THE ACTIONS OF NITROVASODILATORS ON SPONTANEOUSLY ACTIVE 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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Publications

Some aspects of the work described in this thesis have been published :

Smith, A.D. & Muir, T.C. (1991). The involvement of nitric oxide in neuronally mediated relaxation in the rabbit distal colon. Br. J. Pharmacol., **104**, 201P.

Smith, A.D. & Muir, T.C. (1992). The effects of nitric oxide and nitrovasodilators on spontaneous electrical and mechanical activity in the rabbit distal colon. Br. J. Pharmacol., **107**, 86P.

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Abbreviations

ACh	Acetylcholine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BRL 34915	Cromakalim
BRL 38227	Levcromakalim, Lemakalim
BRP	Bovine retractor penis
$[Ca^{++}]_i$	Intracellular free Ca^{++} concentration
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
E	Efflux rate constant
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GABA	γ-amino-n-butyric acid
GTN	Glyceryl trinitrate
^{3}H	Tritium
Hb	Haemoglobin
IAS	Internal anal sphincter
ICJ	Ileocolonic junction
IDN	Isosorbide dinitrate
IF	Inhibitory factor
i.j.p.	Inhibitory junction potential
$K_4Fe(CN)_6$	Potassium ferrocyanide
^{42}K	Radiolabelled potassium
L-NAME	N^{G}-nitro-L-arginine methyl ester

L-NMMA	N^G-monomethyl-L-arginine
L-NNA	N^G-nitro-L-arginine
LOS	Lower oesophageal sphincter
MeB	Methylene blue
NMDA	N-methyl-D-aspartate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NANC	Non-adrenergic non-cholinergic
NO	Nitric oxide
NO_2	Nitrite group
NO_3	Nitrate group
O	Overflow rate constant
P	Probability
PV	Portal vein
^{86}Rb	Radiolabelled rubidium
RDC	Rabbit distal colon
RPV	Rabbit portal vein
SNAC	S-nitroso-N-acetylcysteine
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide

Summary

1. The aim of this study was to investigate the actions of nitric oxide (NO) donors i.e. nitrovasodilators, on the extracellular electrical and mechanical activity recorded from spontaneously active smooth muscle, in particular the rabbit distal colon (RDC), rabbit portal vein (RPV) and rat portal vein (rat PV), *in vitro*, using the Golenhofen apparatus. First, circular strips of RDC were examined for evidence of a "nitrergic" innervation.

2. Addition of carbachol (3×10^{-5}M) to circular strips of RDC mounted in a conventional (25ml) organ bath raised the tone to 7.73 ± 0.67g ($n = 44$). Electrical stimulation (16 pulses, 1-16Hz, 0.1-0.5ms, supramaximal voltage), in the presence of phentolamine and propranolol (each 3×10^{-6}M), produced frequency-dependent, non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxations and rebound contractions.

3. NANC relaxations induced by nerve stimulation were mimicked by authentic NO ($\geq 10^{-5}$M), and partially inhibited by the avid NO binding molecule, haemoglobin (Hb ; 3×10^{-5}M), or by the inhibitor of endogenous NO synthesis, N^G-nitro-L-arginine methyl ester (L-NAME ; 5×10^{-4}M). On some occasions, L-NAME-induced inhibition was reversed by the precursor to NO synthesis, L-arginine. The response to authentic NO (2×10^{-5}M) was abolished by Hb (3×10^{-5}M).

4. Rebound contractions in response to inhibitory NANC nerve stimulation were mimicked by authentic NO ($\geq 10^{-5}$M) and partially inhibited by L-NAME (5×10^{-4}M) or the cyclooxygenase inhibitor, indomethacin (10^{-5}M).

5. Unstretched, longitudinal strips of RDC mounted in the Golenhofen apparatus exhibited spontaneous, calcium-dependent spike bursts that were always accompanied by contraction. The frequency and duration of these

electrical events was 3.77 ± 0.18 bursts min^{-1} (n = 80) and 7.91 ± 0.77s (n = 57) respectively, and each contained 7.84 ± 0.62 spikes (n = 61). The amplitude of the largest spike in each was 248.46 ± 28.37 μV (n = 63) while the amplitude of corresponding contraction was 2.21 ± 0.13g (n = 79). Contractile bursts lasted for 12.23 ± 1.42s (n = 79) and each contained 2.78 ± 0.33 mechanical peaks (n = 79).

6. Under an applied tension of 1g, intact sections of RPV mounted in the Golenhofen apparatus exhibited spontaneous spike bursts that were always accompanied by contraction. The frequency and duration of electrical events was 2.95 ± 0.41 bursts min^{-1} (n = 5) and 7.40 ± 1.94s (n = 4) respectively, and each contained 16.76 ± 3.14 spikes (n = 4). The amplitude of the largest spike in each was 24.67 ± 2.60 μV (n = 3) while the amplitude of corresponding contraction, including the 1g applied, was 1.59 ± 0.2g (n = 5). Contractile bursts lasted for 12.17 ± 1.57s (n = 5) and each contained 1.50 ± 0.2 mechanical peaks (n = 5).

7. Under an applied tension of 1g, intact sections of rat PV mounted in the Golenhofen apparatus exhibited spontaneous, calcium-dependent spike bursts, that were always accompanied by contraction. The frequency and duration of electrical events was 2.61 ± 0.14 bursts min^{-1} (n = 49) and 6.81 ± 0.56s (n = 24) respectively, and each contained 15.08 ± 1.11 spikes (n = 20). The amplitude of the largest spike in each was 39.04 ± 3.69 μV (n = 24) while the amplitude of corresponding contraction, including the 1g applied, was 1.48 ± 0.39g (n = 49). Contractile bursts lasted for 8.31 ± 0.36s (n = 49) and each contained 1.63 ± 0.08 mechanical peaks (n = 49).

8. Nitrovasodilators in the form of sodium nitroprusside (SNP), glyceryl trinitrate (GTN) or isosorbide dinitrate (IDN), each possessed both inhibitory

and excitatory properties in the RDC. SNP was the most effective and, consequently, was used as a model for this group of drugs. Each nitrovasodilator ($\geq 10^{-5}$M) induced a short-lived period of quiescence in unstretched, longitudinal strips of RDC. Following this, an altered pattern of spontaneous electrical and mechanical activity returned abruptly. In tissues with a low initial frequency (< 6 bursts min^{-1}), SNP or IDN (each $\geq 10^{-5}$M) significantly enhanced the frequency of activity. In tissues with a high initial frequency (> 6 bursts min^{-1}), SNP (10^{-5}M) significantly reduced the frequency of activity. Spike amplitude was increased significantly by GTN (10^{-6}M) and, on occasion, by SNP ($\geq 10^{-5}$M), while the number of spikes per burst and the duration of each burst were reduced significantly by SNP ($\geq 10^{-5}$M) or IDN ($\geq 10^{-5}$M and 10^{-4}M respectively). Only SNP ($\geq 10^{-5}$M) significntly reduced the number of mechanical peaks per burst but both SNP ($\geq 10^{-6}$M) and IDN ($\geq 10^{-5}$M) significantly decreased the duration of mechanical events. Each of the nitrovasodilators (each $\geq 10^{-5}$M) significantly lowered the amplitude of spontaneous contraction.

9. In the intact RPV, SNP possessed only inhibitory properties and (10^{-5}M) abolished both electrical and mechanical activity. In the intact rat PV, nitrovasodilators exhibited both excitatory and inhibitory properties. The pattern of spontaneous activity was altered without the prior induction of quiescence. As in the RDC, SNP was most effective. The frequency of activity was increased significantly by SNP ($\geq 10^{-7}$M). Spike amplitude was reduced significantly by SNP (10^{-5}M) but increased by IDN (10^{-5}M), while the number of spikes per burst and the duration of each electrical burst were inhibited by SNP ($\geq 10^{-6}$M) or GTN (10^{-7}M). Only SNP ($\geq 10^{-7}$M) significantly reduced the number of mechanical peaks per burst, but both SNP ($\geq 10^{-6}$M) and GTN (10^{-7}M) significantly decreased the duration of mechanical events. The amplitude of spontaneous contraction was lowered significantly by SNP

($\geq 10^{-7}$M) and by GTN (10^{-6}M).

10. In both RDC and rat PV, the actions of SNP on spontaneous electrical and mechanical activity were mimicked by the analogue of cyclic guanosine monophosphate (cGMP), 8-bromo cGMP, and inhibited by Hb, indicating that these effects were mediated by nitric oxide. Potassium ferrocyanide ($K_4Fe(CN)_6$) which structurally resembles SNP, but which does not release NO, had no effect on the spontaneous electrical or mechanical activity of either tissue.

11. The addition of a subthreshold dose of SNP (10^{-6}M) to the RDC, together with a subthreshold dose of the potassium channel opener, BRL 38227 (10^{-7}M), abolished activity i.e. synergism occurred.

12. The means adopted to test directly for the ability of vasodilators to stimulate an efflux of potassium from gastrointestinal smooth muscle, was to load the RDC with radiolabelled rubidium (a marker for potassium) before mounting it in the Golenhofen apparatus. The muscarinic agonist, carbachol (10^{-5}M), then enhanced the efflux rate constant for rubidium. Neither SNP, BRL 38227 nor isoprenaline (each 10^{-7}-10^{-4}M) had any effect on the basal efflux rate constant.

13. Similarly, the ability of vasodilators to stimulate the efflux of potassium from vascular smooth muscle was examined by loading the rat PV with radiolabelled rubidium, before mounting it in the Golenhofen apparatus. The contractile agonist, noradrenaline, or BRL 38227 (each 10^{-5}M) then enhanced the efflux rate constant of rubidium. Neither SNP nor isoprenaline (each 10^{-7}-10^{-4}M) affected the basal efflux rate constant.

14. In RDC, the SNP (10^{-5}M)-induced reduction in contractile amplitude was reversed by the calcium-channel agonist, BAY K 8644 (10^{-7}M) indicating that this effect of SNP was probably mediated by a fall in the cytosolic free calcium level. When added in reverse order, SNP ($\geq 10^{-5}$M) reversed the desynchronization of activity produced by BAY K 8644 (10^{-7}M).

15. The ability of SNP to alter the cytosolic free calcium concentration was examined on rat aortic vascular smooth muscle cells grown on glass cover slips. Angiotensin II (10^{-7}M)elevated this parameter ; SNP (10^{-5}M) did not.

16. The SNP (10^{-5}M)-induced increase in the frequency of contraction of rat PV was antagonized by the prior addition of phentolamine and propranolol (each 3 x 10^{-6}M) together, suggesting that SNP may have stimulated the release of noradrenaline. The means adopted to test for this in rat PV, was to radiolabel the stores of transmitter in the nerves and examine the effects of SNP on the overflow of noradrenaline from the tissue mounted in the Golenhofen apparatus. The indirect sympathomimetic, tyramine (10^{-5}M), enhanced the overflow of noradrenaline ; SNP (10^{-7}-10^{-5}M) did not increase the overflow of noradrenaline from the rat PV.

17. In conclusion, in spontaneously active smooth muscle, nitrovasodilators possessed both excitatory (i.e. increased frequency of activity, increased electrical spike amplitude) and inhibitory properties (i.e. period of quiescence, reduced amplitude of contraction, reduced number of electrical spikes/mechanical peaks per burst and shorter duration of electrical/mechanical bursts). Each of these actions was mediated through the release of NO and probable stimulation of cGMP. Experiments in the presence of SNP and BAY K 8644 suggested that, at least in RDC, the nitrovasodilator-induced fall in contractile amplitude was mediated through a reduction in cytosolic calcium

levels. Beyond this, the mechanisms are less clear. However, the enhanced frequency of spontaneous activity, the decrease in spikes/peaks per burst and the reduction in the duration of each spontaneous event were possibly a consequence of a direct effect of NO on the smooth muscle pacemaker. Further, in RDC, the nitrovasodilator-induced period of quiescence may have been mediated via a mechanism that was similar to that of BRL 38227 but which did not involve a significant transmembrane efflux of potassium from the smooth muscle as a whole.

The finding that SNP had significantly greater effects than organic nitrates such as GTN and IDN, supported the proposal that this drug was activated differently. The significant excitation observed with SNP suggested that, in some cases, the relaxant properties of this compound could be compromised due to concomitant excitation.

The effects of SNP on the extracellular electrical and mechanical activity recorded from the RDC and the rat PV (+ = increase, o = no change, - = decrease).

Electrical

	Frequency	Spike Amplitude	No. of spikes/burst	Duration
RDC	+	o (sometimes +)	-	-
Rat PV	+	- (sometimes +)	-	-

Mechanical

	Frequency	Peak Amplitude	No. of peaks/burst	Duration
RDC	+	- (sometimes +)	-	-
Rat PV	+	-	-	-

Chapter 1: Introduction

1.1. The Nitrovasodilators

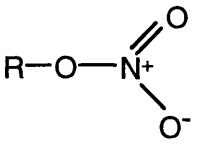
The term 'nitrovasodilator' describes any member of a chemically heterogeneous group of compounds that release nitric oxide (NO) i.e. they are prodrugs of NO. For this reason, they are frequently used, in biological systems, to mimic the effects of the highly unstable, authentic, NO. Nitrovasodilators include organic nitrates, organic and inorganic nitrites, sodium nitroprusside , S-nitrosothiols, sydnonimins and furoxans (Fig. 1). Of particular interest in this study, were the actions of sodium nitroprusside (SNP), glyceryl trinitrate (GTN) and isosorbide dinitrate (IDN).

More than one hundred years have elapsed since amyl nitrite (Brunton, 1867) and GTN (Murrell, 1879) were first advocated for the relief of angina pectoris. Prior to this, therapy for this condition had been largely ineffective ; it included brandy, ether, chloroform or ammonia. The use of nitrovasodilators was a major advance in the therapy of anginal pain. Following his trial of amyl nitrite, Brunton recorded, "On pouring from five to ten drops of the nitrite on a cloth and giving it to the patient to inhale, the physiological action took place in from thirty to sixty seconds ; and simultaneously with the flushing of the face the pain completely disappeared, and generally did not return till its wonted time next night." However, although nitrovasodilators are potent coronary vasodilators their principal benefit arises, not from an action on the heart, but from an action on the systemic circulation where they act to reduce venous return and consequently left ventricular work. Amyl nitrite is no longer in common use, but GTN, IDN and SNP are still used, routinely, to treat angina pectoris, congestive heart failure, percutaneous coronary angioplasty, pulmonary hypertension and hypertensive emergencies (Abrams, 1987 ; see Moncada *et al*, 1991b).

GTN and IDN are organic nitrates, a group of drugs each of which contains one or more functional nitrate groups (NO_3 ; Fig. 1). Amyl nitrite, though an

Fig. 1
The different chemical classes (with examples) of nitrovasodilator compounds.

Organic nitrates



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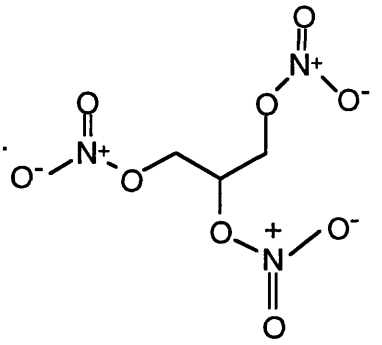
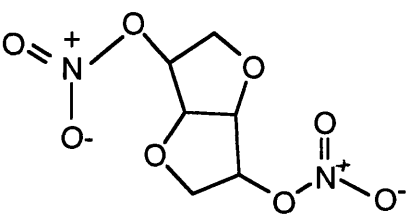
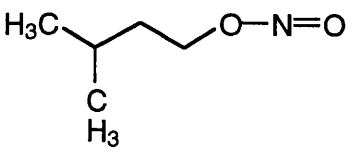


Glyceryl trinitrate



Isosorbide dinitrate

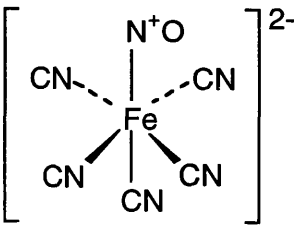
Organic nitrites R - O - N = O

e.g. 

Amyl nitrite

Inorganic nitrite NO_2^-

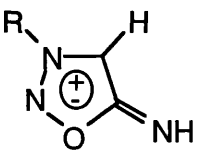
Sodium Nitroprusside $Na_2[Fe(CN)_5(NO)]$



Nitroprusside anion

S-nitrosothiols R - S - N = O

Sydnonimins



Furoxans

organic nitrite, since it contains the nitrite functional group (NO_2 ; Fig. 1), is often also referred to as an organic nitrate due to the similarities in pharmacologic effects to the other nitrates (Fung, 1992). Although SNP causes vasodilatation via the same biochemical pathway as the organic nitrates or nitrites, it is neither, since the nitroprusside anion lacks the requisite functional groups (Fig. 1). Despite the widespread clinical use of the nitrovasodilators, their mechanism of action has become clear only relatively recently (see Ignarro & Kadowitz, 1985 ; Waldman & Murad, 1987).

1.1.1. Mechanism of Action of Nitrovasodilators

The action of GTN was once attributed to its conversion, in the circulation, to NO_2^- which, unlike NO_3^-, had weak vasodilator actions (Hay, 1883). However, an effective vasodilator dose of GTN could not provide sufficient NO_2^- to account for the relaxation observed (Krantz *et al*, 1940).

Other nitrovasodilators (e.g. sodium azide, hydroxylamine and $NaNO_2$), activated soluble guanylate cyclase and raised cyclic guanosine monophosphate (cGMP) levels (Kimura *et al*, 1975 a,b ; Mittal *et al*, 1975), a property now known to be common to all nitrovasodilators, in certain vascular and non-vascular smooth muscles (Schultz *et al*, 1977 ; Katsuki *et al*, 1977 ; Kukovetz *et al*, 1979). Guanylate cyclase activation by nitrovasodilators was augmented by thiols such as cysteine or glutathione (Ignarro *et al*, 1980a ; Ignarro *et al*, 1980b). These compounds reduced nitrovasodilator stability by promoting NO formation. The NO so released, was unstable and reacted with oxygen to form NO_2. In solution, NO_2 formed NO_2^- and NO_3^-, each of which was relatively inactive. Alternatively, NO could react with thiols to form S-nitrosothiols which, although unstable, had half-lives greater than NO and were potent activators of guanylate cyclase (Ignarro & Gruetter, 1980 ; Ignarro *et al*, 1980a,b ; see Ignarro, 1989). Indeed, SNP, GTN and isoamyl nitrite reacted

with certain thiols to form the corresponding S-nitrosothiols (Ignarro *et al*, 1981). Moreover, the haemodynamic profile of action of a series of S-nitrosothiols was almost identical to that of SNP or GTN (Ignarro *et al*, 1981). The relaxant action of the nitrovasodilators was thus attributed, at least in part, to the formation of S-nitrosothiols (Ignarro *et al*, 1981). This, however, is unlikely to be the case. Under physiological conditions, nitrosothiol formation from nitrite and cysteine was negligible (Yeates *et al*, 1985), although this did not discount the possibility that reaction of the organic nitrate with cysteine resulted in the transient formation of a nitrosothiol intermediate that disintegrated rapidly to release NO and the disulphide, cystine. In addition, the most effective nitrosothiols as activators of soluble guanylate cyclase were the least stable (Feelisch, 1991), suggesting that it was not the intact nitrosothiol, but rather a decomposition product (i.e. NO), that was reponsible for enzyme activation. In fact, soluble guanylate cyclase stimulation by the organic nitrates was dependent on the release of NO (Feelisch & Noack, 1987). This was in keeping with earlier findings that NO, itself, raised soluble guanylate cyclase activity (Katsuki *et al*, 1977 ; Arnold *et al*, 1977 ; Craven & DeRubertis, 1978). Together, this evidence indicates that NO *per se* rather than a nitrosothiol is the effector molecule of the nitrovasodilators, though the mechanisms for this release may not always be the same. For example, SNP, once believed to generate NO spontaneously, is now thought to spontaneously liberate NO only in the presence of light (Bates *et al*, 1991). When kept in the dark, SNP released NO only after the nitroprusside anion had been reduced and cyanide lost (Bates *et al*, 1991). Reduction was achieved by the addition of either vascular tissue or agents such as cysteine, sodium dithionite, haemoglobin (Hb) or partially purified cytochrome P450 with a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system. A schematic representation of the release of NO from the nitroprusside anion is shown in Fig 2. The release of NO from SNP differed from the release

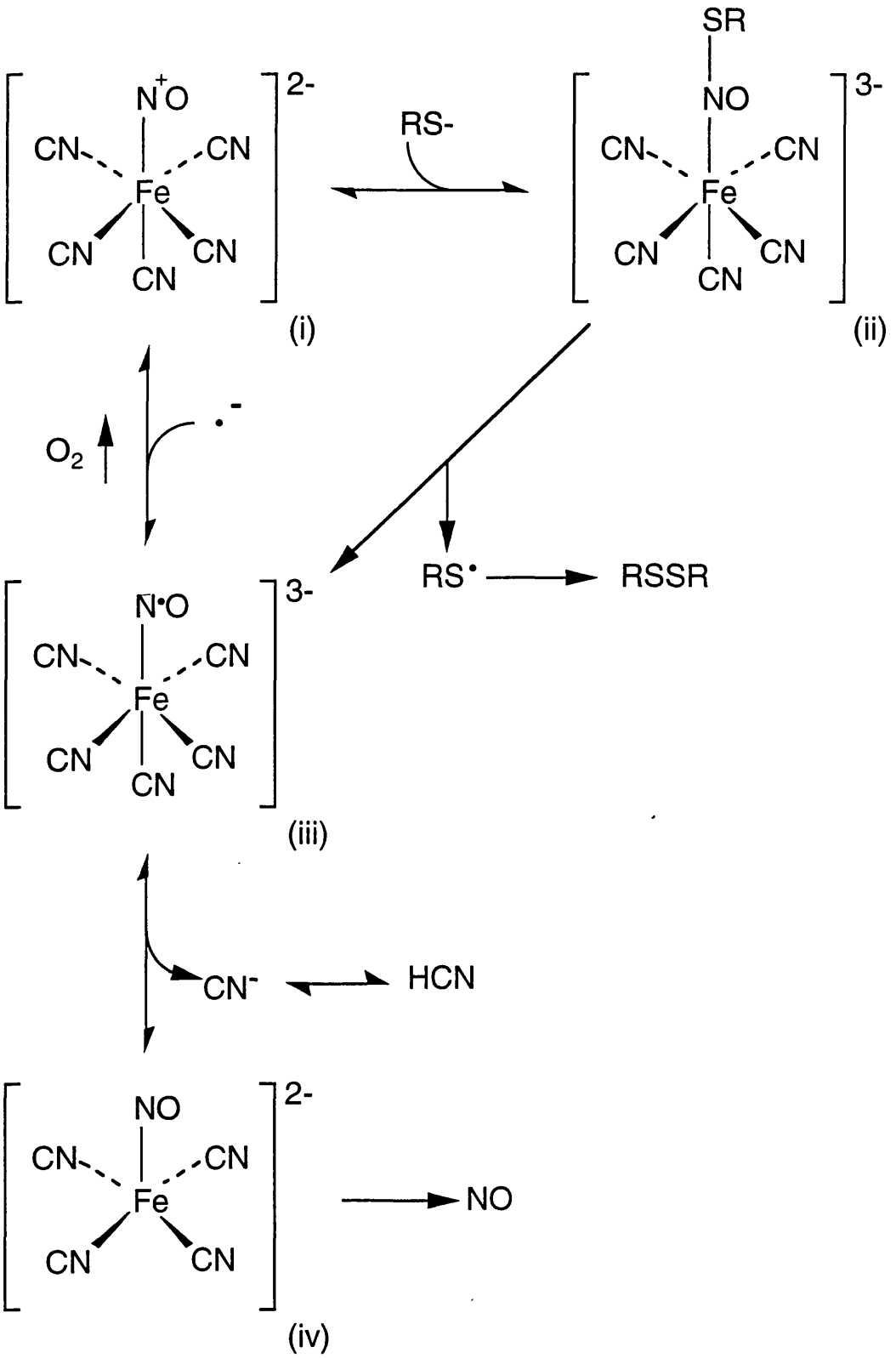


Fig. 2

The release of NO from nitroprusside (adapted from Bates *et al*, 1991). The nitroprusside anion (i) may be reduced directly to (iii) by agents such as haemoproteins or indirectly via (ii) by thiols (R is any organic group). NO is released from product (iv).

of NO from the organic nitrates. In rabbit aorta for instance, inhibitors of NO, such as methylene blue (MeB) and pyocyanin, not only failed to attenuate, but potentiated, SNP-induced relaxations (Gryglewski *et al*, 1992). Relaxations induced by GTN, on the other hand, were attenuated by both MeB and pyocyanin. To explain this, an intracellular site for the biotransformation of SNP was proposed (Gryglewski *et al*, 1992), where MeB and pyocyanin could each activate the transfer of NO from SNP to the active haem centre of the soluble guanylate cyclase. Thus, although SNP is a potent stimulator of soluble guanylate cyclase, it should not be thought of as a donor of NO in the same way as the organic nitrates (Gryglewski *et al*, 1992). Additionally, SNP has properties not mediated via soluble guanylate cyclase ; for example, SNP protected primary cultures of cerebellar granule cells from glutamate- or N-methyl-D-aspartate (NMDA)-induced death (Kiedrowski *et al*, 1991), a property not due to the release of NO, but determined by the ferrocyanide portion of the SNP molecule (Kiedrowski *et al*, 1991).

Organic nitrates, such as GTN or IDN, required bioactivation and failed to stimulate soluble guanylate cyclase in cell-free vascular smooth muscle preparations (Ignarro & Gruetter, 1980 ; Ignarro *et al*, 1981). In broken-cell preparations, organic nitrates reacted with thiols to induce NO formation (Ignarro & Gruetter, 1980 ; Feelisch & Noack, 1987a). All thiols generated NO_2^- from organic nitrates, but only a few (e.g. cysteine, N-acetylcysteine) also liberated NO (Feelisch, 1991). Those thiols that induced the release of NO, acted as coactivators of soluble guanylate cyclase. A schematic representation of the thiol-induced release of NO from the organic nitrates is shown in Fig 3. In intact blood vessels, biotransformation of the organic nitrates was believed to be catalyzed by glutathione S-transferase (Keen *et al*, 1976 ; Brien *et al*, 1988) or by the cytochrome P450 enzyme system (McDonald & Bennett, 1990 ; Schröder, 1992), but this has recently been disputed (Salvemini *et al*, 1993).

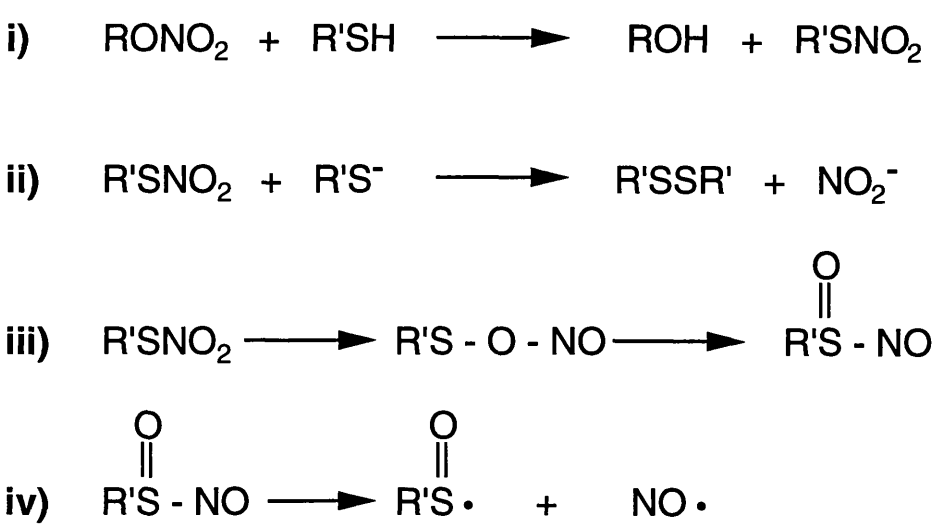


Fig. 3

The thiol-dependent release of NO from organic nitrates (R, R' are any organic groups). The reaction of any thiol with an organic nitrate (i) results in the release of NO_2^- (ii). Only certain thiols e.g. cysteine, N-acetylcysteine, additionally generate NO (iii and iv).

Unlike organic nitrates, organic nitrites did not require **specific** thiols as coactivators. The reaction of organic nitrites with thiols caused the formation of S-nitrosothiols, from which NO was liberated. The reactions leading to the release of NO from the organic nitrites is shown in Fig 4.

The receptor for NO was the active haem centre of soluble guanylate cyclase (Gerzer *et al*, 1988). The resultant increase in cGMP, following NO activation of this enzyme, produced relaxation presumably by reducing cytosolic calcium (Ca^{++}) levels (Collins *et al*, 1986 ; Schini *et al*, 1987 ; Bukoski *et al*, 1989). One, or several, of the following mechanisms could explain this cGMP-evoked relaxation : intracellular Ca^{++} sequestration or binding (Lincoln, 1983) ; enhanced dephosphorylation of myosin light chain (Rapoport *et al*, 1983) ; activation of an ATPase involved in the extrusion of Ca^{++} (Popescu *et al*, 1985) ; inhibition of receptor-operated Ca^{++} channels (Godfraind, 1986) ; membrane hyperpolarization and closure of voltage-dependent Ca^{++} channels (Tare *et al*, 1990).

Following prolonged pretreatment, tolerance developed to the cardiovascular effects of nitrovasodilators (Crandall *et al*, 1931 ; Schelling & Lasagna, 1967 ; Needleman, 1970). In aortae from GTN-pretreated rats, both the relaxant response and the increase in cGMP declined markedly in response to a challenging dose of GTN (Axelsson & Anderson, 1983 ; Waldman *et al*, 1986). Tolerance was attributed to a reduction in the metabolism of GTN (Brien *et al*, 1986). Similar effects occurred in human coronary artery (Waldman *et al*, 1986) possibly due to desensitization of soluble guanylate cyclase to the action of NO. Cardiovascular tolerance to the actions of these drugs could limit their efficacy in the long-term control of conditions such as angina pectoris or congestive heart failure (Abrams, 1986 ; Horowitz & Henry, 1987 ; Packer *et al*, 1987). Interestingly, clinical investigations revealed that in the presence of the sulphydryl donors N-acetylcysteine and methionine, the short-term efficacy of GTN was increased and the GTN-induced tolerance

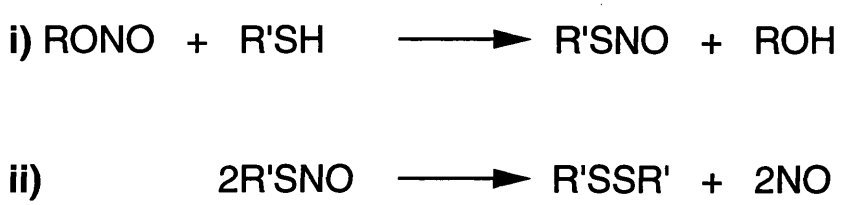


Fig. 4

The thiol-dependent release of NO from organic nitrites (R, R' are any organic groups). All thiols can generate NO (ii) from a reaction with an organic nitrite (i). The degree of NO liberation is dependent on the rate of formation and decomposition of the nitrosothiol intermediate.

reversed (Horowitz *et al*, 1983 ; Packer *et al*, 1987 ; Levy *et al*, 1988), probably through the formation of S-nitroso-N-acetylcysteine (SNAC ; Loscalzo, 1985 ; Fung *et al*, 1988). S-nitrosothiols may, therefore, prove to have a therapeutic use in attenuating organic nitrate-induced tolerance. Experiments using SNAC in bovine coronary artery supported this idea (Henry *et al*, 1989), but prolonged exposure to this compound led to a decrease in vascular responsiveness. Thus, there was likely to be a further site of tolerance in the bioconversion of organic nitrates to NO (Henry *et al*, 1989).

During the last decade, it was shown that, in addition to having an exogenous source, NO was synthesized endogenously in the vascular endothelium, the peripheral nervous system, the central nervous system (CNS) and the immune system (see Moncada *et al*, 1991b). This NO helped mediate a number of biological processes, including relaxation of vascular (see Ignarro, 1989 ; Marín & Sánchez-Ferrer, 1990 ; Feng & Hedner, 1990 ; Moncada, 1990 ; Moncada *et al*, 1991b) and non-vascular smooth muscle (see Gillespie, 1990 ; Rand, 1992 ; Sanders & Ward, 1992 ; Stark & Szurszewski, 1992), inhibition of platelet aggregation (see Ignarro, 1989 ; Moncada *et al*, 1991b), intercellular communication in the central nervous system (see Garthwaite, 1990 ; Moncada *et al*, 1991b), and macrophage cytotoxicity (see Hibbs *et al*, 1990 ; Moncada *et al*, 1991b). To discuss the role of NO in each of these processes would be beyond the scope of this thesis. The role of endogenous NO in the vasculature - where it was first discovered - and as a non-adrenergic, non-cholinergic (NANC) neurotransmitter in the gastrointestinal tract, each of which is relevant to the aims of this thesis, will be discussed in detail.

1.2. Endothelium-derived Relaxing Factor (EDRF)

The well-documented, apparently paradoxical vascular effects of acetylcholine (ACh), *in vitro* and *in vivo*, remained unresolved for many years ; both vasodilator (Dale, 1914) and vasoconstrictor (Hunt, 1915) actions of the transmitter were observed. For example, blood vessels of the isolated perfused rabbit ear dilated to ACh, but this gave way to vasoconstriction over a period of hours (Burn & Robinson, 1951). Similarly, helical strips of rabbit descending thoracic aorta constricted to ACh concentrations greater than 0.1 μM, an effect mediated via muscarinic receptors (Furchgott & Bhadrakom, 1953), but relaxed to lower concentrations of the substance (Jelliffe, 1962). These apparent discrepancies were resolved when it became clear that ACh-induced vascular relaxation required the presence of endothelial cells (Furchgott & Zawadski, 1980). ACh, it is now known, was acting on endothelial cell muscarinic receptors to release an endothelium-derived relaxing factor (EDRF). Results suggesting that ACh was a vasoconstrictor probably arose from unintentional rubbing of the intimal surface and removal of endothelial cells or from a gradual loss of endothelial cells.

The release of EDRF, now demonstrated in both arterial and venous preparations, underlay the relaxant effects of ACh, bradykinin, substance P, adenosine diphosphate (ADP), adenosine triphosphate (ATP) and the calcium ionophore A23187 (see Moncada *et al*, 1991b). In addition to relaxing vascular smooth muscle, EDRF inhibited platelet aggregation and adhesion to vascular endothelium (Azuma *et al*, 1986 ; Radomski *et al*, 1987a,b,c,d). In contrast, other vasorelaxants such as the nitrovasodilators, β-adrenergic agonists and prostacyclin acted independently of the endothelium. EDRF was labile (Furchgott & Zawadski, 1980 ; Griffith *et al*, 1984), with a half-life of only seconds in physiological salt solutions (Griffith *et al*, 1984 ; Cocks *et al*, 1985), and was released at rest or following stimulation (Griffith *et al*, 1984 ; Martin

et al, 1985 ; Rubanyi *et al*, 1985). The half-life of EDRF was species-dependent ; canine EDRF had a longer half-life than that of rabbit (Förstermann e*t al*, 1984).

The chemical nature of the EDRF was not initially obvious. Early evidence suggested that it was a short-lived hydroperoxide, or free radical, formed following oxidation of arachidonic acid by a lipoxygenase pathway (Singer & Peach, 1983 ; Förstermann & Neufang, 1984 ; see Furchgott, 1984), that stimulated vascular smooth muscle soluble guanylate cyclase to elevate cGMP levels and produce relaxation (see Furchgott, 1984). In contrast, endothelium-dependent relaxation was reportedly resistant to lipoxygenase inhibition unless the lipoxygenase inhibitor was also an antioxidant (Griffith *et al*, 1984). It was thus proposed that EDRF, from aortic preparations, was not a lipoxygenase derivative or free radical, but an unstable compound with a carbonyl group at or near its active site (Griffith *et al*, 1984). Neither of these proposals proved to be correct.

Endothelium-dependent relaxation was associated with increased cGMP levels in vascular smooth muscle (Rapoport & Murad, 1983). Both endothelium-dependent vasodilators and nitrovasodilators induced relaxation through identical cGMP-dependent protein phosphorylation and dephosphorylation of myosin light chain (Rapoport *et al*, 1983). MeB and Hb each inhibited both endothelium-dependent and GTN-induced relaxation of rabbit aorta (Martin *et al*, 1985). These compounds bound directly to and thus inactivated the EDRF, although MeB may also have oxidized the active haem centre of soluble guanylate cyclase (Martin *et al*, 1985). The inhibitory actions of MeB and Hb on nitrovasodilator-induced effects were attributed to their NO binding and scavenging effects (Hermann, 1865 ; Gibson & Roughton, 1957 ; Martin *et al*, 1986 ; Marshall *et al*, 1988). In addition, superoxide anions destabilized the EDRF ; an effect that was inhibited by superoxide dismutase (SOD ; Gryglewski *et al*, 1986 ; Rubanyi & Vanhoutte, 1986). Dithiothreitol,

hydroquinone and pyrogallol which formed superoxide anions (Moncada *et al*, 1986), inhibited the EDRF. In 1986, based on these pharmacological similarities between EDRF and NO, two groups suggested independently that the EDRF could be NO (see Furchgott, 1988 ; Ignarro *et al*, 1988). In 1987, proof was obtained that this was indeed the case.

1.2.1. NO in the Vasculature

Measurement of NO as the chemiluminescent product of its reaction with ozone (Palmer *et al*, 1987), provided the first direct evidence that the EDRF was NO. Bradykinin was used to show a concentration-dependent release of NO from cultured endothelial cells in amounts sufficient to account for the biological activity of EDRF (Palmer *et al*, 1987). Bioassay revealed that EDRF-induced relaxations and those induced by NO were indistinguishable ; both were inhibited by Hb and enhanced by SOD to similar degrees (Palmer *et al*, 1987 ; Hutchinson *et al*, 1987). The sensitivity of a number of vascular and non-vascular smooth muscles, including rabbit aorta, rat anococcygeus, guinea pig trachea and guinea pig taenia caeci, to EDRF and NO, were also the same (Gillespie & Sheng, 1988 ; Cocks & Angus, 1990), suggesting that EDRF was NO.

NO has now been identified as the EDRF in a large number of cardiovascular preparations which include porcine aorta (Palmer *et al*, 1987 ; Hutchinson *et al*, 1987 ; Radomski *et al*, 1987a), rabbit aorta (Gillespie & Sheng, 1988), guinea pig uterine artery (Tare *et al*, 1990) and rabbit hepatic arterial vascular bed (Mathie *et al*, 1991). In spite of this, there are some blood vessels where NO may not be the EDRF. For instance, in rat small mesenteric artery, ACh-induced relaxation was endothelium-dependent but was not mediated by NO (Garland & McPherson, 1992). Further, there could be more than one EDRF. Indeed, two EDRF's were allegedly released from porcine

aortic endothelial cells (Boulanger *et al*, 1989) ; one under basal conditions and following stimulation with ADP, the other, which was most probably NO, following stimulation with bradykinin or the calcium ionophore A23187.

It has also been suggested that the EDRF may not be NO itself, but a NO-related compound, since NO is a highly labile gas with a half-life of only a few seconds (Griffith *et al*, 1984 ; Förstermann *et al*, 1984 ; Rubanyi *et al*, 1985 ; Cocks *et al*, 1985), making storage of the preformed molecule difficult. In fact, in cultured bovine aortic endothelial cells, the amount of NO released under basal conditions or in response to either bradykinin or the calcium ionophore A23187 was insufficient to account for the vasorelaxant properties of the EDRF (Myers *et al*, 1990). Similarly, in rat aortic rings, dinitrosyl iron complexes with cysteine, induced relaxations that were more similar to ACh-induced than to NO-induced relaxations (Vedernikov *et al*, 1992). Findings such as these led to speculation that the EDRF could be a hydroxylamine (Thomas & Ramwell, 1989), S-nitrosocysteine (Myers *et al*, 1990) or a dinitrosyl iron complex with cysteine (Mülsch *et al*, 1991 ; Vedernikov *et al*, 1992). The EDRF may well be stored and stabilized in one of these forms, but the majority of the evidence to date implies that the biological effects of EDRF, like those of the nitrovasodilators, are mediated ultimately by the release of NO.

1.2.2. NO Synthesis and Inhibition

Endothelium-derived NO was synthesized from the terminal guanidino nitrogen atoms of L-arginine (Palmer *et al*, 1988a), possibly via a hydroxylamine intermediate (DeMaster *et al*, 1989). This reaction was catalyzed by NO synthase and L-citrulline was formed as a by-product (Fig. 5 ; Palmer & Moncada, 1989). Two similar NO synthases were recognised ; one constitutive the other inducible. Both used L-arginine as their substrate to form

NO and L-citrulline, required NADPH as an electron donor (Mayer *et al*, 1989 ; Mülsch *et al*, 1989 ; Lopez-Jaramillo *et al*, 1990 ; Busse & Mülsch, 1990b) and contained one molecule each of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN ; Bredt *et al*, 1991 ; Stuehr *et al*, 1991). The constitutive NO synthase required Ca^{++} and calmodulin (Busse & Mülsch, 1990a ; Schini & Vanhoutte, 1992) and has been found in, amongst other cell types, the vascular endothelium (Mayer *et al*, 1989 ; Mülsch *et al*, 1989 ; Palmer & Moncada, 1989), the blood platelet (Radomski *et al*, 1990a), the peripheral nervous system (Gillespie *et al*, 1989 ; Li & Rand, 1989) and the CNS (Bredt & Snyder, 1990). In the endothelium, although originally believed to be cytosolic (Palmer & Moncada, 1989), the constitutive enzyme was membrane bound (Boje & Fung, 1990 ; Förstermann *et al*, 1991). In the CNS, this enzyme was cytosolic (Schmidt *et al*, 1989).

The inducible NO synthase was cytosolic (Marletta *et al*, 1988) and required tetrahydrobiopterin as a cofactor (Busse & Mülsch, 1990b). Cytokines such as interferon-γ, tumour necrosis factor, interleukins 1 and 6 or bacterial endotoxin induced its expression (Drapier *et al*, 1988 ; Beasley *et al*, 1991 ; Nussler *et al*, 1991 ; Schini *et al*, 1991) and it synthesized NO for long periods. Other substances such as transforming growth factor β, interleukin-8, platelet-derived growth factor or glucocorticoids (Ding *et al*, 1990 ; Knowles *et al*, 1990 ; Radomski *et al*, 1990b ; Rees *et al*, 1990 ; M^{c}Call *et al*, 1992 ; Schini *et al*, 1992) inhibited the expression of the inducible enzyme. This enzyme was found in macrophages (Hibbs *et al*, 1988 ; Stuehr *et al*, 1989), neutrophils (M^{c}Call *et al*, 1991), vascular endothelial cells (Radomski *et al*, 1990 ; Rees *et al*, 1990) and vascular smooth muscle cells (Busse & Mülsch, 1990b ; Fleming *et al*, 1991 ; Mollace *et al*, 1991).

The activity of both enzymes was impaired *in vitro* by structural analogues of the NO precursor L-arginine (Palmer & Moncada, 1989 ; Busse & Mülsch, 1990b ; Fleming *et al*, 1991). These compounds included N^{G}-monomethyl-L-

arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME ; Fig. 6), but not their D-isomers.

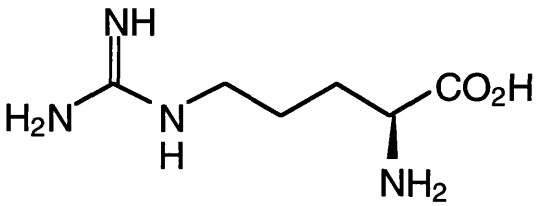
The use of L-NMMA revealed that the basal release of NO, synthesized from L-arginine, was important in the regulation of blood flow and pressure. L-NMMA constricted rings of rabbit aorta when the endothelium was present (Palmer *et al*, 1988b), indicating that NO was continuously released in this tissue. Moreover, intravenous administration of L-NMMA, but not D-NMMA, increased blood pressure in anaesthetized rabbits (Rees *et al*, 1989), demonstrating that basally released NO helped maintain blood pressure at safe levels. This increase in pressure was reversed by L-, but not D-,arginine.

Venous tissues had a lower basal release of endothelium-derived NO (Seidel & LaRochelle, 1987), but an increased sensitivity to nitrovasodilators, when compared with arterial tissues. It was unclear whether this increased sensitivity was due to a greater concentration of soluble guanylate cyclase in venous smooth muscle compared with arterial smooth muscle, or whether the venous soluble guanylate cyclase was more sensitive to stimulation by NO (Moncada *et al*, 1991b).

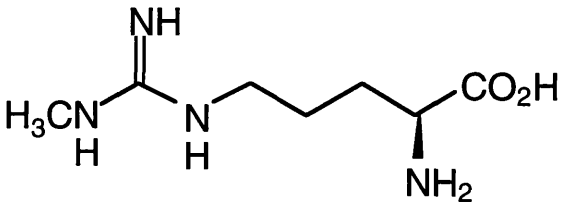
1.2.3. NO-induced Hyperpolarization?

The vasorelaxant properties of NO have been known for some years, but there has recently been some debate on the ability, if any, of NO to induce hyperpolarization (Komori *et al*, 1988 ; Tare *et al*, 1990 ; Rand & Garland, 1992 ; Suzuki *et al*, 1992). ACh hyperpolarized and relaxed a number of vascular smooth muscles including rabbit saphenous artery (Komori & Suzuki, 1987), canine mesenteric artery (Komori *et al*, 1988), rat aorta (Taylor *et al*, 1988 ; Chen *et al*, 1988), rat main pulmonary artery (Chen *et al*, 1988), canine coronary artery (Chen *et al*, 1989), guinea pig uterine artery (Tare *et al*, 1990), rabbit cerebral artery (Brayden, 1990) and rabbit basilar artery (Rand &

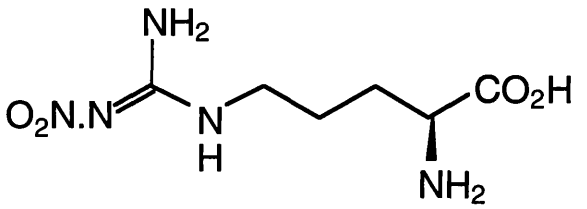
L-arginine



L-NMMA



L-NNA



L-NAME

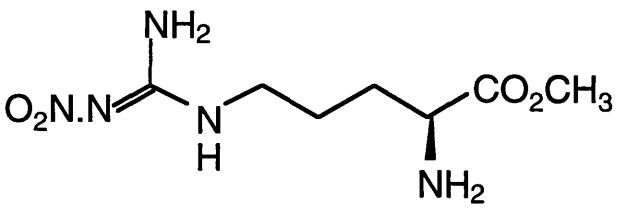


Fig. 6

The structures of the precursor of NO synthesis, L-arginine, and of some analogues which inhibit NO synthesis.

Garland, 1992). These hyperpolarizations required an intact endothelium and the current was probably carried by potassium (K^{+}) ions. This membrane hyperpolarization may have contributed to the ACh-induced dilator response through the closure of voltage-dependent Ca^{++} channels (Taylor *et al*, 1988 ; Tare *et al*, 1990 ; Brayden, 1990), However, the hyperpolarization may not have been due to the actions of EDRF (Taylor *et al*, 1988 ; Huang *et al*, 1988 ; Komori *et al*, 1988 ; Chen *et al*, 1988 ; Chen *et al*, 1989 ; Brayden, 1990 ; Suzuki *et al*, 1992). There was evidence that endothelium dependent vasodilators caused the concomitant release of an endothelium-dependent hyperpolarizing factor (EDHF) that was distinct from the EDRF. For instance, endothelium-dependent relaxation, but not hyperpolarization, of vascular smooth muscle cells was inhibited by the inhibitors of NO, Hb and MeB (Huang *et al*, 1988 ; Chen *et al*, 1988). Exogenous NO did not hyperpolarize either canine mesenteric artery (Komori *et al*, 1988) or rabbit cerebral artery (Brayden, 1990). Moreover, SNP, believed to generate NO in vascular smooth muscle, relaxed rat aorta without increasing radiolabelled rubidium (^{86}Rb ; a marker for K^{+}) efflux (Taylor *et al*, 1988). Thus, in these arteries, NO would appear to be unable to hyperpolarize the smooth muscle.

But,the donors of NO, SNP or GTN, hyperpolarized the rabbit pulmonary artery (Ito *et al*, 1978), rat tail artery (Cheung & MacKay, 1985) and guinea pig uterine artery (Tare *et al*, 1990). ACh-induced hyperpolarization and relaxation of guinea pig uterine artery (Tare *et al*, 1990) and rabbit basilar artery (Rand & Garland, 1992) were reduced by the inhibitors of NO synthesis, L-NMMA and L-NAME, and authentic NO hyperpolarized and relaxed guinea pig uterine, mesenteric and coronary arteries, rabbit saphenous artery and rat tail artery among others (Tare *et al*, 1990). Thus, in some smooth muscles, NO clearly produced hyperpolarization. This increases the probability that in some arteries at least, endothelium dependent hyperpolarization was mediated via the release of NO. In others, it was probably mediated via a separate EDHF, the

release of which required a rise in endothelial cell Ca^{++} levels, that produced an increase in K^+ permeability (Suzuki *et al*, 1992). Why there should be this distinction is unclear ; there are no obvious differences between those arteries in which NO has been reported to cause hyperpolarization, and those in which it was ineffective. At present the identity of the putative EDHF is unknown.

1.3. NO in the Peripheral Autonomic Nervous System

The quest for NO in the peripheral autonomic nervous system stemmed from endeavours to identify the neurotransmitter from non-adrenergic, non-cholinergic (NANC) nerves. The identification of the EDRF as NO, coupled with the unsuitability of several candidates as transmitter substances, led to speculation that NO could be a NANC neurotransmitter.

Classically, sympathetic nerves were considered to release only noradrenaline and parasympathetic nerves were considered to release only ACh. Yet, from an early time, evidence existed that this was an oversimplification. For example, both motor and inhibitory responses were obtained from the stomach following electrical stimulation of the vagus nerve (Langley, 1898) and electrical stimulation of the sacral parasympathetic outflow via the pelvic nerves, led to penile erection that was unaffected by atropine (Langley & Anderson, 1895). Furthermore, atropine only partially blocked motor responses of mammalian urinary bladder following parasympathetic nerve stimulation (Langley & Anderson, 1895 ; Henderson & Roepke, 1934 ; Ambache, 1955). Only Henderson and Roepke, however, concluded that the nerves were releasing a transmitter other than ACh.

It was only after the introduction of selective adrenergic neurone blocking drugs that it became obvious that some smooth muscles were innervated by nerves that were neither adrenergic nor cholinergic. Non-adrenergic inhibitory nerves were first reported in the guinea pig taenia coli (Burnstock *et al*,

1964). Then, following vagus nerve stimulation, relaxations of the cat stomach were shown to be resistant to both atropine and guanethidine (Martinson, 1965). Since this time, NANC responses have been described in a wide range of smooth muscles including both the small and large intestines, the retractor penis muscle, the anococcygeus muscle and the lower oesophageal sphincter muscle (see Rand, 1992 ; Sanders & Ward, 1992). NANC nerves are now known to be prevalent throughout the entire length of the gastrointestinal tract.

NANC nerves do not belong to a single population with only a single neurotransmitter. A number of putative transmitters, including ATP (Burnstock *et al*, 1970 ; Burnstock *et al*, 1972 ; see Burnstock, 1981), 5-hydroxytryptamine (see Gershon, 1979) and several peptides, vasoactive intestinal peptide (VIP), substance P and neuropeptide Y among them (Cuello, 1982 ; Lundberg & Hökfelt, 1983 ; Bartfai *et al*, 1988) were implicated in NANC neurotransmission or in cotransmission along with "classical" transmitters in the gut. Despite this, the NANC transmitter to smooth muscles such as the bovine retractor penis muscle (BRP) or rat anococcygeus remained a mystery. Then, a few years ago, using dilute hydrochloric acid, a novel inhibitory factor was isolated from the BRP (Ambache *et al*, 1975) and rat anococcygeus muscles (Gillespie & Martin, 1978). The study of this smooth muscle inhibitory factor recently provided the first evidence that NO was released from NANC nerves.

1.3.1. Smooth Muscle Inhibitory Factor

The inhibitory factor (IF) from BRP was neither adenosine nor a purine derivative (Bowman *et al*, 1979), nor a peptide (Gillespie *et al*, 1981). It was labile and, following neutralization, mimicked the response to NANC nerve stimulation. It was only activated after a brief exposure to an acidic pH

(Gillespie & Martin, 1978), was soluble in water or methanol, but insoluble in ether, acetone or ethanol, had a molecular weight of approximately 500 daltons and was retained on an anion exchange resin (Gillespie *et al*, 1981). It relaxed isolated blood vessels, yet when injected intravenously in the anaesthetized rat, did not lower blood pressure (Bowman *et al*, 1981). This lack of effect was due to the presence of Hb (Bowman *et al*, 1982). In BRP and rat anococcygeus, Hb blocked the relaxations and increases in tissue cGMP content produced by inhibitory nerve stimulation, SNP and the inhibitory factor (Bowman & Gillespie, 1982 ; Bowman & Drummond, 1984).

With hindsight, it was evident that the IF had a number of similarities to NO, but it was another three years, following the discovery that the EDRF was NO (Palmer *et al*, 1987), before the IF was suggested to be nitrite which released NO upon acidification (Furchgott, 1988b ; Martin *et al*, 1988). This idea fitted well with many of the reported observations. For instance, the relaxant activity of nitrite solutions was known to be greater following acidification (see Gillespie, 1990) ; both nitrite and the IF raised cGMP levels ; Hb inhibited the effects of both substances. But, the ability of the IF to bind to an anion exchange resin and its molecular weight of approximately 500 daltons (Gillespie *et al*, 1981) seemed incompatible with NO being the effector molecule.

Further evidence however, suggested that NO or a NO-releasing compound was the likely NANC inhibitory neurotransmitter in the BRP and rat anococcygeus muscles. Thus, using a cascade apparatus the BRP, rat anococcygeus, rabbit aorta and guinea pig trachea were shown to have an identical ranking order of sensitivity to EDRF, the inhibitory factor and NO (Gillespie & Sheng, 1988), suggesting that NO was the active principle of all three. However, although Hb prevented relaxations to each, a red blood corpuscle suspension containing an equivalent concentration of Hb abolished the responses only to EDRF and NO (Gillespie & Sheng, 1988). Thus, if NO

was responsible for the relaxant properties of the acid-activated inhibitory factor, it probably existed in a form which rendered it unable to pass through the red blood cell membrane. L-NMMA raised tone and blocked NANC nerve responses in the rat anococcygeus - effects reversed by L-arginine - but was without effect in the BRP (Gillespie *et al*, 1989). D-NMMA had no effect on either tissue. The superoxide generator, pyrogallol, also inhibited NANC nerve responses in the rat anococcygeus but not in the BRP (Gillespie & Sheng, 1990). This antagonism was reversed by SOD. The more potent analogue of L-arginine, L-NNA, was not only more effective than L-NMMA in rat anococcygeus but also inhibited NANC nerve responses in the BRP, indicating that NO or a NO-releasing compound was released in each case. Moreover, NO synthase was found in tissue homogenates from rat anococcygeus and BRP (Mitchell *et al*, 1991 ; Sheng *et al*, 1992). Thus, the evidence indicated that in both BRP and rat anococcygeus the IF was NO or a related compound. But, since relaxations of the BRP were unaffected by L-NMMA or pyrogallol, it is possible that there were some differences in synthesis and inactivation of NO in the two tissues (see Gillespie, 1990).

It is perhaps unlikely that NO was transmitted as a gas. More probably it was transmitted bound to a carrier molecule to form a more stable compound, in the fashion of the nitrovasodilators. This would then be broken down in the target cell to release NO. This idea fits better with the "classical" view of neurotransmission (Eccles, 1964), and would allow the storage of preformed transmitter in vesicles, a concept difficult to conceive of with NO itself because of its high membrane permeability (see Gillespie, 1990). Indeed, the NANC neurotransmitter in the BRP has recently been proposed to be a superoxide anion-resistant NO-releasing molecule (Liu, 1993).

NO synthase is concentrated in cell bodies and nerve fibres in the myenteric plexus of the intestine (Bredt *et al*, 1990). NO or a NO-related compound has now been implicated in NANC neurotransmission, or in

cotransmission along with such substances as ATP or VIP, in a wide range of smooth muscles from the gastrointestinal tract. NANC nerves which release NO have been termed 'nitrergic' (Rand & Li, 1990). The evidence for 'nitrergic' transmission or cotransmission in a variety of gastrointestinal smooth muscles is discussed in the following pages.

1.3.2. The Lower Oesophageal Sphincter (LOS)

Inhibitory NANC nerves mediated the LOS relaxation that occurred during swallowing (Goyal & Rattan, 1976). In spite of earlier support for VIP as the transmitter (Goyal *et al*, 1980 ; Biancani *et al*, 1984), NO has emerged as the more likely candidate.

Field-stimulation of LOS muscle strips evoked frequency-dependent tetrodotoxin (TTX)-sensitive NANC relaxations (De Man *et al*, 1991). Authentic NO produced a concentration-dependent relaxation (De Man *et al*, 1991 ; Knudsen *et al*, 1992). NO-induced responses were resistant to TTX. Hb inhibited the responses to electrical stimulation in both canine and opossum LOS (De Man *et al*, 1991 ; Knudsen *et al*, 1992). The inhibitor of NO biosynthesis, L-NNA, also inhibited LOS relaxation - an effect that was reversed by L-arginine (De Man *et al*, 1991 ; Tøttrup *et al*, 1991), but not by D-arginine. Moreover, the inhibitors of guanylate cyclase, cystamine and MeB, blocked the hyperpolarization evoked by nerve stimulation or by SNP in opossum LOS (Conklin & Du, 1992). In opossum sphincter the effects of NO, S-nitroso-L-cysteine or SNP did not exactly match the response to NANC nerve stimulation (Knudsen *et al*, 1992), but the action profile of SNP bore the closest resemblance. This suggests that, in this particular tissue, the NANC neurotransmitter may be a carrier molecule that, like SNP, generates NO intracellularly.

1.3.3. The Gastric Fundus (Forestomach)

Increased intragastric pressure resulted in dilation of the gastric fundus in a process that was mediated via inhibitory NANC nerves (Abrahamsson & Jansson, 1969). This "adaptive relaxation" (Abrahamsson,1973) accommodated the consumption of liquid or food. VIP and NO are the most likely mediators of this response (Li & Rand, 1990 ; D'Amato *et al*, 1992 ; Grider *et al*, 1992 ; Barbier & Lefebvre, 1993).

Electrical stimulation elicited frequency-dependent NANC relaxations of circular and longitudinal muscle strips of gastric fundus from rat (De Beurme & Lefebvre, 1988 ; Li & Rand, 1990 ; D'Amato *et al*, 1992 ; Shimamura *et al*, 1993), guinea pig (Grider *et al*, 1985 ; Grider *et al*,1992) and cat (D'Amato *et al*, 1988 ; Barbier & Lefebvre, 1993). These were abolished by TTX (Li & Rand, 1990). VIP was known to be involved in these inhibitory responses (Grider *et al*, 1985 ; De Beurme & Lefebvre, 1988 ; D'Amato *et al*, 1988 ; Li & Rand, 1990 ; D'Amato *et al*,1992 ; Barbier & Lefebvre, 1993) but, as they were only partially blocked by VIP-antiserum or by the peptidase, trypsin (De Beurme & Lefebvre, 1988 ; D'Amato *et al*, 1988 ; Li & Rand, 1990 ; D'Amato *et al*, 1992 ; Barbier & Lefebre, 1993), another transmitter was likely to contribute.

L-NMMA, L-NNA and L-NAME, but not their D-isomers, inhibited nerve-mediated relaxations of gastric fundus (Li & Rand, 1990 ; D'Amato *et al*, 1992 ; Shimamura *et al*, 1993 ; Barbier & Lefebvre, 1993) in a concentration dependent manner. These inhibitions were reversed by L-arginine, but not by D-arginine. Similarly, in isolated stomach of the guinea pig, "adaptive relaxation" was inhibited by L-NMMA and by MeB (Desai *et al*, 1991). Again the effect of L-NMMA was partially reversed by L-arginine.

NANC nerve stimulation in the circular smooth muscle of rat gastric fundus also induced inhibitory junction potentials (i.j.p.'s) which were depressed by L-NNA (Shimamura *et al*, 1993 ; Kitamura *et al*, 1993). Moreover, hyperpolarizations occurred in response to S-nitrosocysteine, SNP or 8-bromo cGMP (Kitamura *et al*, 1993). Hyperpolarizations following field stimulation or S-nitrosocysteine were partly due to activation of apamin-sensitive but tetraethylammonium-resistant K^+ channels. However, only that part of the response which was resistant to apamin was mediated through an increase in cGMP. The apamin-sensitive part of the response was probably mediated via VIP.

However, a recent study in guinea pig gastric fundus (Grider *et al*, 1992) reported that VIP was the primary NANC transmitter and that this stimulated the production of NO from the target muscle cells. This NO, in turn, amplified the inhibitory response to VIP and presynaptically enhanced the release of VIP (Grider *et al*, 1992).

Thus, in guinea pig gastric fundus, NO and VIP were not simply released as cotransmitters. The mechanisms of release were more complex. This may yet prove to be the case in gastric fundi from other species

1.3.4. The Small Intestine

The release of NO has been demonstrated from NANC nerves innervating the duodenum, jejenum and ileum from a variety of species including dog, rabbit, rat, guinea pig and human (Toda *et al*, 1990 ; Boeckxstaens *et al*, 1990, 1991a ; Maggi *et al*, 1991 ; Kanada *et al*, 1992 ; He & Goyal, 1993). For example :

Longitudinal strips of rabbit duodenum relaxed in response to field stimulation of NANC nerves. The relaxations were blocked by L-, but not

D-NNA and this inhibition was reversed by L-arginine (Toda *et al*, 1990). NANC relaxations were mimicked by NO and GTN, and blocked by Hb.

In circular muscle of the canine jejenum, electrical stimulation evoked NANC i.j.p.'s which resembled the hyperpolarization induced by authentic NO (Stark *et al*, 1991). L-NMMA depressed the i.j.p. amplitude. This depression was reversed by L- but not by D-arginine. Haemoglobin (Hb) reduced the i.j.p. amplitude and abolished the effects of authentic NO.

NANC relaxations in circular muscle from human ileum were reduced by L-NMMA and L-NNA (Maggi *et al*, 1991). This effect was reversed by L-arginine, thereby implicating a role for NO.

NANC nerve stimulation relaxed circular muscle strips of the canine terminal ileum, as did authentic NO. L-NMMA, L-NNA and Hb inhibited the NANC relaxation to electrical stimulation in a concentration-dependent manner. Only Hb inhibited the response to exogenous NO. L-, but not D-arginine, overcame the inhibitory effects of L-NMMA and L-NNA (Boeckxstaens *et al*, 1990 ; 1991a).

Distension of rat ileum with a small balloon, induced dilation of the circular muscle on the anal side of the distended region. L-NNA or MeB inhibited, while SOD enhanced, the amplitude of this relaxation (Kanada *et al*, 1992). L-NNA inhibition was reversed by L-arginine. NO and 8- bromo cGMP relaxed the circular muscle.

Transmural stimulation of longitudinal muscle of the rat ileum produced a relaxation and rebound contraction. The relaxation was accompanied by an increased cGMP content (Kanada *et al*, 1992). Authentic NO also increased the cGMP content in circular and longitudinal muscle of rat ileum - an effect that was prevented by preincubation with MeB.

Electrical field stimulation of guinea pig ileum circular muscle produced a fast, ATP-mediated i.j.p.(He & Goyal, 1993). But, stimulation in the presence of apamin and substance P desensitization produced a slow i.j.p.. The

amplitude of this slow response was reduced by L-NNA, and this inhibition was reversed by L-, but not by D-arginine (He & Goyal, 1993). The responses to exogenous ATP or NO were unaffected by substance P desensitization, but the hyperpolarization in response to VIP was enhanced. Moreover, the VIP-induced hyperpolarization was antagonized by L-NNA. These results indicated that NO and VIP together mediated the slow i.j.p.. The slow i.j.p. was normally masked by a depolarization due to the VIP-induced concomitant release of substance P (He & Goyal, 1993). Thus, NO clearly functions as a NANC neurotransmitter throughout the entire length of the small intestine.

1.3.5. The Ileocolonic Junction (ICJ)

By using specific inhibitors of the actions of NO, the ileocolonic junction was the first gastrointestinal tissue from which the release of NO was demonstrated.

Electrical stimulation produced NANC relaxation and hyperpolarization of the canine ICJ (Bult *et al*, 1990 ; Boeckxstaens *et al*, 1991a,b ; Ward *et al*, 1992a). Both the relaxation and hyperpolarization were mimicked by authentic NO and antagonized by Hb. The inhibitors of NO synthase, L-NMMA and L-NNA, but not D-NMMA, inhibited the relaxations in response to nerve stimulation in a concentration-dependent manner (Bult *et al*, 1990 ; Boeckxstaens *et al*, 1991a,b). This inhibition was antagonized by the precursor for NO synthesis, L-arginine, but not by D-arginine, thus implicating NO in the NANC nerve responses. In support of this, the relaxations were potentiated by SOD and mimicked by GTN (Bult *et al*, 1990 ; Boeckxstaens *et al*, 1991b). M&B 22948, a specific cGMP phosphodiesterase inhibitor, hyperpolarized canine ICJ (Ward *et al*, 1992a) suggesting that, in this tissue, NO was also tonically released. This tonic release may have helped regulate resting membrane potential or tone in the ICJ (Ward *et al*, 1992a).

1.3.6. The Colon

In the canine proximal colon, i.j.p.'s induced by electrical stimulation of NANC nerves, may have been mediated by Ca^{++}-activated K^+ channels (Thornbury *et al*, 1991). These i.j.p.'s were reduced by L-NAME (Dalziel *et al*, 1991) and restored by L-, but not by D-arginine. Hb blocked not only neuronal responses, but also the similar hyperpolarizations that occurred in response to authentic NO or S-nitrosocysteine (Thornbury *et al*, 1991). Since S-nitrosocysteine was degraded at a sufficient rate to cause i.j.p.-like hyperpolarizations, these authors suggested that NO could be stored as a nitrosothiol in nerve terminals.

In the rat, colonic smooth muscle was tonically suppressed by NO or a related substance. L-NNA enhanced the spontaneous contractile activity of circular muscle of rat distal colon (Niklasson *et al*, 1992). Moreover, TTX increased the force of low frequency contractions before, but not after, incubation of the tissue with L-NMMA (Middleton *et al*, 1993). L-NMMA, but not D-NMMA, also increased the force of low frequency contractions. This increase was counteracted by L-arginine, implicating the tonic release of NO.

1.3.7. The Internal Anal Sphincter (IAS)

NANC nerve stimulation induced the IAS to relax (Lim & Muir, 1983). VIP has been suggested as a leading candidate for the role of NANC neurotransmitter in this tissue (Biancani *et al*, 1985 ; Nurko & Rattan, 1988) but, as in gastric fundus, NO is also likely to be involved.

For example, authentic NO relaxed IAS smooth muscle strips from opossums. In addition, L-NNA inhibited NANC relaxations in opossum and in human IAS, and these inhibitions were prevented by L-arginine (Rattan & Chakder, 1992 ; Burleigh, 1992). The superoxide generator hydroquinone,

depressed the inhibitory nerve responses and was reversed by SOD (Chakder & Rattan, 1992). These observations implicated NO as a NANC transmitter in IAS. Interestingly, VIP-induced falls in IAS tension were also suppressed by both L-NNA and hydroquinone. This hydroquinone response was inhibited by SOD. Therefore, in opossum IAS, NO or a NO-like factor did not only play a role in NANC nerve-mediated relaxations but was also, at least partly, responsible for the inhibitory actions of VIP.

In guinea pig IAS, field stimulation gave rise to NANC i.j.p.'s (Rae & Muir, 1994). In the presence of apamin, to block the fast i.j.p. component, a slower i.j.p. component was revealed. This was abolished by L-NAME and reduced by Hb. Following L-NAME-induced abolition, the slow i.j.p. was partially restored by L-arginine (Rae & Muir, 1994). In subsequent experiments, neurones in the myenteric plexus stained positively for NADPH diaphorase, known to label NO synthase (Rae & Muir, 1994). Clearly, the NANC slow i.j.p. in guinea pig IAS was mediated by the release of NO. The fast i.j.p. was proposed to involve purines and/or peptides (Rae & Muir, 1994).

1.4. Pathology of NO

Although direct evidence of a role for NO in the pathology of disease is relatively scarce, its heterogeneity of action and tissue distribution (see Moncada *et al*, 1991b) create a number of opportunities for an abnormality in the synthesis of , or in the tissue response to NO, to lead to disease. Among other conditions, essential hypertension, septic shock and a host of gastrointestinal disorders may be due, at least in part, to such abnormalities.

NO was released under basal conditions in the vasculature (Palmer *et al*, 1988b ; Rees *et al*, 1989). In hypertensive rats, endothelium-dependent relaxation was inhibited (Winquist *et al*, 1984 ; Luscher *et al*, 1987). Further, in patients with essential hypertension, ACh-induced vasodilatation of the

brachial artery was reduced, while the response to SNP was unaffected or even enhanced (Linder *et al*, 1990 ; Panza *et al*, 1990). Findings such as these led to the belief that the loss of NO-induced vasodilator tone could contribute to the development of essential hypertension (see Moncada *et al*, 1991b).

Bacterial endotoxin or a number of cytokines activated the inducible NO synthase in endothelial cells (Radomski *et al*, 1990b ; Gross *et al*, 1991) or in vascular smooth muscle (Busse & Mülsch, 1990b) *in vitro*. *In vivo*, bacterial endotoxin caused septic shock. This condition, mediated by the release of cytokines, was manifest by cardiovascular collapse. The inhibitor of NO synthase, L-NMMA, reversed the vascular response to bacterial endotoxin, an action that could be subsequently reversed by L-arginine (Kilbourn *et al*, 1990), indicating that overproduction of NO aided the development of septic shock. Later studies suggested that the immediate hypotension and vascular hyporeactivity to noradrenaline in this condition, resulted from activation of the constitutive NO synthase (Szabó *et al*, 1993). The delayed hypotension, vascular hyporeactivity and eventual cardiovascular collapse, resulted from activation of the inducible NO synthase (Szabó *et al*, 1993). Inhibitory analogues of L-arginine are currently under study for the treatment of septic shock.

NO was released from inhibitory NANC nerves throughout the gastrointestinal tract (see 1.3.). Consequently, any impairment of this process or in the ability of NO to induce the normal tissue response could lead to disease. One such example, is achalasia. This was described as a failure of the LOS to relax normally, probably due to the selective dysfunction of inhibitory NANC nerves (Feldman, 1991) which, in this tissue, probably released NO (De Man *et al*, 1991 ; Tøttrup *et al*, 1991 ; Conklin & Du, 1992 ; Knudsen *et al*, 1992). In support of this, nitrovasodilators were among the most effective pharmacological therapies for this condition (Feldman, 1991).

For some time, it has been known that acute or chronic inflammation or immune activation altered gut motility. The discovery of the inducible NO synthase in macrophages and neutrophils (Hibbs *et al*, 1988 ; Stuehr *et al*, 1989 ; McCall *et al*, 1991) makes it increasingly likely that NO could be a mediator of this inflammation and/or play a role in the alteration of gastrointestinal motility. For instance, in patients with Crohn's disease, degeneration occurred in the myenteric and submucous plexuses (Dvorak *et al*, 1980). Examination revealed that the plexuses were infiltrated by a number of immunologically active cell types, including macrophages. It seems plausible that cytokines released from these cells could stimulate the inducible NO synthase in surrounding tissues, thereby augmenting the inflammatory response. Indeed, in the mucosa of colonic tissue from patients with ulcerative colitis, inducible NO synthase activity was eightfold greater than controls (Boughton-Smith *et al*, 1993), though the cell type from which this increased activity derived was unclear. Infiltrating inflammatory cells, resident macrophages, or vascular endothelial, smooth muscle, epithelial, or other mucosal cells were all suggested as possible sources (Boughton-Smith *et al*, 1993). This elevation of NO synthase activity could have contributed to the mucosal vasodilatation and increased vascular permeability observed in ulcerative colitis. It remains to be seen whether inhibitors of NO synthesis or compounds such as L-arginine which increase its release, will have a therapeutic value in the treatment of conditions such as these.

1.5. Excitatory Effects of NO-donors

Given that the potent vasorelaxing properties of NO donors i.e. the nitrovasodilators, were employed in the treatment of disease for many years, it was not surprising that the vast majority of research into their actions focused on their inhibitory effects. It was only recently, following the revelation that

NO functioned as a biological mediator, with the consequent elevation of interest in NO and related substances, that any evidence of their excitatory properties came to light. Nonetheless, it is now clear that these substances do possess excitatory effects, particularly in spontaneously active smooth muscle.

For instance, in guinea pig ileum, SNP, GTN or 8-bromo cGMP each enhanced the peristaltic reflex (Sugisawa *et al*, 1991). The SNP-induced stimulation, but not that induced by 8-bromo cGMP, was prevented by MeB. SNP did not alter the baseline tension of the preparation, nor did it affect the circular muscle response to nerve stimulation (Sugisawa *et al*, 1991). These workers postulated that NO was formed from SNP in mechanosensitive neurons and that the resultant elevation in cGMP levels increased the sensitivity of intestinal stretch receptors.

Electrical field stimulation of myenteric plexus-longitudinal muscle preparations from rat ileum evoked TTX-sensitive, NANC-mediated contractions (Barthó *et al*, 1992). The NO synthase inhibitor, L-NNA, inhibited these contractions in a concentration-dependent manner, and this inhibition was antagonized by the precursor for NO synthesis, L-arginine (Barthó *et al*, 1992). SNP produced a short-lived contraction of this smooth muscle preparation. However, these findings did not exclude the possibility that NO, could in fact, have caused the secondary release of an excitatory substance.

Secondary excitation in response to NO was reported in oesophageal longitudinal muscle. In this preparation, SNP, authentic NO or 8-bromo cGMP each evoked a concentration-dependent relaxation followed by a rebound contraction (Saha *et al*, 1993). Neither MeB, nor another guanylate cyclase inhibitor, LY-83583, affected the SNP-induced relaxation, but both abolished the contractions induced by SNP or NO. Likewise, the cyclooxygenase inhibitor, indomethacin, while having no effect on oesophageal smooth muscle relaxation, blocked the contractions due to SNP, NO or 8-bromo cGMP (Saha

et al, 1993). Thus, in oesophageal longitudinal muscle of the opossum, the cGMP-dependent excitatory action of NO donors also involved products of the cyclooxygenase pathway. In the circular muscle of canine proximal colon too, NANC nerve stimulation led to NO-induced inhibition followed by contraction (Ward *et al*, 1992b). Endogenous eicosanoids were involved in the NO-associated rebound depolarization.

Excitatory effects of NO, or related compounds, were not restricted to gastrointestinal smooth muscle. Excitation also occurred in spontaneously active vascular smooth muscle. In rat portal vein, although the amplitude of spontaneous contraction and extracellularly-recorded discharges was decreased, the frequency of spontaneous electrical and mechanical events was increased by GTN (Bray *et al*, 1987 ; Wylie, 1988). This was attributed to a rise in the intracellular cGMP concentration (Wylie, 1988).

Clearly, the mechanisms behind these actions were not the same in every tissue. In guinea pig ileum, the nitrovasodilator-induced increase in cGMP levels may have acted to increase the sensitivity of stretch receptors (Sugisawa *et al*, 1991). In canine proximal colon and opossum oesophagus, the excitation succeeded a preliminary relaxation and may have been partly or wholly mediated by eicosanoids (Ward *et al*, 1992b ; Saha *et al*, 1993). In other tissues, such as rat portal vein or ileum, (Bray *et al*, 1987 ; Wylie, 1988 ; Barthó *et al*, 1992), the mechanisms were less well understood. This thesis represents attempts made to examine the mechanisms behind the actions of the nitrovasodilators, in particular SNP, on pacemaker driven smooth muscle *in vitro*.

1.6. Aims of the thesis

So far, the evidence has implicated NO as the EDRF from vascular tissues (see Moncada *et al*, 1991b) and as a neurotransmitter in many areas of the gastrointestinal tract (see Rand, 1992). To date, the most frequently described effects of NO have been inhibitory. However, many, if not most, established transmitters have demonstrated, either directly or indirectly, both inhibitory and excitatory properties. For instance, noradrenaline acting on α-adrenoceptors constricts vascular smooth muscle but relaxes gastrointestinal smooth muscle. Similarly, ACh's stimulation of muscarinic receptors in the gut causes contraction but, on endothelial cells, mediates the release of EDRF. There is , therefore, the possibility that inhibition is not the only type of physiological response to NO. Perhaps the ability to cause excitation is an inherent, but as yet undiscovered property of this molecule.

On the whole, it must be said that reports of NO-induced excitations are few. Yet, such actions could have important pharmacological, as well as clinical, implications. For instance, is it possible that the excitatory phenomena observed in some tissues could be mediated via a second messenger system that is distinct from guanylate cyclase/cGMP or are they, perhaps, the result of an action of NO on the smooth muscle pacemaker? Further, since the relaxant properties of nitrovasodilators are used in the treatment of a number of conditions including angina pectoris, congestive heart failure, hypertensive emergencies and achalasia, would any concomitant excitation compromise the therapeutic usefulness of these compounds? For example, GTN reduced the amplitude of spontaneous contraction in rat portal vein but the frequency of activity was increased (Bray *et al*, 1987 ; Wylie, 1988). Would this reduce the efficiency of these compounds for the treatment of hypertension or hypertensive emergencies? Moreover, nitrovasodilators, whether directly or indirectly, stimulated contraction in a variety of gut smooth muscles from the

oesophagus to the colon (Sugisawa *et al*, 1991 ; Barthó *et al*,1992 ; Ward *et al*, 1992 ; Saha *et al*, 1993). Would this make them any less useful for the treatment of gut disorders such as achalasia? Would the concomitant administration of a cyclooxygenase inhibitor, to inhibit secondary prostaglandin release, enhance their efficacy? Despite the potential importance of these findings, to date, an extensive study of the mechanisms behind the stimulatory actions of the nitrovasodilators has not been undertaken.

In light of these facts, the effects and underlying mechanisms of the nitrovasodilators were examined on spontaneously active smooth muscle, *in vitro*, from the gastrointestinal and cardiovascular systems ; namely the rabbit distal colon (RDC) and the rat portal vein (rat PV). The excitatory properties, if any, of the nitrovasodilators were of specific interest but their inhibitory properties were also examined. In order to examine these mechanisms, a variety of techniques were employed. These included the testing of agonist and antagonist drugs on the spontaneous extracellular electrical and mechanical activity of each tissue and measuring the effects of SNP on ^{86}Rb efflux, radiolabelled transmitter (^{3}H-noradrenaline) overflow and Ca^{++} mobilization or sequestration.

Chapter 2 : Methods

2.1. Choice of Tissues

Nitrovasodilators are used to treat a number of conditions in both the gastrointestinal and cardiovascular systems e.g. achalasia, angina pectoris, congestive heart failure. It was desirable to examine the effects of these drugs on smooth muscle preparations from each of these systems. When selecting the tissues to be examined, two main factors were considered. Firstly, since much of the evidence for nitrovasodilator-induced excitation has come from spontaneously active smooth muscles (Bray *et al*, 1987 ; Wylie, 1988 ; Sugisawa *et al*, 1991 ; Barthó *et al*, 1992), each tissue should have been spontaneously active. Secondly, for the sake of physiological relevance, NO should have been released from each tissue chosen, either as a neurotransmitter, or as a humoral factor.

The RDC was selected since it was spontaneously active and since the spontaneous electrical activity (Gillespie, 1962 ; see Sanders & Smith, 1989) and motility (see Christensen, 1989) of colonic smooth muscle had been well described. In addition, gut smooth muscle was known to receive a dense NANC innervation (Burnstock *et al*, 1963 ; Martinson, 1965 ; Holman & Hughes, 1965 ; Bianchi *et al*, 1968) which utilized NO (Li & Rand, 1990 ; Toda *et al*, 1990 ; Bult *et al*, 1990 ; Dalziel *et al*, 1991). It was necessary to demonstrate a neural role for NO in the RDC, before commencing the investigation.

The rabbit portal vein (RPV) was originally chosen as the second tissue since, in addition to releasing endothelium-derived NO, it was innervated by NANC nerves (Hughes & Vane, 1967) which released both ATP and NO (Brizzolara *et al*, 1993). Moreover, the portal vein was a spontaneously active blood vessel. However, preliminary experiments revealed the rat portal vein (rat PV) to be more suitable for the study of the actions of the nitrovasodilators on spontaneous electrical and mechanical activity. For these reasons, the RDC

and the rat PV were mainly used during this investigation though, on a few occasions, the effects of SNP were also examined on non-spontaneously active smooth muscle. On these occasions, rat anococcygeus or rabbit abdominal aorta were used.

2.2. Dissection of Tissues

2.2.1. Rabbit Distal Colon (RDC)

The colon lies in the midline of the abdominal and pelvic cavities suspended by the mesocolon which is a continuation of the parietal peritoneum of the dorsal abdominal wall (Gillespie, 1955). The distal colon originates at the junction of the superior and inferior mesenteric arteries (see Christensen, 1989) and extends to the anal canal. Embryologically, development is from the hindgut (Lewis, 1912).

The outermost layer of the colon is the serosa. Beneath this lies an outer longitudinal and an inner circular muscle layer between which is the myenteric plexus. Meissner's plexus is found in the submucosa and the lumen is lined with mucosal cells. The circular muscle layer contracts on the faecal pellets within the lumen, giving the colon a characteristic beaded appearance. Gillespie (1955) found no evidence in the terminal colon of a specialized region for storage or evacuation. Thus, in the rabbit, the entire length of intestine that in humans would include the rectum and anal canal, is here considered simply as the colon.

Adult New Zealand White (NZW) male or female rabbits (1.3-3.5 kg) were killed by CO_2 (BOC) or sodium pentobarbitone (Euthatal ; 200mg ml^{-1}) overdose (140mg kg^{-1}), in a gas chamber or by intra-arterial injection respectively, then bled. When using CO_2, anaesthesia was induced in under 2 min whilst with sodium pentobarbitone, induction generally took less than 5 s.

Immediately following death, a midline incision was made in the abdomen, the peritoneal cavity exposed and the gastrointestinal tract displaced. During dissection, tissues were kept moist with Krebs solution (see 2.9 for formula) bubbled with 95% O_2 and 5% CO_2 (pH-7.4). In male rabbits, the vasa deferentia and seminal vesicles and in females the uterus, were removed. In each case the pubic symphysis was split to reveal the distal colon ; the bladder and urethra were discarded. The colon was ligated 6-8 cm proximal to the anus and this terminal section removed to a Sylgard (Dow-Corning)-coated petri dish containing oxygenated (95% O_2 ; 5% CO_2) Krebs solution at room temperature. The tissue was trimmed free of fat and connective tissue before being opened longitudinally and faeces removed. The tissue was then transferred to a clean petri dish containing fresh oxygenated (95% O_2 ; 5% CO_2) Krebs solution and the mucosa removed by sharp dissection under a microscope (Carl Zeiss). The colon was cut into longitudinal (0.2 x 1.5 cm) or circular strips (0.3 x 2 cm), i.e. they were cut longitudinally or circularly, as required.

2.2.2 Rat or Rabbit Portal Vein (Rat PV or RPV)

Veins have three coats comprising an inner endothelial layer (tunica intima), a central layer of elastic and smooth muscle cells (tunica media) and an outer layer of connective tissue (tunica adventitia). The hepatic portal vein conveys blood from the gut to the liver. This blood vessel originates from the junction of the anterior mesenteric and lienogastric veins and courses ventrally for a short distance before entering the lobes of the liver (see Wells, 1961). The anterior mesenteric vein drains the ileum, caecum and upper part of the rectum, while the lienogastric vein drains the stomach and spleen. Below the junction with the lienogastric vein, the duodenal and posterior mesenteric veins also merge with the anterior mesenteric vein (see Wells, 1961). Like gastrointestinal smooth muscles, the portal vein possesses an outer longitudinal

and an inner circular muscle layer (see Sutter, 1990). This was the first discovered spontaneously active blood vessel.

Adult male Wistar rats (200-400 g) were killed by stunning then bled whilst adult male or female NZW rabbits (1.3-3.5 kg) were killed as previously described (2.2.1.). In each case, following a midline abdominal incision, the peritoneal cavity was exposed and the gastrointestinal tract displaced. During dissection, tissues were kept moist with oxygenated (95% O_2 ; 5% CO_2) Krebs solution. Portal veins were exposed and cleared of surrounding tissue. The length of portal vein from the junction of the anterior mesenteric and lienogastric veins, to the bifurcation of the portal vein into left and right branches (2-3 cm ; Hughes & Vane, 1967), was removed to a Sylgard (Dow Corning)-coated petri dish containing Krebs solution bubbled with 95% O_2 and 5% CO_2 at room temperature. Using a microscope (Carl Zeiss), veins were cleaned free of fat and connective tissue before being used.

2.2.3. Rat Anococcygeus

The anococcygeus consists of parallel bundles of longitudinal smooth muscle fibres, that originate from a tendinous attachment to coccygeal vertebrae 1 and 2. Each muscle runs caudally behind the colon before passing ventrally around either side of the terminal colon where they fuse to form a 'ventral bar' (Gillespie, 1972). Due to its close proximity to the terminal colon, the anococcygeus may be involved in the defaecation reflex (McGrath, 1973).

Following a midline abdominal incision, the vasa deferentia and seminal vesicles were removed from the freshly killed male rat (200-400g ; see 2.2.2.), the pubic symphysis split, and the bladder and urethra discarded. The colon, ligated and cut at the level of the pelvic brim, was pulled forward and the connective tissue behind it removed, until the two anococcygeus muscles were visible. Either muscle was ligated at its point of attachment to the coccygeal

vertebrae and at the 'ventral bar'. Each anococcygeus (2-3 cm) was then transferred to a Sylgard (Dow Corning)-coated petri dish, containing oxygenated (95% O_2 ; 5% CO_2) Krebs solution at room temperature, and trimmed free of fat and connective tissue under a microscope (Carl Zeiss) before being used.

2.2.4. Rabbit Abdominal Aorta

Arteries, including the aorta, like veins, possess intimal, medial and adventitial layers. As blood leaves the heart it enters the aorta. This main artery runs caudally from the left ventricle through the thorax and abdomen to its bifurcation into the common iliac arteries (see Wells, 1961). That stretch of aorta which is enclosed in the thorax is generally known as the thoracic aorta, whilst that which is exposed in the abdomen is generally known as the abdominal aorta.

Adult male NZW rabbits (1.3-3.5 kg) were killed as previously described (see 2.2.1.). Following a midline abdominal incision, the vasa deferentia and seminal vesicles were removed from the freshly killed animal, the abdominal aorta exposed and cleared of surrounding tissue. The length of aorta, from the coeliac artery, to its bifurcation into left and right branches of the common iliac artery (5-6 cm), was transferred to a Sylgard (Dow Corning)-coated petri dish containing Krebs solution aerated with 95% O_2 and 5% CO_2, at room temperature. The aorta was cleaned free of fat and connective tissue under a microscope (Carl Zeiss) before being cut into sections (0.2 x 2 cm) and used.

2.3. Analysis of NANC nerve responses in RDC

Apparatus

Following 1g calibration, circular strips (0.3 x 2 cm) of RDC were mounted in oxygenated (95% O_2 ; 5% CO_2) Krebs solution in a water jacketed (25 ml) organ bath at 37 ± 1°C for isometric tension recording of the response to NANC nerve stimulation and drugs. One end of each tissue was fixed, by thread, to an isometric force-displacement transducer (Grass FT03C) connected to a polygraph (Grass 7D), while the other was tied to a hook on the stimulating electrode (Fig. 7). Where possible, the optimal point of the length-tension relationship was determined by increasing the length of each strip incrementally. At each length, the tissues were stimulated electrically (8 pulses at 8 Hz, 40V supramaximal voltage, 0.2 ms) until the optimal length (i.e. that which gave the maximum contraction) was reached. The tissues were then allowed to equilibrate at this length for at least 30 min before experimentation. However, in a few tissues, only relaxations followed by rebound contractions (rather than initial contractions) were obtained in response to nerve stimulation, no matter the level of tension applied. In these cases, the muscle strips were allowed to equilibrate with a tension of approximately 1g, for at least 30 min. Experiments were conducted in the presence of phentolamine and propranolol (each 3 x 10^{-6}M) to respectively block α- and β-adrenoceptors, thus ensuring that the nerve responses were indeed non-adrenergic. Carbachol (3 x 10^{-5}M) was used to raise tone. Electrical stimulation was achieved using Ag/AgCl ring electrodes (0.5 cm outside diameter) by passing square wave pulses (40V, 0.1-0.5 ms at 0.5-16 Hz) from an electrical stimulator (Grass SD9). Drugs were added directly to the Krebs solution.

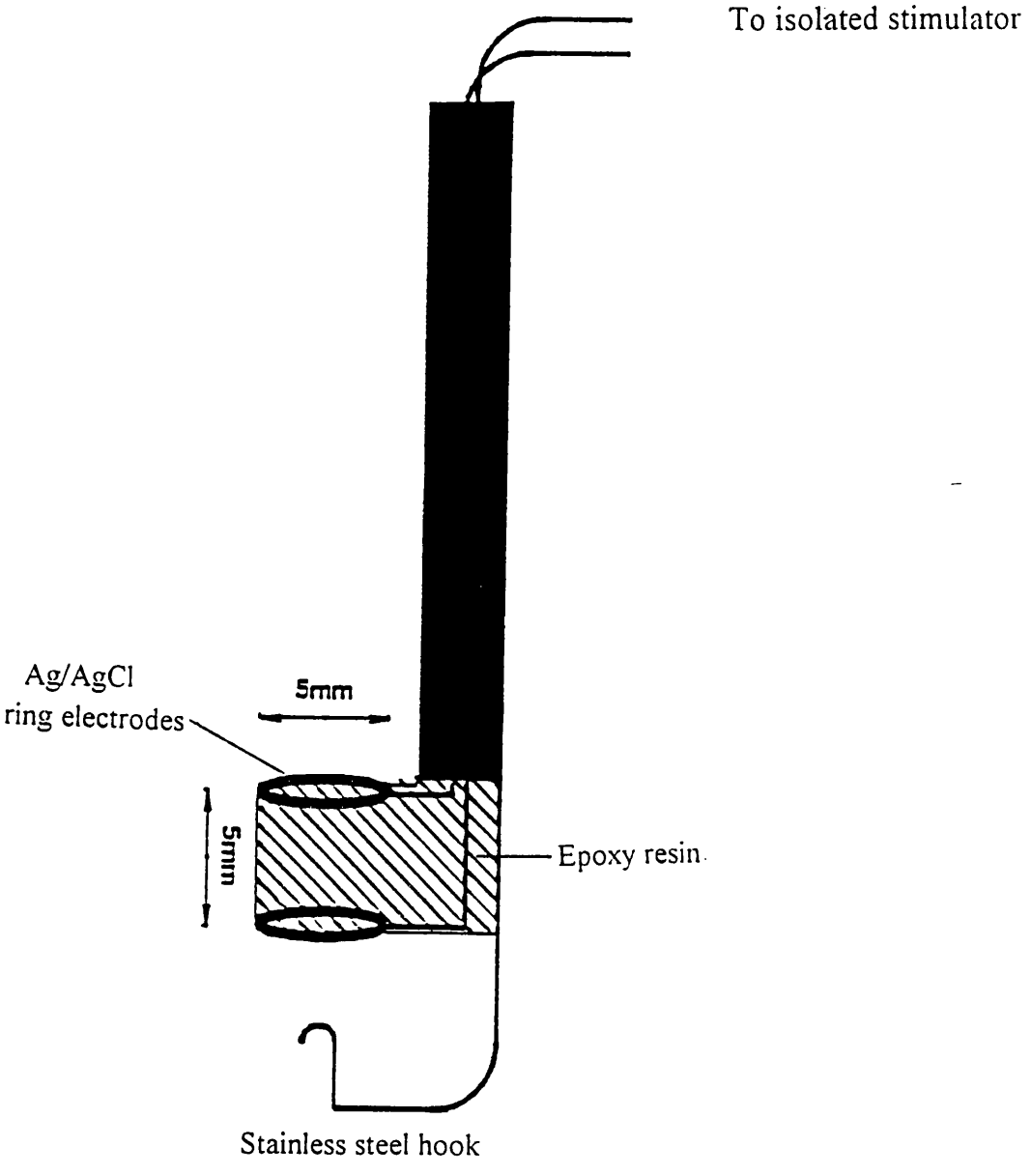


Fig. 7 : Stimulating electrode for the measurement of the response to NANC nerve stimulation (16 pulses, 40V, 0.1-0.5ms, 0.5-16Hz). Circular strips of rabbit distal colon (0.3 x 2 cm) were pulled through the Ag/AgCl ring electrodes and one end was tied to a hook on the electrode assembly while the other was fixed, by thread, to an isometric force displacement transducer (Grass FT03C). Responses were recorded on a polygraph (Grass 7D). Tone was raised by carbachol (3 x 10^{-5}M). Phentolamine and propranolol (each 3 x 10^{-6}M) were present throughout to ensure that responses were non-adrenergic.

2.4. Analysis of activity in spontaneously active smooth muscles

Apparatus

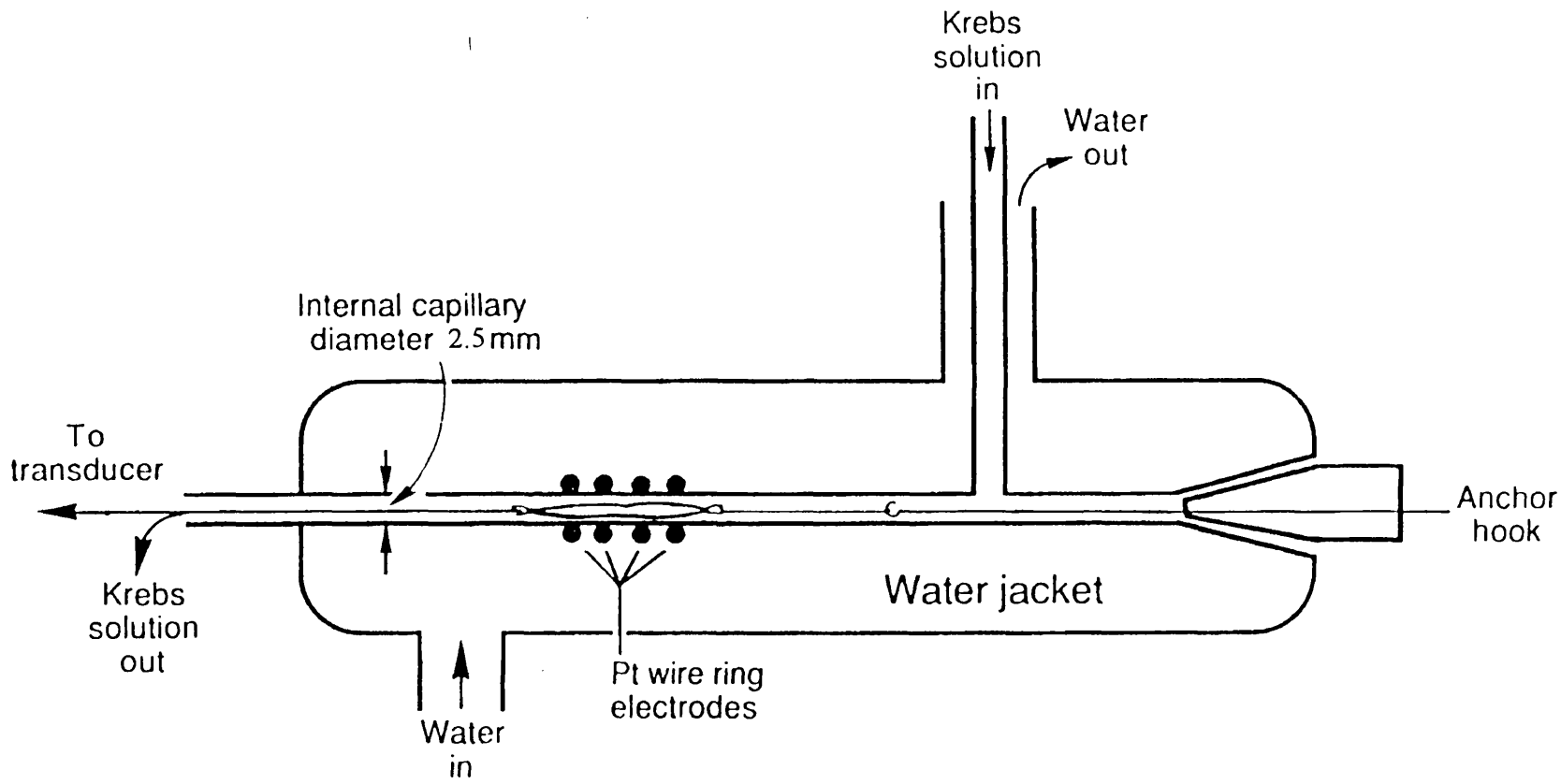
The Golenhofen apparatus (Golenhofen & von Loh, 1970 ; Fig. 8) was used since this allowed the simultaneous recording of extracellular electrical and mechanical events from many muscle cells. This equipment consists of four platinum wire ring electrodes (inside diameter approximately 2mm) contained within a narrow, water jacketed (37 ± 1°C) glass capillary (inside diameter approximately 2.5mm) perfused with oxygenated (95%O_2 ; 5% CO_2) Krebs solution. These electrodes could be used for either recording or stimulating as required. Mechanical signals were calibrated for 1g whilst electrical signals were calibrated by passing 0.1 mV from an A.C. preamplifier (Neurolog NL103).

Longitudinal strips of RDC (0.2 x 1.5cm) or intact segments of rat PV, RPV, rat anococcygeus or rabbit abdominal aorta (each 1-1.5cm in length) were pulled gently through the internal capillary of the Golenhofen apparatus and mounted between the electrodes. One end of each tissue was fixed by thread to an anchor hook (see Fig. 8) while the other was similarly attached to an isometric force displacement transducer (Grass FT03C), connected to a polygraph (Beckman R-611/R-511A or Grass 7D). Strips of RDC were left virtually unstretched (0.1-0.4g tension) while, to the other tissues, a resting tension of 1g approximately was applied. Each tissue was allowed to equilibrate for approximately 45 min before the experiment began.

The recording electrodes were connected to an A.C. preamplifier (Neurolog NL103, low frequency cutoff 0.1Hz) and the signals filtered (Neurolog NL115). Both electrical and mechanical signals were amplified via a cathode ray oscilloscope (Tektronix, 5103N) before being recorded on, and further amplified by, an ultraviolet oscillograph (EMI, SE 6150 Mk II). Responses were permanently recorded on a 4-channel instrumentation tape recorder

Fig. 8 : The Golenhofen apparatus (Golenhofen & von Loh, 1970).

The tissue to be investigated was passed through a narrow, water-jacketed (37 ± 1°C) glass capillary perfused (3 ml min^{-1}) with oxygenated (95% O_2 ; 5% CO_2) Krebs solution, as indicated, and mounted between four platinum wire ring electrodes for extracellular electrical recording. One end of each tissue was tied, by thread, to an anchor hook, while the other was similarly attached to an isometric force displacement transducer (Grass FT03C).


Krebs
solution
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Internal capillary
diameter 2.5mm
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electrodes
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(Racal, Store 4/DS). Drugs were added to oxygenated (95% O_2 ; 5% CO_2) Krebs solution, which was pumped through polythene tubing (inside diameter 3mm) using a Gilson pump (Minipuls 3) at a rate of 3 ml min^{-1}. Before entering the Golenhofen apparatus, the Krebs solution flowed through a heat exchange coil, jacketed with water (37 ± 1°C) pumped by a Tecam (TWB) pump.

Spontaneous activity in RDC, RPV or rat PV, as measured from the Golenhofen apparatus, consisted largely of discrete bursts of action potentials which preceded, or were accompanied by, corresponding bursts of contraction (Figs. 9-11). The following parameters of activity (as indicated in Fig. 9) were measured:

a) frequency of bursts of spiking (bursts min^{-1})
b) maximum spike amplitude per burst of electrical activity (mV)
c) number of electrical spikes per burst (spikes $burst^{-1}$)
d) duration of each electrical burst (s)
e) frequency of contractile bursts (bursts min^{-1})
f) maximum contraction per burst of mechanical activity (g)
g) number of mechanical peaks per burst (peaks $burst^{-1}$)
h) duration of each mechanical burst (s)

For the purpose of this study, discrete electrical events were considered as bursts, even if they contained only a single spike. Similarly, discrete contractions were considered as bursts of mechanical activity, even if they contained only a single mechanical peak.

Each parameter was measured during the four minutes immediately prior to drug addition and compared with those four minutes after addition, when the drug was considered to be having its maximum effect. Washout of drug was always within 20 min. Each measurement was made, as far as possible,

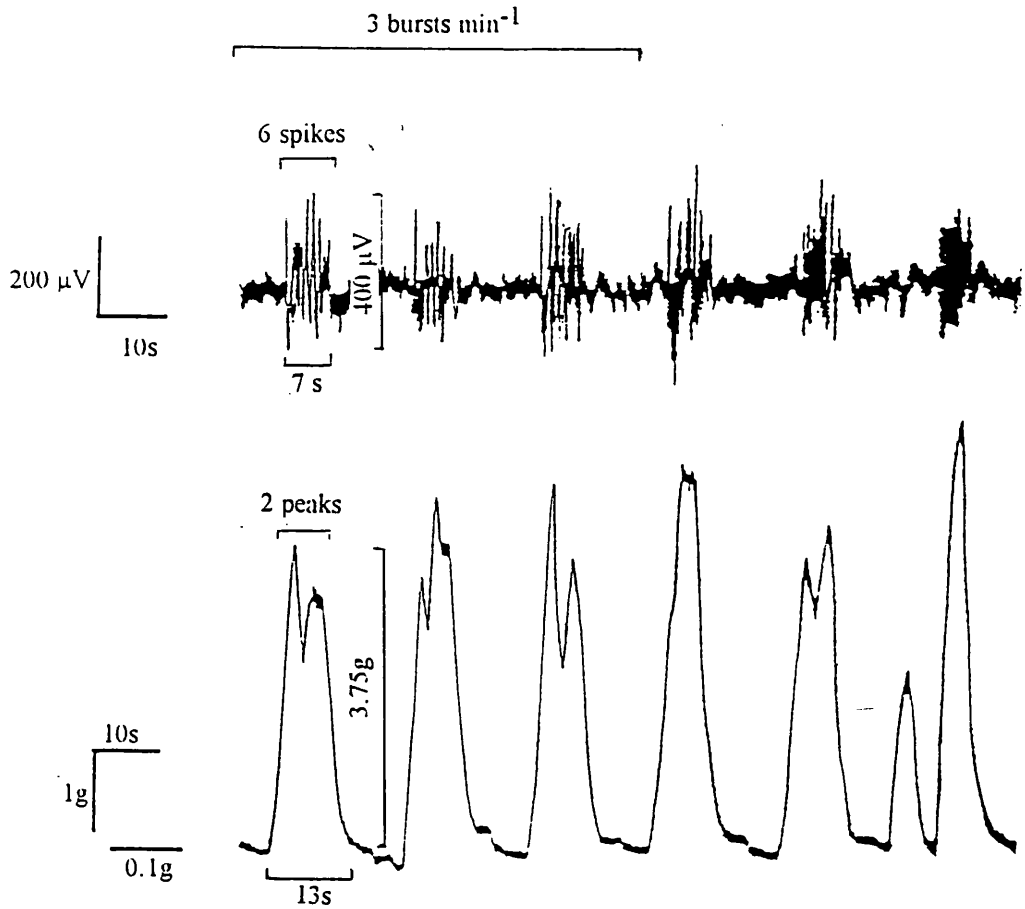


Fig. 9 : The spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. Each burst of electrical spiking was accompanied by contraction i.e. electrical and mechanical activity were synchronized. Each parameter of activity measured is indicated.

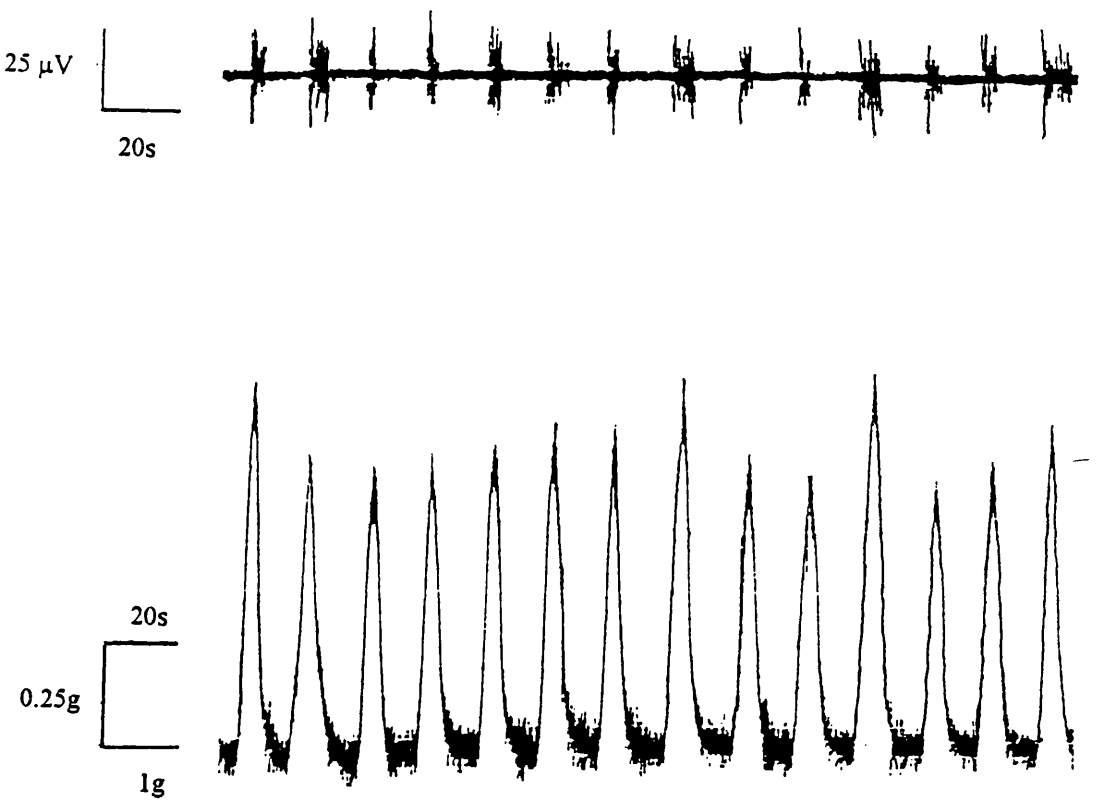


Fig. 10 : The spontaneous electrical (top) and mechanical activity recorded from the rabbit portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. Each burst of electrical spiking was accompanied by contraction i.e. electrical and mechanical activity were synchronized.

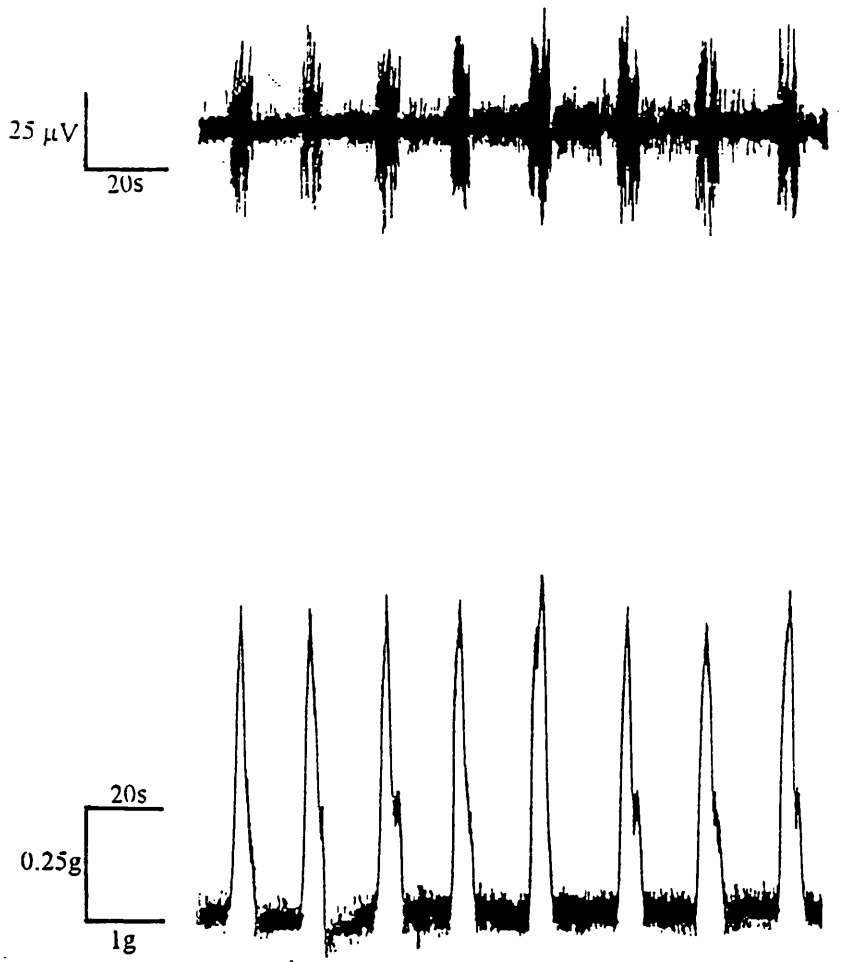


Fig. 11 : The spontaneous electrical (top) and mechanical activity recorded from rat portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. Each burst of electrical spiking was accompanied by contraction i.e. electrical and mechanical activity were synchronized.

visually, by the naked eye. Generally, this presented no problem with any measurement other than that of the number of electrical spikes per burst. Sometimes, spiking was so frequent, particularly in portal vein, that it was extremely difficult to measure this parameter. Rarely in RDC, electrical spiking was continuous not burstlike, and was accompanied by very rapid, frequent contraction (Fig. 12). In these cases, in the RDC, the only parameter measured was the frequency of contraction.

2.5. Radiolabelled Rubidium (^{86}Rb) Efflux

In these experiments, it was intended to measure the movement of K^+ across the cell membrane in response to drugs. Radiolabelled rubidium (^{86}Rb), a marker for K^+ (Imaizumi & Watanabe, 1981 ; Martin & Gordon, 1983 ; Hamilton *et al*, 1986), was used to monitor this movement in strips of RDC and in intact sections of rat PV, simultaneously with measurements of spontaneous electrical and/or mechanical activity. ^{86}Rb was chosen in place of ^{42}K, as the longer half-life of this isotope (18.4 days as compared to 12 hr for ^{42}K) made it more convenient and economical to use. This method has been routinely used to monitor K^+ movement for some time (Imaizumi & Watanabe, 1981 ; Martin & Gordon, 1983 ; Hamilton *et al*, 1986).

Strips of RDC, or intact segments of rat PV, were dissected as previously described (see 2.2.1. and 2.2.2.) and incubated in oxygenated (95% O_2 ; 5% CO_2) Krebs solution (36 ± 1°C) containing $^{86}RbCl$ (5-10 μCi ml^{-1}) for 2-3 h. After incubation, tissues were rinsed for approximately 3 min in ice cold, nonradioactive Krebs solution, to remove excess isotope, before being drawn through and mounted between the platinum wire ring electrodes of the Golenhofen apparatus, as previously described (see 2.4.). The equipment for amplification, filtration and recording of electrical and mechanical signals was also the same as that previously described (see 2.4.). The tissue was perfused

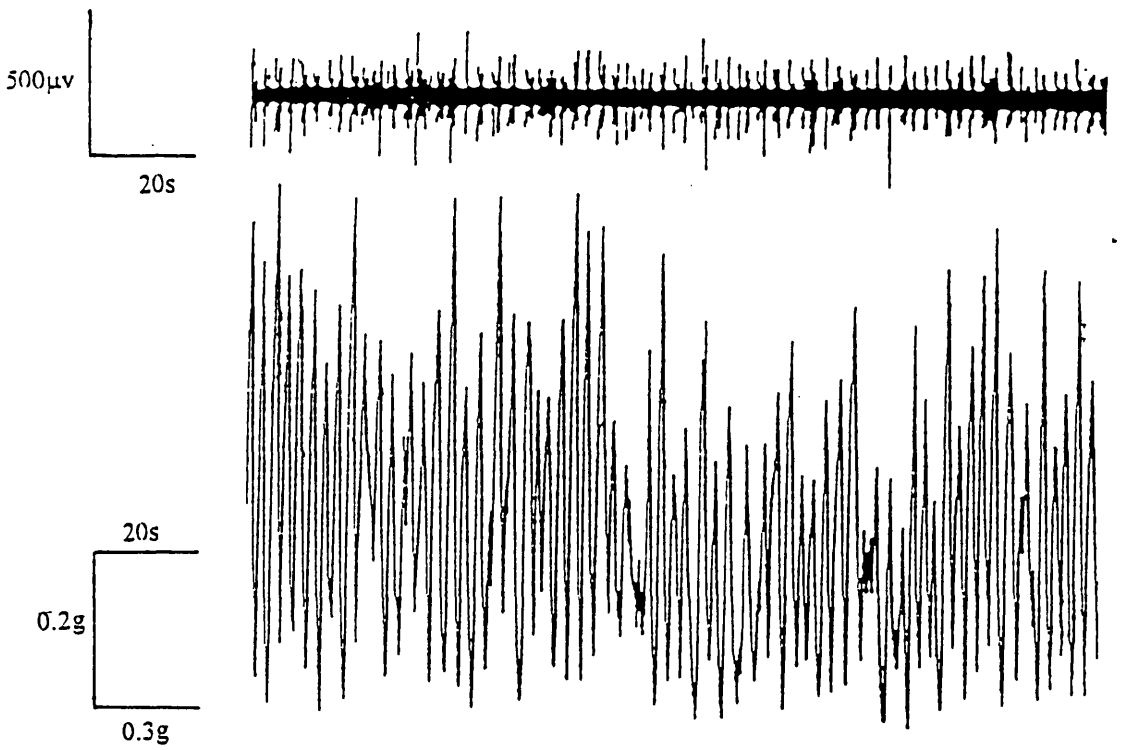


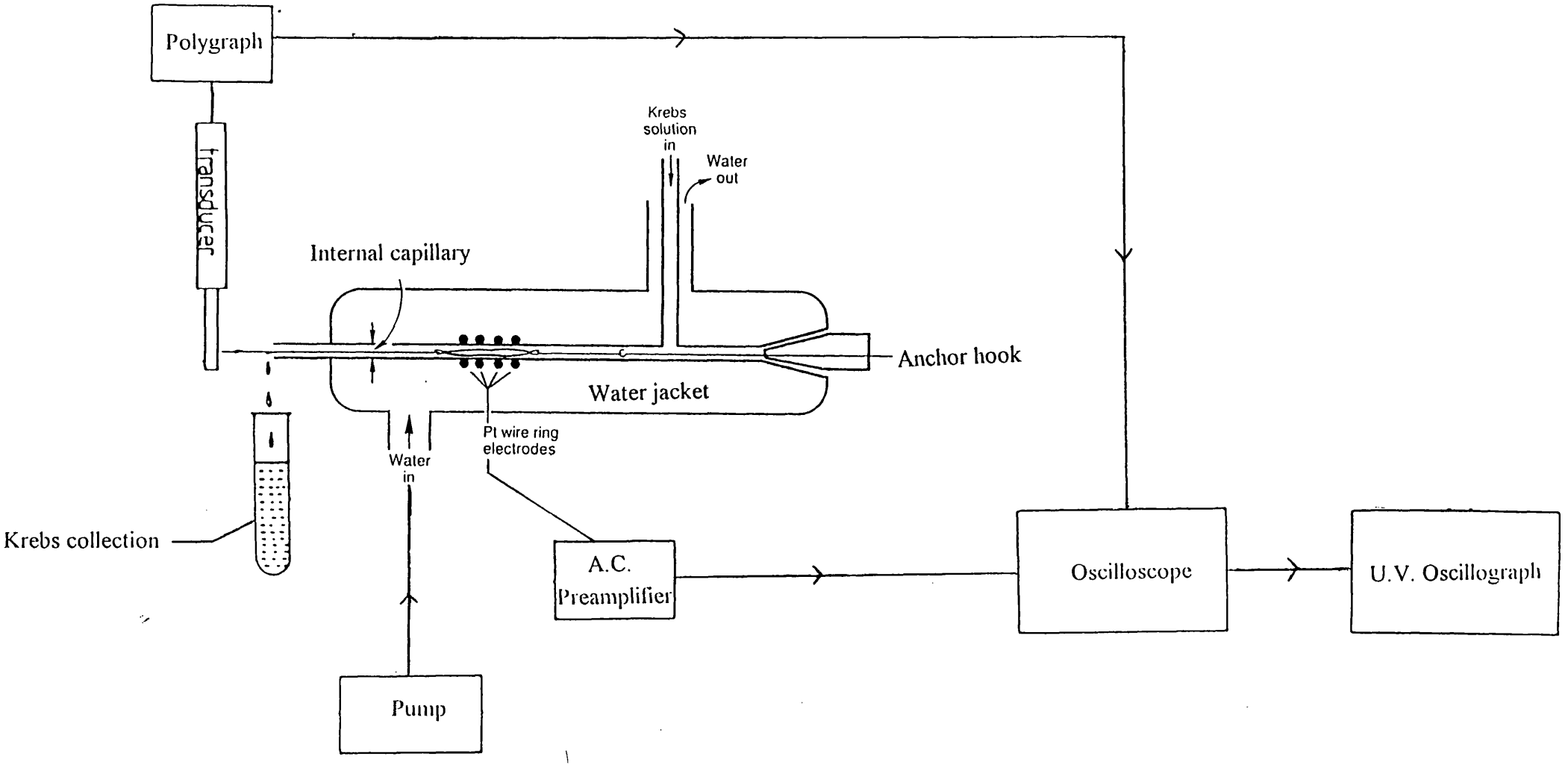
Fig. 12 : The spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. In this case, activity was not burst-like, but was continuous.

(3 ml min^{-1}) with warm (37 ± 1°C) Krebs bubbled with 95% O_2 and 5% CO_2, for the duration of the experiment. Drugs were added to the external Krebs reservoir. A fraction collector (Pharmacia Fine Chemicals, Frac-100) was used to automatically collect samples (6 ml) of superfusate every 2 min throughout the experiment (Fig. 13). Scintillation fluid (10 ml ; Ecoscint A ; National Diagnostics) was added to each fraction collected and the level of ^{86}Rb measured with a scintillation counter (Packard 2000 CA) as counts per minute (cpm). At the end of the experiment, the tissue was dissolved in soluene-100 (1 ml ; Packard) and the amount of ^{86}Rb remaining in the tissue measured. Data were expressed in terms of the efflux rate constant (E ; see 3.3.2.). The mean efflux rate constant during the 10 min prior to drug addition was calculated, and compared with the mean value for the 10 min immediately following drug addition.

2.6. Radiolabelled ^{3}H-Noradrenaline Overflow

The release of noradrenaline from nerve terminals cannot be measured directly (see Starke, 1977). Methods commonly used to measure, indirectly, the release of noradrenaline include evaluating the response of the postsynaptic cell or measuring, either chemically or radiochemically or by bioassay, the quantity of noradrenaline or of noradrenaline plus its metabolites that overflows into the incubation medium (see Starke, 1977). When adopting the radiochemical method, it should be remembered that a large part of the total radioactive release will be due to metabolites of noradrenaline. Though noradrenaline can be separated from its metabolites this is a lengthy process. Fortunately, it has been claimed that the overflow of total radioactivity approximates to release more closely than does the overflow of noradrenaline alone (see Starke, 1977). In this study, the release of noradrenaline and its metabolites from rat portal vein in response to drugs was measured,

Fig. 13 : Apparatus for measuring ^{86}Rb efflux or ^{3}H overflow. Each tissue loaded with either $^{86}RbCl$ or [^{3}H]-noradrenaline was passed through the platinum wire ring electrodes of the Golenhofen apparatus and perfused (3 ml min^{-1}) with oxygenated (95% O_2 ; 5% CO_2) Krebs solution as before. One end of each tissue was tied, by thread, to an anchor hook while the other was similarly attached to an isometric force displacement transducer. The perfusate was collected every 2 min using a fraction collector.


Polygraph
transducer
Krebs solution in
Water out
Internal capillary
Anchor hook
Water jacket
Pt wire ring electrodes
Water in
Krebs collection
A.C. Preamplifier
Oscilloscope
U.V. Oscillograph
Pump

radiochemically, by labelling the stores of transmitter in the nerves. The overflow was then analysed for radioactivity. In this case, the term 'overflow' is defined as the amount of neurotransmitter or its metabolites that escaped the uptake process and was collected from the tissue.

Rat PV's were dissected as previously described (see 2.2.2.) and incubated in oxygenated (95% O_2 ; 5% CO_2) Krebs solution (36 ± 1°C) containing [^{3}H]-noradrenaline (78 μCi ml^{-1}, 2 x 10^{-6}M) for approximately 45 min, then rinsed (3 min) with ice cold Krebs solution to remove excess radioactivity, before being mounted in the Golenhofen apparatus. The apparatus was as previously described for the measurement of ^{86}Rb efflux.

Portal veins, previously incubated with [^{3}H]-noradrenaline, were superfused (3 ml min^{-1}) in the Golenhofen apparatus throughout each experiment with oxygenated (95% O_2 ; 5% CO_2) Krebs solution containing ascorbic acid (1.1 x 10^{-4}M) to inhibit catecholamine oxidation, imipramine (6 x 10^{-7}M) and normetanephrine (10^{-5}M) to repectively block uptake 1 and uptake 2, tranylcypromine (10^{-5}M) to inhibit monoamine oxidase and yohimbine (10^{-7}M) to block presynaptic α_2-adrenoceptors. Use of these conditions, was intended to optimize the presynaptic release of noradrenaline in response to drugs.

Samples (6 ml) of superfusate were collected automatically every 2 min using a fraction collector (see Fig. 13 ; Pharmacia Fine Chemicals, Frac-100) and added to scintillation fluid (10 ml ; Ecoscint A ; National Diagnostics). The amount of ^{3}H per sample was measured with a scintillation counter (Packard 2000 CA). To overcome the problem of radioactive 'quenching', the amount of ^{3}H in cpm was corrected for the efficiency of the counter and converted to disintegrations per minute (dpm). At the end of the experiment, the tissue was dissolved in soluene-100 (1 ml ; Packard) and the amount of ^{3}H remaining in the tissue measured. Data were expressed in terms of the overflow rate constant (O ; see 3.5.3.). The mean overflow rate constant during

In retrospect :

The design of the experimental protocol for experiments that examined the overflow of [^{3}H]-noradrenaline from rat portal vein could have been improved. These experiments showed that tyramine increased ^{3}H overflow in the presence of the uptake-1 blocker, imipramine (6×10^{-7}M). It would have been better to have omitted imipramine since this blocks the uptake mechanism utilized by tyramine to induce its effects. Alternatively, the more lipid soluble amphetamine, which is not entirely dependent upon the uptake-1 mechanism for its effects, could have been used - again without imipramine.

the 10 min prior to drug addition was calculated, and compared with the mean value for the 10 min immediately following drug addition.

2.7. Measurement of $[Ca^{++}]_i$

The measurement of cytoplasmic Ca^{++} concentration $([Ca^{++}])_i$ in mammalian cell populations was not possible until the development of the fluorescent Ca^{++} indicator, quin-2 (Tsien *et al*, 1982a,b). This substance had both Ca^{++}-binding and fluorescence properties and could detect changes in $[Ca^{++}]_i$ in the physiological range (nm-μm). However, quin-2 was far from ideal. This compound did not undergo a very large shift in excitation or emission wavelength following Ca^{++}-binding. Hence, as $[Ca^{++}]_i$ measurement with quin-2 was reliant on absolute fluorescence changes, any changes in fluorescence which were not due to changes in $[Ca^{++}]_i$ could lead to errors. Moreover, a high cytosolic concentration of quin-2 was necessary. Some of these problems were overcome by the development of another class of Ca^{++} indicators which included fura-2 (Grynkiewicz *et al*, 1985). The advantage of fura-2 over quin-2 was in its ability to increase fluorescence emission upon Ca^{++} binding. In this study, fura-2, rather than quin-2 was used to measure $[Ca^{++}]_i$.

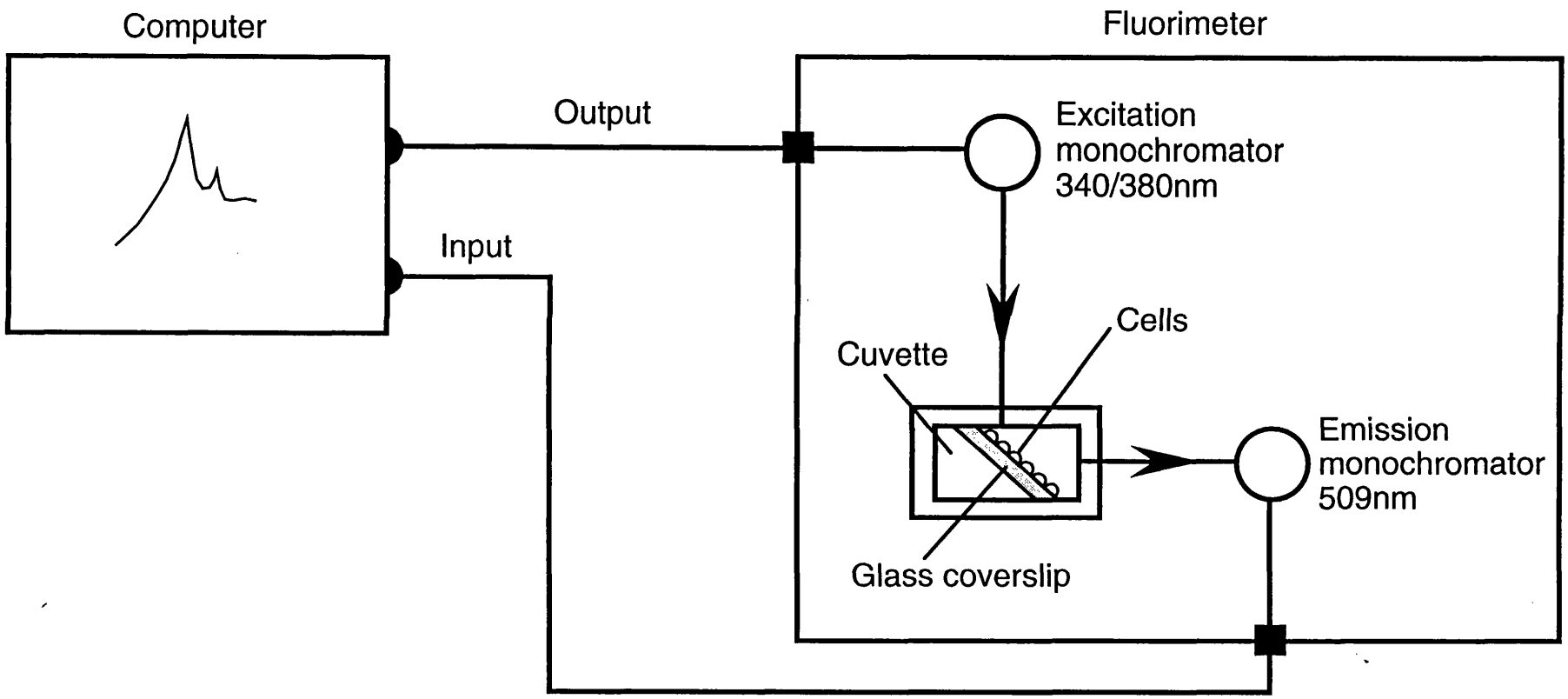
Monolayers of rat aortic vascular smooth muscle cells were incubated for 45 min, at 37°C, with the membrane permeant, penta-acetoxymethyl ester form of fura-2 (2 μM ; fura-2/AM ; Sigma) in HEPES (20 mM)-buffered Dulbecco's modification of Eagle's medium (DMEM ; Gibco brl) containing 1% bovine serum albumin (Fraction V ; Sigma) and cremophor (2 μl ml^{-1} ; Sigma). At the end of this loading period, the cells were transferred to HEPES-buffered Krebs solution. The cells were left in this solution for 30 min at room temperature, to allow the extracellular fura-2/AM to be washed from the cells and the hydrolysis of fura-2/AM to the Ca^{++}-sensitive acid form. Coverslips with

fura-2 loaded cells were placed in a quartz cuvette, which contained HEPES (10 mM)-buffered Krebs solution, in a spectrofluorimeter (Perkin Elmer LS3B), at an angle of 30° to the excitation beam (Fig. 14). This angle minimized the scattering of light towards the emission monochromator. The cuvette was maintained at 37°C and stirred continuously throughout the experiment. In order to check the viability of the cells, they were stimulated with angiotensin II (100 nM). The excitation monochromator was computer driven (IBM-PCAT ; Fig. 14) and alternated between 340 nM and 380 nM every 3.8s. Fluorescence emission was collected at 509 nM. At the end of each experiment (5 min after agonist stimulation), ionomycin (1 μM) and $MnCl_2$ (20 mM) were added, to permeabilize the cells to divalent cations and to quench intracellular fura-2 fluorescence respectively. This allowed the calculation of auto-fluorescence (Hallam *et al*, 1988). Following subtraction of autofluorescence, the corrected fura-2 fluorescence values obtained at 340 nM were divided by those obtained at 380 nM, giving a corrected ratio (R). The equation of Grynkiewicz *et al* (1985) was used to calculate $[Ca^{++}]_i$:

$$[Ca^{++}]_i = K_d \times [(R - R_{min}) / (R_{max} - R)] \times S_{f2} / S_{b2}$$

where K_d is the dissociation constant for the Ca^{++}-fura-2 complex ; R_{min} is the minimal ratio (F_{340} : F_{380}) obtained in Ca^{++}-free medium (with 40 mM EGTA) ; R_{max} is the maximal ratio (F_{340} : F_{380}) obtained in medium containing saturating concentrations of Ca^{++} ; S_{f2} and S_{b2} are the fluorescence emissions, measured at an excitation wavelength of 380 nM in the absence of Ca^{++}, and in the presence of saturating levels of Ca^{++}, respectively. At 37°C, a value of 225 nM was assumed for the K_d of the fura-2-Ca^{++} complex. Smooth muscle cells in suspension (in the presence of 40 mM EGTA the cells were detached from the glass cover slip) gave the following experimental calibration

Fig. 14 : Diagrammatic representation of the experimental apparatus for the measurement of $[Ca^{++}]_i$ in rat aortic vascular smooth muscle cells (adapted from Buchan, 1991). To measure $[Ca^{++}]_i$, fura-2-loaded monolayers of vascular smooth muscle cells on glass cover slips were placed in a quartz cuvette containing HEPES (10mM)-buffered Krebs solution (37°C) in a spectrofluorimeter (Perkin Elmer LS3B). The excitation monochromator was computer driven (IBM-PCAT) between 340nm and 380nm every 3.8s. Fluorescence emission data was collected at 509nm. At the end of each experiment $[Ca^{++}]_i$ was calculated.


Computer
Fluorimeter
Output
Input
Excitation
monochromator
340/380nm
Cells
Cuvette
Emission
monochromator
509nm
Glass coverslip

values that were used throughout to calculate $[Ca^{++}]_i$: R_{min} = 0.84, S_{f2} / S_{b2} = 5.25. R_{max} was calculated at the end of each experiment.

2.8. Statistics

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

2.9 Physiological Solutions

The Krebs physiological saline solution (Krebs & Henseleit, 1932) used during this study had the following ionic composition (mM) : NaCl 118.4, KCl 4.7, $CaCl_2$ 2.7, $MgCl_2$ 1.3, NaH_2PO_4 1.1, $NaHCO_3$ 25.0 and (+)-glucose 11.0. The pH was ~ 7.4.

2.10. Drugs

The following drugs were used : acetylcholine chloride (ACh ; Sigma), adenosine 5'-triphosphate (ATP ; Sigma), angiotensin II (Sigma), (-)-ascorbic acid (Sigma), atropine sulphate (Sigma), BAY K 8644 [methyl 1, 4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate ; kindly donated by Bayer A.G.], bovine haemoglobin (Sigma), BRL 38277 [(-)-6-cyano-3,4- dihydro-2,2- dimethyl- *trans*-4- (2-oxo-1-pyrrolidyl)-2H-1- benzo-pyran -3-ol ; lemakalim ; levcromakalim ; kindly donated by Smith Kline Beecham], 8-bromo cyclic guanosine monophosphate (8-bromo cGMP, sodium salt ; Sigma), carbamylcholine chloride (carbachol ; Sigma), diltiazem hydrochloride (Sigma), forskolin (Sigma), γ-amino-n-butyric acid (GABA ;

Sigma), glyceryl trinitrate (GTN ; kindly donated by Bard Pharmaceuticals), imipramine hydrochloride (Sigma), indomethacin (Sigma), isoprenaline sulphate (Macarthy's), isosorbide dinitrate (IDN ; Sigma), (L)-arginine (Sigma), 2-methylthio adenosine triphosphate (2-methylthio ATP ; tetrasodium salt ; Research Biochemicals Incorporated), N^G-nitro-D-arginine methyl ester (D-NAME ; Sigma), N^G-nitro-L-arginine methyl ester (L-NAME ; Sigma), (-)-7,8-[^{3}H]-noradrenaline (Amersham International, 37 MBq, 1mCi), (-)-noradrenaline bitartrate (Sigma), (±)-normetanephrine hydrochloride (Sigma), phentolamine mesylate (Rogitine ; Ciba), phenylephrine hydrochloride (Sigma), potassium ferrocyanide ($K_4Fe(CN)_6$; Hopkin & Williams), propranolol hydrochloride (ICI), rubidium hydrochloride (^{86}RbCl ; Amersham International, 37MBq, 1mCi), sodium nitroprusside (SNP ; BDH), tetrodotoxin (TTX ; Sigma), tranylcypromine sulphate (Parnate ; Smith Kline & French), tyramine hydrochloride (Sigma), vasoactive intestinal peptide (VIP ; Sigma), yohimbine hydrochloride (Boots).

Drug stock solutions were prepared in distilled water with the exception of BAY K 8644 (70% methanol), BRL 38277 (70% methanol) and forskolin (dimethyl sulphoxide), which were prepared in the solvents indicated. Unless otherwise stated, these solvents, alone, were shown to have no apparent effect on the tissues under examination. Subsequent dilutions were prepared in Krebs solution. Solutions of BAY K 8644 in a volumetric flask were protected from light by covering with aluminium foil. To prevent oxidation, solutions of isoprenaline sulphate and noradrenaline bitartrate each contained (-)-ascorbic acid (50 $\mu g\ ml^{-1}$). (-)-7,8-[^{3}H]-noradrenaline, supplied in a 0.02 M acetic acid : ethanol (9 : 1, v : v) mixture, was resuspended in Krebs solution containing ascorbic acid (50 $\mu g\ ml^{-1}$). (-)-ascorbic acid alone did not have any effect on the chosen tissues.

2.10.1. Preparation of Solutions of NO

Stock solutions of NO (10 μM) were prepared immediately before use. One end of a length of rubber tubing (inside diameter 3 mm) was attached to a NO (99.9%, Air Products Ltd., UK) containing cylinder and the other end submerged under water. Air was ejected from the tubing by passing NO gas from the cylinder. Using a Hamilton air-tight syringe, the tubing wall was pierced close to the cylinder and 4.5 μl of NO gas removed. This was injected immediately through the rubber seal of a brown bottle completely filled with helium (British Oxygen Company)-deoxygenated distilled de-ionized water. The solution of NO was protected from light by storing in the fridge until used, usually within one hour of preparation.

2.10.2. Preparation of Oxyhaemoglobin (Hb)

Commercially available bovine haemoglobin (Sigma) contained a mixture of oxyhaemoglobin and the oxidized derivative, methaemoglobin. Stock solutions of pure haemoglobin (oxyhaemoglobin ; Hb) were prepared by the method of Martin *et al* (1985). Commercially available haemoglobin (1.34 g) was dissolved in 19.5 ml distilled water. A length (30-40 cm) of dialysis tubing which had been boiled for 30 min in distilled water, was knotted at one end and a filter funnel placed in the open end. Sodium hydrosulphite ($Na_2S_2O_4$; 69.6 mg) was dissolved in distilled water (0.5 ml) and added immediately to the haemoglobin solution. After stirring for a few seconds, the mixture was poured into the dialysis tubing. The open end was tied and the dialysis tubing placed in a measuring cylinder containing one litre of distilled water at 4°C. This was inverted gently every hour and left to dialyse for 3 hr. When ready, 1 ml aliquots of Hb were placed in Eppendorf tubes (1.5 ml) and frozen at -25°C. They were stored for up to 1 week before use.

Chapter 3 : Results

3.1. Evidence that NO is a NANC Neurotransmitter in the Rabbit Distal Colon

In circular strips of rabbit distal colon (RDC), carbachol (3 x 10^{-5}M) raised the tone to 7.73 ± 0.67g (n = 44). Following this, field stimulation (16 pulses, 0.1-0.5 ms, supramaximal voltage) produced frequency-dependent (4Hz optimum), tetrodotoxin (TTX)-abolished relaxations and rebound contractions (n = 31; Fig. 15). At a frequency of 4Hz, these relaxations had a mean amplitude of 3.39 ± 0.45g (n = 31) while the rebound contraction had a mean amplitude of 4.23 ± 0.74g (n = 20).

Exogenous NO relaxed carbachol-induced tone in colonic smooth muscle (Fig. 16). NO (≥10^{-5}M) caused pronounced concentration-dependent relaxations that were succeeded by rebound contractions. A concentration of 2 x 10^{-5}M gave the maximum response. At this dose, the NO-induced relaxations had a mean amplitude of 3.85 ± 1.08 g (n = 8) while the rebound responses had a mean amplitude of 1.53 ± 0.44g (n = 7). Haemoglobin (Hb ; 3 x 10^{-5}M) abolished all responses to exogenous NO (2 x 10^{-5}M ; n = 3 ; results not shown). The NO donor, SNP (5 x 10^{-6} M : n = 8) caused a relaxation of almost identical amplitude (3.88 ± 0.88g) to that of exogenous NO (Fig. 17). Unsurprisingly, the nitrovasodilator-induced inhibition was of longer duration, since SNP would continue to release NO until reduction of the nitrovasodilator was complete. The SNP-evoked response was not followed by a rebound contraction.

Hb (3 x 10^{-5}M) significantly reduced ($P < 0.01$), but did not abolish, NANC nerve-mediated relaxations, suggesting that NO, though released, was not wholly responsible for these neuronally-mediated inhibitions (n = 8 ; Fig. 18). At a frequency of 4Hz, the relaxations were reduced from 2.97 ± 0.17g to 2.4 ± 0.26g by Hb (3 x 10^{-5}M ; n = 10) ; a reduction of approximately 20%. On a number of occasions, this concentration of Hb reduced, and sometimes

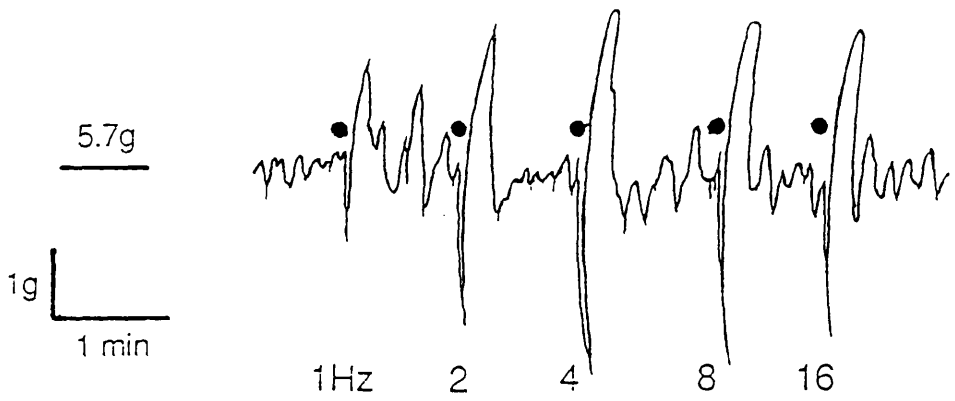
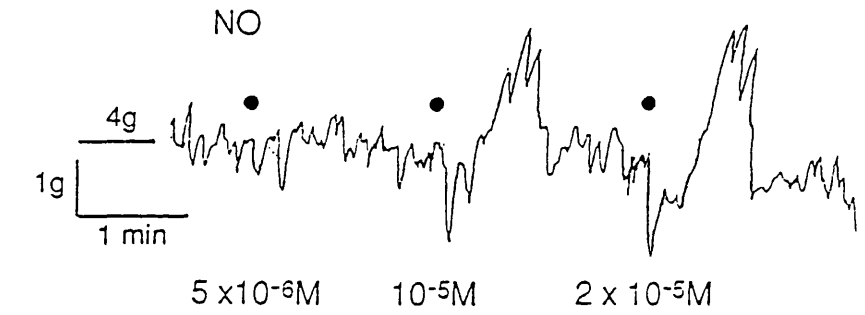


Fig. 15 : Relaxations and rebound contractions recorded from a carbachol (3×10^{-5}M)-contracted circular strip of rabbit distal colon in response to NANC nerve stimulation (●,16 pulses, 1-16Hz, 0.2ms, supramaximal voltage). A frequency of 4Hz gave the optimum response. Phentolamine and propranolol (each 3×10^{-6}M) were present throughout.



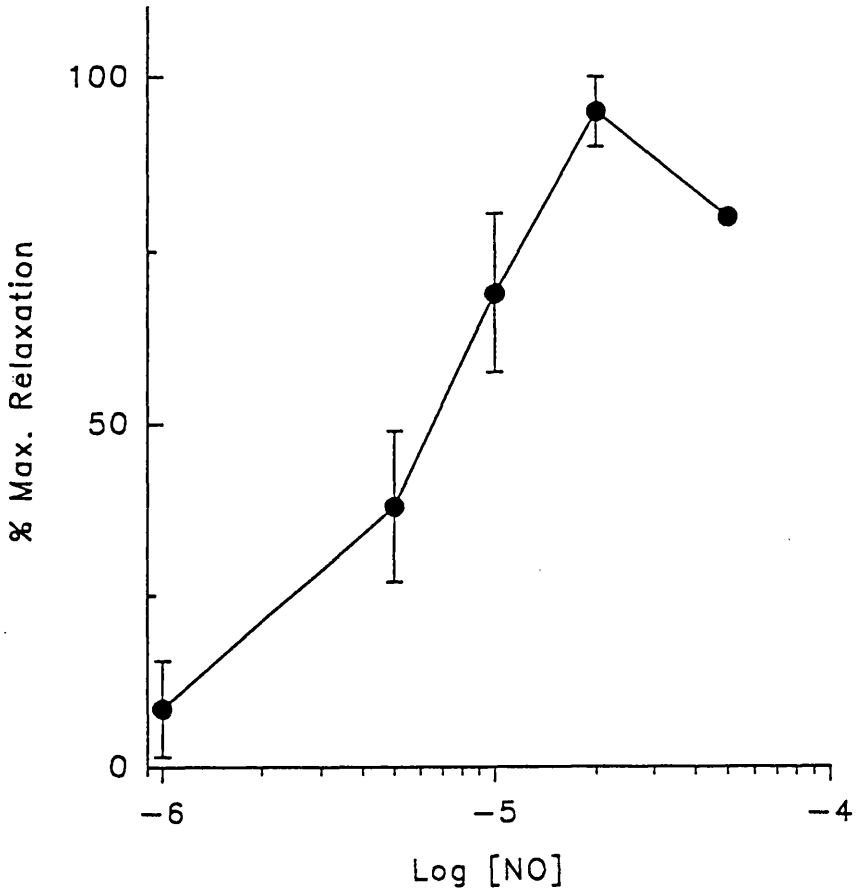


Fig. 16 Top : Dose-dependent relaxations and rebound contractions recorded from a carbachol (3 x 10^{-5}M)-contracted circular strip of rabbit distal colon in response to NO (●,5 x 10^{-6}-2 x 10^{-5}M).
Bottom : The effect (± s.e. mean) of NO (10^{-6}-5 x 10^{-5}M; n = 5, except 5 x 10^{-5}M, n = 2) on carbachol (3 x 10^{-5}M)-contracted circular strips of rabbit distal colon, expressed as a percentage of the maximum relaxation (g) obtained. A concentration of 2 x 10^{-5}M gave the maximum response.

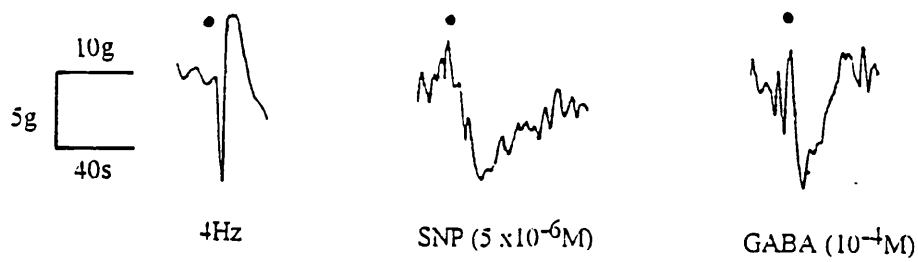


Fig. 17 : Comparison of relaxations in response to NANC nerve stimulation (• ,16 pulses, 4Hz, 0.2ms, supramaximal voltage), SNP (•, 5 x 10^{-6}M) and GABA (•, 10^{-4}M) on a carbachol (3 x 10^{-5}M)-contracted circular strip of rabbit distal colon. Neither SNP- nor GABA-induced relaxations were followed by a rebound contraction.

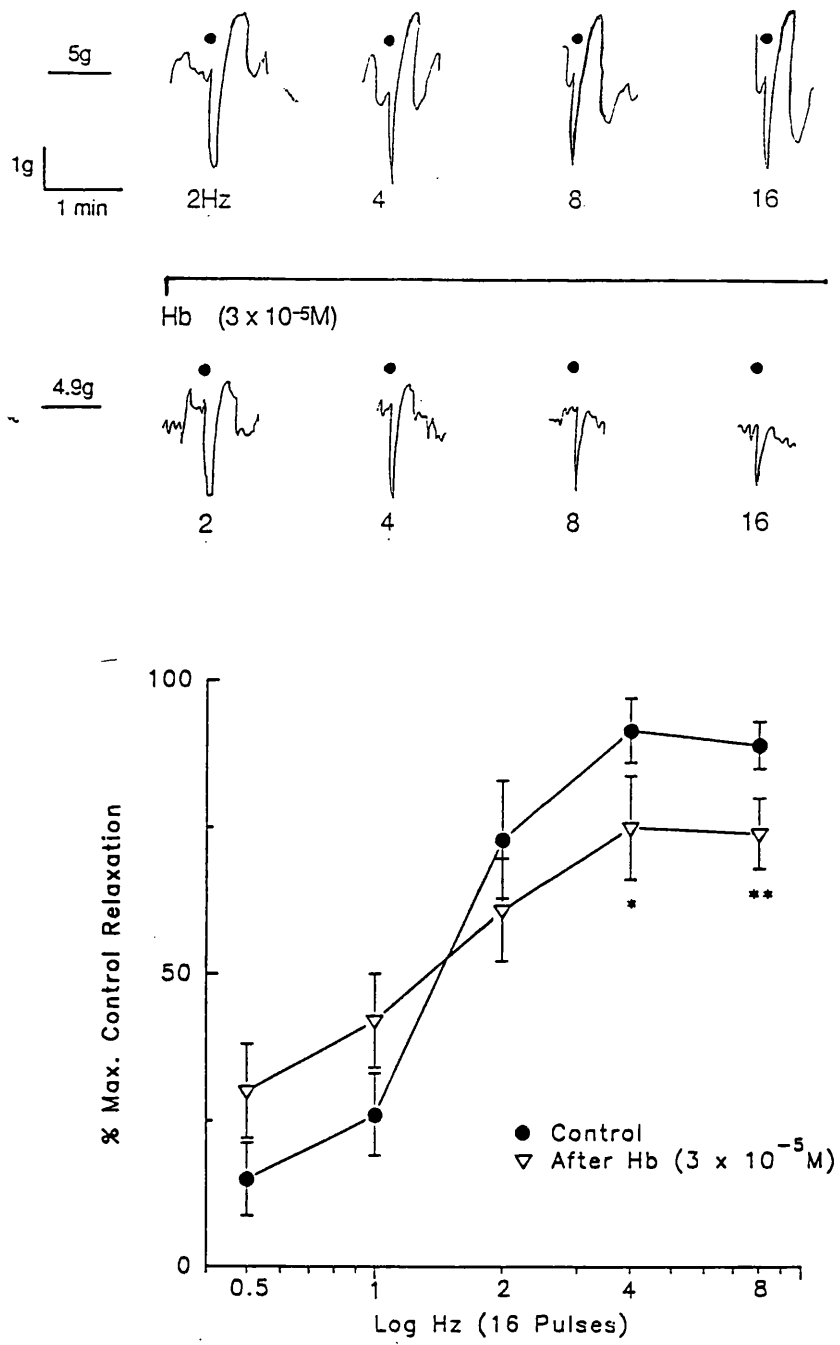


Fig. 18 Top: Frequency-dependent relaxations and rebound contractions recorded from a carbachol (3 x 10^{-5}M)-contracted circular strip of rabbit distal colon in response to NANC nerve stimulation (• ,16 pulses, 2-16Hz, 0.2ms, supramaximal voltage) and their inhibition by Hb (3 x 10^{-5}M ;———). **Bottom** : The effect (± s.e. mean) of Hb (3 x 10^{-5}M ; $n \geq 7$), expressed as a percentage of the maximum control relaxation, on the amplitude (g) of relaxation in response to NANC nerve stimulation (16 pulses, 0.5-8Hz, 0.2ms, supramaximal voltage) on carbachol (3 x 10^{-5}M)-contracted circular strips of rabbit distal colon. Hb reduced significantly (*, $P < 0.05$; **, $P < 0.01$), the amplitude of relaxation.

abolished, the rebound contractions in response to nerve-stimulation (Fig. 18 top). In a few cases, the baseline tension was also slightly reduced (< 0.5g) by Hb (3 x 10^{-5}M).

Similar effects were observed in the presence of the NO synthase inhibitor, L-NAME. NANC nerve responses were significantly reduced in amplitude ($P < 0.001$), but never abolished, by L-NAME (up to 10^{-3}M). L-NAME (5 x 10^{-4}M) caused a reduction from 3.1 ± 0.12g to 2.34 ± 0.21g ; a reduction of approximately 25% (n = 15 ; Fig. 19). Sometimes L-NAME, like Hb, also reduced ($P < 0.01$) the subsequent rebound effect (Fig. 19 top). Following electrical stimulation at a frequency of 4Hz, L-NAME (5 x 10^{-4}M) reduced the rebound amplitude to 2.07 ± 0.39g from 3.05 ± 0.41g (n = 15 ; Fig. 20). The stereoisomer of L-NAME, D-NAME, had no effect on either response (results not shown). After the addition of L-NAME to the carbachol-contracted tissue, a fall in tension, with a mean amplitude of 1.37 ± 0.31g (n = 15), often occurred. This was unexpected, but was not considered to contribute to the inhibitory effects of L-NAME on NANC nerve stimulation since, in a number of experiments, D-NAME induced a comparable fall in tension, but did not reduce the nerve response during carbachol-induced tone (results not shown). The ability of the precursor to NO synthesis, L-arginine, to reverse the L-NAME-induced inhibitory response was examined in 8 experiments. On 2 occasions, L-arginine (3 x 10^{-4}M) reversed the inhibition produced by L-NAME (5 x 10^{-4}M ; Fig. 21), but on the other 6 was ineffective.

It seems likely that inhibitory NANC nerve stimulation in the RDC released NO or a NO-related compound ; inhibition of the nerve response by Hb or L-NAME suggested that a NO component accounted for 20-25% of the response. Alternative putative transmitters were examined in an attempt to identify the other inhibitory transmitter(s) in the RDC. Neither ATP (≤ 5 x 10^{-4}M ; n = 8) nor its analogue 2-methylthioATP (≤ 5 x 10^{-5}M ; n = 2) relaxed circular strips of RDC (results not shown). VIP (5 x 10^{-7}M ; n = 3)

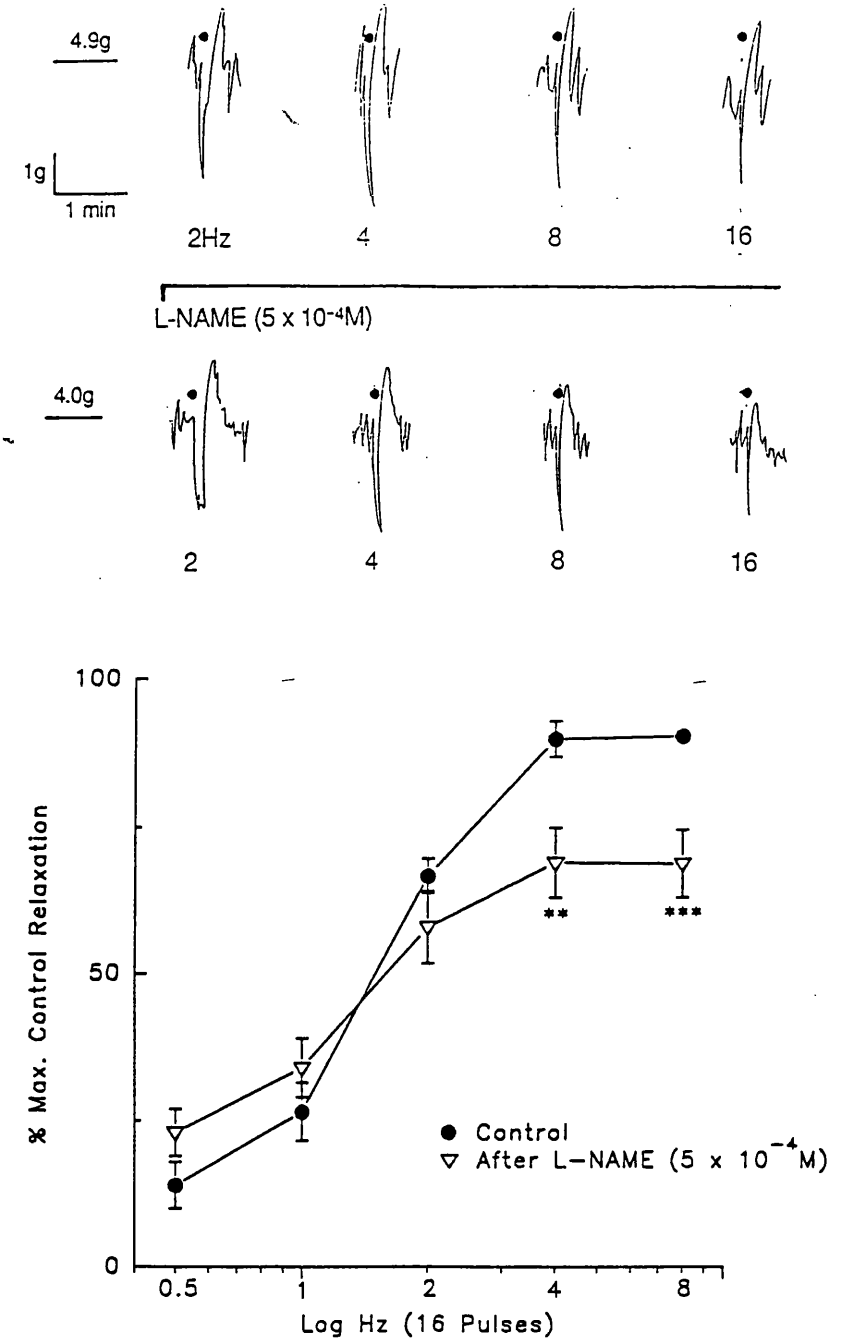


Fig. 19 Top: Frequency-dependent relaxations and rebound contractions recorded from a carbachol (3 x 10^{-5}M)-contracted circular strip of rabbit distal colon in response to NANC nerve stimulation (•,16 pulses, 2-16Hz, 0.2ms, supramaximal voltage) and their inhibition by L-NAME (5 x 10^{-4}M ;——). **Bottom** : The effect (± s.e. mean) of L-NAME (5 x 10^{-4}M ; n ≥ 13), expressed as a percentage of the maximum control relaxation, on the amplitude (g) of relaxation in response to NANC nerve stimulation (16 pulses, 0.5-8Hz, 0.2-0.5ms, supramaximal voltage) on carbachol (3 x 10^{-5}M)-contracted circular strips of rabbit distal colon. L-NAME reduced significantly (**, $P < 0.01$; ***, $P < 0.001$), the amplitude of relaxation.

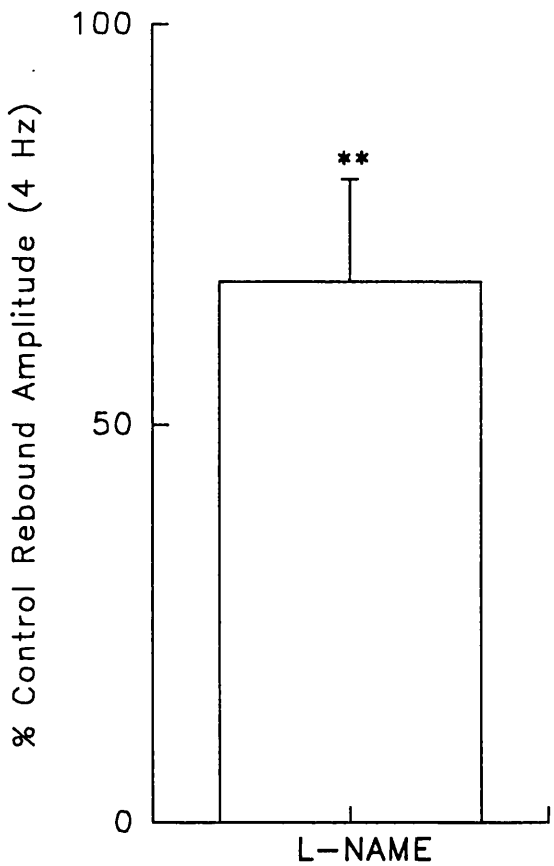


Fig. 20 : The effect (± s.e. mean) of L-NAME (5×10^{-4}M ; n = 15), expressed as a percentage of the control (100%), on the amplitude of the rebound contraction in response to NANC nerve stimulation (16 pulses, 4Hz, 0.1-0.5ms, supramaximal voltage), in carbachol (3×10^{-5}M)-contracted circular strips of rabbit distal colon. L-NAME reduced significantly (**, $P < 0.01$), the amplitude of rebound contraction.

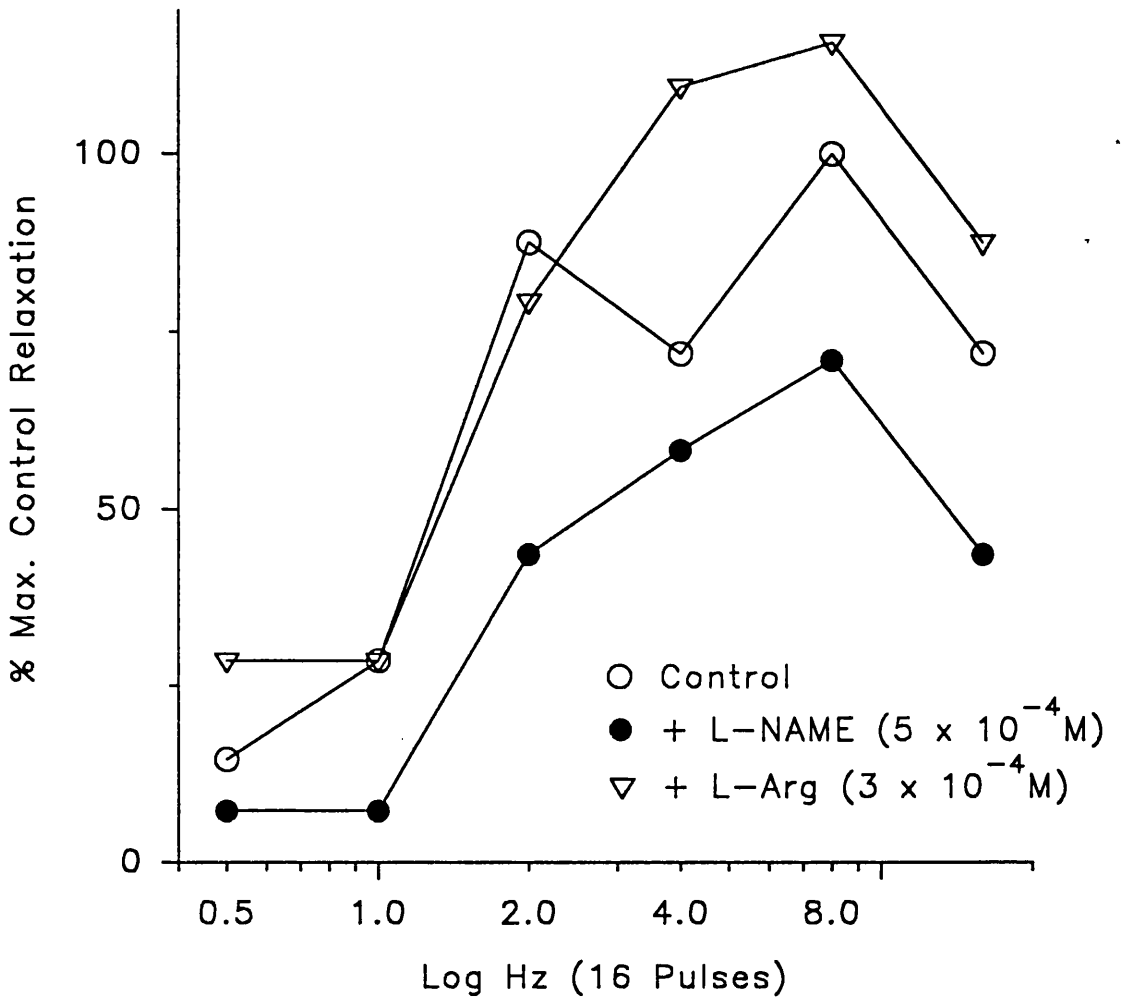


Fig. 21 : The inhibition by L-NAME (●, 5 x 10^{-4}M), expressed as a percentage of the maximum control response (○ , 8 Hz, 100%), of the amplitude (g) of relaxation in response to NANC nerve stimulation (0.5-16 Hz, 0.2ms, supramaximal voltage), in a carbachol (3 x 10^{-5}M)-contracted circular strip of rabbit distal colon, and its reversal by L-arginine (▽, 3 x 10^{-4}M). L-NAME alone reduced the amplitude of relaxation. In the continued presence of L-NAME, L-arginine increased the amplitude of relaxation above control levels, in 2 out of 8 experiments. This graph was plotted from a single experiment.

relaxed the tissue (by 1.1 ± 0.38g), but the onset of this relaxation was much slower, and the duration longer, than that in response to nerve stimulation (results not shown). GABA-induced relaxations (Fig. 17 ; n = 4) were more like those obtained in response to NANC nerve stimulation than were those to VIP. However, GABA induced relaxation only at high concentrations (10^{-4}M) and, overall, the responses were considerably larger (9.05 ± 0.86g ; n = 4) than nerve-evoked relaxations. Thus, ATP, VIP or GABA were unlikely to be involved in NANC nerve responses in this tissue. On a number of occasions, rebound contractions following nerve stimulation (16 pulses, 0.1-0.5ms, supramaximal voltage) were reduced in amplitude by the cyclooxygenase inhibitor, indomethacin (10^{-5}M ; n = 5 ; Fig. 22). This suggested that a product of the cyclooxygenase pathway i.e. a prostaglandin, may have mediated these events.

3.2. Patterns of Spontaneous Activity in the Rabbit Distal Colon

The Golenhofen apparatus was used to examine the spontaneous electrical and mechanical responses in longitudinal strips of the RDC. A number of parameters (see 2.4.) were measured.

Activity in the unstretched (0.1-0.5g tension) colonic strip was generally burst-like in nature (Fig. 9). Each spike-burst was accompanied by a corresponding burst of contraction and periods of relative quiescence existed between individual bursts. These electrical events occurred with a frequency of 3.77 ± 0.18 min^{-1} (n = 80) and each contained 7.84 ± 0.62 spikes (n = 61). The duration of each spike-burst was 7.91 ± 0.77s (n = 57) and the amplitude of the largest spike in each was 248.46 ± 28.37 μV (n = 63). Since the muscle never contracted in the absence of electrical firing, and vice versa, the frequency of contractile bursts was also 3.77 ± 0.18 min^{-1} (n = 80). Each mechanical event

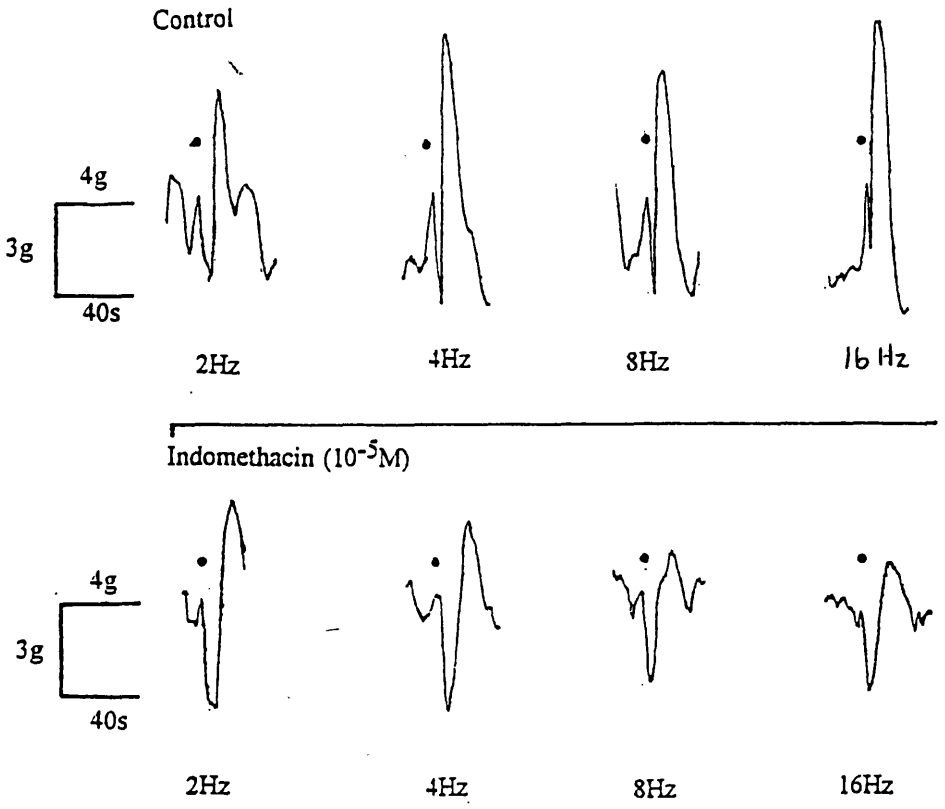


Fig. 22 : Frequency-dependent relaxations and rebound contractions recorded from a carbachol (3 x 10^{-5}M)-contracted circular strip of rabbit distal colon in response to NANC nerve stimulation (•, 16 pulses, 2-16 Hz, 0.2ms, supramaximal voltage) and rebound inhibition by indomethacin (10^{-5}M ; ———).

contained 2.78 ± 0.33 peaks (n = 79) and had a duration of of 12.23 ± 1.42s (n = 79). The maximum tension developed during each was 2.21 ± 0.13g (n = 79).

On rare occasions (< 10%) another pattern of activity was present (Fig. 12). In these cases, the electrical pacemakers did not fire in bursts but were continually active. Consequently, there were as many as 60 contractions in any 4 min period. This made analysis extremely difficult. As a result, experiments were generally conducted on those tissues that exhibited the former, burst-like activity.

As the tissue was stretched from 0-5g, the amplitude of mechanical activity was enhanced ($P < 0.01$; Fig. 23). Under an applied tension of 5g (n = 3) the amplitude of the mean phasic contraction was 5.16 ± 0.81g. This was compared with control values of 2.56 ± 1.15g in the unstretched tissue, or 4.1 ± 0.67g when the tissue was under 3g of stretch. Similarly, from Fig. 23, as the tissue was stretched from 0-3g the frequency of activity and the amplitude of electrical spikes were increased. However, probably because of the small number of samples examined, these actions did not prove to be significant. This enhancement of activity as tension was raised may have been due to a stretch-induced depolarization i.e. more cells would be at, or close to, their firing threshold. However, a further increase in tension to 5g caused the frequency of activity and the maximum spike amplitude to fall. The increased amount of stretch may have led to a form of depolarizing block that resulted in decreased frequency and spike amplitude. Alternatively, stretching could have damaged the tissue - perhaps certain nexuses or close junctions were destroyed - and thus reduced syncytial activity. In most of the following experiments, the unstretched muscle preparation was used.

The RDC was also sensitive to temperature changes (n = 3 ; Fig. 24). As the tissue was cooled from 37°C to 32°C the frequency of activity and the amplitude of electrical spiking was increased. Cooling to 29°C, raised the tone

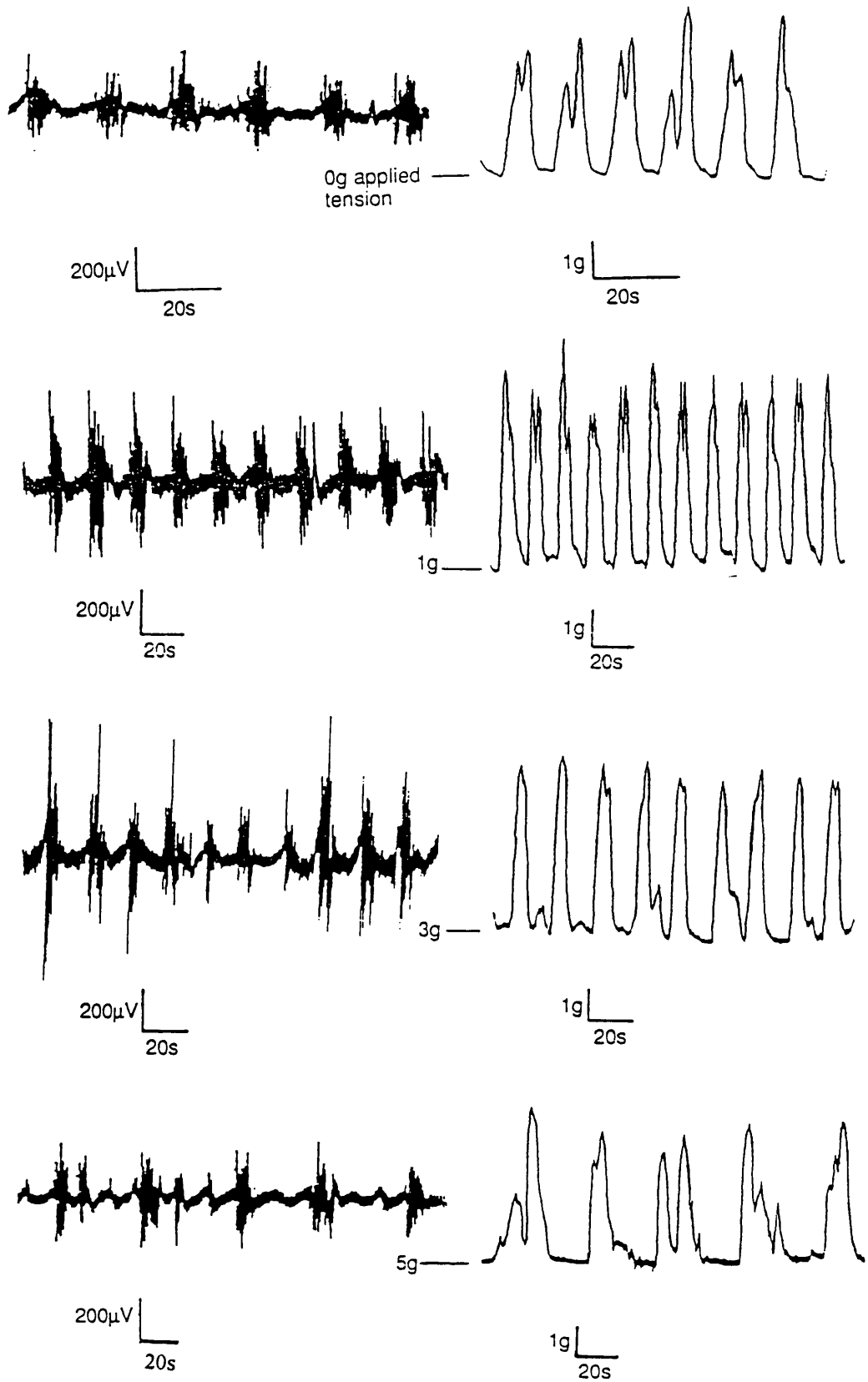


Fig. 23 : The effect of stretch (0-5g) on the spontaneous electrical (left) and corresponding mechanical activity recorded from a single, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. Both the frequency (bursts min^{-1}) and the electrical spike amplitude (mV) were initially enhanced (1-3g) but declined as the tissue was stretched further (5g).

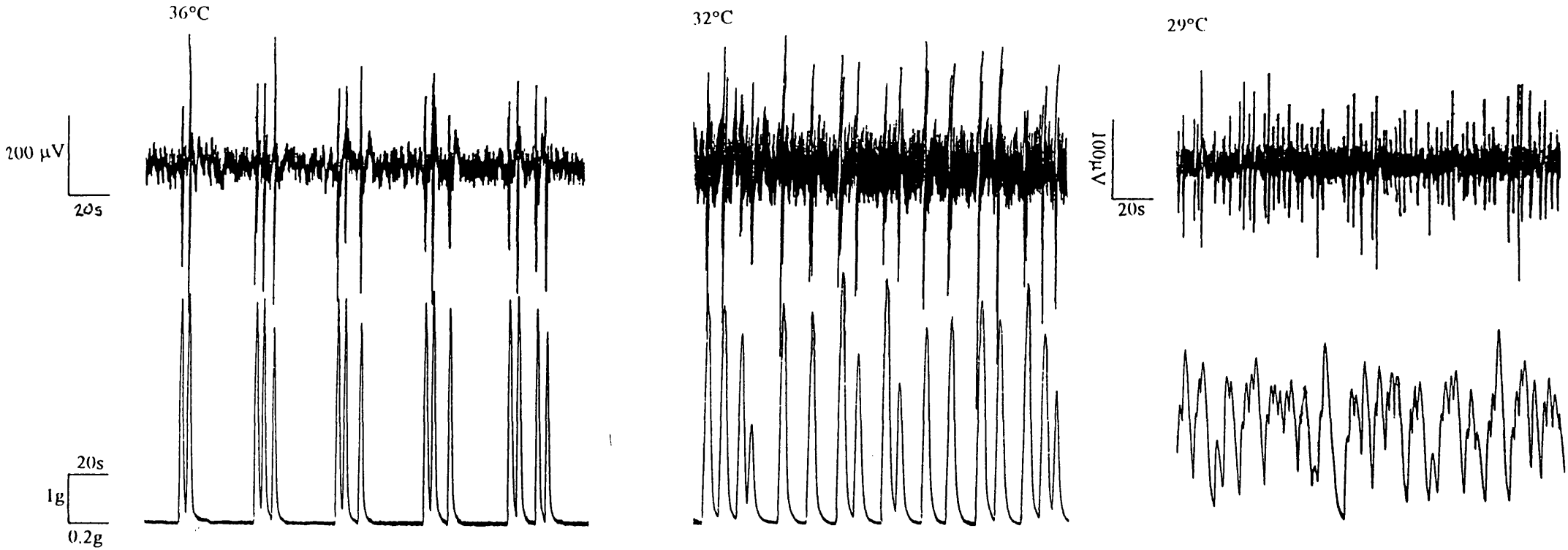


Fig. 24 : The effect of decreases in temperature (from 36°C to 29°C) on the spontaneous electrical (top) and mechanical activity recorded from a single, unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. As the temperature was reduced, the frequency of activity and the resting tone were increased.

of the tissue and caused both spontaneous electrical and mechanical activity to become so rapid that they were continuous, making accurate analysis impossible. All future experiments were conducted at a temperature of 37 ± 1° C.

Electrical, as well as mechanical events in this tissue, were entirely dependent on the presence of extracellular Ca^{++} (Fig. 25). When $MgCl_2$ replaced $CaCl_2$ in the Krebs solution, both spontaneous spiking and contraction were abolished (n = 3). Similarly, the Ca^{++}-channel blocking drug, diltiazem, gradually reduced the frequency of responses until, at a concentration of 10^{-4}M, all activity was abolished (n = 6 ; results not shown). None of the other parameters was significantly affected prior to this abolition of spontaneous activity. TTX failed to abolish responses in the RDC (n = 4 ; results not shown). Thus, pacemaker activity in this tissue relied on the presence of Ca^{++}- rather than Na^{+} -spikes.

3.3. Actions of Nitrovasodilators on the Rabbit Distal Colon

3.3.1. Spontaneous electrical and mechanical responses

The effects of certain nitrovasodilators, namely SNP, GTN and IDN, were examined on spontaneously active, unstretched longitudinal strips of the RDC mounted in the Golenhofen apparatus. The actions of these drugs were both complex and manifold. To further complicate matters, not every effect was produced by every compound. Despite this complexity, a sequence of events has emerged that generally characterizes the nitrovasodilator-induced response.

Each nitrovasodilator tested was able to regulate pacemaker activity to some degree, but SNP was most effective. The primary action of SNP ($\geq 10^{-5}$M) was to induce a short-lived ($\leq$ 10 min) period of quiescence (Fig. 26). Following the addition of SNP (10^{-4}M), the mean duration of this

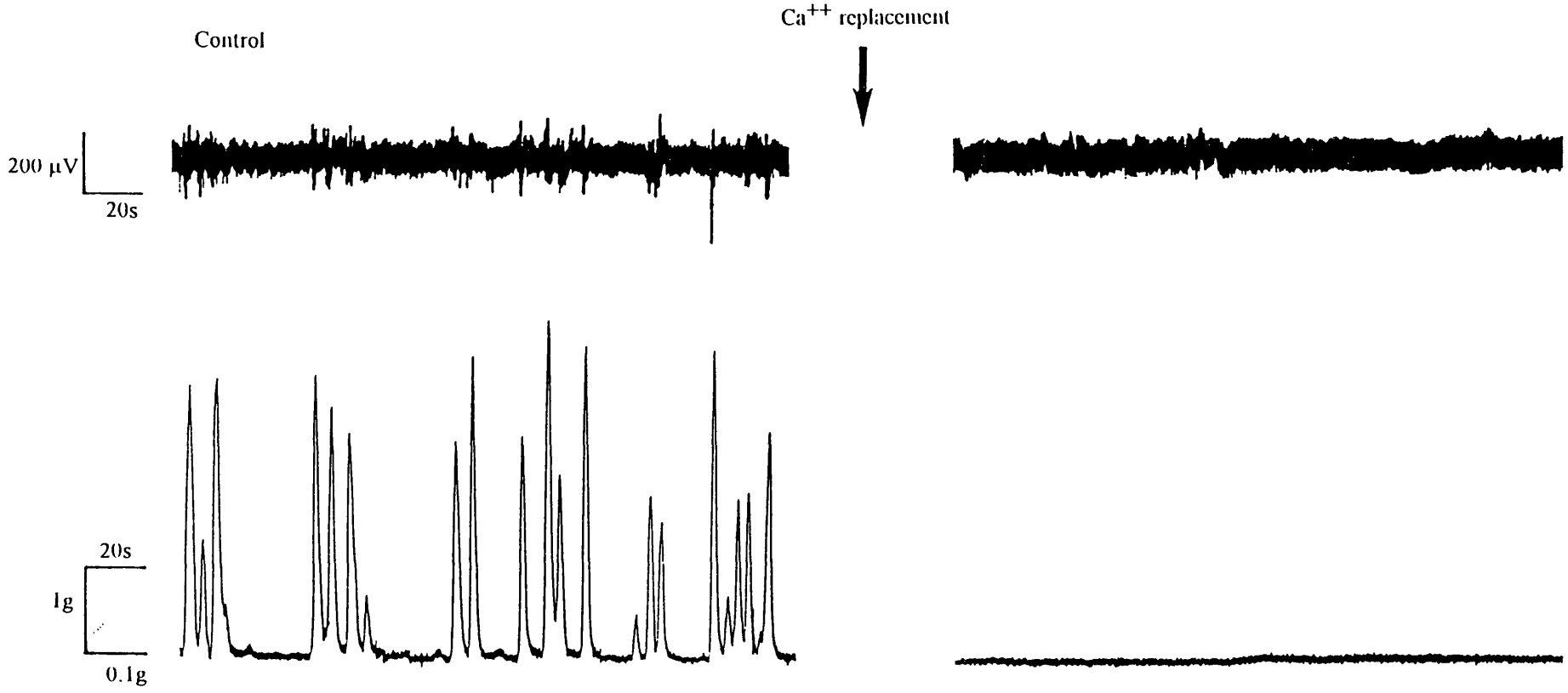


Fig. 25 : The effect of Ca^{++} replacement by Mg^{++} in the Krebs solution, on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. The time between panels was approximately 11 min. Both electrical and mechanical activity were abolished.

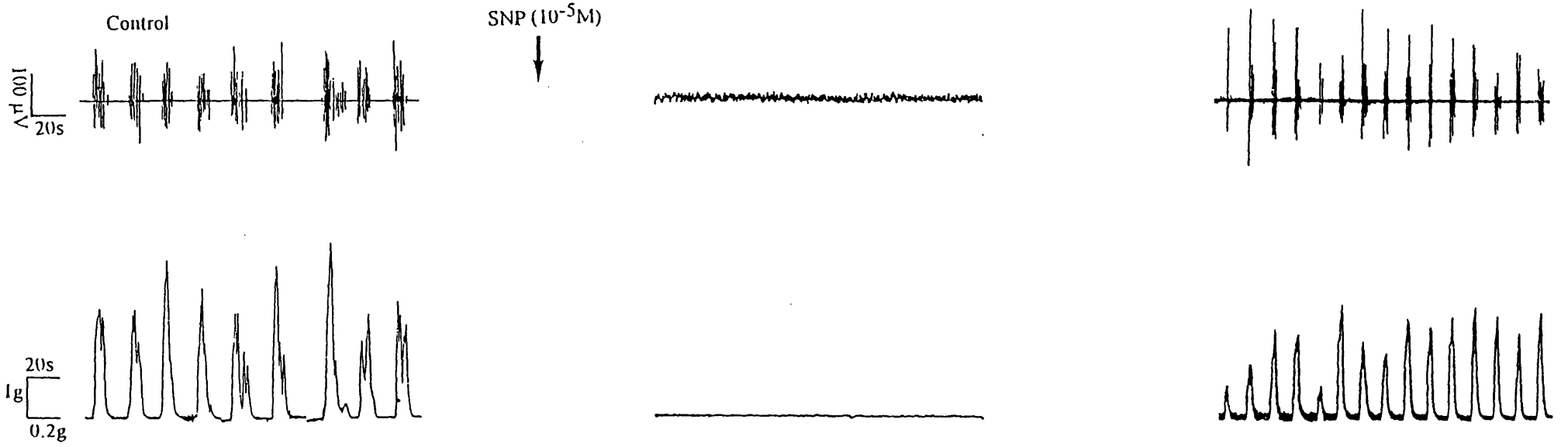


Fig. 26 : Short-lived, SNP (10^{-5}M ; ——)-induced quiescence (centre panel) of the spontaneous electrical (top) and mechanical activity (in each panel) recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. After approximately 2 min (right panel), a more coordinated form of spontaneous activity returned.

period was 2.11 ± 0.61 min (n = 28). This property was shared by both GTN and IDN (each ≥ 10^{-5}M).

At the end of this quiescent period, spontaneous electrical and mechanical activity returned, suddenly, rather than gradually. The pacemaker now was reset to a different level of activity and responses generally had a more coordinated, regular appearance than in the control tissue (Fig. 27). However, the exact effects produced were dependent largely, on the inherent properties of the tissue. For instance, the actions of SNP on the frequency of firing varied according to the initial frequency of activity, before the addition of drug. For this reason, when studying the effects of SNP on this parameter, control tissues were allocated to one of two groups ; those with a low frequency of activity (< 6 bursts min^{-1}) and those with a high frequency of activity (> 6 bursts min^{-1}). In those muscles with a low firing rate, the frequency was significantly enhanced by SNP (≥ 10^{-5}M ; n ≥ 31 ; P < 0.001 ; Fig. 28), without any alteration in the level of baseline tension. The response of a low frequency tissue to SNP (10^{-4}M) can be seen in Fig. 27. In this case, the frequency of activity was increased to 466% of the control value. However, in those muscles where the firing rate was high, the frequency was reduced by SNP (10^{-5}M ; n = 7 ; P < 0.001 ; Figs. 29 and 30). In either case, SNP coordinated the activity so that both electrical and mechanical responses occurred at more regular intervals. Tissues that fell into the high frequency category were rare, and both IDN and GTN were examined only on tissues with a low level of firing. Each of these compounds was capable of imitating the effects of SNP on frequency (Figs. 31 and 32) but, in the case of GTN, this happened only in isolated cases. Overall, GTN (10^{-6}-10^{-4}M), had no effect (n ≥ 5 ; Fig. 28). IDN (≥ 10^{-5}M) significantly increased (P < 0.01) the frequency of spontaneous events in the RDC (n = 7 ; Fig. 28).

Concomitant with this excitatory effect on frequency, the nitrovasodilators had an inhibitory effect on the amplitude of contraction (Figs. 27, 31, 32 and

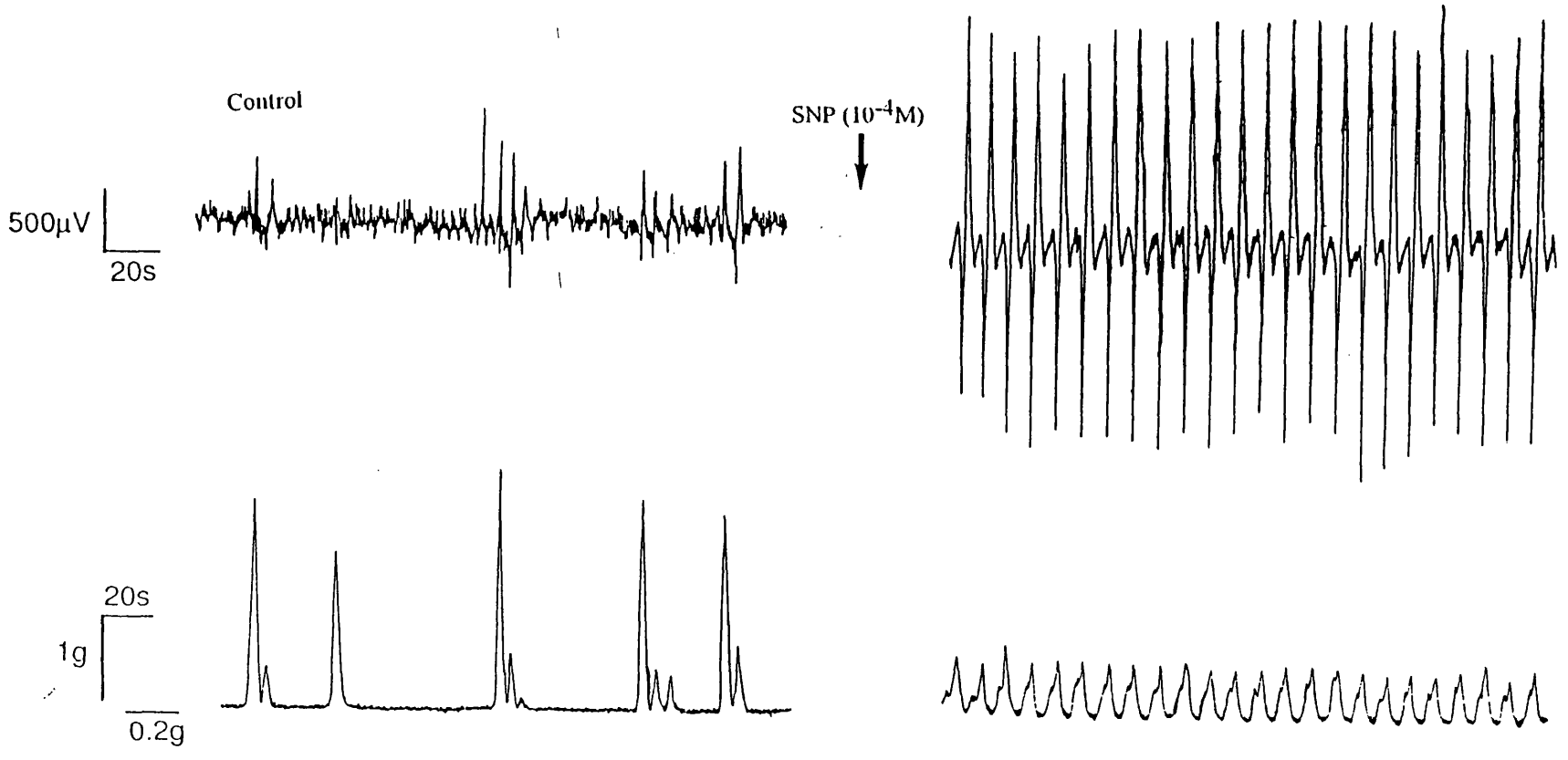


Fig. 27 : The effect of SNP (10^{-4}M) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. SNP increased the frequency of the electrical and mechanical activity and the amplitude of electrical spikes but reduced the amplitude of contraction, the duration of electrical or mechanical events and the number of electrical spikes or mechanical peaks per burst of activity.

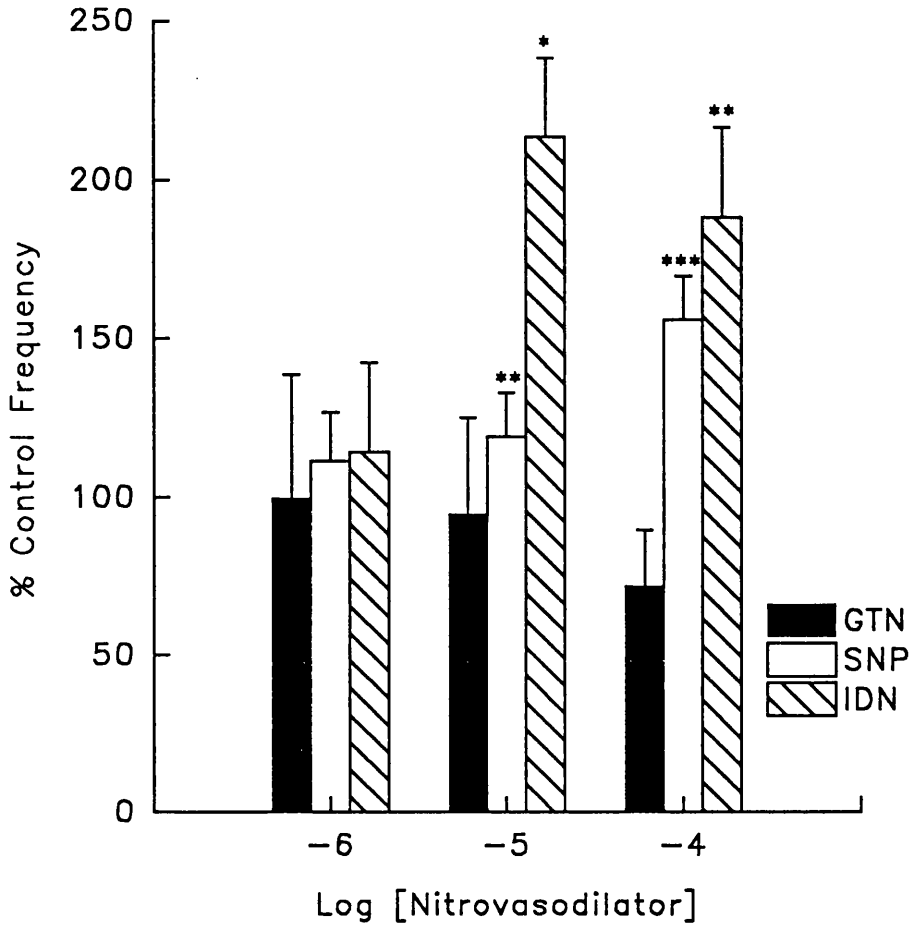


Fig. 28 : The effects (± s.e. mean) of GTN (n = 7), SNP (n ≥ 18) and IDN (n = 6 ; each 10^{-6}–10^{-4}M), expressed as a percentage of the control (100%), on the frequency (bursts min^{-1}) of spontaneous activity, in the rabbit distal colon, where the frequency of control activity was low (< 6 bursts min^{-1}). SNP and IDN each increased significantly (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$), the frequency of activity.

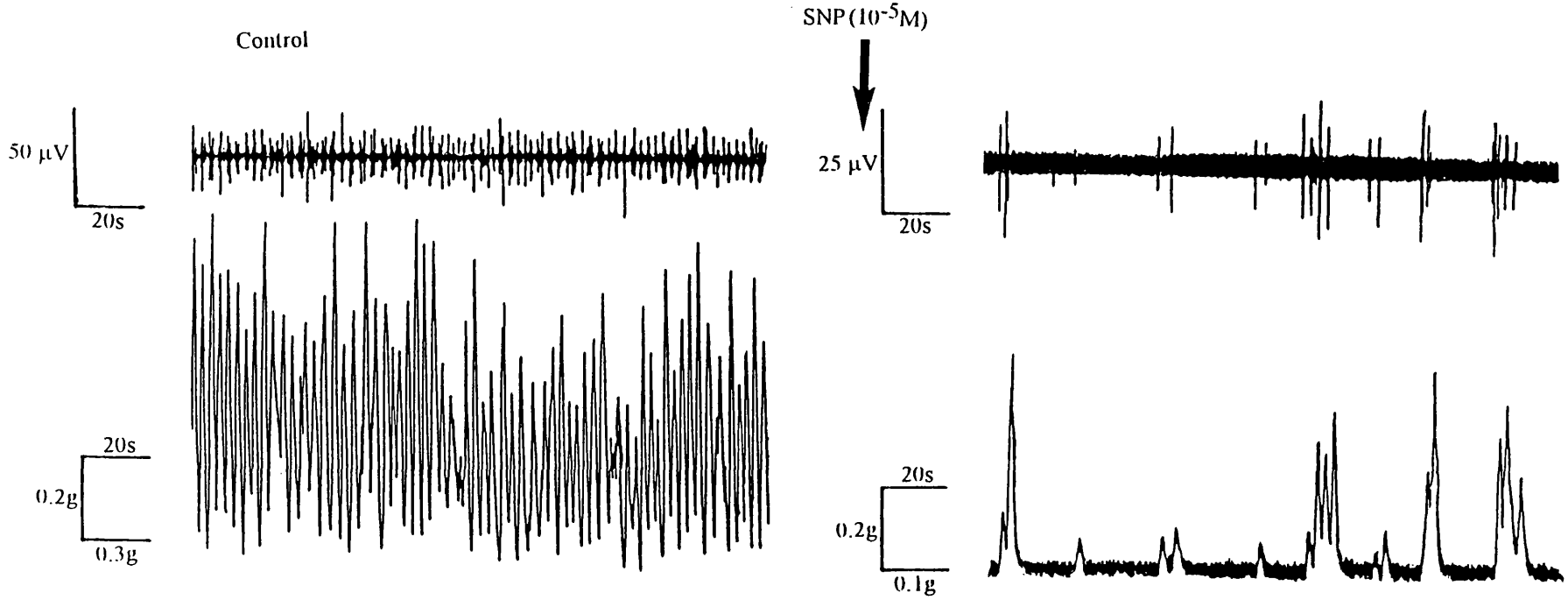


Fig. 29 : The effect of SNP (10^{-5}M) on the spontaneous electrical (top) and mechanical activity of an unstretched, longitudinal strip of rabbit distal colon, in which activity was continuous, in the Golenhofen apparatus. In this case, SNP slowed the frequency of activity and reduced the amplitude of contraction.

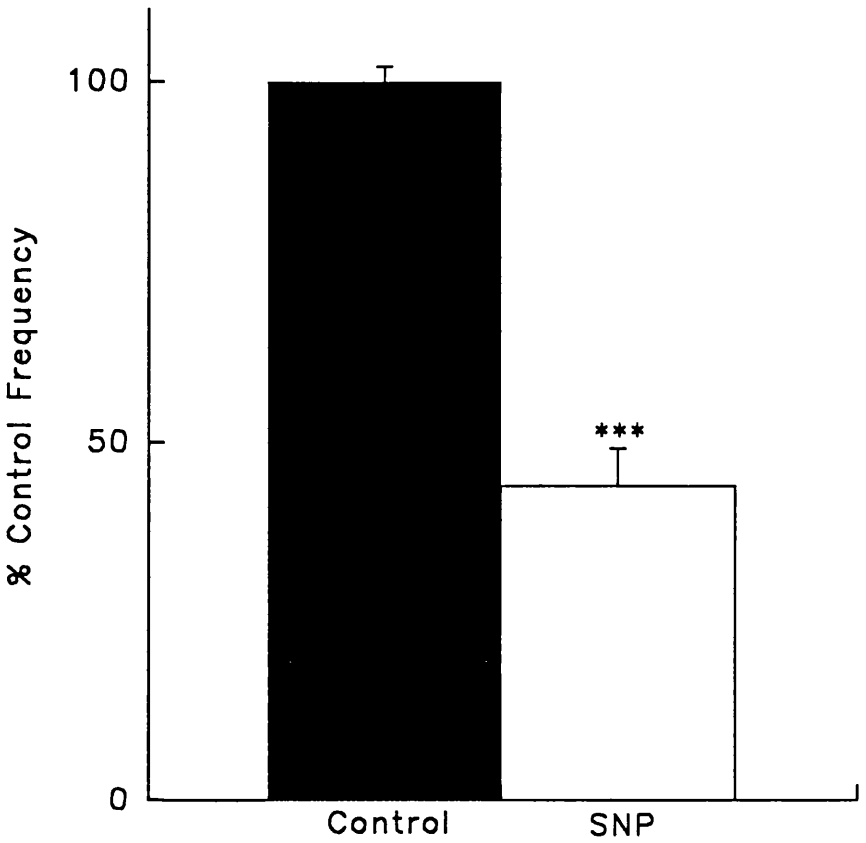


Fig. 30 : The effect (± s.e. mean, n = 7) of SNP (10^{-5}M), expressed as a percentage of the control (100%), on the frequency (bursts min^{-1}) of spontaneous activity, in the rabbit distal colon, where the frequency of control activity was high (> 6 bursts min^{-1}). SNP reduced significantly (***, $P < 0.001$), the frequency of activity.

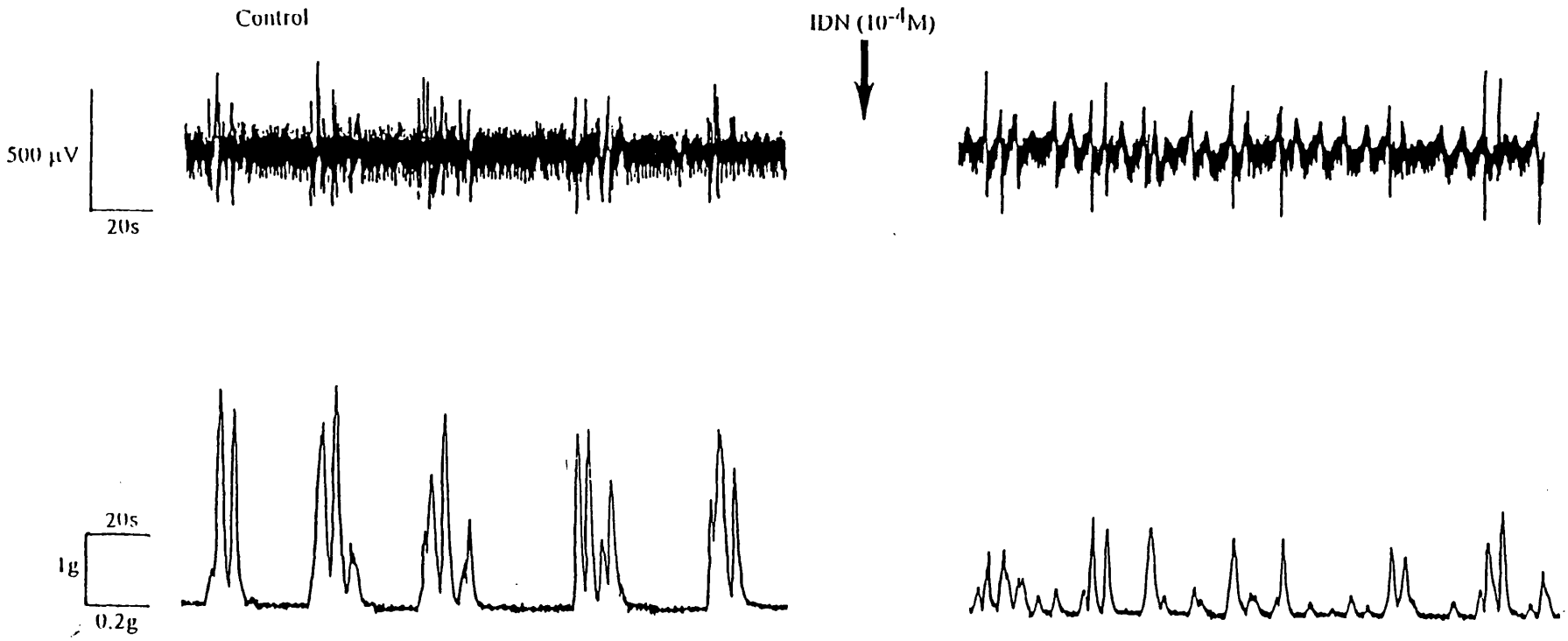


Fig. 31 : The effect of IDN (10^{-4}M) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. IDN increased the frequency of electrical and mechanical activity, but the amplitude of contraction was reduced.

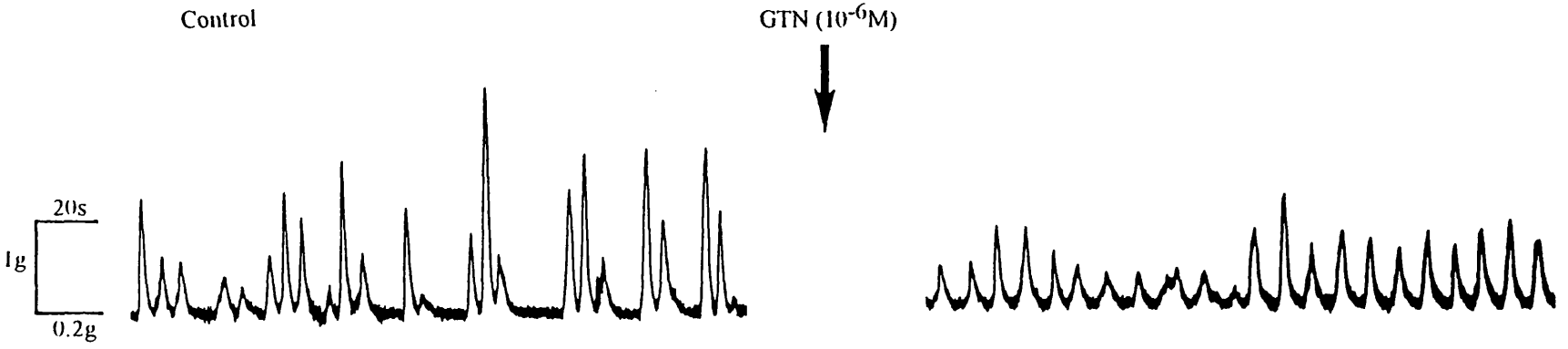


Fig. 32 : The effect of GTN (10^{-6}M) on the spontaneous mechanical activity of an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. The frequency of activity was increased but the amplitude of contraction was reduced.

33). SNP ($\geq 10^{-5}$M ; n $\geq$ 34), IDN ($\geq 10^{-5}$M ; n = 6) and GTN (10^{-5}M ; n = 7) each significantly lowered ($P < 0.05$) the amplitude of spontaneous contraction in longitudinal strips of RDC. However, rarely, at concentrations lower than those required to reduce this parameter, SNP ($\leq 10^{-6}$M) enhanced the amplitude of spontaneous contraction (Fig. 34).

In a number of individual experiments, nitrovasodilators induced a sharp rise in the amplitude of electrical discharge. For example, in Fig. 27, this parameter was increased to 389% of the control value by SNP (10^{-4}M). However, preparations in which SNP produced this effect were rare. Overall, the effect of SNP (10^{-6}-10^{-4}M ; n $\geq$ 13 ; Fig. 35) was not a significant one. Similarly, IDN (10^{-6}-10^{-4}M ; n = 6 ; Fig. 35) did not significantly affect this parameter. GTN (10^{-6}M ; n = 5 ; Fig. 35), however, significantly increased the spike amplitude ($P < 0.05$). In the majority of experiments in which spike amplitude was increased (by any nitrovasodilator), this change occurred simultaneously with the increased frequency and reduced contractile amplitude. But, in a few isolated cases the spike amplitude was increased only after these other changes had occurred (Fig. 36). This suggested that this effect was, in fact, secondary to the increase in frequency and reduction in the amplitude of contraction.

In addition to these effects, SNP (n = 25) and IDN (n = 6 ; each $\geq 10^{-5}$M), significantly reduced the number of electrical spikes per burst ($P < 0.05$; Fig. 37). In the most susceptible tissues, electrical discharges now consisted only of single spikes (Fig. 27) though, for the purpose of this study, these events were still regarded as bursts of activity (see 2.4.). In less susceptible tissues, the frequency was increased but, although the number of spikes per burst was reduced, single spikes did not occur (Fig. 38). This reduction in the number of spikes per burst led to a concomitant reduction in the mean duration of electrical bursts by SNP ($\geq 10^{-5}$M ; n $\geq$ 24 ; $P < 0.001$) and IDN (10^{-4}M ;

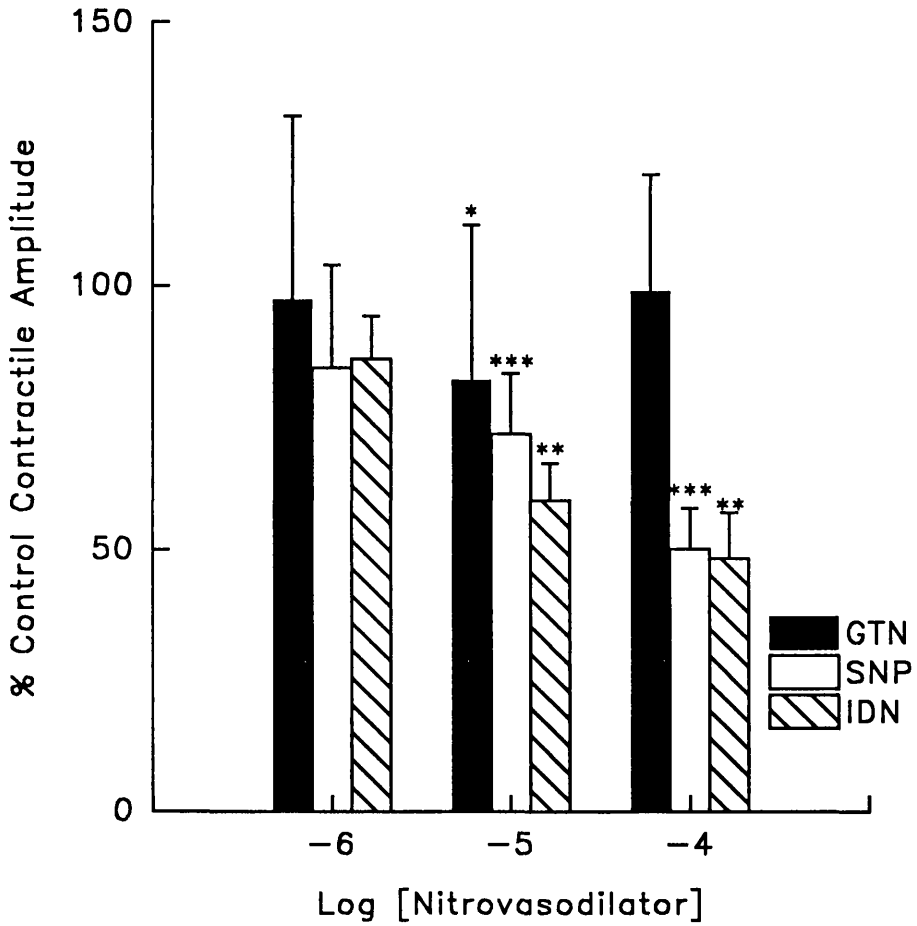


Fig. 33 : The effects (± s.e. mean) of GTN (n = 7), SNP (n ≥18) and IDN (n = 6 ; each 10^{-6}–10^{-4}M), expressed as a percentage of the control (100%), on the maximum contractile amplitude (g) per burst of mechanical activity, in the rabbit distal colon. GTN, SNP and IDN each reduced significantly (*, P < 0.05 ; **, P < 0.01 ; ***, P < 0. 001), the maximum contractile amplitude.

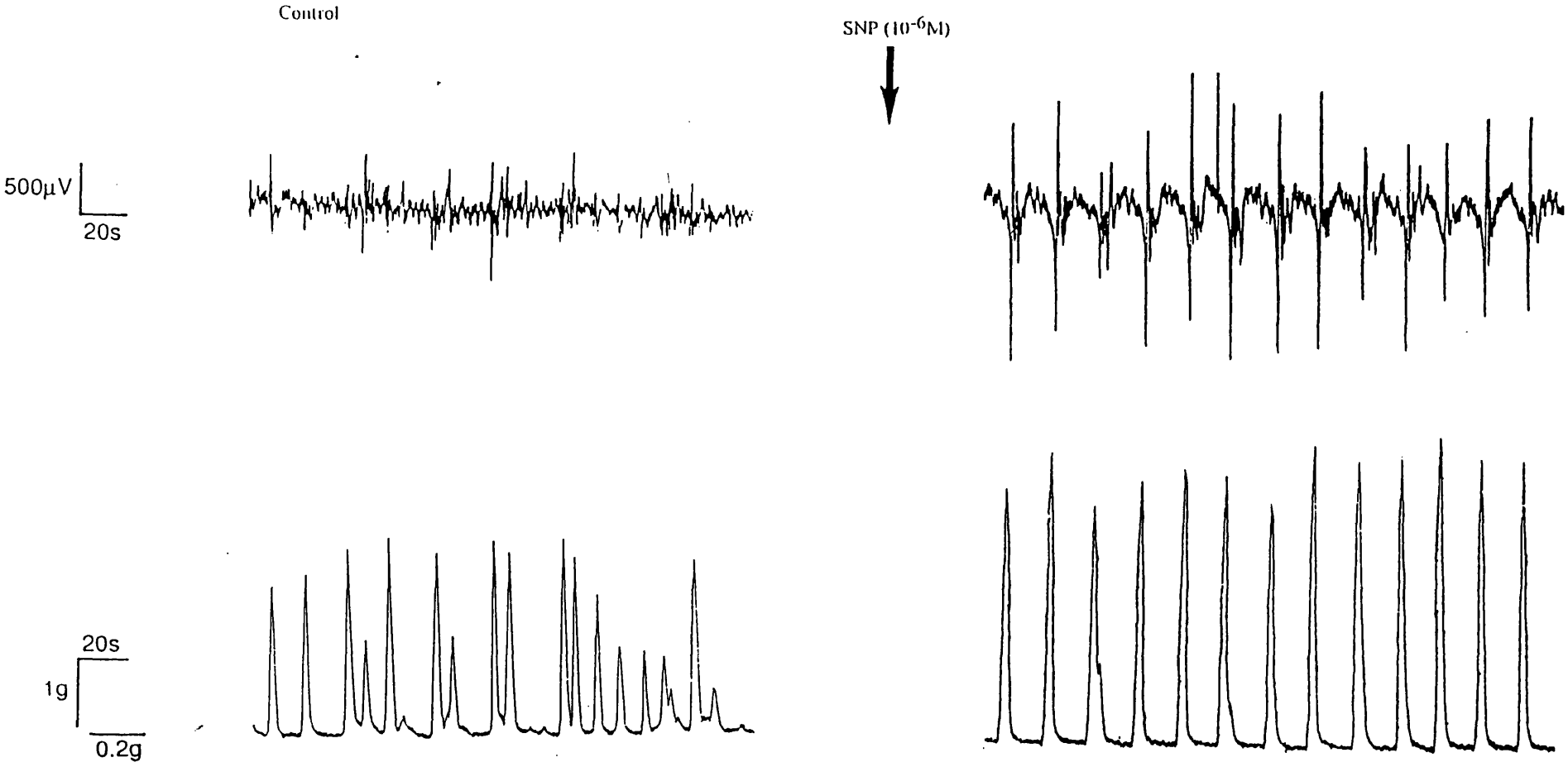


Fig. 34 : The effect of SNP (10^{-6}M) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. SNP coordinated and enhanced the amplitude of both electrical and mechanical activity.

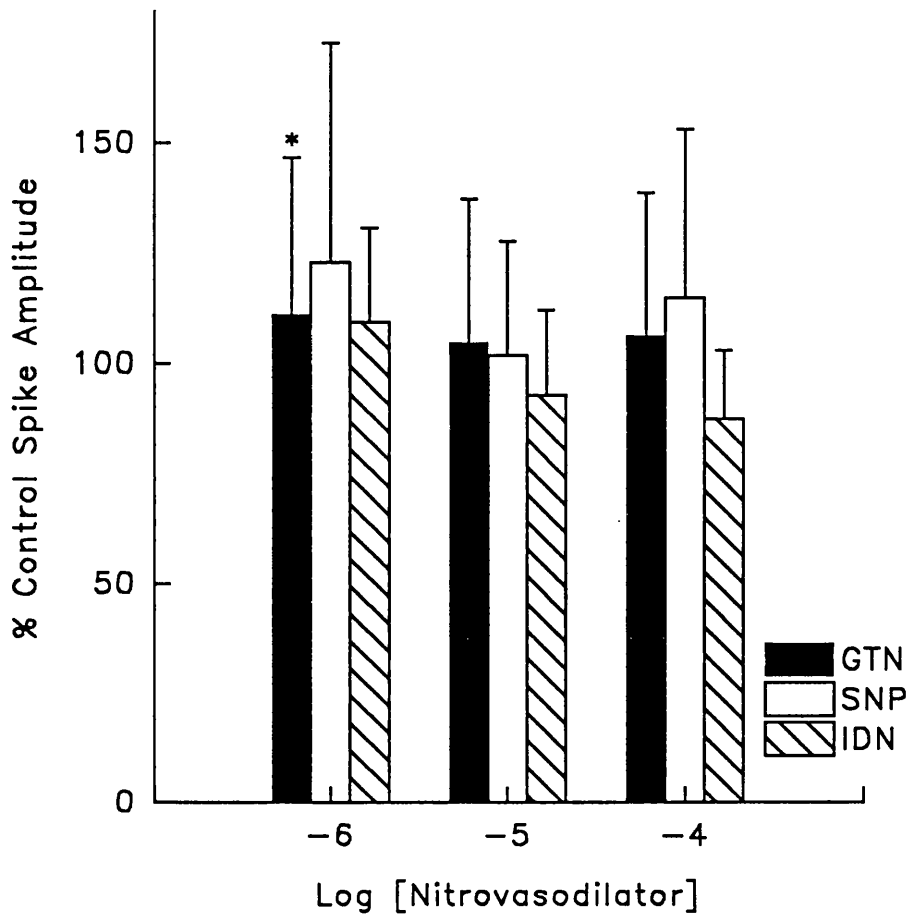


Fig. 35: The effects (± s.e. mean) of GTN (n ≥ 5), SNP (n ≥ 13) and IDN (n = 6 ; each 10^{-6}–10^{-4}M), expressed as a percentage of the control (100%), on the maximum spike amplitude (mV) per burst of electrical activity, in the rabbit distal colon. GTN increased significantly (*, $P < 0.05$), the maximum spike amplitude.

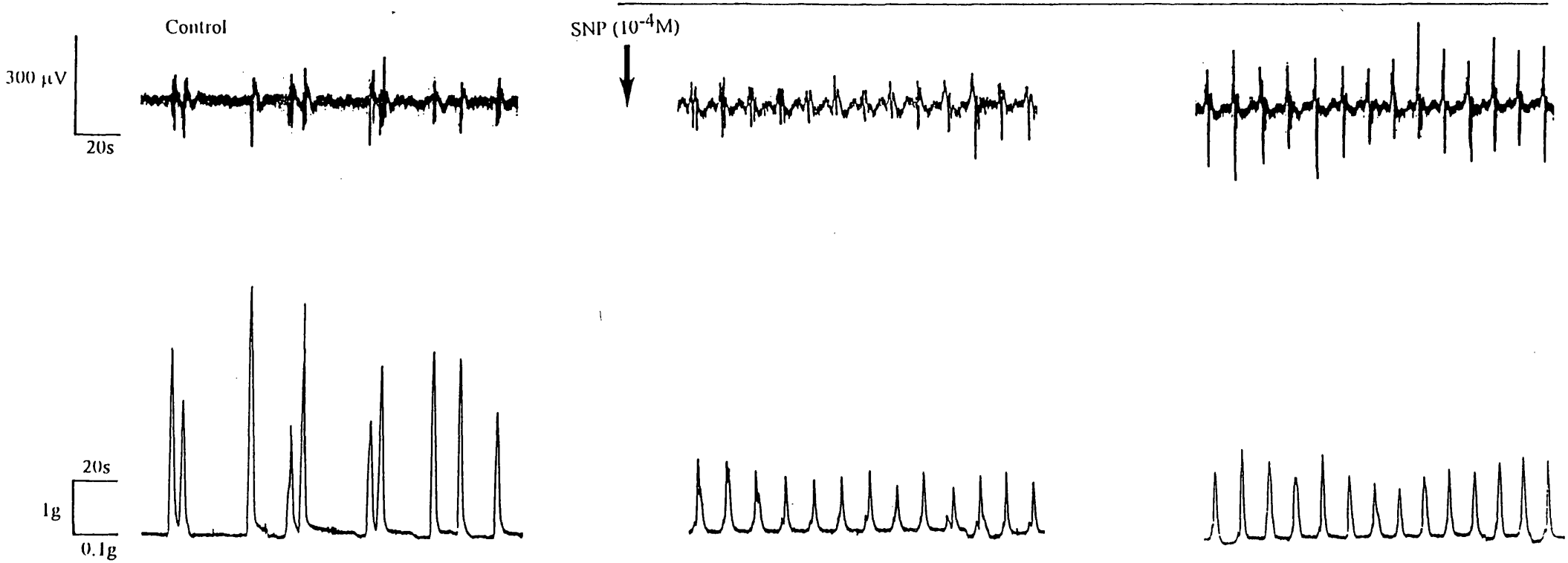


Fig. 36 : The effect of SNP (10^{-4}M ;———) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. After approximately 2 min (centre panel), the frequency of both electrical and mechanical activity was increased and the amplitude of contraction was reduced. After approximately 7 min (right panel), these effects persisted but the amplitude of electrical spiking was also increased.

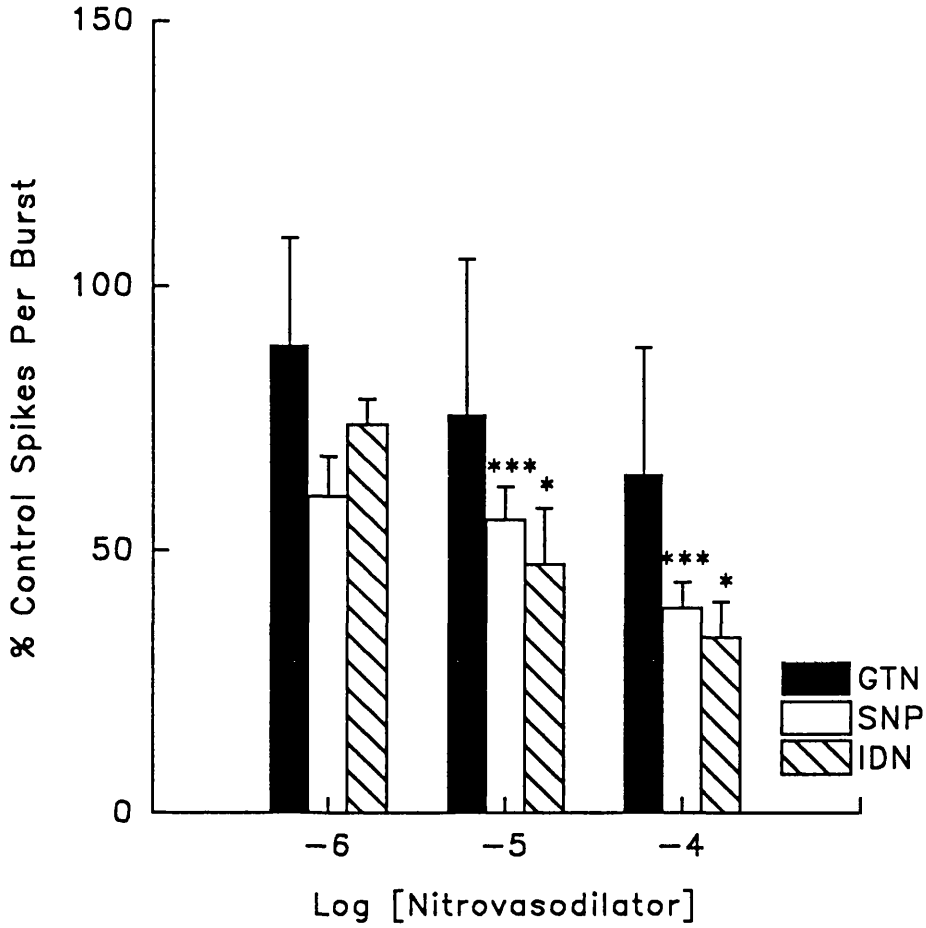


Fig. 37 : The effects (± s.e. mean) of GTN (n = 5), SNP (n ≥ 12) and IDN (n = 6 ; each 10^{-8}–10^{-4}M), expressed as a percentage of the control (100%), on the number of spikes per burst of electrical activity, in the rabbit distal colon. SNP and IDN each reduced significantly (*, P < 0.05 ; ***, P < 0.001), the number of spikes per burst.

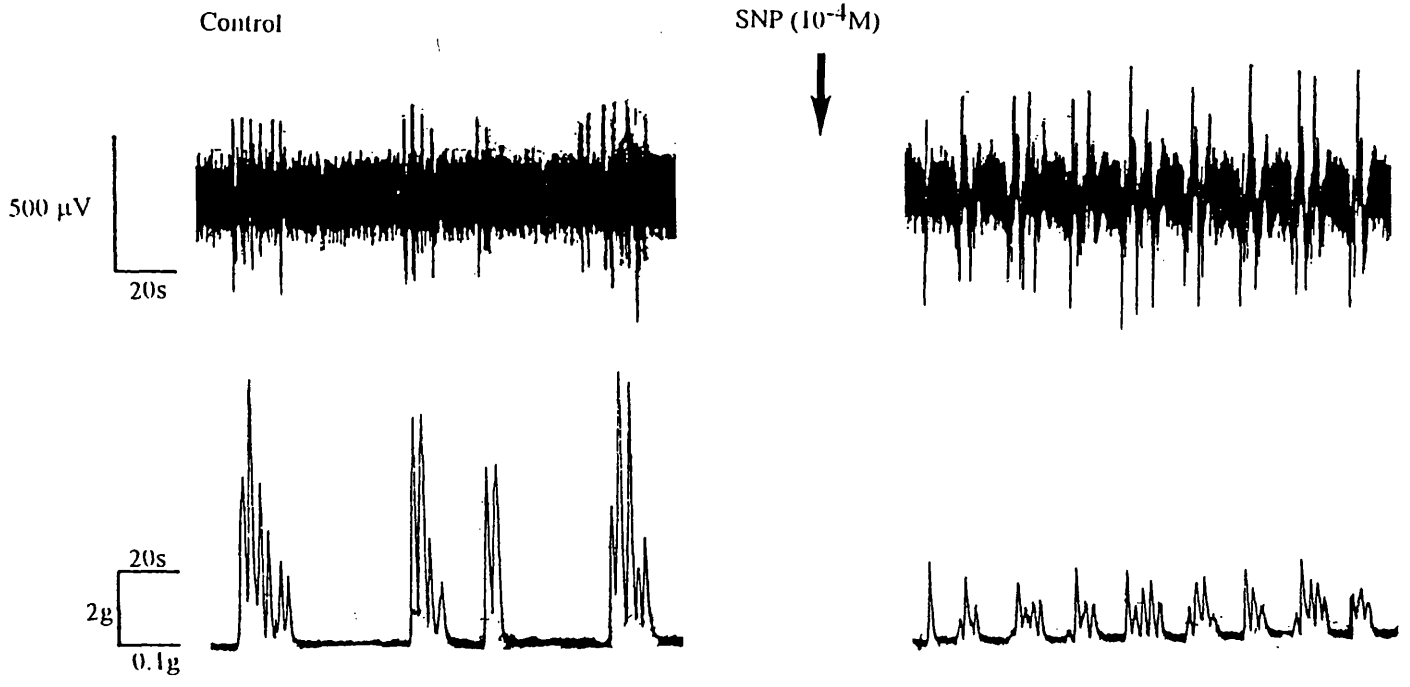


Fig. 38 : The effect of SNP (10^{-4}M) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. SNP increased the frequency of both electrical and mechanical activity and reduced the amplitude of contraction. The number of spikes per burst of electrical activity was reduced but single spikes did not occur.

$n \geq 5$; $P < 0.05$; each Fig. 39). GTN (10^{-6}-10^{-4}M ; $n \geq 5$; Figs. 37 and 39) had no effect on either of these parameters.

The reduction in the number of spikes per electrical burst corresponded, in the case of SNP ($\geq 10^{-6}$M ; $n \geq 32$; Fig. 40), with a significant reduction in the number of peaks in each mechanical event ($P < 0.001$). Consequently, the mean duration of mechanical responses was significantly reduced by SNP ($\geq 10^{-6}$M ; $n \geq 18$; $P < 0.001$; Fig. 41). IDN did not have a significant effect on the number of peaks in each burst of contraction (10^{-6}-10^{-4}M ; $n = 7$; Fig. 40), though there was a downward trend as the concentration of drug increased, but did significantly reduce the duration of mechanical responses ($\geq 10^{-5}$M ; $n = 6$; $P < 0.05$; Fig. 41). GTN (10^{-6}-10^{-4}M ; $n \geq 5$; Figs. 40 and 41) had no effect on either of these parameters. All of these changes attributed to an action of the nitrovasodilators, lasted for the full length of the recording period of up to 20 min.

Clearly, in RDC, the nitrovasodilators produced excitation e.g. an increase in frequency or spike amplitude, in addition to their expected inhibition. In the gut, the main excitatory neurotransmitter is ACh. Experiments were conducted to investigate the possibility that the nitrovasodilators were stimulating the release of ACh, leading to excitation. Since SNP was most effective, in all subsequent experiments on RDC, the effects of this compound were used as a model for the effects of the nitrovasodilators in general. The pattern of activity in response to SNP differed from that in response to the excitatory neurotransmitter, ACh. ACh (5×10^{-8}M ; $n = 3$) induced continuous spiking and enhanced the amplitude and frequency of phasic contraction. But, in contrast to the actions of SNP, in the presence of this agonist, the responses were less regular than controls. ACh ($\geq 10^{-7}$M ; $n = 4$) induced a rise in tone (Fig. 42). Spiking remained continuous, tone was maintained, and the frequency and amplitude of mechanical activity was increased. Atropine ($\geq 10^{-6}$M) prevented these actions of ACh, indicating that they were mediated

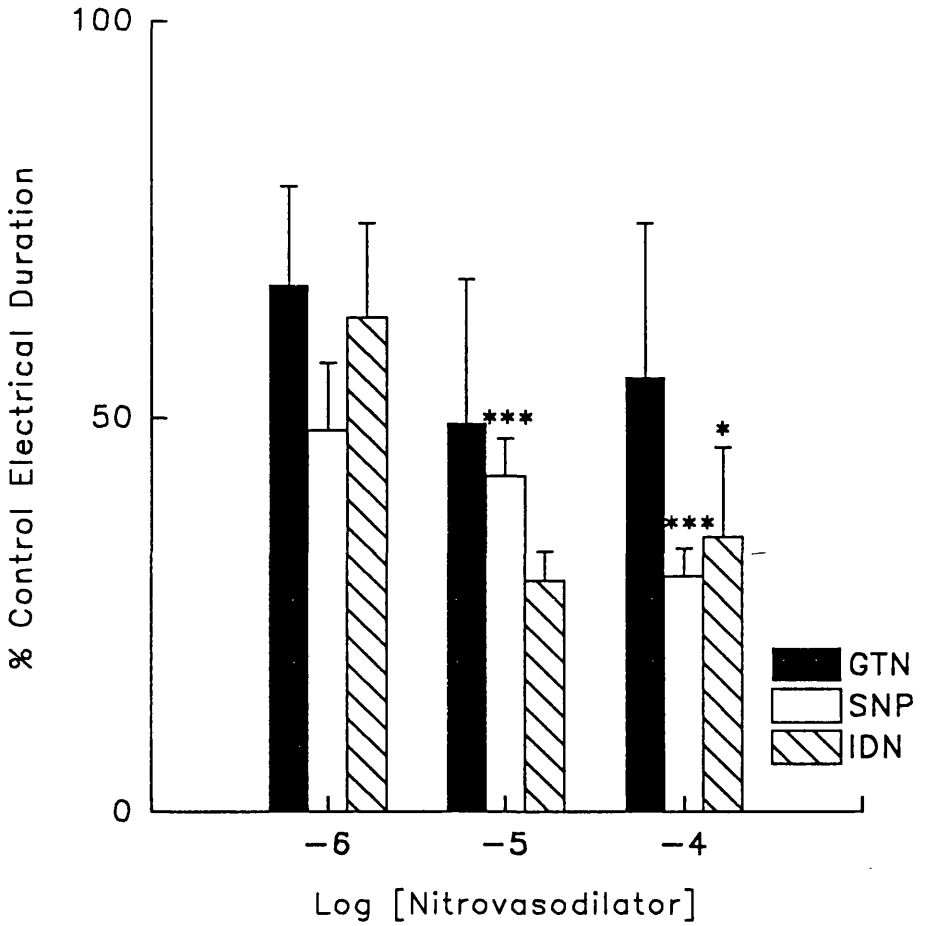


Fig. 39 : The effects (± s.e. mean) of GTN (n = 5), SNP (n ≥ 12) and IDN (n = 5 ; each 10^{-6}–10^{-4}M), expressed as a percentage of the control (100%), on the duration (s) of each burst of electrical activity, in the rabbit distal colon. SNP and IDN each reduced significantly (*, $P < 0.05$; ***, $P < 0.001$), the duration of each burst.

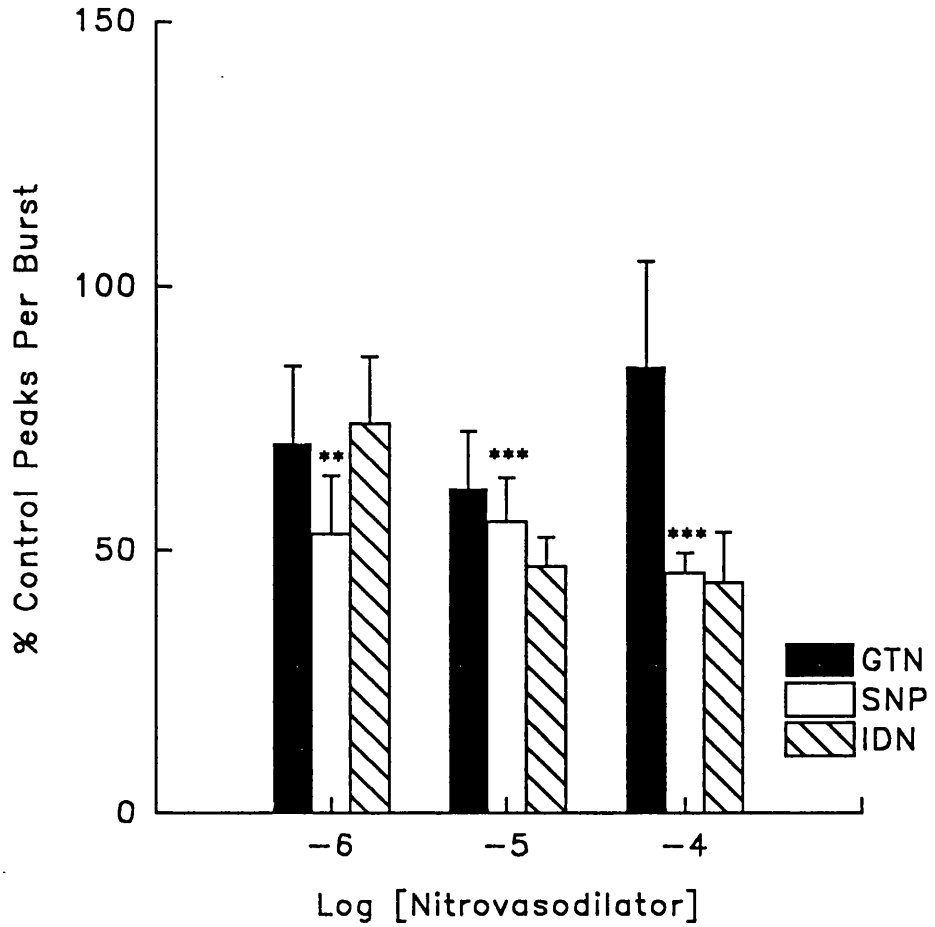


Fig. 40 : The effects (± s.e. mean) of GTN (n ≥ 5), SNP (n ≥ 18) and IDN (n = 6 ; each 10^{-6}–10^{-4}M), expressed as a percentage of the control (100%), on the number of peaks per burst of mechanical activity, in the rabbit distal colon. SNP reduced significantly (**, P < 0.01 ; ***, P < 0.001), the number of peaks per burst.

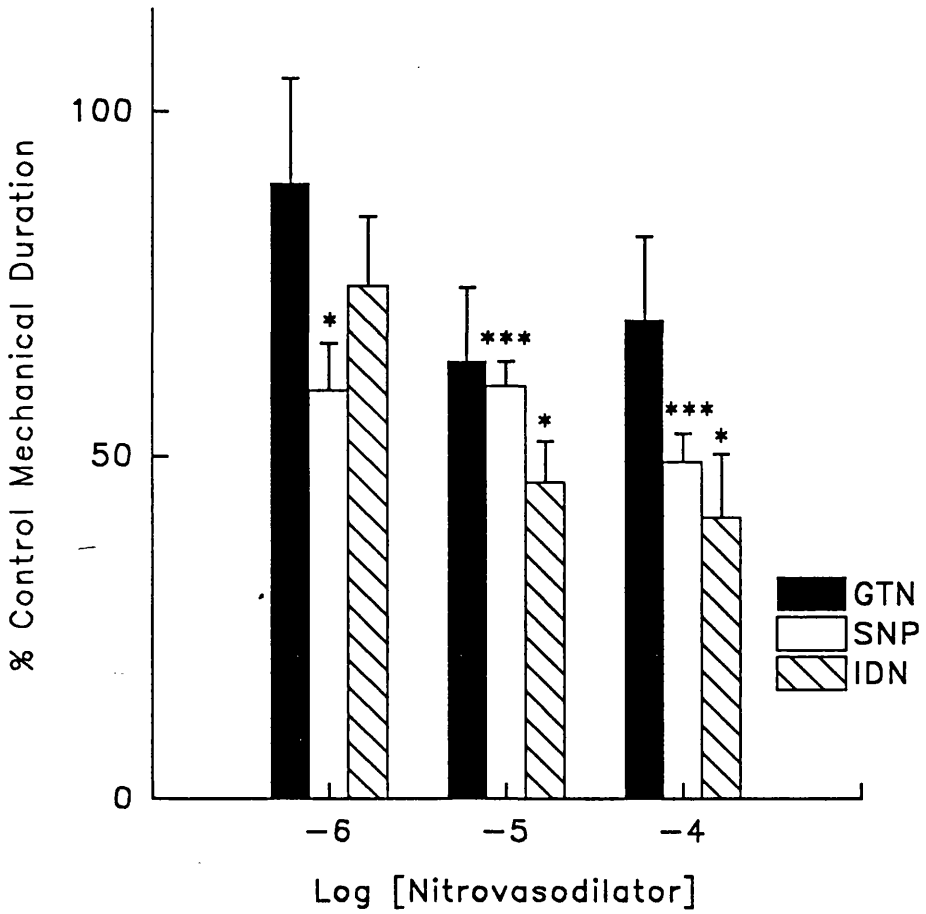


Fig. 41 ; The effects (± s.e. mean) of GTN (n ≥ 6), SNP (n ≥ 18) and IDN (n = 6 ; each 10^{-8}–10^{-4}M), expressed as a percentage of the control (100%), on the duration (s) of each burst of mechanical activity, in the rabbit distal colon. SNP and IDN each reduced significantly (*, $P < 0.05$; ***, $P < 0.001$), the duration of each burst.

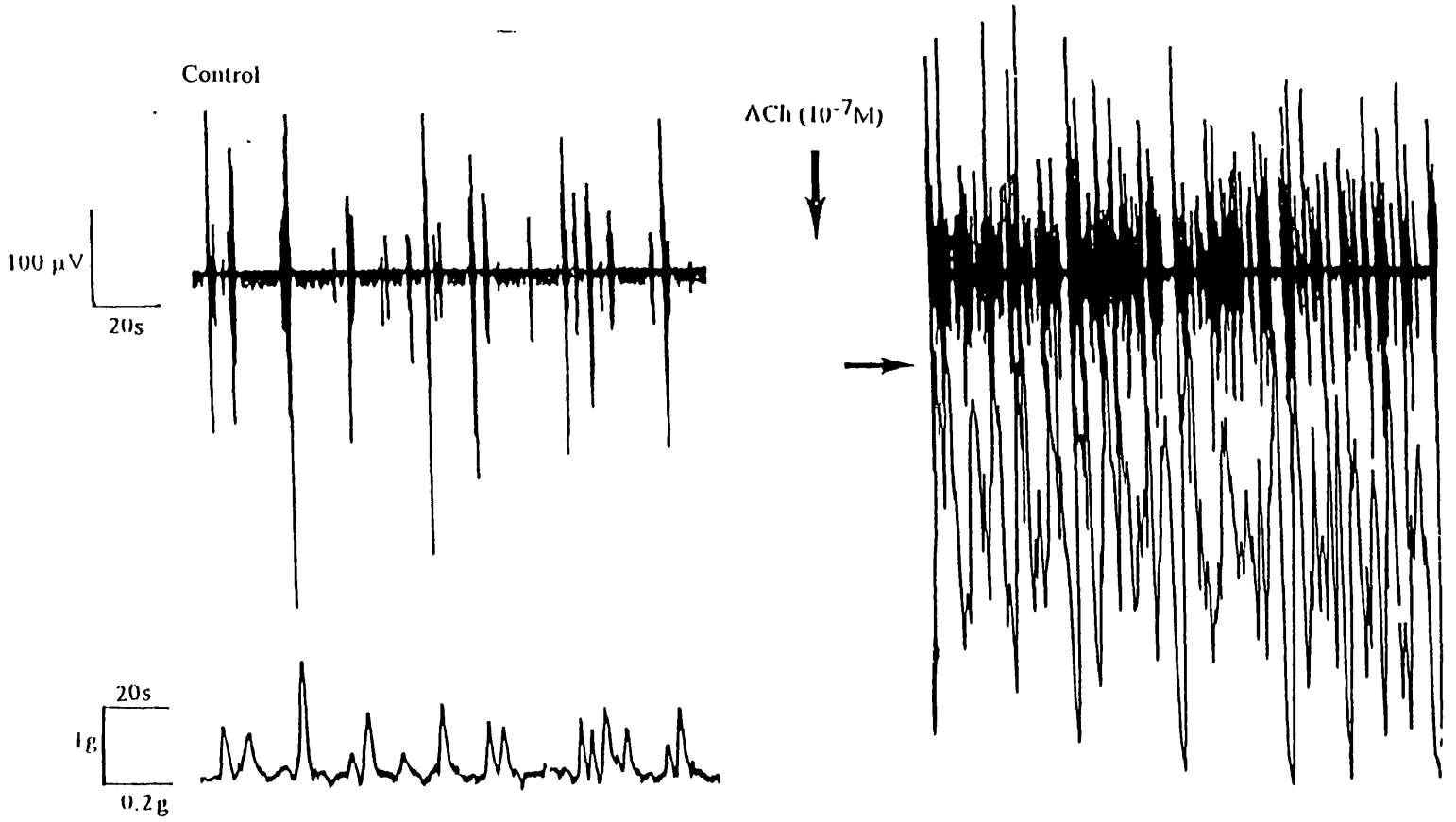


Fig. 42 : The effect of ACh (10^{-7}M) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. ACh raised the tone and caused both electrical and mechanical activity to become continuous. The horizontal arrow (right panel) indicates, approximately, the division between the electrical and mechanical traces.

by muscarinic receptors (results not shown). SNP never raised tone in this preparation nor was its actions inhibited by atropine (n = 3).

An alternative possibility was, that the excitatory properties of the nitrovasodilators were due to antagonism of the inhibitory neurotransmitter, noradrenaline. The α- and β-adrenoceptor antagonists, phentolamine and propranolol, were examined together on the spontaneous activity of RDC. In the presence of these antagonists (each 10^{-5}M) the frequency of activity and the contractile amplitude were enhanced (Fig. 43). Spontaneous activity was desynchronized, became continuous and, on some occasions, baseline tension was slightly increased. This suggested that a basal release of noradrenaline could have moderated pacemaker activity and controlled the level of resting tension. However, although SNP ($\geq 10^{-5}$M ; $n \geq 31$) increased frequency, activity was still burst-like and was more regular than in control tissue. Moreover, even at high concentrations, SNP never elevated baseline tension ; rather than antagonizing the release of inhibitory neurotransmitter it seems more likely that SNP was acting on the pacemaker itself.

The smooth muscle relaxant properties of nitrovasodilators are known to be mediated through NO release and subsequent cGMP stimulation (see Feelisch, 1991 ; Moncada *et al*, 1991b). The possibility that the excitatory and/or inhibitory actions of these compounds on RDC were also mediated via this mechanism was investigated. The rise in frequency ($P < 0.05$) and the fall in contractile amplitude ($P < 0.05$) induced by SNP (10^{-4}M), were completely inhibited by the inhibitor of NO, Hb (10^{-4}M ; n = 4 ; Figs. 44, 45 and 46). Hb (10^{-4}M) itself caused a rise in the frequency of spontaneous activity (Fig. 44) ; in its presence, SNP (10^{-4}M) then caused a fall in frequency, the opposite effect to that of SNP alone. Moreover, in the presence of Hb (10^{-4}M), SNP (10^{-4}M) produced a slight rise in contractile amplitude, yet SNP alone elicited a fall in amplitude. Thus, both the excitatory and inhibitory effects of SNP on RDC were opposed by Hb, suggesting that both were due to NO release. The

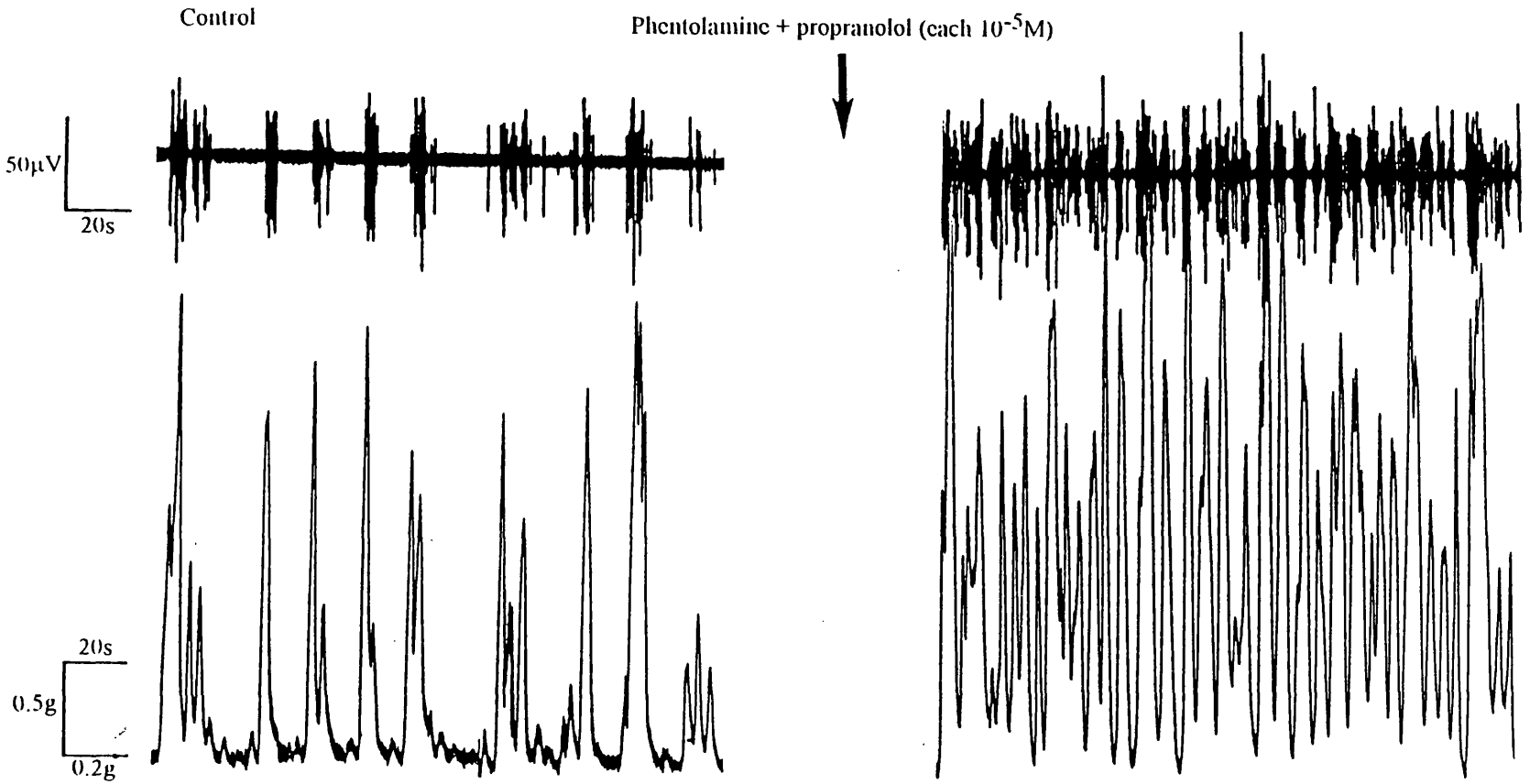


Fig. 43 : The effect of phentolamine and propranolol, together (each 10^{-5}M), on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. Phentolamine and propranolol caused both electrical and mechanical activity to become continuous.

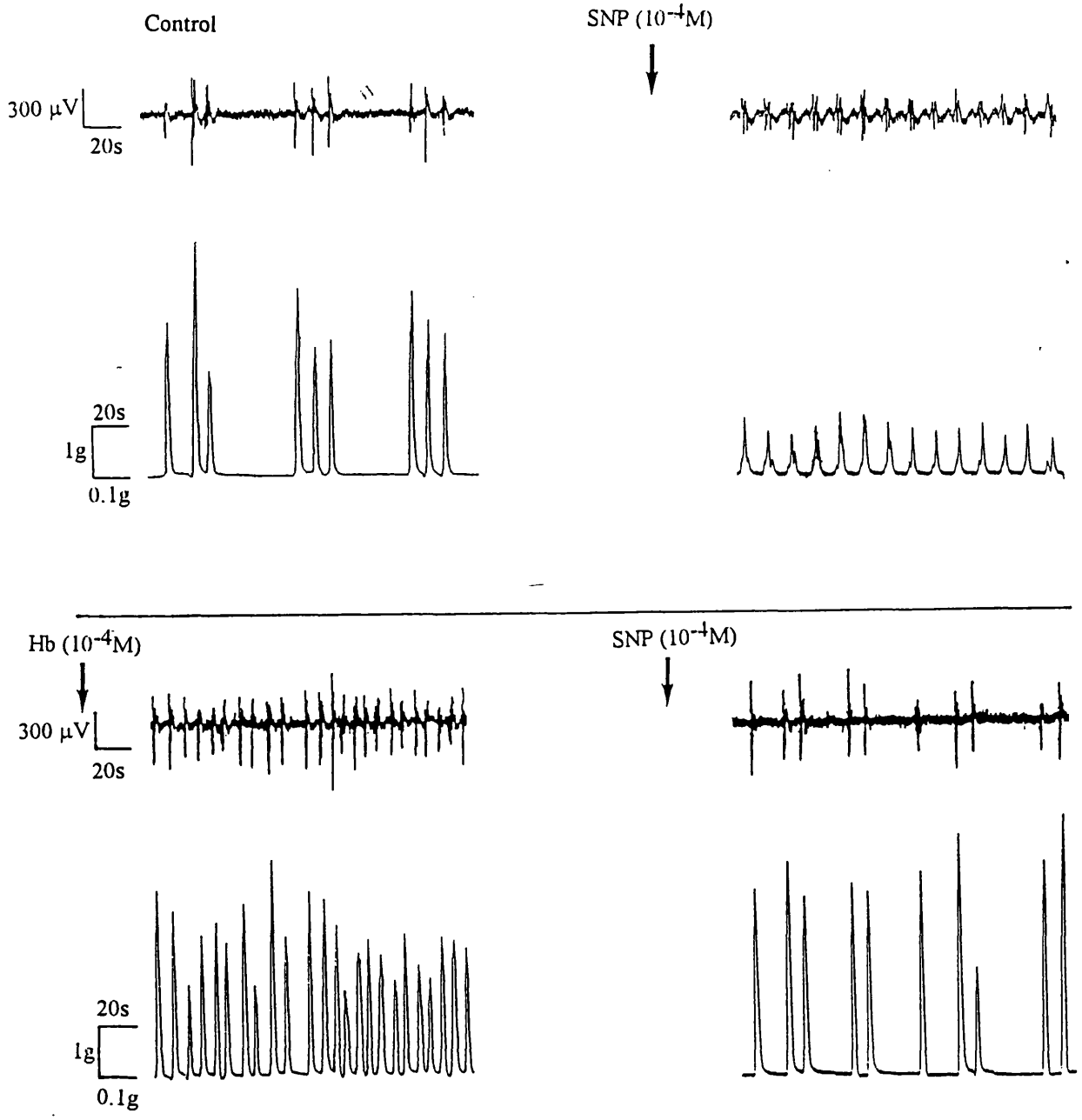


Fig. 44 : The effect of SNP (10^{-4}M) on the spontaneous electrical (top) and mechanical activity (in each panel) recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus, and its inhibition by the prior addition (approximately 10 min before) of Hb (10^{-4}M ;———). SNP alone (top right panel) increased the frequency of activity and reduced the amplitude of contraction. Following the washout of SNP, Hb alone (bottom left panel) also increased the frequency of activity. SNP in the continued presence of Hb (bottom right panel) caused a fall in frequency and an increase in the amplitude of contraction.

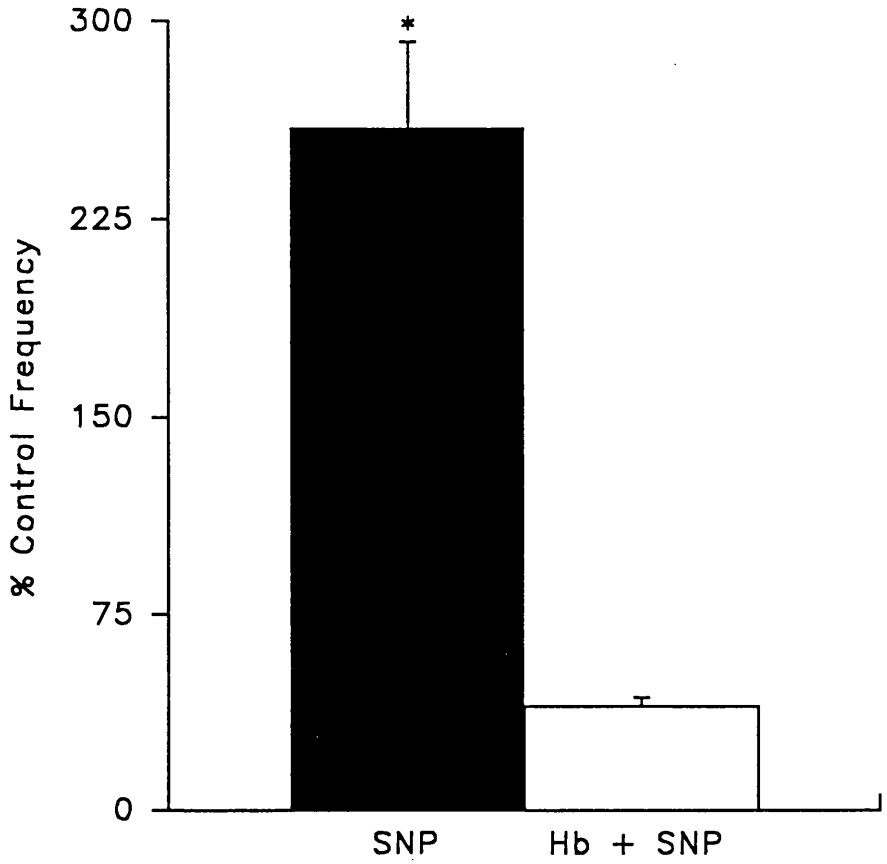


Fig. 45 : The effect (± s.e. mean, n = 4) of SNP (10^{-4}M), expressed as a percentage of the control (100%), on the frequency (bursts min^{-1}) of spontaneous activity, in the rabbit distal colon, where the frequency of control activity was low (< 6 bursts min^{-1}), in the absence and in the presence of Hb (10^{-4}M). Hb abolished the significant excitatory response (*, $P < 0.05$) of the tissue to SNP.

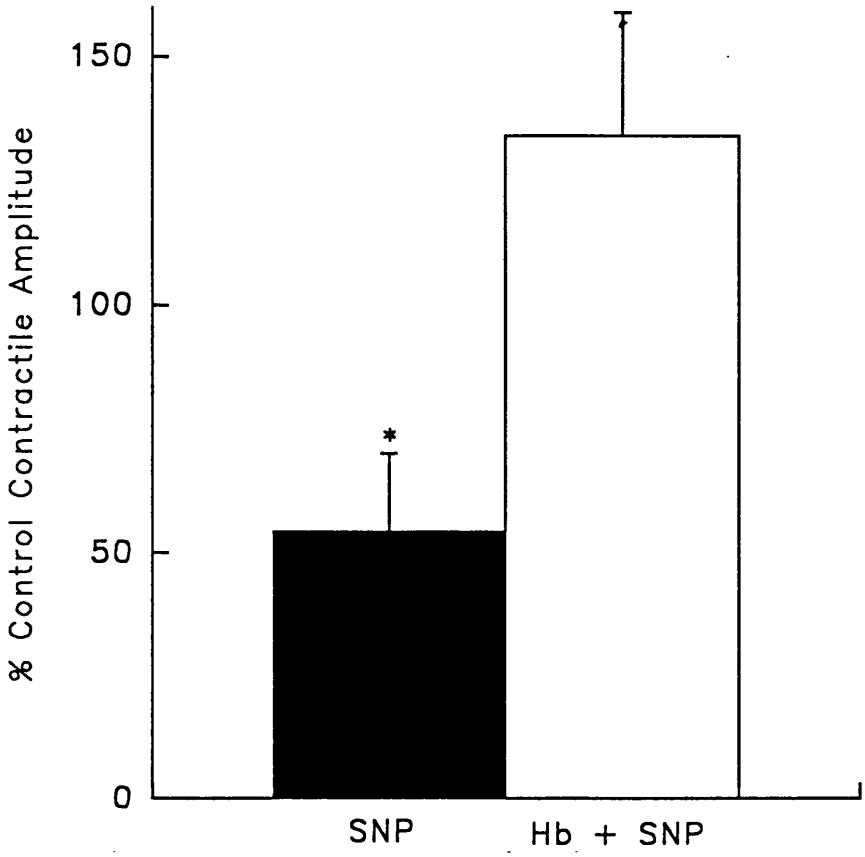


Fig. 46 : The effect (± s.e. mean ; n = 7) of SNP (10^{-4}M), expressed as a percentage of the control (100%), on the maximum contractile amplitude (g) per burst of mechanical activity, in the rabbit distal colon, in the absence and in the presence of Hb (10^{-4}M). Hb abolished the significant inhibitory response (*, $P < 0.05$) of the tissue to SNP.

number of spikes or peaks per burst and the duration of both electrical and mechanical responses were reduced by Hb (10^{-4}M) alone (Fig. 44), making it difficult to assess any effects of SNP on these parameters.

Experiments in which the properties of 8-bromo cGMP (10^{-4}M) were studied, revealed that both the excitatory and inhibitory effects of SNP were likely to be mediated by stimulation of cGMP (Fig. 47). Following a short period of quiescence, this cyclic nucleotide derivative (8-bromo cGMP) increased the frequency of both spike discharge and corresponding contraction. Electrical and mechanical responses now consisted almost wholly of single spikes and single peaks respectively (Fig. 47). By approximately 10 min, the increased frequency of contraction had persisted, but the amplitude of the mechanical response was reduced sharply. In the presence of 8-bromo cGMP (10^{-4}M) the rate of spontaneous electrical and mechanical activity was increased significantly to 858 ± 8.3% ($n = 3$; $P < 0.01$; Fig. 48). SNP (10^{-4}M) caused an increase to 192.9 ± 16.7% ($n = 31$; $P < 0.001$). On another occasion, when the activity was rapid (> 6 bursts min^{-1}), 8-bromo cGMP (10^{-4}M), like SNP, reduced the frequency of activity (results not shown). The contractile amplitude was decreased significantly ($P < 0.05$) to 12.9 ± 6.2 % of controls by 8-bromo cGMP (10^{-4}M ; $n = 3$) and to 50.15 ± 7.83% of controls by SNP (10^{-4}M ; $n = 31$; $P < 0.001$; Fig. 49). This suggests that the effects of SNP, both excitatory and inhibitory, could have been mediated by elevation of cGMP levels.

In support of these suggestions, potassium ferrocyanide ($K_4Fe(CN)_6$), in concentrations up to 10^{-4}M, which is structurally similar to SNP but which does not release NO (Fig. 50), did not alter any of the parameters significantly ($n = 3$; Fig. 51). This indicates that the effects of SNP on the RDC were not due to the ferrocyanide portion of the SNP molecule, but were likely to involve NO.

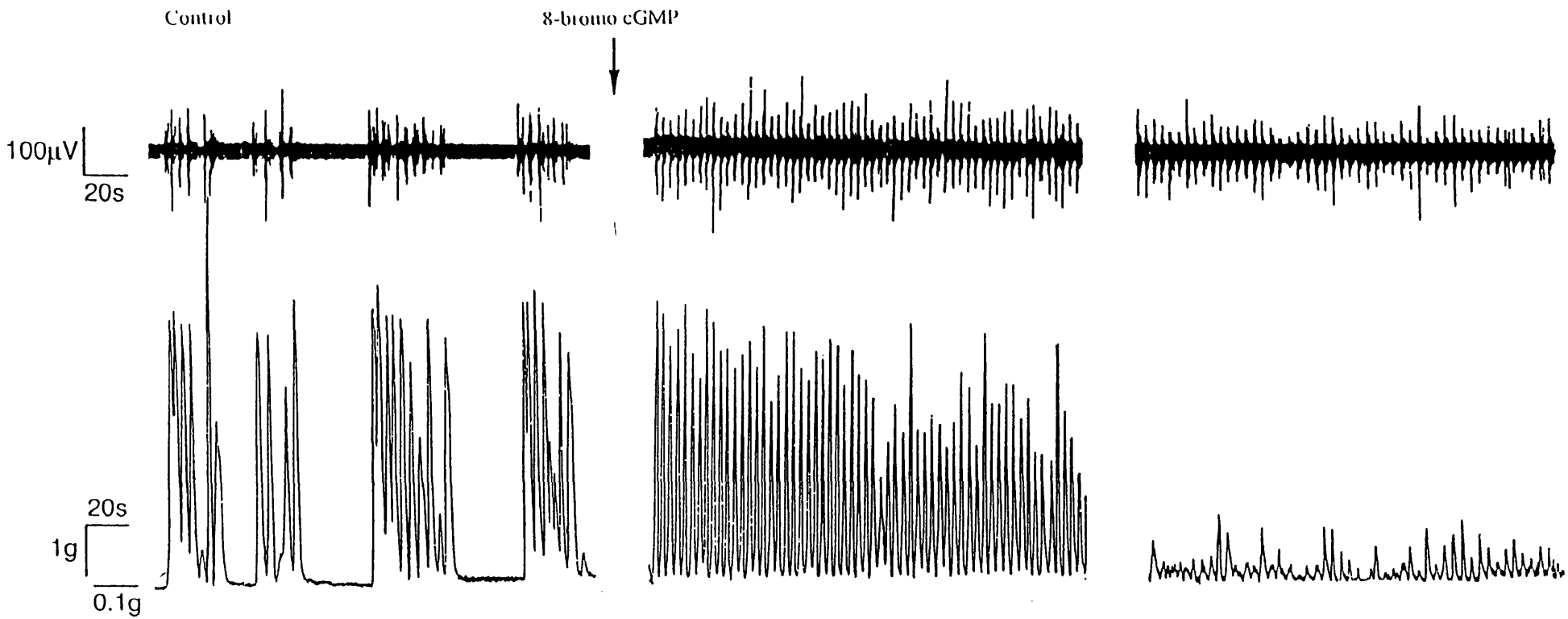


Fig. 47 : The effect of 8-bromo cGMP (10^{-4}M ;——), after 2 min (centre) and 10 min (right), on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. Like SNP, 8-bromo cGMP increased the frequency of both electrical and mechanical activity and reduced the amplitude of contraction.

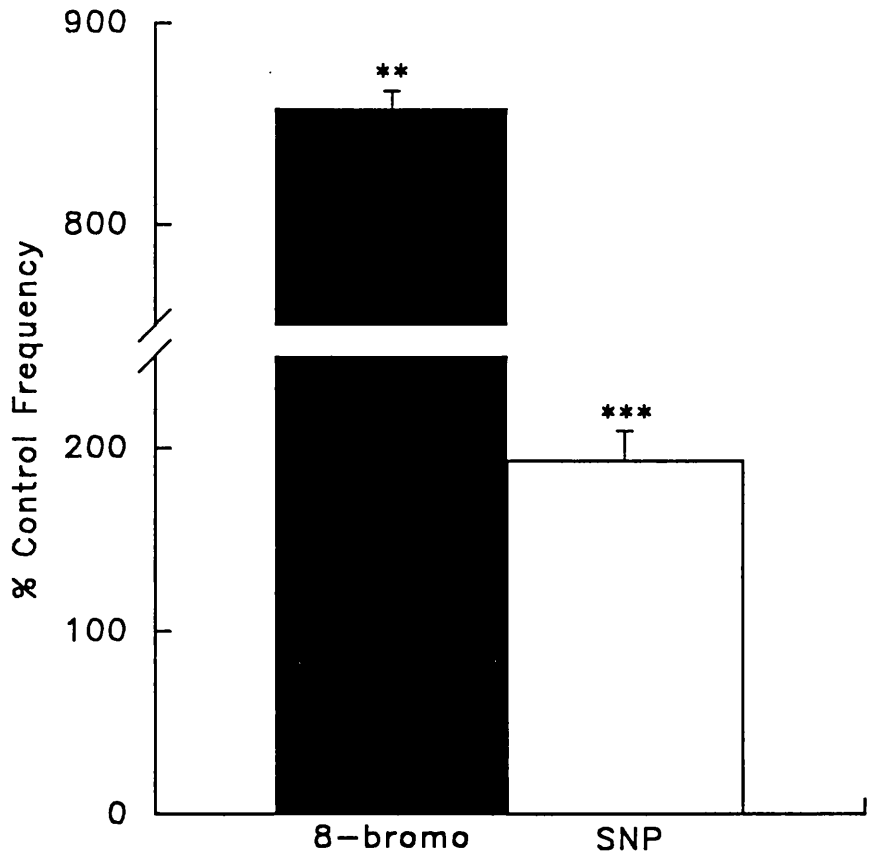


Fig. 48 : The effect (± s.e. mean) of 8-bromo cGMP (10^{-4}M ; n = 3) and SNP (10^{-4}M ; n = 31), each expressed as a percentage of the control (100%), on the frequency (bursts min^{-1}) of spontaneous activity, in the rabbit distal colon, where the frequency of control activity was low (< 6 bursts min^{-1}). Each compound increased significantly (**, $P < 0.01$; ***, $P < 0.001$), the frequency of activity.

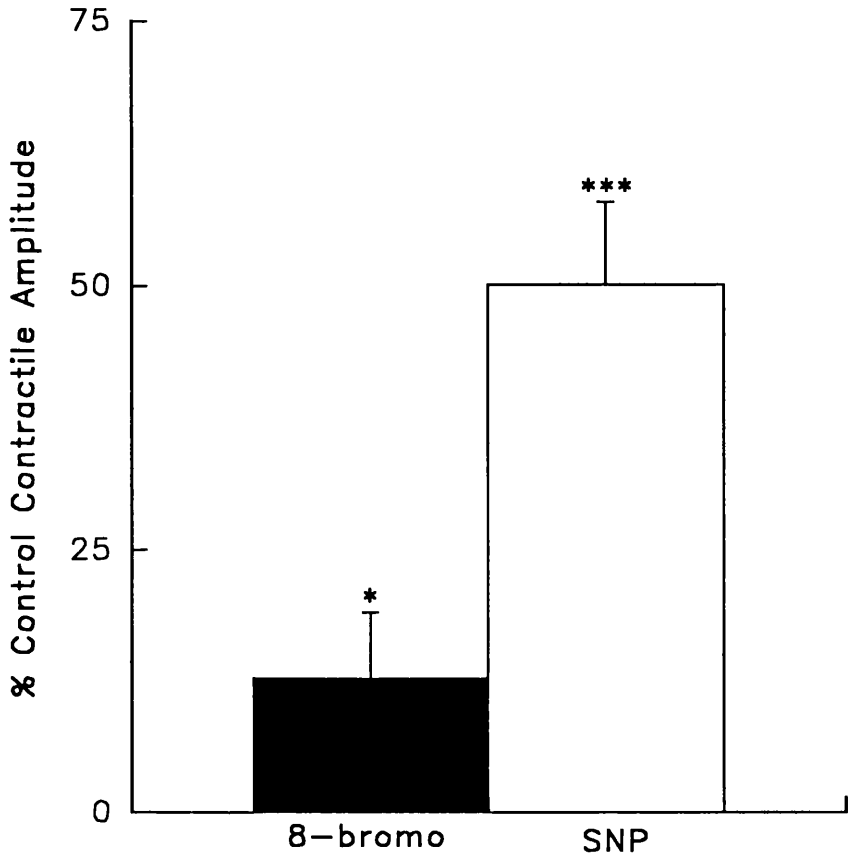
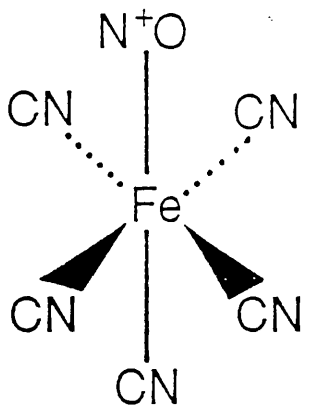
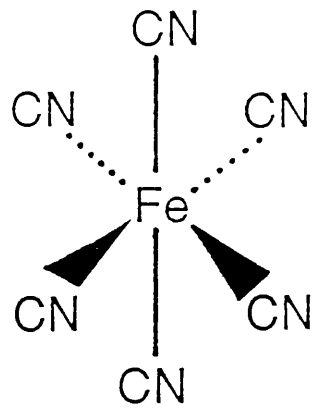


Fig. 49 : The effect (± s.e. mean) of 8—bromo cGMP (10^{-4}M ; n =3) and SNP (10^{-4}M ; n = 31), expressed as a percentage of the control (100%), on the maximum contractile amplitude (g) per burst of mechanical activity, in the rabbit distal colon. Each compound reduced significantly (*, P < 0.05 ; ***, P < 0.001), the amplitude of contraction.



Nitroprusside



Ferrocyanide

Fig. 50 : A comparison of the structure of the nitroprusside anion with that of the ferrocyanide molecule. In ferrocyanide, the NO-releasing group is replaced by cyanide.

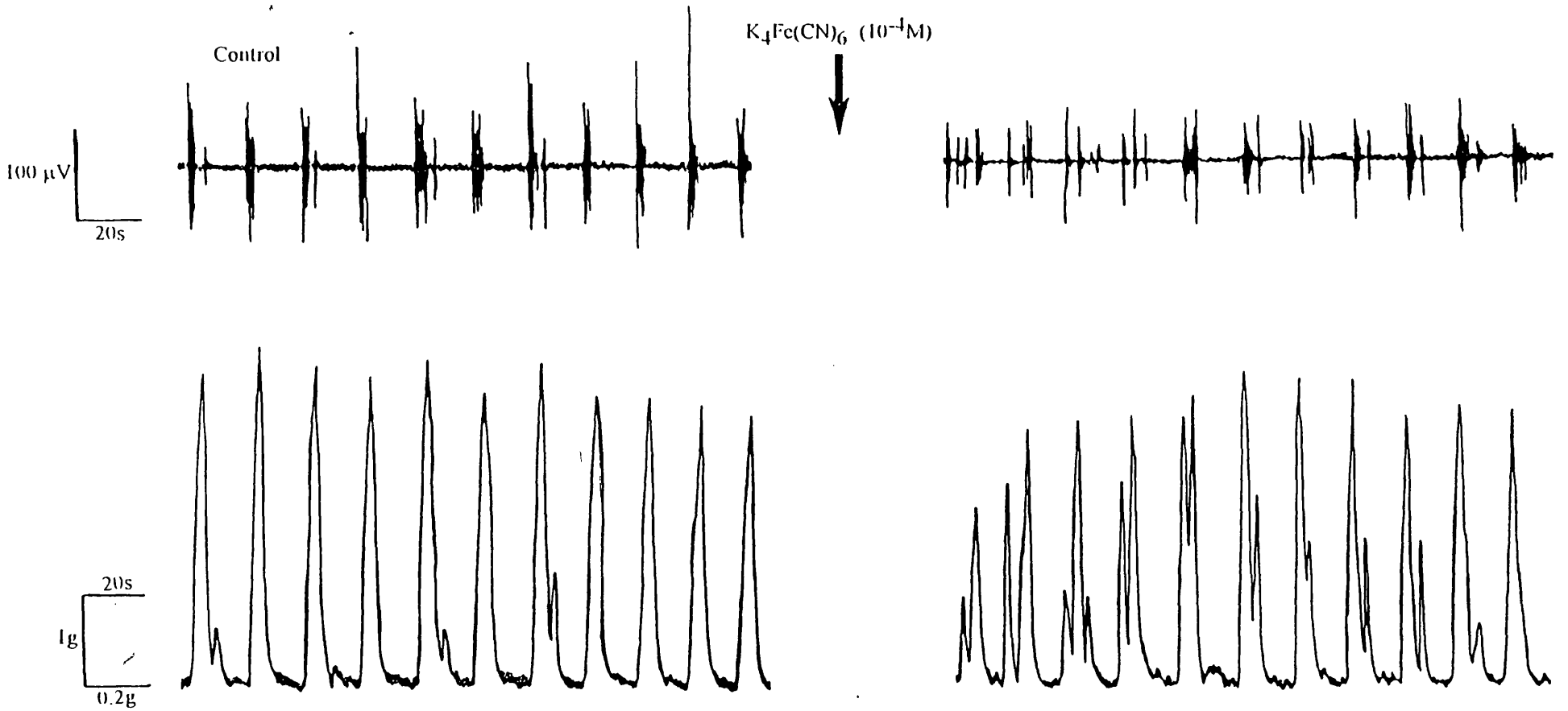


Fig. 51 : The effects of $K_4Fe(CN)_6$ (10^{-4}M) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. $K_4Fe(CN)_6$ neither increased the frequency of activity nor reduced the amplitude of contraction.

Clearly, the observed excitation in response to nitrovasodilators, in the RDC, is not an action that one would normally associate with NO. Indeed, this action would more likely be expected following inhibition of NO. This prompted a comparison of the effects of SNP with those of the inhibitor of NO synthesis, L-NAME. L-NAME (10^{-4}M), when added to the unstretched RDC (Fig. 52), increased the level of baseline tension to 1.58 ± 1.14g (n = 4). and, sometimes, instigated continuous firing. Spontaneous activity now appeared less coordinated than the control. The increase in tension, if great enough, was accompanied by the conversion of spontaneous contraction to relaxation. Since the L-NAME-induced increase in frequency was secondary to an elevation of baseline tension, an effect never produced by SNP, and L-NAME caused activity to become less coordinated, the opposite effect to SNP, the actions of the two compounds were dissimilar.

The effects of cyclic adenosine monophosphate (cAMP) stimulation in RDC were compared with those of cGMP stimulation, by comparing the effects of isoprenaline with those of SNP. Isoprenaline acts on. β-adrenoceptors to stimulate adenylate cyclase and raise cyclic adenosine monophosphate (cAMP) levels. This mediates smooth muscle relaxation. Like the nitrovasodilators, isoprenaline (≥ 10^{-7}M) significantly ($P < 0.001$) reduced the amplitude of spontaneous contraction and the number of spikes per burst of electrical activity (n = 6 ; Fig. 53). However, unlike SNP, isoprenaline did not coordinate the pacemaker activity. In addition, isoprenaline (4 x 10^{-7}M) almost always abolished spontaneous electrical and mechanical activity in RDC. SNP never abolished activity even at concentrations as high as 10^{-3}M. The actions of isoprenaline (2 x 10^{-7}M ; Fig. 53), but not SNP (results not shown), were prevented by propranolol (3 x 10^{-6}M ; n = 3). Thus, a number of differences existed between the actions of isoprenaline and SNP. This made it unlikely that cAMP stimulation was in any way involved in the response to the nitrovasodilators.

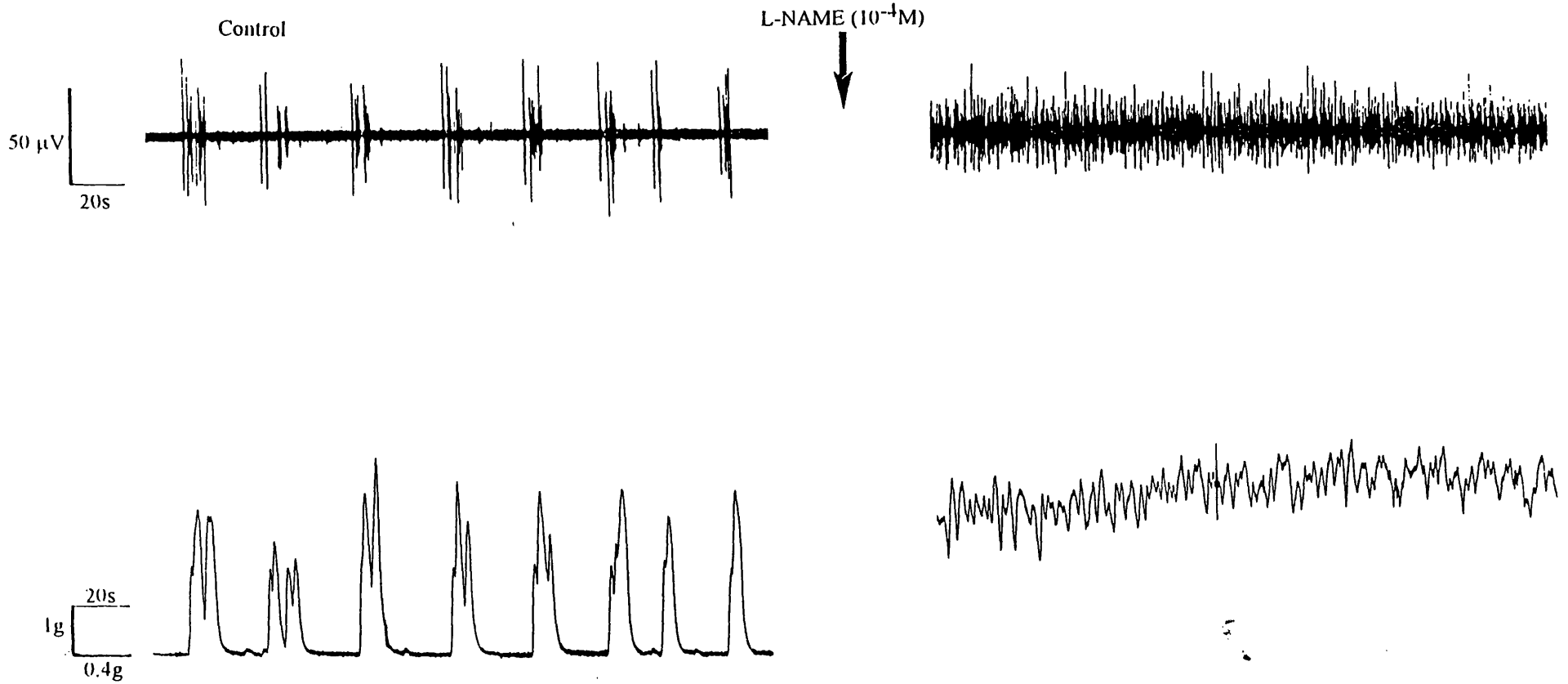


Fig. 52 : The effect of L-NAME (10^{-4}M) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. L-NAME raised the tone of the tissue and induced continuous electrical and mechanical activity.

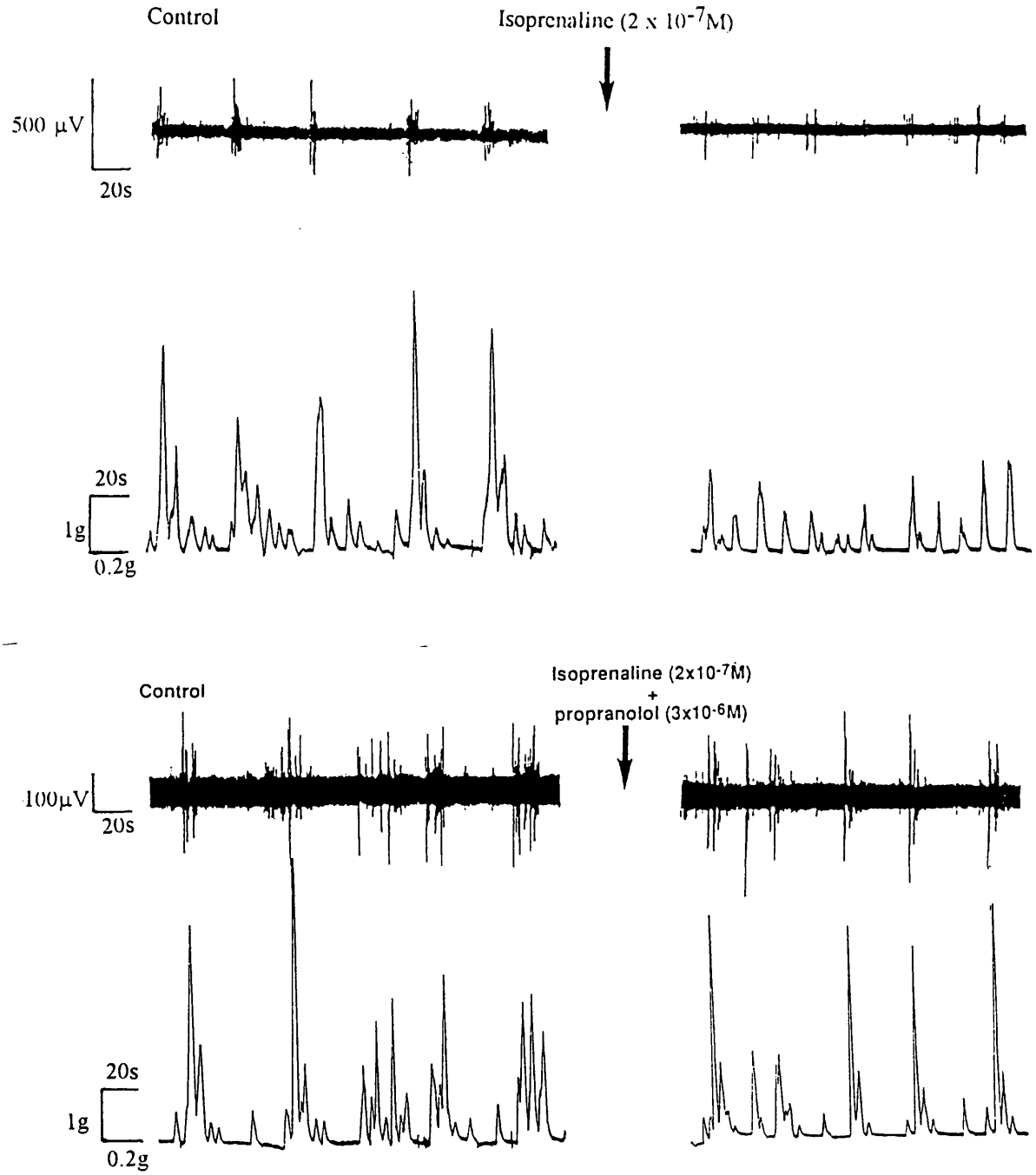


Fig. 53 : The effects of isoprenaline alone (2 x 10^{-7}M) and in the presence of propranolol (3 x 10^{-6}M) on the spontaneous electrical (top) and mechanical activity, in each pair of traces, recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. Isoprenaline alone reduced the amplitude of electrical and mechanical responses. These effects were inhibited by propranolol.

Activation of cGMP is believed to cause smooth muscle relaxation via a reduction in the level of intracellular Ca^{++}. In spite of the fact that, in these experiments, there was no evidence of nitrovasodilator-induced relaxation (possibly because the tissue was unstretched throughout the experiments), the actions of SNP were examined to determine whether some, or all of them, were mediated by a fall in the intracellular Ca^{++} concentration. SNP (10^{-5}M) alone increased the frequency of electrical and mechanical events and reduced the amplitude of contraction (Fig. 54). In the continued presence of SNP, the amplitude of contraction was enhanced above control levels, by the Ca^{++}-channel agonist, BAY K 8644 (10^{-7}M ; n = 3), probably by increasing the concentration of Ca^{++} available for contraction. In this particular case (Fig. 54), SNP (10^{-5}M) also increased the amplitude of electrical spiking - an effect that was reversed by BAY K 8644 (10^{-7}M). If the drugs were added in reverse order, BAY K 8644 (10^{-7}M) alone, often raised the tone of the tissue and increased the firing rate until activity was continuous (Fig. 55). Following the addition of SNP ($\geq 10^{-5}$M), tension returned to normal and both spontaneous electrical and mechanical activity were regulated so that activity was again burstlike. From this evidence, it is likely that the SNP-induced reduction in contractile amplitude and, possibly, the increase in spike amplitude were due to a reduction in the level of intracellular Ca^{++}. Further, the coordination of activity produced by SNP, following the prior addition of BAY K 8644, may have been the result of a fall in the cytosolic Ca^{++} concentration.

The actions of another well known smooth muscle relaxant, the opener of ATP-dependent K^{+}-channels, BRL 38227 (levcromakalim or lemakalim), were also compared with those of the nitrovasodilators. This compound produced a graded, dose-dependent inhibition of spontaneous activity in the RDC (Fig. 56). The frequency and contractile amplitude were significantly reduced from 3.5 ± 1.08 bursts min^{-1} and 2.16 ± 0.28g to 1.56 ± 0.52 bursts min^{-1} and 1.2 ± 0.26g respectively, by BRL 38227 (10^{-5}M ; $n \geq 4$; $P < 0.05$). At other

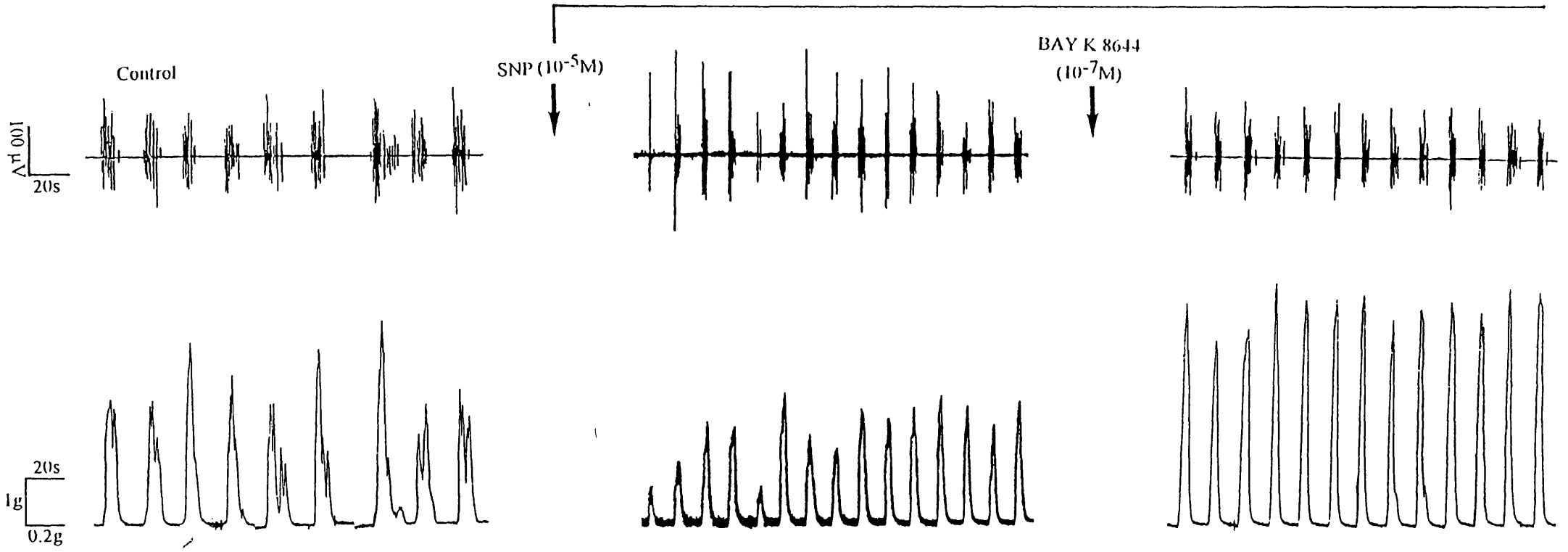


Fig. 54 : Reversal of the effect of SNP (10^{-5}M ;———), by the Ca^{++}-channel agonist, BAY K 8644 (10^{-7}M), on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. In the continued presence of SNP, BAY K 8644 enhanced the amplitude of contraction above control levels.

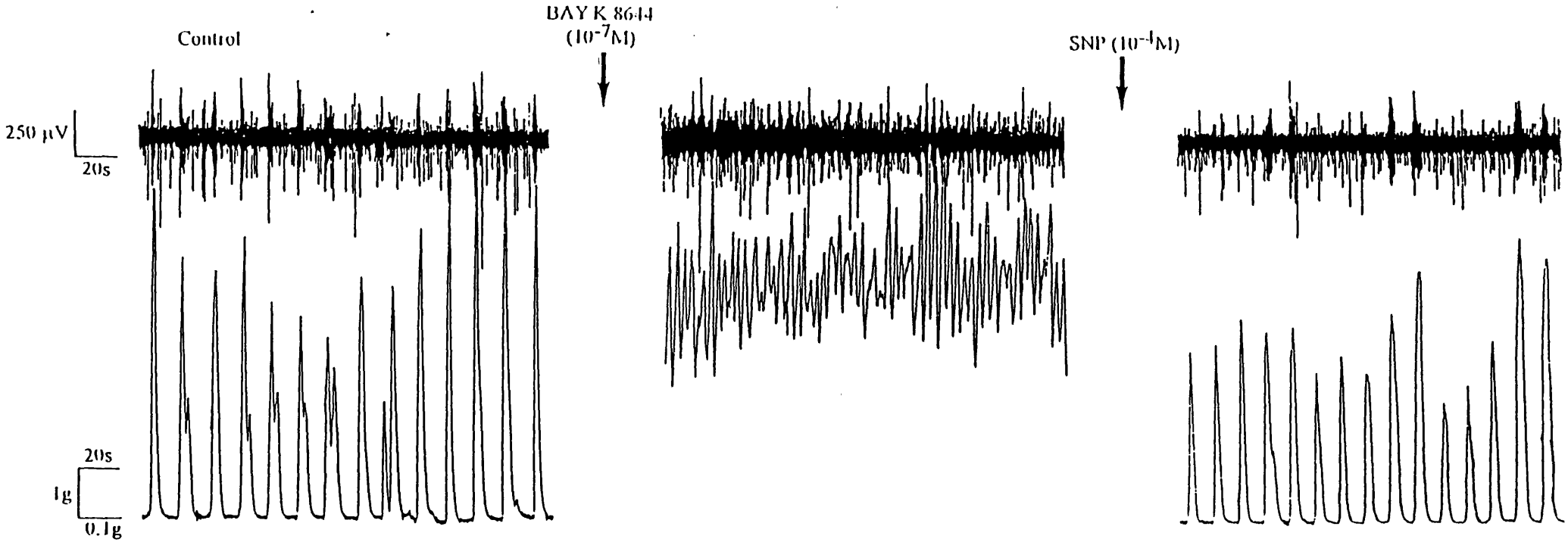


Fig. 55 : Reversal of the effect of the Ca^{++}-channel agonist, BAY K 8644 (10^{-7}M ;———), by SNP (10^{-4}M), on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. BAY K 8644, alone, raised the tone and induced continuous electrical and mechanical activity. SNP lowered the tone and regulated both electrical and mechanical activity so that they were again burst-like.

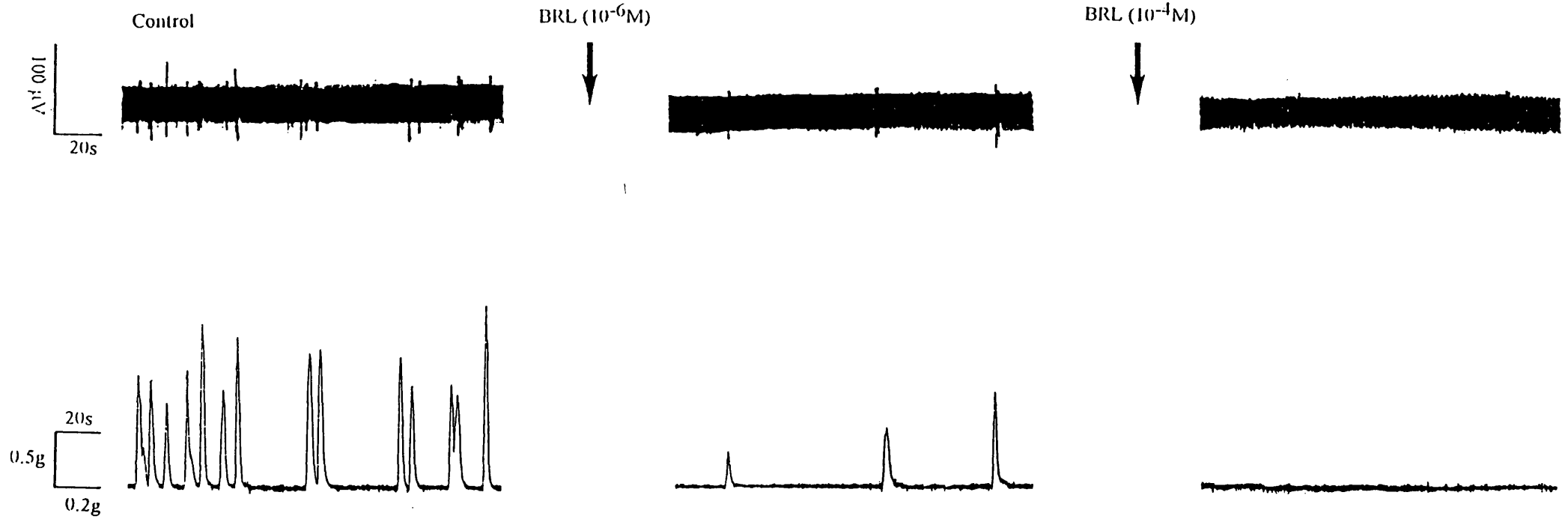


Fig. 56 : The effect of BRL 38227 (BRL ; 10^{-6}M, centre panel ; 10^{-4}M, right panel) on the spontaneous electrical (top) and mechanical activity recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. The frequency of both electrical and mechanical activity and the amplitude of contraction were reduced by BRL 38227 (10^{-6}M). All activity was abolished by BRL 38227 (10^{-4}M).

times, this concentration of drug abolished all activity (n = 3). At higher concentrations, (10^{-4}M), BRL 38227 always abolished activity in this preparation (n = 5). Subthreshold concentrations of SNP and BRL 38277 were synergistic with one another (Fig. 57). Neither SNP (10^{-6}M) nor BRL 38277 (10^{-7}M) induced quiescence when administered alone. However, when added together, both electrical and mechanical activity were abolished for a short period (1-10 min). Possibly, both SNP and BRL 38227 induced a K^+ efflux.

3.3.2. Radiolabelled rubidium efflux

In order to measure the movement of K^+ across the cell membrane, longitudinal strips of RDC were incubated in Krebs solution containing radiolabelled rubidium (^{86}Rb ; a marker for K^+ ; Martin & Gordon, 1983 ; Hamilton *et al*, 1986), before being mounted in the Golenhofen apparatus. Following the commencement of superfusion, the efflux of ^{86}Rb from the RDC declined rapidly (Fig. 58). The rapid loss of isotope observed during the first 5-6 min represented washout from the extracellular space. The loss of ^{86}Rb was then constant indicating the loss of tracer from the cytoplasm of the smooth muscle cells. Data were expressed in terms of the efflux rate constant :

$$\text{Efflux Rate Constant (E)} = \Delta A / \Delta_t A_t$$

Δ_t is the time over which the fraction was collected, ΔA is the number of counts released in that time and A_t is the average number of counts remaining in the tissue at that time. The value of E clearly differs from tissue to tissue. Values of $4.22 \pm 0.14 \times 10^{-3}$ min^{-1} (Martin & Gordon, 1983) or $4.8 \pm 0.4 \times 10^{-3}$ min^{-1} (Bolton & Clapp, 1984) have been reported for E in the rabbit aorta while a value of $11.38 \pm 0.4 \times 10^{-3}$ min^{-1} has been reported in guinea-pig taenia caeci

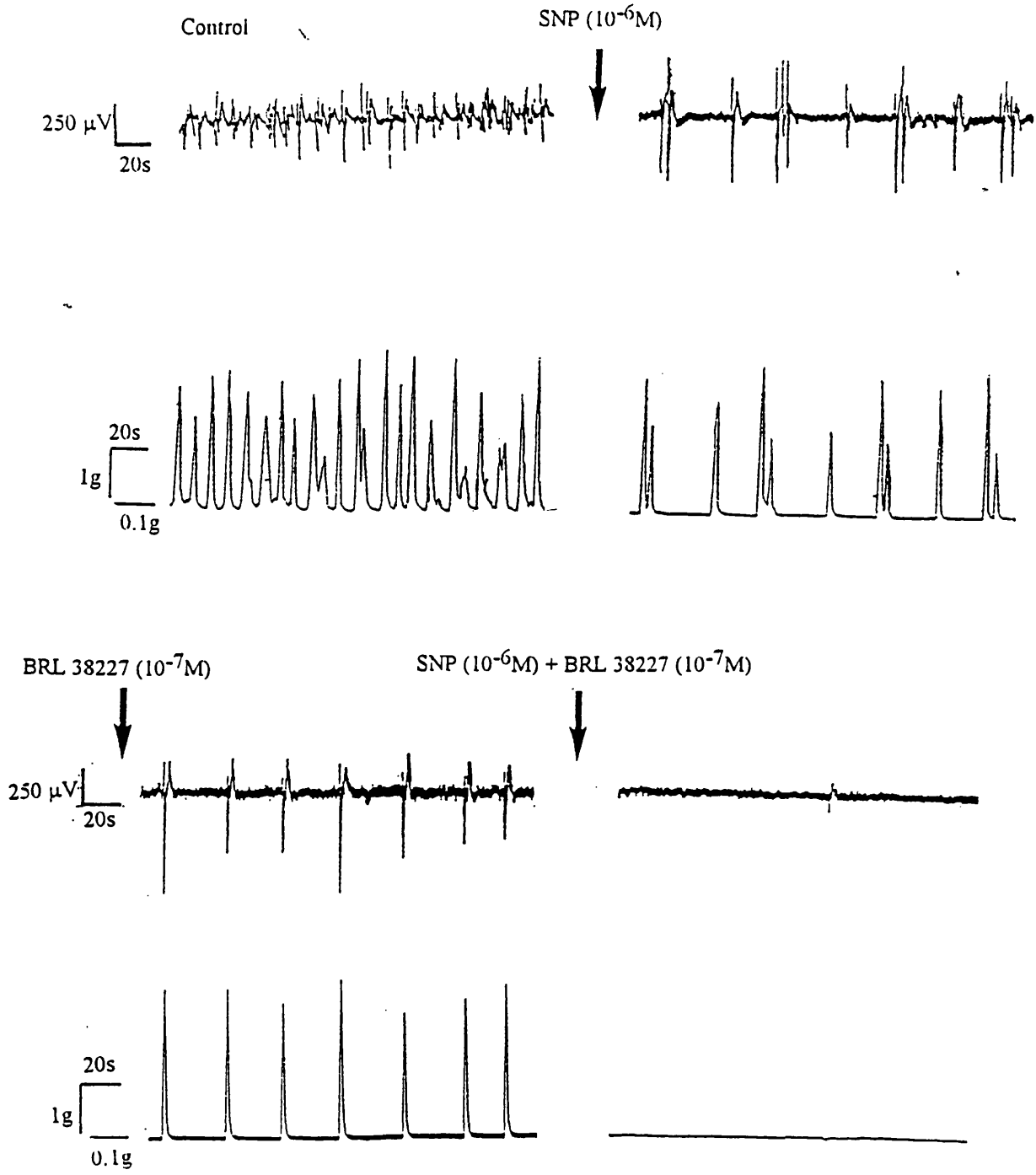


Fig. 57 : The effects of SNP (10^{-6}M) and BRL 38227 (10^{-7}M), alone and together, on the spontaneous electrical (top) and mechanical activity, in each panel, recorded from an unstretched, longitudinal strip of rabbit distal colon in the Golenhofen apparatus. Due to the high frequency of control activity, SNP,alone, caused a reduction in frequency. Drugs were washed out between each panel. These drugs had a greater effect when added together rather than individually.

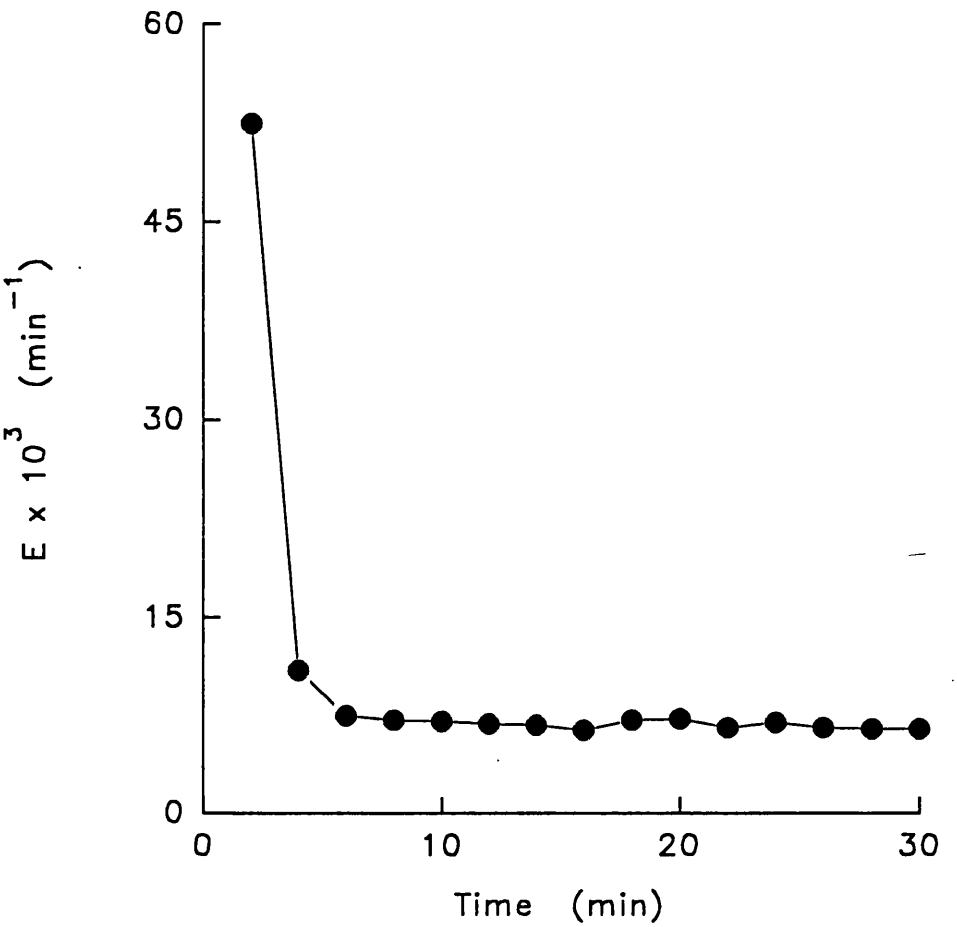


Fig. 58 : The exponential decline with time of the resting ^{86}Rb efflux, expressed as the first–order rate constant (E ; x 10^3), from the rabbit distal colon incubated with $^{86}RbCl$. By 6–8min, approximately, ^{86}Rb efflux had reached a relatively steady value.

(Bolton & Clark, 1981). In the RDC, the mean value of E was 6.59 ± 0.26 x 10^{-3} min^{-1} (n = 20).

Since carbachol was known to significantly increase the rate of ^{42}K or ^{86}Rb efflux from gastrointestinal smooth muscle (Bolton & Clark, 1981), this drug was used to test that the system was functional. Carbachol (10^{-5}M) significantly enhanced the ^{86}Rb efflux rate constant ($P < 0.05$) from 7.4 ± 0.81 x 10^{-3} min^{-1} to 22.7 ± 1.7 x 10^{-3} min^{-1} - an increase of approximately 200% (Fig. 59). This stimulation of ^{86}Rb efflux may have been secondary to carbachol-induced depolarization and subsequent opening of voltage-operated K^+ channels. Similar effects have been reported for contractile agonists in the rabbit aorta (Martin & Gordon, 1983).

Earlier experiments in RDC had indicated that SNP caused initial quiescence and abolished spontaneous activity (see 3.3.1.). Further, the effects of SNP were synergistic with those of the K^+ channel opener, BRL 38227. Both these effects could be explained if SNP induced the opening of membrane K^+ channels. Accordingly, the effects of SNP on the efflux of ^{86}Rb were measured. However, the effects of SNP were disappointing. SNP (10^{-7}-10^{-4}M ; n = 5 ; Fig. 59 & 60) did not significantly affect the rate of ^{86}Rb efflux at any concentration.

The relaxant, BRL 38227 (10^{-7}-10^{-4}M ; $n \geq 4$), which has been reported to open K^+ channels in both vascular (Hamilton *et al*, 1986 ; Weir & Weston, 1986b) and gastrointestinal (Weir & Weston, 1986a) smooth muscles was also examined. It was expected that this compound would significantly increase the efflux of ^{86}Rb from the RDC. As with SNP, the results were disappointing. BRL 38227 (10^{-7}-10^{-4}M ; $n \geq 4$) had no effect on the rate of ^{86}Rb efflux. The effect of BRL 38227 (10^{-5}M) is shown in Fig. 59.

The β-adrenoceptor agonist, isoprenaline, like BRL 38227, abolished all spontaneous activity in the RDC. In this case, it is likely that abolition was mediated by cAMP elevation. Isoprenaline was used to compare the effects of

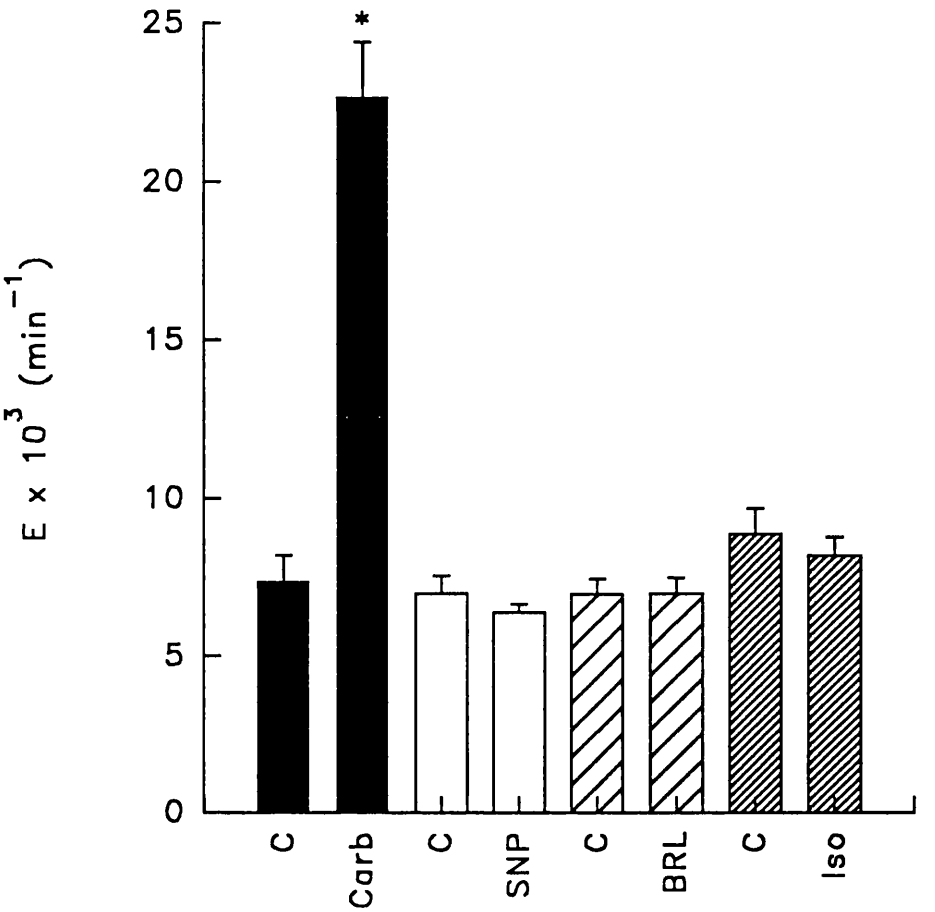


Fig. 59 : The effects (± s.e. mean) of carbachol (Carb ; n = 3), SNP (n = 5), BRL 38227 (BRL ; n = 8) and isoprenaline (Iso ; n = 3 ; each 10^{-5}M), on the resting ^{86}Rb efflux (C), expressed as the efflux rate constant (E ; x 10^{3}), from the rabbit distal colon incubated with ^{86}RbCl. The resting ^{86}Rb efflux was similar in all experiments. Only carbachol increased significantly (*, $P < 0.05$), the value of E.

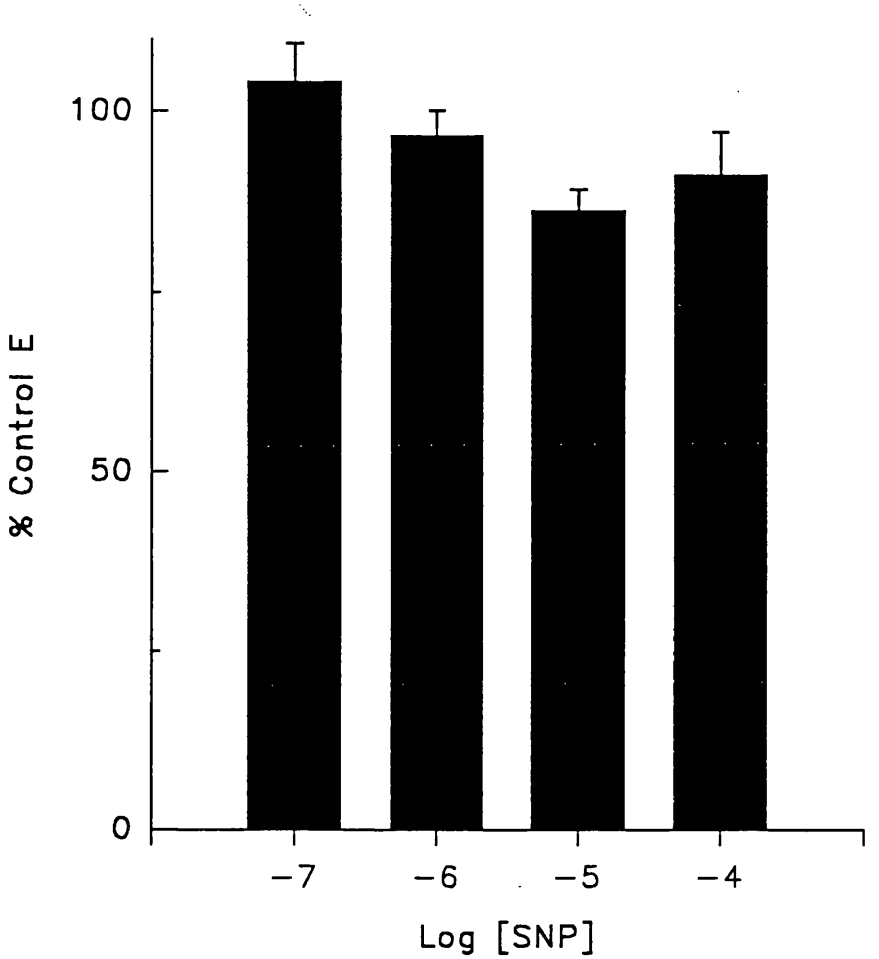


Fig. 60 : The effect (± s.e. mean, n = 5) of SNP (10^{-7}–10^{-4}M), expressed as a percentage of the control (100%), on the resting ^{86}Rb efflux rate (E) from the rabbit distal colon incubated with ^{86}RbCl. SNP did not significantly affect the value of E at any concentration.

cAMP elevation with those of SNP (cGMP elevation) and BRL 38227, on ^{86}Rb efflux. Unfortunately, isoprenaline ($10^{-5}M$; n = 5 ; Fig. 59) was no more effective than these other compounds. Indeed, the rate constant for ^{86}Rb efflux was not enhanced at any concentration of isoprenaline (10^{-7}-$10^{-4}M$).

Excitatory effects such as those observed in RDC could have potentially far reaching implications for the use of the nitrovasodilators. Clearly, excitation is an undesirable property in drugs that are generally used for their smooth muscle relaxant ability - would this compromise their therapeutic usefulness? Since, these drugs are most often used in the cardiovascular system to treat conditions such as angona pectoris, congestive heart failure, pulmonary hypertension or hypertensive emergencies, it was important to examine their actions on spontaneously active vascular smooth muscle. The portal vein was chosen. As in the RDC, spontaneous electrical and mechanical activity in portal veins from rabbits and from rats was burst-like in nature (Figs. 10 and 11).

3.4. Patterns of Spontaneous Activity in the Portal Vein

In rabbit portal vein (RPV), under an applied tension of 1g, bursts of electrical spiking and contraction (Fig. 10) had a frequency of 2.95 ± 0.41 min^{-1} (n = 5). Each spike-burst had a duration of 7.40 ± 1.94s (n = 4) and contained 16.76 ± 3.14 spikes (n = 4). The amplitude of the largest spike in each was 24.67 ± 2.60 μV (n = 3). Contractile bursts contained 1.50 ± 0.22 peaks (n = 5) and had a mean duration of 12.17 ± 1.57s (n = 5). The tension developed during each, including the 1g applied, was 1.59 ± 0.2g (n = 5).

In rat portal vein (rat PV), under an applied tension of 1g, the frequency of spontaneous activity (Fig. 11) was 2.61 ± 0.14 bursts min^{-1} (n = 49). Spike bursts had a mean duration of 6.81 ± 0.56s (n = 24) and consisted of 15.08 ± 1.11 spikes (n = 20). The maximum spike amplitude per burst was

39.04 ± 3.69 μV (n = 24). Each mechanical event had a duration of 8.31 ± 0.36s (n = 49) and possessed 1.63 ± 0.08 peaks (n = 49). The maximum tension developed during each, including the 1g applied, was 1.48 ± 0.39g (n = 49).

Thus, of the three spontaneously active muscles employed, the RDC was generally the most active. For example, in the RDC, the frequency of activity was 3.77 ± 0.18 bursts min^{-1} (n = 80) as compared to 2.95 ± 0.41 bursts min^{-1} (n = 5) in the RPV and 2.61 ± 0.14 bursts min^{-1} (n = 49) in the rat PV. The difference in the frequency of activity between RDC and rat PV was a significant one ($P < 0.001$). Similarly, the electrical spike amplitude in RDC was approximately 10 times as large as that in RPV and 6 times as large as that in rat PV. Further, the maximum tension developed during each burst of mechanical activity in RDC, was more than 0.5g greater than the responses in either RPV or rat PV, despite the fact that each portal vein had 1g applied tension, while the RDC was left unstretched.

As in the RDC, both electrical and mechanical responses in the rat PV required the presence of extracellular Ca^{++} (Fig. 61). When $CaCl_2$ in the Krebs was replaced by $MgCl_2$, there was an initial increase in frequency and the amplitude of contraction and of spiking fell. After approximately 5 min all activity was abolished.

3.5. Actions of Nitrovasodilators on the Portal Vein

3.5.1. Spontaneous electrical and mechanical activity

The effects of SNP, GTN and IDN were studied on the intact, spontaneously active portal vein of either species, mounted under a tension of 1g, in the Golenhofen apparatus.

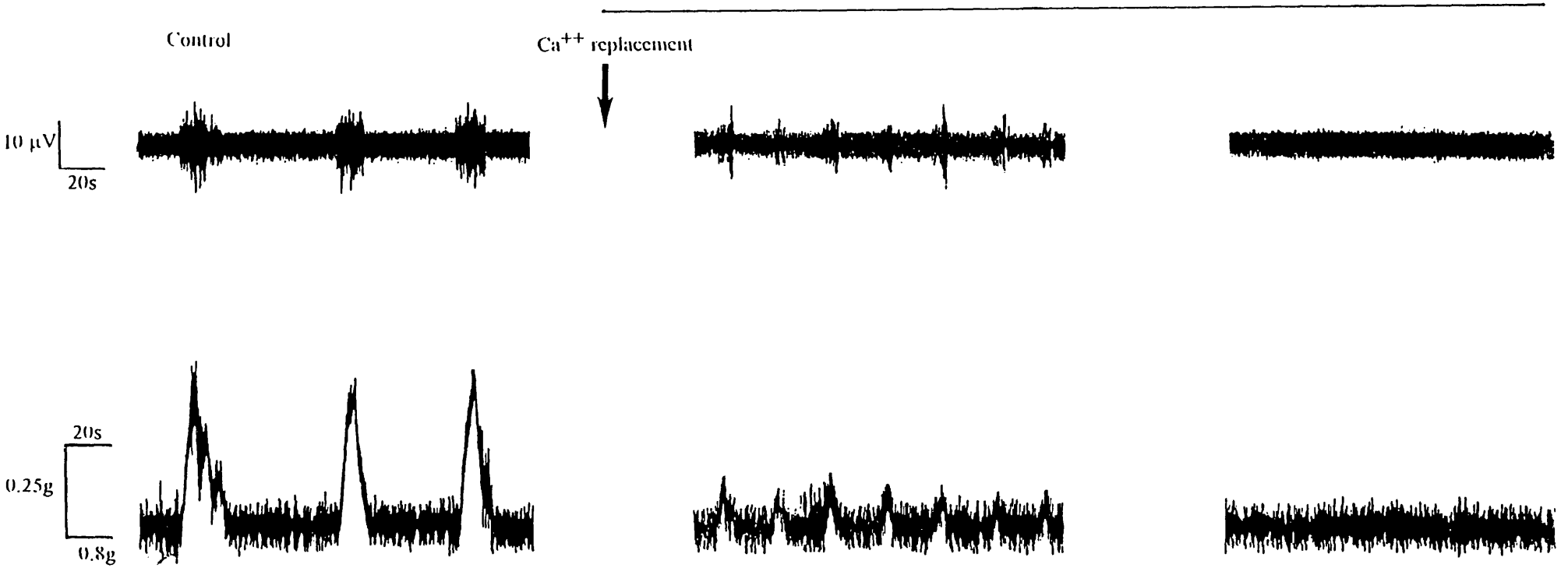


Fig. 61 : The effect of Ca^{++} replacement (———→) by Mg^{++} in the Krebs solution, on the spontaneous electrical (top) and mechanical activity recorded from the rat portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus, after approximately 2 min (centre) and 5 min (right). The frequency of both electrical and mechanical activity was increased, but the amplitude of each reduced, before abolition.

Unlike in the colon, in which SNP increased the frequency of activity, there was no evidence of an SNP-induced frequency increase in the RPV. The firing rate gradually decreased until SNP (10^{-5}M) abolished both electrical and mechanical responses (n = 3 ; Figs. 62 & 63). The maximum contractile amplitude, the number of spikes or peaks per burst and the duration of responses were reduced before abolition. In the RDC, activity was never abolished by SNP even at concentrations as high as 10^{-3}M. Because of these qualitative differences in effect, the RPV was not pursued as a test model on which to study the actions of the nitrovasodilators, but was substituted by the rat PV.

The effects of the nitrovasodilators on the rat PV were similar to those in the RDC, but still differed in two main respects. Firstly, in the rat PV no quiescent period was ever induced by the nitrovasodilators. Secondly, since the frequency of activity was lower than in RDC, no evidence of a reduction in the frequency of activity was ever found (compare this with the effects of SNP on high frequency tissues in the RDC, Figs. 29 and 30). Apart from this, the effects were qualitatively, though not quantitatively, similar.

As in the RDC, SNP was the most effective nitrovasodilator tested. In the rat PV, SNP was more potent than in RDC and, at concentrations as low as 10^{-7}M, significantly enhanced the frequency of spontaneous activity (Fig. 64 ; $P < 0.001$). However, the maximum effects observed in the rat PV were generally less than the corresponding effects in RDC. The effects of SNP (10^{-6}M) are shown in Fig. 65. In this example, the frequency of responses was increased to 233% of the control. In the rat PV, the effects of the other nitrovasodilators were disappointing. Neither GTN (10^{-7}-10^{-5}M ; $n \geq 4$) nor IDN (10^{-7}-10^{-5}M ; n = 4), had a significant effect on the frequency of pacemaker activity (Fig. 64). Baseline tension was unaffected by any of these drugs.

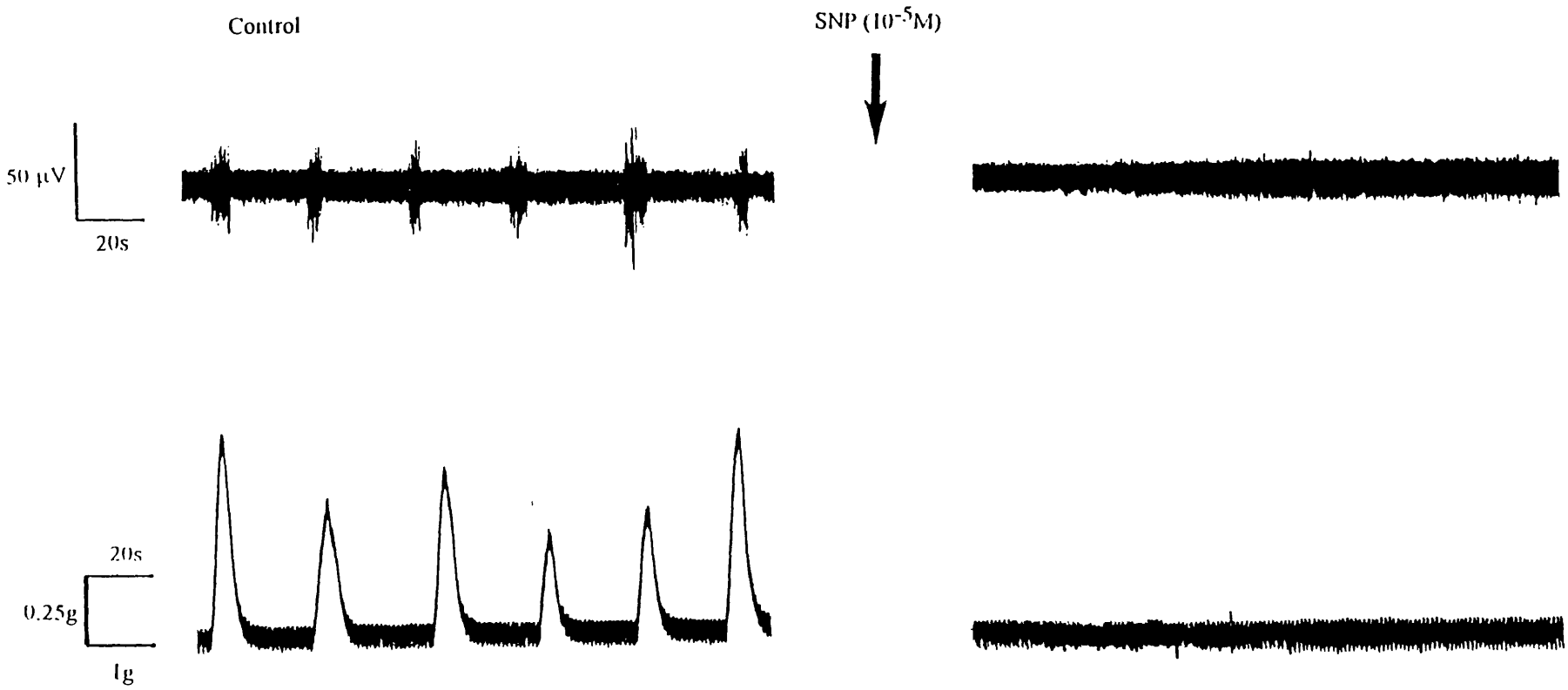


Fig. 62 : The effect of SNP (10^{-5}M) on the spontaneous electrical (top) and mechanical activity recorded from the rabbit portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. SNP abolished both electrical and mechanical activity.

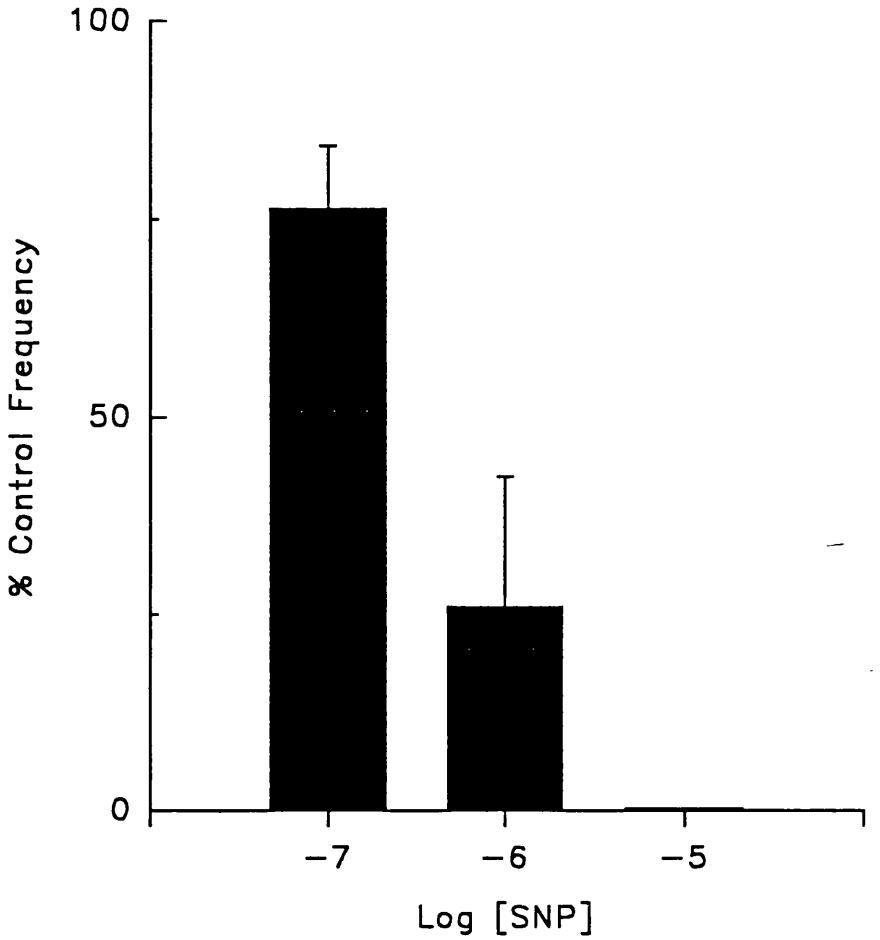


Fig. 63 : The effect (± s.e. mean, n ≥ 3) of SNP (10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the frequency (bursts min^{-1}) of spontaneous activity, in the rabbit portal vein. SNP (10^{-5}M) abolished all activity ; there was no evidence of excitation.

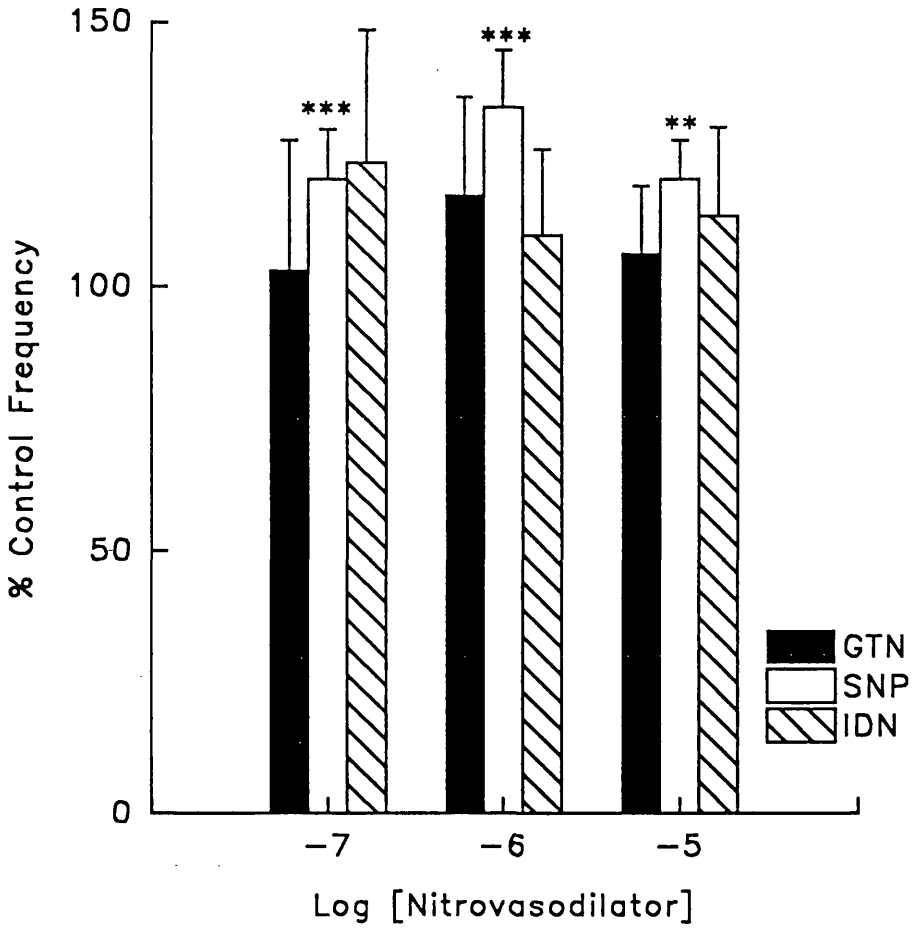


Fig. 64 : The effects (± s.e. mean) of GTN ($n \geq 5$), SNP ($n \geq 29$) and IDN ($n = 4$; each 10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the frequency (bursts min^{-1}) of spontaneous activity, in the rat portal vein. Only SNP increased significantly (**, $P < 0.01$; ***, $P < 0.001$), the frequency of activity.

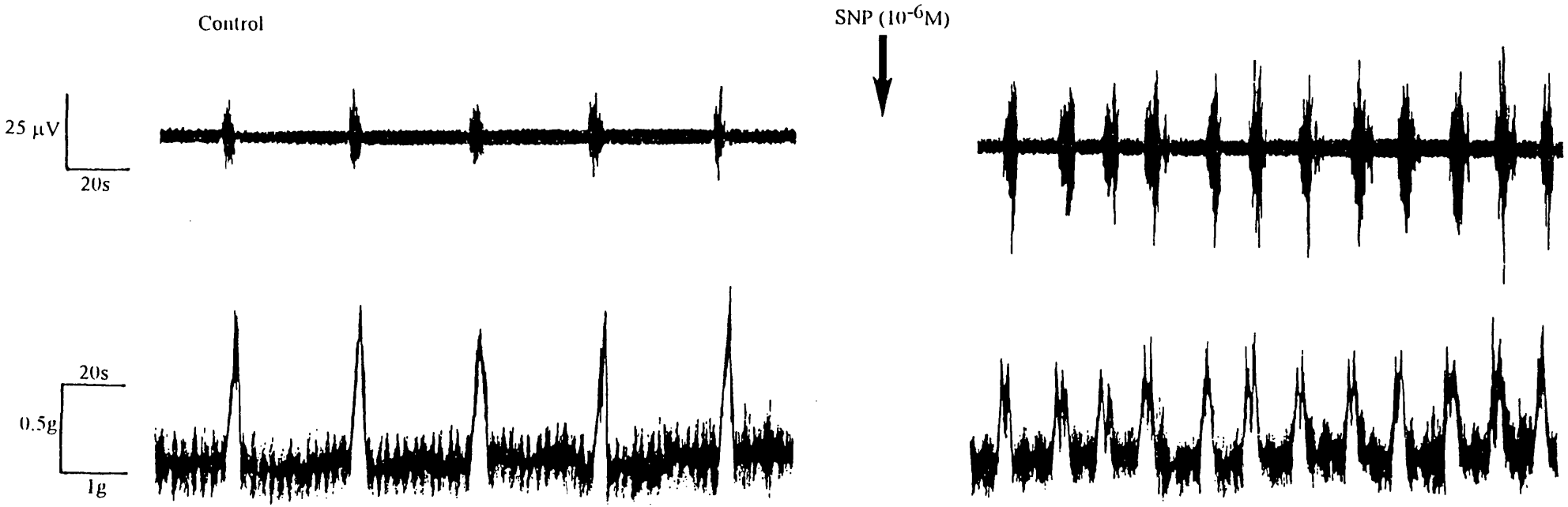


Fig. 65 : The effect of SNP (10^{-6}M) on the spontaneous electrical (top) and mechanical activity recorded from the rat portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. SNP increased the fequency of activity and the amplitude of spiking, but reduced the amplitude of contraction.

Concomitant with the increase in frequency, the amplitude of the spontaneous contractions was significantly decreased by SNP ($\geq 10^{-7}$M ; $n \geq 29$; $P < 0.001$; Figs. 65 & 66). Although GTN did not increase the frequency of activity in rat PV, this drug, at a concentration of 10^{-6}M, also lowered the contractile amplitude (Fig. 66 ; $P < 0.05$). IDN (10^{-7}-10^{-5}M ; $n = 4$; Fig. 66) was without effect.

As in the RDC, in certain individual experiments on the rat PV, the amplitude of spike discharge was augmented (Fig. 65). In this particular case (Fig. 65), the amplitude was increased to 230% of the control by SNP (10^{-6}M). Examples of this effect were, however, in the minority. In the majority of cases, SNP (10^{-7}-10^{-5}M ; $n \geq 17$; Fig. 67) did not increase the spike amplitude. In fact, SNP (10^{-5}M) significantly reduced the maximum spike amplitude ($P < 0.01$; Fig. 67). This was in contrast to the actions of IDN (10^{-5}M ; $n = 4$) which significantly enhanced this parameter ($P < 0.05$; Fig. 67). GTN (10^{-7}-10^{-5}M ; $n \geq 4$; Fig. 67) had no effect. With SNP, any changes in the amplitude of spontaneous spiking occurred simultaneously with the increases in frequency or reductions in contractile amplitude previously described i.e. it was not a gradual effect.

Both SNP ($\geq 10^{-6}$M ; $n \geq 14$; $P < 0.01$) and GTN (10^{-7}M ; $n = 6$; $P < 0.05$) significantly reduced the number of spikes per electrical burst and the mean duration of each burst in rat PV (Figs. 68 and 69 respectively). However, these reductions were not as great as those achieved in response to SNP in RDC (Figs. 37 and 39) - in rat PV, following nitrovasodilator addition the electrical discharge never consisted of single spikes. The effects of IDN were, again, disappointing. This substance (10^{-7}-10^{-5}M ; $n \geq 3$; Figs. 68 and 69) had no effect on either parameter.

Similarly, the number of peaks per mechanical burst was significantly reduced by SNP (10^{-7}M and 10^{-5}M ; $n \geq 29$; $P < 0.05$; Fig. 70). Neither GTN (10^{-7}-10^{-5}M ; $n \geq 5$; Fig. 70) nor IDN (10^{-7}-10^{-5}M ; $n = 4$; Fig. 70) had any

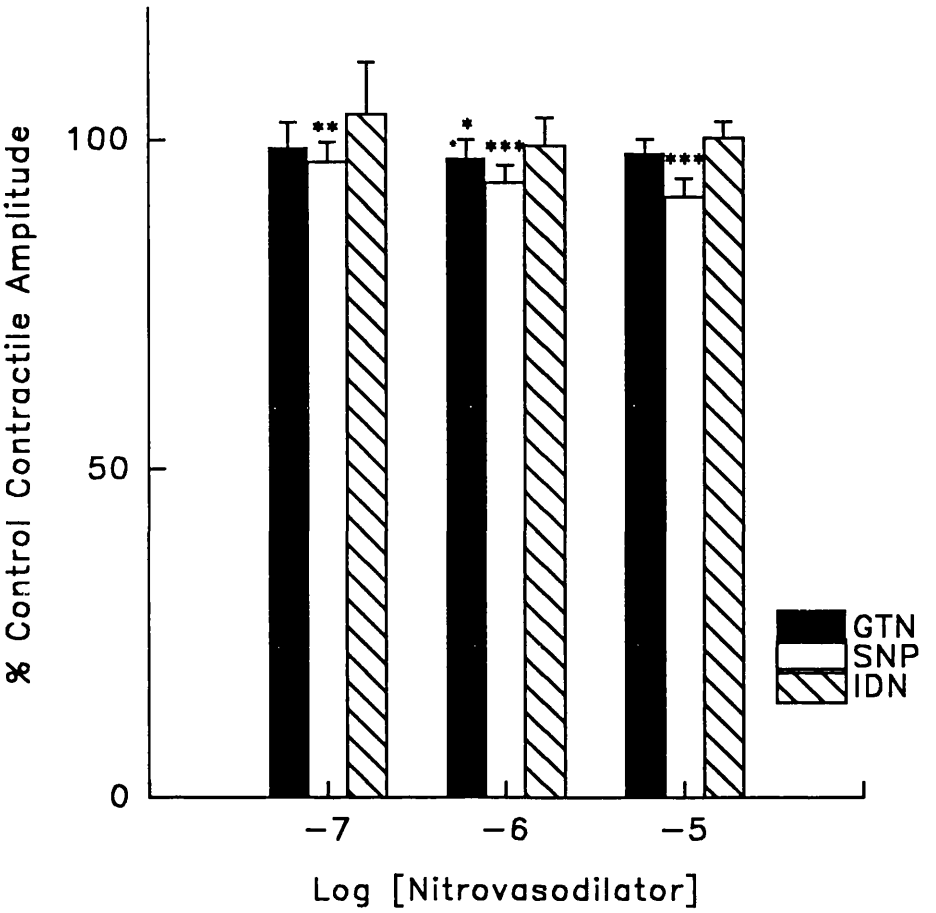


Fig. 66 : The effects (± s.e. mean) of GTN (n ≥ 5), SNP (n ≥ 29) and IDN (n = 4 ; each 10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the maximum contractile amplitude per burst of mechanical activity, in the rat portal vein. GTN and SNP each reduced significantly (*, P < 0.05 ; **, P < 0.01 ; ***, P < 0.001), the maximum contractile amplitude.

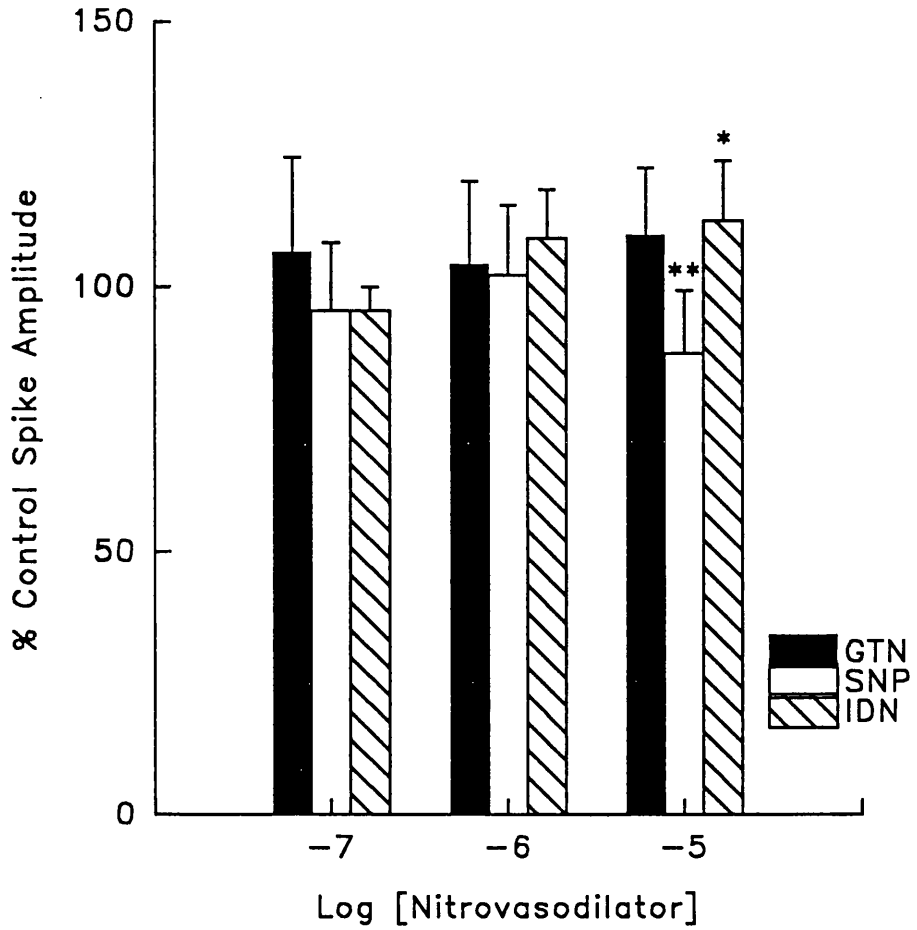


Fig.67 : The effects (± s.e. mean) of GTN (n ≥ 5), SNP (n ≥17) and IDN (n = 4 ; each 10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the maximum spike amplitude (mV) per burst of electrical activity, in the rat portal vein. SNP reduced but IDN increased significantly (*, P < 0.05 ; **, P < 0.01), the maximum spike amplitude.

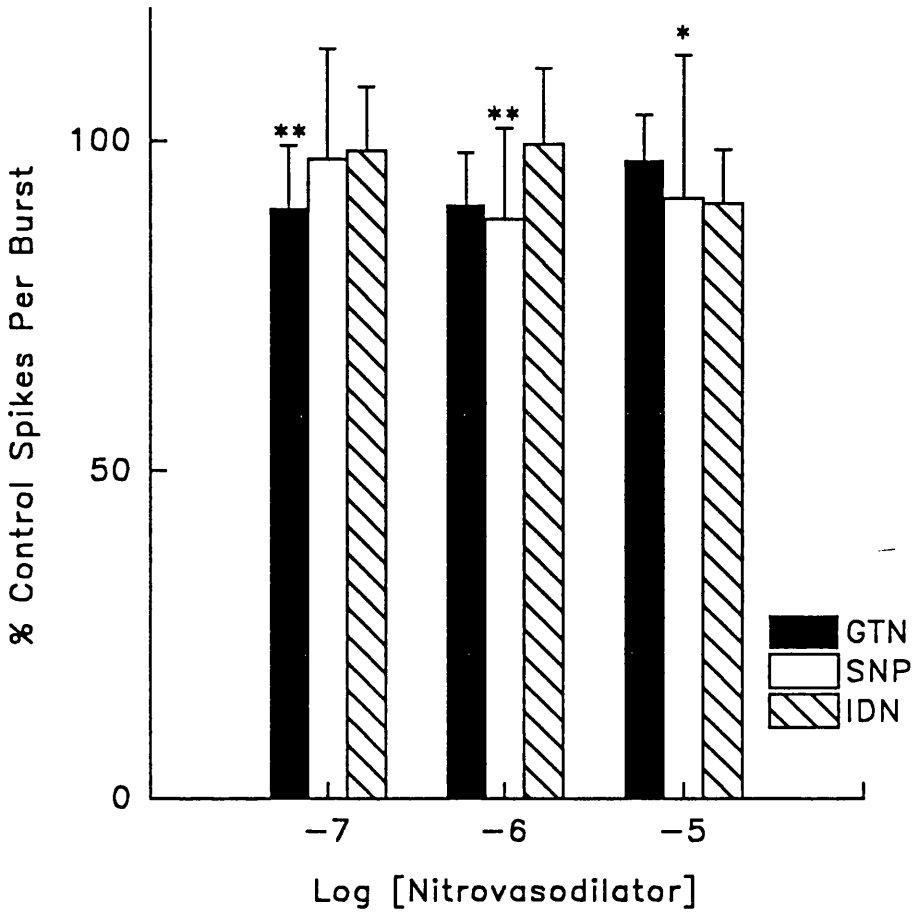


Fig. 68 : The effects (± s.e. mean) of GTN (n ≥ 5), SNP (n ≥ 14) and IDN (n = 4 ; each 10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the number of spikes per burst of electrical activity, in the rat portal vein. GTN and SNP each reduced significantly (*, $P < 0.05$; **, $P < 0.01$), the number of spikes per burst.

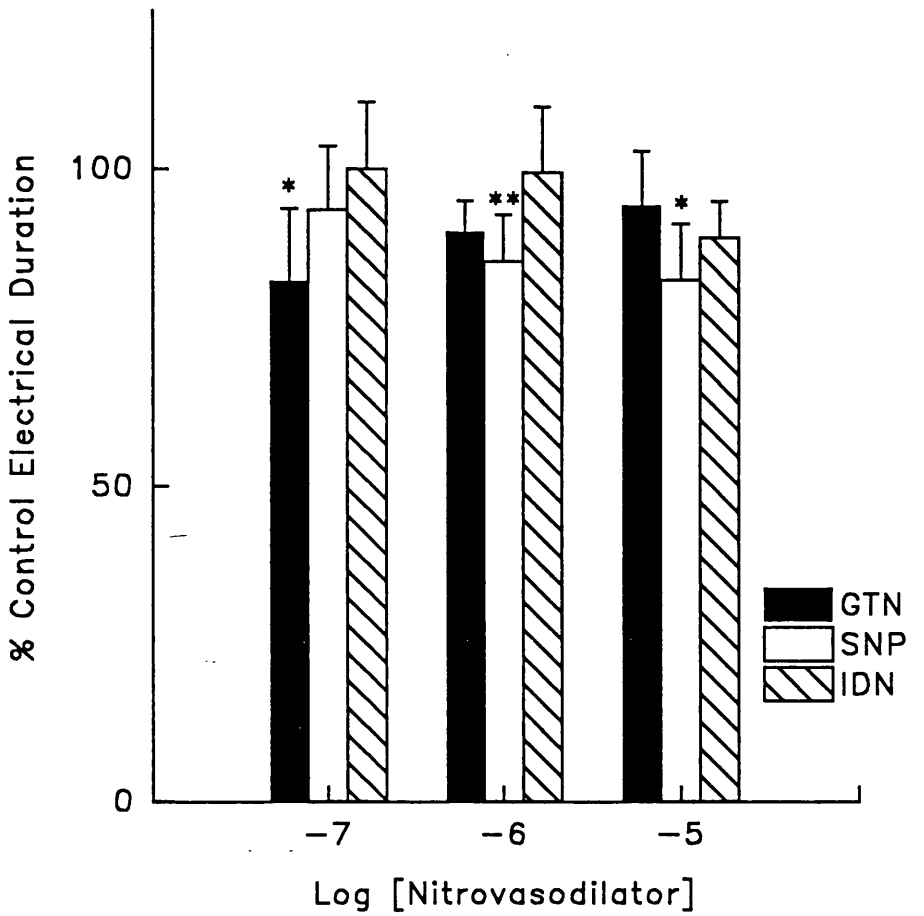


Fig. 69 : The effects (± s.e. mean) of GTN (n ≥ 5), SNP (n ≥ 17) and IDN (n = 4 ; each 10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the duration (s) of each burst of electrical activity, in the rat portal vein. GTN and SNP each reduced significantly (*, P < 0.05 : **, P < 0.01), the duration of each burst.

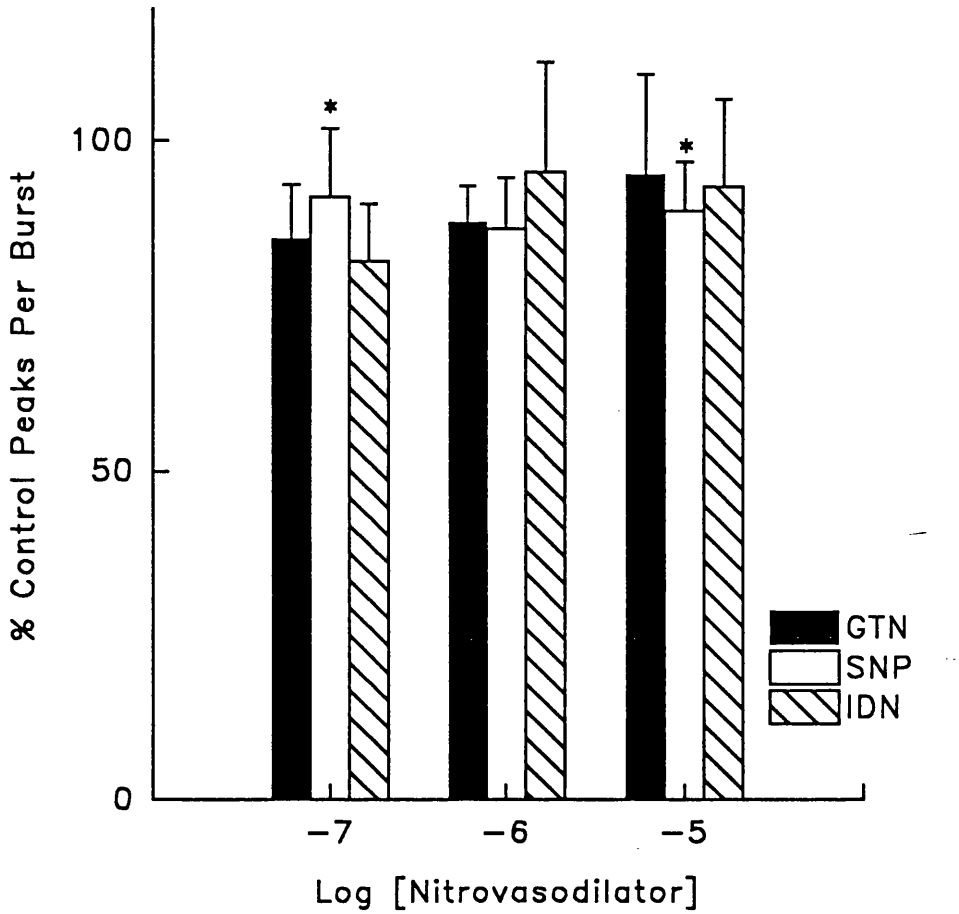


Fig. 70 : The effects (± s.e. mean) of GTN (n ≥ 5), SNP (n ≥ 29) and IDN (n = 4 ; each 10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the number of peaks per burst of mechanical activity, in the rat portal vein. SNP reduced significantly (*, P < 0.05), the number of peaks per burst.

effect. Despite its lack of effect on this parameter, however, GTN (10^{-7}M ; n = 6 ; $P < 0.05$; Fig. 71) significantly decreased the mean duration of mechanical bursts ; an effect also achieved by SNP ($\geq 10^{-6}$M ; $n \geq 33$; $P < 0.001$; Fig. 71). IDN (10^{-7}-10^{-5}M ; $n \geq 3$; Fig. 71) had no effect.

The concept that, as in RDC, these nitrovasodilator-induced effects would be mediated via NO release and cGMP stimulation was investigated. Hb (10^{-5}M) inhibited both the excitatory and inhibitory effects of SNP on spontaneous activity (Fig. 72). The rise in frequency ($P < 0.05$; Fig. 73) and the fall in contractile amplitude ($P < 0.05$; Fig. 74) induced by SNP (10^{-5}M), were antagonized by Hb (10^{-5}M ; n = 4), added 10 min before SNP, indicating that the actions of this nitrovasodilator were mediated through the release of NO. Hb (10^{-5}M), alone, was without effect.

Using 8-bromo cGMP it was established that the actions of SNP could be mediated through cGMP stimulation. This compound mimicked a number of the effects of SNP in the rat PV (Fig. 75). The number of spikes in and the duration of each electrical burst, the maximum contractile amplitude and the duration of each mechanical burst were significantly inhibited by 8-bromo cGMP ($\geq 10^{-5}$M ; $n \geq 3$; $P < 0.05$), suggesting that these actions of SNP could be mediated by a cGMP-dependent mechanism.

In support of the idea that the effects of SNP were, indeed, mediated through NO release and cGMP activation, $K_4Fe(CN)_6$ was found to have no effect on the mechanical responses of rat PV. The frequency, amplitude, number of peaks per burst and duration of the response were unaffected by $K_4Fe(CN)_6$ (10^{-6}-10^{-4}M ; $n \geq 3$; results not shown). Thus, as in RDC, in the rat PV, the effects of SNP on pacemaker activity were not mediated by the ferrocyanide portion of the molecule.

Interestingly, in the rat PV, the actions of SNP were also mimicked by cAMP activation. The effects of cAMP stimulation were examined in two separate experiments using a) isoprenaline and b) forskolin. The β-

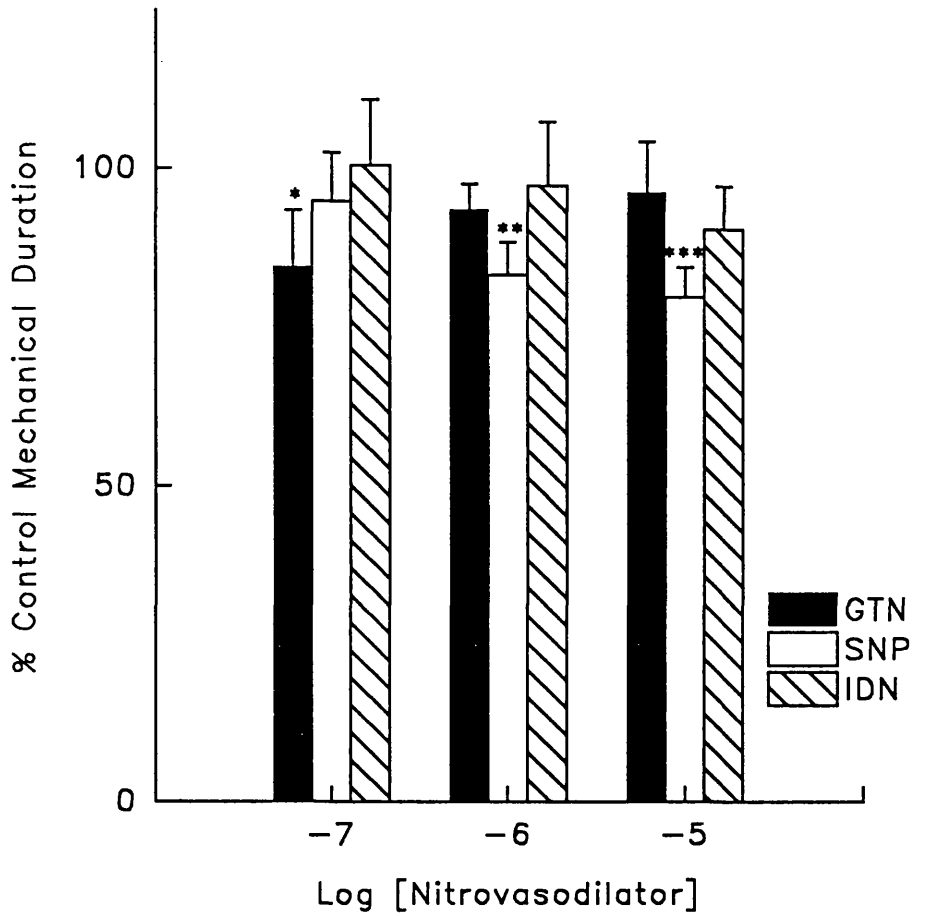


Fig. 71 : The effects (± s.e. mean) of GTN ($n \geq 5$), SNP ($n \geq 29$) and IDN ($n = 4$; each 10^{-7}–10^{-5}M), expressed as a percentage of the control (100%), on the duration (s) of each burst of mechanical activity, in the rat portal vein. GTN and SNP each reduced significantly (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$), the duration of each burst.

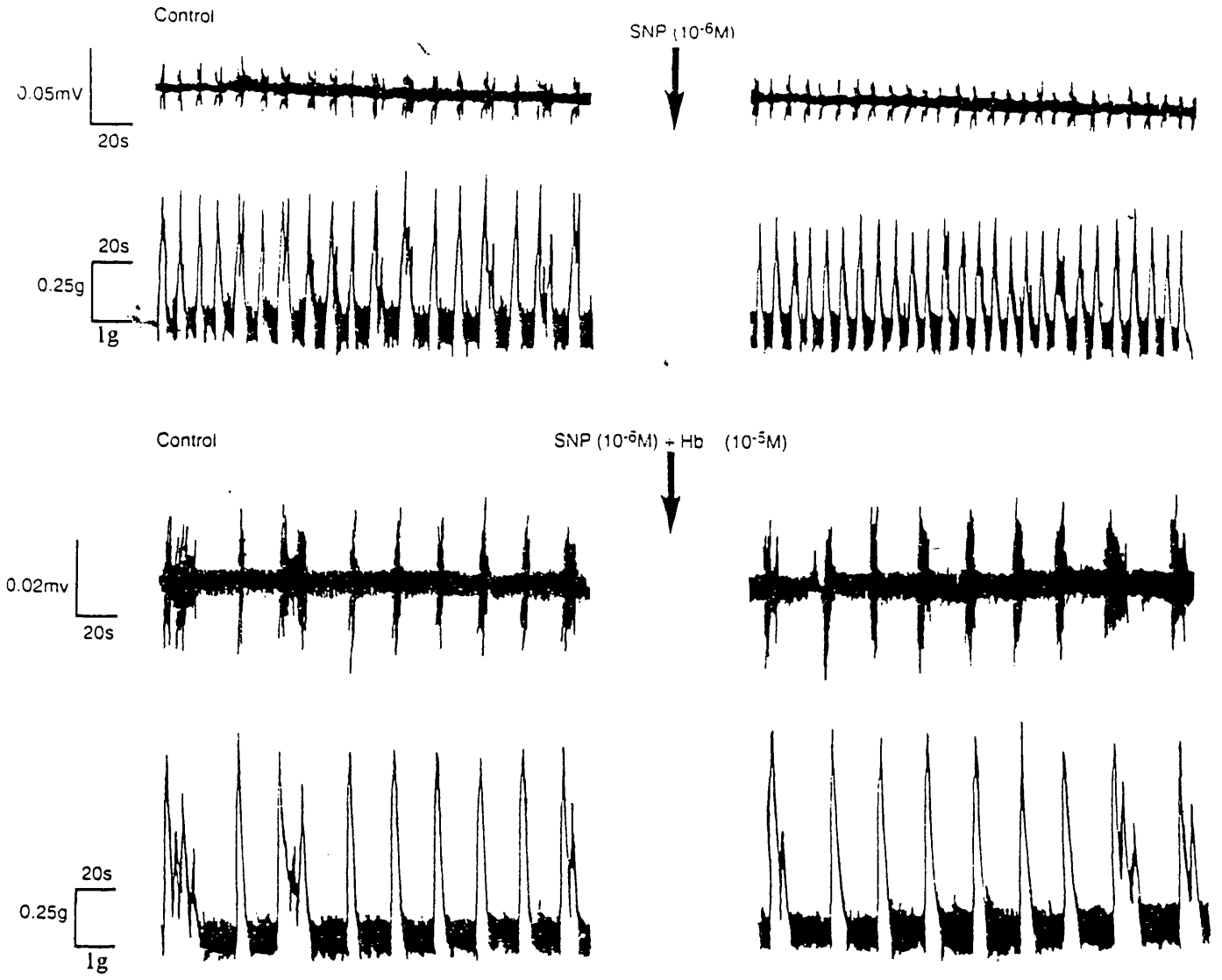


Fig. 72 : Inhibition of the excitatory and inhibitory effects of SNP (10^{-6}M), by Hb (10^{-5}M), on the spontaneous electrical (top) and mechanical activity recorded from the rat portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. SNP, alone, increased the frequency of both electrical and mechanical activity and reduced the amplitude of contraction. In the presence of Hb, SNP had no effect.

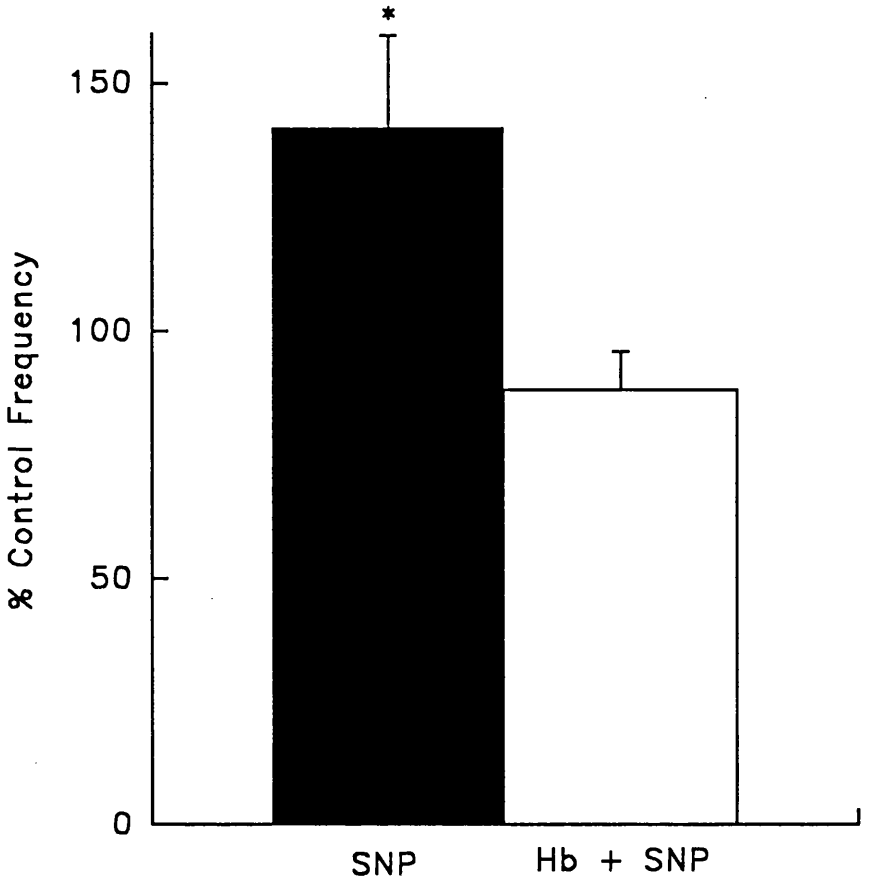


Fig. 73 : The effect (± s.e. mean, n = 4) of SNP (10^{-5}M), expressed as a percentage of the control (100%), on the frequency (bursts min^{-1}) of spontaneous activity, in the rat portal vein, in the absence and in the presence of Hb (10^{-5}M). SNP significantly increased (*, $P < 0.05$) the frequency of activity in the absence, but not in the presence, of Hb.

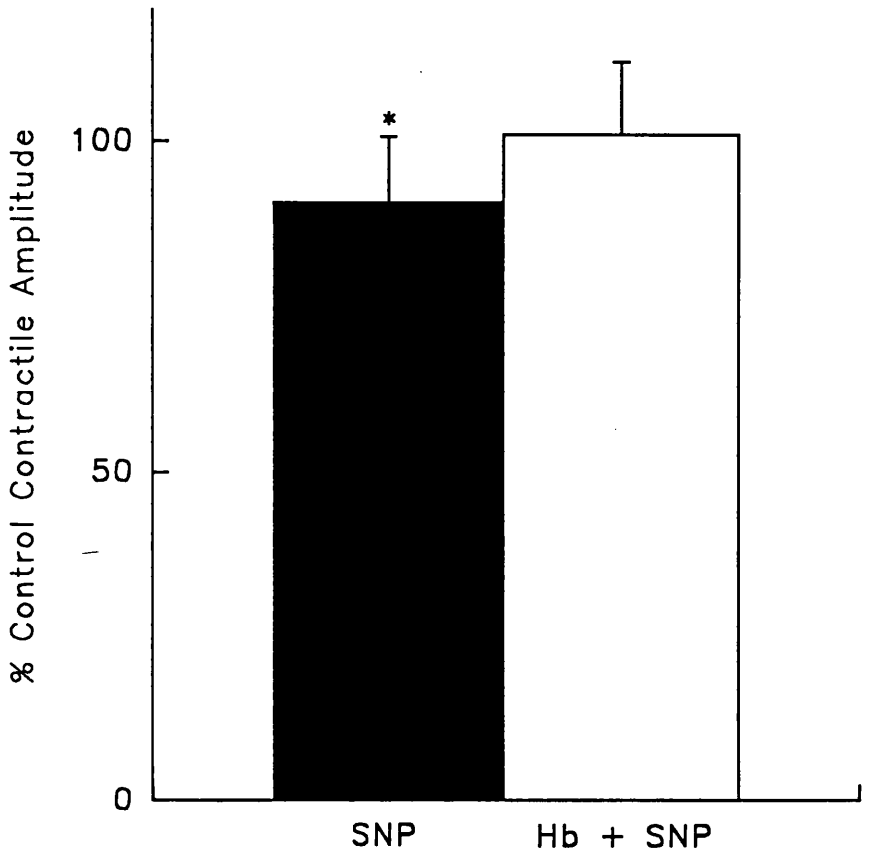


Fig. 74 : The effect (± s.e. mean, n = 4) of SNP (10^{-5}M), expressed as a percentage of the control (100%), on the maximum contractile amplitude (g) per burst of mechanical activity, in the rat portal vein, in the absence and in the presence of Hb (10^{-5}M). SNP significantly reduced (*, $P < 0.05$) the amplitude of contraction in the absence, but not in the presence, of Hb.

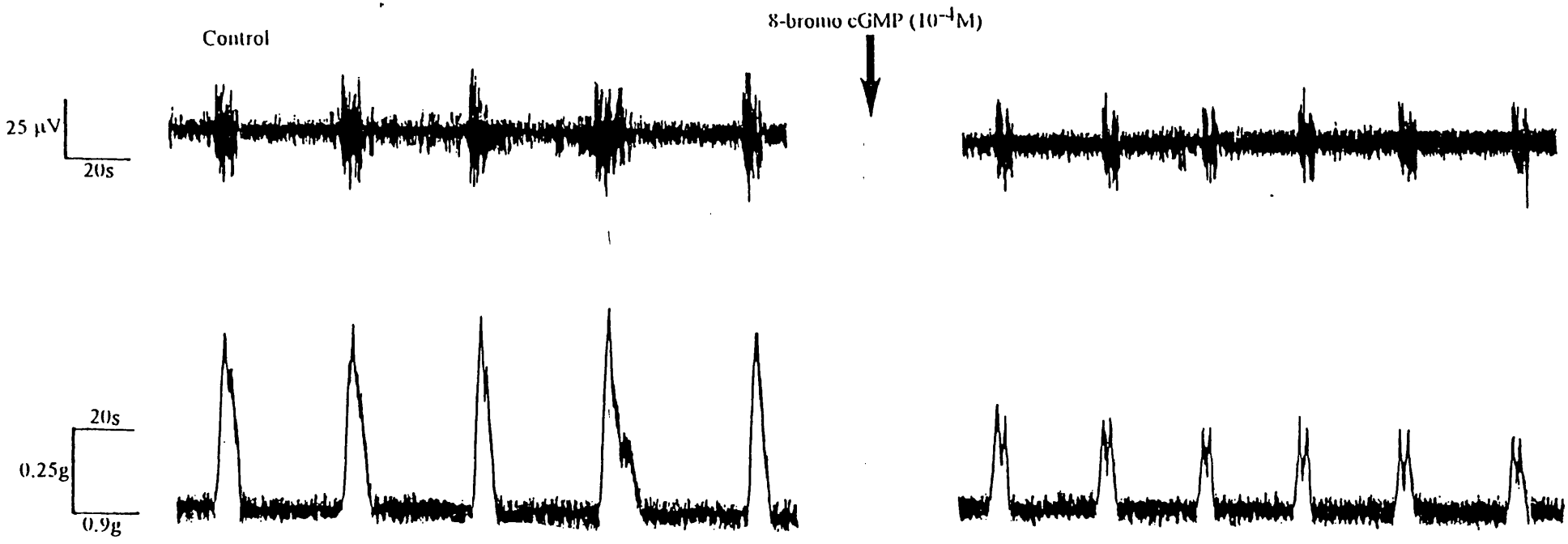


Fig. 75 : The effect of 8-bromo cGMP (10^{-4}M) on the spontaneous electrical (top) and mechanical activity recorded from the rat portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. 8-bromo cGMP increased the frequency of both electrical and mechanical activity and reduced the amplitude of contraction.

adrenoceptor agonist, isoprenaline, like SNP, increased the frequency of spontaneous activity and reduced both the spike and contractile amplitudes, the number of spikes or peaks per burst and the duration of electrical or mechanical responses significantly ($P < 0.05$; Fig. 76). Isoprenaline was, in fact, more potent than SNP, and enhanced the frequency and lowered the amplitude of contraction at concentrations as low as 10^{-8}M (n = 9). The potent adenylate cyclase activator, forskolin, had a similar action to that of isoprenaline on both spontaneous electrical and mechanical activity except that, at higher doses (10^{-6}M), forskolin abolished all activity (Fig. 77). Isoprenaline never abolished activity even at 10^{-5}M. It is, however, unlikely that SNP, itself, was stimulating cAMP. There is no evidence of this in the literature and, besides, the cAMP activators examined were at least ten times as potent as SNP in producing the desired effects. The available evidence indicates that both the excitatory and the inhibitory effects of SNP are probably mediated via NO release and cGMP stimulation.

In theory, the release of a contractile agonist such as noradrenaline or other catecholamine, would be expected to increase the frequency of spontaneous activity in rat PV. In an attempt to shed light on the mechanisms underlying the excitation to SNP, more in hope than in expectation, the effects of SNP (10^{-5}M) were investigated in the presence of phentolamine and propranolol (each 3 x 10^{-6}M) in rat PV (Fig. 78). The results were unexpected. SNP (10^{-5}M), alone, increased the frequency of activity and reduced the contractile amplitude, number of peaks per burst and duration of mechanical bursts. These effects of SNP were antagonized by the α-adrenoceptor antagonist, phentolamine, together with the β-adrenoceptor antagonist, propranolol (each 3 x 10^{-6}M ; Fig. 78), suggesting that SNP was either stimulating the secondary release of noradrenaline or was acting directly on adrenoceptors. These results prompted a study of the ability of SNP to liberate radiolabelled noradrenaline (^{3}H-noradrenaline) from adrenergic nerves in the rat PV (see 3.5.3.).

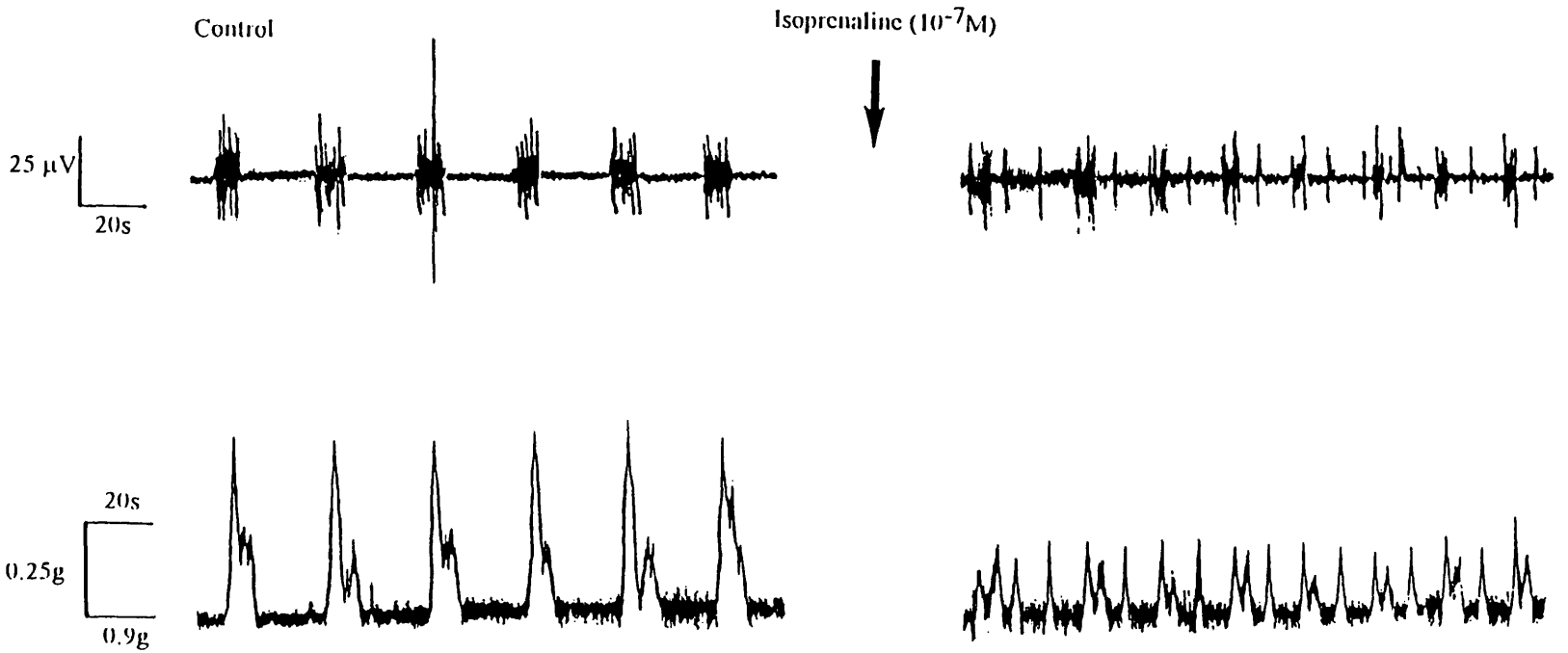


Fig. 76 : The effect of isoprenaline (10^{-7}M) on the spontaneous electrical (top) and mechanical activity recorded from the rat portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. Isoprenaline increased the frequency of both electrical and mechanical activity and reduced the amplitude of contraction.

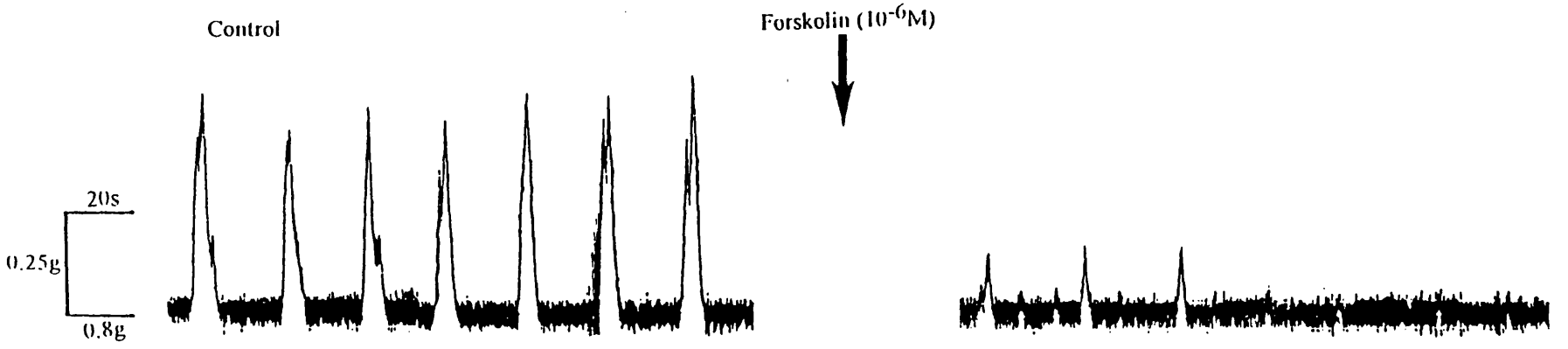


Fig. 77 : The effect of forskolin (10^{-6}M) on the spontaneous mechanical activity recorded from the rat portal vein, under a resting tension of approximately 1g, in the Golenhofen apparatus. Forskolin reduced the amplitude of, then abolished, mechanical activity.

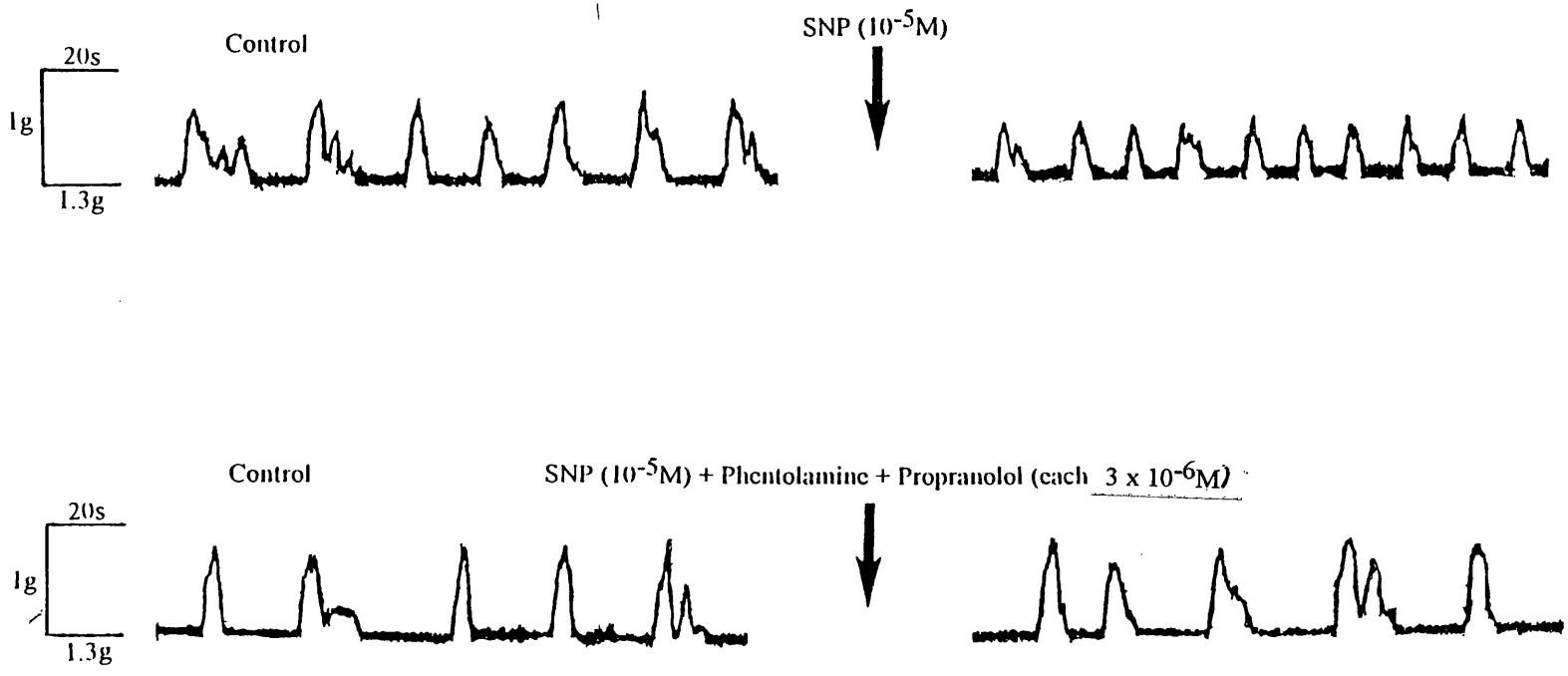


Fig. 78 : Inhibition of the effect of SNP (10^{-5}M) on the spontaneous mechanical activity of the rat portal vein, under a resting tension of approximately 1g, by phentolamine and propranolol together (each 3 x10^{-6}M). SNP alone, but not in the presence of phentolamine and propranolol, increased the frequency of contraction.

3.5.2. Radiolabelled rubidium efflux

The movement of K^+ across the cell membrane was studied by incubating intact sections of rat PV in Krebs solution containing ^{86}Rb (Martin & Gordon, 1983 ; Hamilton *et al*, 1986), before mounting them in the Golenhofen apparatus. Following the commencement of superfusion, the ^{86}Rb efflux rate declined rapidly (Fig. 79) due to the loss of isotope from the extracellular space. The rate was then constant. This constant phase represented the loss of ^{86}Rb from the smooth muscle cells themselves. The mean value of the efflux rate constant (E) was $11.2 \pm 0.74 \times 10^{-3}$ min^{-1} (n = 24). This was considerably larger than the values obtained in RDC ($6.59 \pm 0.26 \times 10^{-3}$ min^{-1}) or rabbit aorta ($4.22 \pm 0.14 \times 10^{-3}$ min^{-1}, Martin & Gordon, 1983 ; $4.8 \pm 0.4 \times 10^{-3}$ min^{-1}, Bolton & Clapp, 1984), but was comparable with the value reported in guinea-pig taenia caeci ($11.38 \pm 0.4 \times 10^{-3}$ min^{-1}, Bolton & Clark, 1981).

In this tissue, both noradrenaline and BRL 38227 were used as controls, to test that the system was functional. These drugs were selected since noradrenaline was known to have significantly increased ^{86}Rb efflux from aortic vascular smooth muscle (Martin & Gordon, 1983), while BRL 38227 is the active (-)-enantiomer of the racemate, BRL 34915 (cromakalim), which had been reported to enhance the ^{86}Rb efflux from rat portal vein (Hamilton *et al*, 1986 ; Weir & Weston, 1986b).

In rat PV, noradrenaline induced a ^{86}Rb efflux (Fig. 80). The value of E was significantly increased ($P < 0.01$) from $13.3 \pm 1.3 \times 10^{-3}$ min^{-1} to $18.04 \pm 2 \times 10^{-3}$ min^{-1} by noradrenaline ($10^{-5}M$; n = 5). This efflux of rubidium in response to noradrenaline, may have been secondary to a primary influx of Ca^{++} which affected the gating mechanism. This has been reported in rabbit aorta (Martin & Gordon, 1983).

BRL 38227 ($10^{-5}M$; n = 7) also enhanced the efflux of ^{86}Rb in rat PV. E was significantly increased ($P < 0.05$) from $10 \pm 1.7 \times 10^{-3}$ min^{-1} to $11.9 \pm 2 \times$

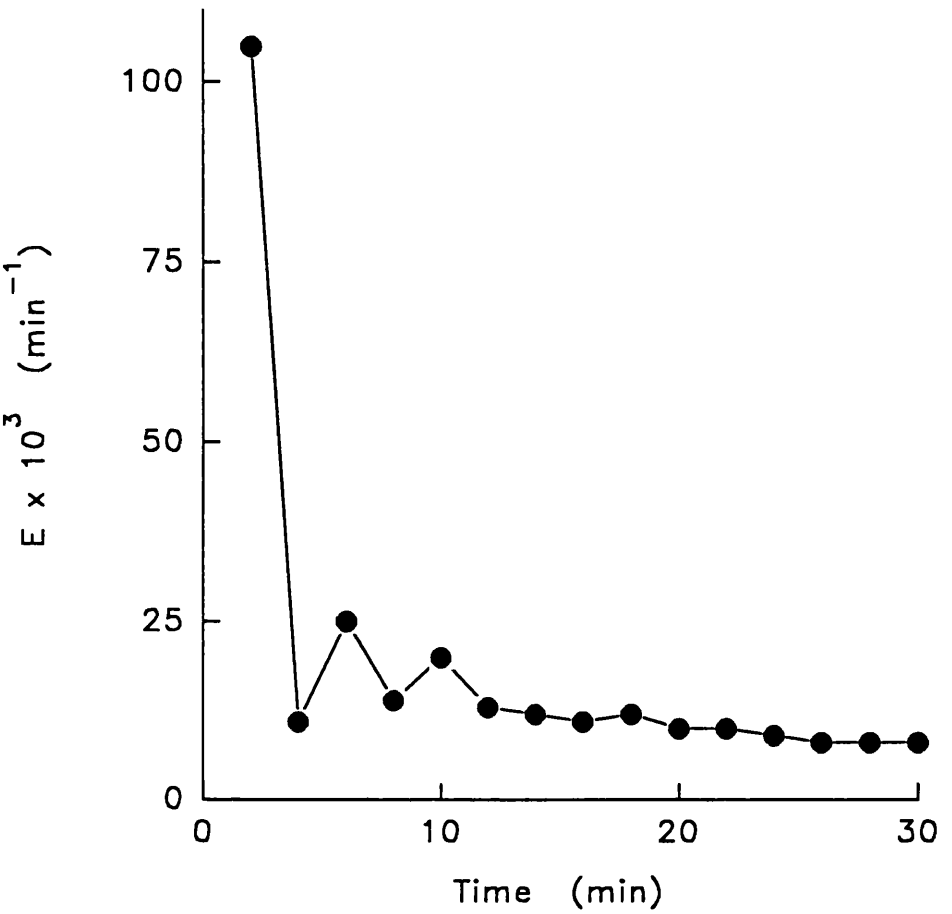


Fig. 79 : The exponential decline with time of the resting ^{86}Rb efflux, expressed as the first-order rate constant (E ; x 10^3), from the rat portal vein incubated with $^{86}RbCl$. By 15 min, approximately, ^{86}Rb efflux had reached a relatively steady value.

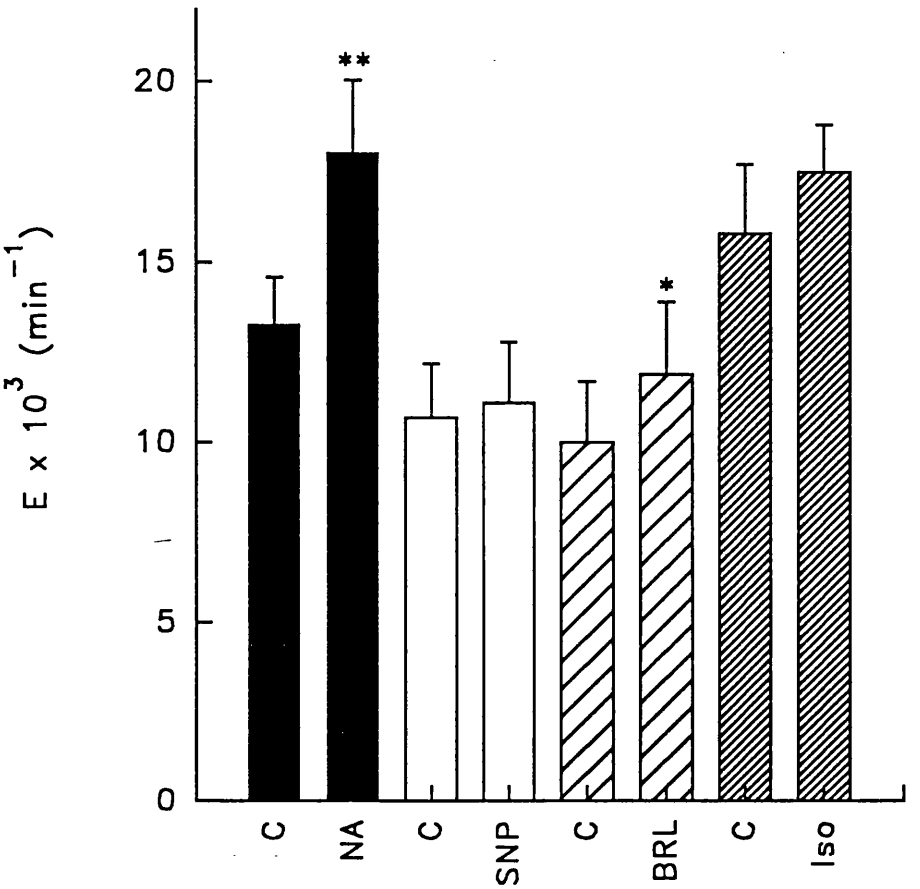


Fig. 80 : The effects (± s.e. mean) of noradrenaline (NA, n = 5), SNP (n = 12), BRL 38227 (BRL ; n = 7) and isoprenaline (Iso ; n = 4 ; each 10^{-5}M), on the resting ^{86}Rb efflux (C), expressed as the efflux rate constant (E ; x 10^3), from the rat portal vein incubated with ^{86}RbCl. The resting ^{86}Rb efflux varied between 10 and 15.8. Noradrenaline and BRL 38227 each increased the value of E significantly (*, $P < 0.05$; **, $P < 0.001$).

10^{-3} min^{-1} by this concentration of drug (Fig. 80). This effect was almost certainly mediated through the primary opening of membrane K^+ channels as has been previously reported (Hamilton *et al*, 1986 ; Weir & Weston, 1986).

The results with SNP were slightly disappointing. SNP ($n \geq 4$) had no effect on the ^{86}Rb efflux rate constant at any concentration (10^{-7}-10^{-4}M) indicating that the effects of cGMP stimulation did not involve an efflux of K^+. The effects of SNP (10^{-5}M) are compared with those of noradrenaline (10^{-5}M) and BRL 38227 (10^{-5}M) in Fig. 80.

The effects of cAMP stimulation, which resembled the response to SNP on electrical and mechanical activity, was also examined on ^{86}Rb efflux using isoprenaline. Like SNP this compound was ineffective. Isoprenaline did not significantly increase the ^{86}Rb efflux rate at any concentration (10^{-7}-10^{-4}M ; $n = 3$; Fig. 80). Thus, neither the response to cGMP stimulation nor the response to cAMP stimulation involved the efflux of K^+.

3.5.3. Radiolabelled (^3H) noradrenaline overflow

Experiments in which the actions of SNP on mechanical activity were inhibited by phentolamine and propranolol, together, suggested that SNP possibly stimulated the release of noradrenaline (see 3.5.1.). To examine this possibility, intact sections of rat PV were incubated with radiolabelled (^3H) transmitter and the effect of drug on the overflow measured. Following the commencement of superfusion, the overflow of ^3H from the rat PV declined exponentially (Fig. 81), until after about 1.5-2 hr it reached a steady level. Drugs were added after three hours, at which time the overflow of ^3H was assumed to be constant. The value of the overflow rate constant (calculated using the same equation as was used for the efflux rate constant, with the exception that d.p.m. rather than c.p.m. were inserted) was $1.89 \pm 0.12 \times 10^{-3}$ min^{-1}.

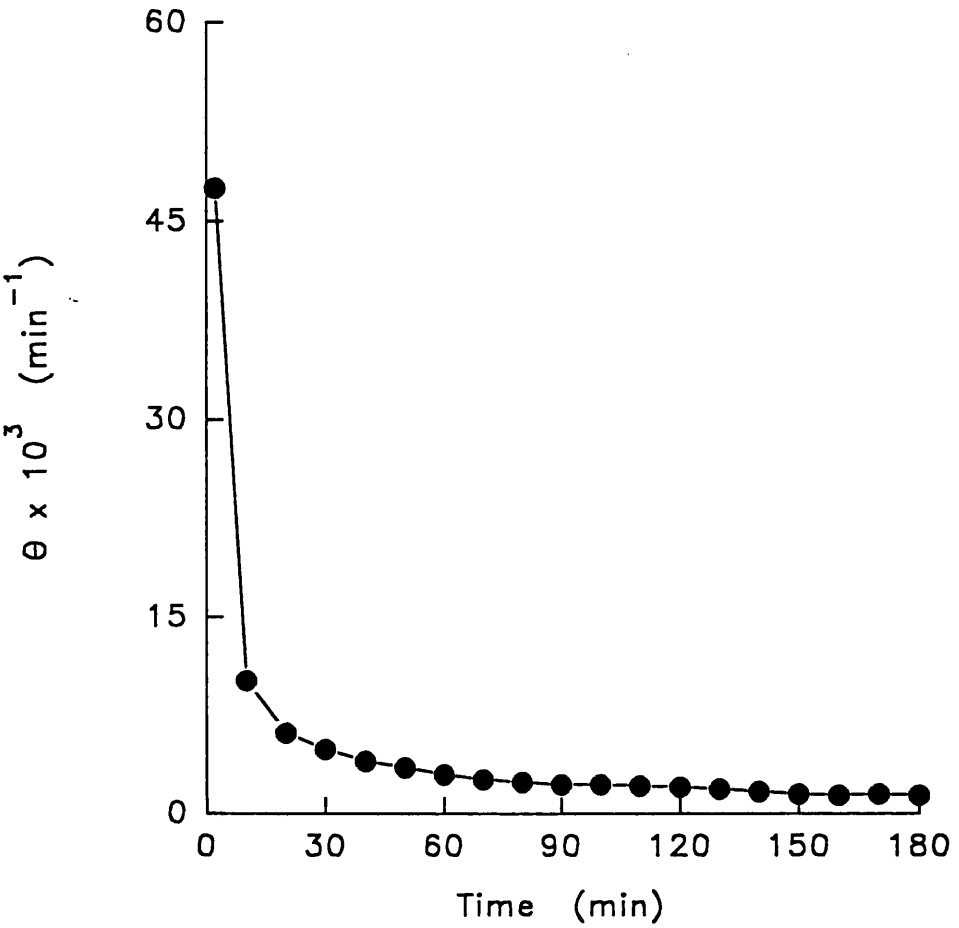


Fig. 81 : The exponential decline with time of the resting ^{3}H overflow, expressed as the overflow rate constant (θ ; x 10^{3}), from the rat portal vein incubated with [^{3}H]–noradrenaline. By 1.5–2 hr, approximately, [^{3}H] overflow had reached a relatively steady value.

The indirect sympathomimetic, tyramine, was used as a control to check that the system was functional. This drug should have released noradrenaline from the nerve terminals, thereby increasing the overflow. This was the case. Tyramine (10^{-5}M ; n = 10) significantly increased ($P < 0.001$) the overflow rate of ^{3}H from $1.29 \pm 0.09 \times 10^{-3}$ min^{-1} to $1.62 \pm 0.11 \times 10^{-3}$ min^{-1} (Fig. 82).

SNP was ineffective. This compound did not significantly increase the rate of ^{3}H overflow at any concentration (10^{-7}-10^{-5}M ; $n \geq 7$; Figs. 82 and 83).

3.6. Effects of SNP on non-spontaneously active smooth muscle

The effects of SNP were examined on non-spontaneously active smooth muscle to determine whether SNP-induced excitation was unique to pacemaker driven tissues. The evidence from the muscles examined, suggested that this was probably the case. When the non-spontaneously active rat anococcygeus or rabbit abdominal aorta were mounted, under a resting tension of 1g, in the Golenhofen apparatus, then SNP did not induce spontaneous activity at any concentration (10^{-7}-10^{-4}M), nor did it have any effect on the resting tension (in each tissue, n = 3 ; results not shown). In each tissue, noradrenaline (10^{-5}M) raised the tone, indicating that the tissues were active and functioning as normal.

3.7. Effects of SNP on $[Ca^{++}]_i$

Experiments in which the Ca^{++} channel agonist BAY K 8644 reversed the actions of SNP, and vice versa, on the spontaneous activity of RDC (see 3.3.1.), provided indirect evidence that the SNP-induced actions (in particular the reduced amplitude of spontaneous contraction) were, at least partly, mediated by a reduction in the level of intracellular Ca^{++} ($[Ca^{++}]_i$). The ability of SNP to reduce $[Ca^{++}]_i$ was examined directly in rat aortic vascular smooth

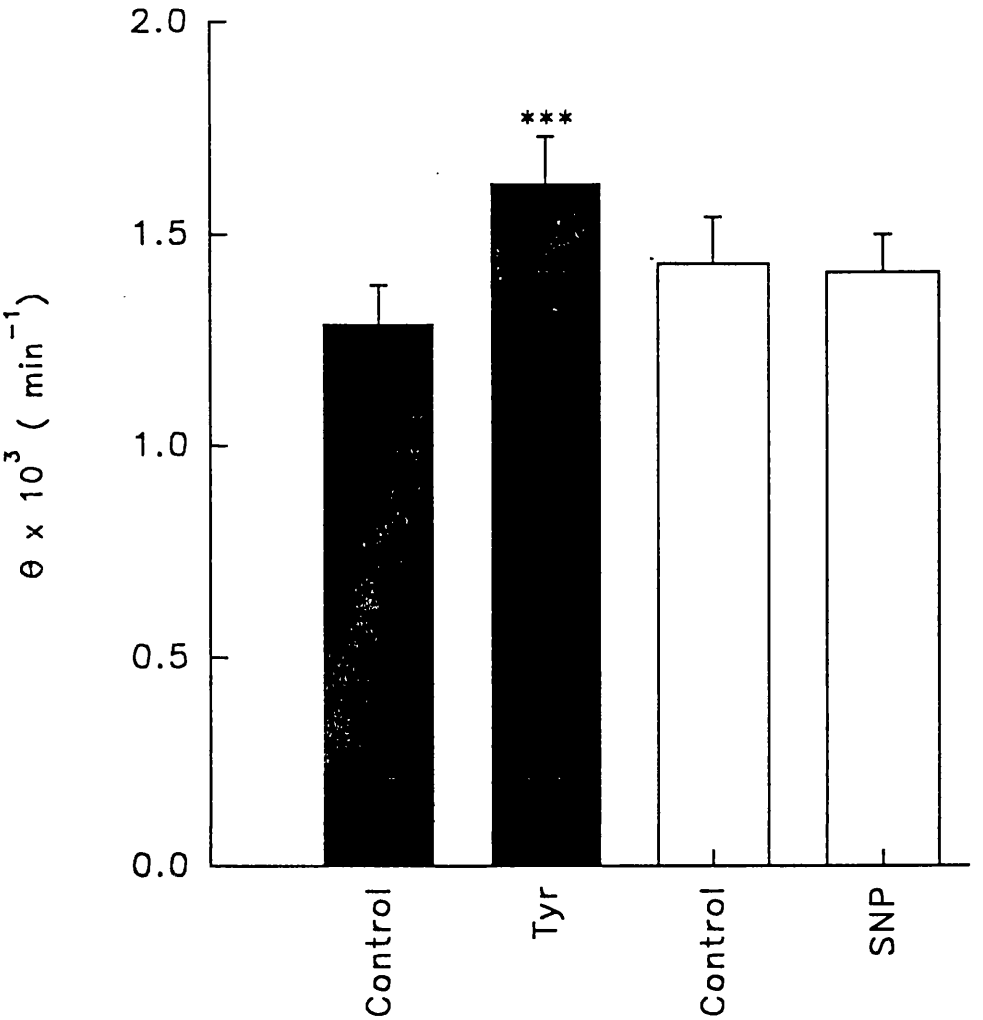


Fig. 82 : The effects (± s.e. mean, n ≥ 7) of SNP compared with those of tyramine (each 10^{-5}M), on the resting ^{3}H overflow, expressed as the overflow rate constant (θ ; x 10^{3}), from the rat portal vein incubated with [^{3}H]–noradrenaline. Only tyramine increased the resting ^{3}H overflow (***, $P < 0.001$). The following drugs were present throughout : ascorbic acid (1.1×10^{-4}M) to prevent catecholamine oxidation, imipramine (6×10^{-7}M) and normetanephrine (10^{-5}M) to respectively inhibit uptake–1 and uptake–2 processes, tranylcypromine (10^{-5}M) to inhibit monoamine oxidase and yohimbine (10^{-7}M) to block presynaptic α_2–adrenoceptors.

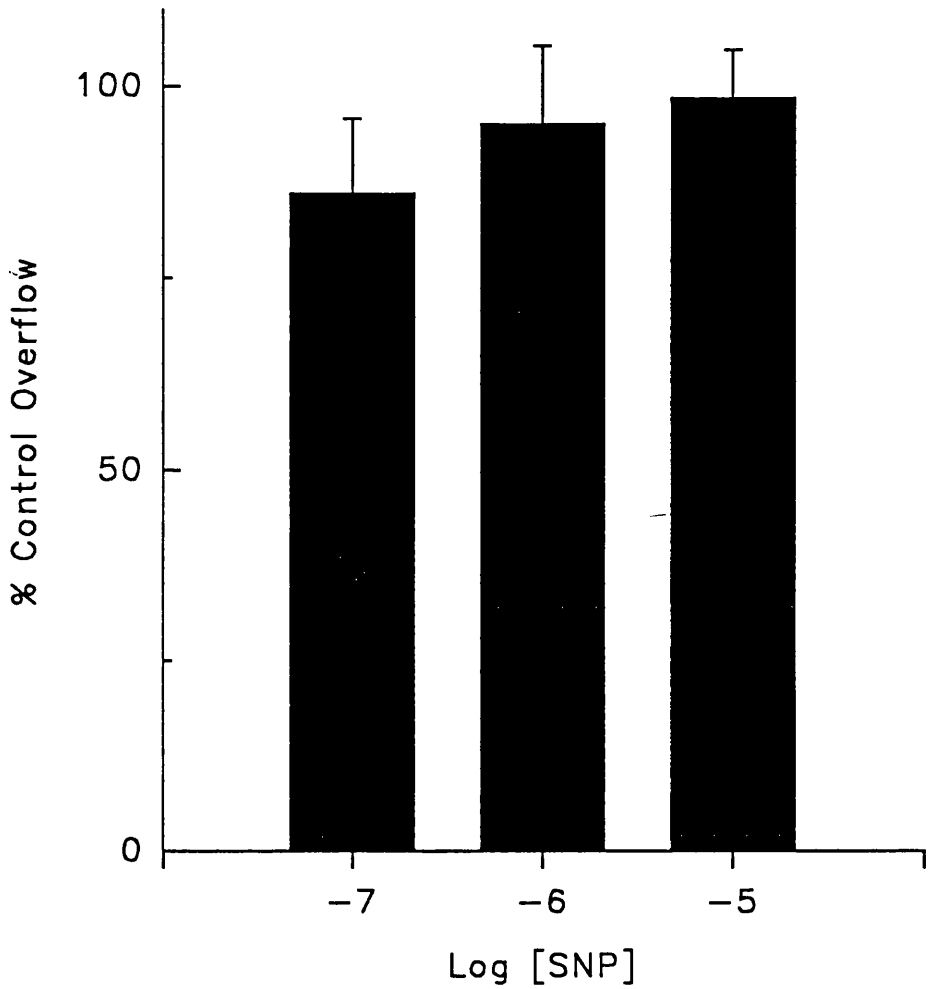


Fig. 83 : The effect (± s.e. mean, n ≥ 3) of SNP (10^{-7}–10^{-5}M) expressed as a percentage of the control (100%), on the resting ^{3}[H] overflow rate from the rat portal vein incubated with ^{3}[H]–noradrenaline. SNP did not significantly affect the rate of overflow at any concentration.

muscle cells, grown on glass cover slips. In these cells the basal concentration of intracellular free Ca^{++} was 119 ± 11.5 nM (n = 6). In order to check the viability of the cells, they were stimulated with angiotensin II (10^{-7}M). This significantly increased ($P < 0.01$) the basal free $[Ca^{++}]_i$ to 250.5 ± 100 nM (Fig. 84), indicating that the cells were viable. The results with SNP were disappointing. Despite SNP (10^{-5}M) significantly reducing the amplitude of spontaneous contraction in RDC ($P < 0.001$), this concentration of drug had no effect on $[Ca^{++}]_i$ (Fig. 84).

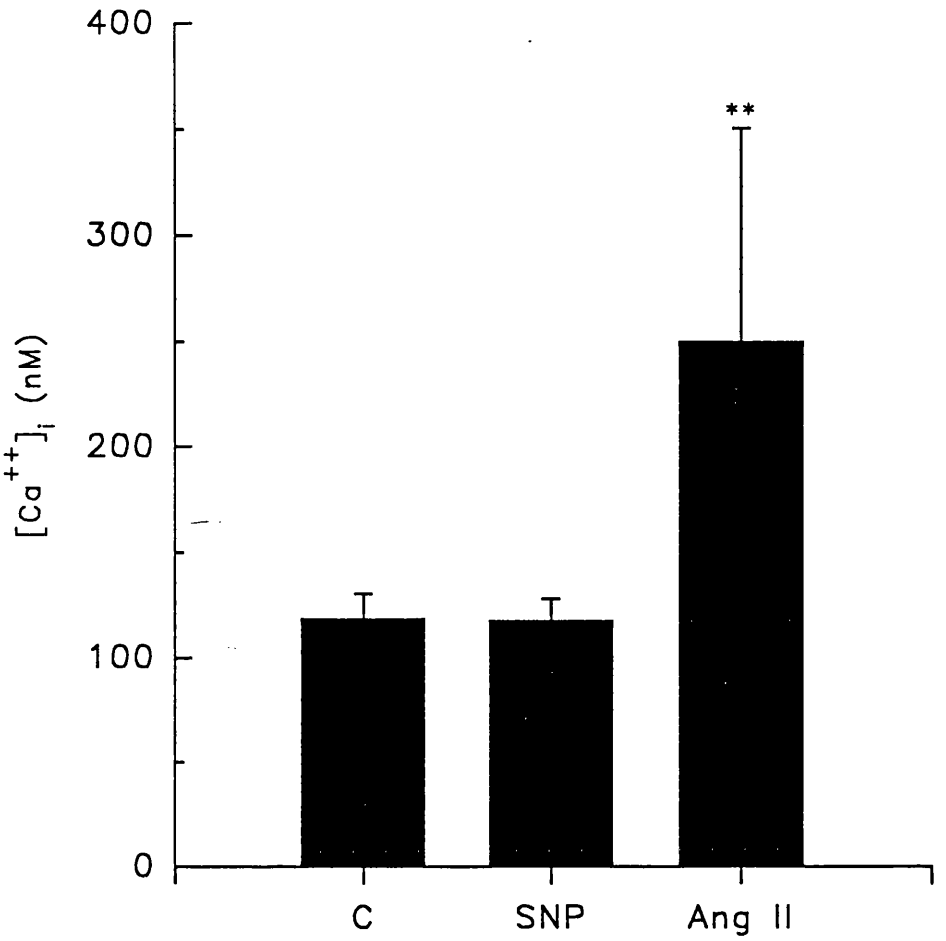


Fig. 84 : The effect of SNP (10^{-5}M) or angiotensin II (Ang II ; 10^{-7}M), expressed as a percentage of the control (100%), on the intracellular free calcium concentration ($[Ca^{++}]_i$; nM ; n = 6), of rat aortic vascular smooth muscle cells. Only angiotensin II increased significantly ($P < 0.01$), the intracellular free calcium concentration.

Chapter 4 : Discussion

Examination of the actions of the nitrovasodilators on spontaneously active smooth muscle revealed a number of distinct effects. There were certain differences in the responses of different tissues to these drugs but, in the main, nitrovasodilator-induced responses in RDC and rat PV followed the same broad pattern. This pattern differed from that in the RPV. Since SNP was the most effective compound tested, this was used as a model for the actions of this group of drugs.

In the RDC, but not in the rat PV, the immediate effect of the nitrovasodilators was to induce a short period of quiescence. This generally lasted between 1 and 5 min although, rarely, up to 10 min. Spontaneous electrical and mechanical activity, the pattern of which differed from the control, then returned abruptly. In response to SNP, IDN or, occasionally, GTN, the frequency of both electrical and mechanical responses was significantly increased, suggesting that these drugs exerted an effect on the pacemaker. Additionally, in the few RDC strips where the initial frequency was high (> 6 bursts min^{-1}), SNP slowed the pacemaker activity. It appeared that SNP could either increase or decrease the pacemaker frequency depending on the level of control activity. Simultaneously, each nitrovasodilator significantly reduced the mean contractile amplitude per burst of mechanical activity. This pattern of spontaneous events was mimicked in the rat PV, in the absence of the prior period of quiescence. In this tissue, SNP significantly increased the frequency of activity while SNP and GTN each significantly decreased the amplitude of spontaneous contractions. In most cases, in either tissue, these effects were accompanied by a reduction in the number of spikes/peaks per burst and the duration of electrical/mechanical bursts and, occasionally, by an increase in the amplitude of electrical spikes. Rarely, the increase in spike amplitude came after the other changes, suggesting that it was secondary to them. In RPV, there was no evidence of any excitation in response to SNP ; the frequency of electrical and mechanical activity, the

contractile amplitude, the number of spikes/peaks per burst and the duration of electrical/mechanical bursts were each reduced then abolished by this drug.

The quiescence observed in RDC in response to the nitrovasodilators, it was presumed, arose from a membrane hyperpolarization which could not be detected by the extracellular recording technique employed. Indeed, nitrovasodilators hyperpolarize 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 guinea pig internal anal sphincter (Rae & Muir, 1993). Since most smooth muscle hyperpolarizations are the result of a K^+ efflux, this mechanism was presumed to operate here. Evidence to support this view was obtained when SNP and the K^+ channel opener, BRL 38227, were found to be synergistic in the abolition of spontaneous activity.

In light of these results from the RDC, K^+ efflux was studied directly by loading both RDC and rat PV with the marker for K^+, ^{86}Rb, mounting the tissues in the Golenhofen apparatus, and analysing the superfusate for radioactivity. In RDC, the efflux rate constant for ^{86}Rb (E) was $6.59 \pm 0.26 \times 10^{-3}$ min^{-1}. This was considerably less than the reported value in guinea-pig taenia caeci of $11.38 \pm 0.4 \times 10^{-3}$ min^{-1} (Bolton & Clark,1981), indicating that the membrane permeability of this tissue for K^+ ions was greater than that of RDC. If SNP induced the opening of membrane K^+ channels, then the value of E, in response to SNP, should have been increased. Under the experimental conditions, SNP did not increase the value of E in RDC. However, BRL 38227 was also ineffective. Even at concentrations that abolished both electrical and mechanical events, BRL 38227 did not enhance ^{86}Rb efflux in RDC. This was unexpected since BRL 38227 is the active (-)-enantiomer of the racemate cromakalim (BRL 34915 ; Clapham *et al*, 1991) which opens K^+ channels in a variety of smooth muscles (Weir & Weston,1986a,b ; Quast, 1987 ; Shetty & Weiss, 1987 ; McPherson & Angus, 1990). The ^{86}Rb efflux rate was, however,

increased by the muscarinic agonist, carbachol. The mechanism for this efflux may have involved a Ca^{++} influx, subsequent depolarization and the secondary opening of voltage-operated K^+ channels, as has been reported for the actions of noradrenaline on ^{86}Rb efflux in rat aorta (Martin & Gordon, 1983).

In rat PV, E was 11.2 ± 0.74 x 10^{-3} min^{-1} ; a value comparable to that (11.38 ± 0.4 x 10^{-3} min^{-1}) found in guinea-pig taenia caeci (Bolton & Clark, 1981). This indicates that the membrane permeabilities of these two tissues to K^+ were roughly equivalent, but almost double that of RDC (6.59 ± 0.26 x 10^{-3} min^{-1}), and some three times that of rabbit aorta (4.22 ± 0.14 x 10^{-3} min^{-1}, Martin & Gordon, 1983 ; 4.8 ± 0.4 x 10^{-3} min^{-1}, Bolton & Clapp, 1984). As in RDC, SNP did not induce a ^{86}Rb efflux. Noradrenaline (10^{-5}M) did, however, augment ^{86}Rb efflux (from 13.3 ± 1.3 x 10^{-3} min^{-1} to 18.04 ± 2 x 10^{-3} min^{-1}) ; probably via the opening of voltage-operated K^+ channels, secondary to Ca^{++} influx and contraction. BRL 38227 (10^{-6}M) abolished both electrical and mechanical activity in rat PV, but did not augment efflux. In higher concentrations (10^{-5}M), BRL 38227 abolished all activity **and** enhanced ^{86}Rb efflux (from 10 ± 1.7 x 10^{-3} min^{-1} to 11.9 ± 2 x 10^{-3} min^{-1}). This was in keeping with earlier observations that the relaxant actions of the racemate, BRL 34915, in the portal vein could be dissociated from its ability to open K^+ channels (Weir & Weston, 1986b ; Shetty *et al*, 1987). In rat PV, BRL 34915 (cromakalim ; 5 x 10^{-7}M) abolished spontaneous activity, but it was only when the concentration was increased tenfold (5 x 10^{-6}M) that any evidence of a K^+ efflux was found (Shetty *et al*, 1987). Perhaps, as has been suggested for BRL 34915 (Weir & Weston, 1986b), at the lower doses of BRL 38227, the anticipated small increase in ^{86}Rb efflux from the rat PV was negated by a concomitant reduction in efflux due to the abolition of spontaneous spiking throughout the tissue. Alternatively, the ineffectiveness of BRL 38227 in RDC and, at lower doses, in rat PV, to enhance ^{86}Rb efflux could have been the result of a preferential action of this drug to enhance ^{86}Rb efflux from

pacemaker cells. Similar effects, in guinea pig portal vein, were explained by this proposal (Quast, 1987).

For this reason, in spite of the fact that, in RDC, neither SNP nor BRL 38227 increased the ^{86}Rb efflux rate constant, the possibility that the synergism between these compounds, in producing quiescence in this tissue, was the result of an enhanced K^+ efflux, cannot be eliminated entirely. However, in light of the high concentrations ($10^{-4}M$) tested, it is, perhaps, unlikely that the ineffectiveness of these drugs on the rate of ^{86}Rb efflux was due to their effect being masked by the cessation of spontaneous electrical activity. At this concentration of BRL 38227, the expected large increase in efflux should have been measurable despite the abolition of spontaneous spiking. In fact, BRL 34915 ($6.4 \times 10^{-6}M$) significantly increased the value of E in guinea-pig taenia caeci (Weir & Weston, 1986a). A more likely explanation is that the synergism observed between SNP and BRL 38227 in RDC, was mediated by a preferential effect of each on the pacemaker cells to hyperpolarize them and abolish both spontaneous electrical and mechanical activity. The overall increase in K^+ permeability required would have been so small as to go undetected.

On the other hand, the nitrovasodilator-induced quiescence of spontaneous activity in RDC, may not have arisen from a membrane hyperpolarization ; hence no ^{86}Rb efflux would be expected. NO failed to hyperpolarize a number of smooth muscles (Taylor *et al*, 1988 ; Huang *et al*, 1988 ; Komori *et al*, 1988 ; Chen *et al*, 1988 ; Chen *et al*, 1989 ; Brayden, 1990 ; Suzuki *et al*, 1992). In the vasculature, an endothelium derived hyperpolarizing factor (EDHF) has been postulated to account for endothelium-dependent hyperpolarization (Chen *et al*, 1988 ; Suzuki *et al*, 1992). However, in a variety of smooth muscles, NO did cause hyperpolarization (Ito *et al*, 1978 ; Cheung & MacKay, 1985 ; Tare *et al*, 1990 ; Rae & Muir, 1993). Clearly the ability of NO to hyperpolarize is a tissue dependent effect. While it is

impossible to discount the possibility that another mechanism may have been responsible for the nitrovasodilator-induced quiescence in RDC, the synergism observed between SNP and BRL 38227 suggests that hyperpolarization was probably involved.

Theoretically, excitation may be induced through the release of an excitatory neurotransmitter or through the antagonism of an inhibitory neurotransmitter. In RDC, it was considered that the increase in frequency following nitrovasodilator-induced quiescence could have been mediated through the release of ACh or through the inhibition of noradrenaline release. However, this was unlikely. Neither ACh, nor the adrenoceptor antagonists phentolamine and propranolol, together, mimicked the actions of the nitrovasodilators. In the presence of these compounds, although the frequency of electrical and mechanical activity was increased, activity was irregular and baseline tension often increased. Nitrovasodilators usually regulated activity and never raised the tone. Moreover, the effects of ACh, but not SNP, were inhibited by atropine. Rather than interfering with neurotransmitter release in RDC, nitrovasodilators were more likely to have acted on the pacemaker itself.

Similarly, in rat PV, the possibility that SNP acted through the release of noradrenaline to increase the frequency of activity was considered. In support, the SNP-induced increase in the frequency of the mechanical response was inhibited by phentolamine and propranolol, together, suggesting the involvement of adrenoceptors. To test this, noradrenaline overflow from the rat PV was studied by loading the nerves with [^{3}H]-noradrenaline, mounting the tissue in the Golenhofen apparatus and analysing the overflow for radioactivity. The results obtained in response to SNP were in contrast to those expected in light of experiments in the presence of phentolamine and propranolol. SNP did not increase the overflow of [^{3}H]-noradrenaline at any concentration (10^{-7}-10^{-4}M). In contrast, the indirect sympathomimetic, tyramine (10^{-5}M),

increased [^{3}H]-noradrenaline overflow from rat PV. Thus, the effects of SNP in rat PV were unlikely to be mediated through the release of noradrenaline.

In RDC, the nitrovasodilator-induced reduction in contractile amplitude, that occurred concurrently with the increase in frequency, was apparently due to a reduction in the intracellular Ca^{++} concentration. The Ca^{++}-channel agonist, BAY K 8644, reversed the SNP-induced reduction in amplitude until contractions exceeded controls. On those occasions when SNP evoked a rise in spike amplitude, this was overcome by the Ca^{++} channel agonist, BAY K 8644. Following SNP addition, the muscle cells apparently sensed the depletion of intracellular Ca^{++} levels and attempted to compensate by transporting more extracellular Ca^{++} across the membrane leading to an increased spike amplitude. BAY K 8644 would presumably increase Ca^{++} influx further and, as cytoplasmic Ca^{++} stores were replenished, enhance the amplitude of contraction and restore spike amplitude. When added in reverse order, i.e. BAY K 8644 before SNP, then SNP reversed the actions of the Ca^{++} channel agonist. BAY K 8644 alone raised the basal tension of RDC and caused both electrical and mechanical activity to become constant. SNP then relaxed the tissue, activity became burst-like and contractile amplitude was slightly smaller than in control. BAY K 8644 presumably evoked a Ca^{++} influx to elevate tension and cause continuous firing. SNP then lowered cytosolic Ca^{++} levels, possibly through an action of NO to raise cGMP levels, tension fell, and activity again became burst-like.

To test directly for an action of SNP on cytosolic Ca^{++} levels, the membrane permeant form of fura-2 was used to measure $[Ca^{++}]_i$ in rat aortic vascular smooth muscle cells. There were disadvantages with the choice of cells. The aorta was not spontaneously active and SNP failed to have any effect on a stretch of aorta mounted under a tension of 1g in the Golenhofen apparatus. On the other hand, since access was available to an established rat aortic vascular smooth muscle cell line and since SNP relaxed rat aorta when

tone was raised (Lincoln, 1983 ; Taylor *et al*, 1988), it was decided to use these cells. It was anticipated that if SNP reduced free cytosolic Ca^{++} levels in RDC, then it may also have done so in aortic cells. Unfortunately, this was not the case. In contrast to angiotensin II which raised $[Ca^{++}]_i$, SNP had no effect. At present, there is no way of telling if the ineffectiveness of SNP was specific to the aortic smooth muscle cells or if it was a more general phenomenon. However, since BAY K 8644 reversed SNP and vice versa, in RDC, it is likely that SNP lowered $[Ca^{++}]_i$, at least in this tissue. Perhaps then, this effect was restricted to spontaneously active smooth muscle or perhaps, as seems more likely, in rat aorta, SNP would only be seen to reduce this parameter following the application of a stimulus to first increase it. In support of this, 8-bromo cGMP lowered $[Ca^{++}]_i$ in cultured aortic cells following prior depolarization with K^+ (Cornwell & Lincoln, 1989) and SNP relaxed noradrenaline contracted rat aorta by a mechanism that probably involved Ca^{++} sequestration or binding (Lincoln, 1983).

Results from experiments in the presence of Hb, 8-bromo cGMP or $K_4Fe(CN)_6$, indicated that both the excitatory and inhibitory properties of the nitrovasodilators on spontaneous electrical and mechanical activity, whether in RDC or rat PV, involved the release of NO. On each tissue examined, both the excitatory and the inhibitory effects of SNP were inhibited by the NO binding substance, Hb. This action was complicated somewhat in RDC, as Hb, itself, sometimes increased the frequency of spontaneous events, reduced the number of spikes/peaks per burst and the duration of electrical/mechanical responses ; effects normally associated with SNP. In the presence of Hb, SNP failed to either further increase frequency or reduce the amplitude of contraction. In rat PV, the effects of Hb on the SNP response were easier to observe as Hb, itself, did not alter the pattern of spontaneous activity. SNP was inactive in the presence of Hb, providing strong evidence that the actions of SNP, on spontaneously active smooth muscle, involved the release of NO from the SNP

which can also act as a 'sink' for intracellular NO (Gryglewski et al, 1992).

molecule. This proposal was lent credence by the fact that $K_4Fe(CN)_6$, which is structurally similar to SNP but which does not release NO, had no effect on the spontaneous activity of either RDC or rat PV.

The NO so released from nitrovasodilators is believed to stimulate the haem centre of soluble guanylate cyclase to raise cGMP levels, lower the cytosolic Ca^{++} concentration and produce relaxation (Collins *et al*, 1986 ; Schini *et al*, 1987 ; Gerzer *et al*, 1988 ; Bukoski *et al*, 1989). Thus, if the SNP-induced excitation was mediated through NO release, analogues of cGMP would be expected to mimic these excitatory effects. This was the case in RDC, where the effects of SNP were similar to those of 8-bromo cGMP, indicating that the actions of SNP were probably mediated via a cGMP-dependent mechanism. In fact, in RDC, 8-bromo cGMP (10^{-4}M) was actually more effective at increasing frequency and reducing contractile amplitude than an identical concentration of SNP ; in rat PV, 8-bromo cGMP was less effective. The contractile amplitude, number of spikes per burst of electrical activity and the duration of both electrical and mechanical events were each reduced significantly by 8-bromo cGMP in rat PV but, unlike SNP, this cyclic nucleotide derivative did not significantly increase the frequency of activity. This apparent ineffectiveness could signify that differences exist in the relative membrane permeabilities of the two tissues to 8-bromo cGMP or SNP. As SNP is believed to be activated intracellularly (Gryglewski *et al*, 1992), it should be ineffective unless it can freely permeate the membrane. Similarly, a rise in the intracellular concentration of cGMP mediates the response to NO. If 8-bromo cGMP was unable to freely permeate the membrane, then it too should be ineffective. As both SNP and 8-bromo cGMP were highly active in RDC, it is probable that both passed easily through the smooth muscle or pacemaker cell membrane. Due to its activity in rat PV, the same is most likely true for SNP in this tissue. However, 8-bromo cGMP did not enhance frequency in rat PV. Although the intracellular concentration of cyclic nucleotide attained was sufficient to reduce

contractile amplitude, spikes per burst and electrical/mechanical duration, perhaps, due to a lower membrane permeability, it was insufficient to increase the frequency of activity.

Surprisingly, in rat PV, the nitrovasodilator-induced response also resembled that following adenylate cyclase activation. The frequency of activity was increased and the other parameters reduced by the activators of this enzyme, forskolin or isoprenaline (each $\geq 10^{-8}$M). But, at higher doses ($\geq 10^{-6}$M) the cAMP activator, forskolin, abolished all spontaneous responses. Similarly, in RDC, the β-adrenoceptor agonist, isoprenaline, reduced the amplitude of contraction and the number of spikes per burst of electrical activity at low concentrations ($\leq 10^{-7}$M) and abolished all activity at higher concentrations (4×10^{-7}M). However, in RDC, the frequency of activity was not increased by isoprenaline. SNP abolished activity in neither RDC nor rat PV. Moreover, in the gastrointestinal smooth muscle, isoprenaline, unlike SNP, did not regulate pacemaker activity. Thus, although the effects of SNP and cAMP activation were similar, there were differences. It is therefore unlikely, on balance, that SNP was inducing cAMP activation sufficiently to explain its effects.

Despite increasing the frequency of activity in RDC and rat PV, SNP and, by implication NO, failed to raise tone. But, there is evidence from experiments in the carbachol-contracted RDC that NO, probably indirectly, caused rebound contraction. Thus, NO can apparently evoke both direct (increased frequency, spike amplitude of spontaneous activity) and indirect (rebound contraction) excitation. For instance, in this study, electrical stimulation of carbachol-contracted colonic smooth muscle evoked NANC nerve-mediated relaxations that were followed by rebound contractions. The relaxations were partially inhibited by the inhibitors of NO, Hb or L-NAME, while the rebounds were partially blocked by L-NAME and, occasionally, by Hb. Each response was mimicked by authentic NO ; the actions of which were

blocked by Hb. This provided strong evidence that NO was at least partially responsible (20-30%) for both the inhibitory NANC nerve response and the rebound excitation, in RDC. However, the rebounds following NANC nerve stimulation were also partially blocked by the inhibitor of prostaglandin synthesis, indomethacin. Thus, in RDC, this excitatory phenomenon could have been due to an indirect action of NO on the smooth muscle, via the secondary release of prostaglandins. Similar mechanisms have been reported for NO-induced rebound contraction in taenia coli and proximal colon (Ward *et al*, 1992b). Moreover, in the oesophagus, SNP or NO induced a rebound contraction, via a cGMP-dependent pathway, that was inhibited by indomethacin (Saha *et al*, 1993). This rebound contraction of oesophagus in response to SNP was in contrast to the effect of SNP in RDC. This drug did not stimulate a rebound effect in the colon, possibly because the long-lasting relaxation to SNP would have masked the effects of any transient prostaglandin release. The identity of the other inhibitory transmitter(s) are at present unknown but, as neither ATP, VIP nor GABA produced relaxations which mimicked those in response to nerve stimulation none of these substances was likely to be involved.

From an examination of the actions of the nitrovasodilators on pacemaker activity, it was clear that these compounds differed in their ability both to enhance and to inhibit activity in the chosen tissues. The excitatory and inhibitory actions of SNP, on either tissue, were greater than those of the other compounds. Despite being highly active in RDC, IDN was without effect in rat PV. Conversely, GTN significantly affected more parameters in rat PV than in RDC, but did not appear to have an obviously dose-dependent effect. My results differed from those of Bray *et al* (1987) and Wylie (1988) in that, in my experiments, GTN did not increase the frequency of activity in the rat PV.

The fact that the effects of SNP were unmatched by GTN or IDN is in keeping with the finding that the biotransformation of SNP differs from that of

the organic nitrates (Gryglewski *et al*, 1992). *In vitro,* at physiological pH, only small amounts of NO were released from SNP (Feelisch & Noack, 1987b ; Gryglewski *et al*, 1989), yet this substance possessed potent vasorelaxant activity. Further, in the case of SNP, there was no correlation between formation of NO and soluble guanylate cyclase activation *in vitro*, as there was with the organic nitrates (Feelisch, 1991). These discrepancies were explained by the proposal of an intracellular site for the biotransformation of SNP (Gryglewski *et al*, 1992). The organic nitrates i.e. GTN, IDN were not activated at this site, but were effectively decomposed to release NO only when they came into contact with cysteine or N-acetylcysteine (Feelisch, 1991). Consequently, there should be less chance of the NO so released coming into contact with guanylate cyclase, perhaps making them less effective than SNP. However, since IDN, but not GTN, was active in the RDC, differences may exist between these two compounds themselves. Based upon stoichiometric principle, if each nitrate grouping was acting independently of the other features of the molecule then IDN should release two molecules of NO and GTN should produce three molecules of NO, thus making GTN the more potent. Indeed, GTN has a greater vasodilator activity than IDN in the Lagendorff heart preparation (Noack, 1984). Similarly, an empirical parameter, F(5.8 Å) - "the frequency with which the molecule can exist in a conformation wherein oxygen atoms in the nitrate group and any other oxygen atoms are separated by a distance of 5.8 Å" - has recently been described (Tzeng & Fung, 1992). This parameter reflects the probability that an organic nitrate will interact with its metabolizing enzyme, since this enzyme is believed to possess at least two attachment sites, separated by a distance of 5.8 Å, which bind to the oxygen atoms in the organic nitrate (Tzeng & Fung, 1992). GTN, with an F(5.8 Å) of 12, should be more potent than IDN, with a value of 7. Yet, in RDC, this was not the case. The reason for this discrepancy is unknown, but there are a number of possibilities. Firstly, if each nitrate grouping in the

organic nitrate was not acting independently of the rest of the molecule, then the stoichiometric principle would not hold. Secondly, when a molecular model which was more accurate in measuring distances within cyclic compounds was used to calculate F(5.8 Å), then IDN gave a value of 11 (Tzeng & Fung, 1992). The F(5.8 Å) for GTN, which is not cyclic, was not calculated using this model but clearly the values obtained are dependent on the model used. Thirdly, assuming that the F(5.8 Å) for GTN is, indeed, greater than that for IDN, it is possible that the metabolizing enzyme in RDC does not have active sites that are 5.8 Å apart. The possibility that, in RDC, the discrepancy in the activities of GTN and IDN was due to migration of GTN into the plastic tubing of the apparatus (Cossum *et al*, 1978) resulting in concentration loss, was unlikely, since GTN had a greater effect than IDN in rat PV.

Differences also existed between the effects of IDN in the RDC and rat PV - IDN was without effect in rat PV - indicating that differences between gastrointestinal and vascular smooth muscles themselves may govern the effects of drugs. It is unclear whether the insensitivity of rat PV to IDN represents a difference, between the two tissues, in the sensitivity to NO or in the biotransformation of IDN to release NO. At first glance, it appears unlikely that the insensitivity of rat PV to IDN is due to an insensitivity to NO since SNP was actually more potent in rat PV than RDC. However, the efficacy i.e. the ability of SNP to produce the desired effect, was greater in RDC than in rat PV. It is possible, therefore, that the vascular smooth muscle is indeed less sensitive to these particular actions of NO.

Clearly, the smooth muscle response to the nitrovasodilators was species dependent. For example, SNP increased the frequency of pacemaker activity in the rat PV but, after a gradual decline in the frequency and amplitude of contraction, SNP (10^{-5}M) abolished activity in the RPV. The precise reasons for this are unclear. Perhaps the pacemakers in RPV were less sensitive to the excitatory actions of SNP or perhaps SNP had a more potent cGMP stimulating

action in RPV, leading to a greater reduction in cytosolic Ca^{++} levels and hence gradual abolition of activity.

The effects of SNP were also examined on non-spontaneously active smooth muscles to determine whether a pacemaker was necessary for excitation to occur. In this study, there was no evidence of nitrovasodilator-induced excitation in the non-spontaneously active rat anococcygeus or rabbit abdominal aorta. However, the use of intracellular recording techniques revealed that SNP, following an initial hyperpolarization, increased the frequency of spiking in guinea pig vas deferens (Rae, unpublished observation). Pacemaker activity such as that seen in RDC or rat PV does not appear, therefore, to be prerequisite for NO-induced excitation to occur, although the ability of the nitrovasodilators to produce this effect is likely to vary among tissues.

The actions of the nitrovasodilators on spontaneously active smooth muscle were both complex and at odds with those normally expected of smooth muscle relaxants. How were these effects mediated ? To complicate matters, examination of the results revealed a number of paradoxes. For example :

1) The Ca^{++}-channel agonist, BAY K 8644, reversed the effects of SNP, and vice versa, in RDC, but use of the fluorescent Ca^{++}-indicator, fura-2, yielded no evidence that SNP reduced $[Ca^{++}]_i$ in rat aortic vascular smooth muscle cells.

2) Again in RDC, SNP was synergistic with the opener of ATP-dependent K^+ channels, BRL 38227, but neither drug increased the efflux of the K^+ marker, ^{86}Rb, from this tissue.

3) The α- and β-adrenergic antagonists, phentolamine and propranolol respectively, together, inhibited the mechanical effects of SNP on rat PV, but

SNP did not enhance the overflow of [^{3}H]-noradrenaline from the nerve terminals.

Despite these paradoxes, by making some simple assumptions, it was possible to predict a model to explain the effects of SNP on RDC. Although there were some differences between RDC and rat PV the responses were basically similar. There are, therefore, likely to be a number of similarities in the mechanisms underlying the actions of SNP in either tissue.

Immediately after drug addition, both electrical and mechanical activity were abolished. Although SNP failed to produce a measurable increase in ^{86}Rb efflux as evidenced from measurements in the whole tissue, it is possible that the effect of the drug was directed to the pacemaker cells alone, leading to inhibition and quiescence. This pacemaker quiescence may have involved membrane hyperpolarization mediated by an enhanced efflux of K^+ (^{86}Rb) but, in the whole tissue, would have been of insufficient size to be detected by the techniques employed in this investigation. This possibility is supported by two observations : a) BRL 38227 and SNP were synergistic in RDC b) a mechanism such as this has been proposed to account for the dissociation between the relaxant and K^+ channel opening potencies of BRL 34915 in guinea pig portal vein (Quast, 1987)

After the period of quiescence, a more coordinated form of rhythmic activity than in untreated tissues returned, usually in under 5 min This coordinated behaviour was indicative of a direct effect of this drug on the pacemakers. Each pacemaker now seemed to be firing in time with the others and each burst of electrical spiking and mechanical contraction was of uniform amplitude. SNP increased the frequency of electrical and mechanical activity and the amplitude of spontaneous contractions was reduced. The rise in frequency was not an effect of SNP on transmitter (i.e. ACh, noradrenaline) release, but was most likely due to a synchronizing action of NO on the

pacemaker. The decline in contractile amplitude was, however, unlikely to have been mediated through an action of SNP on the pacemaker. Early experiments suggested that this effect could have arisen simply from the inability of the muscle cell to provide enough Ca^{++} to cope with the increased pacemaker rate following SNP addition. But, further examination revealed that the amplitude of contraction declined even when the increase in frequency was only slight. In this case, the muscle cell should not have been troubled to satisfy Ca^{++} demand. A more likely explanation for the effect of SNP on mechanical amplitude was that this drug acted on the smooth muscle cell to reduce cytosolic Ca^{++} levels (and hence contractile tension) via the NO mediated stimulation of soluble guanylate cyclase and elevation of cGMP levels (Collins *et al*, 1986 ; Schini *et al*, 1987 ; Bukoski *et al*, 1989). This explanation was supported by BAY K 8644 reversal of the effect of SNP.

Simultaneous with the aforementioned changes, the number of spikes/ peaks $burst^{-1}$ and the duration of electrical/mechanical responses were each reduced. Like the increase in frequency, these effects were probably consequent to an action of SNP on the pacemaker. If SNP induced each pacemaker to fire simultaneously rather than fragmentedly, then the number of electrical spikes per burst would be reduced, since the number of membrane Ca^{++} channels opening in response to a single pacemaker depolarization would be increased. This in turn would lead to a reduction in the number of mechanical peaks per burst and in the duration of electrical/mechanical bursts. In the most susceptible tissues, the rise in frequency could even have contributed to the inhibition and uniformity of electrical and mechanical responses. If the pacemaker cells fired and repolarized at a sufficient rate, this would ensure that the contractile amplitude, number of spikes/peaks $burst^{-1}$ and duration of electrical/mechanical bursts were all reduced. If every pacemaker depolarized simultaneously at regular, frequent intervals then the responses would be uniform (see Fig. 27).

Rarely, a coordinated, increased amplitude of spiking and/or contraction occurred in response to SNP ; the latter only at low concentrations (10^{-6}M). The increase in contractile amplitude at these low doses, like a number of the other effects of SNP, could be explained if the primary action of SNP was on the pacemaker cells, to reset them to the same coordinated rhythm, so that they fired synchronously. At higher concentrations, contractile amplitude was always reduced indicating that SNP was now probably acting on guanylate cyclase to lower Ca^{++} levels. In most cases, the increase in spike amplitude occurred at the same time as the other nitrovasodilator-induced effects (i.e. increased frequency, reduced contractile amplitude) suggesting that this was also a result of pacemaker synchronization. Interestingly though, rarely, the increased spike amplitude was observed only after the other SNP-induced effects had occurred, suggesting that this excitation may, in fact, have been a consequence of the inhibitory response to SNP. For example, in receptive tissues, as cytosolic Ca^{++} levels fell, and a reduction in contractile amplitude occurred, a compensatory mechanism could have been activated, whereby the muscle itself tried to replenish Ca^{++} stores by transporting more extracellular Ca^{++} across the membrane. Since electrical activity in RDC consisted of Ca^{++} spikes, this would lead to the enhanced spike amplitude observed. However, if $[Ca^{++}]_i$ had been reduced sufficiently by SNP, then this mechanism would be unable to provide enough Ca^{++} to restore the contractile ability of the tissue to normal. This could explain why the spike amplitude was dramatically enhanced in some tissues (see Fig. 27) while the amplitude of spontaneous contraction was decreased.

Although the pattern of events in the rat PV was similar, there were exceptions. SNP never abolished activity, even for a short while, in rat PV. If SNP initiated pacemaker hyperpolarization in this tissue, it was of insufficient amplitude to prevent action potential conductance and induce quiescence. Also, phentolamine and propranolol, together, inhibited the actions of SNP on

the mechanical activity of rat PV. The underlying mechanisms are unclear. SNP failed to enhance [^{3}H]-noradrenaline overflow. Thus, it is unlikely that a presynaptic effect of the compound on transmitter release was operative in rat PV. In any case, a presynaptic modification of the noradrenaline release process is unlikely since, in RDC, the response to SNP was excitatory and that to noradrenaline is known to be inhibitory in the gut (Burnstock, 1979). Moreover, when longitudinal strips of RDC were incubated with phentolamine and propranolol, together, the resultant activity did not resemble that induced by SNP. The emphatic response following the addition of 8-bromo cGMP to RDC, in fact, indicated that the effects of nitrovasodilators were due to a direct action of NO on guanylate cyclase. In rat PV, it is more likely that the inhibition of SNP in the presence of phentolamine and propranolol was mediated at a postsynaptic locus, where these antagonists in some way modified the characteristic response to NO.

Care should be applied when selecting NO donors with which to examine the effects of NO. Evidently, these compounds possess a wide spectrum of activity and some drugs are considerably more effective than others. For instance, in RDC or rat PV, SNP was more effective than either of the organic nitrates tested. This may reflect the fact that SNP is likely to be activated differently from either GTN or IDN. In each of these tissues, SNP produced significant dose-dependent changes in every parameter of activity measured, with the exception of the maximum spike amplitude per burst of electrical activity, in RDC. Even then, this parameter was profoundly increased in a number of individual experiments. IDN was highly active in the RDC, but was inactive in the rat PV, while GTN, although mimicking the effects of SNP on a few occasions, was relatively inactive in either tissue. Since, SNP significantly increased the frequency of spontaneous electrical and mechanical activity in both tissues, this compound may not be the most suitable for investigation of

the relaxant properties of nitrovasodilators, particularly in spontaneously active tissues.

Nitrovasodilators have, for some time, been used for their relaxant effects in the cardiovascular system in the treatment of angina pectoris, congestive heart failure and essential hypertension, or in the gastrointestinal system, in the treatment of achalasia. Excitation such as that observed in RDC or rat PV would clearly be an unwanted side effect. In this study, the organic nitrates produced less excitation than SNP in both cardiovascular and gastrointestinal tissues. Consequently, these drugs would appear to be more suited than SNP for the treatment of conditions where there main benefit arises from smooth muscle relaxation. However, in the RDC, although SNP produced a dramatic excitation of frequency this significant change generally occurred only at non-physiological concentrations ($\geq 10^{-5}$M) and would be unlikely ever to be encountered in practice. But, at lower concentrations (10^{-6}M), in certain experiments, SNP increased both the amplitude of spiking and contraction. Thus, in particularly sensitive tissues, the relaxant effects of SNP could quite possibly be compromised by any concomitant excitatory effects. Further work is required to fully elucidate the mechanisms underlying the effects of nitrovasodilators on spontaneously active smooth muscle and to determine whether the usefulness of compounds such as SNP in disease therapy, is indeed compromised by the effects of unwanted excitation.

Conclusions

This project was undertaken to examine the influence of nitrovasodilators .on spontaneously active smooth muscles ; namely the rabbit distal colon (RDC) and the rat portal vein (rat PV). The results indicated that the actions of these drugs were complex. Despite NO being involved in inhibitory NANC

neurotransmission in a number of preparations (see Moncada *et al*, 1991b ; Rand, 1992 ; Sanders & Ward, 1992), including the RDC, nitrovasodilators both excited and inhibited spontaneously active smooth muscle. SNP was the most effective nitrovasodilator tested in either tissue confirming that this drug may be activated differently from the organic nitrates i.e. GTN, IDN.

Inhibition, in the RDC, appeared either as an abolition of rhythmic electrical and mechanical responses (quiescence) and/or as a reduction in the maximum contractile amplitude, number of spikes/peaks per burst and/or duration of electrical/mechanical responses. Quiescence was probably mediated by K^+ efflux while the reduction in the amplitude of contraction was probably mediated through the lowering of $[Ca^{++}]_i$. The effects on the number of spikes/peaks per burst and/or duration of electrical/mechanical responses were possibly the result of a direct action of the nitrovasodilator on the pacemaker. Events in the rat PV were similar with the exception that no quiescence was ever induced.

Excitation, in either tissue, appeared as an increased frequency of spiking and contraction or, more rarely, as an increased amplitude of spiking and/or contraction. The increase in frequency was probably mediated by a direct action on the pacemaker cells whereby they were synchronized to fire simultaneously and more frequently. This would account for the enhanced amplitude of contraction in those cases where it occurred. The increase in spike amplitude may have been a reflex reaction as the tissue attempted to compensate for the reduction in intracellular Ca^{++} (which led to a decreased contractile amplitude), by transporting more Ca^{++} across the membrane. Both the inhibition and the excitation in response to nitrovasodilators were due to the release of NO.

These results confirmed earlier findings of nitrovasodilator-induced excitation in rat PV (Bray *et al*, 1987 ; Wylie, 1988), guinea pig ileum (Sugisawa *et al*, 1991) and rat ileum (Barthó *et al*, 1992). Similar effects have

also recently been identified in our lab in the non-spontaneously active guinea pig vas deferens (Rae, unpublished observations). Intracellular electrical recordings revealed that SNP caused a short-lived hyperpolarization followed by a burst of increased spiking - as in RDC (i.e. quiescence followed by enhanced activity). Perhaps then, these excitatory responses are more ubiquitous than was first imagined. These results suggest that endogenous NO, like other neurotransmitters (i.e. ACh, noradrenaline), may possess both inhibitory and excitatory properties.

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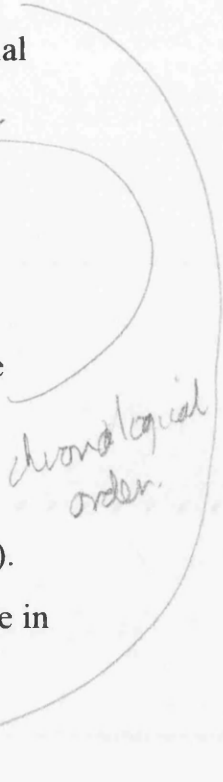

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