Role of Nitric Oxide and Prostacyclin in Modulating Pulmonary Vascular Tone

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By

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Publications

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ELBISHTI,W. & POLLOCK,D. (1994) L-NAME enhances vasorelaxation to sodium nitroprusside in rat pulmonary artery rings precontracted with KCl. Br. J. Pharmacol., **112**, 227P.

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SHAW,A.M., BARFARAZ,A.R., ELBISHTI,W., POLLOCK,D. & McGRATH,J.C. (1992) The effect of L-N-nitro arginine methyl ester and sodium nitroprusside on 5-HT and KCl-induced responses in the perfused lung and pulmonary arteries of the rat. Br. J. Pharmacol., **107**, 406P.

Abbreviations

CARB	Carbachol
cGMP	Cyclic guanosine 3',5'-monophosphate
CRC	Concentration-response curve
5-HT	5-Hydroxytryptamine
ISO	Isoprenaline
KCl	Potassium chloride
L-NAME	N^{ω}-Nitro-L-arginine methyl ester
L-NOARG	N^{ω}-Nitro-L-arginine
MPA	Main pulmonary artery
NO	Nitric oxide
NOS	Nitric oxide synthase
PE	Phenylephrine
SNP	Sodium nitroprusside

Summary

The aim of this study was to investigate the possible role of the endothelium-derived vasorelaxant factors, NO and prostacyclin, in the maintenance of the low pulmonary vascular tone. This was achieved by investigating whether a basal release of NO and prostacyclin is involved in modulating the responsiveness of isolated pulmonary arteries to vasoconstrictors, and by examining the ability of various humoral agents to stimulate NO release in this vascular bed. The extent to which this basal and stimulated release correlates with artery size was also investigated.

The main results obtained are summarised below :

1- Comparison of the concentration-response curves to the vasoconstrictors PE (10^{-9}-10^{-4}M), 5-HT (10^{-9}-10^{-3}M) or KCl (10-50mM) in MPA, 1st and 2nd branches revealed that generally 2nd branches displayed a significantly greater contractility and higher sensitivity to the vasoconstrictors than 1st branches, which showed higher contractility and sensitivity than MPA. The greater contractility of the smaller arteries is thought to be due to their higher smooth muscle content.

2- Pretreatment with the NOS inhibitor L-NAME ($2x10^{-4}$M) significantly potentiated the contractile responses to PE (10^{-9}-10^{-4}M), 5-HT (10^{-9}-10^{-3}M) and KCl (10-50mM) in all of the three arteries. The potentiating effect was not specific to L-NAME as another NOS inhibitor, L-NOARG (10^{-4}M), also caused a similar potentiation of contractile responses to PE (10^{-9}-10^{-4} M) in 2nd branches.

3- Prior incubation with L-arginine (10^{-2}M) completely prevented the ability of L-NAME ($2x10^{-4}$M) and L-NOARG (10^{-4}M) to potentiate PE-induced contractions (10^{-9}-10^{-4} M) in 2nd branches.
These results confirm that the potentiating effects of L-NAME and L-NOARG were due to inhibition of NO synthesis, and suggest that NO exerts a depressant effect on the contractile responses of pulmonary arteries to vasoconstrictors.

4- The α_2-agonist clonidine (10^{-8}-10^{-4} M) failed to produce any relaxant effect in 2nd branches precontracted with the thromboxane A_2 mimetic U 46619 ($8x10^{-10}$ M), even in the presence of the α_1-blocker prazosin (10^{-6} M) to suppress any possible α_1-mediated contractile effect of clonidine. This result excludes the presence of endothelial α_2-receptors that could stimulate NO release.

5- 5-HT (10^{-7}-10^{-4} M) did not evoke any relaxation in 2nd branches precontracted with U 46619 ($8x10^{-10}$ M), even when the contractile effect of 5-HT was suppressed by the 5-HT_2-antagonist ketanserin (10^{-6} M). Similarly, KCl (10-50 mM) failed to produce any degree of relaxation in 2nd branches precontracted with PE ($9.5x10^{-8}$ M).

6- Exposure of 2nd branch rings to PE (10^{-4} M) or 5-HT (10^{-4} M) did not cause any significant change in basal cGMP level. The effect of KCl (25.1 mM) on cGMP level was variable. KCl caused a significant rise in cGMP level in 2 out of 6 rings, with no effect in the 4 remaining rings.
These results generally suggest that the potentiating effects of L-NAME and L-NOARG were due to the loss of modulation of contractile responses by basally-released NO, rather than NO released by vasoconstrictor stimulation.

7- The potentiating effect of L-NAME on the contractile responses to PE, 5-HT and KCl was more pronounced as artery size decreased. this was neither due to a higher sensitivity of soluble guanylate cyclase in the smaller arteries, as the endothelium-independent vasorelaxant SNP (10^{-11}-10^{-4} M) produced similar maximum relaxant effects in the three arteries, nor was it due to the thicker vessel wall in the larger arteries serving as a physical barrier for NO diffusion, as MPA was more sensitive to the relaxant effect of low concentrations of the endothelium-dependent vasorelaxant CARB than were 1st and 2nd branches. The most likely cause for the greater potentiating effect of L-NAME in the smaller arteries, therefore, is a higher basal production of NO in these arteries.

8- This conclusion is further supported by the finding that the small arteries had higher cGMP levels than larger arteries.

9- Pretreatment of 2nd branch rings with the cyclo-oxygenase inhibitor flurbiprofen (10^{-5} M) had no significant effect on contractile responses to PE (10^{-9}-10^{-4} M). This result suggests that prostacyclin is not released spontaneously from the pulmonary endothelium.

10- The endothelium-dependent vasorelaxant CARB (10^{-9}-10^{-4}M) induced concentration-dependent relaxations in PE-precontracted rings (2.2×10^{-7} M or 9.5×10^{-8} M) from MPA, 1st and 2nd branches. However, 2nd branches were more responsive to the relaxant effect of CARB than 1st branches, which were more responsive than MPA. This most likely reflects the capacity of the small arteries to generate greater amounts of NO in response to the stimulatory effect of CARB than larger arteries.

11- Pretreatment with L-NAME ($2x10^{-4}$M) or L-NOARG (10^{-4}M) inhibited CARB-induced relaxations (10^{-9}-10^{-4}M). The inhibitory effects of L-NAME and L-NOARG were only partially prevented by prior incubation with L-arginine (10^{-2} M).

The results demonstrate that only the inhibition by L-NAME and L-NOARG of basal, but not CARB-stimulated, NO release is completely prevented by L-arginine. This raises the possibility that there are different isoenzymes of NOS involved in basal and CARB-stimulated NO release, or alternatively only one form of NOS is present and activation of the enzyme by CARB increases its affinity for the inhibitors.

12- The relaxant effects of CARB (10^{-9}-10^{-4}M) in KCl-precontracted rings (25.1 mM, 27.5 mM or 29.0 mM) were significantly weaker than in PE-precontracted rings ($2.2x10^{-7}$ M or $9.5x10^{-8}$ M). This was not due to an inhibitory action by KCl on the generation of NO, as SNP (10^{-11}-10^{-4} M) also induced significantly weaker relaxations in KCl-precontracted rings.

13- Pretreatment with L-NAME ($2x10^{-4}$ M) had no effect on SNP-induced relaxation in PE-precontracted rings but caused a significant enhancement of the relaxant responses to SNP in KCl-precontracted rings.

These results imply that the limited capacity of CARB and SNP to relax KCl-precontracted rings reflect the ability of KCl to stimulate NO release, thereby reducing the capacity of the endothelium to generate more NO in response to CARB and decreasing the amount of unstimulated guanylate cyclase available for activation by NO.

14- The non-selective β-agonist ISO (10^{-10}-10^{-4} M) induced concentration-dependent relaxations in endothelium-intact 2nd branch rings precontracted with PE ($9.5x10^{-8}$ M). Flurbiprofen (10^{-5} M) had no effect on ISO-induced relaxation, which excludes the contribution of prostacyclin to this effect.

15- Several lines of evidence indicate that the relaxant response to ISO (10^{-10}-10^{-4} M) was partially mediated by stimulation of NO release. Pretreatment with L-NAME ($2x10^{-4}$ M) caused partial inhibition of ISO-induced relaxation, an effect which was completely prevented by L-arginine (10^{-2}M). Moreover, when L-NAME was added during maximum relaxation to ISO, a partial reversal of the relaxation was observed. In addition, ISO (10^{-4} M) caused a rise in cGMP level.

16- In endothelium-denuded rings, ISO (10^{-10}-10^{-4}M) produced concentration-dependent relaxations comparable to those induced in endothelium-intact rings, but in this case L-NAME ($2x10^{-4}$M) had no effect on ISO-induced relaxation.

These results suggest that in the presence of endothelium, the relaxant effect of ISO is mediated partly by stimulation of NO release and partly by a direct effect on smooth muscle cells. In the absence of endothelium, ISO induces comparable relaxations by a direct effect on the smooth muscle, independently of NO release.

17- In endothelium-intact rings, the submaximal responses to ISO were markedly shifted to the right by the non-selective β-antagonist propranolol (10^{-6}M), and to a lesser extent by the selective β_1-antagonist atenolol (10^{-5} M) and the selective β_2-antagonist

ICI 118551 (10^{-6} M), which indicates the involvement of β_1 and β_2 receptors in mediating these responses.

18- In conclusion, this study indicates that NO, but not prostacyclin, is spontaneously released in the pulmonary vascular bed. The release of NO, which can also be stimulated by a variety of vasoactive agents, serves to depress the contractility of pulmonary arteries to various vasoconstrictors and, therefore, might contribute to the maintenance of the low pulmonary vascular tone. The study also shows that in the pulmonary vascular tree, small arteries display a greater contractility to vasoconstrictors and a larger capacity to generate NO than larger arteries, and therefore would have a greater role in regulating pulmonary pressure.

Chapter 1

Introduction

The main function of pulmonary circulation is gas exchange, particularly transport of oxygen from air to blood. For the lungs to perform their gas exchange function efficiently, pulmonary blood vessels must be thin-walled so that the poorly soluble oxygen molecule can more easily diffuse from the alveolar air into the blood. This in turn requires the intravascular pressure to be low to prevent leakage of fluids from the vessels. Indeed, although blood flow to the lungs is higher than that to any other organ of the body, the pressure within pulmonary arteries is only one-fifth of that within systemic arteries. Not only is the pulmonary circulation a high flow,low pressure system in a subject at rest but it can also accommodate increases in cardiac output of up to four times resting value with only a small rise in intravascular pressure.

It is not clearly known how the lung maintains a low pressure. In addition to anatomical and mechanical properties of the pulmonary vascular bed, a variety of vasoactive agents might be contributors. Over the last two decades, it has become increasingly clear that the endothelium plays a major role in the control of vascular tone. However, the extent to which the endothelium contributes to the maintenance of the low pulmonary vascular tone is still under investigation. This introduction will focus on the vasorelaxant substances released by the endothelium, their mechanisms of producing vascular smooth muscle relaxation, and what is known on their role in modulating pulmonary vascular tone.

1.1. Properties of arteries

1.1.1. Structure

Although the basic organisation of the wall of all arteries is similar, pulmonary arteries differ from their systemic counterparts in having far thinner walls as well as some other histological differences. Three concentric layers or *tunicae* can be distinguished in the arterial wall (Bloom & Fawcett, 1975) (Fig.1) :

a) An inner layer, the *tunica intima*, consisting mainly of endothelial cells.

b) An intermediate layer, the *tunica media*, predominantly composed of smooth muscle cells with small bundles of collagen and elastic fibres. The boundary between the media and intima is marked by the internal elastic lamina. On the adventitial side, a thinner external elastic lamina can be found in many arteries.

c) An outer layer, the *tunica adventitia*, made up of elastin, collagen, fibroblasts and occasional Schwann cells with associated nerve axons which are usually confined to the adventitia and do not penetrate the media (Hirst & Edwards, 1989).

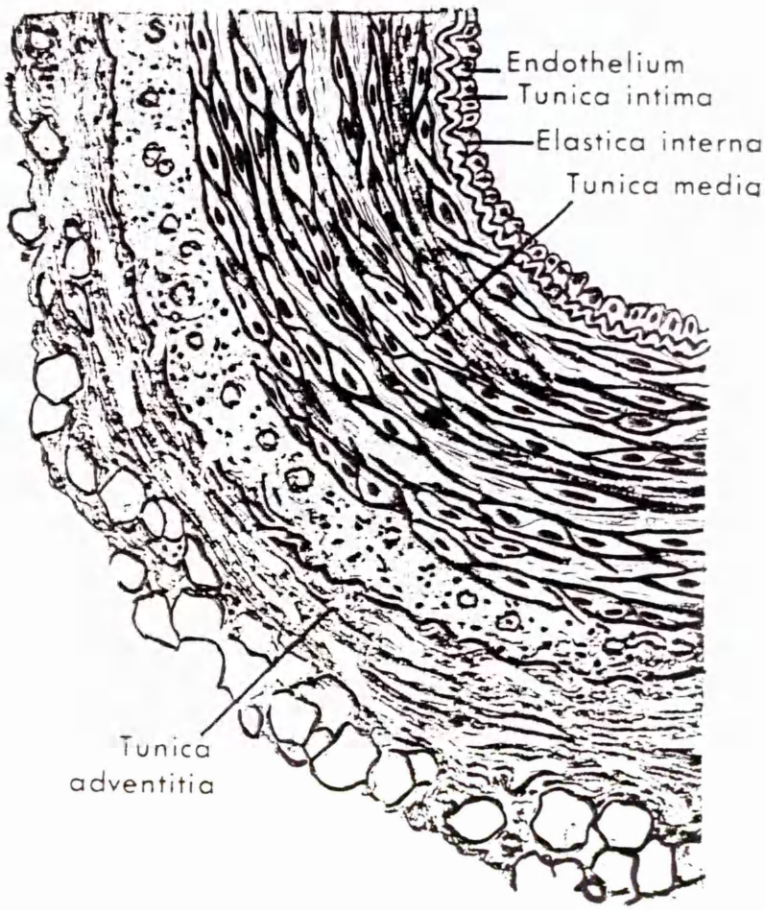


Fig. 1.

A cross sectional area of an artery showing the different layers forming the artery wall (Bloom & Fawcett, 1975).

Three types of pulmonary arteries can be identified in the normal human lung (Murray, 1986) :

i) Elastic arteries :

The pulmonary trunk, its main branches and all extralobular arteries are classified as elastic arteries because in them the media consists predominantly of elastic tissue with little smooth muscle and collagen.

ii) Muscular arteries :

In these vessels, which lie within lung lobules and accompany bronchioles, a compact muscle layer appears with poorly developed internal elastic lamina. In the lung, the transition to muscular arteries occurs gradually, whereas in the systemic circulation this transition occurs in large arteries.

iii) Partially muscular and non-muscular arteries :

The pulmonary vascular bed is also distinctive in that it possesses no counterpart to the small muscular arteries and arterioles of the systemic circulation. In the lung, the muscular arteries give way to partially muscular arteries which have an incomplete muscular coat in which the muscle has a spiral orientation. The non-muscular part of the wall contains cells that are intermediate in structure between mature cells and pericytes (Reid & Meyrick, 1980). The partial layer of muscle gradually disappears until the vessel wall consists only of endothelial cells,

pericytes and elastic lamina. The partially muscular and non-muscular arteries supply alveolar ducts and alveoli

1.1.2. Signal transduction in vascular smooth muscle

In vascular smooth muscle, the cycle of contraction-relaxation is mainly regulated by changes in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), although Ca^{2+}-independent regulation may also play a role (Nishikawa *et al.*, 1984). The major mechanisms of excitation-contraction coupling are electromechanical and pharmacomechanical coupling. Electromechanical coupling involves depolarisation of the plasma membrane leading to opening of voltage-operated Ca^{2+} channels through which Ca^{2+} influx occurs, as well as depolarisation-induced intracellular Ca^{2+} release (Himpens & Somlyo, 1988). Pharmacomechanical coupling, which does not involve membrane potential changes, is initiated by the binding of an excitatory agonist with its particular receptor (Somlyo, 1985). These receptors are coupled by guanine nucleotide-binding regulatory proteins (G proteins) to phospholipase C which when activated causes hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2), resulting in the production of 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C which phosphorylates myosin light chains (Nishikawa *et al.*, 1985). IP_3 releases Ca^{2+} from the sarcoplasmic reticulum (Berridge, 1987). Receptor activation may also stimulate Ca^{2+} influx through receptor-operated Ca^{2+} channels (Bolton, 1979). Ca^{2+} binds to calmodulin and the Ca^{2+}-calmodulin complex subsequently activates myosin light chain kinase (MLCK) (Dabrowska *et al.*, 1977) which phosphorylates myosin light chains leading to an

increase in actin-myosin Mg^{2+}-ATPase activity and, hence, muscle contraction.

Inhibition of excitation-contraction coupling, i.e smooth muscle relaxation, can result from withdrawal of the contractile stimulus leading to a decrease in $[Ca^{2+}]_i$, or can be mediated by an inhibitor of contraction in the continued presence of the contractile agonist. The latter mechanism involves the generation of an intracellular second messenger. The cyclic nucleotides adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) are thought to be the major second messengers mediating relaxation in vascular smooth muscle (Kuriyama *et al.*, 1982 ; Murad, 1986). cAMP is synthesised from adenosine triphosphate by the action of adenylate cyclase. A number of membrane-bound receptors are now known to exert their stimulatory actions on adenylate cyclase via G proteins (Gilman, 1987). The primary mechanism by which cAMP causes relaxation include : (i) phosphorylation of MLCK by cAMP-dependent protein kinase (protein kinase A) resulting in a decreased affinity of MLCK for the Ca^{2+}-calmodulin complex (deLanerolle *et al.*, 1984) ; (ii) reduction of $[Ca^{2+}]_i$ by inhibition of Ca^{2+} influx, augmentation of Ca^{2+} efflux or enhancement of Ca^{2+} sequestration into the sarcoplasmic reticulum (Scheid & Fay, 1984). Both of these events would decrease the extent of myosin light chain phosphorylation and cause relaxation.

cGMP synthesis is catalysed from guanosine triphosphate by guanylate cyclase, which can be activated by a number of agents including nitric oxide (NO) (Arnold *et al.*, 1977). The primary mechanism by which cGMP causes smooth muscle relaxation is by lowering $[Ca^{2+}]_i$ (Collins *et al.*, 1986). cGMP exerts the majority of its actions through

the activation of a protein kinase, termed cGMP-dependent protein kinase. The mechanisms by which cGMP-dependent protein kinase decreases $[Ca^{2+}]_i$ include : (i) inhibition of phospholipase C, presumably through the phosphorylation of a regulatory protein for phospholipase C, resulting in inhibition of IP_3 formation (Hirata *et al.*, 1990); (ii) stimulation of Ca^{2+}-activated K^+ channels leading to hyperpolarisation and inhibition of Ca^{2+} entry through voltage-operated Ca^{2+} channels (Robertson *et al.*, 1993) ; (iii) stimulation of Ca^{2+}-ATPase activity leading to augmentation of Ca^{2+} sequestration into the sarcoplasmic reticulum (Yoshida *et al.*, 1991).

1.1.3. **Endothelium-derived vasoactive agents**

The original conception of the vascular endothelium as a physical and biochemical barrier between the circulating blood and the vascular smooth muscle, with no other functional properties, has been dramatically changed over the past two decades. It is now well established that the endothelial cells are a rich source of substances regulating the tone of underlying vascular smooth muscle as well as coagulation, inflammatory and immunological processes. The first endothelial vasoactive substance was discovered in 1976 (Moncada *et al.*) when it was demonstrated that endothelial cells synthesise prostacyclin (PGI_2), a potent vasorelaxant and platelet inhibitory metabolite of arachidonic acid. In 1980, Furchgott and Zawadzki demonstrated the phenomenon of endothelial-dependent relaxation in vascular tissue and its mediation by a humoral factor, which later became known as endothelium-derived relaxing factor (EDRF).Subsequent

studies have suggested that EDRF may be NO (Ignarro *et al.*, 1986) or a NO-containing compound (Myers *et al.*, 1990).

In addition to the production of relaxing factors, the endothelium can also release vasoconstrictor substances, these include : vasoconstrictor metabolites of arachidonic acid (Miller & vanhoutte, 1985b) ; the contracting factor released by severe hypoxia, the nature of which has not yet been identified (Rubanyi & Vanhoutte, 1985) ; and endothelin-1 (Yanagisawa *et al.*, 1988), which is considered the most potent vasoconstrictor substance yet discovered.

1.2. Nitric oxide

1.2.1. Nitric oxide and EDRF

NO has long been known to account for the vasorelaxant activity of nitrovasodilators (Arnold *et al.*, 1977). Based on the similarities in the pharmacological behaviour of EDRF and NO, Furchgott (1988) and Ignarro *et al.* (1986) suggested that EDRF may be NO or a closely related species. The first evidence for the formation of NO by endothelial cells came from experiments which showed that the concentrations of bradykinin that induced EDRF release from cultured porcine aortic endothelial cells also caused a concentration-dependent release of NO (Palmer *et al.*, 1987). A detailed comparison of the biological actions of EDRF and NO on vascular strips and on platelets also showed that the two compounds were indistinguishable (Moncada *et al.*, 1988). Both EDRF and NO caused a relaxation of vascular strips (Palmer *et al.*, 1987) and inhibition of platelet aggregation (Radomski *et al.*, 1987). These

actions were similarly potentiated by superoxide dismutase and cytochrome C and inhibited by Fe^{2+} and some redox compounds (Hutchinson *et al.*, 1987 ; Palmer *et al.*, 1987 ; Radomski *et al.*, 1987). Furthermore, both EDRF and NO act on vascular smooth muscle and platelets through the stimulation of guanylate cyclase and elevation of cGMP (Mellion *et al.*, 1981 ; Rapoport & Murad, 1983). All of this evidence strongly supported the proposal that EDRF is NO. However several studies have questioned this conclusion, based on observations about variations in the half-life of EDRF (Griffith *et al.*, 1984 ; Cocks *et al.*, 1985), differential binding of EDRF and NO to anion exchange columns (Cocks *et al.*, 1985 ; Long *et al.*, 1987), and stabilisation of EDRF by acidification which would not be expected to stabilise NO (Murray *et al.*, 1986). However, the general line of evidence suggests that even if EDRF is released from endothelial cells as a NO-containing compound, the biological effects of EDRF are mediated ultimately by NO.

1.2.2. **Synthesis**

NO is synthesised from the amino acid L-arginine, with the formation of L-citrulline as a by-product (Palmer *et al.*, 1988). The reaction is stereospecific since a number of analogues of L-arginine, including its D-enantiomer, are not substrates. The synthesis is catalysed by several isoforms of the enzyme NO synthase (NOS) (Forstermann *et al.*, 1991), which catalyse the oxidation of one of the terminal guanidino nitrogen atoms of L-arginine to form NO and L-citrulline, with N^{ω}-hydroxy-L-arginine as an intermediate (Stuehr *et al.*, 1991). NOS utilises O_2 and nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates

and thiol, tetrahydrobiopterin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) as cofactors (Mayer *et al.*, 1990 ; Bredt *et al.*, 1991). Two broad groups of NOS can be identified : a "constitutive" Ca^{2+}-calmodulin dependent enzyme and an "inducible" Ca^{2+} independent enzyme (Forstermann *et al.*, 1991). The constitutive enzyme is present in a wide variety of cells including platelets, endothelial and neuronal cells (Mayer *et al.*, 1989 ; Knowles *et al.*, 1990 ; Radomski *et al.*, 1990). It binds calmodulin and becomes fully active at increased $[Ca^{2+}]_i$. Thus, increased $[Ca^{2+}]_i$ can be linked to increased NO synthesis in cells. The inducible enzyme can be expressed in endothelial cells (Radomski *et al.*, 1990), vascular smooth muscle (Busse & Mulsch, 1990), macrophages (Marletta *et al.*, 1988) and neuronal cells (Salvemin *et al.*, 1992) among other cells. The conditions required for the induction of NOS in these cell types varies, but interferon γ (Lowenstein *et al.*, 1993), endotoxin (Buttery *et al.*, 1993) and interleukin-1 (Suschek *et al.*, 1992) have all been shown to induce the enzyme.

1.2.3. **Mechanisms of release**

Different mechanisms can lead, by activation of the constitutive or inducible NOS, to the release of NO from the vascular endothelium. The inducible release occurs following activation by bacterial endotoxins or cytokines. Two types of constitutive release of NO can be defined : basal and stimulated release. Several lines of evidence suggest that there is a continuous basal release of NO from the endothelium. NOS inhibitors caused endothelium-dependent contractions of isolated vascular rings (Palmer *et al.*, 1988 ; Gold *et al.*, 1990), increased coronary perfusion

pressure in rabbit isolated perfused heart (Amezcua *et al.*, 1989), raised blood pressure when administered to conscious rats (Gardiner *et al.*, 1990), and increased pulmonary arterial pressure and pulmonary vascular resistance in conscious rabbits (Wiklund *et al.*, 1990). Moreover, removal of the endothelium or pretreatment with NOS inhibitors potentiated the contractile responses of isolated arteries to various vasoconstrictors (Martin *et al.*, 1986 ; Trezise *et al.*, 1992 ; Ayotunde *et al.*, 1994). The basal release of NO presumably reflects activation of NOS by the normal resting $[Ca^{2+}]$ within the endothelial cell.

NO release can be stimulated by various agonists such as acetylcholine (Furchgott & Zawadzki, 1980), bradykinin (Palmer *et al.*, 1987), and substance P (Furchgott, 1983) or by receptor-independent compounds such as Ca^{2+} ionophores (Furchgott, 1983). Physical stimuli such as shear stress (Pohl *et al.*, 1986) and pulsatile flow (Hutcheson & Griffith, 1991) also stimulate NO release and may represent the physiologically most important mechanisms of NO release from the endothelium. As the constitutive enzyme in endothelial cells is a Ca^{2+}-calmodulin dependent enzyme, an elevation of $[Ca^{2+}]_i$ is an absolute prerequest for agonist-stimulation of NO release. Numerous studies have shown that the agonist-induced increase in $[Ca^{2+}]_i$ in endothelial cells involves both a transient IP_3-mediated release of intracellular Ca^{2+} and a more sustained extracellular Ca^{2+} influx (Freay *et al.*, 1989). A similar relation between shear stress and increased $[Ca^{2+}]_i$ have been observed (Schwarz *et al.*, 1992).

1.2.4. **Mechanism of action**

Following its release from endothelial cells, NO diffuses into the underlying vascular smooth muscle cells where it binds to the haeme moiety at the catalytic site of guanylate cyclase thereby activating the enzyme, which leads to the generation of cGMP from guanosine triphosphate (Greutter *et al.*, 1981 ; Ignarro *et al.*, 1986). cGMP in turn causes vascular relaxation through the mechanisms described in section 1.1.2.

1.2.5. **Inhibitors of NO**

NO is a highly reactive free radical. It binds with a variety of biological substances, resulting in its rapid inactivation. The most important pathway in the removal of NO is its rapid reaction with oxyhaemoglobin (O_2Hb) to form methaemoglobin and nitrate (Wennmalm *et al.*, 1992):

$$NO + O_2Hb \rightarrow metHb + NO_3^-$$

This reaction ensures that NO diffusing into the lumen of a blood vessel will be very rapidly converted to nitrate. Within the vessel wall, a reaction that has also been shown to be important in removing NO is that with superoxide anion (O_2^-) (Grylewski *et al.*, 1986) :

$$NO + O_2^- \rightarrow ONOO^- \rightarrow NO_3^-$$

In the absence of haemoglobin or superoxide anion, for example in physiological salt solutions, the predominantly reaction of NO is probably that with O_2 (Olbergts, 1985) :

$$2\ NO + O_2 \rightarrow N_2O_4 \rightarrow NO_2^- + NO_3^-$$

Several analogues of L-arginine have been shown to inhibit stereoselectively the synthesis of NO. They act by competitively inhibiting NOS, their effects being reversed by the addition of excess L-arginine (Rees *et al.*, 1989). Among the most commonly used NOS inhibitors are N^G-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1989), N^G-nitro-L-arginine (L-NOARG) (Ishii *et al.*, 1990), and N^G-nitro-L-arginine methyl ester (L-NAME) (Rees *et al.*, 1990). The action of NO can be inhibited by methylene blue which acts by inhibiting guanylate cyclase (Gruetter *et al.*, 1980).

1.3. Prostacyclin

1.3.1. Discovery and synthesis

In 1976, Moncada *et al.* reported that prostaglandin endoperoxides are transformed in blood vessel walls to an unstable substance which they called prostacyclin. Although prostacyclin can be formed in vascular smooth muscle, endothelial cells are the major site of its synthesis (Moncada *et al.*, 1977). Stimuli for prostacyclin synthesis include various agonists such as acetylcholine (Beetens *et al.*, 1983), bradykinin (Hong, 1980) and histamine (Schellenberg *et al.*, 1986); Ca^{2+} ionophores (Wekler *et al.*, 1978), and physical stimuli such as

shear stress and pulsatile flow (van Grondelle *et al.*, 1984 ; Frangos *et al.*, 1985). All of these stimulants activate the enzyme phospholipase A_2 which releases arachidonic acid from membrane phospholipids (Van Den Bosch, 1980). Arachidonic acid is then converted by cyclo-oxygenase to prostaglandin G and subsequently to prostaglandin H (Hamberg & Samuelsson, 1974), from which prostacyclin is formed by the enzyme prostacyclin synthase (De Witt & Smith, 1983).

Prostacyclin synthesis can be inhibited by glucocorticoids which inhibit the enzyme phospholipase A_2 (Hirata *et al.*, 1980), and by nonsteroidal anti-inflammatory drugs which inhibit the enzyme cyclo-oxygenase (Vane, 1976).

1.3.2. **Mechanism of action**

Prostacyclin binds to a specific G protein-coupled receptor, termed the IP receptor, on the membrane of smooth muscle cells (Kennedy *et al.*, 1982), resulting in the activation of adenylyl cyclase (Dembinska-Kiec *et al.*, 1980) which catalyses the conversion of adenosine triphosphate to cAMP. The mechanisms by which cAMP causes vasorelaxation are discussed in section 1.1.2.

1.4. **Regulation of pulmonary pressure**

Several factors might be involved in the regulation of pulmonary pressure. These can be divided into passive and active factors. Passive factors cause changes in pulmonary pressure by imposing a passive

increase or decrease in the calibre of pulmonary blood vessels. In contrast, alterations in pulmonary pressure by active factors imply that contraction or relaxation of vascular smooth muscles has occurred.

1.4.1. **Passive Factors**

i) Pulmonary blood flow

The pulmonary circulation can accommodate increases in cardiac output of up to four times resting values with only a small rise in intravascular pressure. This is thought to be achieved mainly by distension and recruitment of blood vessels (Roos *et al.*, 1961). However, vasoactive agents might be involved. This ability of the lungs to accommodate greatly increased blood flow during exercise obviously conserves the energy of the right side of the heart, and it also prevents a significant rise in pulmonary capillary pressure and therefore prevents development of pulmonary oedema during the increased cardiac output.

ii) Left atrial pressure

Left atrial pressure can rise as a result of left heart failure. A moderate rise in left atrial pressure causes distension and recruitment of pulmonary blood vessels leading to a decrease in vascular resistance, therefore showing almost no change in pulmonary arterial pressure (Borst *et al.*, 1956). However, a significant rise in left atrial pressure causes an almost similar increase in pulmonary pressure. This increases the load on the left side of the heart and leads to pulmonary oedema.

iii) Lung volume

During inspiration, the extra-alveolar vessels (which are not in the alveolar walls) become distended as the lung expands. By contrast, the alveolar vessels (which lie in and around alveolar walls) become flattened by lung inflation because the alveolar pressure tends to increase relative to their intravascular pressure (Howell *et al.*, 1961). Accordingly, pulmonary pressure increases as lung volume rises because of the increase in resistance through the compressed alveolar vessels. The pressure becomes lowest at about the normal resting end-tidal expiratory level, but increases again at lower lung volumes due to an increased resistance in the extra-alveolar vessels which become flattened when the lung is deflated.

1.4.2. **Active factors**

i) Neural effects

The pulmonary vasculature is innervated by both sympathetic and parasympathetic nerves (Downing & Lee, 1980). Despite the presence of a definite vascular nervous plexus which presumably must have functional significance, it has not been possible to demonstrate any influences mediated by the autonomic nervous system on the pulmonary circulation in normal human adults (Widdicombe & Sterling, 1970). In experimental animals, electrical stimulation of the pulmonary sympathetic nerves can increase vascular resistance which can be attributed to α-adrenoceptor stimulation (Kadowitz *et al.*, 1973 ; Hakim & Dawson, 1979). In the presence of α-adrenoceptor blockers, sympathetic stimulation can cause vasodilatation which appears to be mediated by

β-adrenoceptors (Hyman et al.,1981). Stimulation of the vagal nerve in the cat have indicated that the nerve contains both sympathetic and parasympathetic efferent fibres that innervate the pulmonary vascular bed (Nandiwada *et al.*, 1983). Stimulation of the sympathetic fibres in the vagus caused vasoconstriction, whereas stimulation of the parasympathetic fibres caused vasodilatation but only when some degree of vasoconstriction was already present. In more recent studies on the intact-chest cat (McMahon *et al.*, 1992 ; McMahon & Kadowitz, 1992), vagal stimulation caused a decrease in pulmonary arterial pressure which was blocked by atropine as well as L-NAME and methylene blue, suggesting that neurogenically-released acetylcholine induces NO release in the pulmonary vascular bed.

ii) Chemical effects

The pulmonary circulation is distinguished by responding to hypoxia with a vasoconstriction, rather than a vasodilatation as seen in systemic vascular beds (Fishman, 1976 ; Yuan *et al.*, 1990). This hypoxic pulmonary vasoconstriction (HPV) is the principal mechanism that matches local lung perfusion to ventilation by diverting blood from poorly ventilated areas to those areas of the exchange membrane where oxygen concentrations are adequate. The response depends upon the oxygen tension in the alveoli and, to a much lesser extent, on that in the blood stream. A potential disadvantage of HPV is that chronic alveolar hypoxia can lead to chronic pulmonary hypertension (Fishman, 1961). Although the phenomenon has been studied in various *in vivo* and *in vitro* preparations, its mechanism is still poorly understood. The search for a mediator of HPV has generated mostly negative evidence. The response to hypoxia is unaltered by inhibition of the sympathetic nervous system

(Silove & Grover, 1968), and it is not dependent on the release of histamine, 5-hydroxytryptamine or angiotensin II (Nayar *et al.*, 1972 ; Hales & Kazemi, 1975 ; McMurtry, 1984). Recent studies that have addressed the possible role of the endothelium in mediating or modulating HPV have produced conflicting results. Some studies have proposed that hypoxic vasoconstriction is mediated by the release of an endothelium-derived contracting factor (Holden & McCall, 1984 ; Rubanyi & Vanhoutte, 1985). However, HPV does not appear to be mediated by vasoconstrictor metabolites of arachidonic acid since it is not affected by inhibition of cyclo-oxygenase (Rodman *et al.*, 1989), and the fact that return to normoxia results in rapid reversal of hypoxic contraction does not support a role for endothelin as a mediator, since endothelin-induced contractions are characteristically slow to reverse (Vanhoutte *et al.*, 1989). Other studies have proposed that HPV is mediated by a decreased release of NO from the endothelium (Warren *et al.*, 1989 ; Graser & Vanhoutte, 1991). However, it is consistently found that inhibition of NO synthesis markedly enhances the pulmonary pressor response to hypoxic challenges. L-NAME enhanced HPV in open-chest rabbits (Persson *et al.*, 1990), and L-NMMA augmented the vasoconstrictor response to hypoxia in rat perfused lungs and isolated pulmonary arteries (Archer *et al.*, 1989). These results not only rule out the hypothesis of a blunted NO release as the cause of HPV, they also suggest that NO activity is in fact increased during acute hypoxia. This increased activity probably represents an important physiological defence mechanism, enabling the pulmonary vascular bed to limit excessive vasoconstriction during hypoxia.

Pulmonary vasoconstriction occurs also in response to hypercapnia and acidosis (Barer *et al.*, 1967). Although hypercapnia is a weaker

vasoconstrictor stimulus, the hypoxic response is potentiated by hypercapnia or the associated acidosis (Malik & Kidd, 1973). Thus changes in carbon dioxide tension can be important in determining the magnitude of the hypoxic response.

iii) Humoral effects

The pulmonary vasculature responds to numerous biological mediators. However, it remains to be established whether this mediator vasoactivity is significantly involved in the control of either the normal or the abnormal pulmonary pressure. The lung itself is a source of a large number of vasoactive substances. In addition to the relaxing and contracting factors released by pulmonary endothelium, the mast cells contain histamine, 5-hydroxytryptamine, adenosine triphosphate and dopamine (Lewis & Austen, 1977), which can influence pulmonary vascular tone when released. Among the peptides known to occur in lung tissue are angiotensin II, vasoactive intestinal peptide (VIP) and substance P (Said, 1982). Angiotensin II and, to a lesser extent, substance P contract isolated segments of pulmonary arteries. Inhibition of the conversion of angiotensin I to angiotensin II has been reported to reduce pulmonary arterial pressure and vascular resistance in humans (Niarchos *et al.*, 1979). On the other hand, VIP is a potent vasodilator of isolated pulmonary arteries (Hamasaki & Said, 1981). It has also been shown to reduce pulmonary arterial pressure and vascular resistance in cats receiving infusions of a prostaglandin endoperoxide analogue (Said, 1982). Lung tissue can also generate substantial quantities of prostaglandins and thromboxanes especially during pathophysiological response (Demling *et al.*, 1981). Most of these and other vasoactive substances can also reach the pulmonary circulation via

venous blood. However, the pulmonary endothelium has the capacity to inactivate or remove many of these substances and, therefore, their role in modulating pulmonary pressure is difficult to determine. The picture is further complicated by the fact that vasoactive substances can have multiple effects. Histamine can cause direct vasoconstriction or vasodilatation depending on the background vascular tone, as well as an indirect endothelium-dependent vasodilatation (Shaw, 1971 ; Hill, 1990). 5-Hydroxytryptamine can cause direct vasoconstrictor effects, indirect sympathetic effects and also can induce endothelium-dependent as well as endothelium-independent vasodilatation (Hollenberg, 1988 ; Glusa & Richter, 1993). Prostaglandins produce diverse effects on pulmonary vasculature ranging from intense vasoconstriction to vasodilatation. PGH_2 and most of its derivatives (PGD_2, PGE_2, $PGF_{2\alpha}$ and TXA_2) are vasoconstrictors. In contrast, prostacyclin and PGE_1 are vasodilators (Kadowitz *et al.*, 1981).

Since the discovery of the release of prostacyclin and more recently NO from the endothelium, a growing research have focused on investigating their role in the maintenance of the low pulmonary vascular tone. Although prostacyclin is currently used to treat patients with severe primary pulmonary hypertension, its role in the physiology and pathophysiology of the pulmonary circulation is still unclear. The effect of cyclo-oxygenase inhibitors on baseline pulmonary haemodynamics during normoxic ventilation have been highly variable. In the intact anaesthetised dog, indomethacin produced an increase in pulmonary arterial pressure and vascular resistance whereas meclofenamate and ibuprofen had no effect (Rubin *et al.*, 1985). Ogletree (1982) found that only high doses of indomethacin increased pulmonary pressure and vascular resistance, and that meclofenamate did not alter pulmonary

haemodynamics. In contrast, Walker *et al.* (1982) reported that administration of meclofenamate in conscious dogs increased pulmonary pressure and vascular resistance. Prostacyclin have been shown to be produced by the lungs in response to vasoconstriction, and inhibition of prostacyclin production by meclofenamate and indomethacin resulted in potentiation of the pulmonary pressor responses to angiotensin II, $PGF_{2\alpha}$ and hypoxia (Weir *et al.*, 1974 ; Voelkel *et al.*, 1981). Studies in sheep (Newman *et al.*, 1986) have indicated that prostacyclin does not seem to have a major role in the mechanisms that maintain a low pulmonary pressure during exercise.

The relation between chronic hypoxia and development of pulmonary hypertension is well established (Fishman, 1961). Studies investigating the relation between prostacyclin production and the development of pulmonary hypertension have shown that repeated administration of indomethacin in sheep for 3 weeks caused sustained pulmonary hypertension (Meyrick *et al.*, 1985), and that pulmonary hypertension induced by chronic hypoxia in neonatal calves was associated with reduced pulmonary artery production of prostacyclin (Badesch *et al.*, 1989). By contrast, others have found that prostacyclin production was increased in the endothelium and vascular smooth muscle of pulmonary arteries from rats with chronic hypoxic pulmonary hypertension (Shaul *et al.*, 1991).

There is increasing evidence indicating an involvement of NO in the modulation of pulmonary pressure in health and disease. Endothelium-dependent relaxation resulting from NO release has been found in isolated pulmonary arteries (Chand & Altura, 1981 ; Ignarro *et al.*, 1984) and perfused lungs (Archer *et al.*, 1990) from most animal species.

Haemoglobin and the NOS inhibitors N^G-methyl-L-arginine and L-NMMA caused endothelium-dependent contraction of rat and bovine pulmonary arterial rings (Archer *et al.*, 1989 ; Gold *et al.*, 1990). Similarly, methylene blue and L-NOARG significantly increased perfusion pressure in isolated perfused lungs from rats (Archer *et al.*, 1990) and rabbits (Persson *et al.*, 1990). In addition, inhibition of NO synthesis either by endothelium denudation or NOS inhibitors enhanced responsiveness to various vasoconstrictors in isolated pulmonary arteries (Yamaguchi *et al.*, 1989 ; Gold *et al.*, 1990) and perfused lungs (Yamaguchi *et al.*, 1987 ; Mazmanian *et al.*, 1989). While these results are consistent, other contrasting findings have been reported. L-NMMA had no effect on the basal tone of pulmonary arterial rings from rats (Crawley *et al.*,1990), and in isolated perfused lungs from rats and rabbits, L-NMMA and haemoglobin did not alter perfusion pressure under normoxic conditions (Cherry & Gillis, 1987 ; Archer *et al.*, 1989). Despite these contradictory results, it seems likely that basal release of NO occurs in the pulmonary vascular bed, which contributes to the maintenance of the low pulmonary pressure.

This suggestion is further supported by studies in humans. Endogenous NO has been found in the exhaled air of normal humans (Gustafsson *et al.*, 1991), and as in other mammalian species, endothelium-dependent relaxation mediated by NO is also present in the human pulmonary circulation (Greenberg *et al.*, 1987 ; Cremona *et al.*, 1991). In addition, infusion of methylene blue or L-NAME significantly increased pulmonary vascular resistance of isolated perfused lungs from humans (Cremona *et al.*, 1991). Moreover recently, Celermajer *et al.* (1994) carried out a study on children with normal pulmonary haemodynamics who were undergoing cardiac catheterisation, and found that intralobar

infusion of L-NMMA caused a decrease in pulmonary blood flow without altering systemic haemodynamics.

To determine the role of endothelial dysfunction in pulmonary disease, several studies have investigated the effects of chronic hypoxia on endothelium-derived NO activity in the pulmonary circulation of both animals with chronic hypoxic pulmonary hypertension and humans suffering from chronic hypoxic lung disease. Endothelium-dependent relaxation to acetylcholine was markedly reduced in isolated pulmonary arterial rings (Leach *et al.*, 1990) and perfused lungs (Adnot *et al.*, 1991) from rats kept in hypoxic chambers for several weeks. Normal endothelium-dependent relaxation was restored when the animals were returned to normoxia. Studies in humans also produced results consistent with those in animals. Endothelium-dependent relaxation was markedly impaired in isolated pulmonary arterial rings from patients with chronic obstructive lung disease as compared with control subjects (Dinh-Xuan *et al.*, 1991, 1992). These results suggest that NO release and/or activity is impaired in chronic hypoxic pulmonary hypertension.

1.5. Metabolic functions of the lung

It is now well established that the lung has important metabolic functions in addition to its main function of gas exchange, in which the lung takes up, inactivates, or activates certain circulating substances, and synthesises and releases others. The lungs are ideally located and equipped for metabolic functions. First, the lungs are interposed between the venous and arterial sides of the systemic circulation and, therefore, receive the entire cardiac output. Consequently, the lungs are

strategically located to exert a control function over the composition of arterial blood. Second, the pulmonary endothelial cells, which constitute a substantial fraction of all the vascular endothelial cells, are equipped with superficial enzymes and carrier systems that allow entry of selected products for intracellular processing.

Inactivation of 5-HT was the first metabolic function of the lung to be identified (Gaddum *et al.*, 1953). Several vasoactive amines have since been shown to be inactivated to various extents during passage through the pulmonary circulation. The pulmonary inactivation of 5-HT and noradrenaline depends on uptake, followed by enzymatic degradation. The sites of uptake are in the endothelial cells, especially those within arterioles and capillaries (Strum & Junod, 1972 ; Nicholas *et al.*, 1974). In contrast, the lung does not inactivate adrenaline, dopamine and histamine, even though all are substrates for intracellular enzymes. This is due to the lack of specific uptake systems (Alabaster, 1977). The uptake mechanism thus enables the lung to exercise selective control over the metabolism of circulating substances.

The pulmonary inactivation of prostaglandins, like that of vasoactive amines, requires an uptake process before enzymatic degradation. This uptake mechanism exists for PGE_2 and $PGF_{2\alpha}$, but not for prostacyclin (Eling *et al.*, 1977 ; Dusting *et al.*, 1987). Consequently, PGE_2 and $PGF_{2\alpha}$ are inactivated during passage through the pulmonary circulation, whereas prostacyclin is unaffected by the lung.

The fate of several biologically-active peptides in the pulmonary circulation is quite different from that in other vascular beds. While peptides are generally extensively removed from the circulation by other

vascular beds, most peptides pass through the lungs unchanged due to the absence of specific uptake mechanisms. Two physiologically important peptides, bradykinin and angiotensin I, are exceptions. Bradykinin is inactivated and angiotensin I is converted to the biologically active peptide angiotensin II by the action of a dipeptidyl carboxypeptidase, known as angiotensin-converting enzyme, which is localised on the luminal surface of endothelial cells (Ryan *et al.*, 1976). The adenine nucleotides adenosine triphosphate and monophosphate are inactivated across the pulmonary circulation by phosphate esterases which are situated on the luminal surface of endothelial cells (Ryan & Ryan, 1977). In this respect, the pulmonary metabolism of nucleotides resembles that of peptides.

Many of the biologically active compounds already discussed in relation to their pulmonary metabolism may also be synthesised and released by the lung, where they apparently serve as local hormones. The lungs are an important source of synthesis of arachidonic acid metabolites, through both the cyclo-oxygenase pathway which generates prostaglandins and thromboxanes, and the lipoxygenase pathway which leads to the formation of leukotrienes (Hyman *et al.*, 1978). The major tissue sources of cyclo-oxygenase products are alveolar macrophages, fibroblasts, smooth muscle cells, and type II epithelial cells ; endothelial cells are rich sources of prostacyclin, and platelets are active producers of thromboxane A_2. The most important cellular sites of lipoxygenase products are mast cells, basophils and neutrophils.

The lung contains abundant mast cells which are situated mainly within the bronchial mucosa and the deeper connective tissues surrounding pulmonary venules. When stimulated by IgE-mediated immediate

hypersensitivity reactions, mast cells release both preformed and newly synthesised biologically active substances, including: histamine, 5-HT, eosinophil chemotactic factor of anaphylaxis, the sulfidopeptide leukotrienes originally known as slow-reacting substance of anaphylaxis and a platelet-activating factor (Lewis & Austen, 1977). As mentioned previously, the lung synthesises several vasoactive peptides, including vasoactive intestinal peptide, substance P, bradykinin and angiotensin II. The lung also contains endocrine cells which may occur singly or in groups called neuro-epithelial bodies in the epithelium of airways. These cells have been shown to contain bombesin-like peptides, calcitonin and opioid peptides (Becker & Gazdar, 1984).

1.6. **Clinical application of prostacyclin and NO in pulmonary hypertension**

Pulmonary hypertension is defined as a mean pulmonary arterial pressure above 25 mmHg for a cardiac output below 5 $L.min^{-1}$ (Weir, 1984). Usually a cause can be found : commonly, left heart failure, mitral valve disease, congenital heart disease, chronic obstructive or fibrotic lung disease, hypoventilation or repeated pulmonary emboli. When no cause is known, the condition is called primary pulmonary hypertension (PPH) (Weir, 1984).

Vasodilator drug therapy of pulmonary hypertension is often unsuccessful due to concomitant systemic hypotension, which can be fatal (Packer, 1985). For this reason, most of the systemic vasodilators which had been tried in the treatment of pulmonary hypertension were discarded from clinical practice (Packer, 1985 ; Palevsky & Fishman, 1985). Thus,

the " ideal " pulmonary vasodilator must be selective, with its activity restricted to the pulmonary circulation.

Compared with other vasodilators, prostacyclin infusion produces rapid onset but short duration pulmonary vasodilatation, enabling titration of optimal vasodilating dose (Palevsky & Fishman, 1988). It also causes systemic vasodilatation, but due to its short half-life (about 5 min) any systemic hypotension can be reversed by discontinuing the infusion. The disadvantages of prostacyclin is that it is orally inactive, and long-term infusion is expensive and complicated. At present, acute infusion of prostacyclin is used for the initial testing of the pulmonary vascular bed's responsiveness to vasodilatation. If acute infusion of prostacyclin causes a decrease of greater than 30% in pulmonary vascular resistance, oral vasodilators can be used. The calcium channel blockers nifedipine and diltiazem seem to be the favoured (Rich & Brundage, 1987). Long-term infusion of prostacyclin is reserved for the most severely affected PPH patients awaiting heart-lung transplantation.

NO is a gas at room temperature, and therefore can be delivered directly to the lungs via inhalation. When inhaled, NO reaches pulmonary blood vessels primarily through diffusion from alveolar spaces, thereby causing pulmonary vasodilatation. However, no systemic vasodilatation should be seen, since NO is rapidly inactivated by circulating haemoglobin. On this basis, inhaled NO has been tested as a selective means to induce pulmonary vasodilatation in the treatment of pulmonary hypertension. In infants with persistent pulmonary hypertension, short-term inhalation of NO, with doses ranging from 10 to 80 ppm, caused selective pulmonary vasodilatory effects with significant improvement of oxygenation, without causing systemic hypotension or significantly

raising methaemoglobin levels (Kinsella *et al.*, 1992 ; Robe *et al.*, 1992). In another study (Pepke-Zaba *et al.*, 1991), the acute effects of inhaled NO (40 ppm in air) on pulmonary and systemic vascular resistance were compared with those of an intravenous infusion of prostacyclin (24 µg / h) in patients with PPH. Inhaled NO decreased pulmonary vascular resistance without altering systemic vascular resistance. Infusion of prostacyclin also decreased pulmonary vascular resistance, but this was associated with a similar fall in systemic vascular resistance. Similar results with inhaled NO were obtained in patients with chronic obstructive lung disease and pulmonary hypertension (Fratacci *et al.*, 1992). These results show inhaled NO as a selective pulmonary vasodilator agent. Further studies are needed, however, to investigate the development of tolerance and toxicity of NO before it can be considered as a treatment of pulmonary hypertension in humans.

1.7. Aims of study

The aims of this study were :

1- To investigate whether a basal release of NO and prostacyclin is involved in modulating pulmonary vascular tone.

2- To test the ability of various humoral agents to stimulate NO release in this vascular bed.

3- To determine the extent to which this basal and stimulated release correlates with artery size.

Chapter 2

Materials and Methods

2.1. Experimental animals

Adult male Wistar rats (250-300g) were used throughout this study. Rats were housed in a room maintained at 20°C and were exposed to a 12-hour light/dark cycle. All rats were allowed tap water and pellet food *ad libitum*.

2.2. Preparation of pulmonary arterial rings

2.2.1. Dissection of pulmonary arteries

Rats were killed by stunning and exsanguination. The thoracic cavity was exposed, and the lungs and heart were immediately removed and placed in a Petri dish containing Krebs` buffer. The main pulmonary artery (MPA), 1st and 2nd branches were dissected free, cleaned of adherent fat and connective tissue and cut into 3-4 mm rings (Figs. 2 and 3, table 1). The localisation of the 2nd branches was facilitated by the fact that they are found in the same connective sheet as that of the bronchus when entering the lobe. The total dissection was usually accomplished within 30 min.

2.2.2. Removal of endothelium

In some 2nd branch rings, the endothelium was destroyed by perfusing rings with the detergent Triton X-100 (1:5000) for 30s, a technique which had been used in other resistance blood vessels to cause selective destruction of the endothelium (Gaw et al., 1991) (Figs.4 and 5). Endothelial and smooth muscle integrity was determined functionally at the beginning of each experiment by assessing the relaxant responses

to carbachol (CARB, 10^{-4} M) and sodium nitroprusside (SNP, 10^{-4} M) in rings submaximally contracted with phenylephrine (PE, 9.5×10^{-9} M).

2.3. **Recording of mechanical responses**

Arterial rings were suspended between two L-shaped stainless steel wire hooks in 25ml organ baths containing Krebs` buffer (mM: NaCl, 118.5 ; KCl, 4.7 ; $CaCl_2$, 2.5 ; $MgSO_4.7H_2O$, 1.0 ; KH_2PO_4, 1.2 ; $NaHCO_3$, 25.0 ; glucose, 22.2), which was maintained at 37°C and gassed with 95% O_2/ 5% CO_2. The rings were placed under 1g tension, selected on the basis of preliminary experiments (section 2.4). Isometric tension was recorded using force displacement transducers (Grass FTO3) and displayed on a Grass polygraph (model 7). The tissues were allowed to equilibrate for 60 min, during which time the bathing solution was changed once and the resting tension was re-adjusted to 1g if required. At the end of each experiment, the rings were gently blotted dry and weighed.

2.4. **Preliminary experiments on length-tension relationship**

Preliminary experiments were carried out to determine the optimum length (tension) for maximum tension development in MPA, 1st and 2nd branch rings (n = 6). Optimum tension was defined as the minimum level of tension that allowed for development of the largest contractile response to a maximum concentration of PE (10^{-4} M). Various levels of tension (0.25-1.5 g) were applied to the rings in a stepwise manner. At each level of tension, the rings were allowed to equilibrate for 30 min before obtaining contractile responses to PE, which was then washed out and the baseline level re-established. Optimum tension was determined to

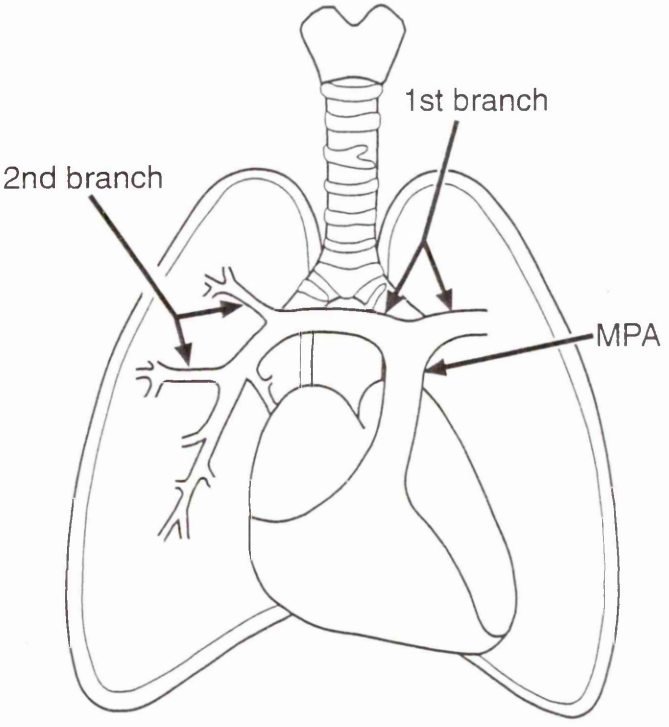
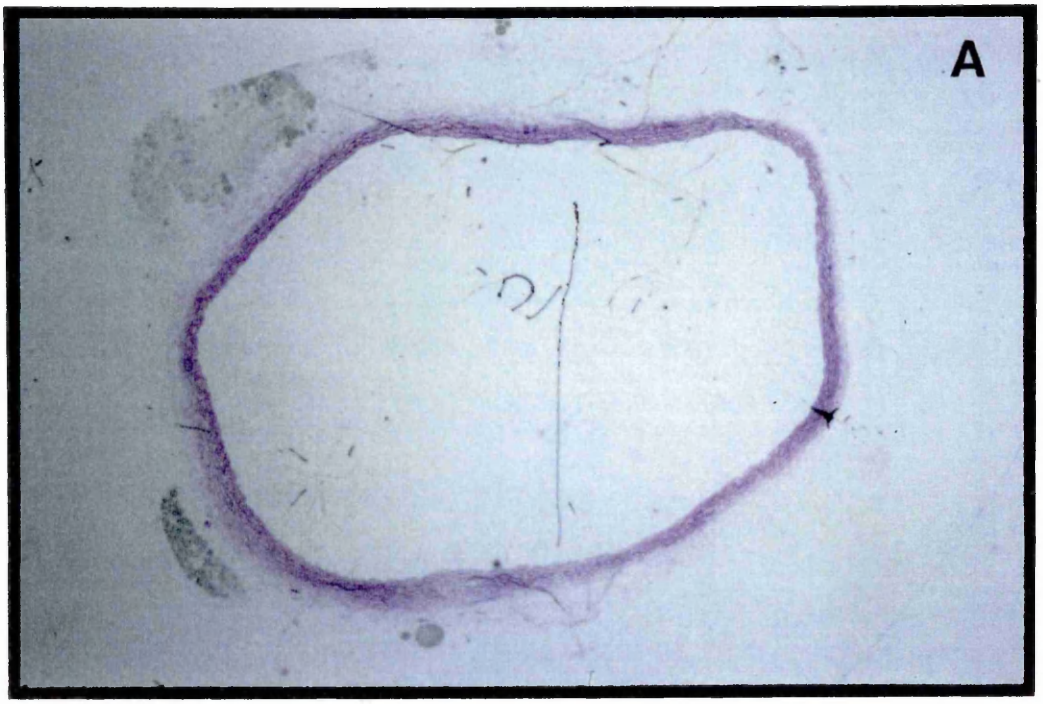


Fig. 2.

Diagram of the lungs and heart. The arrows indicate the position of the MPA, 1st branches (extrapulmonary), and 2nd branches (intrapulmonary).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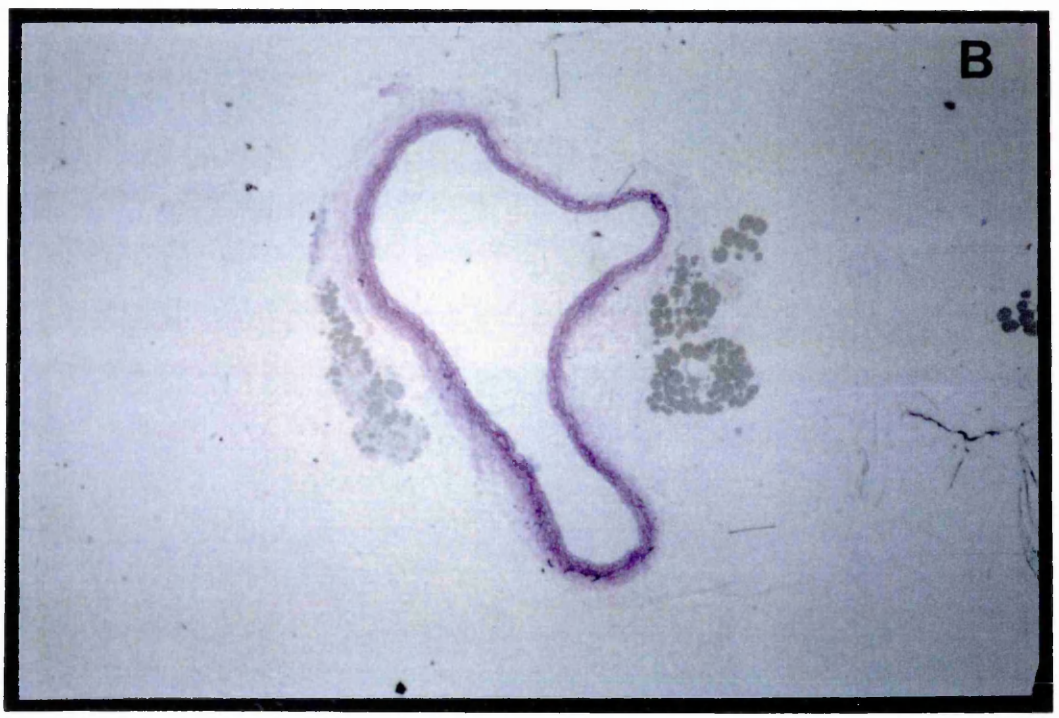
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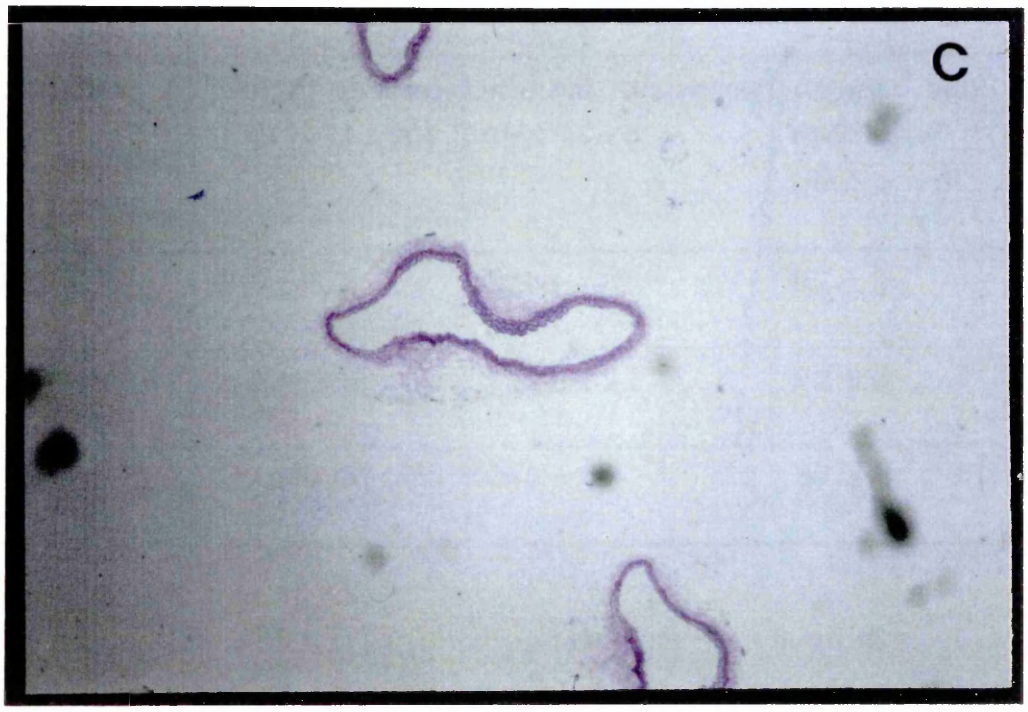


Fig. 3.

Low power micrographs of cross sections from (A) MPA, (B) 1st and (C) 2nd branch rings, showing the size difference between the three arteries and the thin artery wall characteristic to pulmonary arteries. Magnification x40 , Polychrome stain.

Table 1.

Pulmonary artery	Weight of rings (mg) (n = 50)	Internal diameter (μm) (n = 6)	Artery wall thickness (μm) (n = 6)
MPA	6.4 ± 0.3	2236 ± 35	87 ± 9
1st branch	2.3 ± 0.1	1325 ± 21	62 ± 4
2nd branch	1.1 ± 0.04	648 ± 18	37 ± 4

Comparison of some anatomical features of MPA, 1st and 2nd branches. Cross sections of artery rings were projected onto a paper using a slide microprojector (Prado 500), and sketched. Internal diameter and wall thickness were measured from the sketches using the MOP system (Kontron). The system operates by generating magnetic pulses which get intercepted when a stylus is moved between two points, and a pulse count is established. This count is transformed into a distance by a microprocessor. Several measurements were made along each section and averaged to obtain the mean diameter and wall thickness of each ring. Data are presented as mean ± s.e.mean. n represents the number of rings, one ring per rat.

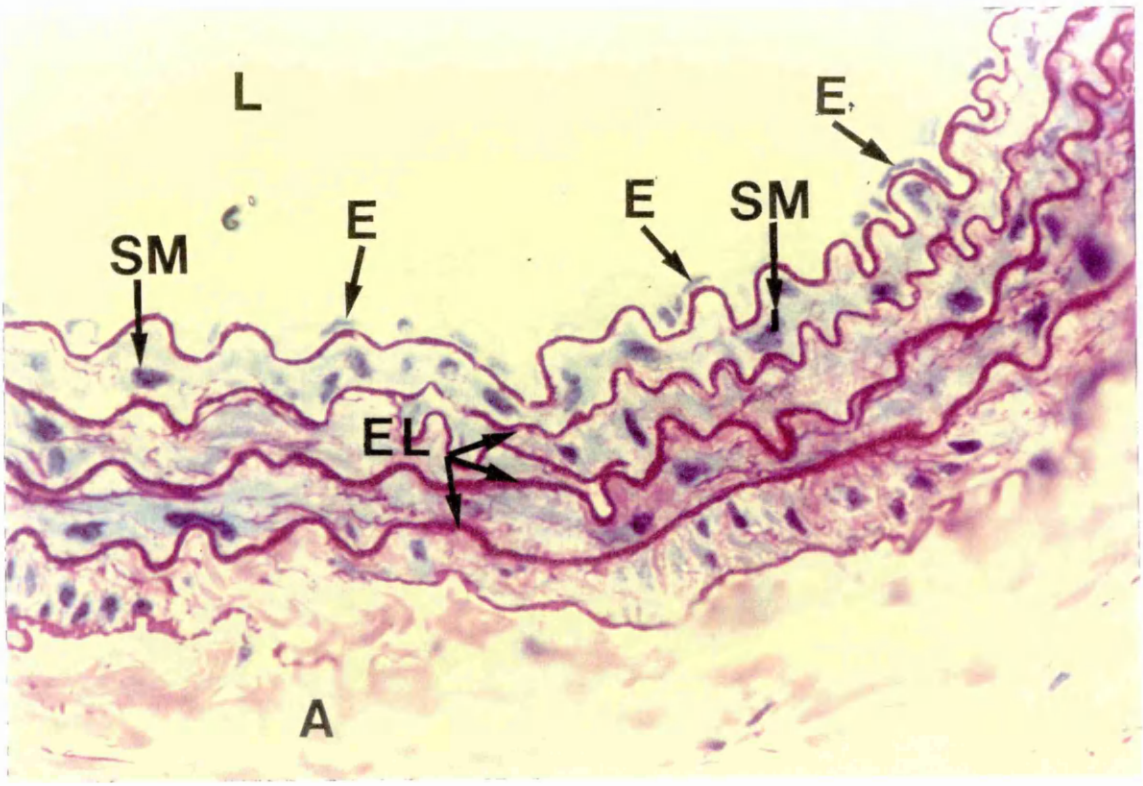


Fig. 4.

A high power micrograph showing a cross sectional area of a 2nd branch artery wall with an intact endothelial cell layer. E, endothelium ; SM, smooth muscle ; EL, elastic lamina ; L, lumen ; A, adventitia. Magnification x160 , Polychrome stain.

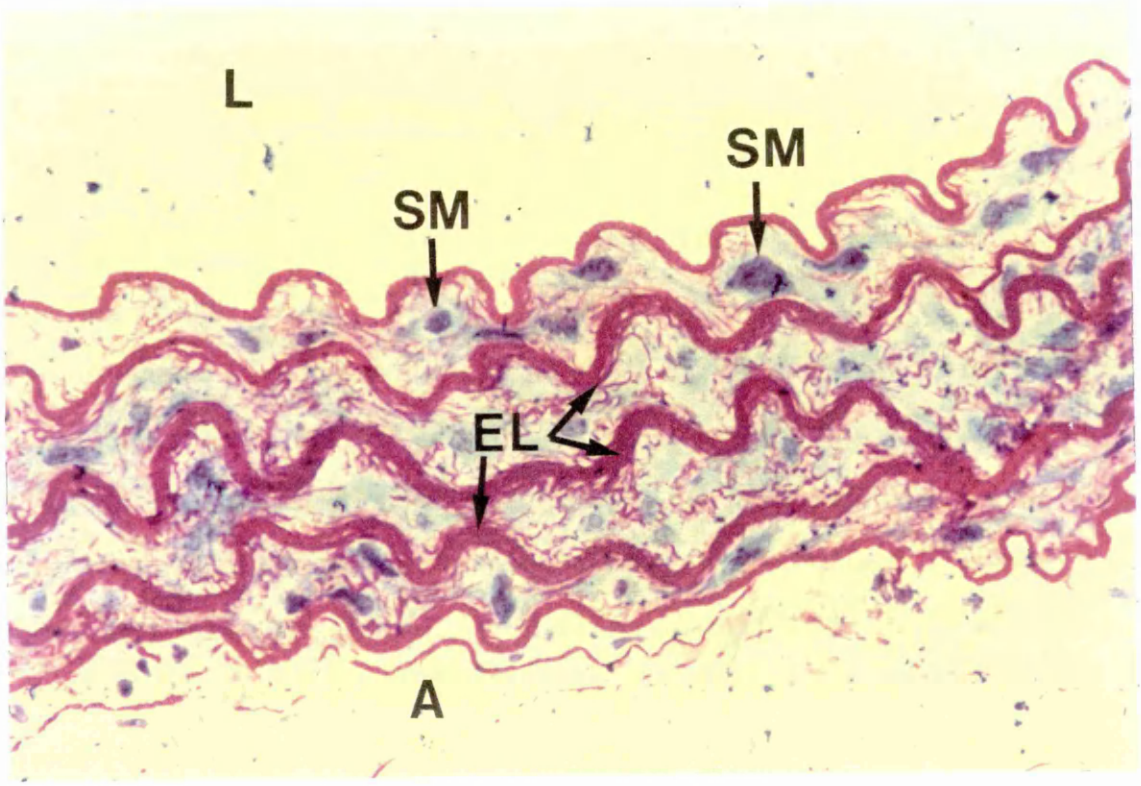


Fig. 5.

A high power micrograph showing a cross sectional area of a 2nd branch artery wall which had been perfused with Triton X-100 (1:5000) for 30s. This procedure resulted in total destruction of the endothelial cell layer, without damaging the underlying smooth muscle cells. SM, smooth muscle ; EL, elastic lamina ; L, lumen ; A, adventitia. Magnification x160 , Polychrome stain.

be 1g for the three arteries, and did not vary as a result of endothelium denudation.

2.5. Experimental protocols

2.5.1. Experiments on contractile responses

Non-cumulative concentration-response curves (CRC) to the vasoconstrictors PE (10^{-9}-10^{-4} M), 5-hydroxytryptamine (5-HT, 10^{-9}-10^{-3} M) and potassium chloride (KCl, 10-50 mM) were obtained in endothelium-intact rings from MPA, 1st and 2nd branches. Preliminary experiments were carried out in which two consecutive CRC to each vasoconstrictor were constructed in the same ring with no treatment between the two curves, to determine whether there was any change in sensitivity of the tissues due to time and/or pre-exposure to the vasoconstrictor. An increase in sensitivity of tissues to all vasoconstrictors was observed, and therefore in all subsequent experiments only one CRC was constructed in each ring.

To investigate the effect of inhibiting NO synthesis on the contractile responses to vasoconstrictors, the NOS inhibitor, L-NAME (N^{ω}-nitro-L-arginine methyl ester, $2x10^{-4}$ M) or L-NOARG (N^{ω}-nitro-L-arginine, 10^{-4} M) was added to the Krebs` reservoir and a CRC was constructed after a 10-min equilibration period. Thus tissues remained exposed to the inhibitor throughout the experiment. In some experiments, tissues were first equilibrated with L-arginine ( $10^{-2}$ M ) for 10 min, after which time L-arginine was added to the Krebs` reservoir containing L-NAME or L-NOARG.

Similar experiments were carried out in which only the cyclo-oxygenase inhibitor, flurbiprofen (10^{-5} M) was added to the Krebs` reservoir, to investigate the effect of inhibition of prostanoid synthesis on responses to vasoconstrictors.

Contractile responses to vasoconstrictors were expressed as mg tension. mg^{-1} tissue.

2.5.2. Experiments on relaxant responses

Cumulative CRC to the vasorelaxants CARB (10^{-9}-10^{-4} M), SNP (10^{-11}-10^{-4} M) and isoprenaline (ISO, 10^{-10}-10^{-4} M) were obtained in the absence and presence of L-NAME ($2x10^{-4}$ M) or L-NOARG (10^{-4} M) alone or with L-arginine (10^{-2} M), in endothelium-intact rings from MPA, 1st or 2nd branches in which the tone had been raised with either PE (EC_{75} $2.2x10^{-7}$ M for MPA , $9.5x10^{-8}$ M for 1st and 2nd branches) or KCl (EC_{75} 27.5 mM for MPA , 25.1 mM for 1st branches and 29.0 mM for 2nd branches). CRC to ISO were also constructed in endothelium-denuded 2nd branch rings precontracted with PE ($9.5x10^{-9}$ M), in the absence and presence of L-NAME ($2x10^{-4}$ M). Where L-NAME or L-NOARG were used, they were equilibrated with tissues for 10 min before induction of tone with PE or KCl. Where L-arginine was used, it was added 10 min before addition of L-NAME or L-NOARG.

In other experiments, CRC to ISO were constructed in the presence of either flurbiprofen (10^{-5} M), propranolol (10^{-6} M), atenolol (10^{-5} M) or ICI 118551 (10^{-6} M), which were allowed to equilibrate with tissues for 30 min prior to exposure to ISO.

Preliminary experiments showed that repeating CRC to CARB, SNP or ISO in the same ring produced similar results. This observation indicated that since vasorelaxant responses to CARB, SNP or ISO were reproducible, it was possible to compare CRC in the absence and presence of antagonists in the same ring.

All drugs were added directly to the bathing solution. Relaxant effects were expressed as % relaxation of PE- or KCl-induced tone.

2.6. Measurement of cGMP

2.6.1. Experimental

cGMP levels were measured in artery rings in which mechanical responses had been recorded. Rings were set up in organ baths as previously described, and measurements were made under three different conditions :

i) Basal levels, in the absence of drug treatments.

ii) During contraction to PE (10^{-4} M, 2 min), 5-HT (10^{-4} M, 2 min) or KCl (25.1 mM, 3 min).

iii) During relaxation to CARB (10^{-4} M, 30s) or ISO (10^{-4} M, 30s) in PE-precontracted rings.

Once responses to drugs had stabilised at the time intervals specified above for each drug, rings were removed from the organ baths while still

attached to the wire hooks and frozen in liquid nitrogen. This step was completed in less than 2 seconds.

2.6.2. **Extraction of cGMP**

The frozen rings were deproteinised by extraction in trichloroacetic acid (TCA) (1.6 ml, 5% W/V). After a period of 2 hours the tissues were detached from the wire hooks, re-immersed in TCA and hand homogenised using Potter-Elvehjem glass-glass homogenisers. The tissue homogenates were then left for a further 18 hours at 4°C. Following this incubation period, the samples were vortex-mixed and centrifuged (1000g, 5 min, 4°C) to remove any non-acid soluble tissue components. The cGMP content of a fraction of the supernatant (400 µl) was extracted by washing four times with three volumes of water-saturated ether. Any remaining ether was evaporated by placing the sample tubes in a water bath at 80°C for 2 min. The samples (300 µl) were neutralised with sodium acetate buffer (100 µl, 200 mM, pH 6.2), vortex-mixed and stored at 4°C until required.

2.6.3. **Radioimmune assay**

Tissue cGMP levels were measured using a commercially available cGMP radioimmune assay kit (Amersham International, UK). The assay was based on the competition between unlabelled cGMP and a fixed quantity of the tritium labelled compound for binding to an antiserum which has a high specificity and affinity for cGMP.

Standard cGMP concentrations (0.5-8 pmol/100 µl) were prepared in Tris/EDTA buffer (50 mM, pH 7.5). Samples of both standard and

unknown tissue concentrations of cGMP (100 µl) were acetylated by the addition of a freshly prepared mixture of acetic anhydride (1g/ml) and triethylamine (100%V/V) 1:2 by volume (5µl). The samples were immediately vortex-mixed. [^{3}H]-labelled cGMP (50 µl) was then added to each sample, followed by anti-cGMP antiserum (50 µl). Samples were vortex-mixed and incubated for 16 hours at 4^{o}C. Following this incubation period, ice-cold ammonium sulphate (1 ml, 0.4g/ml) was added to each sample and vortex-mixed. The samples were allowed to stand for 5 min after the addition of ammonium sulphate to the last sample, before centrifuging (1000g, 5 min, 4^{o}C). The supernatant was decanted and the assay tubes inverted on a tissue to drain. Cold distilled water (1.1 ml) was then added to each tube and vortex-mixed until the precipitate had dissolved. Samples (1 ml) were transferred from the assay tubes to plastic counting vials containing scintillant (Ecoscint, 10 ml), and antibody bound [^{3}H]-cGMP was measured by counting for 5 min in a Packard liquid scintillation counter.

Standard calibration curves were obtained and plotted as C_o/C_x against concentration of cGMP (Fig.6) where C_o and C_x represent the amount of [^{3}H]-cGMP bound in the absence and presence of unlabelled cGMP, respectively. The concentrations of cGMP in unknown samples (100 µl) were determined by reference to the calibration curve. The concentration of cGMP in the original sample (1.6 ml) was expressed as pmol.mg^{-1} tissue, and calculated using the following method :

1) Concentration of cGMP in 100 µl multiplied by 16 gives concentration of cGMP in sample tube (pmol.tube^{-1}).

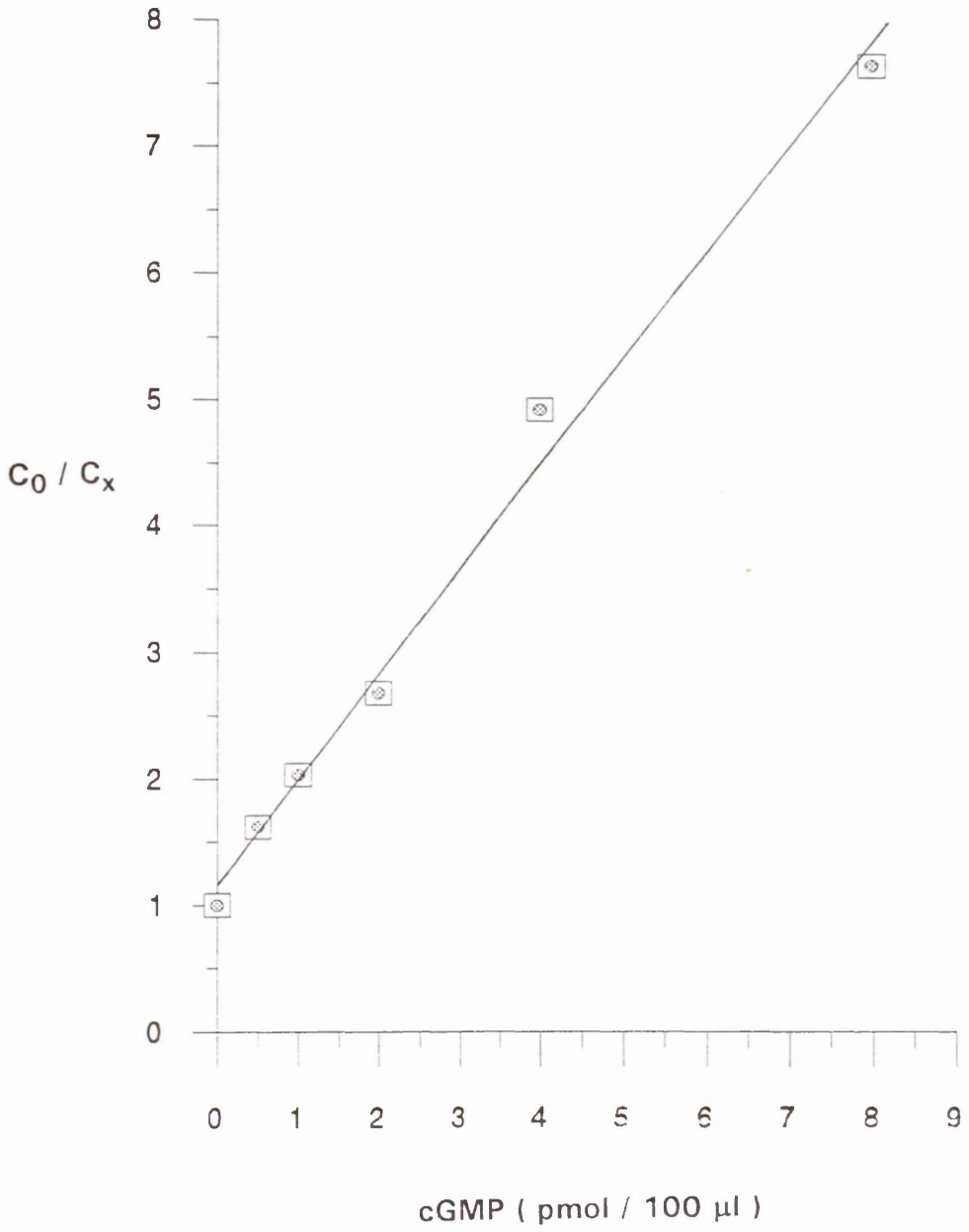


Fig. 6.

Standard calibration curve for cGMP as measured by radioimmuno assay based on the competition between unlabelled cGMP and [^{3}H]-cGMP for binding to a specific anti-cGMP antiserum. C_0 and C_x represent the amount of [^{3}H]-cGMP bound in the absence and presence of unlabelled cGMP, respectively. The line of best fit through the points is a regression line fitted by computer.

2) Concentration of cGMP in sample tube divided by weight of artery ring (mg) gives the concentration of cGMP per weight of tissue ($pmol.mg^{-1}$ tissue).

2.7. **Materials**

The following drugs were used :

L-Arginine hydrochloride, atenolol, carbamylcholine chloride (carbachol), clonidine hydrochloride, flurbiprofen, hydrocortisone, 5-hydroxytryptamine creatinine sulphate, (±)-isoprenaline sulphate, Ketanserin tartrate, N^{ω}-nitro-L-arginine, N^{ω}-nitro-L-arginine methyl ester hydrochloride, phenylephrine hydrochloride, prazosin hydrochloride, (±) -propranolol hydrochloride, sodium nitroprusside (Sigma) ; BRL 37344 (sodium-4-[2-{ 2-hydroxy-2-(3-chlorophenyl) ethylamino }propyl] phenoxyacetate) was kindly supplied by Dr. A. MacDonald (Glasgow Caledonian University) ; ICI 118551 (erythro-DL-1-[7-methylindan-4-yloxy-3-isopropylaminobutan-2-ol) (ICI) ; U 46619 (9,11-dideoxy-11_{α},9_{α}-methanoprostaglandin $F_{2\alpha}$) was kindly supplied by Dr. A. Shaw (Glasgow Caledonian University). The cGMP radioimmunoassay kit (TRK 500) was obtained from Amersham, U.K. potassium chloride (Fisons)

Stock solutions of drugs were prepared daily and kept on ice during the experiment. Atenolol, ICI 118551 and isoprenaline sulphate were dissolved and diluted in distilled water containing ascorbic acid (50 μg/ml). Flurbiprofen, hydrocortisone and ketanserin tartrate were dissolved in absolute ethanol and diluted in distilled water. Vehicles had no effect on tissue responses. All other drugs were dissolved in distilled

water and diluted in Krebs` solution. Concentrations of drugs are expressed as the final molar concentration in the organ bath.

2.8. **Statistical analysis**

Results are expressed as mean ± s.e.mean. EC_{50} values were estimated graphically by plotting the logarithm of drug concentration versus percentage of maximum response, and determining the concentration of drug required to produce 50% of the maximum response. Statistical comparison between two groups of data was performed using Student's paired t-test for data within rings, and unpaired t-test for data between rings. For multiple comparison of data, one way analysis of variance was used and the modified t-test calculated for comparison between individual means. A probability (P) value of less than 0.05 was considered significant.

Chapter 3

Results

3.1. Studies on contractile responses

3.1.1. Comparative effects of PE, 5-HT and KCl in MPA, 1st and 2nd branch rings

In the systemic circulation, the properties of arteries alter with decreasing diameter. Small resistance arteries have different receptor properties and sensitivity to vasoconstrictors when compared with large conduit arteries (Bevan & Bevan, 1985 ; Leher & Bevan, 1985). Although the reactivity of small pulmonary arteries may have a profound influence on pulmonary resistance, there has been comparatively little investigation of these arteries. Data from *in vitro* studies of isolated conduit pulmonary arteries are often at variance with the findings documented in whole-lung perfusion experiments, and may reflect differences in the vasoreactivity of large and small pulmonary arteries. In this series of experiments, the responsiveness of MPA, 1st and 2nd branches to vasoconstrictors was compared.

The results generally show that 2nd branches were more responsive to the contractile effects of vasoconstrictors than 1st branches, which were more responsive than MPA.

PE induced concentration-dependent contractions with EC_{50} values and maximum responses of 5.6×10^{-8} M and 179.2 ± 14.9 $mg.mg^{-1}$ tissue in MPA , 8.3×10^{-9} M and 311.0 ± 12.9 $mg.mg^{-1}$ tissue in 1st branches, and 2.3×10^{-9} M and 323.6 ± 23.4 $mg.mg^{-1}$ tissue in 2nd branches, respectively (Fig. 7).

Fig.8. shows CRC to 5-HT in the three arteries. The EC_{50} values and maximum responses for 5-HT were $4.3x10^{-6}$ M and 133.5 ± 3.0 $mg.mg^{-1}$ tissue in MPA, $3.2x10^{-6}$ M and 189.7 ± 10.1 $mg.mg^{-1}$ tissue in 1st branches, and $2.2x10^{-6}$ M and 315.9 ± 22.1 $mg.mg^{-1}$ tissue in 2nd branches, respectively.

KCl induced concentration-dependent contractions with EC_{50} values and maximum responses of 22.9 mM and 142.5 ± 14.9 $mg.mg^{-1}$ tissue in MPA, 19.5 mM and 239.6 ± 27.3 $mg.mg^{-1}$ tissue in 1st branches, and 21.9 mM and 435.0 ± 24.9 $mg.mg^{-1}$ tissue in 2nd branches, respectively (Fig. 9).

3.1.2. **Effect of NOS inhibitors on responsiveness of MPA, 1st and 2nd branches to vasoconstrictors.**

Several studies have shown that endothelium denudation or inhibition of NO synthesis increases vascular tone and potentiates contractile responses to vasoconstrictors (Martin *et al.*, 1986 ; Trezise *et al.*,1992). Most of these studies however have been conducted on systemic arteries, and little information is available on the relation between these effects and artery size. Therefore, the aim of the present experiments was to investigate the role of NO in modulating the responsiveness of pulmonary arteries to vasoconstrictors, and the extent to which this modulation correlates with artery size. Inhibition of NO synthesis was achieved with the NOS inhibitor L-NAME and, in some experiments, L-NOARG was used.

Inhibition of NO synthesis significantly enhanced the contractile responses to PE, 5-HT and KCl in the three arteries. The enhancement

was more profound at low concentrations of the vasoconstrictors, and was more pronounced as artery size decreased. There were, however, no significant differences in the maximum responses.

Pretreatment with L-NAME potentiated PE-induced contractions, yielding EC_{50} values of $1.4x10^{-8}$ M in MPA, $1.3x10^{-9}$ M in 1st branches and $2.8x10^{-10}$ M in 2nd branches, representing a 4.0, 6.4 and 8.2-fold increase in sensitivity, respectively. The maximum responses induced by PE were 203.3 ± 8.8, 346.8 ± 12.8 and 370.7 ± 22.8 $mg.mg^{-1}$ tissue, respectively, which were not significantly different from those obtained in control tissues (Fig. 10). The EC_{50} values were significantly different from control values ($0.01>P>0.001$, $P<0.001$, $P<0.001$, respectively).

The EC_{50} values for 5-HT in the presence of L-NAME were $1.1x10^{-6}$ M in MPA, $6.6x10^{-7}$ M in 1st branches and $5.1x10^{-7}$ M in 2nd branches, representing a 3.9, 4.8 and 4.3-fold increase in sensitivity, respectively. The maximum responses induced by 5-HT in MPA, 1st and 2nd branches (143.2 ± 13.6, 227.5 ± 17.3 and 367.4 ±13.2 $mg.mg^{-1}$ tissue) did not differ significantly from those obtained in control tissues (Fig. 11). The EC_{50} values were significantly different from control values ($0.01>P>0.001$).

Similarly, L-NAME potentiated KCl-induced contractions yielding EC_{50} values of 15.1 mM in MPA, 11.5 mM in 1st branches and 10.0 mM in 2nd branches, representing a 1.5, 1.7 and 2.2-fold icrease in sensitivity, respectively. There was no significant difference in the maximum responses induced by KCl (179.6 ± 22.5, 309.4 ± 11.5 and 447.9 ± 24.5 $mg.mg^{-1}$ tissue, respectively) when compared with those obtained in control tissues (Fig. 12). The EC_{50} values were significantly different from control values ($0.05>P>0.01$,$0.01>P>0.001$,$0.01>P>0.001$, respectively).

In some experiments, the effect of L-NOARG on PE-induced contractions in 2nd branch rings was investigated (Fig.14). In a similar manner to

L-NAME, L-NOARG enhanced the contractile responses to PE producing a 5.6-fold increase in sensitivity ($P < 0.001$), with no significant effect on the maximum response. L-NAME and L-NOARG per se had no effect on basal tone in any of the three arteries.

3.1.3. Effect of L-arginine on L-NAME and L-NOARG-induced potentiation of contractile responses

To confirm the involvement of NO in the depression of contractile responses to vasoconstrictors, the effect of prior incubation with L-arginine on L-NAME and L-NOARG-induced potentiation of contractile responses to PE in 2nd branch rings was studied. Addition of excess L-arginine to the system generally prevents the actions of NOS inhibitors by competing for the enzyme (Mulsch & Busse, 1990).

Prior incubation of tissues with L-arginine completely prevented the ability of L-NAME and L-NOARG to potentiate PE-induced contractions (Figs.13 and 14). In the presence of both L-NAME and L-arginine or L-NOARG and L-arginine, the EC_{50} values for PE were 1.8×10^{-9} M and 3.0×10^{-9} M, and the maximum responses were 338.3 ± 10.8 and 314.9 ± 20.7 $mg.mg^{-1}$ tissue, respectively, which were not significantly different from those obtained in control tissues.

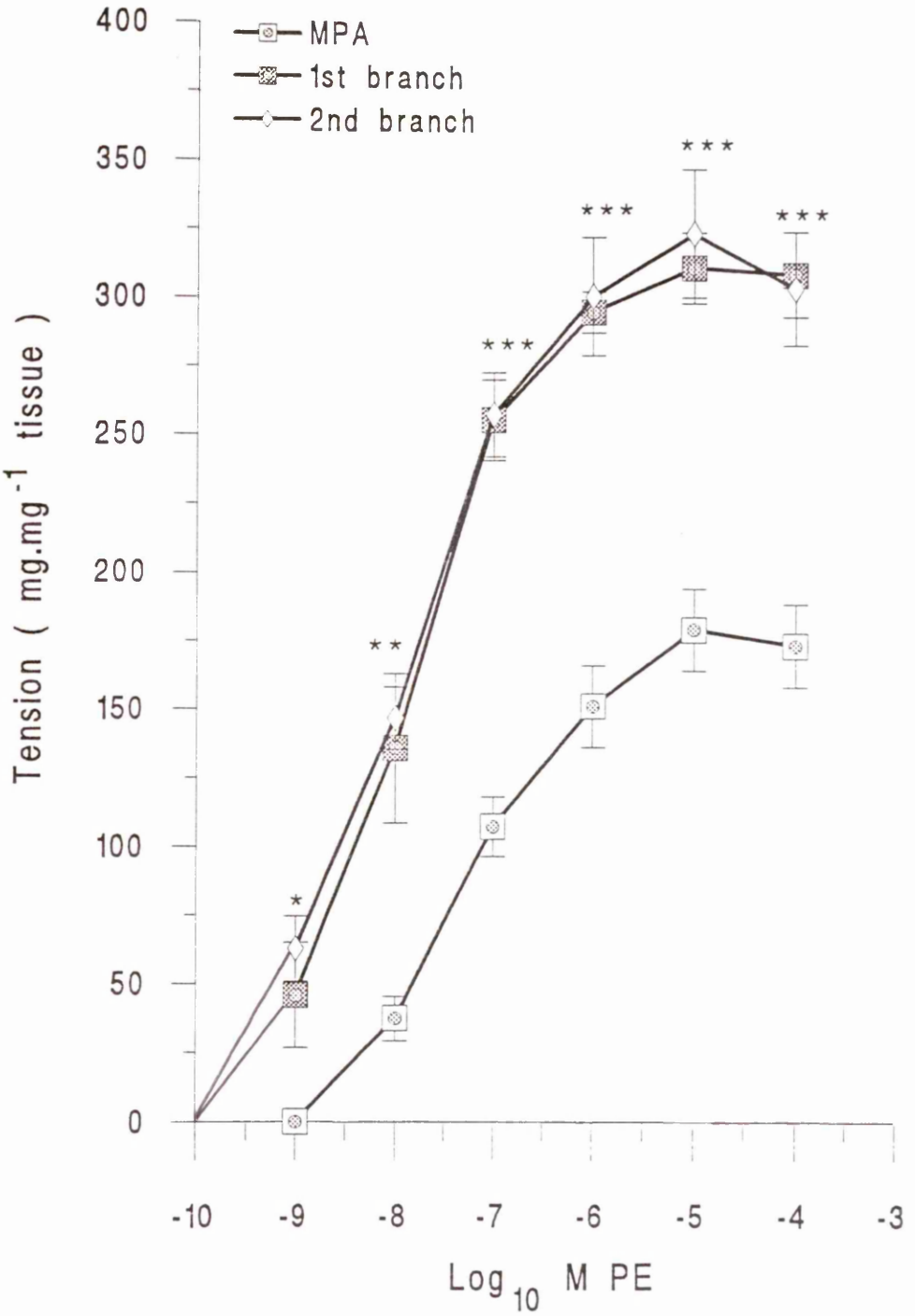


Fig. 7.

Contractile responses induced by non-cumulative concentrations of PE in MPA, 1st and 2nd branches. Each point is the mean ± s.e.mean of 6-8 observations. Statistical comparison between the three arteries was performed using one way analysis of variance, followed by modified t-test for comparison between individual means. Asterisks denote significant difference as compared with MPA ($*P<0.05$, $**P<0.01$, $***P<0.001$).

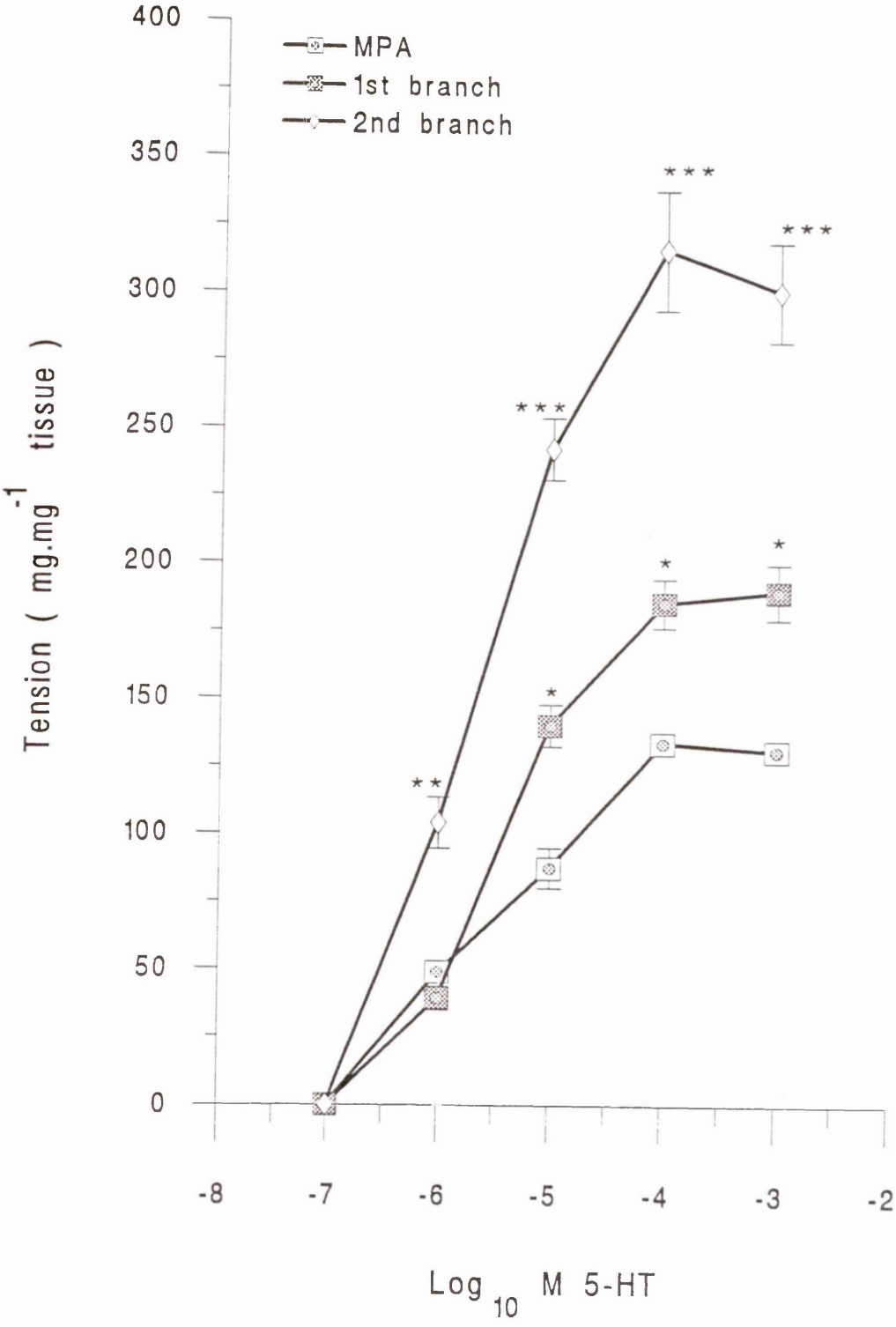


Fig. 8.

Contractile responses induced by non-cumulative concentrations of 5-HT in MPA, 1st and 2nd branches. Each point is the mean ± s.e.mean of 6-9 observations. Statistical comparison between the three arteries was performed using one way analysis of variance, followed by modified t-test for comparison between individual means. Asterisks denote significant difference as compared with MPA (*P<0.05, **P<0.01, ***P<0.001).

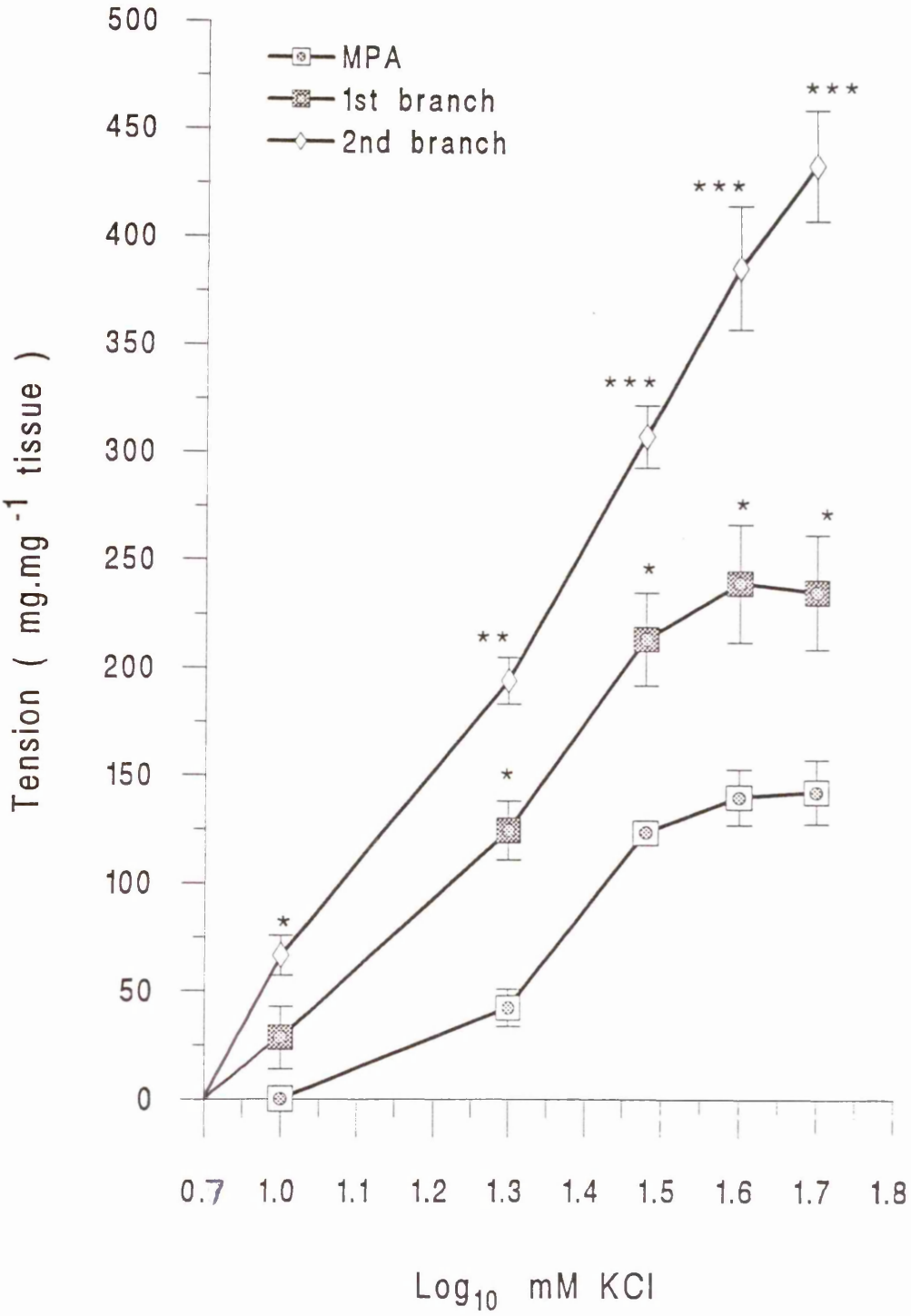
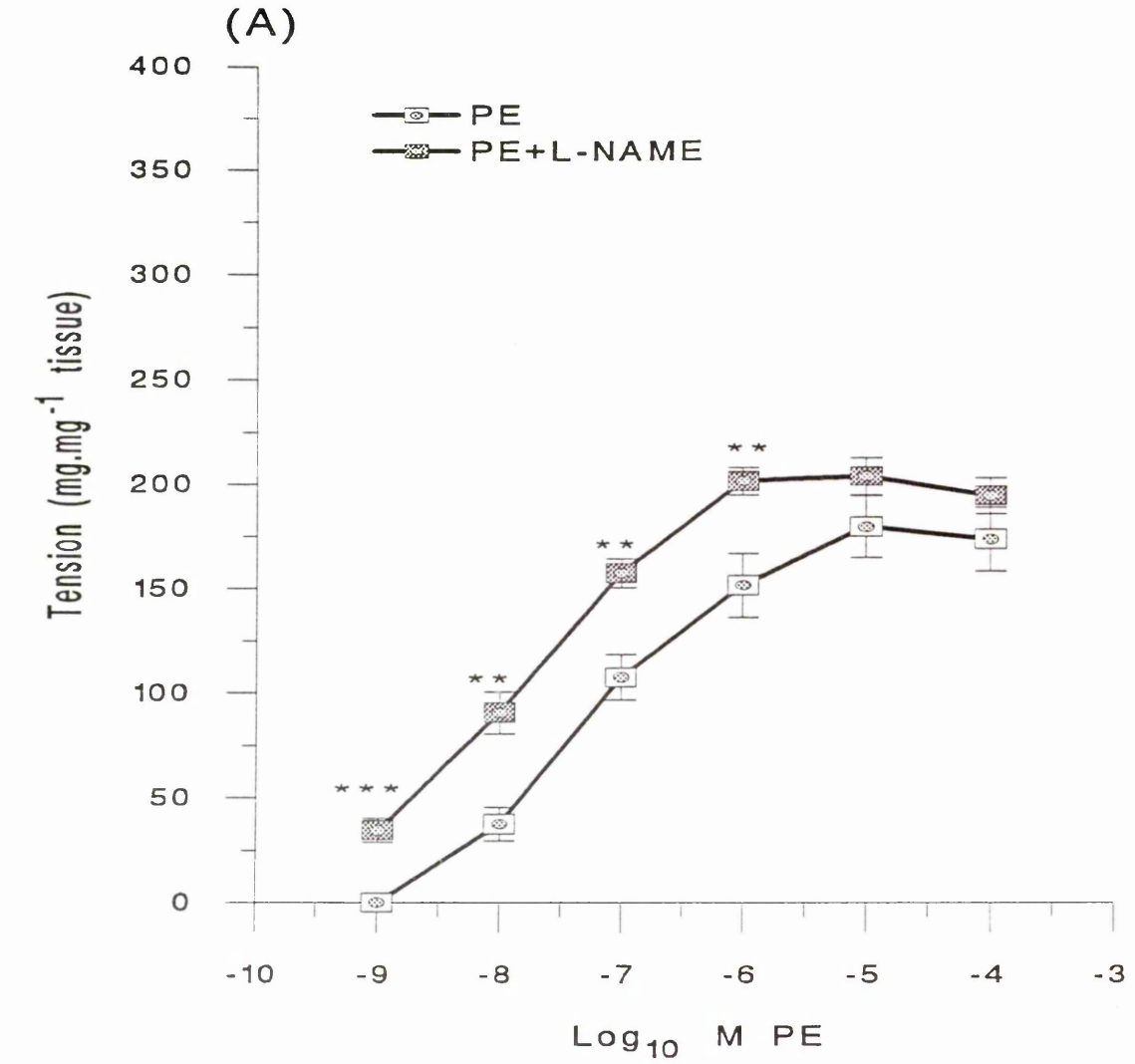
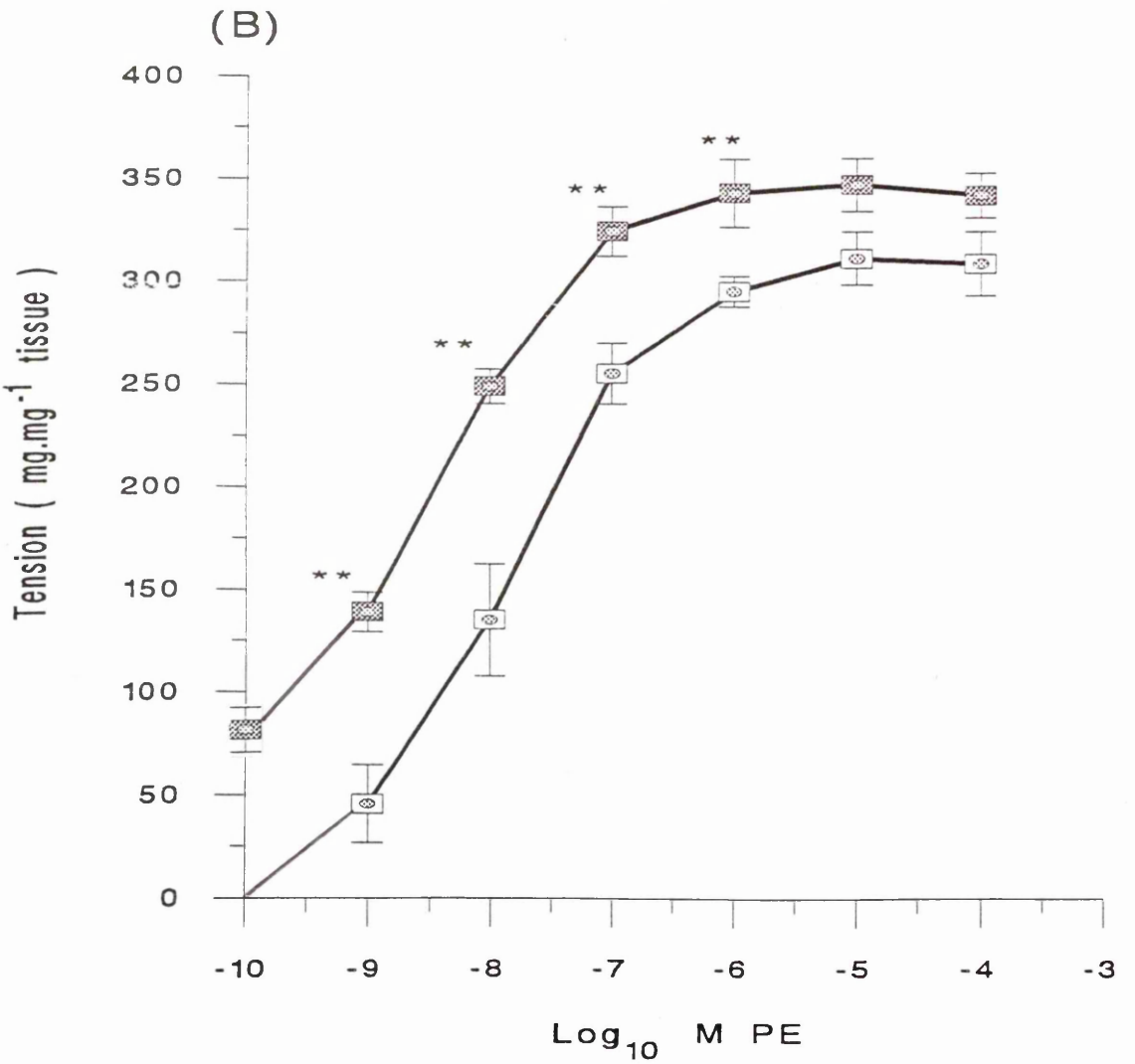


Fig. 9.

Contractile responses induced by non-cumulative concentrations of KCl in MPA, 1st and 2nd branches. Each point is the mean ± s.e.mean of 6-10 observations. Statistical comparison between the three arteries was performed using one way analysis of variance, followed by modified t-test for comparison between individual means. Asterisks denote significant difference as compared with MPA (*P<0.05, **P<0.01, ***P<0.001)


(A)
PE
PE+L-NAME
Tension (mg.mg-1 tissue)
400
350
300
250
200
150
100
50
0
* * *
* *
* *
* *
-10
-9
-8
-7
-6
-5
-4
-3
Log10 M PE


(B)
Tension (mg.mg-1 tissue)
400
350
300
250
200
150
100
50
0
* *
* *
* *
* *
-10
-9
-8
-7
-6
-5
-4
-3
Log10 M PE

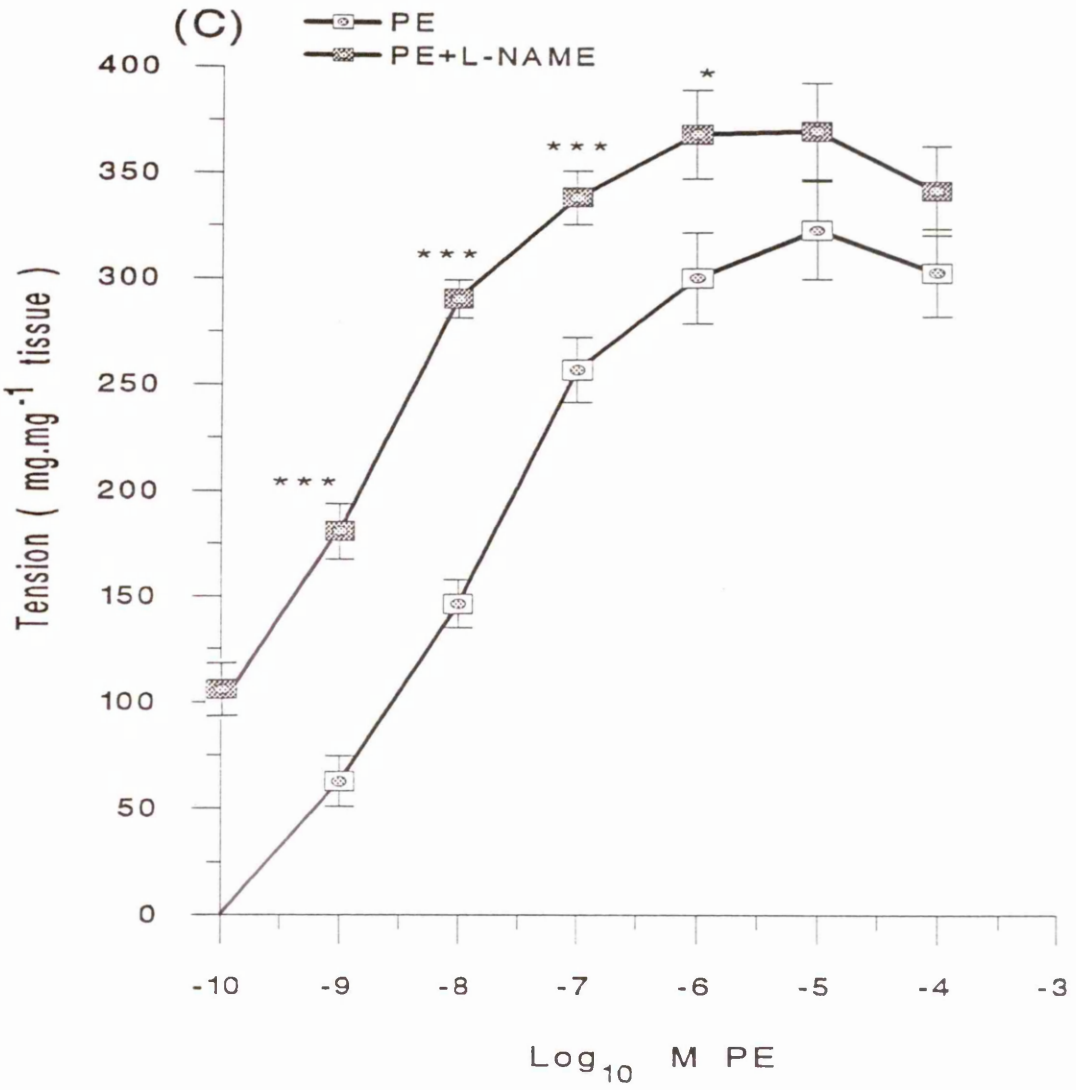
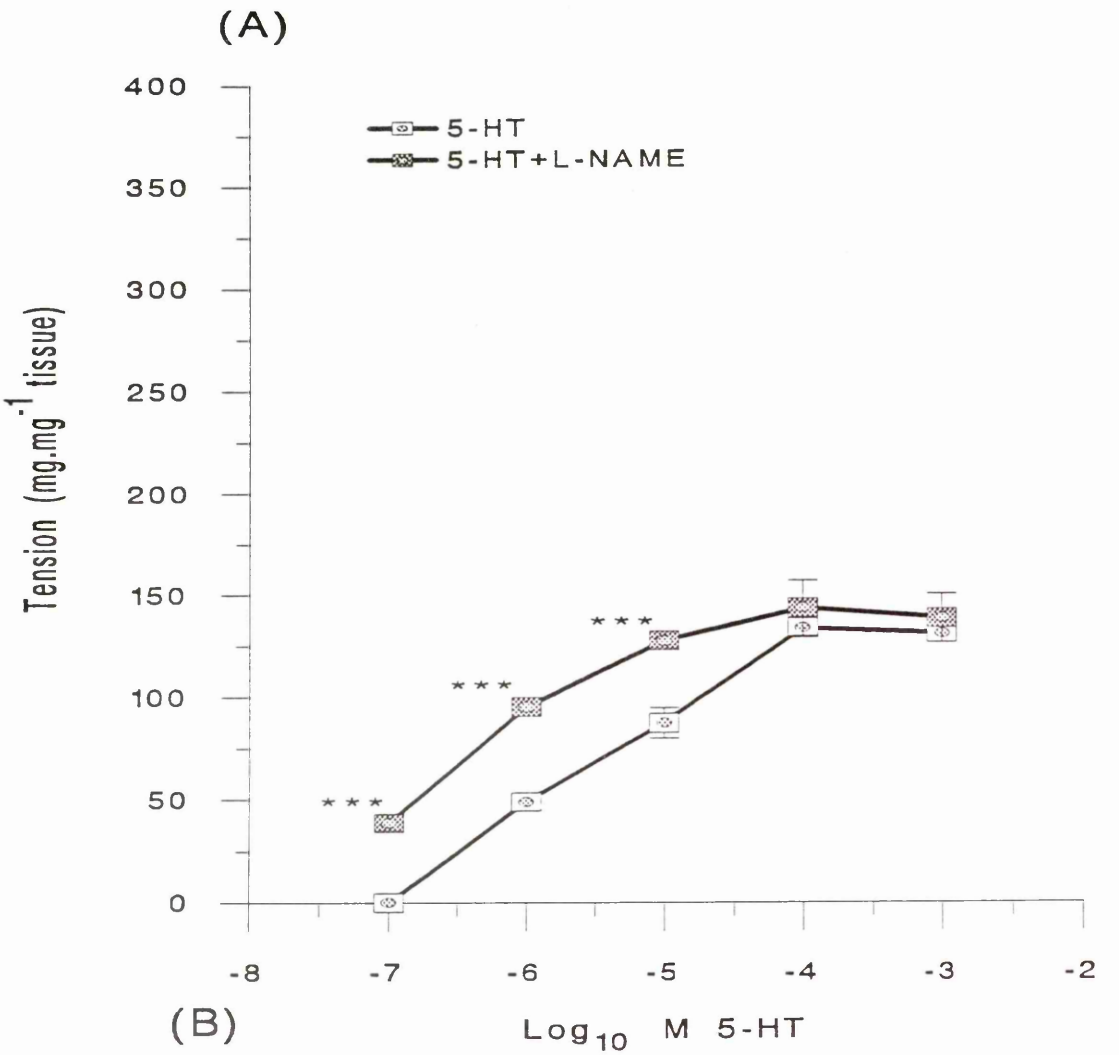
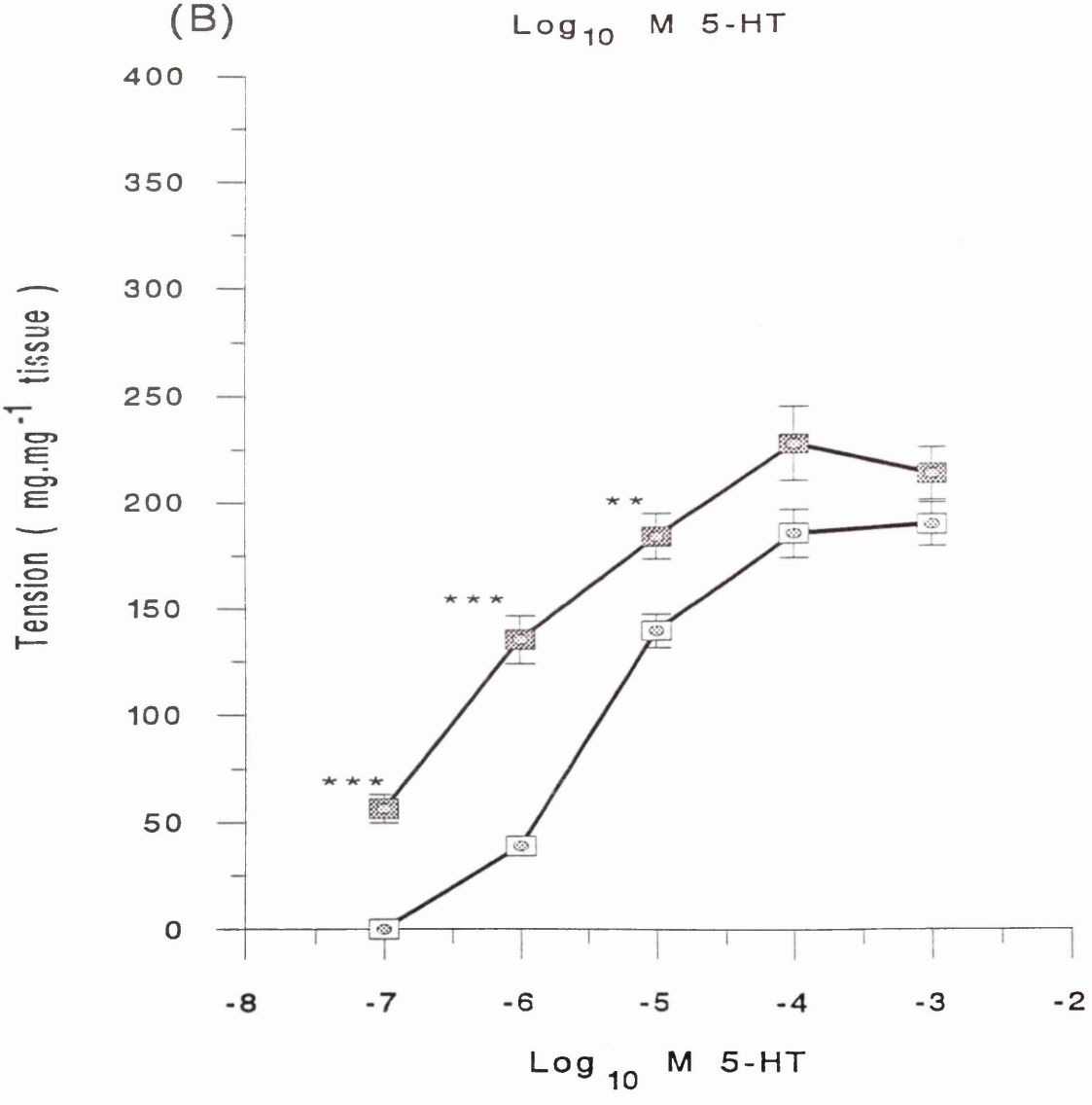


Fig. 10.

Effect of pretreatment with L-NAME ($2x10^{-4}$ M) on concentration-response curves to PE in (A) MPA, (B) 1st and (C) 2nd branches. Each point is the mean ± s.e.mean of 6-8 observations. The potentiating effect of L-NAME on PE-induced contractions was more pronounced as artery size decreased. Asterisks denote significant difference from untreated rings (*$P<0.05$, **$P<0.01$, ***$P<0.001$; unpaired t-test).


(A)
5-HT
5-HT+L-NAME
Tension (mg.mg-1 tissue)
400
350
300
250
200
150
100
50
0
* * *
* * *
* * *
-8
-7
-6
-5
-4
-3
-2
Log10 M 5-HT


(B)
Tension (mg.mg-1 tissue)
400
350
300
250
200
150
100
50
0
* * *
* * *
* *
-8
-7
-6
-5
-4
-3
-2
Log10 M 5-HT

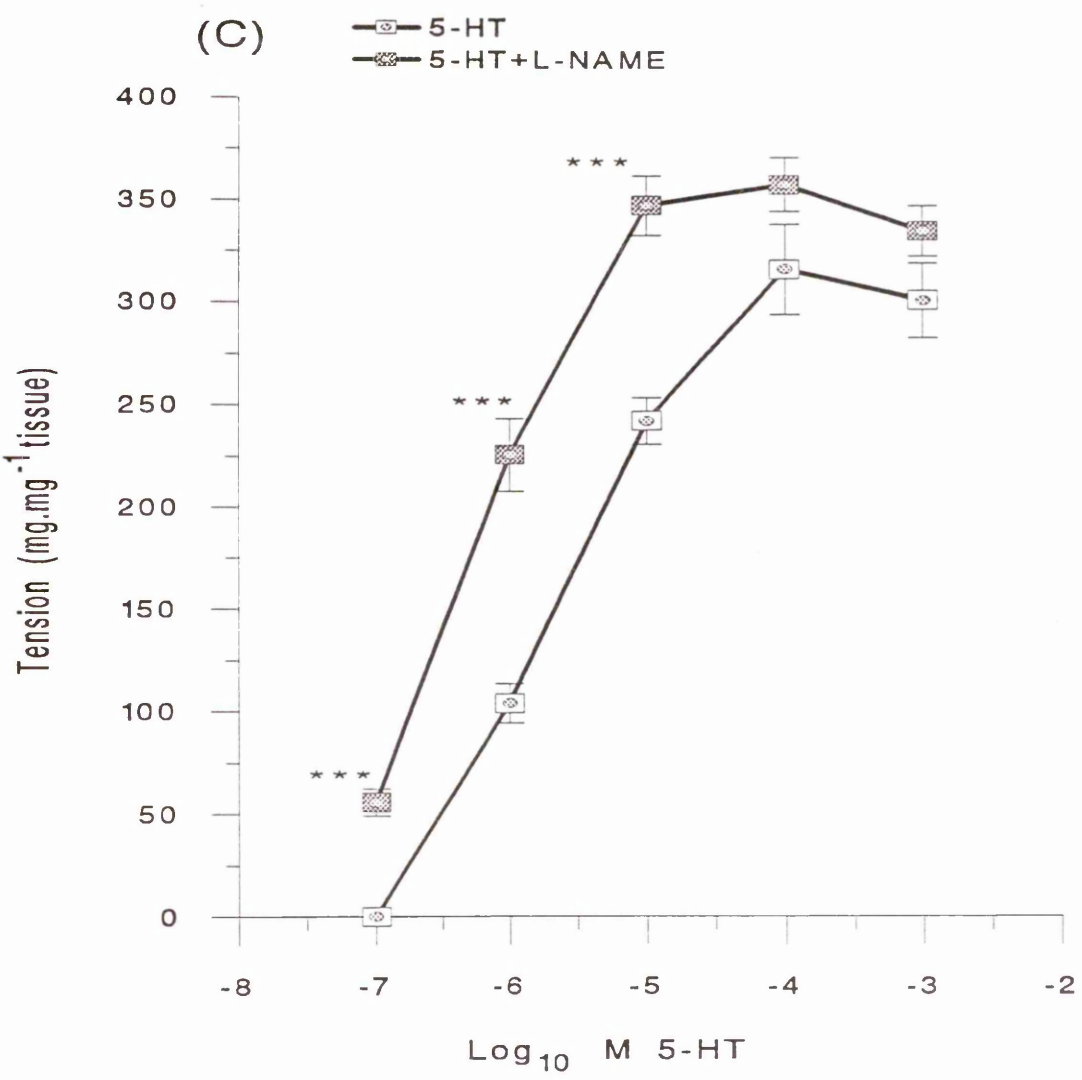
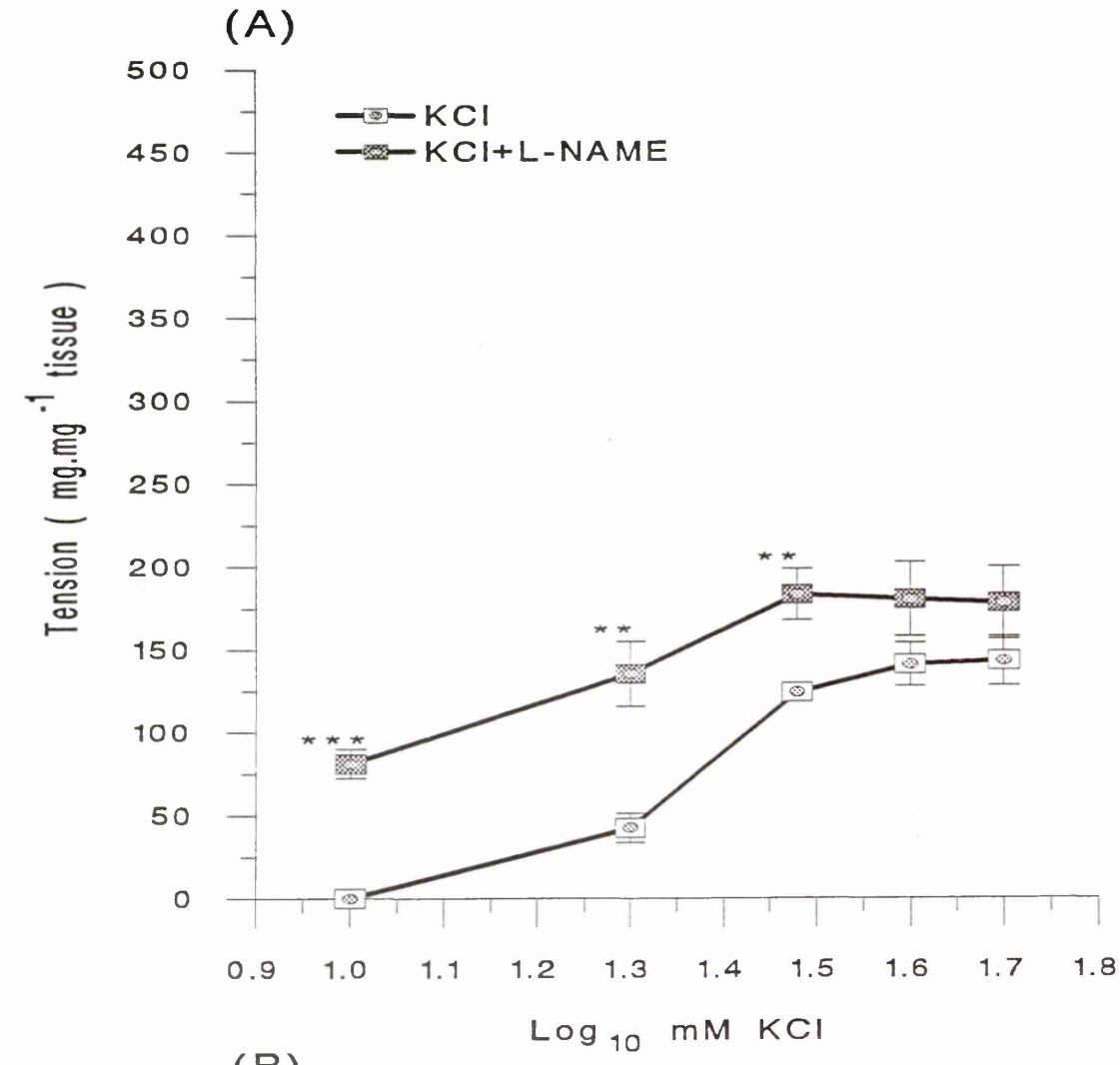
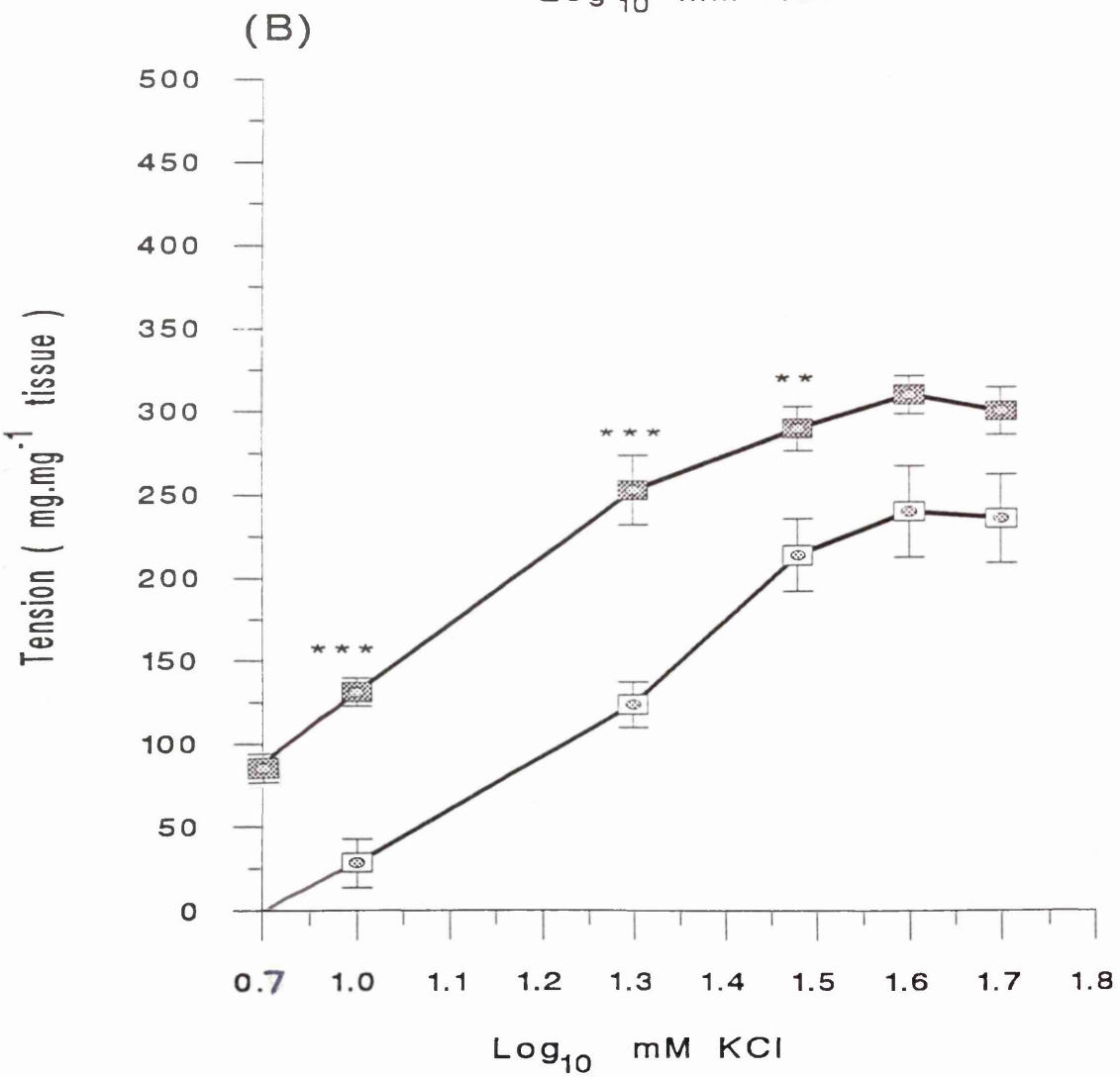


Fig. 11.

Effect of pretreatment with L-NAME ($2x10^{-4}$ M) on concentration-response curves to 5-HT in (A) MPA, (B) 1st and (C) 2nd branches. Each point is the mean ± s.e.mean of 6-9 observations. The potentiating effect of L-NAME on 5-HT-induced contractions was more pronounced as artery size decreased. Asterisks denote significant difference from untreated rings (**$P<0.01$, ***$P<0.001$; unpaired t-test).


(A)
KCl
KCl+L-NAME
Tension (mg.mg -1 tissue)
500
450
400
350
300
250
200
150
100
50
0
* * *
* *
* *
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
Log 10 mM KCl


(B)
Tension (mg.mg -1 tissue)
500
450
400
350
300
250
200
150
100
50
0
* * *
* * *
* *
0.7
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
Log10 mM KCl

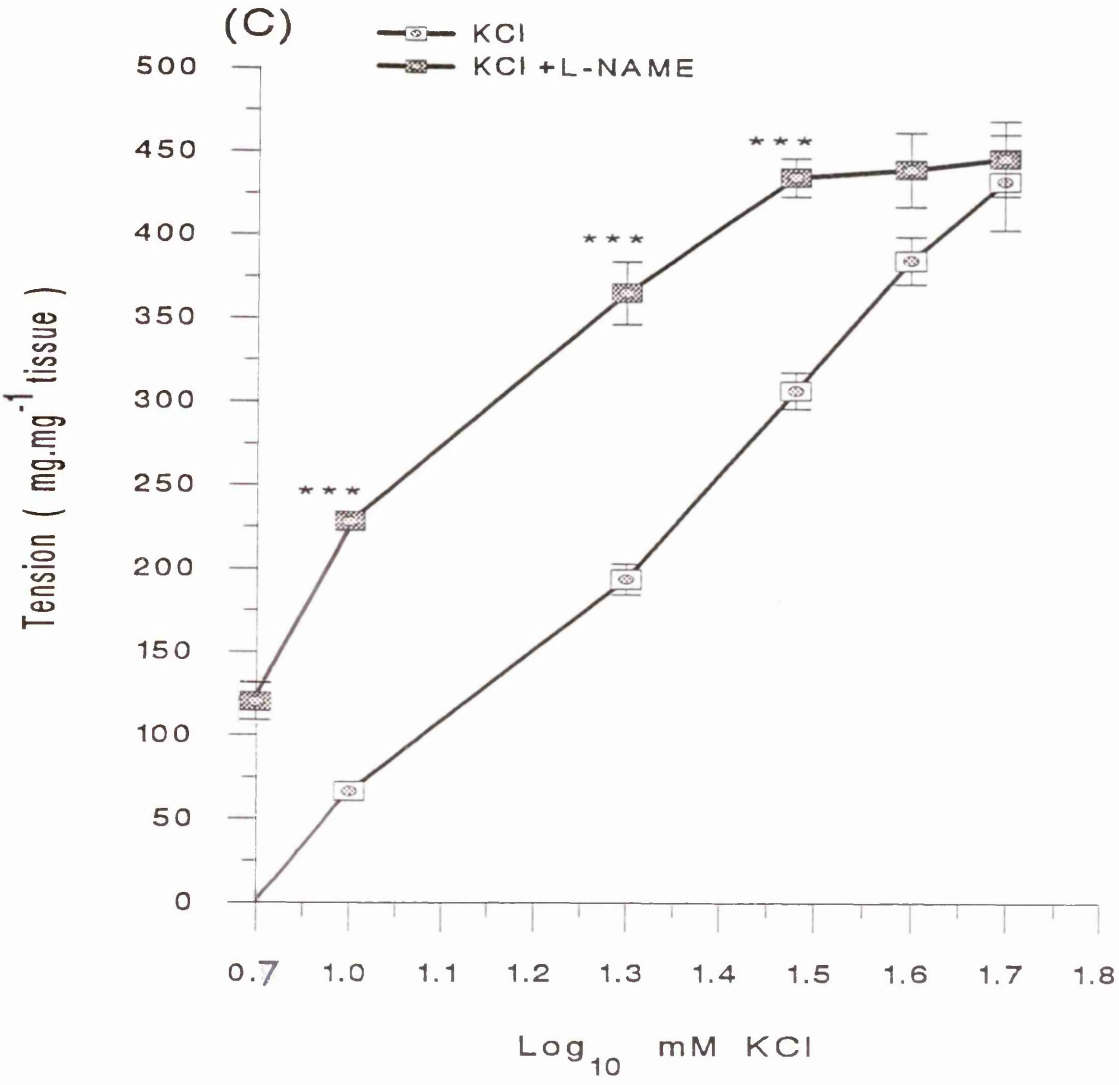


Fig. 12.

Effect of pretreatment with L-NAME ($2x10^{-4}$ M) on concentration-response curves to KCl in (A) MPA, (B) 1st and (C) 2nd branches. Each point is the mean ± s.e.mean of 6-10 observations. The potentiating effect of L-NAME on KCl-induced contractions was more pronounced as artery size decreased. Asterisks denote significant difference from untreated rings ($^{**}P<0.01$, $^{***}P<0.001$; unpaired t-test).

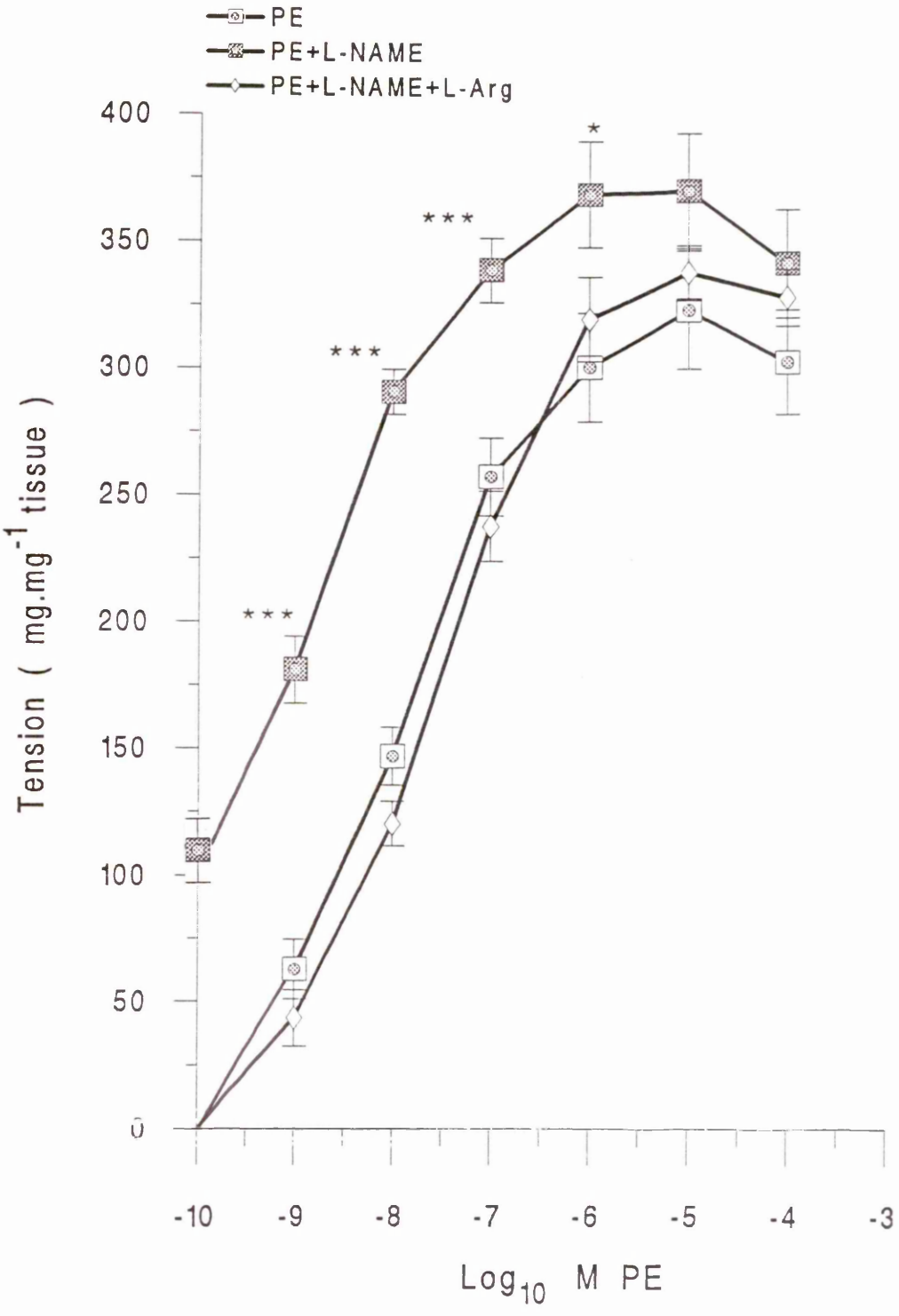


Fig. 13.

Concentration-response curves to PE in 2nd branches, in the absence and presence of L-NAME ($2x10^{-4}$ M) or L-NAME + L-arginine (L-Arg, 10^{-2} M). Each point is the mean ± s.e.mean of 8 observations. L-NAME potentiated PE-induced contractions, an effect which was completely prevented by pretreatment with L-arginine. Asterisks denote significant difference from PE alone (*P<0.05, ***P<0.001 ; unpaired t-test).

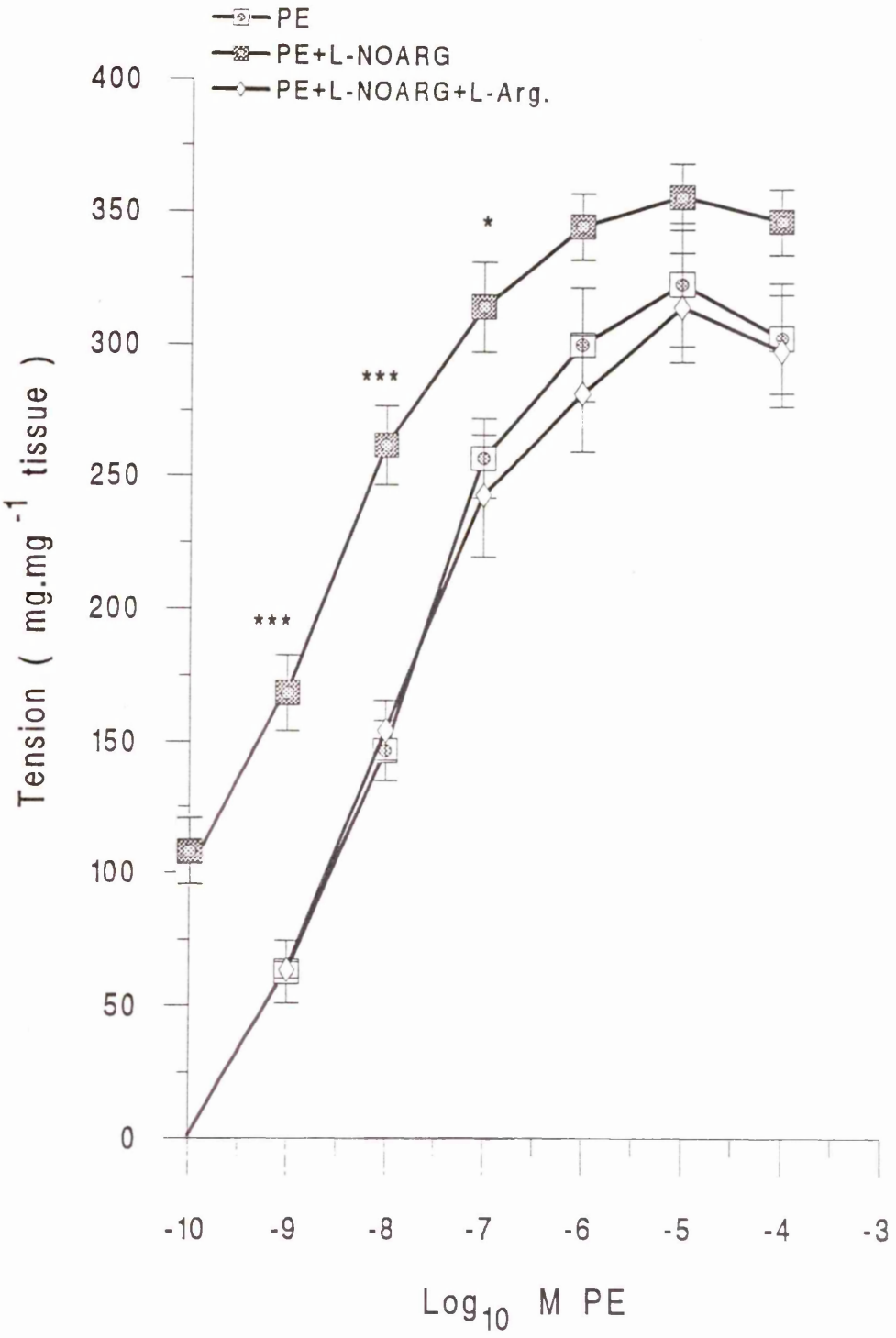


Fig. 14.

Concentration-response curves to PE in 2nd branches, in the absence and presence of L-NOARG (10^{-4} M) or L-NOARG + L-arginine (L-Arg, 10^{-2} M). Each point is the mean ± s.e.mean of 8 observations. L-NOARG potentiated PE-induced contractions, an effect which was completely prevented by pretreatment with L-arginine. Asterisks denote significant difference from PE alone ($*P<0.05$, $***P<0.001$; unpaired t-test).

3.1.4. **Effect of inhibition of prostanoid synthesis on contractile responses to PE**

The present experiments investigated the possible role of vasorelaxant prostanoids, especially prostacyclin, released by the endothelium in modulating the responsiveness of pulmonary arteries to vasoconstrictors. Prostanoid synthesis was inhibited by pretreatment of tissues with the cyclo-oxygenase inhibitor flurbiprofen.

In 2nd branch rings pretreated with flurbiprofen, PE induced concentration-dependent contractions with an EC_{50} value of 1.4×10^{-9} M and a maximum response of 334.8 ± 13.0 $mg.mg^{-1}$ tissue (Fig. 15). These values were not significantly different from those obtained in control tissues.

3.1.5. **Investigation of relaxant responses to clonidine, 5-HT and KCl**

As endothelium-dependent relaxations to α_2-agonists, 5-HT and KCl have been described in some vascular beds including pulmonary arteries from other species (Miller & Vanhoutte, 1985a ; Rubanyi & Vanhoutte, 1988 ; Glusa & Richter, 1993), attempts were made to determine whether the previously described depression of contractile responses to

vasoconstrictors was a consequence of a basal release or a vasoconstrictor-stimulated release of NO. In these experiments, cumulative CRC to the α_2-agonist clonidine or 5-HT were constructed in 2nd branch rings precontracted with the thromboxane A_2 mimetic U 46619 (EC_{75} $8x10^{-10}$ M), in the presence of the α_1-antagonist prazosin (10^{-6} M) or the 5-HT_2-antagonist Ketanserin (10^{-6} M), respectively. Cumulative CRC to KCl were obtained in PE-precontracted 2nd branch rings.

Clonidine, 5-HT and KCl failed to produce any degree of relaxation (Figs. 16, 17 and 18).

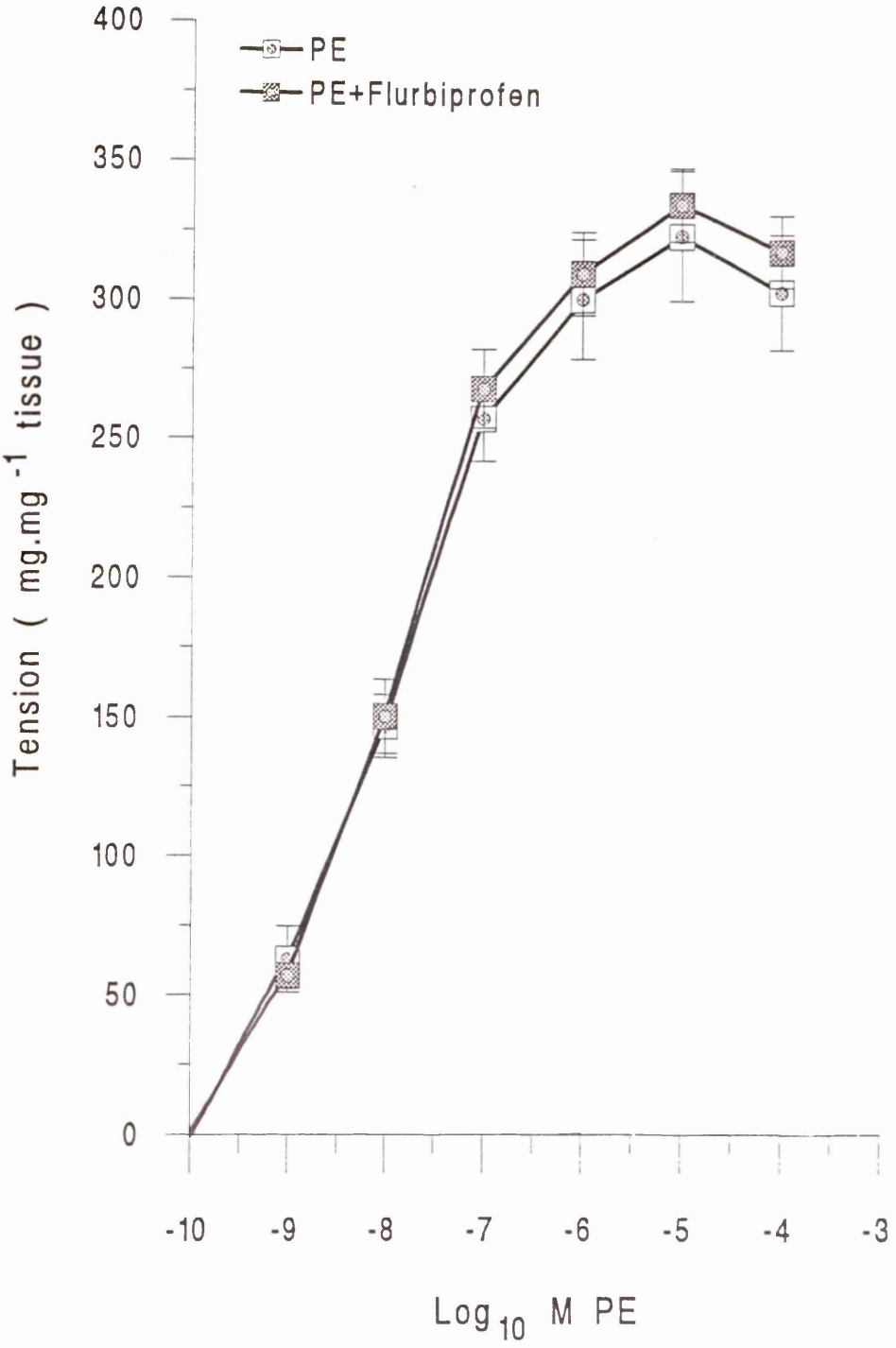


Fig. 15.

Concentration-response curves showing the lack of effect of pretreatment with flurbiprofen (10^{-5} M) on PE-induced contractions in 2nd branches. Each point is the mean ± s.e.mean of 8 observations.

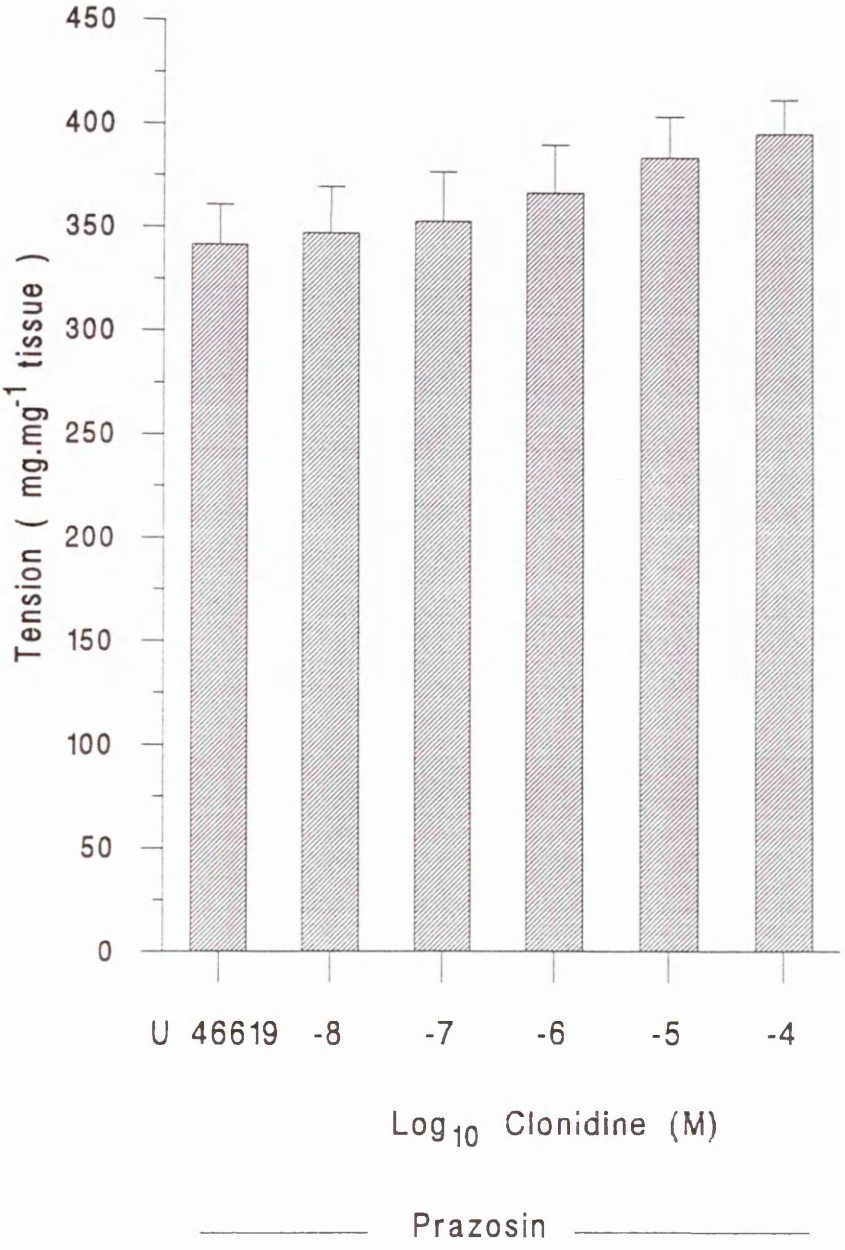


Fig. 16.

Histogram showing the lack of a relaxant effect to increasing concentrations of clonidine in 2nd branches in which tone had been raised with U 46619 (EC_{75} $8x10^{-10}$ M). Prazosin (10^{-6} M) was present throughout. Each bar is the mean ± s.e.mean of 6 observations.

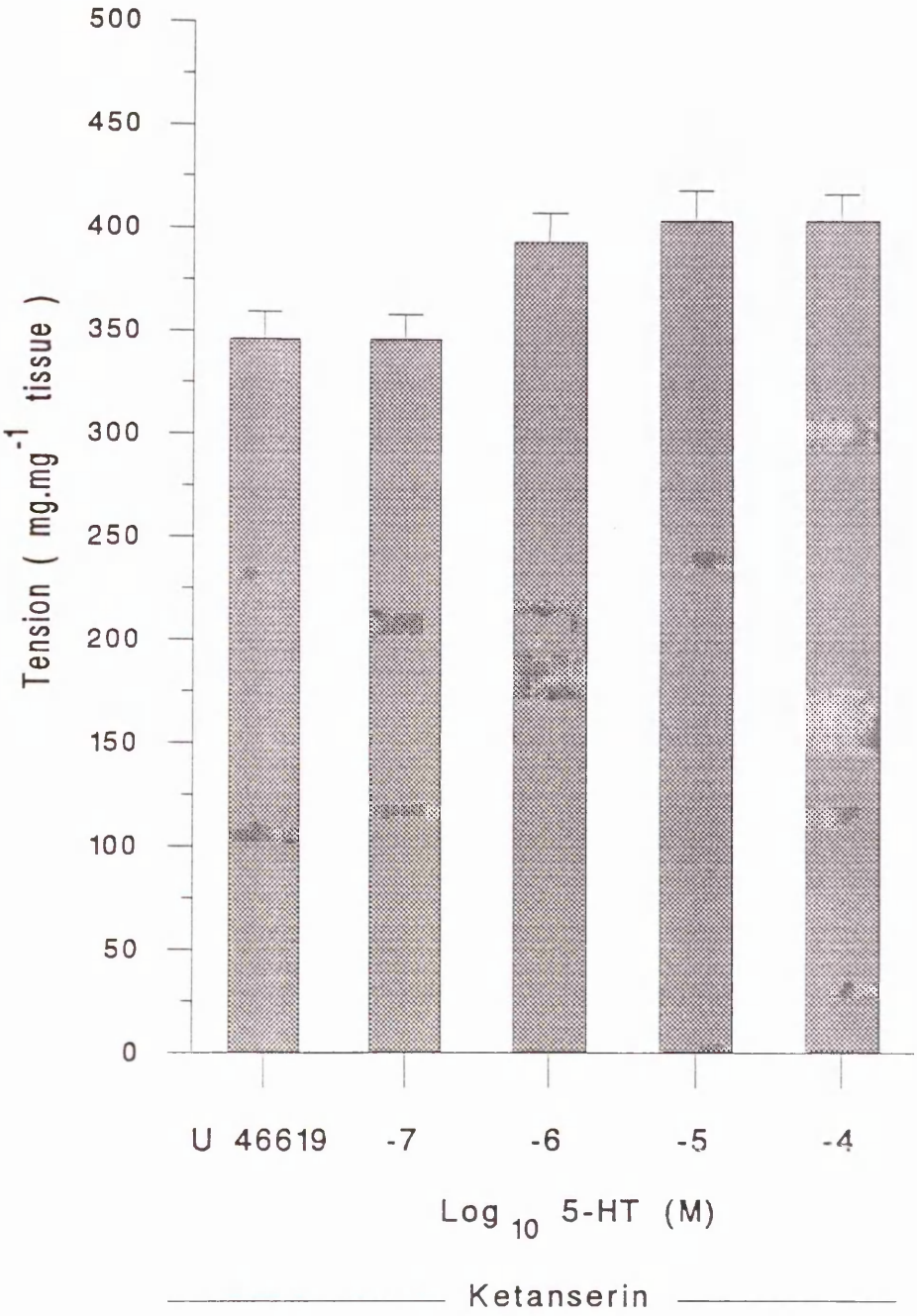


Fig. 17.

Histogram showing the lack of a relaxant effect to increasing concentrations of 5-HT in 2nd branches in which tone had been raised with U 46619 (EC_{75} $8x10^{-10}$ M). Ketanserin (10^{-6} M) was present throughout. Each bar is the mean ± s.e.mean of 6 observations.

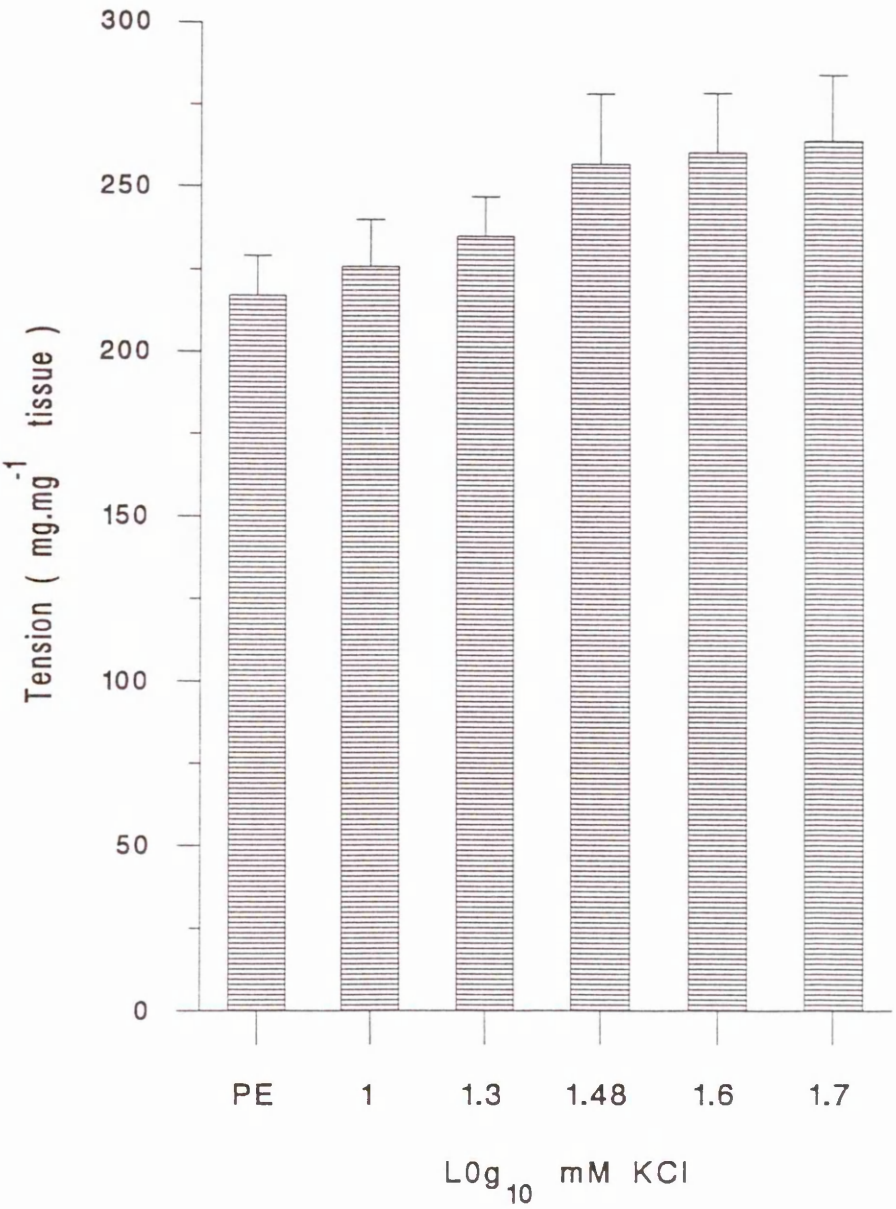


Fig. 18.

Histogram showing the lack of a relaxant effect to increasing concentrations of KCl in 2nd branches in which tone had been raised with PE (EC_{75} $9.5x10^{-8}$ M). Each bar is the mean ± s.e.mean of 6 observations.

3.1.6. Comparison of basal cGMP levels in MPA, 1st and 2nd branches

The finding that the potentiation of contractile responses after inhibition of NO synthesis was more pronounced as artery size decreased prompted an analysis of basal cGMP levels in the three arteries.

Basal cGMP levels inversely correlated with the vessel size. Basal cGMP level in 2nd branches (1.84 ± 0.14 pmol.mg^{-1} tissue) was 1.4 times higher than in 1st branches (1.34 ± 0.11 pmol.mg^{-1} tissue), which was 3 times higher than in MPA (0 .46 ± 0.04 pmol.mg^{-1} tissue) (Fig.19).

3.1.7. Influence of PE, 5-HT and KCl on cGMP levels

The cGMP levels measured in 2nd branch rings during contractions to PE or 5-HT were 1.60 ± 0.21 and 2.5 ± 0.30 pmol.mg^{-1} tissue, respectively (Fig.20 and 21). These levels were not significantly different from the basal level.

The effect of KCl on cGMP level in 1st branch rings was variable. KCl caused a significant rise in cGMP level in 2 out of 6 rings, with no effect in the 4 remaining rings. Taken together, the overall effect of KCl on cGMP level was not statistically significant (Fig. 22).

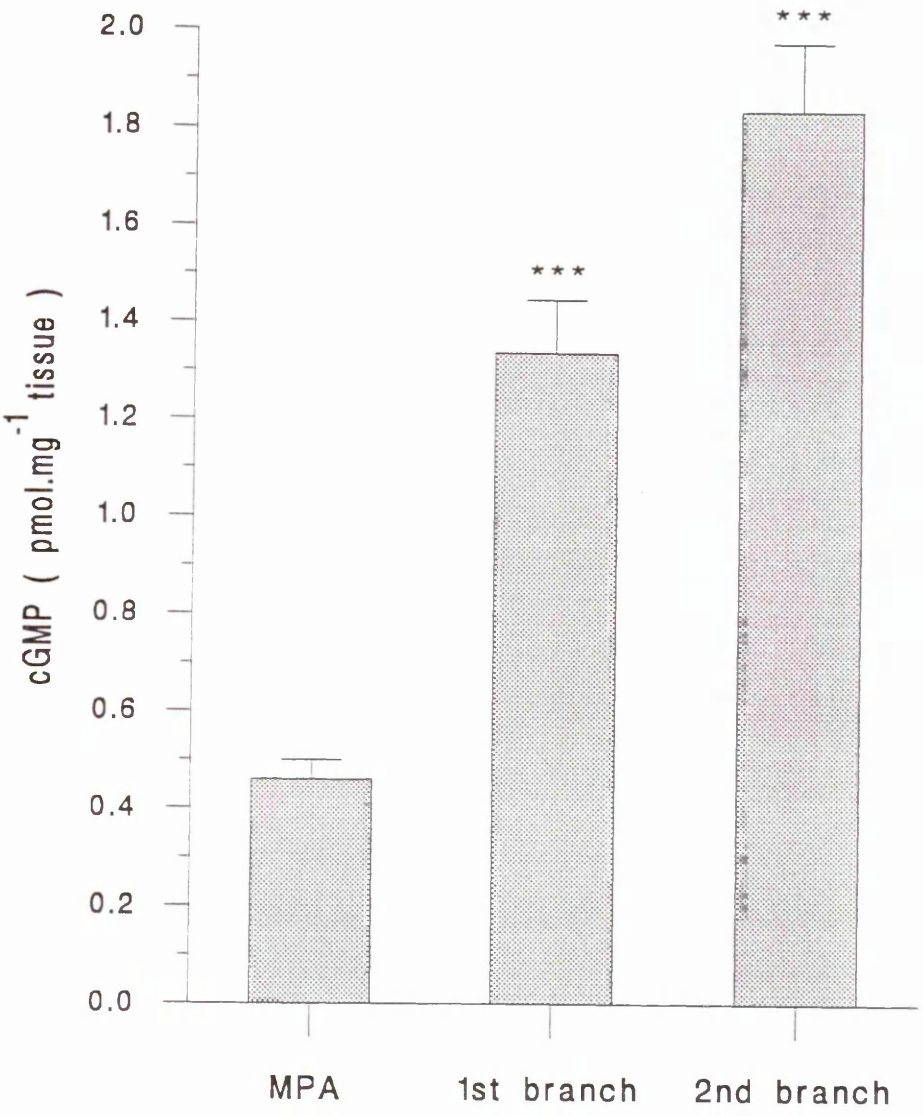


Fig. 19.

Comparison of basal cGMP levels in MPA, 1st and 2nd branches. Each bar is the mean ± s.e.mean of 6 measurements. cGMP levels were measured in artery rings which had been set up in organ baths and allowed to equilibrate for 60 min under 1g tension. Statistical comparison between the three arteries was performed using one way analysis of variance, followed by modified t-test for comparison between individual means. Asterisks denote significant difference as compared with MPA (***$P<0.001$).

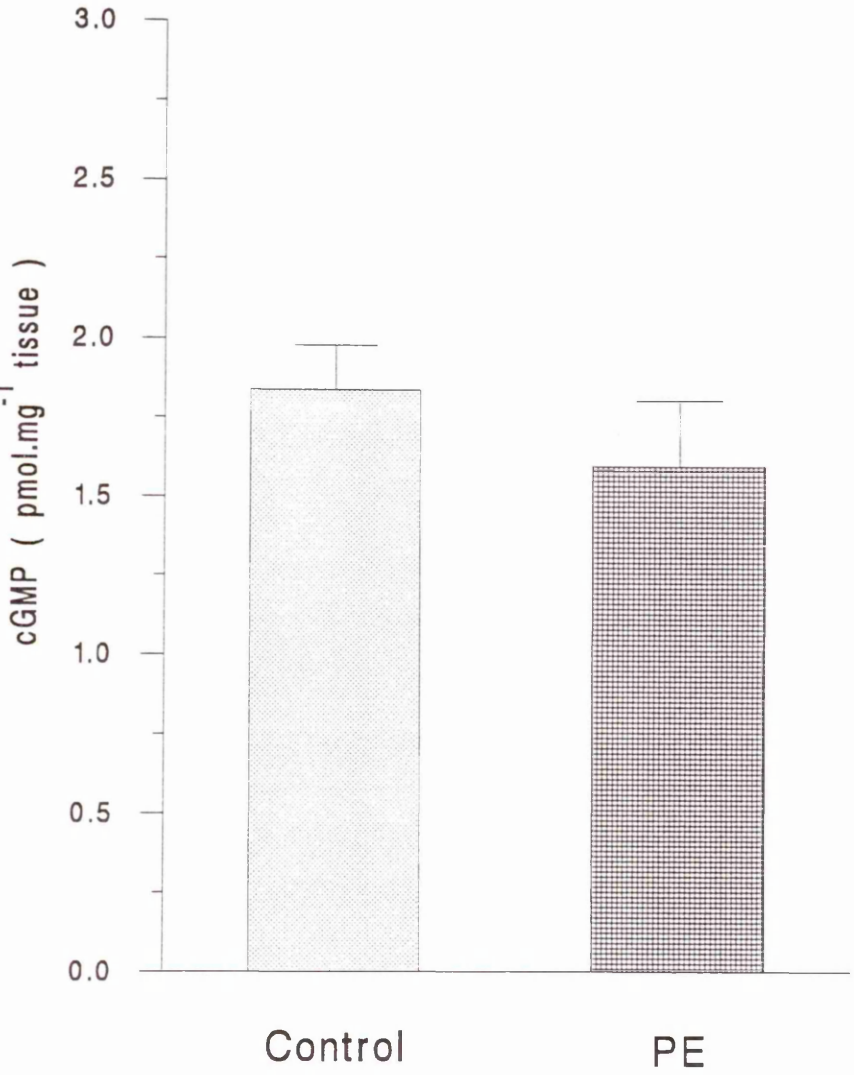


Fig. 20.

Effect of PE (10^{-4} M) on cGMP level in 2nd branches. Each bar is the mean ± s.e.mean of 6 measurements. Artery rings were set up in organ baths and allowed to equilibrate for 60 min under 1g tension before being exposed to PE for 2 min. PE did not significantly alter cGMP level as compared with the basal level (control).

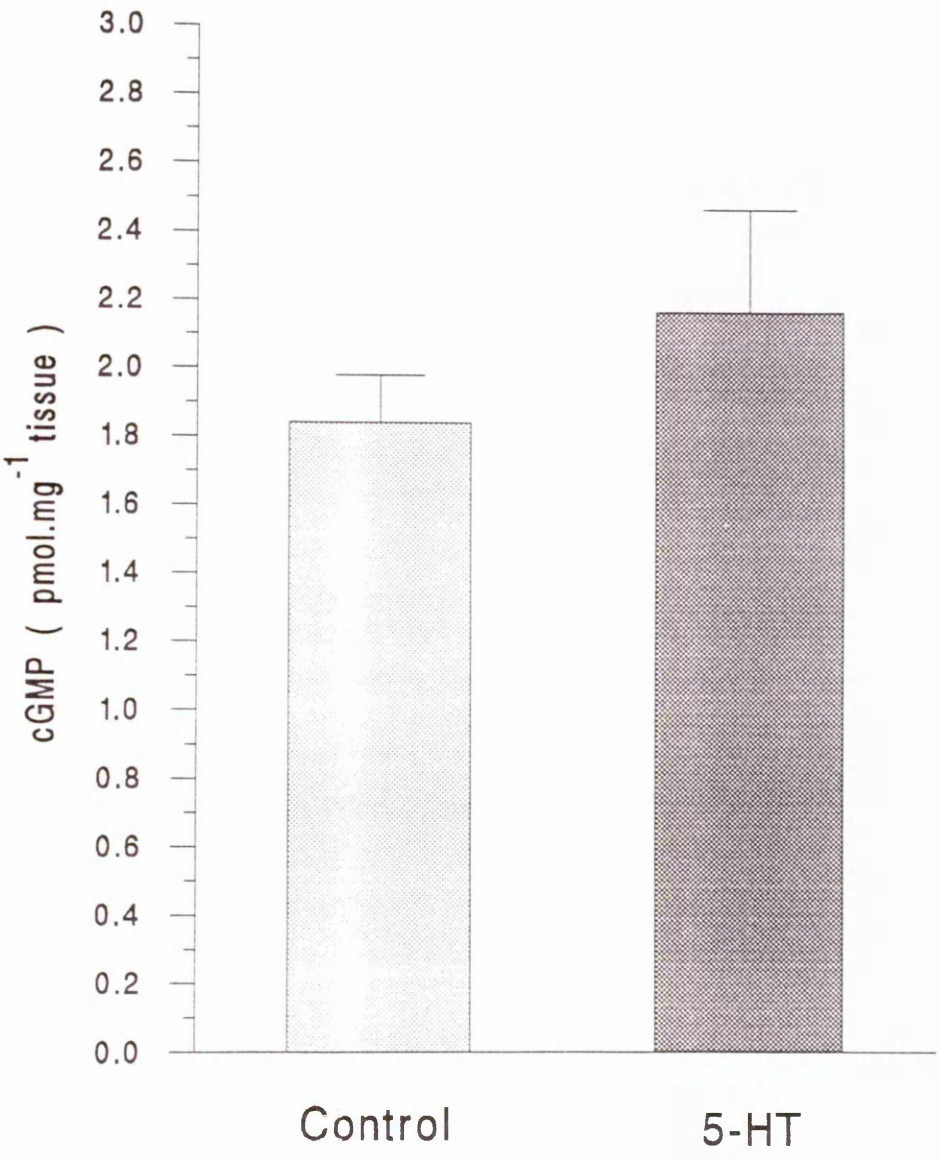


Fig. 21.

Effect of 5-HT (10^{-4} M) on cGMP level in 2nd branches. Each bar is the mean ± s.e.mean of 6 measurements. Artery rings were set up in organ baths and allowed to equilibrate for 60 min before being exposed to 5-HT for 2 min. 5-HT did not significantly alter cGMP level as compared with the basal level (control).

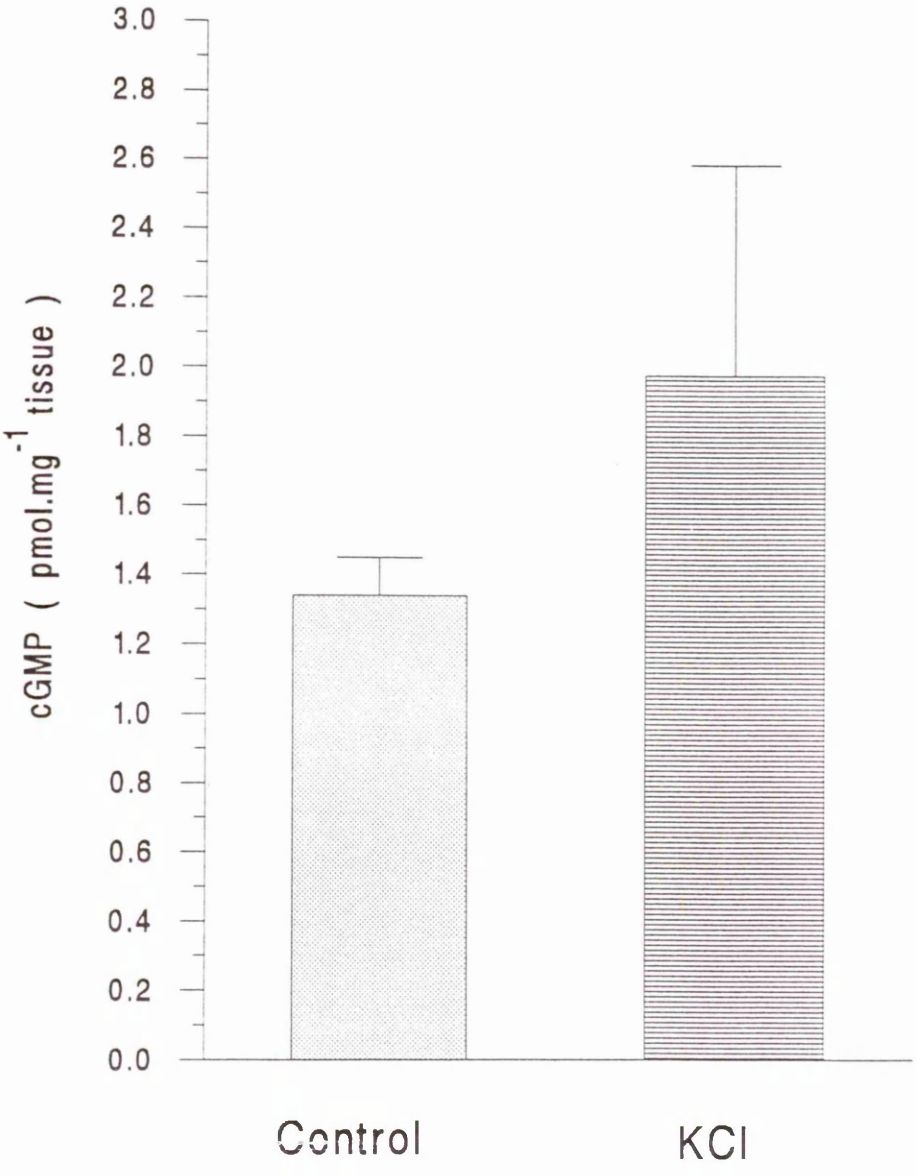


Fig. 22.

Effect of KCl (25.1 mM) on cGMP level in 1st branches. Each bar is the mean ± s.e.mean of 6 measurements. Artery rings were set up in organ baths and allowed to equilibrate for 60 min under 1g tension before being exposed to KCl for 3 min. Although KCl apparently increased cGMP level, the difference was not statistically significant as compared with the basal level (control).

3.2. Studies on relaxant responses

3.2.1. Endothelium-dependent relaxations in MPA, 1st and 2nd branches

Several studies have suggested that NO released by the pulmonary endothelium may play an important role in the maintenance of the low pulmonary vascular tone (Peach et al., 1989 ; Wiklund et al., 1990). However, most of the studies on isolated pulmonary arteries have used large conduit arteries, and the results may not reflect the significance of NO in the more functionally important small resistance arteries. This study has already demonstrated that the extent to which NO modulates responses to vasoconstrictors increases as artery size decreases. Therefore, further experiments were performed to compare endothelium-dependent relaxations in MPA, 1st and 2nd branches.

i) PE-precontracted rings

The endothelium-dependent vasorelaxant CARB induced concentration-dependent relaxations in PE-precontracted rings from MPA,1st and 2nd branches (Figs.23 and 24). An inverse relationship between CARB-induced relaxations and artery size was observed, as evident by the maximum relaxations obtained in MPA, 1st and 2nd branches which were 49.4 ± 5.1%, 81.6 ± 2.4% and 102.1 ± 4.5%, respectively. However, MPA rings were more sensitive than smaller artery rings to the relaxant effect of the low 10^{-8} M concentration of CARB.

ii) KCl-precontracted rings

CRC to CARB were also constructed in MPA, 1st and 2nd branch rings precontracted with KCl to investigate whether, as has been found in aortic rings (Collins *et al.*, 1988 ; Morrison & Pollock, 1988), the degree of relaxation of pulmonary artery is influenced by the nature of the contractile agent used to raise muscle tone.

Relaxant responses to CARB in KCl-precontracted rings from the three arteries displayed a similar difference to that observed in PE-precontracted rings (Fig.25). However, KCl-precontracted rings showed a significantly smaller magnitude of relaxation in response to CARB than rings precontracted with PE. This was most striking in MPA where CARB, at all concentrations tested, failed to produce any degree of relaxation. In KCl-precontracted 1st and 2nd branch rings, the maximum relaxations obtained were 25.6 ± 2.1% and 45.4 ± 2.8%, respectively, representing approximately 30% and 40% of the maximum relaxations obtained in PE-precontracted rings.

CRC to CARB in 1st and 2nd branch rings precontracted with either PE or KCl are shown for comparison in Figs. 26 and 27.

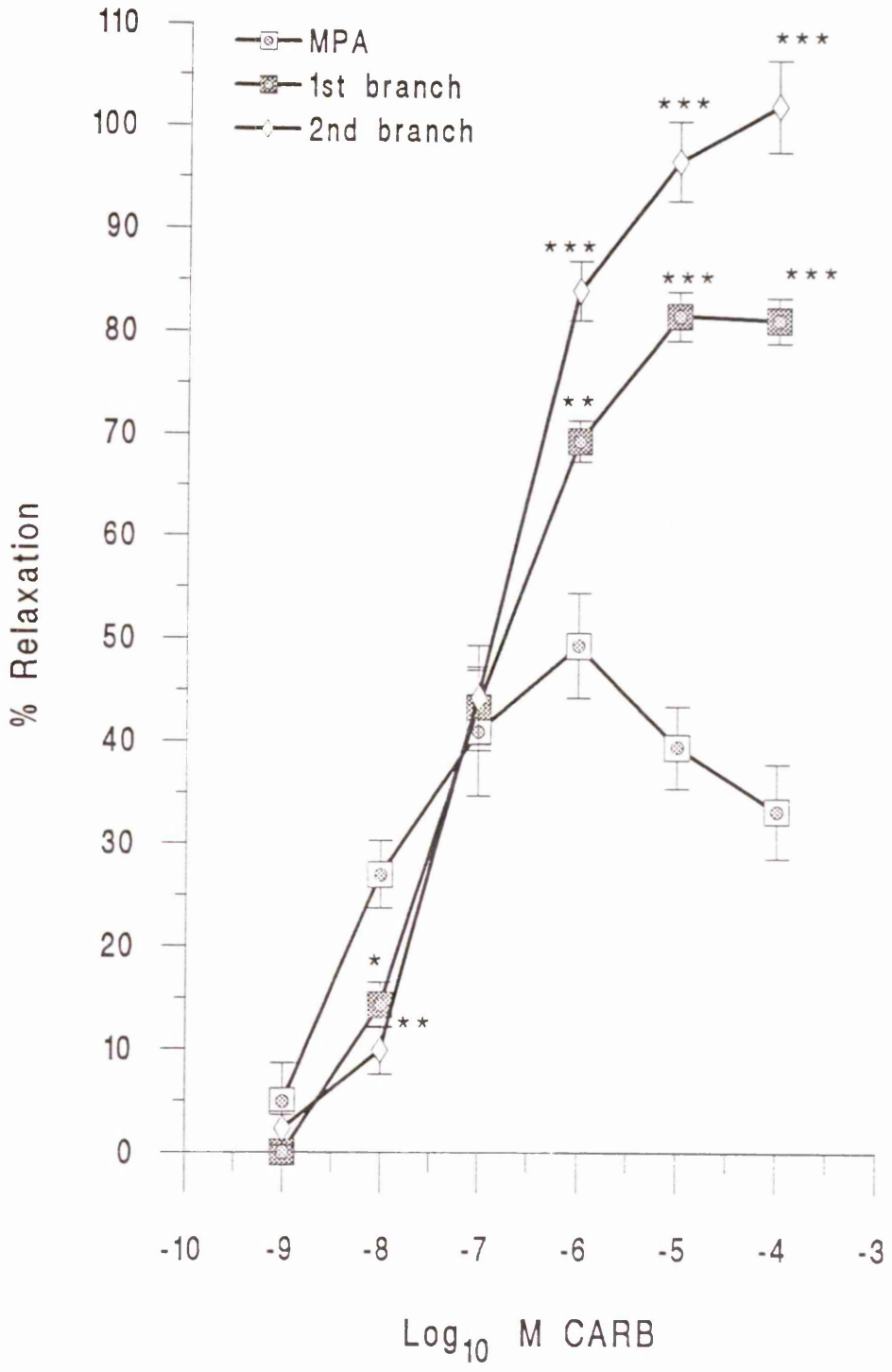


Fig. 23.

Relaxant effects induced by cumulative concentrations of CARB in MPA, 1st and 2nd branches precontracted with PE (EC_{75} 2.2×10^{-7} M for MPA, 9.5×10^{-8} M for 1st and 2nd branches). Each point is the mean ± s.e.mean of 6-9 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. Statistical comparison between the three arteries was performed using one way analysis of variance, followed by modified t-test for comparison between individual means. Asterisks denote significant difference as compared with MPA ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

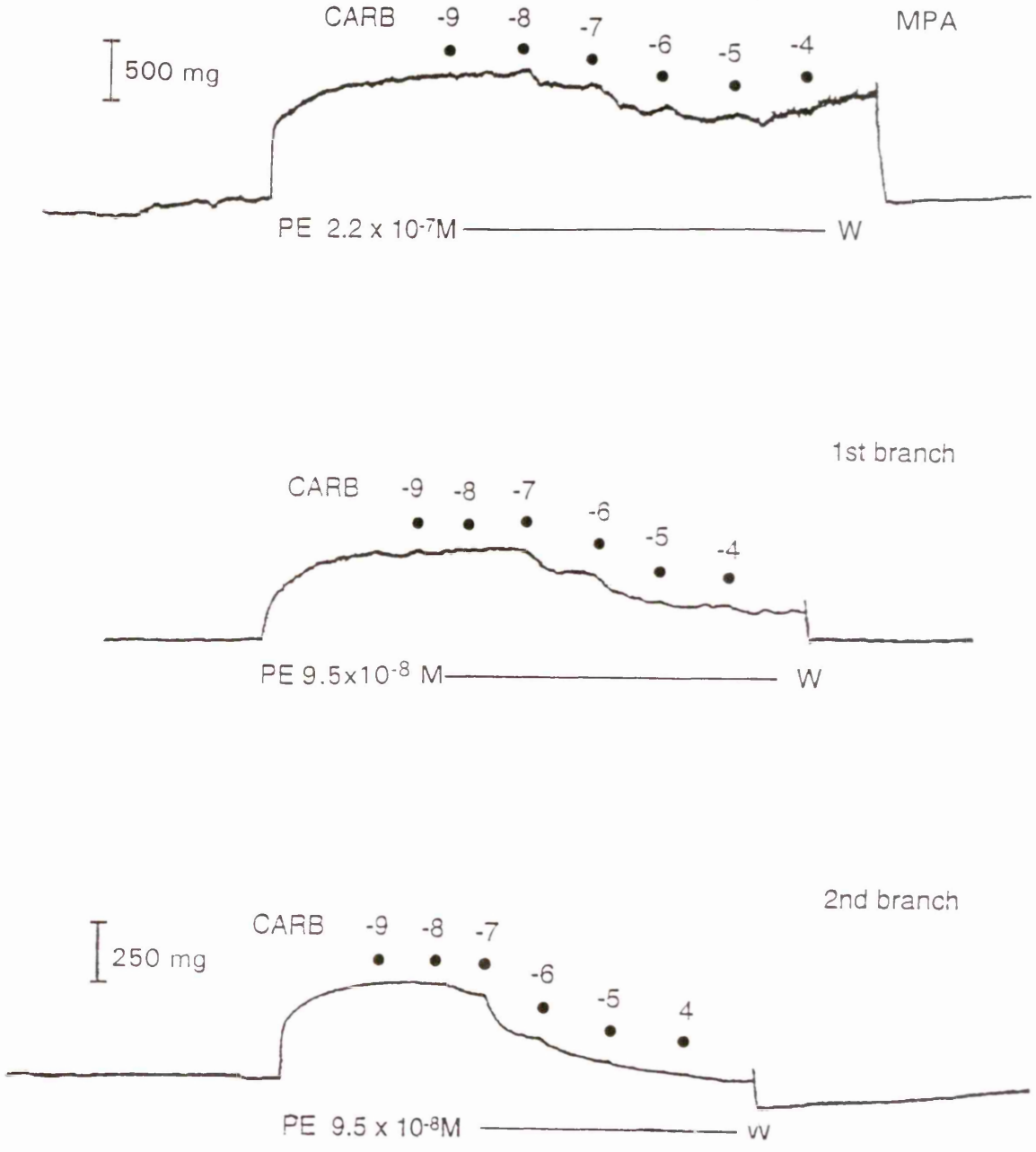


Fig. 24.

Individual traces showing relaxant effects induced by cumulative concentrations of CARB in MPA, 1st and 2nd branches in which tone had been raised with PE. 2nd branches were more responsive to the relaxant effect of CARB than 1st branches, which were more responsive than MPA.

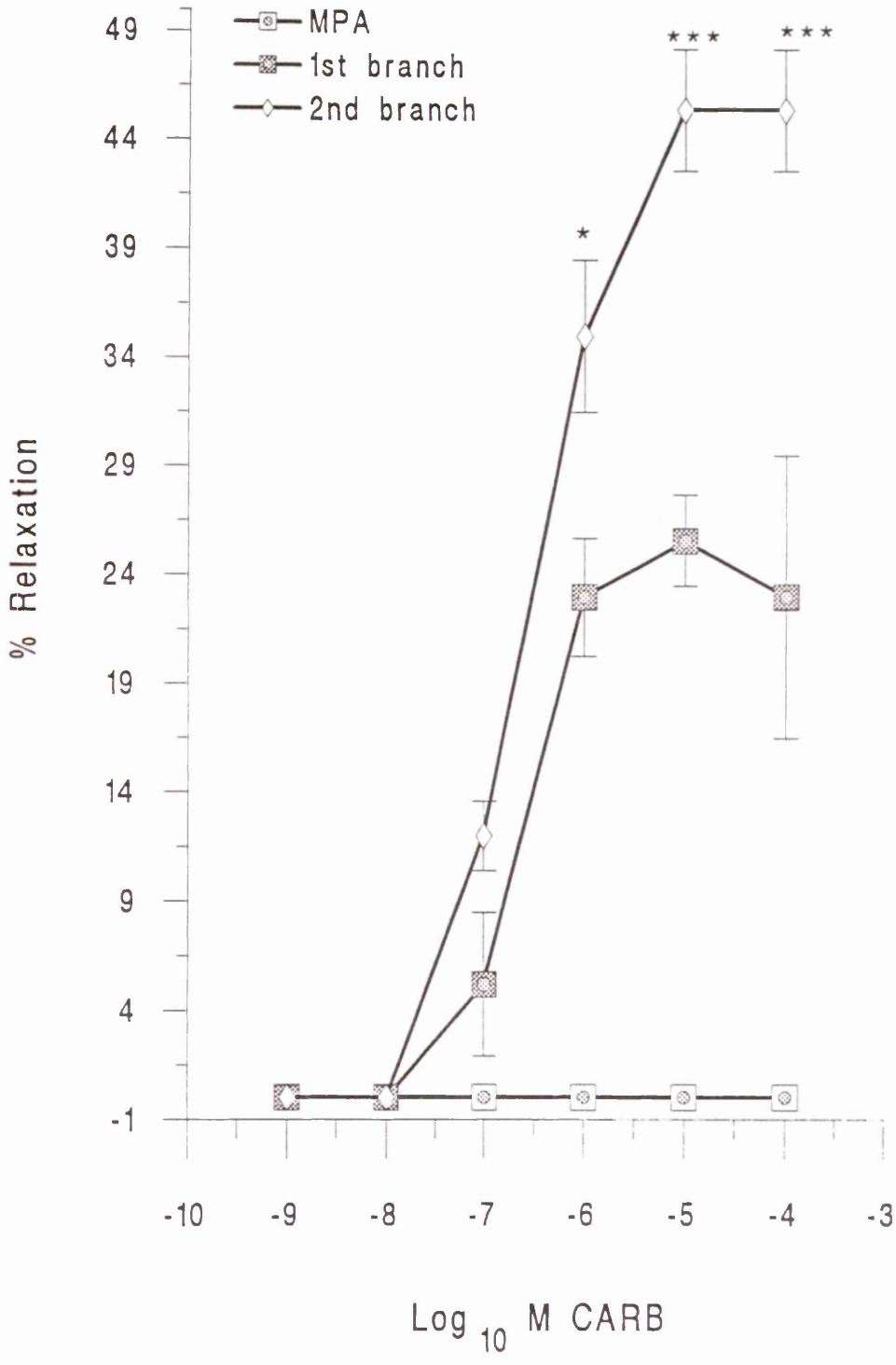


Fig. 25.

Cumulative concentration-response curves to CARB in MPA, 1st and 2nd branches precontracted with KCl (EC_{75} 27.5 mM for MPA, 25.1 mM for 1st branches and 29.0 mM for 2nd branches). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of KCl-induced tone. CARB failed to produce any degree of relaxation in KCl-precontracted MPA. Asterisks denote significant difference as compared with 1st branches (*$P<0.05$, ***$P<0.001$; unpaired t-test).

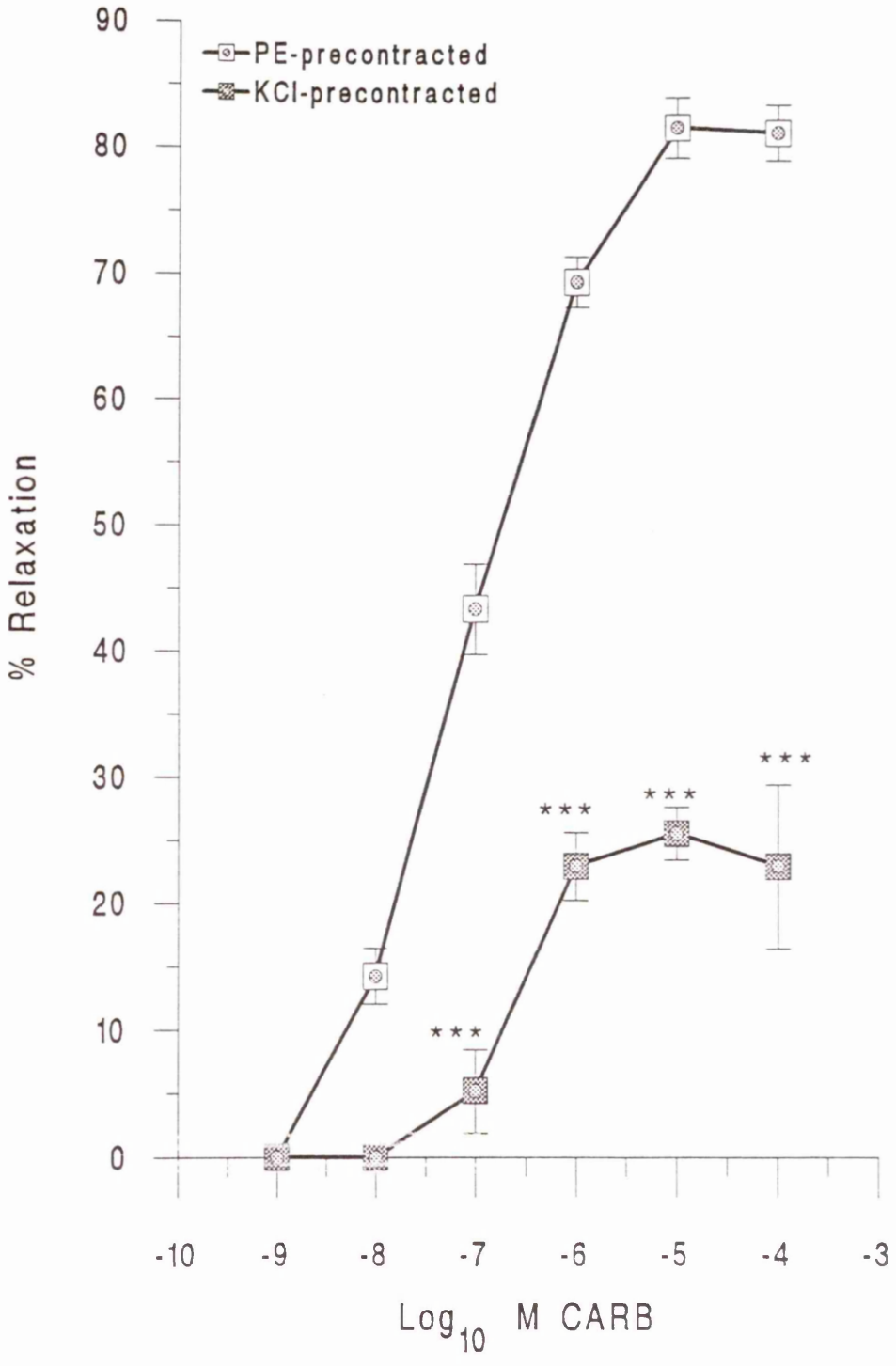


Fig. 26.

Comparison of CARB-induced relaxations in 1st branches precontracted with either PE (EC_{75} 9.5×10^{-8} M) or KCl (EC_{75} 25.1 mM). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE- or KCl-induced tone. CARB induced significantly weaker relaxations in KCl-precontracted rings than in PE-precontracted rings. Asterisks denote significant difference (***$P<0.001$; unpaired t-test).

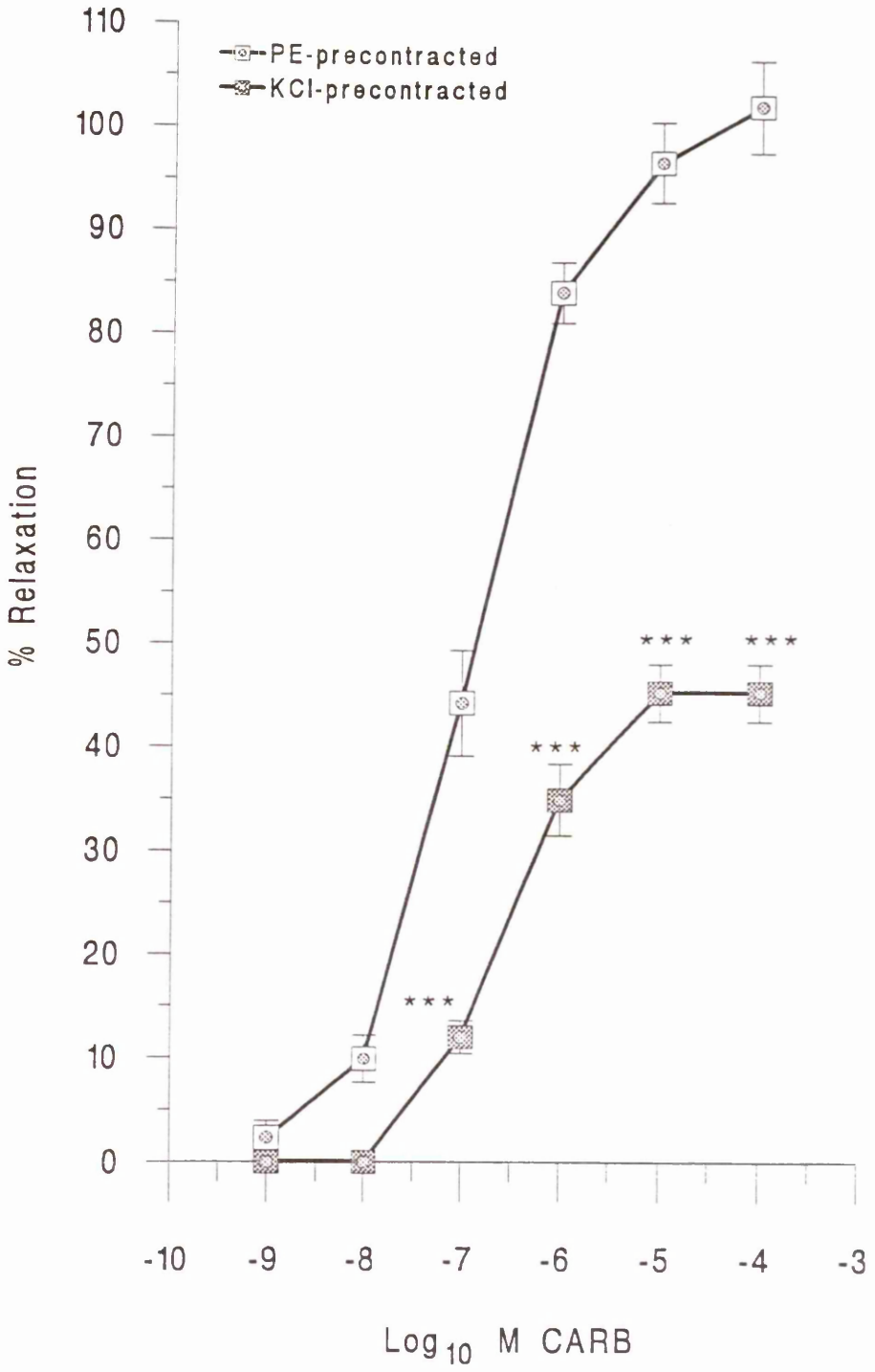


Fig. 27.

Comparison of CARB-induced relaxations in 2nd branches precontracted with either PE (EC_{75} 9.5×10^{-8} M) or KCl (EC_{75} 29.0 mM). Each point is the mean ± s.e.mean of 6-9 observations. Relaxant responses are expressed as % relaxation of PE- or KCl-induced tone. CARB induced significantly weaker relaxations in KCl-precontracted rings than in PE-precontracted rings. Asterisks denote significant difference (***$P < 0.001$; unpaired t-test).

3.2.2. Effect of NOS inhibitors on CARB-induced relaxation

In the early experiments of this study, L-NAME was used to investigate the effect of NOS inhibitors on CARB-induced relaxation. However recently, Buxton *et al.* (1993) have reported that L-NAME may act as a muscarinic antagonist, and therefore is a poor choice as a NOS inhibitor in studies in which muscarinic receptors are not blocked. In contrast to the effect of L-NAME on NOS, muscarinic blockade cannot be reversed by an excess of L-arginine. This led us to compare the effects of L-NAME and L-NOARG on CARB-induced relaxation, and their prevention by L-arginine. L-NOARG was chosen because it does not block muscarinic receptors (Buxton *et al.*, 1993) and, unlike L-NMMA, does not act as a partial agonist for NO synthesis (Archer & Hampl, 1992). Since L-NAME and L-NOARG both potentiate PE-induced contraction, a lower concentration of PE (2.2×10^{-8} M for MPA , 9.5×10^{-9} M for 1st and 2nd branches) was used to obtain a similar degree of contraction to that obtained in the absence of both agents.

Pretreatment with L-NAME abolished CARB-induced relaxations in MPA and 1st branches, and almost completely inhibited responses to CARB in 2nd branches. In this case, the maximum relaxation induced by CARB was $6.6 \pm 1.8\%$ (Fig.28).

Pretreatment with L-NOARG caused complete inhibition of CARB-induced relaxations in 2nd branch rings precontracted with PE (Fig.29).

3.2.3. Effect of L-arginine on L-NAME and L-NOARG-induced inhibition of CARB-induced relaxation

In PE-precontracted 2nd branch rings, pretreatment with L-arginine only partially prevented the inhibitory effects of L-NAME and L-NOARG on CARB-induced relaxations, resulting in maximum relaxations of 44.1 ± 1.8% and 37.6 ± 1.3%, respectively (Figs.28 and 29).

3.2.4. Influence of CARB on cGMP levels in MPA, 1st and 2nd branches

CARB increased cGMP levels in MPA, 1st and 2nd branches from 0.46 ± 0.04, 1.34 ± 0.11 and 1.84 ± 0.14 pmol.mg^{-1} tissue to 1.82 ± 0.23, 7.30 ± 0.95 and 10.47 ± 2.15 pmol.mg^{-1} tissue, respectively (Fig.30), which is 4.0, 5.4 and 5.7 times higher than basal levels, respectively.

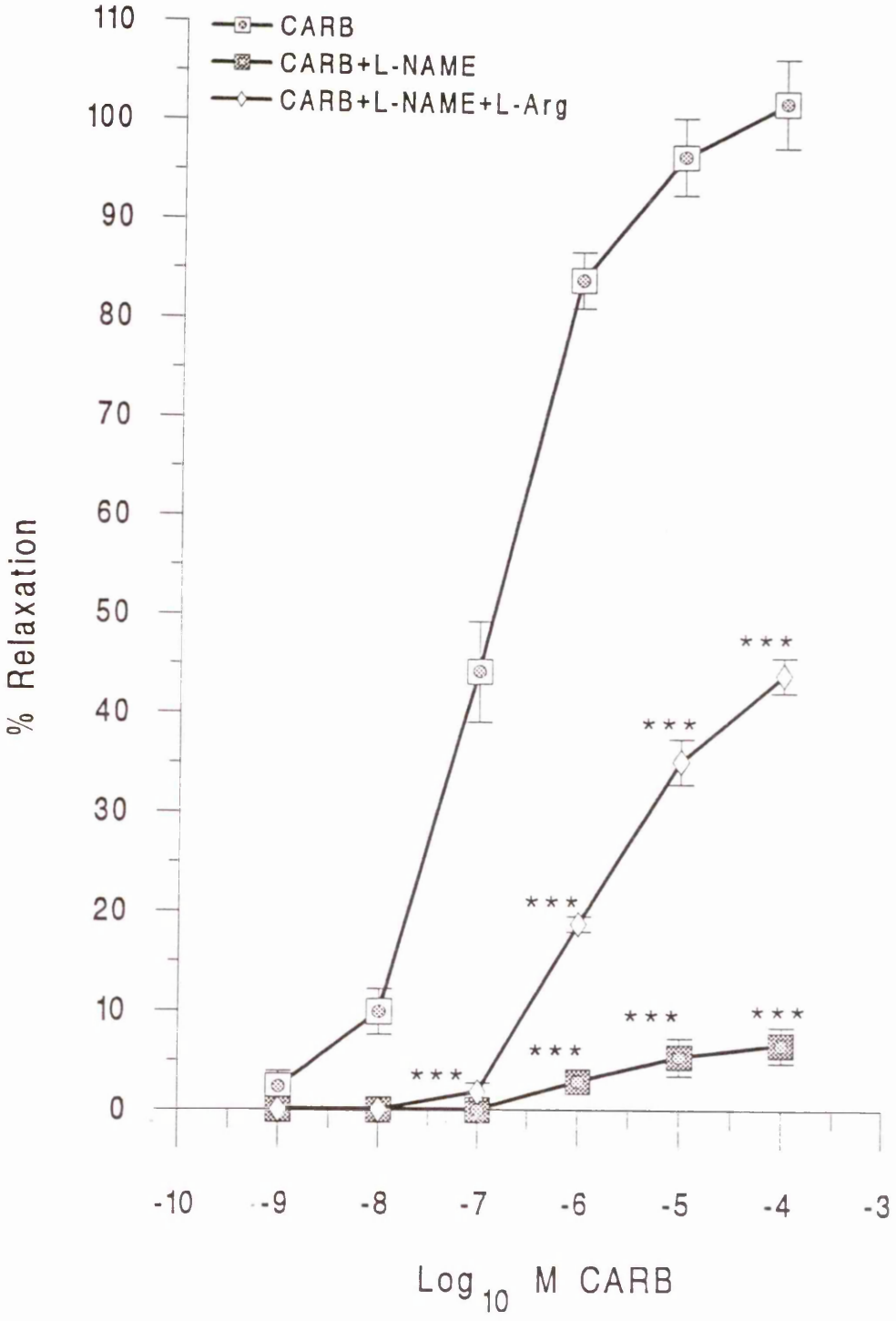


Fig. 28.

Cumulative concentration-response curves to CARB in 2nd branches precontracted with PE (9.5×10^{-8} M or 9.5×10^{-9} M), in the absence and presence of L-NAME (2×10^{-4} M) or L-NAME + L-arginine (L-Arg, 10^{-2} M). Each point is the mean ± s.e.mean of 9 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. L-NAME markedly inhibited the relaxant effect of CARB, an effect which was only partially prevented by pretreatment with L-arginine . Asterisks denote significant difference from CARB alone (***$P < 0.001$; unpaired t-test).

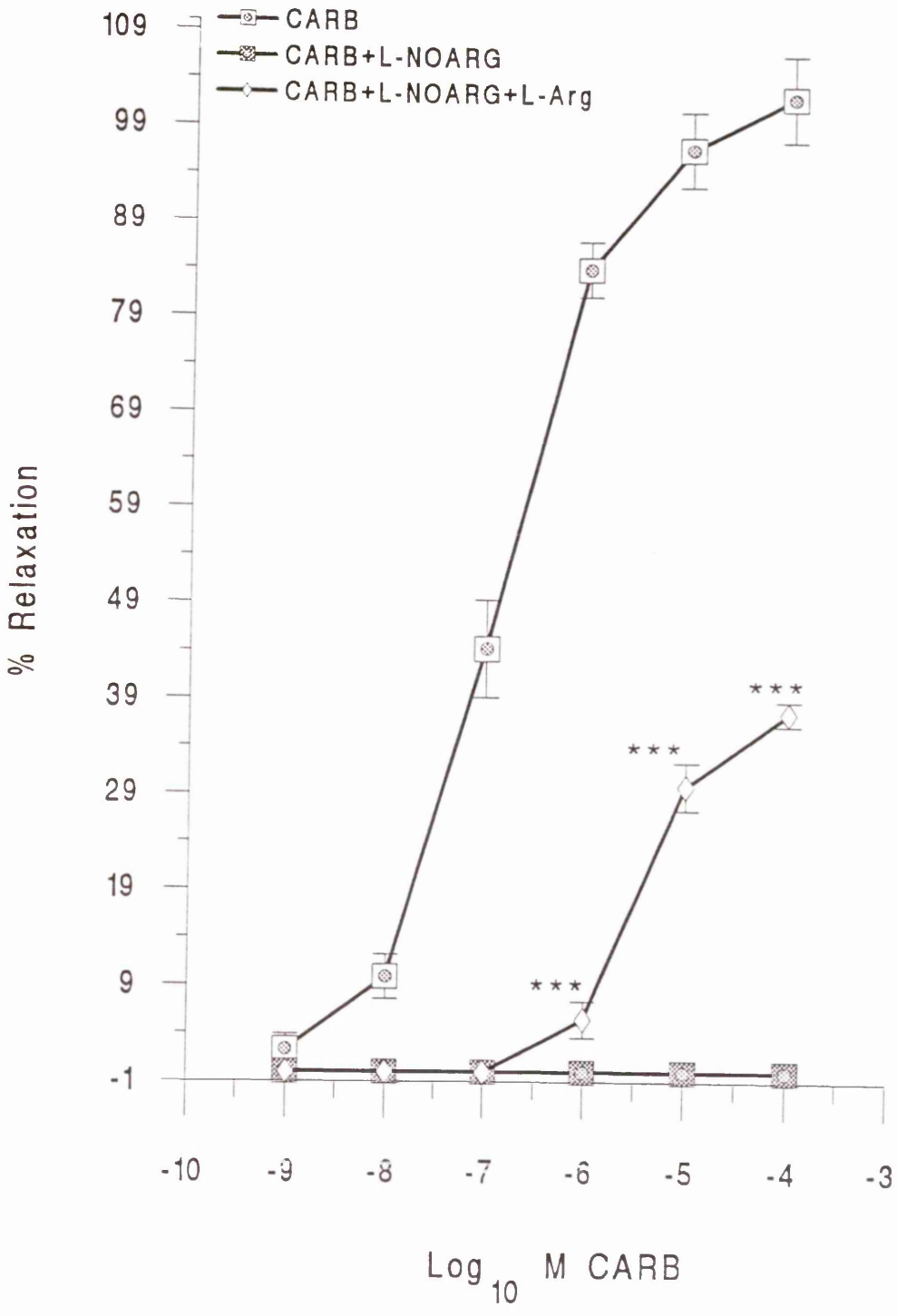


Fig. 29.

Cumulative concentration-response curves to CARB in 2nd branches precontracted with PE (9.5×10^{-8} M or 9.5×10^{-9} M), in the absence and presence of L-NOARG (10^{-4} M) or L-NOARG + L-arginine (L-Arg, 10^{-2} M). Each point is the mean ± s.e.mean of 9 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. L-NOARG completely inhibited the relaxant effect of CARB, an effect which was only partially prevented by L-arginine pretreatment. Asterisks denote significant difference from CARB alone (***$P < 0.001$, unpaired t-test).

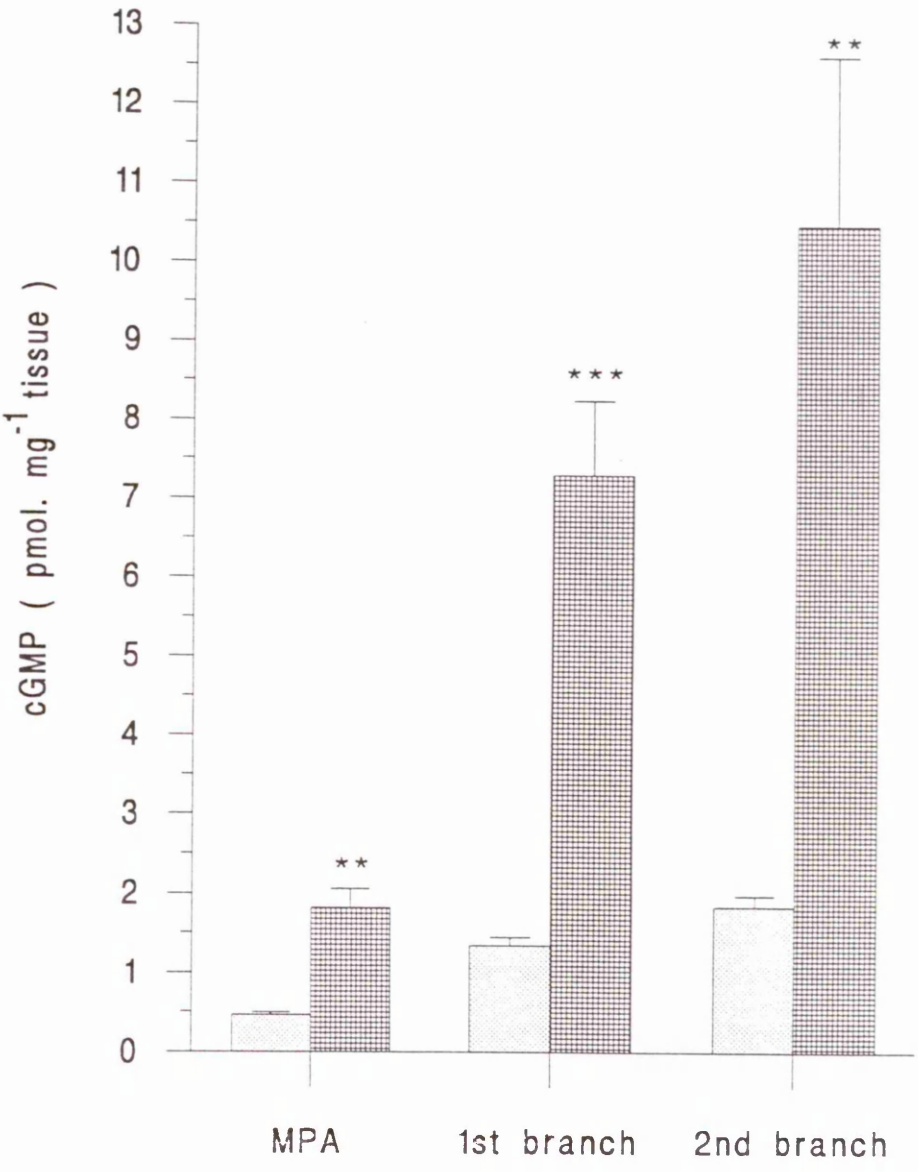


Fig. 30.

Effect of CARB (10^{-4} M) on cGMP levels in MPA, 1st and 2nd branches. Each bar is the mean ± s.e.mean of 6 observations. Artery rings were precontracted with PE (EC_{75} 2.2×10^{-7} M for MPA, 9.5×10^{-8} M for 1st and 2nd branches) before being exposed to CARB for 30s. CARB significantly increased cGMP levels in the three arteries. Asterisks denote significant difference from the basal level (control) ($^{**}P<0.01$, $^{***}P<0.001$; unpaired t-test).

3.2.5. Endothelium-independent relaxations in MPA, 1st and 2nd branches

The endothelium-independent vasorelaxant SNP relaxes vascular smooth muscle by spontaneously releasing NO in aqueous solutions (Ignarro *et al.*, 1981). The effect of SNP in MPA, 1st and 2nd branches was investigated in an attempt to determine whether the previously described difference in CARB-induced relaxations between the three arteries could be accounted for by a higher guanylate cyclase sensitivity in the smaller arteries. If this was the case, SNP-induced relaxations in the three arteries would be expected to display a similar difference to that observed with CARB-induced relaxations.

i) PE-precontracted rings

SNP caused a complete inhibition of PE-induced tone in the three arteries, with maximum relaxations of 100% in MPA and 1st branches, and 102.2 ± 3.0% in 2nd branches. Relaxant responses to submaximal concentrations of SNP were almost identical in MPA and 1st branches, but significantly weaker in 2nd branches (Fig.31).

ii) KCl-precontracted rings

In view of the earlier observation that relaxant responses to CARB in KCl-precontracted rings were markedly attenuated when compared to responses in rings precontracted with PE, we investigated whether this difference could be attributed to an inhibitory action by KCl on the

generation of NO in response to CARB. If this was the case, SNP would be expected to produce similar relaxations in both PE- and KCl-precontracted rings.

In KCl-precontracted MPA and 1st branch rings, SNP induced significantly weaker relaxations when compared with those obtained in PE-precontracted rings, with maximum relaxations reaching only 74.1 ± 8.7% and 65.7 ± 5.4%, respectively.

CRC to SNP in MPA and 1st branch rings precontracted with either KCl or PE are shown for comparison in Figs.32 and 33.

3.2.6. **Effect of L-NAME on SNP-induced relaxation**

In PE-precontracted MPA and 1st branch rings, pretreatment with L-NAME had no effect on SNP-induced relaxations except for the response to the lowest SNP concentration tested which was slightly enhanced (Figs.34 and 36). In contrast in KCl-precontracted MPA and 1st branch rings, L-NAME caused a significant enhancement of the relaxant responses to SNP increasing the maximum relaxations to 111.7 ± 3.5% and 95.6 ± 5.9%, respectively, which did not differ significantly from those obtained in rings precontracted with PE (Figs.35 and 37).

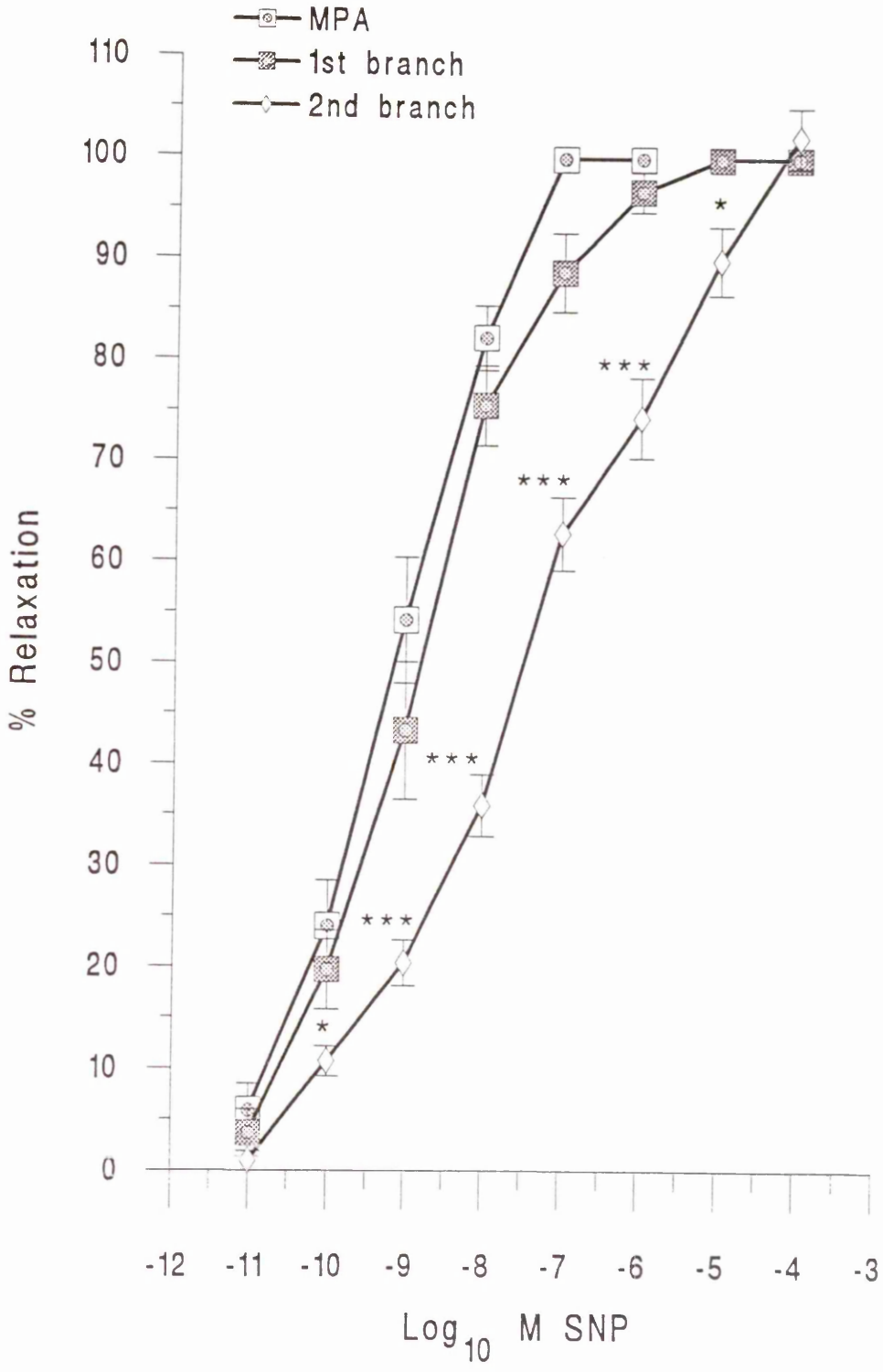


Fig. 31.

Cumulative concentration-response curves to SNP in MPA, 1st and 2nd branches precontracted with PE (EC_{75} 2.2×10^{-7} M for MPA, 9.5×10^{-8} M for 1st and 2nd branches). Each point is the mean ± s.e.mean of 6-12 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. Statistical comparison between the three arteries was performed using one way analysis of variance, followed by modified t-test for comparison between individual means. Asterisks denote significant difference as compared with MPA (*$P<0.05$, ***$P<0.001$).

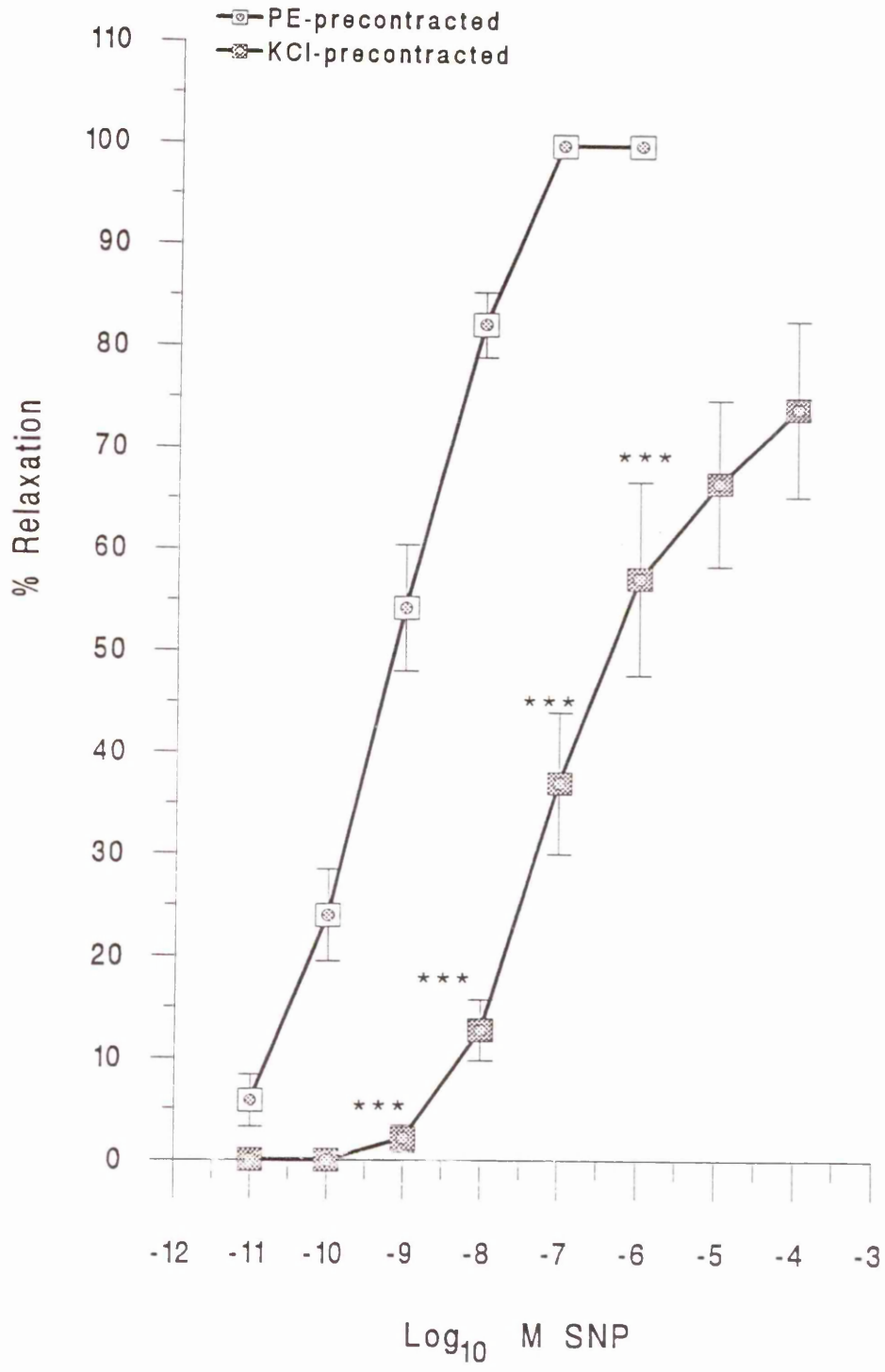


Fig. 32.

Comparison of SNP-induced relaxations in MPA precontracted with either PE (EC_{75} $2.2x10^{-7}$ M) or KCl (EC_{75} 27.5 mM). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE- or KCl-induced tone. SNP induced significantly weaker relaxations in KCl-precontracted rings than in PE-precontracted rings. Asterisks denote significant difference (***$P<0.001$; unpaired t-test).

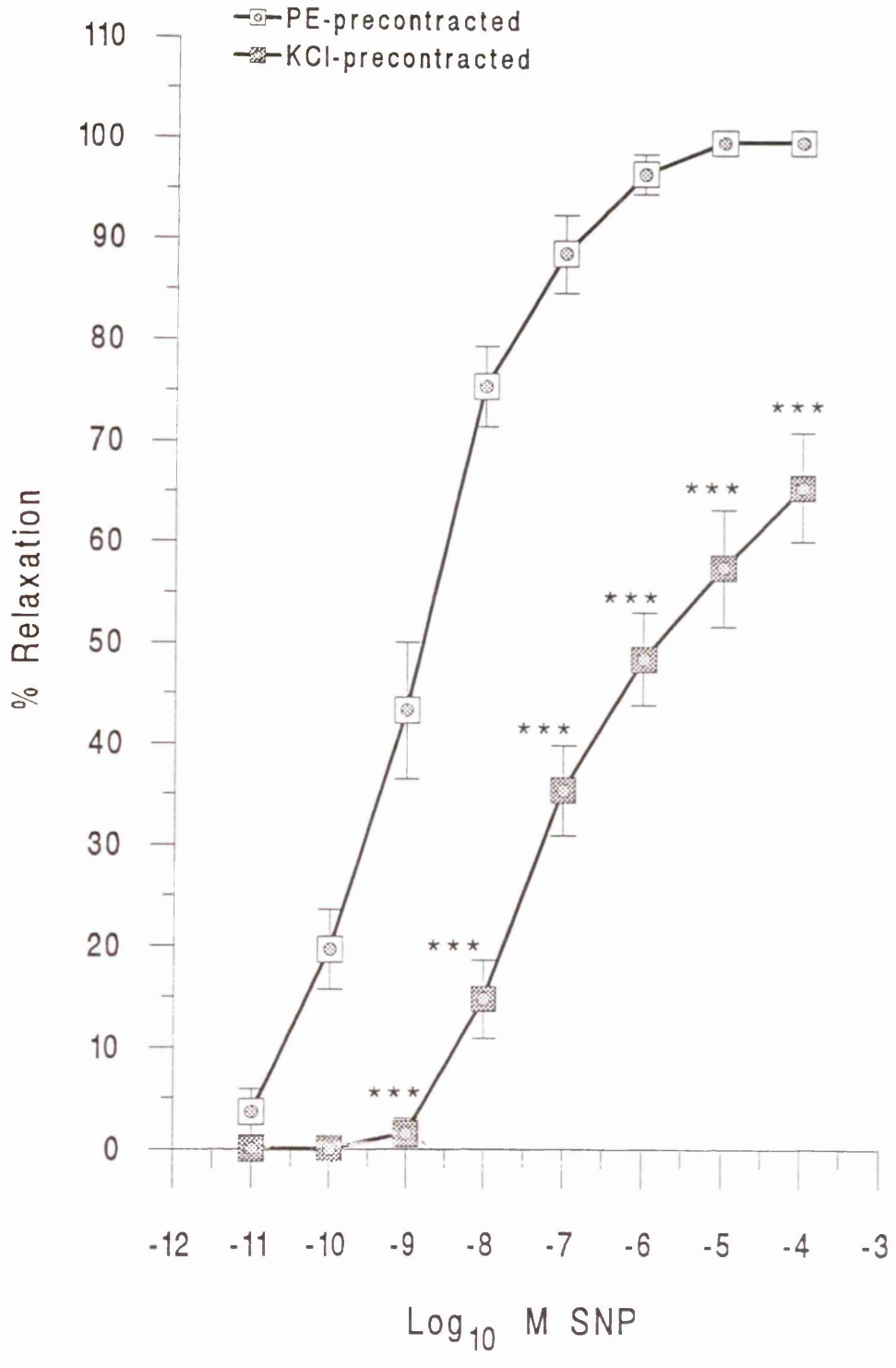


Fig. 33.

Comparison of SNP-induced relaxations in 1st branches precontracted with either PE (EC_{75} 9.5×10^{-8} M) or KCl (EC_{75} 25.1 mM). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE- or KCl-induced tone. SNP induced significantly weaker relaxations in KCl-precontracted rings than in PE-precontracted rings. Asterisks denote significant difference (***$P < 0.001$; unpaired t-test).

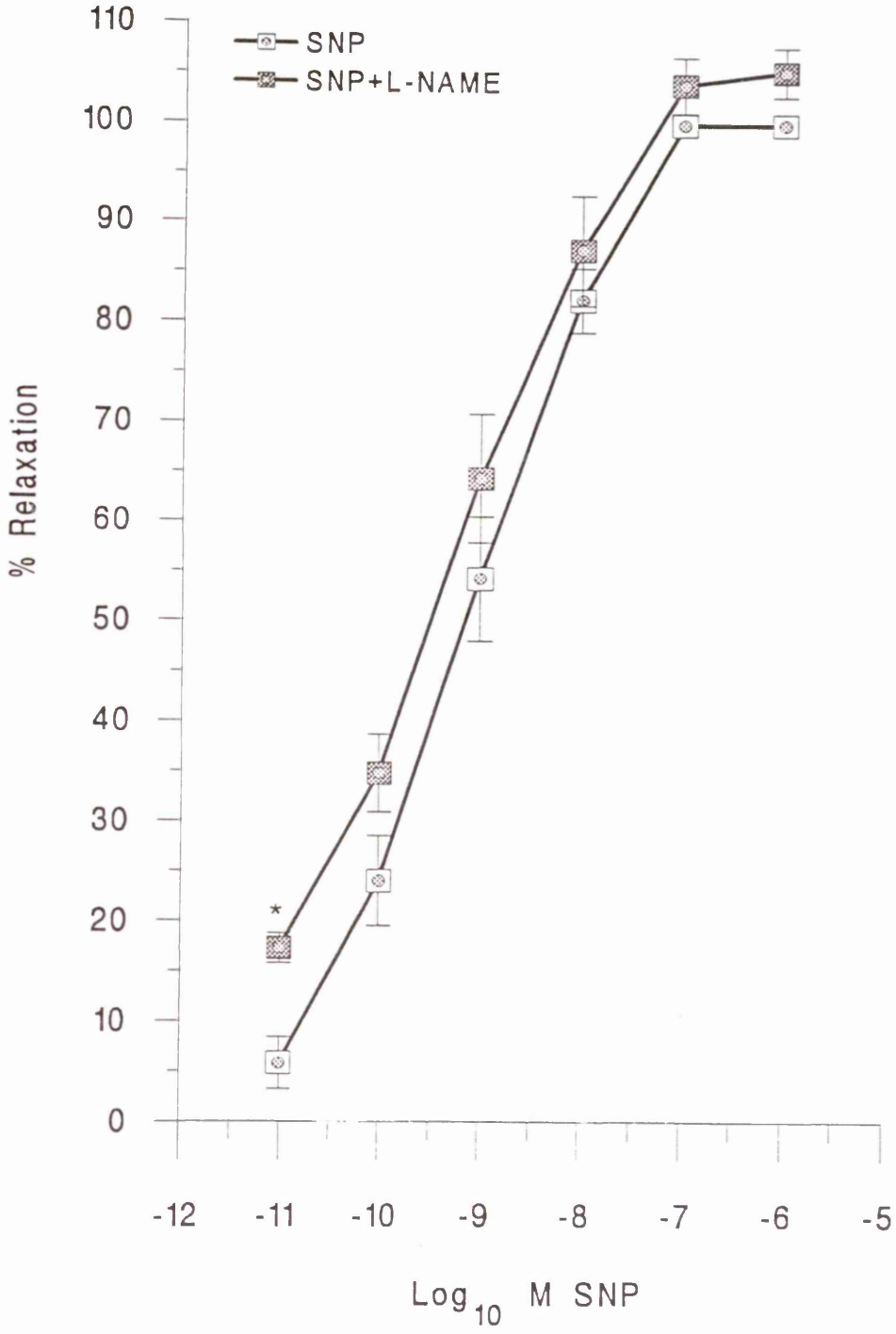


Fig. 34.

Cumulative concentration-response curves to SNP in MPA precontracted with PE (2.2×10^{-7} M or 2.2×10^{-8} M), in the absence and presence of L-NAME (2×10^{-4} M). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. Pretreatment with L-NAME did not significantly alter relaxant responses to SNP except to the lowest concentration which was slightly enhanced ($^{*}P < 0.05$, paired t-test).

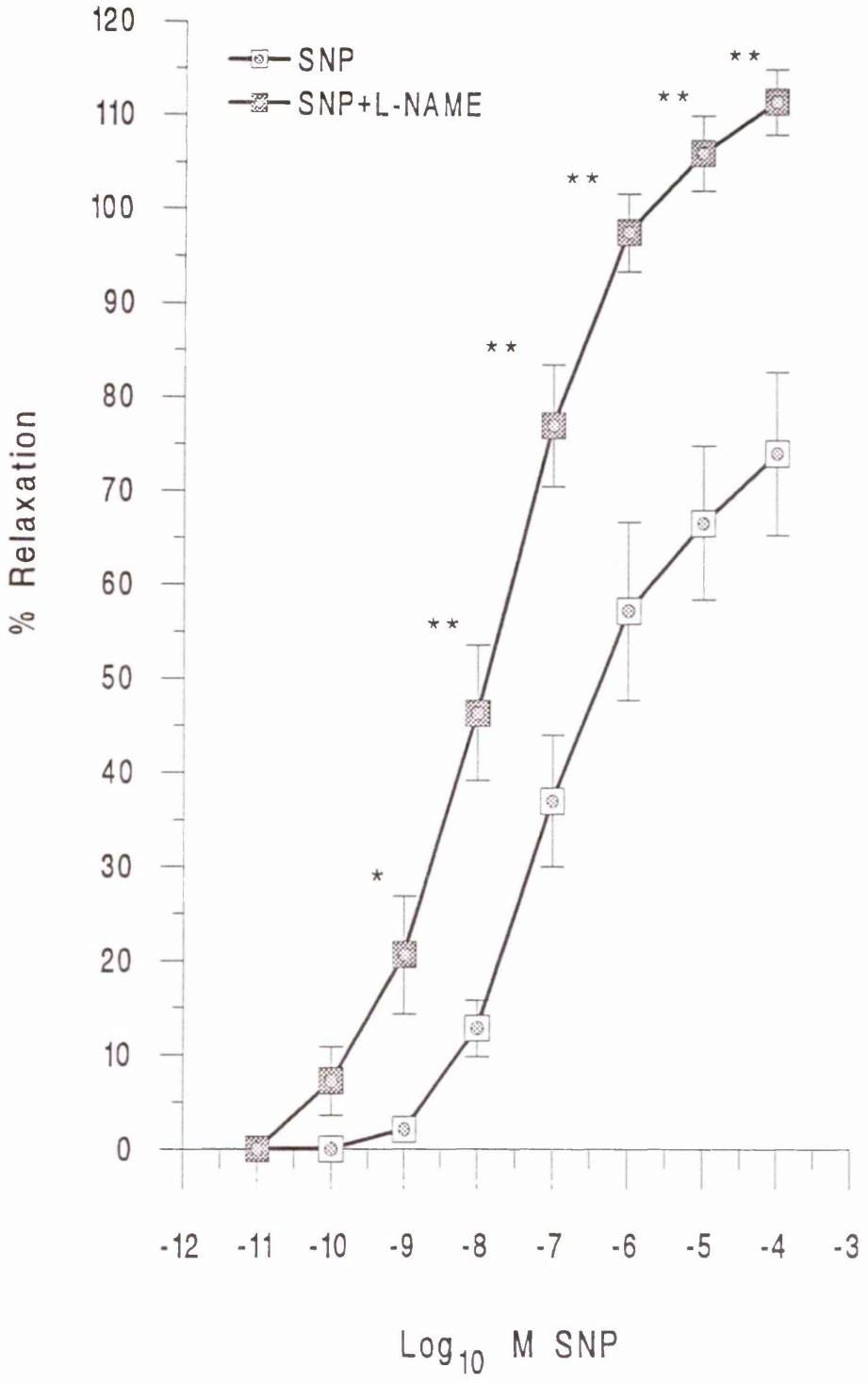


Fig. 35.

Cumulative concentration-response curves to SNP in MPA precontracted with KCl (27.7 mM or 17.5 mM), in the absence and presence of L-NAME (2×10^{-4} M). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of KCl-induced tone. Pretreatment with L-NAME significantly enhanced SNP-induced relaxations. Asterisks denote significant difference from untreated rings ($*P<0.05$, $**P<0.01$; paired t-test).

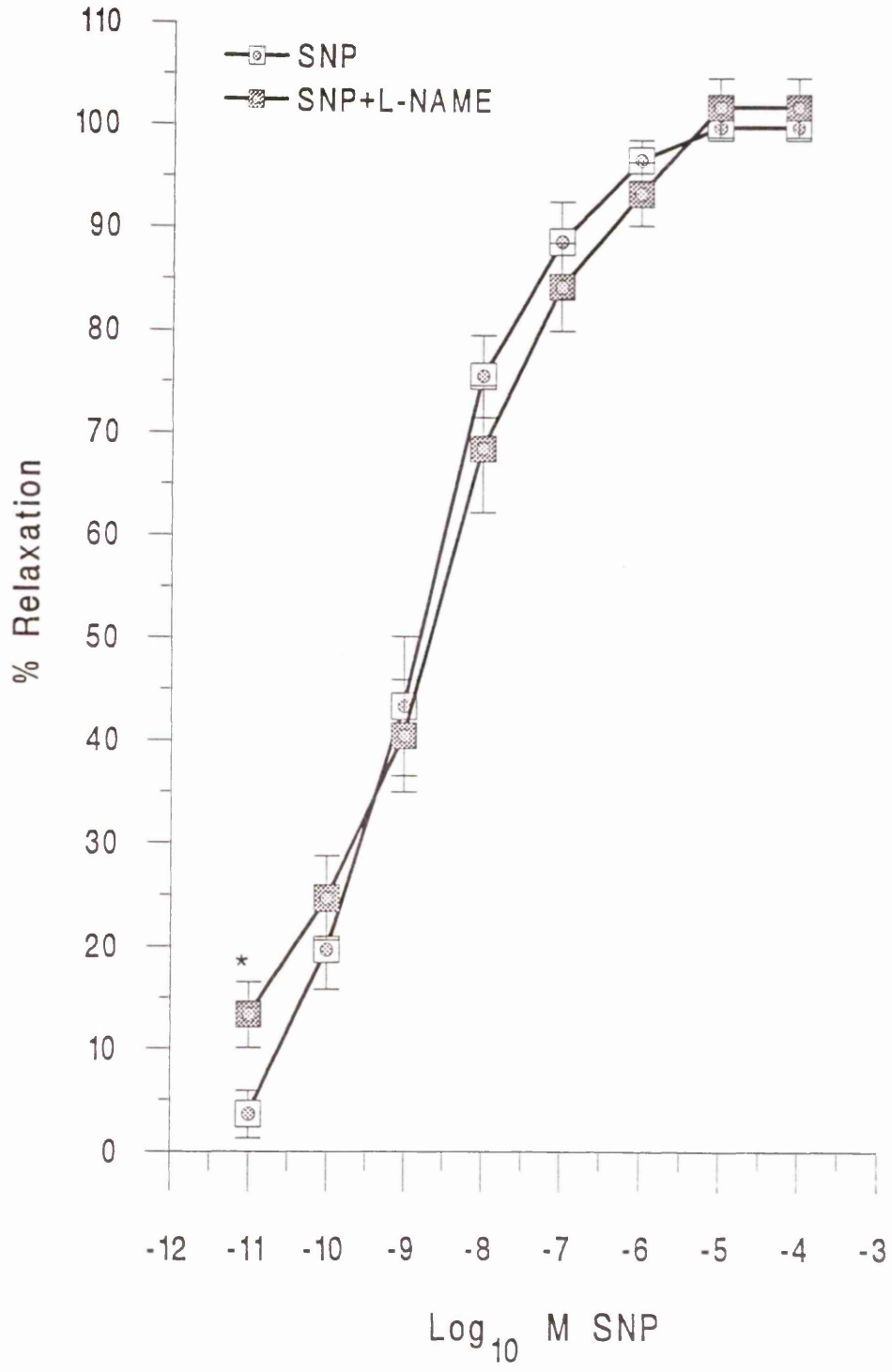


Fig. 36.

Cumulative concentration-response curves to SNP in 1st branches precontracted with PE (9.5×10^{-8} M or 9.5×10^{-9} M), in the absence and presence of L-NAME (2×10^{-4} M). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. Pretreatment with L-NAME did not significantly alter relaxant responses to SNP except to the lowest concentration which was slightly enhanced ($^{*}P < 0.05$, paired t-test).

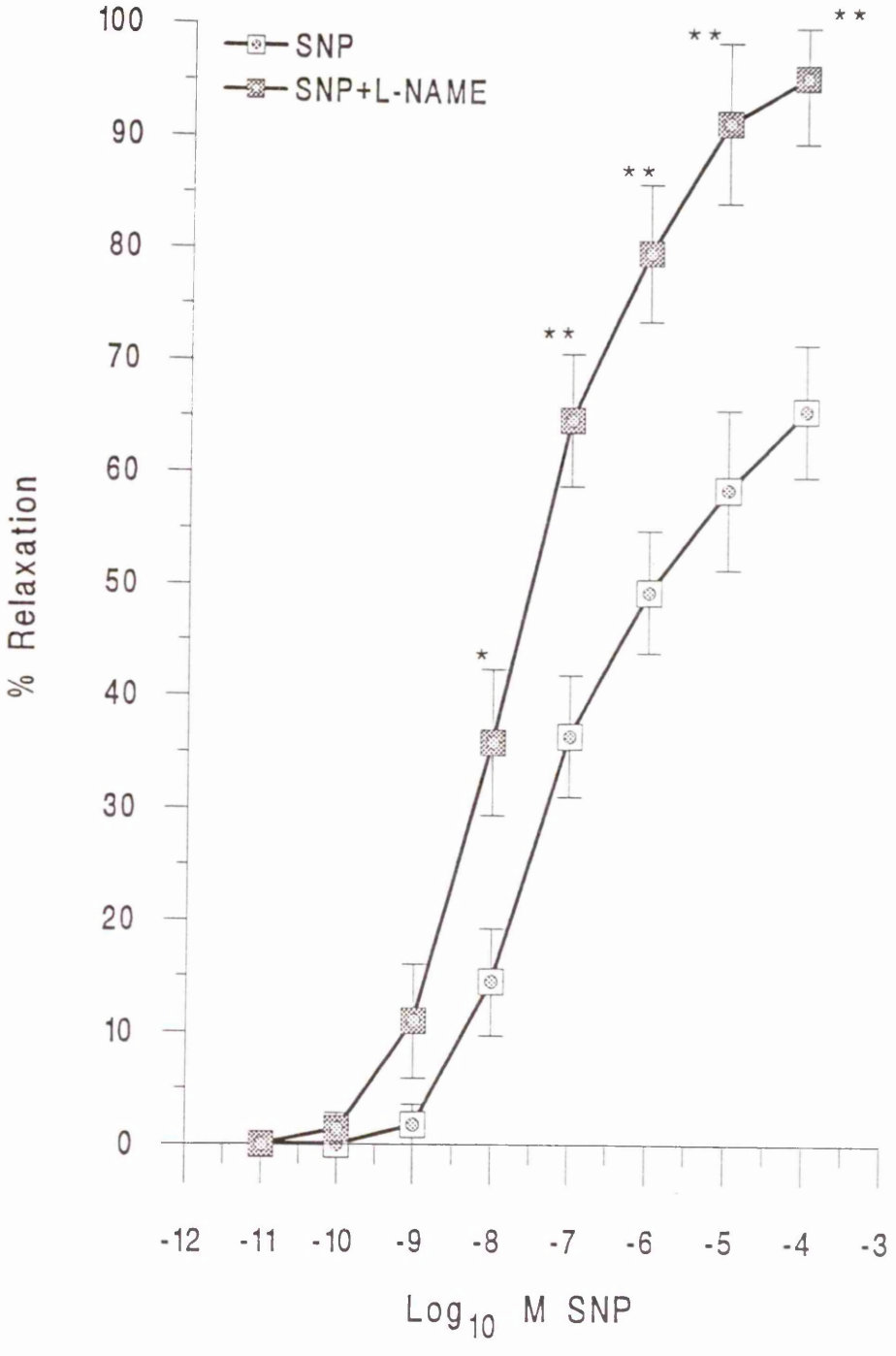


Fig. 37.

Cumulative concentration-response curves to SNP in 1st branches precontracted with KCl (25.1 mM or 15.1 mM), in the absence and presence of L-NAME ($2x10^{-4}$ M). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of KCl-induced tone. Pretreatment with L-NAME significantly enhanced SNP-induced relaxations. Asterisks denote significant difference from untreated rings (*$P<0.05$, **$P<0.01$, paired t-test).

3.2.7. Role of endothelium in mediating ISO-induced relaxation

β-Adrenoceptor agonists have been widely believed to cause endothelium-independent vasorelaxation via increasing cAMP production in the smooth muscle. However recently, there have been conflicting reports regarding the contribution of the synthesis of NO to β-adrenoceptor mediated vasorelaxation (Jackson & Busse, 1991 ; Gray & Marshall, 1992). Since a high density of β-adrenoceptors have been detected in bovine pulmonary endothelial cells (Ahmed et al., 1990) and endothelial cells are in direct contact with blood-borne catecholamines, it is highly possible that catecholamines could contribute to the regulation of pulmonary vascular tone through β-adrenoceptor-stimulated NO release.

3.2.7.1 Effect of ISO and BRL 37344 in endothelium-intact rings in the absence and presence of flurbiprofen, L-NAME or L-NAME + L-arginine

In endothelium-intact 2nd branch rings precontracted with PE, ISO induced concentration-dependent relaxations with a maximum relaxation of 102.4 ± 1.8% (Fig.38). Pretreatment with flurbiprofen had no effect on the relaxant responses to ISO resulting in a maximum relaxation of 101.7 ± 2.5%. In contrast, L-NAME caused a partial inhibition of ISO-induced relaxations with a reduction in the maximum response to 60.0 ± 6.4%. The inhibitory effect of L-NAME was completely prevented by prior incubation of rings with L-arginine (Fig.38). Furthermore, when L-NAME was added during

maximum relaxation to ISO, a partial reversal of the relaxation (45.7 ± 4.0%) was observed, and addition of L-arginine markedly reversed the effect of L-NAME (Figs.39 and 43).

The β_3-agonist BRL 37344, at all concentrations tested, failed to produce any degree of relaxation in PE-precontracted 2nd branch rings (Fig.40).

3.2.7.2. Effect of ISO in endothelium-denuded rings, in the absence and presence of L-NAME

To further investigate the role of endothelium in mediating ISO-induced relaxations, CRC to ISO were obtained in 2nd branch rings which had been perfused with Triton X-100. This procedure resulted in successful removal of the endothelium as indicated by absence of relaxant responses to a maximum concentration of CARB, without causing major damage to the underlying smooth muscle as indicated by normal relaxant responses to SNP and contractile responses to PE. Since endothelium-denudation potentiates PE-induced contraction, a lower concentration of PE (9.5×10^{-9} M) was used to obtain a similar degree of contraction to that obtained in endothelium-intact rings.

In endothelium-denuded rings, ISO induced concentration-dependent relaxations comparable to those produced in endothelium-intact rings, with a maximum relaxation of 100.4 ± 1.4% (Fig.41). In contrast to endothelium-intact rings, pretreatment of endothelium-denuded rings with L-NAME had no effect on ISO-induced relaxations (Fig.41), and when L-NAME was added during maximum relaxation to ISO, no reversal of the relaxation was observed (Figs.42 and 43).

3.2.7.3. **Influence of ISO on cGMP level**

The level of cGMP in endothelium-intact 2nd branch rings was increased by ISO from 1.84 ± 0.14 pmol.mg^{-1} tissue to 4.48 ± 0.87 pmol.mg^{-1} tissue (Fig.44), which is 2.4 times higher than the basal level.

3.2.7.4. **Effect of β-antagonists on ISO-induced relaxation**

In an attempt to classify the β-adrenoceptor subtypes mediating ISO-induced relaxation, CRC to ISO were constructed in the absence and presence of the non-selective β-antagonist propranolol, the selective β_1-antagonist atenolol and the selective β_2-antagonist ICI 118551. In these experiments, extraneuronal uptake of ISO was blocked by prior incubation of rings with hydrocortisone ($3x10^{-5}$ M) for 30 min. This had no effect on the potency of ISO in either endothelium-intact or denuded rings.

In endothelium-intact rings, the submaximal responses to ISO were markedly shifted to the right by propranolol, and to a lesser extent by atenolol and ICI 118551 (Fig.45). The EC_{50} values for ISO alone and in the presence of either propranolol, atenolol or ICI 118551 were $3.2x10^{-8}$ M, $6.3x10^{-6}$ M, $2.5x10^{-7}$ M and $5.0x10^{-7}$ M, respectively. However, neither of the antagonists caused any shift in the maximum response.

A similar rightward shift of submaximal responses to ISO in the presence of propranolol was observed in endothelium-denuded rings (Fig.46).

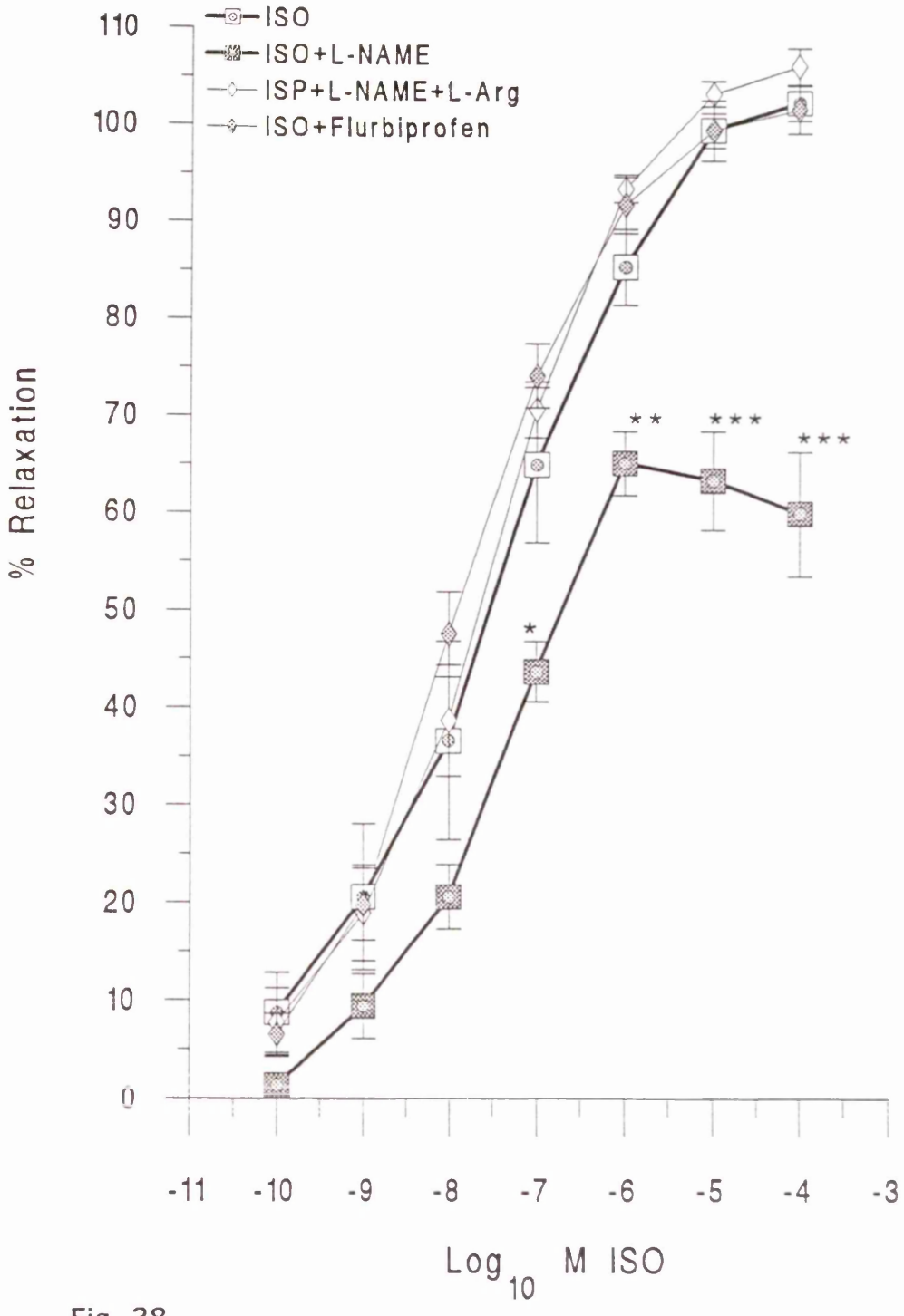


Fig. 38.

Cumulative concentration-response curves to ISO in endothelium-intact 2nd branches precontracted with PE (9.5×10^{-8} M or 9.5×10^{-9} M), in the absence and presence of flurbiprofen (10^{-5} M), L-NAME (2×10^{-4} M) or L-NAME + L-arginine (L-Arg, 10^{-2} M). Each point is the mean ± s.e.mean of 9 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. ISO-induced relaxations were not significantly altered by flurbiprofen, but partially inhibited by L-NAME. Pretreatment with L-arginine completely prevented the inhibitory effect of L-NAME. Asterisks denote significant difference from untreated rings ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; unpaired t-test).

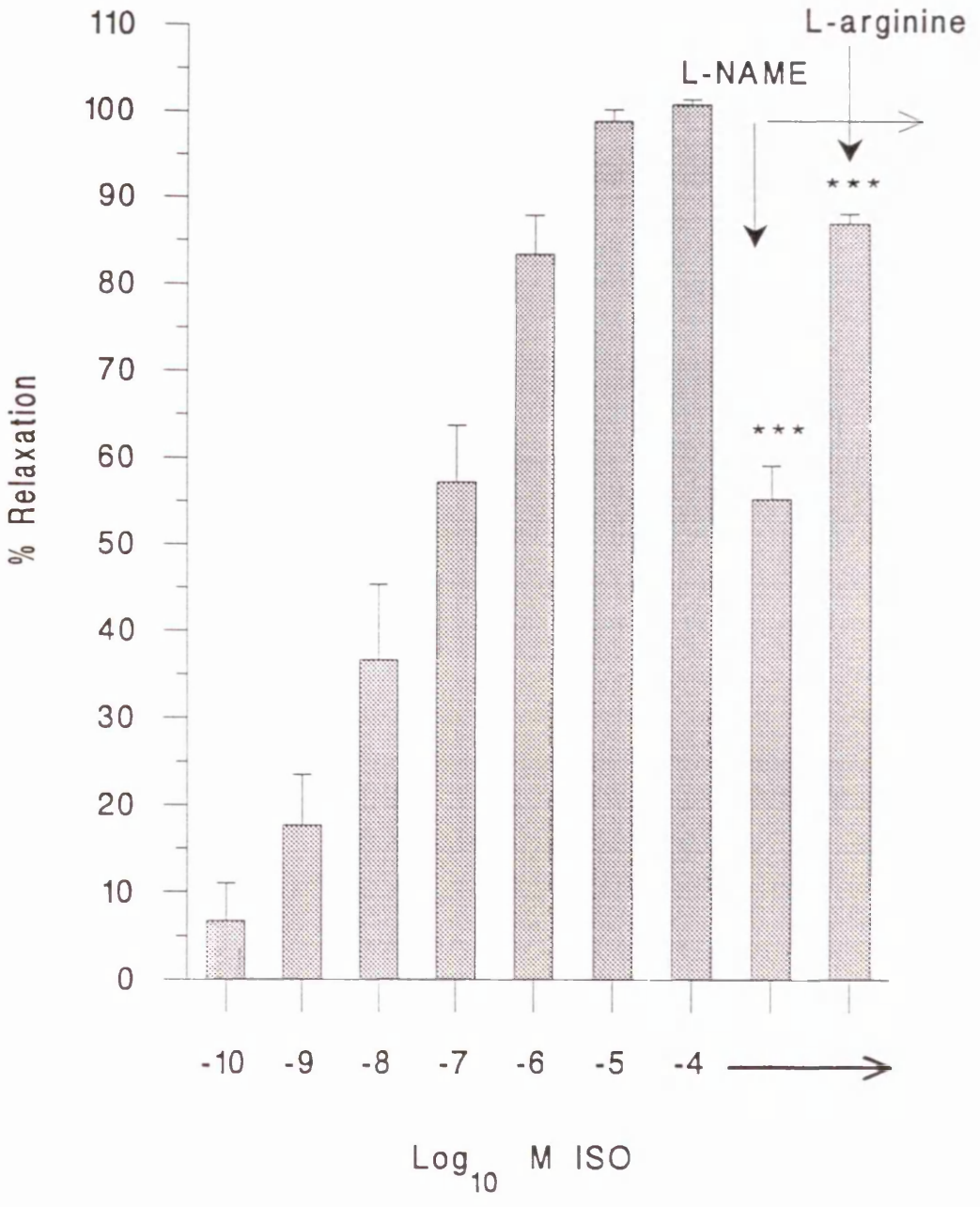


Fig. 39.

Histogram showing ISO-induced relaxations in endothelium-intact 2nd branches precontracted with PE (EC_{75} $9.5x10^{-8}$ M), and the effect of L-NAME and L-arginine added at the arrows. Each bar is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. L-NAME partially reversed ISO-induced relaxation, an effect which was markedly reversed by L-arginine. Asterisks denote significant difference as compared with the maximum relaxation to ISO (***$P<0.001$, paired t-test).

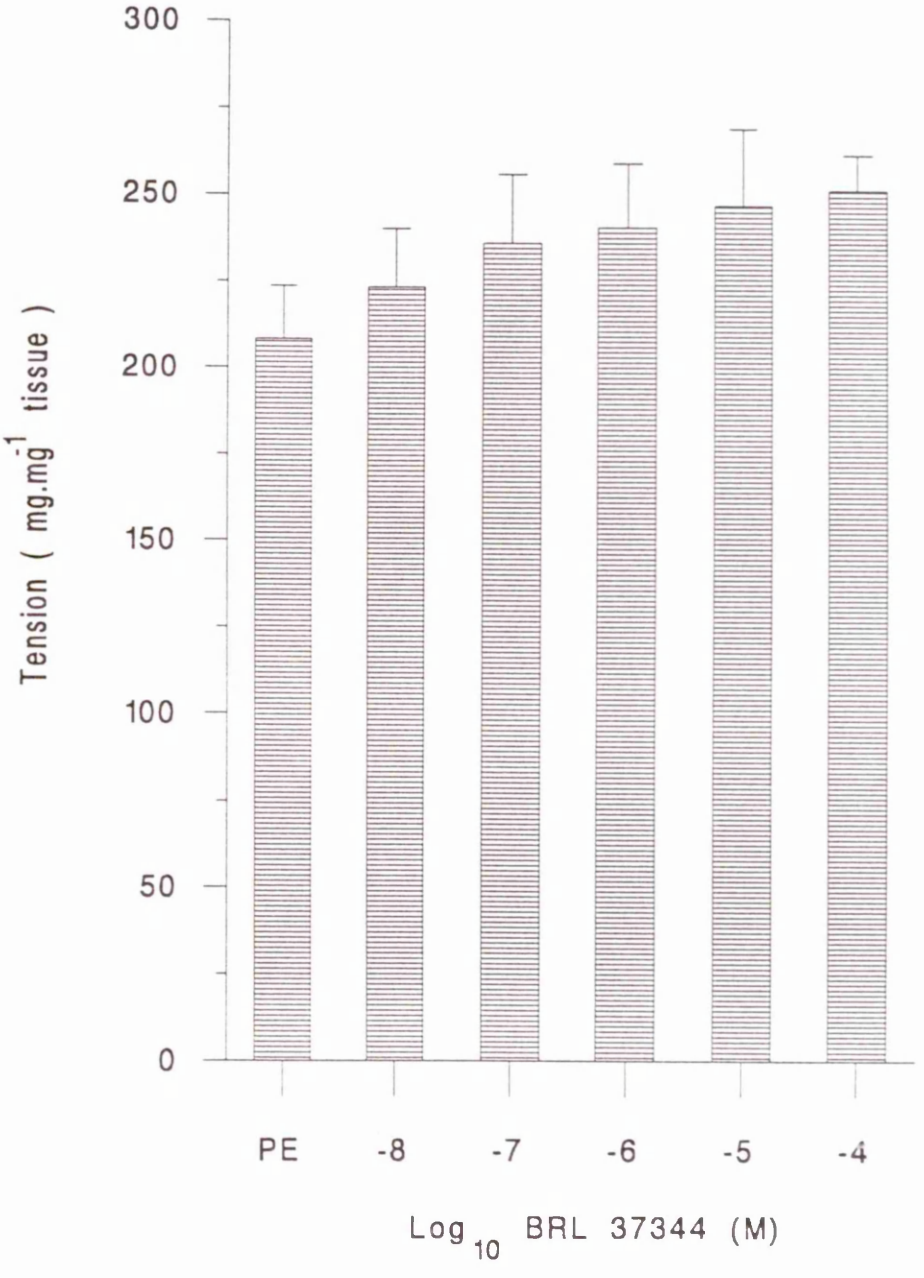


Fig. 40.

Histogram showing the lack of a relaxant effect to increasing concentrations of BRL 37344 in endothelium-intact 2nd branches in which tone had been raised with PE (EC_{75} $9.5x10^{-8}$ M). Each bar is the mean ± s.e.mean of 6 observations .

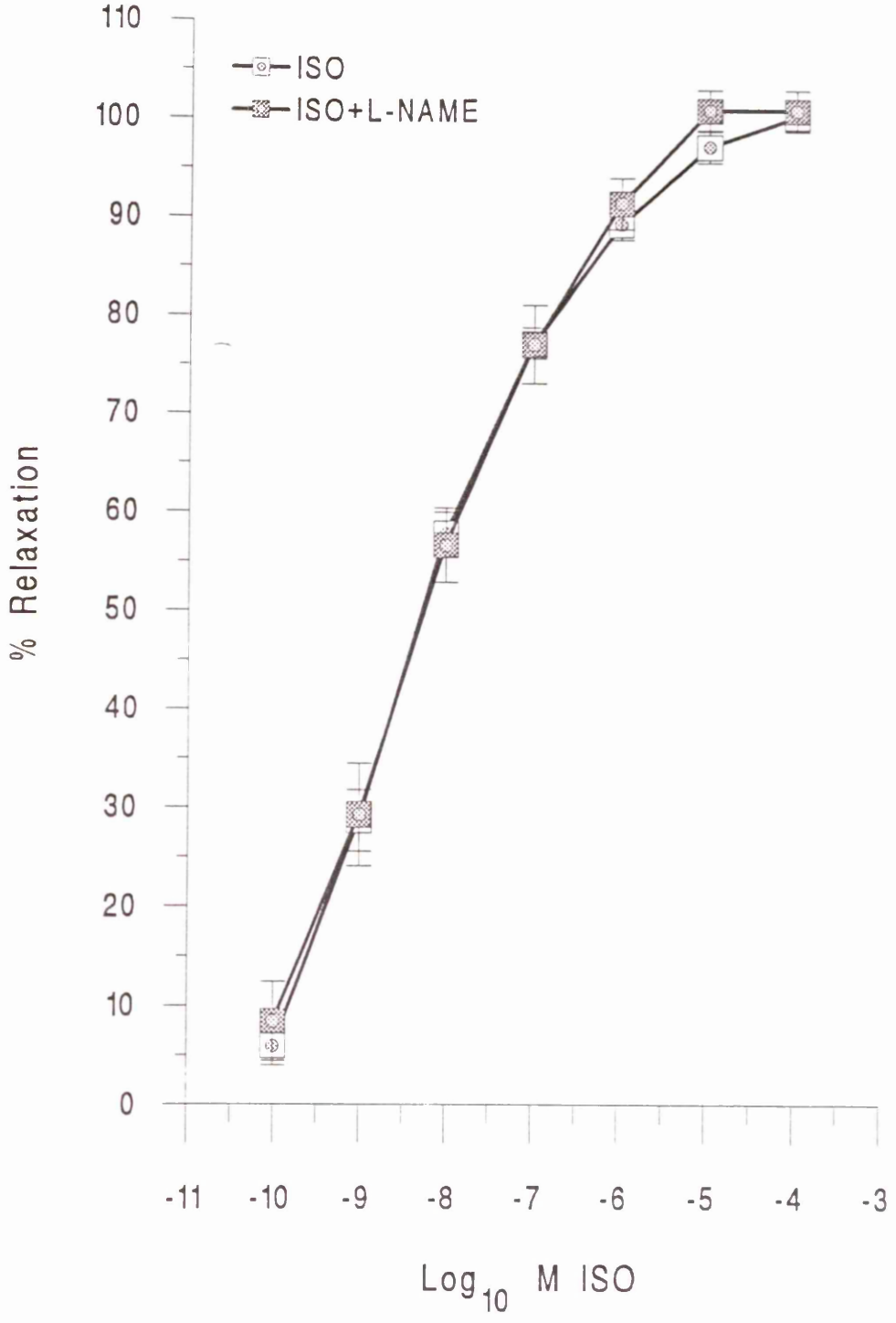


Fig. 41.

Cumulative concentration-response curves to ISO in endothelium-denuded 2nd branches precontracted with PE (9.5×10^{-9} M), in the absence and presence of L-NAME (2×10^{-4} M). Each point is the mean ± s.e.mean of 6 observations. Relaxant effects are expressed as % relaxation of PE-induced tone. ISO induced concentration-dependent relaxations comparable to those obtained in endothelium-intact rings. Pretreatment with L-NAME had no effect on ISO-induced relaxations.

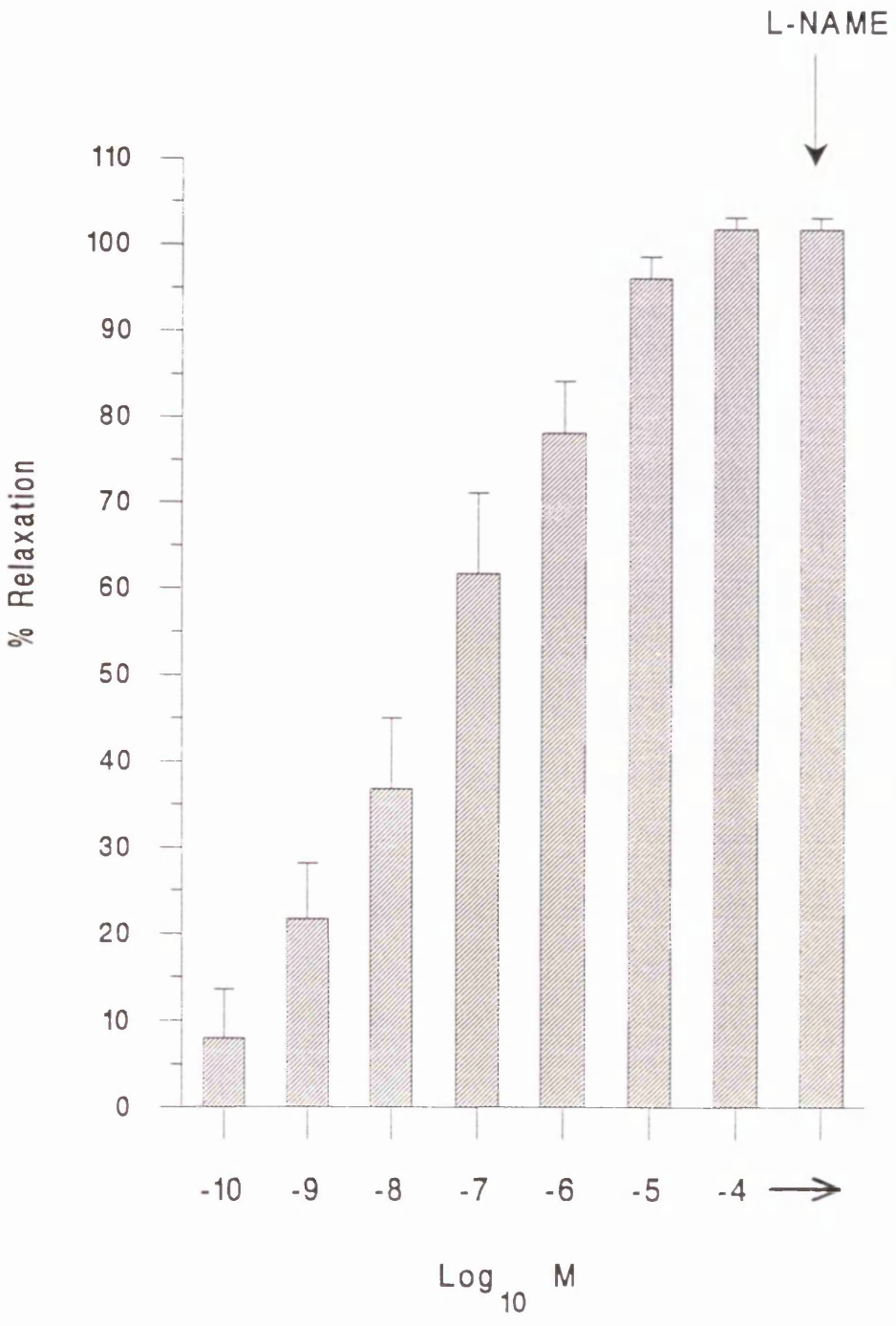
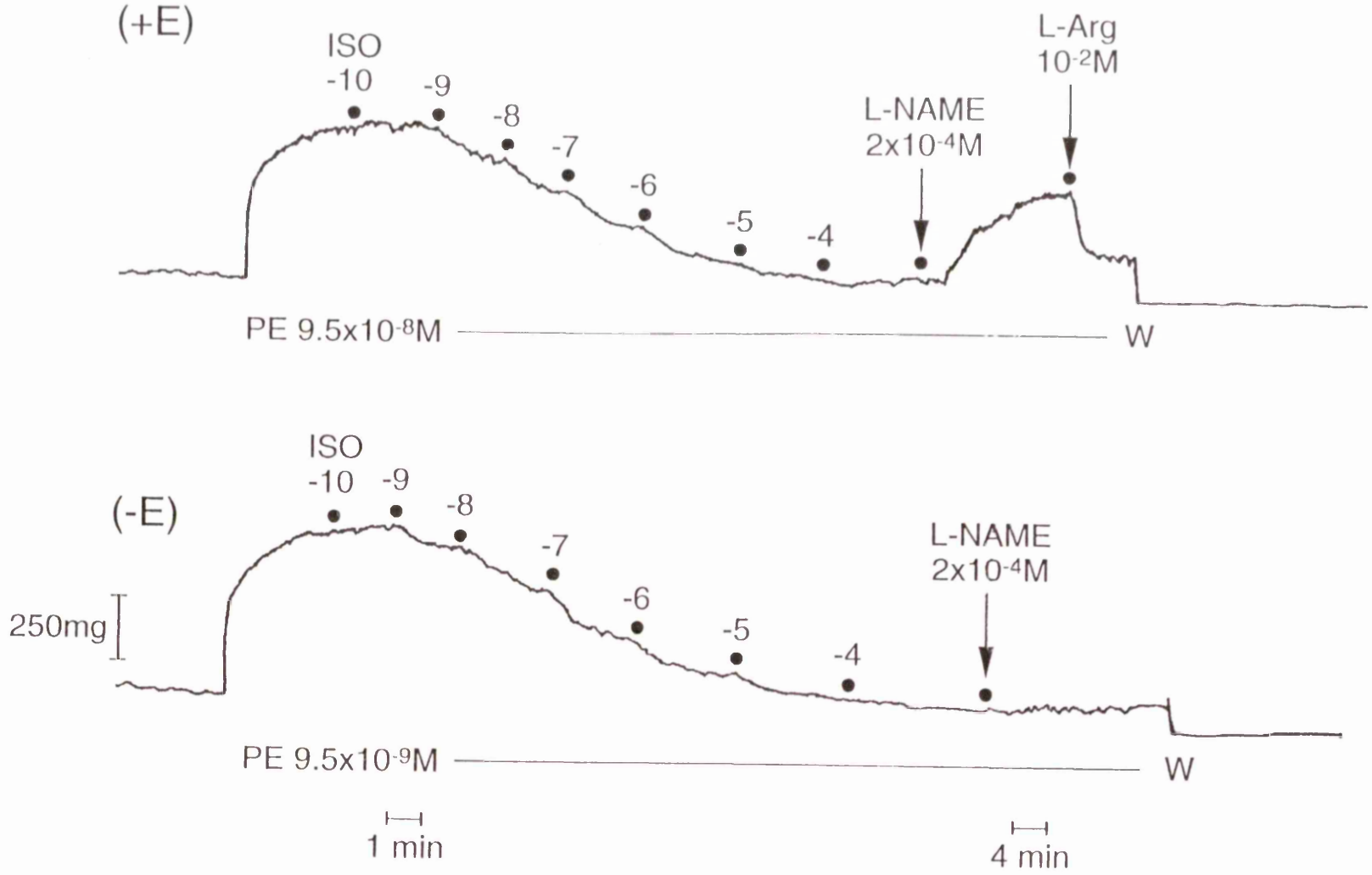


Fig. 42.

Histogram showing ISO-induced relaxations in endothelium-denuded 2nd branches precontracted with PE (9.5×10^{-9} M), and the lack of effect of L-NAME (2×10^{-4} M) when added at the arrow. Each bar is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE-induced tone.

Fig. 43.

Individual traces showing ISO-induced relaxation in endothelium-intact (+E) and endothelium-denuded (-E) 2nd branch rings in which tone had been raised with PE. L-NAME, added at the arrow, partially reversed the relaxant effect of ISO in endothelium-intact but not endothelium-denuded rings. Addition of L-arginine (L-Arg) markedly reversed the effect of L-NAME.


(+E)
ISO
-10
-9
-8
-7
-6
-5
-4
L-NAME
2x10-4M
L-Arg
10-2M
PE 9.5x10-8M
W
(-E)
ISO
-10
-9
-8
-7
-6
-5
-4
L-NAME
2x10-4M
250mg
PE 9.5x10-9M
W
1 min
4 min

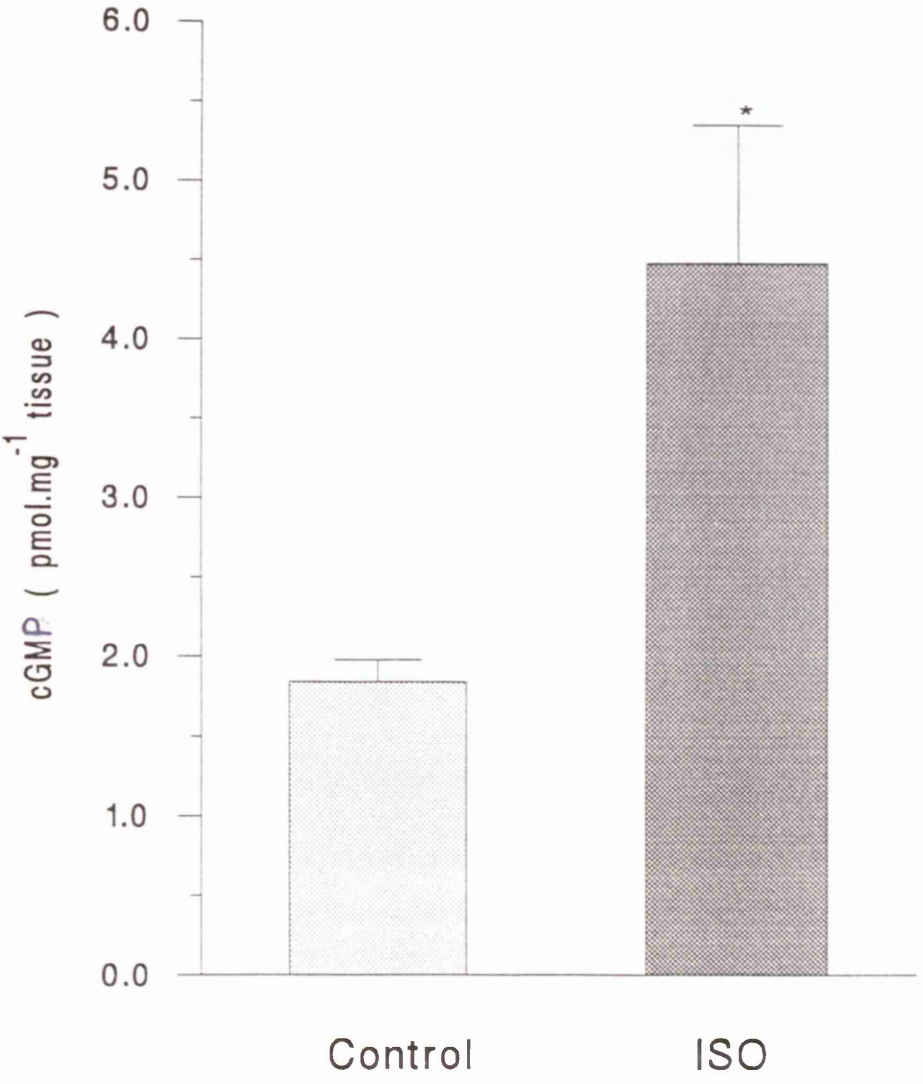


Fig. 44.

Effect of ISO (10^{-4} M) on cGMP level in endothelium-intact 2nd branches. Each bar is the mean ± s.e.mean of 6 measurements. Artery rings were precontracted with PE (EC_{75} 9.5×10^{-8} M) before being exposed to ISO for 30s. ISO significantly increased cGMP level as compared with the basal level (control) ($*P < 0.05$, unpaired t-test).

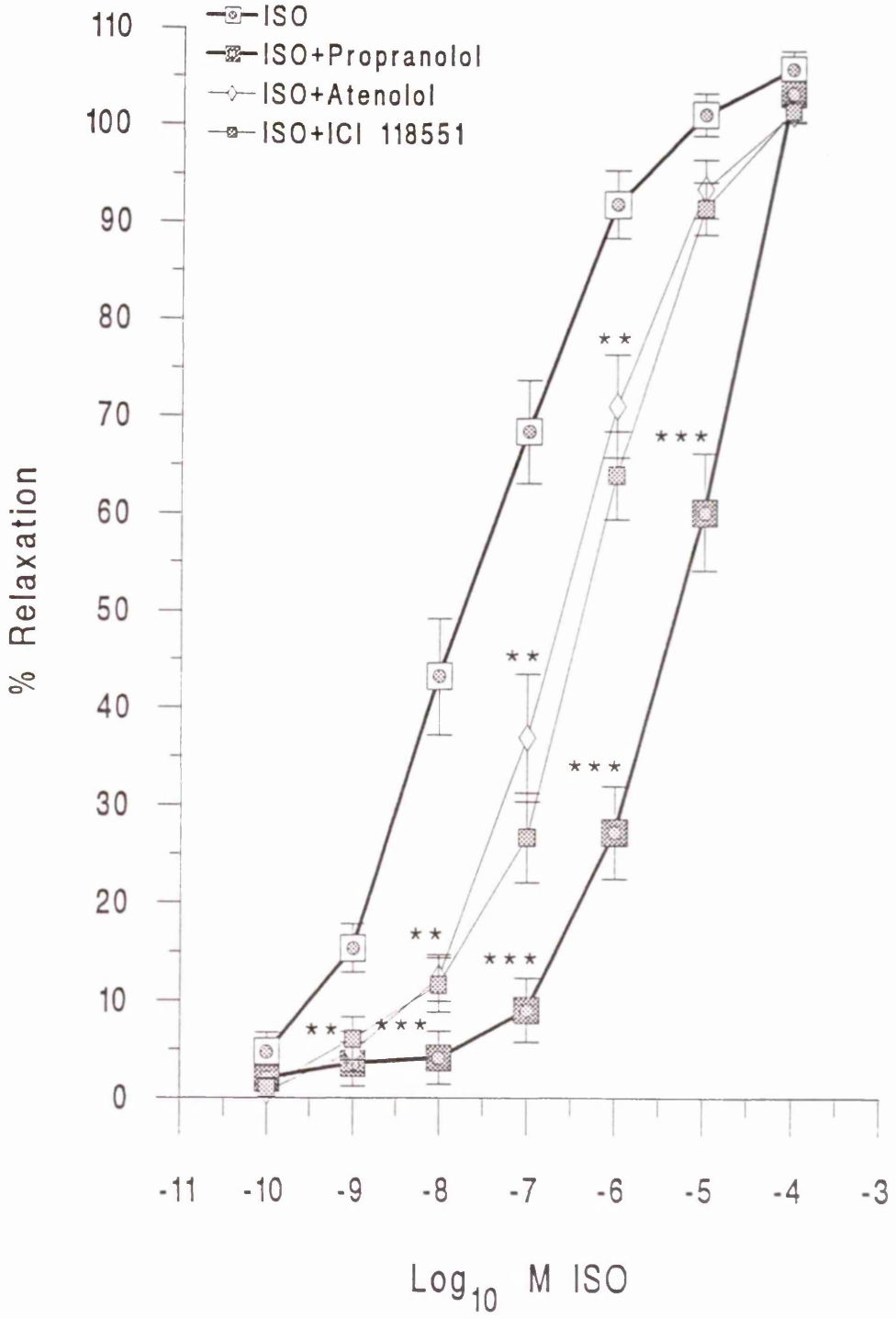


Fig. 45.

Cumulative concentration-response curves to ISO in endothelium-intact 2nd branches precontracted with PE (EC_{75} 9.5×10^{-8} M), in the absence and presence of propranolol (10^{-6} M), atenolol (10^{-5} M) or ICI 118551 (10^{-6} M). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. Asterisks denote significant difference from untreated rings ($^{**}P<0.01$, $^{***}P<0.001$; unpaired t-test). For clarity, statistical significance for ICI 118551 is not shown.

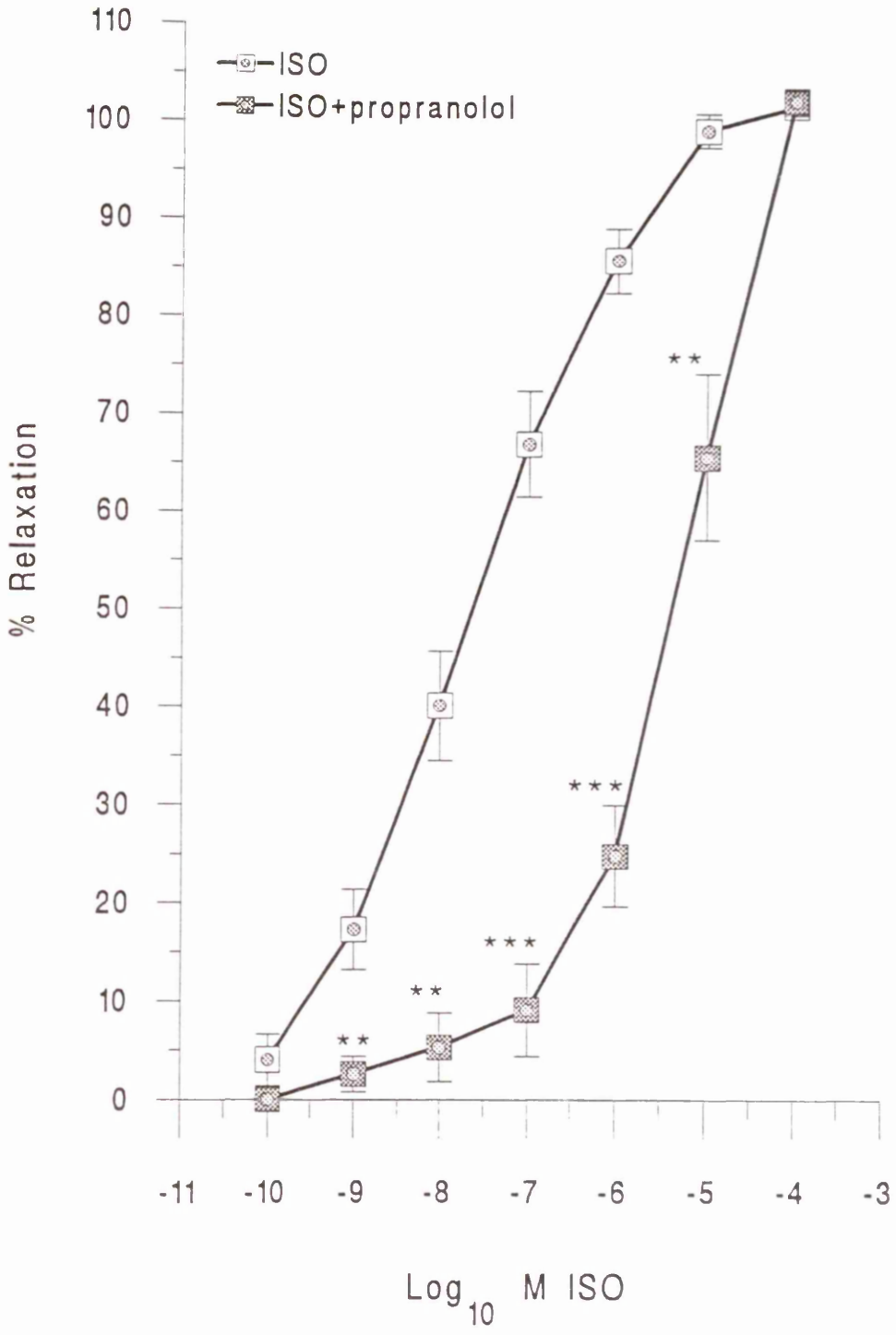


Fig. 46.

Cumulative concentration-response curves to ISO in endothelium-denuded 2nd branches precontracted with PE (9.5×10^{-9} M), in the absence and presence of propranolol (10^{-6} M). Each point is the mean ± s.e.mean of 6 observations. Relaxant responses are expressed as % relaxation of PE-induced tone. Pretreatment with propranolol caused a rightward shift of submaximal responses to ISO but had no effect on the maximum response. Asterisks denote significant difference from untreated rings (**$P<0.01$, ***$P<0.001$; paired t-test).

Chapter 4

Discussion

The pulmonary circulation is characterised by being a low pressure, low resistance system with a special capacity to accommodate large increases in blood flow with only a small rise in intravascular pressure. The mechanisms by which the pulmonary vasculature maintains a low vascular tone are still unclear. However, since the discovery of the release by endothelial cells of the two potent relaxing factors, NO and prostacyclin, they have become potential candidates for such a function. Although the role of the endothelium in modulating the systemic circulation has been extensively studied, less is known about its role in modulating pulmonary circulation. Previous studies that have addressed this subject have produced conflicting results. Whereas some studies have suggested an important role for NO or prostacyclin in maintaining the low pulmonary vascular tone (Voelkel *et al.*, 1981 ; Walker *et al.*, 1982 ; Archer *et al.*, 1990 ; Gold *et al.*, 1990), others have found no evidence for such a role (Ogletree, 1982 ; Rubin *et al.*, 1985 ; Archer *et al.*, 1989 ; Crawley *et al.*, 1990). Furthermore, most of the *in vitro* studies have used strips or rings of large conduit pulmonary arteries where the results, as demonstrated in the systemic circulation, may not be applicable to the smaller arteries which may account for the major part of the pulmonary vascular resistance. Therefore, the purpose of the present study was :

(i) To further investigate whether a basal release of NO and prostacyclin is involved in modulating pulmonary vascular tone.

(ii) To test the ability of various humoral agents to stimulate NO release in this vascular bed.

(iii) To determine the extent to which this basal and stimulated release correlates with artery size.

In this study, isolated rat pulmonary arteries were used. The normal structure and pattern of branching of rat pulmonary arteries have been described by Hislop and Reid (1978), and similarities between rat and human arteries assessed. In the rat, the main pulmonary artery (MPA) divides into left and right branches (1st branches). The left lung has a single lobe, the right lung has four. Each lobe has one axial artery, from which side branches arise at right angles. The wall of the rat pulmonary artery is similar to the human in possessing an adventitia, muscular media with circularly arranged muscle cells bounded with internal and external elastic laminae with central elastic laminae in between, and an intima consisting of a single layer of endothelial cells. Furthermore, the percentage wall thickness is similar in rat and human arteries. Unlike the human lung, there are no elastic arteries in the rat lung i.e. arteries with more than four central elastic laminae. The MPA has only four central laminae, thus falling within the definition of muscular arteries. As the diameter of arteries decreases, the central laminae decrease in number until they disappear, and the wall gradually becomes partially muscular or non-muscular in structure. The distribution of size range for muscular,

partially muscular and non-muscular arteries is similar in rat and human lungs. All arteries over 150 μm (stretched external diameter) are muscular in structure, and all arteries less than 50 μm non-muscular (Hislop & Reid, 1978). The many similarities between rat and human pulmonary arteries makes it appropriate to use the rat for the study of pulmonary vasculature.

The first series of experiments compared the contractility of large and small pulmonary arteries by investigating the responsiveness of MPA, 1st and 2nd branches to vasoconstrictors acting by different mechanisms - namely PE, 5-HT and KCl. Activation of α_1-adrenoceptors by PE and of 5-HT_2-receptors by 5-HT stimulates the hydrolysis of membrane phosphoinositides (de Chaffoy de Courcelles *et al.*, 1985 ; Minneman, 1988) resulting in the production of DAG and IP_3 which, through the mechanisms described in section 1.1.2., cause smooth muscle contraction. On the other hand, KCl induces smooth muscle contraction through membrane depolarisation and opening of voltage-operated Ca^{2+} channels (Bolton, 1979). Comparison of the concentration-response curves to either PE, 5-HT or KCl in MPA, 1st and 2nd branches revealed that generally 2nd branches displayed a significantly greater contractility and higher sensitivity to the vasoconstrictors than 1st branches, which showed higher contractility and sensitivity than MPA. These results are at variance with those reported by Leach *et al.* (1992),

who found that noradrenaline and 5-HT were more powerful vasoconstrictors in large than in small pulmonary arteries. The discrepancies in the results could be due to differences in the size of arteries used (100-2000 μm) and in the expression of developed tension (force per unit vessel length). The greater responsiveness of the smaller arteries to the vasoconstrictors could be accounted for by a greater smooth muscle content, a higher affinity and/or density of α_1-adrenoceptors and 5-HT_2-receptors, and a more positive smooth muscle cell membrane potential. However, it has been shown that smooth muscle cells in large and small pulmonary arteries show similar resting membrane potentials and a similar degree of membrane depolarisation in response to increased extracellular [K^+] (Suzuki & Twarog, 1982). This finding together with the fact that the difference in the responsiveness of the three arteries was not restricted to receptor-mediated contractions but extended to depolarisation-mediated contractions suggest that the greater contractility of the smaller arteries is mainly due to their higher smooth muscle content.

Pretreatment with the NOS inhibitor L-NAME significantly potentiated the contractile responses to PE, 5-HT and KCl in all of the three arteries. The potentiating effect was not specific to L-NAME as L-NOARG also caused a similar potentiation of contractile responses to PE in 2nd branches. Prior incubation with L-arginine completely prevented the ability of

L-NAME and L-NOARG to potentiate PE-induced contractions in 2nd branches. Since addition of L-arginine generally prevents the actions of NOS inhibitors by competing for the enzyme, this result confirms that the potentiating effects of L-NAME and L-NOARG were due to inhibition of NO synthesis.

Having confirmed that NO exerts a depressant effect on the contractile responses of pulmonary arteries to vasoconstrictors, the question remained whether the NO was released spontaneously or as a consequence of stimulation by the vasoconstrictors. If the latter assumption is true, the vasoconstrictors would be expected to induce endothelium-dependent relaxations in these arteries. Since α_2-agonists have been shown to induce endothelium-dependent relaxation in various blood vessels including canine pulmonary arteries (Miller & Vanhoutte, 1985), it could be inferred that the NO, which mediated the depression of PE-induced contractions, was released in response to stimulation of endothelial α_2-adrenoceptors by PE. However, we failed to detect any relaxant effect to the α_2-agonist clonidine in 2nd branches precontracted with the thromboxane A_2 mimetic U 46619, even in the presence of the α_1-blocker prazosin to suppress any possible α_1-mediated contractile effect of clonidine. This result contrasts with that of Miller and Vanhoutte (1985) who reported that the selective α_2-agonist UK 14,304 produced a relaxant effect in canine pulmonary arteries precontracted with PE.

However recently, α_2-agonists have been shown to competitively antagonise α_1 activation by α_1-agonists (Skrbic & Chiba, 1993) and, therefore, the reported inhibition of PE-induced tone by UK 14,304 could merely be a manifestation of its antagonistic action at α_1-receptors. Further evidence against PE being able to stimulate NO release was obtained from experiments involving measurements of cGMP. NO causes vasorelaxation via stimulation of intracellular cGMP production, and thus cGMP level has been widely used as an indication of NO release. Exposure of 2nd branch rings to PE did not cause any significant change in basal cGMP level.

Endothelium-dependent relaxations to 5-HT in various vascular beds including porcine pulmonary arteries have also been reported (Glusa & Richter, 1993). However, we found no evidence that 5-HT stimulates NO release in rat pulmonary arteries. In 2nd branches precontracted with U 46619, 5-HT did not evoke any relaxation even when the contractile effect of 5-HT was suppressed by the 5-HT_2-antagonist ketanserin. Furthermore, 5-HT failed to cause any significant rise in cGMP level.

Elevations in extracellular K^+ concentration have previously been shown to indirectly stimulate NO release in canine femoral arteries (Rubanyi & Vanhoutte, 1988), and to cause relaxation associated with a rise in cGMP level in duodenal smooth muscle (Toda *et al.*, 1992). In this

study, we were unable to demonstrate any relaxant effect to KCl in 2nd branches precontracted with PE. It is possible, however, that any relaxant effect of KCl was simply masked by the accompanying contractile effect. The effect of KCl on cGMP level was variable. KCl caused a significant rise in cGMP level in 2 out of 6 rings , with no effect in the 4 remaining rings. These results do not rule out a stimulant effect of KCl on NO release.

In general, these results support the view that the potentiating effects of L-NAME and L-NOARG were due to the loss of modulation of contractile responses by basally-released NO, rather than NO released by vasoconstrictor stimulation. The source of NO is most likely the endothelium, but a contribution of NO released from smooth muscle cells or neuronal cells cannot be ruled out.

This conclusion is further supported by several observations. The potentiating effects of L-NAME and L-NOARG were more profound at low concentrations of the vasoconstrictors, with no significant change in the maximum responses. This is consistent with a basal release of a stable amount of NO which would be more effective in counteracting contractile responses to low rather than high concentrations of vasoconstrictors. Moreover, the modulatory effect of NO was a general phenomenon, occurring with receptor-mediated contractions as well as

depolarisation-mediated contractions. Although L-NAME and L-NOARG, per se, did not cause a rise in baseline tone in any of the three arteries, this does not refute the presence of a basal release of NO. When arteries possess intrinsic tone, this provides an appropriate background for NO to exert its relaxant effect. Inhibition of NO synthesis removes this effect and leads to an increase in basal tone. Since, in this study, the maximum relaxations induced by various vasorelaxants in MPA, 1st and 2nd branches were not significantly greater than 100%, this was considered as an indication of absence of an intrinsic tone in these arteries.

The presence of a continuous basal release of NO from pulmonary endothelium would serve to depress the contractility of pulmonary arteries to the various vasoconstrictors that are either released locally or reach the pulmonary vasculature via venous blood. Therefore, NO may play a major role in maintaining the normally low pulmonary vascular tone.

The potentiating effect of L-NAME on the contractile responses to PE, 5-HT and KCl was more pronounced as artery size decreased. This could be accounted for by :

(i) Higher basal production of NO in the smaller arteries.

(ii) Higher sensitivity of soluble guanylate cyclase in the smaller arteries.

(iii) Assuming similar amounts of NO released in the three arteries, the smooth muscle cells in the smaller arteries may acquire higher concentrations of NO since the thinner arterial wall would constitute less of a diffusion barrier for NO than in larger arteries.

The possibility of a higher guanylate cyclase sensitivity in the smaller arteries is excluded by the observation that the endothelium-independent vasorelaxant SNP, which acts by spontaneously releasing NO in aqueous solutions, produced similar maximum relaxant effects in MPA, 1st and 2nd branches. Moreover, relaxant responses to submaximal concentrations of SNP in 2nd branches were weaker than in MPA and 1st branches. It is tempting to speculate that this was due to desensitisation of guanylate cyclase as a result of a higher basal release of NO in 2nd branches. The third suggestion is equally unlikely, since MPA was more sensitive to the relaxant effect of low concentrations of the endothelium-dependent vasorelaxant CARB than were 1st and 2nd branches, indicating that wall thickness does not prevent NO from reaching the underlying smooth muscle layer. The most likely cause for the greater potentiating effect of L-NAME in the smaller arteries, therefore, is a higher basal production of NO in these arteries.

This conclusion is further supported by the finding that the small arteries had higher basal cGMP levels than larger arteries. This result is in

agreement with a previous study on bovine pulmonary arteries of different sizes, in which higher cGMP levels were found in smaller branches (Ignarro *et al.*, 1987). It would seem reasonable that NO release should become greater as arteries become smaller and more muscular, to counteract the accompanying increase in contractility and, therefore, maintain a low vascular tone.

Pulmonary endothelium can also modulate pulmonary vascular tone via the release of vasodilator prostanoids, especially prostacyclin. To test this possibility, we examined the effect of inhibition of prostanoid synthesis on contractile responsiveness of pulmonary arteries to vasoconstrictors, and found that pretreatment of 2nd branch rings with the cyclo-oxygenase inhibitor flurbiprofen had no significant effect on contractile responses to PE. From this result we can conclude that prostacyclin is not released spontaneously from the pulmonary endothelium and, therefore, does not contribute significantly to the maintenance of the low pulmonary vascular tone.

Having established that NO is basally-released in pulmonary arteries, and that this release correlates inversely with artery size, the next step was to investigate agonist-stimulation of NO release in the three arteries. The endothelium-dependent vasorelaxant CARB induced concentration-dependent relaxations in MPA, 1st and 2nd branches precontracted with

PE. However, 2nd branches were more responsive to the relaxant effect of CARB than 1st branches, which were more responsive than MPA. Similar results have been reported by Owen and Bevan (1985) who found that isolated resistance vessels of the rabbit ear relax by proportionally greater amount to acetylcholine than the central artery. Since we have already excluded the possibility of a higher guanylate cyclase sensitivity in the smaller arteries and established that wall thickness does not act as a physical barrier for NO diffusion, the difference between the three arteries most likely reflects the capacity of the small arteries to generate greater amounts of NO in response to the stimulatory effect of CARB than larger arteries. In line with this conclusion is the observation that high concentrations of CARB, while causing further relaxations in 1st and 2nd branches, tended to evoke contractile responses in MPA. This suggests that the inability of MPA to generate more NO in response to increasing concentrations of CARB unmasked its direct contractile effect on smooth muscle cells. Whether these results can be extrapolated to even smaller arteries in the pulmonary vascular bed can only be speculated on.

The physiological significance of the stimulatory effect of muscarinic agonists on NO release is not yet clear. The pulmonary vasculature is innervated by cholinergic nerves which are situated in the adventitial-medial border. Since acetylcholine is destroyed very rapidly *in vivo*, it

seemed unlikely that neuronally-derived acetylcholine could diffuse across the media to stimulate the endothelium. However, recent studies on the intact-chest cat (McMahon *et al.*, 1992 ; McMahon & Kadowitz, 1992) have shown that vagal stimulation causes pulmonary vasodilatation which is blocked by atropine as well as L-NAME and methylene blue, suggesting that neuronally-released acetylcholine could diffuse to the endothelium where stimulation of muscarinic receptors results in the release of NO. The possibility that endothelial cells themselves could synthesise acetylcholine was first proposed in 1985, when Parnavelas *et al.* reported that choline acetyltransferase, the enzyme responsible for the synthesis of acetylcholine, could be localised in vascular endothelial cells of the rat cortex and mesenteric arteries.

Pretreatment with L-NAME abolished CARB-induced relaxations in MPA and 1st branches, and almost completely inhibited its effect in 2nd branches. Recently, Buxton *et al.* (1993) have reported that L-NAME may act as a muscarinic antagonist, and therefore is a poor choice as a NOS inhibitor in studies in which muscarinic receptors are not blocked. In contrast to the effect of L-NAME on NOS, muscarinic blockade cannot be reversed by an excess of L-arginine. The same study showed that L-NOARG does not block muscarinic receptors. In the present study it was possible, therefore, that the inhibitory effect of L-NAME on CARB-induced relaxations was partly due to inhibition of NO synthesis

and partly due to blocking of muscarinic receptors. Consequently, further experiments were undertaken to clarify this point by comparing the ability of L-arginine to prevent the inhibitory actions of both L-NAME and L-NOARG.

In a similar manner to L-NAME, L-NOARG abolished the relaxant response to CARB in 2nd branches. Prior incubation with L-arginine only partially prevented the inhibitory effects of L-NAME and L-NOARG on CARB-induced relaxations. The similarity in the inhibitory effects of L-NAME and L-NOARG on CARB-induced relaxations and in their partial prevention by L-arginine suggests that L-NAME has no significant antagonistic activity on the M_3 muscarinic receptor subtype which has been shown to mediate the relaxant effect of muscarinic agonists in rat pulmonary artery (McCormack *et al.*, 1988). In this respect, Sideso *et al.* (1994) also have found that L-NAME lacks antagonistic activity on the M_3 muscarinic receptor mediating contraction of the mouse anococcygeus.

In addition to stimulation of NO release, acetylcholine and related cholinomimetics also cause hyperpolarisation of smooth muscle cells in some blood vessels via an endothelium-dependent mechanism (Chen *et al.*, 1988 ; Keef & Bowen, 1989). The hyperpolarisation is not mimicked by NO, nor can it be blocked by NOS inhibitors (Garland &

McPherson, 1992). These observations have led to the suggestion that a factor distinct from NO is also released from the endothelium. This factor has been termed " endothelium-derived hyperpolarising factor " or EDHF (Chen *et al.*, 1988). However in this study, the association of CARB-induced relaxation with a significant rise in cGMP level and the inhibition of this relaxation by L-NAME and L-NOARG indicates that NO is the sole mediator of the relaxant effect of CARB in rat pulmonary artery.

The finding that L-arginine completely prevented the potentiating effects of L-NAME and L-NOARG on PE-induced contractions but only partially prevented their inhibitory effects on CARB-induced relaxations demonstrates that only the inhibition by L-NAME and L-NOARG of basal, but not CARB-stimulated, NO release is completely prevented by L-arginine. These results raise the possibility that there are different isoenzymes of NOS involved in basal and CARB-stimulated NO release, where perhaps L-NAME and L-NOARG bind more tightly to the isoform mediating the effect of CARB that their inhibitory action cannot be completely overcome by L-arginine. Alternatively, only one form of NOS is present and activation of the enzyme by CARB increases its affinity for the inhibitors. Several studies have suggested differences in the mechanisms responsible for basal and agonist-stimulated NO release. In the perfused vascular bed of the rabbit ear, L-NAME inhibited both basal and acetylcholine-stimulated release of NO, but only in the former case

was inhibition reversed by L-arginine (Randall & Griffith, 1991). In rat aortic rings, L-NMMA inhibited basal but not acetylcholine-stimulated NO release (Frew *et al.*, 1993). N-Ethylmaleimide, a sulphydryl alkylating agent, inhibited agonist-stimulated but not basally-released NO from cultured endothelial cells (Siegle *et al.*, 1991). Furthermore, aortic rings from rats with heart failure showed an impaired basal production of NO despite the presence of a normal agonist-stimulated release of NO (Teerlink *et al.*, 1994).

Several studies have found endothelium-dependent vasorelaxants to be more powerful inhibitors of agonist-induced contractions than of KCl-induced contractions in aortic rings (Collins *et al.*, 1988 ; Morrison & Pollock, 1988). In an attempt to investigate whether the degree of endothelium-dependent relaxation in pulmonary artery is influenced by the nature of the contractile agent used to raise muscle tone, relaxant responses to CARB were also obtained in artery rings precontracted with KCl. CARB-induced relaxations in KCl-precontracted MPA, 1st and 2nd branches displayed a similar difference to that observed in PE-precontracted rings. However, KCl-precontracted rings showed a significantly smaller magnitude of relaxation in response to CARB. This was most striking in MPA where CARB, at all concentrations tested, failed to produce any degree of relaxation. This was surprising as basally-released NO had the ability to depress KCl-induced contractions in MPA.

The attenuation of CARB-induced relaxations in KCl-precontracted rings cannot be attributed to an inhibitory action by KCl on the generation of NO, as SNP also induced significantly weaker relaxations in KCl-precontracted than in PE-precontracted rings. It has been suggested to be due to the ability of cGMP to reduce Ca^{2+} influx through receptor-operated but not voltage-operated Ca^{2+} channels (Morrison, 1988). However, NO has been shown to selectively inhibit KCl-stimulated Ca^{2+} influx through voltage-operated Ca^{2+} channels in neuronal cells via a cGMP-dependent mechanism (Desole *et al.*, 1994). Pretreatment with L-NAME had no effect on SNP-induced relaxation in PE-precontracted rings but caused a significant enhancement of the relaxant responses to SNP in rings precontracted with KCl. These results imply that the limited capacity of CARB and SNP to relax KCl-precontracted rings reflect, at least in part, the ability of KCl to stimulate NO release, thereby reducing the capacity of the endothelium to generate more NO in response to CARB and decreasing the amount of unstimulated guanylate cyclase available for activation by NO. It is unlikely that KCl could stimulate NO release in large vessels by a direct action on endothelial cells, as voltage-operated Ca^{2+} channels have not been detected in aortic endothelial cells, despite their presence in capillary endothelium (Colden-Stansfield *et al.*, 1987 ; Bossu *et al.*, 1989).

In addition to muscarinic-agonists, a wide variety of biological mediators stimulate the release of NO. β-Adrenoceptor agonists, on the other hand, have been regarded as endothelium-independent vasorelaxants mediating their effects by increasing cAMP production in vascular smooth muscle. However recently, there have been conflicting reports regarding the contribution of NO release to β-adrenoceptor mediated vasorelaxation. Endothelium-denudation did not alter relaxation to β-agonists in hamster thoracic aorta and canine coronary arteries (Macdonald *et al.*, 1987 ; Jackson & Busse, 1991), but completely abolished β-adrenoceptor relaxation in rat thoracic aorta (Gray & Marshall, 1992). Treatment with NOS inhibitors reduced salbutamol or isoprenaline-induced relaxation in rat cerebral arteries and hindquarters (Gardiner *et al.*, 1991 ; Hempelmann & Ziegler, 1993), but not in feline hindquarters (Bellan *et al.*, 1991). Since a high density of β-adrenoceptors have been detected in bovine pulmonary endothelial cells (Ahmed *et al.*, 1990) and endothelial cells are in direct contact with blood-borne catecholamines, it is highly possible that catecholamines could contribute to the regulation of pulmonary vascular tone through stimulation of NO release. Therefore, we sought to investigate the possible role of the endothelium in mediating vasorelaxant responses to the non selective β-agonist ISO in pulmonary artery.

ISO induced concentration-dependent relaxations in endothelium-intact 2nd branch rings precontracted with PE. The lack of effect of flurbiprofen excludes a contribution of prostacyclin to the ISO-induced relaxation. Several lines of evidence indicate that the relaxant response to ISO was partially mediated by stimulation of NO release. Pretreatment with L-NAME caused partial inhibition of ISO-induced relaxation, an effect which was completely prevented by prior incubation with L-arginine. Moreover, when L-NAME was added during maximum relaxation to ISO, a partial reversal of the relaxation was observed. In addition, ISO caused a rise in cGMP level, which was moderate in comparison with CARB. Gray and Marshall (1992) have suggested that the rise in cAMP induced by ISO could lead to stimulation of NO release. In this respect, it is noteworthy that NOS exhibits sites for cAMP-dependent phosphorylation (Dinermann *et al.*, 1993). Surprisingly however, the relaxant effect of ISO was not at all affected by endothelium-denudation. This was not due to incomplete removal of endothelial cells, since denudation abolished CARB-induced relaxation. In contrast to endothelium-intact rings, L-NAME had no effect on ISO-induced relaxation in endothelium-denuded rings, which excludes the involvement of NO in mediating this effect. Regardless of the mechanisms involved, these results suggest that in the presence of endothelium, the relaxant effect of ISO is mediated partly by stimulation of NO release and partly by a direct effect on smooth muscle cells. In the absence of endothelium, ISO induces comparable relaxations

by a direct effect on the smooth muscle, independently of NO release. However, what remains unexplained at this point is the differential effect of L-NAME and endothelium-denudation on ISO-induced relaxation.

To characterise the receptors involved in mediating ISO-induced relaxation, antagonists with a certain degree of selectivity for β-adrenoceptor subtypes were used. Pharmacological studies as well as receptor binding studies have provided evidence for the existence of three main classes of β-adrenoceptors : the classical β_1 and β_2 receptors, and the atypical β_3 receptor which is resistant to blockade by classical β-antagonists (Lands *et al.*, 1967 ; Minneman *et al.*, 1981 ; Emorine *et al.*, 1989). In endothelium-intact rings, the submaximal responses to ISO were markedly shifted to the right by the non-selective β-antagonist propranolol, and to a lesser extent by the selective β_1-antagonist atenolol and the selective β_2-antagonist ICI 118551. A similar rightward shift of submaximal responses to ISO in the presence of propranolol was observed in endothelium-denuded rings. Thus, submaximal relaxant responses to ISO seem to be mediated by both β_1 and β_2 receptors. The presence of a mixed population of β_1 and β_2 adrenoceptors in rat pulmonary artery has previously been demonstrated (O'Donnell & Wanstall, 1981). However, neither of the antagonists caused any shift in the maximum response, implicating the involvement of an atypical β-receptor. The failure of the selective β_3-agonist BRL 37344 to induce

any relaxation suggests that this receptor differs from the previously recognised β_3-receptor.

In conclusion, this study demonstrates the presence of a continuous basal release of NO, but not prostacyclin, in the pulmonary vascular bed. The basal release of NO serves to depress contractile responses to vasoconstrictors and, therefore, can play a major role in maintaining the low pulmonary vascular tone. NO release can be also stimulated by a variety of vasoactive agents such as CARB, ISO and possibly KCl. The basal and CARB-stimulated NO release appear to be mediated by different isoenzymes of NOS. The study also demonstrates important differences between small and large pulmonary arteries. The small arteries are more responsive to vasoconstrictors and have a greater capacity to generate NO under basal conditions as well as in response to agonist stimulation than the larger arteries and, therefore, would have a greater role in regulating pulmonary pressure.

References

ADNOT,S., RAFFESTIN,B., EDDHAHIBI,S., BRAQUET,P.,CHABRIER,P.E. HMED,S., CHRETIEN,P., DANIEL,E.E. & SHEN,S.H. (1990) Characterisation of beta adrenoceptors on cultured endothelial cells by radioligand binding. Life Sci., **47**, 2365-2370.

ALABASTER,V.A. (1977) Inactivation of endogenous amines in the lung. In: Metabolic functions of the lung, ed. Bakhle,Y.S. & Vane,J.R., Marcel Dekker, New York, pp3-31.

AMEZCUA,J.L., PALMER,R.M.J., DeSOUZA, B.M. & MONCADA,S. (1989) Nitric oxide synthesised from L-arginine regulates vascular tone in the coronary circulation of the rabbit. Br. J. Pharmacol., **97**, 1119-1134.

ARCHER,S.L. & HAMPL,V. (1992) N-Monomethyl-L-arginine causes nitric oxide synthesis in isolated arterial rings: Trouble in paradise. Biochem. Biophys. Res. Commun., **188**, 590-596.

ARCHER,S.L., RIST,K., NELSON,D.P., DEMASTER,E.G., COWAN,N. & WEIR,E.K. (1990) Comparison of the haemodynamic effects of nitric oxide and endothelium-dependent vasodilators in intact lungs. J. Appl. Physiol., **68**, 735-747.

ARCHER,S.L., TOLINS,J.P., RAIJ,L. & WEIR,E.K. (1989) Hypoxic pulmonary vasoconstriction is enhanced by inhibition of the synthesis of an endothelium-derived relaxing factor. Biochem. Biophys. Res. Commun., **164**, 1198-1205.

ARNOLD,W.P., MITAL,C.K., KATSUKI,S. & MURAD,F. (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc. Natl. Acad.Sci. USA., **74**, 3203-3207.

AYOTUNDE,S.O., TABRIZCHI,A.R. & TRIGGLE,C.R. (1994) The effects of perfusion rate and N-nitro-L-arginine methyl ester on cirazoline- and KCl-induced responses in the perfused mesenteric arterial bed of rats. Br. J. Pharmacol., **111**, 13-20.

BADESCH,D.B., ORTON,E.C., ZAPP,L.M.,WESTCOTT,J.Y., HESTER,J., VOELKEL,N.F. & STENMARK,K.R. (1989) Decreased arterial wall prostaglandin production in neonatal calves with severe chronic pulmonary hypertension. Am. J. Respir. Cell. Mol. Biol., **1**, 489-498.

BARER,G.R., HOWARD,P. & McCURRIE,J.R. (1967) The effect of carbon dioxide and changes in blood pH on pulmonary vascular resistance in cats. Clin. Sci., **32**, 361-376.

BECKER,K.L. & GAZDAR,A.F. (1984) In: The endocrine lung in health and disease. ed. Saunders,W.B., Philadelphia.

BEETENS,J.R., HOVE,C.V., RAMPART,M. & HERMAN,A.G. (1983) Acetylcholine stimulates the release of prostacyclin by rabbit aorta endothelium. J. Pharm. Pharmacol., **35**, 251-252.

BERRIDGE,M.J. (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem., **56**, 159-193.

BEVAN,J.A. & BEVAN,R.D. (1988) Changes in arteries as they get smaller. In: Vascular smooth muscle, peptides, autonomic nerves and endothelium. ed. Vanhoutte,P.M., pp 55-60, New York, Raven Press Ltd.

BLOOM,W. & FAWCETT,D. (1975) In: Textbook of Histology. ed. Saunders,W.B., Philadelphia, pp 396-403.

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

BORST,H.G., McGREGOR,M., WHITTENBERGER,J.L. & BERGLUND,E. (1956) Influence of pulmonary arterial and left atrial pressures on pulmonary vascular resistance. Circ. Res., **4**, 393-399.

BOSSU,J.L., FELTZ,A., RODEAU,J.L. & TANZI,F. (1989) Voltage-dependent transient calcium currents in freshly dissociated capillary endothelial cells. FEBS letters, **255**, 377-380.

BREDT,D.S., HWANG,P.M., GLATT,C.E., LOWENSTEIN,C., REED,R.R. & SNYDER,S.H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature, **351**, 714-718.

BUTTERY,L.D.K., SPRINGALL,D.R., CARPENTER,A., RIVEROS-MORENO,V., MONCADA,S., COHEN,J. & POLAK,J.M. (1993) Distinct localisation of inducible nitric oxide synthase in endotoxin-treated rats. Endothelium (suppl), 1,S27.

BUXTON,I.L.O., CHEEK,D.J., ECKMAN,D., WESTFALL,D.P., SANDERS,K.M. & KEEF,K.D. (1993) N-Nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. Circ. Res., **72**, 387-395.

CELERMAJER,D.S., DOLLERY,C., BURCH,M., DEANFIELD, J.E. & BCHIR,M.B. (1994) Role of endothelium in the maintenance of low pulmonary vascular tone in normal children. Circulation, **89**,2041-2044.

CHAND,N. & ALTURA, B.M. (1981) Acetylcholine and bradykinin relax intrapulmonary arteries by acting on endothelial cells: role in lung vascular disease. Science, **213**, 1376-1379.

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

CHERRY,P.D. & GILLIS,C.N. (1987) Evidence for the role of endothelium-derived relaxing factor in acetylcholine-induced vasodilatation in the intact lung. J. Pharmacol. Exp. Ther., **241**, 516-520.

COCKS,T.M., ANGUS,J.A., CAMPBELL,J.H. & CAMPBELL,G.R. (1985) Release and properties of endothelium-derived relaxing factor (EDRF) from endothelial cells in culture. J. Cell. Physiol., **123**, 310-320.

COLDEN-STANSFIELD,M., SCHILLING,W.P., RITCHIE,A.K., ESKIN,S.G., NAVARRO,L.T. & KUNZE,D. (1987) Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. Circ.Res., **61**, 632-640.

COLLINS,P., GRIFFITH,T.M., HENDERSON,A.H. & LEWIS,M.J. (1986) Endothelium-derived relaxing factor alters calcium fluxes in rabbit aorta: A cyclic guanosine monophosphate-mediated effect. J. Physiol. (Lond)., **381**, 427-437.

COLLINS,P., HENDERSON,A.H., LANG,D. & LEWIS,M.J. (1988) Endothelium-derived relaxing factor and nitroprusside compared in noradrenaline- and K^+-contracted rabbit and rat aorta. J. Physiol., **400**, 395-404.

CRAWLEY,D.E., LIU,S.F., EVANS,T.W. & BARNES,P.J. (1990) Inhibitory role of endothelium-derived relaxing factor in rat and human pulmonary arteries. Br. J. Pharmacol., **101**, 166-170.

CREMONA,G., HIGENBOTTAM,T.W., DINH-XUAN,A.T., WELLS,F.C., LARGE,S.R., STEWART,S. & WALLWORK,J. (1991) Inhibitors of endothelium-derived relaxing factor increase pulmonary vascular resistance in isolated perfused human lungs. Eur. Respir. J., **4** (suppl. 14), 336s.

DABROWSKA,R., AROMATORIO,D., SHERRY,J.M.F., HARTSHORNE,D.J. (1977) Composition of the myosin light chain kinase from chicken gizzard. Biochem. Biophys. Res. Commun., **78**. 1263-1272.

DELANEROLLE,P., NISHIKAWA,M., YOST,D.A. & ADELSTEIN,R.A. (1984) Increased phosphorylation of myosin light chain kinase after an increase in cyclic AMP in intact smooth muscle. Science., **223**, 1415-1417.

DEMBINSKA-KIEC,A., RUCKER,W. & SCHONHOFER,P.S. (1980) Effects of PGI_2 and PGI analogues on cAMP levels in cultured endothelial and

smooth muscle cells derived from bovine arteries. Nauyn-Schmied. Arch. Pharm., **311**, 67-70.

DEMLING,R., SMITH,M., GUNTHER,R., FLYNN,J. & GEE,M. (1981) Pulmonary injury and prostaglandin production during endotoxemia in conscious sheep. Am. J. Physiol., **240**, H348-H353.

DESOLE,M.S., KIM,W.K., RABIN,R.A. & LAYCHOCK,S.G. (1994) Nitric oxide reduces depolarisation-induced calcium influx in PC12 cells by a cyclic GMP-mediated mechanism. Neuropharmacology, **33**, 193-198.

DeWITT,D.L. & SMITH, W.L. (1983) Purification of prostacyclin synthase from bovine aorta by immunoaffinity chromatography. Evidence that the enzyme is a haemoprotein. J. Biol. Chem., **258**, 3285-3293.

DINERMANN,J.L., LOWENSTEIN,C.J. & SNYDER,S.H. (1993) Molecular mechanisms of nitric oxide regulation: potential relevance to cardiovascular disease. Circ. Res., **73**, 217-222.

DINH-XUAN,A.T., HIGENBOTTAM,T.W., CLELLAND,C.A., PEPKE-ZABA,J., CREMONA,G., BUTT,A.Y., LARGE,S.R., WELLS,F.C. & WALLWORK,J. (1991) Impairment of endothelium-dependent pulmonary artery relaxation in chronic obstructive lung disease. N. Engl. J. Med., **324**, 1539-1547.

DINH-XUAN,A.T., HIGENBOTTAM, T.W. & WALLWORK,j. (1992) Relationship between chronic hypoxia and *in vitro* pulmonary relaxation mediated by endothelium-derived relaxing factors in human chronic obstructive lung disease. Angiology., **43**, 350-356.

DOWNING,S.E. & LEE,J.C. (1980) Nervous control of the pulmonary circulation. Annu. Rev. Physiol., **42**, 199-210.

EMORINE,L.J., MARULLO,S., BRIEND-SUTREN,M.M., PATEY,G., TATE,K., DELAVIER-KLUTCHKO,C. & STROSBERG,D. (1989) Molecular characterisation of the human β_3-adrenergic receptor. Science, **245**, 1118-1121.

FISHMAN,A.P. (1961) Respiratory gases in the regulation of the pulmonary circulation. Physiol. Rev., **41**, 214-280.

FISHMAN,A.P. (1976) Hypoxia on the pulmonary circulation: How and where it acts. Circ. Res., **38**, 221-231.

FORSTERMANN,U., SCHMIDT,H.W., POLLOCK,J.S., SHENG,H., MITCHELL,J.A., WARNER,T.D., NAKANE,M. & MURAD,R. (1991) Isoforms of nitric oxide synthase. Characterisation and purification from different cell types. Biochem. Pharmacol., **42**, 1849-1857.

FRANGOS,J.A., ESKIN,S.G., MCINTIRE,L.V. & IVES,C.L. (1985) Flow effects on prostacyclin production by cultured human endothelial cells. Science, **227**, 1477-1479.

FRATACCI,M.D., DEFOUILLOY,C., ANDRIVET,P., KOUYOUMADJIAN,C., SEDIAME,S., HERIGAUT,R. & ADNOT,S. (1992) Responses to infusion of acetylcholine and chronic inhalation of nitric oxide in patients with chronic obstructive lung disease and pulmonary hypertension. Am. Rev. Respir. Dis., **145** (suppl), A722.

FREAY,A., JOHNS,A., ADAMS,D.J., RYAN,U.S. & VANBREEMEN,C. (1989) Bradykinin and inositol 1,4,5-trisphosphate-stimulated calcium release from intracellular stores in cultured bovine endothelial cells. Pflugers. ARCH., **414**, 377-384.

FREW,J.D., PAISLEY,K. & MARTIN,W. (1993) Selective inhibition of basal but not agonist-stimulated activity of nitric oxide in rat aorta by N-monomethyl-L-arginine. Br. J. Pharmacol., **110**, 1003-1008.

FURCHGOTT,R.F. (1983) Role of endothelium in responses of vascular smooth muscle. Circ. Res., **53**, 557-573.

FURCHGOTT,R.F. (1988) In: Vasdilatation: vascular smooth muscle, peptides, autonomic nerves and endothelium. ed. Vanhoutte,P.M., pp401-414. 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.

GADDUM,J.H., HEBB,C.O., SILVER,A., SWAN,A.A.B. (1953) 5-Hydroxytryptamine. Pharmacological action and destruction in perfused lungs. Q. J. Exp. Physiol., **38**, 255-262.

GARDINER,S.M., COMPTON,A.M., KEMP,P.A. & BENNETT,T. (1990) Regional and cardiac haemodynamic effects of N-nitro-L-arginine methyl ester in conscious, long Evans rats. Br. J. Pharmacol., **101**, 625-631.

GARLAND,C.J. & McPHERSON,G.A. (1992) Evidence that nitric oxide does not mediate the hyperpolarisation and relaxation to acetylcholine in the rat small mesenteric artery. Br. J. Pharmacol., **105**, 429-443.

GAW,A.J., ABERDEEN,J., HUMPHREY,P.P.A., WADSWORTH,R.M. & BURNSTOCK,G. (1991) Relaxation of sheep cerebral arteries by vasoactive intestinal polypeptide and neurogenic stimulation: inhibition by L-N-monomethyl arginine in endothelium-denuded vessels. Br. J. Pharmacol., **102**, 567-572.

GILMAN,A.G. (1989) G proteins and regulation of adenylyl cyclase. J.A.M.A., **262**, 1819-1825.

GLUSA,E. & RICHTER,M, (1993) Endothelium-dependent relaxation of porcine pulmonary arteries via 5-HT_{1C}-like receptors. Naunyn-Schmied. Arch. Pharmacol., **347**, 471-477.

GOLD,M.E., WOOD,K.S., BYRNS,R.E., FUKUTO,J. & IGNARRO,L.J. (1990) N-Methyl-L-arginine causes endothelium-dependent contraction and inhibition of cyclic GMP formation in artery and vein. Proc. Natl. Acad. Sci. USA., **87**, 4430-4434.

GRASER,T. & VANHOUTTE,P.M. (1991) Hypoxic contraction of canine coronary arteries: role of endothelium and cGMP. Am. J. Physiol., **261**, H1769-H1777.

GRAY,D.W. & MARSHALL,I. (1992) Novel signal transduction pathway mediating endothelium-dependent β-adrenoceptor vasorelaxation in rat thoracic aorta. Br. J. Pharmacol., **107**, 684-690.

GREENBERG,B., RHODEN,K. &BARNES,P.J. (1987) Endothelium-dependent relaxation of human pulmonary arteries. Am. J. Physiol. 252, H434-H438.

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.

GRUETTER,C.A., GRUETTER,D.Y., LYON,J.E., KADOWITZ,P.J. & IGNARRO,J. (1981) Relationship between cyclic guanosine 3':5'-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryltrinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methaemoglobin. J. Pharmacol. Exp. Ther., **219**, 181-186.

GRUETTER,C.A., KADOWITZ,P.J. & IGNARRO,L.J. (1980) Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite, and amyl nitrate. Can. J. Physiol. Pharmacol., **59**, 150-156.

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

GUSTAFSSON,L.E., LEONE,A.M., PERSSON,M.G., WIKLUND,N.P. & MONCADA,S. (1991) Endogenous nitric oxide is present in the exhaled

air of rabbits, guinea pigs and humans. Biochem. Biophys. Res. Commun., **181**, 852-857.

HAKIM,T.S. & DAWSON,C.A. (1979) Sympathetic nerve stimulation and vascular resistance in a pump-perfused dog lung lobe. Proc. Soc. Exp. Biol. Med., **160**, 38-41.

HALES,C.A. & KAZEMI,H. (1975) Role of histamine in the hypoxic vascular response of the lung. Respir. Physiol., **24**, 81-88.

HAMASAKI,Y. & SAID,S. (1981) Vasodilator effects of vasoactive intestinal peptide and prostacyclin on isolated rat pulmonary artery. Am. Rev. Respir. Dis., **122**, 239.

HAMBERG,M. & SAMUELSSON,B. (1974) Prostaglandin endoperoxides. Novel transformation of arachidonic acid in guinea pig lung. Biochem. Biophys. Res. Commun., **61**, 942-949.

HEMPELMANN,R.G. & ZIEGLER,A. (1993) Endothelium-dependent noradrenaline-induced relaxation of rat isolated cerebral arteries: pharmacological characterisation of receptor subtypes involved. Br. J. Pharmacol., **110**, 1321-1328.

HILL,S.J. (1990) Distribution, properties and functional characteristics of three classes of histamine receptor. Pharmacol. Rev., **42**, 45-83.

HIMPENS,B. & SOMLYO,A.P. (1988) Free-calcium and force transients during depolarisation and pharmacochemical coupling in guinea-pig smooth muscle. J. Physiol., **395**, 507-530.

HIRATA,M., KOHSE,K.P., CHANG,C.H., IKEBE,T. & MURAD,F. (1990) Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. J. Biol. Chem., **265**, 1268-1273.

HIRATA,F., SCHIFFMAN,E., VENKATASUBRAMANIAN,K., SALOMON,K. & AXELROD,D. (1980) A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. Proc. Natl. Acad. Sci. USA., **77**, 25-33.

HIRST,G.D.S. & EDWARDS,F.R. (1989) Sympathetic neuroeffector transmission in arteries and arterioles. Physiol. Rev., **69**, 546-604.

HISLOP,A. & REID,L. (1978). Normal structure and dimensions of the pulmonary arteries in the rat. J. Anat., **125**, 71-83.

HOLDEN,W.E. & MCCALL,E. (1984) Hypoxia-induced contractions of porcine pulmonary artery strips depend on intact endothelium. Exp. Lung Res., **7**, 101-112.

HOLLENBERG,N.K. (1988) Serotonin and vascular responses. Annu. Rev. Pharmacol. Toxicol., **28**, 41-59.

HONG,S.L. (1980) Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical vein. Thrombos Res., **18**, 787-795.

HOWELL, J.B.L., PERMUTT,S., PROCTOR,F. & RILEY,R.L. (1961) Effect of inflation of the lung on different parts of the pulmonary vascular bed. J. Appl. Physiol., **16**, 71-76.

HUTCHESON,I.R. & GRIFFITH,T.M. (1991) Release of endothelium-derived relaxing factor is modulated both by frequency and amplitude of pulsatile flow. Am. J. Physiol., **261**, H257-H262.

HUTCHINSON,P.J.A., PALMER,R.M.J. & MONCADA,S. (1987) Comparative pharmacology of EDRF and nitric oxide on vascular strips. Eur. J. Pharmacol., **141**, 445-451.

HYMAN,A.L., NANDIWADA,P., KNIGHT,D.S. & KADOWITZ,P.J. (1981) Pulmonary vasodilator responses to catecholamines and sympathetic nerve stimulation in the cat. Circ. Res., **48**, 407-415.

HYMAN,A.L., SPANNHAKE,E.W. & KADOWITZ,P.J. (1978) Prostaglandins and the lung. Am. Rev. Respir. Dis., **117**, 111-136.

IGNARRO,L.J., ADAMS,J.B., HORWITZ,P.M. & WOOD,K.S. (1986) Activation of soluble guanylate cyclase by NO-haemoprotein involves NO-haeme exchange. J. Biol. Chem., **261**, 4997-5002.

IGNARRO,L.J., BURKE,T.M., WOOD,K.S., WOLIN,M.S. & KADOWITZ,P.J. (1984) Association between cyclic GMP accumulation and acetylcholine-elicited relaxation of bovine intrapulmonary artery. J. Pharmacol. Exp. Ther., **228**, 682-690.

IGNARRO,L.J., BYRNS,R.E. & WOOD,K.S. (1986) Pharmacological and biochemical properties of endothelium-derived relaxing factor (EDRF): Evidence that it is closely related to nitric oxide (NO) radical. Circulation, **74**(suppl II), 287.

IGNARRO,L.J., LIPPTON,H., EDWARDS,J.C., BARICOS,W.H., HYMAN,A.L., KADOWITZ,P.J. & GRUTTER,C.A. (1981) Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of

S-nitrosothiols as active intermediates. J. Pharmacol. Exp. Ther., **218**, 739-749.

ISHII,K., CHANG,B., KERWIN,J.F., HUANG,Z.J. & MURAD,F. (1990) L-N-Nitro-L-arginine: a potent inhibitor of endothelium-derived relaxing factor. Eur. J. Pharmacol., **179**, 219.

JACKSON,W.F. & BUSSE,R. (1991). Elevated guanosine 3':5'-cyclic monophosphate mediates the depression of nitrovasodilator reactivity in endothelium-intact blood vessels. Naunyn-Schmied. Arch. Pharmacol., **344**, 345-350.

KADOWITZ,P.J., GRUTTER,C.A., SPANNHAKE,E.W. & HYMAN,A. (1981) Pulmonary vascular responses to prostaglandins. Fed. Proc., **40**, 1991-1996.

KADOWITZ,P.J., JOINER,P.D. & HYMAN,A.L. (1973) Differential effects of phentolamine and bretylium on pulmonary vascular responses to norepinephrine and nerve stimulation. Proc. Soc. Exp. Biol. Med., **144**, 172-176.

KEEF,K.D. & BOWEN,S.M. (1989) Effect of acetylcholine on electrical and mechanical activity in guinea pig coronary arteries. Am. J. Physiol., **257**, H1096-H1103.

KENNEDY,I., COLEMAN,R.A., HUMPHREY,P.P.A., LEVY,G.P. & LUMLEY,P. (1982) Studies on the characterisation of prostanoid receptors: a proposed classification. Prostaglandins, **24**, 667-689.

KISELLA,J.P., NEISH,S.R., SHAFFER,E. & ABMAN,S.H. (1992) Low-dose inhalational nitric oxide in persistent pulmonary hypertension of the newborn. Lancet, **340**, 819-820.

KNOWLES,R.G., PALACIOS,M., PALMER,R.M.J. & MONCADA,S. (1990) Kinetic characteristics of nitric oxide synthase from rat brain. Biochem. J. **269**, 207-210.

KURIYAMA,H., ITO,Y., SUZUKI,H., KITAMURA,K. & ITOH,T. (1982) Factors modifying contraction-relaxation cycle in vascular smooth muscles. Am. J. Physiol., **243**, H641-H662.

LAHER,I. & BEVAN,R.D. (1988) Changes in arteries as they get smaller. In: vascular smooth muscle, peptides, autonomic nerves and endothelium. ed. Vanhoutte,P.M., pp 55-60, New York, Raven Press Ltd.

LANDS,A.M., LUDUENA,F.P. & BUZZO,H.J. (1967) Differentiation of receptors responsive to isoproterenol. Life Sci., **6**, 2241-2249.

LEACH,R.M., TWORT,C.H.C., CAMERON,I.R. & WARD,J.P.T. (1992) A comparison of the pharmacological and mechanical properties *in vitro* of large and small pulmonary arteries of the rat. Clin, Sci., **82**, 55-62.

LEACH,R.M., TWORT,C.H.C., WARD,J.P.T. & CAMERON,I.R. (1990) Endothelial dysfunction in isolated pulmonary arteries from chronically hypoxic rats. Thorax, **45**, 808P.

LEWIS,R.A. & AUSTIN,K.F. (1977) Nonrespiratory functions of pulmonary cells: the mast cell. Federation Proc., **36**, 2676-2683.

LONG,C.J., SHIKANO,K. & BERKOWITZ,B.A. (1987) Anion exchange resins discriminate between nitric oxide and EDRF. Eur. J. Pharmacol., **142**, 317-318.

LOWENSTEIN,C.J., ALLEY,E.W., RAVAL,P., SNOWMAN,A.M., SNYDER,S.H., RUSSELL,S.W. & MURPHY,W.J. (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon-gamma and lipoplysaccharide. Proc. Natl. Acad. Sci. USA., **90**,9730-9734.

MALIK,A.B. & KIDD,B.S.L. (1973) Independent effects of changes in H^+ and CO_2 concentrations on hypoxic pulmonary vasoconstriction. J. Appl. Physiol., **34**, 318-323.

MARLETTA,M.A., YOON,P.S., IYENGAR,R., LEAF,C.D. & WISHNOK,J.S. (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry, **27**, 8706-8711.

MARTIN,W., FURCHGOTT,R.F., VILLANI,G.M. & JOTHIANANDAN,D. (1986) Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. J. Pharmacol. Exp. Ther., **237**, 529-538.

MAYER,B., JHON,M. & BOHME,E. (1990) Purification of Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum: cofactor role of tetrahydrobiopterin. FEBS Letters, **277**, 215-219.

MAYER,B., SCHMIDT,K., HUMBERT,R., & BOHME,E. (1989) Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. Biochem. Biophys. Res. Commun., **164**, 678-685.

MAZMANIAN,G.M.,BAUDET,B.,BRINK,C.,CERRINA,J.,KIRKIACHARIAN,S. & WEISS,C., (1989) Methylene blue potentiates vascular reactivity in isolated rat lungs. J. Appl. Physiol., **66**, 1040-1045.

McCORMACK,D.G., MAK,J.C., MINETTE,P. & BARNES,P.J. (1988) Muscarinic receptor subtypes mediating vasodilation in the pulmonary artery. Eur. J. Pharmacol., **158**, 293-297.

McMAHON,T.J., HOOD,J.S. & KADOWITZ,P.J. (1992) The pulmonary vasodilator response to vagal stimulation is blocked by N-nitro-L-arginine methyl ester in the cat. Circ. Res., **70**, 364-369.

McMAHON,T.J. & KADOWITZ,P.J. (1992) Methylene blue inhibits neurogenic cholinergic vasodilator responses in the pulmonary vascular bed of the cat. Am. J. Physiol., **263**, 575-584.

McMURTRY,I.F. (1984) Angiotensin is not required for hypoxic constriction in salt solution-perfused rat lungs. J. Appl. Physiol., **56**, 375-380.

MELLION,B.T., IGNARRO,L.J., OHLSTEIN,E.H., PONTECORVO,E.G., HYMAN,A.L. & KADOWITZ,P.J. (1981) Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. Blood, **57**, 946-955.

MEYRICK,B., NIEDERMEYER,M.E., OGLETREE,M.L. & BRIGHAM,K.L. (1985) Pulmonary hypertension and increased vasoreactivity caused by repeated indomethacin in sheep. J. Appl. Physiol., **59**, 443-452.

MILLER,V.M. & VANHOUTTE,P.M. (1985a) Endothelial α_2-adrenoceptors in canine pulmonary and systemic blood vessels. Eur. J. Pharmacol., **118**, 123-129.

MILLER,V.M. & VANHOUTTE,P.M. (1985b) Endothelium-dependent contractions to arachidonic acid are mediated by products of cyclo-oxygenase . Am. J. Physiol., **248**, H432-H437.

MINNEMAN,K.P., PITTMAN,R.N. & MOLINOFF,P.B. (1981) β-Adrenergic receptor subtypes: properties, distribution and regulation. Am. Rev. Neurosci., **4**, 419-461.

MONCADA,S., GRYGLEWSKI,R., BUNTING,S. & VANE,J.R. (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation, Nature, **263**, 663-665.

MONCADA,S., HERMAN,A.G., HIGGS,E.A. & VANE,J.R. (1977) Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the anti-thrombic properties of vascular endothelium. Thromb. Res., **11**, 323-344.

MONCADA,S., RADOMSKI,M.W. & PALMER,R.M.J. (1988) Endothelium-derived relaxing factor: Identification as nitric oxide and role in the control of vascular tone and platelet function. Biochem. Pharmacol., **37**, 2495-2501.

MORRISON,K.J. (1988) A study of smooth muscle relaxation in rat vasculature. Ph.D. thesis, University of Glasgow.

MORRISON,K.J. & POLLOCK,D. (1988) Effects of vasodilators on tone, cyclic monophosphate levels and phosphatidylinositol hydrolysis in rat rings precontracted with noradrenaline, potassium chloride or phorbol 12-myristate-13-acetate. J. Muscle Res. Cell Motil., **9**, 460.

MULSCH,A.& BUSSE,R. (1990) N-Nitro-L-arginine(N^5-[imino-(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. Naunyn-Schmied. Arch. Pharmacol., **341**, 143-147.

MURAD,F. (1986) Cyclic guanosine monophosphate as a mediator of vasodilation. J. Clin. Invest., **78**, 1-5.

MURRAY,J.F. (1986) In: The normal lung, the basis for diagnosis and treatment of pulmonary diseases. ed. Saunders,W.B., Philadelphia, pp35-39.

MURRAY,J.J., FRIDOVICH,I., MAKHOUL,R.G. & HAGEN,P.O. (1986) Stabilisation and partial characterisation of endothelium-derived relaxing factor from cultured bovine aortic endothelial cells. Biochem. Biophys. Res. Commun., **141**, 689-696.

MYERS,P.R., MINOR,R.L.J., GUERRA,R.J., BATES,J.N. & HARRISON,D.G. (1990) Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature, **345**, 161-163.

NANDIWADA,P.A., HYMAN,A.L. & KADOWITZ,P.J. (1983) Pulmonary vasodilator response to vagal stimulation and acetylcholine in the cat. Circ. res., **53**, 86-95.

NAYER,H.S., MATHUR,R.M. & RANADE,V.V. (1972) The role of serotonin (5-hydroxytryptamine) in the pulmonary arterial pressor response during acute hypoxia. Indian J. Med. Res., **60**, 1665-1673.

NEWMAN,J.H., BUTKA,B.J. & BRIGHAM,K.L. (1986) Thromboxane A_2 and prostacyclin do not modulate pulmonary hemodynamics during exercise in sheep. J. Appl. Physiol., **61**, 1706-1711.

NIARCHOS,A., ROBERTS,A. & LARAGH,J. (1979) Effects of the converting enzyme inhibitor (SQ 20881) on the pulomnary circulation in man. Am. J. Med., **67**, 785-791.

NICHOLAS,T.E., STRUM,J.M., ANGELO,L.S. & JUNOD,A.F. (1974) Site and mechanism of uptake of ^{3}H-norepinephrine by isolated perfused rat lungs. Circ. Res., **35**, 670-680.

NISHIKAWA,M., DELANEROLLE,P., LINCOLN,T.M. & ADELSTEIN,R.S. (1984) Phosphorylation of mammalian myosin light chain kinases by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. J. Biol. Chem., **259**, 8429-8436.

NISHIKAWA,M., SHIRAKAWA,S., ADELSTEIN,R.S. (1985) Phosphorylation of smooth muscle myosin light chain kinase by protein kinase C. Comparative study of the phosphorylated sites. J. Biol. Chem., **260**, 8978-8983.

O'DONELL,S.R. & WANSTALL,J.C. (1981) Demonstration of both β_1- and β_2-adrenoceptors mediating relaxation of isolated ring preparations of rat pulmonary artery. Br. J. Pharmacol., **74**, 547-552.

OGLETREE,M.L. (1982) Pharmacology of prostaglandins in the pulmonary microcirculation. Ann. NY. Acad. Sci., **384**, 191-206.

OLBERGTS,J. (1985) Termolecular reaction of nitrogen monoxide and oxygen: a still unsolved problem. Intl. J. Chem. kinetics., **17**, 835-848.

OWEN,M.P. & BEVAN,J.A. (1985) Acetylcholine-induced endothelial-dependent vasodilation increases as artery diameter decreases in the rabbit ear. Experientia, **41**, 1057-1058.

PACKER,M. (1985) Vasodilator therapy for primary pulmonary hypertension: Limitations and hazards. Ann. Intern. Med., 103, 258-270.

PALEVSKY,H.I. & FISHMAN,A.P. (1985) Vasodilator therapy for primary pulmonary hypertension. Annu. rev. Med., **36**, 563-578.

PALEVSKY,H.I. & FISHMAN,A.P. (1988) Comparison of acute hemodynamic responses to prostacyclin with standard vasodilators in patients with primary pulmonary hypertension. Chest, **93**(suppl.), 179.

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. (1988) L-Arginine is the physiological precursor for the formation of nitric oxide

in endothelium-dependent relaxation. Biochem. Biophys. Res. Commun., **153**, 1251-1256.

PARNAVELAS,J.G., KELLY,W. & BURNSTOCK,G. (1985) Ultrastructural localisation of choline acetyltransferase in vascular endothelial cells in rat brain. Nature, **316**, 724-725.

PEACH,M.J., JOHNS,R.A. & ROSE,C.E. (1989) The potential role of interactions between endothelium and smooth muscle in pulmonary vascular physiology and pathophsiology. In: The lung: Pulmonary vascular physiology and pathophysiology. ed. Weir.k., vol 38, pp643-697. New York, Marcel Dekker.

PEPKE-ZABA,J., HIGENBOTTAM,T.W., DINH-XUAN,A.T., STONE,D. & WALL,J. (1991) Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. Lancet, **338**, 1173-1174.

PERSSON,M.G., GUSTAFSSON,L.E., WIKLUND,N.P., MONCADA,S. & HEDQVIST,P. (1990) Endogenous nitric oxide as a probable modulator of pulmonary circulation and hypoxic pressor response *in vivo*. Acta. Physiol. Scand., **140**, 449-457.

POHL,U., HOLTZ,J., BUSSE,R. & BASSENGE,E. (1986) Crucial role of endothelium in the vasodilator response to increased flow *in vivo*. Hypertension, **8**, 37-44.

RADOMSKI,M.W., PALMER,R.M.J. & MONCADA,S. (1987) Comparative pharmacology of endothelium-derived relaxing factors, nitric oxide and prostacyclin, in platelets. Br. J. Pharmacol., **92**, 181-187.

RADOMSKI,M.W., PALMER,R.M.J. & MONCADA,S. (1990) Characterisation of the L-arginine/ nitric oxide pathway in human platelets. Br. J. Pharmacol., **101**, 325-328.

RADOMSKI,M.W., PALMER,R.M.J. & MONCADA,S. (1990) Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc. Natl. Sci. USA., **87**, 10043-10047.

RANDALL,M.D. & GRIFFITH,T.M. (1991) Differential effects of L-arginine on the inhibition by N-nitro-L-arginine methyl ester of basal and agonist-stimulated EDRF activity. Br. J. Pharmacol., **104**, 743-749.

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

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

REES,D.D., PALMER,R.M.J., SCHULZ,R., HODSON,H.F. & MONCADA,S. (1990) Characterisation of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. Br. J. Pharmacol., **101**, 746-752.

REID,L. & MEYRICK,B. (1980) In: Metabolic activities of the lung. Ciba Found. Sympos. (#78). Amesterdam, Excerpta Medica, pp147-164.

RICH,S. & BRUNDAGE,B.H. (1986) High-dose calcium channel blocking therapy for primary pulmonary hypertension: evidence for long-term reduction in pulmonary arterial pressure and regression of right ventricular hypertrophy. Circulation, **76**, 135-141.

ROBERTS,J.D., POLANER,D.M., LANG,P. & ZAPOL,W.M. (1992) Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. Lancet, 340, 818-819.

ROBERTSON,B.E., SCHUBERT,R., HESCHELER,J. & NELSON,M.T. (1993) cGMP-dependent protein kinase activates Ca^{2+}-activated K^+ channels in cerebral artery smooth muscle cells. Am. J. Physiol., **265**, C299-C303.

RODMAN,D.M., YAMAGUCHI,T., O'BRIEN,R.F. & McCURTRY,I.F. (1989) Hypoxic contraction of isolated rat pulmonary artery. J. Pharmacol. Exp. Ther., **248**, 952-959.

ROOS,A., THOMAS,L.J., NAGEL,E.L. & PROMMAS,D.C. (1961) Pulmonary vascular resistance as determined by lung inflation and vascular pressures. J. Appl. Physiol,. **16**, 77-84.

RUBANYI,G.M. & VANHOUTTE,P.M. (1985) Hypoxia releases a vasoconstrictor substance from canine vascular endothelium. J. Physiol., **364**, 45-56.

RUBANYI,G.M. & VANHOUTTE,P.M. (1986) Potassium-induced release of endothelium-derived relaxing factor from canine femoral arteries. Circ. Res., **62**, 1098-1103.

RUBIN,L.J., HUGHES,J.D. & LAZAR,J.D. (1985) The effects of eicosanoid synthesis inhibitors on normoxic and hypoxic pulmonary vascular tone in dogs. Am. Rev. Respir. Dis. **132**, 93.

RYAN,J.W. & RYAN,U.S. (1977) Pulmonary endothelial cells. Fed. Proc., **36**, 2683-2691.

RYAN,U.S., RYAN,J.W., WHITAKER,C. & CHIU,A. (1976) Localisation of angiotensin converting enzyme (kininase II). Immunocytochemistry and immunofluorescence. Tissue Cell, **8**, 125-145.

SAID,S.I. (1982) Vasoactive peptides and the pulmonary circulation. Ann. NY. Acad. Sci., **384**, 207-212.

SALVEMINI,D., MOLLACE,V., PISTELLI,A., ANGGARD,E. & VANE,J. (1992) Cultured astrocytoma cells generate a nitric oxide-like factor from endogenous L-arginine and glyceryl trinitrate: effect of E.Coli lipopolysaccharide. Br. J. Pharmacol., **106**, 931-936.

SCHEID,C.R. & FAY,F.S. (1984) Beta-adrenergic effects on transmembrane Ca^{45} fluxes in isolated smooth muscle cells. Am. J. Physiol., **246**, C431-C438.

SCHELLENBERG,R.R., DUFF,M.J., FOSTER,A. & PADDON,H.B. (1986) Histamine releases PGI_2 from human pulmonary artery. Prostaglandins, **32**, 201-209.

SCHWARZ,G., CALLEWAERT,G., DROOGMANS,G. & NILIUS,B. (1992) Shear stress-induced calcium transients in endothelial cells from human umbilical cord veins. J. Physiol., **458**, 527-538.

SHAUL,P.W., KINANE,B., FARRAR,M.A., BIYA,M. & MAGNESS,R.R. (1991) Prostacyclin production and mediation of adenylate cyclase activity in the pulmonary artery: alterations after prolonged hypoxia in the rat. J. Clin. Invest., **88**, 447-455.

SHAW,J.W. (1971) Pulmonary vasodilator and vasoconstrictor actions of histamine. J. Physiol., **215**, 34-35p.

SIDESO,E., TUCKER,J.F. & GIBSON,A. (1994) Lack of antagonist action of L-NAME on muscarinic (M_3) receptors in the mouse anococcygeus. Br. J. Pharm., **112**, 233p.

SIEGLE,I., HECKER,M., SESSA,W.C. & VANE,J.R. (1991) Sulfhydryl reagents inhibit stimulated but not basal EDRF release from cultured endothelial cells. Br. J. Pharmacol., **102**, 119p.

SILOVE,E.D. & GROVER,R.F. (1968) Effects of alpha adrenergic blockade and tissue catecholamine depletion on pulmonary vascular responses to hypoxia. J. Clin. Invest., **47**, 274-285.

SKRBIC,R. & CHIBA,S. (1993) Dominant antagonistic action of α_2-adrenoceptor agonists on α_1-agonist-induced vasoconstriction. Eur. J. Pharmacol., **230**, 131-137.

SOMLYO,A.P. (1985) Excitation-contraction coupling and the ultrastructure of smooth muscle. Circ. Res., **57**, 497-507.

STRUM,J.M. & JUNOD,A.F. (1972) Radioautographic demonstration of ^{3}H-5-hydroxytryptamine uptake by pulmonary endothelial cells. J. Cell. Biol. **54**, 456-467.

STUEHR,D., KWAN,N.S., NATHAN,C., GRIFFITH,O., FELDMAN,P. & WISEMAN,J. (1991) N-Hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J. Biol. Chem., **266**, 6259-6263.

SUSCHEK,C., ROTHE,H., FEHSEL,K., ENCZMANN,J. & KOLB-BACHOFEN,V. (1993) Induction of a macrophage-like nitric oxide synthase in cultured rat aortic endothelial cells: IL-1 beta-mediated induction regulated by tumor necrosis factor-alpha and IFN-gamma. J. Immunol., **151**, 3283-3291.

SUZUKI,H. & TWAROG,B.M. (1982) Membrane properties of smooth muscle cells in pulmonary arteries of the rat. Am. J. Physiol., **242**, H900-H906.

TEERLINK,J.R., GRAY,G.A., CLOZEL,M. & CLOZEL,J.P. (1994) Increased vascular responsiveness to norepinephrine in rats with heart failure is endothelium dependent: Dissociation of basal and stimulated nitric oxide release. Circulation, **89**, 393-401.

TODA,N., BABA,H., TANOBE,Y. & OKAMURA,T. (1992) Mechanism of relaxation induced by K^{+} and nicotine in dog duodenal longitudinal muscle. J. Pharmacol. Exp. Ther., **260**, 697-701.

TREZISE,D.J., DREW,G.M. & WESTON,A.H. (1992) Analysis of the depressant effect of the endothelium on contractions of rabbit isolated basilar artery to 5-hydroxytryptamine. Br. J. Pharmacol., **106**, 587-592.

VAN DEN BOSCH,H. (1980) Intracllular phospholipases A. Biochem. Biophys. Acta., **604**, 191-246.

VANGRONDELLE,A., WORTHEN,G.S., ELLIS,D., MATHIAS,M.M., MURPHY,R.C., STRIEFL,R.J., REEVES,J.T. & VOELKEL,N.F. (1984) Altering hydrodynamic variables influences PGI_2 production by isolated lungs and endothelial cells. J. Appl. Physiol., **57**, 388-395.

VOELKEL,N.F., GREBER,J.G., McMURTRY,I.F., NIES,A.S. & REEVES,J.T. (1981) Release of vasodilator prostaglandin, PGI_2, from isolated lung during vasoconstriction. Circ. Res., **48**, 207-213.

WALKER,B.R., VOELKEL,N.F. & REEVES,J.T. (1982) Pulmonary pressor response after prostaglandin synthesis inhibition in conscious dogs. J. Appl. Physiol., **52**, 705-709.

WARREN,J.B., MALTBY,N.H., MACCORMACK,D. & BARNES,P.J. (1989) Pulmonary endothelium-derived relaxing factor is impaired in hypoxia. Clin. Sci., **77**, 671-676.

WEIR,E.K. (1984) Diagnosis and management of primary pulmonary hypertension. In: Pulmonary hypertension. eds. Weir,E.K. & Reeves,J.T., pp115-168.

WEIR,E.K., REEVES,J.T. & GROVER,R.F. (1974) Meclofenamate and indomethacin augment the pulmonary pressor response to hypoxia and exogenous prostaglandin $F_{2\alpha}$. Physiologist, **17**,355p.

WEKSLER,B.B., LEY,C.W. & JAFFE,E.A. (1978) Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and the ionophore A 23187. J. Clin. Invest., **62**, 923-930.

WENNMALM,A., BENTHIN,G. & PETERSSON,A.S. (1992) Dependence of the metabolism of nitric oxide (NO) in healthy human whole blood on the oxygenation of its red cell haemoglobin. Br. J. Pharmacol., **106**, 507-508.

WIDDICOMBE,J.G. & STERLING,G.M. (1970) The autonomic nervous system and breathing. Arch. Intern. Med., **126**, 311-329.

WIKLUND,N.P., PERSSON,M.G., GUSTAFSSON,L.E., MONCADA,S. & HEDQVIST,P. (1990) Modulatory role of endogenous nitric oxide in pulmonary circulation *in vivo*. Eur. J. Pharmacol., **185**, 123-124.

YAMAGUCHI,T., RODMAN,D.M., O'BRIEN,R.F. & McMURTY,I.F. (1987) Potentiation of pulmonary vasoconstriction by inhibitors of endothelium-derived relaxing factor. Annu. rev. Respir. Dis., **135**, A131.

YAMAGUCHI,T., RODMAN,D.M., O'BRIEN,R.F. & McMURTRY,I.F. (1989) Modulation of pulmonary contraction by endothelium-derived relaxing factor. Eur. J. Pharmacol., **161**, 259-262.

YANAGISAWA,M., KURIHARA,H., KIMURA,S., MITSUI,Y., KOBAYASHI,M., WATANABE,T.X. & MASAKI,T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature, **332**, 411-415.

YOSHIDA,Y., SUN,H.T., CAI,J.Q. & IMAI,S. (1991) Cyclic GMP-dependent protein kinase stimulates the plasms membrane Ca^{2+} pump

ATPase of vascular smooth muscle via phosphorylation of a 240-KDa protein. J. Biol. Chem., **266**, 19819-19825.

YUAN,X.J., TOD,M.L., RUBIN,L.J. & BLAUSTEIN,M.P. (1990) Contrasting effects of hypoxia on tension in rat pulmonary and mesenteric arteries. Am. J. Physiol., **259**, H281-H289.

VANHOUTTE,P.M., AUCH-SCHWELK,W., BOULANGER,C., JAMES,P.A., KATUSIC,Z.S., KOMORI,K., MILLER,V.M., SCHINI,N. & VIDAL,M. (1989) Does endothelin-1 mediate endothelium-dependent contractions during anoxia?. J. Cardiovas. Pharmacol., **13** (suppl 5), S124-S128.