ATP ($\beta\gamma$; $30\mu gkg^{-1}$,i.v.) in the anaesthetised guinea-pig <u>in vivo</u>. Each bar represents the mean ± S.D. of at least 6 observations.

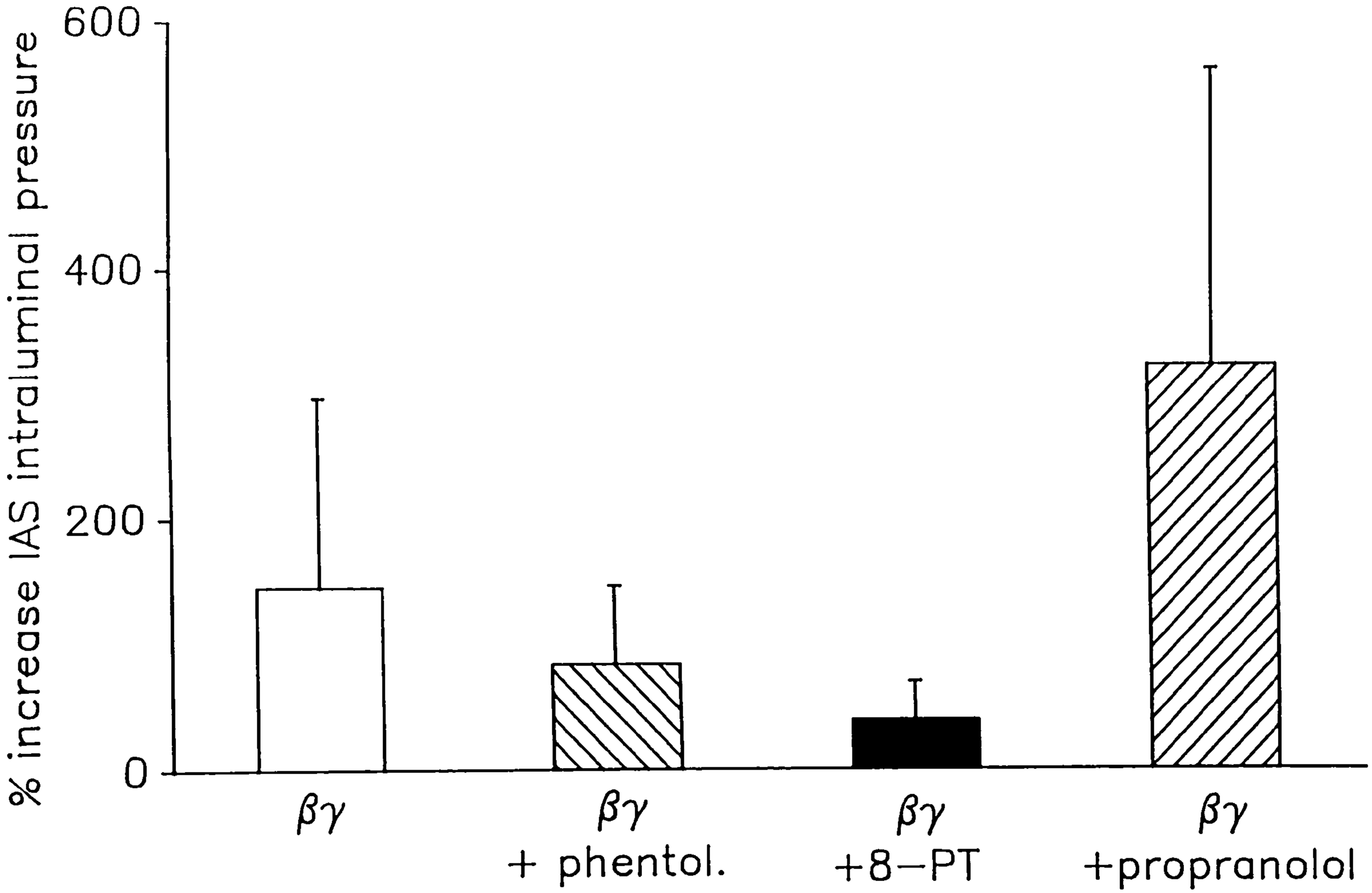

% increase IAS intraluminal pressure
600
400
200
0
βγ
βγ + phentol.
βγ +8–PT
βγ +propranolol

FIGURE 69:

The dose-dependent decrease in blood pressure (mmHg) produced by sodium nitroprusside (NaNP; 0.02- .3μgkg^{-1},i.v.) in the anaesthetised guinea-pig in vivo. Each point represents the mean ± S.D. of at least 4 observations.

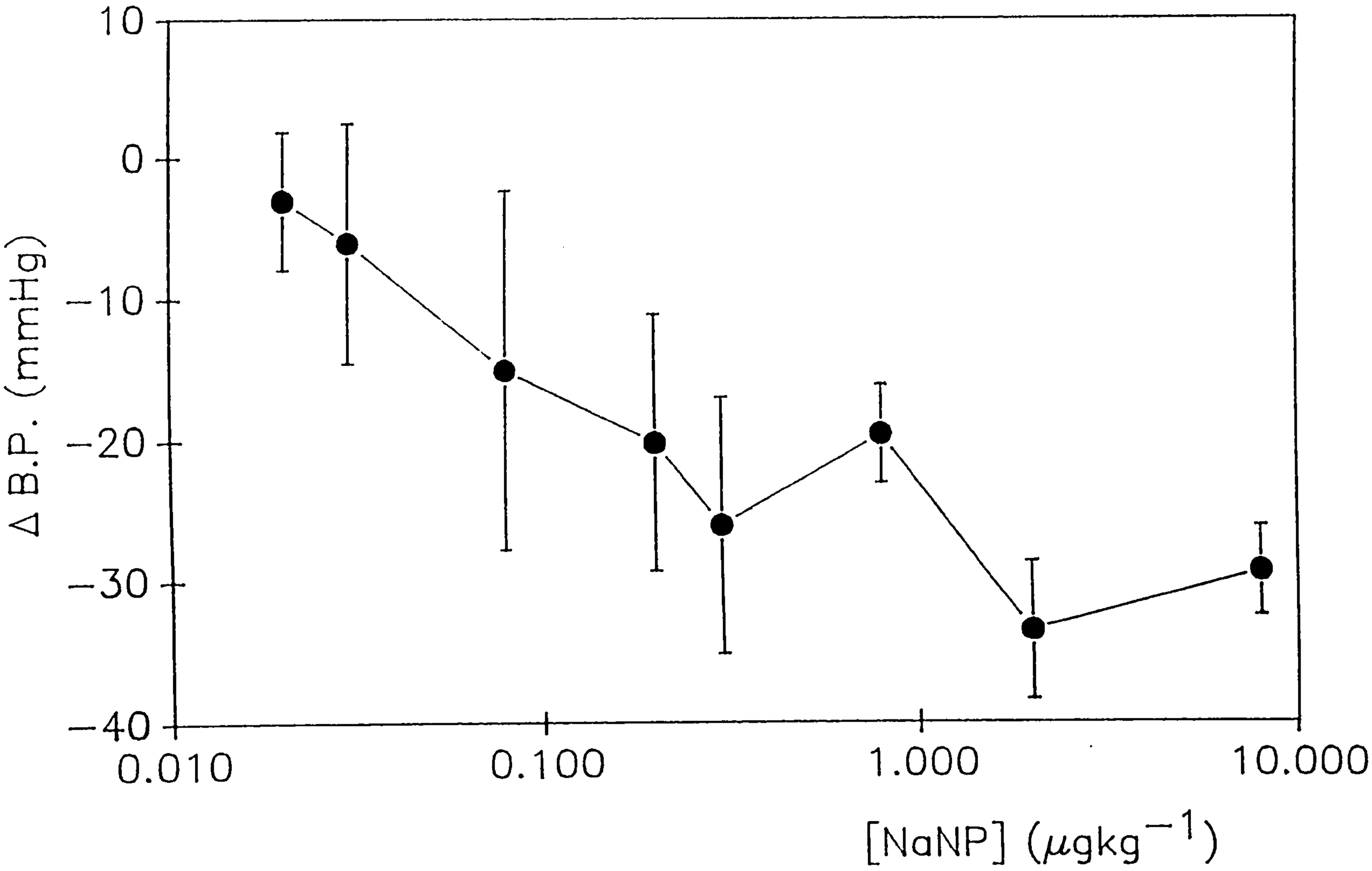
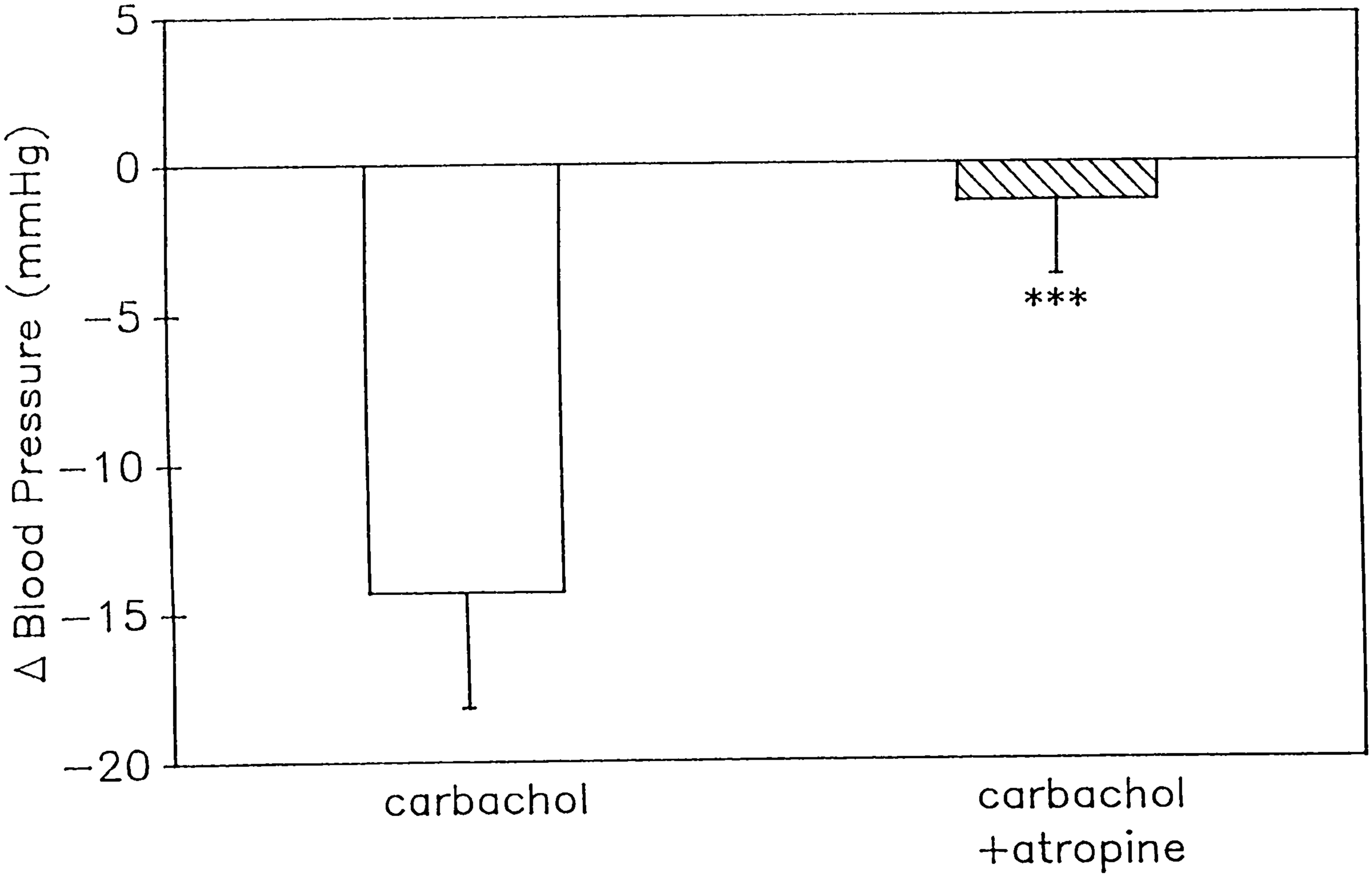

10
0
−10
−20
−30
−40
Δ B.P. (mmHg)
0.010
0.100
1.000
10.000
[NaNP] (μgkg⁻¹)

FIGURE 70:

The inhibitory effect of atropine (100μgkg^{-1},i.v.) on the depressor response in blood pressure (mmHg) produced by carbachol (0.2μgkg^{-1},i.v.) in the anaesthetised guinea-pig <u>in vivo</u>. Each bar represents the mean ± S.D. of at least 4 observations.


5
0
−5
−10
−15
−20
Δ Blood Pressure (mmHg)
***
carbachol
carbachol
+atropine

(100μgkg^{-1},i.v.) also inhibited the spontaneous wave patterns which were superimposed on the basal pressure within the anal canal. (Figure 71).

The action of carbachol *in vivo* is quite different to that *in vitro* in the anal sphincter, since *in vitro* cholinergic agents are known to be more active on longitudinal muscle rather than the circular muscle of the anal sphincter (Costa & Furness, 1973).

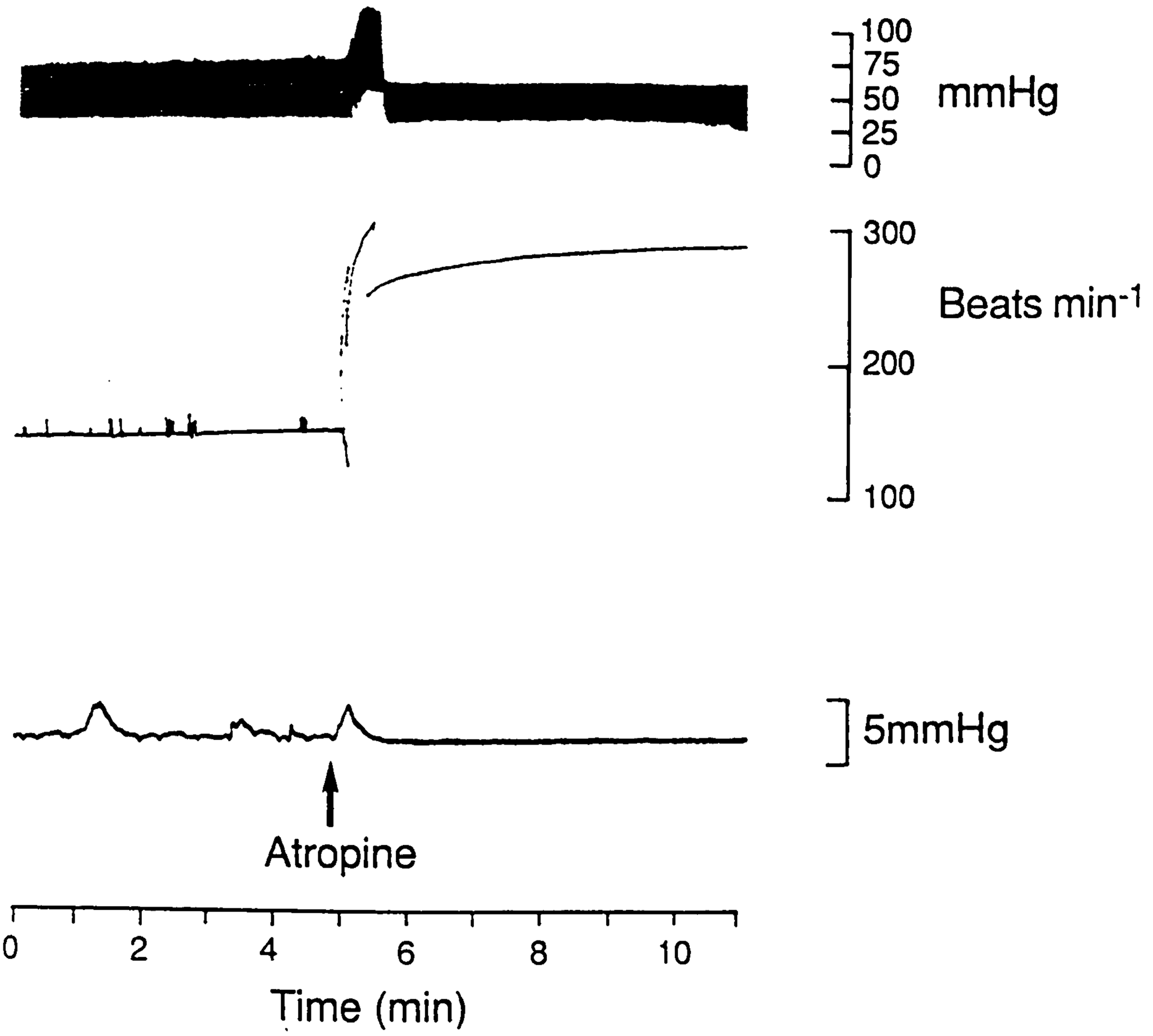


FIGURE 71:

The effect of atropine (100μgkg^{-1}, i.v.) on the blood pressure (mmHg, top panel), heart rate (beatsmin^{-1}, middle panel) and anal sphincter pressure (mmHg, bottom panel). Resting heart rate was increased by atropine whilst blood pressure showed a transient increase followed by a decrease in response to atropine. The spontaneous oscillations superimposed on the resting anal sphincter pressure were abolished by atropine.

CHAPTER IV

DISCUSSION

Smooth muscle must be able to relax to perform physiologically. For example, vascular smooth muscle in order to maintain blood pressure, the iris to alter the amount of light entering the eye and gastrointestinal smooth muscle to move and aid the digestion of a bolus of food each must relax. Relaxation of smooth muscle can be produced in different ways. It can be regarded as inhibition of spontaneous contractile activity, where that exists. For example, isoprenaline inhibits spontaneous activity of the membrane of the guinea-pig portal vein and suppresses contraction (Ito & Kuriyama, 1971). Alternatively, relaxation can be expressed as an inhibition of tone where the latter is induced physiologically, for example the action of atropine on the tone produced by light in the sphincter pupillae, or in disease, for example in pseudo obstruction of the gastrointestinal tract.

It is the second type of relaxation which has been the main concern of this thesis where relaxation occurs as an inhibition of existing tone. This action is predominant in the smooth muscle of sphincters of the gastrointestinal tract where transient and complete relaxation must be achieved when required. Relaxation of sphincters of the gastrointestinal tract is produced physiologically by the action of NANC inhibitory neurones on smooth muscle cells, for example in the lower oesophageal sphincter (Torphy et al., 1986) and in the ileo-caecal junction (Bult et al., 1990). These inhibitory NANC neurones are found throughout the gastrointestinal tract and associated muscles such as the anococcygeus and rectococcygeus muscles (for reviews see Gillespie, 1982; Burnstock, 1972; 1979).

Many gastrointestinal disorders are disorders of motility arising often from a failure of mechanisms which control relaxation of smooth

muscle, for example irritable bowel syndrome, achalasia and pseudo obstruction. Treatment of such conditions by drugs represents attempts to replace failing control systems or compensate for their loss. To achieve success in this, it is necessary to understand basic processes by which inhibitory nerves control smooth muscle and especially how neurotransmitters produce their effects. Lack of knowledge of this is the main obstacle to the development of specific treatments of the many disorders known and the reason for non-selective symptomatic remedies. Such a lack of knowledge is also a challenge to determine how relaxation is produced. The aim of the following discussion is to assess the mechanisms underlying relaxation of the smooth muscle of the guinea-pig IAS in terms of electrical and biochemical events.

The results of the in vitro studies clearly show an involvement of both so called electrical and biochemical events in relaxation produced by NANC nerve stimulation and drugs. Any consideration of the electrical changes accompanying relaxation of the IAS is immediately confronted by the magnitude of the membrane hyperpolarisation. Field stimulation of the intramural inhibitory NANC nerves of the IAS, even with a single pulse, produced large transient ijps of up to 15mV in amplitude. Large membrane hyperpolarisations, together with relaxations, were also produced by ATP and isoprenaline.

The magnitude of the hyperpolarisations produced by inhibitory stimuli suggest that large alterations in the concentrations of ions within the smooth muscle cells must be taking place to account for such changes. The ion which has been especially implicated in the mediation of hyperpolarisation and subsequent relaxation in the IAS is K^+ (Lim & Muir, 1985). The electrochemical gradient across the cell membrane for

K^+ is large, since the external concentration of the ion (5mM) is much lower than its internal concentration (150mM). Thus, during inhibitory stimulation, K^+ ions leave the smooth muscle cell which takes the membrane potential towards the K^+ equilibrium potential of -90mV. The membrane channels through which K^+ leaves the cell cannot be ascertained without channel analysis by patch clamp techniques. Some indication of the K^+ channels involved can be obtained from the use of certain antagonists. For example, the hyperpolarisations and relaxations produced by both field stimulation and ATP were blocked by apamin and TEA which suggests that Ca^{2+}-mediated K^+ channels (Benham & Bolton, 1986; Inoue et al., 1985; 1986) are involved in the response. However this may involve more than one type of Ca^{2+}-mediated K^+ channel. Apamin, for example, produces selective blockade of a small conductance (10-15pS) channel which is volatage- and TEA- insensitive. TEA, on the other hand, blocks most K^+-channels (for review see Cook, 1988). Nevertheless, inhibitory NANC nerve stimulation and ATP produce similar electrical and mechanical changes in the IAS.

Relaxation produced by isoprenaline was accompanied by a membrane hyperpolarisation. The induced change was qualitatively different to that seen in response to NANC nerve stimulation and ATP; it was slow and prolonged. Hyperpolarisation has already been noted in the responses of the guinea-pig taenia coli (Bülbring & Den Hertog, 1980), mouse uterus (Magaribuchi & Osa, 1971), pig coronary artery (Ito et al., 1979), rabbit facial vein (Prehn & Bevan, 1983) and guinea-pig portal vein (von Loh, 1971) in response to the catecholamine. The actions of isoprenaline were mediated by β-adrenoceptors, since propranolol effectively antagonised both the hyperpolarisation and the relaxation. Moreover, the sensitivity

of the response produced by isoprenaline to TEA, which blocked the hyperpolarisation and the relaxation induced by isoprenaline, suggested that an increase in K^+ conductance is a major part of the response. However, the increase in K^+ conductance is not mediated by the apamin-sensitive K^+ channel, since the neurotoxin was an ineffective antagonist against isoprenaline. Clearly, hyperpolarisation is an integral part of the inhibitory response produced by nerve stimulation, ATP or isoprenaline. That is, in these three cases relaxation cannot be produced without an accompanying change in membrane potential of the IAS.

Further investigation of the contribution of K^+ to membrane hyperpolarisation and relaxation of the IAS was carried out using cromakalim. One of the primary mechanisms by which the drug is proposed to act is to increase the K^+ conductance which results in membrane hyperpolarisation and closure of voltage-operated Ca^{2+} channels with subsequent relaxation (Hamilton et al., 1986; Weir & Weston, 1986b; Allen et al., 1986c). In the IAS cromakalim produced large slow membrane hyperpolarisations and relaxations. Thus it appeared that the action of this drug was mediated by K^+ efflux moving the membrane potential towards the K^+ equilibrium potential. This was substantiated by altering the external concentration of K^+ surrounding the IAS and hence the equilibrium potential. When the normal external K^+ concentration was lowered, shifting the K^+ equilibrium potential towards a more negative value, the hyperpolarisation produced by cromakalim in the IAS increased in size. On the other hand, increasing the external K^+ concentration from normal values to move the K^+ equilibrium potential in a positive direction, reduced the hyperpolarisation produced by cromakalim in the IAS. Furthermore, both the hyperpolarisation and relaxation

produced by the drug were inhibited by TEA, although not by apamin in the IAS. The latter findings are consistent with those obtained in guinea-pig taenia coli (Weir & Weston, 1986a), where apamin was found to be ineffective against the relaxant action of cromakalim.

Because the response produced by cromakalim was insensitive to apamin (Weir & Weston, 1986a; present investigation), it was concluded that the small conductance Ca^{2+}-mediated K^+ channel was not involved. Hollingsworth et al., (1987) suggested that the inhibitory action of the compound in rat uterus was mediated by the K^+ channels involved in the production of pacemaker activity. There are Ca^{2+}-mediated K^+ channels sensitive to TEA (Beech & Bolton, 1987; Inoue et al., 1985; 1986) and proposed to function in repolarisation of action potentials which could mediate the actions of cromakalim. However recently compounds related to cromakalim have been proposed to act by opening ATP-sensitive K^+-channels (Quast & Cook, 1989) which in resting conditions remain closed. Channel activity recorded from dispersed rabbit and rat mesenteric arterial smooth muscle cells showed the presence of a K^+ channel which was closed in the presence of ATP but reopened by cromakalim and this was inhibited by glibenclamide, a selective ATP-sensitive K^+ channel blocker (Standen et al., 1989). Regardless of the K^+ channel involved, membrane hyperpolarisation always precedes the relaxation produced by cromakalim. The electrical and mechanical responses, like others produced by inhibitory stimuli in the IAS, cannot be separated by antagonists.

This relaxation of the IAS, which is mediated by K^+ efflux and manifest by a large membrane hyperpolarisation, involves at least three types of K^+ channels; one, sensitive to apamin; a second insensitive to

apamin but sensitive to internal ATP concentrations; and a third sensitive to neither apamin nor ATP. Where relaxation is accompanied by a large K^+-mediated hyperpolarisation in response to field stimulation and drugs, then these two aspects of the response cannot be separated by antagonists. This suggests that the hyperpolarisation in these cases is a necessary prerequisite for relaxation in the IAS. A similar situation exists where hyperpolarisation, mediated by K^+ efflux, is an important facet of the mechanical response of the guinea-pig taenia coli to field stimulation of NANC inhibitory nerves, ATP (Tomita & Wantanabe, 1973; Maas et al., 1980; Axelsson & Holmberg, 1969), isoprenaline (Bülbring & Den Hertog, 1980) and cromakalim (Weir & Weston, 1986a). Moreover, the hyperpolarisation and relaxation produced in the guinea-pig taenia coli could not be separated by antagonists (Maas et al., 1980; Weir & Weston, 1986a). In the BRP muscle, however, which is a non spontaneously-active smooth muscle, stimulation of inhibitory NANC nerves produced a prominent relaxation with a small preceding ijp (Byrne & Muir, 1984, 1985; present investigation). Indeed, in the BRP muscle it was observed that the ijp was unnecessary for relaxation since, in the presence of TEA, the ijp produced in response to nerve stimulation was abolished but the powerful relaxation remained unaffected (Byrne et al., 1984; Byrne & Muir, 1984). However, a relaxation of the BRP muscle accompanied by a large hyperpolarisation was achieved with application of cromakalim. In the IAS and BRP muscle, cromakalim appeared to act in a similar manner by producing dose-dependent hyperpolarisations and accompanying relaxations (present investigation). The hyperpolarisations were slow and prolonged in both the IAS and the BRP muscle, although they were much more prolonged in the BRP muscle. Such a difference may have arisen because the IAS, a

spontaneously active muscle which relaxes by voltage-dependent mechanisms (Lim & Muir 1985; 1986; present investigation), would have an efficient facility for repolarising the cell membrane. For example, the inward rectification current contributed to by Na^+ and K^+ demonstrated in dispersed rabbit jejunal smooth muscle cells (Benham et al., 1987) was proposed to account for the rebound excitation observed following inhibitory NANC nerve stimulation and may be present in IAS but not BRP muscle. Hyperpolarisation which accompanies relaxation of the IAS can be small and slow as, for example, the response produced by isoprenaline. This, however, is not similar to the hyperpolarisation produced by nerve stimulation in the BRP muscle which accompanies relaxation (Byrne & Muir, 1984; 1985) since the electrical and mechanical responses to isoprenaline in the IAS cannot be separated by antagonists. In the BRP muscle, on the other hand, this is possible by the use of TEA which blocks the hyperpolarisation, but not the relaxation produced by nerve stimulation (Byrne et al., 1984).

Clearly, relaxation of the IAS is accompanied by a large hyperpolarisation, which presumably closes voltage-operated Ca^{2+} channels, thereby reducing the internal Ca^{2+} concentration and producing relaxation. However, relaxation of smooth muscle is much more complex than simply closing Ca^{2+} channels to reduce the Ca^{2+} concentration within cells. There are many examples of relaxation of smooth muscle where no, or very little, accompanying membrane hyperpolarisation has been recorded. These examples include the aforementioned BRP muscle (Byrne & Muir, 1984; 1985) and rat anococcygeus (Creed et al., 1975) in response to field stimulation, the BRP muscle in response to isoprenaline (Klinge & Sjöstrannd, 1974) and the rat aorta in response to sodium nitroprusside

(Lincoln, 1983). In all these cases relaxation has been attributed to an intracellular biochemical change; namely an increase in cyclic nucleotide levels. Even in tissues, for example the IAS, where relaxation is accompanied by hyperpolarisation, the membrane potential change may arise as a consequence of primary alterations in biochemical parameters.

In the IAS, relaxation produced by inhibitory NANC nerve stimulation and ATP was accompanied by raised levels of cyclic AMP. The increase in cyclic AMP produced by field stimulation was sensitive to TTX and apamin, implying that the rise had neurogenic origins and involved a K^+ efflux. In response to an increase in cyclic AMP levels, cyclic AMP-dependent phosphorylation also takes place and subsequent activation of Na^+-K^+-ATPase and hence activation of the Na^+-K^+ electrogenic pump in smooth muscle (Scheid et al., 1979). As a consequence of the activation of the Na^+ pump, the Na^+-Ca^{2+} exchange is stimulated which lowers intracellular Ca^{2+} with subsequent relaxation. The electrogenic Na^+-K^+ pump is inhibited by nanomolar concentrations of apamin (Zemková et al., 1988) which suggested that this toxin inhibited both the small conductance Ca^{2+}-dependent K^+ channel and the electrogenic Na^+-K^+ pump with the same efficiency. Since apamin cannot cross the cell membrane (Habermann, 1972), it must act outside the membrane at a site different to that for ouabain or cation binding because [^{3}H]-ouabain binding was unaffected by the neurotoxin. Zemková et al., (1988) therefore suggested that apamin acted by decreasing the turnover rate of the pump without changing the number of pumping sites. Thus, in the IAS, apamin may block the Ca^{2+}-mediated K^+ efflux and the electrogenic Na^+-K^+ pump which is activated by cyclic AMP. Consequently, the rise in cyclic AMP could be reduced by a feedback system, where cyclic AMP no

longer becomes necessary because the turnover rate of the pump has been inhibited.

The relaxation produced by isoprenaline in the IAS is accompanied by an increase in cyclic AMP. A rise in this cyclic nucleotide is also part of the response to isoprenaline in other tissues, including rabbit portal vein (Collins & Sutter, 1975), rat myometrium (Diamond & Holmes, 1975), guinea-pig trachea (Murad & Kimura, 1974) and intestinal smooth muscle (Andersson, 1972; Andersson & Nilsson, 1972). It is believed that the action of the catecholamine is mediated mainly by the action of cyclic AMP in a voltage-independent manner where membrane hyperpolarisation is a side effect (Meisheri & van Breemen, 1982; Rüegg & Paul, 1982; Miller et al., 1983). Such voltage-independent mechanisms have already been described where cyclic AMP accelerates Ca^{2+} extrusion from the cell and promotes storage of Ca^{2+} and myosin light chain dephosphorylation to produce relaxation (Itoh et al., 1985).

Isoprenaline may not act solely via cyclic AMP. In rat myometrium, relaxation produced by isoprenaline is associated with a modest increase of cyclic AMP (6-12 $pmol\,mg^{-1}$ of protein), whilst forskolin, which activates adenylate cyclase (Seamon & Daly, 1981), produced larger rises in cyclic AMP (80-120 $pmol\,mg^{-1}$ of protein) during relaxation similar in magnitude to that produced by isoprenaline. Furthermore, prostaglandin E_2 elevated cyclic AMP but produced contractions which were antagonised by isoprenaline (do Khac et al., 1986). From these results it was suggested that the relaxant effects of isoprenaline were mediated by two mechanisms: a cyclic AMP-dependent process sensitive to low concentrations of cyclic AMP and a cyclic AMP-independent process which may involve a mechanism at the level of the cell membrane which ultimately

reduces cystolic Ca^{2+} levels. In the IAS a similar situation may exist since both hyperpolarisation and an elevation of cyclic AMP are evident during relaxation in response to isoprenaline. It may be that the change in membrane potential and the rise in cyclic AMP produced by the catecholamine are related. Activation of the electrogenic Na^{+}-K^{+} pump by an elevation of cyclic AMP content resulting in hyperpolarisation could occur. TEA, which inhibited the isoprenaline-induced hyperpolarisation and relaxation in the IAS (present investigation), attenuates the Na^{+}-K^{+} pump (Zemková et al., 1988). This explanation, however, seems unlikely since apamin, which also inhibits the Na^{+}-K^{+} pump (Zemková et al., 1988), had no effect on the isoprenaline-induced hyperpolarisation and relaxation in the IAS. Therefore in the IAS two mechanisms may indeed co-exist to mediate the intracellular effects of isoprenaline; one cyclic AMP-dependent and one voltage-dependent. These mechanisms may or may not be interdependent. The membrane change may have been produced in response to a rise in cyclic AMP. On the other hand, the increase in the cyclic AMP level may be independent of a membrane potential change. These present experiments did not separate the electrical and biochemical events.

Significantly increasing the levels of cyclic AMP by forskolin produced relaxation of the IAS without an accompanying membrane potential change. However, when tone was abolished by forskolin, small hyperpolarisations (10mV) were noted; this was presumably due to activation of the electrogenic Na^{+}-K^{+} pump by raised levels of cyclic AMP.

Relaxation of the IAS, however, is accompanied not only by a rise in cyclic AMP levels but also by a rise in levels of cyclic GMP. Inhibitory

NANC nerve stimulation produced a significant increase in the tissue cyclic GMP level in the IAS which was TTX-sensitive (present investigation) and suggested that the elevation was neurogenic. Apamin, which blocked the electrical and mechanical effects of inhibitory NANC nerve stimulation (Lim & Muir, 1985; 1986; present investigation), did not abolish the rise in cyclic GMP content produced by NANC nerve stimulation, although the mechanical effects were inhibited. This anomaly may have arisen because apamin blocks the effects of receptor occupation, not the receptor itself, i.e. it antagonises the Ca^{2+}-mediated K^{+} efflux alone. The rise in cyclic GMP may be due to the increase in intracellular Ca^{2+} necessary to open the K^{+} channels, since it has been shown that formation of cyclic GMP is Ca^{2+}-dependent (see Goldberg & Haddox, 1977). However, the electrical and mechanical effects would be antagonised because K^{+} efflux could not take place because of external blockade of the channel by apamin. Thus, in the IAS, relaxation is accompanied not only by hyperpolarisation but also the accumulation of both cyclic AMP and cyclic GMP.

Relaxation without accompanying electrical change in the IAS was achieved by sodium nitroprusside and M&B22948. Sodium nitroprusside directly stimulates guanylate cyclase and M&B22948 is a cyclic GMP-specific phosphodiesterase inhibitor; both produce an increase in the accumulation of cyclic GMP within the cells. Hence, relaxation is produced without hyperpolarisation in the IAS by stimulation of the accumulation of cyclic GMP. This is similar to the relaxation produced by forskolin in the IAS, where cyclic AMP levels are raised but there is no membrane potential change. Thus, changes in the levels of the cyclic nucleotides alone can produce relaxation.

During almost complete inhibition of tone of the IAS, further doses of sodium nitroprusside, M&B22948, 8-Br-cyclic GMP and forskolin abolished spike potentials and hyperpolarised the membrane of the IAS a little. The membrane change is presumably a side effect of the elevation of cyclic nucleotide content within the cells by stimulation with very large doses of agonists and has been noted previously in response to sodium nitroprusside in arterial muscle (Haeusler, 1975; Haeusler & Thorens, 1976). The change in membrane potential could have been due to the action of nitroprusside on Cl^- efflux. It was shown that in a Cl^--free solution the relaxant effect of nitroprusside was abolished and $^{36}Cl^-$ efflux from preloaded smooth muscle in response to nitroprusside was inhibited (Kreye et al., 1977). Such an alteration of Cl^- efflux may not be a direct action of sodium nitroprusside on the cell membrane, but rather that of cyclic GMP. In the BRP muscle, inhibitory NANC nerve stimulation produced an ijp (Byrne & Muir, 1984) and an increase in cyclic GMP content (Bowman & Drummond, 1984) to accompany relaxation. Reduction or removal of Cl^- from the Krebs' solution depressed or abolished the ijp, implying that it may be mediated by change in Cl^- permeability (Byrne & Muir, 1985). Therefore it would seem that the change in Cl^- conductance is produced by the actions of cyclic GMP, since both sodium nitroprusside and the inhibitory NANC neurotransmitter increase accumulation of the cyclic nucleotide. It is not surprising that sodium nitroprusside and the inhibitory NANC neurotransmitter act via cyclic GMP to produce similar effects, since sodium nitroprusside activates guanylate cyclase by releasing NO (see Waldman & Murad, 1987) and the NANC neurotransmitter in the BRP muscle is proposed to be NO (Gillespie & Sheng, 1988a).

TEA attenuated the inhibitory effects of sodium nitroprusside in the IAS (present investigation) which further suggested that inhibition of Cl^- efflux was important in the relaxant response. This is because TEA inhibits not only K^+ channels (see Cook, 1988; Tomita, 1988) but also the Na^+-K^+ pump at a site similar to that at which ouabain is active (Zemková et al., 1988), with a subsequent reduction in the Cl^- gradient across the membrane (Brading, 1980). Previously it was proposed that the Na^+-K^+ pump induced smooth muscle relaxation by activation with cyclic GMP (Rapoport & Murad, 1983) via cyclic GMP-dependent protein kinase (Fiscus et al., 1983) leading to protein phosphorylation (Rapoport et al., 1983a & b; 1982; Rapoport & Murad, 1983). Later Rapoport et al., (1985) showed that agents and procedures which inhibited the Na^+-K^+ pump also inhibited smooth muscle relaxation produced by nitroprusside. Cyclic GMP, via the Na^+-K^+ pump, therefore, may mediate the ionic permeability changes seen in smooth muscle (Kreye et al., 1977; Byrne & Muir, 1985) which accompany relaxation. Notwithstanding, an increase in the levels of either cyclic AMP or cyclic GMP can produce relaxation without a change in the membrane potential of the IAS.

The evidence presented here suggests that relaxation of the IAS can be achieved by electrical and/or biochemical stimulation depending on the stimulus. The rise in tissue content of both cyclic nucleotides, together with the membrane hyperpolarisation in response to field stimulation, suggests that more than one mechanism is responsible for the transient mechanical inhibition seen in the IAS. Hyperpolarisation would simply close voltage-operated Ca^{2+} channels thus lowering the amount of free Ca^{2+} within the cell, initiating relaxation. The actions of the cyclic nucleotides are much more complex. A rise in cyclic GMP would

accelerate the amount of Ca^{2+} extruded from the cell and consequently lower the amount of stored and free Ca^{2+} in the myoplasm (Itoh et al., 1985). In addition, a rise in cyclic AMP content would promote the increased storage of Ca^{2+} in the sarcoplasmic reticulum and other storage sites (Itoh et al., 1985), as well as augmenting Ca^{2+} extrusion from the plasmalemma by activating the electrogenic Na^{+}-K^{+} pump (Schied et al., 1979). The latter action of cyclic AMP would also induce some hyperpolarisation in addition to that produced by opening Ca^{2+}-dependent K^{+} channels. Furthermore, both cyclic nucleotides, in conjunction with their cyclic nucleotide-dependent protein kinases, would produce dephosphorylation of myosin light chain and a reduction in the sensitivity of the contractile elements to Ca^{2+} (Itoh et al., 1985; Draznin et al., 1983; 1986). All of these actions initiated by an increase in tissue cyclic nucleotide content would contribute to smooth muscle relaxation.

From the work carried out in IAS and other smooth muscles with an inhibitory NANC innervation, for example the guinea-pig taenia caeci, BRP muscle and LOS, it would seem that irrespective of the muscle type, membrane hyperpolarisation and second messenger system changes are similar in response to field stimulation. However, it would appear that the degree of change in these post-junctional responses is distinct, depending on the tissue. That is, inhibitory NANC nerve stimulation in the IAS produced a large ijp (Lim & Muir, 1985; 1986; present investigation) and a rise in the tissue content of cyclic AMP and cyclic GMP (present investigation), whilst in the LOS there is a large ijp (Gonella et al., 1977) but a rise in cyclic GMP alone (Torphy et al., 1986). On the other hand, in the BRP muscle, field stimulation produced a small ijp and an

elevation of cyclic GMP concentration. In all of these cases the end result of nerve stimulation is relaxation. However, such diversity in the post-synaptic responses which translate the external signal into an intracellular effect may arise from the different NANC neurotransmitters present in these tissues. For example, in the ERP muscle the neurotransmitter is proposed to be NO or a related substance (Gillespie & Sheng, 1988a). In the LOS the transmitter is allegedly vasoactive intestinal polypeptide (Rattan et al., 1977), although this has been disputed since the peptide does not produce a change in membrane potential similar to the ijp (Daniel et al., 1989). In the IAS on the other hand, the neurotransmitter has been proposed to be ATP (Lim & Muir, 1986; present investigation).

The evidence presented here shows that adminstration of ATP from a blunt micropipette by hydrostatic pressure ejection or from a microsyringe produced rapid and brief membrane hyperpolarisations with relaxations. The hyperpolarisations had similar characteristics to those produced by field stimulation, i.e rate of onset, decline and duration, they were dose-dependent and sensitive to apamin and TEA. Since the electrical and mechanical responses of the IAS to ATP were blocked by both apamin and TEA, this would suggest that, like the response to field stimulation, hyperpolarisation is a necessary facet of the inhibitory response produced by the nucleotide. In the case of ATP, relaxation cannot be produced without membrane hyperpolarisation. Furthermore, the effectiveness of apamin and TEA, (which are both K^+ channel antagonists) at blocking the inhibitory response produced by ATP, suggested that it was mediated by an increase in K^+ conductance. Previously it was proposed that the response produced by field stimulation of inhibitory nerves was mediated

by an increase in K^+ conductance (Lim & Muir, 1985). Therefore the implication from this evidence is that ATP and the NANC neurotransmitter are the same substance.

Furthermore, since neither adenosine nor αβMeATP were effective, the inhibitory response of the IAS to ATP, and by implication the inhibitory NANC neurotrasmitter, appeared to be mediated by P_{2y}-purinoceptors (see Burnstock & Kennedy, 1985). ATP which is the putative inhibitory NANC neurotransmitter in the guinea-pig taenia caeci (Gough et al., 1973; Satchell & Maguire, 1975) was proposed to act via P_{2y}-purinoceptors (Burnstock et al., 1983). Both the taenia coli and IAS of the guinea-pig are spontaneously active tissues where the physiological response of the muscles to NANC nerve stimulation is a large hyperpolarisation and a powerful relaxation (Bennett et al., 1966a; Lim & Muir, 1985; present investigation). Therefore, it would not be surprising if the neurotransmitter, ATP, were the same in both tissues, resulting in relaxation.

Relaxation produced by the proposed inhibitory neurotransmitter, ATP, was also accompanied by a rise in cyclic AMP and cyclic GMP. This biochemical response was similar to that produced by field stimulation and further suggests that ATP is the inhibitory NANC neurotransmitter. Thus relaxation of the IAS in response to ATP, acting on P_{2y}-purinoceptors, involves a complex combination of electrical and biochemical changes. Classically, relaxation produced by ATP via P_{2y}-purinoceptors is recognised as the NANC inhibitory response of the guinea-pig taenia coli (see Burnstock & Kennedy, 1985). Not only are these purinoceptors evident on the IAS and taenia caeci but also on vascular endothelial cells (Martin et al., 1985). The action of ATP on vascular endothelial cells

was noted to increase the production of inositol polyphosphates (Pirotton et al., 1987) by a phospholipase C-catalysed mechanism. This action of ATP on P_{2y}-purinoceptors has also been shown in hepatocytes (Charest et al., 1985; Okajima et al., 1987) and turkey erythrocytes (Berrie et al., 1989). In turkey erythrocytes, taking inositol polyphosphate production as an endpoint, the potency series was 2-methylthioATP>>ATP>$\beta\gamma$MeATP which is similar to that noted for guinea-pig taenia caeci (Berrie et al., 1989). Thus purinergic receptors stimulated a phospholipase C that was activated by a G-protein, since GTP s and NaF both caused potent activation of inositol phosphate production (Harden et al., 1988).

Inositol polyphosphate production itself is associated with smooth muscle relaxation. The production of inositol polyphosphates leads to the mobilization of Ca^{2+} (Berridge & Irvine, 1984; Putney, 1987) which appears at first sight to be counterproductive in causing relaxation. However, in the guinea-pig taenia caeci Ca^{2+} is necessary for the relaxation (Bülbring & Tomita, 1977) produced by α_1-adrenoceptor stimulation. Furthermore, α_1-adrenoceptor stimulation in guinea-pig taenia caeci was associated with an increased formation of inositol triphosphate (Nelemans & Den Hertog, 1987a). Thus mobilization of Ca^{2+} may be involved in relaxation as, for example, in the control of the K^+ channel as occurs in Helix neurones (Meech, 1974) to cause membrane hyperpolarisation. Moreover, a receptor-regulated Ca^{2+} pool bound to the plasma membrane, as in the parotid gland and hepatocytes (Aub et al., 1982; Poggioli & Putney, 1982; Burgess et al., 1983), may also facilitate the release of Ca^{2+} from the endoplasmic reticulum via formation of inositol triphosphate. This process has been described in the guinea-pig taenia caeci (Nelemans & Den Hertog, 1987b).

ATP-induced relaxations in the IAS were not however accompanied by an increased inositol polyphospate formation while noradrenaline, which contracted the IAS, increased the inositol phosphate turnover. It is possible that a rise in inositol phosphate production in response to ATP was not detected because the nucleotide was degraded in the incubation medium to adenosine which is known to inhibit agonist-induced inositol phosphate production (Long & Stone, 1987). However, a lack of inositol polyphosphate production does not necessarily imply that Ca^{2+} was not required for relaxation of the IAS.

Calcium required to mediate the K^+ efflux involved in relaxation may not be released from intracellular stores by inositol triphosphate, but rather may come from the extracellular space via a receptor-operated Ca^{2+} channel. An ATP activated receptor-operated channel which is selective for Ca^{2+} over Na^+ ions has been demonstrated in dispersed cells from rabbit ear artery (Benham & Tsien, 1987). Nevertheless, a small increase in intracellular Ca^{2+}, probably local to the K^+ channels in the plasmalemma, would be necessary to activate K^+ efflux. This is evident from the use of apamin which blocks Ca^{2+}-mediated K^+ channels (Banks et al., 1979).

Changes in cyclic nucleotide levels may also explain, at least in part, some of the effects of agonists which act primarily by other mechanisms. For example, cromakalim, proposed to produce relaxation by membrane hyperpolarisation, induced an increase in cyclic AMP levels. However, that cromakalim produces its mechanical effects by voltage changes alone has been questioned. This was because little change in the sensitivity of cromakalim-induced inhibition of tissues with differing dependence on voltage-operated Ca^{2+} channels was found (Gillespie &

Sheng, 1988b). These workers looked towards cyclic nucleotides as an alternative to membrane hyperpolarisation to produce relaxation but found that neither cyclic AMP nor cyclic GMP was raised in response to cromakalim in the BRP muscle (see Kauffman et al., 1986; Coldwell & Howlett, 1987; Taylor et al., 1988). In contrast to these findings, cromakalim elevated the tissue content of cyclic AMP in the IAS (present investigation). The use of spontaneous and quiescent smooth muscles may explain this paradox. The lack of variability in sensitivity of the response to cromakalim in different tissues (Gillespie & Sheng, 1988b) may have been due to activation of two mechanisms in the spontaneously active tissues such as guinea-pig taenia coli and IAS, but only one mechanism in quiescent tissues like the BRP muscle. Thus the increase in cyclic AMP could be the second mechanism for relaxation sought after, but absent from the tissue selected by Gillespie & Sheng (1988b). Thus, in addition to closing voltage-operated Ca^{2+} channels, cromakalim could also have produced relaxation via an increase in cyclic AMP levels which would promote calcium extrusion and storage and myosin light chain dephosphorylation.

The logical step was then taken to apply the findings of the in vitro studies to the in vivo situation. Therefore a set of experiments was designed to investigate the properties of the IAS in vivo and to assess the effects in vivo of drugs which were found to be active in vitro, and hence by implication to deduce their mechanism of action. Accordingly, a method was devised to measure the effects of drugs on sphincteric motility in the anaesthetised guinea-pig. This method involved placing a small pressure transducer - Millar pressure transducer - which was less than 3mm broad into the anal canal to measure endoluminal

pressure. The sphincter region was designated as that in which the endoluminal pressure measured was higher than elsewhere in the rectum (Duthie & Bennett, 1963; Hill et al., 1960). This method avoided the problem of irritation of the sphincter wall encountered when balloons are used to measure pressure in the anal canal in humans (Duthie & Watts, 1965; Schuster et al., 1963), vervet monkey (Rayner, 1979) and cat (Kerremans & Penninckx, 1970). As far as can be ascertained this is the first measurement of anal sphincter pressure in the anaesthetised guinea-pig.

Any in vivo method must have differences from the in vitro situation. These differences are due to two main factors. Firstly, the presence of adjacent tissues and organs, for example longitudinal smooth muscle in the anal region, the skeletal muscle of the external anal sphincter and the rectum itself. However, the external anal sphincter is known to contribute little to the total pressure within the anal canal (Schuster, 1968). Furthermore, nerve pathways from ganglia such as the inferior mesenteric ganglion will be present in vivo but lost during dissection of the in vitro preparation. Secondly, cardiovascular effects could directly affect pressure within the anal region and also limited the amount of agonist used. This was a result of intravenous injection because injected agents initially produced cardiovascular changes and were vulnerable to degradation and uptake into the other organs before reaching the anal sphincter.

Notwithstanding, measurement of anal sphincter intraluminal pressure showed that a basal tone existed which was partly myogenic and partly neurogenic, since phentolamine reduced but did not abolish it. These results confirm those found in the cat (Garrett et al., 1974). The

sympathetic control of resting intraluminal pressure of the sphincter *in vivo* may explain previous findings of Lim (1985) who demonstrated a dense noradrenergic innervation in the IAS using Falck histochemistry *in vitro* but could not elicit responses to sympathetic nerves via field stimulation. Peristaltic contractions measured *in vivo* superimposed on resting intraluminal sphincter pressure were abolished by atropine like that in the vervet monkey (Rayner, 1979), suggesting that some excitatory cholinergic nerves are also present *in vivo*.

Results from the study of relaxations *in vivo* complemented the results from the *in vitro* investigations. Reductions in sphincter endoluminal pressure could be obtained *in vivo* to similar agents which produced relaxation of the IAS *in vitro* with a few exceptions. An important feature of the *in vivo* study was to identify the purinergic receptors in the sphincteric region *in vivo*. Adenosine decreased pressure in the anal canal presumably by P_1-purinoceptors, since the inhibitory response was attenuated by 8-phenyltheophylline. These results are different to those *in vitro* where the nucleoside applied by hydrostatic pressure injection produced no effect on the IAS (present investigation). Lim & Muir (1986) showed that adenosine applied by microsyringe produced hyperpolarisation and relaxation of the IAS. This suggests that adenosine may have a pharmacological effect but not a physiological one, because discrete locally applied amounts are unable to produce an effect. It may be that only a few P_1-purinoceptors exist on the smooth muscle cells and more than one cell must be stimulated in order to produce electrical and mechanical changes. ATP decreased the pressure within the anal region *in vivo*, probably by its action on P_{2y}-purinoceptors, since apamin reduced the inhibitory effects of the

purine nucleotide. These results, together with those obtained from in vitro studies (present investigation; Lim & Muir, 1986) in the IAS, support the hypothesis that ATP is the inhibitory neurotransmitter in this tissue.

The stable ATP analogues, αβMeATP, which was ineffective on the IAS in vitro, and βγMeATP each raised the pressure within the anal canal in vivo. The direction of pressure change is not surprising since both αβMeATP and βγMeATP are proposed to act via P_{2x}-purinoceptors which mediate contraction (see Burnstock & Kennedy, 1985). However, the action of the nucleotides on the anal canal in vivo suggested that they are acting on receptors outwith the circular muscle on some of the other muscles in that region of the anal canal. These other muscles could include the longitudinal smooth muscle or the skeletal muscle of the external anal sphincter which could contribute to the measured pressure within the anal region. Thus it would seem that in vivo P_1-, P_{2x}-, and P_{2y}- purinoceptors are present in the anal sphincter region.

The actions of sympathomimetics were similar in the anal region in vivo to those in the IAS in vitro. Noradrenaline, acting on α-adrenoceptors, produced an increase in intraluminal pressure of the sphincter which was sensitive to phentolamine. An α-adrenoceptor-mediated effect of noradrenaline was found also in the cat (Bouvier & Gonella, 1981) and the vervet monkey (Rayner, 1979). A reduction in anal sphincter intraluminal pressure of the guinea-pig was produced by isoprenaline acting on β-adrenoceptors and has also been described in humans (Friedmann, 1968; Parks et al., 1969) and cats (Garrett et al., 1974) on the anal sphincter after α-blockade. Thus, in the guinea-pig in vivo as in vitro the anal sphincter has excitatory α-adrenoceptors and

inhibitory β-adrenoceptors.

From the results obtained in the study of the response to agonists *in vivo* in the anal sphincter in general, a similar qualitative change is produced to that in the IAS *in vitro* of the guinea-pig.

CONCLUSIONS

Relaxation of smooth muscle can be achieved by voltage-dependent and voltage-independent mechanisms. For example, relaxation of the IAS produced physiologically by nerve stimulation is voltage-dependent like non-sphincteric gastrointestinal smooth muscle such as the taenia caeci of the guinea-pig. However, the electrical and mechanical relationship appears to be a feature of the stimulus rather than the muscle, since the BRP muscle which does not require a voltage change to relax can be relaxed by hyperpolarisation. Furthermore, relaxation of the IAS produced by sodium nitroprusside is unaccompanied by a change in membrane potential. Thus voltage-independent mechanisms clearly exist in the IAS which mediate relaxation. However, as with electrical changes, the biochemical changes involved are also stimulus dependent. The rapid transient relaxation produced by nerve stimulation is accompanied by an accumulation in the level of both cyclic nucleotides whilst slower mechanical responses involve only one cyclic nucleotide.

Electrical and biochemical events appear to be different facets of relaxation in the IAS which are not mutually exclusive. Both mechanisms are ultimately involved in the management of calcium within the smooth muscle cells. That is, Ca^{2+} must be moved away from the contractile

machinery of the cells for mechanical inhibition to occur.

A further important conclusion from the present in vitro studies of electrical and biochemical events and in vivo studies, together with previous results (Lim, 1985), suggest that ATP is the neurotransmitter involved in the physiological response of the IAS. Thus, in order to provide relief for conditions such as achalasia of the anal sphincter, effort should be put into the discovery of stable ATP compounds which are more selective for the inhibitory receptors of sphincteric smooth muscle.

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