THE MECHANISM OF SMOOTH MUSCLE RELAXANTS

A thesis presented for the degree of

Doctor of Philosophy

by

Hong Sheng

Department of Pharmacology

University of Glasgow

September 1989

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor, Professor John S. Gillespie for accepting me as a Ph.D student in his department, for suggesting the subject of this investigation and for affording me the facilities of his laboratory. I also wish to express my appreciation to him for sharing his brilliant ideas and wide experience with me and for his great patience and help especially during the preparation of this thesis. To him, my gratitude is far beyond expression.

A special word of thanks to Dr. W. Martin for his very helpful discussion and suggestion during the last two years and also for his extreme patience in reading through my poorly written thesis.

I am also grateful to Miss Hannah McCaffery not only for her excellent technical assistance but also for her special friendship. When things were not going so well, she was always there to listen to my probelms.

I would like to thank all members of staff in the Pharmacology Department: especially Professor T. W. Stone for allowing me the facilities to prepare this thesis; Drs. T.C. Muir and D. Pollock for their help and friendship; Mr. Trevor Clarke for making me special equipment; Mr. Adam Richie for his help any time when I asked; Mr. Iain Gibson and Mr. Robert Auld for making jokes and teaching me Glaswegian at tea time. I would also like to thank Mrs. Majory Wright in the departmental library for allowing me to use her office for

printing this thesis; Mrs. Edith McNab, Mrs. Judith Brooker and Miss Sheena Ogilvie for answering my questions out of office time; Mr. Jim Younger and Miss Tricia Feely for supplying me with healthy animals; Mrs. Josie Odber, my fellow Ph.D student, for her good company, help and friendship; Mr. Naill Smith for all his help, especially for correcting my English mistakes.

My thanks are due to the staff of Medical Illustration for their excellent assistance in preparing the illustrations; the helpful assistance of Glasgow abattoir for supplying the BRP is greatly appreciated. Thanks also to Glasgow University and to the ORS award for the Postgraduate scholarship.

Finally, I would like to express my extreme gratitude to my family: to my husband for his understanding and support both in finance and in spirit; to my parents, my sister and my brother-in-law for their invaluable, endless help and unselfish support; and to my little baby who spent the first three months of her life with me in Glasgow and gave me so much joy.

CONTENTS

SUMMARY

The subject of this thesis was an investigation of the mechanism of action of smooth muscle relaxants and included two projects.

I. the Nature of the NANC Neurotransmitter in the Bovine Retractor Penis and the Rat Anococcygeus Muscles

(1) The relaxant action of endothelium-derived relaxing factor (EDRF), the smooth muscle inhibitory factor (IF) isolated from the bovine retractor penis (BRP) muscle, nitric oxide (NO) and sodium nitroprusside (NaNP) were examined on four isolated smooth muscle preparations; the rabbit aortic strip, the BRP muscle, the rat anococcygeus muscle and the guinea-pig trachea. EDRF (released by acetylcholine), the IF and NO produced powerful relaxation of the rabbit aortic strip and the BRP muscle but had little or no effect on the rat anococcygeus muscle or the guinea-pig trachea. The same rank order of potency of these stimuli in all four muscles suggests all might owe their effects to NO. NaNP, however, produced complete relaxation of the rabbit aortic strip, the rat anococcygeus and the BRP muscles at low concentrations but the guinea-pig trachea was much less sensitive. This suggests an action in addition to the release of NO.

(2) Haemoglobin added to solutions of the IF, NO or EDRF released from a donor aorta, completely abolished their relaxant properties.

Haemoglobin added to the bath solution also greatly reduced effects of NANC nerve stimulation in the BRP and the rat anococcygeus muscles and that of EDRF released in situ, consistent with all acting by virtue of NO.

(3) Suspensions of erythrocytes with an intracellular haemoglobin concentration equivalent to that in (2) were as effective as free haemoglobin in abolishing the relaxant effect of NO or EDRF released from the donor aorta. Erythrocytes were less effective in abolishing the response to EDRF released in situ. Erythrocytes had no effect on the inhibitory response induced by the IF or NANC nerve stimulation in the BRP or the rat anococcygeus muscles.

(4) The superoxide anion generators, pyrogallol and hydroquinone, both significantly reduced the response to NANC nerve stimulation in the rat anococcygeus muscle but had no effect in the BRP muscle. The inhibition of the NANC response in the rat anococcygeus muscle by pyrogallol was completely reversed by superoxidedismutase.Pyrogallol also inhibited the response to NO in the rat anococcygeus muscle but not that to 3-isobutyl-1-methyl-xanthine (IBMX) confirming this is a selective action.

(5) These results suggest the neurotransmitter in the rat anococcygeus muscle may be NO or a substance releasing NO. The transmitter in the BRP muscle which is unaffected by superoxide anions or the environment in which it acts may be different. In addition, EDRF, the IF and the NANC transmitter in the BRP and the rat

anococcygeus muscles may rely on NO for activity, but the IF, like the NANC transmitter, may be a less diffusible substance than NO itself.

II. The Effects of Cromakalim on Spasmogen- or Stretch-induced Contraction of a Variety of Smooth Muscle Preparations

(1) The relaxant effect of cromakalim has been examined on four isolated preparations, the rabbit aortic strip, the BRP muscle, the guinea-pig trachea and the guinea-pig taenia coli contracted by either histamine, carbachol, noradrenaline or KCl. Even though the contribution of external calcium entering through voltage-operated channel (VOC) in these tissues varied, the expected greater effectiveness of cromakalim on tissues using this pathway was not observed.

(2) Desensitization was observed in the guinea-pig trachea when the tissue was exposed to cromakalim 30 min before the spasmogen was added. This did not happen when cromakalim was added at the peak of contraction. Such desensitization was not found in the rabbit aortic strip.

(3) Verapamil significantly reduced the relaxant effect of cromakalim in the guinea-pig trachea. In contrast, cromakalim potentiated the relaxant effects of isoprenaline and NaNP in the guinea-pig trachea.

(4) The relaxant effects of cromakalim on the BRP muscle and the guinea-pig taenia coli were unaffected by haemoglobin (3.3 μM) or apamin

(0.5 μM) in concentrations which block completely the relaxant effect of NANC nerve stimulation in these tissues respectively. Methylene blue (30 μM) greatly reduced the response to both cromakalim and NANC nerve stimulation in the BRP suggesting a non-specific action.

(5) Cromakalim in concentrations causing maximal relaxation did not increase the tissue level of either cyclic AMP or cyclic GMP in the BRP muscle, although the appropriate synthetic enzymes were present and could be stimulated by forskolin or NaNP.

(6) In the BRP muscle, isoprenaline (30 μM) caused maximal relaxation but did not raise the level of cyclic AMP, even though lower concentrations of 2 μM did raise the level of this nucleotide in the rabbit uterus.

(7) A preparation was developed in which contraction could be induced by a controlled stretch. In the rabbit femoral and renal arteries removal of calcium from the Krebs' saline or adding the non-selective calcium channel blocker cobalt abolished stretch-induced contractions. Stretch-induced contraction, particularly that of the renal artery was not sensitive to blockade by verapamil even at a concentration of 300 μM.

(8) Stretch-induced contraction in renal and femoral arteries is only partially inhibited by cromakalim. The renal artery was particularly resistant with a maximal inhibition of 10%. The femoral artery on first application of the drug showed a larger inhibition averaging 35% but this effect declined with time to about 10%.

(9) In conclusion, cromakalim can produce relaxation in a variety of smooth muscle preparations contracted by different spasmogens. Cromakalim is relatively ineffective against contractions produced by stretch in renal and femoral arteries. Desensitization to cromakalim was found in the guinea-pig trachea and rabbit femoral artery. The relaxant effect of cromakalim is not related in raising the level of either cyclic AMP or cyclic GMP in the BRP muscle.

THE NATURE OF THE NANC NEUROTRANSMITTER IN THE BOVINE RETRACTOR PENIS & THE RAT ANOCOCCYGEUS MUSCLES

INTRODUCTION

The original identification of the unique anatomical features of the autonomic nervous system by Gaskell (1916) and Langley (1921) was accompanied by its separation into two divisions, the sympathetic and parasympathetic. The ganglion cells of the former are a considerable distance from the tissue innervated, the fibres leave the spinal cord in the thoracic-lumber region. The parasympathetic, in contrast, has ganglion cells close to or within the organ innervated; the nerve fibres leave the central nervous system either from the brain stem as parts of several cranial nerves or from the sacral spinal cord. In the 1930's, as a result of the work of Dale and his collaborators, another method of classifying nerves according to the neurotransmitters they released was introduced (Dale, 1933). With a few exceptions sympathetic nerves liberate adrenaline (now recognized as noradrenaline) and parasympathetic nerves acetylcholine. The terms adrenergic and cholinergic become exchangable with the older sympathetic and parasympathetic terminology.

Even at this early time, however, findings of "atropine resistance" of parasympathetic responses were reported. Such effects were observed in the gastrointestinal tract and genito-urinary system, especially the stomach and the urinary bladder (Langley & Anderson, 1895; Langley, 1898; Henderson & Roepke, 1934). At this time no equivalent blocking agent to atropine for the sympathetic system was available so it was not possible to determine if a single neurotransmitter was responsible for all sympathetic responses. The introduction of the highly selective adrenergic neurone blocking agents such as guanethidine changed this.

With these it was shown there were inhibitory fibres in the sympathetic nerves to the gut which were not blocked (Burnstock et al., 1963; Martinson & Muren, 1963). From these observations sprang the belief that there exist, in peripheral autonomic nerves, a group of neurones whose transmitter was neither noradrenaline nor acetylcholine, the non-adrenergic non-cholinergic (NANC) nerve fibres.

Several groups found that when the responses to both adrenergic and cholinergic nerve stimulation had been blocked in some gastrointestinal preparations, transient hyperpolarizations and relaxations were still produced. Since these responses were abolished by tetrodotoxin, they were established as inhibitory junction potentials (IJPs) resulting from stimulation of non-adrenergic, non-cholinergic (NANC) neurones (Burnstock et al., 1963; Martinson & Muren, 1963). Since then, NANC nerves have been found in wide variety of smooth muscle preparations including urinary bladder, lung, trachea and parts of the vascular system (Campbell, 1970).

Although Holton & Holton (1954) in their studies of antidromically elicited cutaneous vasodilatation were the first to suggest that ATP might be a neurotransmitter, Burnstock and his colleagues demonstrated that adenosine triphosphate (ATP) mimicked the nerve-mediated response in several tissues and was the most likely candidate as NANC neurotransmitter to the smooth muscle of the gastrointestinal tract and bladder (Burnstock et al., 1970; Burnstock et al.,1972). In 1971, nerves utilizing ATP as the principal transmitter were termed "purinergic" (Burnstock, 1971) and a tentative model of the storage,

release and inactivation of ATP in purinergic nerves was proposed in 1972 (Burnstock, 1972). Not all evidence was in favour of such a scheme. For example, Gillespie (1982) indicated that in some preparations in the gastrointestinal system, the concentration of ATP needed was too high for it to be a transmitter, for example, in the guinea-pig taenia coli, and it produced contraction in the rat anococcygeus muscle where the NANC nerve response is inhibition. Stone (1981) indicated that in the central nervous system, the pharmacological agents which block responses to exogenous purines had little effect on synaptic responses.

Between 1975 and 1977, important new findings suggested that it was likely that some NANC nerves utilize transmitter substances other than ATP. These findings were followed by numerous investigations based on the new immunohistochemical methods for localizing various biologically active polypeptides. Autonomic nerves containing enkephalins, substance P, vasoactive intestinal polypeptide (VIP), neurotensin, somatostatin, gastrin releasing peptide (GRP), bombesin (BN), cholecystokinin (CCK), neuropeptide Y (NPY)/pancreatic polypeptide (PP), calcitonin gene-related peptide (CGRP), galanin (GAL) have been reported both in central and peripheral nervous system (for review see Burnstock, 1986).

Some nerve profiles contained a complex mixture of vesicles, suggesting that they may contain more than one transmitter, giving rise to the term co-transmission. A variety of co-transmission system have been found in recent years (for review see Campbell, 1987). For

example, NA-ACh co-transmission in rat exocrine sweat glands (Landis & Keefe, 1983); ACh-LHRH (luteinizing hormone-releasing hormone) in frog sympathetic ganglia (Jan & Jan, 1982); ACh-SOM (somatostain) in the heart (Campbell & Jackson, 1985): and ACh-VIP (vasoactive intestinal peptide) in exocrine glands (e.g. nasal, salivary, pancreatic and sweat glands) (Lundberg et al., 1980; Lundberg, 1981) and ATP-NA in certain smooth muscle preparations (for review see Burnstock, 1986).

The clearest evidence for ATP and NA as co-transmitters is in the vas deferens. The neurogenic response of this tissue is biphasic (Swedin, 1971; McGrath, 1978), consisting of an initial twitch response and a slow second component. The electrical responses underlying these mechanical events could be separated by nifedipine which abolishes the smooth muscle action potential and the initial twitch response without reducing the excitatory junction potential (Blakeley et al., 1981). The second phase is adrenergic in nature, whereas the twitch response has been shown to be non-adrenergic, non-cholinergic in nature and is due to the release of ATP (Levitt et al., 1984; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984a,b; Kirkpatrick & Burnstock, 1987; Kasakov et al., 1988). ATP and NA are stored together in the same vesicles in sympathetic nerve terminals (Carlsson et al., 1963). ATP and NA released from the vas deferens has been shown to be of neuronal origin, as release is not affected when the mechanical and electrical responses are blocked by treatment with α,β-methylene ATP and prazosin (Kasakov et al., 1988; Muir & Wardle, 1988). Furthermore, ATP release is unaffected following depletion of NA by reserpine pretreatment,

suggesting that ATP release is not secondary to NA release (Kirkpartrick & Burnstock, 1987).

Although such NANC nerve fibres have been recognized and widely studied for about twenty years, the transmitter in some tissues is still unknown. The interest in this project is in the transmitter of one particular group of nerves which have their origin in the sacral spinal cord and innervate such tissues as the anococcygeus muscle, the retractor penis muscles and the penile artery.

The rat anococcygeus muscle receives a dense motor adrenergic innervation via lumbar sympathetic nerves and an inhibitory innervation via the sacral parasympathetic nerves (Gillespie & Maxwell, 1971; Gillespie, 1972; Gillespie & McGrath, 1973). The retractor penis muscle also receives an adrenergic motor innervation (Klinge & Sjöstrand, 1974; Ambache & Killick, 1978) via the lumbar sympathetic nerves (Langley & Anderson, 1895) and an inhibitory innervation via the sacral parasympathetic nerves (Langley & Anderson, 1895). In view of the anatomical continuity of the anococcygeus and retractor penis muscles the similarity of their innervation, the demonstration from ultrastructural evidence of nerve profiles in both tissues (Eränkö et al., 1976; Gibbins & Haller, 1979) morphologically similar to NANC nerve profiles described elsewhere, it has been assumed that they share a common inhibitory neurotransmitter (Gillespie, 1982). The NANC inhibitory response of the anococcygeus and retractor penis muscles can also be obtained in stimulation of the extrinsic nerves either *in vitro* or *in vivo* (Gillespie, 1972; Gillespie & McGrath, 1973; Langley &

Anderson, 1895). Inhibitory responses to electrical field stimulation in isolated preparations of both rat anococcygeus and BRP muscles are of neural origin since they are blocked by tetrodotoxin (Gillespie, 1972; Klinge & Sjöstrand, 1974). Like other NANC nerve responses, the inhibitory effects produced by field stimulation in both tissues cannot be blocked by any conventional receptor or neurone blocking agents such as α- or β-adrenoceptor antagonists, guanethidine, atropine, hexamethonium, d-tubocurarine, lysergic acid diethylamide, mepyramine or burimamide (Gillespie, 1972; Klinge & Sjöstrand, 1974; Gillespie & McGrath, 1975). In contrast to others, the response to NANC nerve stimulation in both tissues can be blocked by haemoglobin (Bowman & Gillespie, 1981, 1982; Bowman et al., 1982) and anoxia (Bowman & McGrath, 1985). Evidence from the rat anococcygeus muscle shows that the inhibitory innervation is not an example of co-transmission from adrenergic nerves since the origin of the inhibitory fibres in the sacral cord is several spinal segments away from the sympathetic outflow (Gillespie & McGrath, 1973) and destruction of the adrenergic nerves with 6-hydroxydopamine does not impair inhibitory responses to nerve stimulation (Gibson & Gillespie, 1973). Attempts to detect the release of inhibitory transmitter in response to nerve stimulation in a Loewi type experiment or to extract it into buffered saline failed (Gillespie, 1987). Furthermore, the inhibitory response to NANC nerve stimulation cannot be mimicked satisfactorily in either tissue by any known substance. The majority of proposed inhibitory transmitter substances have been tested on the rat anococcygeus muscle because this tissue

offers the unique advantage that most potential relaxant transmitters are either ineffective or cause contraction. For example, both NA and ACh cause contraction in this tissue. Isoprenaline produces inhibitory response in the BRP muscle (Klinge & Sjöstrand, 1974) but not the rat anococcygeus muscle (Gillespie, 1972). Substance P can produce inhibitory responses in the latter but its action is slower and less effective than the response to nerve stimulation (Gillespie & McKnight, 1978). Bradykinin relaxes the anococcygeus muscle of the rat, rabbit and cat (Gillespie & McKnight, 1978) but produces a motor response on the BRP at low tone (Klinge & Sjöstrand, 1974). Evidence suggesting that ATP is the inhibitory neurotransmitter in the rat anococcygeus muscle has been presented (Burnstock et al., 1978), but in these experiments, the inhibitory effect of ATP was seen only when the preparation was pretreated with indomethacin to block prostaglandin synthesis. It is difficult to visualize how, in the absence of indomethacin, the inhibitory nerves, through the release of ATP could produce a powerful inhibition without the simultaneous production of prostaglandins. In addition, ATP is known to contract both the rat anococcygeus (Gillespie, 1972) and the BRP muscles (Klinge & Sjöstrand, 1974).

In 1975, Ambache and his colleagues reported the presence of a smooth muscle relaxant in acid extracts of the BRP muscle which may be the neurotransmitter (Ambache et al., 1975). This finding was confirmed by other workers (Gillespie & Martin, 1980; Gillespie et al., 1981) and this relaxant substance termed inhibitory factor (IF) has been

the subject of extensive investigation (Gillespie, 1987). IF from the BRP or the rat anococcygeus muscles is soluble in water and methanol and has a molecular weight about 500 (Gillespie et al., 1981).

Early experiments showed that the IF, unlike ATP, is not retained on alumina columns at pH 9.0 and therefore, is presumably not ATP (Bowman et al., 1979). Further evidence suggests that it is not a polypeptide since a group of non-specific proteases—trypsin, subtilisin and pepsin—together with the peptidases, pyroglutamate aminopeptidase, leucine aminopeptidase and carboxypeptidase had no effect on the IF (Gillespie et al., 1981). In addition, the effects of the IF and of NANC inhibitory nerve stimulation in rat anococcygeus and BRP muscles are not potentiated by aprotinin, a general tissue protease inhibitor, or by SQ20881, a specific angiotensin converting enzyme inhibitor (Gillespie et al., 1981). The IF relaxes both BRP and rat anococcygeus muscles though the latter is less sensitive (Gillespie & Martin, 1980). In the BRP muscle, both the IF and NANC nerve stimulation produce membrane hyperpolarization and mechanical relaxation (Byrne & Muir, 1985). Relaxation to IF or nerve stimulation is associated with a selective rise in cyclic GMP content and is blocked by haemoglobin or N-methylhydroxylamine, two agents known to block stimulation of soluble guanylate cyclase (Bowman & Drummond, 1984). The IF also produces relaxation of spiral strips of various arteries from ox, cat, rabbit or rat: this occurs whether tone is induced with noradrenaline, K^+ or Ba^+ (Bowman et al., 1981). Furthermore, relaxation is not dependent on the presence of endothelial cells, so it does not arise indirectly following

release of EDRF (Bowman et al., 1986). Thus, it is possible that the NANC neurotransmitter in the BRP and rat anococcygeus muscles and the IF extracted from both tissues are similar substances.

EDRF was accidentally discovered by Furchgott and his colleague in 1980 (Furchgott & Zawadzki, 1980). They found that the relaxation of isolated preparations of rabbit arteries by ACh was strictly dependent on the presence of endothelial cells on the intimal surface of the preparations. They showed that endothelial cells exposed to ACh release a diffusible substance(s), demonstrated by transfer experiments where aortic strips without endothelium were layered with strips containing it, and the endothelium-free strips regained the ability to relax in response to ACh (Furchgott & Zawadzki, 1980). Further evidence that endothelial cells release a humoral substance comes from bioassay experiments in which the perfusate flowing through blood vessels with endothelium was passed over or through another endothelium-denuded blood vessel causing it to relax (Förstermann et al., 1984; Griffith et al., 1984). From those experiments the half-life of EDRF released by ACh could be measured and was shown to be short, from 6 to 49 sec, depending upon the experimental conditions and the species examined (Förstermann et al., 1984; Griffith et al., 1984; Rubanyi et al.,1985). Subsequent to these experiments with ACh, other agents known to possess a vasodilator action in vivo, were demonstrated to produce all or part of their relaxing effects by release of EDRF from endothelial cells. Some of these agents including the calcium ionophore A23187, ATP, substance P, bradykinin, thrombin, etc. have been

widely used as tools for testing EDRF on various blood vessels (Furchgott, 1984).

The relaxation by EDRF was found to be associated with increased production of cyclic GMP in smooth muscle (Holzmann, 1982; Rapoport & Murad, 1983a; Diamond & Chu, 1983). Removal of the endothelium abolished the relaxation and the accumulation of cyclic GMP (Rapoport & Murad, 1983b). The phosphodiesterase inhibitors M&B 22948, papaverine, and 3-isobutyl-1-methyl-xanthine (IBMX) potentiate the relaxant effects of spontaneously released EDRF on both rat and rabbit aorta supporting the hypothesis that it is the rise in cyclic GMP which is responsible for the mechanical inhibition (Martin et al., 1986). Haemoglobin and methylene blue at high concentrations completely abolish endothelium-dependent relaxation and the rise in cyclic GMP (Martin et al., 1985). The likely mechanism of action of haemoglobin is to bind EDRF, and that of methylene blue to oxidize the heme component of guanylate cyclase. Thus, it appears likely that the inhibitory effect of EDRF is mediated by soluble guanylate cyclase via the production of cyclic GMP and the activation of cyclic GMP-dependent protein kinase in vascular smooth muscle.

In addition to haemoglobin and methylene blue, a number of other agents have been reported to block the effects of EDRF. These include: the phospholipase A_2 inhibitor quinacrine (Furchgott & Zawadzki, 1981) and manoalide (Long et al., 1987); lipoxygenase inhibitors such as nordihydroguaiaretic acid (NDGA), 5,8,11,14-eicostatetraynoic acid (ETYA) and phenidone (Furchgott &

Zawadzki, 1980; Singer & Peach, 1983); cytochrome P-450 mono-oxygenase inhibitors such as SKF 525A, metyrapone, and cimitidine (Förstermann et al., 1988); low density lipoproteins (Andrews et al., 1987); superoxide anion generator pyrogallol (Moncada et al., 1986); and a free radical scavenger hydroquinone (van de Voorde & Lensen, 1983; Furchgott, 1981) etc. The early interpretation of some of these effects was that EDRF was a fatty acid derivative of arachidonic acid (Furchgott & Zawadzki, 1980). This interpretation was incorrect: it is now known that a common property of many of these substances is ability to generate superoxide anions which accelerate the destruction of EDRF (Moncada et al., 1986). Early studies ruled out adenosine, adenosine monophosphate (AMP), and prostacyclin as possible candidates for EDRF (Furchgott & Zawadzki, 1980; Gryglewski et al., 1986a). Both adenosine and adenosine monophosphate induce smaller maximal relaxation than ACh (Furchgott & Zawadzki, 1980). Prostacyclin has no effect on rabbit and pig aorta (Furchgott & Zawadzki, 1980; Gordon & Martin, 1983) whereas EDRF does and its action is unaffected by indomethacin or asprin (Furchgott & Zawadzki, 1980). The nitrovasodilators are believed to produce smooth muscle relaxation by releasing nitric oxide (NO) (Waldman & Murad, 1987). NO, like EDRF, relaxes smooth muscle by stimulating soluble guanylate cyclase (Rapoport & Murad, 1983a,b) this and the evidence that antioxidants or nonspecific superoxide anion generators greatly reduce endothelium-dependent relaxation (Förstermann & Neufang, 1984; Griffith et al., 1984; Rubanyi et al.,1985) and that superoxide dismutase prolongs the half-life of

EDRF (Gryglewski et al.,1986b; Rubanyi & Vanhoutte, 1986) prompted Furchgott (1988) to propose that EDRF might be NO.

In 1987, Moncada and his colleagues finally confirmed this suggestion by releasing EDRF from endothelial cells in culture and measuring the appearance of NO (Palmer et al., 1987). In their experiments NO was determined as the chemiluminescent product of its reaction with ozone. They found that NO could always be detected when EDRF was released and that the amount of NO was sufficient to account for the relaxant effects observed. The relaxations induced by EDRF and NO were inhibited by haemoglobin and enhanced by superoxide dismutase to a similar degree. Thus NO released from endothelial cells is indistinguishable from EDRF in terms of biological activity, stability and susceptibility to inhibitors and potentiators. Their conclusion was that EDRF and NO are identical. However, some reports are disagree with this conclusion. Shikano et al. (1987, 1988) found that guinea-pig taenia coli relaxed to NO but not to EDRF released by ACh or A23187. Similar report from Dusting et al. (1988) was that the guinea-pig trachea was sensitive to NO but not to EDRF released by bradykinin. In addition, Guerra et al. (1988) reported that using chemiluminescence, the amount of NO detected from bovine aortic endothelial cells and pig carotid artery was approximately 1/10th that required to account for EDRF-induced relaxation. Greenberg et al. (1988) reported that in the experiment when EDRF released from intact artery was collected under vacuum through a column of methemoglobin-agarose and samples assayed by electron paramagnetic

responace, EDRF did not give an NO spectral shift but NO did. However, more recent work by Moncada and his colleagues has shown that NO can be synthesized from L-arginine by porcine aortic endothelial cells in culture (Palmer et al., 1988a). They found that the release of NO from endothelial cells cultured in the absence of L-arginine was reversibly enhanced by infusions of L-arginine and L-citrulline, but not by the steroisomer D-arginine. Mass spectrometry studies using ^{15}N-labelled L-arginine also indicated that this enhancement was due to the formation of NO from the terminal guanidino nitrogen atom(s) of L-arginine. In addition, the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA), inhibited both the release of NO from these cells and the endothelium-dependent relaxation of rabbit aorta (Palmer et al., 1988b; Rees et al., 1989). Other workers in Germany found that the endothelium-dependent relaxation induced by ATP in rat thoracic aorta was reduced following pretreatment with L-canavanine, another guanidinoxy analogue of L-arginine which inhibits various L-arginine-utilizing enzymes (Schmidt et al., 1988). The conclusion from these results is that L-arginine is the physiological precursor for NO synthesis by vascular endothelial cells.

Another factor that appears to act by stimulating soluble guanylate cyclase in a number of neural tissues is contained in the brain extract described by Deguchi and his colleagues (Deguchi, 1977; Deguchi et al., 1978). Further work from this group has suggested that the active constituent is L-arginine (Deguchi & Yoshioka, 1982). Recently, Garthwaite et al. (1988) showed that a substance, similar to EDRF and

NO is released through activation of glutamate receptors in the cerebellum.

These results together with others suggested the possibility that EDRF and the IF from the BRP or rat anococcygeus muscles are chemically related. Both are vasodilators, both are unstable, both stimulate the formation of cyclic GMP, both effects are blocked by haemoglobin and both, during their formation, appear to be sensitive to anoxia (Bowman & McGrath, 1985; Furchgott & Zawadzki, 1980).

As EDRF has been identified, though not yet perhaps to everyone's satisfaction, as the very simple molecule NO and since cells in the cerebellum can apparently liberate the same substance, it was of interest to determine whether the NANC neurotransmitter in the rat anococcygeus and BRP muscles and IF extracted from the BRP muscle could also be acting through NO.

The first experimental approach was based on the assumption that if they were all the same substance then the rank order of sensitivity of different smooth muscles to each would be the same. The second was to test a variety of inhibitors to see again whether they would be equally sensitive to the blocking actions.

METHODS

APPARATUS

In this project the response of tissues to drugs and nerve stimulation was examined in two different kinds of *in vitro* apparatus.

(1) Simple 10 ml organ baths.

(2) A cascade system in which EDRF released from a length of perfused donor rabbit aorta could be rapidly transferred to an adjacent chamber containing the test tissues.

Simple Organ Bath Studies

In these experiments tissues were suspended with braided silk thread (5-0) on stainless steel hooks under 1 g tension in 10 ml jacketed organ baths containing Krebs' saline at 36°C and oxygenated with 95%O_2 + 5%CO_2. The composition of the Krebs saline in mM was: Na^+, 145; K^+, 5.9; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^-, 127; HCO_3^-, 25; HPO_4^{2-}, 1.2; SO_4^{2-}, 1.2; and dextrose, 11. In experiments stimulating intramural nerves, the lower end of the preparation was tied to a Ag/AgCl hook which served as one electrode and the tissue threaded through a second Ag/AgCl ring electrode embedded in araldite. The thread on the other end of the tissue was tied to a Grass FT03C force transducer the output of which was displayed on a Grass Polygraph Model 7D. Intramural nerves were field stimulated by a Grass S4 stimulator with 1 ms duration pulses for 10 seconds at frequences between 0.2 and 10 Hz at

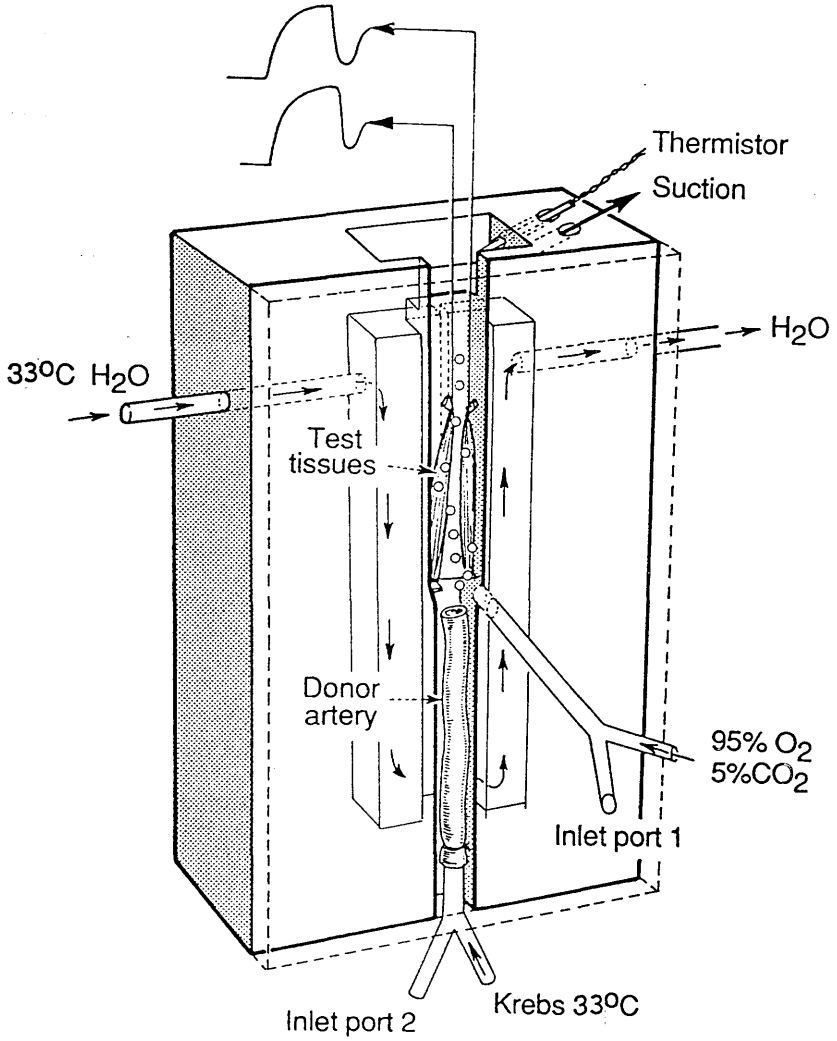

Figure 1 A diagrammatic representation of the closed cascade system used to release EDRF from a donor rabbit aorta and detect this on test tissues in a chamber immediately above the perfused aorta. Drugs can be introduced either into the upper chamber alone through inlet port 1 or into the Krebs' saline perfusing the aorta (inlet port 2). The entire cascade is jacketed on three sides with channels through which water at 33°C is pumped to maintain a constant temperature.

supramaximal voltages. All preparations were allowed to equilibrate for 60 minutes before beginning the experiment. When agonist drugs were used to induce tone, the concentration used was one which produced between 60-80% of the maximal tone. These concentrations were based on cumulative concentration-response curves in a preliminary set of experiments. When noradrenaline was used as the agonist, the Krebs' saline contained ascorbic acid 0.23 mM and EDTA 0.06 mM to stabilise it. Stock solutions of drugs (10^{-2}M) were made in distilled water and final dilutions made in normal saline (0.9%). The maximal volume of saline added to baths was 0.3 ml. Drug solutions were kept on ice during the experiment and the final bath concentrations quoted are in molar terms.

Cascade Studies

The perfusion cascade apparatus used to study EDRF is illustrated in figure 1. A 6 cm length of donor rabbit aorta with intact endothelium was mounted on a cannula in the lower chamber. The oxygenated Krebs' saline perfusing this artery then passed to an upper chamber containing the test tissues. The intention was to test whether smooth muscle other than blood vessels is sensitive to EDRF. At the time the experiments were done there was no information on whether any other smooth muscle responded to EDRF, it was therefore essential to have present at all times, a preparation known to be relaxed by EDRF. For this reason the upper chamber of the cascade system always contained two

test preparations, an endothelium-free rabbit aortic strip plus one of the other smooth muscle preparations being tested: the BRP muscle, rat anococcygeus muscle or guinea-pig trachea. EDRF is an unstable substance with a half-life in Krebs' saline at 36°C of about 6-8 seconds. For this reason it was essential to transfer the released EDRF from the perfused donor aorta to the test chamber as rapidly and with as little dilution as possible. This was achieved by keeping the volumes of the two chambers as low as possible (the total volume of two chambers is 1.6 ml), by arranging the test chamber immediately above the donor aorta chamber and determining experimentally the optimal rate of perfusion. This was found to be relatively fast (4 ml min^{-1}). Some experiments to test the optimal temperature were also made. The sensitivity to temperature variation was less than that to flow rate and 33°C was adopted for all experiments. The temperature in the cascade was maintained by pumping warm water from a thermostatially controlled reservoir through the water jacket surrounding three sides of the chambers. The Krebs' saline was oxygenated with 95%O_2 + 5%CO_2 in the reservoir but, in addition, the upper chamber was separately bubbled with the same gas mixture. After a 60 minutes period of equilibration the tone of the preparations in the upper chamber was raised by the continuous infusion of 5-HT and ACh to produce a concentration of 10^{-5}M of each in the Krebs' saline entering the chamber. This mixture of agonists was more effective than either alone in producing maintained tone in all the test preparations. The infusion was regulated by a B.Braun continuous infusion apparatus so that only small volumes of drug

were infused. Once tone had stabilized in the test preparations, ACh in concentrations from 10^{-7}M to 10^{-4}M was infused into the Krebs' saline perfusing the donor artery to release EDRF. Tension in test tissues was detected by Grass FT03C isometric transducers and displayed on a Grass Polygraph Model 7D. Indomethacin (5×10^{-6}M) was added to the Krebs' saline to prevent the formation of prostaglandins and the development of spontaneous tone in the non-vascular smooth muscle preparations.

PREPARATION OF TISSUES

(1) Rabbit Aorta New Zealand Rabbits (2-3 kg, either sex) were killed by exposure to carbon dioxide followed by exsanguination. The aorta was removed carefully and cleaned gently of surrounding connective tissue and fat. The abdominal aortic strips were cut at an angle of 30° to the line of the artery to produce preparations 1.5 cm long by 2-3 mm wide under 1 g tension in the bath. The retention of endothelium on these strips was confirmed by testing the ability of ACh (10^{-6}M) to relax 10^{-5}M 5-HT-induced tone by over 90%. The endothelium was removed from some preparations by gentle abrasion of the luminal surface with filter paper. Such preparations did not relax with ACh.

As the donor preparation for EDRF in the cascade system, a 6 cm length of thoracic aorta was tied to a cannula in the lower chamber of the cascade.

(2) Bovine Retractor Penis Muscle Bovine retractor penis muscles were obtained from Glasgow Abattoir and kept in Krebs' saline until required. They were normally used within 3 hours of the bullock's death. Strips of muscle 2-3 mm wide and 15 mm long were dissected from the middle portion of the bovine retractor penis, cutting along the lines of cleavage separating the smooth muscle bundles. Some preparations were kept in Krebs saline at 4°C overnight.

(3) Rat Anococcygeus Muscle Male Wistar rats (200-350 g) were killed either by a blow on the head or by exposure to carbon dioxide followed by exsanguination. The abdomen was opened in the mid-line, the pelvis split, bladder and urethra removed exposing the colon until the two anococcygeus muscles came into view. The muscles were cleaned, and after cutting through the ventral bar, each muscle was tied at either end and then removed into Krebs' saline. Indomethacin (5×10^{-6}M) was present in Krebs' saline for cascade studies.

(4) Guinea-Pig Trachea Guinea-pigs (200-300 g, either sex) were killed either by a blow on the head or by exposure to carbon dioxide followed by exsanguination. The trachea was exposed, cleaned and removed and paired rings of trachea prepared. The cartilaginous bars were then cut through in the mid-line and the preparation tied at both cartilaginous ends. When using this preparation, Indomethacin was

present in Krebs' saline for both the organ bath (10^{-6}M) and cascade studies (5 x 10^{-6}M) to reduce the background tone.

OTHER TECHNIQUES

Extracting the IF from the Bovine Retractor Penis Muscle

Fresh BRP muscles were weighed and minced by passing them through a Moulinex mincer. The minced muscle mixed with glass beads was ground in a pestle and mortar for 10 min with five times its volume of 100% methanol. The whole operation was performed on ice. After stirring in an MSE homogenizer for 15 minutes, the methanol extract was filtered through Whatman No.1 filter paper under reduced pressure, chilled with liquid nitrogen and centrifuged at 4000 rpm at 4°C for 10 minutes to remove lipids. The supernatant was passed through a C18 HPLC preparation cartridge, degassed and applied to a freshly prepared 10 x 1 cm Bio Rad anion exchange column which formed part of an HPLC system. After applying the sample, the column was washed with H_2O then eluted with 150 mM sodium nitrate ($NaNO_3$) at a flow rate of 5 ml min^{-1}. A sample was collected between 15-18 minutes where the IF is known to elute. 2 ml aliquots containing the IF were freeze dried and sealed in glass ampoules. The dried extracts were weighed and reconstituted, usually in distilled water, but sometimes in half-normal saline (0.45%), particularly for experiments involving erythrocyte binding. The

concentration of IF stock solution was approximately 20 mg/ml which was then activated at pH 2 with 1N HCl, then neutralized with 1N NaOH after 10 min and kept on ice till tested.

Erythrocyte Suspensions and Haemoglobin Solutions

Male rats (250-350 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and a cannula was inserted into a carotid artery. Blood was collected from this cannula into tubes containing heparin (20 IU ml^{-1} blood), centrifuged at 1000 rpm for 20 min (4°C), and the plasma and buffy coat removed carefully by aspiration. The erythrocytes were washed three times in sodium phosphate buffer at pH 7.4 and then resuspended in phosphate-buffered isotonic saline to restore the solution to its original volume. This erythrocyte suspension solution could be stored in the fridge (4°C) and was used up to three days after preparation. Such washed erythrocytes proved more fragile than unwashed cells, so that when added to an oxygenated organ bath the turbulence caused a variable degree of lysis. The extent of haemolysis was tested at the end of each experiment by removing the bath fluid, centrifuging to remove unlysed cells, then assaying spectrophotometrically the haemoglobin content of the supernatant. The released haemoglobin caused interference in several experiments and limited the concentration of erythrocytes which could be used. Whole blood was much more stable and, since previous experiments (Bowman &

Gillespie, 1982) showed plasma to be quite ineffective against the NANC relaxation, suspensions of erythrocytes were produced by adding an appropriate volume of heparinised whole blood to the bath. The concentration of free haemoglobin in the bath fluid was measured at the end of each experiment and on all occasions was below the level of detection (10^{-7}M).

Haemoglobin solutions were freshly prepared every second day. Blood was removed as before into heparinised tubes then haemolysed by pipetting 1 ml of the washed erythrocyte suspension into 19 ml of hypotonic phosphate buffer (20 mM). The resulting solution was centrifuged at 20,000 rpm for 40 min (4°C); the supernatant constituted the haemolysate and the concentration of haemoglobin in it was measured spectrophotometrically at 540 nm after conversion to methaemoglobin. The standard was made up from haemoglobin supplied by Sigma which was converted to methaemoglobin by adding Drabkin's solution which contains sodium bicarbonate, 100 parts, potassium ferricyanide, 20 parts and potassium cyanide, 5 parts (also supplied by Sigma).

Erythocyte and Haemoglobin Binding Experiment

Freshly prepared solutions of haemoglobin or erythrocyte suspensions were mixed with NO stock saline solution or IF both activated and unactivated. The IF was dissolved in half-saline to allow for the increased osmolarity during the activation procedure.

The final concentration of haemoglobin was 10^{-5}M and the erythocyte suspensions contained an equivalent amount. The mixtures were left for 10 minutes at room temperature. Haemoglobin was then removed by filtration through an Amicon YM 10 ultrafiltration membrane with a retention value of 10,000 Daltons. In experiments with NO special care was taken to prevent its loss by processes other than its binding to haemoglobin. Two problems in particular caused difficulties. Firstly, in spite of deoxygenating solutions with helium, the stirrer action in the Amicon cell caused a near complete loss of activity. No stirring was therefore done in these experiments. Secondly, the membrane of these filters can bind NO. The latter problem was controlled by using a solution of NO one half of which was filtered alone and the other after mixing with haemoglobin. The difference between the two was attributed to binding to haemoglobin. Separating erythrocytes from the mixtures was done by centrifugation at 2000 rpm for 10 minutes (4°C). The unactivated IF was activated after separation. All these solutions were then assayed on endothelium-free rabbit aortic strips.

Such techniques could not be applied to EDRF because the humoral substance released from aorta in the oxygenated cascade system has very short half-life. For this compound therefore, haemoglobin or erythrocytes were infused into the Krebs' saline in the cascade system in the upper chamber immediately above the donor aorta. This allowed a short period of time for interaction between EDRF and its blockers.

DRUGS AND SOLUTIONS

Nitric Oxide Solution

The method used was similar to that described by Palmer et al. (1987). Briefly, gaseous nitric oxide (99% pure), was obtained from BDH. A soft rubber tube was attached to the gas cylinder with its other end under water and enough gas allowed to escape to expel all air from the tubing. With the gas still flowing, the tubing wall close to the cylinder was pierced with a fine hypodermic needle attached to a gas-tight syringe and 6.5 µl of gas was removed. This was then injected into a rubber-sealed 65 ml brown bottle completely filled with normal saline previously purged for 1 hour with helium. This produced a 4.4 µM stock solution of nitric oxide which was made freshly each day and used within 10 hours. In some experiments, particularly with the rat anococcygeus, which is rather insensitive to NO, a five- or ten-times more concentrated stock solution was prepared.

Buffer solutions

The following solutions were used for preparing erythrocytes and haemoglobin. Sodium phosphate buffer was made as described by Dodge et al. (1963). Stock solutions of sodium dihydrogen orthophosphate (NaH_2PO_4, 0.155M) and disodium hydrogen orthophosphate, (Na_2HPO_4,

0.103M) were prepared and stored at 4°C. Isotonic phosphate buffer was made by mixing appropriate volumes of these solutions to give a pH of 7.4. Hypotonic phosphate buffer, 20 mM, was made by diluting isotonic phosphate buffer 1 with 15.5 distilled water. Isotonic phosphate buffered saline was made by mixing four volumes of 0.9% NaCl with one volume of isotonic phosphate buffer.

Drugs

Drugs used during the project were acetylcholine chloride (Sigma); atropine sulphate (Sigma); guanethidine sulphate (CIBA); hydroquinone (Sigma); 5-hydroxytryptamine sulphate (Sigma); indomethacin (Sigma); 3-Isobutyl-1-methyl-xanthine (Sigma); noradrenaline bitartrate (Koch-Light); pyrogallol (BDH); sodium nitroprusside (BDH); superoxide dismutase from bovine erythrocytes (Sigma).

Statistical Analysis

Results are expressed as mean ± standard error. N=number of experiments. Data were analysed using Student's t-test and $P < 0.05$ was considered significant.

RESULTS

THE SENSITIVITY OF DIFFERENT SMOOTH MUSCLE PREPARATIONS TO EDRF, THE IF AND NO

Because of the similarities in the responses and in the mode of action of EDRF, the IF, and NANC nerve stimulation it is possible that all are mediated by a common substance, i.e. NO or something which readily liberates NO. If this is true then the rank order of the sensitivity of different smooth muscles to each of these stimuli would be the same. At the time the present studies were begun there were no reports on the effects of EDRF on non-vascular smooth muscle. However, when the experiments were almost complete, and at a time when the high sensitivity of the BRP to EDRF had been demonstrated, a report did appear describing the failure of EDRF to relax guinea-pig taenia coli (Shikano et al., 1987). This paper suggested that only vascular smooth muscle was sensitive to EDRF. As the present work will show this is not so. It would be helpful if the NANC transmitter, like EDRF, could have been detected in the perfusing fluid. If this had been possible then the sensitivity of the different smooth muscle preparations to the transmitter could also have been tested. Unfortunately, it was impossible to demonstrate any smooth muscle relaxant activity in the fluid bathing a preparation of BRP following nerve stimulation (Gillespie, unpublished observation). This section therefore describes the differential sensitivity of four smooth muscle preparations, the rabbit aortic strip, the BRP muscle, the rat anococcygeus muscle and the guinea-pig tracheal strip, to four stimuli: EDRF; the IF; NO; and

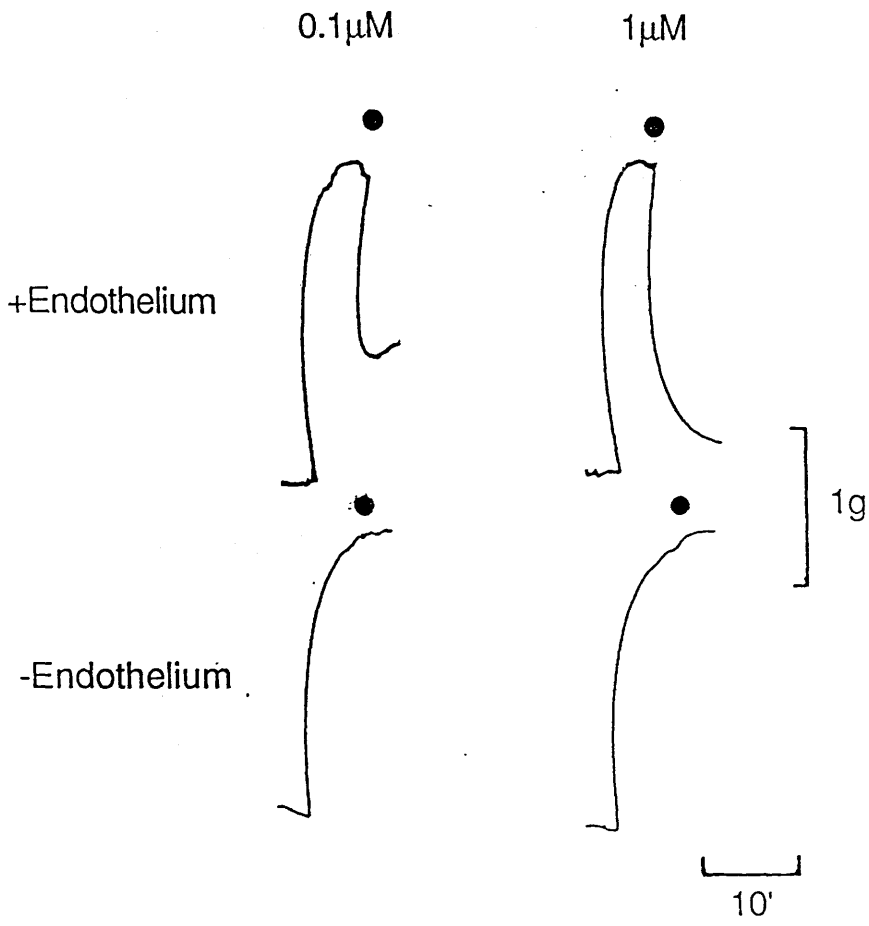

Figure 2 The relaxant responses of isolated rabbit aortic strips, with or without endothelium, to EDRF released by different concentrations of acetylcholine (ACh). Tone was raised by 5-hydroxytryptamine (5-HT, 10^{-5}M). ACh (10^{-7}M or 10^{-6}M) produced a concentration-related relaxation in the aortic strip retaining its own endothelium (upper panel) but had no effect or gave a small contraction in the endothelium-free aortic strip (lower panel).

sodium nitroprusside (NaNP).

(1) The Release of EDRF from Aorta Retaining Its Endothelium The effects of different concentrations of ACh on rabbit aortic strips, with or without endothelium, was tested simply to confirm that EDRF could be released from the intact aorta. The experiment was carried out in 10 ml organ baths and tone was raised with 5-HT at a concentration of 10^{-5}M. In preparations retaining their endothelium ACh produced a concentration-related relaxation with a threshold at about 3×10^{-8}M and a maximum at 10^{-6}M. High concentrations of ACh (>10^{-6}M) usually produced a slightly smaller relaxation. In preparations devoid of endothelium, as a result of gentle rubbing, ACh never produced relaxation. Indeed concentrations of 10^{-6}M and higher regularly produced small contractions (Fig 2). Both relaxation and contraction were abolished by atropine 10^{-6}M (data is not shown). These results are similar to those reported by Furchgott & Zawadzki (1980) who suggested that the relaxation was due to the release of EDRF from endothelial cells and the contraction was due to a direct stimulation of smooth muscle cells. Presumably it is the direct smooth muscle stimulation which reduces the EDRF-mediated relaxation at very high concentration of ACh.

(2) EDRF Released from the Perfused Rabbit Aorta These experiments were performed in the closed cascade system (as shown in Fig

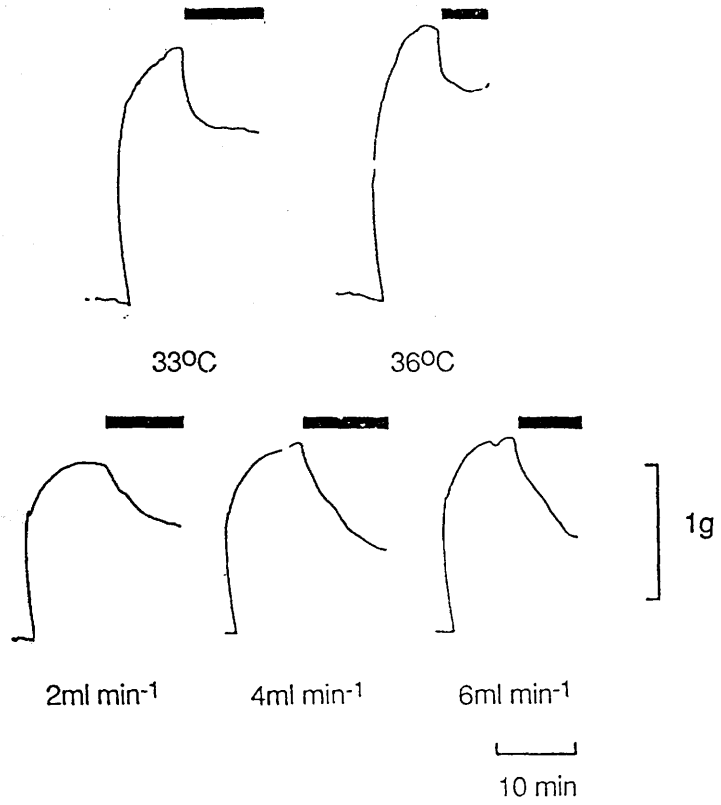

Figure 3 The response of isolated rabbit aortic strips to EDRF liberated by acetylcholine (ACh) from a donor aorta and superfused over the test tissues at different temperatures and perfusion flow rates. Tone was raised in the test preparations by infusing ACh and 5-hydroxytryptamine (5-HT) into the upper chamber to give a concentration of 10^{-5}M of each. In all experiments, ACh ($5x10^{-6}$M) was infused into the Krebs' saline perfusing the donor aorta for a period shown by the black bar. Various temperatures (upper panel) and perfusion flow rates (lower panel) were tested as shown under each response. The Krebs' saline contained indomethacin ($5x10^{-6}$M).

1). Since ACh at high concentration produced a small contraction in endothelium-free aortic strips (Fig 2), this might reduce the relaxant response to EDRF when using high concentrations of ACh. To overcome this problem ACh (10^{-5}M) was combined with 5-HT at the same concentration and infused into the upper chamber only. The test preparation was therefore already contracted with ACh and the additional low concentration of ACh from the donor preparation would have no additional effect. The other reason for using this combination of spasmogens is that in some preparations the tone is more stable than with 5-HT alone. Indomethacin at a concentration of 5×10^{-6}M was present in the Krebs' saline in all cascade experiments.

As only a limited concentration of EDRF could be released from the donor aorta, it was first necessary to optimise the conditions to give the maximal concentration of EDRF. This requires the maximal release of EDRF, which is dependent on the ACh concentration, the minimal dilution and the maximal rate of transport from the donor aorta to the test tissues. These last two conditions place contradictory requirements on the system, fast transport requires a rapid flow rate while minimal dilution requires a slow flow rate. It is also desirable to reduce EDRF destruction which is temperature dependent.

With these precautions it was relatively easy to demonstrate a concentration-related release of EDRF from the donor aorta in terms of a graded relaxation of the rabbit aortic strip. Water temperature in the reservoir and the perfusion flow rate were varied and the effects of relaxation studied. Figure 3 shows that a temperature of 33°C and flow

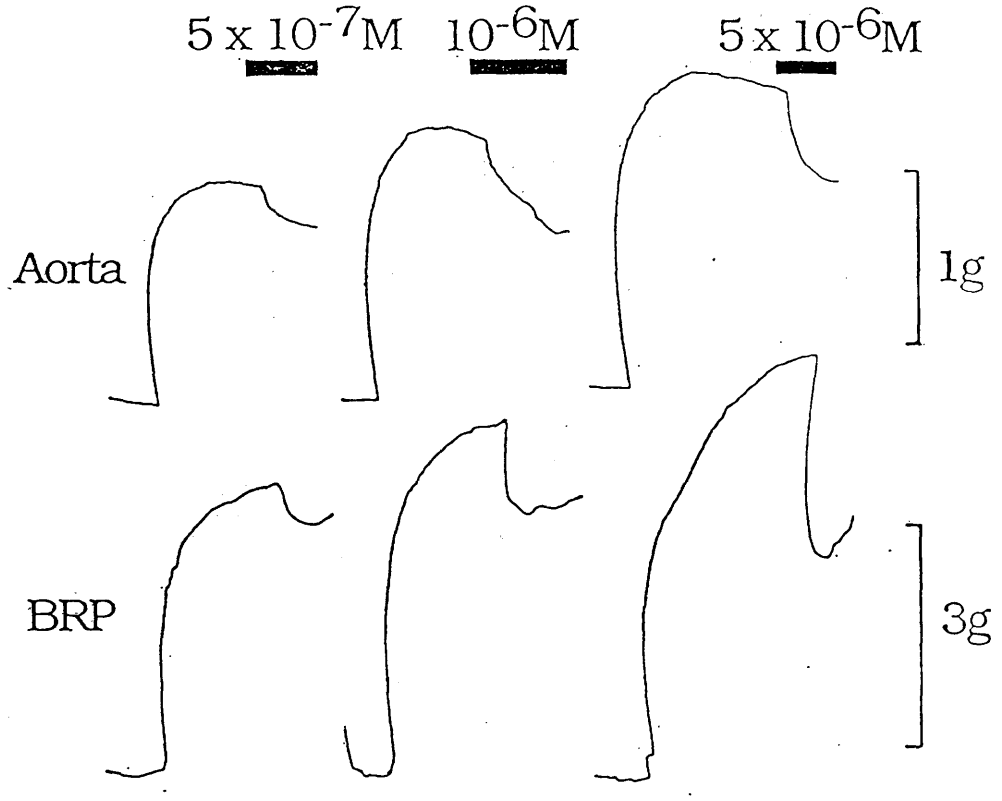

Figure 4 A comparison of the responses of an endothelium-free rabbit aortic strip (Aorta) and bovine retractor penis (BRP) to EDRF liberated by acetylcholine (ACh) from a donor rabbit aorta and superfused over test tissues by the method shown in Figure 1. Tone was induced in test preparations by infusing ACh and 5-hydroxytryptamine (5-HT) into the upper chamber to give a concentration of 10^{-5}M of each. Indomethacin ($5x10^{-6}$M) was present in the Krebs' saline throughout. ACh was infused into the Krebs' saline perfusing the donor aorta for the period shown by the black bar to produce concentrations of ACh shown above each record. EDRF liberated from the donor aorta produced concentration-related relaxation of tone in both the rabbit aortic strip and the BRP preparations.

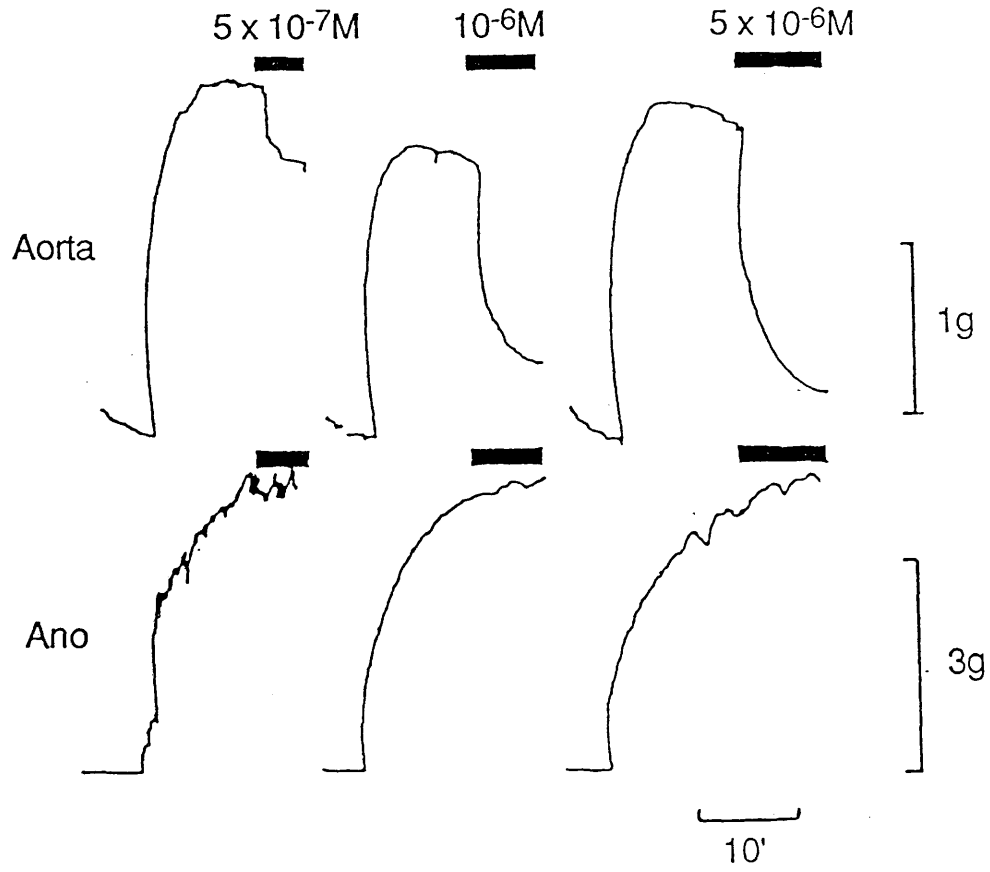

Figure 5 A comparison of the responses of an endothelium-free rabbit aortic strip and the rat anococcygeus (ANO) to EDRF liberated by acetylcholine from a donor aorta and perfused over test tissues by the technique shown in Figure 1. Tone was induced in test preparations by infusing ACh and 5-hydroxytryptamine (5-HT) into the upper chamber to give a concentration of 10^{-5}M of each. Indomethacin ($5x10^{-6}$M) was present in the Krebs' saline throughout. ACh was infused into the Krebs' saline perfusing the donor aorta for the period shown by the black bar to give concentrations shown above each record. While EDRF liberated from the donor aorta produced concentration-related relaxation of tone in the rabbi aortic strip, it had no effect on the rat anococcygeus.

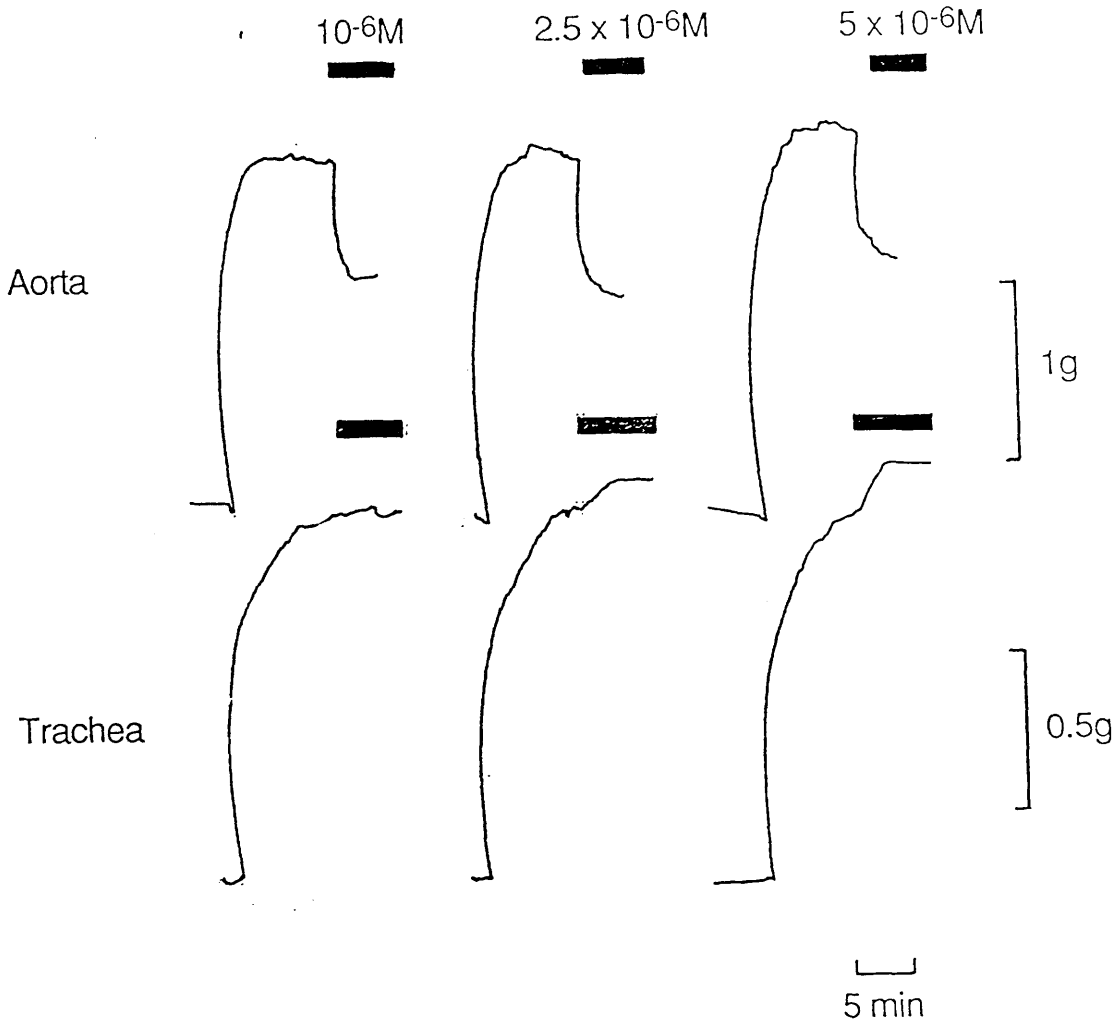

Figure 6 A comparison of the responses of an endothelium-free rabbit aortic strip and guinea-pig trachea (Trachea) to EDRF liberated by acetylcholine (ACh) from a donor rabbit aorta superfused over test tissues by the technique shown in Figure 1. Tone was induced in test preparations by infusing ACh and 5-hydroxytryptamine (5-HT) into the upper chamber to give a concentration of 10^{-5}M of each. Indomethacin ($5x10^{-6}$M) was present in the Krebs' saline throughout. ACh was infused into the Krebs' saline perfusing the donor aorta for the period shown by the black bar to give concentrations shown above each record. EDRF liberated from the donor aorta produced concentration-related relaxation of tone in the rabbit aortic strip but not in the guinea-pig trachea.

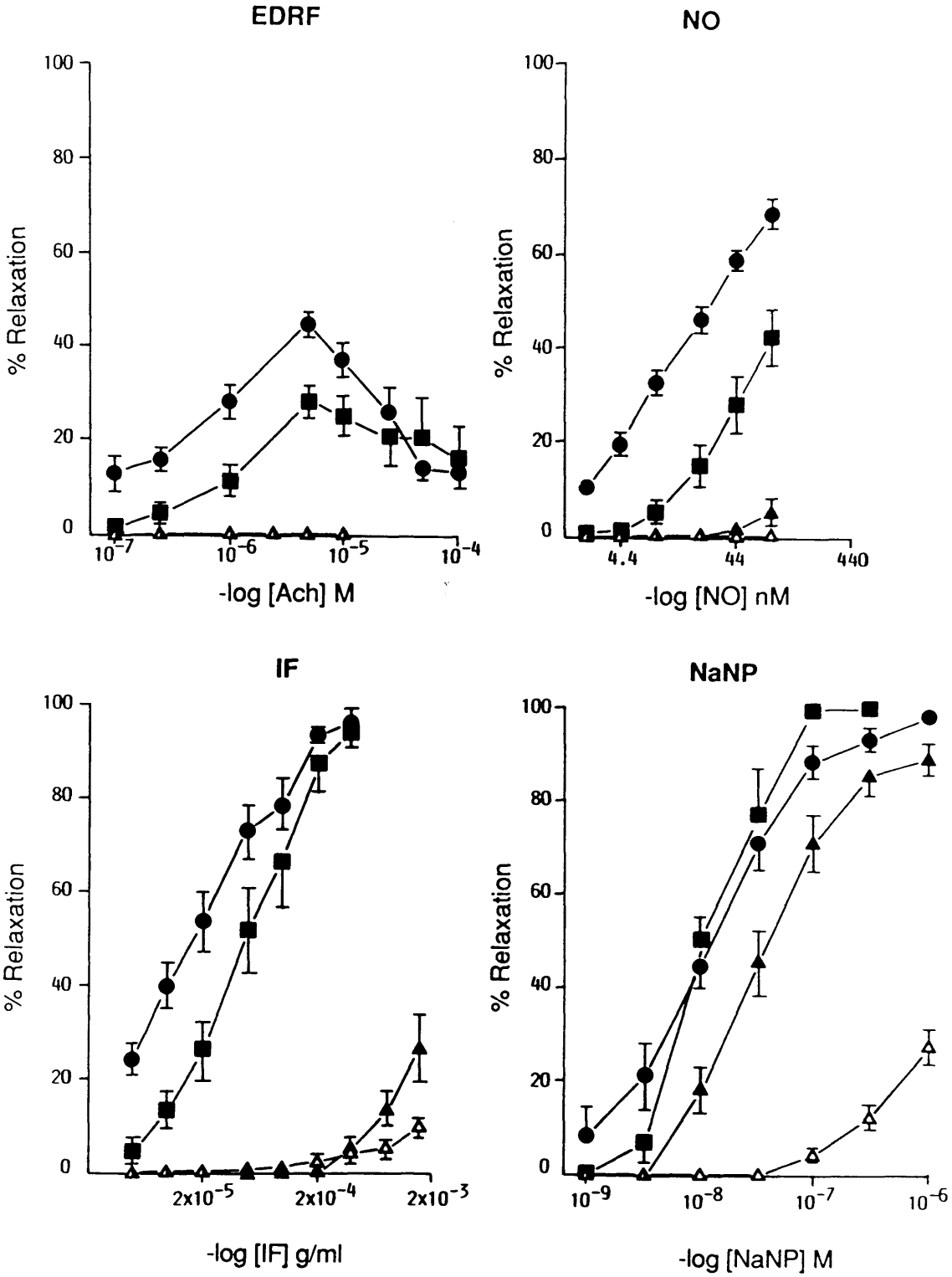
EDRF
% Relaxation
100
80
60
40
20
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
-log [Ach] M
NO
% Relaxation
100
80
60
40
20
0
4.4
44
440
-log [NO] nM
IF
% Relaxation
100
80
60
40
20
0
$2x10^{-5}$
$2x10^{-4}$
$2x10^{-3}$
-log [IF] g/ml
NaNP
% Relaxation
100
80
60
40
20
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
-log [NaNP] M

Figure 7 Log concentration-response curves for the relaxant effect induced by acetylcholine (EDRF), nitric oxide (NO), the inhibitory factor (IF) extracted from the bovine retractor penis and sodium nitroprusside (NaNP) on four smooth muscle preparations: spiral strips of endothelium-free rabbit aorta (●); strips of BRP (■); the rat anococcygeus (▲); and the guinea-pig trachea (Δ). EDRF caused a concentration-related relaxation of the rabbit aortic strip and the BRP but had no effect on the rat anococcygeus or guinea-pig trachea. NO and the IF also relaxed the rabbit aortic strip and the BRP but had no effect on the rat anococcygeus or the guinea-pig trachea except at high doses. No concentration of NO tested relaxed the guinea-pig trachea. NaNP was equally effective in relaxing the rat anococcygeus, the rabbit aortic strip and the BRP, producing a maximal response of equal magnitude in all three tissues, however, it was much less effective in the guinea-pig trachea. Each point is the mean of between 6 and 42 observations and the bars represent ± s.e. Indomethacin ($5x10^{-6}M$) was present in the Krebs' saline throughout.

rate of 4 ml min^{-1} were optimal for this experiment. All subsequent experiments were performed under these conditions.

(3) Effects of EDRF on the Four Smooth Muscle Preparations Since there was no information on whether non-vascular smooth muscle was sensitive to EDRF, in every experiment an endothelium-free rabbit aortic strip was always included as one of a pair of test preparations in the upper chamber. In this way the release of EDRF could always be measured. Examples of the effect of EDRF on the three non-vascular smooth muscle preparations compared with aortic strips are illustrated in Figure 4-6. Figure 7 summarizes all of the information.

Different concentrations of ACh perfused through the donor aorta produced a concentration-related relaxation on endothelium-free aortic strips. The maximal relaxation of the aortic strip, on average, was 45% at a concentration of 5×10^{-6}M ACh. Greater relaxation was obtained in aortic strips retaining their own endothelium as shown in Figure 2, presumably because the short diffusional distance between the endothelial and smooth muscle cells allows EDRF to act before it is destroyed; the concentration of EDRF could also be expected to be higher than in cascade experiments. An alternative explanation is that higher concentrations of ACh were producing a motor effect, by the direct action on the muscarinic receptors of the smooth muscle of the test tissues, which masked the relaxant effect induced by the release of EDRF. This would also explain the decline in the relaxant effect of ACh at concentrations greater than 5×10^{-6}M which is seen in Figure 7.

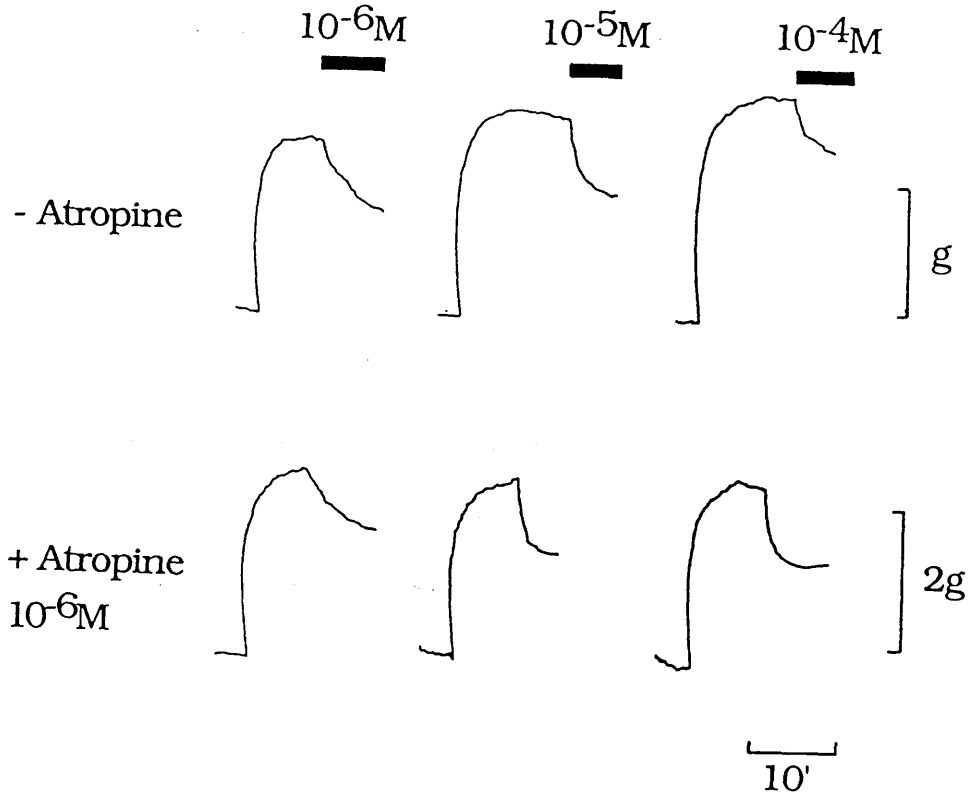

Figure 8 The effect of atropine on the response of an endothelium-free rabbit aortic strip to EDRF liberated from a donor aorta. Tone was induced by 5-hydroxytryptamine (5-HT, 10^{-5}M) infused into the upper chamber. Acetylcholine (ACh) was infused into the Krebs' saline perfusing the donor aorta for the period shown by the black bar to give concentrations shown above each response. In the absence of atropine, the relaxation to the highest concentration of ACh fell slightly (upper panel). The lower panel shows that in the presence of atropine infused only into the upper chamber, the response to EDRF did not fall at high concentrations of ACh. The Krebs' saline contained indomethacin (5×10^{-6}M).

Nevertheless, the relaxation at a concentration of 5 x 10^{-6}M ACh in the cascade probably did represent the maximal release of EDRF and therefore the maximal concentration of EDRF which could be obtained in this type of experiment. This was confirmed in a few experiments in which atropine 10^{-6}M was infused into the upper chamber and at the same time using 5-HT 10^{-5}M alone to induce tone in rabbit aortic strips. Under these conditions there was no decline in the response of the test tissue to concentrations of ACh above 5 x 10^{-6}M, suggesting the motor effect on the smooth muscle was responsible for the decline. However, the maximal relaxation was still observed at this concentration (Fig 8). This result further proved that the relaxation of endothelium-free aortic strip was due to the release of EDRF from the donor aorta because the same concentration of atropine completely abolished this relaxant effect when it was infused through the donor aorta in the lower chamber (data is not shown).

With this background information it was possible to compare the effects of EDRF on each non-vascular smooth muscle preparation in comparison with the rabbit aortic strip set in the same chamber as a control. EDRF relaxed the BRP muscle in a concentration-related manner in response to ACh being perfused through the donor aorta. The BRP muscle was almost as sensitive to EDRF as the aortic strip (Fig 4). This relaxation was due to the action of EDRF from the donor aorta rather than to ACh itself stimulating the smooth muscle directly. This was shown by infusing atropine (10^{-6}M) into the upper chamber only; this did not change the relaxant effect of EDRF on the BRP muscle.

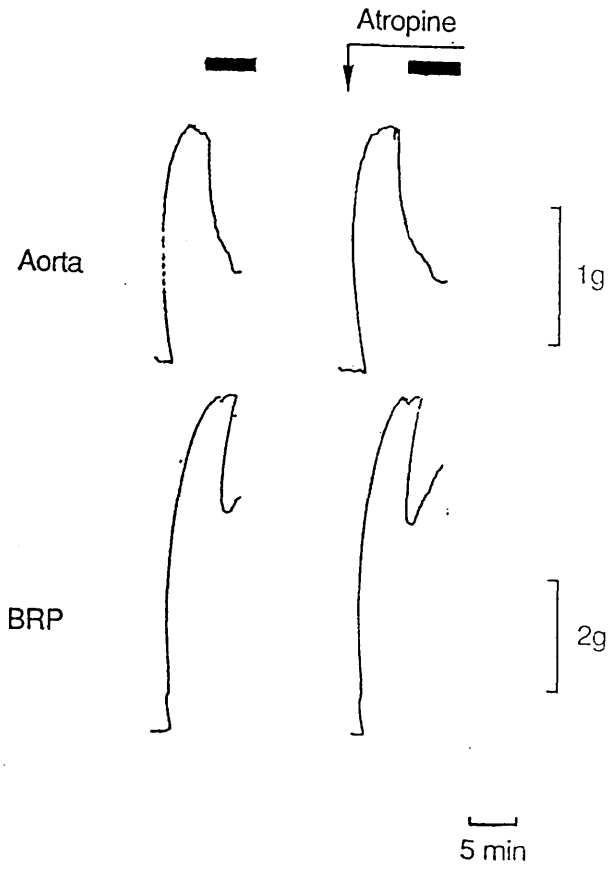

Figure 9 The effect of atropine on the response of the bovine retractor penis (BRP) and an endothelium-free rabbit aortic strip to EDRF liberated from a donor aorta. Tone was induced in the test preparations by infusing acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) into the upper chamber to give a concentration of 10^{-5}M of each. ACh ($5x10^{-6}$M) was infused into Krebs' saline perfusing the donor aorta for the period shown by the black bar to release EDRF which produced relaxation in both preparations (left panel). The right panel shows the same responses in the presence of atropine (10^{-6}M) infused into the upper chamber only. The Krebs' saline contained indomethacin ($5x10^{-6}$M).

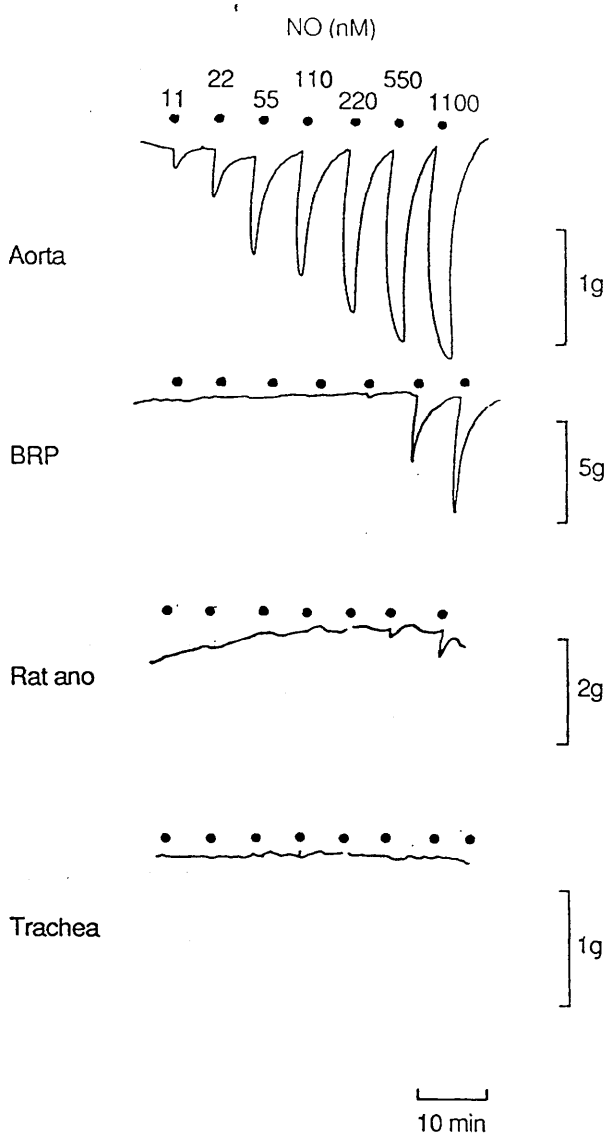

Figure 10 The responses of four smooth muscle preparations, the endothelium-free rabbit aortic strip (Aorta), the bovine retractor penis (BRP), the rat anococcygeus (ANO) and the guinea-pig trachea (Trachea), to nitric oxide (NO). In all the preparations, tone was induced by acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) each at a bath concentration of 10^{-5}M. NO, at concentrations ranging from 11 to 1100 nM, produced relaxation in the aorta and BRP; only higher concentrations of NO relaxed the rat anococcygeus and even the highest concentration had little effect on the guinea-pig trachea.

Atropine perfused through the donor aorta abolished the response (Fig 9). Similar experiments were also carried out in the rat anococcygeus muscle and guinea-pig trachea, but neither was relaxed by EDRF (Fig 5,6). The possibility that this failure to relax was due to a limited concentration of EDRF was considered, and therefore, the effects of other related relaxants were tested on these preparations.

(4) Effects of NO, IF and NaNP on Smooth Muscle Preparations

Three relaxants, NO, the IF and NaNP were tested on the four muscles in 10 ml organ baths rather than the cascade system. In all these tissues, tone was induced with the same mixture of 10^{-5}M 5-HT and 10^{-5}M ACh as used in the cascade experiment for assaying EDRF. Indomethacin (5 x 10^{-6}M) was present in the Krebs' saline to inhibit prostaglandins synthesis. An endothelium-free rabbit aortic strip or BRP preparation, previously shown to be sensitive to EDRF, was always included in each experiment as a control.

Both the rabbit aortic strip and the BRP muscle relaxed in a concentration-dependent manner to the freshly prepared NO solution whereas the rat anococcygeus muscle and the guinea-pig trachea gave little or no response (Fig 10). A further increase in the concentration of NO stock solution by five times did produce small relaxations in the rat anococcygeus muscle, but even the highest concentration (1.1 μM) tested did not relax the guinea-pig trachea.

The effect of the IF on these four preparations was similar to the

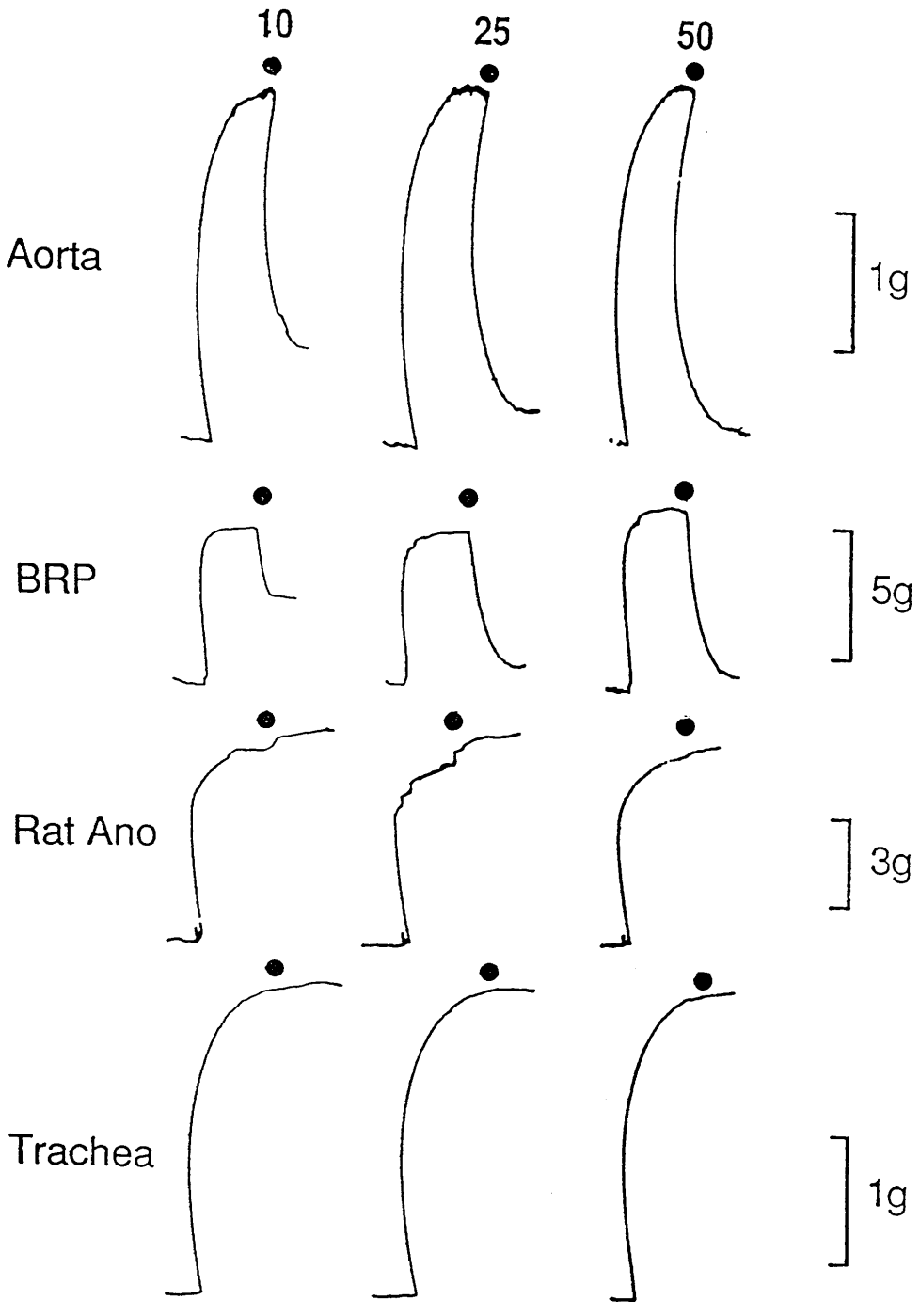
IF (µl)
10
25
50
Aorta
1g
BRP
5g
Rat Ano
3g
Trachea
1g

Figure 11 The responses of four smooth muscle preparations, an endothelium-free rabbit aortic strip (Aorta), the bovine retractor penis (BRP), the rat anococcygeus (ANO) and the guinea-pig trachea (Trachea) to the inhibitory factor (IF) extracted from the BRP. Tone was induced by acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) each at a bath concentration of 10^{-5}M. The IF was dissolved in distilled water to a concentration of 20 mg ml^{-1} and was acid-activated. The values above each response represent the volume in µl of the IF solution added to the bath. The IF produced concentration-related relaxation of both aorta and BRP but had no effect on the rat anococcygeus or guinea-pig trachea.

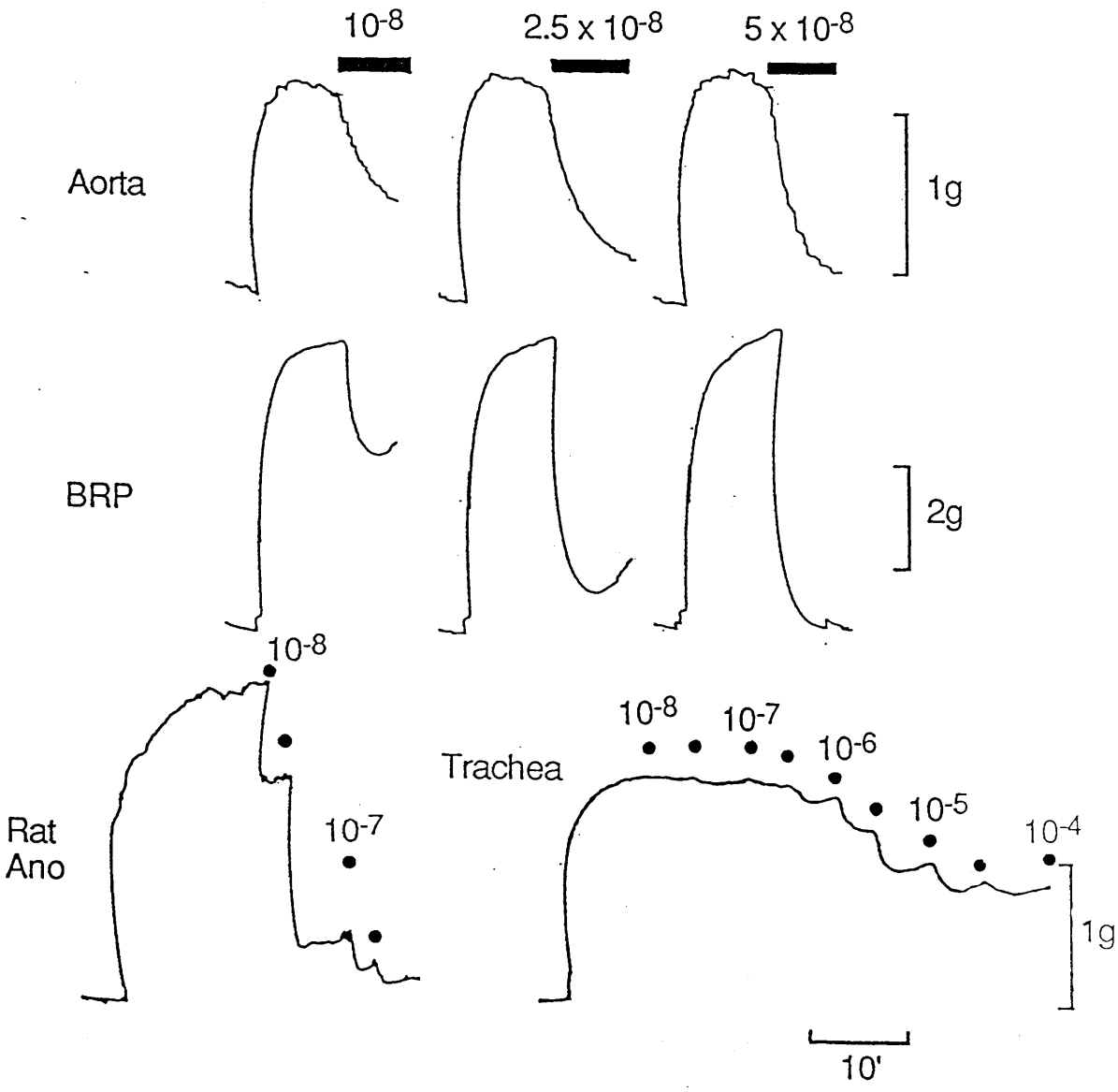
NaNP(M)
10-8
2.5 x 10-8
5 x 10-8
Aorta
1g
BRP
2g
10-8
10-7
Rat
Ano
Trachea
10-8
10-7
10-6
10-5
10-4
1g
10'

Figure 12 The responses of four smooth muscle preparations, an endothelium-free rabbit aortic strip (Aorta), the bovine retractor penis (BRP), the rat anococcygeus (ANO) and the guinea-pig trachea (Trachea), to sodium nitroprusside (NaNP). The rabbit aortic strip and BRP were tested in the upper chamber of the cascade apparatus, as shown in Figure 1, with no donor aorta in the lower chamber. The rest of the tissues were tested in organ baths. Tone was induced by acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) each at a concentration of 10^{-5}M. In the aortic strip and BRP preparations, NaNP was infused at the concentrations shown above each black bar representing the perfusion period. In the rat anococcygeus and guinea-pig trachea, NaNP was added to the bath solution cumulatively. Unlike the other relaxants, NaNP produced powerful relaxation in the rat anococcygeus, as in the aortic strip and BRP, but its effect on the guinea-pig trachea was still very poor.

effect of NO. Both the rabbit aortic strip and the BRP muscle relaxed in a concentration-dependent manner with a similar sensitivity but even the concentration of IF which gave maximal relaxation on these preparations had little effect on either the rat anococcygeus muscle or the guinea-pig trachea (Fig 11). Further increases in the concentration of the IF again produced some relaxation in the rat anococcygeus muscle but had no effect in the guinea-pig trachea.

To sodium nitroprusside (NaNP), the rabbit aortic strip and the BRP muscle were tested in the cascade system, the remaining tissues were tested in the organ baths. The sensitivities of the aortic strip and the BRP muscle were high. The pattern of response of the rat anococcygeus muscle however was quite different from the other relaxants. While the rat anococcygeus muscle gave little or no response to EDRF, NO and IF it was readily relaxed by NaNP (Fig 12). The guinea-pig trachea remained the least sensitive.

The concentration-response curves of the four stimuli on each of the four smooth muscle preparations are summarized in Fig 7. The sensitivities of four muscle preparations to EDRF, NO and the IF were the same, consistent with NO being the final mediator in relaxation induced by both EDRF and the IF as suggested by Furchgott (1988) and Martin et al. (1988). The effect of NaNP on the rat anococcygeus muscle was unexpected. Either the liberation of NO within the muscle cells in this tissue is much more effective than NO added to the bath, or NaNP has some additional mechanism of action independent of the release of NO.

EFFECTS OF HAEMOGLOBIN AND ERYTHROCYTES

Haemoglobin is known to block the responses to EDRF in the rabbit aorta (Martin et al, 1985), to NANC nerve stimulation and to the IF in the BRP and rat anococcygeus muscles (Bowman & Gillespie, 1981; 1982; Bowman et al, 1982). As NO is known to be a rapidly diffusible substance, it should easily pass through cell membranes. If this is so and all these stimuli were acting through NO, then erythrocytes should be as effective as haemoglobin in blocking responses to these stimuli, since NO will rapidly penetrate the membrane and bind to haemoglobin within the cells. According to this hypothesis, erythrocytes would be as effective as solutions of haemoglobin in blocking these responses. This was tested in a variety of ways depending on the nature of each relaxant. The effect on the response to NANC nerve stimulation was tested in 10 ml organ baths by adding haemoglobin or erythrocytes to the bath solution. The responses to the IF and NO were also assayed in organ baths but the IF or NO solutions were previously exposed to either haemoglobin or erythrocytes and then separated by ultrafiltration or centrifugation before assay. EDRF was tested both in organ baths, where the preparations were aortic strips retaining their endothelium, and in the cascade system where EDRF was liberated from a donor aorta and detected on a separate endothelium-free aortic strip. Haemoglobin solutions or erythrocyte suspensions were either added to the bath fluid or, in the cascade system, infused into the Krebs' saline between the

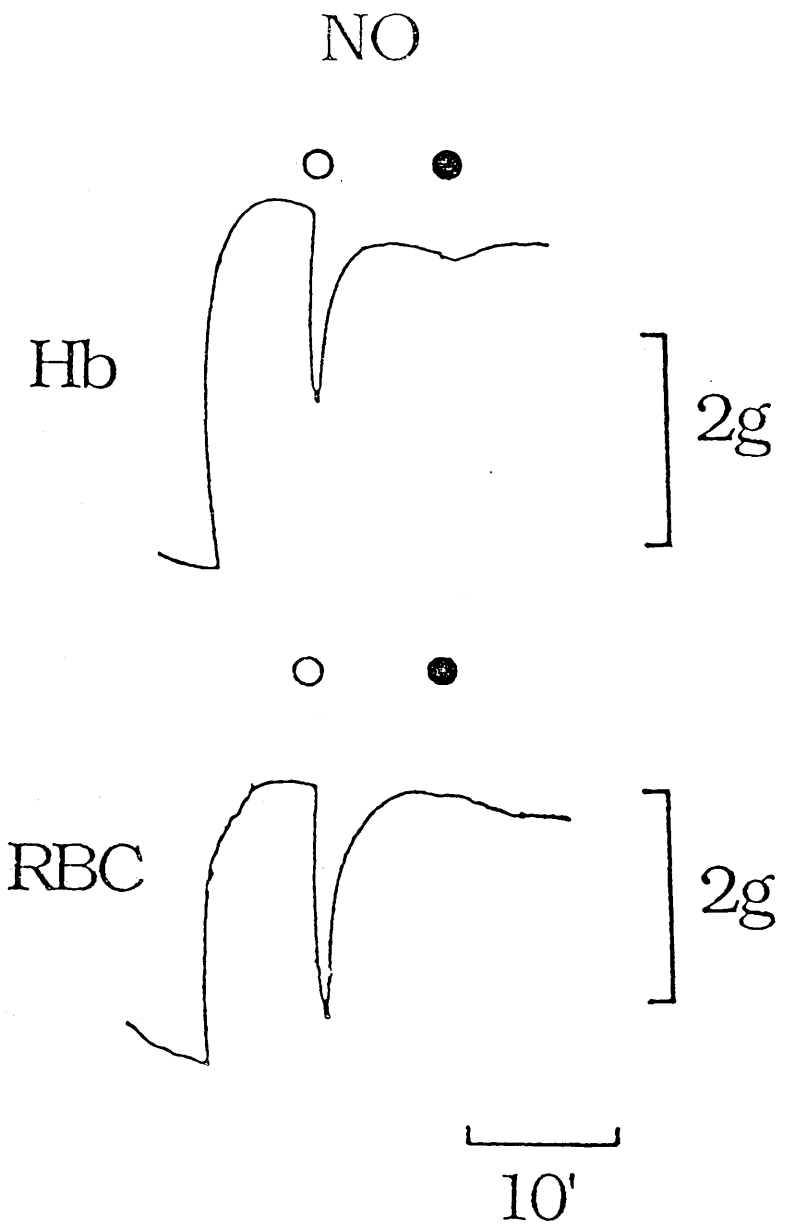

Figure 13 The binding of nitric oxide (NO) by 10^{-5}M haemoglobin (Hb, upper records) or an equivalent concentration of erythrocytes (RBC, lower records). The first marker (o) in each pair indicates the control response of an endothelium-free rabbit aortic strip precontracted with 5-HT (10^{-5}M), to NO and the second (•) the response to the same solution of NO previously mixed either with haemoglobin or erythrocytes, left for 10 min, and then separated either by ultrafiltration (Hb) or centrifugation (RBC). Both haemoglobin and erythrocytes completely abolished the relaxant effect of a solution of NO.

donor aorta and test tissues.

(1) Effects on NO-induced Relaxation Theoretically, haemoglobin which binds NO, should not only abolish the response to NO when it was added to the organ bath but, if added to a solution of this relaxant and then removed, it should also abolish the relaxant effect when it was subsequently assayed on the test preparation. This was tested by mixing haemoglobin with the NO solution at a haemoglobin concentration of 10^{-5}M and allowing 10 min for binding. Haemoglobin was then removed by ultrafiltration and the haemoglobin-free filtrate was then assayed on the rabbit aortic strip. An example of this type of experiment is shown in Figure 13. Haemoglobin completely abolished the relaxant effect of NO.

In a separate experiment, erythrocytes at a concentration equivalent to a 10^{-5}M solution of haemoglobin were added to the NO solution and again allowed 10 min for binding. Erythrocytes were then removed by centrifugation and the activity of the supernatant solution assayed on the endothelium-free rabbit aortic strip. Figure 13 shows that erythrocytes, as expected, completely abolished the effect of NO.

(2) Effects on IF-induced Relaxation In this experiment the IF was used in two forms:

1. Acid-activated, where the IF was acidified to pH 2 for 10 min by

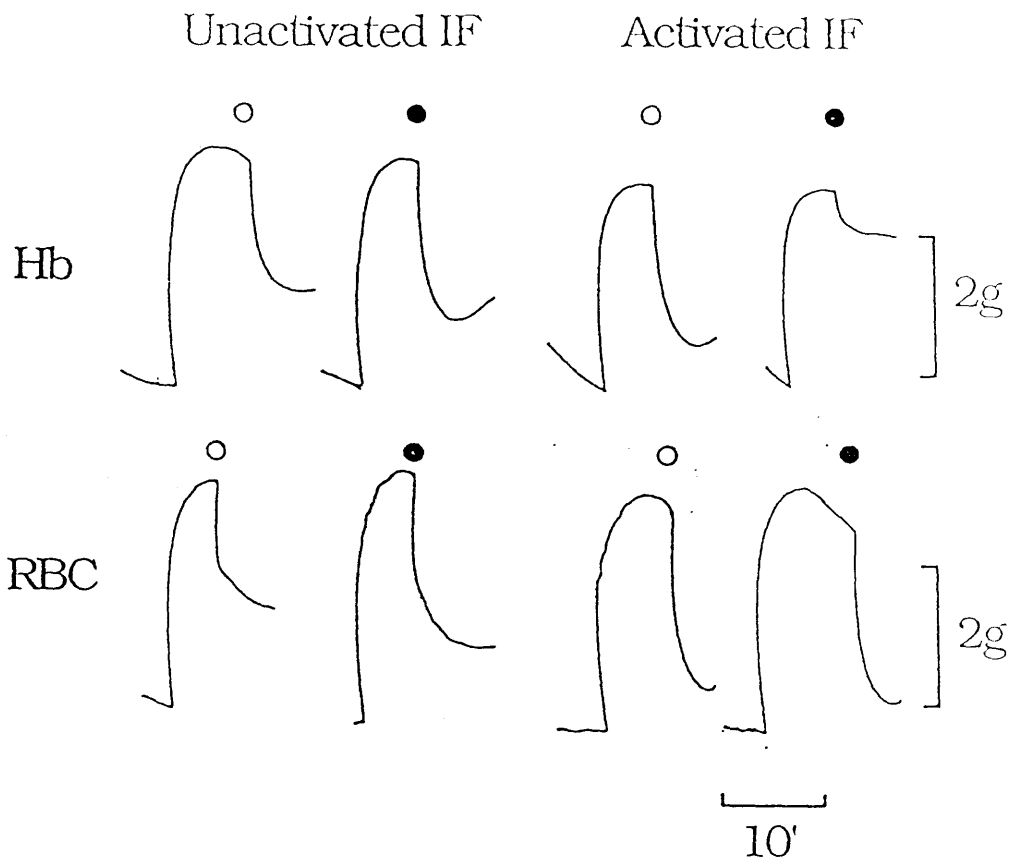

Figure 14 The binding of the inhibitory factor (IF) extracted from the bovine retractor penis by 10^{-5}M haemoglobin (Hb, upper records) or an equivalent concentration of erythrocytes (RBC, lower records). In each pair of responses the first (o) is the control response of an endothelium-free rabbit aortic strip precontracted with 5-HT (10^{-5}M), to the IF and, the second (•) the response after exposure of the IF either to haemoglobin or erythrocytes, left for 10 min, and then separated either by ultrafiltration (Hb) or centrifugation (RBC). The unactivated IF was exposed to solutions of haemoglobin or erythrocytes, prior to separation, activation, and assay. Haemoglobin had no effect on the unactivated IF but greatly reduced the relaxant activity of acid-activated IF. Erythrocytes had no effect on the relaxant action of either activated or unactivated IF.

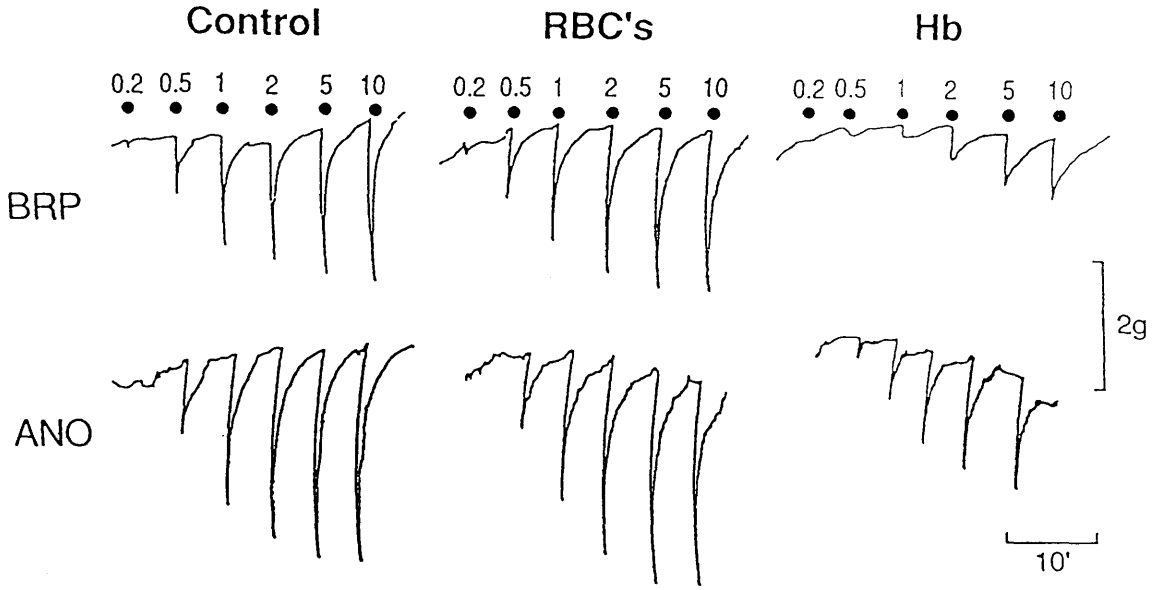

Figure 15 The responses of the bovine retractor penis (upper records) and the rat anococcygeus (lower records) to field stimulation of their NANC nerves. The nerves were stimulated for 10 seconds at the marker (•) at the frequencies shown. Guanethidine ($3x10^{-5}M$) was present throughout to block the motor adrenergic nerves and raise tone. Haemoglobin (Hb, $3x10^{-6}M$) as seen in the last panel, reduced responses at all frequencies in both preparations by more than 50%, whereas a comparable concentration of haemoglobin, but held within erythrocytes (RBC's, middle panel) was without effect.

using 1N HCl and then neutralized to pH 7 by using 1N NaOH prior to the binding experiment.

2. Unactivated, where the IF was activated only after separation from haemoglobin or erythrocytes. The activated IF was then assayed.

Both forms of the IF were tested on the same day and from a single aliquot of the same sample of extract. The results are shown in Figure 14. Haemoglobin greatly reduced the effect of the activated IF but had no effect on the unactivated extract which, after separation from haemoglobin followed by activation, retained all of its activity. Erythrocytes, which abolished the effect of NO, were without effect on either activated or unactivated IF suggesting the acid activated principle is not identical with NO.

(3) Effects on NANC Nerve Stimulation Field stimulation at supramaximal voltages produced a frequency-related NANC relaxation (frequencies between 0.2 and 10 Hz) in both BRP and rat anococcygeus muscles whose tone has been raised by guanethidine (3×10^{-5}M). The same stimulation was repeated immediately after adding haemoglobin (3×10^{-6}M) or a suspension of erythrocytes, at an equivalent concentration of haemoglobin to the bath solution. An example of this type of experiment is illustrated in Figure 15. In early experiments with washed erythrocytes a variable degree of inhibition of the NANC relaxation was observed. This was due to haemolysis of the erythrocytes and the liberation of free haemoglobin. For this reason

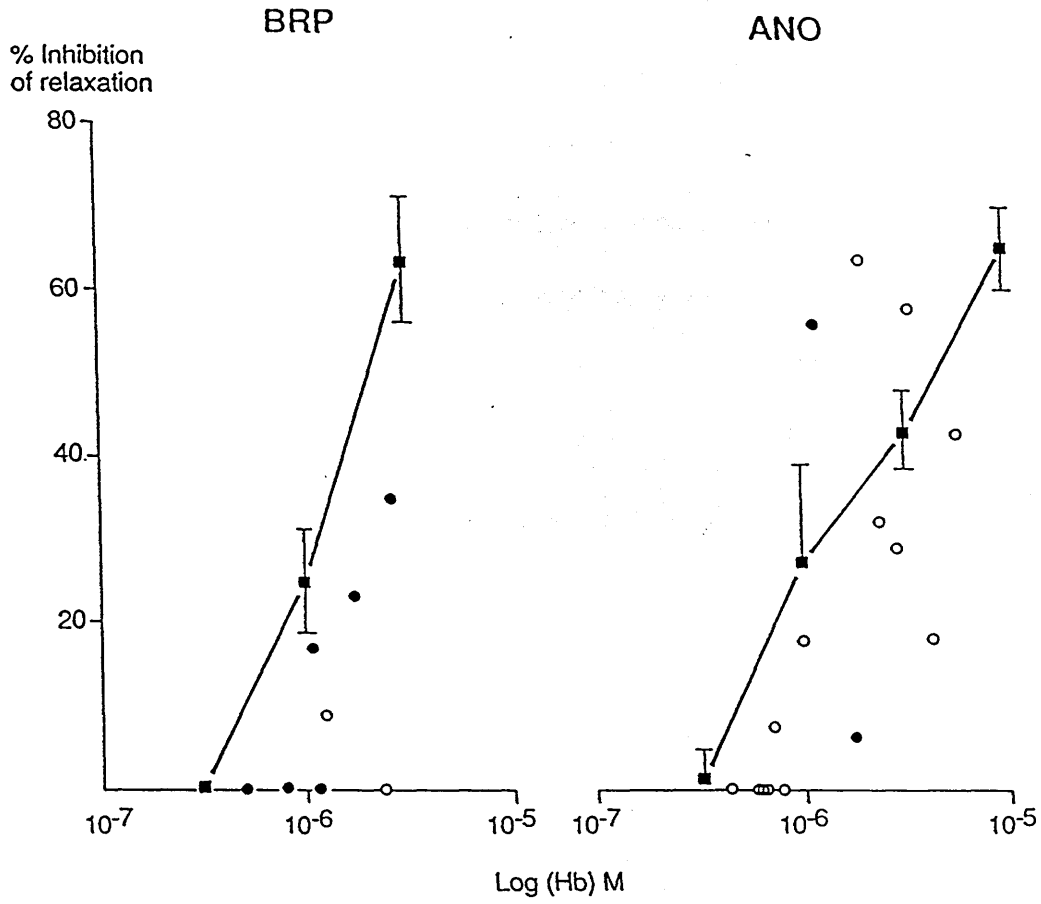
BRP
ANO
% Inhibition
of relaxation
80
60
40
20
10^{-7}
10^{-6}
10^{-5}
10^{-7}
10^{-6}
10^{-5}
Log (Hb) M

Figure 16 Comparison of inhibitory effects of different concentrations of haemoglobin solution on the response of the BRP and rat anococcygeus to field stimulation of their NANC nerves, with the inhibition produced by suspensions of erythrocytes which have undergone variable degrees of haemolysis. The solid line (■——■) represents the inhibitory effect of solutions of haemoglobin at the concentrations shown, on the BRP and rat anococcygeus muscles. The individual points indicate the levels of free haemoglobin found in the bath solution at the end of experiments in which suspensions of erythrocytes had been added to the bath. The values are a measure of the degree of haemolysis which had taken place. Open circles are from experiments in which the concentration of erythrocytes was equivalent to 10^{-5}M haemoglobin and the closed circles equivalent to $3x10^{-6}$M haemoglobin. The inhibition of the NANC response by the erythrocyte suspensions correlated reasonably well with the free haemoglobin they gave rise to though most values lay below the haemoglobin concentration-response curve presumably because some additional cell rupture took place during centrifugation. The NANC nerves in these experiments were stimulated at 5 Hz for 10 seconds.

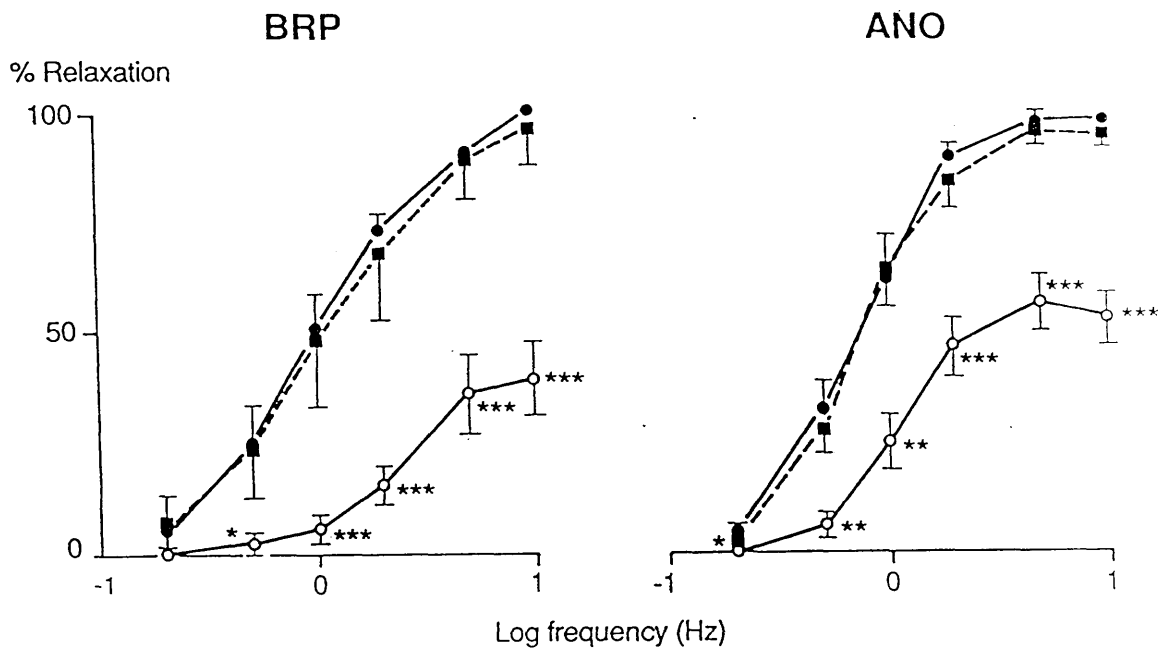

Figure 17 Log frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz for 10 seconds in the BRP (left panel) and the rat anococcygeus (right panel). Control responses (●——●) and responses in the presence of erythrocytes with a haemoglobin concentration equivalent to $3x10^{-6}M$ (■- - -■) were indistinguishable whereas haemoglobin solutions of $3x10^{-6}M$ (○——○) produced a significant reduction in response at almost all frequencies. Tone was raised by guanethidine ($3x10^{-5}M$). Bars represent ± s.e.. N=5, *P<0.05, **P<0.01, ***P<0.001.

the level of free haemoglobin was measured at the end of each experiment and as Figure 16 shows these levels correlate reasonably well with the degree of inhibition of the NANC nerve responses. Erythrocytes are made more fragile by repeated washing and centrifugation. If whole blood rather than washed cells were used, much less haemolysis occurred. Since previous experiments have shown plasma to be without effect on the response to NANC nerve stimulation (Bowman & Gillespie, 1982), whole blood was used on subsequent experiments. In these circumstances the concentration of free haemoglobin in the bath fluid at the end of the experiment was below the level of detection by spectrophotometry (10^{-7}M). Figure 17 summarizes the effects of haemoglobin and a haemoglobin-equivalent suspension of erythrocytes. While haemoglobin produced a 50% or greater inhibition of relaxation in both the rat anococcygeus and BRP muscles, equivalent suspensions of erythrocytes were completely without effect. Comparison of these results with the effect of erythrocyte suspensions on the action of NO suggests that both the IF and the NANC neurotransmitter in both tissues either cannot penetrate the erythrocyte membrane or cannot reach the erythrocytes in the bath fluid.

(4) Effects on EDRF-mediated Relaxation Because of the short half-life of EDRF, it is impossible to perform the binding experiment in the same way as with NO and the IF. The blocking actions of haemoglobin and erythrocytes were therefore tested first in the cascade

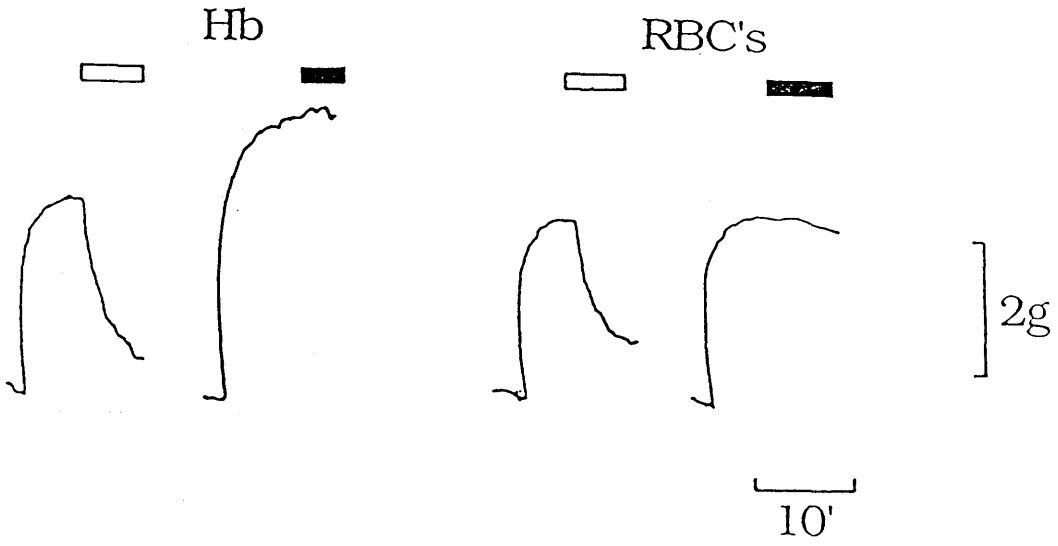

Figure 18 The effect of superfusing haemoglobin (Hb, 10^{-6}M), or an equivalent concentration of erythrocytes (RBC's) over an endothelium-free rabbit aortic strip, on the tissue's response to EDRF released by infusing acetylcholine (ACh, $5x10^{-6}$M) through the donor aorta. Tone was raised by infusion of 5-HT and ACh at a concentration of 10^{-5}M of each. The open bars show the control responses to ACh infused in the absence of Hb or RBC's; the solid bars show the responses in the presence of either Hb or RBC's; the RBC's were as effective as Hb in abolishing the relaxant effects of EDRF.

system infusing the haemoglobin or erythrocytes into the Krebs' saline between the donor aorta and the test aortic strips. Secondly, effects were examined on aortic strips retaining their endothelium in organ baths. Figure 18 shows a typical response in the cascade experiment. Both haemoglobin (10^{-6}M) or that present in an equivalent concentration of erythrocytes perfused into the upper chamber completely abolished the response of the test tissues to EDRF released by infusion of 5 x 10^{-6}M ACh through the donor aorta.

The results just described for the NANC neurotransmitter and EDRF were quite different. Erythrocytes readily blocked the response to EDRF but had no effect on the response to NANC nerve stimulation. One conclusion could be that the NANC transmitter, unlike EDRF, cannot penetrate easily into the erythrocyte membrane. The second, and more obvious, explanation lies in the experimental conditions. EDRF in these cascade experiments is liberated into the perfusing saline and comes into close contact with the erythrocytes. In the BRP and the rat anococcygeus muscles the transmitter is liberated within the tissue and must diffuse to the muscle/bath-fluid boundary before it is exposed to the erythrocytes. This argument assumes that few, if any, erythrocytes will penetrate into the muscle itself. A closer comparison with the BRP and the rat anococcygeus would be the rabbit aortic strip retaining its own endothelium. EDRF liberated in these circumstances can diffuse directly to the underlying smooth muscle and is separated from the erythrocytes by the thickness of the endothelial cell. The effects of haemoglobin and erythrocytes on such preparations was tested in 10 ml

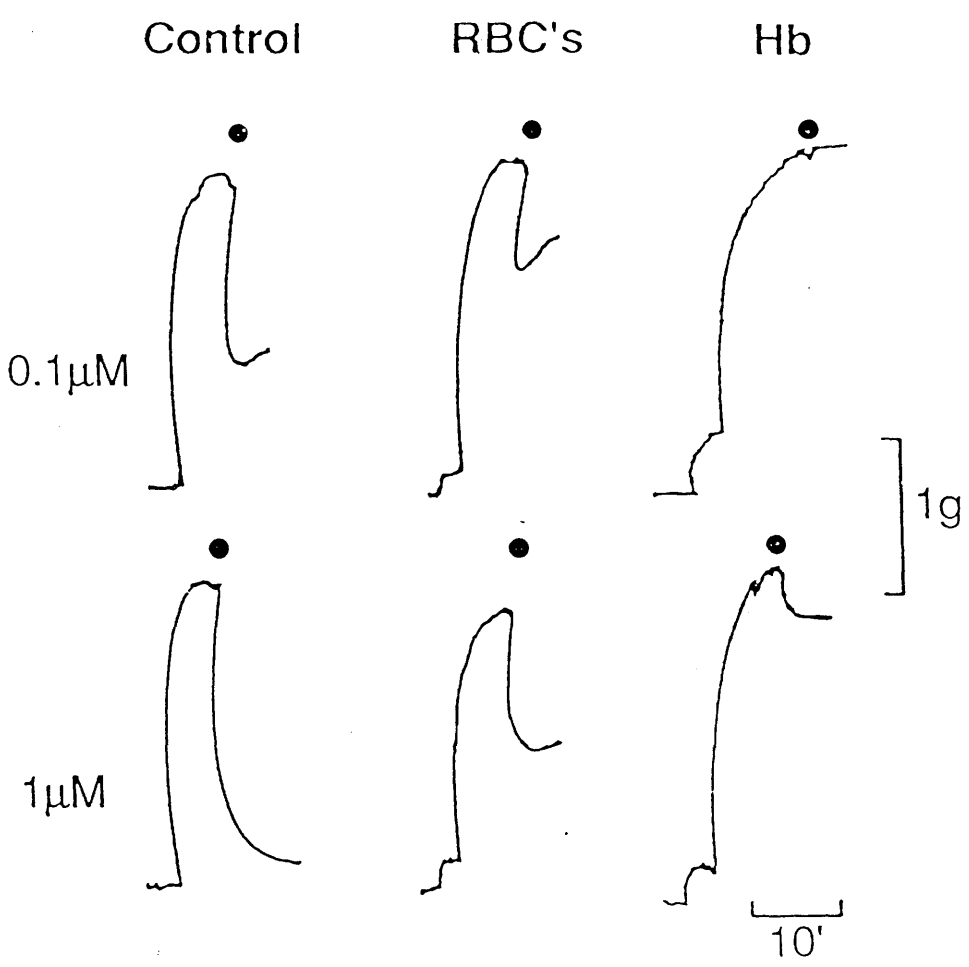

Figure 19 The response of a rabbit aortic strip with intact endothelium to EDRF liberated by two dose levels of acetylcholine (0.1 and 1 μM), and the effect on this of haemoglobin (Hb, 3 μM) or a suspension of erythrocytes (RBC's) with an equivalent haemoglobin content. Haemoglobin abolished the response to the low concentration of acetylcholine and greatly reduced that to the high concentration (end panel). Erythrocytes also reduced the responses at both concentrations (middle panel), although the reduction was less than with haemoglobin. Tone was raised with 5-HT. In the presence of erythrocytes or haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration used in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes and to 0.1 μM in the presence of haemoglobin solutions.

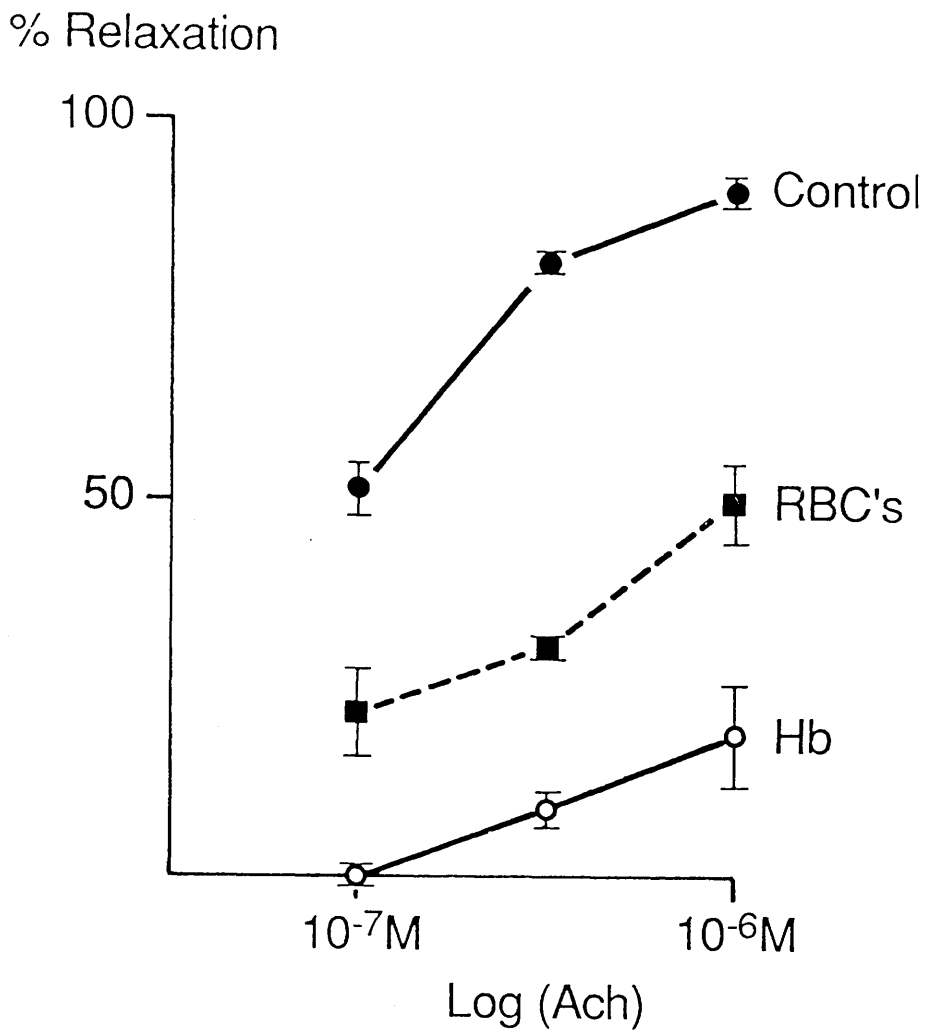

Figure 20 Log concentration-response curves to EDRF in the rabbit aortic strip with its endothelium intact and the effect on these curves of haemoglobin solutions and erythrocyte suspensions. EDRF was liberated by acetylcholine at concentrations shown. Haemoglobin (Hb, 3 μM) (o——o) and its equivalent concentration of erythrocytes (RBC's) (■----■) greatly reduced the control response to EDRF (●——●). Tone was raised by 5-HT. In the presence of erythrocytes and haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration used in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes and to 0.1 μM in the presence of haemoglobin solutions. Bars represent ± s.e.. N=9-15.

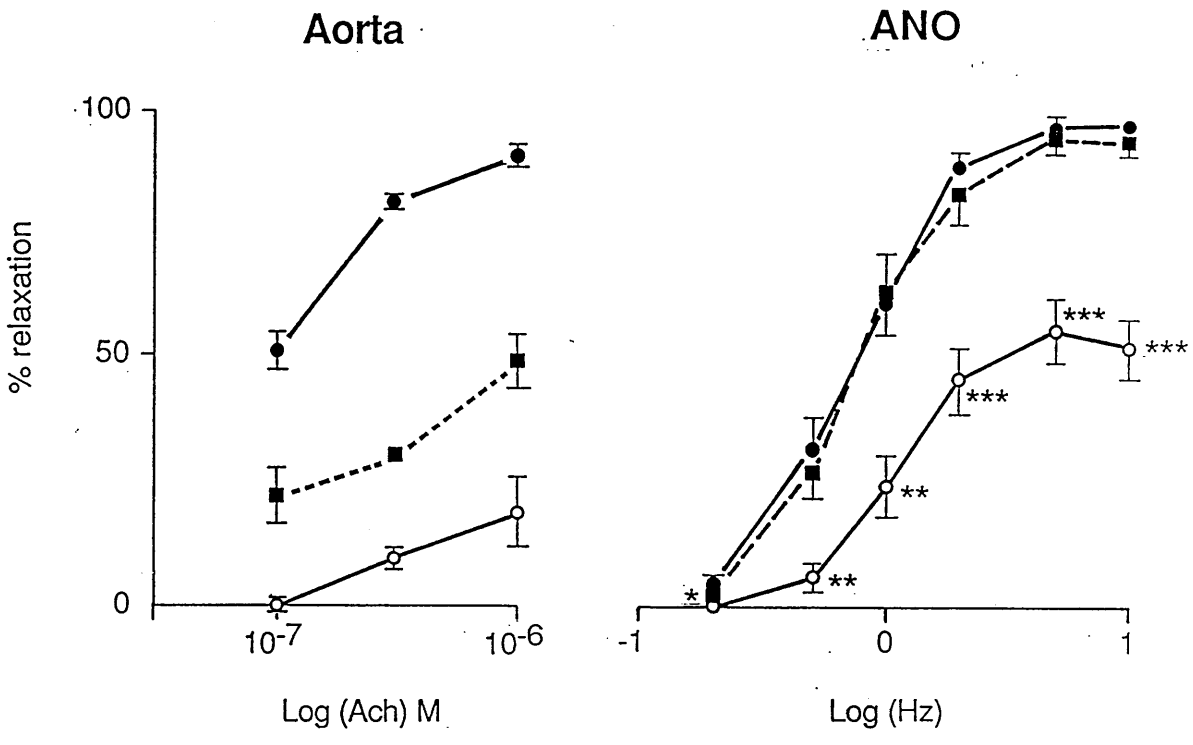
Aorta
ANO
100
50
0
% relaxation
10^{-7}
10^{-6}
Log (Ach) M
-1
0
1
Log (Hz)
*
**
**
***
***
***

Figure 21 A comparison of the effects of free haemoglobin (o——o) and a suspension of erythrocytes with an equivalent haemoglobin content (■- - - -■) on the response of the rabbit aortic strip to EDRF (●——●, left panel) and the rat anococcygeus to NANC nerve stimulation (●——●, right panel). These results have already appeared in Figures 17 and 20. EDRF was liberated by acetylcholine at the concentration shown; the NANC nerves were supramaximally stimulated between 0.2 and 10 Hz for 10 seconds. Tone was raised using 5-HT (10 μM) in the aortic strip and with guanethidine (30 μM) in the rat anococcygeus. Haemoglobin (3 μM) reduced the response to EDRF more than it reduced the response to NANC nerve stimulation. Erythrocytes reduced the response to EDRF but had no effect on the response to NANC nerve stimulation. In the presence of erythrocytes or haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration used in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes and to 0.1 μM in the presence of haemoglobin solutions. Bars represent ± s.e.. N=5 for the rat anococcygeus and 9-15 for the aorta.

organ baths with 10^{-5}M 5-HT as spasmogen. As Figure 19 shows, the relaxation to EDRF released by 10^{-7}M ACh was completely abolished by haemoglobin (3 x 10^{-6}M) but reduced, not abolished, by an equivalent suspension of erythrocytes. A higher concentration of ACh (10^{-6}M) produced a greater relaxation which, though greatly reduced, could not be completely blocked even by the solution of haemoglobin. Again relaxation was much less affected by the suspension of erythrocytes (Fig 19). The results of all experiments with EDRF in the organ bath studies are summarized in Figure 20. In all these experiments, the lysis of erythrocytes in the bath fluid was measured at the end of experiment and the concentration of haemoglobin was found to be below the level of detection (10^{-7}M). Both haemoglobin and erythrocytes significantly reduced the EDRF-mediated relaxation at all concentrations but free haemoglobin was clearly more effective than that contained within erythrocytes.

(5) Effects of Higher Concentrations of Erythrocytes Figure 21, brings together the effects of haemoglobin and erythrocytes on responses of the rabbit aortic strip to EDRF and the rat anococcygeus muscle to NANC nerve stimulation. Haemoglobin is clearly a more effective inhibitor of the response to EDRF than to NANC nerve stimulation. Since even in the aorta erythrocytes are less effective than haemoglobin, it is possible that in the rat anococcygeus muscle, the effect of erythrocytes is simply below threshold. To test this

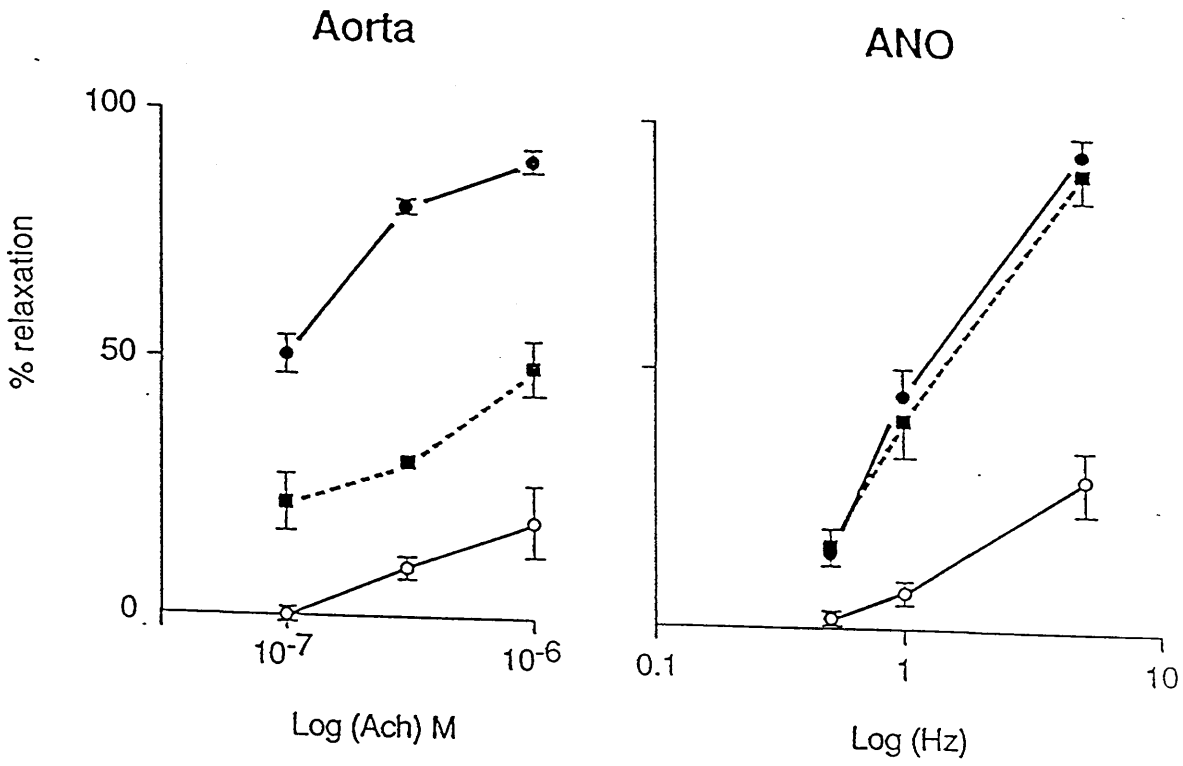
Aorta
ANO
100
50
0
% relaxation
10-7
10-6
Log (Ach) M
0.1
1
10
Log (Hz)

Figure 22 A comparison of the effects of free haemoglobin (o——o) and a suspension of erythrocytes with an equivalent haemoglobin content (■- - - -■) on the response of the rabbit aortic strip to EDRF (left panel) and the rat anococcygeus to NANC nerve stimulation (●——●, right panel). EDRF was liberated by acetylcholine at the concentrations shown; the NANC nerves were supramaximally stimulated at 0.5, 1 and 5 Hz for 10 seconds. In these experiments the concentration of haemoglobin, and the erythrocyte equivalent, remained at $3x10^{-6}M$ for the rabbit aortic strip but was raised to $10^{-5}M$ for the rat anococcygeus. As a result the inhibition of the response by haemoglobin in the two tissues was roughly equal (compared with Fig 21). In spite of the greater inhibition by haemoglobin, erythrocytes in a comparably high concentration had no effect on the response to NANC nerve stimulation. Tone was raised with 5-HT (10 μM) in the aortic strip and with guanethidine (30 μM) in the rat anococcygeus. In the presence of erythrocytes or haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration used in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes, and to 0.1 μM in the presence of haemoglobin solutions. Bars represent ± s.e.. N=6 for the rat anococcygeus and 9-15 for the aorta.

possibility it would be necessary to use haemoglobin concentrations in the rat anococcygeus muscle which produced as great an inhibition of the NANC response as 3 x 10^{-6}M haemoglobin gave of EDRF in the aortic strip. It would then be possible to ask the question whether erythrocyte suspensions, with an equivalent high concentration of haemoglobin, were still ineffective against the response to NANC nerve stimulation. There were t echnical difficulties in doing this experiment because it was found that erythrocytes in a vigorously bubbled organ bath can be partially haemolysed and the high concentrations of haemoglobin they release may interfere with the results. This problem was solved by cutting the time course of the experiment and reducing the degree of bubbling. Three frequencies between 0.5 and 5 Hz, instead of six (from 0.2 to 10 Hz) were tested by adding 10^{-5}M haemoglobin or its equivalent in erythrocytes to the bath solution. Figure 22 shows the results. Haemoglobin then produced a degree of inhibition of the NANC nerve response in the rat anococcygeus muscle equal to its effect on EDRF in the aortic strip but still erythrocytes, containing the same concentration of haemoglobin, had no effect on the rat anococcygeus muscle response to NANC nerve stimulation.

The ineffectiveness of erythrocytes on the response to NANC nerve stimulation in both the BRP and the rat anococcygeus muscles suggests that the NANC neurotransmitter, despite its action being blocked by haemoglobin, is not as diffusible a substance as NO or EDRF.

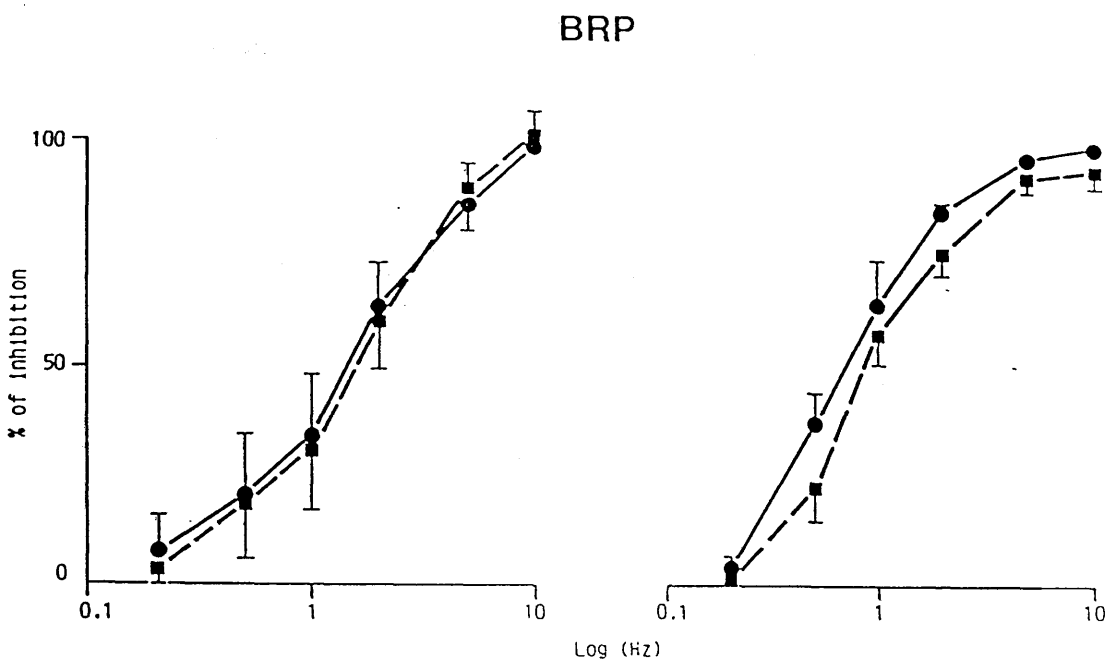

Figure 23 Log frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz for 10 seconds in the BRP. Tone was induced by guanethidine (30 μM). Control responses (●——●) and responses in the presence of pyrogallol (left panel) or hydroquinone (right panel) both at 0.1 mM (■– – –■) are indistinguishable. Bars represent ± s.e.. N=4 for with pyrogallol, N=5 with hydroquinone.

THE EFFECT OF SUPEROXIDE ANION ON NANC RELAXATION

Moncada et al. (1986) reported that a number of substances which were believed to block the effect of EDRF were generators of superoxide anion and their effects could be reversed by superoxide dismutase. Two of these substances, pyrogallol and hydroquinone, have been tested on the response to NANC nerve stimulation in both the BRP and the rat anococcygeus muscles to see whether they inhibited responses to the NANC nerve stimulation and if so whether, as with EDRF, this effect could be reversed by superoxide dismutase.

(1) Effects of Superoxide Anions on the Response of the BRP muscle to NANC Nerve Stimulation The effects of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the BRP muscle are illustrated in Figure 23. The NANC nerves were field stimulated for 10 seconds with supramaximal voltages at a range of frequencies between 0.2 and 10 Hz. The same field stimulation repeated in the presence of pyrogallol or hydroquinone, produced results similar to the controls: both drugs (10^{-4}M) had no effect on the frequency-response curves to NANC nerve stimulation.

(2) Effects of Superoxide Anions on the Response of the Rat Anococcygeus muscle to NANC Nerve Stimulation The effects of

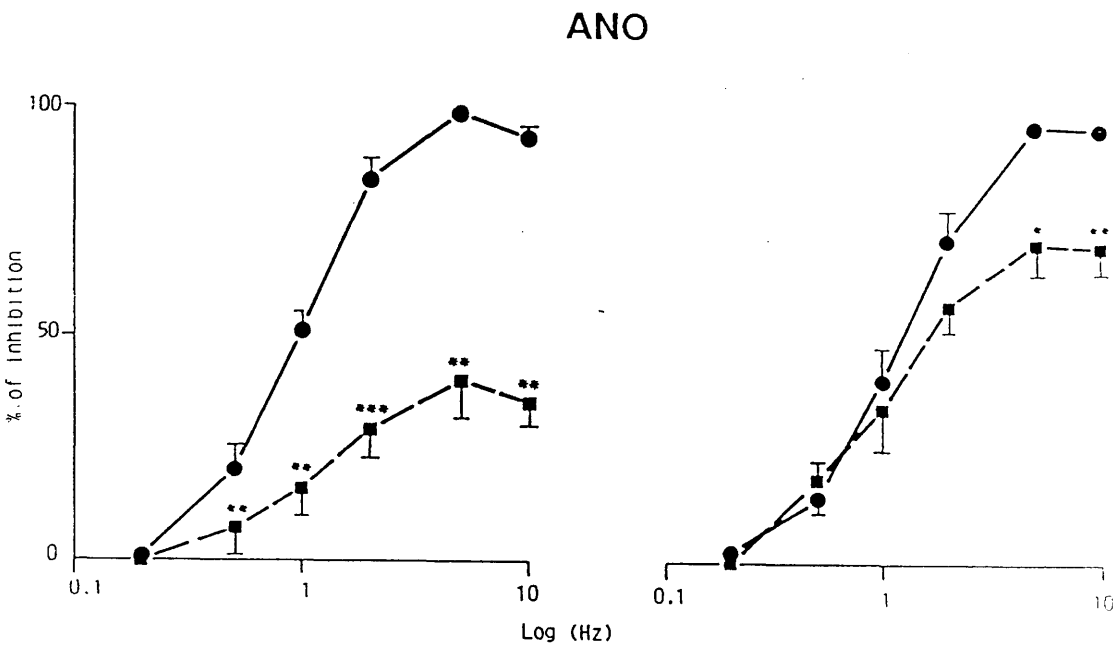

Figure 24 Log frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz for 10 seconds in the rat anococcygeus. Tone was induced by guanethidine (30 μM). Comparison of control responses (●——●) and responses in the presence of pyrogallol (left panel) or hydroquinone (right panel), both at 0.1 mM (■- - -■), show a significant reduction particularly with pyrogallol. Bars represent ± s.e. N=5. *P<0.05, **P<0.01, ***P<0.001.

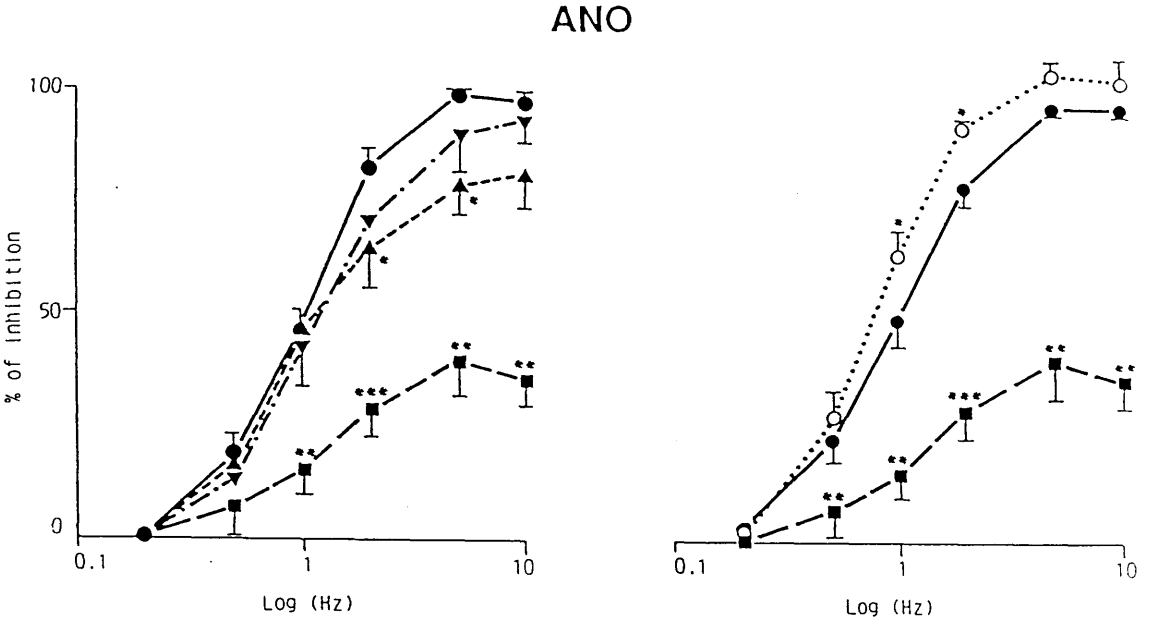

Figure 25 Log frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz for 10 seconds in the rat anococcygeus. The left panel shows the graded inhibitory effect of pyrogallol at 10 μM (▼-·-·-▼), 30 μM (▲-----▲) and 100 μM (■- - -■) in comparison with the control (●————●). The right panel shows the control responses (●————●), their inhibition by pyrogallol (100 μM, ■- - -■) and the complete reversal of that inhibition by superoxide dismutase (100 U ml^{-1}, o·····o). Tone was induced by guanethidine (30 μM). Bars represent ± s.e.. N=4-6. *P<0.05, **P<0.01, ***P<0.001.

pyrogallol and hydroquinone were also tested on the response of the rat anococcygeus muscle to NANC nerve stimulation. The experimental procedures were the same as for the BRP muscle. NANC nerves were stimulated at frequencies between 0.2 and 10 Hz with supramaximal voltages for 10 seconds. The same range of stimulation was repeated in the presence of pyrogallol or hydroquinone. The results however were quite different. At a high concentration pyrogallol (10^{-4}M) reduced the tone in the rat anococcygeus muscle though in most cases the tone returned to the original level after a few minutes. More importantly, both drugs (10^{-4}M) significantly inhibited the response to NANC nerve stimulation (Fig 24). Pyrogallol was more effective than hydroquinone. If the inhibitory effect of both drugs is due to the generation of superoxide anion, it should be reversed by superoxide dismutase, a superoxide anion scavenger. This was tested by adding superoxide dismutase (100 U ml^{-1}) to the bath solution together with pyrogallol (10^{-4}M). The results are illustrated in Figure 25. Pyrogallol inhibited the response to NANC nerve stimulation and this inhibition was completely reversed by superoxide dismutase. Indeed the responses in the presence of both pyrogallol and superoxide dismutase exceeded the control response suggesting that, even in the absence of pyrogallol, the generation of superoxide radicals in a vigorously oxygenated solution is sufficient to produce some reduction of the response. These results confirm the suggestion that the effect of pyrogallol on the response to NANC nerve stimulation in this tissue is due to the generation of superoxide anions.

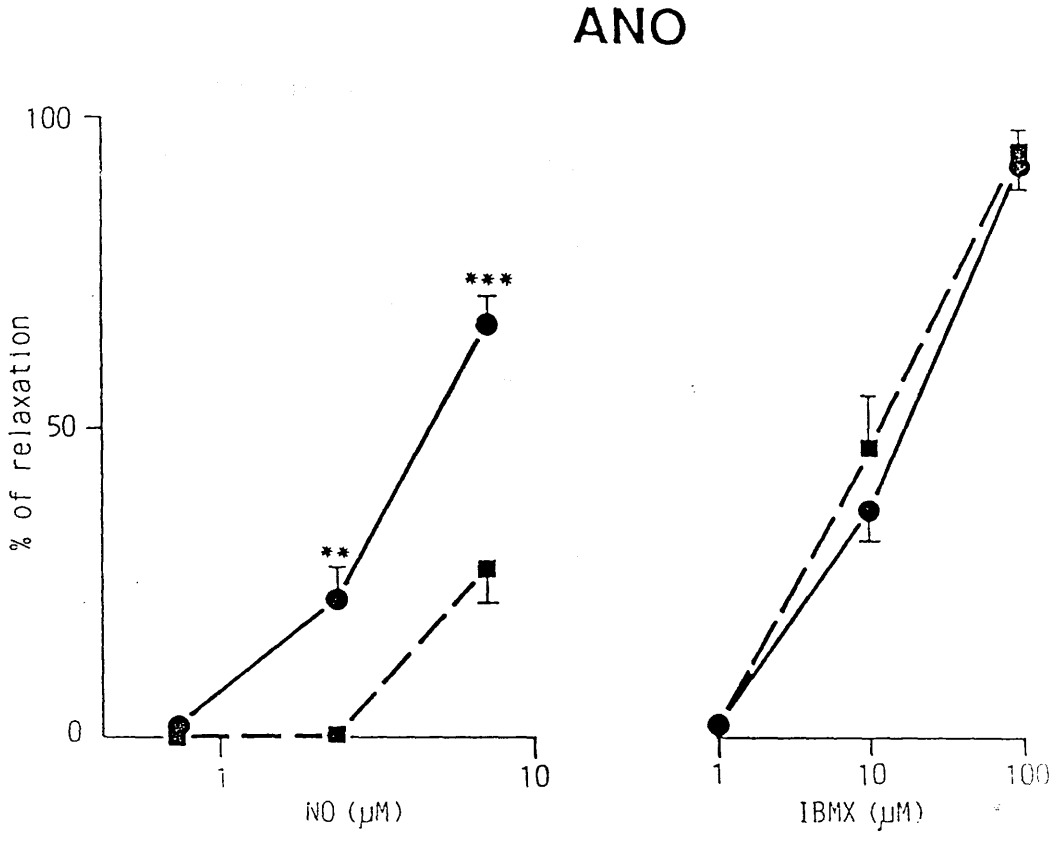

Figure 26 Log concentration-response curves of the rat anococcygeus to nitric oxide (NO) and 3-isobutyl-1-methyl-xanthine (IBMX) and the effect on these of pyrogallol (0.1 mM). The control curve (●——●) for nitric oxide is shifted to the right by pyrogallol (■- - - -■) consistent with its accelerated destruction by superoxide anions whereas the curve for IBMX is unaffected by pyrogallol. Tone was induced by guanethidine (30 μM). Bars represent ± s.e. N=6.

(3) Responses to Other Relaxants The effect of pyrogallol on the response to some relaxants other than NANC stimulation was also tested in the rat anococcygeus muscle. NO, a direct stimulant of soluble guanylate cyclase, and IBMX, an inhibitor of phosphodiesterase, both produced concentration-related relaxation in the rat anococcygeus muscle. As shown in Figure 26, pyrogallol (10^{-4}M) shifted the concentration-response curve of NO to the right but had no effect on that of IBMX. These results are further confirmation that pyrogallol acts by superoxide anion generation and has no effect on cyclic nucleotides formation.

DISCUSSION

At the time when this project was started the chemical nature of EDRF was unknown and there was no information on its effects on any smooth muscle other than blood vessels. Recently, Shikano et al. (1987, 1988) tested the sensitivity of the rabbit and guinea-pig taenia coli to EDRF released by ACh or A23187. EDRF was ineffective on this non-vascular smooth muscle and they concluded that EDRF may selectively relax vascular smooth muscle. Since authentic NO could relax the taenia coli they further concluded that EDRF is not NO. In their experiment, NO was tested in the preparations in the absence of EDRF stimulator ACh or A23187. Similar results were reported by another group (Dusting et al., 1988), who found that guinea-pig trachea relaxed to NO but not to EDRF released by infusing bradykinin through columes of cultured bovine aortic endothelial cells on microcarrier beads. The experiments in testing the effects of EDRF, NO, the IF and sodium nitroprusside in this thesis showed that sensitivity to EDRF is not confined to vascular smooth muscle; the BRP muscle is only slightly less sensitive than the rabbit aortic strip. Furthermore, by looking at concentration-response curves rather than a single concentration of each relaxant the origin of the misinterpretation in the paper of Shikano et al. (1987) is clear. The maximal concentration of EDRF which can be achieved by increasing concentrations of ACh is limited. In the present experiments the maximal relaxation in the aortic strip was about 45%. With this restriction only those tissues with the highest sensitivity to EDRF will be relaxed, e.g. the aortic strip and the BRP muscle. A similar restriction in the sensitivity of all these

tissues to NO was also found. Even when a greater degree of relaxation of the most sensitive tissues of between 70% and 100% is induced, the rat anococcygeus muscle and guinea-pig trachea were apparently insensitive to NO. Yet if the concentration of NO was increased tenfold, relaxations of rat anococcygeus muscle were produced. When these effects of differential sensitivity are taken into account, it is clear that the rank order of sensitivity of the four different smooth muscles was similar for EDRF, the IF and NO, consistent with the first two stimuli acting through NO. Sodium nitroprusside was also tested on these four muscles in the early experiment as an example of a nitrovasodilator, which is believed to owe its relaxant properties to the liberation of NO within the smooth muscle cells (Waldman & Murad, 1987). The sensitivity of different muscles to this agent was expected to parallel that to NO. This is not so, however, the rat anococcygeus muscle relaxes only slightly to concentrations of NO which almost completely relax tone in the rabbit aorta and the BRP muscle, but is highly sensitive to sodium nitroprusside. The reasons for this are not clear. One possibility is that extracellular NO could be rapidly destroyed in the oxygenated bath solution. NO released intracellularly might be protected by superoxide dismutase within the cell. The high sensitivity to sodium nitroprusside would therefore represent the true sensitivity of the tissue to NO. If this was true then all nitrovasodilators would show a similar high potency in the rat anococcygeus muscle. This is not so. In experiments not reported in this thesis sodium nitrite was found to be a poor relaxant of rat

anococcygeus muscle but a powerful relaxant of the rabbit aortic strip. Other explanations such as easier access of sodium nitroprusside to the guanylate cyclase or preferential accumulation of sodium nitroprusside are unlikely given the high membrane permeability of NO. At present the most likely explanation is that in addition to releasing NO, sodium nitroprusside can directly relax smooth muscle by an alternative unknown mechanism.

Furchgott (1988) and Martin (1988) and their colleagues recently suggested that the IF extracted from the BRP muscle is nitrite, which yields NO when the extract was acid activated. The similar sensitivity of the IF compared with NO and EDRF of four smooth muscle preparations seems consistent with this view. Furchgott's evidence was essentially that the muscle-relaxing potency of solutions of nitrite was greatly enhanced by acidification, which would be expected to release NO, and the additional activity is, like the IF, evanescent. A criticism of this work was that while acidification undoubtedly increased the relaxant activity of solution of nitrite, the actual quantities of nitrite (10 μM) necessary even after acidification to produce a response were unreasonably high. Martin et al. (1988) have measured the levels of nitrite in extracts of the BRP muscle and showed that they fall upon acidification. They attributed this to the presence of a stabilizing factor in the extracts.

The present experiments comparing haemoglobin solutions with an equivalent concentration of haemoglobin in erythrocyte suspensions on the activity of EDRF, the IF and NO were not entirely consistent with

these explanation. If the activated IF owed its relaxant properties to NO released into solution by the acid then this NO should diffuse as easily as EDRF or a solution of pure NO into the erythrocytes and be bound to haemoglobin within the cells. This did not happen. Erythrocytes were quite ineffective in abolishing the relaxant properties of activated IF even though they could abolish the effects of EDRF and NO. If the explanation of this difference lies in NO in the solutions of activated IF remaining bound to the stabilizing factor postulated by Martin et al. (1988), then the molecular weight of the combination might be too large to allow easy passage into the erythrocytes. Such a theory postulates that subsequent to release of NO from nitrite, the released NO combines with a stabilizing factor in the solution to produce a complex analogous to a nitrovasodilator.

An alternative suggestion would be that the IF is some other compound which as a precursor, can liberate NO or have effects similar to NO. Higher concentration of the IF in the high-speed sediment from homogenized BRP muscle (Gillespie & Hunter, 1982), a molecular weight about 500, irreversible destruction by boiling (Gillespie et al., 1981) and the ability to isolate a material with similar properties from connective tissue and the vitreous humour of the bovine eye (Gillespie, 1987) are properties difficult to reconcile with nitrite as precursor.

Unlike the IF, responses to EDRF were abolished equally well by haemoglobin or erythrocytes in cascade-type experiments. This is consistent with the concept of Palmer et al. (1987) that EDRF is NO. However, the organ bath experiments showed that erythrocytes were

significantly less effective than haemoglobin in abolishing the response of an aortic strip to the release of EDRF from its own intact endothelium. The most obvious explanation is the inability of haemoglobin in erythrocytes to gain access to the interstitial space in the artery. Nevertheless, erythrocytes (containing 3 x 10^{-6}M haemoglobin) did reduce by more than 50% the response to even the highest concentration of ACh (10^{-6}M). In contrast, the same concentration of erythrocytes was completely ineffective in reducing the response to NANC nerve stimulation in either BRP or rat anococcygeus muscle. In the rat anococcygeus muscle even higher concentration of erythrocytes (containing 10^{-5}M haemoglobin) still had no effect on the NANC-induced relaxation. Does this mean the transmitter, while it is able to react with haemoglobin, is unable to penetrate the erythrocyte membrane, or is it simply that the sites of release are too deeply buried within the muscle for erythrocytes to be effective? The effectiveness of erythrocytes will depend on two factors: first, the diffusional distance between the endothelium or nerve varicosity and the muscle layer and, secondly, the distance between the endothelial cells or varicosities and the bath fluid. The mean gap between nerve varicosities and muscle cells in the rat anococcygeus muscle is about 260 nm (Gillespie & Lüllmann-Rauch, 1974) and between the deep surface of the endothelial cells and the underlying muscle in aorta 50-100 nm (Simionescu & Simionescu, 1977). In terms of the immediate diffusional distance, therefore, the endothelium would be the more difficult site to block. On the other hand, the total distance between erythrocytes and

luminal surface of the endothelium is negligible, whereas there is a substantial distance between the muscle surface and the nerve varicosities in the BRP and anococcygeus muscles. Nevertheless, the rat anococcygeus is a flat and relatively thin tissue averaging 250-300 µM in thickness and the nerves are uniformly distributed through the tissue (Gillespie & Lüllmann-Rauch, 1974). Some reduction in response as a result of diverting transmitter from superficial muscle bundles, might have been expected. The results may indicate that either the NANC transmitter is not NO or the NO is bound in a form not easily diffusible through the erythrocyte membrane.

The concentration of haemoglobin or erythrocytes will also affect the rate of loss by binding. In the cascade experiments this was usually at 10^{-6}M, in the organ bath experiments with EDRF it was 3 x 10^{-6}M and in experiments with NANC stimulation it was 3 x 10^{-6}M and 10^{-5}M. In many ways EDRF released in the cascade experiments had the shortest time for binding before reaching the test tissues yet this response was greatly reduced by erythrocytes in lower concentration. This suggests, especially for the endothelial cell in situ, that it is the relative closeness of the endothelial cell to the underlying smooth muscle which prevents the erythrocytes from inactivating all of the released NO, since haemoglobin was more effective and capable of completely blocking the response to EDRF in situ. This may indicate that haemoglobin can permeate into the interstitial space behind the endothelium. The highest concentration of erythrocytes (10^{-5}M) with a relatively long time allowed for binding still showed no effect on the

response to NANC nerve stimulation in the rat anococcygeus muscle. This may suggest the transmitter in this tissue is not free NO.

The ability of erythrocytes with a haemoglobin equivalent of 3 μM, i.e. about 600 times less than whole blood, to reduce the response to EDRF raises a question mark against the physiological role of EDRF. To determine the extent to which concentrations of erythrocytes closer to physiological levels might reduce the response, much higher concentrations of haemoglobin equivalent 500 μM were used. This concentration abolished EDRF-induced responses stimulated by low concentrations of ACh (10^{-8}M-10^{-6}M), though occasionally a transient small relaxation could be seen if concentrations of ACh higher than 1 μM were used. Unfortunately, interpretation of these results is difficult since some haemolysis always occurred as measured by the haemoglobin content of the solution at the end of each experiment. Nevertheless, in some preparations the free concentration of haemoglobin was less than 3 μM, this is the level used in previous experiments yet the inhibition of the EDRF-induced response was much greater. It is almost certain that ACh reduces blood pressure in vivo by stimulating EDRF released from the endothelium. However, the results from this project seems against this view since the response to EDRF, as well as to NO, could be greatly reduced by erythrocytes in physiological circumstances. There are several possible explanations for this paradox. For example, there may be more than one substance released from endothelium stimulated by ACh. Prostacyclin could be one of the substances and responses to it are resistant to haemoglobin, on the other hand, it does not relax the

aorta. Recently, several groups have reported that in various arteries ACh may release another substance (Bolton & Clapp, 1986; Komori & Suzuki, 1987; Taylor et al., 1987), endothelium-derived hyperpolarizing factor (EDHF) which is resistant to haemoglobin and methylene blue (Chen et al., 1988). The response to EDHF can only be seen by using a relatively high concentration of ACh (>1 μM, occasionally, >10 μM) (Chen et al., 1988). This hyperpolarization is transient which may explain the small transient relaxation which can be detected in high concentration of erythrocytes in present study. This explanation is unlikely since treatment with gossypol, a nonspecific inhibitor of endothelium-dependent relaxant abolish the response to endothelium-dependent vasodilators in vivo (Dudel & Förstermann, 1988). More recently, Rees et al. (1989) reported that N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of its formation from L-arginine, but not its D-enantiomer, increased blood pressure in the aneasthetized rabbit. This hypertensive action of L-NMMA is reversed by L-arginine which is believed to be a precursor of EDRF (Palmer et al., 1988b). Other possibilities could be that for anatomical reasons, EDRF itself is most easily blocked in large arteries and the blocking action of erythrocytes demonstrated in this thesis could not be demonstrated if the experiments were performed on the small vessels which constitute the peripheral resistance vessels. It is also possible that prolonged exposure to Krebs' saline rather than the normal blood results in loss of endothelial cells. For example, at the end of some experiments, the artery had only retained 60-75% of its endothelial coverage although the

percentage of relaxation did not change (Furchgott & Zawadzki, 1980). In this case either some erythrocytes can get behind the endothelium or more likely the diffusional path from the endothelium to the smooth muscle is increased, and the balance between diffusion to the bath fluid and to the muscle is tilted in favour of the former.

A similar problem does not arise with the bovine retractor penis muscle since erythrocytes had no ability to reduce the NANC response in this tissue. This has been interpreted in this thesis as suggesting a transmitter different from EDRF. It would of course be possible that if the erythrocytes were brought into closer proximity with the nerve/muscle junction, for example, by perfusing the capillary bed with Krebs' saline containing erythrocytes, some reduction would be seen. Such experiments are technically possible and the results would be very interesting.

Although there is no direct evidence that the NANC inhibitory nerves present in the BRP and rat anococcygeus muscles are identical, some experiments showed that they have very similar properties. First, the anococcygeus and the retractor penis muscles in dog, pig and sheep are innervated by sacral parasympathetic nerves (Gillespie & McGrath, 1973; Langley & Anderson, 1895) produce inhibitory response to field stimulation (Gillespie, 1972; Klinge & Sjöstrand, 1974). Secondly, the inhibitory response to NANC nerve stimulation in both tissues can be blocked by haemoglobin (Bowman et al., 1982) or hypoxia (Bowman & McGrath, 1985), but not by erythrocytes. Finally, the anatomical continuity of the anococcygeus and retractor penis muscles also

suggested that they share a common inhibitory nerve releasing the same transmitter (Gillespie, 1982). Certain results are not consistent with this hypothesis. Both pyrogallol and hydroquinone significantly reduced the response to NANC nerve stimulation in the rat anococcygeus muscle but had no effect in the BRP muscle. Pyrogallol and hydroquinone both inactivate EDRF through the formation of superoxide anion (Moncada et al., 1986; Ignarro et al., 1988). The inhibition of NANC nerve stimulation in the rat anococcygeus muscle is not a non-specific effect since pyrogallol at the same concentration did not affect the inhibitory action of IBMX. Furthermore, this inhibitory action on NANC nerve stimulation was completely reversed by superoxide dismutase. The lack of effect on IBMX also suggests pyrogallol did not interfere with the spontaneous synthesis of cyclic nucleotides. Two possible explanations could be offered. First, the neurotransmitter in these two test preparations is not the same. In the rat anococcygeus muscle the transmitter is susceptible to destruction by superoxide anion and destroying these with superoxide dismutase restores the response. NO would readily fit the bill as such a transmitter. Other evidence supporting this view has been obtained in the laboratory. The response to NANC nerve stimulation in the rat anococcygeus but not the BRP muscle is blocked by L-NMMA (Gillespie & Xiaorong, 1989; Gillespie et al., 1989). The inhibition was stereospecific; D-LNMMA was ineffective. The inhibition was reversed by L-arginine. The simplest explanation would be to assume that the neurotransmitters in two tissues are different and only in the rat anococcygeus muscle is free NO

involved. A major difficulty with this explanation is that the BRP muscle is much more sensitive to NO as well as to EDRF and the IF than the rat anococcygeus muscle yet the experimental evidence for NO as transmitter lies entirely with the rat anococcygeus muscle. An alternative proposition would be to assume that the neurotransmitter is the same but the interaction with superoxide dismutase differs in some way in the two muscles. One possibility is to assume a neurotransmitter which readily liberates NO within the tissue, i.e. a substance functionally related to the nitrovasodilator. If this neurotransmitter liberates NO intracellularly, then the ability of superoxide anion to inhibit its effect will depend on the ease of penetration of the superoxide anion into the smooth muscle cells and the concentration of endogenous superoxide dismutase within the cell. The failure of superoxide anion generators to block the BRP muscle response could be attributed to high concentration of endogenous superoxide dismutase. This might also explain the high sensitivity of this tissue to NO, EDRF and the IF compared with the rat anococcygeus muscle. In these experiments, the rat anococcygeus muscle was set up in the organ bath as an intact muscle but the BRP muscle was cut as a strip from the retractor penis. This cutting could damage a large number of muscle cells surrounding the surface of the BRP muscle strip and endogenous superoxide dismutase from these cells might be released into the bath solution. In the rat anococcygeus muscle a careful dissection may not damage the cells so that probably only small amounts of superoxide dismutase are released. If this is the case, the concentration of

endogenous superoxide dismutase in both tissues might be not very different but the higher concentration of this enzyme from damaged cells on the surface of the BRP muscle may be the reason for this tissue having a sensitivity to NO and low sensitivity to blockade by the superoxide anion. One problem with this explanation is that it is inconsistent with the results of work with L-NMMA which on this scheme should be as effective in the BRP muscle as in the rat anococcygeus muscle. Clearly more experimental work is required to explain the difference between the BRP and the rat anococcygeus muscles.

It is obvious that the NANC transmitter in the rat anococcygeus muscle, is not as diffusible a substance as NO since erythrocytes even at high concentration had no effect on its effect. An explanation, though perhaps an improbable one, which might reconcile some of these conflicting observations is to assume the NANC transmitter is some unstable compound capable of liberating NO. If this decayed to NO in the bath fluid in certain circumstances and was also taken up into muscle cells to liberate even more NO intracellularly, then the effect of pyrogallol or hydroquinone would depend on the relative importance of these two sources of NO. It has been reported that effects of some nitrovasodilators in stimulating guanylate cyclase is increased in the presence of reducing agents (Murad et al., 1979). This could be due to more NO being released in such circumstances. If the NANC neurotransmitter is a compound like nitrovasodilator, then more NO could be released extracellularly in the presence of reducing agents such as pyrogallol or hydroquinone in the bath solution. NO in the

extracellular fluid would be highly susceptible to superoxide anion, this would be a major source in the rat anococcygeus and would explain that tissues susceptibility to pyrogallol and hydroquinone. The BRP muscle would be more dependent on NO liberated intracellularly and less affected by pyrogallol and hydroquinone. Such an explanation suggests that any drugs whose relaxant action was solely dependent on the release of NO intracellularly would be as effective in the two tissues. This immediately explains the sodium nitroprusside results where both tissues were equally sensitive. Unfortunately sodium nitrite which might have been expected to be similar was not particularly effective on the rat anococcygeus muscle.

In conclusion, the response to NANC stimulation in both BRP and rat anococcygeus muscles can be blocked by haemoglobin but not erythrocytes suggesting that if the transmitter in both tissues acts through NO it is present as a less diffusible substance. The similar rank order of potency of EDRF, NO, and the IF on four different smooth muscles suggest, however, that NO may be the common mediator. The effects of all three can be blocked by haemoglobin but those of the IF, unlike the other two, is not blocked by erythrocytes suggesting it may be related to the NANC neurotransmitter. There is a difference between the BRP and rat anococcygeus muscles since pyrogallol and hydroquinone can only block the response to NANC nerve stimulation to the latter. The reason for this is not clear but it may indicate that in the rat anococcygeus muscle NO can be released in the extracellular environment.

THE EFFECT OF CROMAKALIM ON THE RESPONSE TO SPAMOGEN- & STRETCH-INDUCED CONTRACTION

INTRODUCTION

Smooth muscle cells, unlike skeletal muscle cells, possess a wealth of membrane receptors which allow them to respond to a large number of external stimuli. The coupling of these receptor-mediated events to changes in cell activity can be brought about either electrically through changes in membrane potential or biochemically by the production of a second messenger. As well as responding to chemical stimuli, smooth muscle cells respond to stretch, acting in effect as mechano-electrical transducers. Smooth muscles may also be spontaneously active in the absence of any external stimulus. In many tissues this spontaneous mechanical activity is dependent upon changes in electrical activity, usually slow waves of depolarization with action potentials superimposed on the crest of the wave (Bülbring, 1954, 1955; Marshall, 1962; Gillespie, 1962; Small, 1982). In other smooth muscles, for example, some blood vessels, tone may be produced with very little evidence of electrical activity (Droomans et al., 1977; Casteels et al., 1977a,b).

Whatever the coupling mechanism, electrical or biochemical, there is a general belief that contraction is usually accompanied and indeed caused by a rise in the free, ionized calcium levels in the cell cytoplasm (Bolton, 1979). Intracellular calcium concentrations can be increased from two major sources: an influx of extracellular calcium as a result of opening membrane calcium channels, and a release of calcium from intracellular stores within which it is bound and therefore not available for contraction.

Intracellular calcium release from the stores is associated mainly

with the production of the second messenger inositol triphosphate (IP_3) (Berridge & Irvine, 1984). A variety of substances such as NA or ACh, in addition to stimulating receptor-operated calcium channels, also stimulate calcium release from these calcium stores. For example, in vascular smooth muscles, NA-induced contraction has two phases: an initial phasic component followed by a tonic contraction. The former is believed mainly due to the stimulation of calcium release intracellularly as a result of increased phospho-inositol metabolism and the latter the entry of extracellular calcium. Removal of external calcium can reduce the tonic contraction but does not interfere with the phasic one (Golenhofen, 1981).

Calcium crossing the cell membrane is believed to pass through calcium channels. Two channels have been widely studied and accepted: the receptor-operated calcium channels (ROC) and the voltage-operated (VOC) calcium channels (Bolton, 1979). The former is opened by drugs or neurotransmitters acting on appropriate receptors, for example, NA and ACh, acting on α-adrenoceptors and muscarinic receptors respectively. Activation of these channels is blocked by receptor antagonists but is relatively insensitive to inhibition by calcium channel antagonists such as verapamil (Bolton, 1979). The voltage-operated calcium channel can be activated by a variety of substances which change the membrane potential. Potassium-induced contractions in various smooth muscle preparations are due solely to stimulation of this channel and completely blocked by calcium channel antagonists (Bolton, 1979). Recent electrophysiological studies in the

neurone of chick dorsal root ganglion has suggested the existence of three types of voltage-operated calcium channels, L-, T-, and N-type which differ in their conductance and rate of activation and inactivation (Nowycky et al., 1985). Moreover, L- but not T- and N-type calcium channels are sensitive to dihydropyridines, and L- and N- but not T-type calcium channels are blocked by ω-conotoxin, a 27-amino acid peptide neurotoxin fraction isolated from Conus geographus venom (Nowycky et al., 1985; McCleskey et al., 1986). In the rabbit ear artery, two types of voltage-operated channels have been found, a T-type calcium channel which is resistant to dihydropyridines and the L-type calcium channel which is completely blocked by PN 202-791 and potentiated by the calcium channel agonist BAY K 8644 (Benham & Tsien, 1987). In fact, calcium entry through VOC and ROC can be influenced in a variety ways. VOC are primarily altered by changes in membrane potential which in turn may be due to alteration in membrane permeability to other ions such as potassium, sodium or chloride. In addition, the Nernst potential for any one of these ions may be altered by altering its external concentration; the most frequent example of this is the depolarizing effect of raised external potassium. The probability of the VOC being in the open configuration may be altered by phosphorylation as for example in the heart as a result of raised cyclic AMP dependent kinase (Osterrieder et al., 1982) but in vascular smooth muscle cyclic AMP increased calcium binding in stores (Baudoum-Legros & Meyer, 1973). ROC are primarily affected by the particular receptor coupled to the calcium channel so that receptor agonists and antagonists

are the obvious point of attack. At present no drug exists that blocks ROC in a manner analogous to the effects of calcium channel blocking agents at VOC, but the diffusion gradient for calcium will influence the membrane potential and increase the rate of entry by stimulating of VOC. However, calcium channel antagonists would be expected to be most effective in blocking VOC but in some preparations, the opposite results can be obtained although the mechanism is not clear. For example, Church & Zsotér (1980) reported that diltiazem and nifedipine caused significantly greater reduction of NA- than of potassium-induced contraction in the rat portal vein and rabbit mesentery artery. Walus et al. (1981) reported that nifedipine is very effective in tissues contracted with either NA or KCl and produced almost complete relaxation of NA-induced contraction even when no extracellular calcium was present in the medium presumably by antagonizing the release of intracellular calcium. In addition to these, van Breeman et al. (1979) reported that there is a significant influx of calcium in quiescent vascular smooth muscle in the absence of depolarization or an appropriate receptor ligand, a report which has received little attention. Supporting this view Kuno et al. (1986) and Rosenberg & Tsien (1987) found that in helper T lymphoctes and cardiac sarcolemma there is a similar calcium leak channel open at resting membrane potentials.

Another stimulus that induces contraction in smooth muscle independent of external chemical stimulation is stretch. One of the first to report a response of smooth muscle to stretch was Bozler

(1947), who found that stretch-induced contraction in the dog ureter was associated with a depolarization of the membrane. Bülbring (1954, 1955) also reported that in the guinea-pig taenia coli a similar response was observed. This was further confirmed by Burnstock & Prosser (1960) who examined the response of a variety of smooth muscle preparations to stretch. Most of these, including the pig oesophagus muscularis mucosae, guinea-pig and rabbit taenia coli, cat circular and longitudinal intestinal muscle, rat and guinea-pig ureter, guinea-pig vas deferens, rabbit bladder and dog retractor penis, gave contractions with an fall in membrane potential in response to quick stretch. However, in the cat nictitating membrane, pig renal vein and carotid artery, no response was elicited to stretch. Evidence of a link between contraction and depolarization in response to stretch was also reported by Gillespie (1962) who measured both the electrical and mechanical activity of the smooth muscle cells of the longitudinal muscle coat of the rabbit colon. The stretched cells showed a membrane potential lower than average and with strong stretch the depolarization produced was itself sufficient to keep the membrane potential permanently in the firing zone and as a consequence the discharge of spike potentials was continuous.

There is good evidence that the active response to stretch in smooth muscle preparations is a myogenic response. For example, the chick amnion (Evans & Schild, 1956) and the human umbilical artery strip (Sparks Jr, 1964), are both nerve-free preparations, and both contract in response to stretch. Further support is provided by the inability

of tetrodotoxin to block pressure-induced depolarization in rabbit and cat cerebral arteries (Nakayama, 1982; Harder, 1984).

Bayliss in 1902 first suggested that the arterial vessel wall contracts in response to the stretching force of the blood pressnre. This may be the mechanism involved in autoregulation to maintain blood flow constant in the face of changes in the level of the blood pressure (Bayliss, 1902). Since the tone induced by stretch may be the basis of the basal vascular tone that develops in response to increase in transmural pressure in vivo (Folkow, 1949; Winquist & Bevan, 1981), the response of a variety of vascular smooth muscle preparations to stretch has been studied by several groups. An early investigation in helically cut strips of the wall of small branches of dog superior mesenteric artery in response to a stepwise increase in stretch was reported by Harvey et al (1962). Contraction (tension developed) increased with stretch, until a certain optimal length was reached, after which the response decreased with further stretch. Other blood vessels including rabbit basilar artery (Nakayama, 1982; Bevan, 1982; Nakayama et al., 1986), facial vein (Laher & Bevan, 1987; Laher et al., 1988), coronary artery (Nakayama, 1982), ear artery (Hwa & Bevan, 1986a,b), renal artery (Nakayama, 1982), canine basilar artery (Toda et al., 1978; Katusic et al., 1987) and rat portal vein (Johansson & Mellander, 1975) have also been examined. All these blood vessel preparations contracted to stretch, and in most cases the contraction was not completely abolished by calcium antagonists.

Several possible mechanisms underlying this stretch-induced

contraction have been considered. First, the contractile mechanism may be directly activated by stretch (Peiper et al., 1974); secondly, the plasma membrane is depolarized when it is mechanically deformed by stretch, and the resulting calcium influx activates the contractile mechanism (Bohr, 1964); and finally, upon stretch, calcium is released from intracellular sources and this calcium activates the myosin light chain kinase producing phosphorylation of the light chain and contraction (Ledvora et al., 1983). The stretch-induced contraction does not take place in chemically skinned rabbit basilar artery suggesting that the first possibility seems to be unlikely (Nakayama et al., 1986). Removal of extracellular calcium or the presence of calcium antagonists can reduce or abolish the stretch-induced contraction (Winquist & Bevan, 1981; Nakayama, 1982; Bevan, 1983; Hwa & Bevan, 1986a,b; Nakayama et al., 1986) consistent with the idea that not only is the contraction to stretch calcium dependent, but the source is extracellular. Supporting this is the recent observation that stretch-dependent myogenic tone in the rabbit facial vein is accompanied by an increased uptake of $^{45}Ca^{2+}$ (Laher et al., 1988). This influx, however, does not seem to occur through the conventional voltage-gated calcium channels since neither the extent of myogenic tone nor $^{45}Ca^{2+}$ influx is affected by 1,4-dihydropyridine (PN200-110) though this drug reduces tone and $^{45}Ca^{2+}$ influx in response to depolarization by potassium. Similarly, in the rabbit ear artery, the myogenic tone induced by stretch is also resistant to PN200-110 or nimodipine, but can be completely abolished by the inorganic calcium antagonist Mn^{2+},

suggesting that entry is through a calcium channel (Hwa & Bevan, 1986a,b). The influx of calcium in response to stretch seems therefore to be through channels distinct from voltage-operated channels (Winquist & Baskin, 1983; Hwa & Bevan, 1986b). Only very few authors suggest that intracellular calcium can be involved in stretch-induced contraction. Nakayama et al.(1986) assumed that the stretch-induced mechanical response is associated with calcium release from the inner surface of the plasma membrane since dantrolene and ryanodine, drugs known to prevent calcium release from the endoplasmic reticulum of skeletal muscle, inhibited stretch-induced contraction in rabbit basilar artery. There is some doubt about this interpretation, however, as dantrolene and ryanodine have been found to inhibit calcium movement across the membrane in cardiac (Sutko et al., 1979; Sutko & Willerson, 1980) and smooth muscles (Graves, 1978; Mahmoudian et al., 1981). Ledvora, who also believed intracellular calcium could be released by stretch demonstrated stretch-induced phosphorylation of the light chain of myosin in porcine caroid artery, however this was not accompanied by contraction in this tissue (Burnstock & Prosser, 1960; Ledvora et al., 1983). In conclusion, from the literature, stretch-induced contraction in smooth muscle is myogenic in origin. In vascular smooth muscle the active response to stretch is calcium dependent and is relatively resistant to the organic calcium channel antagonists but blocked by inorganic antagonists such as manganese. Thus, the stretch-induced mechanism involves a calcium channel system different from either the conventional receptor operated or voltage-operated calcium channels.

Although the calcium channel antagonists, as a major new class of therapeutic drugs, have been available for more than twenty years, and potassium channel blockers such as TEA or 4-aminopyridine for even longer, potassium channel activators which open potassium channels are relatively new. The first drug to be studied in this group was nicorandil (Furukuwa et al., 1981). This vasodilator possesses an ability to hyperpolarize smooth muscle cell membranes in both porcine and guinea-pig coronary arteries (Furukuwa et al., 1981). Based on electrophysiological studies, an increase in the membrane K^+ conductance was suggested to be responsible for this hyperpolarization (Furukawa et al., 1981; Karashima et al., 1982). The subsequent demonstration that nicorandil can elicit $^{86}Rb^+$ efflux from preloaded tissues (Weir & Weston, 1986a,b) supported this conclusion. However, the vasorelaxant effects of nicorandil are not due solely to an opening of membrane K^+ channels. The nitro group within its structure also contributes to its vasodilatory action (Holzmann, 1983). A significant advance in this field of research has been the discovery that the benzopyran compound cromakalim (BRL 34915) which was reported to lower blood pressure both in animals and humans *in vivo* (Ashwood et al., 1984; Buckingham et al., 1984 a,b; Vandenburg et al., 1986, 1987) and appears to relax a variety of smooth muscles by opening K+ channels. Weston and his colleagues were the first to provide evidence *in vitro* that in the rat aorta, portal vein and guinea-pig taenia coli, cromakalim produces mechanical relaxation and this is associated with cell membrane hyperpolarization and an increase of 86Rb+ efflux in all three test preparations (Hamilton

et al., 1986; Weir & Weston, 1986a,b). Very recently, a number of other antihypertensive drugs have been reported to have similar properties, i.e. the ability to activate potassium channels. They include pinacidil (Bray et al., 1987; Hermsmeyer 1988 ; Southerton et al., 1988; Longman et al., 1988; Steingerg et al., 1988; Weston et al., 1988), minoxidil sulfate (Meisheri et al., 1988; Winquist et al., 1989) and diazoxide (Trube et al., 1986).

Since cromakalim was identified initially as a novel antihypertensive agent (Ashwood et al., 1984; Buckingham et al., 1984a, b), not surprisingly the effect of this drug has been tested on a variety of vascular smooth muscle preparations which include the rat and rabbit aorta (Weir & Weston, 1986b; Bray et al., 1988a,b,; Cook et al., 1988a,b; Chiu et al., 1988; Taylor et al., 1988; Quast & Baumlin, 1988), guinea-pig portal vein (Quast, 1987), rat portal vein (Hamilton et al., 1986; Shetty & Weiss, 1987; Beech & Bolton, 1987; Quast & Cook, 1988a; Winquist et al., 1989), and rabbit mesenteric artery and vein (Coldwell & Howlett, 1986, 1988; Clapham & Wilson, 1987; Nakao et al., 1988). The results of these studies suggest that cromakalim produces relaxation of vascular smooth muscle preparations by opening membrane potassium channels. The evidence is that muscle relaxation is accompanied by an increase in $^{86}Rb^{+}$ or $^{42}K^{+}$ efflux and at the same time hyperpolarization of the cell membrane occurs (Hamilton et al., 1986; Weir & Weston, 1986a,b; Kreye et al., 1987a,b; Southerton et al., 1987; Quast, 1987, 1988; Quast & Cook, 1988a; Cook et al., 1988b). Electrophysiological studies with cultured rabbit and bovine

aortic cells (Kusano et al., 1987) and enzymatically dispersed smooth muscle from rabbit portal vein (Beech & Bolton, 1987) and human inferior mesenteric artery (Trieschmann et al., 1988) provide evidence that cromakalim induces an increase in membrane potassium conductance. Additional support for the concept of in creased potassium permeability comes from studies in which potassium channel blockers such as TEA and 4-aminopyridine inhibit the relaxant effect and the stimulation of $^{86}Rb^{+}$ or $^{42}K^{+}$ efflux by cromakalim (Coldwell & Howlett, 1986; Quast, 1987, 1988).

Cromakalim has also been tested on a variety of non-vascular smooth muscles. In the guinea-pig trachea, cromakalim suppressed spontaneous tone, depressed the foot of the concentration-response curve for KCl, and caused minor rightward shifts in the concentration-response curves of ACh and histamine (Allen et al., 1986). In this tissue cromakalim also produced a marked hyperpolarization and increased $^{86}Rb^{+}$ efflux. The relaxant action of cromakalim can be inhibited by non-specific potassium channel blockers such as procaine and 4-aminopyridine, it is depressed by TEA at high concentrations but unaffected by apamin. In chemical-skinned preparations, tension development induced by low calcium was unaffected by cromakalim (Allen et al.,1986; Small et al., 1988) suggesting it has no effect on the contractile machinery or the ability of calcium to activate that machinery. In vivo, cromakalim inhibits 5-HT-induced bronchospasm for at least 60 min in anaesthetized guinea-pig whereas the calcium blocker nifedipine shows very weak or no protective effect (Arch et al., 1988). In human, pig, and guinea-pig

urinary bladder cromakalim abolishes spontaneous activity, increases membrane conductance and causes a concentration-dependent hyperpolarization of the cell membrane, consistent with its reported actions on other smooth muscles (Foster et al., 1989a,b). In the guinea-pig taenia coli cromakalim abolishes spontaneous tone and increases $^{86}Rb^{+}$ efflux. An effect resistant to apamin (Weir & Weston, 1986a). Cromakalim can also reduce motility of the small intestine in mice both in vivo and vitro (Buchheit & Berthrolet, 1988). It can also suppress the contraction evoked by field stimulation or by TEA and increase $^{86}Rb^{+}$ efflux in the rat oesophagal tunica muscularis mucosa (Akbarali et al., 1988). Not all tissues, however, show this pattern of mechanical inhibition, increased potassium efflux and membrane hyperpolarization. For example, in the rat uterus, cromakalim produces concentration-dependent inhibition of spontaneous contractions but shows little hyperpolarization of the cell membrane and no detectable increase in $^{86}Rb^{+}$ or $^{42}K^{+}$ efflux (Hollingsworth et al., 1987; Hollingsworth et al., 1988). The inhibitory effects of cromakalim in the uterus may involve an action only on pacemaker cells and the depolarization responsible for the pacemaker potentials. Such cells may possess other kind of potassium channels. It also produces long lasting inhibition of uterine contraction but there appears to be tolerance to the uterine relaxant action of cromakalim in vivo (Downing et al., 1989).

Cromakalim was also tested in tissues other than smooth muscle. In the heart it has been examined in the guinea-pig (Cain & Metzler, 1985; Scholtysik, 1987; Sanguinetti et al., 1988) and in dog (Gotanda et al.,

1988). As in smooth muscles, cromakalim can activate potassium channels in cardiac muscle and as a result shorten action potential duration and produce a negative inotropic action. Fortunately, cromakalim is about 10-30 times less potent in cardiac muscle than in vascular smooth muscles (Sanguinetti et al., 1988). Such a discrimination will be essential if it is to be used to treat hypertension.

The action of cromakalim on nervous tissue has also been examined. In guinea-pig hippocapal slices it can reduce the spontaneous activity and the rate of spike generation (Alzheimer & Bruggencate, 1988), an effect consistent with hyperpolarization. It has only slight affinity to monoamine binding sites in the rat neocortex and corpus striatum (Coldwell & Howlett, 1987). There is also a report that cromakalim can antagonize a behavioural response to pilocarpine in the mouse which may suggest that cromakalim probably can influence the central cholinergic system (Tricklebank et al., 1988).

Almost all of this experimental work suggests that the effect of cromakalim on smooth and cardiac muscles and on the central nervous system is due to the activation of potassium channels. Several types of potassium channels have been identified by the combination of pharmacological and single channel electrical studies. Calcium-activated potassium channels are sensitive to both potential and to the intracellular concentration of free ionized calcium. Two of them have been well studied. One potassium channel with a high-conductance is sensitive to charybdotoxin (Miller et al., 1985;

Gimenez-Gallego et al., 1988), while another channel with a low-conductance can be blocked by apamin (Maas et al., 1980). The ATP-activated potassium channel is sensitive to the level of intracellular ATP and has been found in pancreatic and cardiac tissues (Cook & Hales, 1984; Noma, 1985). Sulphonyureas such as glyburide have been shown to block this type of potassium channel (Schmid-Antomarchi et al., 1987). Non-selective potassium channel blockers such as TEA, 4-aminopyridine and procaine can partly blocked the relaxant effect of cromakalim in vascular smooth muscle, guinea-pig trachea and rat uterus (Coldwell & Howlett, 1986; Allen et al., 1986; Hollingsworth et al., 1987; Quast, 1987; Wilson et al., 1988b). These drugs, however, provide little information on the type of potassium channel involved. Clearly cromakalim does not stimulate an apamin-sensitive potassium channel since several reports using different preparations agree that apamin does not prevent the response to cromakalim (Weir & Weston, 1986a; Allen et al., 1986; Cook & Hof, 1988). The high conductance calcium-activated potassium channel at first sight looks to be a more likely candidate. Charybdotoxin which has been isolated from the venom of of the Israeli scorpion (Leinurus quinquestriatus) by Miller et al. (1985) blocks this channel in mammalian skeletal muscle T-tubules (Bolton & Clark, 1981). This venom is also a potent blocker of the action of cromakalim in stimulating $^{86}Rb^{+}$ efflux in the rat portal vein (Quast & Cook , 1988a). Unfortunately purified charybdotoxin itself has little or no effect on the action of cromakalim, and the only other toxin isolated from the

venom by Castle & Strong (1986) acts to block apamin-sensitive calcium activated potassium channels which are known not to be involved in the action of cromakalim. Clearly, both of these toxins can be ruled out as responsible for the inhibitory effect of the venom (Winquist et al., 1989). It is likely that the scorpion venom contains some other selective toxin capable of blocking the action of cromakalim. Only when this material is isolated and its effect on determined will it be possible to use these venom-derived proteins to analyse the potassium channels acted on by cromakalim.

More interesting perhaps are the ATP-sensitive potassium channels. Very recently, several groups reported that sulphonylureas such as glibenclamide, glyburide and glypizide can antagonize the relaxant action of cromakalim in vivo and in vitro. (Quast & Cook, 1988b; Escande et al., 1988; Wilson et al., 1988a; Winquist et al., 1989; Buckingham et al., 1989; Wilson, 1989; Cavero et al., 1989). These drugs also prevent the hypotensive action of cromakalim (Cavero et al., 1989). In cardiac muscle these drugs inhibit the ATP-sensitive potassium channels (Sanguinetti et al., 1988; Escande et al., 1988). There are several publications linking the ability of sulphonylureas to stimulate insulin secretion from the α-cells of the pancreatic islets to the blockade of ATP-sensitive potassium channels (Cook & Hales, 1984; Schmid-Antomarchi, et al., 1987). It is tempting to conclude that the site of action of cromakalim is on these ATP-sensitive channels, and that when the toxin in scorpoin venom is isolated it too will be found to act at this site. Caution, however is needed in that in rats the

sensitivity of vascular smooth muscle and of the pancreatic islct cells to glybenclamide is quite different (Buckingham et al., 1989; Cavero et al., 1989).

Although most of a large body of experimental evidence supports the hypothesis that cromakalim acts by increasing potassium permeability, hyperpolarizing the membrane, and as a result preventing the entry of external calcium there are some inconsistencies. For example, in the guinea-pig portal vein, spontaneous activity was modified by cromakalim at concentrations below those at which a stimulation of $^{86}Rb^{+}$ efflux could be detected (Quast, 1987; Cook et al., 1988a; Shetty & Weiss, 1987). In the guinea-pig urinary bladder again the concentration of cromakalim required to reduce the spontaneous activity was lower than that required to change the membrane potential and increase $^{43}K^{+}$ efflux (Foster et al., 1989a). In the rat uterus, cromakalim produces inhibitory effect similar to those observed in other tissues, but at concentrations up to 10 μM has no effect on $^{86}Rb^{+}$ or $^{42}K^{+}$ efflux and produced only minor (5mv) hyperpolarization of the membrane (Hollingsworth et al., 1987, 1988). Recently, Nakao et al. (1988) reported that the hyperpolarization induced by cromakalim in rabbit mesenteric vein comprised two components, one of which was Mn^{2+} sensitive. In the mesenteric artery, however, the hyperpolarization induced by cromakalim was relatively insensitive to Mn^{2+} (Nakao et al., 1988). In addition, during prolonged exposure to cromakalim, both hyperpolarization and relaxation were observed during the early exposure period, but in mesenteric artery the hyperpolarization decayed while the

relaxation was maintained, suggesting that hyperpolarization is not essential for relaxation. The early reports also showed that there is not much difference in the effect of cromakalim on the concentration-response curves of KCl, ACh and histamine in the guinea-pig trachea (Allen et al., 1986). Similar evidence was observed in vascular smooth muscles contracted by NA or KCl (Hamilton et al., 1986; Weir & Weston, 1986b). Clearly some of these agonists are believed to stimulate receptors and increase the calcium concentration mainly by activating receptor-operated channels and by releasing calcium from the intracellular stores. If the effect of cromakalim is solely to open potassium channels it would be expected to inhibit more effectively contractions induced by KCl than by receptor-dependent agonists.

Based on these information, the first approach in this project is to compare the relaxant activity of cromakalim on different smooth muscle preparations contracted by different stimuli. The possible involvement of second messengers was also examined.

METHODS

APPARATUS

In this project the response of tissues to drugs and stretch was examined in two different kinds of in vitro apparatus.

(1) Simple 10 ml organ baths.

(2) A Golenhofen organ bath so arranged as to allow controlled stretch to be applied to the muscle and its contractile response measured.

Simple Organ Bath Studies

All the simple organ bath studies were conducted as described in previous project.

Apparatus for Controlled Stretch

In studying the smooth muscle relaxant effect of cromakalim, the distinction between tone induced by biochemical coupling and that due to depolarization was important since cromakalim is believed to act by hyperpolarization. The usual method of causing depolarization is by adding potassium. At all but small increases in potassium concentration, this is unsatisfactory since high potassium so alters the Nernst potential for this ion that even if a relaxant drug opens

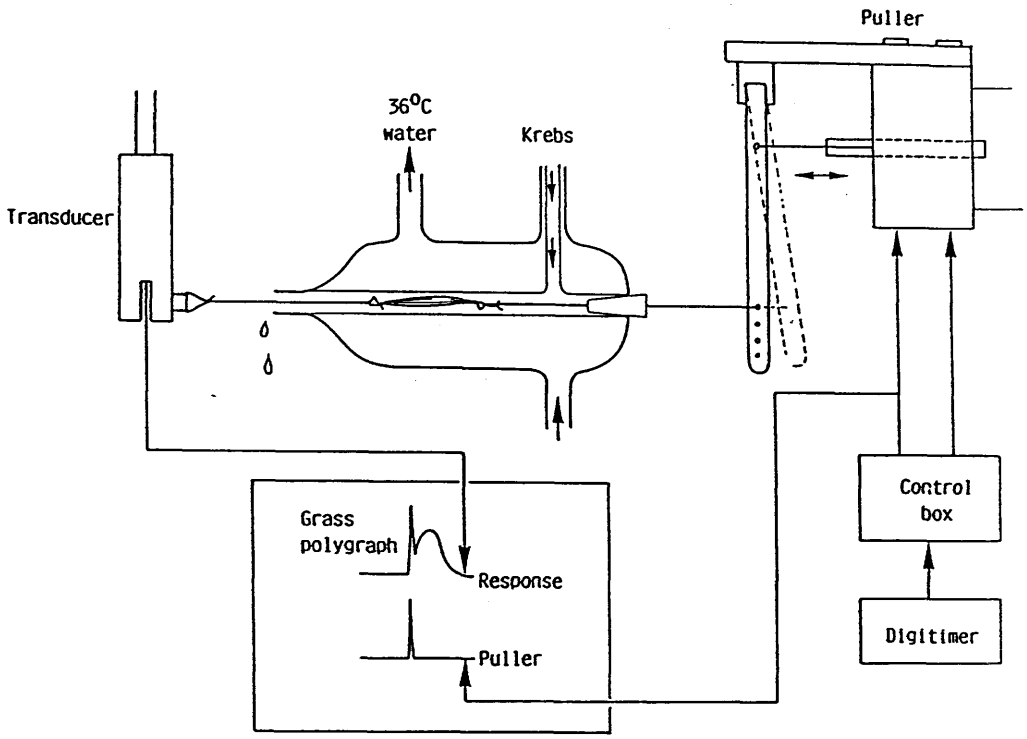

Figure 27 A diagrammatic representation of the apparatus used to apply controlled stretch to smooth muscle preparations. This consists of electromagnetic puller controlled by variable current pulses from a control box. The fraction of the current pulses can be controlled by the digitimer. The tissue was tired to a stainless-steel wire connected to the puller in the Golenhofen jacketed glass chamber. The other end of the muscle was connected to the Grass transducer. The movement of the puller was recorded on one channel of the Grass Polygraph and on a second channel the pull stimulus transmitted through the muscle plus the muscle response was recorded.

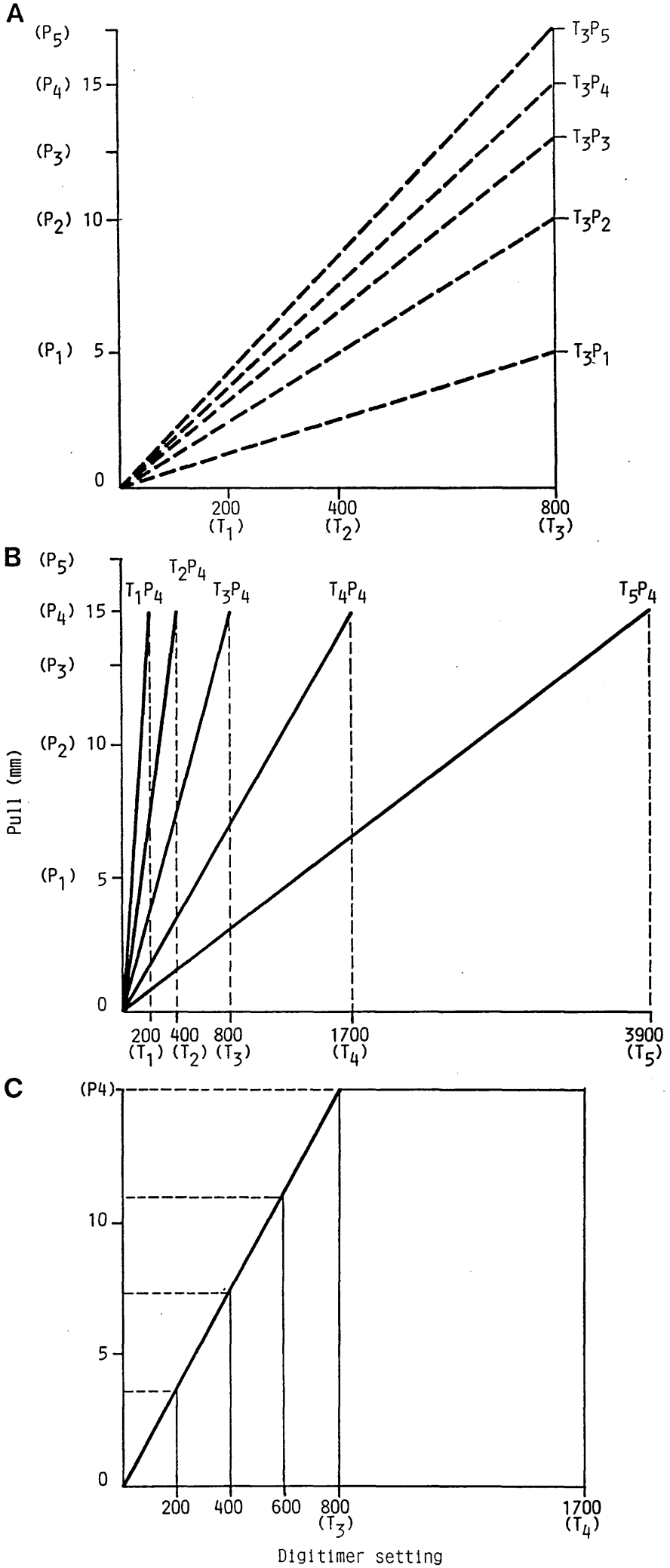
A
(P5)
(P4) 15
(P3)
(P2) 10
(P1) 5
0
T3P5
T3P4
T3P3
T3P2
T3P1
200 (T1)
400 (T2)
800 (T3)
B
(P5)
(P4) 15
(P3)
(P2) 10
(P1) 5
0
Pull (mm)
T1P4
T2P4
T3P4
T4P4
T5P4
200 (T1)
400 (T2)
800 (T3)
1700 (T4)
3900 (T5)
C
(P4)
10
5
0
200
400
600
800 (T3)
1700 (T4)
Digitimer setting

Figure 28 This shows the effect of varying the "Pull" setting at a constant time (a) or of varying "Time" setting at a constant pull (b) on the stretch stimulus applied to the tissue. Variation in either setting also varies the rate of stretch. With a fixed setting of both (T3P4) the digitimer can be used to stretch on the pull for a controlled time (c); this produces a constant rate of stretch in which increases in the degree of stretch are accompanied by proportional increases in time. If the digitimer setting exceeds the T setting the final stretch is maintained until the end of the digitimer pulse.

P(Pull)	P1	P2	P3	P4	P5
(mm)	5	10	13	15	17
T(Time)	T1	T2	T3	T4	T5
(ms)	200	400	800	1700	3900

Table 1 This shows the distances corresponding to the five pull settings and the duration of stretch corresponding to the five time settings geberated by the control box.

potassium channels, it is eventually ineffective in causing hyperpolarization.

Another method of causing depolarization is by stretch and this has been observed in a variety of smooth muscle preparations (Bülbring, 1954, 1955; Burnstock & Prosser, 1960; Gillespie, 1962; Harder, 1984). The apparatus illustrated in Figure 27 is designed to produce a controlled stretch stimulus. The artery strip was horizontally mounted in a central chamber of a Golenhofen organ bath jacketed with water at 36°C and perfused with Krebs' saline gassed with 95%O_2 + 5%CO_2 at a rate of 2 ml min^{-1}. Each end of the test tissue was tied with silk thread (5-0), one attached to a Grass FT03C transducer and the other end to a fine stainless steel rod whose other end was attached to an electromagnetic puller (the central core of a large diameter loudspeaker). The electro-magnetic coil was activated by current pulses from a specially designed control box. This produced a saw-tooth wave form so that two parameters could be controlled: first, the eventual height of the saw-tooth determined the distance pulled (the P setting); secondly, the duration of the saw-tooth controlled the duration of the pull (the time, or T setting). The combination of P and T determined another important variable, the rate of the pull, for example, the fastest rate of pulling was to combine P5, the longest pull, with T1, the shortest time. The number of settings available and the corresponding distances and durations are given in Table 1, and Figure 28(a,b) illustrates the effects of changing P and T on the rate of pull. These two variables alone produced insufficient control since

changing P to increase the stretch stimulus also changed the rate of stretch. The only way to avoid this would be simultaneously to change the duration of the pull, so that the rate was kept constant. This was difficult to do since the settings of both P and T were fixed. The rate of pull is an important variable and for this reason a third method of control was used. In this case the gate of a digitimer was used to produce pulses of variable duration. These pulses were then used to trigger the central control box so as to allow only a fraction of the waveform generated by the puller unit to reach the electromagnetic coil. The effect is illustrated in Figure 28(c) which shows that with the help of the digitimer, it was possible to maintain a constant rate of pull but vary the distance pulled. For example, with T3 which produces an 800 ms saw-tooth the digitimer might allow only half of this to reach the puller coil. The rate of pull would be appropriate to the T3 setting, but the distance travelled would be only half whatever setting was chosen for P. The digitimer could also be set to exceed the T value, in which case the coil would remain activated and the pull "held" till the end of the digitimer pulse. The first saw-tooth wave form was available for display on a second channel of the polygraph and it magnitude and rate of rise was a measure of the stretch stimulus applied to the muscle.

The effect of stretch is likely to be influenced by the initial length and cross-sectional area of the muscle. To control this, tissues were dissected to give a 10 mm long preparation when placed under a resting tension of 500 mg. The actual length was measured in

preparation	wet wt (mg)	length (mm)	maximum % of inhibition			
			BRL 10^{-5}M	VER 10^{-4}M	Co^{2+} 30 mM	Ca^{2+} 0mM EGTA 1mM
renal artery (N)	3.2±0.3 (20)	9.7±0.3 (27)	16.5±4.3 (4)	27±5.2 (6)	96.5±2.6 (4)	100 (5)
femoral artery (N)	2.7±0.3 (17)	10.3±0.3 (23)	34.7±8.7 (6)	60.6±5 (5)	99±1 (5)	100 (5)

Table 3 This shows a degree to which the length of the two preparations varied around the intended length of 10 mm. Also shown is the percentage inhibition of the response to stretch produced by cromakalim (BRL), verapamil (VER), cobalt bromide (Co^{2+}) and the removal of calcium plus the addition of EGTA to the Krebs' saline. While cobalt and zero calcium in effect abolished the response, verapamil and cromakalim produce more modest inhibition. Values are mean ± s.e. N=number of experiments.

each experiment and the results are shown in Table 3. At the end of the experiment the tissue's wet weight was determined. This measurement was made only as a check on the degree to which standardization of preparations was achieved.

PREPARATION OF TISSUES

(1) Rabbit Arteries New Zealand Rabbits (2-3 kg, either sex) were killed by exposure to carbon dioxide followed by exsanguination. The aorta, and renal and femoral arteries were removed carefully and cleaned gently of surrounding connective tissue and fat. Since the percentage stretch of the tissue depends on the initial length, considerable care was taken to obtain preparations all of a similar length and this was measured once the preparations were mounted under tension in the bath. Abdominal aortic strips, and renal and femoral arteries were 10 mm long by 1.5 mm wide.

(2) Bovine Retractor Penis Muscle (see Methods in previous project).

(3) Guinea-Pig Trachea (see Methods in previous project).

(4) Guinea-Pig Taenia Coli Animals were killed as previously described. The abdomen of the animal was opened in the midline and 15 mm long preparations of the taenia dissected from the caecum and tied at each end.

OTHER TECHNIQUES

Haemoglobin Solutions

(see Methods in previous project).

Measurement of Cyclic Nucleotides

The cyclic AMP and cyclic GMP contents of bovine retractor penis muscles were measured by radioimmunoassay. Tissues were set up in organ bath as usual and removed at various times after exposure to different concentrations of relaxant drugs, but always after relaxation had reached a maximum. The tissue was immediately frozen in liquid nitrogen and stored in 5% trichloracetic acid at 4°C overnight. Aliquots of the acid-soluble fraction were freed of trichloracetic acid by extracting the samples four times with three volumes of water-saturated diethyl ether.

Cyclic AMP in the samples was measured after acetylation with acetic anhydride followed by exposure to goat antiserum raised to cyclic AMP.

Radioactive iodine-labelled cyclic AMP was then added for 1 hour to bind to sites not already occupied by cyclic AMP from the sample and the excess unbound radioactivity absorbed by activated charcoal. The radioactivity remaining in the supernatant was then measured on a gamma counter and compared with a standard curve for known concentrations of cyclic AMP.

Cyclic GMP was similarly measured by radioimmunoassay with a kit supplied by Amersham. The tritium-labelled cyclic GMP was used as competitor for the cyclic GMP in the extracts. The amount of radioactivity bound to the antibody was measured by precipitating the antibody complex with ammonium sulphate, separating the precipitate by centrifugation in a refrigerated centrifuge at 4°C, redissolving the precipitate in water and measuring its radioactivity on a beta counter.

DRUGS AND SOLUTIONS

Drugs

Drugs used during the section were apamin (Sigma); carbachol (Sigma); cobaltous bromide (BDH); cromakalim (Beecham Pharmaceuticals); forskolin (Sigma); guanethidine sulphate (CIBA); histamine acid phosphate (Sigma); indomethacin (Sigma); isoprenaline sulphate (Burroughs Wellcome); noradrenaline bitartrate (Koch-Light);

prazosin (Pfizer); sodium nitroprusside (BDH); verapamil chloride (Sigma).

Solutions

Calcium-free Krebs' saline was identical to normal Krebs' except that $CaCl_2$ was omitted and ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 1 mM was added.

Statistical Analysis

Results were expressed as mean ± standard error. In stretch studies, the peak of the maximal response was considered to be 100% contraction. N=number of experiments. Data was analysed using Student's t-test and $P < 0.05$ was considered significant.

RESULTS

Cromakalim is believed to cause relaxation by opening membrane potassium channels, hyperpolarizing the membrane, and as a consequence, closing voltage operated calcium channels previously opened by agonist action (Cook, 1988). Although there is much evidence in support of this, other effects of the drug seem inconsistent with this scheme. For example, cromakalim causes relaxation of tissues contracted by agonists which act mainly through biochemical coupling and which do not cause depolarization, for instance, noradrenaline on some vascular smooth muscles (Casteels et al., 1977a,b; Droogmans et al., 1977). It is therefore possible that hyperpolarization is not the only, or indeed the main mechanism of inhibition induced by cromakalim. The experiments in this project were intended to explore more fully the mechanisms of relaxation. In particular to examine whether cyclic nucleotides were involved, whether contraction induced by potassium depolarization was more sensitive to the relaxant action of cromakalim than that induced by agonists coupled biochemically, and finally whether contraction induced by stretch, which decreases the membrane potential in a variety of smooth muscles (Bülbring, 1954, 1955; Burnstock & Prosser, 1960; Gillespie, 1962; Harder, 1984), could be reduced by cromakalim.

TISSUE AND AGONIST SENSITIVITY TO CROMAKALIM

Cromakalim was tested on four smooth muscle preparations, the BRP muscle, the rabbit aortic strip, the guinea-pig taenia coli and the

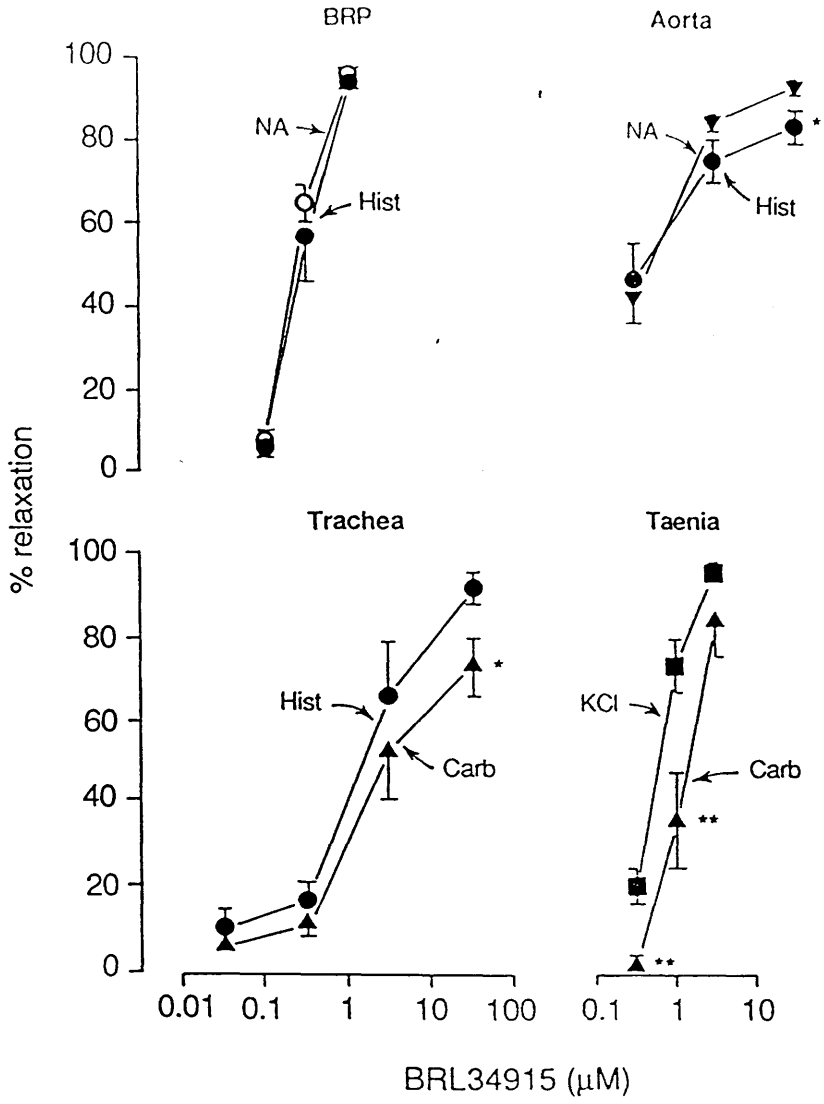

Figure 29 Log concentration-response curves for the relaxant effect of cromakalim (BRL 34915) on the bovine retractor penis (BRP, upper left) contracted by noradrenaline (O ; guanethidine 1μM) or histamine (● ; 3μM), the rabbit aortic strip (Aorta, upper right) contracted by either noradrenaline (▼ ; 50 nM) or histamine (● ; 3 μM), the guinea-pig trachea (Trachea, lower left) contracted by histamine (● ; 3μM) or carbachol (▲ ; 0.1 μM) and the guinea-pig taenia coli (Taenia, lower right) contracted by KCl (■ ; 10mM) or carbachol (▲ ; 30 nM). Each point is the mean of at least six preparations. Indomethacin (10^{-6}M) was present in the Krebs' saline in experiments with the guinea-pig trachea. Bars represent ± s.e. *P<0.05, **P<0.01.

Table 2 IC_{50} values (in μM) for relaxation of agonist-induced spasm by cromakalim

agonist	BRP	Aorta	Trachea	Taenia
NA	0.27±0.04 (15)	0.52±0.11 (19)	—	—
Hist	0.32±0.05 (11)	0.69±0.38 (6)	3.32±1.64 (6)	—
CCh	—	—	9.72±5.16 (6)	1.51±0.38 (8)
KCl	—	—	—	0.65±0.07 (12)

Four tissues, the bovine retractor penis (BRP), rabbit aortic strip (Aorta), guinea-pig trachea (Trachea) and taenia coli (Taenia), were contracted with either noradrenaline (NA), histamine (Hist), carbachol (CCh) or potassium chloride (KCl). The values are the means ± s.e. of the number of preparations indicated by the number in parentheses. Within any tissue there was no significant difference between the IC_{50} values of cromakalim to against agonists, with the exception of the taenia coli where $P<0.05$.

guinea-pig trachea; and against tone induced by four agonists, histamine, carbachol, guanethidine (noradrenaline) and low concentrations of potassium (10mM). Preliminary concentration-response curves to each spasmogen were constructed and a standard concentration producing about 60%-80% of maximal contraction in each tissue was selected for studies with cromakalim. Cromakalim relaxed all four preparations. The concentration-response curves are shown in Fig 29 and IC_{50} values for inhibition in Table 2. In the BRP muscle the concentration-response curves for relaxation of histamine (3×10^{-6}M) or guanethidine (noradrenaline, 10^{-6}M) induced tone were almost identical, as were the IC_{50} values. IC_{50} values for cromakalim were also similar in rabbit aortic strips precontracted with noradrenaline (5×10^{-8}M) or histamine (3×10^{-6}M), and in guinea-pig trachea precontracted with histamine (3×10^{-6}M) or carbachol (10^{-7}M). In each tissue the relaxation induced by cromakalim was similar for each spasmogen except the maximal relaxation by cromakalim in the trachea with carbachol-induced tone was less than with tone induced by histamine and in the aorta where histamine-induced tone was less reduced than that induced by noradrenaline (Fig 29). In the guinea-pig taenia coli the IC_{50} value for relaxation of contractions induced by a low concentrations of potassium (10 mM) was significantly lower than that for 3×10^{-8}M carbachol-induced contraction (Fig 29). Although for each spasmogen, there was only a slight difference in the sensitivity to relaxation in any given tissue, there was some difference between tissues as measured by the IC_{50} values (Table 2).

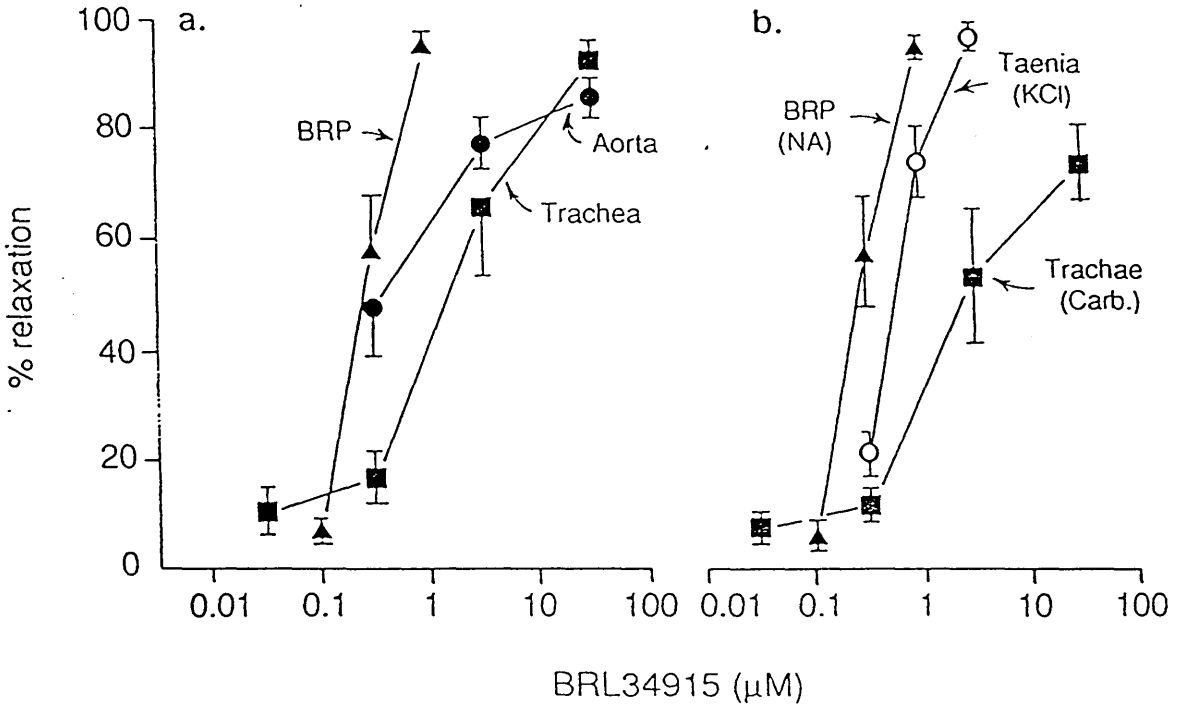

Figure 30 In (a) the concentration-response curves for the relaxant action of cromakalim (BRL 34915) have been compared in three tissues, the bovine retractor penis (▲; BRP), guinea-pig trachea (■) and rabbit aortic strip (●), all contracted by the same concentration of agonist, histamine (3 μM). There was a tissue difference in sensitivity. In (b) the concentration-response curve for relaxation in the guinea-pig taenia coli contracted with 10 mM KCl (o) has been compared with the most sensitive preparation, the BRP contracted by noradrenaline (▲), and the least sensitive, the guinea-pig trachea contracted by carbachol (■). Contractions to a low concentration of potassium in the taenia coli were intermediate in sensitivity between the other two tissues. Indomethacin (10^{-6}M) was present in the Krebs' saline for the guinea-pig trachea. Each point represents the mean and bars represent ± s.e.

The BRP muscle was marginally the most sensitive tissue to the relaxant action of cromakalim but there was little difference between it and the rabbit aorta or guinea-pig taenia coli. However, the guinea-pig trachea was ten times less sensitive than the BRP muscle (Fig 30). It was not possible to use all of the agonists on each tissue since not all produced maintained contraction to each spasmogen. Histamine (3×10^{-6}M) was generally the most effective agonist and Figure 30a illustrates the concentration-response relationships for relaxation by cromakalim on three of the tissues, the BRP muscle, the rabbit aorta and the guinea-pig trachea all contracted by this agonist. The relatively low sensitivity of trachea is clear. Figure 30b also illustrates the extremes of sensitivity to relaxation by cromakalim. The most sensitive combination was the BRP muscle contracted with noradrenaline and the least sensitive the guinea-pig trachea contracted by carbachol. For comparison, the concentration-response curve for the guinea-pig taenia coli contracted by 10mM KCl has been superimposed and, as can be seen in Figure 30b, there is no evidence that it is particularly sensitive to the relaxant effect of cromakalim.

DESENSITIZATION TO CROMAKALIM IN THE GUINEA-PIG TRACHEA

Recently, there have been a few reports that desensitization occurs in response to cromakalim in some smooth muscle preparations. For example, Nakao et al. (1988) reported desensitization to cromakalim

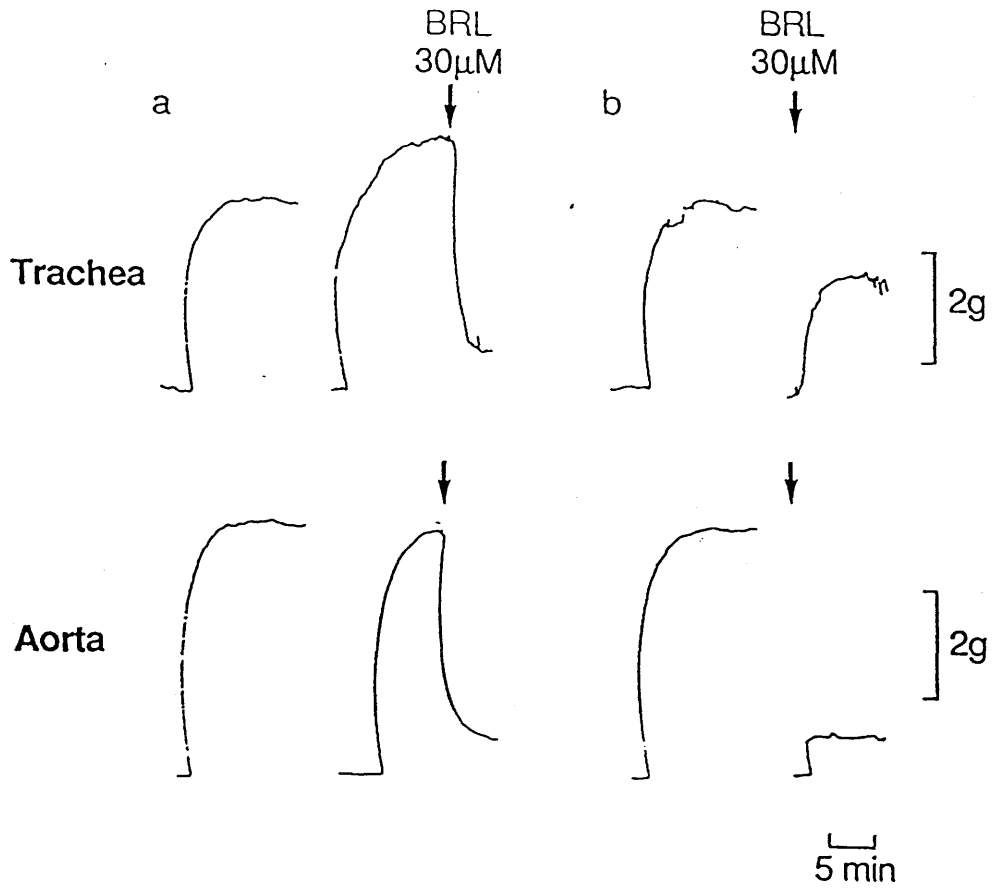

Figure 31 The response to cromakalim (BRL) of the guinea-pig trachea (Trachea) and rabbit aortic strip (Aorta). In both tissues, tone was induced by histamine (3 μM). In each pair of records, the first is the control response to histamine and the second is in the presence of cromakalim either added at the peak of the response to histamine (a) or 30 min before adding the agonist (b). In (a) cromakalim (30 μM) caused about 80% relaxation of histamine-induced tone in both tissues. In (b) cromakalim (30 μM) produced almost the same degree of inhibition of the response to histamine as in (a) but in the trachea cromakalim produced only about 40% inhibition. Indomethacin (10^{-6}M) was present in the Krebs' saline in experiments with the guinea-pig trachea.

evoked membrane hyperpolarization in the guinea-pig mesenteric artery and Downing et al. (1989) reported tolerance to cromakalim in the rat uterus motility *in vivo*. However, desensitization of mechanical responses to cromakalim *in vitro* have not been discovered.

(1) Differential responses of the guinea-pig trachea & rabbit aorta to cromakalim In the course of constructing the concentration-response curves described in the previous chapter, it was found that relaxation to high concentrations of cromakalim developed fairly rapidly, whereas low concentrations took up to 30 minutes to reach equilibrium. Measurements of small relaxations developing over such long periods are difficult to make. A different regime of drug exposure was therefore studied. Instead of adding the spasmogen first and cromakalim at the plateau of contraction, cromakalim was added 30 minutes before the agonist, and its inhibitory effect measured by the difference between the response to spasmogen in the absence or in the presence of cromakalim. This method worked well in the rabbit aortic strip, where the inhibitory effect of cromakalim added 30 minutes before the spasmogen produced a very similar degree of inhibition to that produced at the height of the contraction, but in the guinea-pig trachea quite different results were obtained. This is shown in Figure 31, where in both test preparations histamine (3×10^{-6}M) was used as the spasmogen. In the rabbit aortic strip, cromakalim produced about 85% inhibition whether added at the peak of contraction or 30 minutes before

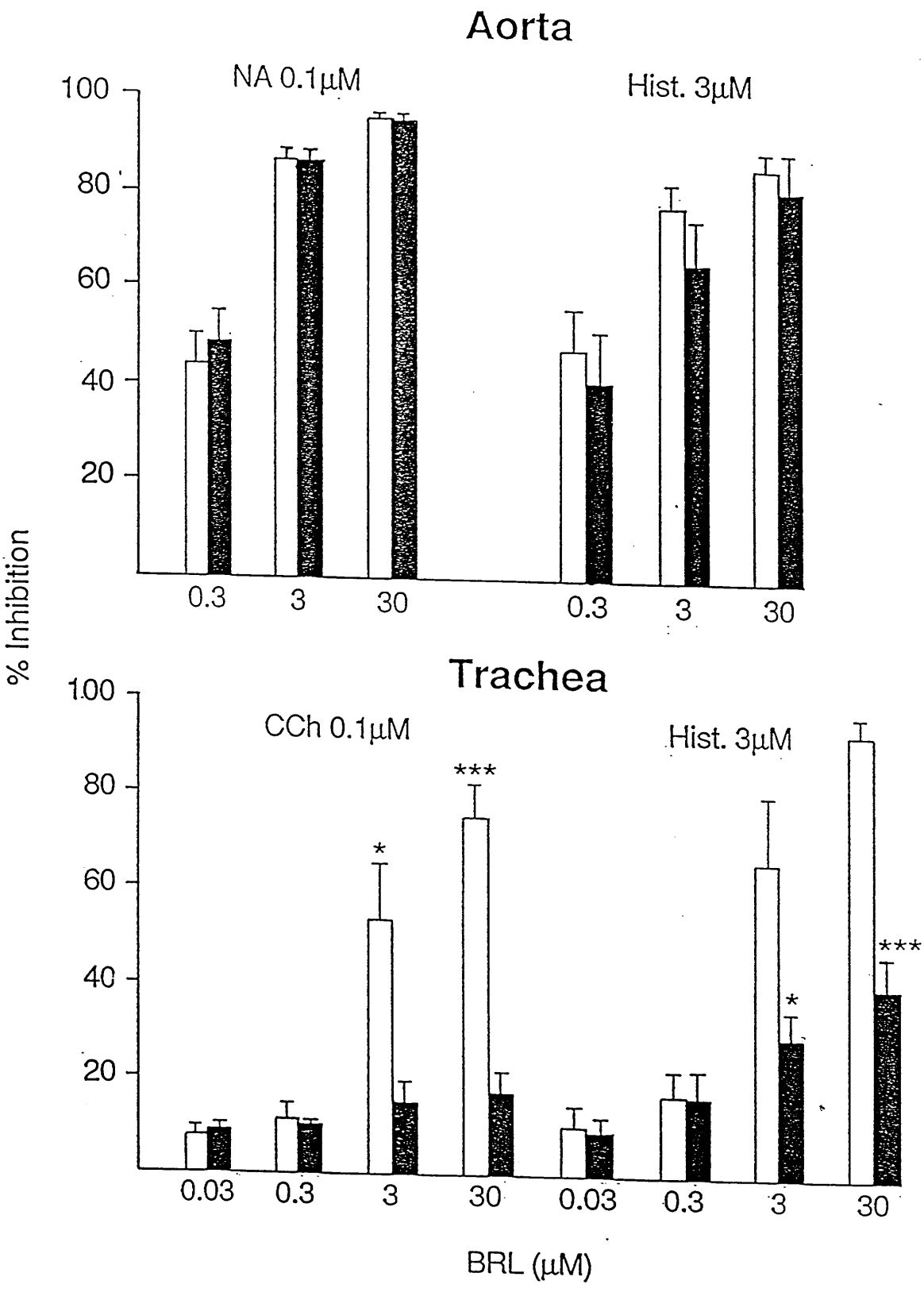
Aorta
NA 0.1µM
Hist. 3µM
100
80
60
40
20
% Inhibition
0.3
3
30
0.3
3
30
Trachea
CCh 0.1µM
Hist. 3µM
100
80
60
40
20
*
***
*
***
0.03
0.3
3
30
0.03
0.3
3
30
BRL (µM)

Figure 32 A histogram of the inhibitory effects of different concentrations of cromakalim (BRL) on the rabbit aortic strip (Aorta) contracted by noradrenaline (NA, 0.1 μM) or histamine (Hist, 3 μM) and on the guinea-pig trachea (Trachea) contracted by carbachol (CCh, 0.1 μM) or histamine (Hist, 3 μM). The open columns show the inhibition by cromakalim when it was added in the presence of spasmogens and the closed columns that obtained when cromakalim was added 30 min before spasmogens. Cromakalim produced a similar inhibition of the tone in the aortic strip whether it was added before or during contraction induced by spasmogens, but in the trachea cromakalim added before spasmogens produced only a small inhibition compared with its effect when added at the peak of the contraction. Indomethacin (10^{-6}M) was present in Krebs' saline in experiments with the guinea-pig trachea. N=6. Bars represent ± s.e. N=6. *P<0.05, **P<0.01.

the spasmogen whereas in the trachea, cromakalim added at the peak of the contraction again produced a similar 85% inhibition but when added 30 minutes before, reduced the response to only 30%. In both tissues the effects of cromakalim were independent of the nature of the spasmogen, either noradrenaline (10^{-7}M) or histamine (3 x 10^{-6}M) was used in the rabbit aortic strip, and carbachol (10^{-7}M) or histamine (3 x 10^{-6}M) in the guinea-pig trachea. The inhibitory action of cromakalim added 30 minutes before either agonist in the trachea was very much reduced, compared with similar concentrations added after the contraction had reached a plateau. The histograms in Figure 32 summarize all of these experiments.

These results indicated that desensitization to cromakalim appears to occur in the guinea-pig trachea but not in the rabbit aortic strip. The explanation of how this desensitisation is brought about is much less clear.

(2) Effects of cromakalim on the guinea-pig trachea in the presence of verapamil In guinea-pig trachea, organic calcium channel blockers, such as verapamil, have little effect on contractions induced by carbachol, whereas they suppress contractions induced by excess potassium, although both are dependent on the external calcium (Baba et al., 1985). The contraction due to excess potassium is the consequence of depolarization and opening of voltage-operated calcium (VOC) channels. In most cases, verapamil is known to be particularly

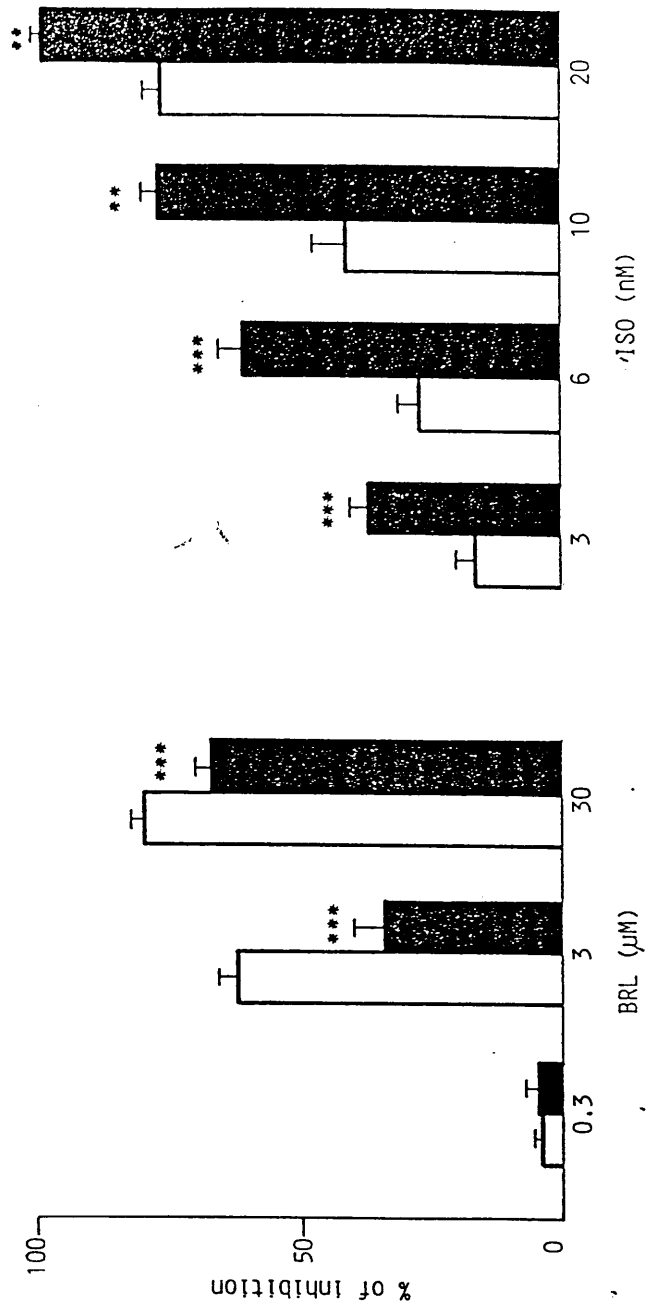

Figure 33 A histogram of the inhibitory effects of different concentrations of cromakalim (BRL) and isoprenaline (ISO) on the guinea-pig trachea contracted by carbachol (0.1 μM, open column) compared with the responses in the presence of verapamil (1 μM, closed column). The Krebs' saline contained indomethacin (10^{-6}M). Bars represent ± s.e. N=8 for cromakalim and N=6 for isoprenaline. ** P< 0.01, *** P< 0.001.

effective on such channels. Cromakalim is believed to produce relaxation in the guinea-pig trachea by opening potassium channels, resulting in hyperpolarization and closure of the voltage-operated calcium channels on the cell membrane (Allen et al., 1986). According to this scheme, if verapamil is used in this tissue to block VOC channels and tone is then raised with carbachol, the effect of cromakalim should be reduced. This was tested in the guinea-pig trachea, contracting the tissue with carbachol (10^{-7}M) in the presence of verapamil (10^{-6}M). As expected, the concentration-related relaxation induced by cromakalim was significantly reduced in the presence of verapamil (Fig 33). Isoprenaline also produced concentration-related inhibition of the tone induced by carbachol (10^{-7}M). In the presence of verapamil, however, the response to isoprenaline was greatly potentiated (Fig 33). Since the inhibitory effect of cromakalim is reduced in the presence of verapamil, this suggests that verapamil-sensitive calcium channels play a role in the effect of cromakalim. The residual inhibitory effect suggests other mechanisms may also participate in the relaxant action. With isoprenaline it is equally clear that verapamil-sensitive calcium channels are not involved in the response. Presumably cyclic AMP acting through cyclic AMP kinase is responsible. What is difficult to explain is why the relaxant effect of isoprenaline should be enhanced.

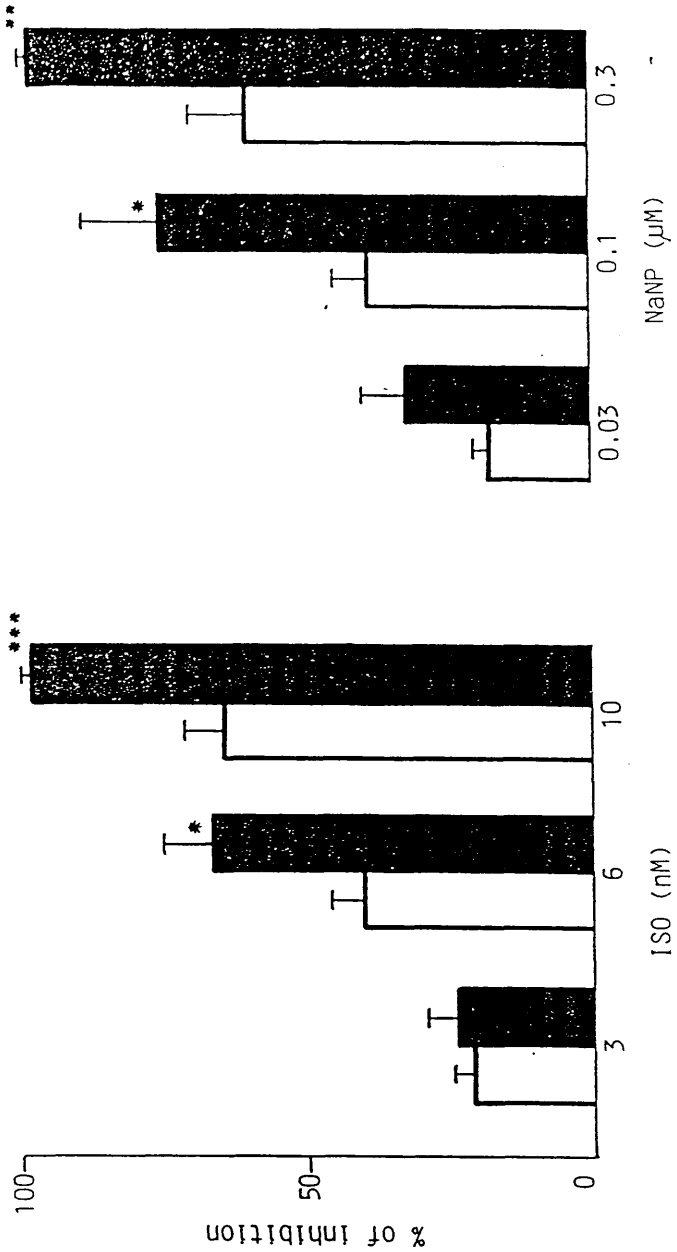

Figure 34 A histogram of the inhibitory effects of different concentrations of isoprenaline (ISO) and sodium nitroprusside (NaNP) on the guinea-pig trachea contracted with histamine (3 μM) in the absence (open column) and presence of cromakalim (10 μM, closed column). Cromakalim significantly potentiated the inhibitory effect of both relaxants. The Krebs' saline contained indomethacin (10^{-6}M). Bars represent ± s.e. N=10 for isoprenaline and N=6 for sodium nitroprusside. * $P< 0.05$, ** $P< 0.01$ and *** $P< 0.001$.

(3) Influence of cromakalim on responses to isoprenaline & sodium nitroprusside The effects of cromakalim on the guinea-pig trachea declined if the drug was added 30 minutes before the spasmogen, i.e. desensitization occurred. The present experiments were intended to determine whether the desensitization extended to other relaxants whose mode of action is different from cromakalim. Two were chosen, isoprenaline and sodium nitroprusside. It is generally accepted that isoprenaline acts through membrane a-adrenoceptors coupled to adenylate cyclase resulting in raised levels of cyclic AMP. Sodium nitroprusside acts inside the cell where it is believed to release NO which activates soluble guanylate cyclase with a consequent increase in cyclic GMP. These, therefore, are examples of biochemical coupling, mechanisms different from membrane hyperpolarization or electro-mechanical coupling observed with agents such as cromakalim.

Since the aim was to produce desensitization to cromakalim, the drug (3×10^{-5}M) was added 30 minutes before testing the other two relaxants. Histamine (3×10^{-6}M) in these circumstances can still produce about 60% of the contraction it would have produced in the absence of cromakalim. Concentration-response curves for isoprenaline or sodium nitroprusside were obtained in the presence or absence of cromakalim. As Figure 34 shows there was no desensitization of the relaxant action of these compounds, indeed both were significantly potentiated, an effect reminiscent of the potentiation of isoprenaline described above and illustrated in Figure 33.

APAMIN, HAEMOGLOBIN AND METHYLENE BLUE ON THE RESPONSE TO CROMAKALIM

Bowman et al. (1981,1982) have demonstrated the selective action of haemoglobin and apamin in blocking the relaxant effect of NANC nerve stimulation in different smooth muscle preparations. In the BRP muscle haemoglobin blocks the response to NANC nerve stimulation and apamin is without effect, whereas the reverse is true in the guinea-pig taenia coli. Haemoglobin inhibits stimulation of soluble guanylate cyclase (Murad et al., 1978) and this is probably its mechanism of action in the BRP muscle (Bowman & Drummond,1984), whereas apamin blocks one of the calcium-operated potassium channels (Banks et al., 1979). The effects of methylene blue which blocks soluble guanylate cyclase (Ignarro & Kadowitz, 1985) were also examined. From previous results, it was known that both the BRP muscle and guinea-pig taenia coli were sensitive to the relaxant effect of cromakalim. The effects of apamin, haemoglobin and methylene blue were then tested in both tissues to see whether they could interfere with the relaxation induced by cromakalim.

(1) Effects of Apamin Guinea-pig taenia coli preparations were set up within ring electrodes for field stimulation. Tone was raised with 10 mM KCl and the cholinergic and adrenergic nerves blocked by adding atropine (3×10^{-7}M) and guanethidine (3×10^{-6}M) to the bath. NANC nerves were stimulated for 10 seconds with supramaximal voltages at a range of frequencies followed by a control concentration-response

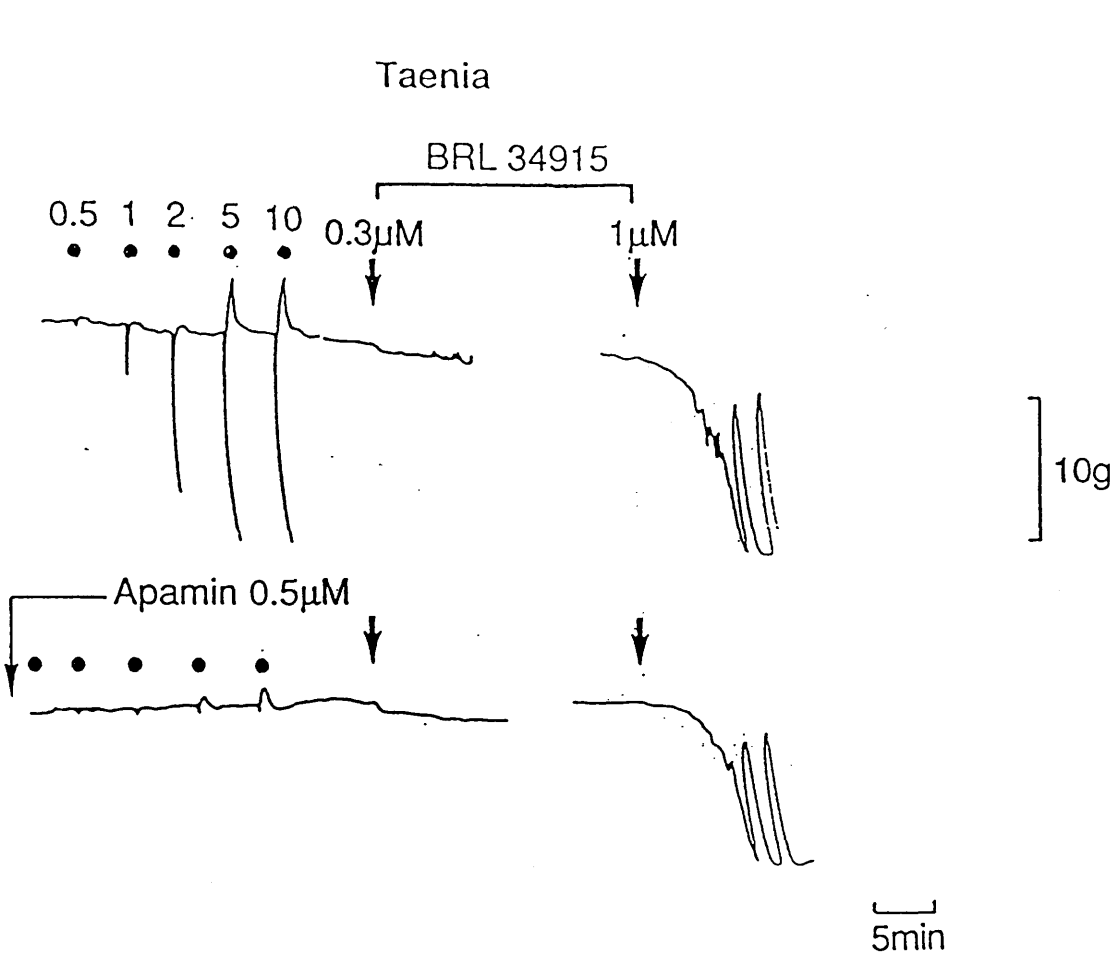

Figure 35 Representative records of the effects of apamin on the relaxant responses to NANC nerve stimulation and to two concentrations of cromakalim (BRL 34915) in the guinea-pig taenia coli. The upper records show the control response to supramaximal field stimulation of the NANC nerves for 10 sec. The frequency of stimulation in Hz is shown above each response. The relaxations produced by 0.3 μM and 1 μM cromakalim are also shown. The lower records from the same experiment show the responses to NANC nerve stimulation and to cromakalim in the presence of apamin (0.5 μM). Apamin completely abolished the relaxation to nerve stimulation but had no effect on the relaxation produced by cromakalim. Tone was raised with 10 mM KCl and Krebs' saline contained atropine ($3x10^{-7}M$) and guanethidine ($3x10^{-6}M$).

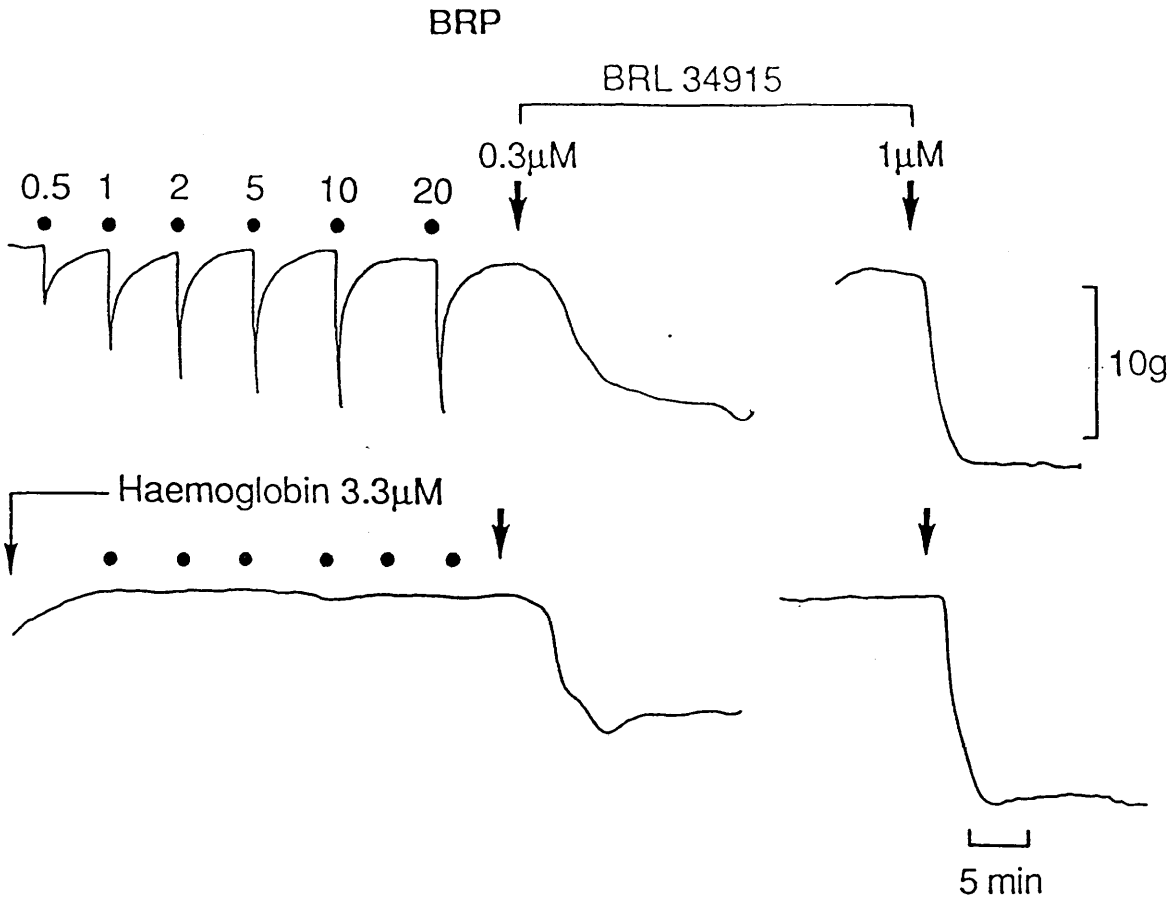

Figure 36 Representative records of the effects of haemoglobin on the relaxant responses to NANC nerve stimulation and to two concentrations of cromakalim (BRL 34915) in the bovine retractor penis (BRP). The upper records show the control responses to supramaximal field stimulation of the NANC nerves for 10 sec (the motor adrenergic nerves were blocked by 1 μM guanethidine). The frequency of stimulation in Hz is shown above each response. The relaxations produced by 0.3 μM and 1 μM cromakalim are also shown. The lower records, from the same experiment show the responses to NANC nerve stimulation and to cromakalim in the presence of freshly prepared guinea-pig haemoglobin (3.3 μM). Haemoglobin completely abolished the response to nerve stimulation without affecting the response to cromakalim. Atropine ($3x10^{-7}M$) was present in the bath solution.

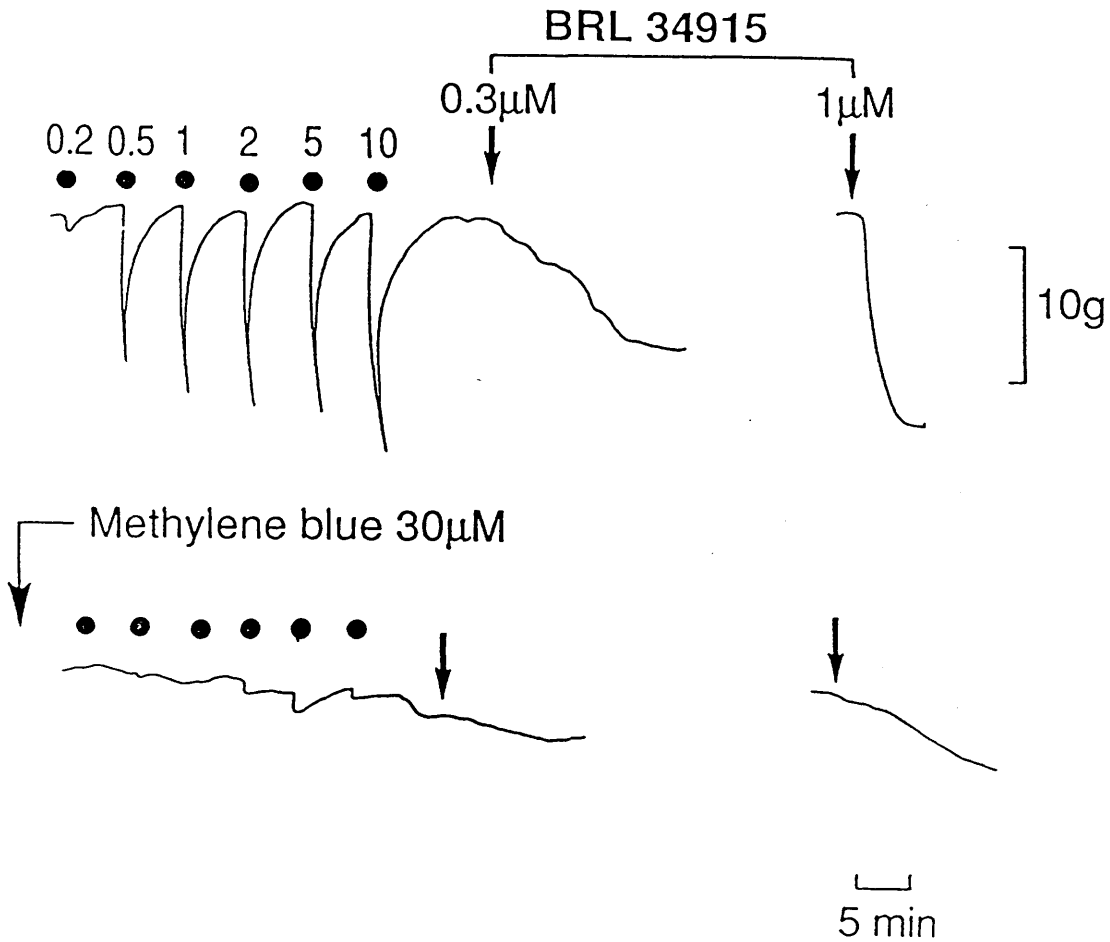

Figure 37 Representative records of the effects of methylene blue on the relaxant responses to NANC nerve stimulation and to two concentrations of cromakalim (BRL 34915) in the bovine retractor penis (BRP). The upper records show the control responses to supramaximal field stimulation of the NANC nerves for 10 sec (the motor adrenergic nerves were blocked by 1 μM guanethidine). The frequency of stimulation in Hz is shown above each response. Relaxations produced by 0.3 μM and 1 μM cromakalim are also shown. The lower records show the response to NANC nerve stimulation and to cromakalim in the presence of methylene blue (30 μM). Methylene blue almost completely blocked the responses to nerve stimulation and to cromakalim. Atropine ($3x10^{-7}M$) was present in the bath solution.

curve to cromakalim. Apamin was then added to the bath solution and the frequency-response curve to NANC nerve stimulation and the concentration-response curve to cromakalim repeated in its presence. Apamin (5×10^{-7}M) completely abolished relaxation to the NANC nerve stimulation but had no effect on that to cromakalim in the same preparation (Fig 35). This result suggests that cromakalim does not act through apamin-sensitive calcium-operated potassium channels.

(2) Effects of Haemoglobin and Methylene blue These were tested in the BRP muscle where the NANC nerve response is sensitive to both haemoglobin and methylene blue. The experimental procedure was the same as with the guinea-pig taenia coli but tone was raised with guanethidine (10^{-6}M) with atropine (3×10^{-7}M) in the Krebs' saline. Haemoglobin (3.3×10^{-6}M) greatly reduced the NANC nerve response in the BRP muscle but had no effect on the relaxation to cromakalim (Fig 36).

Similar experiments using methylene blue (3×10^{-5}M) to block the NANC nerve response were performed in the BRP muscle. In these experiments, the responses to both NANC inhibitory nerve stimulation and cromakalim were reduced in the presence of methylene blue (Fig 37). Similar results on rabbit mesenteric arteries have been reported by another group (Coldwell & Howlett, 1986). The reason for this may be some non-specific action of methylene blue which has been reported by Bowman et al.(1986). The results of all the experiments with both blockers on NANC nerve stimulation and cromakalim responses are shown in

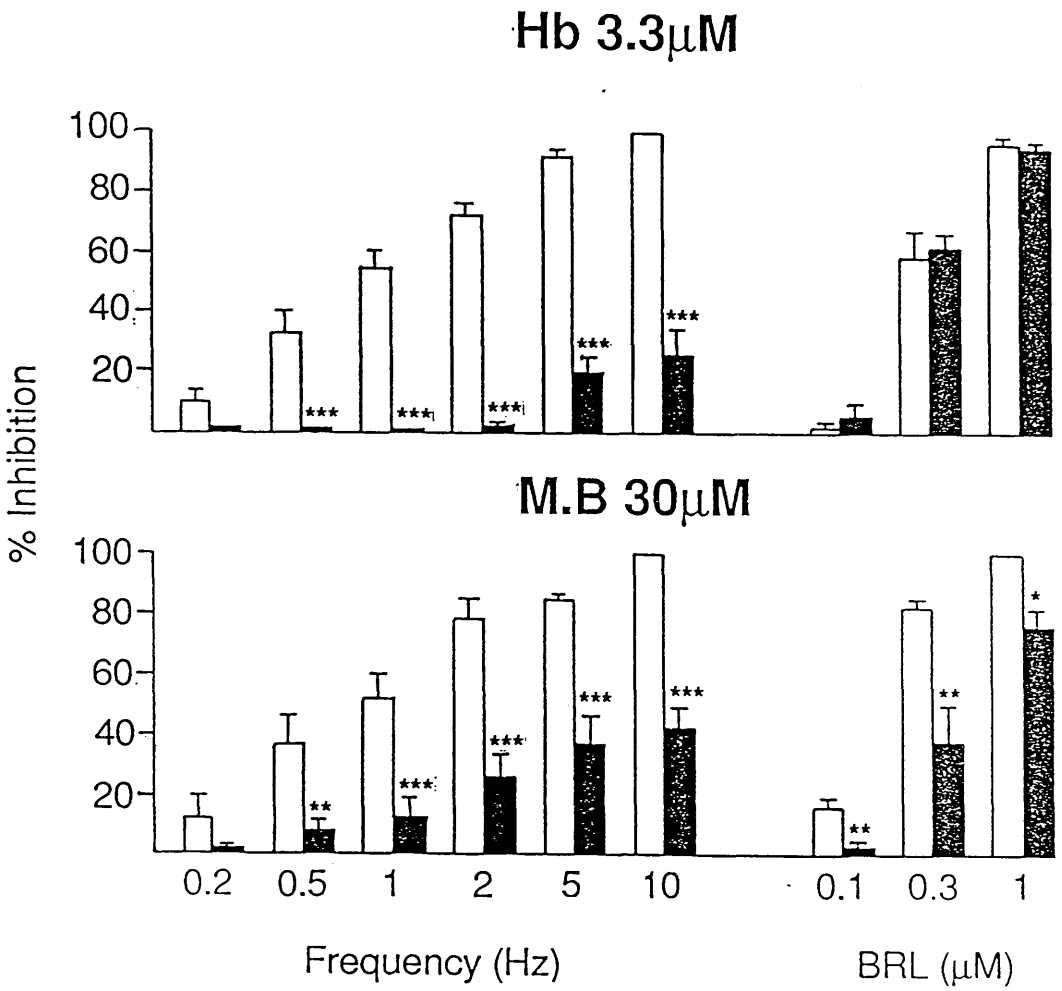

Figure 38 A histogram of the responses of BRP to superamaximal field stimulation of the NANC nerves at the frequencies shown and to three concentrations of cromakalim (BRL) in the absence (open column) and presence of either haemoglobin (3.3 μM) or methylene blue (30 μM, (closed column). Tone was induced with guanethidine (1 μM). Haemoglobin greatly reduced the response to NANC field stimulation but had no effect on the response to cromakalim. Methylene blue significantly reduced responses to both stimuli. Bars represent ± s.e. N=6. *P<0.05, **P<0.01 and ***P<0.001.

Figure 38. The frequency-response curves of NANC nerve stimulation in the BRP muscle were significantly reduced by both haemoglobin and methylene blue, whereas the concentration-response curves for cromakalim were unaffected by haemoglobin but reduced by methylene blue. The possible involvement of cyclic GMP or cyclic AMP was next investigated directly by measuring the effects of cromakalim on the levels of these second messengers.

EFFECTS OF CROMAKALIM ON CYCLIC NUCLEOTIDES LEVELS

From the results described earlier in this chapter the BRP muscle is the most sensitive tissue to cromakalim. In this preparation, both isoprenaline and sodium nitroprusside induce a powerful relaxation (Gillespie et al., 1982; Bowman et al., 1982), the former through β-adrenoceptors coupled presumably to adenylate cyclase and the latter by direct stimulation of soluble guanylate cyclase. The BRP muscle was therefore used to see whether cromakalim raises the level of either cyclic nucleotide. As controls, isoprenaline was used to stimulate cyclic AMP production and sodium nitroprusside was used to stimulate cyclic GMP production. Both cromakalim and isoprenaline at the highest concentrations used caused complete relaxation of the BRP muscle. The measurements in the early experiments were made 2 minutes after adding the relaxant drug to the bath and at a time, at least with the higher concentrations of cromakalim (3×10^{-6}M, 3×10^{-5}M) when the relaxation

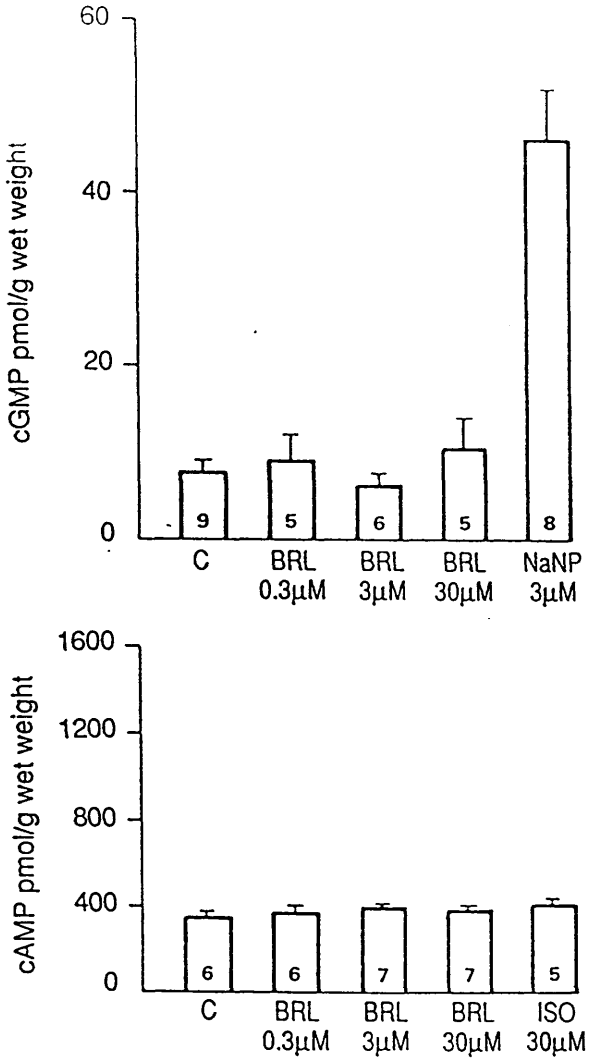

Figure 39 A histogram to show the effects of increasing concentrations of cromakalim (BRL) on the levels of cyclic GMP (upper) and cyclic AMP (lower) in the bovine retractor penis. Concentrations of cromakalim between 0.3 and 30 μM had no effect on the levels of either nucleotide. The control levels of cyclic AMP and cyclic GMP in tissues without adding drugs (c) are also shown. By contrast, sodium nitroprusside (NaNP, 3 μM) increased cyclic GMP by about six times. Isoprenaline (Iso, 30 μM) failed to increase cyclic AMP even though it produced complete relaxation in all preparations. The numbers in each column show the number of animals used and the vertical lines the s.e. mean. In the experiments with isoprenaline, prazosin ($3x10^{-7}M$) was added to the bath to block α-adrenoceptors.

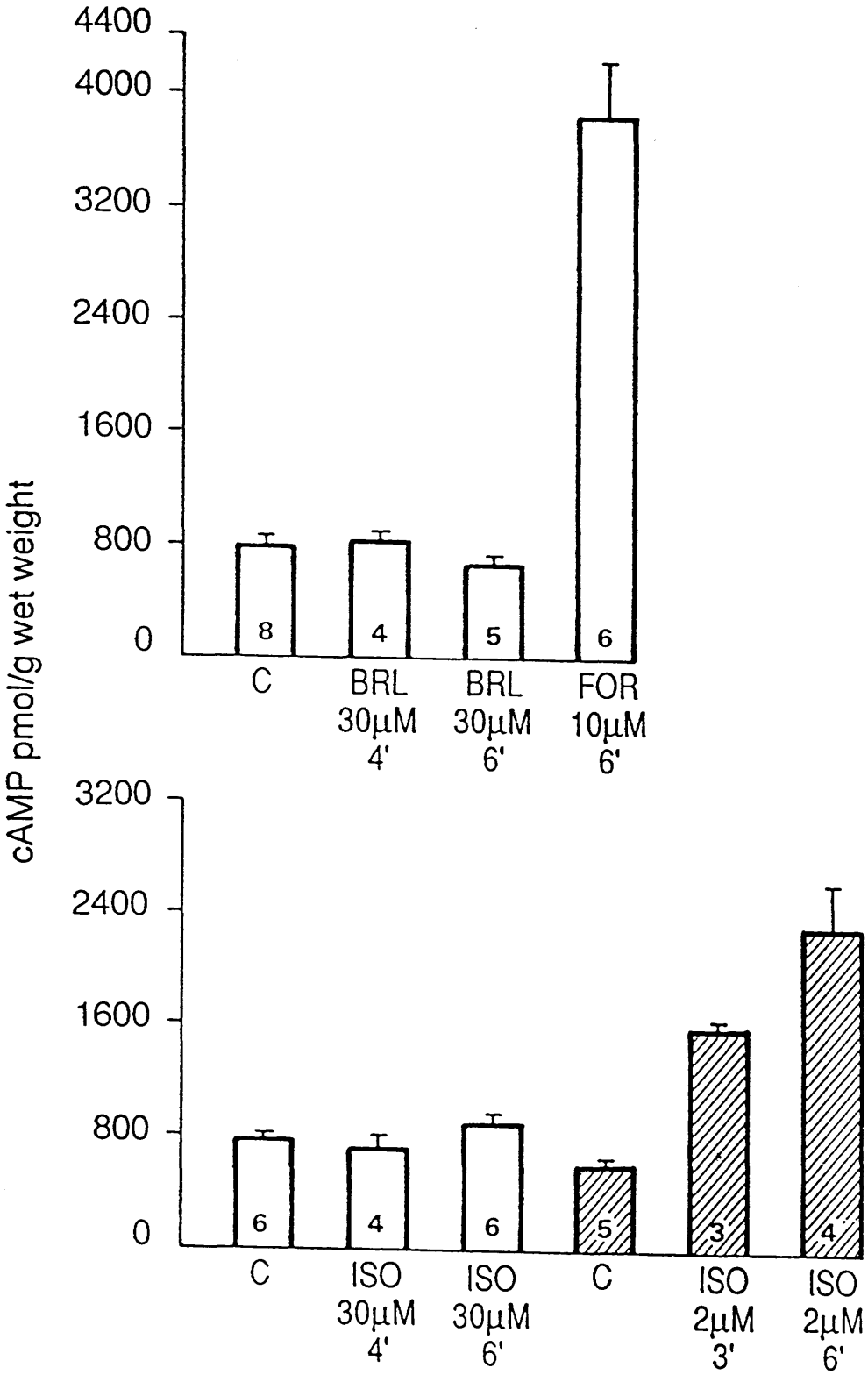
cAMP pmol/g wet weight
4400
4000
3200
2400
1600
800
0
8
4
5
6
C
BRL 30µM 4'
BRL 30µM 6'
FOR 10µM 6'
3200
2400
1600
800
0
6
4
6
5
3
4
C
ISO 30µM 4'
ISO 30µM 6'
C
ISO 2µM 3'
ISO 2µM 6'

Figure 40 A histogram to show the effects of cromakalim (BRL), forskolin (For) and isoprenaline (Iso) at different times after addition on the levels of cyclic AMP in the bovine retractor penis (open columns). The effects of isoprenaline on the levels of cyclic AMP in the rabbit uterus are also shown (hatched columns). The control levels of cyclic AMP in tissues where no drugs were added (c) are also shown. Neither cromakalim nor isoprenaline, each a at concentration of 30 μM, increased the levels of cyclic AMP either after 4 or 6 min exposure. In contrast, forskolin at 6 min incubation time increased the levels of this nucleotide, by approximately five times in the BRP. In the rabbit uterus, isoprenaline in an even lower dose significantly increased the levels at 3 min and had a greater effect at 6 min. The numbers in each column show the number of experiments and the vertical lines the s.e. mean. In all experiments with isoprenaline, including controls, Krebs' saline contained prazosin (3×10^{-7}M) to block α-adrenoceptors.

was at its maximum. As Figure 39 shows, cromakalim at all the concentrations tested raised neither cyclic AMP nor cyclic GMP levels. Sodium nitroprusside (3×10^{-6}M), by contrast, increased cyclic GMP content about six fold (Fig 39a). Surprisingly, isoprenaline (3×10^{-5}M), in spite of relaxing the muscle completely, had no effect on the levels of cyclic AMP (Fig 39b). The inability to show an effect of this drug on cyclic AMP threw doubt on the significance of the negative results with cromakalim.

It was considered that 2 minutes incubation period was not optimal for observing cyclic AMP increases since in the rabbit uterus, for example, the levels reached depends on the incubation time (Nesheim et al., 1975). For this reason, the effects of both cromakalim and isoprenaline on levels of cyclic AMP were examined at different incubation times. Also, a new control, forskolin, which directly stimulates adenylate cyclase was introduced. As Figure 39 shows neither cromakalim nor isoprenaline raised cyclic AMP levels but forskolin (10 μM), increased the cyclic AMP levels about five fold (Fig 40a). As an additional control the effects of isoprenaline on cyclic AMP levels in the rabbit uterus was examined. As expected, isoprenaline at 3 μM produced an increase in cyclic AMP content which was maximum at 6 minutes (Fig 40b), a time already examined in the experiments on the BRP muslce.

In conclusion, cromakalim can produce powerful relaxation in a variety of smooth muscle preparations. This effect is not linked to changes in the levels of cyclic nucleotides.

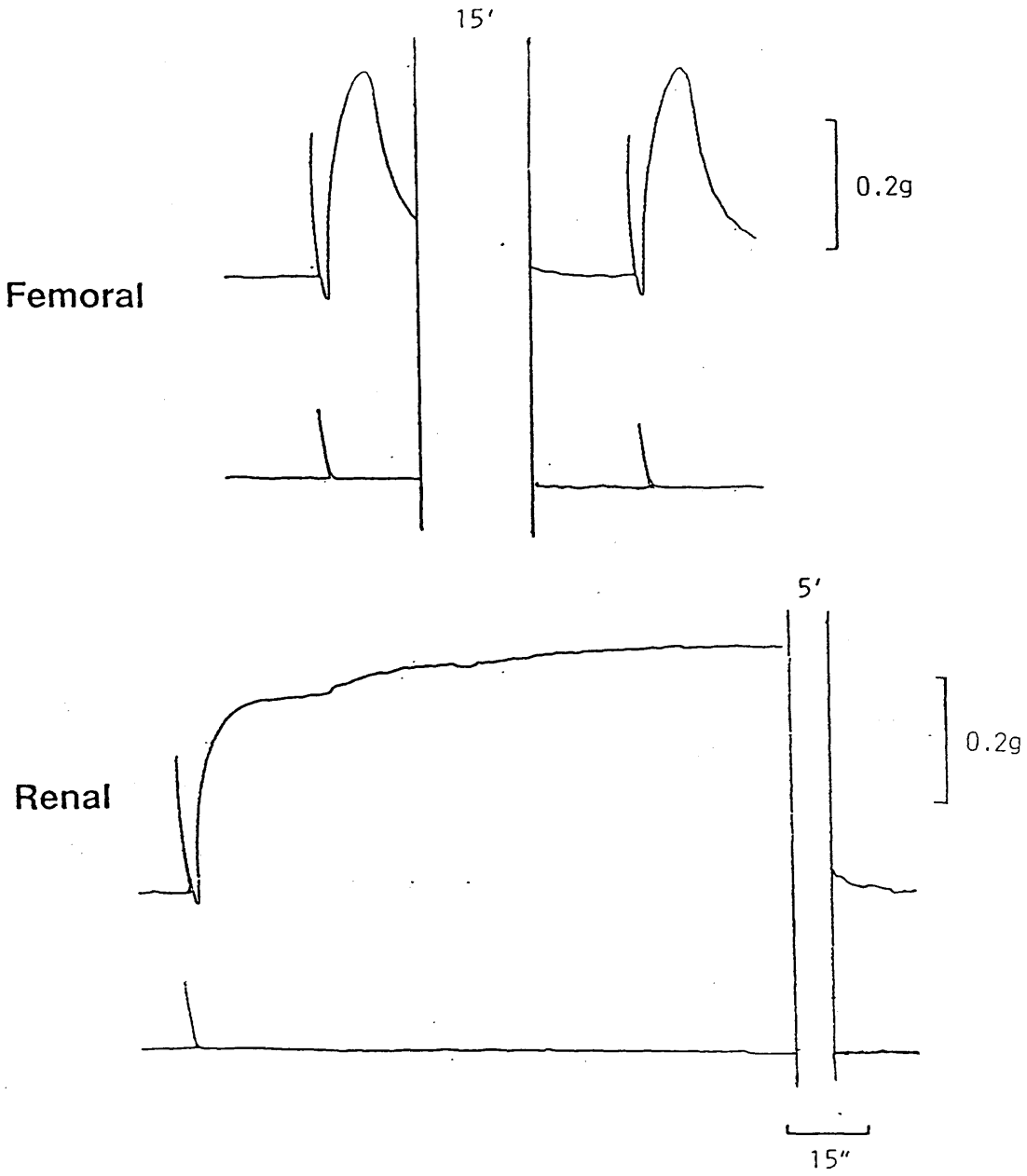

Figure 41 This shows typical responses to stretch in the rabbit femoral artery (upper records) and renal artery (lower records). A 100 ms stretch with a setting of T1P4 produced contractions in both muscles. In the femoral artery the response was phasic, but in the renal it was long-lasting and returned to the baseline only after five minutes. Both phasic and tonic responses were repeatable if sufficient time (15 min) was allowed between stimuli.

STRETCH-INDUCED CONTRACTION

These experiments were performed on vascular smooth muscle since this is known to be relaxed by cromakalim and unlike the guinea-pig trachea, desensitization does not occur. Three blood vessels were chosen, the rabbit abdominal aortic strip and femoral and renal arteries. These were prepared as spiral strips and with their endothelium removed. These were chosen because the aorta is a large elastic artery, the femoral is a muscular artery and the renal artery is a vessel known to display autoregulation. It was felt the latter might show a higher sensitivity to stretch since that is presumably the stimulus for autoregulation. The abdominal aorta rarely contracted in response to stretch. This may be because of the large proportion of elastic and collagen fibres in its wall which probably take most of the strain of the pull. Both femoral and renal arteries consistently responded with a contraction in response to stretch. Typical responses for each vessel to T1P4 at 100 ms are shown in Figure (41). In the femoral artery the usual response was a phasic contraction reaching its maximum after 5-10 seconds and gradually decaying back to the baseline. In the renal artery the response to stretch was normally maintained for up to 5 minutes or sometimes even longer (see Fig 41) and this may be some measure of its autoregulatory capacity. The response to a given magnitude of stretch was repeatable. The responses to stretch in both tissues are not due to the stimulation of nerve fibres, since tetrodotoxin (5×10^{-7}M) was without effect on both kinds of

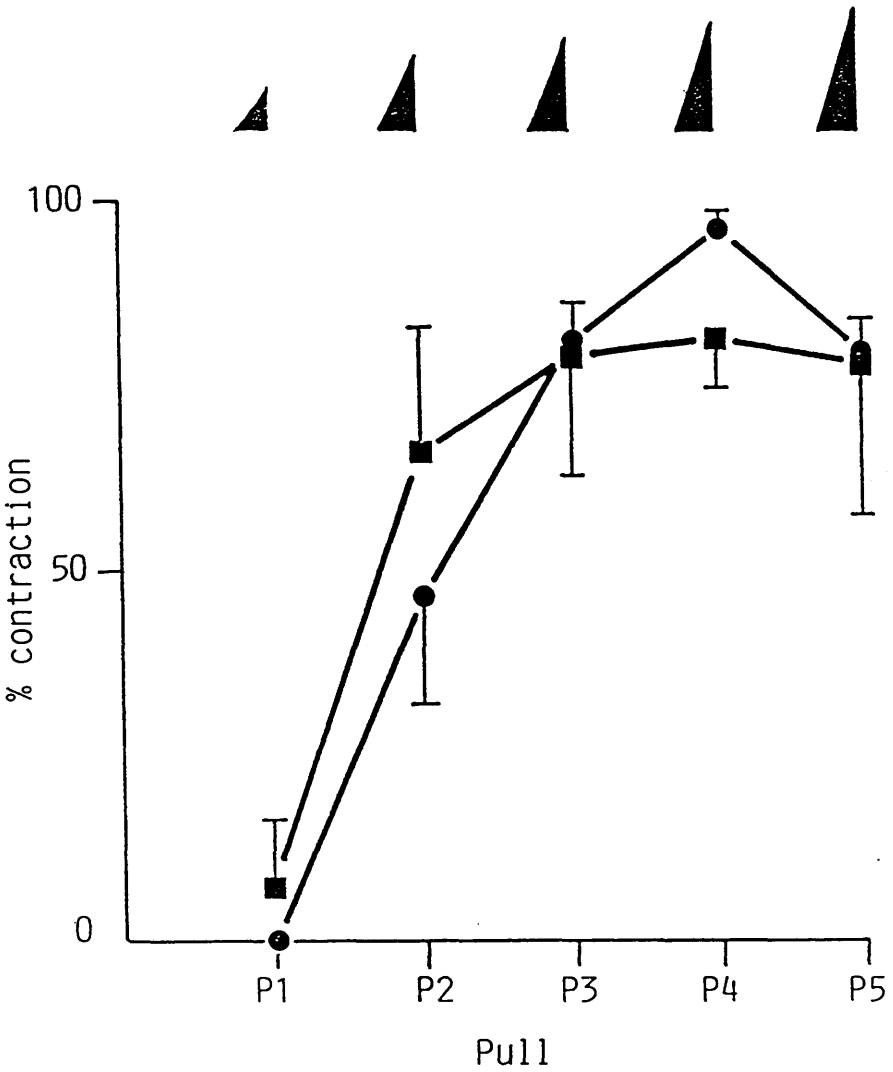

Figure 42 Stretch-response curves to different degrees of stretch in rabbit renal and femoral arteries. Stretches at a constant T1 (200 ms) here with different pull distances as shown diagramatically above each response produced contractions in both renal (•) and femoral arteries (■). Bars represent ± s.e. N=5 for renal artery and N=3 for femoral artery.

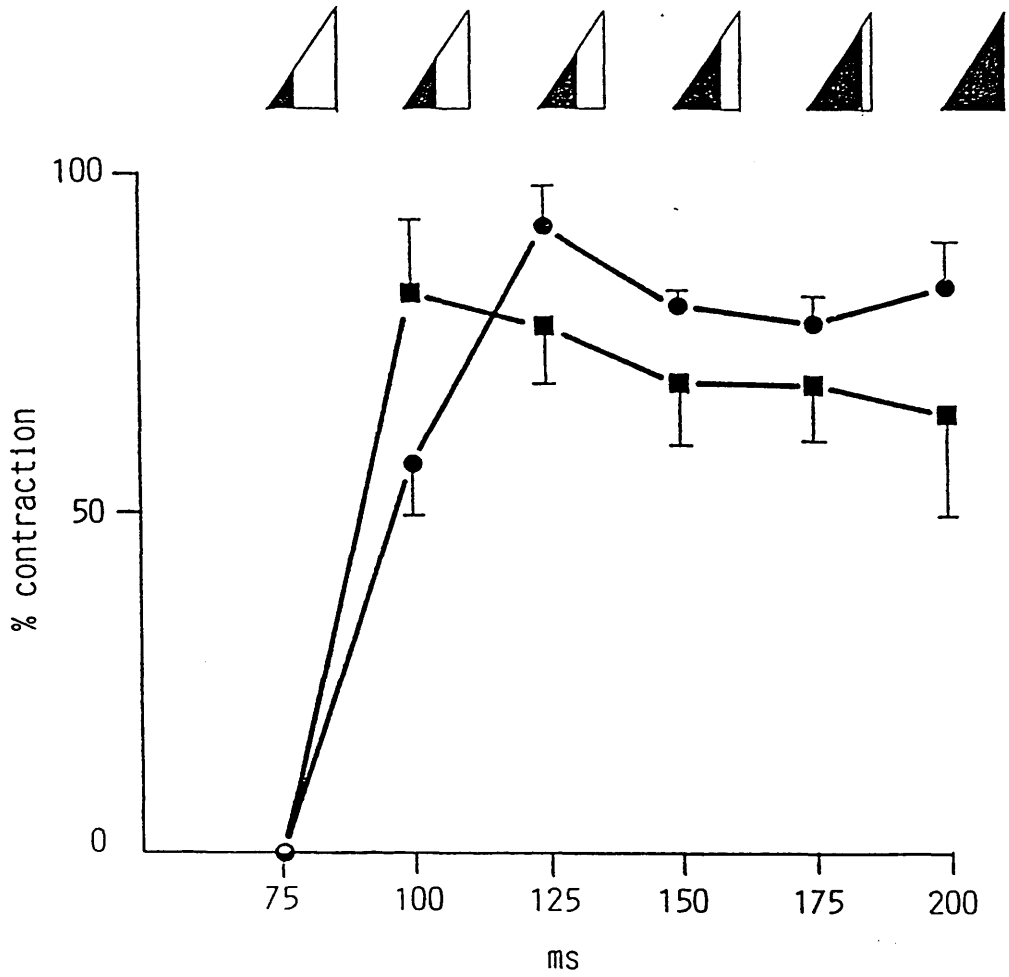

Figure 43 Stretch-response curves of the rabbit femoral (■) and renal (●) arteries at a constant rate of stretch. The control unit was set for T1P4 and fractions of the total pull selected by varying the digitimer between 75 ms and 200 ms. The degree of stretch is shown diagramatically above each response. Both arteries contracted in response to stretch and there was little difference in their thresholds. Bars represent ± s.e. N=5.

contractions. The responses to stretch are also not due to the stimulation or release of endothelium-derived contractile factor (EDCF) (Katusic et al., 1987) since the endothelium was removed in both test preparations. The effects of varying the rate and the magnitude of stretch from T1 (200 ms) to T5 (3900 ms) was investigated. In preliminary experiments, different durations of stretch were tested (as shown in Fig 28b). A quick pull at T1 was found most effective and T5 showed little or no effect in both tissues. This short duration of T1 (200 ms) was then used in experiments in which the length of the pull was varied by altering the P setting. The maximal response was reached with a fairly long pull of 15 mm (P4). The renal and femoral arteries were equally sensitive to stretch. This is illustrated in Figure 42 where with a fast pull (T1), the distance was steadily increased from 5 mm (P1) to 17 mm (P5). The responses were graded, with a threshold at or just above P1 and rapidly increasing to reach a maximum at P4. However, in this case the rate of stretch was increased with the distance pulled (as Fig 28a shown). To avoid this, the experiment was repeated by using the digitimer to select increasing fraction of a fixed saw-tooth waveform. In these experiments the rate of stretch was constant and the length of the pull was controlled by the time setting of the digitimer which determined how much of the full P value was used (as Fig 28c shows). The results of the two tissues in response to this kind of stretch are illustrated in Figure 43. In comparison with Figure 42, the response to a constant rate of stretch shows little change with time and distance of the pull. With a pull lasting only 75

ms there was no response to stretch in both arteries. Increasing this to 100 ms produced a maximal response in the femoral artery and a near maximum in the renal artery. A comparison of the results in Figures 42 and 43 suggests that the rate of stretch is an important variable. Consistent with this was the observation that slow stretches produced no active response from the muscle suggesting some adaptation can take place.

The Mechanism of the Contractile Response to Stretch

From the above preliminary results it was decided to use the T1P4 which corresponds to a maximal stretch with 200 ms every 15 minutes. Smaller stretches but at the same rate, which produced nearly the maximal response in both tissues, were then selected by the digitimer. A digitimer setting of 100 ms corresponding to a 7 mm stretch was used as the standard stimulus. With a preparation length of 10 mm, this corresponded to a stretch of 70% of the initial length. This stimulus elicited a stable contractile response which, with suitable rest periods, could be continued for several hours without decline.

The intention in developing these preparations were to have an alternative to raised potassium as a method of depolarizing smooth muscle and causing contraction through voltage-operated calcium channels. The assumption was that stretch caused depolarization allowing extracellular calcium to enter through channels opened by the

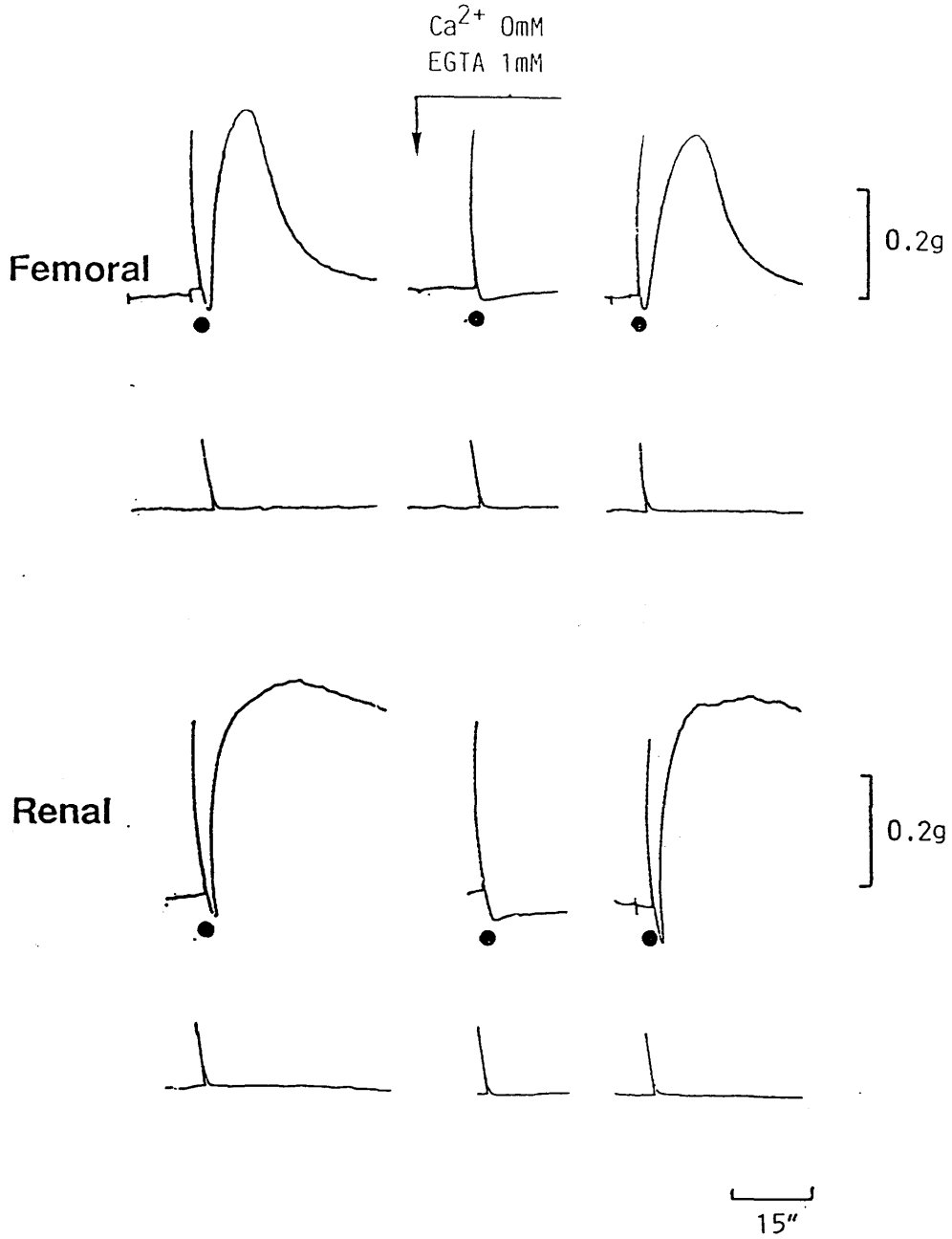

Figure 44 The responses to stretch of the rabbit femoral (upper records) and renal (lower records) arteries and the effect of removing calcium on this response. In each set of records the first response is the control, the second in the presence of zero calcium and the third, 15 min after reintroduction of normal calcium-containing Krebs' saline. The zero calcium Krebs' saline contained EGTA 1 mM. The stretch stimulus was 100 ms of a pull at T1P4. Removing calcium completely abolished the response in both arteries and this effect was completely reversed by reintroduction of calcium containing Krebs' saline.

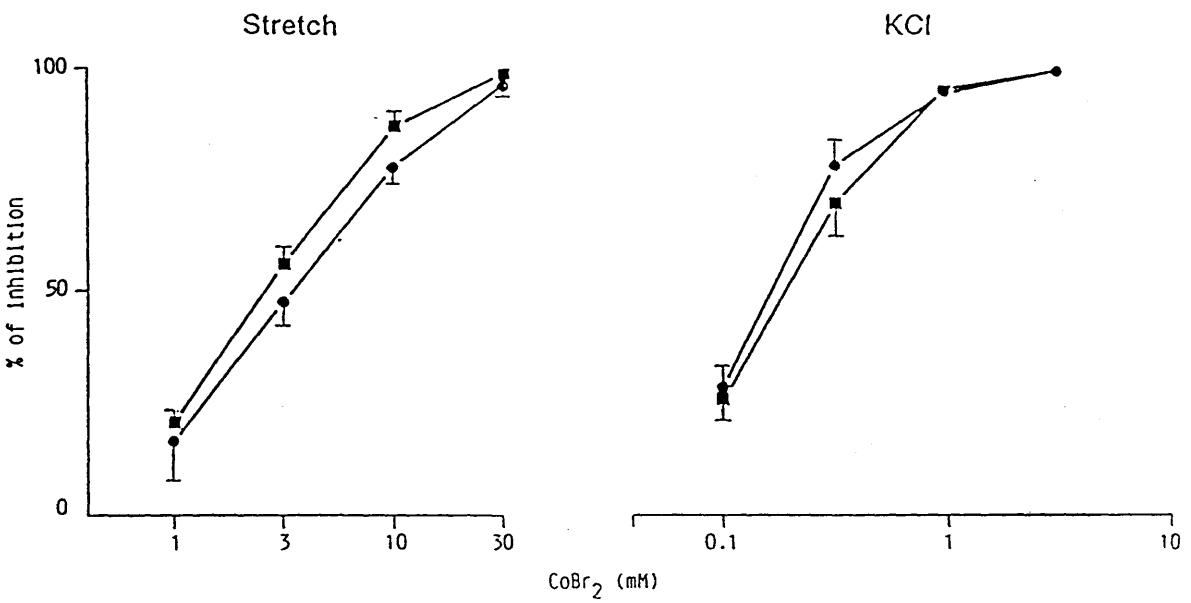

Figure 45 Log concentration-response curves for the effects of cobalt bromide on the responses of the rabbit femoral (■) and renal (●) arteries to stretch (a) and to 30 mM KCl (b). The stretch stimulus was 100 ms with a puller setting of T1P4. Cobalt inhibited the response in both arteries to either stretch- or potassium-induced tone. The tone raised by potassium was about ten times more sensitive to inhibition than that obtained to stretch. Bars represent ± s.e. N=4 except for the potassium stimulated femoral artery where N=5.

lowered membrane potential. The first experiment, therefore, was to confirm the dependence on external calcium entry and whether the channels involved had similar properties to those opened by raised potassium.

(1) The Effect of Removing External Calcium After control responses to the standard pull stimulus had been produced, the normal Krebs' saline was replaced by Krebs' saline containing no calcium and to which EGTA 1 mM had been added. An example of the results obtained under these conditions is shown on Figure 44. The omission of calcium from the Krebs' saline plus the chelation of any residual calcium in every instance resulted in a complete loss of the response to stretch which was completely reversed by returning to normal calcium-containing Krebs' saline. This data suggests that contractions induced by stretch in both renal and femoral arteries are entirely dependent on the presence of external calcium.

(2) The Effect of Cobalt Bromide Cobalt can block non-specifically all calcium channels (Anderson et al., 1971). Its effect on the response to stretch and to raised potassium is illustrated in Figure 45. In concentrations between 1.0 and 30 mM it blocked completely the response to stretch, and at lower doses of between 0.1 and 3 mM it completely abolished the contractile response to raised

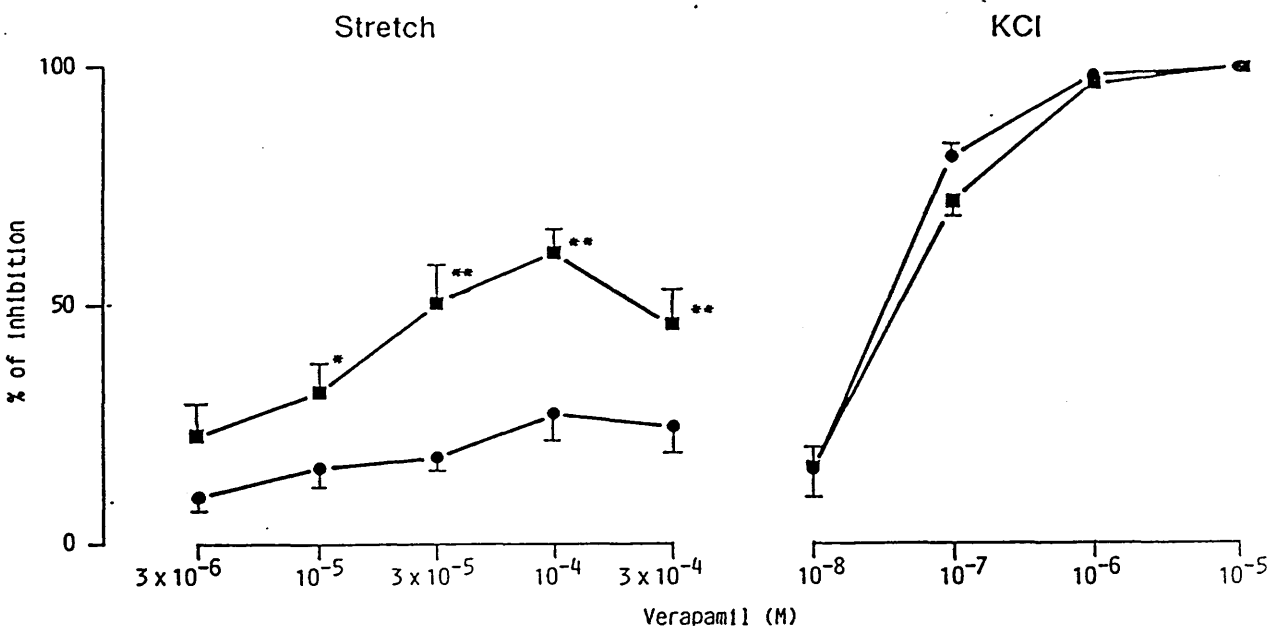

Figure 46 Log concentration-response curves for the effects of verapamil on the responses of the rabbit femoral (■) and renal (●) arteries to stretch (a) and to 30 mM KCl (b). The stretch stimulus was a 100 ms setting of the digitimer at a T1P4 setting of the puller. Verapamil effectively and equally abolished the tone raised by potassium but was much less effective on the tone induced by stretch. Stretch-induced contraction of the renal artery was much less susceptible to the blocking action of verapamil than was that of the femoral artery. Bars represent ± s.e. N=6 with the exception of the potassium-stimulated renal artery where N=5. *P< 0.05, **P<0.01.

potassium (30 mM). There was no significant difference in sensitivity between the renal and femoral arteries to blockade by cobalt bromide. Taken with the previous results with zero calcium these results confirm the complete dependence of the response to stretch and to raised potassium on the entry of external calcium. The greater sensitivity of the calcium channels opened by raised potassium to blockade by cobalt might indicate a difference between the channels opened by the two stimuli. Alternatively, it could be due to a greater number of channels being opened by stretch though this is unlikely since the magnitude of the response is smaller than the response to raised potassium.

(3) The Effect of Verapamil Verapamil is an organic calcium blocker which is considered to be relatively selective for voltage-operated channels. Its effects on the contractile response to stretch and to raised potassium are shown in Figure 46. Verapamil's effects revealed a considerable difference between the channels opened by potassium and those opened by stretch. The response to raised potassium was completely blocked in both arteries at a concentration of 10^{-6}M verapamil. In contrast, the response to stretch in the femoral artery was reduced by a maximum of 60% and this required a concentration of 10^{-4}M verapamil. In the renal artery where the contraction is maintained for a few minutes, as described previously, it contains two components, the tonic and the phasic. The difference to the inhibition

effect of verapamil between these two components was marked. The tonic component of contraction in the renal artery was more sensitive than the phasic one to verapamil. The inhibition of the tonic component by verapamil was concentration-dependent. Since the percentage inhibition was calculated by measuring the peak response, the inhibition of the tonic component did not show on concentration-response curves to verapamil. The phasic component of the renal artery was less sensitive than that of the femoral artery to verapamil in which high concentrations of verapamil produced even less inhibition of this response and gave a maximum of 28%. This difference between the two arteries together with the prolonged contractile response to stretch in the renal artery may indicate the special properties of this vessel in promoting autoregulation.

Effects of Cromakalim on Contraction Induced by Stretch

From previous results, the contractions induced by stretch in both renal and femoral arteries are due to the influx of extracellular calcium. The nature of the channel controlling influx is not clear, but it is obviously not a verapamil-sensitive calcium channel. As cromakalim can open potassium channels in a variety of smooth muscles, it was of interest to see whether it reduced contractions induced by stretch. This drug proved difficult to study particularly in the femoral artery because its effectiveness declined with time. Often the

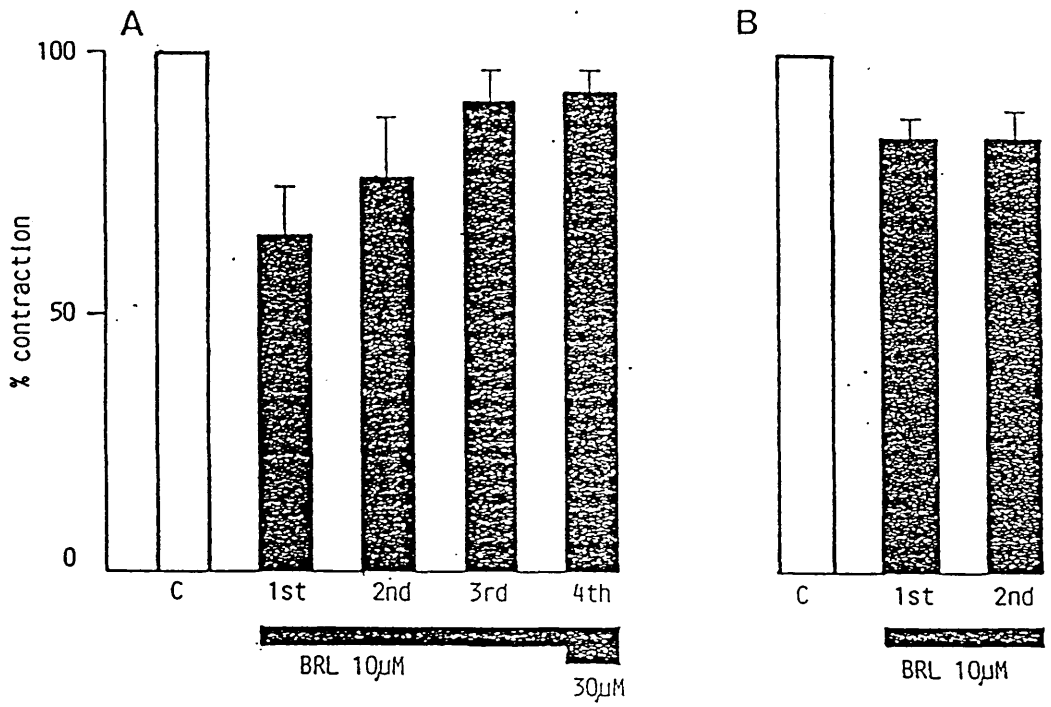

Figure 47 A histogram of the effects of cromakalim on stretch-induced contractions in the rabbit femoral (A) and renal (B) arteries. The open columns show the control response to stetch. The closed columns show the response to the next four stretches in the presence of cromakalim (10 μM). The initial inhibition of contraction in the femoral artery diminished and almost disappeared with time. Even increasing the concentration of cromakalim to 30 μM did not increase the blockade. The renal artery gave a smaller initial inhibition with cromakalim but this shows less evidence of desensization. Bars represent ± s.e. N=6 for femoral artery and N=4 for renal artery.

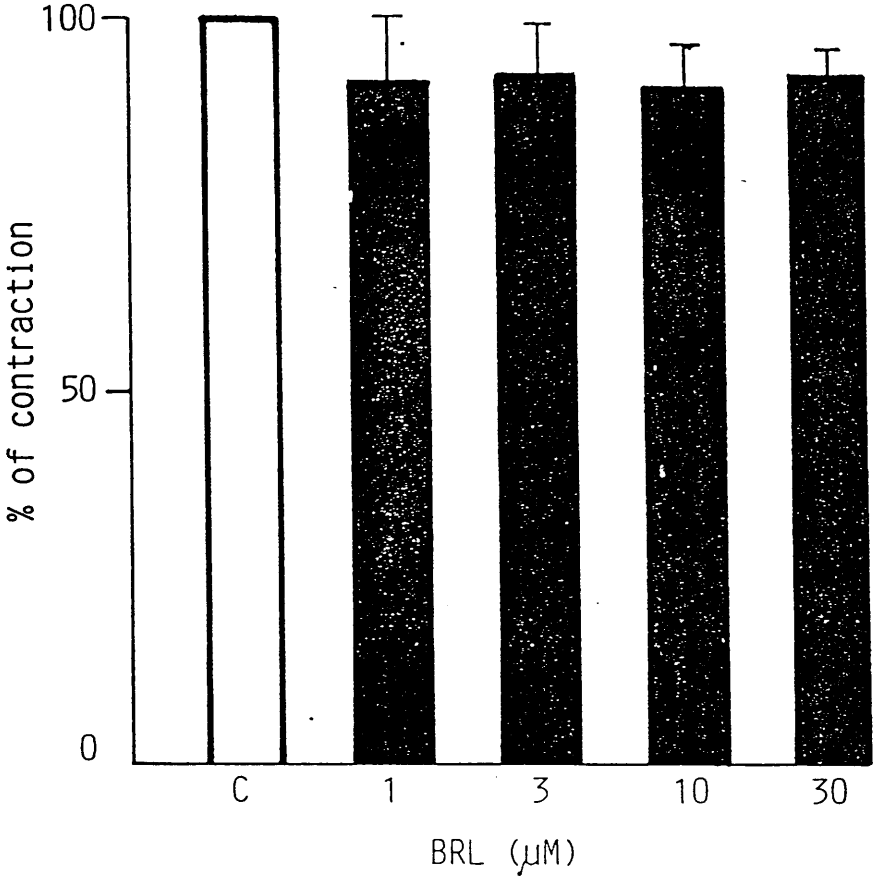

Figure 48 A histogram of the effects of cumulative additions of cromakalim on stretch-induced contraction in the rabbit femoral artery. The concentrations of cromakalim are shown. A low concentration (1 μM) of cromakalim produced only 10% inhibition of the contraction. Increasing the concentration of cromakalim did not increase the magnitude of the inhibition. Bars represent ± s.e. N=6.

most effective concentration was the first irrespective of concentration. For this reason, a rather high concentration (10^{-5}M) was chosen and the effect of this single concentration measured. This therefore, reduced the time available for desensitization. Figure 47a summarises all the experiments in which cromakalim (10^{-5}M) was added after the control response to stretch. In the femoral artery the first contraction in the presence of cromakalim was reduced by about 35%, the second by 24% and the third and subsequent contraction by less than 10% (Fig 47a). In the renal artery the effects of cromakalim were slight. The first contraction in the presence of cromakalim (10^{-5}M) was reduced by 15% and the degree of inhibition of the second contraction was similar (Fig 47b). As the inhibitory effect of cromakalim on stretch in this preparation was so small, it was difficult to construct concentration-response curves. Figure 48 summarizes the responses to stretch in the presence of cumulative concentrations of cromakalim ranging from 1 to 30 μM. Because desensitization to cromakalim develops gradually, no concentration-dependent relationship could be seen (Fig 47). The results with cromakalim resemble those obtained with verapamil in so far as both were more effective in the femoral than the renal artery but with the difference that there was no evidence of desensitization with time to verapamil. The results with cromakalim again point to the difference between the two blood vessels. The small magnitude of the relaxation and the evidence of desensitization also throw doubt on whether the effects demonstrated here are related to the hypotensive action of cromakalim.

DISCUSSION

The first approach to the mode of action of cromakalim was to study the influence of the mechanism underlying contraction on the efficacy of the drug. Smooth muscle may be induced to contract through either voltage-dependent or voltage-independent mechanisms (Bolton & Large, 1986). In some smooth muscles such as the vas deferens and certain blood vessels both mechanisms operate. In the present experiments four different smooth muscles were examined and four different agonists were used to induced tone. The guinea-pig taenia coli is spontaneously active, displaying waves of depolarization culminating in spike potentials and contraction. Acetylcholine and histamine can augment this spontaneous depolarization (Bülbring, 1955). The BRP muscle and the guinea-pig trachea show no spontaneous spike potentials or rhythmic mechanical activity but they do develop spontaneous tone probably because their membrane potential is at a level at which voltage-sensitive calcium channels are open. Finally, the rabbit aortic strip which in the absence of agonist has a high steady membrane potential and no tone (Casteels et al., 1977a,b). The four agonists, though not all active on each preparation, were chosen to provide as wide a range of coupling mechanism as possible. Potassium contractures are entirely dependent on membrane depolarization and the opening of voltage-sensitive calcium channels; consistent with this was the observation in this thesis and by others that organic calcium antagonists such as verapamil and nifedipine block these contractures in several smooth muscles including the guinea-pig and the rat tracheal muscle (Cheng & Townley, 1983; Cerrina et al., 1983; Foster et al.,

1984; Baba et al., 1985; Nielsen-Kudsk et al., 1986). Both acetylcholine and histamine depolarize guinea-pig tracheal muscle through muscarinic and histamine H_1-receptors, respectively (Ahmed et al., 1984). To this extent they might be expected to resemble the effects of raised potassium. Organic calcium antagonists and removal of external calcium do not, however, abolish the contractions induced by acetylcholine and histamine (Ahmed et al., 1984; Ahamd, 1985; Foster et al., 1984; Cerrina et al., 1983), suggesting other forms of coupling and other sources of calcium must be important. Most authors conclude that muscarinic agonists and histamine act mainly by releasing intracellular calcium. A likely mechanism particularly for acetylcholine is the production of inositol polyphosphates, especially inositol triphosphate (IP_3). An increase in IP_3 and phosphatidic acid in dog tracheal muscle stimulated with acetylcholine has been demonstrated (Hashimoto et al., 1985). The mechanism of action of histamine on this tissue is less clear. In the guinea-pig ileum histamine increases phosphatidyl-inositol turnover through H_1 receptor stimulation (Jafforji & Michell, 1976). On the dog tracheal smooth muscle, however, histamine failed to increase PI turnover (Hashimoto et al., 1985). An additional complication is the presence of H_2 receptor for histamine mediating relaxation (Opkako et al., 1978) through the activation of adenylate cyclase in the guinea-pig lung (Foreman et al., 1986). The action of the fourth agonist, noradrenaline is also complex, at least on vascular smooth muscles. In some arteries, endogenous neurotransmitter liberated by sympathetic nerve stimulation

gives rise to excitatory junction potential (EJP's) and even spike potentials which are insensitive to α-adrenoceptor blocking drugs. This fast EJP is followed by a small, slow depolarization which is sensitive to α-adrenoceptor blocking drugs and seems to be more important than the EJP in promoting muscle contraction (Bolton & Large, 1986). Exogenous noradrenaline ($<10^{-7}$M) added to the bath mimics well the contractile effect of sympathetic nerve stimulation but does not cause depolarization or spike potentials (Casteels et al., 1977a,b). Two explations have been offered for this difference between exogenous and endogenous (nerve-liberated) noradrenaline. Noradrenaline liberated by the nerves only may have access to receptors (junctional receptors) which can open ion channels and depolarize the membrane potential whereas exogenous noradrenaline has access only to extrajunctional receptors which are coupled biochemically (Hirst & Nield, 1981). Alternatively, the nerves liberate a second transmitter probably ATP (Sneddon & Burnstock, 1984a,b). ATP acting on purinergic receptor is responsible for opening ion channels for membrane depolarization. Evidence in supporting of this last hypothesis is provided by the stable ATP analogue α,β-methylene ATP which reduces or abolishes EJP's in the guinea-pig vas deferens and the rat tail artery (Snedden & Burnstock, 1984a,b) and the rabbit ear artery (Allcorn et al., 1985). Stimulation of α_1-adrenoceptors is known to activate PI turnover (Jafforji & Michell, 1976; Burnstock, 1986) and this may be the form of biochemical coupling which controls contraction. Recently, Takuwa & Rasmussen (1987) have reported that both noradrenaline and

histamine can act via the phosphoinositide system and lead to a rise in free cytoplasmic calcium as measured by aequorin in the rabbit aorta. The increase in cytoplasmic free calcium could be divided into a transient peak due to mobilization of calcium from a caffine-sensitive intracellular calcium and a plateau after the peak which was due to influx of extracellular calcium since it could be abolished by removing calcium from the solution. These authors also reported the interesting observation that the atrial natriuretic peptide (ANP) could block the plateau phase induced by both agonist and the transient phase produced by noradrenaline but was ineffective against the transient phase due to histamine. Their explanation was that ANP acted to inhibit the coupling of the α-adrenoceptor to phospholipase C but had no similar effect on the coupling of the histamine H_1 receptor. On the BRP muscle, noradrenaline added to the bath, liberated by nerve stimulation, or released indirectly by guanethidine causes small depolarization and contraction (Samuelson et al., 1983; Byrne & Muir, 1984). Samuelson et al. (1983) found no difference between the effects of guanethidine (endogenous noradrenaline) and exogenous noradrenaline though Byrne & Muir (1984) found the electrical effects of nerve stimulation were not blocked by prazosin whereas those of exogenous noradrenaline were.

Given these differences in the mode of action of agonists on the four muscles some notable differences in the efficacy of cromakalim might have been expected. In particular, given its proposed mode of action through hyperpolarization, cromakalim would be expected to be particularly effective against raised potassium in all tissues and the

least effective against exogenous noradrenaline in blood vessels where there is little or no depolarization. Such results were not found. Potassium contractures were not particularly sensitive to cromakalim and the aortic strip contracted with noradrenaline was not particularly insensitive.

The stretch-induced tone in the rabbit renal and femoral arteries, as in some other smooth muscle preparations is independent of innervation since tetrodotoxin at 5×10^{-7}M is without effect. The myogenic tone in both test preparations is dependent on the influx of calcium from extracellular space since removal of calcium from the Krebs' saline or in the presence of the non-specific calcium channel blocker, cobalt, completely abolished the active response to stretch. In the femoral artery the stretch-induced tone was more sensitive than renal artery to verapamil, although higher concentrations were required than to block the tone induced by potassium (30 mM). Desensitization to cromakalim was rapid in the femoral artery: the first stretch-induced response was blocked by about 40% but the third response was reduced by only 10%. However, both cromakalim and verapamil had little effect on stretch-induced tone in the renal artery. The reason for the difference between these two tissues are not clear. Very recently, several groups have reported that cromakalim may stimulate an ATP-sensitive potassium channel since the sulphonylureas such as glibenclamide, glyburide and glypizide, all block its effects on both mechanical and electrical activity in various tissues (Quast, 1988b; Escande et al., 1988; Winquist et al., 1989; Buckingham et al., 1989;

Wilson, 1989; Cavero et al., 1989). It is possible that the contraction induced by stretch in the renal artery does not involve ATP-sensitive potassium channels. Another possibility is that the calcium channels opened in these two test preparations by stretch are different. Similar results were reported by Bevan and his colleague who found that the calcium antagonist, diltiazem, blocks stretch-induced tone in the rabbit ear resistance artery and portal vein (Bevan, 1983; Hwa & Bevan, 1986b) but not in the rabbit mesenteric, renal, or basilar arteries etc. (Bevan, 1983). Since the myogenic tone in many blood vessels is resistant to organic calcium channel antagonists such as nimodipine, a potent calcium antagonist of potassium-induced contraction, PN200-110 and nifedipine, the selective antagonists to potassium-induced contraction (Hwa & Bevan, 1986b; Laher et al., 1988), the calcium channel opened by stretch is probably different from the voltage-sensitive calcium channel. If this is the case, then the different sensitivities of stretch-induced contraction in renal and femoral arteries to inhibition might reflect a difference in the ratio of the two ion channels.

Little is known about stretch-activated ion channels. Recently, stretch activated ion channels have been reported in single smooth muscle cells from the stomach of the toad bufo marinus (Kirber et al., 1987), yeast (Gustin et al., 1987), amphiuma red blood cells, xenopus myocytes (Yang & Sachs, 1987), vascular endothelial cells (Lansman et al., 1987) and chick skeletal muscle (MacGregor et al., 1989) etc. The properties of these channels are still not clear. In the case of

myogenically active vascular smooth muscle, mechanical distension probably causes influx of calcium through these channels.

The most significant finding in the present study was the desensitization to cromakalim in two test preparations, the guinea-pig trachea and the rabbit femoral artery.

A report by Allen et al. (1986) indicated cromakalim to be relatively ineffective against agonist-induced contractions but more effective against spontaneous tone. Their experiments were performed on the guinea-pig trachea, but instead of varying the concentration of cromakalim as was done in the experiments in this thesis, they used a fixed concentration of 10^{-5}M cromakalim and varied the concentration of agonist. The former was added some time before the concentration-response curve was established. As a result, cromakalim caused minor rightward shifts in the concentration-response curves of ACh and histamine. The explanation for the apparent insensitivity in their experiments may be that desensitization to cromakalim occurred when it was added before the agonist. In this thesis, cromakalim was tested in two different ways and each gave different results. When added at the height of the contracture it was a powerful relaxant but when added 30 minutes before adding the spasmogens such as carbachol or histamine, it lost half or more of its relaxant effect. The loss of sensitivity (or desensitization) to cromakalim occurred only in certain tissues such as the guinea-pig trachea; in the rabbit aortic strip no difference was detected when cromakalim was tested both ways. Clearly, the desensitization is not related to the spasmogen since the effect was

observed with both carbachol and histamine. In contrast, no desensitization to the effect of cromakalim was seen in the rabbit aorta when it was contracted with noradrenaline or histamine. The reason for the difference between these two tissues is not clear. It has been reported that in both tissues, cromakalim produced membrane hyperpolarization and increased $^{86}Rb^{+}$ efflux in the absence of spasmogen (Allen et al., 1986; Kreye et al., 1987a,b). In these experiments the longest time in measuring the membrane potential was 8 minutes after test tissues exposure cromakalim (Allen et al., 1986). If desensitization is time dependent, 8 minutes could be too short for "escape" to occur. This was ruled out in the present study, since cromakalim added 2 minutes before adding the spasmogen, still produced desensitization (data is not shown). Another possibility could be that the relaxant action of cromakalim may not always be associated with membrane hyperpolarization, i.e. desensitization affects only the mechanical response to cromakalim but not the electrical response. However, a opposite report from Nakao et al. (1988) showed that in the guinea-pig isolated mesenteric artery the hyperpolarizing action of high concentrations of cromakalim steadily diminished in the continuing present of drug for 13 minutes but the mechanical response was maintained. In addition, several authors have shown that the increase in $^{86}Rb^{+}$ efflux from the rat isolated portal vein induced by cromakalim is not sustained during prolonged contact with the drug (Coldwell & Howlett, 1986; Quast, 1987; Cook et al., 1988a). If this is the case, the question is therefore raised why this desensitization is only

observed in the guinea-pig trachea but not the rabbit aorta. There is no experimental evidence to explain this from present study. It is possible that in the guinea-pig trachea, the relaxant effect of cromakalim may depend on the concentration of intracellular calcium. When the cytoplasmic concentration of calcium is high, for example, in the presence of spasmogens such as carbachol or histamine, the enhanced calcium concentration may sensitize the ion channel to cromakalim in trachea. It is not clear in the rabbit aorta whether the higher resting membrane potential is due to the higher concentration of cytoplasmic calcium which reaches the threshold to open potassium channels. If this is true then it would be difficult to explain the desensitization in the femoral artery as it is a branch of the aorta. Whether at least a certain level of cytoplasmic calcium is required for the action of cromakalim cannot be deduced from the present result. In this thesis there was no observation of desensitization in the rabbit aortic strips stimulated either by noradrenaline or histamine. It is unfortunate that this tissue did not respond to stretch. If it had it might have been possible to test whether cromakalim's effect on this stretch response desensitized. If it did then clearly the desensitization could not be related to the tissue but to the mechanism of causing contraction. Equally, if it was known that the effect of cromakalim on rabbit femoral artery stimulated by agonists did not desensitize then a similar conclusion could be drawn. Unfortunately the last type of experiment was not done. There is, however, evidence from a similar potassium channel activator, nicorandil, which in addition to

sharing the same mode of action as cromakalim, also showed cross-desensitization in the guinea-pig mesenteric artery (Nakao et al., 1988). This drug did not show desensitization in either the rabbit renal or femoral artery contracted by noradrenaline (Shibata & Satake, 1987). If these results hold for cromakalim, which seems likely, then it does seem that while cromakalim can acutely cause relaxation of smooth muscle whatever the mechanism responsible for contraction, this effect is very different from that of other drugs active on ion channels. For example, the effect of cobalt or verapamil on the stretch responses, showed no evidence of desensitization even though the effect of verapamil was relatively weak. Furthermore drugs which block potassium channels such as tetraethylammonium (TEA), 4-aminopyridine or apamin do not show desensitization in a variety of test preparations. Desensitization may be a unique feature of this new group of potassium channel opening drugs rather than a characteristic of the potassium channels involved.

Clearly, the relaxant effect of cromakalim is not mediated through apamin-sensitive potassium channels. The results of this thesis are consistent with those of Weir & Weston (1986) who found in guinea-pig taenia coli that apamin failed to block the effects of cromakalim. In the guinea-pig trachea, where the relaxant action is also unaffected by apamin (Allen et al., 1986), verapamil induced a blockade. Kreye et al.(1987a,b) reported that in the rabbit aorta where the increases in $^{86}Rb^{+}$ efflux induced by cromakalim are reduced by D600, nifedipine, trifluperazine or exposure to Ca^{2+}-free solution containing 2 mM EGTA.

Following treatment with cromakalim, in the rabbit mesenteric artery, action potentials evoked by outward current pulses ceased before initiation of hyperpolarization, and in high K^+ solution, a small but significant inhibition of contraction occurred (Nakao et al., 1988). This also suggests that cromakalim can inhibit voltage-dependent calcium entry. Chiu et al. (1988), using the $^{45}Ca^{2+}$ flux techniques in rabbit aortic strips, reported that cromakalim (1-10 μM) inhibited contraction and calcium entry in response to noradrenaline and angiotensin II. Cain & Metzler (1985) showed that cromakalim is capable of reducing the slow inward (Ca^{2+}) current in guinea-pig papillary muscle. However, several pieces of evidence suggest that inhibition of a calcium current plays little role in the inhibitory action of cromakalim. Coldwell & Howlett (1987) have shown that cromakalim did not interact with 1,4-dihydropyridine binding sites in rabbit mesenteric artery. Clapham (1988) tested the cardioaccelerator response in pithed rat that distinguishes calcium entry blockers from other agents which have modes of action not involving direct blockade of calcium entry; they found that drugs such as nifedipine, verapamil, and diltiazem reduced stimulation-evoked sustained cardioaccelator responses in the pithed rat but cromakalim and nicorandil were without effect. This suggest cromakalim and nicorandil do not act through voltage-operated calcium channels. The results in this thesis are consistent with this interpretation but since only part of the action of cromakalim is lost in the presence of verapamil. The residual component may indicate an action of cromakalim on other potassium channels.

While verapamil reduced the relaxant effect of cromakalim on the guinea-pig trachea it enhanced that of isoprenaline. In this tissue isoprenaline is known to cause hyperpolarization; an effect reduced by TEA or procaine and due therefore to opening of potassium channels (Allen et al., 1985). However, while TEA blocked the hyperpolarization it did not prevent the relaxant effect suggesting that hyperpolarization does not play a large part role in the relaxant action of isoprenaline (Allen et al., 1985). In addition, slow waves in tracheal muscle may represent nascent action potentials (Kirkpatrick, 1981) and it might therefore be proposed that the relaxation induced by isoprenaline in this tissue results from the reduced frequency or abolition of slow waves. However, organic inhibitors of Ca^{2+} influx such as nifedipine and verapamil can abolish slow waves without lowering the spontaneous tone of guinea-pig trachea (Foster et al., 1984; Ahmed et al., 1985). Since the link between slow wave discharge and the maintenance of spontaneous tone is tenuous, slow wave suppression cannot be envisaged as playing a major role in the relaxation which follows β-adrenoceptor activation. If isoprenaline owes its relaxant effect to a cyclic AMP mediated sequestration of calcium inside the cell, then it may be that verapamil cuts off calcium entry through ion channels opened either by agonists used to induce tone or by the spontaneous fall in membrane potential underlying spontaneous tone. Ito & Itoh (1984) have reported that isoprenaline enhances the sequestration of free Ca^{2+} into the intracellular store sites in the cat trachea. In the case when calcium entry was blocked by verapamil, the action of isoprenaline would be

enhanced.

More difficult to explain is the increase in the relaxant effect of both isoprenaline and sodium nitroprusside in the presence of cromakalim. These results certainly show that desensitization to cromakalim did not extend to relaxants acting by other mechanisms. They also indicated that cromakalim does not inhibit adenylate cyclase and guanylate cyclase, a point of some importance in view of the failure of cromakalim to raise the level of either of these cyclic nucleotides. There is insufficient evidence to analyse the reason for the potentiation of isoprenaline and sodium nitroprusside. It may be that in the presence of cromakalim the activation of adenylate and guanylate cyclase is greater. Alternatively the effectiveness of the cyclic nucleotides on the appropriate kinases may be enhanced or the phosphorylation of target proteins by these kinases could be increased. In the absence of such information further speculation is pointless.

The levels of both cyclic AMP and GMP were measured under the influence of cromakalim, isoprenaline or sodium nitroprusside. Cromakalim had no effect on either nucleotide and this was consistent with the failure of haemoglobin to alter its effects. Methylene blue, which unlike haemoglobin enters the cell to act directly on the guanylate cyclase (in review see Ignarro & Kadowitz, 1985) did reduce the effect of cromakalim but this appears to be a non-specific action since it also lowered both inhibitory and motor nerve responses in the BRP muscle. This non-specific effect may explain an observation that methylene blue inhibited the cromakalim-induced increase in $^{86}Rb^{+}$ efflux

(Coldwell & Howlett, 1986). Cromakalim has also been reported to have no effect on cyclic GMP or AMP levels in the rabbit mesenteric artery or rat heart (Coldwell & Howlett, 1987).

An unexpected finding was the failure of isoprenaline in concentrations producing complete relaxation of the BRP muscle to raise the levels of cyclic AMP. This is unlikely to be a technical failure since increases in responses to forskolin could easily be measured as could the rise in the rabbit uterus with isoprenaline. There are an increasing number of references in the literature to similar discrepancies between a rise in cyclic AMP and the relaxant effect of isoprenaline. For example, in the bovine trachea and rat uterus propranalol has been reported to block the relaxant effect of isoprenaline but not the rise in cyclic AMP (Palocek & Daniel, 1971; Lau & Lum, 1983). In the rabbit uterus the reverse has reported, propranalol blocked the rise in cyclic AMP but not the relaxant effect (Nesheim et al., 1975). In the rabbit uterus both isoprenaline and prostaglandin E_2 (PGE_2) raised cyclic AMP and activated cyclic AMP dependent kinase but only isoprenaline caused relaxation; PGE_2 was a spasmogen. If contraction was produced by PGE_2, isoprenaline subsequently caused relaxation with no further change in cyclic AMP levels (Do Khac et al., 1986). The conclusion seems to be that not only is relaxation by isoprenaline independent of the hyperpolarization it sometimes produces, but it may also be independent of the rises in cyclic AMP levels which it often but not invariably produces. There may be a third mechanism of relaxation by isoprenaline acting through

β-adrenoceptors and the BRP muscle is one other tissue where this mechanism operates.

In conclusion, cromakalim relaxes four smooth muscle preparations contracted by four spasmogens. There was little corresponding variation in sensitivity to cromakalim. The relaxation of BRP muscle induced by cromakalim is not associated with an increasing in either cyclic nucleotide. An expected finding that isoprenaline caused maximal relaxation but did not raise the levels of cyclic AMP in the BRP muscle. The response to cromakalim in the guinea-pig trachea is partly lost in the presence of verapamil but it is unaffected by apamin in the guinea-pig taenia coli.

Stretch-induced contractions in both rabbit renal and femoral arteries are myogenic and calcium-dependent. Neither verapamil nor cromakalim can abolish the stretch-induced contraction in both tissues. The renal artery is less sensitive to these blockers than the femoral one.

Desensitization to cromakalim was occurred in the guines-pig trachea and rabbit femoral artery.

AHMED, F., FOSTER, R.W., SMALL, R.C. & WESTON, A.H. (1984). Some features of the spasmogenic actions of acetylcholine and histamine in guinea-pig isolated trachealis. Br. J. Pharmacol., 83, 227-233.

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

AKBARALI, H.I., BIEGER, D., OHIA, S.E. & TAIGGLE, C.R. (1988). Similarity of relaxations evoked by BRL 34915, pinacidil and field-stimulation in rat oesophageal tunica muscularis mucosae. Br. J. Pharmacol., 95, 519-525.

ALLCORN, R.J., CUNNANE, T.C., MUIR, T.C. & WARDLE, K.A. (1985). Dose contraction in the rabbit ear artery require excitatory junction potentials (ejp's) and 'spikes'? J. Physiol., 362, 30P.

ALLEN, S.L., BEECH, D.J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1985). Electrophysiological and other sapects of the relaxant action of isoprenaline in guinea-pig isolated trachealis. Br. J. Pharmacol., 86, 843-854.

ALLEN, S.L., BOYLE, J.P., CORTIJO, J., FOSTER, R.W., MOGEN, G.P. & SMALL, R.C. (1986). Electrical and mechanical effects of BRL 34915 in guinea-pig isolated trachealis. Br. J. Pharmacol., 89, 395-405.

ALZHEIMER, C. & BRUGGENCATE, G. (1988). Actions of BRL 34915 (cromakalim) upon convulsive discharges in guinea-pig hippocampal slices. Naunyn Schmiedebergs Arch Pharmacol., 337, 429-434.

AMBACHE, N. & KILLICK, S.W. (1978). Species differences in postganglionic motor transmission to the retractor penis muscle.

Br. J. Pharmacol., 63, 25-34.

AMBACHE, N., KILLICK, S.W. & ZAR, M.A. (1975). Extraction from ox retractor penis of an inhibitory substance which mimics its atropine-resistant neurogenic relaxation. Br. J. Pharmacol., 54, 409-410.

ANDERSON, N.C., RAMON, F. & SNYDER, A. (1971). Studies on calcium and sodium uterine smooth muscle excitation under current-clamp and voltage-clamp conditions. J. Physiol., 58, 322-339.

ANDREWS, H.E., BRUCKDORFER, K.R., CUNN, R.C. & JACOBS, M. (1987). Low-density lipoproteins inhibit endothelium-dependent relaxation in rabbit aorta. Nature, 327, 237-239.

ARCH, J.R., BUCKLE, D.R., BUMSTEAD, J., CLARKE, G.D., TAYLOR, J.F. & TAYLOR, S.G. (1988). Evaluation of the potassium channel activator cromakalim (BRL 34915) as a bronchodilator in the guinea-pig: comparison with nifedipine. Br. J. Pharmacol., 95, 763-770.

ASHWOOD, V.A., CASSIDY, F., EVANS, J.M.,FARUK, E.A. & HAMILTON, T.C. (1984). Trans-4-cyclic-amido-3,4-dihydro-2H-1-benzopyran-3-ols as antihypertensive agents. In Proc 8th Int. Symp. Med. Chem. ed. Dahlbom, R. & Nilsson, J.L.G., Vol. 1, 316-317, Stockholm: Swedish Pharmaceutical Press.

BABA, K., KAWANISHI, M., SATAKE, T. & TOMITA, T. (1985). Effects of varapamil on the contractions of guinea-pig tracheal muscle induced by Ca, Sr and Ba. Br. J. Pharmacol., 84, 203-211.

BANKS, B.E.C., BROWN, C., BURGESS, G.M., BURNSTOCK, G., CLARET, M., COCKS, T.M. & JENKINSON, D.H. (1979). Apamin blocks certain

neurotransmitter-induced increases in potassium permeability. Nature, 282, 415-417.

BAUDOUIN-LEGROS, M. & MEYER, P. (1973). Effects of angiotensin, catecholamines and cyclic AMP on calcium storage in aortic microsomes. Br. J. Pharmacol., 47,377-385.

BAYLISS, W.M. (1921). On the local reactions of the arterial wall to changes of internal pressure. J. Physiol., 28, 221-231.

BEECH, D.J. & BOLTON ,T.B. (1987). Effects of BRL 34915 on membrane currents recorded from single smooth muscle cells from the rabbit portal vein. Br. J. Pharmacol., 92, 550P.

BENHAM, C.D. & TSIEN, R.W. (1987). Calcium-permeable channels in vascular smooth muscle: voltage-activated, receptor-operated, and leak channels. In Cell Calcium and the Control of Membrane Transport, ed. Mandel, L.J. & Eaton, D.C., pp 46-64.

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

BEVAN, J.A. (1982). Selective action of diltiazem on cerebral vascular smooth muscle in the rabbit: Antagonism of extrinsic but not intrisic maintained tone. Am. J. Cardiol., 49, 519-524.

BEVAN, J.A. (1983). Diltiazem selectively inhibits cerebrovascular extrinsic but not intrinsic myogenic tone. Circ. Res., 52 (suppl), 104-109.

BLAKELEY, A.G.H., BROWN, D.A., CUNNANE, T.C., FRENCH, A.M., McGRATH, J.C. & SCOTT, N.C. (1981). Effects of nifedipine on electrical and

mechanical responses of the rat and guinea-pig vas deferens. Nature, 294, 759-761.

BOHR, D.F. (1964). Description of the myogenic hypothesis (in discussion on the paper by Folkow, B.). Circ. Res., 15, suppl.1. 279-285.

BOLTON, T.B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev., 59, 606-718.

BOLTON, T.B. & CLARK, J.P. (1981). Actions of various muscarinic agonists on membrane potential, potassium efflux, and contraction of longitudinal muscle of guinea-pig intestine. Br. J. Pharmacol., 72, 319-334.

BOLTON, T.B. & CLAPP, L.H. (1986). Endothelial-dependent relaxant actions of carbachol and substance P in arterial smooth muscle. Br. J. Pharmacol. 87, 713-723.

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

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

BOWMAN, A. & GILLESPIE, J.S. (1981). Differential blockade of non-adrenergic inhibitory machanisms in bovine retractor penis and guinea-pig taenia caeci. J. Physiol., 317, 92-93P.

BOWMAN, A. & GILLESPIE, J.S. (1982). Block of some non-adrenergic inhibitory responses of smooth muscle by a substance from haemolysed

erythrocytes. J. Physiol., 328, 11-25.

BOWMAN, A., GILLESPIE, J.S. & MARTIN, W. (1979). The inhibitory material in extracts from the bovine retractor penis muscle is not an adenine nucleotide. Br. J. Pharmacol.,67, 327-328.

BOWMAN, A., GILLESPIE, J.S. & MARTIN, W. (1981). Actions on the cardiovascular system of an inhibitory material extracted from the bovine retractor penis. Br. J. Pharmacol., 72, 365-372.

BOWMAN, A., GILLESPIE, J.S. & POLLOCK, P.(1982). Oxyhaemoglobin blocks non-adrenergic non-cholinergic inhibition in the bovine retractor penis muscle. Eur. J. Pharmacol., 85, 221-224.

BOWMAN, A., GILLESPIE, J.S. & SOARS-DA-SILVA, P. (1986). A comparison of the action of the endothelium-derived relaxant factor and the inhibitory factor from the bovine retractor penis on rabbit aortic smooth muscle. Br. J. Pharmacol., 87, 175-181.

BOWMAN, A. & McGRATH, J.C. (1985). The effect of hypoxia on neuroeffector transmission in the bovine retractor penis and rat anococcygeus muscles. Br. J. Pharmacol., 85, 869-875.

BOZLER, E. (1947). The response of smooth muscle to stretch. Am. J. Physiol., 149, 299-301.

BRAY, K.M., NEWGREEN, D.T., SMALL, R.C., SOUTHERTON, J.S., TAYLOR, S.G., WEIR, S.W. & WESTON, A.H. (1987). Evidence that the mechanism of the inhibitory action of pinacidil in rat and guinea-pig smooth muscle differs from that of glycerl trinitrate. Br. J. Pharmacol., 91, 421-429.

BRAY, K.M., WESTON, A.H., McHARG, A.D., NEWGREEN, D.T. & SOUTHERTON,

J.S. (1988a). Comparison of the inhibitory effects of BRL 34915 and nifedipine on noradrenaline- and KCl-induced contractions in rabbit aorta. Br. J. Pharmacol. 93, 205P.

BRAY, K.M., WESTON, A.H., McHARG, A.D., NEWGREEN, N.T. & SOUTHERTON, J.S. (1988b). Analysis of the inhibitory action of BRL 34915 on responses to noradrenaline in rabbit aorta. Br. J. Pharmacol., 93, 206P.

BUCHHEIT, K.H. & BERTHROLET, A. (1988). Inhibition of small intestine motility by cromakalim (BRL 34915). Eur. J. Pharmacol., 154, 335-337.

BUCKINGHAM, R.E., CLAPHAM, J.C., COLDWELL, M.C., HAMILTON, T.C. & HOWLETT, D.R. (1984a). Differences in vascular effects of BRL 34915 and nifedipine in vitro. 9th IUPHAR Congress, (abstr) London, 1702P. Macmillan, London.

BUCKINGHAM,R.E., CLAPHAM, J.C., HAMILTON, T.C., LONGMAN, S.D., NORTON, J. & POYSER, R.H. (1984b). BRL 34915—a novel peripherally acting antihypertensive agent. 9th IUPHAR Congress, (abstr), London, 287P. Macmillan, London.

BUCKINGHAM, R.E., HAMILTON, T.C., HOWLETT, D.R., MOOTOO, S. & WILSON, C. (1989). Inhibition by glibenclamide of the vasorelaxant action of cromakalim in the rat. Br. J. Pharmacol., 97, 57-64.

BÜLBRING, E. (1954). Membrane potentials of smooth muscle fibres of the taenia coli of the guinea-pig. J. Physiol., 125, 302-315.

BÜLBRING, E. (1955). Correlation between membrane potential, spike discharge and tension in smooth muscle. J. Physiol., 128, 200-221.

BURNSTOCK, G. (1971). Neural nomenclature. Nature, 229, 282-283.

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

BURNSTOCK, G. (1986). The changing face of autonomic neurotransmission. Acta. Physiol. Scand., 126, 67-91.

BURNSTOCK, G., CAMPBELL, G., BENNETT, M. & HOLMAN, M.E. (1963). Inhibition of the smooth muscle of the taenia coli. Nature, 200, 581-582.

BURNSTOCK, G., CAMPBELL, G., SATCHELL, D.G. & SMYTHE, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. Br. J. Pharmacol., 40, 668-688.

BURNSTOCK, G., COCKS, T. & CROWE, R. (1978). Evidence for purinergic innervation of the anococcygeus muscle. Br. J. Pharmacol., 64, 13-20.

BURNSTOCK, G. DUMSDAY, B.H. & SMYTHE, A. (1972). Atropine-resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. Br. J. Pharmacol., 44, 451-461.

BURNSTOCK, G. & PROSSER, C.L. (1960). Response of smooth muscles to quick stretch; relation of stretch to conduction. Am. J. Physiol., 198, 921-925.

BYRNE, N.G. & MUIR, T.C. (1984). Electrical and mechanical responses of the bovine retractor penis to nerve stimulation and to drugs. J. Auton. Pharmacol., 4, 261-271.

BYRNE, N.G. & MUIR, T.C. (1985). Mechanisms underlying electrical and mechanical responses of the bovine retractor penis to inhibitory nerve stimulation and to an inhibitory extract. Br. J. Pharmacol., 85, 149-161.

CAIN, C.R. & METZLER, V. (1985). Electrophysiological effects of the antihypertensive agent BRL 34915 on guinea-pig papillary muscle. Naunyn-Schmiedebergs Arch. Pharmacol., 329, R53.

CAMPBELL, G. (1970). Autonomic nervous supply to effect tissues. In Smooth Muscle, ed. Bülbring, E., Brading, A., Jones, A.W. & Tomita, T., pp 451-495, Edward Arnold, London.

CAMPBELL, G. (1987). Cotransmission. Ann. Rev. Pharmacol. Toxicol., 27, 51-70.

CAMPBELL, G. & JACKSON, F. (1985). Independent co-release of acetylcholine and somatostatin from cardiac vagal neurones in toad. Neurosci. Lett., 60, 47-50.

CARLSSON, A., HILLARP, N.Å. & WALDECK, B. (1963). Analysis of the Mg^{2+}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. Acta. Physiol. Scand., 59, Suppl. 215, 1-38.

CASTEELS, R., KITAMURA, K., KURIYAMA, H. & SUZUKI, H. (1977a). The membrane properties of the smooth muscle cells of the rabbit main pulmonary artery. J. Physiol., 271, 41-61.

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

CASTLE, N.A. & STRONG, P.N. (1986). Identification of two toxins from

scorpion (Leiurus quinquestriatus) venom which block distinct classes of calcium-activated potassium channel. F.E.B.S. Lett., 209, 117-121.

CAVERO, I., MONDOT, S. & MESTRE, M. (1989). Vasorelaxant effects of cromakalim in rats are mediated by glibenclamide-sensitive potassium channels. J. Pharmacol. exp. Ther., 248, 1261-1268.

CERRINA, J., ANDREIER, P., RENIER, A., FLOCH, A. & DUROUX, P. (1983). Effects of diltiazem and other Ca^{2+} antagonists on guinea-pig tracheal muscle. Eur. J. Pharmacol., 94, 241-249.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarization factor and EDRF from rat blood vessels. Br. J. Pharmacol., 95, 1165-1174.

CHENG, J.B. & TOWNLEY, R.G. (1983). Pharmacological characterisation of effects of nifedipine on isolated guinea-pig and rat tracheal smooth muscle. Archs. Int. Pharmacodyn., 263, 228-244.

CHIU, P.J., TETZLOFF, G., AHN, H.S. & SYBERTZ, E.J. (1988). Effects of BRL 34915, a putative K channel opener, on transmembrane ^{45}Ca movements in rabbit aortic smooth muscle. Eur. J. Pharmacol., 155, 229-237.

CHURCH, J. & ZSOTÉR, T.T. (1980). Calcium antagonistic drugs, mechanism of action. Can. J. Physiol. Pharmacol., 58, 254-264.

CLAPHAM, J.C. (1988). A method for in vivo assessment of calcium slow channel blocking drugs. J. Cardiovasc. Pharmacol., 11, 56-60.

CLAPHAM, J.C. & WILSON, C. (1987). Anti-spasmogenic and spasmolytic effects of BRL 34915: a comparison with nifedipine and nicorandil.

J. Auton. Pharmacol., 7, 233-242.

COLDWELL, M.C. & HOWLETT, D.R. (1986). BRL 34915 induced potassium channel activation in rabbit isolated mesenteric artery is not calcium dependent. Br. J. Pharmacol., 88, 443P.

COLDWELL, M.C. & HOWLETT, D.R. (1987). Specificity of action of the novel antihypertensive agent, BRL 34915, as a potassium channel activator comparison with nicorandil. Biochem. Pharmacol., 36, 3663-3669.

COLDWELL, M.C. & HOWLETT, D.R. (1988). Potassium efflux enhancement by cromakalim (BRL 34915) in rabbit mesenteric artery: an indirect effect independent of calcium? Biochem. Pharmacol., 37, 4105-4110.

COLLIER, J. & VALLANCE, P. (1989). Endothelium-derived relaxing factor is an endogenous vasodilator in man. Br. J. Pharmacol., 97, 639-641.

COOK, N.S. (1988). The pharmacology of potassium channels and their therapeutic potential. T.I.P.S., 9, 21-28.

COOK, D.L. & HALES, C.N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic cells. Nature, 311, 271-273.

COOK, N.S. & HOF, R.P. (1988). Cardiovascular effects of apamin and BRL 34915 in rats and rabbits. Br. J. Pharmacol., 93, 121-131.

COOK, N.S., QUAST, U., HOF, R.P., BAUMLIN, Y. & PALLY, C. (1988a). Similarities in the mechanism of action of two new vasodilator drugs: pinacidil and BRL 34915. J. Cardiovasc. Pharmacol., 11, 90-99.

COOK, N.S., WEIR, S.W. & DANZEISEN, M.C. (1988b). Antivasoconstrictor

effects of the K^+ channel opener cromakalim on the rabbit aorta — comparison with the calcium antagonist isradepine. Br. J. Pharmacol., 95, 741-752.

DALE, H.H. (1933). Nomenclature of fibres in the autonomic system and their effects. J. Physiol., 80, 10-11P.

DEGUCHI, T. (1977). Endogenous activating factor for guanylate cyclase in synaptosomal-soluble fraction of rat brain. J. Biol.Chem., 252, 7617-7619.

DEGUCHI, T., TAITO, M. & KONO, M. (1978). Blockade by N-methylhydroxylamine of activation of guanylate cyclase and elevations of guanosine 3'5'-monophosphate levels in nervous tissues. Biochim. Biophys. Acta., 544, 8-19.

DEGUCHI, T. & YOSHIOKA, M. (1982). L-arginine identified as an endogenous activator for soluble guanylate cyclase from neuroblastoma cells. J. Biol. chem., 257, 10147-10151.

DIAMOND, J. & CHU, E.B. (1983). Possible role for cyclic GMP in endothelium-dependent relaxation of rabbit aorta by acetylcholine. Comparison with nitroglycerin. Res. Commun. Chem. Pathol. Pharmacol., 41, 369-381.

DO KHAC, L., MOKHTARI, A. & HARBON, S. (1986). A reevauated role for cyclic AMP in uterine relaxation. Differential effect of isoproterenol and foskolin. J. Pharmacol. Exp. Ther., 239, 236-242.

DOWNING, S.J., MILLER, M.& HOLLINGSWORTH, M. (1989). Tolerance to cromakalim in the rat uterus in vivo. Br. J. Pharmacol., 96,

732-738.

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

DUDEL, C. & FÖSTERMANN, U. (1988). Gossypol attenuates selectively the blood pressure lowering effect of endothelium-dependent vasodilators in the rabbit in vivo. Eur. J. Pharmacol.,145, 217-221.

DUSTING, G.L., READ, M.A. & STEWART, A.G. (1988). Differential bioassay of endothelium-derived relaxaing factor and nitric oxide on the rabbit aorta and guinea-pig trachea. Br. J. Pharmacol., 93, 101P.

ERÄNKÖ, O., KLINGE, E. & SJÖSTRAND, N.O. (1976). Different types of synaptic vesicles in axons of the retractor penis muscle of the bull. Experientia 32, 1335-1337.

ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. Biochem. Biophys. Res. Commun., 154, 620-625.

EVANS, D.H.L. & SCHILD, H.O. (1956). Reactions of chick amnion to stretch and electrical stimulation. J. Physiol., 132, 31P.

FOLKOW, B. (1949). Introvascular pressure as a factor regulating the tone of the small vessels. Acta. Physiol. Scand., 17, 289-310.

FOREMAN, J.C., NORRIS, D.B., RISING, T.J. & WEBBER, S.E. (1986). A study of the H_2 receptor for histamine stimulating adenylate cyclase in homogenates of guinea-pig lung parenchyma. Br. J. Pharmacol.,

87, 37-44.

FÖRSTERMANN, U., ALHEID, U., FRÖLICH, J.C. & MÜLSCH, A. (1988). Mechanisms of action of lipoxygenase and cytochrome P-450-mono-oxygenase inhibitors in blocking endothelium-dependent vasodilatation. Br. J. Pharmacol., 93, 569-578.

FÖRSTERMANN, U., TROGISCH, G. & BUSSE, R. (1984). Species-dependent differences in the nature of endothelium-derived vascular relaxing factor. Eur. J. Pharmacol. 106, 639-643.

FÖSRTERMANN, U. & NEUFANG, B. (1984). The endothelium-dependent relaxation of rabbit aorta: Effects of antioxidants and hydroxylated eicosatetraenoic acids. Br. J. Pharmacol. 82, 765-767.

FOSTER, C.D., FUJII, K., KINGDON, J. & BRADING, A.F. (1989a). The effect of cromakalim on the smooth muscle of the guinea-pig urinary bladder. Br. J. Pharmacol., 97, 281-291.

FOSTER, C.D., SPEAKMAN, M.J. FUJII, K. & BRADING, A.F. (1989b). The effects of cromakalim on the detrusor muscle of human and pig urinary bladder. Br. J. Urol., 63, 284-294.

FOSTER, R.W., OKPALUGO, B.I. & SMALL,R.C. (1984). Antagonisms of Ca^{2+} and other actions of varapamil in guinea-pig isolated trachealis. Br. J. Pharmacol.,81, 499-507.

FURCHGOTT, R.F. (1981). The requirement for endothelial calls in the relaxation of arteries by acetylcholine and some other vasodilators. Trends Pharmacol. Sci., 2, 173-176.

FURCHGOTT, R.F. (1984). The role of endothelium in the responses of vascular smooth muscle to drugs. Ann. Rev. Pharmacol. Toxicol.,

24, 175-197.

FURCHGOTT, R.F., (1988). Studies on the relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Mechanisms of Vasodilatation. ed. Vanhoutte, P.M. New York: Raven Press.

FURCHGOTT, R.F., & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature, 288, 373-376.

FURCHGOTT, R.F., ZAWADZKI, J.V. & CHERRY, P.D. (1981). Role of endothelium in the vasodilator response to acetylcholine. In : Vasodilation. ed. Vanhoutte, P. & Leusen, I., pp 49-66. New York; Revan.

FURUKAWA, K., ITOH, T., KAJIWARA, M., KITMURA, K., SUZUKI, H., ITO, Y. & KURIYAMA, H. (1981). Vasodilating actions of 2-Nicotinamidoethyl nitrate on porcine and guinea-pig coronary arteries. J. Pharmacol. exp. Ther., 218, 248-259.

GARTHWAITE, J., CHARLES, S.L. & CHESS-WILLIAMS, R. (1988). Endothelium-derived relaxaing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature, 336, 385-388.

GASKELL, W.H. (1916). The Involuntary Nervous System, London, Longmans, Green & Co.

GIBBINS, I.L. & HALLER, C.J. (1979). Ultrastructural identification of

non-adrenergic, non-cholinergic nerves in the rat anococcygeus muscle. Cell. Tiss. Res., 200, 257-271.

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

GILLESPIE, J.S. (1962). Spontaneous mechanical and electrical axtivity of stretched and unstretched intestinal smooth muscle cells and their response to sympathetic nerve stimulation. J. Physiol., 162, 54-75.

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

GILLESPIE, J.S. (1982). Non-adrenergic, non-cholinergic inhibitory control of gastrointestinal motility. In: Motility of the Digestive Tract, pp 51-66. ed. M.Wienbeck Raven Press. New York.

GILLESPIE, J.S. (1987). Searching for the non-adrenergic non-cholinergic autonomic transmitter. In: Plenary lecture Xth International Congress of Pharmacology. ed. Rand, M.J. & Raper, C. pp. 161-170. Amsterdam: Elsevier Science Publishers B.V.

GILLESPIE, J.S. & HUNTER, J.C. (1982). Subcellular localisation of smooth muscle inhibitory factor isolated from the bovine retractor penis muscle. Br. J. Pharmacol. 76, 189P.

GILLESPIE, J.S., HUNTER, J.C. & MARTIN, W. (1981). Some physical and chemical properties of the smooth muscle inhibitory factor in

extracts of the bovine retractor penis muscle. J. Physiol., 315, 111-125.

GILLESPIE, J.S. & LIU, X. & MARTIN, W. (1989). The effect of L-arginine and N^G-monomethyl-L-arginine on the response of the rat anococcygeus to NANC nerve stimulation. Br. J. Pharmacol., (submitted).

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

GILLESPIE, J.S. & MARTIN, W. (1980). A smooth muscle inhibitory material from the bivine retractor penis and rat anococcygeus. J. Physiol., 309, 55-64.

GILLESPIE, J.S., MAXWELL, J.D. (1971). Adrenergic innervation of sphincteric and nonsphincteric smooth muscle in the rat intestine. J. Histochem. Cytochem., 19, 676-681.

GILLESPIE, J.S. & McGRATH, J.C. (1973). The spinal origin of the motor and inhibitory innervation of the rat anococcygeus muscle. J. Physiol. 230, 659-672.

GILLESPIE, J.S. & McGRATH, J.C. (1975). The effects of lysergic acid diethylamide on the response to field stimulation of the rat vas deferens and the rat and cat anococcygeus muscles. Br. J. Pharmacol. 54, 481-488.

GILLESPIE, J.S. & McKNIGHT, A.T. (1978). The actions of some vasoactive polypeptides and their antagonists on the anococcygeus muscle. Br. J. Pharmacol. 62, 267-274.

GILLESPIE, J.S. & XIAORONG, L. (1989). The effect of arginine and

L-N^Gmonomethyl arginine on the response of the bovine retractor penis to stimulation of its NAMC nerve. Br. J. Pharmacol., 97, 453P.

GIMENEZ-GALLEGO, G., NAVIA, M.A., REUBEN, J.P., KATZ, G.M., KACZOROWSKI, G.J. & GARCIA, M.L. (1988). Purification, sequence and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. Proc. Natl. Acad. Sci. USA, 85, 3329-3333.

GOLENHOFEN, K. (1981). Differentiation of calcium activation processes in smooth muscle using selective antagonists. In: Smooth Muscle, ed. Bülbring, E., Brading, A.F., Jones, A.W. & tomita, T. pp 157-170. Edward Arnold, London.

GORDON, J.L. & MARTIN, W. (1983). Stimulation of endothelial prostacyclin production plays no role in endothelium-dependent relaxation of the pig aorta. Br. J. Pharmacol., 80, 179-186.

GOTANDA, K., SATOH, K. & TAIRA, N. (1988). Is the cardiovascular profile of BRL 34915 characteristic of potassium channel activators. J. Cardiovasc. Pharmacol.,12, 239-246.

GRAVES, S., DRETCHEN, K.L. & KRUGER, G.O. (1978). Dantrolene sodium: effects on smooth muscle. Eur. J. Pharmacol., 47, 29-35.

GREENBERG, S.S., WILCOX, D. & RUBANYI, G.M. (1988). Endothelium-derived relaxing factor (EDRF) released from canine femoral artery cannot be identified as nitric oxide by electron paramagnetic responance (EPR). Circulation, 78, suppl.II-182.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON,

A.H. (1984). The nature of endothelium-derived vascular relaxant factor. Nature, 308, 645-647.

GRYGLEWSKI, R.J., MONCADA, S. & PALMER, R.M.J. (1986a). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. Br. J. Pharmacol. 87, 685-694.

GRYGLEWSKI, R.J. PALMER, R.M.J. & MONCADA, S. (1986b). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature, 320, 454-456.

GUERRA, R., MYERS, P.R. & HARRISON, D.G. (1988). The relationship between nitric oxide release and endothelium-derived relaxing factor induced vascular relaxation. Circulation, 78, Suppl. II-181.

GUSTIN, M.C., ZHOU, X.L. MARTINAC, B., CULBERTSON, M.R. & KUNG, C. (1987). Stretch-activated cation channel in yeast. Biophys. J., 51, 251a.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and varapamil on electrical and mechanical activity in rat portal vein. Br. J. Pharmacol., 88, 103-111.

HARDER, D.R. (1984). Pressure-dependent membrane depolarization in cat middle cerebral artery. Circ. Res., 55, 197-202.

HASHIMOTO, T., HIRATA, M. & ITO, Y. (1985). A role for inositol 1,4,5 triphosphate in the initiation of agonist-induced contractions of dog tracheal smooth muscle. Br. J. Pharmacol., 86, 191-199.

HENDERSON, V.E. & ROEPKE, M.H. (1934). The role of acetylcholine in a bladder contractile mechanisms and in parasympathetic ganglia. J. Pharmacol. exp. Ther., 51, 97-111.

HERMSMEYER, R.K. (1988). Pinacidil actions on ion channels in vascular muscle. J. Cardiovasc. Pharmacol., 12, Suppl.II. S17-22.

HIRST, G.D.S. & NIELD, T.O. (1981). Localisation of specialised noradrenaline receptors at neuromuscular junctions on arterioles of the guinea-pig. J. Physiol., 313, 343-350.

HOLTON, F.A. & HOLTON, P. (1954). The capillary dilator substances in dry powders of spinal roots: a possible role of ATP in chemical transmission from nerve endings. J. Physiol., 126, 124-140.

HOLZMANN, S. (1982). Endothelium-induced relaxation by acetylcholine associated with large rises in cyclic GMP in coronary arterial strips. J. Cyclic. Nucl. Res., 8, 409-419.

HOLZMANN, S. (1983). Cyclic GMP as possible metiator od coronary arterial relaxation by nicorandil (SG-75). J. Cardiovac. Pharmacol., 5, 364-370.

HOLLINGSWORTH, M., AMÉDÉE, T., EDWARDS, D., MIRONNEAU, J., SAVINEAU, J.P., SMALL, R.C. & WESTON, A.H. (1987). The relaxant action of BRL 34915 in rat uterus. Br. J. Pharmacol., 91, 803-813.

HOLLINGSWORTH, M., EDWARDS, D., RANKIN, J.R. & WESTON, A.H. (1988). BRL 34915 and relaxation of rat uterus——role of K^+ channels. Br. J Pharmacol., 93, 199P.

HWA, J. & BEVAN, J. (1986a). Stretch-dependent (myogenic) tone in rabbit ear resistance arteries. Am. J. Physiol., 250, H87-95.

HWA, J. & BEVAN, J. (1986b). A nimodipine-resistant Ca^{2+} pathway is involved in myogenic tone in a resistance artery. Am. J. Physiol., 251, H182-189.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M., WOOD, K.S. & CHAUDHURI, G. (1988). Pharmacological evidence that endothelium-derived relaxing factor is nitric oxide: use of pyrogallol and superoxide dismutase to study endothelium-dependent and nitric oxide-elicited vascular smooth muscle relaxation. J. Pharmacol. exp Ther., 244, 181-189.

IGNARRO, L.J. & KADOWITZ, P.J. (1985). The pharmacological and physiological role of ciclci GMP in vascular smooth muscle relaxation. Ann. Rev. Pharmacol. Toxicol., 25, 171-191.

ITO, Y. & ITOH, T. (1984). The roles of stored calcium in contractions of cat tracheal smooth muscle produced by electrical stimulation, acetylcholine and high K^+. Br. J. Pharmacol., 83, 667-679.

JAFFORJI, S.S. & MICHELL, R.H. (1976). Stimulation of phosphatidylinositol turnover in various tissues by cholinergic and adrenergic agonists, by histamine and by caerulein. Biochem. J., 182, 669-676.

JAN, L.Y. & JAN, Y.N. (1982). Peptidergic transmission in sympathetic ganglia of the frog. J. Physiol., 327, 219-246.

JOHANSSON, B. & MELLANDER, S. (1975). Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. Circ. Res., 36, 76-83.

KARASHIMA, T., ITOH, T. & KURIYAMA, H. (1982). Effects of 2-Nicotinamidoethy nitrate on smooth muscle cells of the guinea-pig mesenteric and portal veins. J. Pharmacol. exp. Ther., 221, 472-480.

KASAKOV, L., ELLIS, J., KIRKPATRICK, K., MILNER, P. & BURNSTOCK, G. (1988). Direct evidence for concomitant release of noradrenaline, adenosine 5'-triphosphate and neuropeptide Y from sysmathetic nerves supplying the guinea-pig vas deferens. J. Auton. Nerv. Syst., 22, 75-82.

KATUSIC, Z., SHEPHERD, J.T. & VANHOUTTE, P.M. (1987). Endothelium-dependent contraction to stretch in canine basilar arteries. Am. J. Physiol., 252, H671-H673.

KIRBER, M.T., SINGER, J.V. & WALSH, J.V. (1987). Stretch-activated channels in freshly dissociated smooth muscle cells. Biophys. J., 51, 252a.

KIRKPATRICK, C.T. (1981). Tracheobronchial smooth muscle. In Smooth Muscle. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp385-395, Edward Arnold, London.

KIRKPATRICK, K. & BURNSTOCK, G. (1987). Sympathetic nerve-mediated release of ATP from the guinea-pig vas deferens is unaffected by reserpine. Eur. J. Pharmacol., 138, 207-214.

KLINGE, E. & SJÖSTRAND, N.O. (1974). Contraction and relaxation of the retractor penis muscle and the penile artery of the bull. Acta. Physiol. Scand. Suppl. 420, 1-88.

KOMORI, K. & SUZUKI, H. (1987). Electrical responses of smooth muscle cells during cholinergic vasodilation in the rabbit saphenous artery. Circ. Res., 61, 586-593.

KONO, M., GORONZY, J., WEYAND, C.M. & GARDNER, P. (1986). Single-channel and whole-cell recordings of mitogen-regulated inward

currents in human cloned helper T lymphocytes. Nature, 323, 269-273.

KREYE, V.A.W., GERSTHEIMER, F. & WESTON, A.H. (1987a). Effect of BRL 34915 on resting membrane potential and ^{86}Rb efflux in rabbit tonic vascular smooth muscle. Naunyn Schemiedebergs Arch. Pharmacol., 335, R64.

KREYE, V.A.W., GERSTHEIMER, F. & WESTON, A.H. (1987b). Effects of antihypertensive, BRL 34915, on membrane potential and ^{86}Rb efflux in rabbit tonic vascular smooth muscle. Pflugers. Arch., 408, R79.

KUSANO, K., BARROS, F., KATZ, G., GARCIA, M., KACZOROWSKI, G. & REUBEN, J.P. (1987). Modulation of K channel activity in aortic smooth muscle by BRL 34915 and a scorpion toxin. Biophys. J., 51, 54a.

LAHER, I. & BEVAN, J. (1987). Protein kinase C activation selectively augments a stretch-induced, calcium-dependent tone in vascular smooth muscle. J. Pharmacol. exp. Ther., 242, 566-572.

LAHER, I., VAN BREEMEN, C.V. & BEVAN, J. (1988). Stretch-dependent calcium uptake associated with myogenic tone in rabbit facial vein. Circ. Res., 63, 669-772.

LANDIS, S.C. & KEEFE, D. (1983). Evidence for neurotransmitter plasticity in vivo: developmental changes in properties of cholinergic sympathetic neurons. Dev. Biol., 98, 349-372.

LANGLEY, J.N. (1898). On inhibitory fibres in the vagus to the end of the oesophagus and stomach. J. Physiol., 23, 407-414.

LANGLEY, J.N. (1921). The Autonomic Nervous System, Cambridge; W. Heffer & sons Ltd.

LANGLEY, J.N. & ANDERSON, H.K. (1895). The innervation of the pelvic and adjoining viscere. J. Physiol., 19, 85-121.

LANSMAN, J.B., HALLAM, T.J. & RINK, T.J. (1987). Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers. Nature, 325, 811-813.

LAU, Y.S. & LUM, B.K.B. (1983). Role of cyclic AMP in adrenergically-induced tracheal muscle relaxation. Archs. Int. Pharmacodyn., 261, 36-50.

LEDVORA, R.F., BARANY, K., VAN DER MEULEN, D.L., BARRON, J.T. & BARANY, M. (1983). Stretch-induced phosphorylation of the 20,000-dalton light chain of myosin in arterial smooth muscle. J. Biol. Chem., 258, 14080-14083.

LONG, C.J., SARAU, H.M. & BERKOWITZ, B.A. (1987). The inhibition of release of endothelium-derived relaxant factor by manoalide, a potent inhibitor of phospholipase A_2. Br. J. Pharmacol., 92, 843-849.

LONGMAN , S.D., CLAPHAM, J.C., WILSON, C. & HAMILTON, T.C. (1988). Cromakalim, a potassium channel activator: a comparison of its cardiovasular haemodynamic profile and tissue specificity with those of pinacidil and nicorandil. J. Cardiovasc. Pharmacol., 12, 535-542.

LUNDBERG, J.M. (1981). Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. Morphological, biochemical and functional tissues. Acta. Physiol. Scand., Suppl. 496, 1-57.

LUNDBERG, J.M. ÄNGGÅRD, A., FAHRENKRUG, J., HÖKFELT, T. & MUTT, V. (1980). Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: functional significance of co-existing transmitters for vasodilatation and secretion. Proc. Natl. Acad. Sci. USA., 77, 1651-1655.

McCLESKEY, E.W., FOX, A.P., FELDMAN, D., OLIVERA, B.M., TSIEN, R.W. & YOSHIKAMI, D. (1986). The peptide toxin -CgTx blocks particular types of neuronal Ca channels. Biophys. J., 49, 431a.

MACGREGOR, R.D., SACHS, F. 7 HUNT, C.A. (1989). Mechanisms for opening stretch-activated ion channels. Biophys. J., 55, 245a.

McGRATH, J.C. (1978). Adrenergic and "non-adrenergic" components of the contractile response of the vas deferens to a single indirect stimulus. J. Physiol., 283, 23-39.

MAAS, A.J.J., DEN HERTOG, A., RAS, R. & VAN DER AKKER, J. (1980). The action of apamin on the guinea-pig taenia coli. Eur. J Pharmacol., 67, 265-274.

MAHMOUDIAN, M., DEHPOUR, A.R. & MOFAKHAM, S. (1981). Effect of dantrolene sodium on the contractility of rabbit jejunum in vitro. Eur. J. Pharmacol., 70, 287-292.

MARSHALL, J.M. (1962). Regulation of activity in uterine smooth muscle. Physiol. Rev., 42, suppl. 5, 213-227.

MARTIN, W.,FURCHGOTT, R.F., VILLANI, G.M. & JOTHIANADAH, D. (1986). Phosphodiesterase inhibitors induce endothelium-dependent relaxation of rat and rabbit aorta by potentiating the effects of spontaneously released endothelium-derived relaxing factor. J. Pharmacol. exp.

Ther., 237, 539-547.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and methylene blue in the rabbit aorta. J. Pharmacol. Exp. Ther., 232, 708-716.

MARTIN, W., SMITH, J.A., HENDERSON, A.H. (1988). Evidence that inhibitory factor extracted from bovine retractor penis is nitrite, whose acid-activated derivative is stabilized nitric oxide. Br. J. Pharmacol., 93, 579-586.

MARTINSON, J. & MUREN, A. (1963). Excitatory and inhibitory effects of vagus stimulation on gastric motility in the cat. Acta. Physiol. Scand., 57, 309-316.

MEISHERI, K., CIPKUS, L.A. & TAYLOR, C.J. (1988). Mechanism of action of minoxidil sulfate-induced vasodilation; a role for increased K^+ permeability. J. Pharmacol. exp. Ther., 245, 751-760.

MILLER, C., MOCZYDLOWSKI, E., LATORRE, R. & PHILLIPS, M. (1985). Charybotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. Nature, 313, 316-318.

MONCADA, S., PALMER, R.M.J. & GRYGLEWSKI, R.J. (1986). Mechanism of action of some inhibitors of endothelium-derived relaxing factor. Proc. Natl. Acad. Sci. USA, 83, 9164-9168.

MUIR, T.C. & WARDLE, K.A. (1988). The electrical and mechanical basis of co-transmission in some vascular and non-vascular smooth muscles. J. Auton. Pharmac., 8, 203-218.

MURAD, F. ARNOLD, W.P., MITTAL, A.C. & BRAUGHLER, J.M. (1979).

Properties and regulation of guanylate cyclase and some proposed functions for cyclic GMP. Adv. cyclic. Nucleotide Res., 11, 175-204.

MURAD,F., MITTAL, C.K., ARNOLD, W.P., KATSUKI,S. & KIMURA, H. (1978). Guanylate cyclase: Activation by azide, nitro compounds, nitric oxide and hydroxyl radical and inhibition by haemoglobin and myoglobin. Adv. Cyclic Nucleotide Res., 9, 145-158.

NAKAO, K., OKABE, K., KITAMURA, H., KURIYAMA, H. & WESTON, A.H. (1988). Characteristics of cromakalim-induced relaxations in the smooth muscle cells of guinea-pig mesenteric artery and vein. Br. J. Pharmacol., 95, 795-804.

NAKAYAMA, K. (1982). Calcium-dependent contractile activation of cerebral artery produced by quick stretch. Am. J. Physiol., 242, H760-768.

NAKAYAMA, K., SUZUKI, S. & SUGI, H. (1986). Physiological and ultrastructural studies on the mechanism of stretch-induced contractile activation in rabbit cerebral artery smooth muscle. Jan. J. Physiol., 36, 745-760.

NESHEIM, B.-I., OSNES, J.-B. & OYE, I. (1975). Role of cyclic adenosine 3'5'-monophosphate in isoprenaline-induced relaxation of the oestrogen dominated rabbit uterus. Br. J. Pharmacol., 53, 403-407.

NIELSEN-KUDSK, J.E., KARLSSON, J.A. & PERSSON, C.G.A. (1986). Relaxant effects of Xanthines, a β receptor agonist and Ca^{2+} antagonists in guinea-pig tracheal preparations contracted by potassium or

carbachol. Eur. J. Pharmacol., 128, 33-40.

NOMA, A. (1983). ATP-regulated K channels in cardiac muscle. Nature, 305, 147-148.

NOWKCKY, M.C., FOX, A.P. & TSIEN, R.W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. Nature, 316, 440-443.

OKPAKO, D.T., CHAND, N. & EURE,P. (1978). The presence of inhibitory hitamine H_2-receptors in guinea-pig tracheobronchial muscle. J. Pharm. Pharmac., 30, 181-182.

OSTERRIEDER, W., BRUN, G., HESCHELER, J., TRAUTWEIN, W., FLOCKERZI, V. & HOFMANN, R. (1982). Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca^{2+} current. Nature, 298, 576-578.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S., (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature, 333, 664-666.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature, 327, 524-526.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem. Biophys. Res. Commun., 153, 1251-1256.

PALOCEK, I. & DANIEL, E.E. (1971). Effects of α- and β-adrenergic stimulation on the uterine motility and adenosine 3'5'-monophosphate

level. Can. J. Physiol. Pharmacol., 49, 988-998.

PEIPER, U., GRIEBEL, L. & WENDE, W. (1971). Activation of vascular smooth muscle of rat aorta by noradrenaline and depolarization: two different mechanisms. Pflügers Arch., 330, 774-789.

QUAST, U. (1987). Effect of the K^+ efflux stimulating vasodilator BRL 34915 on $^{86}Rb^+$ efflux and spontaneous activity in guinea-pig portal vein. Br. J. Pharmacol., 91, 569-578.

QUAST, U. (1988). Comparison of $^{86}Rb^+$ and $^{42}K^+$ effluxes stimulated by the K^+ channel opener BRL 34915 in vascular smooth muscle. Br. J. Pharmacol., 93, 19P.

QUAST, U. & BAUMLIN, Y. (1988). Comparison of the effects of $^{42}K^+$ and $^{86}Rb^+$ elicited by cromakalim (BRL 34915) in tonic and phasic vascular tissue. Naunyn Schmiedebergs Arch Pharmacol., 338, 319-326.

QUAST, U. & COOK, N.S. (1988a). Leiurus quinquestriatus venom inhibits BRL 34915-induced $^{86}Rb^+$ efflux from the rat portal vein. Life Sci., 42, 805-810.

QUAST, U. & COOK, N.S. (1988b). Potent inhibitors of the effects of the K^+ channel opener BRL 34915 in vascular smooth muscle. Br. J. Pharmacol., 93, 204P.

RAPOPORT, R.M. & MURAD, F. (1983a). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. Circ. Res., 52, 352-357.

RAPOPORT, R.M. & MURAD, F. (1983b). Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role

of cyclic GMP. J. Cyclic. Nucl. Protein. Phosph. Res., 9, 281-296.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-argine attenuates endothelium-dependent relaxation. Br. J. Pharmacol., 96, 418-424.

ROSENBERG, R.L. & TSIEN, R.W. (1987). Calcium permeable channels from cardiac sacolemma open at resting membrane potential. Biophys. J., 51, 29a.

RUBANYI, G.M., LORENZ, R.R. & VANHOUTTE, P.M. (1985). Bioassay of endothelium-derived relaxing factor(s). Inactivation by catecholamines. Am. J. Physiol. 249, H95-101.

RUBANYI, G.M. & VANHOUTTE, P.M. (1986). Superoxide anion and hyperoxia inactivate endothelium-derived relaxing factor. Am. J. Physiol., 250, H822-827.

SAMUELSON, U., SJÖSTRAND, N.O. & KLINGE, E. (1983). Correlation between electrical and mechanical activity in myogenic and neurogenic control of the bovine retractor penis muscle. Acta. Physiol. Scand., 119, 335-345.

SANGUINETTI, M.C., SCOTT, A.L., ZINGARO, G.L. & SIEGL, P.K. (1988). BRL 34915 (cromakalim) activates ATP-sensitive K^+ current in cardiac muscle. Proc. Natl. Acad. Sci. USA, 85, 8360-8364.

SCHMIDT, H.H.H.W., NAU, H., WITTFOHT, W., GERLACH, J., PRESCHER, K. KLEIN, M.M., NIROOMAND, F. & BÖHME, E. (1988). Arginine is a physiological precursor of endothelium-derived nitric oxide. Eur. J. Pharmacol., 154, 213-216.

SCHMID-ANTOMARCHI, H., DEWEILLE, J., FOSSET, M. & LAZDUNSKI, M. (1987). The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel in insulin-secreting cells. J. Biol. Chem., 262, 15840-15844.

SCHOLTYSIK, G. (1987). Evidence for inhibition by ICS 205-930 and stimulation by BRL 34915 of K^+ conductance in cardiac muscle. Naunyn-Schmiedebergs Arch Pharmacol., 335, 692-696.

SHETTY, S.S. & WEISS, G.B. (1987). Dissociation of actions of BRL 34915 in the rat portal vein. Eur. J. Pharmacol., 141, 485-488.

SHIBATA, S. & SATAKE, N. (1987). Differential inhibitory effects of nicorandil, a new antiangina agent, on the contractile responses to alpha-1- and alpha-2- adrenoceptor agonists in isolated rabbit renal and femoral arteries. Pharmacology, 34, 213-224.

SHIKANO, K., OHLSTEIN, E.H. & BERKOWITZ, B.A. (1987). Differential selectivity of endothelium-derived relaxing factor and nitric oxide in smooth muscle. Br. J. Pharmacol., 92, 483-485.

SHIKANO, K., LONG, C.J., OHLSTEIN, E.H. & BARKOWITZ, B.A. (1988). Comparative pharmacology of endothelium-derived relaxing factor and nitric oxide. J. Pharmacol. exp. Ther., 247, 873-881.

SIMIONESCU, N. & SIMIONESCU, M. (1977). The cardiovascular system. In Histology, pp373-431, ed. Weiss, L. & Greep, R.O., McGraw-Hill Book Company, U.S.A.

SINGER, H.A. & PEACH, M.H. (1983). Endothelium-dependent relaxation of rabbit aorta II. Inhibition of relaxation stimulated by methacholine and A23187 with antagonists of arachidonic acid metabolism. J.

Pharmacol. exp. Ther., 226, 796-801.

SMALL, R.C. (1982). Electrical slow waves and tone of guinea-pig isolated trachealis muscle: effects of drugs and temperature changes. Br. J. Pharmacol., 77, 45-54.

SMALL, R.C., FOSTER, R.W. & BOYLE, J.P. (1988). K^+-channel opening as a mechanism for relaxing airways smooth muscle. Agents Actions, Suppl. 23, 89-94.

SNEDDON, P. & BURNSTOCK, G. (1984a). Inhibition of excitatory junction potentials in guinea-pig vas deferens by α,β-methylene ATP: further evidence for ATP and noradrenaline as cotransmitters. Eur. J. Pharmacol., 100, 85-90.

SNEDDON, P. & BURNSTOCK, G. (1984b). ATP as cotransmitter in rat tail artery. Eur. J. Pharmacol., 106, 149-152.

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

SOUTHERTON, J.S., TAYLOR, S.G. & WESTON, A.H. (1987a). Comparison of the effects of BRL 34915 and of acetylcholine-liberated EDRF on rat isolated aorta. J. Physiol., 382, 50P.

SOUTHERTON, J.S., TAYLOR, S.G., WEIR, S.W. & WESTON, A.H. (1987b). An investigation into the mechanism of action of pinacidil in rat blood vessels. Br. J Pharmacol., 90, 126P.

SOUTHERTON, J.S., WESTON, A.H., BRAY, K.M., NEWGREEN, D.T. & TAYLOR, S.G. (1988). The potassium channel opening action of pinacidil: studies using biochemical, ion flux and microelectrode techniques.

Naunyn Schmiedebergs Arch Pharmacol., 338, 310-318.

SPARKS, H.V. (1964). Effect of quick stretch on isolated vascular smooth muscle. Circ. Res., 15, suppl. I: 254-260.

SPARKS, H.V. & BOHR, D.F. (1962). Effect of stretch on passive tension and contractility of isolated vascular smooth muscle. Am. J. Physiol., 202, 835-840.

STEINBERG, M.I., ERTEL, P., SMALLWOOD, J.K., WYSS, V. & ZIMMERMAN, K. (1988). The relation between vascular relaxant and cardiac electrophysiological effects of pinacidil. J. Cardiovasc. Pharmacol., 12, (Suppl. 2). S30-S40.

STONE, T.W. (1981). Physiological roles for adenosine and adenosine 5'-triphosphate in the nervous system. Neuroscience, 6, 523-555.

SUTKO, J.L., WILLERSON, J.T., TEMPLETON, G.H., JONES, T.L. & BESCH, H.R. (1979). Ryanodine: its alterations of cat papillary muscle contractile state and responsiveness to inotropic and interventions and a suggested mechanism of action. J. Pharmacol. exp. Ther., 209, 37-47.

SUTKO, J.L. & WILLERSON, J.T. (1980). Ryanodine alteration of the contractile state of rat ventricular myocardium, comparison with dog, cat and rabbit ventricular tissues. Circ. Res., 46, 332-343.

SWEDIN, G. (1971). Studies on neurotransmission mechanics in the rat and guinea-pig vas deferens. Acta. Physiol. Scand., 83, Suppl. 369.

TAKUWA, Y. & RASMUSSEN, H. (1987). Measurement of cytoplasmic free Ca^{2+} concentration in rabbit aorta using the photoprotein,

aequorin. J. Clin. Invest., 80.248-257.

TAYLOR, S.G., SOUTHERTON, J.S. & WESTON, A.H. (1988). Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim. Br. J. Pharmacol., 94, 853-863.

TODA, N., HATANO, Y. & HAYASHI, S. (1978). Modifications by stretches of the mechanical response of isolated cerebral and extracerebral arteries to vasoactive agents. Pflügers Arch., 374, 73-77.

TRICKLEBANK, M.D., FLOCKHART, G. & FREEDMAN, S.B. (1988). The potassium channel activation, BRL 34915, antagonises a behavioural response to the muscarinic receptor agonist, pilocarpine. Eur. J. Pharmacol., 151, 349-350.

TRIESCHMANN, U., PICHLMAIER, M., KLÖCKNER, U. & ISENBERG, G. (1988). Vasorelaxation due to K-agonists. Single channel recordings from isolated human vascular myocytes. Pflügers Arch., 411, R199.

TRUBE, G., RORSMAN, P. & OHNO-SHOSAKU, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic cells. Pflügers Arch., 407, 493-499.

VAN BREEMEN, C., AARONSON, P. & LOUTZENHIZER, R. (1979). Sodium-calcium interactions in mammalian smooth muscle. Pharmacol. Rev., 30, 167-208.

VANDERBURG, M.J., WOODWARD, S.A., HASSIEN, M., STUART-LONG, P. & TASKER, T. (1986). Potassium channel activators lower blood pressure—an initial study of BRL 34915 in patients. 11th Scientific Meeting of the International Society of Hypertension, Heidelberg. J.

Hypertens., 4, S166-S167.

VANDENBURG, M.J., WOODWARD, S.A., TASKER, T., STUART-LONG, P., FAIRHURST, G.,STEPHENS, J. & WOOD, B. (1987). Potassium channel activators: hypotensive activity and adverse effect profile: the first study with BRL 34915. Third European Meeting on Hypertension, Milan. J. Hypertens., 5, S193-S195.

VAN DE VOORDE, J. & LENSEN, I. (1983). Role of endothelium in the vasodilator response of rat thoracic aorta to histamine. Eur. J. Pharmacol., 79, 531-541.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. Pharmacol. Rev., 39, 163-196.

WALUS, K.M., FONDACARO, J.D. & JACOBSON, E.D. (1981). Effect of calcium and its antagonists on canine mesenteric circulation. Circ. Res., 48, 692-700.

WEIR, S.W. & WESTON, A.H. (1986a). Effect of apamin on responses to BRL 34915, nicorandil and other relaxants in the guinea-pig taenia caeci. Br. J. Pharmacol., 88, 113-120.

WEIR, S.W. & WESTON, A.H. (1986b). The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. Br. J. Pharmacol., 88, 121-128.

WESTON, A.H., SOUTHERTON, J.S., BRAY, K.M., NEWGREEN, D.T. & TAYLOR, S.G. (1988). The mode of action of pinacidil and its analogs P1060 and P1368: results of studies in rat blood vessels. J. Cardiovasc. Pharmacol., 12, Suppl. II. S10-16.

WILSON, C. (1989). Inhibition by sulphonylureas of vasorelaxation

induced by K^+ channel activators in vitro. J. Autonom. Pharmacol., 9, 9-16.

WILSON, C., BUCKINGHAM, R.E., MOOTOO, S., PARROT, L.S., HAMILTON, T.S., PRATT, S.C. & CAWTHORNE, M.A. (1988a). In vivo and in vitro studies of cromakalim (BRL 34915) and glibenclamide in the rat. Br. J. Pharmacol., 93, 126P.

WILSON, C., COLDWELL, M.C., HOWLETT, D.R., COOPER, S.M., HAMILTON, T.C. (1988b). Comparative effects of K^+ channel blockade on the vasorelaxant activity of cromakalim, pinacidil and nicorandil. Eur. J. Pharmacol., 152, 331-339.

WINQUIST, R.J. & BASKIN, E.P. (1983). Calcium channels resistant to organic calcium entry blockers in rabbit vein. Am. J. Physiol., 245, H1024-H1030.

WINQUIST, R.J. & BEVAN, J.A. (1981). In vitro model of maintained myogenic vascular tone. Blood Vessel, 18, 134-138.

WINQUIST, R.J., HEANEY, L.A., WALLACE, A.A., BASKIN, E.P., STEIN, R.B., GARCIA, M.L. & KACZOROWSKI, G.J. (1989). Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulfate and diazoxide in vascular smooth muscle. J. Pharmacol. exp. Ther., 248, 149-156.

YANG, X.C. & SACHS, F. (1987). Stretch activated channels in several tissues. Biophys. J., 51, 252a.