INHIBITORY TRANSMISSION IN GASTROINTESTINAL SMOOTH MUSCLE

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow.

by

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CHAPTER 1

Abbreviations

Ach	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
BRP	Bovine retractor penis
$[Ca^{2+}]_i$	Intracellular free Ca^{2+}
cAMP	Cyclic adenosine 3`, 5`-monophosphate
$[cAMP]_i$	Intracellular cyclic adenosine 3`, 5`-monophosphate
CCK	Choleocystokinin
cGMP	Cyclic guanosine 3`, 5`-monophosphate
$[cGMP]_i$	Intracellular cyclic guanosine 3`, 5`-monophosphate
DAG	Diacylglycerol
DMPP	Dimethylphenylpiperazinium
EDRF	Endothelium-derived relaxing factor
gpIAS	Guinea-pig internal anal sphincter
GI	Gastrointestinal
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
HbO	Oxyhaemoglobin
IAS	Internal anal sphincter
ICS	Ileocolonic sphincter
IDN	Isosorbide dinitrate
IF	Inhibitory factor
IJP	Inhibitory junction potential

IP_3	Inositol 1,4,5-triphosphate
$[K^+]_o$	Extracellular potassium concentration
$^{42}K^+$	Radiolabelled potassium
K_{ATP}	ATP-activated potassium channels
L-Arg	L-arginine
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NMMA	N^{ω}-monomethyl-L-arginine
L-NNA	N^{ω}-nitro-L-arginine
LOS	Lower oesophageal sphincter
MeB	Methylene blue
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NA	Noradrenaline
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NANC	Non-cholinergic, non-adrenergic
NO	Nitric oxide
NOS	Nitric oxide synthase
P	Probability
PACAP	Pituitary adenylate cyclase activating peptide
PDE	Phosphodiesterase
PI	Inositol phosphate
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PLC	Phospholipase C
PS	Pyloric sphincter
RAc	Rat anococcygeus
RB2	Reactive blue 2

SNP	Sodium nitroprusside
S.R.	Sarcoplasmic reticulum
TEA	Tetraethylammonium
TTX	Tetrodotoxin
VIP	Vasoactive intestinal polypeptide

SUMMARY

1) The responses of the guinea-pig internal anal sphincter (gpIAS) to non-adrenergic, non-cholinergic (NANC) inhibitory nerve stimulation have been measured using electrical and mechanical recording, as well as biochemical, techniques in an attempt to determine the nature of the neuronally-released, relaxation-inducing substances.

2) The first method of study involved the use of intracellular electrical, and simultaneous mechanical recording techniques, to measure the effects of electrical field stimulation (EFS; supramaximal voltage, 0.1ms, single stimuli and 5 stimuli at 5, 10, 20 and 40Hz) of the gpIAS on NANC-evoked IJPs. These were frequency-dependent, could reach up to 60mV in amplitude, accompanied by relaxations of up to 80-90% of muscle tone and abolished by tetrodotoxin (TTX; 1μM).

3) The characteristics of the evoked IJPs were analysed using drugs. The bee venom apamin (0.3μM) uncovered two separate TTX-sensitive IJP components; a large, fast-to-peak (361±31ms, n=25 cells from 8 preparations following a single stimulus) component abolished by the venom, and a second, slower-to-peak (530±17ms, n=16 cells from 4 preparations following a single stimulus) apamin-insensitive component.

4) The apamin-sensitive component appeared to be mediated by adenosine 5'-triphosphate (ATP). The evidence supporting this view was;

a) Exogenous ATP (0.1-1mM) added either by pressure injection or microsyringe, into the bath hyperpolarized and relaxed the gpIAS and these effects mimicked, in amplitude and duration, the responses to EFS.

b) The hyperpolarization and relaxations produced by ATP were antagonized by apamin (0.3μM).

c) The P_2-purinoceptor antagonist suramin (100μM) reduced IJP amplitude; reactive blue 2 (100μM) hyperpolarized the membrane.

5) The apamin-insensitive component was responsible for up to 80% of the relaxation produced by EFS and was mediated by nitric oxide (NO) or a NO-related substance, acting via guanylyl cyclase. The evidence supporting this view was;

a) The NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100μM) stereospecifically antagonized the IJPs and relaxations, an effect reversed by the NO precusor L-arginine (100μM).

b) The NO scavenger, oxyhaemoglobin (HbO; 10μM), reduced, but did not abolish, the IJP component and relaxation.

c) Sodium nitroprusside (SNP; 50μM), which releases NO, also hyperpolarized and relaxed the gpIAS, effects which were mimicked, to a lesser degree, by the membrane-permeable cGMP analogue, 8-bromo-cGMP (100μM) and the cGMP phosphodiesterase inhibitor, methylene blue (MeB; 30μM).

d) The guanylyl cyclase inhibitor LY83583 (10μM) abolished the apamin-insensitive IJPs.

e) NADPH-diaphorase, which is a marker for nitric oxide synthase (NOS), staining of transverse sections (25μM) of gpIAS revealed NOS-containing neurons within the myenteric plexus.

6) The P_2-purinoceptor antagonist suramin (100μM) aslo revealed smaller, slower-to-peak (510±9ms, n=17 cells from 7 tissues following a single stimulus) suramin-insensitive IJPs which were abolished by L-NAME (each 100μM) and significantly reduced, but not abolished, by HbO (10μM). This indicated that the suramin-insensitive IJPs, like the apamin-insensitive IJPs, were also mediated by NO.

7) These results suggest that the neuronally-mediated IJPs and relaxations consist of two components; a) a large, fast, apamin- and suramin-sensitive component mediated by ATP or a closely related analogue and b) a smaller, slower, apamin- and suramin-insensitive component mediated by NO or a closely related substance.

8) In addition to measuring responses with electrical and mechanical techniques, changes in the intracellular levels of the second messengers cyclic adenosine 3`, 5`-monophosphate (cAMP) and cyclic guanosine 3`, 5`-monophosphate (cGMP) were also measured using a radioimmunoassay technique.

9) Field stimulation produced significant increases in both cAMP and cGMP. These increases were not attenuated by apamin (0.5μM) or suramin (100μM) (which each abolished the purinergic component of the relaxation), but were each abolished by a combination of either apamin or suramin, and L-NAME (100μM). Field stimulation in the presence of L-NAME alone, also abolished increases in both cyclic nucleotides. Both cyclic nucleotides therefore appear to be involved in neuronally-mediated relaxation.

10) Exogenous SNP (10μM), stimulated significant increases in both cAMP and cGMP, mimicking the effects of field stimulation. Exogenous ATP (10μM) produced only a small, but significant, elevation in cAMP. These results imply that the neurogenically-mediated increase in cyclic nucleotide levels may be due to the action of NO alone as electrically-evoked IJPs, in the absence of apamin or suramin, remain unaffected by L-NAME, although increases in both cGMP and cAMP are apparently abolished. This implies that ATP may produce relaxation in a cyclic nucleotide-independent manner.

INTRODUCTION

SMOOTH MUSCLE RELAXATION

1) PREFACE

Smooth muscle embraces a wide variety of tissue types, capable of performing numerous different functions within the body. These range from the precision, second by second, control exhibited by tissues such as the iris, in the control of the amount of light entering the eye, to the contractions of the uterus at the end of gestation. However, two features are common to all smooth muscles regardless of their anatomical location, time scale or function, namely, the ability to contract and relax. This thesis is concerned with the process of smooth muscle relaxation, specifically within the gastrointestinal (GI) tract.

Smooth muscle relaxation can be induced both physiologically (either by nerve stimulation or hormones) and pharmacologically. However, despite nearly a century of research, it is a process which is still relatively poorly understood. The last 50 years have exemplified the difficulties encountered by researchers who have tried to describe the mechanisms by which neuronally-mediated smooth muscle relaxation is produced. The success of investigators such as Dale, Loewi and Von Euler in establishing the existence of the so-called "classical" transmitters has not been repeated by those investigating the mediators of neuronal smooth muscle relaxation. As a result, several mediators have been proposed, each greeted with differing degrees of acceptance and scepticism by the scientific community. These mediators, or putative transmitters, each with substantial experimental support, have introduced to the scientific vocabulary the so-called purinergic, peptidergic and nitrergic theories of transmission (see Burnstock, 1972; Fahrenkrug, 1978 a,b; Moncada *et al*, 1991). Hence today, the investigation of smooth muscle relaxation has revealed a complex and not yet fully understood process involving several different substances and mechanisms.

The lack of scientific understanding of smooth muscle relaxation has had a knock-on effect on dependent disciplines. This is felt nowhere more than in clinical medicine where a wide range of pathophysiological disorders such as achalasia, intestinal colic, irritable bowel syndrome and spasm of the internal anal sphincter, which often derive from an inability of precontracted smooth muscle to relax, are poorly understood. The inability to successfully treat, or even allay, these conditions stems from a lack of understanding of those factors controlling contraction and the mechanisms underlying smooth muscle relaxation.

In discussing smooth muscle relaxation, for the purposes of this thesis, a distinction will be drawn between the processes by which smooth muscle relaxation occurs and the activators of these processes. The latter will be discussed initially.

2) MECHANISMS OF SMOOTH MUSCLE RELAXATION

At a molecular level, the mechanics of smooth muscle relaxation are reasonably well understood. Smooth muscle tone depends on the interaction of the intracellular macromolecules actin and myosin. Contraction is triggered when the actin and myosin filaments are able to form cross-bridges and slide over one another after the myosin light chain becomes phosphorylated by myosin light chain kinase (MLCK). This process is driven by the hydrolysis of adenosine triphosphate (ATP) and is dependent on Ca^{2+} and calmodulin (for review see Harthstone, 1987; see Fig. 1). Relaxation on the other hand, is thought to occur when either the affinity of MLCK for myosin light chain is reduced by the phosphorylation of MLCK (Conti & Adelstein, 1980; Adelstein *et al*, 1982), or enhanced dephosphorylation of myosin light chain occurs due to an increased

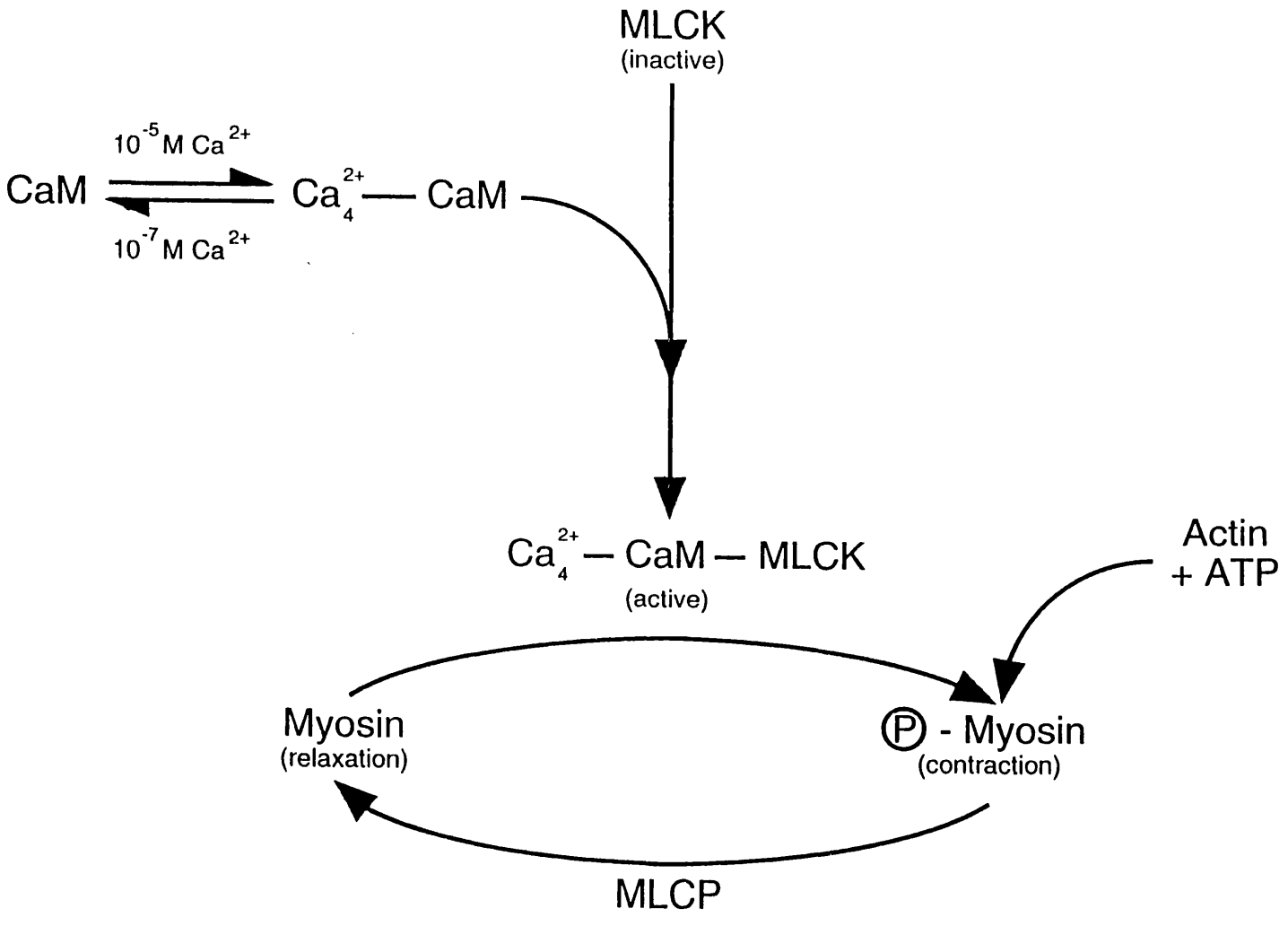


Figure 1: Myosin light chain kinase (MLCK) regulation of smooth muscle relaxation and contraction. MLCK is activated by the binding of calcium-saturated calmodulin (Ca^{2+}_4-CaM). Activated MLCK phosphorylates (P) myosin light chain which, with actin, induces a contraction. Relaxation is induced by the myosin light chain phosphatase (MLCP) which dephosphorylates myosin light chain. (Adapted from Conti & Adelstein, 1980)

activity of the myosin light chain phosphatases (Driska *et al*, 1989; Adelstein *et al*, 1982; see Fig. 1).

This thesis is specifically concerned with the mechanisms by which the processes regulating smooth muscle relaxation are activated. Such mechanisms involve prostaglandins, hormones and, in particular, autonomic nerves. Interference with those neuronal processes using drugs has advanced our understanding of the contractile process, and has been particularly rewarding in clinical terms. On the other hand our incomplete and often fragmentary understanding of the neuronal control of relaxation has prevented the activation of relaxation to be modified for clinical advantage; the following pages describe the major steps taken towards the discovery of the mechanisms controlling smooth muscle relaxation, beginning with neuronal control.

I) Neuronal Control of Relaxation in the GI Tract

This is exercised by the release of substances from autonomic sympathetic and parasympathetic nerves including the "classical" transmitters acetylcholine and noradrenaline, as well as the less well identified substances released from so-called non-adrenergic, non-cholinergic (NANC) nerves which appear to be responsible for the majority of neuronally-mediated relaxant events within GI smooth muscle. It is not clear, however, whether all neuronally-released substances fulfil the accepted criteria for the establishment of a neurotransmitter (Eccles, 1964) nor, in many cases, is there agreement as to whether transmission relies on one, or more than one, of these "neuromediators" (see Bartfai *et al*, 1988) acting in consort.

Within this thesis, it is convenient to describe the effects of neuronally-released substances, particularly the classical transmitters, in terms of two main types; direct and indirect. Direct effects can be considered as those which involve the stimulation of post-synaptic receptors situated on smooth muscle cells, whereas

indirect effects are those which involve the activation of pre-synaptic receptors on nerve terminals, which in turn modify contractile (usually cholinergic in the GI tract) events.

a) Cholinergically-Mediated Relaxation

Cholinergically-mediated effects are not commonly employed as mechanisms of inducing smooth muscle relaxation. However, one well known example of this phenomenon, but which cannot be classed as neuronal, although it concerns the cholinergic transmitter, occurs within the vasculature where acetylcholine (Ach) effects relaxation by stimulating endothelial cells to produce and release endothelium-derived relaxing factor (EDRF; Furchgott & Zawadski, 1980), which may be nitric oxide (NO; Moncada *et al*, 1988). On the other hand, examples of direct cholinergically-mediated relaxation within the GI tract are rare, whether in response to nerve stimulation or exogenous Ach. Evidence, albeit contradictory, exists for such a role within the cat lower oesphageal sphincter (LOS; Clark & Vane, 1961) and human internal anal sphincter (IAS; Parks *et al*, 1969; Burleigh *et al*, 1979; Paskins *et al*, 1982; Carpenedo *et al*, 1983). Both sphincters relax, in an atropine-sensitive manner, to exogenous Ach and, in the IAS, to other cholinergic agonists. Doubt has been cast, however, on each observation. Within the cat LOS contraction, as well as relaxation, to Ach has also been observed (Schenck *et al*, 1961) and within the human IAS part of the response may have been indirect (Burleigh & D`Mello, 1983) because tetrodotoxin (TTX) inhibited the response by varying degrees. Although no further evidence has so far been uncovered to support the hypothesis, it may imply that Ach, in the human IAS, stimulates pre-synaptic muscarinic receptors, located on non-adrenergic nerves, to release an inhibitory transmitter(s).

Most examples of cholinergically-mediated inhibition in the GI tract are attributable to the stimulation of muscarinic receptors located pre-synaptically and pre-junctionally. These inhibitory receptors are situated on the enteric (Sawynok & Jhamandas, 1977; Kilbinger & Wessler, 1980 Morita *et al*, 1982; Kilbinger & Nafziger, 1985) and parasympathetic nerves (Gilbert *et al*, 1984). When activated, they block Ach release from cholinergic motor neurons supplying the gut, so causing the latter to relax. The mechanism underlying this inhibition by muscarinic receptors is probably an increase in membrane K^+ conductance, leading to hyperpolarization and inhibition of Ach release (Hartzell *et al*, 1977; Dodd & Horn, 1983).

b) <u>Adrenergically-Mediated Relaxation</u>

In the main, with only a few exceptions, such as rabbit, cat and guinea-pig ilea (Gonella & Lecchini, 1971; Silva *et al*, 1971; Furness & Costa, 1974) and certain sphincters (Howard & Garret, 1973; see Papasova, 1989), sympathetic nerves in the GI tract do not directly innervate smooth muscle. Most post-ganglionic sympathetic fibres, all of which are noradrenergic (Costa & Furness, 1982), form perivascular nerve trunks running parallel to the blood vessels (Norberg, 1964). These nerves branch within the intestinal wall and terminate around the nerve cell bodies of the intramural plexuses supplying the GI tract. Stimulation of noradrenergic nerves usually causes indirect relaxation of GI smooth muscle by inhibiting acetylcholine release, for example in guinea-pig colon (Beani *et al*, 1969), ileum (Wikberg, 1977), rabbit jejenum (Vizi, 1970; Wikberg, 1977), and canine ileum and duodenum (Sakai *et al*, 1984), thereby inducing relaxation (see also Christ & Nishi, 1971; Knoll & Vizi, 1971; Gabella, 1979). This inhibition may occur as a result of a hyperpolarization of the enteric cholinergic neurons, thus reducing Ach release and tone (Bauer, 1981; Morita &

North, 1981; Costa & Furness, 1982). However, this hypothesis has been questioned by findings which demonstrate that a) cholinergic block by atropine had no effect on responses to sympathetic nerve stimulation (Gershon, 1967), and b) sympathetic nerve stimulation did not affect electrical activity recorded from ganglia in the myenteric plexus (Takayanagi *et al*, 1977).

In the absence of direct innervation, smooth muscle cells may be stimulated by catecholamines that are either present in the circulation, or released following high frequency nerve stimulation by overflow from sympathetic nerve terminals within the enteric plexuses (Gillespie, 1962; Gillespie, 1982).

The inhibitory effects of neuronally-released or circulating/exogenous catecholamines are mediated by receptors originally classified as α- and β-adrenoceptors (Ahlquist, 1948). Both adrenoceptor subtypes were subsequently further subdivided into α_1- and α_2-, and β_1- and β_2-adrenoceptors. Now, with the aid of molecular cloning techniques, further subtypes of receptor have also been proposed (see Ruffolo *et al*, 1991; Van Zwieten, 1991). However, the exact function and location of most of these receptors has yet to be determined, so only the effects of α_1- and α_2-, and β_1- and β_2-adrenoceptor stimulation will be discussed here.

i) <u>α-Adrenoceptor-Mediated Relaxation</u>

The inhibitory α-action is usually only seen in spontaneously active smooth muscles such as the guinea-pig taenia caeci (Bülbring, 1979), proximal or terminal ileum (Fagbemi & Salako, 1980, 1982), rabbit jejenum and colon (Andersson, 1972; Wikberg, 1977) and human colon (Huizingza *et al*, 1986), but can also be witnessed in less excitable tissues such as the guinea-pig and rabbit stomach (Guimarès, 1969; Bailey, 1971; Haffner, 1971).

Both α_1- and α_2-adrenoceptor sub-types are capable of mediating relaxation of the GI tract and can be located both pre- (Bauer, 1981; Wikberg, 1977, 1979) and post- (Bülbring, 1979; Huizinga *et al*, 1986; Bauer, 1982 a) synaptically. Inhibitory effects, produced by stimulation of these receptors, can be seen in several GI smooth muscles;

Taenia Caeci

The effects of α-adrenoceptor stimulation have been studied most intensively in this longitudinal muscle. Activation of taenia caeci α_1-adrenoceptors causes a hyperpolarization of the membrane by increasing K^+ (and possibly Cl^-) conductance which leads to a cessation of spontaneous activity resulting in relaxation (Jenkinson & Morton, 1967 a, b; Bülbring & Tomita, 1969).

The mechanism(s) underlying the increase in K^+ conductance is still a matter of debate, but results suggest that α_1-adrenoceptors, by activating phospholipase C (PLC) via a pertussis toxin-insensitive G_S protein, stimulates diacylglycerol (DAG) as well as inositol 1,4,5 trisphosphate (IP_3) formation which increases intracellular Ca^{2+} levels ($[Ca^{2+}]_i$; Nelemans & Den Hertog, 1987 a,b; Minneman, 1988; for PLC/IP_3 pathway see **Relaxant Transduction Mechanisms**). This process is also dependent on a Ca^{2+} influx, probably to replenish the intracellular Ca^{2+} stores (Bülbring & Tomita, 1977; Den Hertog, 1981, 1982; Timmermans & Van Zwieten, 1981; Tomita *et al*, 1985).

It is clear that the intracellular mechanisms mediating α_1-adrenoceptor-induced relaxation of GI smooth muscle, are different from those mediating α_1-adrenoceptor-induced contraction, for example, in vascular smooth muscle (Minneman, 1988), although both adrenoceptor effects apparently involve the hydrolysis of inositol phosphates and mobilization of intracellular Ca^{2+}.

Small and Large Intestine

The longitudinal muscle of the small and large intestine has similar properties to taenia caeci (Bolton, 1971, 1972; Suzuki & Kuriyama, 1975) in that it produces spike-type action potentials and the spontaneous activity occurs in bursts. Within the guinea-pig, the main action of catecholamines on the small and large intestine is likely to be an α_2-adrenoceptor-mediated suppression of Ach release from cholinergic nerve fibres (Paton & Vizi, 1969; Kosterlitz *et al*, 1970; Wikberg, 1977; Broadley & Grassby, 1985). However, direct inhibitory actions on the smooth muscle have been observed (Bowman & Hall, 1970, Broadley & Grassby, 1985). In the proximal and terminal ileum these are mediated by α_1-receptors (Fagbemi & Salako, 1980, 1982), although α_2-receptors have also been implicated in the proximal ileum (Bauer, 1982 a).

The mechanisms underlying these responses have not been investigated as intensively as in the taenia caeci but it appears that the α_2-adrenoceptors in the guinea-pig proximal ileum produce a hyperpolarization (Bauer, 1982 a). Thus, the inhibitory action of catecholamines is apparently similar to that observed in the taenia but, in the small intestine is mediated by α_2- rather than α_1-adrenoceptors. In the rabbit jejenum and colon, as in the guinea-pig taenia, α_1-adrenoceptor stimulation also produces a hyperpolarization attributable to a Ca^{2+}-dependent increase in K^+ conductance (Andersson, 1972).

Stomach

In the guinea-pig and rabbit stomach, stimulation of α-adrenoceptors produces both excitatory and inhibitory effects depending on the area of the stomach, the degree of existing mechanical activity and the concentration of agonist used (Guimarès, 1969; Bailey, 1971; Haffner, 1971). However, it seems that in most species relaxation is mediated mainly by α_1-adrenoceptors

(Magaribuchi *et al*, 1972; Sahyoun *et al*, 1982) probably via a K^+-dependent hyperpolarization (Haffner, 1972; Magaribuchi *et al*, 1972; Haffner *et al*, 1973; El-Sharkaway & Szurszewski, 1978).

The mechanisms underlying the post-junctional effects of α_2-adrenoceptors are not known, although they would appear to be fundamentally different from those in the vasculature which cause a constriction (Medgett & Rajanayagam, 1984).

The mechanism(s) responsible for the pre-synaptic α_2-adrenoceptor-mediated inhibition of neurotransmitter release is equally unclear but may involve decreasing neuronal Ca^{2+} influx, as release of transmitter is dependent on this ion. This phenomenon has been observed in rat cortical synaptosomes (Adamson *et al*, 1987).

ii) β-Adrenoceptor-Mediated Relaxation

As with the α-adrenoceptors, β-adrenoceptor -mediated events within the GI tract have been investigated mainly on spontaneously active tissues such as guinea-pig taenia caeci (Bulbring & Tomita,1969) and rabbit small intestine (Van Rossum & Mujic, 1965; Lands *et al*, 1967 a,b; Bowman & Hall, 1970) although less spontaneously active tissues like the guinea-pig stomach (Bailey, 1971) have also been utilised.

Both β_1- and β_2-adrenoceptor sub-types are capable of mediating relaxation of the GI tract (Bülbring *et al*, 1981; Huizinga *et al*, 1986). The inhibitory effects produced by stimulating these receptors are also best illustrated by describing β-mediated responses in different GI smooth muscles.

Taenia Caeci

Isoprenaline-induced activation of β-adrenoceptors in this tissue causes relaxation, by suppressing the spike activity, and a small membrane

hyperpolarization (Bülbring & Tomita, 1969). These responses are mediated by both β_1- and β_2- receptors. The β_2-mediated response is proposed to activate events at the membrane, resulting in spike inhibition and hyperpolarization (Bauer, 1982 b) whereas β_1-adrenoceptor activation influences intracellular events, probably raising intracellular cyclic adenosine 3`, 5`-monophosphate (cAMP; Kimura *et al*, 1983; Stiles *et al*, 1984). The hyperpolarization is probably due to an increase in K^+ conductance (Tomita *et al*, 1985) but may not be necessary for relaxation to take place (Bowman & Hall, 1970; Bülbring & Den Hertog, 1980; Bülbring *et al*, 1981; Chow & Huizinga, 1987). The mechanisms mediating the relaxation will be discussed at the end of this section.

Small and Large Intestine

The activity of the longitudinal muscle of the guinea-pig ileum, as well as rabbit duodenum and jejenum, was also suppressed by activation of β_1-adrenoceptors on the smooth muscle cells (Lands *et al*, 1967 a, b; Kosterlitz *et al*, 1970; Grassby & Broadley, 1984; Broadley & Grassley, 1985). However, within the rabbit large intestine and rat and cat colon β-adrenoceptors are predominantly of the β_2-subtype (Sim & Lim, 1983; Ek, 1985).

The fundamental mechanisms causing relaxation through β-receptor activation are probably the same as in the taenia caeci; a small K^+-dependent hyperpolarization accompanied by a second messenger-mediated relaxation (Bülbring & Tomita, 1987).

Stomach

Although this is the least well investigated of the tissues in terms of β-adrenegically-mediated events it is known that adrenergic inhibitory reponses within the stomach are mediated solely by β-adrenoceptors (Guimarès, 1969;

Bailey, 1971; Haffner, 1971) and that both β_1- and β_2-adrenoceptors are probably involved, at least in the rat gastric fundus (Lefebvre *et al*, 1985).

The intracellular events leading to the actual instigation of relaxation by β_1- and β_2-adrenoceptors are believed to involve stimulation of adenylyl cyclase, producing an increase in cyclic adenosine 3`, 5`-monophosphate (cAMP; Stiles *et al*, 1984) which, presumably, leads to an alteration in the cells` Ca^{2+} handling capacity (see **Relaxant Transduction Mechanisms**). However, β-adrenoceptor-stimulated cAMP increases are not always correlated with relaxation; in the guinea-pig taenia caeci for example, isoprenaline-induced relaxation preceded an increase in tissue cAMP (Honda *et al*, 1977). Furthermore, prostaglandins produced a similar rise in cAMP levels to that of isoprenaline in the rat uterus, but caused a contraction (Harbon *et al*, 1976). These findings may refute a relationship between the relaxation and increased cAMP levels but, could also indicate a complex compartmentalization of the cAMP within the cells, such that the production of relaxation or contraction depends on which area of the cell that the increase occurs.

Although catecholamine- and cholinergically-induced relaxation both have important roles to play within the GI tract they do not constitute the full range of neuronal inhibitory mediators believed to control GI relaxation. Overwhelming evidence now suggests that one or more substances released from NANC nerves are responsible for controlling a wide range of inhibitory processes throughout the length of the alimentary canal.

c) Non-Adrenergic, Non-Cholinergic (NANC) Mediated-Relaxation

As early as 1898 Langley demonstrated that vagally-induced relaxation of the rabbit stomach was potentiated by the muscarinic antagonist atropine (Langley, 1898). Atropine-resistant inhibitory responses were also demonstrated in several other tissues such as the dog small intestine (Bayliss & Starling, 1899), the rabbit cardiac sphincter (May, 1904), the cat stomach (Veach, 1925; McSwiney & Wadge, 1928; McSwiney & Robson, 1929), the rabbit and dog stomach (McSwiney & Robson, 1929) and the cat oesophagus (Veach, 1925). Various hypotheses were proposed to explain these "heretical" findings within the existing framework of the sympathetic and parasympathetic nervous system; perhaps the Ach receptors were inaccessible to atropine at the neuroeffector junction (Dale & Gaddum, 1930) because of very tight junctions proposed to separate nerve and muscle.This theory has been largely discounted by electron-microscopy which showed large gaps between the nerve endings and smooth muscle (Dumsday, 1971). Atropine-resistant responses were proposed to have been due to stimulation of sympathetic nerves (known to be inhibitory) within the vagus (Harrison & McSwiney, 1936), or to a "peripheral mechanism" (McSwiney & Wadge, 1928) where the degree of muscle tone determined the response to nerve stimulation, *i.e.* in high tone, either sympathetic or parasympathetic stimulation would cause relaxation but, in low tone, contraction. Notwithstanding the attempts made to accommodate these observations within existing theories, Henderson and Roepke (1934) perceptively proposed that these autonomic nerves released a transmitter other than acetylcholine.

In the absence of specific antagonists however, it was impossible to determine the role of the inhibitory sympathetic nerves in these atropine-resistant effects. This confusion persisted until the advent of the adrenergic neurone blocking drugs such as guanethidine and bretylium, and the adrenoceptor

antagonist phentolamine, in the late 1950's. The ability to block the actions of both classical neurotransmitters using these drugs and atropine allowed, for the first time, the unequivocal identification of a new class of inhibitory, non-adrenergic, non-cholinergic (NANC) neurogenic response. These were characterised initially by two groups of workers, almost simultaneously, but using different techniques, in the early 1960's. First, using extracellular (sucrose-gap) recording, large tetrodotoxin-sensitive, neuronally-induced hyperpolarizations were elicited in the presence of atropine and bretylium (Burnstock *et al* 1963 a, b; 1964). Secondly, vagally-induced, atropine-resistant relaxations in the cat stomach which were unaffected by guanethidine, were seen (Martinson & Muren, 1963; Martinson, 1965). NANC nerves have now been found at almost every level of the GI tract in most mammalian species investigated. Principally, although not invariably (*e.g.* Shuttleworth *et al*, 1993; Zagorodnyuk *et al*, 1993), concerned with relaxation in the GI tract, they allow the passage of material along the tract; they control the "receptive relaxation" of the stomach, descending inhibition, which precedes contraction, in intestinal peristalsis (Burnstock & Costa, 1973) and the reflex opening of the sphincters. Although NANC nerves mediating these responses are intrinsic to the GI tract (Burnstock *et al*, 1966), they are linked to nerves by fibres from a) the vagus nerve (in the small intestine) b) the vagus and pelvic nerves (in the colon), the relative contribution of each being dependent on species, and c) the pelvic nerves (in the rectum and internal anal sphincter; see Gonella *et al*, 1987).

The electrical basis of the NANC responses was found to be membrane hyperpolarizations, or inhibitory junction potentials (IJPs), which preceded the relaxations (Bennett *et al*, 1966 b). Evoked NANC IJPs, recorded from anatomically distinct areas of the alimentary canal, such as the guinea-pig stomach (Kuriyama *et al*, 1970; Beani *et al*, 1971; Beck & Osa, 1971), ileum (Kuriyama *et al*, 1967), jejenum (Hidaka & Kuriyama, 1969), taenia ceaci (Bennett *et al*, 1966 a,

b) and colon (Furness, 1969 a, b) are fundamentally different from those evoked by adrenergic nerve stimulation (Gillespie, 1962). Perivascular sympathetic nerve stimulation of guinea-pig taenia caeci, for example, using trains of stimuli greater than 10Hz, produced relatively small (maximum amplitude 16mV), bretylium-sensitive hyperpolarizations and relaxations (Bennett *et al*, 1966 a). In contrast, stimulation of intramural NANC nerves with only a single pulse, in the same tissue, evoked relaxations and accompanying IJPs which could reach 25mV in amplitude (Bennett *et al*, 1966 b) and up to 35mV in response to trains of stimuli around 8Hz which were unaffected by atropine or bretylium (Bennett *et al*, 1966 b). All NANC electrical responses are not similar however; the large IJPs found in the GI tract, which accompany relaxation, differ markedly from the much smaller ones found in the gastrointestinally-related accessory muscles of reproduction, such as the bovine retractor penis muscle (BRP; Klinge & Sjöstrand, 1974; Byrne & Muir, 1984, 1985), rat anococcygeus (RAc; Gillespie, 1972, Creed *et al*, 1975; Gibson & Yu, 1983; Li & Rand, 1989) and rabbit rectococcygeus (King & Muir, 1981). These IJPs may, however, play a less important role in relaxation (Creed *et al*, 1975; Byrne & Muir, 1984, 1985; Byrne *et al*, 1984). This may indicate either fundamental tissue-specific differences between the inhibitory transmitter(s) or, in the receptors mediating the responses (see **Relaxant Transduction Mechanisms**).

The identification of NANC nerves had a profound impact on scientific insight and research into autonomic systems. The success which had accompanied the establishment of Ach (Dale, 1937) and noradrenaline (NA; Von Euler, 1946) as classic (often excitatory) transmitters provided a template which encouraged the scientific community to investigate the mediators of those NANC nerve-mediated effects. There was no shortage of candidates, including prostaglandins, amino acids and peptides (see Hökfelt *et al*, 1980 a, b; Burnstock, 1981; Furness *et al*, 1989). However, for a substance to be considered an inhibitory transmitter it

should satisfy each of the following five criteria (Eccles, 1964); it must 1) be synthesised and stored in nerve terminals 2) be released by a Ca^{2+}-dependent process during nerve stimulation; 3) have a transmitter inactivation process present; 4) have its effects antagonized or potentiated by drugs which affect the responses to nerve stimulation in the same way; and 5) mimic the responses to nerve stimulation. Within the framework of these criteria, the only likely candidates to emerge from the many postulated inhibitory transmitters were, first, the purines, in particular ATP (see Burnstock, 1979), secondly, the peptides, such as vasoactive intestinal polypeptide (VIP; see Fahrenkrug, 1979) and finally, NO or a closely-related substance (see Rand, 1992). This is not to imply that each candidate satisfies all of the aforementioned criteria. The evidence for representatives of these three classes as inhibitory transmitters within the GI tract will be discussed.

i) <u>Adenosine 5`-Trisphosphate (ATP)</u>

Although smooth muscle sensitivity to adenine nucleotides was recognised in 1929 (Drury & Szent-Györgi, 1929) it took a further twenty years before ATP was proposed as a transmitter (Holton & Holton, 1953, 1954; Holton, 1958). The case for ATP being a NANC neurotransmitter has since strengthened (for reviews see Burnstock 1972, 1975, 1979, 1981, 1986 a, 1990 a) and there is now evidence that ATP largely satisfies the criteria for the establishment of a neurotransmitter (Eccles, 1964) in that;

1) it is synthesised (Su *et al*, 1971) and stored in nerves (see for example Burnstock *et al*, 1978);

2) it is released by nerve stimulation (Su *et al*, 1971; Burnstock *et al*, 1978 a, b). However, because of its ubiquitous nature as a cell metabolite, concern was expressed that the electrically-stimulated ATP release may have been from the

muscle. Against this was the finding that while a 2-6 fold increase in ATP release from the guinea-pig taenia caeci and urinary bladder occurred after NANC nerve stimulation, no significant rise in ATP could be detected after direct muscle stimulation (Burnstock *et al*, 1978);

3) efficient inactivation processes exist for ATP in the form of ecto-ATPases and a 5`-nucleotidase. It is eventually deaminated to inosine or taken back up into smooth muscle or neurons (see Burnstock, 1972; 1979);

4) with regard to inhibitory effects, several putative purinoceptor antagonists such as reactive blue 2 (RB2; Crema *et al*, 1983; Manzini *et al*, 1986; Soediono & Burnstock, 1994) and suramin (Den Hertog *et al*, 1989; Ohno *et al*, 1993; Brizzolara *et al*, 1993) reduced relaxations to ATP and/or evoked NANC-mediated IJPs. Indirect evidence also came from the use of apamin, a bee venom which blocks small conductance Ca^{2+}-activated K^+ channels (Banks *et al*, 1979; Blatz & Magleby, 1986; Capoid & Ogden, 1989). Although not a purinoceptor antagonist *per se* apamin blocked the inhibitory effects of both exogenous ATP and NANC nerve stimulation in various GI smooth muscles such as the guinea-pig taenia caeci (Maas & Den Hertog, 1979; Müller & Baer, 1980; Shuba & Vladimirova, 1980; Costa *et al*, 1986) and gpIAS (Lim & Muir, 1986);

5) the responses to ATP and inhibitory NANC nerve stimulation are very similar. These similarities, and their common resistance to adrenergic and cholinergic block, are perhaps the strongest indicators of an endogenous inhibitory transmitter role for ATP or a related nucleotide. Inhibitory NANC nerve stimulation and ATP both produce relaxations within the mammalian GI tract (see, *inter alia*, guinea-pig taenia caeci- Jager, 1974; rabbit intestine- McKenzie *et al*, 1977; guinea-pig stomach- Huizinga & Den Hertog, 1980; guinea-pig internal anal sphincter (gpIAS); Lim & Muir, 1986) which are often followed by rebound excitations (Tomita & Watanabe, 1973; MacKenzie & Burnstock, 1980). The

concentration-dependent, rapid and relatively brief membrane hyperpolarizations induced by exogenous ATP are also similar to IJPs evoked by NANC nerve stimulation in several tissues such as the guinea-pig taenia caeci (Axelsson & Holmberg, 1969; Tomita & Watanabe, 1973; Jager, 1974; Jager & Schevers, 1980), gpIAS (Lim & Muir, 1986) and rabbit caecum (Small, 1974). However, doubts regarding ATP's mimicry of inhibitory NANC stimulation persist; first, very high (0.1-1mM) concentrations of ATP were used to mimic NANC responses (*e.g.* Tomita & Watanabe, 1973; Maas & Den Hertog, 1979; Lim & Muir, 1986; Zagorodnyuk & Shuba, 1986). This was explained, for the guinea-pig urinary bladder at least, as being due to the rapid breakdown of ATP to adenosine. In support of this view, a stable ATP analogue, βγ-methylene ATP, which was not degraded to adenosine, was found to be more potent than ATP in producing contraction (Brown *et al*, 1979). Secondly, in many tissues, the responses to ATP did not mimic NANC nerve stimulation, for example, in the pig stomach (Ohga & Taneika, 1977), oesophageal smooth muscle (Daniel *et al*, 1983), dog stomach (Sakai & Daniel, 1984) and RAc (Gillespie, 1972) but in these cases another NANC transmitter, possibly related to ATP, could have been responsible for the inhibitory responses. Finally, in certain smooth muscles, such as the guinea-pig fundus, (Baer & Frew, 1979), ileum (Weston, 1973 a), taenia caeci (Ambache *et al*, 1977), pig stomach (Ohga & Taneika, 1977) and rabbit duodenum (Weston, 1973 b) responses to repeated application of ATP desensitized, whereas those to NANC nerve stimulation did not. Although this has been contradicted (Burnstock *et al*, 1970; Kasakov & Burnstock, 1983) it remains a significant barrier to acceptance of ATP as an inhibitory transmitter.

The investigation of a proposed transmitter role for ATP led, not surprisingly, to studies on the receptors involved. Based on rank order of potency studies, purinergic receptors were initially divided into 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) than adenosine (Burnstock, 1978). Purinoceptors have since been further subdivided; the P_1-division into A_1- and A_2-purinoceptors (Van Calker *et al*, 1979) and the P_2-division into P_{2x}- and P_{2y}-purinoceptors (see Burnstock & Kennedy, 1985; Kennedy, 1990). The subdivision of P_2-purinoceptors was also based on studies of rank orders of potency of structural analogues of ATP (see Burnstock & Kennedy, 1985). However, differences between P_{2x}- and P_{2y}-purinoceptors seem to exist at a more basic level than relative sensitivities to nucleotide analogues, *i.e.* P_{2x}-receptors mediate contraction in tissues such as the rat vas deferens (Taylor *et al*, 1983; Burnstock *et al*, 1985) and guinea-pig urinary bladder (Brown *et al,* 1979; Dahlén & Hedquist, 1980), whereas P_{2y}-purinoceptors mediate relaxation in the guinea-pig taenia caeci (Satchell & Maguire, 1975; Burnstock *et al*, 1983) and gpIAS (Lim, 1985). Furthermore, P_{2y}-purinoceptors constitute G protein-linked receptors (Fredholm *et al*, 1994) whereas P_{2x} -purinoceptors represent an intrinsic ion channel permeable to Na^+, K^+, and Ca^{2+} (Bean, 1992).

Following the discovery of receptors activated by ATP and uridine triphosphate, but not by P_{2x}- or P_{2y}-purinoceptor agonists (Brown *et al*, 1991; Dubyak *et al*, 1988; Okajima *et al*, 1989; Stutchfield & Cockroft, 1990), it was postulated that further subtypes of P_{2x}- or P_{2y}-purinoceptors may exist. This would explain why proposed P_{2y}-receptors could apparently couple to two separate second messenger systems, either inhibition of adenylyl cyclase (Boyer *et al*, 1993, 1994; Lin & Chuang, 1993; Okajima *et al*, 1987; Pianet *et al*, 1989) or stimulation of the polyphosphoinositide system (see *inter alia* Pirotton *et al*, 1987; Berrie *et al*, 1989; Boyer *et al*, 1989; Cooper *et al*, 1989; Van der Merwe, 1989; Harden *et al*, 1990; Flitz *et al*, 1994; for second messenger systems see **Relaxant Transduction**

Mechanisms), and has been supported by studies using novel selective agonists for P_{2x}- and P_{2y}-purinoceptors (Fischer *et al*, 1993; Burnstock *et al*, 1994).

ii) <u>Vasoactive Intestinal Polypeptide (VIP)</u>

More than twenty peptides have now been identified in mammalian brain, spinal cord and peripheral neurons, principally using immunohistochemical or radioimmunoassay techniques (see Hökfelt *et al*, 1980 a). Of these, only VIP has emerged as a likely inhibitory transmitter within smooth muscle, as it more adequately fulfills, than any other peptide, the criteria required to establish the existence of a substance as a neurotransmitter (Eccles, 1964), thus;

1) it is synthesised and stored in nerves throughout the GI tract (Alumets *et al*, 1979; Hökfelt *et al*, 1980 a,b; Fahrenkrug *et al*, 1985; Biancani *et al*, 1988; Wattchow *et al*, 1988; Lynn *et al*, 1994; Bandyopadhyay *et al*, 1994).

2) it is released from NANC-innervated tissues such as the taenia caeci (Fahrenkrug *et al*, 1978 a), LOS (Goyal & Cobb, 1981) and small intestine (Fahrenkrug *et al*, 1978 b; Bloom & Edwards, 1980).

3) it is inactivated by relatively slowly (4-15 minutes) acting peptidases (Fahrenkrug, 1979). However, no specific process comparable to that of the classical transmitters exists for VIP (Hökfelt *et al*, 1980 a) which means that the action of exogenous VIP is much more prolonged than that of inhibitory nerve stimulation in certain tissues such as the guinea-pig taenia caeci (Cocks & Burnstock, 1979) and gpIAS (Lim & Muir, 1986).

4) its effects are inhibited by several putative antagonists; α-chymotrypsin, VIP antiserum and the C-terminal VIP fragment, VIP (10-28). α-Chymotrypsin, which degrades certain peptides, abolishes responses to exogenous VIP in the guinea-pig taenia caeci (MacKenzie & Burnstock, 1980). VIP antiserum reduces the relaxations of several tissues in response to exogenous VIP and electrical or

chemical stimulation of NANC nerves (Goyal *et al*, 1980; Ito & Takeda, 1982; Biancani *et al*, 1985 a, b). VIP (10-28) antagonized VIP-induced hyperpolarizations in the guinea-pig ileum (Grider & Rivier, 1990; Crist *et al*, 1992). However, strong doubts exist regarding the specificity of these aforementioned VIP antagonists. α-Chymotrypsin reduced responses to exogenous VIP but had no effect on NANC nerve-mediated reponses in the taenia caeci (MacKenzie & Burnstock, 1980). The inhibitory effect of VIP antiserum on NANC nerve stimulation in cat (Ito & Takeda, 1982; Biancani *et al*, 1985 a) and opossum (Goyal *et al*, 1980) oesophageal tissues was not shown in human oesophageal and gastric tissues (De Carle & Pye, 1982), while VIP (10-28) shows low affinity for VIP receptors and may act as a partial agonist in some systems (Robberecht *et al*, 1990). A final point concerns the ability of apamin to block NANC neuronally-mediated events, but not those of exogenous VIP, in certain tissues such as the guinea-pig taenia caeci (MacKenzie & Burnstock, 1980), which suggests that VIP is not the inhibitory transmitter in these cases.

5) VIP, among the peptides, is uniquely able to relax and hyperpolarize virtually all areas of the GI tract such as the rat colon (Grider & Rivier, 1990), rat, guinea-pig and cat gastric fundus (Grider *et al*, 1985; D`Amato *et al*, 1988, 1992; De Beurme & Lefebvre, 1988; Grider & Rivier, 1990; Li & Rand, 1990) and guinea-pig taenia caeci, circular smooth muscle of the stomach and ileum (Kamata *et al*, 1988; Grider *et al*, 1983; Crist *et al*, 1992) as well as the sphincters (LOS-Biancani *et al*, 1984; 1989; Goyal *et al*, 1980; sphincter of Oddi-Wiley *et al*, 1988; IAS-Burleigh, 1983; Biancani *et al*, 1985; Nurko & Rattan, 1988; Nurko *et al*, 1989; Lim & Muir, 1986). However, probably due to the absence of a rapid inactivation system, the time course of the VIP-mediated events does not correlate well with the much faster (in both onset and recovery) NANC nerve-mediated responses in most tissues (Cocks & Burnstock, 1979; Lim & Muir, 1986).

Notwithstanding the doubts regarding its proposed transmitter role, two VIP receptors have been proposed (VIP_1 and VIP_2; Robberecht *et al*, 1990). This classification was based on the binding of VIP, peptide histidine isoleucine (PHI), which is processed from a common precursor peptide (Itoh *et al*, 1983), and helodermin, a lizard peptide belonging to the VIP-PHI-secretin-GRF family (Robberecht *et al*, 1984), to a human lymphoblast cell line (SUP T1; Robberecht *et al*, 1988; Robberecht *et al*, 1990). Further classification has been restricted by the lack of selective synthetic ligands (Robberecht *et al*, 1990). However, all VIPergic inhibition is induced via stimulation of adenylyl cyclase leading to increased intracellular cAMP levels (Schwartz *et al*, 1974; Simon & Kather, 1978; Frandsen *et al*, 1978; Torphy *et al*, 1986; Ito *et al*, 1990; for cAMP actions see **Relaxant Transduction Mechanisms**).

iii) Nitric Oxide (NO)

EDRF is released from vascular endothelial cells in response to a variety of stimuli such as Ach, ATP and shear stress (Furchgott & Zawadzki, 1980; see Kerwin & Heller, 1994). EDRF has many pharmacological features in common with the gas nitric oxide (NO); each relaxes both vascular and non-vascular tissues, raises cyclic guanosine 3`,5`-monophosphate (cGMP) levels, and is antagonized by methylene blue (MeB), oxyhaemoglobin (HbO) and superoxide anions (Martin *et al*, 1985 a,b; Griffith *et al*, 1984; Förstermann *et al*, 1986; Grylewski *et al*, 1986; Rubanyi & Vanhoutte, 1986). That EDRF was indeed NO was demonstrated by Palmer *et al* (1987) when NO was measured as the chemiluminescent product of EDRF`s reaction with ozone. The similarities between the actions of EDRF and NO have now been demonstrated in both vascular and non-vascular tissues (Hutchinson *et al*, 1987; Gillespie & Sheng, 1988; Cocks & Angus, 1990; Tare *et al*, 1990; Mathie *et al*, 1991).

Outwith the vasculature, following the identification of the "inhibitory factor" (IF), released by the BRP and the RAc (Ambache *et al*, 1975; Gillespie & Martin, 1980), as NO or a closely related substance (Furchgott, 1988; Martin *et al*, 1988), it was proposed that NO may also be a peripheral inhibitory transmitter as it is a very potent and ubiquitous relaxant agent. However, NO is probably released as a highly labile gas and has a short half-life of around 5s (although values of up to 30s have been reported; see Rand, 1992), making it unique in comparison to other proven and putative neurotransmitters. Because of this uniqueness it is perhaps unlikely that it can satisfy all of the criteria previously proposed for the establishment of a substance as a transmitter (Eccles, 1964). Thus:-

1) in a reaction catalysed by nitric oxide synthase (NOS) NO is synthesised from the terminal guanido nitrogen atoms of L-arginine in a stereospecific manner (Palmer & Moncada, 1989). Perhaps not surprisingly, bearing in mind its gaseous and labile nature, no evidence exists for the pre-packaging of NO or a NO precursor within nerve terminals, suggesting that it is synthesised only on demand (see Stark *et al*, 1991). However, in the absence of stored NO, the presence of NOS within neurons is often accepted as proof of the presence of NO. Constitutive, as opposed to inducible, NOS is found in many tissues both centrally (Bredt *et al*, 1990) and peripherally (Gillespie *et al*, 1989; Li & Rand, 1989), particularly concentrated within cell bodies and nerve fibres of the myenteric plexus (Bredt *et al*, 1990; Llewellyn-Smith *et al*, 1992; Ward *et al*, 1992 d; O`Kelly *et al*, 1994);

2) to date, only one group has actually demonstrated its release from smooth muscle. TTX-sensitive, calcium-dependent electrical and DMPP nerve stimulation of the canine ileocolonic sphincter (ICS) and rat gastric fundus, released NO, as demonstrated by bioassay (Bult *et al*, 1990; Boeckxstaens *et al*, 1991 a, b, c; Boeckxstaens *et al*, 1993).

3) with regard to an inactivation process, none is actually required since NO has a very short half-life in physiological solutions (Griffith *et al*, 1984; Förstermann *et al*, 1984; Rubanyi *et al*, 1985; Cocks *et al*, 1985; Palmer *et al*, 1987; Ignarro, 1990), and it is perhaps unnecessary to assume such a conventional process exists.

4) its effects can be antagonized a) at source, b) in transit or c) at its receptor;

a) Synthesis of NO can be blocked by inhibition of NOS activity. Structural analogues of the NO precursor, L-arginine, such as N^{ω}-monomethyl-L-arginine (L-NMMA), N^{ω}-nitro-L-arginine (L-NNA) and N^{ω}-nitro-L-arginine methyl ester (L-NAME), (Palmer & Moncada, 1989; Hobbs & Gibson, 1990; Fleming *et al*, 1991) can each stereoselectively inhibit NOS activity in a reversible manner.

b) By binding to NO, after its proposed neuronal release into the extracellular fluid, HbO is able to antagonize its effects (Martin *et al*, 1985 a, b; Förstermann *et al*, 1986; Thornbury *et al*, 1991). Superoxide anions, which are present in physiological solutions, are also capable of rapidly oxidizing and inactivating NO (Grylewski *et al*, 1986; Rubanyi & Vanhoutte, 1986).

c) Unlike other neuromediators, the receptor for NO, cytosolic guanylyl cyclase, is located intracellularly. Inhibition of guanylyl cyclase activity can be accomplished with MeB (Gruetter *et al*, 1981 a, b; Ignarro *et al*, 1986; Martin *et al*, 1985 a) or LY83583 (Mülsch *et al*, 1988; Brandt & Conrad, 1991) thereby antagonizing the responses to NO.

5) Exogenous NO mimics the inhibitory NANC nerve-mediated responses. It hyperpolarizes and relaxes almost all smooth muscles, effects which are resistant to cholinergic and adrenergic block. Electrical and mechanical responses to both NANC nerve stimulation and exogenous NO are rapid in onset and decline (for reviews see Rand, 1992; Sanders *et al*, 1992; Sanders & Ward, 1992; Stark &

Szurszewski, 1992). In addition increased cGMP levels follow NANC inhibitory nerve stimulation (in the opossum LOS - Torphy *et al*, 1986; opossum and guinea-pig IAS - Baird & Muir, 1990; Chakder & Rattan, 1993 b) and also exogenous NO (see Waldman & Murad, 1987; Baird & Muir, 1990; Chakder & Rattan, 1993 b).

iv) Synergistic Transmitter-Mediated Relaxation

Current views on autonomic transmission are no longer restricted to consideration of only one mediator. There is now widespread acceptance that neuronally-mediated relaxation and contraction, in many smooth muscles, involves the release of more than one substance (*e.g.* Lundberg, 1981; Hökfelt *et al*, 1980 b; Campbell, 1987; Furness *et al*, 1989). This does not imply that all such substances can automatically be considered as transmitters even though this could be the case. In the main, a transmitter role has not been proven for many neuronally-released substances and for this reason, where two or more transmitters are found within the same nerve ending, the terms co-existence, co-localization or co-release, rather than co-transmission, are often preferred.

The release of more than one substance during nerve stimulation has led to a re-examination of "Dale`s Principle", mistakenly ascribed to Sir Henry Dale by Eccles in the mid-1950`s, which asserts that "at all the axonal branches of a neurone, there is liberation of the same transmitter substance or substances" (see Eccles, 1976). This principle has been interpreted, some would say incorrectly, to mean that one nerve fibre makes and releases only one transmitter. This interpretation was widely held for a number of years before Burnstock (1976) questioned its validity by suggesting that, since more than one substance appeared to be released during nerve stimulation, some nerve fibres synthesised and released more than one substance. This dissenting viewpoint has gained support with the identification of numerous examples of co-localized substances within neurons.

Both central and peripheral neurons appear to contain, and probably release, several different combinations of substances such as two or more peptides (Furness *et al*, 1989); peptides and classical transmitters (Hökfelt *et al*, 1980 a,b; Furness *et al*, 1989, 1992); purines and classical transmitters (Burnstock, 1985, 1986 b, 1990 a); or, more recently, peptides or purines with NOS (Furness *et al*, 1992; Costa *et al*, 1992; Lynn *et al*, 1994; Soediono & Burnstock, 1994; Ward *et al*, 1994). Notwithstanding, in only in a very few instances has the co-release of these substances been demonstrated, for example, Ach and VIP in the cat exocrine glands (Lundberg, 1981; Lundberg *et al*, 1980, 1982 a, b; see also Campbell, 1987). Indeed, where dual or multiple mediators are proposed to act synergistically, co-transmission, and contradiction of "Dale`s Principle" have yet to be demonstrated unequivocally.

Notwithstanding this controversy, the weight of evidence suggests that in many cases more than one substance can be released from autonomic nerves onto smooth muscle. Unlike the majority of examples of proposed dual transmission which are excitatory/contractile, such as NA and ATP release from sympathetically-innervated blood vessels (Burnstock, 1990 b; Burnstock, 1988) or Ach and ATP release from somatic and autonomic nerves (Richardson & Brown, 1987; Burnstock, 1986 b), inhibitory processes can apparently also be regulated by more than one substance.

Three inhibitory transmitter combinations have been proposed a) ATP and NO, b) VIP and NO and c) VIP, PACAP and NO.

ATP and NO

The first convincing evidence for the involvement of two inhibitory transmitters mediating NANC nerve-mediated responses was obtained when, using apamin, the nerve-evoked large, fast IJPs of the guinea-pig ileum or distal colon,

were converted to slower, smaller, distinct, so-called, "apamin-insensitive" IJPs (Niel *et al*, 1983 a,b; Smith & Bywater, 1983). Verification soon followed in the same (see *inter alia* Costa *et al*, 1986; Crist *et al*, 1991 c; Crist & He, 1991; Maggi & Giuliani, 1993) and other tissues such as guinea-pig stomach, small and large intestine (Vladimirova & Shuba, 1984), taenia caeci (Bridgewater *et al*, 1995) and human colonic circular muscle (Keef *et al*, 1993).

The apamin-resistant IJP component in the guinea-pig ileum (Humphreys *et al*, 1991; Lyster *et al*, 1992 a, b; Bywater *et al*, 1993; He & Goyal, 1993) and colon (Zagorodnyuk & Maggi, 1994) but not, interestingly, in the guinea-pig taenia caeci (Bridgewater *et al*, 1995), was abolished by NOS inhibitors, indicating its mediation by NO. Attempts to identify the "apamin-sensitive" component soon followed. Most evidence implicates ATP; apamin is a recognised antagonist of ATP-mediated responses in certain tissues (MacKenzie & Burnstock, 1980; Shuba & Vladimirova, 1980; Costa *et al*, 1986; Boeckxstaens *et al*, 1993). In addition, antagonists of P_2-purinergically-mediated responses, such as reactive blue 2 (RB2; Kerr & Krantis, 1979, Choo, 1980, Crema *et al*, 1983) and suramin (Dunn & Blakeley, 1988; Den Hertog *et al*, 1989; Ohno *et al*, 1993), together with NOS inhibitors, abolished the inhibitory responses to NANC nerve stimulation in the rat pyloric sphincter (PS; Soedino & Burnstock, 1994), guinea-pig colon (Maggi & Guiliani, 1993; Zagorodnyuk & Maggi, 1994) and rabbit portal vein (Brizzolara *et al*, 1993). ATP and NO may also be responsible for the separate IJP components seen in the circular muscle of the guinea-pig ileum (Humphreys *et al*, 1991; Lyster *et al*, 1992 a, b; Bywater *et al*, 1993; He & Goyal, 1993), stomach, small and large intestine (Vladimirova & Shuba, 1984) and human colonic muscle (Keef *et al*, 1993; Boeckxstaens *et al*, 1993). The question remains as to whether these putative inhibitory transmitters are released from the same or different neuronal sources. In the guinea-pig taenia caeci, the fast, but not the slow IJP, was selectively abolished

by ω-conotoxin GVIA which blocks neuronal Ca^{2+} channels, suggesting that the two IJP components were probably mediated by different populations of nerves although different release mechanisms within the same nerves could also account for such a finding (Bridgewater *et al*, 1995). Whether or not NO and ATP play a synergistic role within certain GI smooth muscles and if so, how this is achieved, remains unclear.

VIP and NO

There is increasing evidence in favour of a synergistic relationship between VIP and NO within GI smooth muscles (see Makhlouf, 1994, Makhlouf & Grider, 1993). Their roles are closely interlinked but in a different manner from that for ATP and NO together, such that VIP release may be dependent on NO production and *vice versa*.

Electrically or *DMPP-stimulated VIP release from isolated intestinal ganglia, it has been proposed, depends on NO production (Grider & Jin, 1993 a), as L-NAME abolished, and exogenous NO-stimulated, VIP production. In innervated muscles such as the guinea-pig gastric fundus (Grider *et al*, 1992) and rat colon (Grider, 1993) however, while NO production facilitated VIP release, liberation of the peptide could still occur independently. Thus, VIP antagonists, such as VIP (10-28), inhibited NO production (Grider *et al*, 1992; Jin & Grider, 1993). This implies that VIP stimulates NO production within the target muscle cells (Grider *et al*, 1992; Jin & Grider, 1993; Grider & Jin, 1993 b), potentiating the relaxant effects of the peptide and enhancing neuronal VIP release. This also occurs with isolated, nerve free, gastric smooth muscle cells (Murthy *et al*, 1993 b). VIP reportedly stimulated NO production by interacting with a membrane bound NOS (Murthy *et al*, 1994; Murthy & Makhhlouf, 1994).

* Ganglion stimulant

Electrical studies have lent further weight to the proposed synergism between VIP and NO. Apamin-resistant IJPs in guinea-pig and canine ileum involve both NO and VIP; the effects of exogenous VIP in these tissues are suppressed by NOS inhibitors, implying VIP-induced NO release (Grider & Jin, 1993 b; He & Goyal, 1992, 1993). If this were so then at least three inhibitory mediators could be involved in mediating inhibitory responses in these tissues, an apamin-sensitive component, VIP and NO.

However, if NO and VIP act synergistically, then a number of problems arise. If, following VIP stimulation, NO is synthesised within muscle cells rather than in neurons, it cannot be acting as a neurotransmitter in these tissues. This contrasts with the finding that HbO, which is unable to cross cell membranes, greatly reduces NANC responses in most GI smooth muscles (see Sanders & Ward, 1992; Sanders *et al*, 1992; Rand, 1992). Presumably, VIP is unaffected by HbO and, as the major effect of NO would be produced intracellularly on the NO-synthesising cells, it is unclear how HbO could exert its antagonistic effects. Importantly, TTX blocks the portion of the VIP relaxation attributed to NO (Huizinga *et al*, 1992) and ω-conotoxin GVIA, which blocks neuronal calcium channels (McCleskey *et al*, 1987), reduces the contractile response to L-NAME in canine ileal segments (Daniel *et al*, 1994). Each finding indicates a neuronal origin for NO. One might also ask why such a system should exist; the very short half-life of NO makes it extremely unlikely that it would reach many cells that remained untouched by VIP.

PACAP, VIP and NO

A novel brain-gut peptide with a high sequence homology to VIP, pituitary adenylate cyclase-activating peptide (PACAP; Schwörer *et al*, 1992, 1993) relaxes guinea-pig taenia caeci and human colon in an apamin-sensitive manner (Schwörer

et al, 1992, 1993; Jin *et al*, 1994 a, b; McConalogue *et al*, 1994). PACAP may regulate the descending phase of peristalsis in conjunction with VIP and NO, in rat ileum (Grider *et al*, 1994) and be an inhibitory substance in the guinea-pig taenia caeci where VIP and PACAP are proposed co-transmitters (Jin *et al*, 1994 b), however, further information regarding this transmitter combination is still sparse.

The discussion of relaxation has so far concentrated on mediators which are either proposed to be released from nerves or mimic the effects of neuronal mediators. There are ways, however, of inducing smooth muscle relaxation in the absence of any neuronal stimulation.

II) Non-Neuronal Control of Relaxation in the GI tract

Compounds that can be classed as non-neuronal relaxants are either, a) endogenous substances that are released from non-neuronal sources (*e.g.* circulating hormones) or b) drugs whose actions are mediated in ways other than by mimicking the action of known or putative transmitters, or transduction pathways (*e.g.* ATP activated K^+ channel (K_{ATP}) openers). Research into non-neuronal control of smooth muscle relaxation has not been extensive. This is probably because of its perceived relative unimportance in comparison to neuronal relaxation. Probably the most widely investigated endogenous non-neuronal inhibitory agents are the prostaglandins.

Prostaglandins mediate both excitatory and inhibitory effects (see Sanders, 1984 b) and are synthesised by GI smooth muscle cells (Ali & McDonald, 1980; Bennett *et al*, 1968; LeDuc & Needleman, 1979; Sanders, 1978). They may act as local regulators, with their effects being mediated on, or near, the cell that produces them (Needleman, 1978; Sanders, 1984 a). The most likely candidates as

inhibitory prostanoids are PGI_2 and PGE_2 (Sanders, 1984 a, b; Daniel, 1979). They allegedly act by decreasing the ability of calcium channels to open, thereby limiting membrane excitability (Sanders, 1984 a). An inhibitory role for prostaglandins within certain tissues is supported by the fact that indomethacin and other prostaglandin synthesis blockers enhance contractile activity, and suggests that sufficient quantities are produced to be of physiological relevance (Duthie & Kirk, 1978; El-Sharkawy, 1983; Sanders, 1984 a, b). However, in the opossum LOS (Daniel *et al*, 1979) a prostaglandin, released from intestinal interstitial cells during inhibitory nerve stimulation, facilitated the additional release of the NANC transmitter (Daniel *et al*, 1979), but was not necessary for relaxation to be induced (Daniel, 1979).

Circulating hormones represent another form of non-neuronal inhibitory substances. As with the prostaglandins, hormones such as gastrin and choleocystokinin (CCK) each mediate excitation and inhibition in GI smooth muscle (see Bloom, 1977). However, examples of inhibitory effects on GI smooth muscles are relatively scarce; secretin and pancreatic glucagon each inhibit gastric motility (Bloom, 1975; Hubel, 1972); secretin alone inhibits tone in the human small intestine (Hubel, 1972) and motor activity in the human duodenum (Gutiérrez *et al*, 1974). CCK reduces LOS pressure (Resin *et al*, 1973; Fisher *et al*, 1975; Rehfeld, 1978) by inhibiting the effects of endogenous gastrin (Fisher *et al*, 1975). As with the final example, the majority of hormonal effects are probably the result of these compounds acting to either facilitate or inhibit the release of a secondary transmitter (*e.g.* Wiley *et al*, 1988; Bauer *et al*, 1991).

Within the vasculature the dilation induced by a large number of substances such as ATP and bradykinin (see Kerwin & Heller, 1994) could be described as non-neuronal since the relaxation itself is due to the release of NO from the vascular endothelium rather than the "primary transmitter" (see Kerwin & Heller,

1994; Moncada *et al*, 1991), although this may, initially, be released from a neuronal source. However, where mechanical stimuli, such as shear stress, stimulates endothelial NO production (Pohl *et al*, 1991; Shen *et al*, 1992) this may be considered as true non-neuronally-mediated relaxation.

One example of non-neuronally-acting drugs, *i.e.* drugs that do not mimic any known transmitter or transduction mechanism, are the so-called K^+ channel opening drugs such as cromakalim (Hamilton *et al*, 1986) and other compounds based on a benzopyran nucleus (see Edwards & Weston, 1990). The ability of these drugs to hyperpolarize, and induce relaxation of, smooth muscle has direct therapeutic implications, for example, in the treatment of hypertension. They are believed to mediate their inhibitory effects by, as their name suggests, opening K^+ channels, possibly K_{ATP} (Sanguinetti *et al*, 1988; Buckingham *et al*, 1989; Garrino *et al*, 1989), although this has been disputed (Weston & Edwards, 1992; Carl *et al*, 1992; Quast, 1993).

One other group of pharmacological agents that may modify membrane responses to both excitatory and inhibitory stimuli are the so-called membrane "stabilizers" and "labilizers" (Shanes, 1958). These compounds were first described in the context of neuronal activity, but the term has now been extended to also include muscle activity. Membrane stabilizers such as quinidine, procaine, cocaine, lignocaine and lanthanum (Shanes, 1958; Bowman & Hall, 1970; Bülbring & Kuriyama, 1973; Marshall & Kroeger, 1973) as their name suggests, stabilize the membrane by inhibiting changes in its permeability to either Ca^{2+} (Marshall & Kroeger, 1973) and/or K^+ ions (Shanes, 1948 a) thereby inhibiting voltage-dependent changes in tone. For example, procaine and lignocaine inhibited α-, but not β-adrenoceptor-mediated relaxation, of rabbit intestine (Bowman & Hall, 1970) and cocaine inhibited the potassium depolarization of muscle (Shanes, 1948 a, b). Labilizers, on the other hand, are the reverse of stabilizers and

accentuate electrical change. These include veratrum alkaliods, calcium chelating agents and low calcium. As with the stabilizers, labilizers do not generally alter membrane potential *per se* but accentuate changes in membrnae potential such as CO_2-induced hyperpolarization (Shanes, 1951), and potassium and sodium-induced depolarization, of nerves (Stämpfli & Nishie, 1956).

3) RELAXANT TRANSDUCTION MECHANISMS

The neuronal control of smooth muscle relaxation has so far been discussed in terms of inhibitory chemical messengers liberated from neurons. However, before relaxation occurs in response to these chemical messengers, mechanisms function to translate the primary signal, delivered by the chemical mediator, into a cellular response. Initially, this is achieved through the interaction of the messenger with cellular receptors located either in the cell membrane or in the cytosol. A number of mechanisms exist to transduce the agonist message into a cellular response (summarised in Fig. 2) but, with the exception of guanylyl cyclase, the receptor for NO, inhibitory receptors within the GI tract initially trigger relaxation only following an agonist-induced conformational change. This enables the receptors to interact with, and activate, specific membrane-bound proteins, G proteins, following the binding of guanosine triphosphate. The G proteins in turn, stimulate the formation of specific intracellular second messengers via the activation of membrane-associated enzymes (Ross, 1989). The second messengers trigger relaxation by reducing free $[Ca^{2+}]_i$ within the smooth muscle cell (summarised in Fig. 3).

Of the known second messengers systems, the inositol phosphates, diacyl glycerol (DAG) and the cyclic nucleotides, cAMP and cGMP are the best

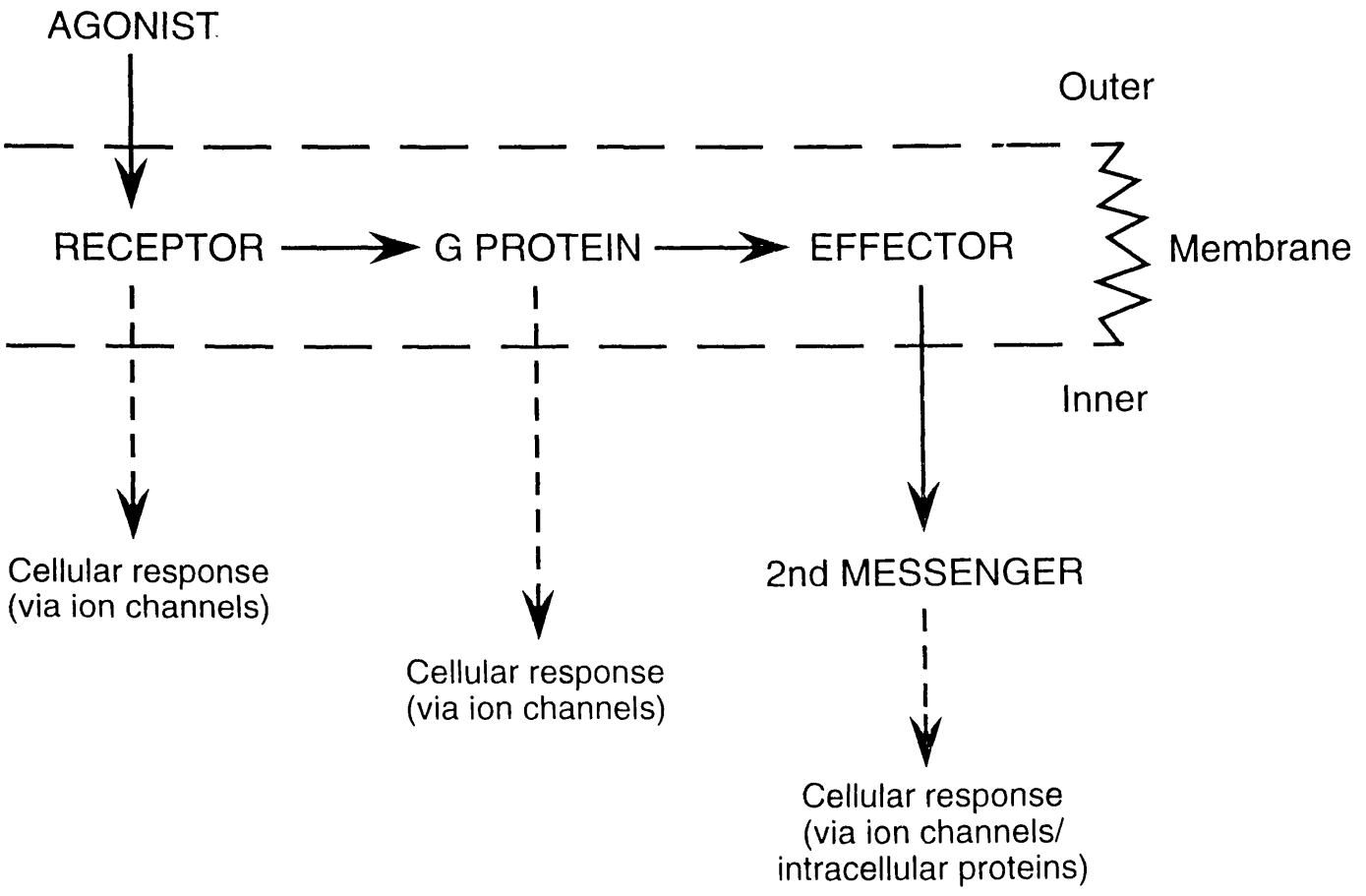


Figure 2: Representation of alternative signal cascades involved in agonist-receptor interactions.

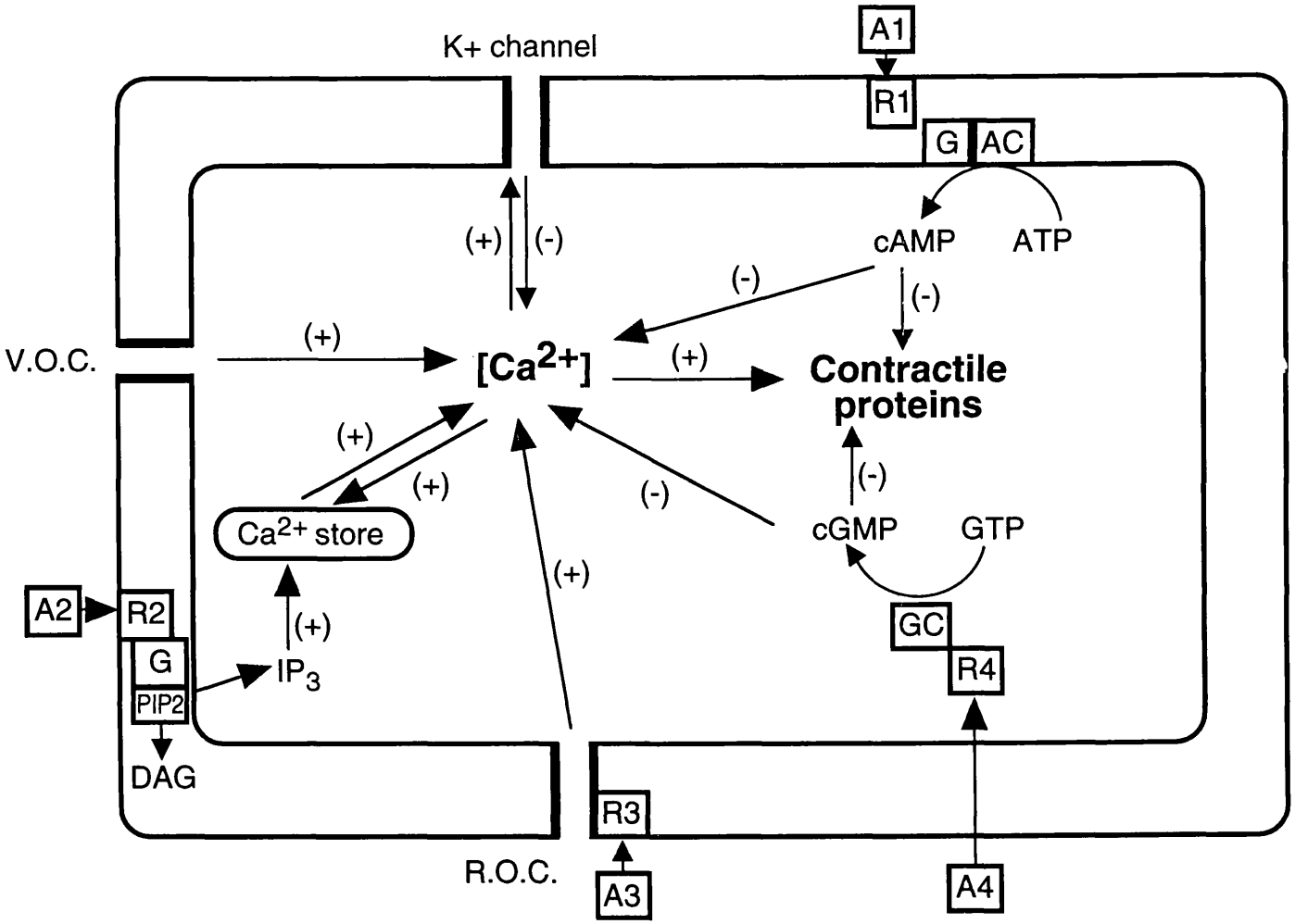


Figure 3: Representation of a smooth muscle cell with possible mechanisms involved in controlling $[Ca^{2+}]_i$. Abbreviations used: A1-4, agonist; R1-4, receptor; G, G protein; AC, adenylyl cyclase; GC, guanylyl cyclase; PIP2, phosphatidyl inositol 4,5,-bisphosphate; DAG, diacylglycerol; V.O.C., voltage operated channel; R.O.C., receptor operated channel; +, stimulatory effect; -, inhibitory effect.

understood. How these second messengers are activated and manipulate free $[Ca^{2+}]_i$ levels will be discussed.

I) <u>The Phosphoinositide System and Ca^{2+} regulation</u>

Four decades have passed since the discovery that stimulation of muscarinic acetylcholine receptors provoked a striking enhancement in the turnover of the minor cell membrane constituent phosphatidyl inositol (PI) and its precursor phosphatidic acid (Hokin & Hokin, 1953). It was not until the mid 1970`s however, that the role of polyphosphoinositides in receptor function, and their direct link with $[Ca^{2+}]_i$ mobilization and PI turnover levels was fully acknowledged (see Michell, 1975). Stimulation of over twenty different types of cell-surface receptors leads to the hydrolysis of phosphatidyl 4,5-bisphosphate (Kirk *et al*, 1981), yielding the biologically active second messengers inositol 1,4,5-trisphosphate (IP_3), DAG (Berridge, 1987; Berridge & Irvine, 1989; Chuang, 1989; Joseph & Williamson, 1989) and, possibly, inositol 1,3,4,5-tetrakisphosphate (IP_4; Joseph & Williamson, 1989; Benham, 1992). This hydrolysis is catalysed by the membrane-associated enzyme phospholipase C (PLC) which is coupled to the receptor by a transducing G_s protein (Smrcka *et al*, 1993).

DAG is a lipid soluble second messenger molecule which remains in the plane of the plasma membrane to activate another membrane-bound enzyme, protein kinase C. This kinase phosphorylates a number of proteins in cell-free systems (Benham, 1992) but *in vivo* it is likely that its physiological targets are more limited. Postulated protein kinase C actions on acute signalling processes can be broken down into processes that are synergistic to the parallel Ca^{2+} mobilization, such as maintained smooth muscle contraction (Benham, 1992; Rasmussen, 1989), and a negative feedback role, which either involves inhibiting

inositol phospholipid breakdown or an increase in the rate of Ca^{2+} efflux from cells (Nishizuka, 1986).

IP_3 stimulates the release of Ca^{2+} from a non-mitochondrial store (Joseph & Willaimson, 1989; Nahorski, 1988; Nahorski & Potter, 1989), probably the sarcoplasmic or endoplasmic reticulum (Ross *et al*, 1989), by coupling to a specific intracellular receptor protein (Furuichi *et al*, 1989). The Ca^{2+} so released is generally associated with smooth muscle contraction (Ochs, 1986), *i.e.* via α_1-adrenoceptors in the vasculature (Minneman, 1988). Conversely however, α_1-adrenoceptor stimulation within GI smooth muscle, despite also causing IP_3 generation (Nelemans & Den Hertog 1987 a, b), hyperpolarizes and relaxes smooth muscle. This may be due to elevated IP_3-stimulated Ca^{2+} levels, which are probably restricted to the membrane, and which cause a membrane hyperpolarization by increasing the open probability of Ca^{2+}-activated K^+ channels (Bennett *et al*, 1963; Holman, 1970; Tomita, 1972; Den Hertog, 1981, 1982; Maas *et al*, 1980; Nelemans & Den Hertog 1987 a,b). This hypothesis is supported by patch-clamp studies which have demonstrated that noradrenaline application, to the outside of single cells, increased the open probability of these channels, an effect similar to that produced by increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$; see Bülbring & Tomita, 1987).

The question arises however, as to how hyperpolarization of the cell membrane, results in relaxation. $[Ca^{2+}]_i$ is the prime regulator of smooth muscle tone, both in the vasculature and GI tract (Rembold & Murphy, 1988, Bülbring & Tomita, 1987), and maintenance of tone depends on the long term balance between the entry of extracellular Ca^{2+} and its extrusion (Ashida & Blaustein, 1987; Quayle *et al*, 1990). Hyperpolarization, in order to produce relaxation, probably interferes with this $[Ca^{2+}]_i$ homeostasis. Within spontaneously active smooth muscle, calcium entry is largely regulated by Ca^{2+} channels sensitive to membrane

potential (see Sanders, 1989), such that lower (depolarized) membrane potentials increase the open probability (Droogmans & Callewaert, 1986) of these channels and *vice versa* for more negative membrane potentials. As a consequence of this coupling, any stimulus causing even a slight hyperpolarization of the membrane such as inhibitory NANC nerve stimulation or K_{ATP} openers blocks Ca^{2+} entry and therefore reduces tone.

Of the proposed NANC neuronally-released substances ATP, via activation of P_{2y}-purinoceptors, stimulates PLC activation and $[Ca^{2+}]_i$ mobilization within several systems (*e.g.* primary cultures of sheep anterior pituitary cells - Van Der Merwe *et al*, 1989; turkey erythrocyte membranes - Berrie *et al*, 1989; Boyer *et al*,1989; Cooper *et al*, 1989, Harden *et al*, 1990; and vascular and adrenal endothelial cells - Forsberg *et al*, 1987; Pirotton *et al*, 1987; Boeynaems & Pearson, 1990). Within GI smooth muscle, P_{2y}-purinoceptors, like α_1-adrenoceptors, are associated with hyperpolarization and relaxation (see Burnstock & Kennedy, 1985; Lim, 1985; Kennedy, 1990). To date however, only one study, using canine kidney cells, has directly implicated an ATP-induced, IP_3-stimulated rise in $[Ca^{2+}]_i$ in producing membrane hyperpolarization (Paulmichl & Lang, 1991). This system could also account for the ATP-induced hyperpolarization and relaxation of GI smooth muscle.

II) <u>Cyclic Adenosine 3`,5`-Monophosphate (cAMP)</u>

Relaxation of smooth muscle is more commonly associated with elevations of cAMP, as well as cGMP (see later). The identification of the second messenger, cAMP (Sutherland & Rall, 1958), was a landmark step in the discovery of how a number of neurotransmitters, hormones and drugs produced their inhibitory effects on target tissues. Cyclic AMP is synthesised within cells by the enzyme adenylyl

cyclase via the catalytic formation of cAMP from ATP (see Greengard *et al*, 1972) following receptor, and subsequent G protein, activation.

Initially, although there was a clear correlation between a rise in intracellular cAMP levels ($[cAMP]_i$) and relaxation (see Andersson *et al*, 1972), the mechanism of cAMP-induced relaxation remained obscure until the discovery of an intracellular receptor protein for cAMP; cAMP-dependent protein kinase (PKA), in skeletal muscle (Walsh *et al*, 1968) and liver (Langan, 1968). PKA, which exists as several different isozymes (Francis & Corbin, 1994), is found in all mammalian tissues (Francis & Corbin, 1994) and mediates the majority (Beebe & Corbin, 1986; Barnette *et al*, 1990), although not all (Nakamura & Gold, 1987), of the effects of cAMP.

The inhibitory effects of cAMP/PKA are attributable to the regulation of $[Ca^{2+}]_i$. This regulation can take several forms. Either by:-

a) <u>Promoting Ca^{2+} Extrusion from the Cell</u>: Calcium extrusion is probably achieved by activation of the two major energy-linked Ca^{2+} efflux pathways across the plasma membrane; the Ca^{2+}-$2H^+$-ATPase (or Ca^{2+} pump) (Niggli *et al*, 1982; Smallwood *et al*, 1983) and the $3Na^+/Ca^{2+}$ exchanger, which is driven by the $[Na^+]$ gradient that exists across the plasma membrane, maintained by the Na^+/K^+-ATPase (Carafoli, 1987; Nicholls, 1986; Borle, 1981; Rasmussen & Rasmussen, 1990). Both ATPases may undergo a cAMP/PKA-dependent increase in activity (Scheid *et al*, 1979), enhancing the extrusion of $[Ca^{2+}]_i$. Isoprenaline, which relaxes and promotes increased intracellular cAMP levels in most smooth muscles (Andersson *et al*, 1972), is proposed to produce its inhibitory effects by promoting Ca^{2+} extrusion (Marshall & Kroeger, 1973; Bülbring & Den Hertog, 1980).

b) Promoting Intracellular Sequestration or Binding of Ca^{2+}: Cyclic AMP and PKA have been implicated in the accumulation of Ca^{2+} into an intracellular storage site within smooth muscle (Andersson & Nilsson, 1972; Casteels & Raeymakers, 1979). The most likely intracellular structure to be involved in this process is the sarcoplasmic reticulum (S.R.). The S.R. accumulates Ca^{2+} from the cytosol through a specific ATPase (Tada & Katz, 1982; Carafoli, 1987), therefore its activation, possibly by PKA, would increase Ca^{2+} sequestration.

c) Modifying the Activity of the Contractile Apparatus: Cyclic AMP is also proposed to induce relaxation via the suppression of Ca^{2+} binding, required to activate the contractile proteins actin and myosin (Adelstein *et al*, 1982; Nishimura & Van Breeman, 1989; Smith *et al*, 1993). Phosphorylation of the Ca^{2+}-calmodulin-dependent enzyme, MLCK, by PKA (Conti & Adelstein, 1980; Adelstein *et al*, 1981), led to a shift in the Ca^{2+} dependency of MLCK, such that a higher Ca^{2+} concentration was required to activate the enzyme (Adelstein *et al*, 1982). Although this has been demonstrated in skinned smooth muscle fibres (Meisheri & Rüegg, 1983; Meisheri *et al*, 1986 a) it has yet to be clearly demonstrated *in vivo* (Miller *et al*, 1983; Obara & De Lanerolle, 1989).

d) Inhibiting the Formation and Action of Inositol Phosphates: Cyclic AMP inhibited the agonist-induced hydrolysis of polyphosphoinositides (Hall *et al*, 1989; Hall & Hill, 1988; Jones *et al*, 1987; Madison *et al*, 1988). It also antagonized agonist-induced, IP_3-dependent, Ca^{2+} release (Murthy *et al*, 1993 a) probably as a result of PKA-dependent phosphorylation of the IP_3 receptor situated on the S.R. (Supattapone *et al*, 1988).

e) Reducing Extracellular Ca^{2+} Entry by Channel Closure: This occurs in several smooth muscles such as guinea-pig taenia caeci (Den Hertog *et al*, 1985, Bülbring & Tomita, 1987), dispersed smooth muscle cells (Yamaguchi *et al*, 1988) and the canine colon (Smith *et al*, 1993; Du *et al*, 1994) where forskolin-stimulated adenylyl cyclase activity produced membrane hyperpolarization and relaxation. Membrane hyperpolarization, probably a result of K^+ efflux (Carl *et al*, 1991; Smith *et al*, 1993; Du *et al*, 1994), would decrease the open probability of voltage-dependent Ca^{2+} channels (Langton *et al*, 1989) thereby inhibiting Ca^{2+} entry.

III) Cyclic Guanosine 3`, 5`-Monophosphate (cGMP)

Although cGMP was discovered shortly after cAMP (Ashman *et al*, 1963), its role in biological regulation has been, until recently, considerably less well understood than that of cAMP. It is a ubiquitous second messenger which regulates many cell functions including smooth muscle relaxation (for review see Waldman & Murad, 1987; Tremblay *et al*, 1988;). Cyclic GMP formation, from guanosine triphosphate, is catalysed by the action of an enzyme, guanylyl cyclase, which exists in both particulate and soluble forms (Hardman & Sutherland, 1969; Schultz *et al*, 1969; White & Aurbach, 1969). The importance of cGMP as a smooth muscle relaxant has recently come to prominence with the identification of the putative transmitter NO (see **Non-Adrenergic, Non-Cholinergic (NANC)-Mediated Relaxation**) which mediates its widespread inhibitory effects by increasing intracellular cGMP levels (see Kerwin & Heller, 1994).

It was originally thought (Goldberg *et al*, 1973) that cGMP stimulated the contraction of smooth muscle (Dunham *et al*, 1974; Clyman *et al*, 1975; Amer & McKinney, 1975; Andersson *et al*, 1975), however the contractions described in these cases preceded the rises in cGMP by as much as fifteen seconds (Diamond &

Hartle, 1976) indicating that the cGMP elevation was a result of, rather than the cause of, the contractions (Diamond & Hartle, 1974; Diamond & Holmes, 1976). It is now accepted that cGMP relaxes, rather than contracts, smooth muscle. Evidence for this was obtained using nitrovasodilators, which release the smooth muscle relaxant NO (Ignarro & Kadowitz, 1985; Murad, 1986; Waldman & Murad, 1987; Kerwin & Heller, 1994; see below), and produced relaxation concomitantly with increased intracellular cGMP levels ($[cGMP]_i$; *e.g.* in rat myometrium (Diamond & Holmes, 1975) canine femoral artery (Diamond & Blisard, 1976) and several other smooth muscles (Schultz *et al*, 1977; Katsuki *et al*, 1977 a, b)). NO increases cGMP levels via activation of soluble guanylyl cyclase (Murad & Aurbach, 1977; Katsuki *et al*, 1977 a; Arnold *et al*, 1977; Murad *et al*, 1978, 1981) by binding to its active haem centre (Gerzer *et al*, 1988). The evidence is now such, that it is almost universally accepted that cGMP is the inhibitory second messenger for NO (see Nakatsu & Diamond, 1989; Ward *et al*, 1992 a).

The intracellular receptor protein for cGMP, cGMP-dependent protein kinase (PKG), was first described in 1970 (Kuo & Greengard, 1970). PKG is closely related in structure and function to PKA, discussed earlier, but is less widely distributed, with highest concentrations found in smooth muscles (Francis & Corbin, 1994). PKG is probably responsible for mediating the majority, but not all (Wrenn & Kuo, 1981; Hartzell & Fischmeister, 1986; Hofmann *et al*, 1992; Altenhofen *et al*, 1991), of the inhibitory effects of cGMP within smooth muscles (see Francis & Corbin, 1994) by modulating smooth muscle $[Ca^{2+}]_i$ metabolism (Collins *et al*, 1986; Schini *et al*, 1987; Bukoski *et al*, 1989; Morgan & Morgan, 1984). One, or several, of the following mechanisms could explain the cGMP/PKG-evoked relaxation. Either by:-

a) Promoting Ca^{2+} Extrusion from the Cell: Ca^{2+} efflux may be achieved by PKG-dependent activation of the plasma membrane associated, calmodulin-stimulated Ca^{2+}-ATPase (Popescu *et al*, 1985; Rashatwar *et al*, 1987; Yoshida *et al*, 1991) although a direct phosphorylation of the ATPase has yet to be demonstrated (Vrolix *et al*, 1988). A second mechanism could be the stimulation of the $3Na^{+}/Ca^{2+}$-exchanger, possibly via stimulation of the Na^{+}/K^{+}-ATPase, which has been demonstrated in vascular smooth muscle cells (Furukawa *et al*, 1991).

b) Promoting Intracellular Ca^{2+} Sequestration or Binding: Most evidence for this mechanism comes from vascular smooth muscle. Nitroprusside and the membrane permeable cGMP analogue, 8-bromo-cGMP, each inhibited noradrenaline-induced contractions of rat aortic rings in Ca^{2+}-free medium (Lincoln, 1983; Karaki *et al*, 1988), suggesting intracellular sequestration of Ca^{2+}, stimulated by cGMP. The intracellular store for this sequestered Ca^{2+} is probably the S.R.. Cyclic GMP directly increased Ca^{2+} uptake into the S.R. of rat aortic cells (Twort *et al*, 1988) and selective inhibitors of the S.R. Ca^{2+}-ATPase, inhibited nitroglycerin-induced relaxations (Luo *et al*, 1993). These processes are probably regulated by PKG (Cornwell & Lincoln, 1989). Indeed, PKG catalyzed the phosphorylation of the S.R. Ca^{2+}-ATPase regulatory protein, phospholamban, *in vitro* (Sarcevic *et al*, 1989; Cornwell *et al*, 1991; Karczewski *et al*, 1992) which was correlated with its activation and reduced $[Ca^{2+}]_i$.

c) Modifying the Activity of the Contractile Apparatus: As with cAMP and PKA, cGMP-activated PKG is probably involved in decreasing the Ca^{2+}

sensitivity of MLCK (Adelstein *et al*, 1982; Pfitzer *et al*, 1982; Karaki *et al*, 1988; Rapoport & Murad, 1983; Nishimura & Van Breeman, 1989; McDaniel *et al*, 1992; Smith *et al*, 1993) although, to date, this hypothesis lacks direct experimental support. PKG may also activate protein phosphatases, resulting in dephosphorylation of the contractile protein, myosin light chain, thereby eliciting a relaxation (Driska *et al*, 1989; see Fig 1).

d) Inhibiting the Formation and Action of Inositol Phosphates: Several investigators have suggested that cGMP elevations inhibit the release of Ca^{2+} from IP_3-sensitive storage sites in smooth muscle cells (Collins *et al*, 1986; Godfraind, 1986; Meisheri *et al*, 1986 b; Rapoport, 1986; Jin *et al*, 1993; Murthy *et al*, 1993 a). Several lines of evidence support this; transfected PKG inhibited IP_3 formation and thrombin-evoked $[Ca^{2+}]_i$ mobilization in chinese hamster ovary cells (Ruth *et al*, 1993). PLC activation, which stimulates IP_3 formation, was also inhibitied due to the phosphorylation of one or more G proteins by PKG thereby inhibiting their GTPase activity (Hirata *et al*,1990; Light *et al*,1990). Recently PKG, like PKA, has been shown to phosphorylate the IP_3 receptor, inhibiting IP_3 receptor-dependent Ca^{2+} activation (Komalavilas & Lincoln, 1994).

e) Reducing Extracellular Ca^{2+} Entry by Channel Closure: NO, and NO-donors, presumably acting via cGMP, and possibly PKG, hyperpolarized certain vascular and non-vascular tissues such as the canine proximal colon (Thornbury *et al*, 1991; Ward *et al*, 1992 a; Koh *et al*, 1995) and pyloric sphincter (Bayguinov & Sanders, 1993), opossum oesophagus (Du *et al*, 1991; Conklin & Du, 1992), rabbit basilar and canine and porcine coronary arteries (Tare *et al*, 1990; Rand & Garland, 1992; Taniguchi *et al*, 1993). Recent evidence suggests that within the canine colon there are three types of K^+ channel which are activated

by NO or NO donors (Koh *et al*, 1995). One of these channel types is the BK, Ca^{2+}-activated K^+ channel, which corresponds with previous studies on the

channel-opening effects of NO (Thornbury *et al*, 1991; Murray *et al*, 1994; Khan *et al*, 1993). When activated, these channels hyperpolarize the membrane and inhibit Ca^{2+} entry through voltage-activated Ca^{2+} channels, thereby producing a relaxation. Significantly, Ca^{2+}-activated K^+ channels are also targets for PKG (Robertson *et al*, 1993; Taniguchi *et al*, 1993; White *et al*, 1993).

The relative importance of these various $[Ca^{2+}]_i$ control systems probably varies within different tissues and it may be that, in certain cases, more than one of these systems is activated at a time to induce relaxation.

IV) Cyclic Nucleotide Cross-Activation of Protein Kinases

Smooth muscle relaxation is not always due to the actions of one biochemical process acting in isolation from other intracellular mediators. The phenomenon of cross-activation, defined as a cross interaction between two or more second messenger pathways under physiological conditions, is believed to occur with cyclic nucleotides and their respective protein kinases.

Originally, the cyclic nucleotide specificities of PKA and PKG were thought to serve as precise means of regulating cellular function, allowing the changes in only one cyclic nucleotide, followed by activation of its protein kinase, to control a particular regulatory pathway (Lincoln & Corbin, 1983). However, PKA and PKG are homologous proteins that share similar functions (Gill *et al*, 1976; Corbin & Lincoln, 1978; Lincoln & Corbin, 1983; Takio *et al*, 1984; Hanks *et al*, 1988; Scott, 1991; Hoffmann *et al*, 1992) and a structural similarity between their cyclic nucleotide binding sites (Weber *et al*, 1987, 1989). Because of this

structural similarity PKG is able to bind cAMP, although with an affinity some 50 times lower than that of PKA for cAMP. However, autophosphorylation of PKG improves its affinity for cAMP by up to 10 times (Foster *et al*, 1981; Landgraff *et al*, 1986). This, paired with the fact that cAMP levels in most smooth muscles can be up to 5 times higher than cGMP levels (Francis *et al*, 1988) means that small elevations in $[cAMP]_i$ could cross-activate PKG, possibly producing relaxation. Support for this has recently emerged; relaxing agents which raised $[cAMP]_i$ in gastric muscle cells and pig coronary arteries, besides activating PKA, also increased PKG activity (Jiang *et al*, 1992; Jin *et al*, 1993). Indeed, in some cases PKG may mediate the relaxation of both cyclic nucleotides (Cornwell & L incoln, 1989; Lincoln *et al*, 1990; Lincoln & Cornwell 1991), for example;

1) PKG-specific analogues, but not PKA-specific ones, relaxed guinea-pig coronary arterial and tracheal smooth muscle (Francis *et al*, 1988).

2) Restoration of PKG, but not PKA, to PKG-depleted aortic smooth muscle cells restored the Ca^{2+}-lowering (*i.e.* relaxing) effects elicited by either cGMP or cAMP (Lincoln *et al*,1990).

3) Low concentrations of cAMP increased, whereas cGMP and higher concentrations of cAMP inhibited, Ca^{2+} channel opening in rabbit portal vein cells (Ishikawa *et al*, 1993), indicating a cAMP concentration-dependent activation of PKG.

PKA may also be cross-activated by cGMP, but this is rare and not directly applicable to smooth muscle relaxation. For example, the cGMP-induced accumulation by a heat stable enterotoxin in intestinal epithelial cells, increased chloride conductance (Forte *et al*, 1992), an effect mimicked by cAMP analogues that potently activate PKA. This contrasts with the ineffectiveness of cGMP analogues. PKA is present within these cells whereas there is no measurable PKG

(Forte *et al*, 1992). This type of cross-activation may also occur *in vivo* for example in Leydig cells where atrial natriuretic factor-induced cGMP increases are proposed to increase testosterone production by activating PKA (Schumacher *et al*, 1992).

In general, although the effector specificity of protein kinases provides a mechanism for defined and precise regulation of cellular function, cross-activation of these enzymes permits some flexibility in this regulation, perhaps reflecting an opportunist mechanism of the cell, *i.e.* the utilisation of an additional second messenger to regulate a pathway that is already in place. This may be analogous to the situation whereby two inhibitory transmitters, which perform essentially the same function, appear to be released together within the same tissue (see **Synergistic Transmitter-Mediated Relaxation**).

4) AIMS

Clearly, relaxation of smooth muscle involves several different processes, from the initial agonist-receptor interaction to the ultimate movement of the actin and myosin filaments creating the relaxation event. This thesis has targeted, for study, the identification of the transmitter processes underlying relaxation, which encompasses both the putative transmitter substance(s), and the intracellular transduction process(es) activated by the transmitter(s). It represents my contribution to furthering the understanding of one particular aspect of neuronally-mediated relaxation namely, NANC-mediated relaxation.

This can no longer be regarded as involving a single inhibitory mediator and is now commonly accepted as being the result of two or more substances. The added complexity of such a system requires definitive investigation to determine

the nature of the substances involved. For this purpose electrical, mechanical and biochemical procedures were employed utilising the spontaneously active, non-propulsive smooth muscle of the gpIAS, eminently suited to this purpose, possessing as it did, a well defined and developed NANC inhibitory innervation which was manifested as large IJPs and relaxations in response to nerve stimulation.

A study of the membrane responses to neuronally-released transmitters, using intracellular electrical recording, simultaneously with corresponding mechanical events, was undertaken. The electrical changes produced by nerve stimulation were measured as IJPs, the initial responses to neuronally released inhibitory transmitters. The electrically-evoked IJPs comprised two components, the nature of which, by pharmacological analysis, were found to be ATP or a closely-related analogue and NO, or a related substance.

The IJPs produced by nerve-stimulated release of these substances acting on the tissue are a result of large changes in ionic fluxes across the cell membrane. The identity of the ion(s) responsible for the separate IJP components, as revealed pharmacologically and by altering the external ionic enviroment of the tissue, probably involve K^+ but not Cl^-.

The electrical responses which preceded relaxation were, however, only one aspect of the inhibitory response. Accompanying these were changes in the levels of the intracellular cyclic nucleotides most often associated with relaxation, cAMP and cGMP. These changes, in response to drugs and field stimulation, were studied a) indirectly, using drugs which modulated the activity of the cyclic nucleotides and b) directly, by measuring the levels of the intracellular cyclic nucleotides using radioimmunoassay.

By combining the information obtained from the two aspects of this study, a direct correlation could be made between the electrical, mechanical and

biochemical events involved in the relaxation of the gpIAS, thereby producing a more integrated picture of relaxation in this tissue and fulfilling the aims of this thesis.

METHODS AND MATERIALS

1) SPHINCTERIC SMOOTH MUSCLE

The GI sphincters are located at various levels of the alimentary canal, separating specialised regions of the tract, where they function to prevent the reflux of contents and ensure their distal propulsion. No major anatomical differences distinguish sphincteric from non-sphincteric tissues, apart from a greater density of circular muscle in the former. However, due to their dense inhibitory NANC innervation, each sphincter responds to nerve stimulation with a profound relaxation. This inhibitory innervation, combined with the inherent tone displayed by most sphincters, makes them especially useful for the study of relaxation (for general review of sphincteric function see Papasova, 1989). Within this context the gpIAS was chosen for further study because of its particularly well developed inhibitory innervation, even in relation to other sphincters, which is manifested as large inhibitory junction potentials and reductions in tone in response to nerve stimulation.

I) Guinea-Pig Internal Anal Sphincter (gpIAS)

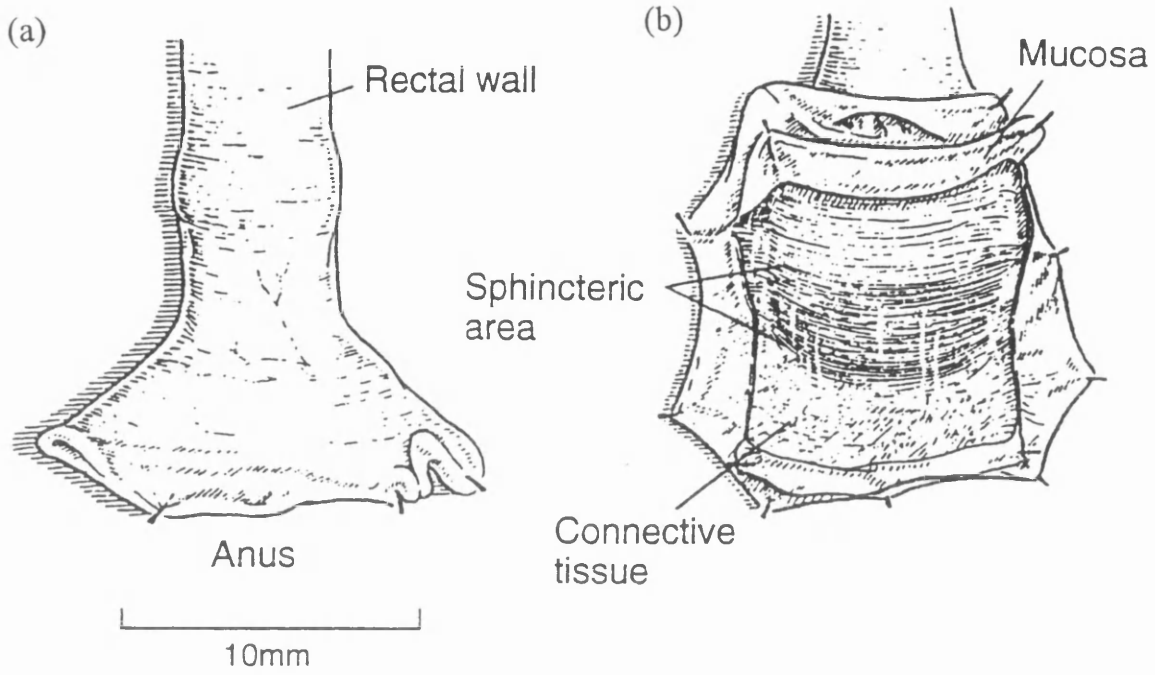
The sphincteric region at the distal end of the digestive tract actually consists of two sphincters, the internal and external anal sphincters. However the external anal sphincter is skeletal rather than smooth muscle. The internal anal sphincter (IAS) is responsible for the involuntary retention of contents in the anal canal and is nearly always in a contracted state, resulting in a higher intraluminal pressure than the rectum (see Papasova, 1989).

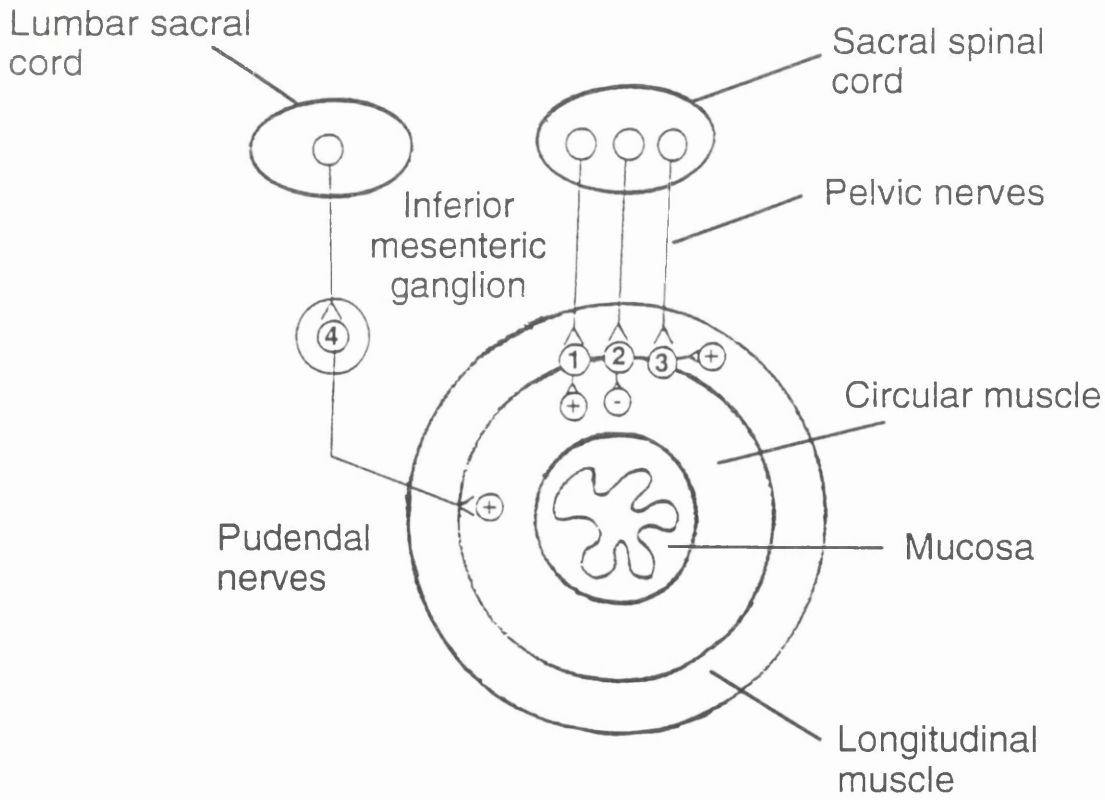
The anatomy of the anal region in the guinea-pig has been described by Furness and Costa (1973). The guinea-pig IAS coincides with a thickening of the rectal wall (approximately 15-20mm long x 5mm broad), which can usually be recognised externally, and consists of a band of circular fibres more densely arranged than elsewhere in the rectoanal region (Fig. 4). The rectum and IAS consists of two muscle layers, the outer longitudinal muscle extending over the inner circular sphincteric muscle. Each muscle layer terminates some 5-10 mm

short of the anal margin and is separated from the skin by a band of largely connective tissue with a few circular muscle fibres. The term gpIAS throughout this thesis refers to the circular muscle free from the longitudinal muscle layer (Fig. 4).

The gpIAS is innervated by three types of efferent nerves. First, sympathetic noradrenergic excitatory innervation. Pre-ganglionic axons emanating from the lumbar spinal cord synapse in the inferior mesenteric ganglion with postganglionic noradrenergic neurons. The latter directly innervate the gpIAS via the pudendal nerves. Secondly, cholinergic excitatory nerves running via the pelvic nerves enter both the circular and longitudinal muscles of the IAS and synapse with postganglionic intramural neurons. The third type of efferent nerves 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 neurons in the pelvic plexuses which then enter the IAS (Costa & Furness, 1973) (see Fig. 4). *In vivo* the inhibitory motor responses emanating from the myenteric plexus are activated only during the rectoanal inhibitory reflex caused by rectal distension (Costa & Furness, 1973; Frenckner, 1975; Frenckner & Ihre, 1976; Tamura *et al*, 1989). *In vitro* electrical stimulation of the IAS results in a profound relaxation in this (Crema *et al*, 1983; Lim & Muir, 1983, 1985, 1986) and other species such as humans (Burleigh *et al*, 1973, 1979; Burleigh, 1991, 1992) and monkeys (Rayner, 1979). Proposed mediators of these inhibitory responses are ATP (Lim & Muir, 1986), VIP (Biancani *et al*, 1984, 1985; Rattan & Chakder, 1992) and, more recently, NO (Burleigh, 1991, 1992; Chakder & Rattan, 1992, 1993 a, b; Craig & Muir, 1991; McKirdy, 1992; O'Kelly *et al*, 1992, 1993; Rattan *et al*, 1992; Rattan & Chakder, 1992; Tøttrup *et al*, 1992).

Figure 4 Top: Position of the guinea-pig internal anal sphincter (x5). In the stretched, intact 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 submucosa removed. The sphincter can be identified as a band of circular smooth muscle in which the fibres are more densely arranged than elsewhere in the rectal wall. The sphincter is thus easily differentiated from the connective tissue, which is adjacent to the anus. **Bottom**: Schematic representation of the innervation of the internal anal sphincter. 1 & 3 are cholinergic intramural excitatory neurons; 2 is a intramural inhibitory NANC neuron; and 4 is a sympathetic excitatory neuron. (Adapted from Gonella, Bouvier & Blanquet, 1987).





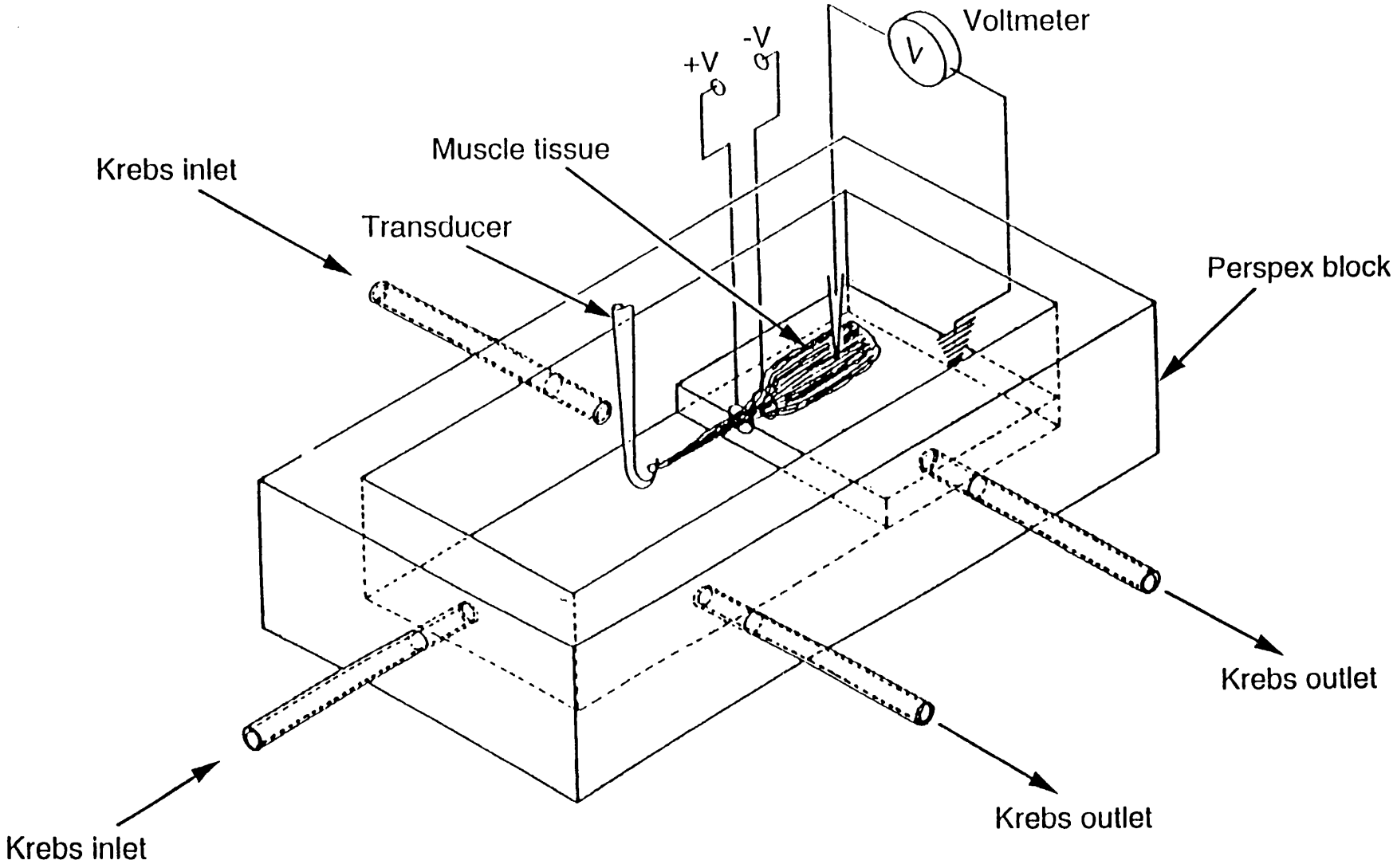
1 & **3** - Cholinergic intramural neurones
2 - Intramural inhibitory NANC neurone
4 - Sympathetic excitatory neurone

2) DISSECTION

I) Guinea-Pig Internal Anal Sphincter

Dunkin-Hartley guinea-pigs (250-500g) of either sex were killed by cervical dislocation and bled. Following a mid-line incision, the peritoneal cavity was opened, and the descending colon exposed as it passed into the pelvic cavity. The pubic symphisis was split and a segment of the rectum, including the anal region (~5cm), removed and transferred to a Sylgard-coated petri dish containing oxygenated Krebs solution (95% O_2, 5% CO_2). A longitudinal incision was made in the rectoanal canal from the anal end. The rectum was pinned out, and the skeletal and connective tissue forming the external sphincter, removed. The mucosa and submucosa were removed and the sphincter identified with the aid of a dissecting microscope (Fig. 3). The longitudinal muscle layer was carefully removed to leave only the circular muscle of the IAS. The gpIAS was then transferred to a horizontal organ bath and pinned to the Sylgard-coated base (Fig. 5). It was continually perfused with oxygenated Krebs solution, using a pump (Gilson minipuls 3; 4ml min^{-1}), at 37±0.5°C, containing atropine and phentolamine (each 1μM), except when stated otherwise. The gpIAS was attached by a thread to an isometric force displacement transducer (Grass FT03C) to measure mechanical activity. The other end was threaded through a bipolar Ag/AgCl ring electrode (o.d. 2mm and 2mm apart) for electrical field stimulation (EFS) which was provided by a Devices type 2521 stimulator, triggered by a Devices Digitimer (0.1ms, 1-40Hz, supramaximal voltage).

Figure 5: Organ bath for simultaneous measurements of intracellular electrical and mechanical activity. 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 (o.d. 2mm) for Krebs` solution perfusion and two outlets for drainage. One end of the tissue was pinned to the Sylgard base of the bath, the other attached, by a thread, to an isometric transducer. Field stimulation by an isolated stimulator was affected via Ag/AgCl ring electrodes (V^+ - V^-). The bath was perfused (4ml min^{-1}), using a pump, with oxygenated, pre-heated Krebs` solution (37±0.5°C), via the two inlets. The polythene tubing (o.d. 2mm) containing the Krebs` solution was surrounded by an outer tubing (o.d. 10mm) containing heated liquid paraffin, circulated by a thermostatically controlled pump (Tempette TE-7).


Voltmeter
V
+V
-V
Muscle tissue
Krebs inlet
Transducer
Perspex block
Krebs outlet
Krebs outlet
Krebs inlet

3) APPARATUS AND TECHNIQUES

I) Intracellular Electrical and Simultaneous Mechanical Recording

The perspex horizontal organ bath (50 x 10 x 10mm; volume 3ml; Figure showing organ bath) was mounted on two non-conducting Bakelite supports attached to a steel plate (200kg) which in turn rested on a marble table, supported by anti-vibration dampers.

The bath was perfused with Krebs' solution, using a pump (Gilson minipuls 3; 4ml min^{-1}) contained in polythene tubing (o.d. 2mm) surrounded by an outer polythene tube (o.d. 10mm) containing liquid paraffin, pumped and heated to 40±0.5°C by a modified Tempette (TE-7) pump in order to maintain the Krebs at the constant temperature stated above.

Other apparatus included Ag/AgCl ring electrodes (o.d. 3mm), capillary glass electrodes (Clark Electromedical Instruments, GC 200F-15), Ag/AgCl plated indifferent electrode, D.C. preamplifier (Neurolog NL102), dual beam storage oscilloscope (Tekronix 5103N), voltmeter and gated pulse generator (Devices type 2521), digital recorder (CRC VR-100B, Instrutech Corp., U.S.A.), VHS video recorder (Sharp VC-A30HM), U.V. oscillograph (6150 Mk II, Thorn EMI) and computer (Dell 486P/33).

Intracellular electrical recordings were made using conventional capillary glass microelectrodes (Clark Electromedical Instruments, GC 200F-15, resistance 10-80MΩ) filled with 3M KCl, connected to a probe. The indifferent electrode was a Ag/AgCl pellet attached to the side of the organ bath and placed in the bathing solution. Electrical signals were passed to a preamplifier (Neurolog NL102) and displayed on a storage oscilloscope (Tektronix 5103N). Electrical and mechanical signals were digitized (CRC VR-100B, Instutech Corp., U.S.A.) and stored on a video cassette recorder (Sharp VC-A30HM) and played back, when required, onto a U.V. oscillograph (6150 Mk II, Thorn EMI)(Fig. 6).

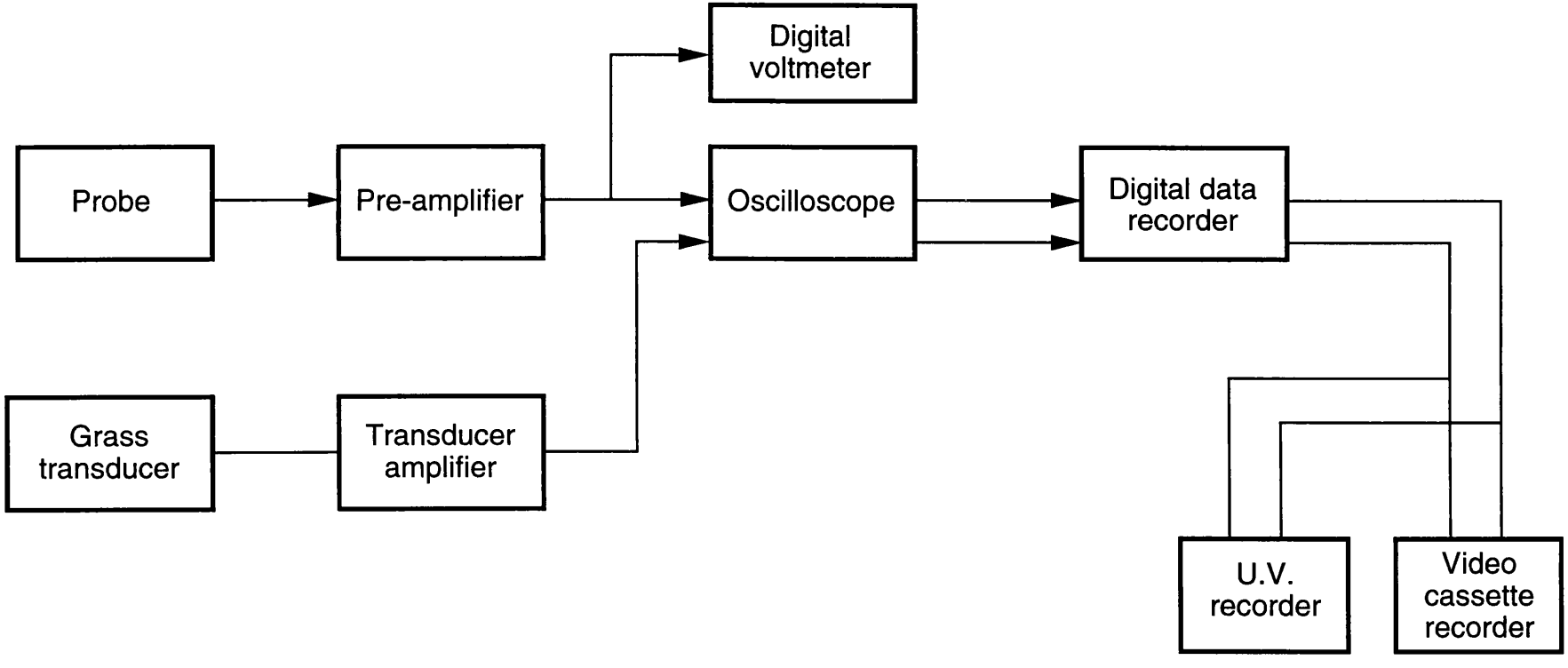


Figure 6: Arrangement of apparatus used to record intracellular electrical and mechanical responses of the gpIAS to drugs and EFS.

II) Criteria For Cell Penetration

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

a) the penetration was sharp and the membrane potential stable, varying by not more than 2mV, over an initial period of approximately 10s,

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

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

III) Administration of Drugs

Drugs were administered in three ways:

1) By addition to the reservoir containing the Krebs' perfusing the organ bath. Drugs were prepared in distilled water before dilution with Krebs' solution and subsequent addition to the reservoir, 2) from a microsyringe (volume 25μl) placed directly into the bath close to the recording site, or 3) by pressure injection using a Picospritzer (Picospritzer II, General Valve Corporation, N.J., U.S.A.). Using this technique, although the concentration of drug may be high, the applied substances are less likely to cause desensitization of the entire tissue because of the small area over which the drug is added. However, for the same reason, no changes in tension are detectable. Using this method drugs were applied, from micropipettes which had their tips broken back to 2-20μM diameter. The pipette tip was placed to within approximately 1mm of the recording site before application of the drugs (20-30 psi). The variation in ejection duration is indicated in the text.

IV) NADPH-Diaphorase Staining

NADPH-diaphorase, is proposed to be nitric oxide synthase (NOS; Hope *et al*, 1991; Valtschanoff *et al*, 1992). Histochemical staining for this enzyme is now routinely used to detect the presence of NOS within tissues. NOS containing regions stained dark blue using this technique.

Following dissection the gpIAS was placed in fixative containing 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS) for 1 hour at 37°C. The tissues were then transferred to a cryoprotectant solution containing 30% sucrose in 0.1M PBS until they sank to the bottom (~15 minutes), signifying that the tissues were saturated with the cryoprotectant, after which they were snap frozen in liquid nitrogen. Transverse and longitudinal sections (20μM) were cut using a Cryocut 1800 (Reichert-Jung). The slivers of tissue were transferred to glycerine-coated slides and incubated in 0.1M PBS containing 0.25% Triton X-100 for 10 minutes. The slides were then placed in a 0.1M PBS solution containing 0.2mg/ml nitro blue tetrazolium and 0.5mg/ml β-NADPH and incubated in the dark at 37°C for one to two hours. The reaction was terminated by washing the slides in 1M PBS. The slides were then dehydrated in increasing concentrations of ethanol (75%, 95% and 100%) for 1 minute at a time and then transferred to increasing concentrations of Histoclear (50%, 75% and 100%) for approximately five minutes each. The slides were allowed to dry before being mounted.

V) Measurement of Cyclic Nucleotides

Levels of both cyclic adenosine 3`, 5`-monophosphate (cAMP) and cyclic guanosine 3`, 5`-monophosphate (cGMP) in the gpIAS were measured by radioimmunoassay (RIA; Steiner *et al*, 1972), using the scintillation proximity assay (SPA) technique. The effects on cyclic nucleotide levels of drugs and field stimulation were measured.

a) Sample Collection

Tissues were dissected out as previously described, weighed, mounted between a pair of platinum electrodes (o.d. 4mm) and placed in a heated vertical organ bath (15ml) containing oxygenated Krebs' solution (95% O_2, 5% CO_2) at 37±0.5°C. Atropine and phentolamine (each 1μM) were always present. The organ bath temperature was maintained using an electric pump (Grant W6).

Tension on the gpIAS was monitored using an isometric force transducer (Maywood, Type 49034). Tissues were stimulated by agonists or an isolated stimulator (Pfizer; 0.1ms, 1-10Hz, supramaximal voltage), with mechanical activity measured and displayed on a pen recorder (Grass or Multitrace 8, Lectromed MT8P).

Upon field stimulation or drug addition, after maximal relaxation had been reached, the tissues were removed rapidly from the organ bath and snap frozen with a pair of stainless steel clamps which had previously been immersed in liquid nitrogen. After clamping, the tissues were immediately immersed in the liquid nitrogen to ensure freezing of the entire tissue. The whole process, from tissue removal to freezing took less than five seconds.

b) Preparation of Samples

Frozen gpIAS samples were thawed in trichloroacetic acid (0.5ml; 10% w/v) and homogenised using a ground glass/glass homogeniser. Cellular debris and precipitated proteins were removed by centrifugation (3000g; 10 min; 4°C). The acid-soluble fraction (the supernatant) was removed and the pellet discarded. The supernatant was washed with water-saturated diethyl ether (4 times with 5 ml), the upper ether layer being discarded each time. Remaining traces of ether were driven off by placing the glass sample tubes in a heated water bath (80°C for 5 mins). The samples were then frozen (-20°C) until required for assay.

c) Radioimmunoassay for cAMP and cGMP

After thawing, an aliquot of each sample was made up to the appropriate volume with sodium acetate buffer (50mM, pH 5.8, 0.01% (w/v) sodium azide; 55µl of sample diluted to 110µl with buffer, for cGMP assay and 10µl of sample diluted to 150µl with buffer for cAMP assay). Both cAMP and cGMP levels were measured using the acetylation method of Harper and Brooker (1975). The assay involves the competition between the unlabelled cyclic nucleotides and a fixed quantity of radioactively (^{125}I)-labelled cAMP or cGMP for a limited number of binding sites on a cGMP specific antibody and a method to separate the antibody-bound from the unbound material. Unlabelled antigens, in this case cAMP or cGMP from samples or standards, together with a fixed amount of radioactively-labelled cyclic nucleotides are allowed to react with a constant amount of primary antibody raised to the appropriate cyclic nucleotide. An inverse relationship therefore exists between the amount of bound radioactivity and the amount of cAMP or cGMP present in the sample, *i.e.* the higher the levels of cyclic nucleotide in the sample the smaller the degree of radioactively-labelled cyclic nucleotide binding to the primary antibody. The antibody-bound cAMP or cGMP is then reacted with the SPA reagent, which contains anti-rabbit second antibody bound to fluomicrospheres. Any radioactively-labelled cyclic nucleotide bound to the primary rabbit antibody will therefore be immobilized on the fluomicrosphere, which then produces light which can be measured using a scintillation counter. The acetylation method was used since it markedly increased 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 10µl of the acetylating reagent; a mixture of acetic anhydride and triethylamine (1:2, v/v), directly into the solution, and vortexed immediately. Either guanosine 3`, 5`-cyclic phosphoric acid 2`-0-succinyl-3-[^{125}I] iodotyrosine methyl ester or adenosine

3`, 5`-cyclic phosphoric acid 2`-0-succinyl-3-[^{125}I] iodotyrosine methyl ester was then added (50µl), as the tracer, to each sample. Subsequently, either the cAMP or cGMP antiserum (50µl), followed by the SPA anti-rabbit second antibody (50µl) were added. The samples were mixed on an orbital shaker for 15-20 hours at room temperature (15-30°C) at 200 rpm. The radioactivity bound to the fluomicrospheres was then determined using a β-scintillation counter (Fig. 7).

A standard curve was constructed for each assay (Figs. 8 & 9); this consisted of a duplicate sample of each of the following concentrations of cAMP (expressed as fmol):

2, 4, 8, 16, 32, 64, 128

or concentrations of cGMP (expressed as fmol):

8, 16, 32, 64, 128, 256, 512

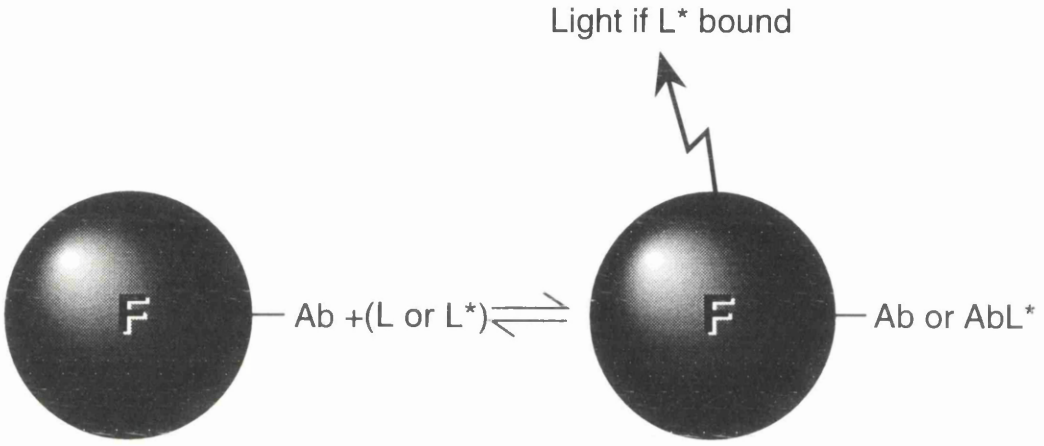
The standards were assayed as described above. The standard curves were used to obtain the unknown cAMP or cGMP content present in each sample.

4) SOLUTIONS AND DRUGS

I) Physiological Salt Solution

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.

Cl^--free ($0[Cl^-]_o$) Krebs solution was made by replacement of NaCl with sodium glucuronate, $CaCl_2$ with calcium gluconate, KCl with potassium gluconate (Saha *et al*, 1992) and $MgCl_2$ with $MgSO_4$. It contained (mM) sodium glucuronate, 118.4; potassium gluconate, 4.7; calcium gluconate, 12.0, $MgSO_4$, 6.0; $NaHCO_3$, 25.0; NaH_2PO_4, 1.13; glucose, 11.0; pH 7.4. The concentration of divalent ions was raised to compensate for binding of Ca^{2+} by organic anions (Aickin & Brading, 1984) as described by Saha *et al* (1992).



F = fluomicrosphere with anti-rabbit second antibody
Ab = primary antibody
L* = labelled ligand
L = unlabelled ligand

Figure 7: Representation of the scintillation proximity assay (SPA). The assay is based on the competition between radioactively (^{125}I) labelled (L*) and unlabelled cyclic nucleotide (L; cAMP or cGMP) for a limited number of binding sites on a cyclic nucleotide-specific primary rabbit antibody. The antibody-bound cyclic nucleotide is reacted with the "SPA reagent" which contains anti-rabbit second antibody (Ab) bound to fluomicrospheres (F). Any labelled cyclic nucleotide bound to the primary rabbit antibody is immobilised on the fluomicrosphere, which then produces light. (Adapted from Amersham SPA handbook).

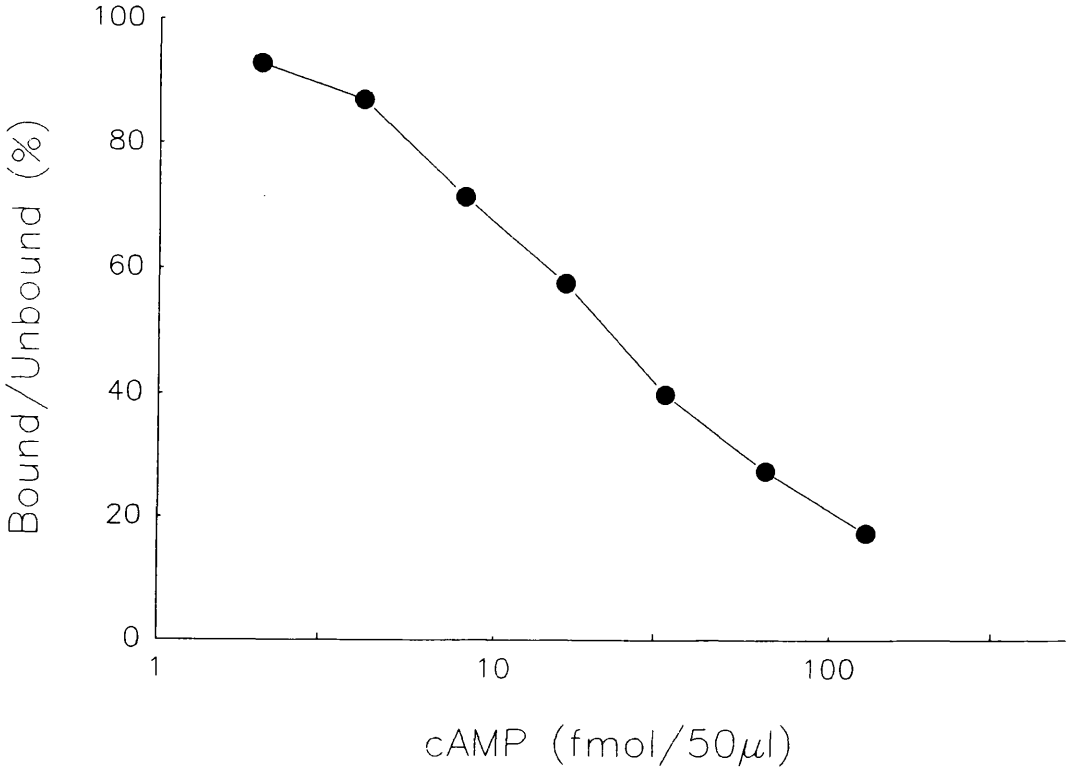


Figure 8: Standard curve for the radioimmunoassay of cAMP. The concentration of cAMP (fmol/50µl) is plotted against the percentage bound for each standard (%B/B_0). The concentration of cAMP is inversely proportional to %B/B_0.

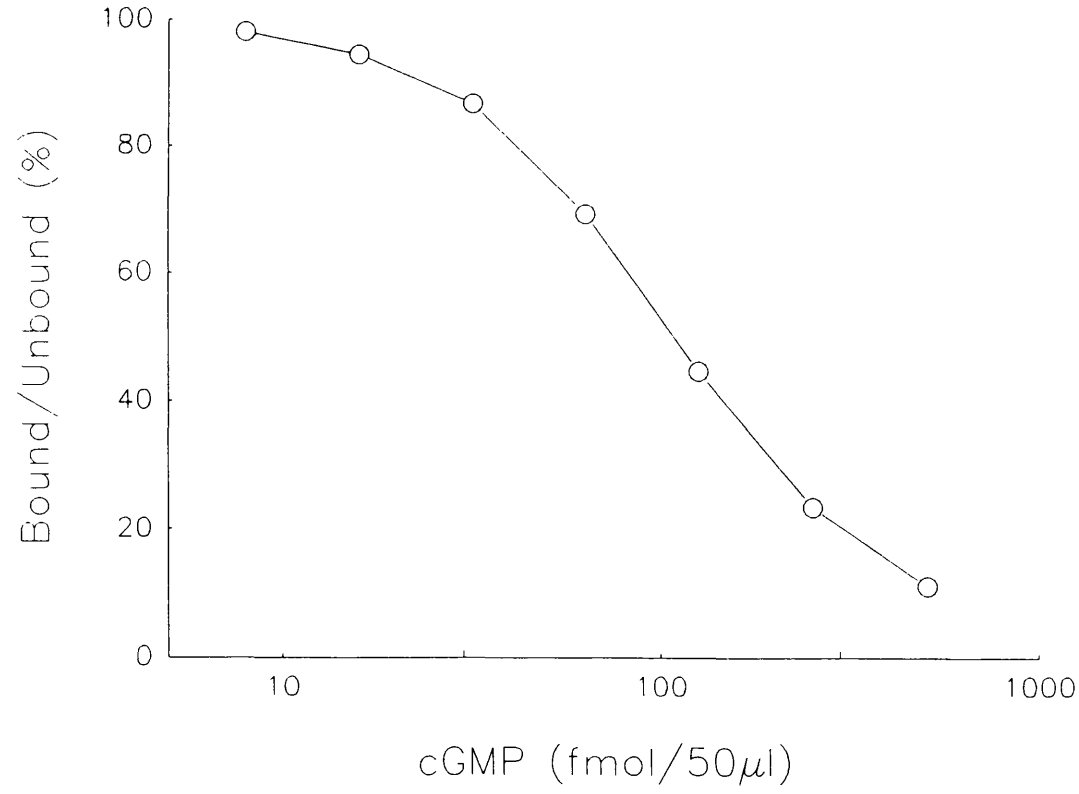


Figure 9: Standard curve for the radioimmunoassay of cAMP. The concentration of cGMP (fmol/50µl) is plotted against the percentage bound for each standard (%B/B_0). The concentration of cGMP is inversely proportional to %B/B_0.

On occasion, nifedipine (1μM) was added to the Krebs` perfusate to reduce spontaneous or evoked movement of the muscle. The organic calcium entry blockers such as nifedipine have been shown to produce no significant effect on neuro-neuronal synaptic transmission (Smith & Furness, 1988; Smith *et al*, 1988; Wood, 1989) and neuro-muscular synaptic transmission enteric nerves of the guinea-pig small intestine (Bywater & Taylor, 1986).

Atropine and phentolamine (each 1μM) were always present except when stated otherwise.

II) Drugs

adenosine 5`-triphosphate disodium salt (ATP; Sigma), apamin (Sigma), L-arginine (L-arg; Sigma), atropine sulphate (Sigma), bovine haemoglobin (HbO; Sigma), BRL 38227, [(-)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-1-benzo-pyran-3-ol (lemakalim; kindly donated by Smith Kline Beecham), 8-bromoguanosine 3`, 5` cyclic monophosphate (sodium salt) (8-Br-cGMP; Sigma), calcium gluconate (Sigma), R(+)-IAA-94, [(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy] (Semat), cyclic AMP scintillation proximity assay radioimmunoassay kit (Amersham), cyclic GMP scintillation proximity assay radioimmunoassay kit (Amersham), (2,2`-di-p-nitrophenyl-5,5`-diphenyl-3,3`-[3,3`-dimethoxy-4,4`-diphenylene] ditetrazolium chloride (nitro blue tetrazolium; Sigma), glibenclamide (Semat), Histoclear (National Diagnostics), isosorbide dinitrate (IDN; Sigma), β-nicotinamide adenine dinucleotide phosphate diaphorase (β-NADPH; Sigma), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma), LY-83583, [6-(phenylamino)-5,8-quinolinedione] (Semat), phentolamine mesylate (Ciba-Geigy), phenylephrine hydrochloride (Sigma), pituitary adenylate cyclase activating polypeptide (PACAP; kindly donated by Dr. J. Morrison), potassium gluconate (Sigma), M&B 22948, [2-O-propoxyphenyl-8-azapurin-6-one] (May & Baker), nifedipine (Sigma), reactive

blue 2 (procion blue; RB2; Sigma), sodium glucuronate (Sigma), sodium nitroprusside (SNP; BDH), suramin (kindly donated by Bayer A.G.), tetrodotoxin (TTX; Sigma), Triton X-100 (Sigma), vasoactive intestinal polypeptide (porcine; VIP, Sigma). Concentrations in the bath refer to the salts except TTX, apamin, VIP, PACAP and HbO, which are expressed as concentrations of the base.

With the following exceptions, stock solutions of drugs were prepared in distilled water before dilution with Krebs' solution prior to use. The following were prepared as 1mM stock solutions as indicated; R(+)-IAA-94 (50% ethanol), lemakalim (50% methanol), LY83583 (10% dimethyl sulphoxide), M&B 22948 (50% ethanol), nifedipine (50% methanol), before dilution with Krebs' solution prior to use. Oxyhaemoglobin was prepared by reduction of bovine haemoglobin as described previously (Martin *et al*, 1985 a,b).

5) ANALYSIS OF RESULTS

Where appropriate, results were expressed as mean ± S.E.M., of n (a number of) cells or preparations. Statistical analyses were performed by means of Student's *t* test for paired or unpaired data to test for significance between means. A t-value of $P<0.05$ was taken as being significant. A minimum of three tissues were used to investigate each drug or combination of drugs. Significance values, relative to control, are shown as asterisks, where $P<0.05$ is denoted by *, $P<0.01$ by ** and $P<0.001$ by ***. In figs. 55 & 56 significance between separate histograms, other than contol, where $P<0.05$ is denoted by #.

RESULTS

1) ELECTRICAL AND MECHANICAL

I) Resting Properties

Following pinning out in the organ bath the IAS, initially, exhibited no tone. However upon application of stretch (1g) and a 40 minute equilibration period, varying degrees of additional tone (1-3g) developed. This tone could be maintained for several hours without additional stretch or drugs. Prior to experimentation, phenylephrine (1μM) was added to the bath; a contraction confirmed the presence of sphincteric muscle, as non-sphincteric tissue relaxes to α-adrenoceptor stimulation. Tone was dependent on the maintenance of a spontaneous action potential discharge - in the absence of tone action potentials were not obtained. The maintenance of tone was unaffected by muscarinic or α-adrenoceptor blockade, or tetrodotoxin (1μM) confirming its myogenic origin.

Circular smooth muscle cells of the gpIAS had membrane potentials of -44.2±0.2 mV (n=1119 cells from 117 preparations; Fig. 10). Electrically, they displayed either spontaneous oscillations in membrane potential and tone accompanied by a discharge of action potentials (maximum amplitude 60mV, 1-2 Hz frequency; Fig. 11) or were relatively quiescent (*e.g.* see Fig. 13).

II) Response to Electrical Field Stimulation (EFS)

EFS (supramaximal voltage, 0.1ms, single stimulus (ss) or trains of 5 stimuli at 5, 10, 20 or 40 Hz), inhibited spike discharge and evoked IJPs and relaxations (Fig. 12). IJP amplitude was not significantly affected by atropine and phentolamine (each 1μM; Fig. 13). The ability to elicit them in the presence of both atropine and phentolamine indicated that the IJPs were mediated by NANC inhibitory nerves. Following a single stimulus, the IJPs peaked in 361±31 ms and lasted approximately 1s (n=25 from 8 preparations). IJP amplitude increased with increasing frequency of stimulation (ss 19.7±1.1mV, n=165, 33 tissues; 5Hz 22.3±1.4mV, n=115, 23 tissues; 10 Hz 27.5±1.2mV, n=145, 29 tissues; 20 Hz 29.6±1.3mV, n=151, 29 tissues; 40 Hz

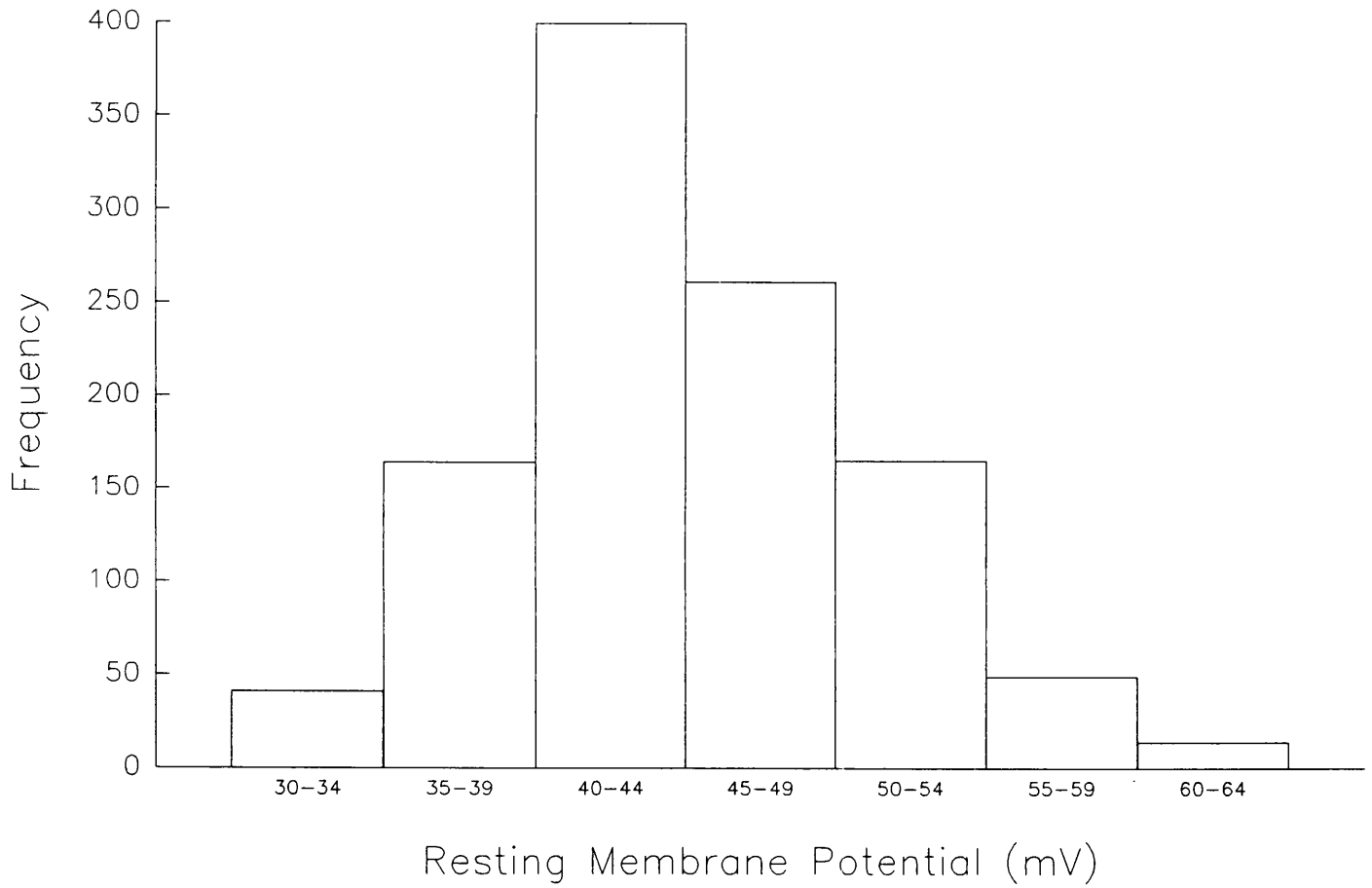


Figure 10: Population distribution of the resting membrane potential of cells impaled for intracellular electrical recording in the gpIAS.

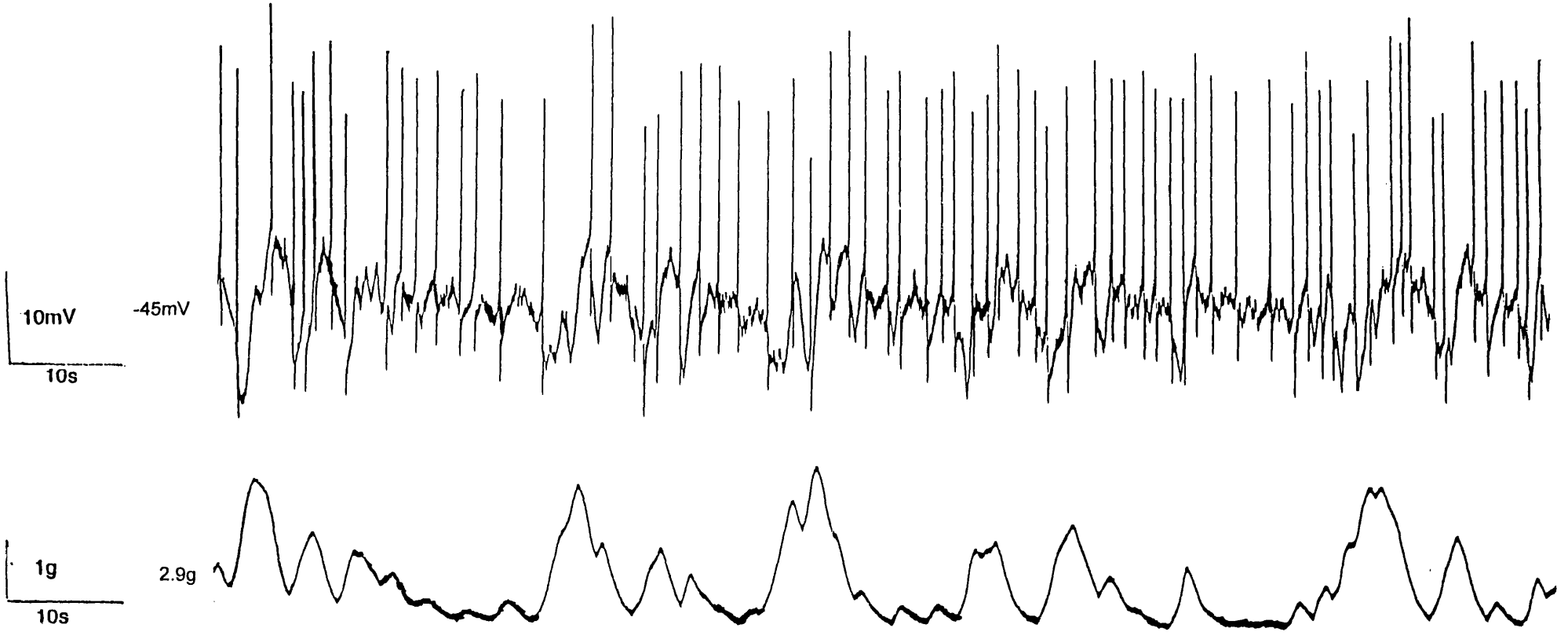


Figure 11 : Intracellular electrical (upper trace) and simultaneously recorded mechanical activity of the gpIAS under 2.9g stretch. The tissue exhibits spontaneous electrical and mechanical activity under these conditions. Record was obtained from a single cell.

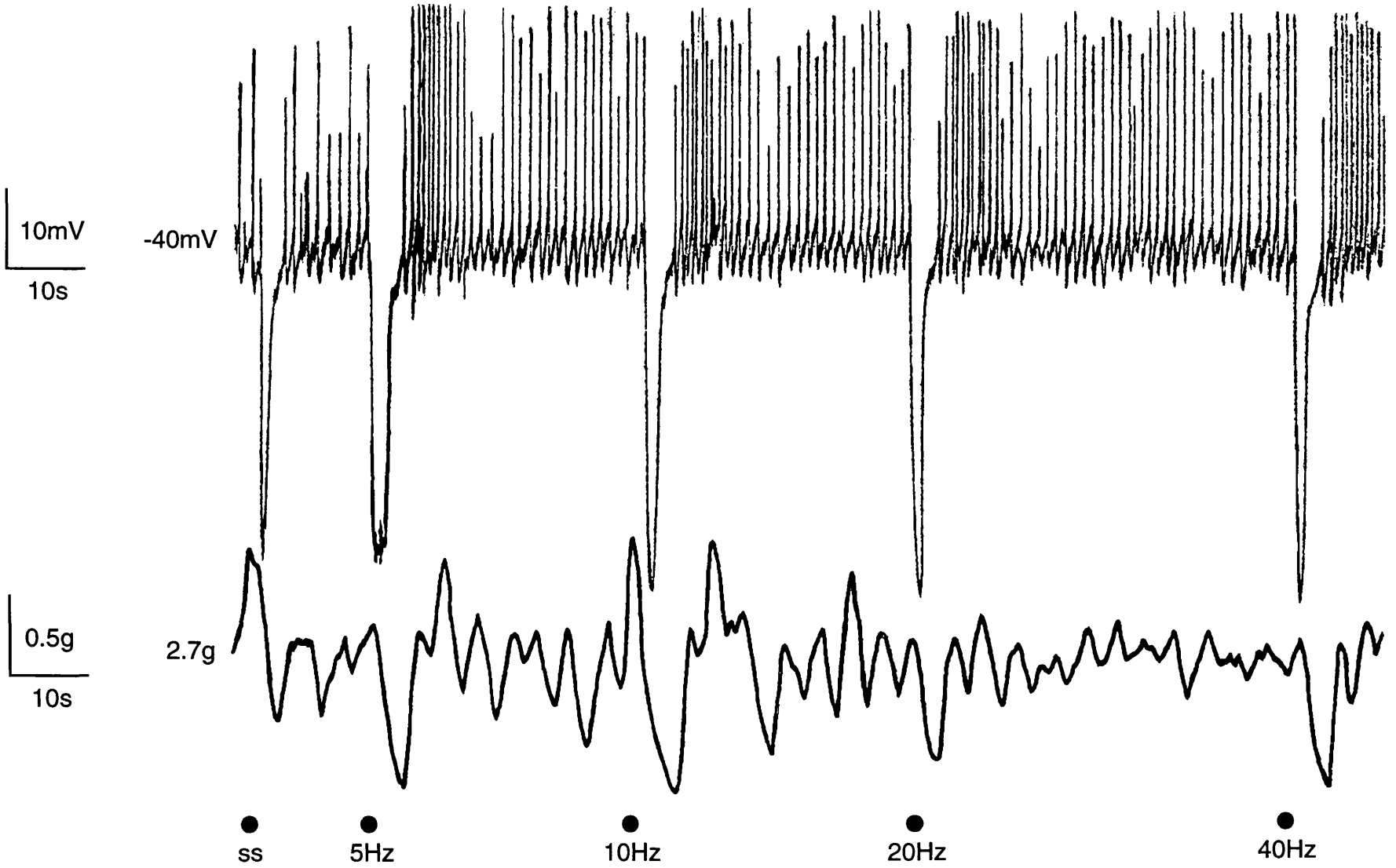


Figure 12: Intracellular electrical (upper trace) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz). EFS inhibited spiking, evoked frequency-dependent IJPs and relaxed the muscle. Record was obtained from a single cell.

30.3±1.5mV, n=130, 26 tissues) and lasted between 1 and 2 seconds depending on the stimulation frequency (maximum duration at 5Hz). Following trains of stimuli, IJPs were usually, but not invariably, followed by a rebound increase in action potential discharge, a common feature of NANC nerve-evoked IJPs in GI smooth muscle (Bennett *et al*, 1966 b; Ward *et al*, 1992 a). This rebound may be mediated by prostaglandins (Burnstock *et al*, 1975) or tachykinins (Zagorodynuk *et al*, 1993; Shuttleworth *et al*, 1993), although no evidence exists for this in the gpIAS. Responses to EFS were abolished by TTX (1μM; Fig. 14).

On occasion, the Ca^{2+} channel antagonist nifedipine (1μM) was used to ease impalement of cells by abolishing tone and spikes. The drug did not affect IJP amplitude (Fig. 15).

III) <u>Response to EFS in the Presence of L-NAME</u>

NO is a widely distributed inhibitory transmitter within the GI tract (Sanders & Ward, 1992; Sanders *et al*, 1992; Rand, 1992; Stark & Szurszewski, 1992). The effect of the NOS inhibitor L-NAME (100μM) was examined. L-NAME failed to affect resting membrane potential, as found by He & Goyal (1993) and Bywater *et al* (1993) in the guinea-pig ileum, or to significantly change the IJP response to EFS (supramaximal voltage, 0.1ms, single stimulus and trains of 5 stimuli at 10 & 20 Hz; n=10 from 5 preparations, $P>0.05$; Fig. 16). These results apparently failed to implicate NO in the electrical response to EFS. However, L-NAME in the presence of high tone (~3-4g), significantly reduced relaxations to EFS (Fig. 17). This result confirmed the findings of Craig and Muir (1991) which implicated NO in neuronally-mediated relaxations in this tissue.

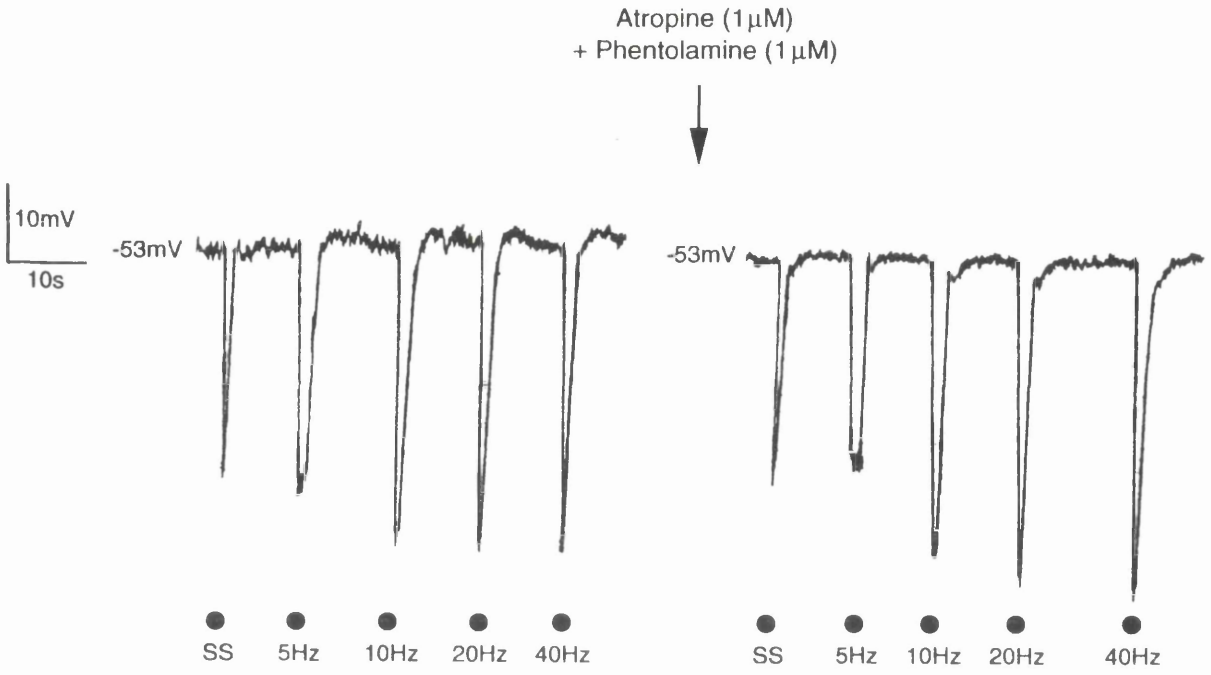


Figure 13: Failure of atropine and phentolamine (each 1μM) to significantly affect either the resting membrane potential or the intracellular electrical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40Hz) in the presence of nifedipine (1μM). Records were obtained from approximately adjacent cells.

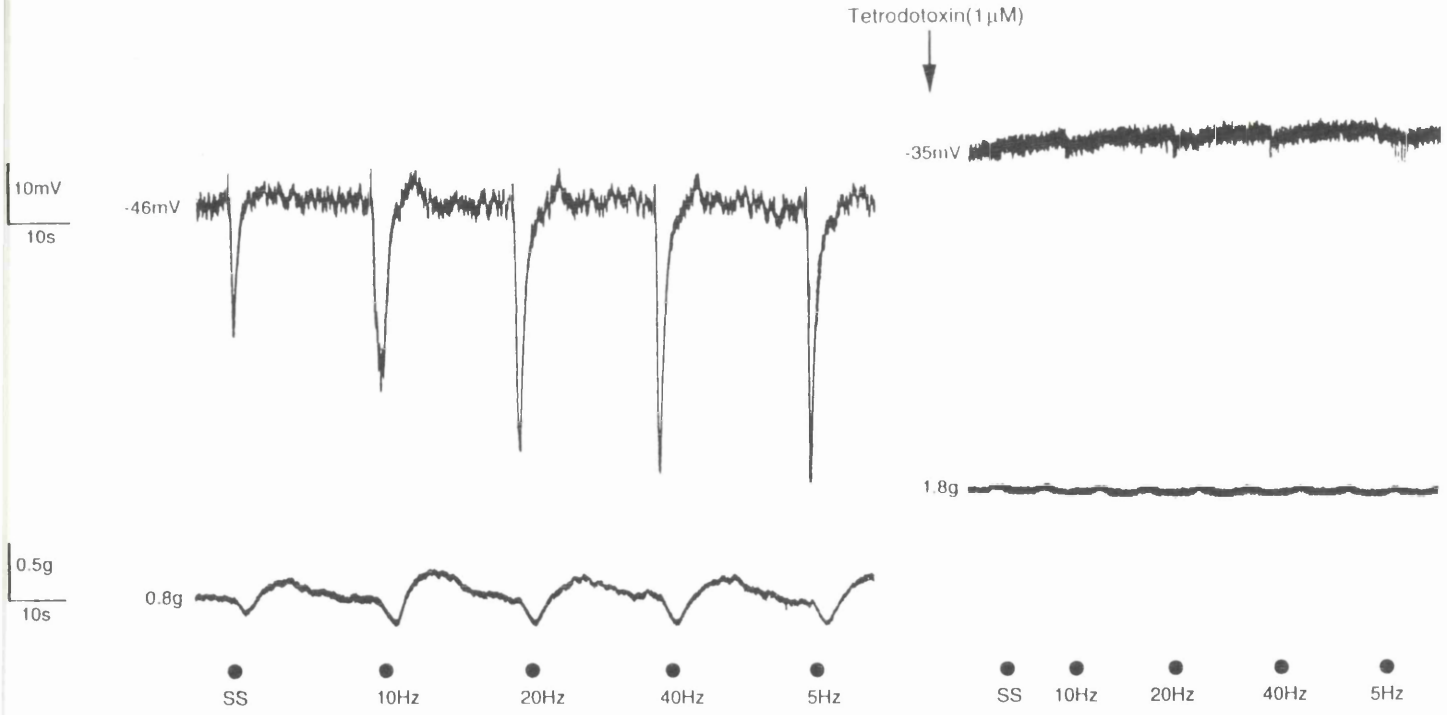


Figure 14: Effect of tetrodotoxin (TTX; 1μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40Hz). TTX depolarized the membrane, increased tone and abolished the nerve-mediated IJPs and relaxations at each frequency. Records were obtained from approximately adjacent cells.

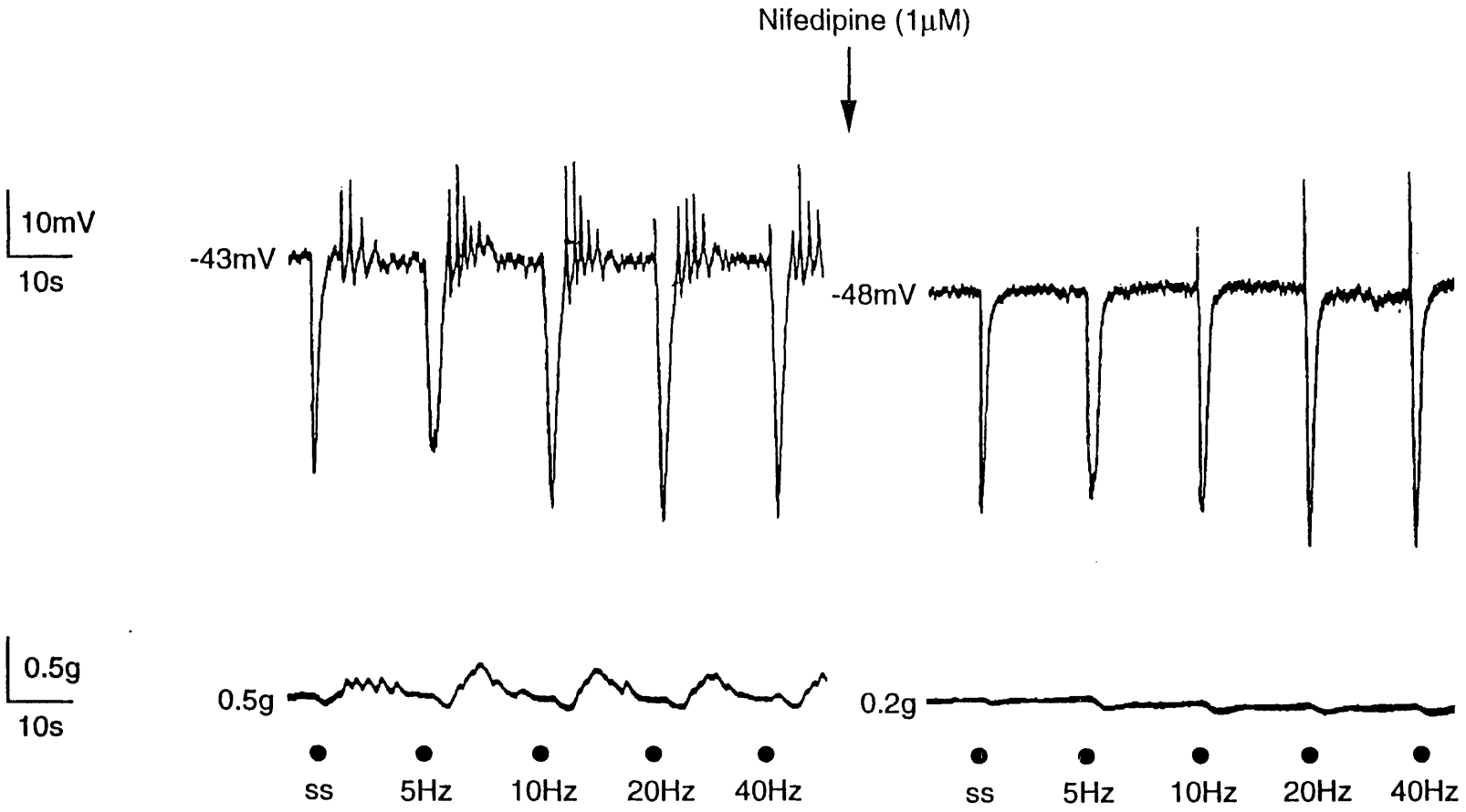


Figure 15 : Effect of nifedipine (1μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40Hz). Nifedipine abolished the "rebound" electrical spiking and reduced mechanical activity, but did not affect the amplitude of the IJPs. Records were obtained from the same cell.

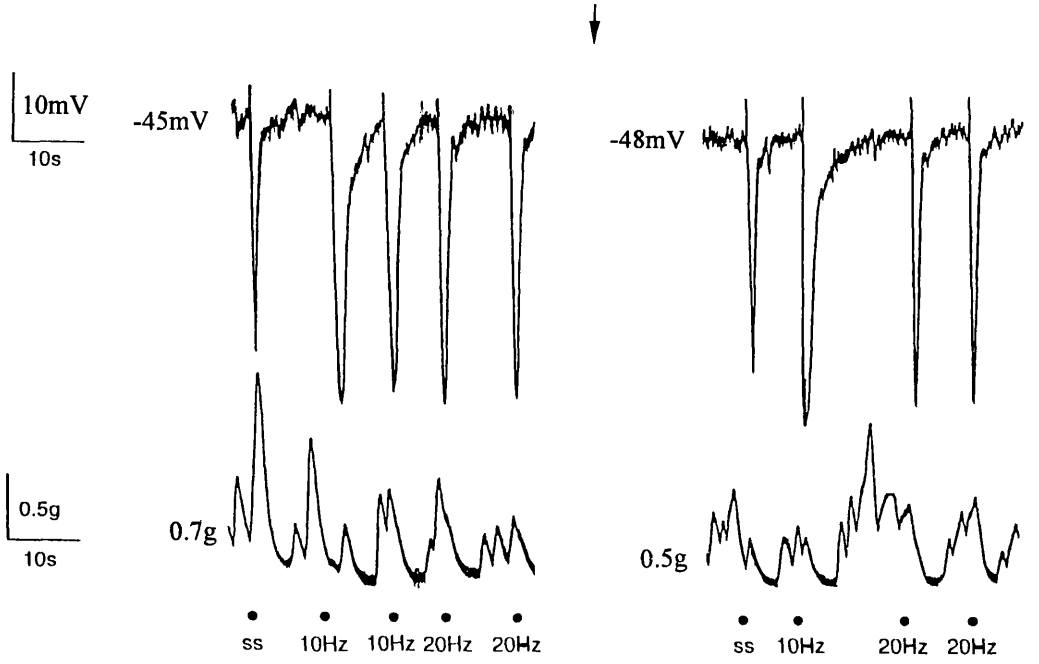


Figure 16: Effect of L-NAME (100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10 and 20Hz). L-NAME had no significant effect on either the amplitude of the IJPs or on the relaxations. Records were obtained from approximately adjacent cells.

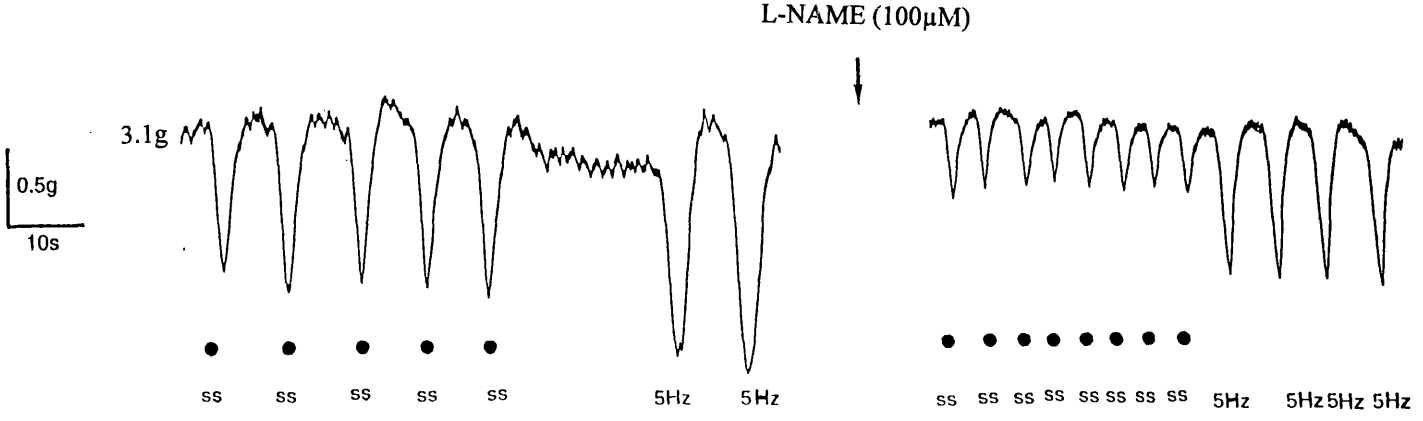


Figure 17: Effect of L-NAME (100μM) on the mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5Hz). L-NAME significantly reduced the amplitude of the relaxations in response to both ss and trains of stimuli.

IV) Response to EFS in the Presence of Oxyhaemoglobin (HbO)

HbO (10μM), a proposed scavenger of NO (Martin *et al*, 1985 a, b), also failed to significantly attenuate the responses, either electrical or mechanical, to EFS (Fig. 18) ($n=4$).

V) Response to EFS in the Presence of K^+ Channel Modulators

In the gpIAS, the responses produced by inhibitory NANC nerve stimulation are mediated by an increase in K^+ conductance (Lim & Muir, 1985) and are abolished by the the non-specific K^+ channel blocker, tetraethylammonium (Lim, 1985; Baird, 1990). In the present study, the effects of lemakalim (50μM), a proposed K_{ATP} channel opener (Weston & Edwards, 1992), glibenclamide (10μM), a K_{ATP} channel blocker (Buckingham *et al*, 1989) and the bee venom apamin, which blocks small conductance Ca^{2+}-activated K^+ channels (Banks *et al*, 1979; Shuba & Vladimirova, 1980) were examined to try define, more fully, the K^+ channel involved.

Lemakalim (30-50μM) slowly hyperpolarized the membrane and reduced tone. IJP amplitude was reduced, but this was probably due to the membrane hyperpolarization rather than an effect on the IJPs *per se*. Upon washout of the drug, the membrane potential and IJPs were restored (Fig. 19) ($n=3$). Glibenclamide (10μM) had no effect on IJPs or relaxations (Fig. 20) ($n=3$). These results indicated that the K_{ATP} channels did not mediate the neuronally evoked hyperpolarization of the gpIAS.

Previous studies had shown that apamin blocked evoked IJPs (Lim, 1985; Baird, 1990), however, in contrast, the present investigation demonstrated that apamin failed to abolish either the IJPs or relaxations in response to EFS. Possible reasons for this anomaly will be discussed later.

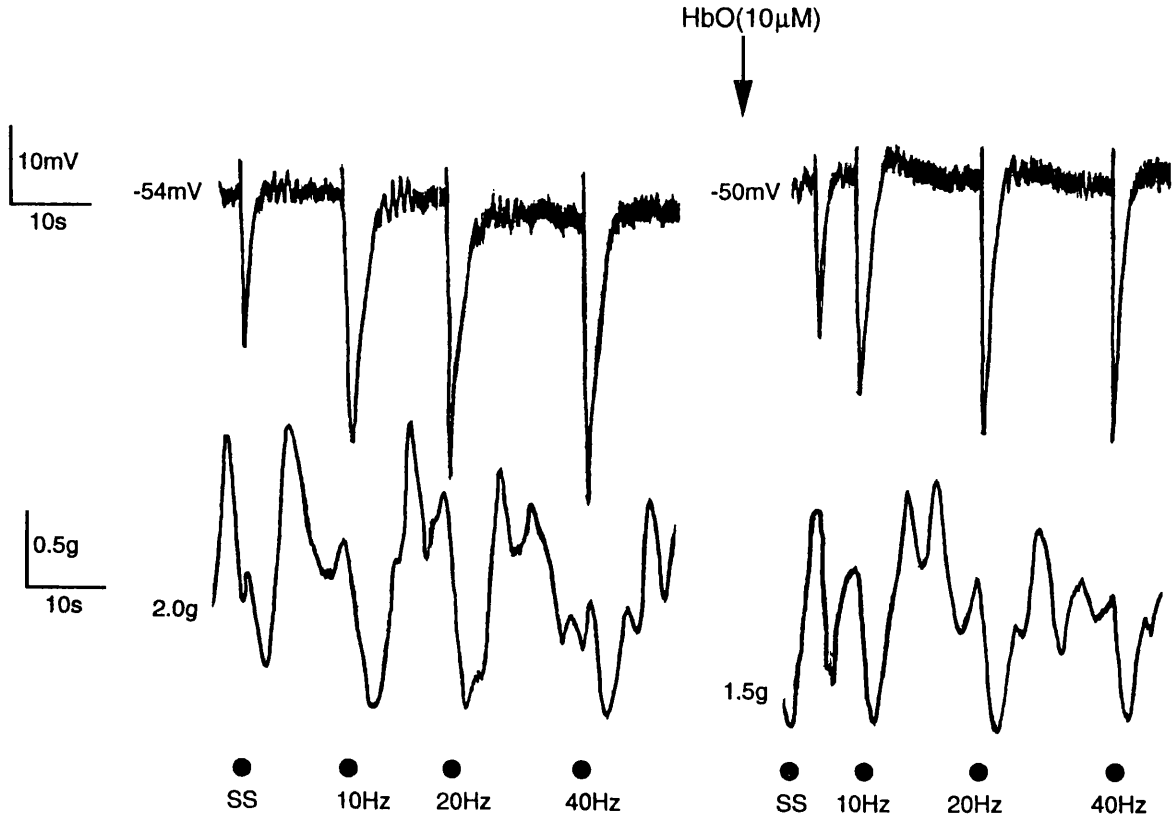


Figure 18: Effect of oxyhaemoglobin (HbO; 10μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz). HbO did not significantly affect IJP amplitude or relaxations. Records obtained from the same cell.

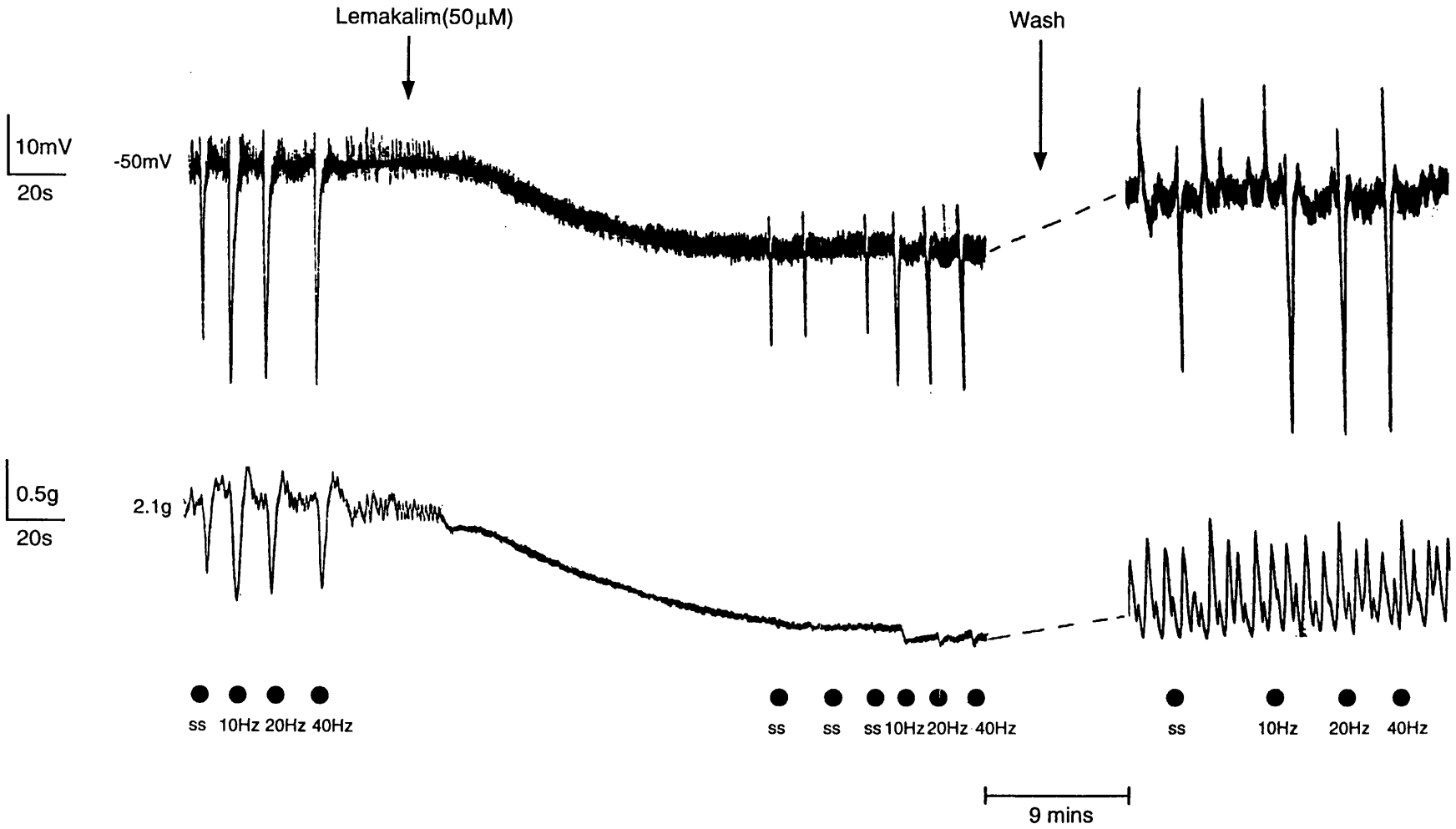


Figure 19: Effect of lemakalim (50μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40 Hz). Lemakalim hyperpolarized the membrane and reduced both IJP amplitude and tone. The reduction in IJP amplitude is dependent on the increase in membrane potential as IJP was restored after restoration of the membrane potential by washing. Records obtained from the same cell.

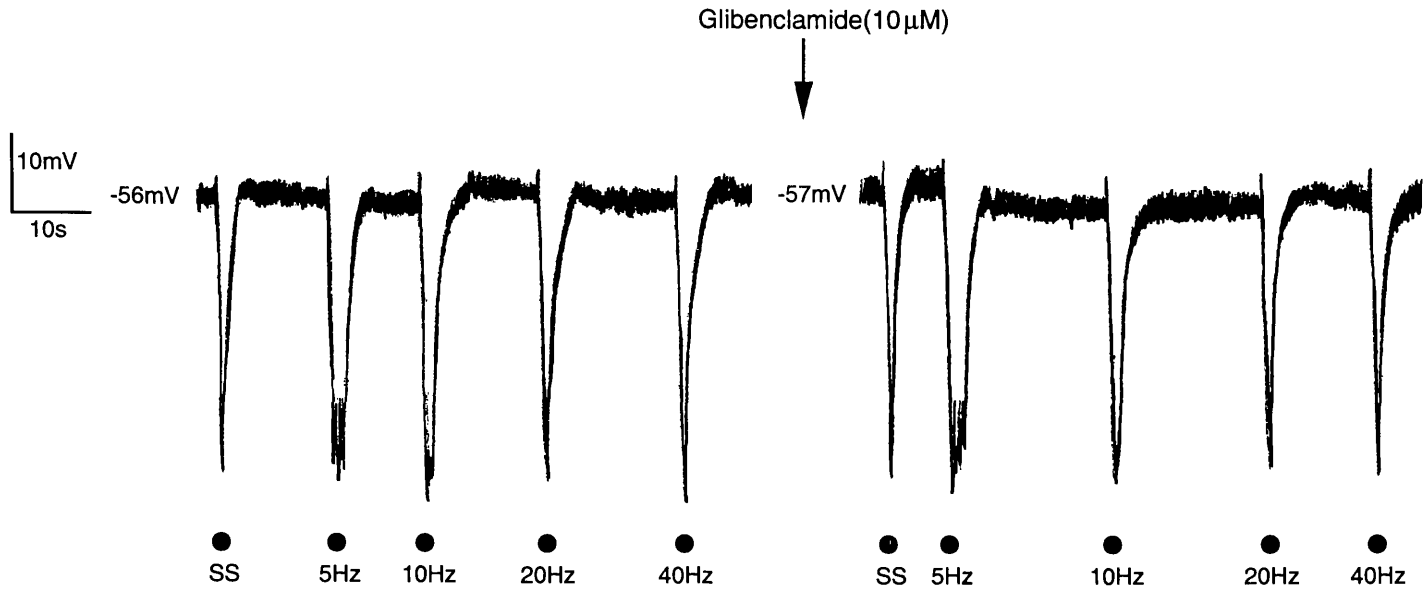


Figure 20: Effect of glibenclamide (10μM) on the intracellular electrical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40Hz). Glibenclamide had no significant effect on evoked IJPs. Records were obtained from the same cell.

VI) Response to EFS in the Presence of Apamin

Apamin (0.3μM) evoked a transient depolarization (Fig. 21) and significantly ($P<0.001$) reduced IJP amplitude, evoked by EFS (supramaximal voltage, 0.1ms, single stimuli, 5 stimuli at 5, 10, 20 and 40Hz), at all frequencies (Figs. 21 & 22). Following a single stimulus (0.1ms, supramaximal voltage) IJPs could be resolved into two components. A fast-to-peak (361±31ms, n=25 cells from 8 preparations) IJP component, obtained in the absence of apamin was changed and a significantly ($P<0.001$) slower-to-peak (530±17ms, n=16 cells from 4 preparations) component revealed (Fig. 23). Higher concentrations of apamin (1μM), far in excess of that required to block Ca^{2+}-activated K^+ channels (Capoid & Ogden, 1989), failed to further significantly reduce the electrical and mechanical responses to EFS (supramaximal voltage, 0.1ms, single stimuli, 5 stimuli at 5, 10, 20 and 40Hz). This would apparently indicate that two inhibitory substances mediate the IJP in this tissue.

VII) The Apamin-Sensitive Component of the IJP

The mediator of the fast, apamin-sensitive component, of the IJP was investigated using two purinoceptor antagonists, suramin (Dunn & Blakeley, 1988; Ohno *et al*, 1993; Hoyle *et al*, 1990) and reactive blue 2 (Kerr & Krantis, 1979; Choo, 1980; Crema *et al*, 1983; Manzini *et al* 1986). The reason for this choice was the evidence for ATP involvement in the evoked IJP in this tissue shown previously (Lim & Muir, 1986). Suramin has been claimed to be an effective P_2-purinoceptor antagonist in reducing IJP amplitude in the guinea-pig taenia caeci (Den Hertog *et al*, 1989), although this has been disputed (McConalogue *et al*, 1994). Suramin (100μM) reduced the mean amplitude of the IJP significantly ($P<0.001$) following EFS (supramaximal voltage, 0.1ms, single stimuli, 5 stimuli at 5, 10, 20 and 40Hz), at all frequencies (Figs. 24 & 25). This smaller suramin-insensitive component bore a marked resemblance to

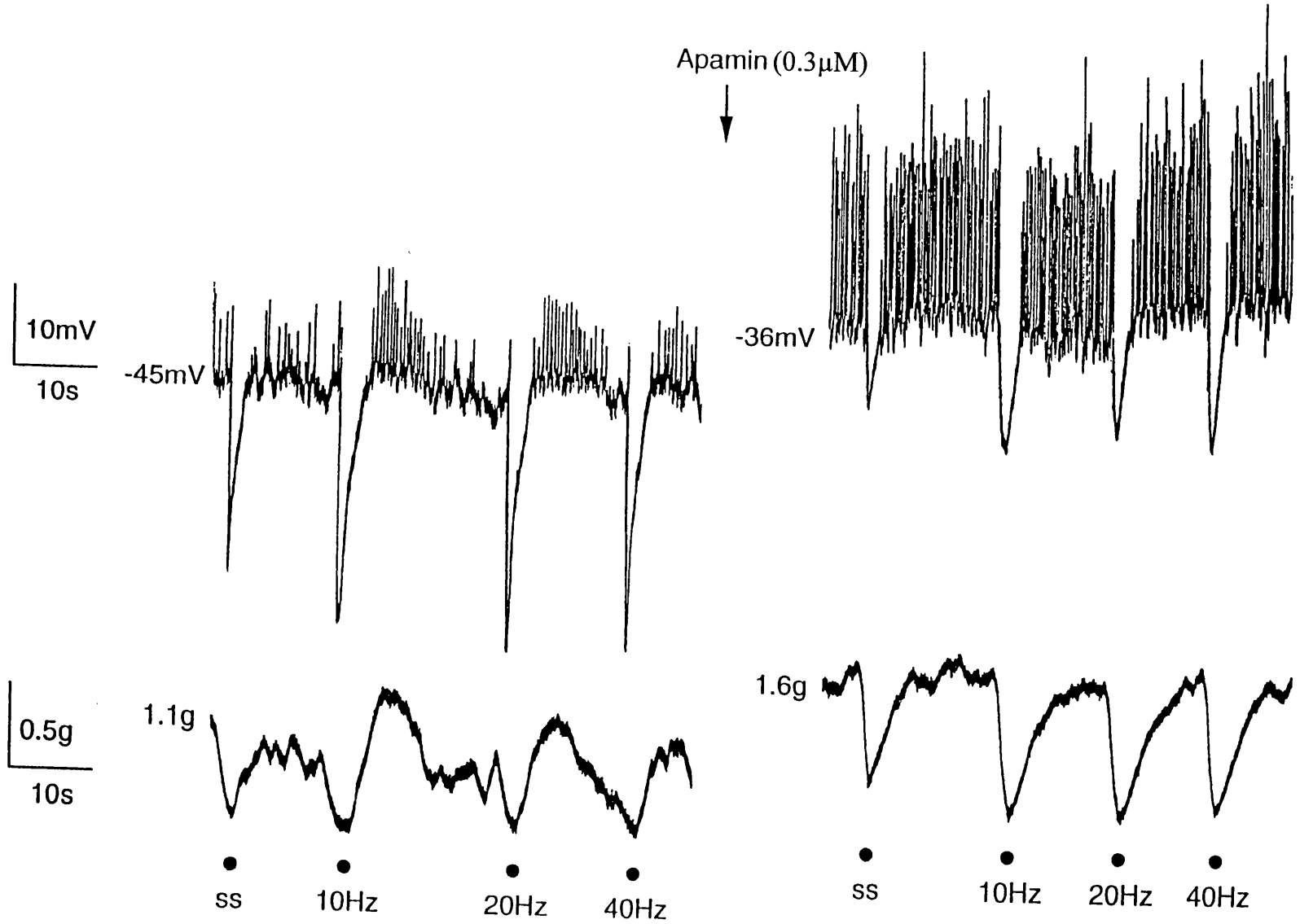


Figure 21: Effect of apamin (0.3μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40 Hz). Apamin produced a transient depolarization and increased tone. IJP amplitude was reduced but relaxations were unaffected. Records were obtained from approximately adjacent cells.

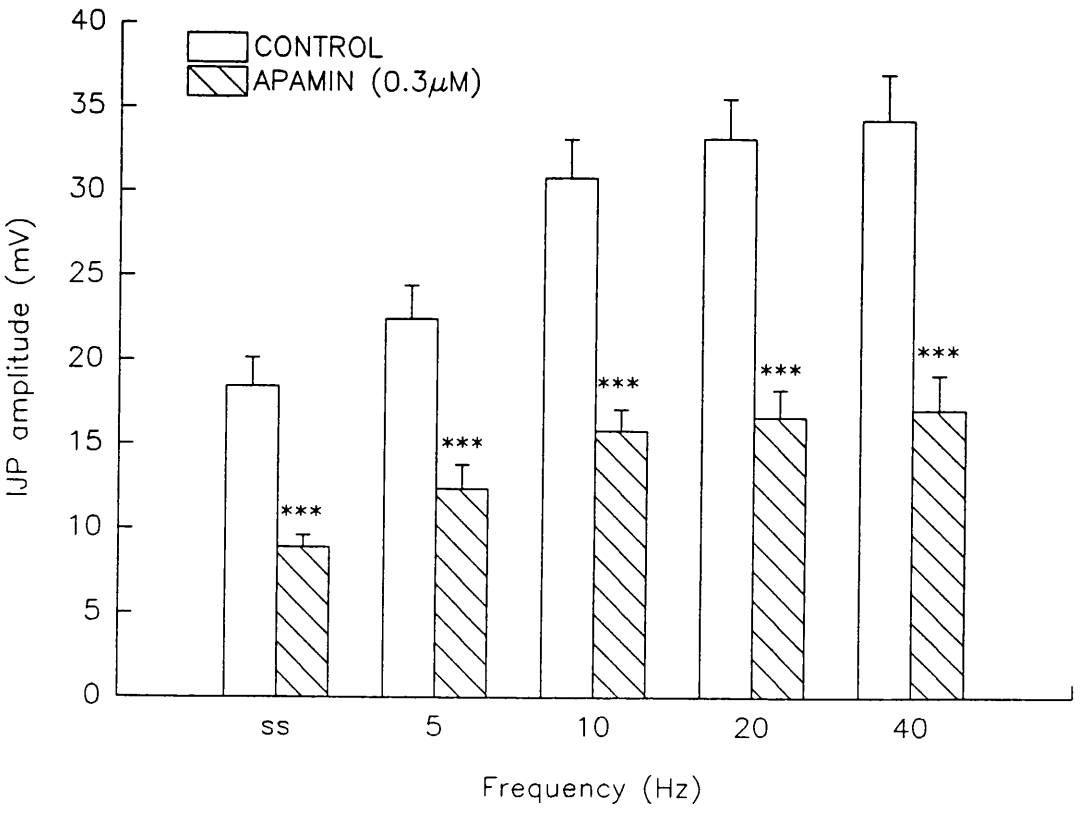


Figure 22: Effect of apamin (0.3μM) on IJP amplitude (mV) in response to increasing stimulation frequency (Hz; single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz, supramaximal voltage, 0.1ms). Each bar represents the mean ± S.E.M., n = 27 (minimum), from 10 different tissues (minimum). Apamin significantly ($P<0.001$) reduced the amplitude of the IJPs at all frequencies.

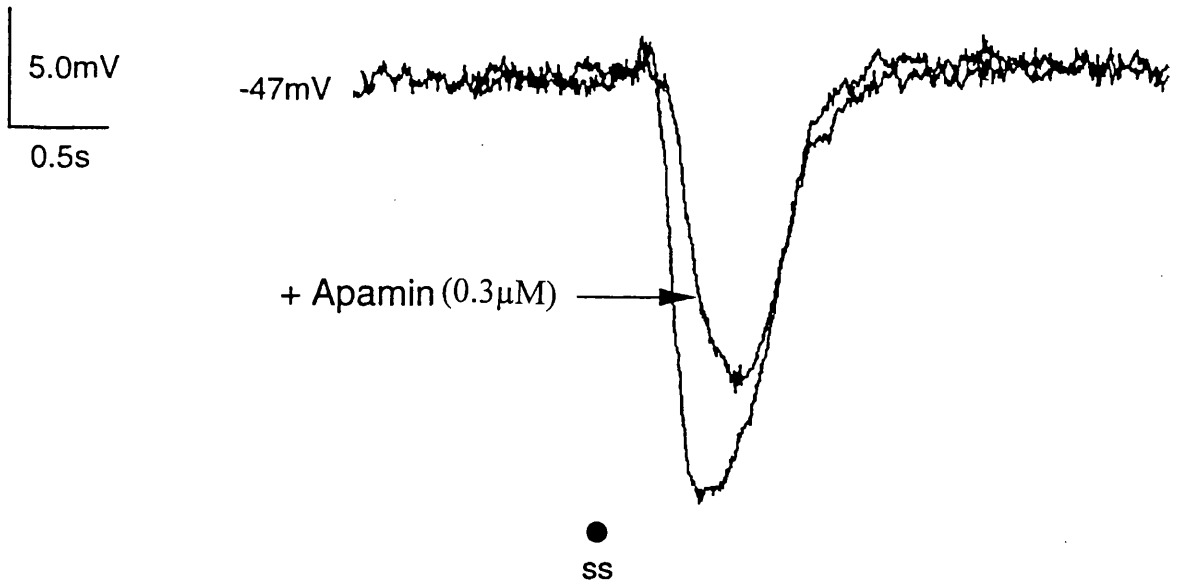


Figure 23: Effect of apamin (0.3μM) on time-to peak of two evoked IJPs, from approximately adjacent cells (~1mm), superimposed by coincidence of their stimulus artefacts, in response to a ss (supramaximal voltage, 0.1ms) from the same experiment. Apamin significantly ($P<0.05$) increased the IJP time-to-peak, but reduced IJP amplitude.

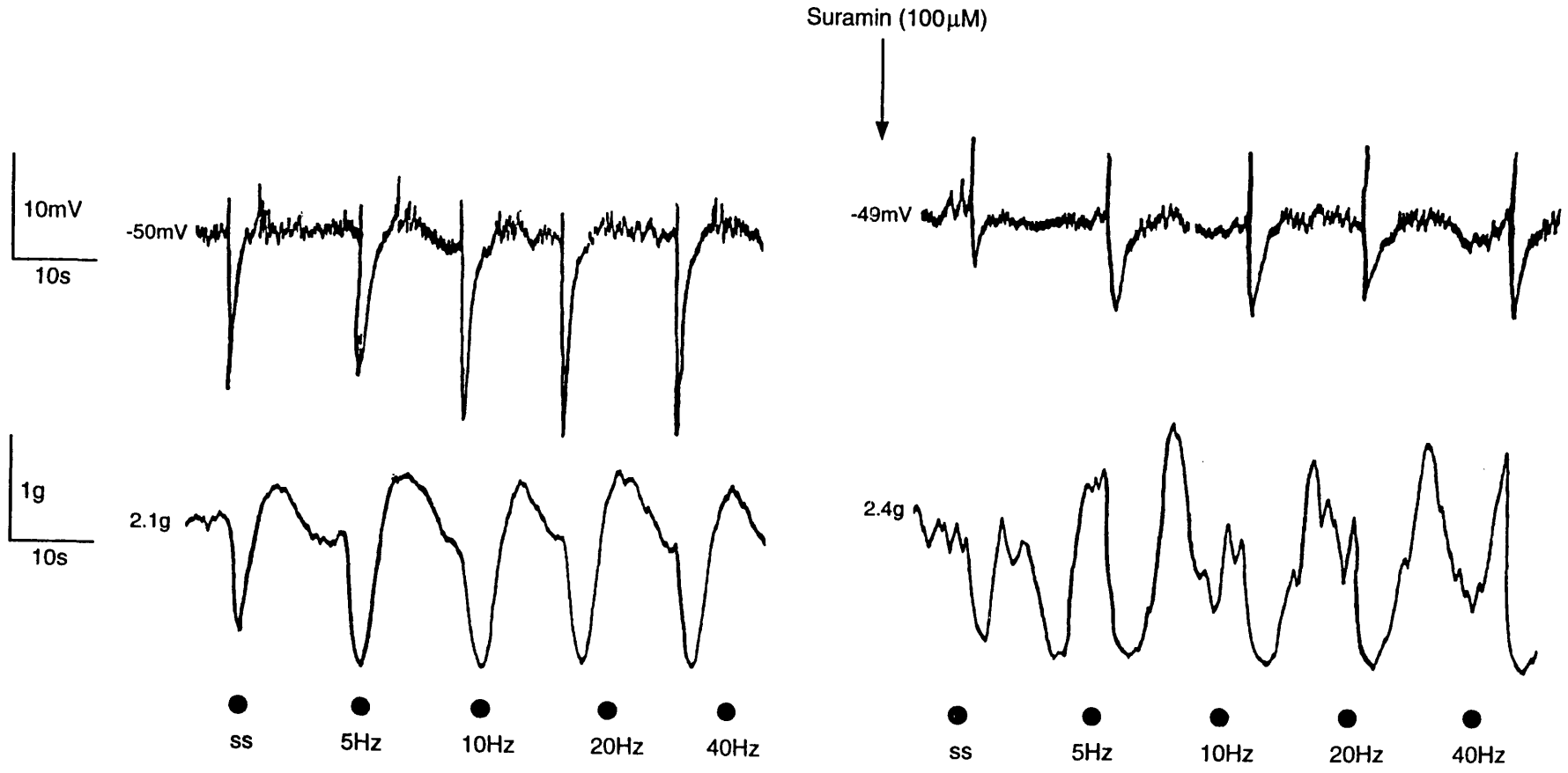


Figure 24 : Effect of suramin (100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz). Suramin reduced, but did not abolish, IJP amplitude at all frequencies. Suramin also desynchronized the accompanying mechanical activity but relaxations were still visible. Records were obtained from approximately adjacent cells.

the apamin-insensitive component, not only in amplitude, but also in its significantly slower time to peak; following a single stimulus (0.1ms, supramaximal voltage) the fast-to-peak (349±4ms, n=23 cells from 7 preparations) IJP component, obtained in the absence of suramin was changed, and a significantly ($P<0.001$) slower-to-peak (510±9ms, n=17 cells from 7 preparations) IJP component revealed (Fig. 26). The accompanying mechanical activity usually became irregular in the presence of suramin.

Reactive blue 2 (100μM) also reduced the amplitude of the apamin-sensitive IJP component but hyperpolarized the membrane and lowered tone irreversibly. These effects could, themselves, have accounted for the reduction in IJP amplitude and could not, unequivocally, be related to the action of the antagonist (Fig. 27).

Together, these results suggested that purinoceptors, probably P_{2y} (Lim, 1985; Burnstock & Kennedy, 1985; Kennedy, 1990), were involved in the fast apamin-sensitive component of the IJP. Support for this view came from experiments using ATP itself applied either by microsyringe injection (1mM-injection volume 3μl) or by hydrostatic pressure ejection (100μM). Because of the small volume of the drug applied to the tissue using pressure ejection, mechanical effects were restricted to a small number of muscle bundles which did not affect the overall tone of the tissue and therfore could not be monitored. ATP hyperpolarized the membrane and relaxed the gpIAS. The hyperpolarization was dose-dependent (Fig. 28). ATP (1mM), injected directly onto the tissue by microsyringe, resembled the fast apamin-sensitive IJPs in amplitude, time-to-peak, and time course (16.9±1.1 mV, n=23, from 5 preparations; 1.7±0.2s, n=27, from 5 preparations and 5.3±0.4s, n=23 cells from 5 preparations respectively, (Fig. 29; see page 70-71 for control IJP values). These hyperpolarizations were also accompanied by relaxations. Significantly, the membrane hyperpolarization and relaxation produced by ATP

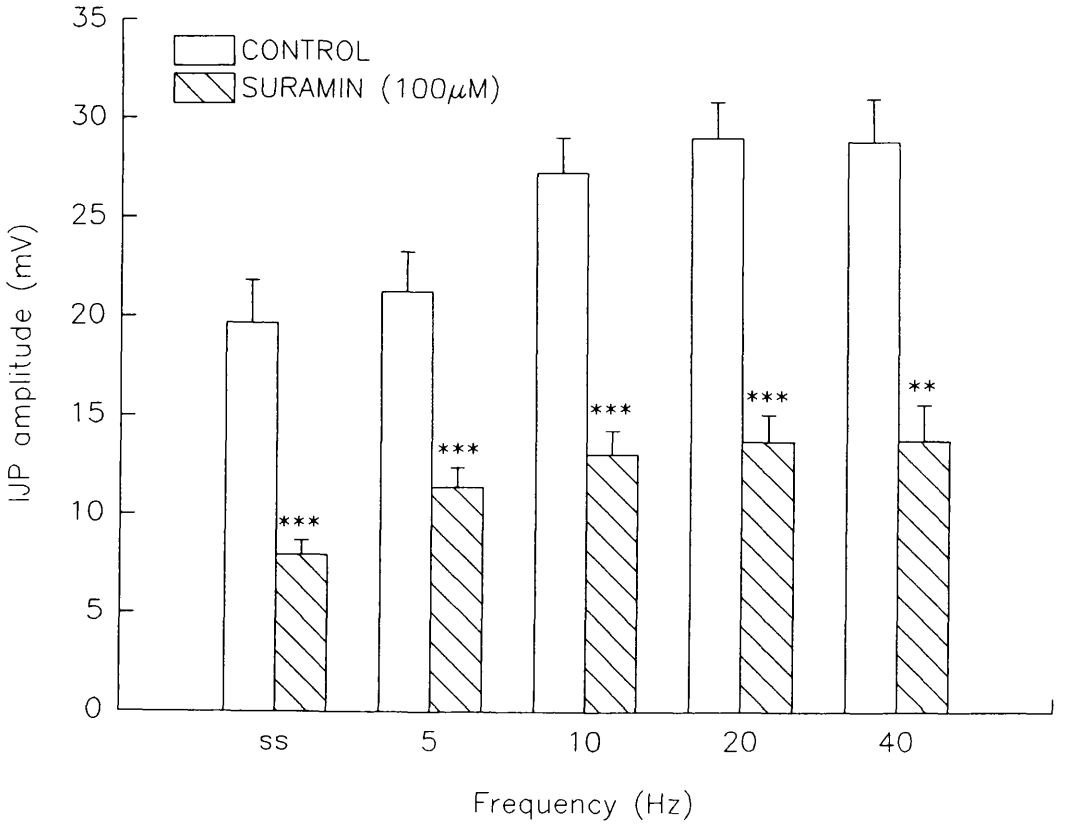


Figure 25: Effect of suramin (100μM) on IJP amplitude (mV) in response to increasing stimulation frequency (Hz; supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz). Each bar represents the mean ± S.E.M., n = 9 (minimum), from 4 different tissues (minimum). Suramin significantly ($P < 0.01$) reduced the amplitude of the IJPs at all frequencies.

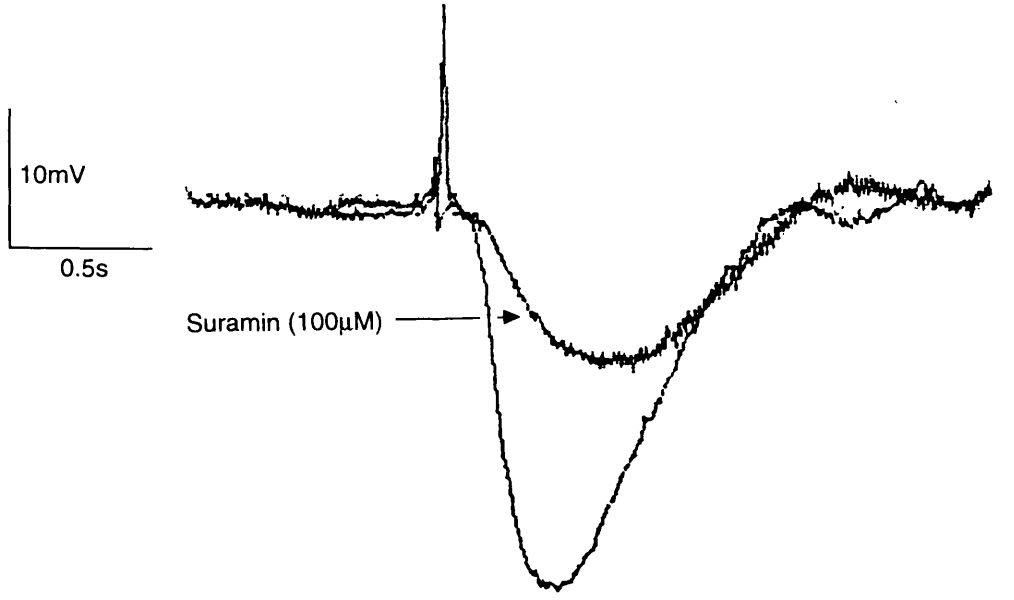


Figure 26: Effect of suramin (100μM) on time-to-peak of two IJPs, from approximately adjacent cells (~1mm), superimposed by coincidence of their stimulus artefacts, in response to a ss from the same experiment. suramin increases the IJP time-to-peak but reduces IJP amplitude.

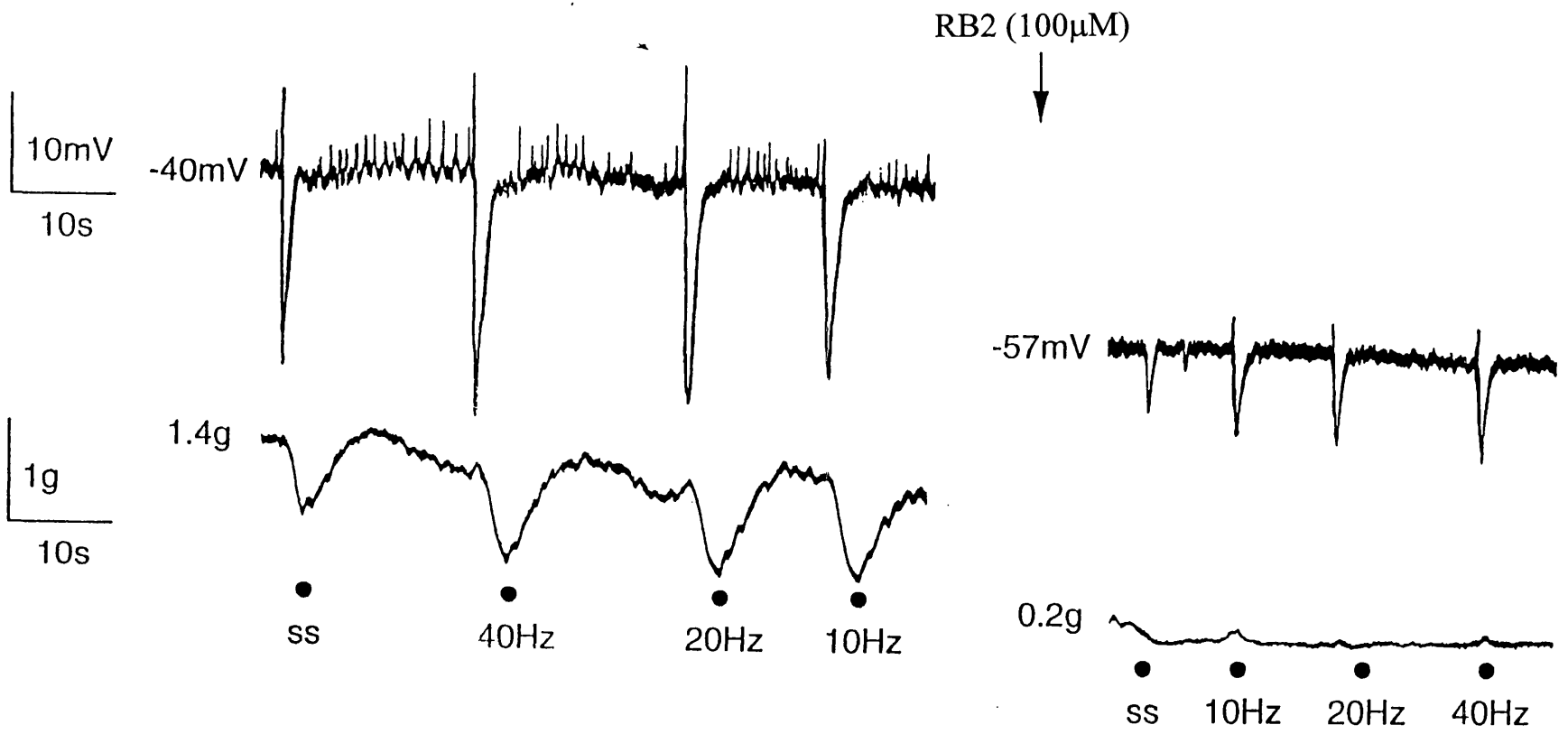


Figure 27: Effect of reactive blue 2 (RB2, 100μM) on the intracellular electrical (upper trace in each pane[1]) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40 Hz). RB2 increased membrane potential, almost abolished tone and relaxations. These effects were not reversed by washing. Records were obtained from approximately adjacent cells.

● ATP (100μM)

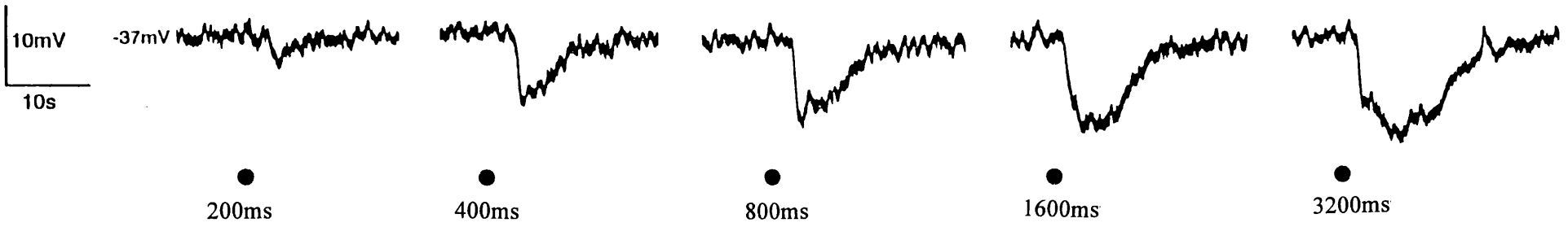


Figure 28: Effect of ATP (100μM), applied locally by pressure ejection (tip diameter 2-10μM; ~ 40 p.s.i.; 200-3200ms), on the intracellular electrical responses of the gpIAS. ATP dose-dependently hyperpolarized the membrane potential, which returned to control shortly after termination of the injection. All records obtained from the same cell.

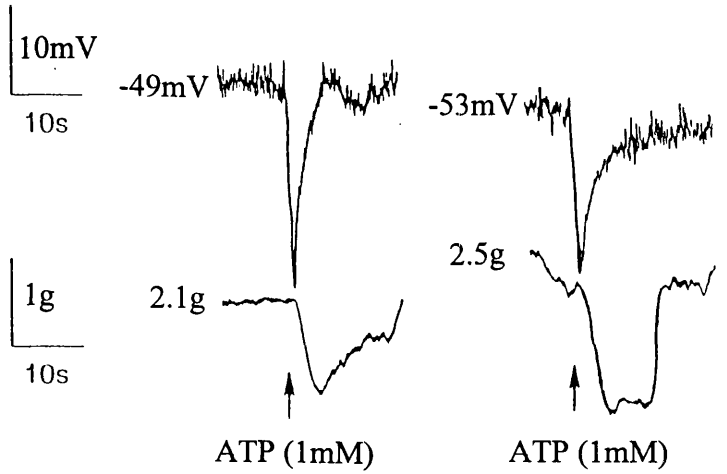


Figure 29: Effect of ATP (1mM), injected by micosyringe (25μl; injection volume 3μl), on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical activity of the gpIAS. ATP produced large, transient membrane hyperpolarizations and relaxed the sphincter. Records were obtained from approximately adjacent cells.

were blocked by apamin (0.3μM), an effect noted previously in several other tissues (Maas & Den Hertog, 1979; MacKenzie & Burnstock, 1980; Shuba & Vladimirova, 1980; Vladimirova & Shuba, 1978; Fig. 30). However, attempts to desensitize the tissue, by superperfusing it with ATP (100μM) and L-NAME (100μM), failed to abolish the evoked IJPs (Fig. 31).

VIII) <u>The Apamin-Insensitive IJP</u>

The possibility that the slow apamin-insensitive, TTX (1μM)-sensitive (Fig. 32) IJP component, responsible for the major component of relaxation (79.1 ± 4.5%, n=5 preparations), may be mediated by NO was then investigated. NO is produced from L-arginine by NOS (Palmer & Moncada, 1989) by a stereospecific process inhibited by the L-arginine analogue, L-NAME (Hobbs & Gibson, 1990). For this reason, the evoked IJPs were examined in the presence of apamin and L-NAME. In contrast to its lack of effect on the fast IJP component, L-NAME abolished the slow apamin-insensitive component of the IJPs and relaxations to EFS (supramaximal voltage, 0.1ms, single stimuli, 5 stimuli at 10, 20 and 40Hz; Fig. 33).

The inhibitory effect of L-NAME was not mimicked by its stereoisomer D-NAME (Fig. 34) and was partially reversed by perfusion (30 minutes) with the NO precursor L-arginine (to 62.7 ± 7.1% of control, n=8 from 3 preparations, single stimulus, supramaximal voltage, 0.1ms; Fig. 33).

Support for the involvement of NO in the slow component of the IJP came also from the inhibitory effect of HbO, a NO scavenger (Martin *et al*, 1985 a, b). HbO (10μM), despite having no effect on the amplitude of the apamin-sensitive IJP component (Fig. 18), significantly ($P<0.001$) reduced, reversibly, the amplitude of the slow IJP component at all frequencies (0.1ms, supramaximal voltage, single stimulus and trains of 5 stimuli at 5, 10, 20 and 40 Hz; Fig. 35 & 36).

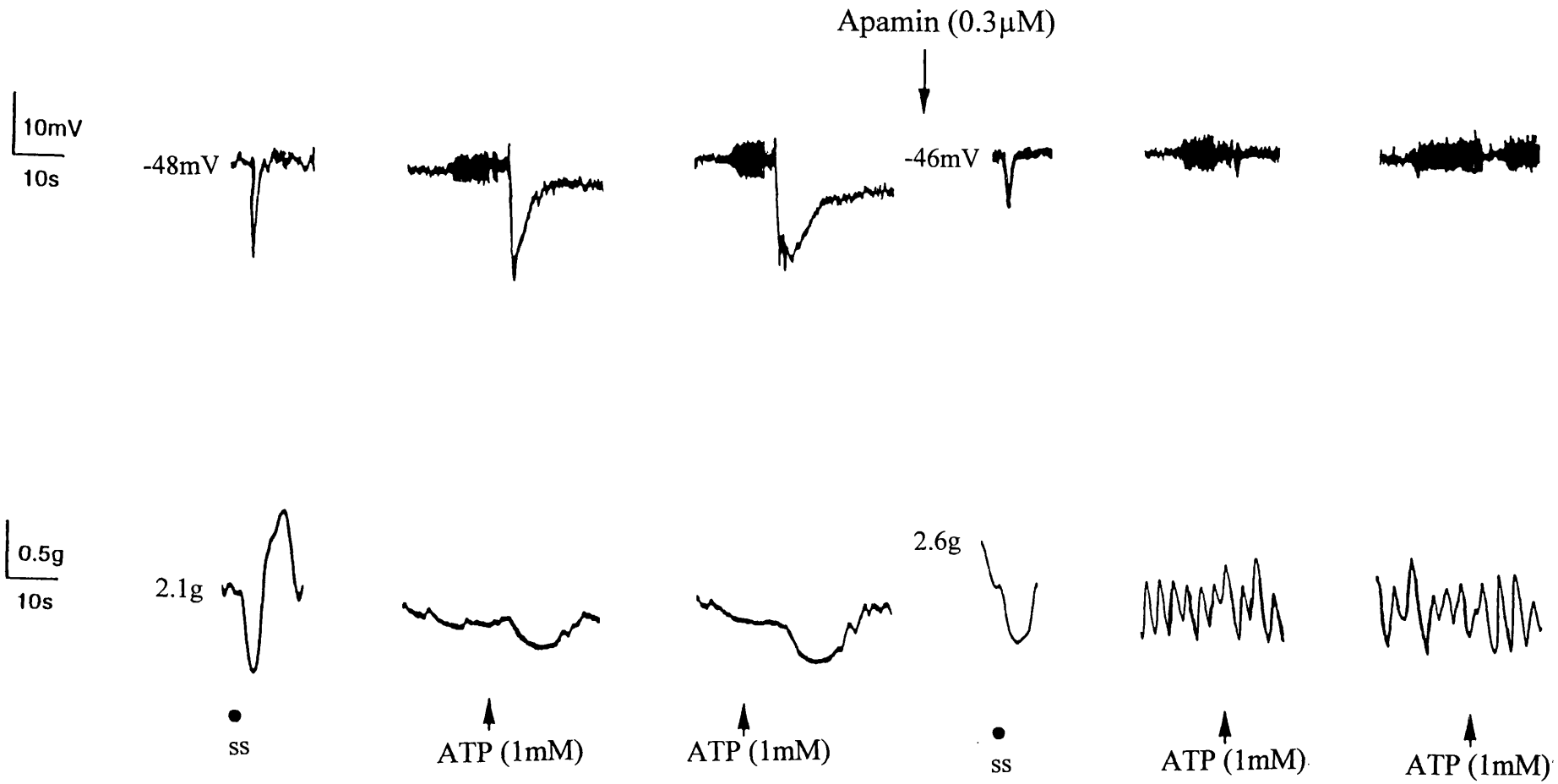


Figure 30: Effect of apamin (0.3μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to ATP (1mM), injection by microsyringe (25μl; injection volume 3μl), and to EFS (single stimulus (ss), supramaximal voltage, 0.1ms). ATP and EFS each hyperpolarized the membrane and reduced tone. The rate of onset and amplitude of the electrical changes to ATP and EFS were similar. The electrical and mechanical responses to ATP were abolished and those to EFS reduced, by apamin, leaving only spontaneous activity. Injection artefacts account for thickening on electrical records. Records were obtained from approximately adjacent cells.

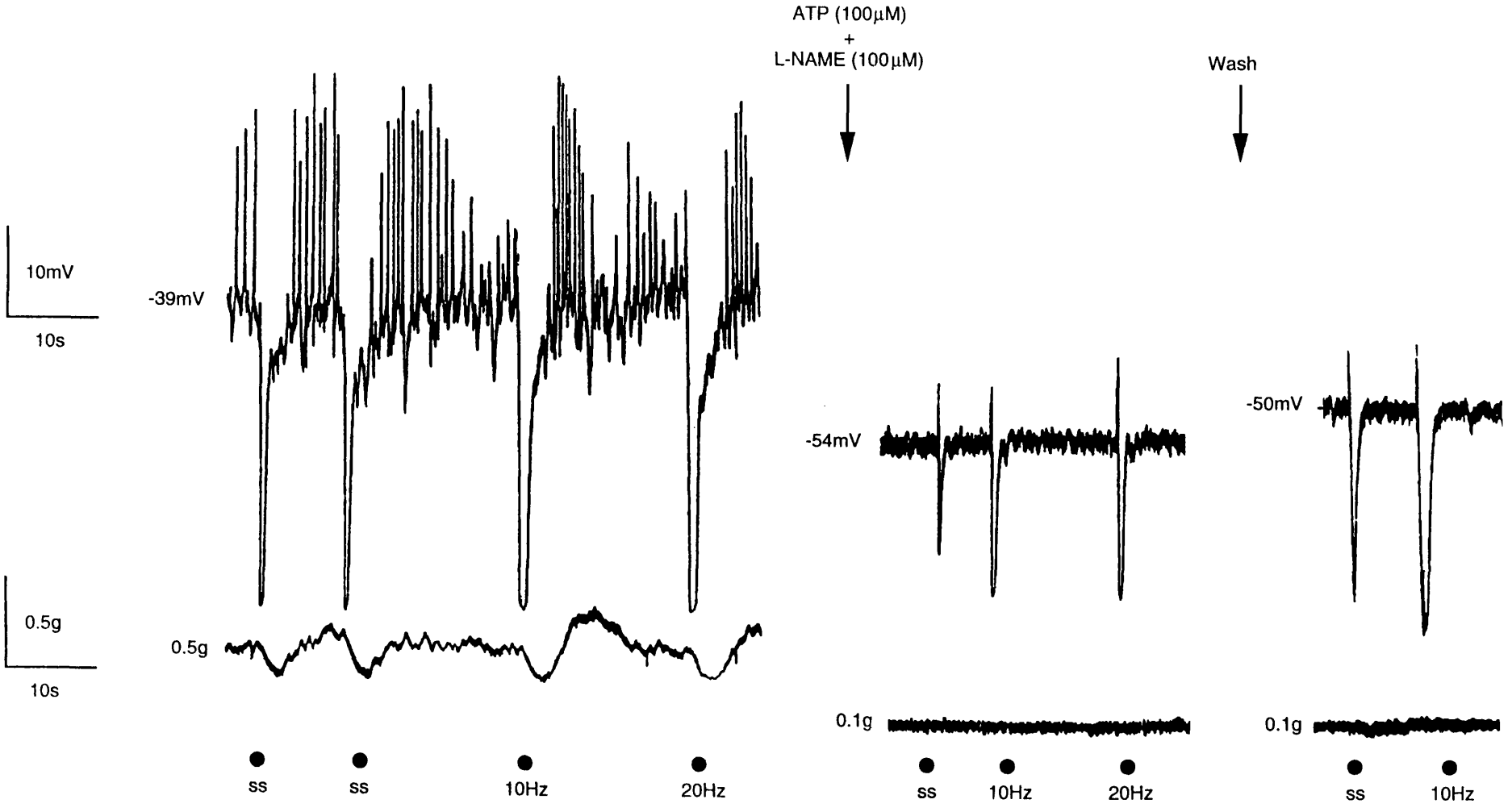


Figure 31: Effect of ATP (100μM) perfusion, in the presence of L-NAME (100μM), on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10 and 20Hz). ATP, in the presence of L-NAME, hyperpolarized and relaxed the tissue. IJP amplitude was reduced, but the extent of this reduction was dependent on the membrane potential, as evidenced by the partial restoration of IJP amplitude as the membrane potential began to return to control. Records were obtained from approximately adjacent cells.

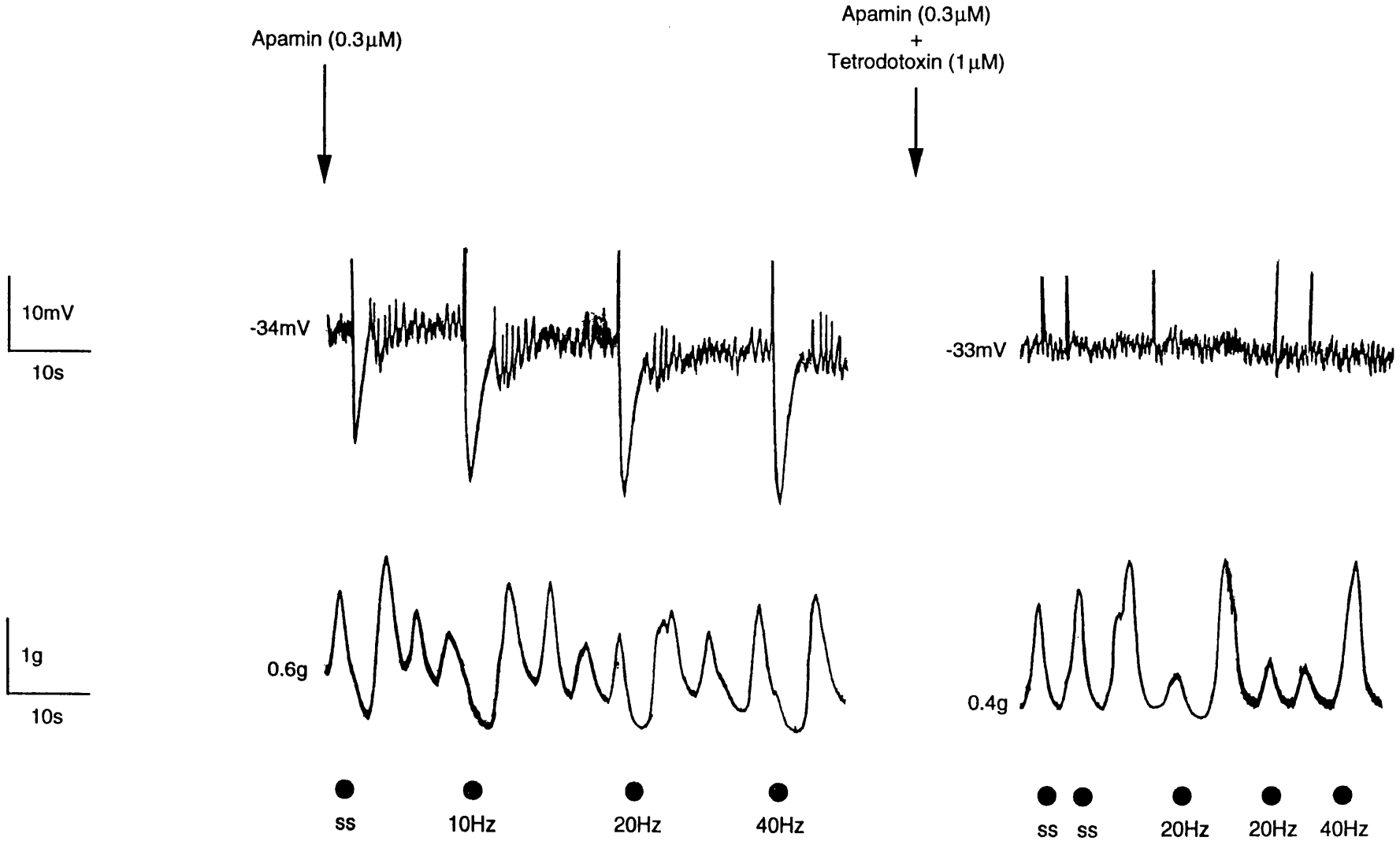


Figure 32: Effect of tetrodotoxin (TTX; 1μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical apamin-insensitive responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40Hz). TTX abolished evoked IJPs and relaxations leaving only stimulus artefacts. Records obtained from the same cell.

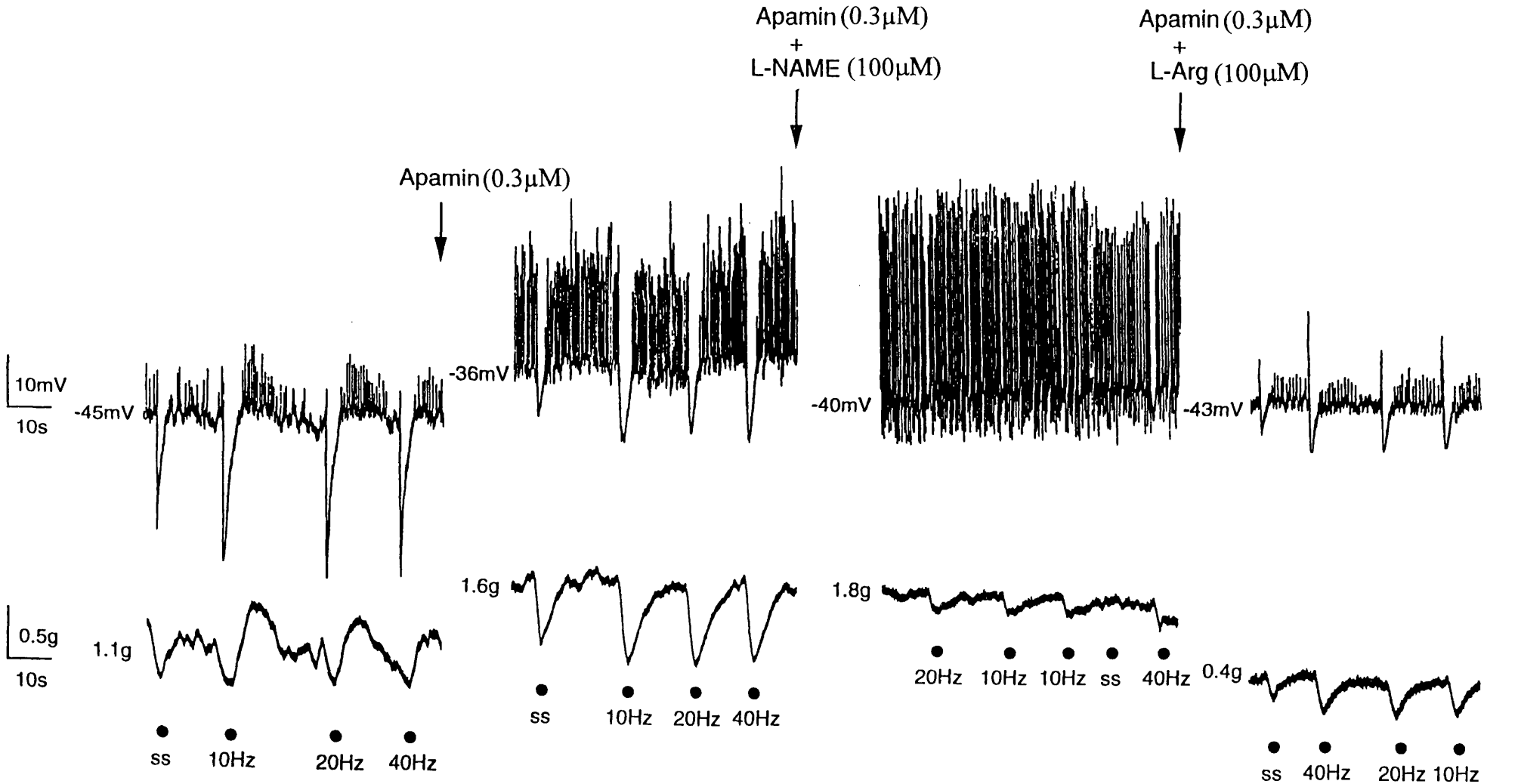
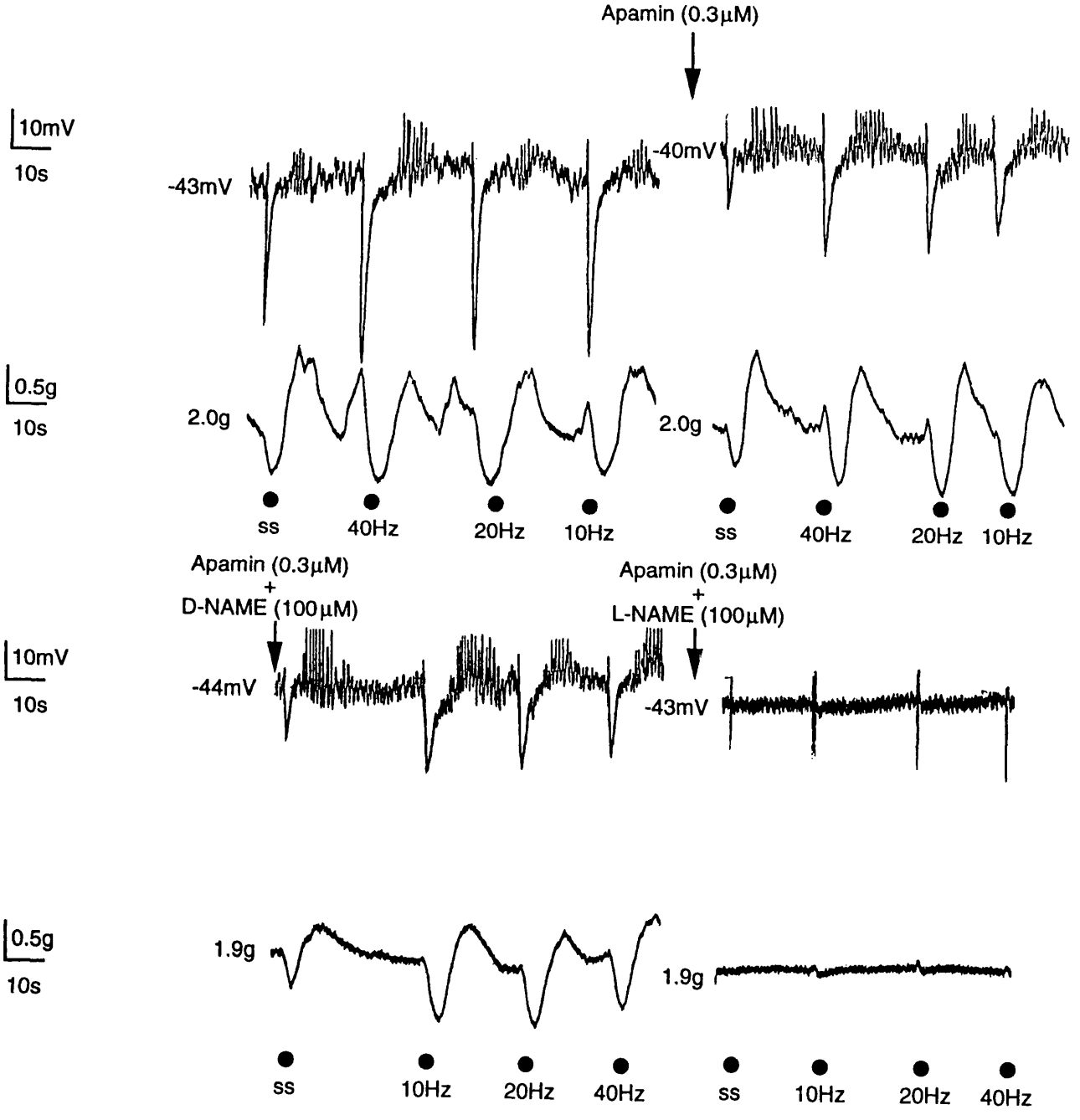


Figure 33: Effect of apamin (0.3μM) alone, together with L-NAME (100μM) and in the additional presence of L-arginine (L-Arg; 100μM) on the intracellular electrical (upper traces in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40 Hz). Apamin itself transiently depolarized the membrane. It reduced, and together with L-NAME, abolished the IJPs and accompanying relaxations. Both IJPs and relaxations were partially restored in the presence of apamin following L-arginine. Records were obtained from approximately adjacent cells.

Figure 34: Effect of apamin (0.3μM) alone, and in the presence of D-NAME or L-NAME (each 100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40 Hz). Apamin itself, transiently depolarized the membrane and reduced both the amplitude of the IJPs and tone. These effects were not significantly altered in the combined presence of apamin and D-NAME. Apamin and L-NAME together abolished IJPs and relaxations leaving only stimulus artefacts. Records were obtained from approximately adjacent cells.


Apamin (0.3μM)
10mV
10s
-43mV
-40mV
0.5g
10s
2.0g
2.0g
ss
40Hz
20Hz
10Hz
ss
40Hz
20Hz
10Hz
Apamin (0.3μM)
+
D-NAME (100μM)
Apamin (0.3μM)
+
L-NAME (100μM)
10mV
10s
-44mV
-43mV
0.5g
10s
1.9g
1.9g
ss
10Hz
20Hz
40Hz
ss
10Hz
20Hz
40Hz

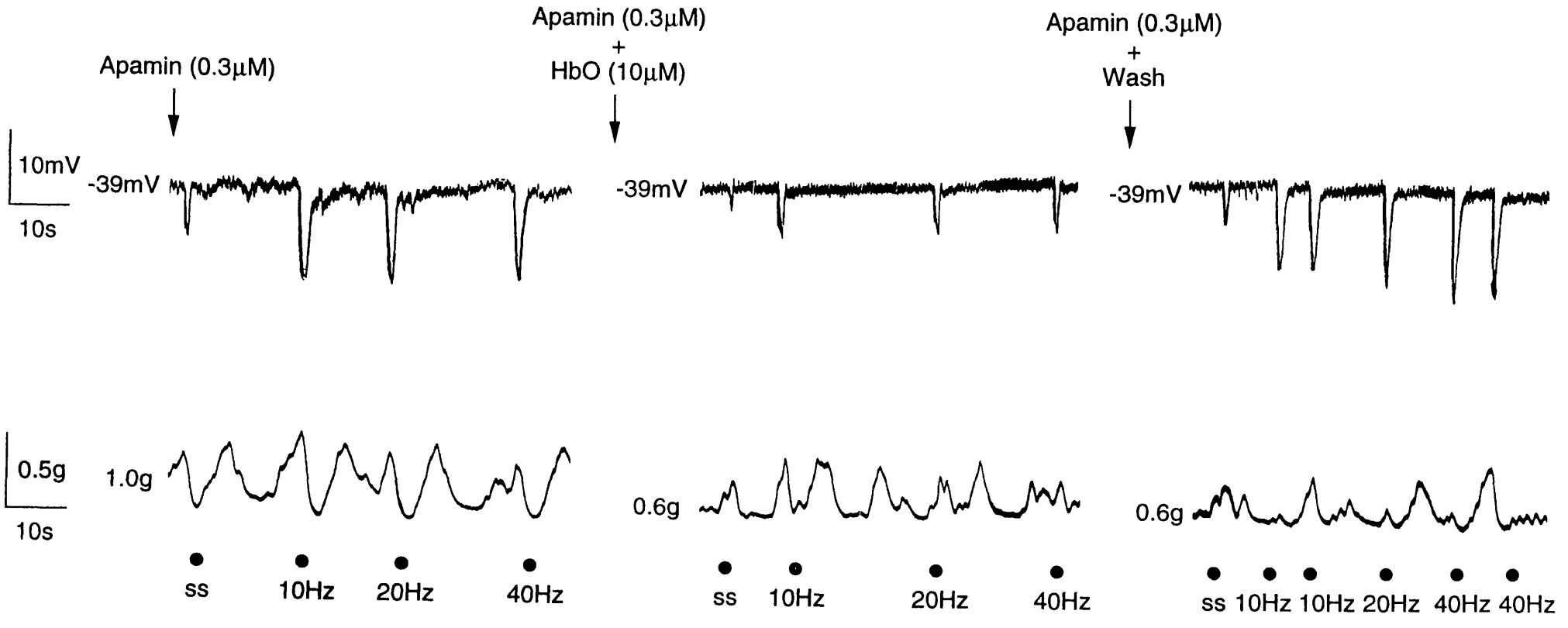


Figure 35 : Effect of oxyhaemoglobin (HbO, 10μM) on the apamin (0.3μM)-insensitive intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40 Hz). At all frequencies investigated, apamin (0.3μM), (after the initial depolarization had worn off) alone reduced the amplitude of the IJPs. This effect was enhanced by HbO, and reversed upon washing with apamin-containing Krebs` solution. Records were obtained from same cell.

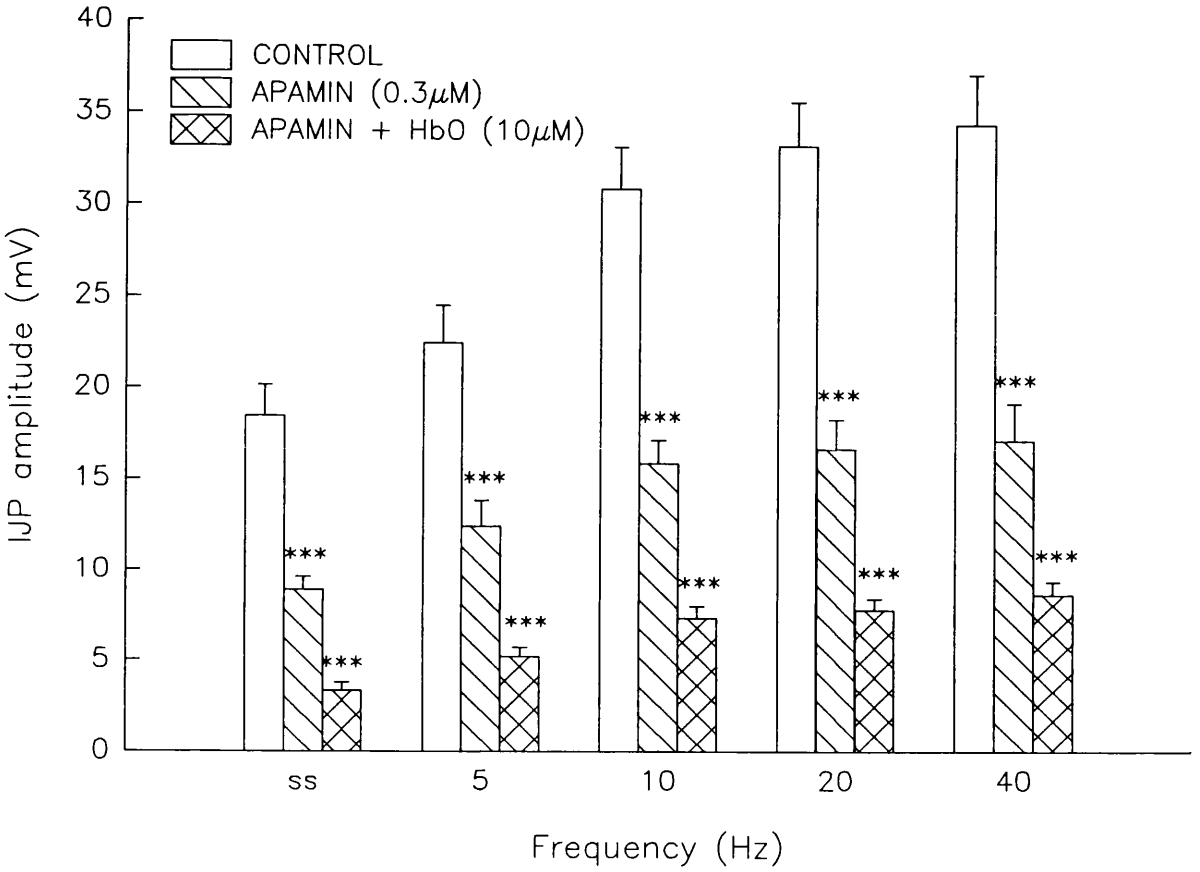


Figure 36: Effect of apamin (0.3μM) alone and in combination with oxyhaemoglobin (HbO; 10μM) on IJP amplitude (mV) in response to increasing stimulation frequency (Hz; single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz, supramaximal voltage, 0.1ms). Each bar represents the mean ± S.E.M., n= 26 (minimum), from 4 different tissues (minimum). Significance refers to comparisons between controls and apamin and between apamin alone and with HbO. Apamin itself significantly reduced IJP amplitude at all frequencies ($P<0.001$). This reduction was significantly ($P<0.001$) enhanced by HbO at all frequencies.

These observations implicated NO in the slow, apamin-insensitive IJP component. If this view was correct, the smaller, slower-to-peak IJP component, revealed by suramin, might also be mediated by NO and a combination of suramin and L-NAME should abolish IJPs and accompanying relaxations. This was indeed the case (Fig. 37). The inhibitory effect of L-NAME on the suramin-insensitive IJP component was reversed by L-arginine (100μM; Fig. 37). The effect of L-NAME on IJPs and relaxations was not mimicked by its stereoisomer D-NAME (Fig. 38).

RB2 (100μM), when combined with L-NAME (100μM), failed to abolish the "RB2-insensitive" IJPs, indicating a difference between the mechanism of action of RB2 and that of suramin (Fig. 39).

The effect of HbO upon the suramin-insensitive component of the IJP was also examined. In the presence of suramin, HbO further reduced IJP amplitude (reversibly) at all frequencies studied (ss and trains of 5 stimuli at 5, 10, 20 and 40 Hz, 0.1ms, supramaximal voltage; Fig. 40 & 41).

IX) <u>Effects of Drugs Affecting Guanylyl Cyclase Activity</u>

If NO was involved in the IJPs and relaxations, then guanylyl cyclase modulators should affect IJPs and relaxations, as the effects of NO are mediated via this enzyme (Rapoport & Murad, 1983). The guanylyl cyclase inhibitor LY83583 (10μM; Mülsch *et al*, 1988; Jin *et al*, 1993) reduced IJP amplitude and relaxations in the presence (Fig. 43) of apamin (0.3μM). The cGMP phophodiesterase inhibitor M&B 22948 (MeB; 30μM) and the membrane permeable cGMP analogue 8-bromo cGMP (8-Br-cGMP; 100μM), each of which may have been expected to enhance NO activity, significantly hyperpolarized the membrane (from -43.4±0.9 mV, n=30 from 3 preparations, to -61.9±1.0 mV, n=30 from 3 preparations, $P<0.001$, and from -49.9±1.1 mV, n=36 from 3 preparations, to -64.4±0.8 mV, n=23 cells from 3 preparations respectively, $P<0.001$) and relaxed the tissue as anticipated

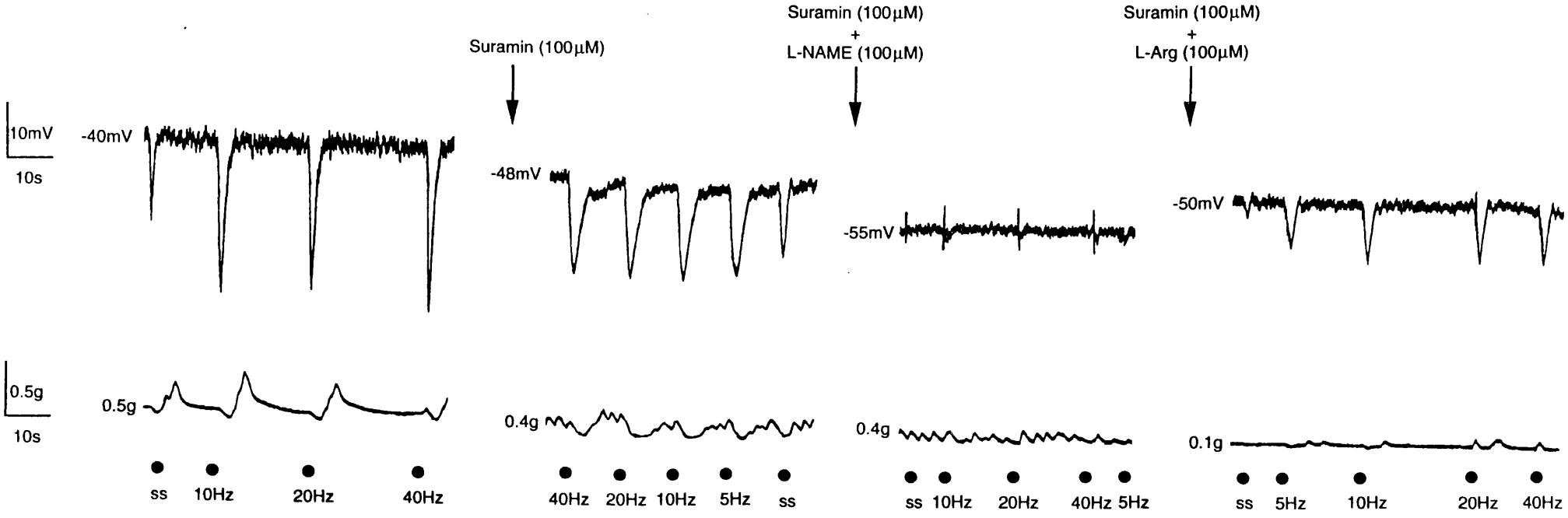


Figure 37: Effect of suramin (100μM) alone and in the presence of L-NAME or L-arginine (each 100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms pulse width, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz). Suramin itself reduced, but did not abolish the IJPs at all frequencies. Relaxations were still visible. Suramin together with L-NAME abolished both electrical and mechanical responses, leaving only stimulus artefacts and spontaneous activity. L-arginine, in the presence of suramin, partially restored evoked IJPs but not the mechanical responses. Records were obtained from approximately adjacent cells.

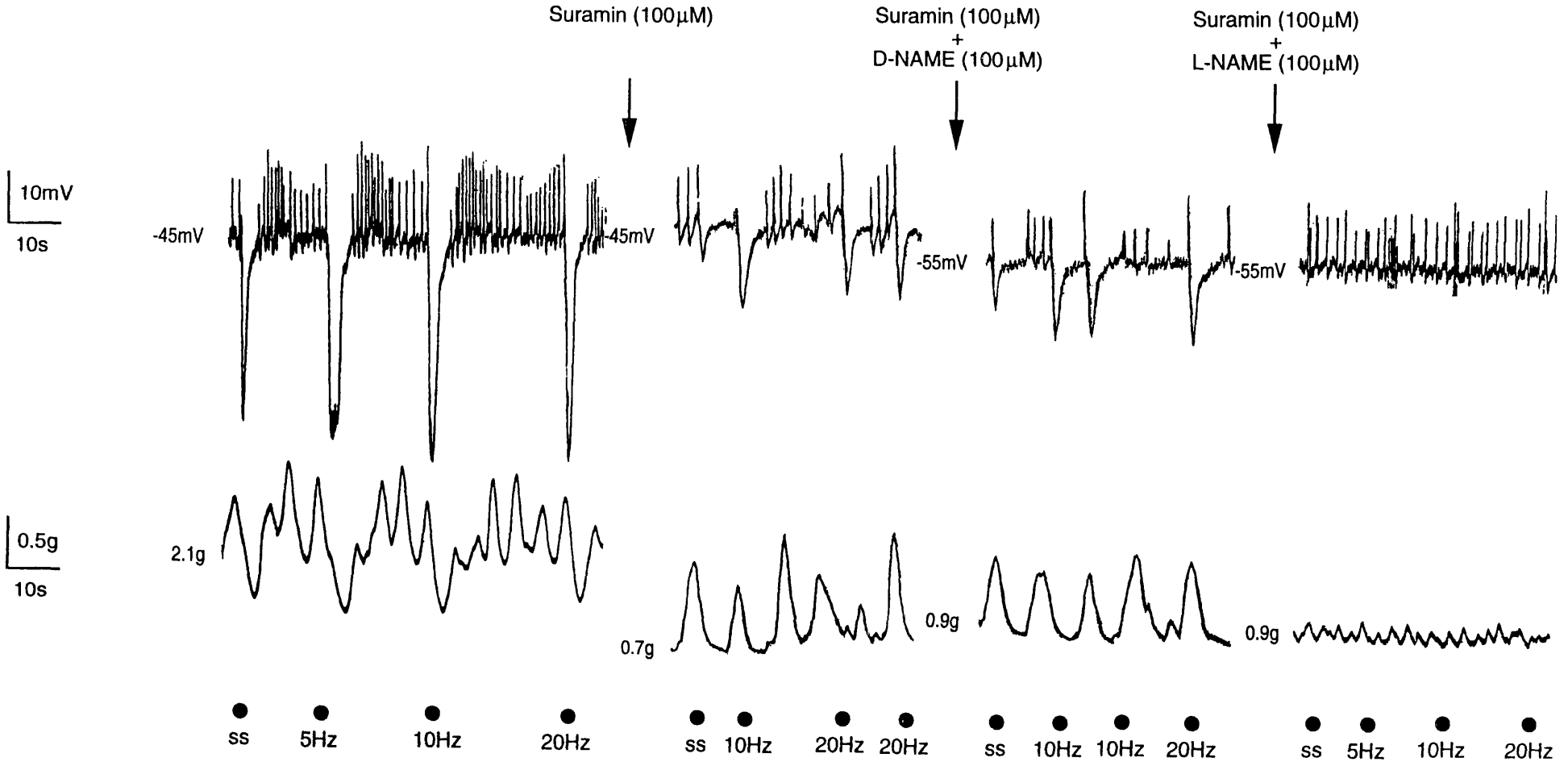


Figure 38 : Effect of suramin (100μM) alone and in the presence of D-NAME or L-NAME (each 100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, and 20 Hz). Suramin itself reduced the amplitude of the IJPs and tone, effects which were not significantly altered in the combined presence of suramin and D-NAME. Suramin and L-NAME together abolished IJPs and relaxations leaving only stimulus artefacts. Records were obtained from approximately adjacent cells.

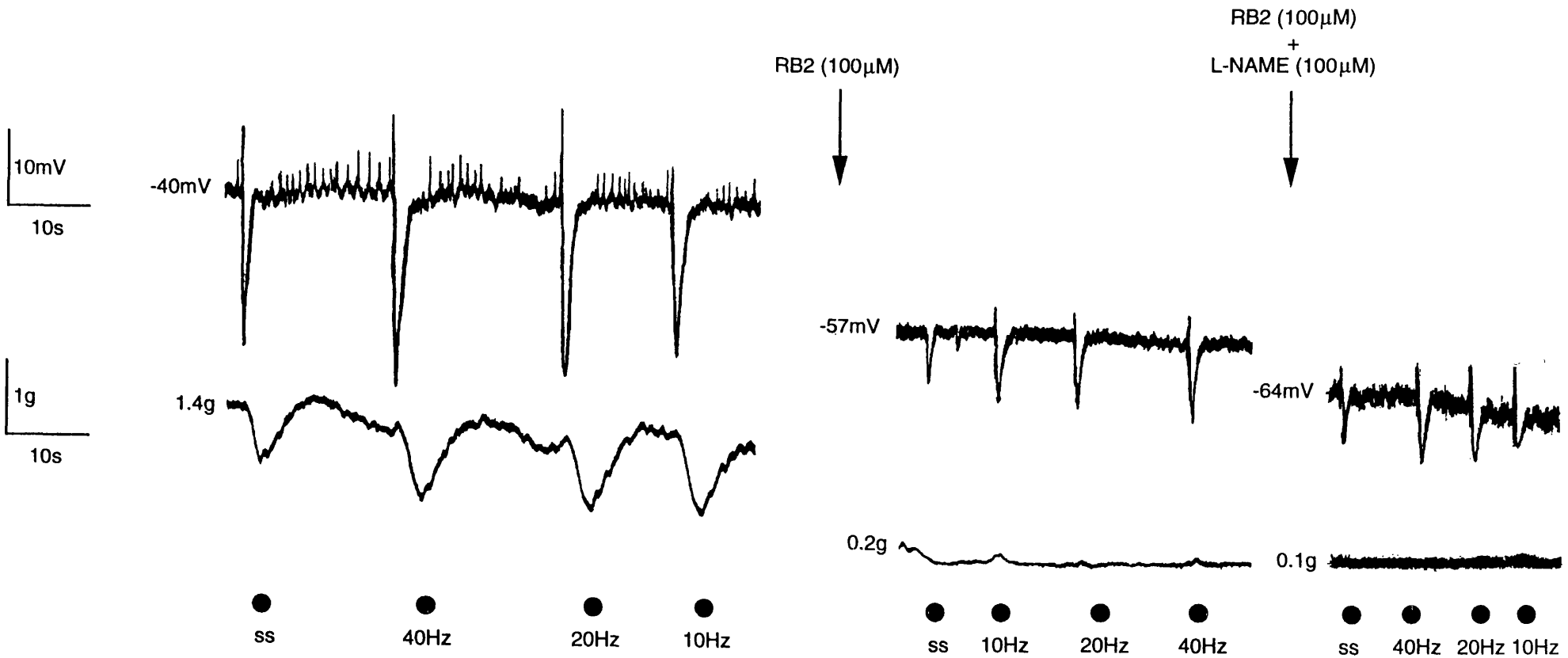


Figure 39: Effect of reactive blue 2 (RB2; 100μM) alone and in the presence of L-NAME (100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 10, 20 and 40 Hz). RB2 alone hyperpolarized the membrane, almost abolished tone and relaxations. The addition of L-NAME, in the presence of RB2 further hyperpolarized the membrane but did not significantly affect IJP amplitude. Records were obtained from approximately adjacent cells.

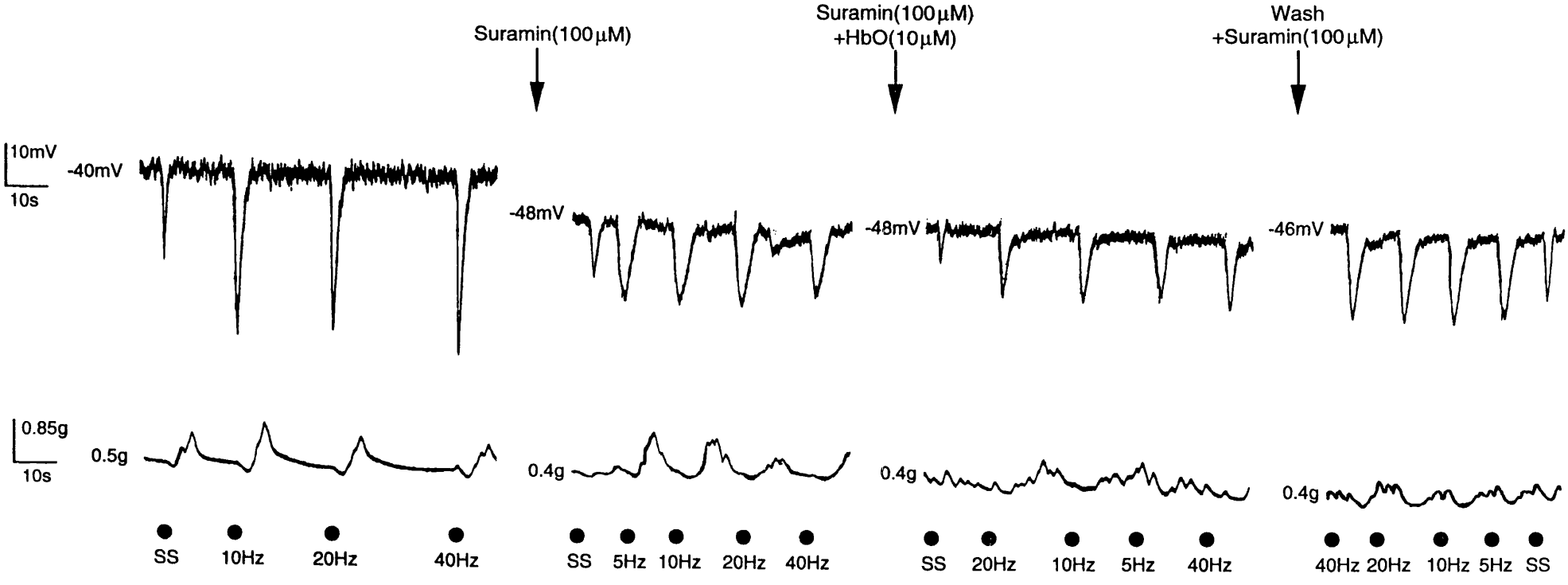


Figure 40: Effect of oxyhaemoglobin (HbO; 10μM) on the suramin (100μM)-insensitive intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz). Suramin alone reduced the amplitude of the IJPs and increased spontaneous activity. The inhibition was enhanced by HbO, an effect reversed upon washing with suramin-containing Krebs` solution. Records were obtained from approximately adjacent cells.

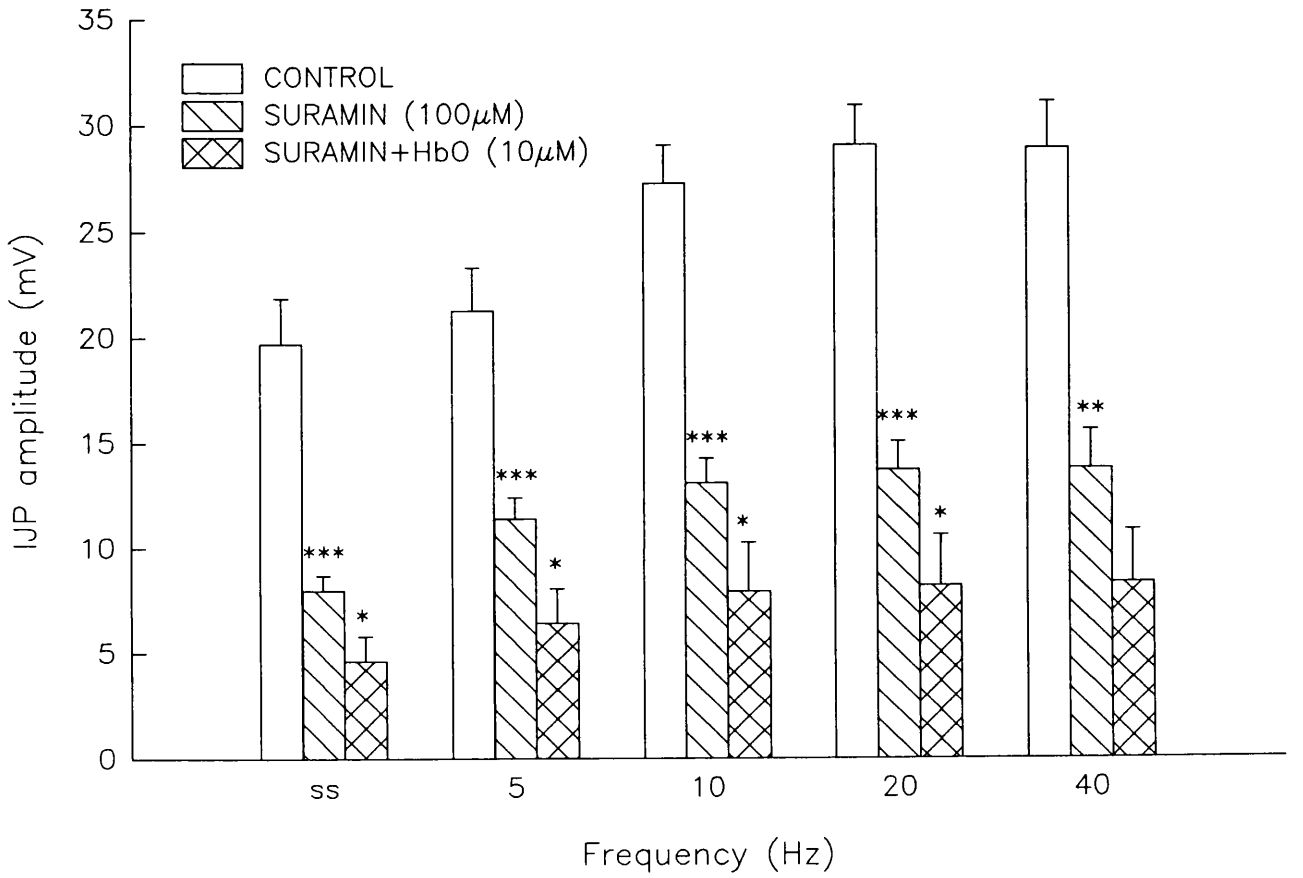


Figure 41: Effect of suramin (100μM) alone and in combination with oxyhaemoglobin (HbO; 10μM) on IJP amplitude (mV) in response to increasing stimulation frequency (Hz; single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz, supramaximal voltage, 0.1ms). Each bar represents the mean ± S.E.M., n = 9 (minimum), from 4 different tissues (minimum). Significance refers to comparisons between controls and suramin, and between suramin alone and with HbO. Suramin alone significantly reduced IJP amplitude at all frequencies ($P<0.01$); this reduction was significantly ($P<0.05$) enhanced by HbO at all frequencies except 40Hz.

(Figs. 44 & 45). Neither drug increased the duration of the IJPs as has been proposed (Ward *et al*, 1992 c). These results suggested that apamin- and suramin-insensitive IJPs and relaxations to NANC nerve stimulation and the drugs which increase guanylyl cyclase activity were acting by a similar mechanism. Sodium nitroprusside (SNP; 50 μM), a NO donor (Bates *et al*, 1991), also significantly hyperpolarized the membrane (from -43.4±0.9 mV, n=15 from 3 preparations to -66.4±1.6 mV, n=8 from 3 preparations, $P<0.001$) and abolished tone. The effects were transient and declined in the presence of the drug; however, the NO-generating mechanism rather than NO itself may be responsible for this decline. During hyperpolarization IJP amplitude was also reduced but recovered upon restoration of the membrane potential (Fig. 46).

X) Effects of VIP

Although a combination of L-NAME and, either apamin or suramin, abolished the evoked IJPs, suggesting the participation of only two mediators, the effects of VIP, proposed as an inhibitory transmitter in other gastrointestinal tissues (Goyal *et al*, 1980; Grider *et al*, 1983, 1992; He & Goyal, 1993; Grider 1993; Makhlouf & Grider, 1993), were examined. Alone, VIP reversibly hyperpolarized the membrane and relaxed the gpIAS. The membrane potential, in the absence of VIP, was -48±1.0 mV (n=23 from 4 preparations) and in its presence (0.01-0.25μM) was -67.9±2.7 mV (n=13 from 4 preparations, $P<0.001$). The duration of the VIP-induced hyperpolarizations were prolonged compared with those produced by ATP and EFS. The response to VIP was not modified by either L-NAME (Fig. 47) or apamin (Fig. 48) suggesting that the hyperpolarizations and relaxations produced by VIP were not mediated by the release of NO from enteric nerves as proposed (Grider *et al* 1992; Grider, 1993; Makhlouf & Grider, 1993; Murthy *et al*, 1994), or that VIP was the apamin-sensitive mediator.

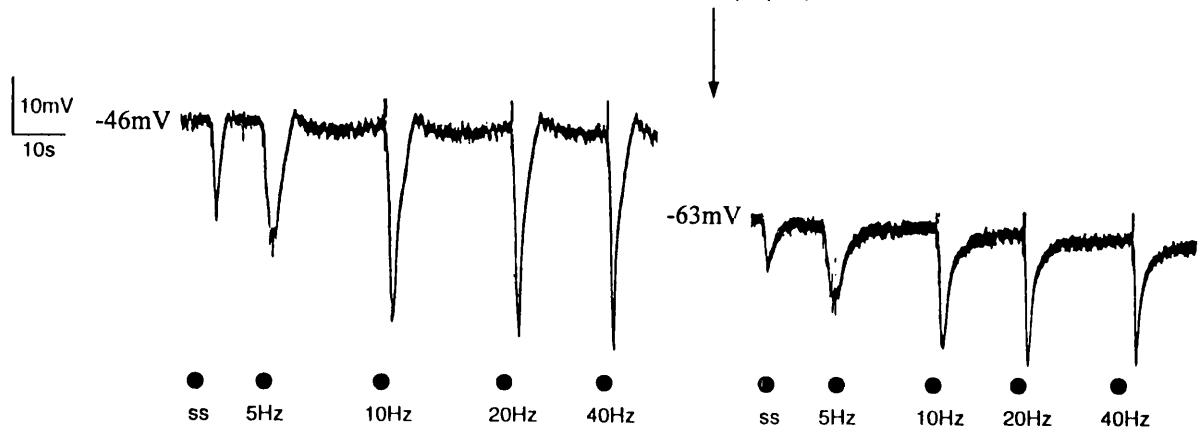


Figure 42: Effect of LY83583 (10μM) on the intracellular electrical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz) in the presence of nifedipine (1μM). LY83583 hyperpolarized the membrane. Records were obtained from the same cell.

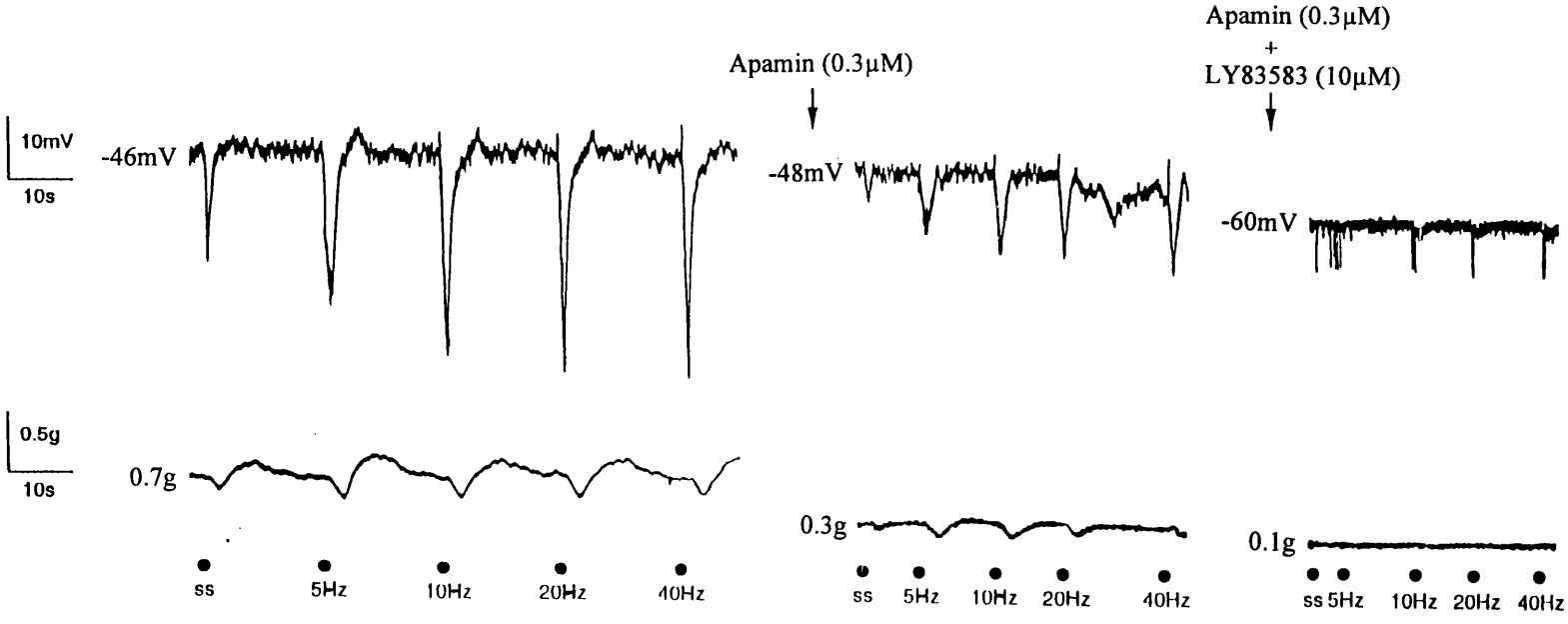


Figure 43: Effect of apamin (0.3μM) alone and in the presence of LY83583 (10μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40Hz). Apamin alone reduced and, in the additional presence of LY83583 abolished, the IJPs leaving only stimulus artefacts. Records were obtained from approximately adjacent cells.

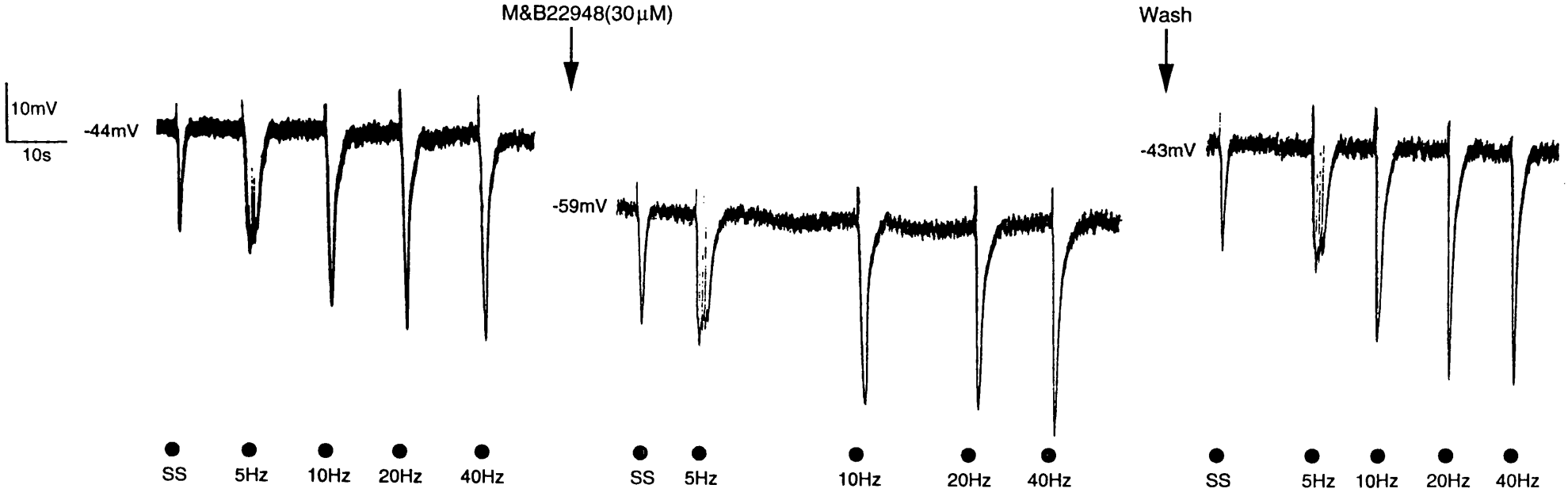


Figure 44: Effect of M&B 22948 (30μM) on the intracellular electrical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40Hz) in the presence of nifedipine (1μM). M&B 22948 hyperpolarized the membrane but did not significantly affect IJP amplitude. The hyperpolarization was reversed on washing. Records obtained from adjacent cells.

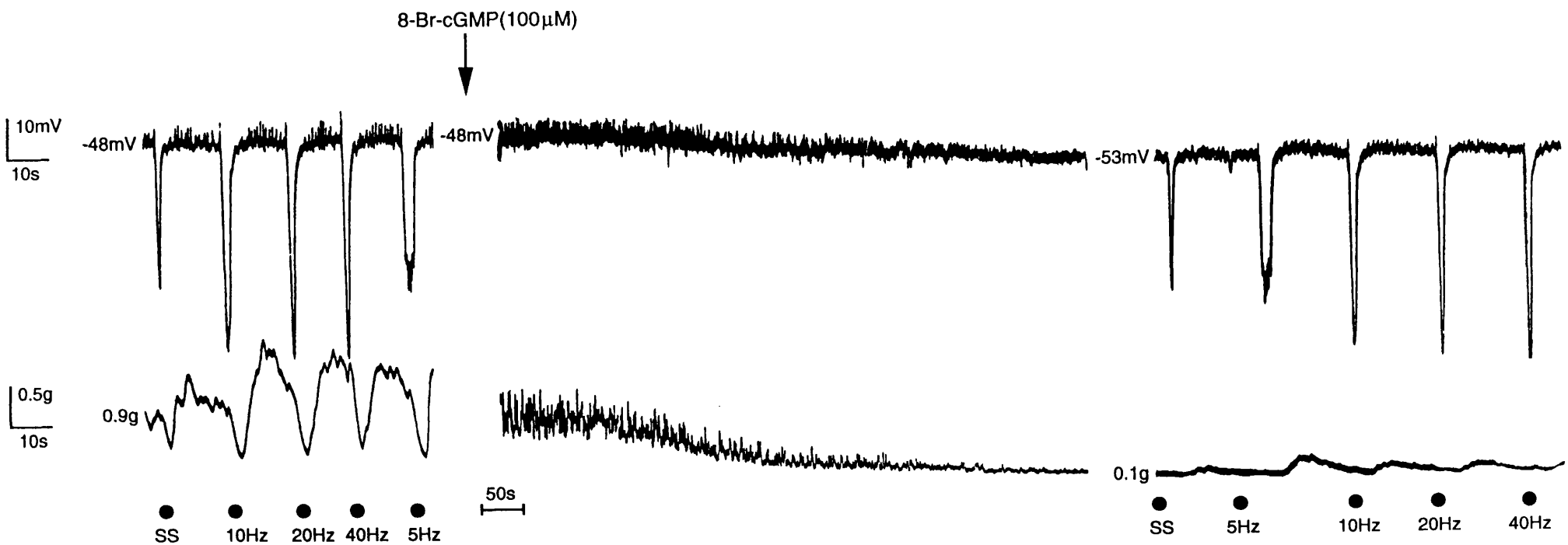


Figure 45: Effect of 8-bromo-cGMP (8-Br-cGMP; 100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40Hz). 8-Br-cGMP reduced tone and increased membrane potential slightly but did not affect the evoked IJPs. Records were obtained from the same cell.

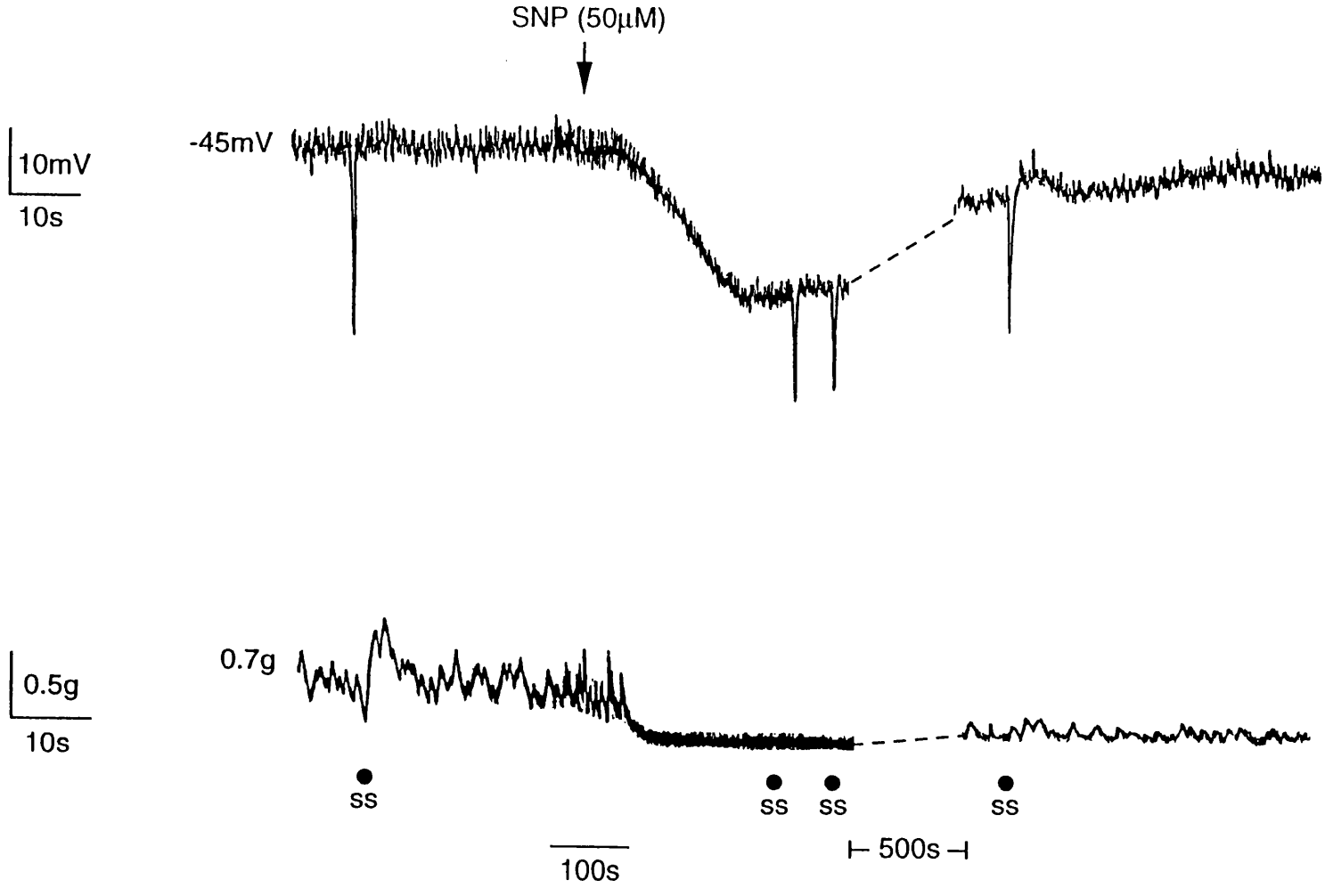


Figure 46: Effect of sodium nitroprusside (SNP; 50μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss). SNP hyperpolarized the membrane and abolished tone. Both parameters were restored in the presence of the drug. IJP amplitude was also reduced by SNP but the extent of this reduction was dependent upon membrane potential. Note different time scales. Records were obtained from the same cell.

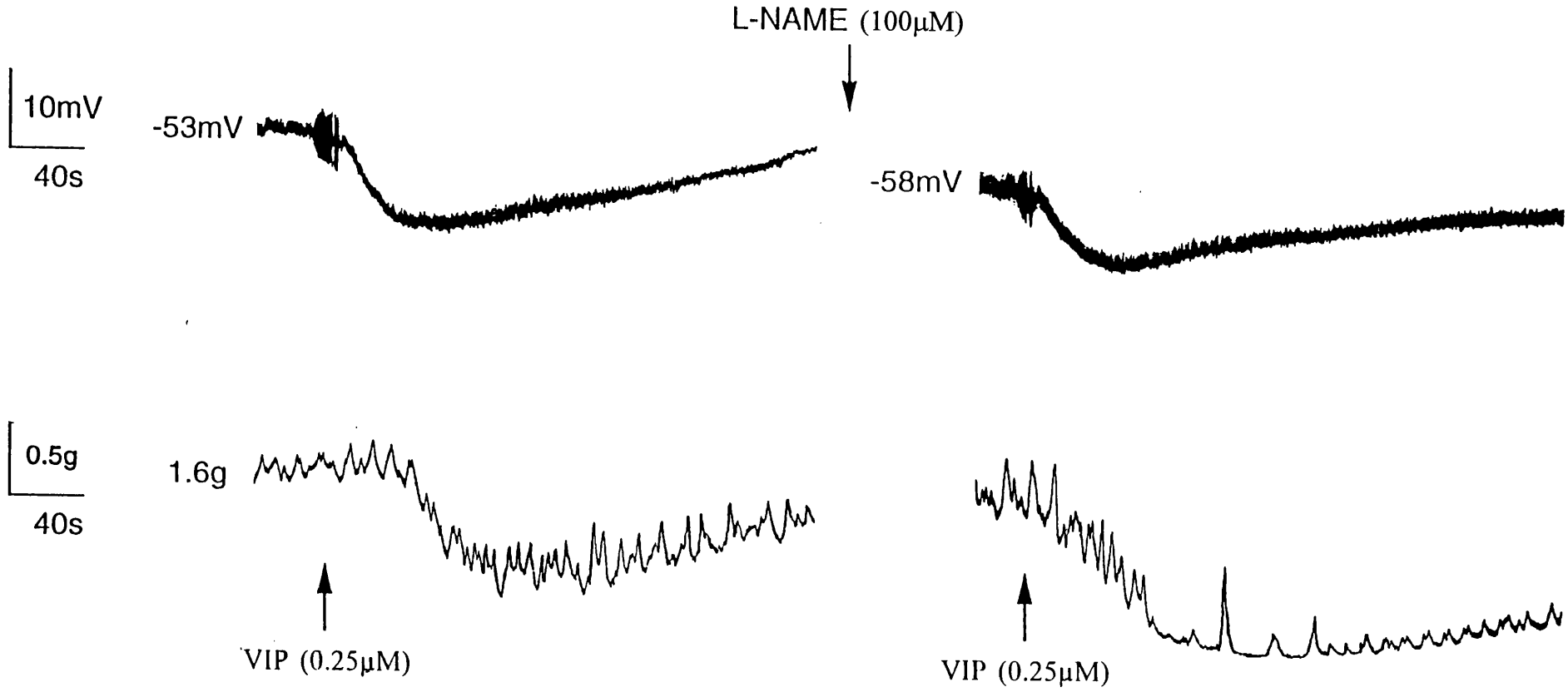


Figure 47: Effect of VIP (0.25μM) in the presence and absence of L-NAME (100μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS. VIP hyperpolarized the membrane and relaxed the tissue; these effects were unaffected by L-NAME, nor did they resemble, in onset or duration, the responses to nerve stimulation in this tissue (*e.g.* see Fig. 12). Records were obtained from approximately adjacent cells.

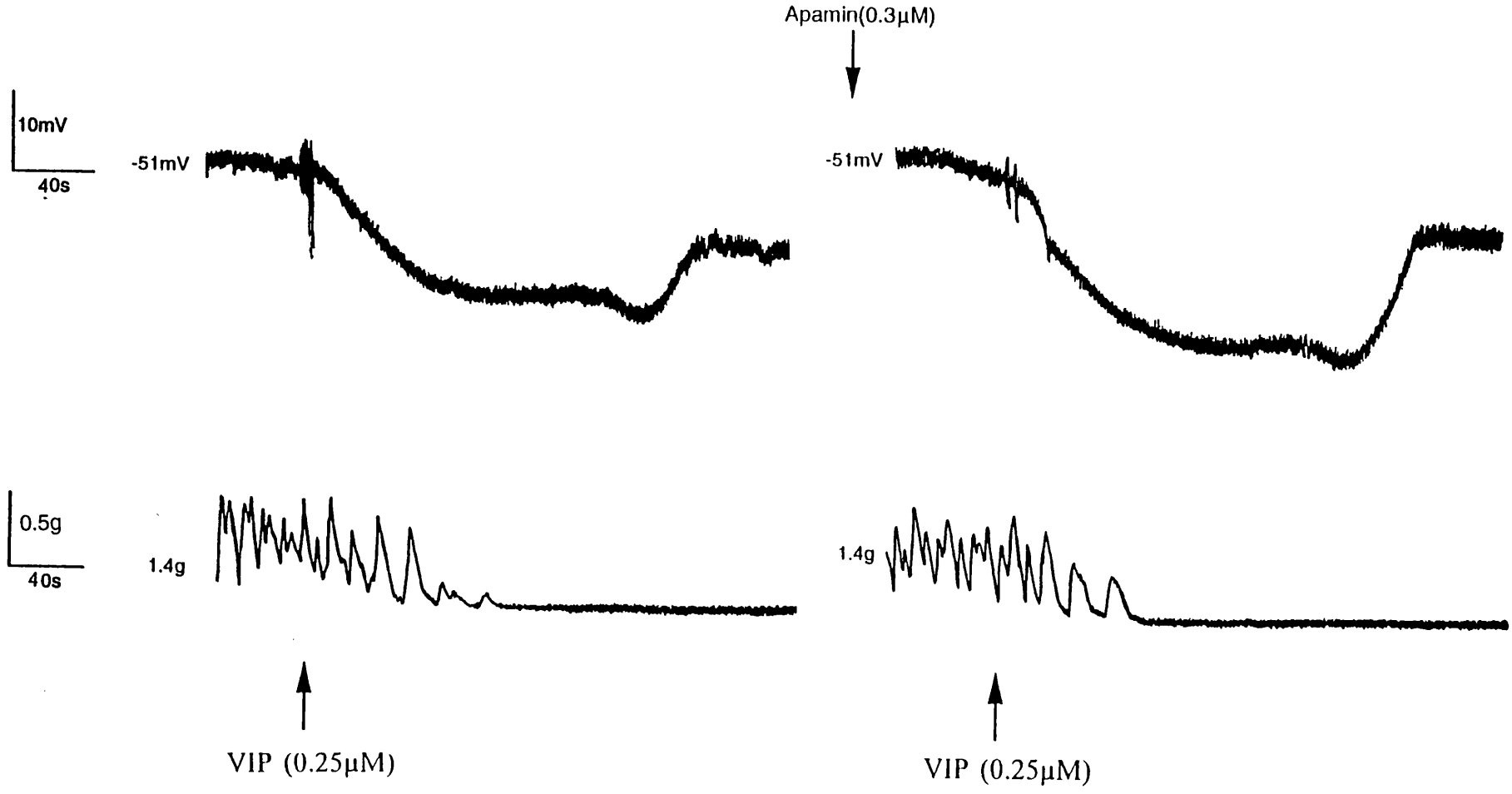


Figure 48: Effect of VIP (0.25μM) in the presence and absence of apamin (0.3μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS. VIP hyperpolarized the membrane and relaxed the tissue; these effects were not affected by apamin, nor did they resemble, in onset or duration, the responses to nerve stimulation in this tissue (*e.g.* see Fig. 12). Records were obtained from approximately adjacent cells.

XI) Effects of $[Cl^-]_o$ Substitution

Elimination of $[Cl^-]_o$ ($0[Cl^-]_o$) with a mixture of sodium glucuronate, potassium gluconate and calcium gluconate hyperpolarized the membrane from -46.9±0.7 mV (n=28 from 5 preparations) to -60.0±1.1 mV (n=17 from 5 preparations, $P<0.001$) and reduced both apamin-sensitive and -insensitive IJP amplitude and tone. These effects were reversible on washing (Figs. 49 & 50).

$0[Cl^-]_o$ almost abolished the apamin (0.3μM)-insensitive IJPs and tone, leaving only spontaneous depolarizations and contractions.

XII) Effects of the Chloride Channel Antagonist, IAA-94

In the presence of the chloride channel antagonist IAA-94 (30-50μM; Landry *et al*, 1987, 1989), alone, the membrane potential increased from -42.7±1.1 mV (n=30 from 6 preparations) to -56.6±1.2 mV (n=20 from 6 preparations, $P<0.001$) and the IJPs were reduced. These effects were reversible on washing (Fig. 51). IAA-94 reduced the amplitude of the suramin-insensitive IJPs, the effect being concentration-dependent. Washing partially restored suramin-insensitive IJP amplitude (Fig. 52).

2) HISTOLOGY

I) NADPH-Diaphorase Staining

Distinct, non-diffuse, blue stained areas could clearly be seen between the circular and longitudinal muscles of the gpIAS following NADPH-diaphorase staining, indicating the presence of NOS-containing elements, probably neurons, within the myenteric plexus (Fig. 53).

3) CYCLIC NUCLEOTIDE CONTENT

I) Effects of Electrical Field Stimulation

Both the cAMP and cGMP levels of the gpIAS were significantly ($P<0.001$) raised from control values of 461.0±60.8 fmol/mg (n=8) to

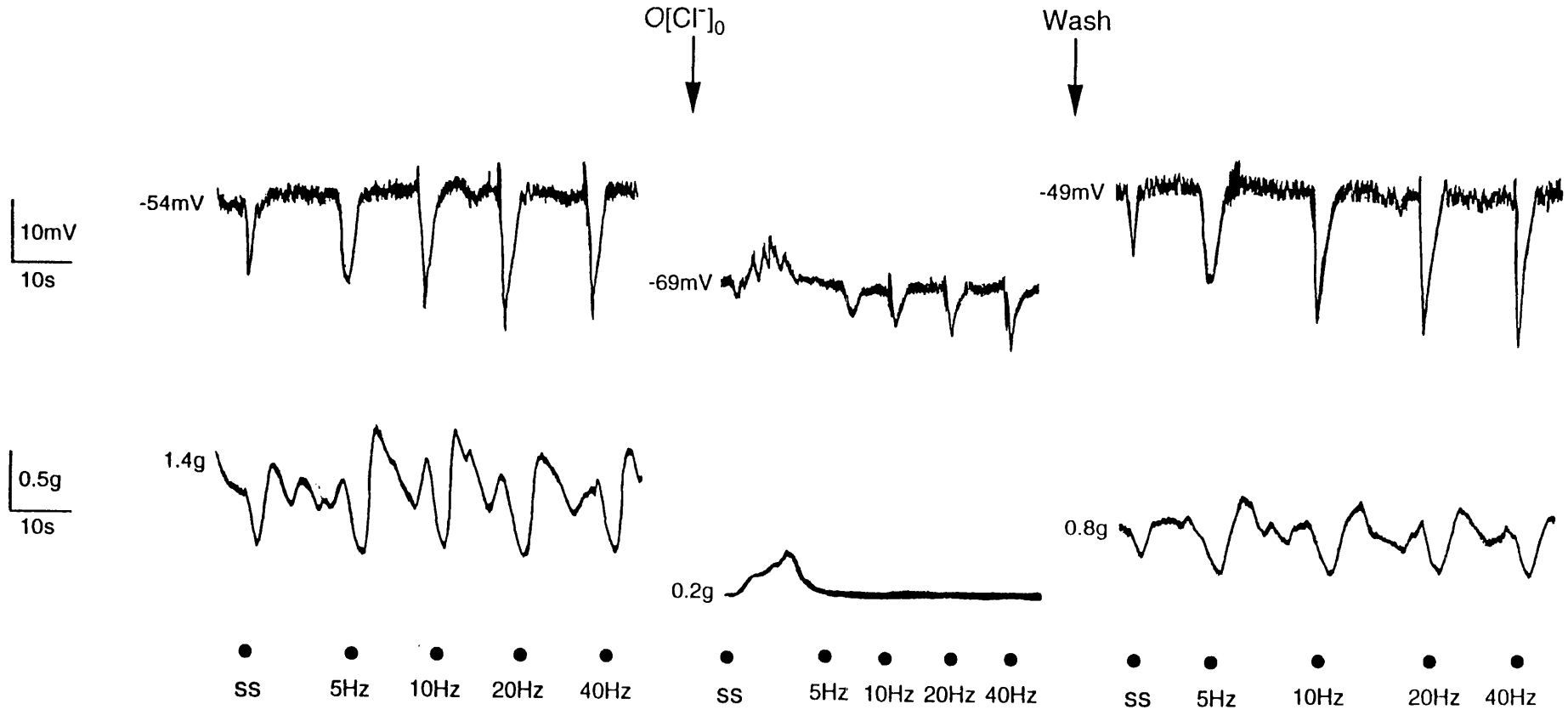


Figure 49: Effect of Cl^- replacement ($0[Cl^-]_0$) by sodium glucuronate, potassium gluconate and calcium gluconate, on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40Hz). The $0[Cl^-]_0$ solution hyperpolarized the membrane, reduced tone and IJP amplitude. These effects were reversed on washing. Records were obtained from approximately adjacent cells.

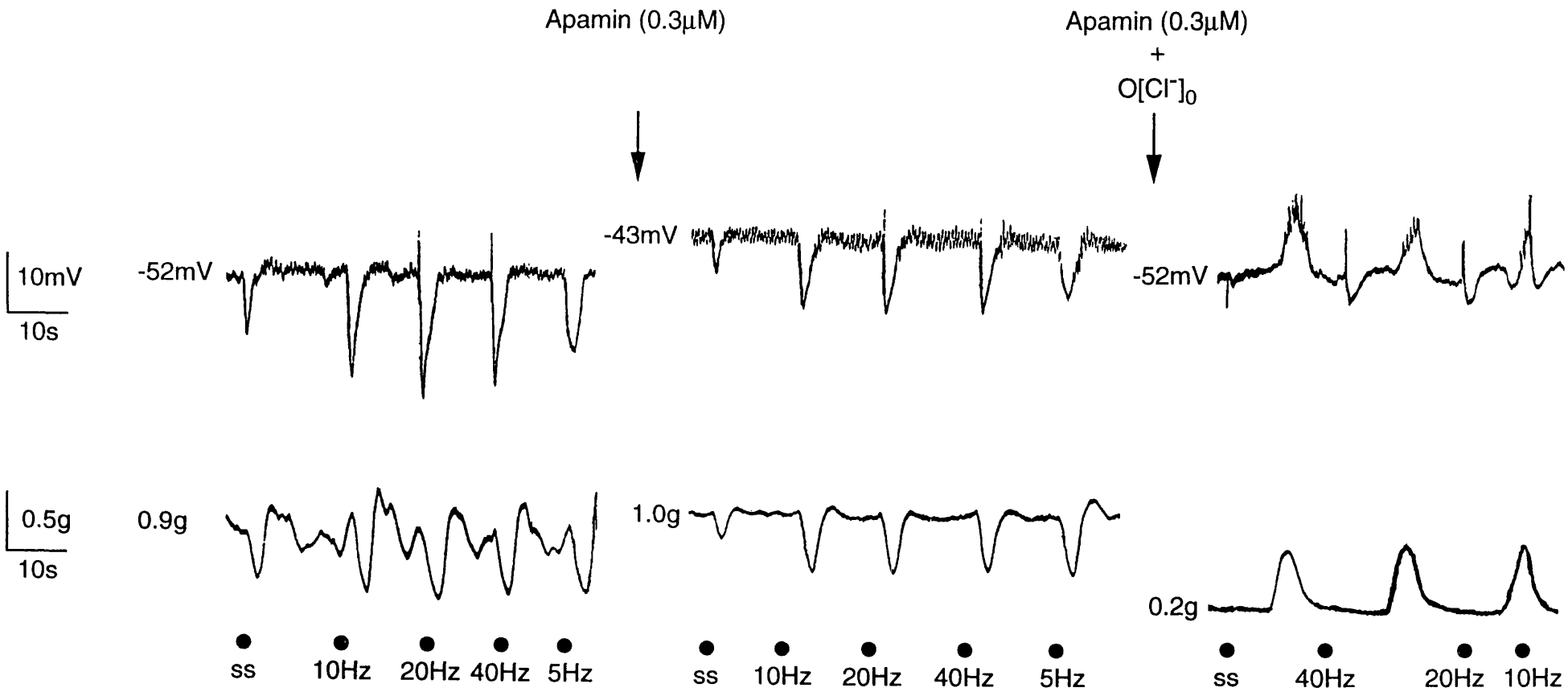


Figure 50: Effect of apamin (0.3μM) alone and in the presence of chloride replacement ($0[Cl^-]_0$) by sodium glucuronate, potassium gluconate and calcium gluconate on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz). Apamin alone transiently depolarized the membrane and increased tone. IJP amplitude was reduced but relaxations are unaffected. Apamin and the $0[Cl^-]_0$ solution hyperpolarized the membrane, abolished tone and further reduced IJP amplitude. Records were obtained from the same cell.

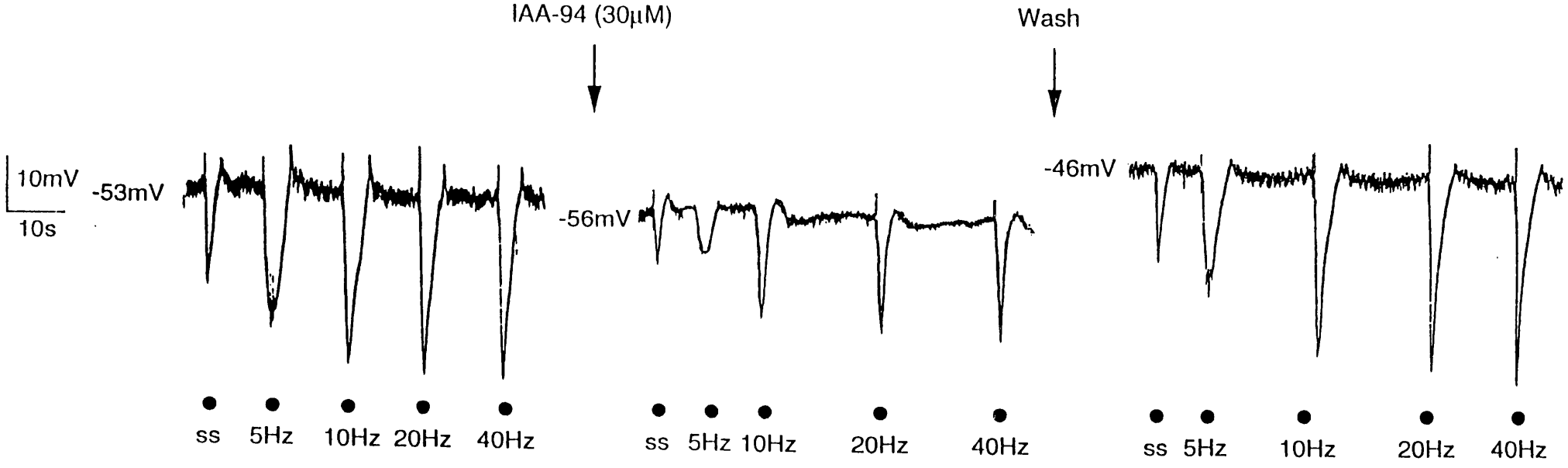


Figure 51: Effect of IAA-94 (30μM) on the intracellular electrical (upper trace in each panel) and simultaneously recorded mechanical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz) in the presence of nifedipine (1μM). IAA-94 hyperpolarized the membrane and reduced IJP amplitude, effects reversed on washing. Records were obtained from approximately adjacent cells.

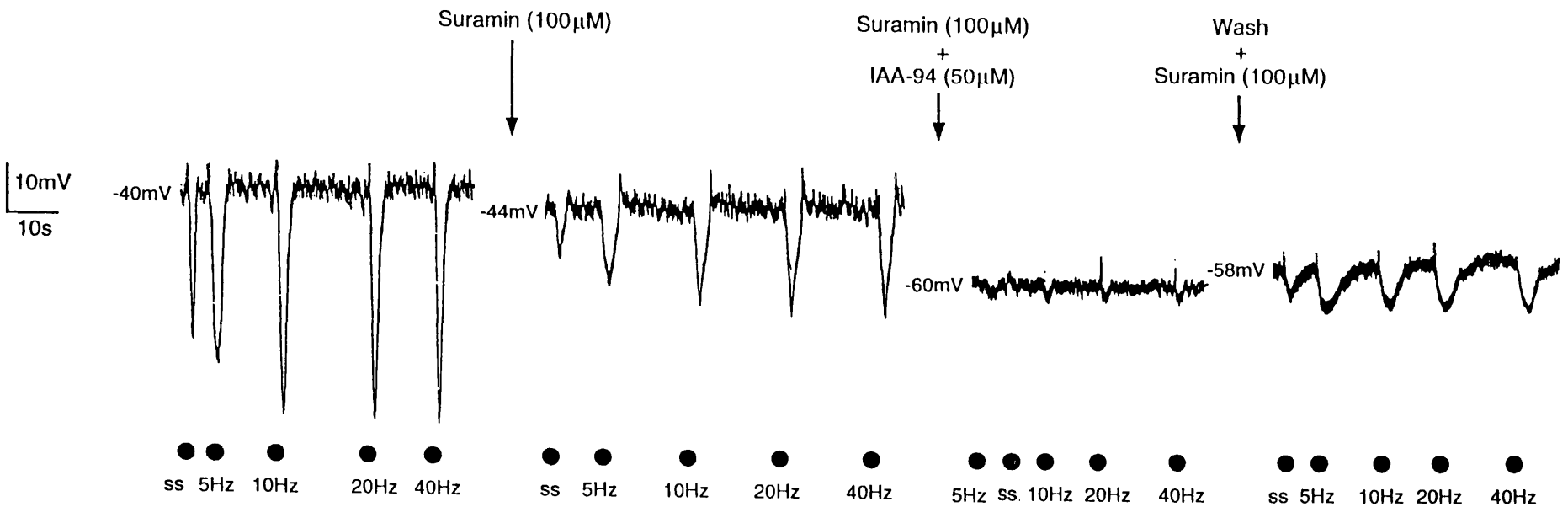


Figure 52: Effect of suramin (100μM) alone and in the presence of IAA-94 (50μM) on the intracellular electrical responses of the gpIAS to EFS (supramaximal voltage, 0.1ms, single stimulus (ss) and 5 stimuli at 5, 10, 20 and 40 Hz) in the presence of nifedipine. Suramin alone reduced the amplitude of the IJPs. Suramin with IAA-94 hyperpolarized the membrane and further reduced IJP amplitude, effects which were partially reversed upon washing. Records were obtained from approximately adjacent cells.

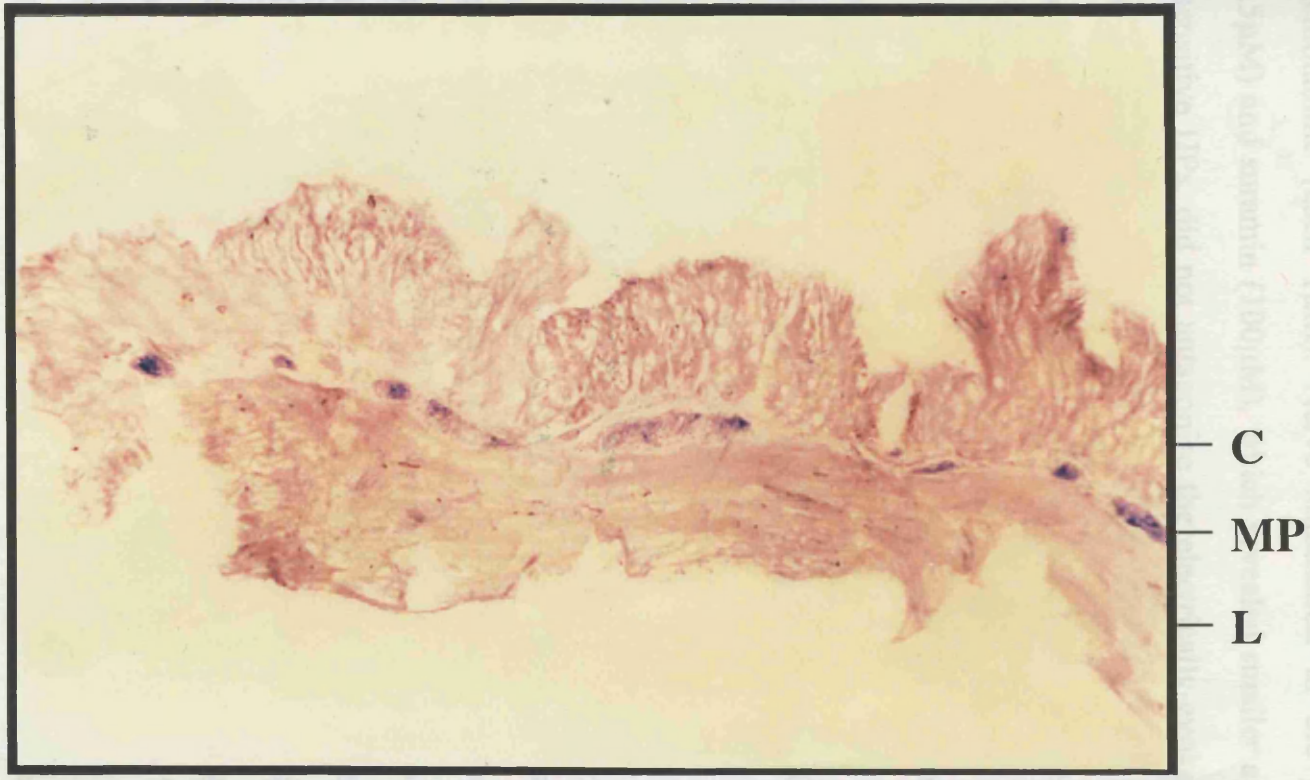


Figure 53: Transverse section (x25) of the guinea-pig anal sphincteric region stained for NADPH-diaphorase, showing longitudinal (L) and circular (C) muscle layers, with the myenteric plexus (MP). The heavily stained areas within the myenteric plexus are nitric oxide synthase containing neurons.

1078.0±64.6 fmol/mg (n=14) and from 30.9±3.4 fmol/mg (n=14) to 164.3±22.0 fmol/mg (n=12), respectively, in response to electrical field stimulation (~ 60 pulses at 10Hz, supramaximal voltage, 0.1 ms) in the presence of atropine and phentolamine (each 1μM). Nerve stimulation in the presence of apamin (0.5μM) and suramin (100μM), which revealed smaller apamin- and suramin-insensitive IJPs, did not antagonize the electrically-evoked increases in either cyclic nucleotide, and, in fact, increased cGMP levels significantly ($P<0.05$) above that produced by nerve stimulation alone in the case of suramin (Figs. 54 & 56).

The electrically-evoked increases in both cAMP and cGMP levels were abolished by a combination of apamin (0.5μM) plus L-NAME (100μM), suramin (100μM) plus L-NAME (100μM) and L-NAME (100μM) alone (Figs. 54 & 56).

II) <u>Effects of Exogenous Drugs</u>

SNP (10μM), which releases NO, was the only drug that significantly increased both cAMP and cGMP levels from control values of 461.0±60.8 fmol/mg (n=8) to 819.2±47.9 fmol/mg (n=12; $P<0.01$) and from 30.9±3.4 fmol/mg (n=14) to 154.3±23.8 fmol/mg (n=8; $P<0.001$) respectively. ATP (10μM), VIP (0.5μM) and VIP+L-NAME (100μM) also significantly ($P<0.05$) elevated cAMP levels from a control of 461±60.8 fmol/mg (n=8) to 720.6±78.6 fmol/mg (n=13), 705.8±53.2 fmol/mg (n=10) and 1178.2±151.6 fmol/mg (n=11) respectively. These results indicate that it is only NO that mimics the effect of nerve stimulation by increasing the levels of both cyclic nucleotides (Figs 55 & 57). Interestingly, the combination of VIP+L-NAME significantly ($P<0.05$) raised cAMP levels above those produced by VIP alone.

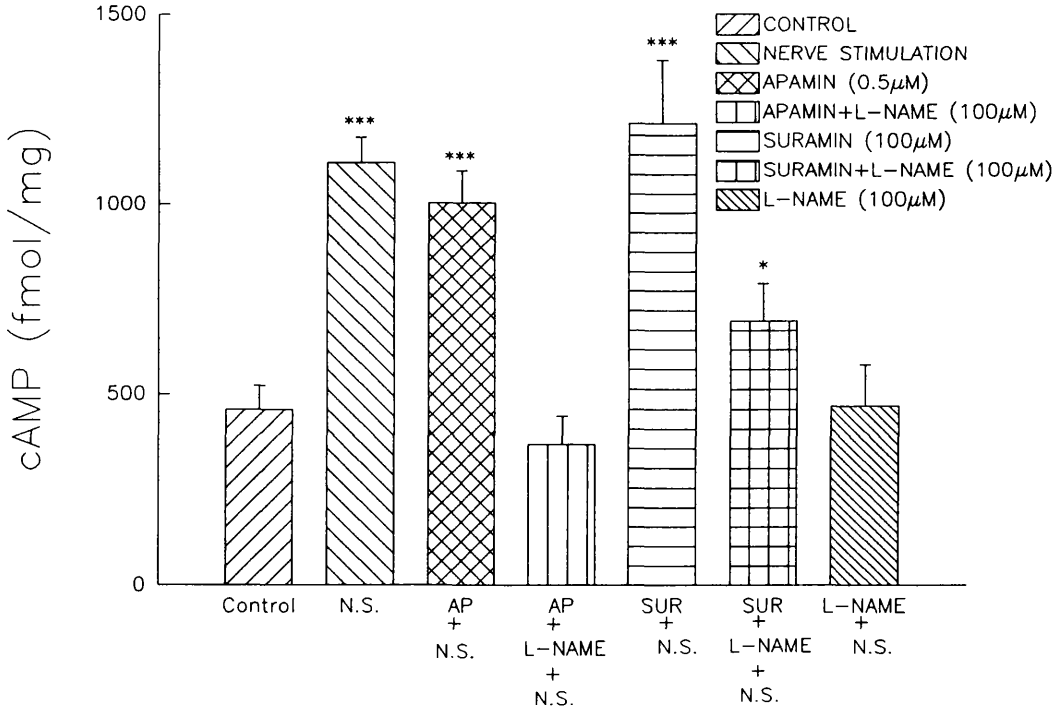


Figure 54: Effect of nerve stimulation (N.S.; ~60 pulses, supramaximal voltage, 0.1ms, 10Hz) alone and in the presence of apamin (AP; 0.5μM), apamin and L-NAME (100μM), suramin (SUR; 100μM), suramin and L-NAME (100μM) and L-NAME alone (100μM) on cAMP levels (fmol/mg) in the gpIAS. Each bar represents the mean ± S.E.M., n = 8 tissues (minimum). Nerve stimulation alone, nerve stimulation plus apamin and nerve stimulation plus suramin significantly (P<0.001) raised cAMP levels relative to control.

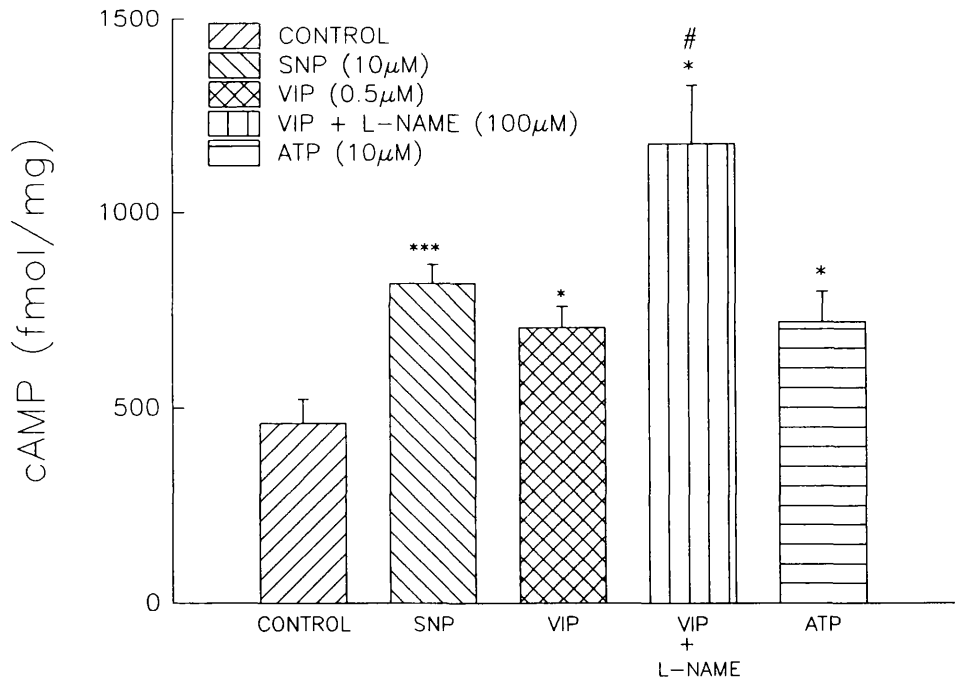


Figure 55: Effect of SNP (10μM), VIP (0.5μM), VIP+L-NAME (100μM) and ATP (10μM) on cAMP levels (fmol/mg) in the gpIAS. Each bar represents the mean ± S.E.M., n = 11 tissues (minimum). All of the drugs produced a significant (P<0.05) increase in cAMP levels relative to control. The increase produced by VIP+L-NAME was significantly (P<0.05) greater than that produced by VIP alone as denoted by #.

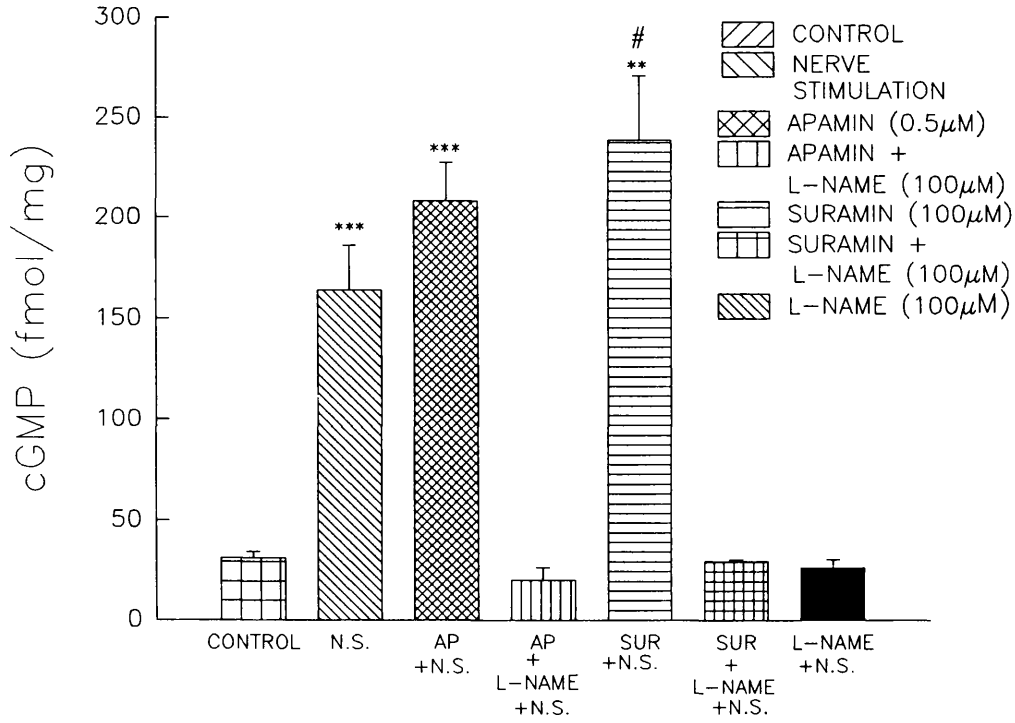


Figure 56: Effect of nerve stimulation (N.S.; ~60 pulses, supramaximal voltage, 0.1ms, 10Hz) alone and in the presence of apamin (AP; 0.5μM), apamin and L-NAME (100μM), suramin (SUR; 100μM), suramin and L-NAME (100μM) and L-NAME alone (100μM) on cGMP levels (fmol/mg) in the gpIAS. Each bar represents the mean ± S.E.M., n=8 tissues (minimum). Nerve stimulation alone, nerve stimulation plus apamin and nerve stimulation plus suramin significantly ($P<0.01$) raised cGMP levels relative to control. The increase produced by nerve stimulation in the presence of suramin was significantly ($P<0.05$) greater than that produced by nerve stimulation alone as denoted by #.

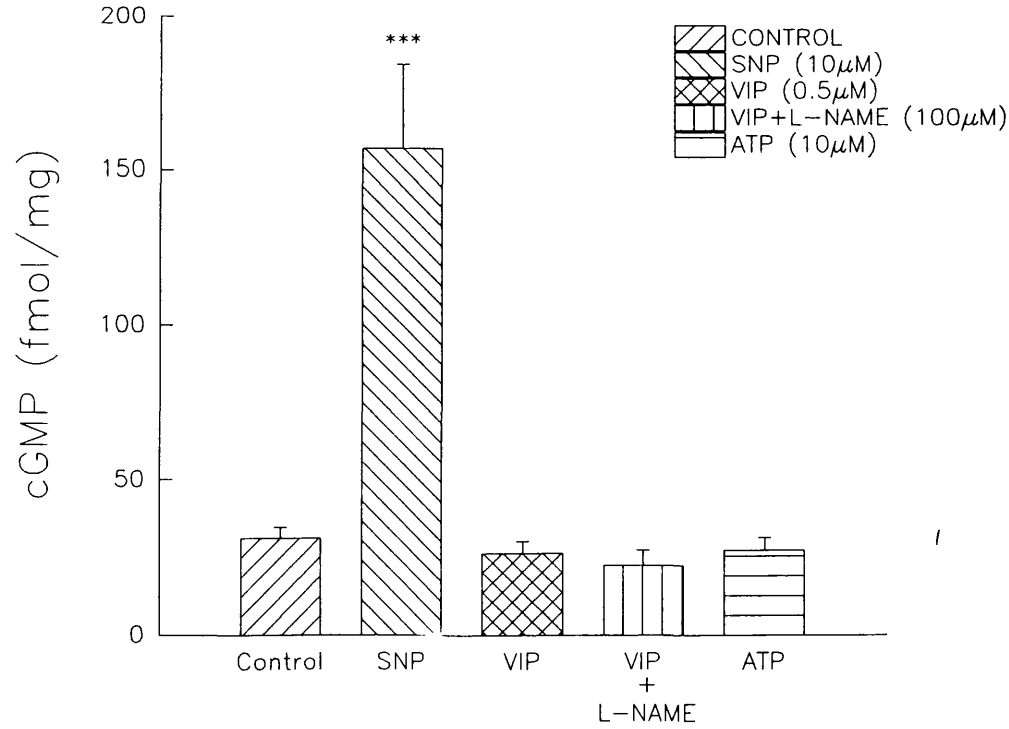


Figure 57: Effect of SNP (10μM), VIP (0.5μM), VIP+L-NAME (100μM) and ATP (10μM) on cGMP levels (fmol/mg) in the gpIAS. Each bar represents the mean ± S.E.M., n = 7 tissues (minimum). Only SNP significantly ($P<0.001$) raised cGMP levels relative to control.

DISCUSSION

Relaxation of the gpIAS was studied using two approaches; a) intracellular electrical, combined with simultaneous mechanical, recording and b) biochemical measurements.

The use of intracellular electrical and mechanical recording has proved a very valuable and powerful means of analysing relaxation in the gpIAS. Using this technique detailed analysis of the electrical responses from individual cells, representative of the entire smooth muscle preparation, together with the mechanical activity of the whole tissue, was made. The technique was also of great benefit as it allowed the prolonged analysis of membrane phenomena, essential for monitoring transmitter-mediated events, without damaging the cells. Furthermore, it is unlikely that the two separate components of relaxation, or their characteristics (see later), would have been discovered without the aid of intracellular electrical recording.

The well maintained tone (0.5-3g) of the gpIAS enabled the inhibitory NANC-mediated, electrical and mechanical effects of nerve stimulation to be easily seen. In this respect the tissue is rather unusual and compares favourably with other tissues such as the taenia caeci, where spontaneous tone is sporadic and often only lasts for short periods without drugs (Bennett & Rogers, 1967), and the BRP, where no tone exists in the absence of drugs (Gillespie, 1972; Creed *et al*, 1975). The ability to maintain tone in the gpIAS is presumably influenced by Ca^{2+} influx during the action potential rather than diffusion of the ion into the muscle at rest (Ward *et al*, 1992 c), as both action potential discharge and tone were abolished by the Ca^{2+} channel antagonist nifedipine. The membrane potential which, under resting conditions was -44.2±0.2mV (n=1119 cells), very similar to that previously reported (Lim & Muir, 1983, 1985, 1986), was also within the range where, in other GI smooth muscles (Ward *et al*, 1992 c), Ca^{2+} channels are activated. Tone may also be influenced, to a small degree, by NO as L-NAME,

which inhibits NOS (Hobbs & Gibson, 1990), raised tone (0.35±0.2g, n=6 from 20 tissues). On the other hand, it was my experience that cell impalement was easier when spontaneous activity was absent. This could be achieved by recording from areas of low stretch tension where non-firing cells predominated.

Electrically-stimulated relaxation of the gpIAS was manifested electrically as NANC-mediated IJPs. These have long been utilised as a means of studying relaxation mechanisms and differ widely in duration and amplitude throughout the GI tract (see for example Ward *et al*, 1992 c; He & Goyal, 1993; Zagorodnyuk & Maggi, 1994) and between GI and non-GI smooth muscles, such as the BRP and the RAc (Creed *et al*, 1975; Byrne & Muir, 1984, 1985; Byrne *et al*, 1984). This is probably a reflection of different inhibitory processes among the tissues. Within the gpIAS, the NANC, TTX-sensitive IJPs were very large in comparison to those from other tissues, exceeding 40mV in response to a single pulse, significantly greater than that previously reported (Lim & Muir, 1985; Baird, 1990), possibly because of more advanced recording techniques. Pharmacological dissection and manipulation of the IJPs was made considerably easier by their large amplitude. As a result of this "dissection", using the bee venom apamin, two distinct IJP components indicated the involvement of at least two transmitters. This result extends previous investigations in this tissue (Lim & Muir, 1985; Baird, 1990) in which apamin abolished the IJPs. The high concentrations (0.1-5μM) of apamin used in the previous studies may have produced non-specific effects by blocking other channels besides small conductance Ca^{2+}-activated K^+ channels.

Previous studies proposed that ATP, or a related nucleotide, was involved as a mediator of the IJPs in this tissue as it could mimic them, both in amplitude and duration (Lim & Muir, 1986; present investigation) and in its ability to produce a K^+ efflux (Lim & Muir, 1985). Nerve stimulation also increased the release of [^{3}H] adenine nucleotides (Beattie *et al*, 1985). Importantly, the ATP-

induced hyperpolarizations and relaxations were antagonized by apamin (0.3μM), an effect noted in several other tissues (Vladimirova & Shuba, 1978, Maas & Den Hertog, 1979; Nelemans & Den Hertog, 1987 a) and a prerequisite for ATP's proposed transmitter role.

The evidence for purine involvement was further investigated using the P_2-purinoceptor antagonists reactive blue 2 (RB2; Kerr & Krantis, 1979; Crema *et al*, 1983; Manzini *et al*, 1986; Crist *et al*, 1992) and suramin (Dunn & Blakeley, 1988; Den Hertog *et al*, 1989; Ohno *et al*, 1993; Soediono & Burnstock, 1994; Zagorodnyuk & Maggi, 1994). Their effectiveness differed markedly. RB2 (procion blue) reduced IJP amplitude, but irreversibly hyperpolarized the membrane; this could have accounted for its inhibitory effect on the IJPs. Suramin on the other hand, despite reservations concerning its specificity (Hoyle *et al*, 1990; McConalogue *et al*, 1995), was the more effective; membrane potential was unaffected and both NANC inhibitory responses and those of ATP were antagonized in parallel. Suramin, like apamin, also revealed an apparently distinct, slower-to-peak and smaller IJP (see later). Together these findings support and extend the evidence already presented (Lim & Muir, 1986) for the involvement of ATP in the neuronally-mediated IJPs of the gpIAS. However, one major discrepancy persists in this proposal; prolonged application of a desensitizing concentration of the drug failed to block the evoked IJPs. Similar results in other tissues has led to the rejection of ATP as a transmitter candidate (Ward *et al*, 1992 c). Clearly, either desensitization may have been incomplete, by the present procedure, or the substance released by NANC nerves may be a closely related analogue of ATP rather than the nucleotide itself.

The emergence of NO as a mediator of inhibitory transmission in several sphincteric tissues, such as the opossum lower oesophageal (Tøttrup *et al*, 1991), canine ileocolonic (Ward *et al*, 1992 c), rat pyloric (Soediono & Burnstock, 1994)

and human, rabbit and opossum internal anal (Burleigh, 1991, 1992; Tøttrup *et al*, 1992, 1993) and the gpIAS (Craig & Muir, 1991), suggested a high probability for NO involvement in the evoked IJPs of the gpIAS. Although initial investigations seemed to rule this out, with the IJPs unaffected by the NOS inhibitor, L-NAME, the identification of NO as the mediator of the apamin-insensitive IJP in the guinea-pig ileum (Lyster *et al*, 1992 a, b; Bywater *et al*, 1993; He & Goyal, 1993) was encouraging in this context. Subsequent experiments demonstrated that the apamin-insensitive IJPs were:-

a) evoked by narrow (0.1 ms) pulse widths, indicating their neuronal origin,

b) stereospecifically abolished by L-NAME, the NOS inhibitor,

c) inhibited by HbO, the NO scavenger, and

d) restored by L-arginine, the NO precursor.

Furthermore, histological studies demonstrated the presence of NOS-containing neuronal elements in the myenteric plexus of this tissue. These provided strong evidence in favour of neuronally-released NO as being the mediator of this IJP component.

Confirmation that the suramin-insensitive IJP component was also mediated by NO suggested that suramin and apamin each revealed what appeared to be identical NO-mediated IJP components and that the two drugs, although acting at different locations (receptor and channel respectively), produced essentially the same effect - antagonism of purinergically-mediated events. It is not obvious why apamin or suramin treatment was required to uncover the NO-mediated component of the IJP. It may be that during the IJP, conductance of the small Ca^{2+}-activated K^+ channels dominates other conductances, necessitating their blockade to enable the contribution of those other channels to be seen. It is equally unclear why L-NAME, which reduced NANC nerve-induced relaxations (Craig & Muir, 1991;

present investigation), did not, in the absence of either apamin or suramin, significantly reduce the amplitude of the apamin- and suramin-sensitive IJPs.

In addition to TTX, evidence to support a neuronal source of NO came with the virtual elimination of the muscle as an alternative; L-NAME failed to affect VIP-induced relaxations. Thus, VIP-mediated NO generation from smooth muscle cells (see Makhlouf & Grider, 1993; Makhlouf, 1994) seemed unlikely.

The proposed combination of ATP and NO as mediators of inhibitory responses is not unique to this tissue or species. A similar combination, based on both electrical and mechanical observations, reportedly accounts for NANC nerve-mediated responses in the rabbit portal vein (Brizzolara *et al*, 1994), rat pyloric sphincter (Soedino & Burnstock, 1994), guinea-pig colon (Zagorodnyuk & Maggi, 1994) and rabbit IAS (together with a "non-L-NNA, non-apamin-sensitive agent") (Tøttrup *et al*, 1993). The fast apamin-sensitive IJP component revealed within the circular muscle of the guinea-pig ileum (Humphreys *et al*, 1991; Lyster *et al*, 1992 a, b; Bywater *et al*, 1993; He & Goyal, 1993), stomach, small and large intestine (Vladimirova & Shuba, 1984) and human colonic muscle (Keef *et al*, 1993; Boeckxstaens *et al*, 1993) may also be ATP, therefore joining NO (the apamin-insensitive transmitter) as an inhibitory mediator in these tissues.

Are ATP and NO co-mediators released from the same nerves? Within the gpIAS several observations suggested that ATP and NO may indeed be released from the same nerves thus, a) both components were abolished by TTX, indicating their neuronal origin, b) the latencies of the two IJP components did not differ significantly and c) the IJPs could not be selectively evoked by varying stimulus parameters. Two recent studies have also investigated the possibility of co-mediation in the guinea-pig colon (Zagorodnyuk & Maggi, 1994) and taenia caeci (Bywater *et al*, 1993) using the N-type voltage-dependent calcium channel blocker ω-conotoxin GVIA (McCleskey *et al*, 1987). Like the gpIAS, these tissues also

displayed distinct apamin-sensitive and -insensitive IJP components which, in the case of the guinea-pig colon, were also believed to be mediated by ATP and NO respectively. ω-conotoxin GVIA blocked or reduced the apamin-sensitive component leaving the apamin-insensitive component unaffected in both tissues. This implied that different release mechanisms were utilised for each substance and that separate nerves may control the release of the apamin-insensitive mediator. The fact that the separate IJP components in the guinea-pig taenia caeci could be evoked selectively and appeared to have different latencies, the apamin-insensitive slow IJP being elicited at smaller (0.06 ms) pulse widths and having a latency in excess of 200 ms with the fast IJP predominating at larger (0.3 ms) pulse widths and having a significantly shorter latency of 144 ms (Bridgewater *et al*, 1994), further supported the proposal for differential release. Fundamental differences apparently exist between the taenia caeci and the IAS of the guinea-pig which merit further investigation.

Although the question of co-mediation remains to be fully resolved there is clear evidence that both substances are involved in the relaxation. The relative contribution of each mediator however, appears to be different; the ATP-mediated IJP, although some 50% larger in amplitude than the NO-mediated IJP, evokes only 20% of the mechanical response. In the light of this, the question arises as to the function of this purinergic component. Few studies have investigated possible ATP involvement in inhibitory co-transmission/co-mediation. In contrast, its role as an excitatory co-transmitter, in conjunction with noradrenaline, for example, in the rat tail artery (*e.g.* Sneddon & Burnstock, 1984; Muir & Wardle, 1988, 1989) and guinea-pig vas deferens (*e.g.* Sneddon & Westfall, 1984), has been well documented. Although not directly comparable, a distinct parallel exists between the inhibitory and excitatory actions in the gpIAS and the aforementioned tissues, in that ATP appeared to evoke the major component of the electrical events but

appeared to play only an ancillary role in the mechanical response to nerve stimulation. In both cases ATP may act as a "primer" for the other transmitter, be it noradrenaline or NO, possibly acting via an additional or separate transduction mechanism as in the rabbit saphenous artery where, in addition to the phosphoinositide hydrolysis promoted by NA, ATP also stimulated phosphatidylcholine hydrolysis (Nally *et al*, 1992).

Although the nature of the mediators of the two IJP components had been determined the identity of the ions involved was still unclear. The IJPs in this tissue had been associated with an increased K^+ conductance (Lim & Muir, 1985). The IJP component abolished by apamin implied its mediation by K^+ ions, as apamin blocks small conductance Ca^{2+}-activated K^+ channels (Banks *et al*, 1979; Capoid & Ogden, 1989). ATP-activated K^+ channels (K_{ATP}) were unlikely to also be involved in the IJPs as the hyperpolarization and relaxation produced by the K_{ATP} channel opener, lemakalim, was slow in onset and recovery and unaffected by apamin (Baird, 1990), unlike the evoked IJPs. Glibenclamide, the K_{ATP} channel antagonist, failed to affect IJPs or relaxations.

Although the role of K^+ in the apamin-insensitive IJP component remained undetermined, evidence suggested possible Cl^- involvement. For example, the relationship between membrane potential and $[K^+]_o$ in the gpIAS was not linear (Lim, 1985) and NO, the mediator of the apamin-insensitive IJP component, hyperpolarizes several tissues, such as arterial smooth muscle (Kreye *et al*, 1977), BRP (Byrne & Muir, 1985), opossum lower oesophageal sphincter (Saha & Goyal, 1992) and oesphageal circular muscle (Crist *et al*, 1991 b), by decreasing resting Cl^- conductance. Additionally, in the guinea-pig ileum, in which an apamin-sensitive and NO-mediated, apamin-insensitive IJP also exists (Lyster *et al*, 1992 a, b; Bywater *et al*, 1993; He & Goyal, 1993), the latter was also the result of decreased Cl^- conductance (Crist *et al*, 1991 a).

For an IJP to involve Cl^- this ion must already contribute to the resting membrane potential (Holman, 1958; Kuriyama, 1963). As the chloride channel antagonist IAA-94 (Landry *et al*, 1987, 1989) produced a reversible hyperpolarization and relaxation, chloride ions appeared to be involved in the gpIAS. On the other hand, the apparent ability of IAA-94 to block K^+ channels as well as Cl^- conductance (see later) casts doubt on the specificity of the drug. The results of experiments in which $[Cl^-]_o$ was substituted with glucuronate/gluconate raised further doubts regarding the role of chloride in maintaining resting membrane potential. This substitution also hyperpolarized the membrane. Normally it would have been expected to favour an increase in the driving force for the efflux of Cl^-, therefore initially depolarizing the cells (Aickin & Brading, 1983), before subsequently decreasing the driving force, by reducing $[Cl^-]_i$, following continued perfusion with $0[Cl^-]_o$ (Aickin & Brading, 1982, 1983, 1984), thereby hyperpolarizing the tissue. As only hyperpolarization was seen it suggested that Cl^- was <u>not</u> involved in regulating membrane potential in contrast to the result with IAA-94. Together, these results are apparently contradictory but the lack of specificity of IAA-94 however, suggested that the Cl^- substitution experiments may have been the more meaningful and that chloride ions were not involved in the resting membrane potential.

If a decreased Cl^- conductance contributes to the NO-mediated IJPs then the amplitude of the IJP should initially increase with the increased driving force, resulting from $[Cl^-]_o$ depletion (Crist *et al*, 1991 a). No such increase was observed and $0[Cl^-]_o$ invariably reduced the amplitude of both the apamin-sensitive and -insensitive IJPs. In this context IAA-94 also reduced the amplitude of the suramin-insensitive IJPs although the ability of the drug *per se* to hyperpolarize the membrane complicates the significance of this finding. In addition to its effects on Cl^- conductances, IAA-94 also reduced the amplitude of the K^+-mediated apamin-

and suramin-sensitive component of the IJP indicating some degree of non-selectivity of the drug. The reduction of IJP amplitude seen using either IAA-94 or $0[Cl^-]_o$ is unlikely to have arisen from a decrease in transmitter release from nerve terminals as chloride conductance is not critically involved in pre-synaptic events, such as α-autoinhibition (Alberts *et al*, 1981).

Together, these results suggest that chloride ions are probably not involved in either maintaining the resting membrane potential or in the mediation of the apamin- or suramin-insensitive IJPs. In order to verify this latter proposal it would be necessary to demonstrate that this IJP component did not occur as a result of a decrease in membrane conductance, that it did not have a reversal potential similar to that of the chloride equilibrium potential (approximately -25mV) and that the reduction in IJP amplitude produced by chloride channel antagonists, like IAA-94, was not due to a membrane hyperpolarization.

These membrane changes associated with transmitter release are however, only one aspect of the inhibitory process. There is now little doubt that these membrane changes are triggered by the inhibitory transmitter signal via the participation of an intracellular transduction system, possibly the phosphoinositide system or, more commonly, the cyclic nucleotides (see **Relaxant Transduction Mechanisms**).

Within the gpIAS there was already evidence for cyclic nucleotide involvement in the inhibitory response to NANC nerve stimulation (Baird & Muir, 1990). It was therefore important to determine whether, and to what degree, changes in cyclic nucleotide levels accompanied each of the two IJP components. Indirect observations, obtained by pharmacologically altering intracellular cGMP levels ($[cGMP]_i$), *i.e.* using M&B 22948, the cGMP phosphodiesterase inhibitor, 8-br-cGMP, the membrane-permeable cGMP analogue and the NO-donor SNP,

each of which hyperpolarized and relaxed the gpIAS, suggested cyclic nucleotide involvement in relaxation.

Direct confirmation was obtained using a radioimmunoassay (RIA) binding technique (Steiner *et al*, 1972). This method is simple, highly specific for both cyclic nucleotides and allows absolute quantification of basal and stimulated cAMP and cGMP levels to be made. In contrast to other techniques, such as the incorporation of ^{32}P into cellular proteins, where drug-induced responses, rather than cyclic nucleotide quantities, produced by analogues of the cyclic nucleotides are investigated, RIA allows the direct measurement of cAMP and cGMP levels following treatment. This technique confirmed that both cAMP and cGMP were involved in the NANC neuronally-mediated relaxation of the gpIAS; both cyclic nucleotide levels increased concomitantly with the point of maximum relaxation of the tissue.

During the present investigation it was notable that the quantities of cyclic nucleotides varied markedly in comparison to previous studies of sphincteric tissue where electrically- or pharmacologically-stimulated increases in the cyclic nucleotides have been described (Baird & Muir, 1990; Chakder & Rattan, 1993 b). With the possible exception of the study of Chakder & Rattan (1993 b), where cyclic nucleotide content was calculated as a function of protein content rather than wet tissue weight, it is unclear why such large discrepancies should exist as each study utilised the RIA technique, albeit with slight modifications among the studies. Direct comparison among the studies was made possible however, by measuring the stimulated percentage increase in cyclic nucleotide content relative to control.

Inhibitory NANC nerve stimulation increased $[cGMP]_i$ levels ($[cGMP]_i$) by ~ 430%, more than one and half times greater than that previously reported for both the gpIAS (Baird & Muir, 1990) and opossum IAS (Chakder & Rattan, 1993

b). Differences in techniques are, however, unlikely sources of this discrepancy as changes in cAMP levels among the different studies were similar (see later). $[cGMP]_i$ levels were further enhanced by apamin (see also Baird & Muir, 1990) and by suramin suggesting that the purinergic transduction process antagonized this additional electrically-stimulated cGMP rise, possibly by inhibiting the activity of guanylyl cyclase. Because of the inhibitory effect of L-NAME on nerve-stimulated $[cGMP]_i$ and the enhancing effect of SNP on basal $[cGMP]_i$, NO involvement was implied. Interestingly, neither ATP, in contrast to a previous study (Baird & Muir, 1990), nor VIP, stimulated $[cGMP]_i$.

In comparison to the effects on $[cGMP]_i$, field stimulation raised intracellular cAMP levels ($[cAMP]_i$) by only ~130%, almost identical to that witnessed both in the gpIAS (Baird & Muir, 1990) and opossum IAS (Chakder & Rattan, 1993 b). Significantly, this increase was abolished by L-NAME. Although this may have been a non-specific effect of L-NAME on adenylyl cyclase, no evidence exists for such a proposal, and it is therefore tempting to speculate that L-NAME blocked an electrically-induced rise in $[cAMP]_i$ mediated by neuronally-released NO. In support, SNP-released NO increased $[cAMP]_i$, an effect also seen in the opossum IAS (Chakder & Rattan, 1993 b), canine lower oesophageal sphincter (Barnette *et al*, 1990) and in the non-sphincteric rat gastric fundus (Ito *et al*, 1990). Furthermore, apamin, as well as suramin, which each antagonize the purinergically-mediated IJP, had no effect on the electrically-evoked rise in $[cAMP]_i$. The latter finding also contrasts with the earlier study of Baird & Muir (1990) in which apamin blocked the neuronally-stimulated increase in $[cAMP]_i$. This, however, was probably due to a non-specific inhibitory effect caused by the very high apamin concentration (5μM) used in the earlier study.

How then does NO stimulate cAMP production? In the absence of direct NO stimulation of adenylyl cyclase, the $[cAMP]_i$ rise could have been due to the

ability of guanylyl cyclase to catalyse the formation of both cGMP and cAMP (Mittal & Murad, 1977; Braughler *et al*, 1979; Gerzer *et al*, 1981) and/or to cGMP-dependent inhibition of cAMP hydrolysis by type III PDE (a cAMP selective isozyme) (Harrison *et al*, 1986). Interestingly VIP, although unlikely to play any role in this tissue (Lim & Muir, 1986; present investigation), increased $[cAMP]_i$, an effect enhanced by L-NAME, possibly by antagonizing a NO-mediated, negative feedback effect on adenylyl cyclase production.

The involvement of both cAMP and cGMP in the neuronally-mediated relaxation of the gpIAS and in the opossum IAS (Chakder & Rattan, 1993 b), contrasts with prior investigations in the opossum LOS (Torphy *et al*, 1986; Barnette *et al*, 1989) and in the canine IAS (Joslyn *et al*, 1990) where only cGMP levels were raised by nerve stimulation; apparently not all sphincters utilise the same second messenger systems.

In light of both the biochemical and electrophysiological findings of the present study, what can be deduced about the respective roles of the two putative mediators of relaxation in this tissue? The apamin-insensitive component, which is probably mediated by NO, or a related substance, was responsible for the major component of relaxation (~80%) and appeared to be the main instigator of both cGMP and cAMP production. As the IJP is dependent on the generation of cGMP and/or cAMP it may explain why it is significantly slower to peak than the apamin-sensitive IJP. On the other hand, purinergic block, using apamin or suramin, failed to significantly reduce the nerve-evoked rise in either cyclic nucleotide and L-NAME had no effect on the apamin-sensitive IJP but antagonized the elevation of both $[cAMP]_i$ and $[cGMP]_i$. This suggested that the apamin-sensitive purinergic inhibitory component, responsible for the large electrically-evoked IJP component, appeared to mediate its effects by mechanisms other than via the cyclic nucleotides. The question arises then as to the identity of

the transduction mechanism for ATP. The majority of evidence suggests that stimulation of P_{2y}-purinoceptors, which mediate relaxation in the gpIAS (Lim, 1985), is linked to increased phosphoinositide turnover (see *inter alia* Pirotton *et al*, 1987; Berrie *et al*, 1989; Boyer *et al*, 1989; Cooper *et al*, 1989; Van der Merwe, 1989; Harden *et al*, 1990; Flitz *et al*, 1994). However, if this is so in the gpIAS, the ability of exogenous ATP to significantly increase $[cAMP]_i$ levels remains unexplained. One possibility is that ATP is able to activate two second messenger systems as in the rabbit saphenous artery (Nally *et al*, 1992). In the gpIAS a Ca^{2+}-sensitive adenylyl cyclase (Mollner & Pfeuffer, 1988; Pfeuffer *et al*, 1989; Feinstein *et al*, 1991) may be stimulated by increased $[Ca^{2+}]_i$, via increased ATP-induced inositol phosphate metabolism, thereby producing a rise in $[cAMP]_i$. However, if this were the case then the increase should be antagonized by apamin or suramin. Perhaps the simplest explanation is, however, that the apamin-sensitive transmitter is not ATP but a closely related analogue which does not raise $[cAMP]_i$.

Whatever the mechanism involved in mediating the effects of the purinergically-mediated IJP component it is clear that it plays a role, albeit apparently minor, in the inhibitory response to field stimulation. The ability of ATP to maximally relax the gpIAS (present investigation) clearly demonstrates the propensity of this compound to be the apamin-sensitive inhibitory transmitter in this tissue.

Conclusions

It was the aim of this thesis to investigate and identify the transmitter processes underlying the neuronally-mediated relaxation of the gpIAS using electrical and mechanical recording and biochemical measurements. To this end, this study has revealed that the neuronally-mediated relaxation is initiated by the

release of two substances, probably ATP and NO, which mediate two distinct components of relaxation involving increases in intracellular cyclic nucleotide levels.

ATP, or a closely-related analogue, is responsible for the large, fast apamin- and suramin-sensitive IJP component which mediates about 20% of the overall relaxation. It triggers relaxation by an as yet unidentified second messenger system which appears to increase the open probability of K^+ channels probably via the release of $[Ca^{2+}]_i$, thereby hyperpolarizing the membrane, reducing Ca^{2+} entry and eliciting relaxation.

NO mediates the smaller, slower-to-peak IJP component which accounts for the other 80% of the relaxation, in response to field stimulation. NO appears to decrease tone by activating cAMP- and cGMP-dependent intracellular cascade systems which in turn hyperpolarize the gpIAS. Chloride ions do not appear to be the ionic mediators of this IJP component.

Both mediators are released simultaneously by nerve stimulation, possibly from the same nerves. However the question of their co-transmission must await further investigation.

Relaxation of the gpIAS is a process that can be achieved by the activation of multiple pathways capable of acting synergistically to attain this end-point.

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CHAPTER 2

Abbreviations

Ach	Acetylcholine
ATP	Adenosine triphosphate
8-br-cGMP	8-bromo-cyclic guanosine 3`,5`-monophosphate
$[Ca^{2+}]_i$	Intracellular free Ca^{2+}
cGMP	Cyclic guanosine 3`, 5`-monophosphate
$[cGMP]_i$	Intracellular cyclic guanosine 3`, 5`-monophosphate
DA	Dopamine
DTZ	Diltiazem
EDRF	Endothelium-derived relaxing factor
EJP	Excitatory junction potential
gpIAS	Guinea-pig internal anal sphincter
GI	Gastrointestinal
GTN	Glyceryl trinitrate
HbO	Oxyhaemoglobin
5-HT	5-Hydroxytryptamine
IAS	Internal anal sphincter
IDN	Isosorbide dinitrate
$[K^+]_o$	Extracellular potassium concentration
NANC	Non-adrenergic, non-cholinergic
NO	Nitric oxide
RDC	Rabbit distal colon
RPV	Rat portal vein
SNP	Sodium nitroprusside
TTX	Tetrodotoxin

SUMMARY

1) The effect of the nitrovasodilators sodium nitroprusside (SNP), glyceryl trinitrate (GTN) and isosorbide dinitrate (IDN), which each release nitric oxide (NO), on the spontaneous extracellular electrical and mechanical activity of the guinea-pig and human internal anal sphincter and the intracellular electrical activity of the mouse vas deferens, has been investigated.

2) Under resting conditions, the guinea-pig internal anal sphincter (gpIAS), mounted in a Golenhofen apparatus, exhibited two distinct patterns of activity. One consisted of almost continuous spontaneous electrical spiking accompanied by rapid mechanical oscillations in tone. The other was also a continuous spike discharge upon which bursts of spikes were superimposed.

3) SNP, GTN and IDN each possessed stimulatory and inhibitory effects on the gpIAS. SNP was the most effective and was used as a model for this type of drug. At lower concentrations (10^{-8}-10^{-6} M) it reduced tone and decreased the frequency of the bursts of electrical and mechanical activity. It increased the maximum amplitude of contractions and, occasionally, of the electrical spikes. At higher concentrations (>10^{-6} M) SNP reduced or abolished all spontaneous activity.

4) The actions of SNP were mediated by NO as its effects were a) mimicked by the membrane permeable cyclic guanosine 3`, 5`-monophosphate (cGMP) analogue, 8-bromo-cGMP and b) inhibited by the nitric oxide scavenger oxyhaemoglobin (HbO). Significantly, the effects of SNP were not mimicked by potassium ferrocyanide, which structurally resembles SNP, but does not release NO.

5) Calcium (Ca^{2+}) availability was a determinant of SNP` s effects; the Ca^{2+} channel antagonist, diltiazem (10^{-4}M), abolished, whereas the Ca^{2+} channel agonist, BAY K 8644 (10^{-6}M), enhanced, the stimulatory actions of the drug.

6) Membrane hyperpolarization, possibly due to potassium ion efflux, may have accounted for the effects of SNP. Raising extracellular potassium ion concentration ($[K^+]_o$) from 4.7mM to 14.1mM enhanced, whereas eliminating $[K^+]_o$ abolished, the stimulatory response to SNP. In addition, SNP and the potassium channel opener, lemakalim each, in subthreshold concentrations, had no effect on it, but together abolished spontaneous activity, indicating that a K^+ efflux was involved in the responses to SNP.

7) Increased intracellular cGMP ($[cGMP]_i$) levels may produce an influx of extracellular Ca^{2+} accounting for the initial stimulation by SNP. However, the Ca^{2+} influx may also increase the open probability of Ca^{2+} -activated K^+ channels thereby hyperpolarizing the tissue and reducing tone. As the degree of membrane hyperpolarization increases with the increasing concentration of SNP, closure of voltage-dependent Ca^{2+} channels ensues, and both spontaneous electrical and mechanical activity declines and is eventually abolished.

8) The effects of SNP, added by pressure ejection, on the mouse vas deferens were examined using intracellular electrical recording. SNP produced two types of effects; an increase in the amplitude of the spontaneous excitatory junction potentials (EJPs) and a membrane hyperpolarization without any change in EJP amplitude.

9) The effect of SNP on the mechanical activity of the human internal anal sphincter was also examined. SNP relaxed the tissue but did not produce any stimulatory effect.

INTRODUCTION

The classical neurotransmitters, acetylcholine (Ach) and noradrenaline (NA), each produce excitatory and inhibitory effects in different smooth muscles. For example, Ach contracts most gastrointestinal (GI) smooth muscles but relaxes vascular tissues by stimulating endothelial cells to release endothelium-derived relaxing factor (EDRF; Furchgott & Zawadski, 1980). Similarly, NA activation of α_1-adrenoceptors in vascular smooth muscle raises tone but relaxes most GI smooth muscles (see Bülbring & Tomita, 1987). Knowledge of drug-receptor interactions enables one to understand these phenomena. It is clear that drugs can interact with different, specific receptors to produce, in some cases, opposite responses; in the case of vascular and GI smooth muscle this is usually because of differences in the signal transduction mechanisms among the receptors. Thus, α_1-adrenoceptor activation, in the vasculature increases $[Ca^{2+}]_i$ levels causing a contraction, but in the GI tract the reverse occurs, with a lowering of $[Ca^{2+}]_i$ levels and a reduction in tone (Bülbring & Tomita, 1987).

The more recently discovered putative transmitters, dopamine (DA), 5-hydoxytryptamine (5-HT), and adenosine triphosphate (ATP) are also each capable of producing apparently opposite effects. For example, DA stimulates or inhibits the activity of adenylyl cyclase by acting either on D_1 or D_2 receptors respectively (Kebabian & Colne, 1979). In the dog, 5-HT elicits both coronary vasodilatation and vasoconstriction via 5-HT_1-like and 5-HT_2 receptors respectively (Cocks & Angus, 1983; Houston & Vanhouette, 1988; Toda & Okamura 1990). ATP vasoconstricts the mesenteric arteries of several species (Ishikawa, 1985; Kügelgen & Starke, 1985; Muramatsu & Kigoshi, 1987; Machalay *et al*, 1988; Muramatsu *et al*, 1989) via P_{2x}-purinoceptors, but also vasodilates the rabbit mesenteric artery and portal vein (Mathieson & Burnstock, 1985; Burnstock & Warland, 1987; Reilly *et al*, 1987) via P_{2y}-purinoceptors.

The newly discovered transmitter nitric oxide (NO), or a closely related substance (Myers *et al*, 1987; Rubanyi *et al*, 1988; Vedernikov *et al*, 1992), mediates a large variety of biological processes such as intracellular communication in the central nervous system (Garthwaite, 1990), macrophage cytotoxicity (Hibbs *et al*, 1990) and, importantly in terms of this thesis, relaxation of vascular (Moncada *et al*, 1991) and non-vascular (Rand, 1992; Sanders & Ward, 1992; Sanders *et al*, 1992) smooth muscle. In its proposed transmitter role, the overwhelming majority of evidence suggests that the effects of NO are purely inhibitory (for reviews see Moncada *et al*, 1991; Sanders & Ward, 1992; Rand, 1992). NO however, may be unique among the proposed named transmitters. It is a highly labile gas, with a half-life as short as ~ 0.1s (Kelm & Schroder, 1990) making its conventional storage prior to release unlikely. Its receptor target, the cytosolic enzyme guanylyl cyclase (Arnold *et al*, 1977), is intracellularly located, in contrast to the extracellular membrane-bound receptors, with which the more "classical" neurotransmitters interact. NO may therefore not behave like other "conventional" transmitters and may not display such a spectrum of excitatory and inhibitory effects.

On the other hand, using nitrovasodilators, such as sodium nitroprusside (SNP) and glyceryl trinitrate (GTN), which release NO (see Kerwin & Heller, 1994), an increasing number of studies have revealed that NO has excitatory properties, particularly on spontaneously active smooth muscle. For example, nitrovasodilators, in particular SNP, increased the frequency and amplitude of spontaneous electrical and mechanical events in the rabbit distal colon (RDC; Smith, 1994) and stimulated the peristaltic reflex in isolated guinea-pig ileal segments in a cGMP-dependent manner (Sugisawa *et al*, 1991). In the latter case it was postulated that free $[Ca^{2+}]_i$ may have been decreased by SNP as a result of an elevation of intracellular cGMP (Greutter *et al*, 1981) which in turn would increase neuronal excitability by decreasing K^+ efflux and depolarizing the membrane.

The electrically-evoked, NANC-mediated contractions of rat ileal longitudinal muscle were antagonized by NO synthase inhibitors and restored by the NO precursor L-arginine. In addition, SNP also produced contractions of this tissue (Barthò *et al*, 1992). However, it was possible that these effects could have been caused by the secondary release of an excitatory transmitter. Such a secondary excitation response to NO was witnessed in oesophageal smooth muscle where NO, SNP and the membrane permeable cGMP analogue, 8-bromo-cGMP (8-br-cGMP), each evoked a concentration-dependent relaxation followed by a rebound contraction (Saha *et al*, 1993). The rebound contraction however, also involved products of the cyclooxygenase pathway, as was also the case in the canine and feline colon in response to NANC nerve stimulation (Ward *et al*, 1992; Venkova & Krier, 1994).

Excitatory effects of NO are also seen in non-GI spontaneous smooth muscle. For example, in the rat portal vein (RPV), the frequency of spontaneous contractions and extracellularly-recorded discharges was increased by GTN (Bray *et al*, 1987; Wylie, 1988; Smith, 1994), SNP and isosorbide dinitrate (IDN; Smith, 1994) although the amplitude of both parameters was decreased.

Evidence for an excitatory role for NO clearly exists, although the underlying mechanisms appear to differ. These findings modify our concept of NO as simply an inhibitory transmitter and have possible significant clinical consequences for the use of nitrovasodilators. For example, would the increase in spontaneous activity seen in the rat portal vein in response to nitrovasodilators (Bray *et al*, 1987; Wylie, 1988; Smith, 1994) make these compounds any less effective for the treatment of hypertension? Would the excitatory effects of these compounds, acting directly or indirectly, on a variety of gut smooth muscles (Sugisawa *et al*, 1991; Barthó *et al*, 1992; Ward

et al, 1992; Saha *et al*, 1993) be significant in the treatment of conditions such as achalasia?

The existence of NO-mediated excitatory events led to my examination of its effects, using nitrovasodilators, on the extracellular electrical and mechanical responses of the spontaneously active guinea-pig internal anal sphincter (gpIAS) and human IAS, where the principal response to nerve stimulation is inhibitory (Burleigh & D`Mello 1983; Lim & Muir, 1985) and where NO has already been implicated as a putative inhibitory transmitter in these and other species (Burleigh, 1991; Craig & Muir, 1991; Chakder & Rattan, 1992, 1993 a, b; Rattan & Chakder, 1992). Possible neuronal mechanisms underlying the NO excitatory effects were investigated in the spontaneously active mouse vas deferens using intracellular electrical recording techniques.

AIMS

To date, the most frequently described effects of NO as a transmitter in smooth muscle have been inhibitory. However, examples of NO-induced excitation, although still relatively rare and poorly understood, are emerging more frequently. Such findings could have important pharmacological and clinical implications, particularly with regard to the therapeutic usefulness of the NO-donating nitrovasodilators which are often used clinically as relaxants. The ability of nitrovasodilators to stimulate contraction, either directly or indirectly, in a variety of GI smooth muscles, such as the ileum (Sugisawa *et al*, 1991) and colon (Ward *et al*, 1992), might affect their usefulness for the treatment of gut disorders such as achalasia and spasm of the IAS. Additionally, most, if not all, of the established transmitters, such as NA and Ach, have demonstrated both excitatory and inhibitory properties. It is possible therefore that NO too has the, as yet largely undiscovered, ability to cause excitation as well as inhibition. In spite of the potential importance of such

questions the phenomenon of possible NO-induced excitation has largely been ignored.

Due to this lack of knowledge and previous results regarding NO-associated excitatory phenomena produced within this laboratory (Wylie, 1988; Smith, 1994), the effects and possible underlying mechanisms of nitrovasodilator action were examined on the spontaneously active smooth muscle, *in vitro*, of the gpIAS, the human IAS and the mouse vas deferens. Both intracellular and extracellular electrical and simultaneous mechanical recording techniques were employed for this purpose.

METHODS AND MATERIALS

1) ANATOMY AND FUNCTION

I) Guinea-Pig Internal Anal Sphincter

The anatomy and function of the guinea-pig internal anal sphincter (gpIAS) was as previously described (see **Chapter 1-Methods and Materials, p. 58-59**).

II) Human Internal Anal Sphincter

The anal sphincteric region in man, like most mammalian species, consists of two sphincters, internal and external. The internal anal sphincter (IAS) is responsible for the involuntary retention of contents in the anal canal. To this end, it is nearly always in a contracted state, resulting in a very high intraluminal pressure (some 85% of the total pressure in the anal canal (Freckner & Von Euler, 1975)) relative to the rectum (Duthie & Bennett, 1963; Bennett & Duthie, 1964; Collins *et al*, 1967; Schuster, 1975).

The IAS is situated at the level of the dentate line where the circular muscle of the rectum becomes considerably thickened (5-8mm) to form the sphincter. The sphincter surrounds the upper three quarters (~30mm) of the anal canal and terminates below the level of the white line (*vide infra*) (Warwick & Williams, 1973).

The IAS receives its excitatory sympathetic innervation via the hypogastric nerves from the fifth lumbar segment and its parasympathetic supply via the first, second and third sacral segments, although the parasympathetic division appears to have very little influence on the tone or function of the sphincter (see Burleigh & D`Mello, 1983). The human IAS also possess an intrinsic inhibitory non-adrenergic, non-cholinergic (NANC) innervation thought to be mediated by NO (Burleigh, 1991, 1992; O`Kelly *et al*, 1994).

III) Mouse Vas Deferens

The vas deferens (~3cm long) is responsible for the transport of spermatozoa and seminal fluid from the epididymus of the testis to the seminal vesicle duct near the prostate gland. It comprises three smooth muscle layers; an outer and inner longitudinal, which surround a circular layer. The smooth muscle cells are surrounded by connective tissue and overlap in an interweaving fashion (Merrillees, 1968).

The tissue receives, principally, an excitatory sympathetic innervation by the hypogastric nerves, which originate at the T9-T10 level of the spinal cord and run to the inferior mesenteric ganglion. The hypogastric nerves, containing mainly preganglionic sympathetic fibres, pass from the inferior mesenteric ganglion to a plexus of ganglia located within 1cm of the prostatic end of the vas deferens (Sjöstrand, 1965; Burnstock, 1970). From this plexus, postganglionic, mainly non-myelinated sympathetic nerve fibres, run first to the prostatic and then towards the epididymal end, sending off radial branches into the muscle, which form preterminal axons from which transmitter is released. The preterminal axons are predominantly non-myelinated, surrounded by a Schwann cell sheath and beaded, due to the varicosities of nerve endings (see Taylor, 1987).

2) DISSECTION

I) Guinea-Pig Internal Anal Sphincter

The gpIAS was dissected as previously described (see **Chapter 1- Methods & Materials, p. 60**) and placed into the Golenhofen apparatus (Golenhofen & Von Löh, 1970; see Apparatus and Techniques; Fig. 1) between the four platinum wire ring electrodes, perfused, using a pump (Gilson Minipuls 3; 4ml min^{-1}), with oxygenated Krebs' solution (95% O_2, 5% CO_2) maintained at 37±0.5°C. The gpIAS was attached by a thread to an isometric force displacement transducer (Grass FT03C) and a fixed anchor

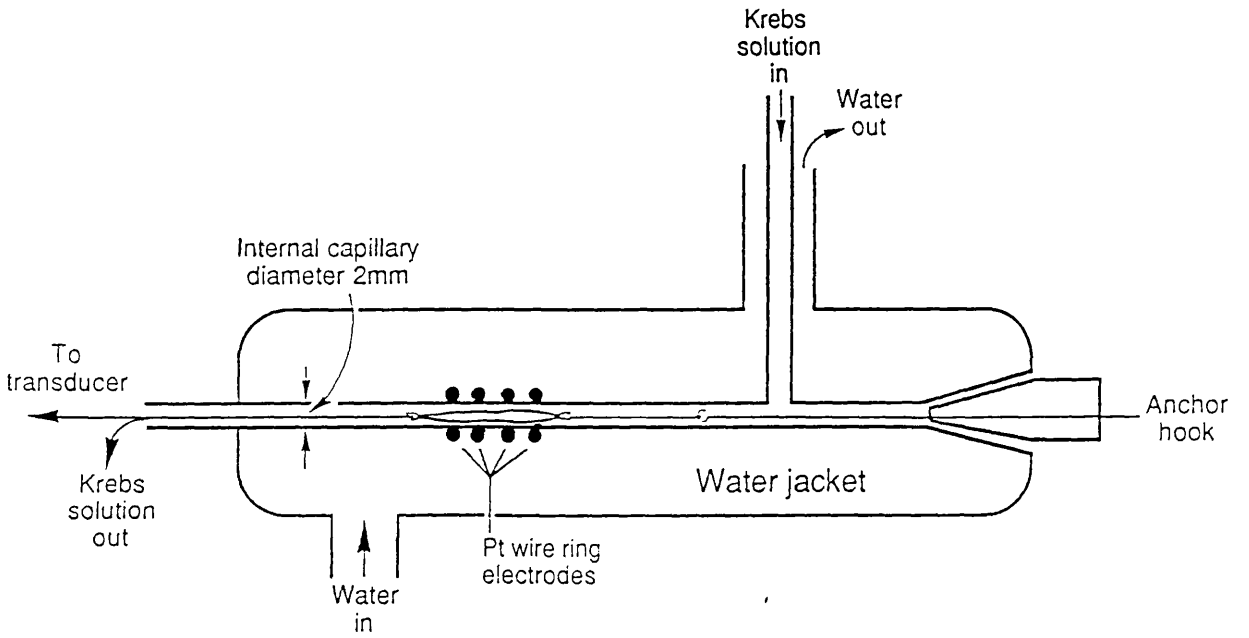


Figure 1: Golenhofen apparatus.

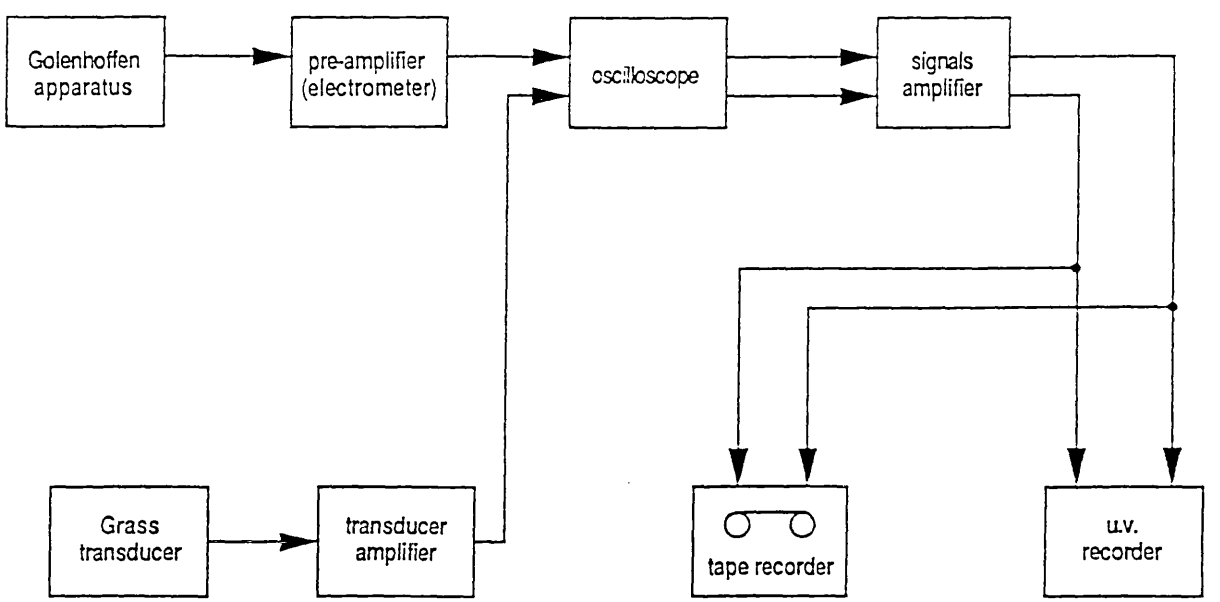


Figure 2: Arrangement of apparatus used to record mechanical and electrical activity from the Golenhofen apparatus.

hook at the opposite end, to measure mechanical activity. It was initially placed under 1g stretch and allowed to equilibrate for at least 40 minutes prior to experimentation. On most occasions during this time, the IAS` developed spontaneous additional tone (0.8±0.5g, n=14). Phenylephrine (10^{-5}-10^{-3} M), injected onto the tissue within the Golenhofen apparatus, contracted the sphincter.

II) Human Internal Anal Sphincter

Five IAS tissues, from patients undergoing abdomino-perineal resection of the rectum and anal canal for low lying rectal carcinoma were available.

After removing fat, connective tissue and mucosa, strips of the anal sphincter (2cm long x 0.5cm wide) containing both longitudinal and circular muscle, with the circular muscle bundles running lengthways, were mounted in the Golenhofen as previously described for the gpIAS. Tissues were initially placed under 1g stretch and allowed to equilibrate for at least one hour prior to experimentation. Two of the five IAS` developed spontaneous, active tone in addition to the stretch placed upon them (1.0g & 3.1g respectively). One tissue failed to contract to phenylephrine (10^{-3}M) and was discarded.

III) Mouse Vas Deferens

Male adult Theiler`s Original mice (20-35g) were killed by a blow to the head and exsanguinated. The abdominal cavity was opened by a midline incision and the testes pushed into the peritoneal cavity. The epididymal end of the vas deferens was tied and the surrounding connective tissue and fat carefully removed. The vas deferens was then cut at the prostatic end and transferred to a Sylgard-coated petri dish containing oxygenated Krebs` solution (95% O_2, 5% CO_2) where any remaining fat and connective tissue were removed under a dissecting microscope. The tubular vas deferens was then transferred to a horizontal organ bath and pinned to the Sylgard-coated

base. It was continually superfused with oxygenated Krebs' solution, using a pump (Gilson Minipuls 3; 4ml min^{-1}), at 37±0.5°C. The tissue was attached by a thread to an isometric force displacement transducer (Grass FT03C) to measure mechanical activity. The tissue was allowed to equilibrate for at least 40 minutes prior to experimentation.

3) APPARATUS AND TECHNIQUES

I) Extracellular Electrical and Simultaneous Mechanical Recording.

Extracellular electrical and simultaneous mechanical activity was recorded from the gpIAS and human IAS, separately, using the Golenhofen apparatus (Golenhofen & Von Löh, 1970; Fig. 1). This equipment consists of four platinum wire ring electrodes (i.d. ~2.5mm) contained within a narrow, water jacketed (37±0.5°C) glass capillary (i.d. ~2.3mm). These electrodes could be used for either recording or stimulating, as required. Electrical signals were amplified (x1000, Neurolog A.C. preamplifier NL104) and filtered (Neurolog NL115, low frequency cut-off 10 Hz, high frequency cut-off 10kHz). Mechanical activity was monitored using an isometric force-displacement transducer (GRASS FT03). Electrical and mechanical activity were displayed on a storage oscilloscope (Hitachi VC-6023) and recorded on an instrumentation tape recorder (Racal, Store 4DS) and U.V. oscillograph (Thorn EMI 6150-Mk II; Fig 2).

The Krebs' solution superfusing the tissue flowed through a heat exchange coil, jacketed with water (37±0.5°C), before entering the Golenhofen apparatus.

II) Intracellular Electrical Recording

The apparatus and techniques used for intracellular electrical recording from the mouse vas deferens were the same as those described previously (see **Chapter 1-Methods and Materials, p. 61**) except that in the present study the

electrical signals were amplified by a unity gain high impedance ($10^{10}\Omega$) D.C. preamplifier (W.P.I. M4A) and monitored on a digital voltmeter. The electrical and mechanical signals in this case were recorded on an instrumentation tape recorder (Racal, Store 4DS) and U.V. oscillograph (Thorn EMI 6150-Mk II).

III) Criteria For Cell Penetration

The criteria for accepting a cell for electrophysiological investigation were the same as those described previously (see **Chapter 1-Methods and Materials, p. 62**).

IV) Administration of Drugs

In the case of the Golenhofen apparatus drugs were added to oxygenated (95% O_2, 5% CO_2) Krebs' solution, which was pumped (Gilson Minipuls 3; 4ml min^{-1}) through polythene tubing (i.d. 3mm) to the Golenhofen apparatus.

Each drug under investigation was allowed to superfuse the tissue until equilibration had been reached, usually between 7-15 minutes after addition. Electrical and mechanical activity was recorded prior to the drug reaching the tissue and for approximately 15 minutes thereafter. A representative example of tissue activity (~2 minute duration), after equilibration, was recorded on the U.V. oscillograph.

For intracellular recording, drugs were administered to the vas deferens by pressure injection using a Picospritzer (Picospritzer II, General Valve Corporation, N.J., U.S.A.). Using this technique the applied substances are less likely to cause desensitization of the entire tissue because of the small area over which the drug is added. However, for the same reason, no changes in tension are detectable. Drugs were applied from micropipettes which had their tips broken back to 2-20μM diameter. The pipette tip was placed to within

~1mm of the recording site before application of the drugs (20-30 psi). The duration of ejection is indicated in the text.

4) SOLUTIONS AND DRUGS

I) Physiological Salt Solution

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.

When altering the $[K^+]$ of the Krebs` solution, the isotonicity of the solution was maintained by substituting KCl with an equivalent amount of NaCl. Thus, the $[K^+]_o$-free Krebs` solution was of the following ionic composition (mM): NaCl, 123.1; $NaHCO_3$, 25.0; NaH_2PO_4, 1.13; $CaCl_2$, 2.7; $MgCl_2$, 1.3; glucose 11.0; pH 7.4.

The 14.1mM $[K^+]_o$-containing Krebs` solution was of the following ionic composition (mM): NaCl, 109.0; $NaHCO_3$, 25.0; NaH_2PO_4, 1.13; KCl, 14.1; $CaCl_2$, 2.7; $MgCl_2$, 1.3; glucose 11.0; pH 7.4.

In the case of Ca^{2+}-free solutions, no ionic substitution was made.

II) Drugs

BAY K 8644 (Bayer A.G.), bovine haemoglobin (HbO; Sigma), 8-bromoguanosine 3`, 5`-cyclic monophosphate (sodium salt) (8-br-cGMP; Sigma), diltiazem hydrochloride (DTZ; Sigma), glyceryl trinitrate (GTN; kindly donated by Napp Laboratories), isosorbide dinitrate (IDN; Sigma), phenylephrine hydrochloride (Sigma), lemakalim (LMK; BRL 38227; kindly donated by Dr. W. Martin), potassium ferrocyanide (Hopkin and Williams), sodium nitroprusside (SNP; BDH), tetrodotoxin (TTX; Sigma).

With the exception of tetrodotoxin and haemoglobin, the concentrations in the text refer to those of the salts. With the following exceptions, stock solutions of drugs were prepared in distilled water before dilution with Krebs`

solution prior to use. The following were initially prepared as 10^{-3}M stock solutions as indicated before dilution with Krebs` solution; lemakalim (50% methanol), BAY K 8644 (70% ethanol). Oxyhaemoglobin was prepared by reduction of bovine haemoglobin as described previously (Martin *et al*, 1985 a, b).

5) ANALYSIS

I) Guinea-pig Internal Anal Sphincter

Two distinct patterns of activity were evident in the gpIAS. One consisted of a continuous discharge of spikes of roughly comparable amplitude accompanied by small oscillations in tone. Under these conditions spike frequency (spikes/min.) and contraction frequency (contractions/min) were measured. The second pattern of activity was also a continuous spike discharge upon which was superimposed bursts of spikes. In these cases the frequency of the bursts (bursts/min) and changes in tone (contractions/min) were measured). In both situations results were expressed as mean±S.E.M. of n (a number of) tissues. Drugs modified the pattern and the degree of tone. Their effects were usually assessed visually since the increase in spike frequency frequently precluded measurement of individual spikes.

II) Human Internal Anal Sphincter

Two of the human IAS` exhibited spontaneous mechanical activity. The frequency of contractions, the degree of spontaneous tone and the extent of relaxation produced by SNP were expressed as mean±S.E.M. of n (a number of) tissues.

III) Mouse Vas Deferens

Where appropriate, results, *i.e.* resting membrane potential and spontaneous EJP frequency and amplitude, were expressed as mean ± S.E.M.,

of n (a number of) cells. Statistical analysis of the EJP amplitude and frequency was performed by means of Student's *t* test for paired data for significance between means. A t-value of $P<0.05$ was taken as being significant. A minimum of three tissues were used to investigate the effects of SNP.

RESULTS

1) GUINEA-PIG INTERNAL ANAL SPHINCTER

I) Characteristics of Spontaneous Activity.

After equilibration, bursts of spontaneous electrical and mechanical activity were always seen. This activity was usually rapid (33.8±10.4 contractions/min., n=39; maximum contraction strength 0.9±0.5g, n=39) with the electrical spiking almost continuous (430 ± 40.6 spikes/min., n=10; Fig. 3), or burst-like (15.8 ± 0.8 contractions/min) with bursts of spikes (17.1 ± 6.8 bursts/min., n=6; *e.g.* Fig. 8) superimposed on the almost continual discharge.

Spontaneous electrical and mechanical activity was myogenic and Ca^{2+}-dependent; it was unaffected by tetrodotoxin ($5x10^{-6}M$; Fig. 4) and abolished after removal of $[Ca^{2+}]_o$ from the perfusing Krebs' solution (n=3; Fig. 5).

II) Effect of Temperature and Stretch

Decreasing the temperature of the perfusing Krebs' solution from 37.5°C to 26°C reversibly decreased the tone and the frequency of spontaneous electrical and mechanical activity (n=3; Fig. 6), probably by decreasing tissue metabolism, and therefore the activity of the ion pumps controlling spontaneous activity.

Increasing stretch on the gpIAS, by up to 6g, had no significant effect on spontaneous activity relative to control, but a tension-dependent reduction in the frequency of spontaneous activity and spike amplitude occurred between 7g and 15g stretch (n=3; Fig. 7). The increased amount of stretch may have led to a form of depolarizing block which decreased, before it eventually abolished, spontaneous activity. On the other hand, stretching the tissue beyond a certain tension may have damaged the tissue and the close junctions between the cells reducing synchronised activity.

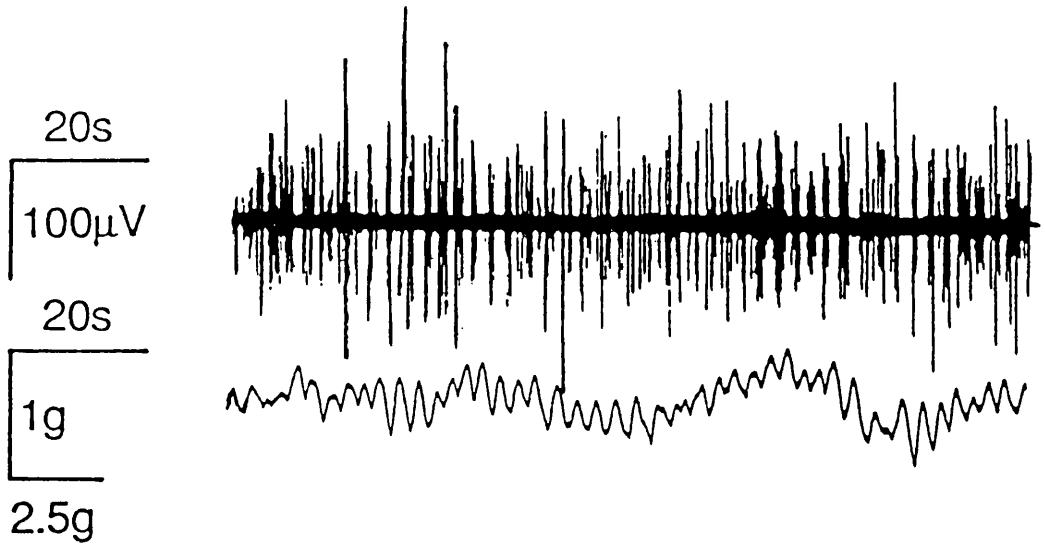


Figure 3: Control trace from U.V. oscillograph showing spontaneous electrical (upper trace) and mechanical activity from the gpIAS.

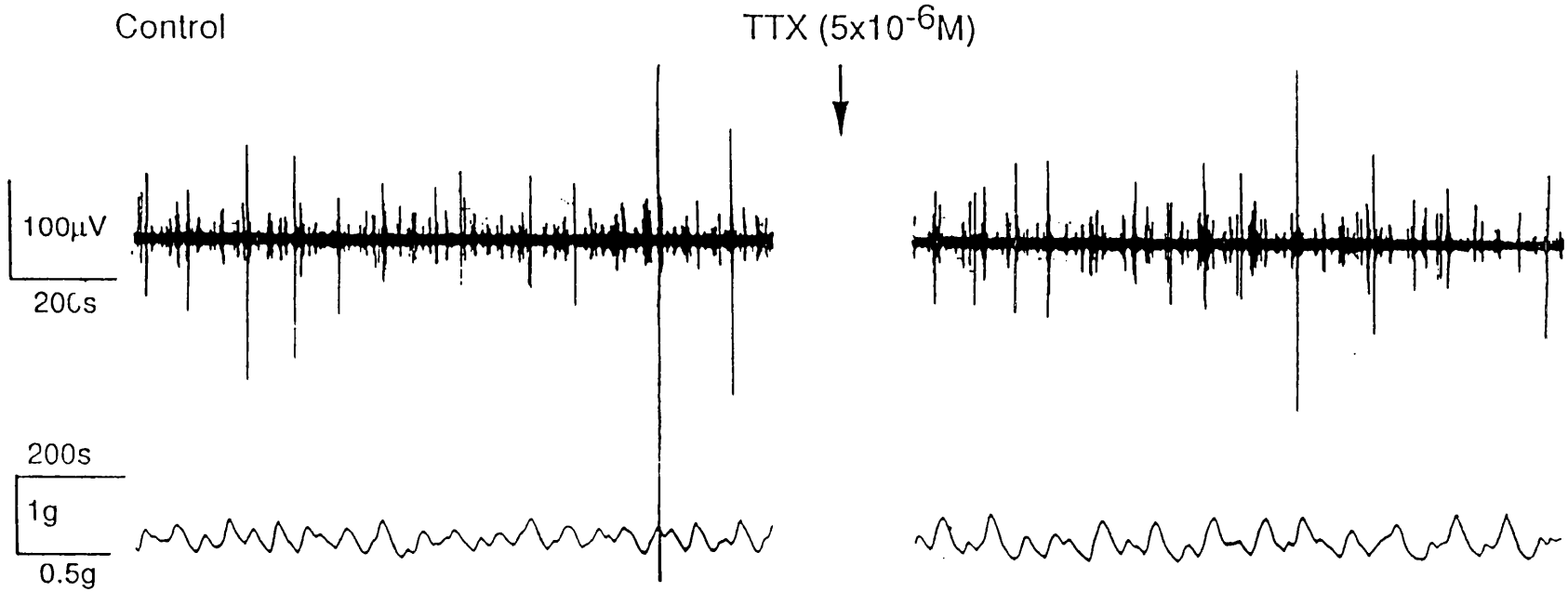


Figure 4: The lack of effect of tetrodotoxin (TTX; $5x10^{-6}$ M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS.

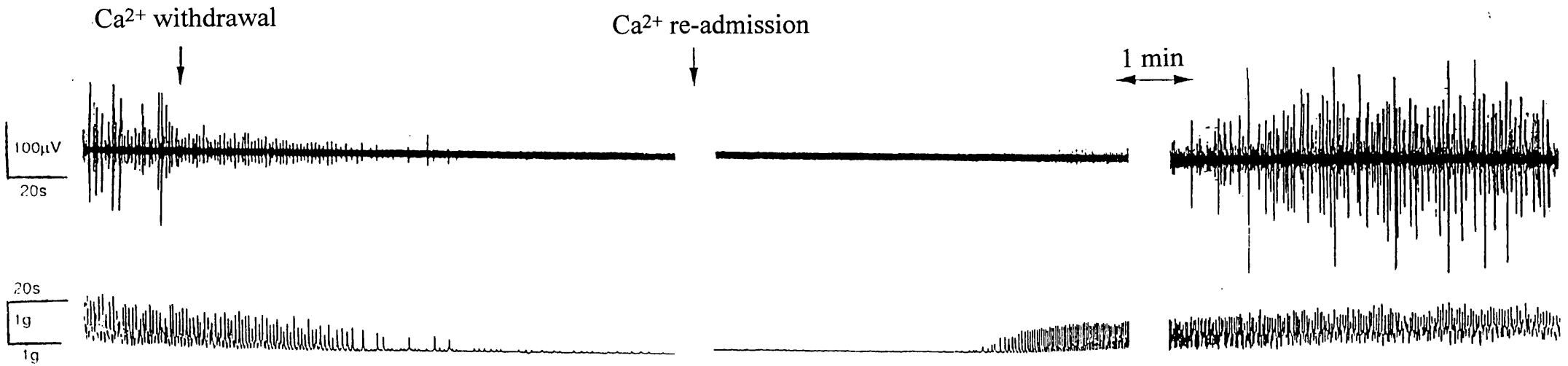


Figure 5: The effects of Ca^{2+} withdrawal and re-admission on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. Ca^{2+} withdrawal abolished, while re-admission restored, spontaneous activity.

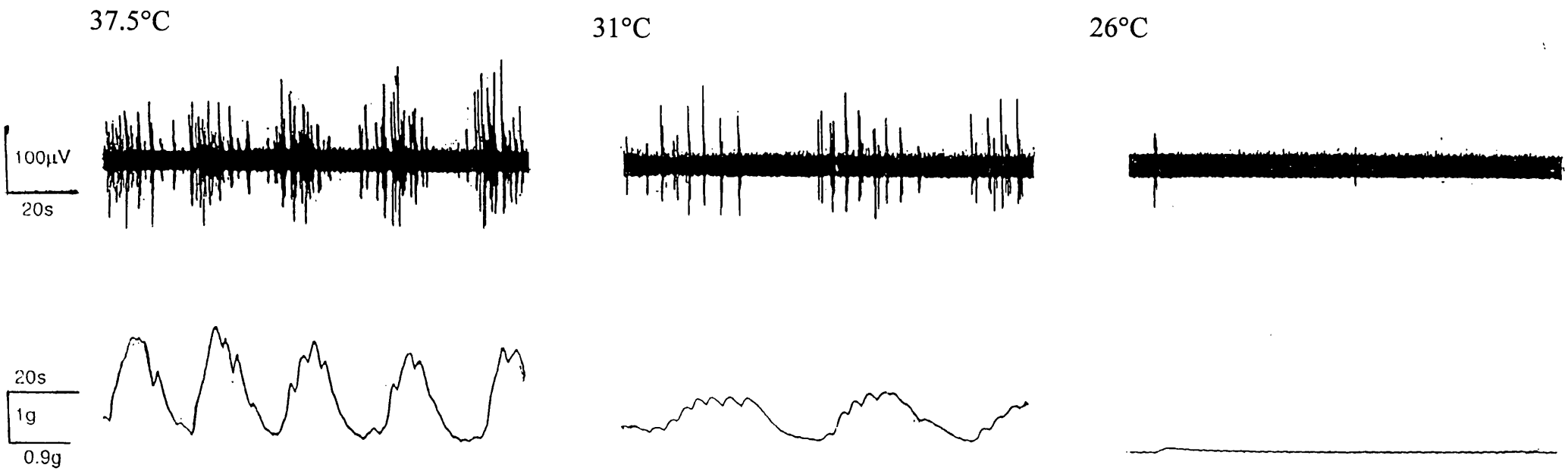


Figure 6: The effect of temperature on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. As temperature decreased the spontaneous activity also decreased relative to that at 37.5°C.

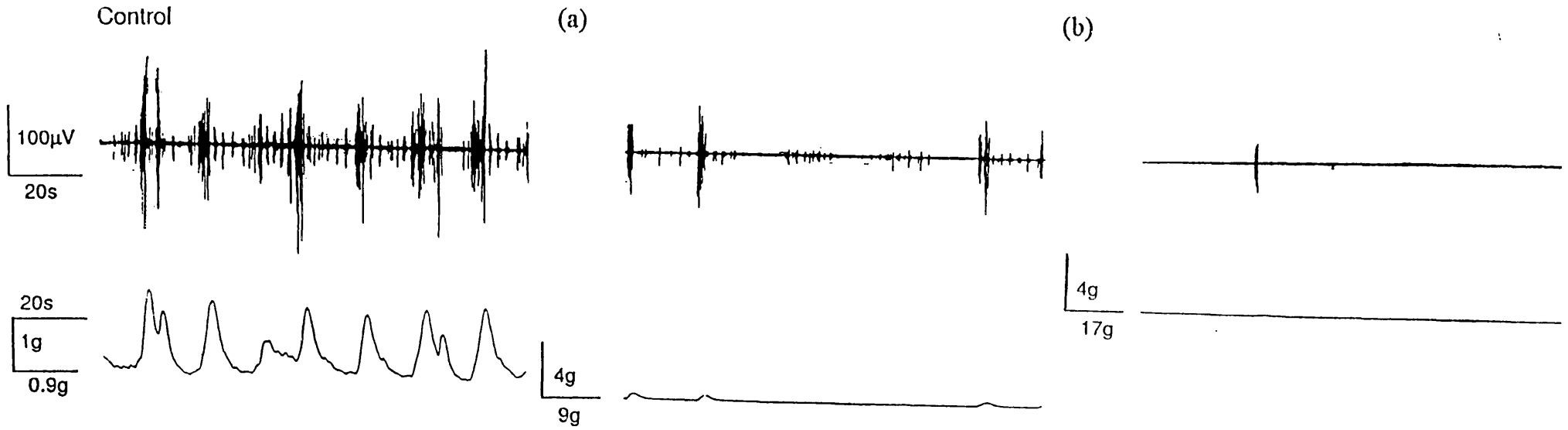


Figure 7: The effect of increasing stretch on the spontaneous electrical (upper traces) and mechanical of the gpIAS. Increasing tension up to 7g had no significant effect on either electrical or mechanical activity. (a) Increasing stretch to 9g reduced electrical and mechanical activity relative to control. (b) Above 15g both electrical and mechanical activity was abolished. Note the alteration in the tension calibration bars in traces (a) and (b).

III) Effects of Nitrovasodilators

The effects of the following nitrovasodilators on the spontaneous activity of the gpIAS were studied:

Sodium nitroprusside (SNP; $5x10^{-8}M$-$10^{-4}M$) Fig. 8

Glyceryl trinitrate (GTN; $10^{-6}M$-$10^{-3}M$) Fig. 9

Isosorbide dinitrate (IDN; $10^{-5}M$-$10^{-3}M$) Fig. 10

These nitrovasodilators, upon addition to the perfusing Krebs' solution, each produced a reversible, qualitatively similar, concentration-dependent alteration in the spontaneous electrical and mechanical activity of the gpIAS with a rank order of potency SNP > GTN > IDN. SNP (10^{-8}-$10^{-4}M$), GTN (10^{-6}-$10^{-3}M$) and IDN (10^{-5}-$10^{-3}M$) each lowered tone and altered the pattern of continual discharge (minimum n=3), initially producing a more synchronised pattern of mechanical and electrical activity before abolishing, or reducing, both.

At lower concentrations, SNP (10^{-8}-$10^{-6}M$), GTN (10^{-6}-$10^{-5}M$) and IDN (10^{-5}-$10^{-4}M$), after relaxing the gpIAS (by 77.4±13.6% from control, n=13, using $5x10^{-7}M$ SNP), increased the maximum amplitude of spontaneous contractions and, on occasion, spikes, as well as the number of spikes in each burst of activity. They also produced a more intermittent pattern of activity. At higher concentrations [SNP (>$10^{-6}M$), GTN (>$10^{-5}M$), IDN (>$10^{-4}M$)] the frequency and amplitude of both spikes and contractions were reduced or abolished.

IV) Effects of SNP

The most potent nitrovasodilator, SNP, was chosen for further study.

As expected, SNP appeared to exert its effects via NO-dependent cGMP stimulation. Oxyhaemoglobin (HbO; $10^{-5}M$), which binds NO (Keilin

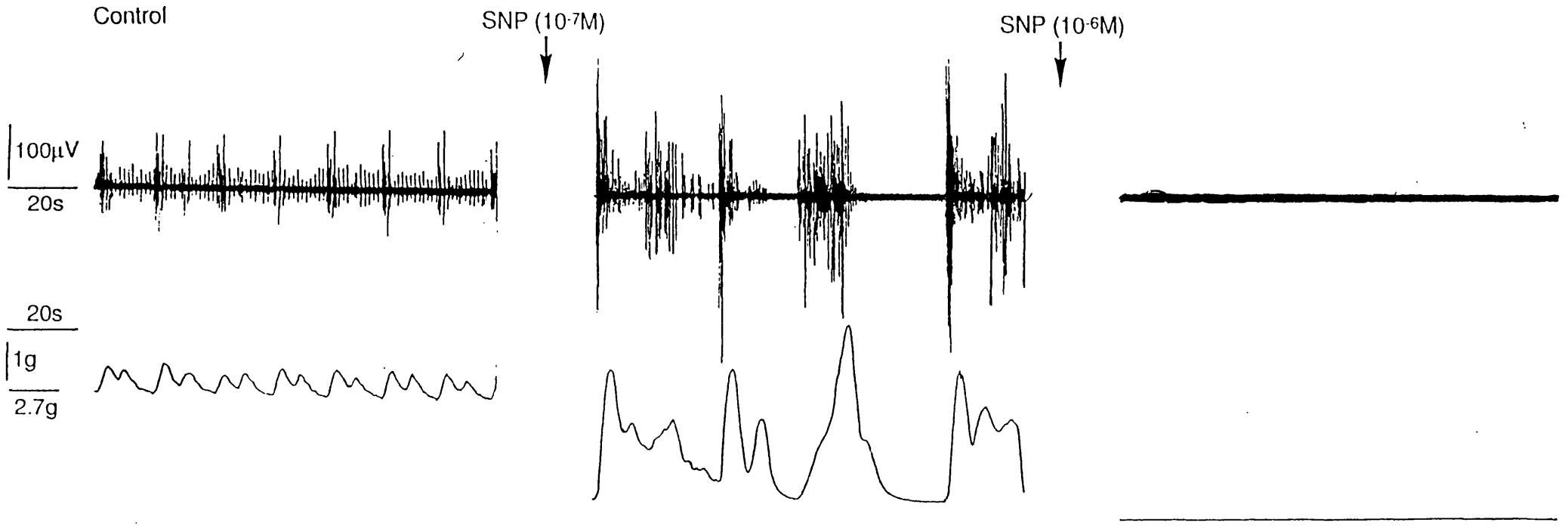


Figure 8: The effect of sodium nitroprusside (SNP; 10^{-7} & 10^{-6} M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. SNP (10^{-7} M) reduced tone (by 88%) and the frequency of spontaneous activity but increased the amplitude of both contractions and spikes and the number of spikes per burst of electrical activity relative to control. At 10^{-6} M, SNP further reduced tone and abolished electrical and mechanical activity.

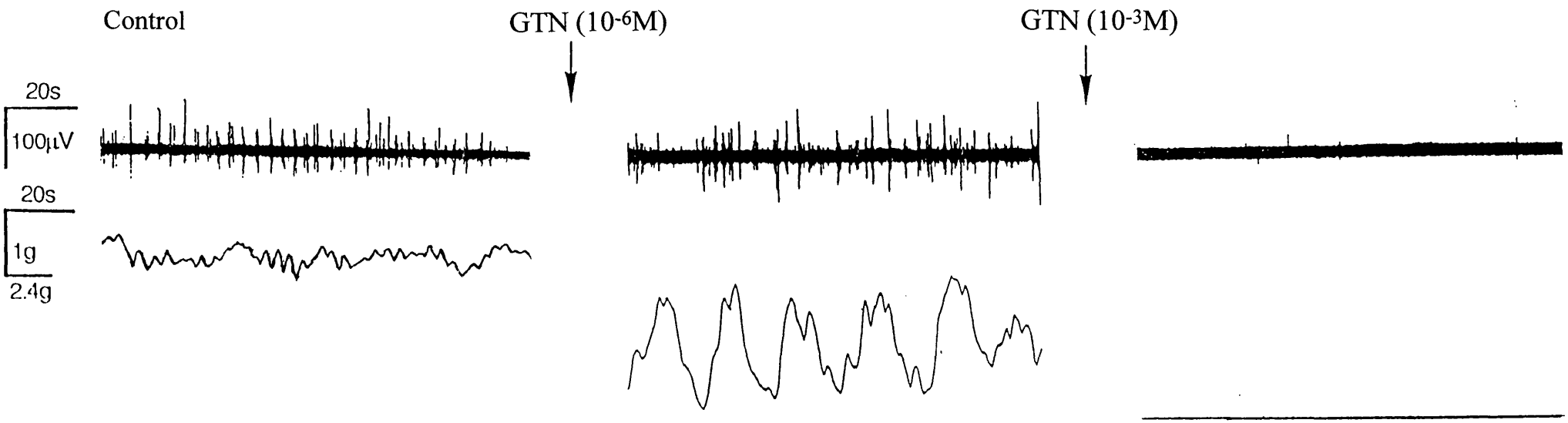


Figure 9: The effect of glyceryl trinitrate (GTN; 10^{-6} & 10^{-3}M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. GTN (10^{-6}M) reduced tone (by 81%) and the frequency of spontaneous activity but increased the amplitude of contractions relative to control. At 10^{-3} M, GTN further reduced tone (by 89% relative to control) and abolished electrical and mechanical activity.

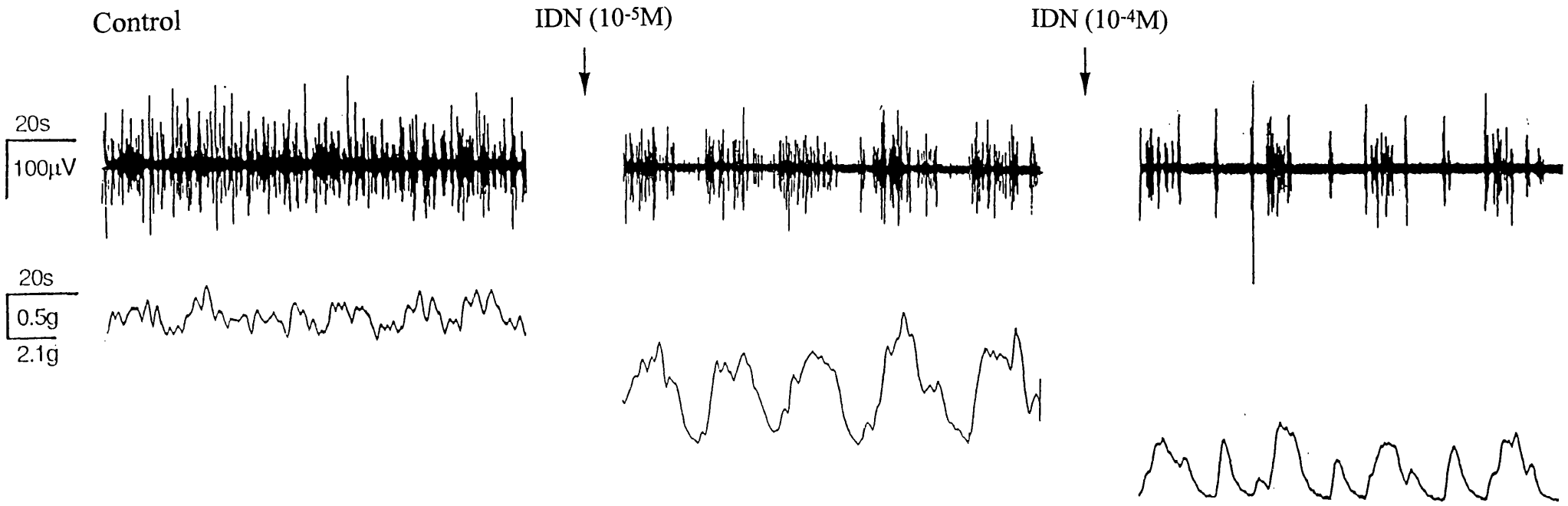


Figure 10: The effect of isosorbide dinitrate (IDN; 10^{-5} & 10^{-4}M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. IDN (10^{-5} M) reduced tone (by 35%) and the frequency of spontaneous activity, but increased the amplitude of contractions and number of spikes per burst of electrical activity relative to control. At 10^{-4}M, IDN further reduced tone (79% relative to control), frequency, amplitude of contractions and number of spikes per burst of electrical activity relative to 10^{-5} M IDN.

& Hartree, 1937; Martin *et al*, 1985 a, b; 1986), antagonized the reduction in tone and the increase in the maximum amplitude of contractions and spikes produced by SNP (10^{-6}M; n=4; Fig. 11). 8-br-cGMP ($2x10^{-5}$-$3x10^{-5}$M), the membrane-permeable cGMP analogue, which, by raising $[cGMP]_i$ levels, mimics the effects of NO stimulation of cytosolic guanylyl cyclase, also reproduced the excitatory (83.0±18.6%, n=3, increase in the maximum amplitude of contractions) and inhibitory effects of SNP (Fig. 12). Potassium ferrocyanide, a compound structurally similar to the nitroprusside molecule but devoid of NO (Fig. 13), failed to mimic the effects of SNP (n=3; Fig. 14), indicating that the effects of SNP on the gpIAS were not due to the ferrocyanide portion of the molecule and were likely to involve NO.

The question remained however, as to how SNP, via NO, exerted its effects on the gpIAS. That a hyperpolarization was responsible was investigated by varying the extracellular concentration of K^+ ($[K^+]_o$). Raising $[K^+]_o$ from 4.7mM to 14.1mM, enhanced the amplitude of contractions and the number of spikes per burst of electrical activity produced by a concentration of SNP ($5x10^{-7}$ -10^{-6}M) which, under control conditions ($[K^+]_o$ = 4.7mM), reduced tone and mechanical and electrical activity (n=4; Fig. 15). So by raising extracellular $[K^+]_o$, and, presumably, depolarizing the gpIAS, the inhibitory effects of SNP were antagonized. The "stimulatory" effect of increased $[K^+]_o$ was reversed by eliminating $[K^+]_o$ (n=4; Fig. 16). This presumably hyperpolarized the gpIAS and enhanced the inhibitory effects of the drug.

K^+ involvement was further investigated using the ATP-dependent K^+-channel opener, lemakalim (see Weston & Edwards, 1992). SNP and lemakalim each, in subthreshold concentrations (10^{-8}M and $5x10^{-8}$M respectively), had no significant effect on spontaneous activity but together abolished spontaneous activity (n=3; Fig. 17), indicating that K^+ was involved in the response to SNP.

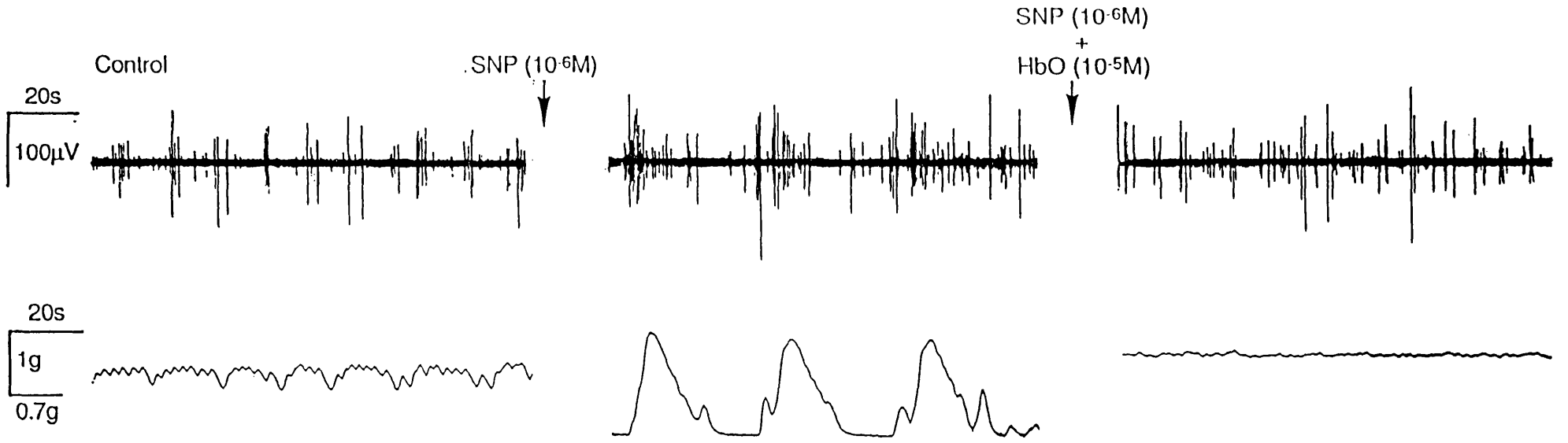


Figure 11: The effect of sodium nitroprusside (SNP; 10^{-6}M) alone and in the presence of oxyhaemoglobin (HbO; 10^{-5}M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. SNP (10^{-6}M) alone reduced tone (by 83%), and the frequency of spontaneous activity but increased the amplitude of the contractions and the number of spikes in each burst of relative to control. HbO (10^{-5}M) antagonized the effects of SNP (10^{-6}M).

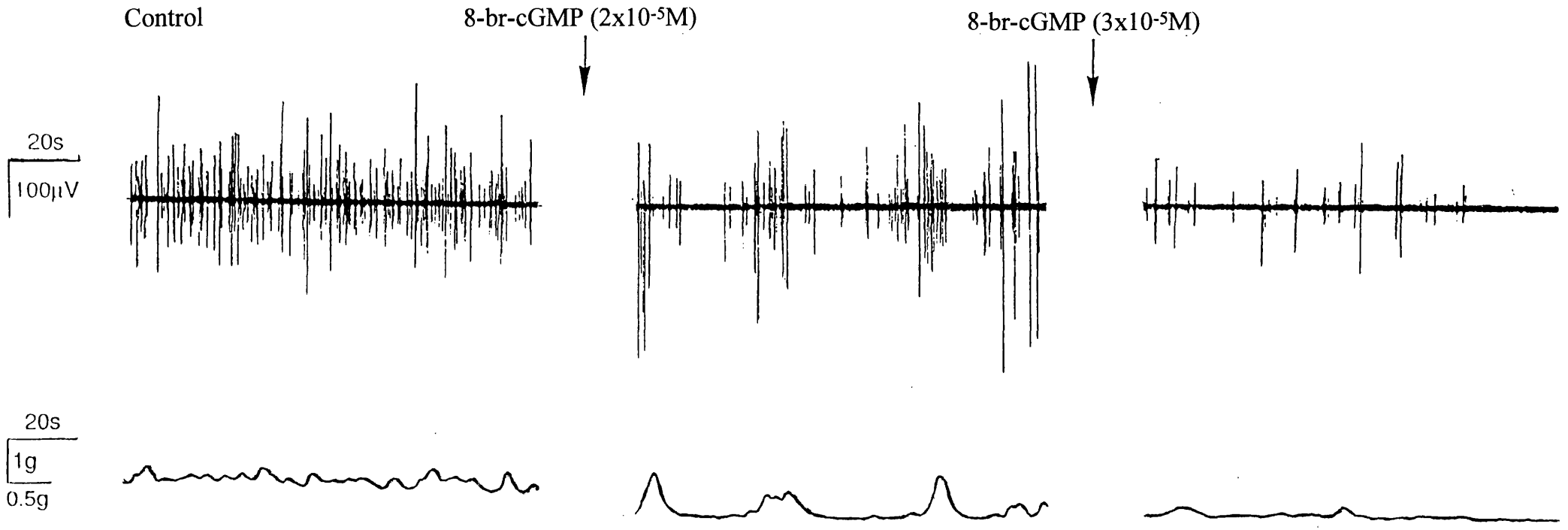


Figure 12: The effect of 8-bromo cGMP (8-br-cGMP; $2x10^{-5}$ & $3x10^{-5}$M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. 8-br-cGMP($2x10^{-5}$M) reduced tone (by 98%) and the frequency of spontaneous activity but increased the amplitude of both contractions and spikes and the number of spikes per burst of electrical activity relative to control. $3x10^{-5}$ M, 8-br-cGMP significantly reduced the amplitude and frequency of both electrical and mechanical activity relative to control.

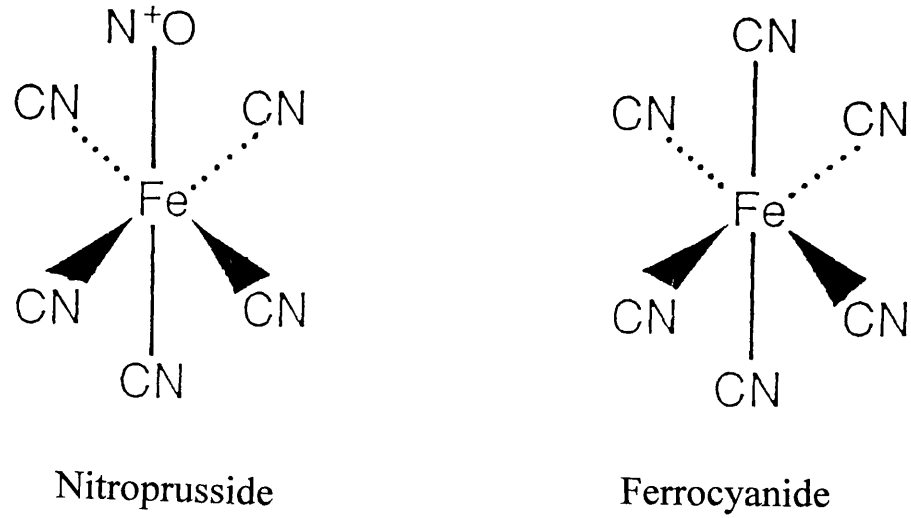


Figure 13: Representation of the nitroprusside and ferrocyanide molecules. The structures are very similar but the ferrocyanide molecule is devoid of NO.

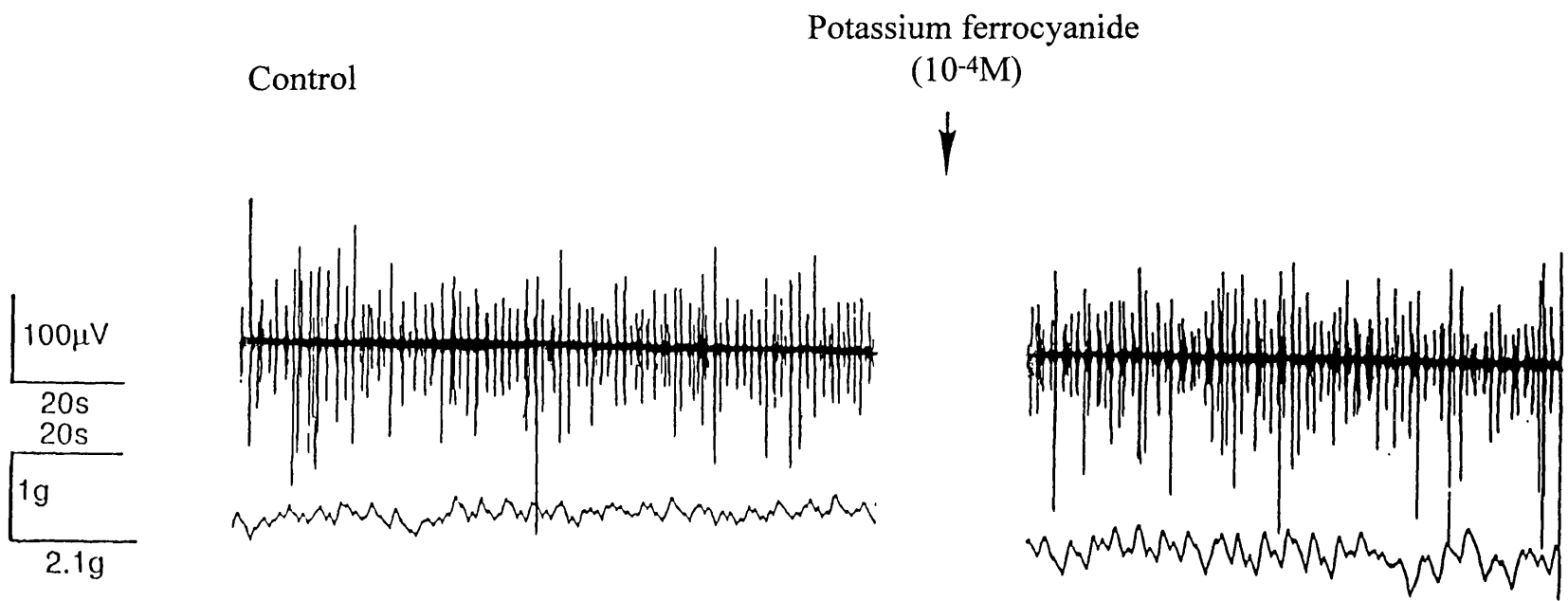


Figure 14: The lack of effect of potassium ferrocyanide (10^{-4} M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS.

Figure 15: The effect of raising $[K^+]_o$ in the absence and presence of sodium nitroprusside (SNP; $5x10^{-7}M$) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. In 4.7mM $[K^+]_o$ Krebs` solution, SNP ($5x10^{-7}M$) produced minimal excitatory effects on the gpIAS, increasing the amplitude of contractions but reducing tone (by 87%) and frequency of spontaneous activity relative to control (a). 14.1mM $[K^+]_o$ Krebs` solution, increased tone (by 33%) and the amplitude of contractions and spikes relative to control (a). SNP ($5x10^{-7}M$), in 14.1mM $[K^+]_o$ Krebs` solution, increased the amplitude of contractions but reduced tone (by 68%) and frequency of spontaneous activity relative to control (b) indicating that increasing $[K^+]_o$ antagonized the inhibitory effects of SNP.

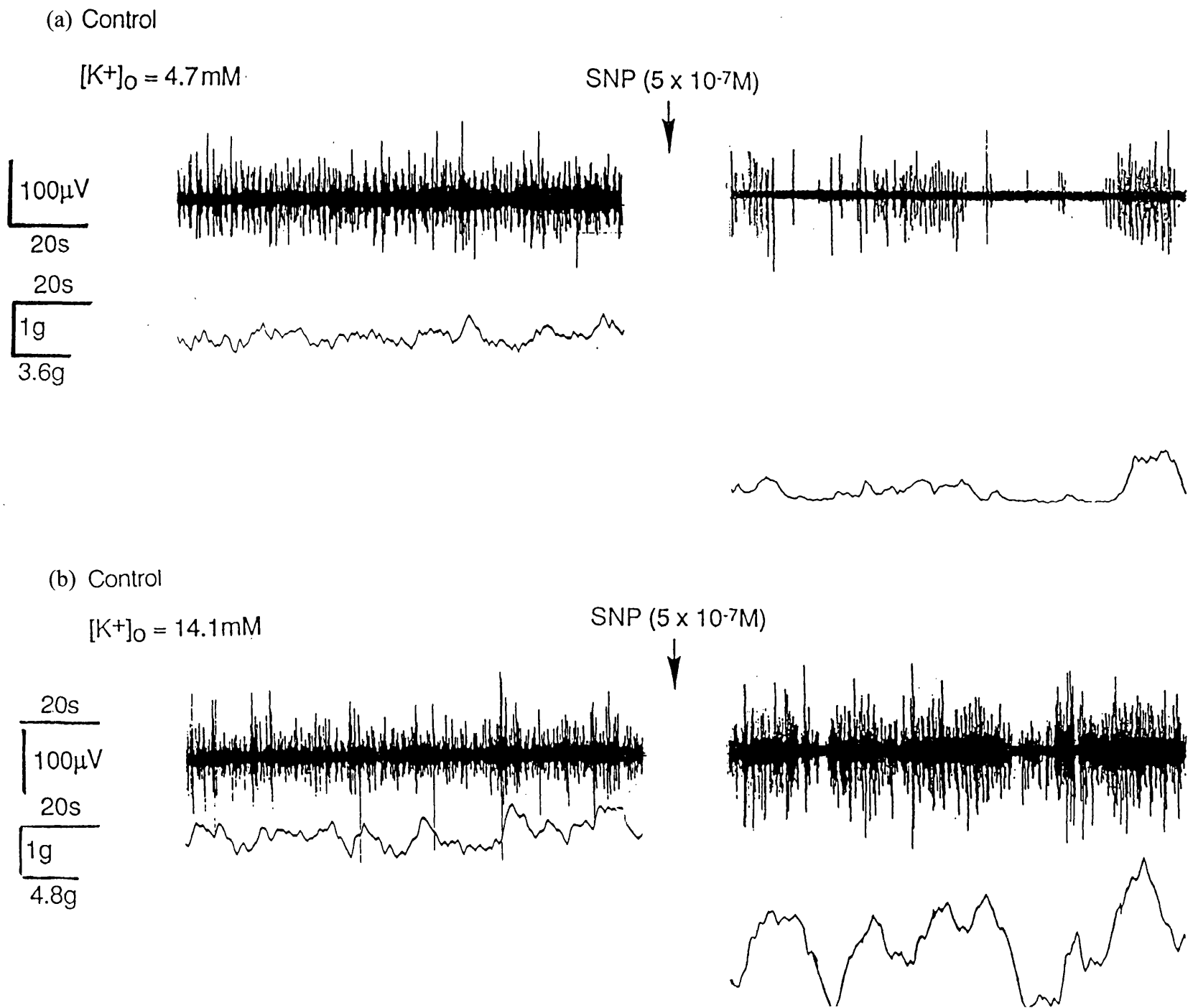

(a) Control
$[K^+]_o$ = 4.7mM
SNP (5 x 10^{-7}M)
100μV
20s
20s
1g
3.6g
(b) Control
$[K^+]_o$ = 14.1mM
SNP (5 x 10^{-7}M)
20s
100μV
20s
1g
4.8g

Figure 16: The effect of eliminating $[K^+]_o$ in the absence and presence of sodium nitroprusside (SNP; $5x10^{-7}M$) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. In 4.7mM $[K^+]_o$ Krebs` solution, SNP ($5x10^{-7}M$) produced minimal excitatory effects on the gpIAS, increasing the amplitude of contractions but reducing tone (by 87%) and frequency of spontaneous activity relative to control (a). 0mM $[K^+]_o$ Krebs` solution, reduces tone (by 83%) and the amplitude of contractions relative to control (a). SNP ($5x10^{-7}M$) in 0mM $[K^+]_o$ Krebs` solution abolished spontaneous activity indicating that eliminating $[K^+]_o$ enhanced the inhibitory effects of SNP.

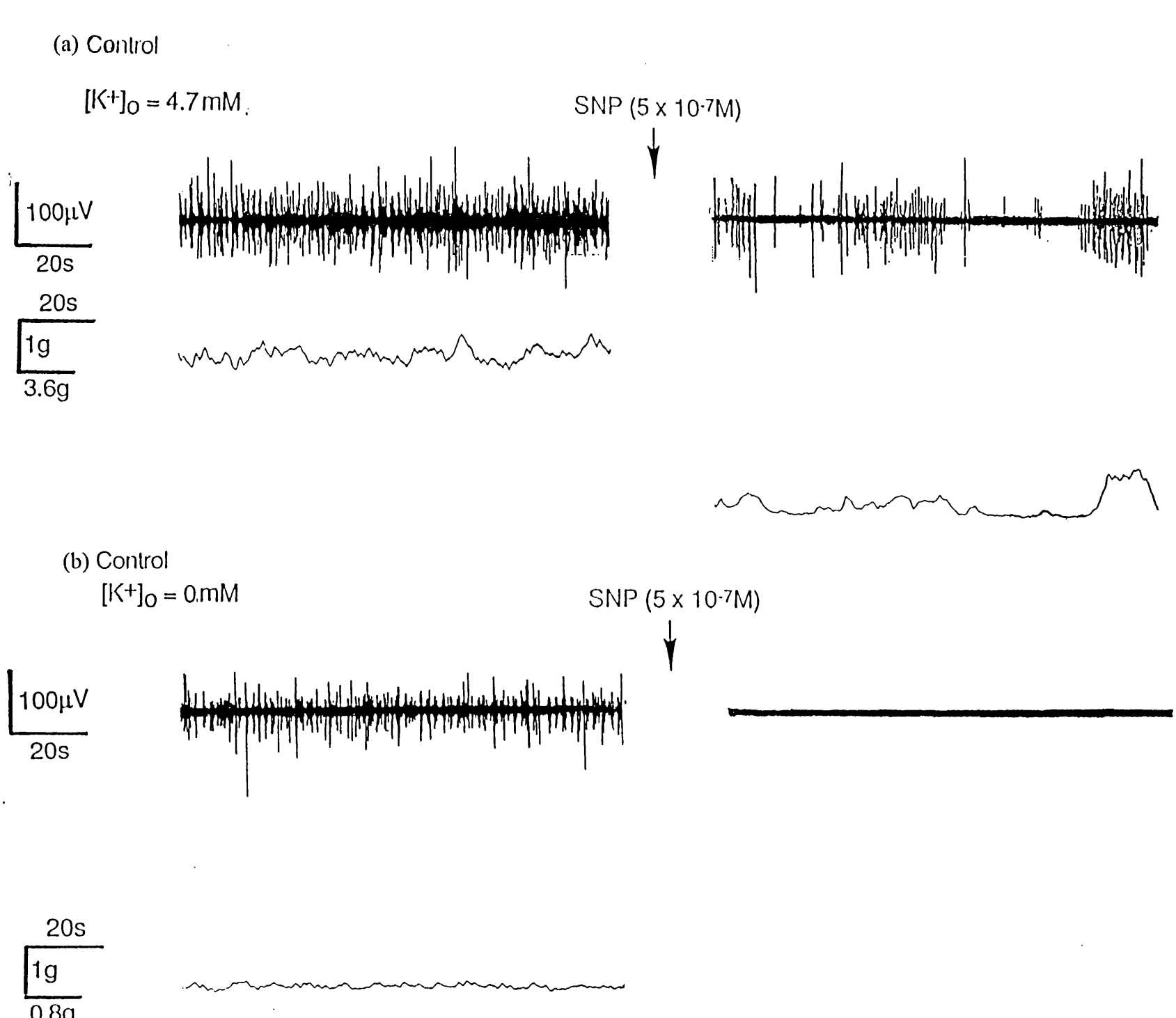
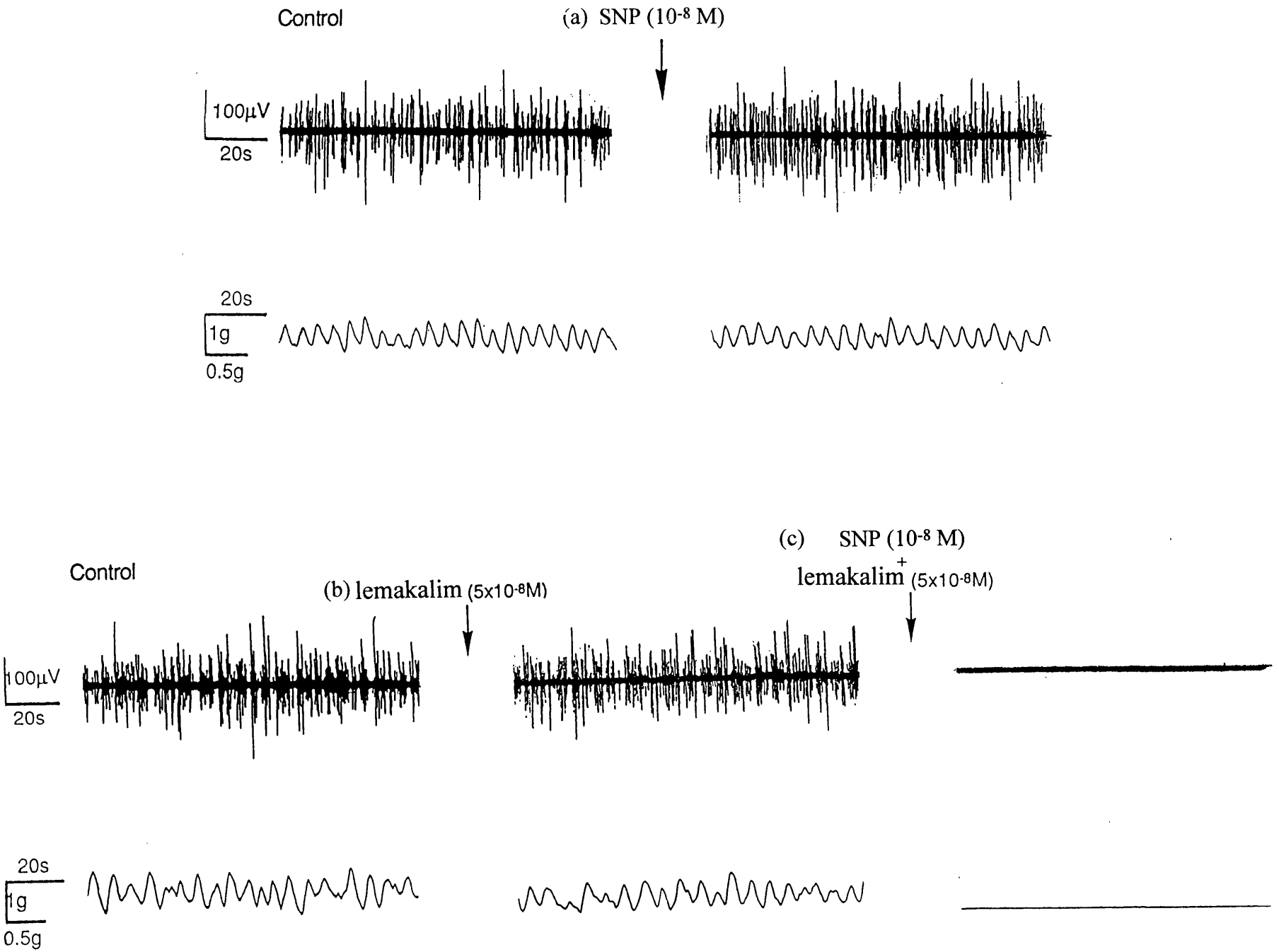

(a) Control
$[K^+]_O$ = 4.7mM
SNP (5 x 10^{-7}M)
100μV
20s
20s
1g
3.6g
(b) Control
$[K^+]_O$ = 0mM
SNP (5 x 10^{-7}M)
100μV
20s
20s
1g
0.8g

Figure 17: The effects of sodium nitroprusside (SNP; 10^{-8}M) alone and with lemakalim ($5x10^{-8}$M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. Neither (a) SNP (10^{-8}M) nor (b) lemakalim ($5x10^{-8}$M) alone was effective, but (c) together they abolished spontaneous activity implying a synergistic action between the two compounds.


Control
(a) SNP (10-8 M)
100μV
20s
20s
1g
0.5g
Control
(b) lemakalim (5x10-8M)
(c) SNP (10-8 M)
+
lemakalim (5x10-8M)
100μV
20s
20s
1g
0.5g

$[Ca^{2+}]_o$ was also involved. The calcium channel antagonist diltiazem (DTZ; 10^{-4} M) reduced or abolished spontaneous activity in combination with SNP ($5x10^{-7}$M; n=3; Fig. 18). The calcium channel agonist BAY K 8644 (10^{-6}M), which increases the open probability of Ca^{2+} channels, enhanced the maximum amplitude of both contractions (by 215.7±163.2%, n=3) and spikes produced by SNP ($5x10^{-7}$ -$2x10^{-6}$ M; Fig. 19).

2) HUMAN INTERNAL ANAL SPHINCTER

I) Effects of SNP

Four of the five tissues responded electrically, with the firing of action potentials (maximum amplitude ~240μV), and mechanically, with a contraction, to phenylephrine (10^{-3} M; Fig 20 (b)). Two of the five tissues exhibited spontaneous tone (4.1g & 2.0g) and mechanical activity (8.8±0.8 contractions min^{-1}, n=2; Fig. 20). SNP ($5x10^{-8}$ M) relaxed the two IAS` (by 69.3±3.2% , n=2, from control); no stimulatory effects were seen (Fig. 21).

3) MOUSE VAS DEFERENS

I) Resting Properties

The mean resting membrane potential of the longitudinal smooth muscle cells of the mouse vas deferens was -72.7±0.5mV (n=345 cells from 31 preparations). Electrically they displayed spontaneous excitatory junction potentials (EJPs) with a frequency of 32.0±6.4 min^{-1} (n=11 cells from 9 preparations) and a mean amplitude of 7.0±0.6mV (n=13 cells from 10 preparations). No spontaneous mechanical activity could be detected.

II) Effects of SNP

SNP (10^{-3} M), applied by pressure ejection (20-30 p.s.i., 50-1000ms duration), produced two kinds of activity on the mouse vas deferens; a

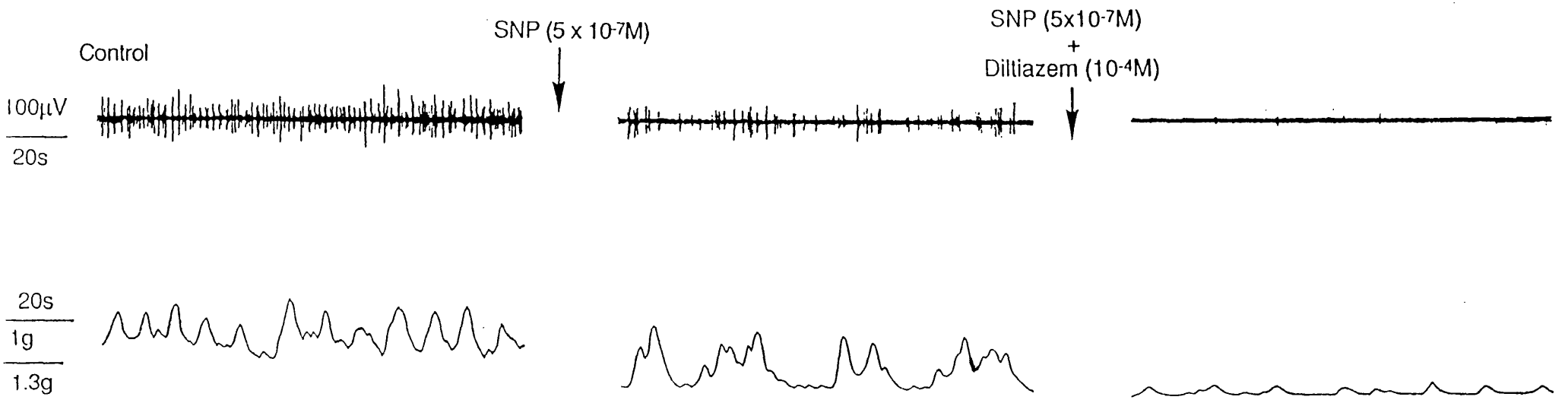


Figure 18: The effects of sodium nitroprusside (SNP; $5x10^{-7}$ M) alone and with diltiazem (DTZ; 10^{-4} M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. SNP ($5x10^{-7}$ M) reduced tone (by 55%) and made spontaneous activity more burst-like but increased the amplitude of contractions relative to control. SNP ($5x10^{-7}$ M), in the presence of DTZ (10^{-4} M) abolished all electrical and most mechanical activity.

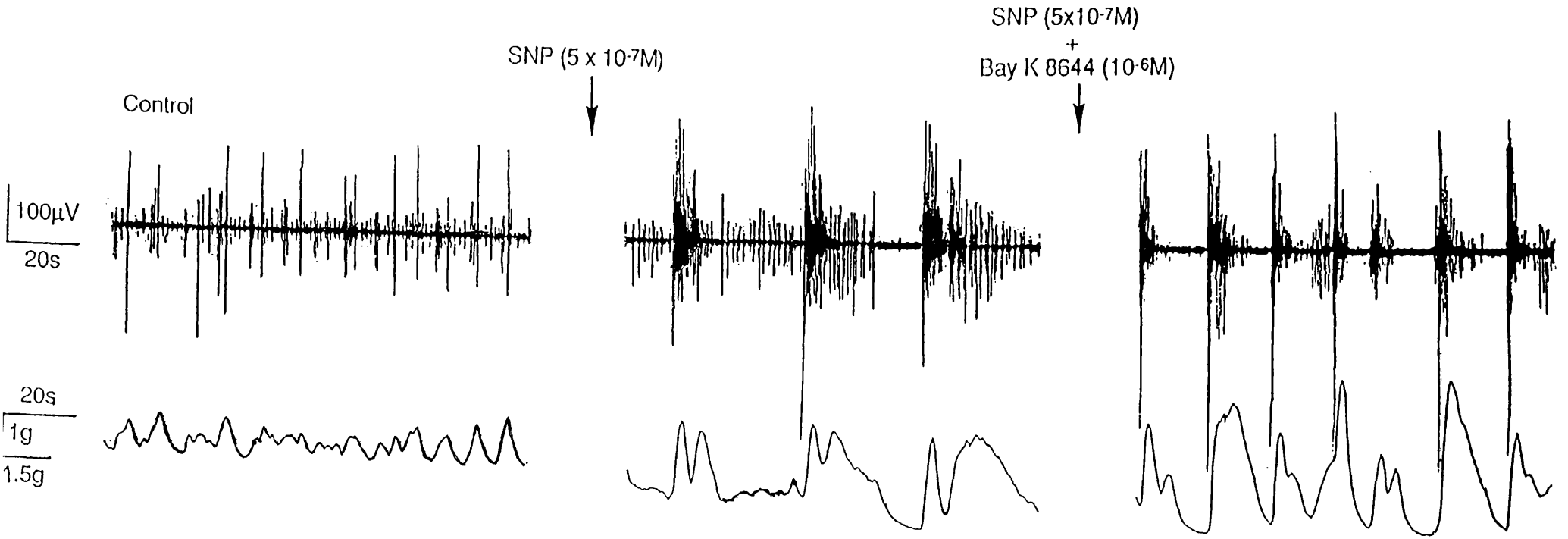


Figure 19: The effects of sodium nitroprusside (SNP; $5x10^{-7}$ M) alone and with BAY K 8644 (10^{-6} M) on the spontaneous electrical (upper traces) and mechanical activity of the gpIAS. SNP ($5x10^{-7}$ M) reduced tone (by 92%), made the spontaneous activity more burst-like and increased the amplitude of contractions, spikes and the number of spikes in each burst relative to control. SNP ($5x10^{-7}$ M) in the presence of BAY K 8644 (10^{-6} M) further increased the amplitude of both contractions and spikes and the frequency of spontaneous activity indicating the dependence of SNP on Ca^{2+} to exert its stimulatory effect.

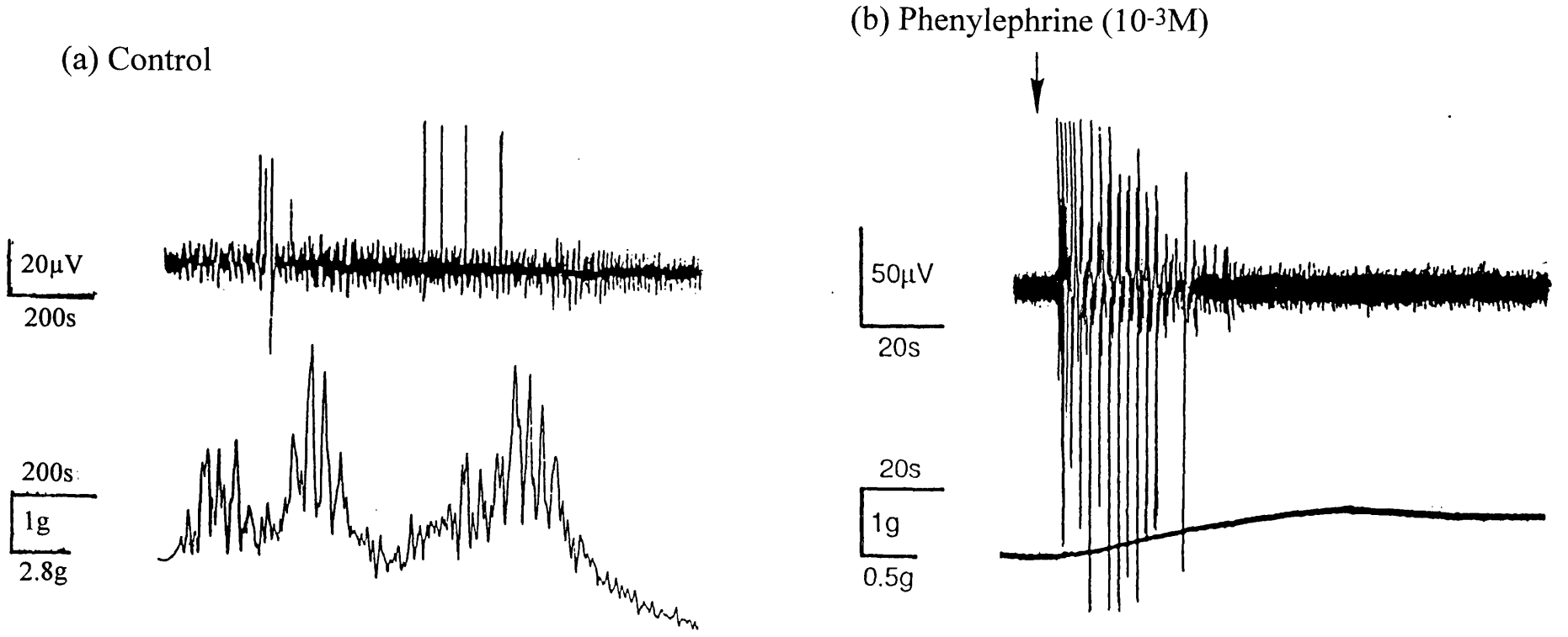


Figure 20: (a) Spontaneous, and (b) phenylephrine (10^{-3}M)-induced electrical (upper traces) and mechanical activity in the human IAS. (a) Spontaneous mechanical accompanied by irregular bursts of electrical activity. (b) Phenylephrine (10^{-3}M) triggered a burst of electrical activity and increased tone (by 0.5g) indicating that the tissue was sphincteric.

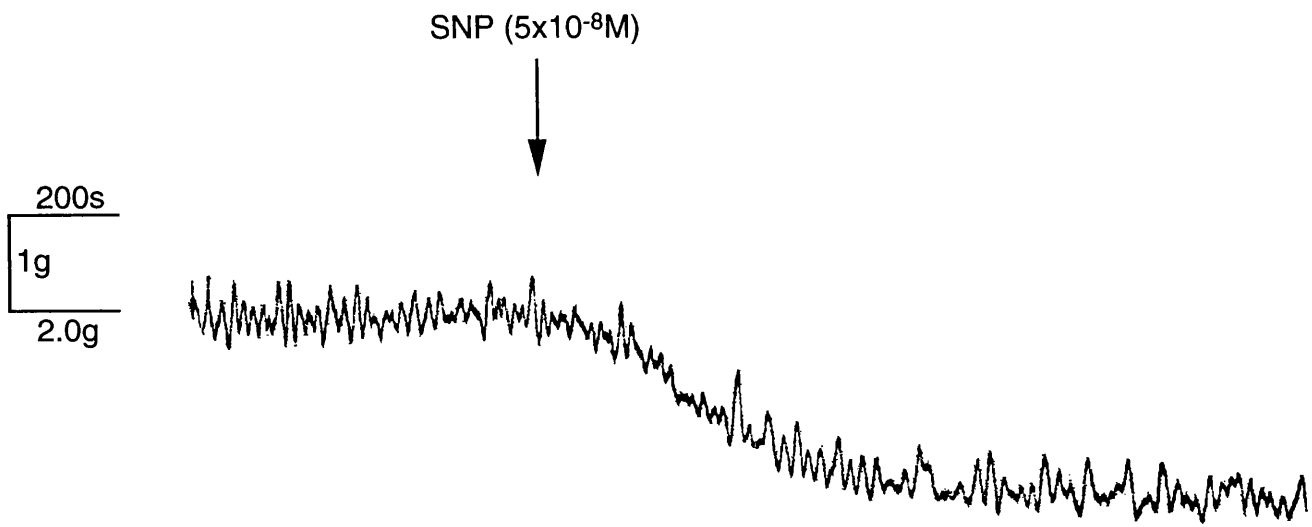


Figure 21: The reduction in tone produced by sodium nitroprusside (SNP; $5x10^{-8}$ M) on the human internal anal sphincter.

membrane hyperpolarization (4.9±0.2mV, n=13 cells from 10 preparations) with or without a significant increase in the amplitude or frequency of excitatory junction potentials, indicating a post-synaptic locus of action, and b) a significant ($P<0.001$) increase in amplitude (by 377.0±9.6% above control, n=10 cells from 8 preparations) of spontaneous EJPs, indicating a pre-synaptic enhancement of transmitter release, sometimes accompanied by a membrane depolarization (Fig. 22). No contractions were measured during these depolarizations. Occasionally, in response to SNP, a hyperpolarization would precede the increase in spontaneous EJP amplitude indicating that both inhibitory and excitatory events could occur simultaneously (see Fig. 22 b).

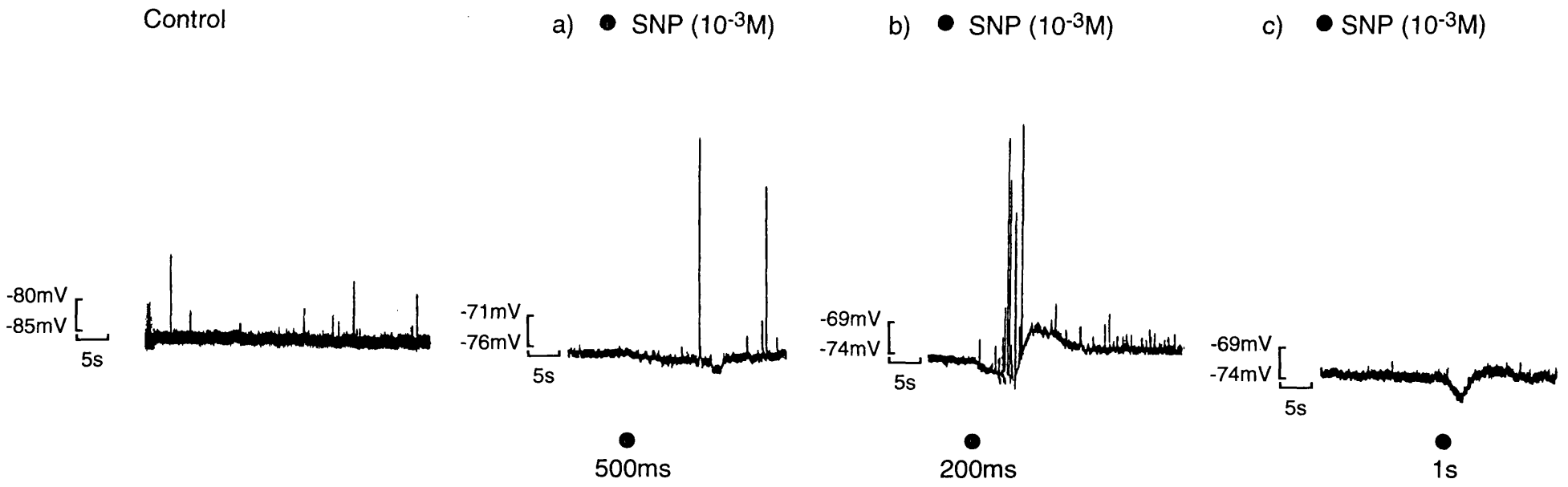


Figure 22: The effect of sodium nitroprusside (SNP; 10^{-3} M), applied locally by pressure ejection (tip diameter 2-10μM; ~ 30 p.s.i.; 200-1000ms) on the intracellular electrical activity of the mouse vas deferens. SNP significantly ($P<0.001$) increased spontaneous EJP amplitude (a) & (b), sometimes accompanied by a membrane hyperpolarization followed by a depolarization (b). SNP also hyperpolarized the tissue without increasing spontaneous EJP amplitude (c).

DISCUSSION

In contrast to the vast majority of studies investigating the role of NO within smooth muscle, which substantiate its inhibitory role (see Rand, 1992; Sanders *et al* 1992, Sanders & Ward, 1992), this investigation demonstrates that nitrovasodilators, which release NO, also exert excitatory effects on gpIAS and mouse vas deferens smooth muscle.

1) GUINEA-PIG INTERNAL ANAL SPHINCTER

Under resting conditions the gpIAS exhibits a high degree of almost continuous myogenic spontaneous electrical and mechanical activity which is probably related to its physiological role as a regulator of rectoanal continence (Gowers, 1887; Denny-Brown & Robertson, 1935; Papasova, 1989).

The nitrovasodilators SNP, GTN and IDN each affected this resting activity. However, the potency of these drugs to effect changes in activity differed markedly; SNP was significantly more potent than the others, a similar finding to those in the rabbit distal colon (RDC) and rat portal vein (RPV; Smith, 1994). The biotransformation of SNP differs from that of the organic nitrates (Gryglewski *et al*, 1992) in that it is probably activated intracellularly (Gryglewski *et al*, 1992) whereas the organic nitrates IDN and GTN are effectively decomposed to release NO only when they come in contact with cysteine or N-acetylcysteine (Feelisch, 1991). Consequently, the chances of NO so released coming into contact with guanylyl cyclase are reduced, so making them less effective than SNP. Differences may also exist between the organic nitrates themselves as IDN is more potent than GTN in the RDC and *vice versa* in the RPV (Smith, 1994) and gpIAS (present investigation). The reason for this is unclear but may be related to their respective affinities for the metabolizing enzyme, possibly cytochrome P-450 (Schröder, 1992), due to their different chemical structures (Tzeng & Fung, 1992). As SNP was the most effective compound, it was used as a model for the actions of this type of drug in the present study.

The release of NO (by SNP) and its subsequent stimulation of guanylyl cyclase, to increase intracellular ($[cGMP]_i$) levels, appeared responsible for both aspects of activity; potassium ferrocyanide, which lacks NO, had no effect on the spontaneous activity of the gpIAS, while HbO inhibited SNP`s activity and 8-br-cGMP mimicked both the stimulatory and inhibitory actions of the drug.

The question remains as to how the increase in NO-stimulated $[cGMP]_i$ mediates the effects of the nitrovasodilators seen here. The increase in this cyclic nucleotide may evoke a membrane hyperpolarization, although this cannot be measured using the Golenhofen apparatus. Nitrovasodilators hyperpolarize and relax a number of smooth muscles including rabbit pulmonary artery (Ito *et al*, 1978), rat tail artery (Cheung & MacKay, 1985) guinea pig uterine artery (Tare *et al*, 1990) and, importantly, the gpIAS (Baird & Muir, 1990; present investigation-see **Chapter 1-Results, pp.76-77**). This hyperpolarization was manifested as a decrease in tone and a resetting of the pacemaker activity, resulting in more synchronised, burst-like activity.

K^+ replacement experiments and lemakalim, supported the proposal that a membrane hyperpolarization may be involved in mediating the actions of SNP; removal of $[K^+]_o$ and lemakalim each enhanced the inhibitory effects of the drug. Although not seen in tissues such as the RDC and RPV (Smith, 1994), this may have been mediated by a K^+ efflux, a well known mechanism of inducing smooth muscle hyperpolarization (*e.g.* guinea-pig taenia caeci - Bennett *et al*, 1963; jejenum -Hidaka & Kuriyama, 1969; gpIAS -Lim & Muir, 1985). Cl^- may also be involved in the hyperpolarizing response to SNP, as evidenced in the opossum LOS (Saha & Goyal, 1992), although experiments have not been carried to investigate this possibility in the gpIAS.

Ca^{2+} availability was necessary for maintaining both normal spontaneous tone and rhythm, and the stimulatory effects of SNP (Smith, 1994; present investigation). Diltiazem (DTZ) which antagonizes, and BAY K

8644 which opens, Ca^{2+} channels blocked and enhanced, respectively, the excitatory effects of SNP.

How does the interaction between K^+ and Ca^{2+} fluxes contribute to the excitatory and inhibitory effects of SNP? In ganglia, dibutryl cGMP which, like SNP, increases $[cGMP]_i$, produced a concentration-dependent, transient inward Ca^{2+} current (Nishimura *et al*, 1992). If so, then this may account for the enhancing effect of SNP in the gpIAS. On the other hand, one might expect that an inward, stimulatory flux of Ca^{2+} would increase, rather than decrease, tone as seen here. However, raised $[cGMP]_i$ levels cause a decrease in $[Ca^{2+}]_i$ levels by increasing the binding, sequestration or efflux of the ion (Lincoln & Johnson, 1984) which may explain why tone decreased in response to SNP.

The proposed membrane hyperpolarization produced by SNP probably results from the opening of Ca^{2+}-activated K^+ channels due to the influx of Ca^{2+}. As the degree of hyperpolarization, induced by higher concentrations of SNP increased, the open probability of voltage-dependent Ca^{2+} channels would decrease (Nelson *et al*, 1990), leaving the muscle increasingly unable to respond to this challenge until, eventually, all activity ceases.

2) HUMAN INTERNAL ANAL SPHINCTER

Two IAS` displayed spontaneous mechanical activity. In these tissues SNP produced a relaxation but did not reproduce the excitatory effects seen in the gpIAS. This may have been due to several reasons; a) the effects witnessed in the gpIAS may have been species-dependent; b) inappropriate concentrations of SNP may have been used to produce excitatory effects in the human IAS, although this is unlikely as the effects of a wide concentration range were examined in both tissues, or c) the tissues may have been unable to respond in such a way to the nitrovasodilator, possibly because the tissues were only available several hours into the operations, during which time they

had undergone relatively severe mechanical stresses which may have affected their physical condition.

3) MOUSE VAS DEFERENS

Intracellular electrical analysis of the mouse vas deferens confirmed the post-synaptic hyperpolarizing effect of SNP, also proposed to occur in the gpIAS (present investigation). However, this study also demonstrated a pre-synaptically-mediated excitatory effect of the drug on spontaneous transmitter release.

Although not investigated, it is possible that the mechanism underlying the inhibitory hyperpolarizing response to SNP is similar to that proposed for the gpIAS, namely, a K^+ efflux, possibly induced by an initial Ca^{2+} influx.

The stimulatory effect of SNP in the vas deferens, manifested as an increase in spontaneous EJP amplitude, indicated that the drug enhanced the release of an excitatory transmitter. How SNP produced this increase is unclear. It can be speculated however, that, as transmitter release is a Ca^{2+}-dependent process, SNP enhances the neuronal Ca^{2+} influx. This could be achieved indirectly by raising intracellular cGMP levels, which in turn may transiently increase Ca^{2+} influx, as in parasympathetic ganglia (Nishimura *et al*, 1992), or, less likely, the nitroprusside molecule, by blocking pre-synaptic receptors, such as α_2-adrenoceptors, which regulate the release of transmitter (see Illes & Starke, 1983), could prevent negative feedback and thereby enhance transmitter release.

Such an enhancement of excitatory transmitter release may explain some excitatory effects of the drug in other tissues. Indeed, previous work in this laboratory has shown that the SNP-induced increase in frequency of both electrical and mechanical activity in the RPV was blocked by a combination of phentolamine and propranolol (Smith, 1994). Within the gpIAS, SNP, under

certain circumstances may also enhance the release of a transmitter, such as noradrenaline, which is a known excitatory transmitter in this tissue.

Clearly, from these and other studies, the nitrovasodilators possess a spectrum of activity, with some drugs more effective than others depending on the tissue and species utilised. Nitrovasodilators are used clinically for their relaxant properties, as in the treatment of angina pectoris or achalasia. The excitation observed in tissues such as the gpIAS, mouse vas deferens (present investigation), RDC and RPV (Smith, 1994), in response to the nitrovasodilators, would clearly be an unwanted side effect in the treatment of conditions where relaxation is required and may therefore warrant a re-assessment of the clinical usage of such drugs.

Further work is required to fully elucidate the mechanism of action involved in mediating the excitatory effects of these drugs within spontaneously active smooth muscle and to determine the possible consequences of these effects in disease therapy.

4) CONCLUSIONS

This part of my work was undertaken to further investigate possible excitatory effects of nitrovasodilators on spontaneously active smooth muscles first demonstrated in this laboratory by Wylie (1988), in the RPV, and Smith (1994) in the RDC and RPV. The results indicate that within the gpIAS and the mouse vas deferens the actions of these drugs were complex.

Although NO is regarded mainly as an inhibitory NANC transmitter within the majority of smooth muscles (see Rand, 1992; Sanders & Ward, 1992; Sanders *et al*, 1992) the nitrovasodilators both enhanced and inhibited, spontaneous electrical and mechanical activity in certain smooth muscles. Inhibition in the gpIAS appeared as a reduction in tone and a decrease in the amplitude of both the contractions and accompanying electrical activity.

Within the mouse vas deferens it was manifested as a membrane hyperpolarization. Both effects were probably the result of a K^+ efflux leading to reduction in $[Ca^{2+}]_i$ levels.

Excitation in the gpIAS was manifested as an increase in both the maximum amplitude of spontaneous contractions and electrical spikes and within the mouse vas deferens as an increase in the amplitude of spontaneous EJPs. The reasons underlying the excitation are unclear but, in the gpIAS may be due to a NO/cGMP-induced influx of Ca^{2+} across the membrane. In the mouse vas deferens, the enhanced transmitter release may also be due to a transient Ca^{2+} influx into the nerves.

These results support previous findings of nitrovasodilator-induced excitation responses in this and other laboratories, *i.e.* in the RPV (Bray *et al*, 1987), guinea-pig ileum (Sugisawa *et al*, 1991) and rat ileum (Barthó *et al*, 1992) suggesting that endogenous NO may, like most other neurotransmitters, possess both inhibitory and excitatory properties.

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