5-Hydroxytryptamine, phenylephrine and endothelium-dependent relaxant factor in the rat aorta: effects and interactions.

A thesis presented for the degree of

Doctor of Philosophy

by

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Dedication

I would like to dedicate this thesis to my mother who was always there and to my father who never knew.

"Only at the end is it known what should have been done at the beginning"

Dr V.G. Wilson, 1988

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Declaration

The experimental work and other research contained within this thesis was undertaken wholly by myself except for the work contained in Figures 2.12, 2.13 , 2.14, 2.15, 2.16, 2.17 and 2.18 which was carried out as part of a collaborative project with A.G.B. Templeton. The data shown in Figures 5.15 and 5.16 is presented here by the kind permission of Dr M. Spedding. None of the material has been previously presented for any other degree. Some of the results have been published during the period of this study, details of which are given below.

Publications

DAINTY, I.A., EGLEN, R.M., MCGRATH, J.C. & SPEDDING, M., (1986). Differences in the relaxant effects of 5-HT, 5-CT, and 8-OH-DPAT on the rat aorta and guinea-pig ileum. *Br. J. Pharmacol.*, **88**, 369P

DAINTY, I.A., MCGRATH, J.C. & SPEDDING, M., (1986). A simple, inexpensive method of storage and analysis of data from Pharmacolacological concentration responses. *Br. J. Pharmacol.*, **89**, 884P

DAINTY, I.A., MCGRATH, J.C., MILLER, D.J. & SPEDDING, M., (1987). Ca^{2+} Re-addition distinguishes between phenylephrine and 5-HT in rat aortic rings: differences in α_1- and 5-HT receptor coupling ? *Br. J. Pharmacol.*, **91**, 387P

DAINTY, I.A., DALY, C.J., MCGRATH, J.C., SINCLAIR, J. & SPEDDING, M., (1987). Measurement and analysis of analogue signals from a Linseis chart recorder using a BBC microcomputer. *Br. J. Pharmacol.*, **91**, 506P

DAINTY, I.A., MCGRATH, J.C., SPEDDING, M. & TEMPLETON, A.G.B. (1987). Optimisation of conditions for the demonstration of the influence of endothelium derived relaxing factor on rings of blood vessels. *J. Physiol.*, **392**, 21P

DAINTY, I.A., DALY, C.J., MCGRATH, J.C., SINCLAIR, J. & SPEDDING, M., (1987). A simple, inexpensive method of collection, storage and analysis of data for the BBC microcomputer. *J. Physiol.*, **392**, 22P

DAINTY, I.A., EGLEN, R.M., MCGRATH, J.C. & SPEDDING, M., (1987). Effect of endothelium on responses of rat aorta to 5-HT receptor agonists. Proceedings of the Xth international Congress of Pharmacolacology (September, 1987).

DAINTY, I.A., MCGRATH, J.C. & SPEDDING, M., (1987). The effect of palmitoyl-carnitine on endothelial-dependent relaxations of rat aorta. *Blood Vessels*, **24**, 203

DAINTY, I.A., MCGRATH, J.C. & SPEDDING, M., (1987). The length-tension relationship critically affects the quantitative demonstration of the effects of endothelium on aortic smooth muscle. *Blood Vessels*, **24**, 203-204

DAINTY, I.A., MCGRATH, J.C., SPEDDING, M. & TEMPLETON, A.G.B. (1987). Experimental conditions affect the quantitative demonstration of the effect of basal and stimulated release of EDRF. *Br. J. Pharmacol.*, **92**, 619P

DAINTY, I.A., EGLEN, R.M., MCGRATH, J.C. & WHITING, R.L., (1988). Analysis of the muscarinic receptor on the rat aortic endothelium. *Br. J. Pharmacol*, **93**, 240P

DAINTY, I.A., MCGRATH, J.C., MILLER, D.J. & SPEDDING, M., (1988). Further observation on the effect of Ca^{2+} re-addition in the presence of phenylephrine and 5-HT in rat aortic rings. *Br. J. Pharmacol.*, **95**, 693P

EGLEN, R.M., MONTGOMERY, W.W., DAINTY, I.A., DUBUQUE, L.K. & WHITING, R.L., (1988). The interaction of methoctramine and himbacine at atrial, smoth muscle and endothelial muscarinic receptors in vitro. *Br. J. Pharmacol.*, **95** (**4**), 1031-1038

Summary

The purpose of the work presented in this thesis was to examine the influence of the vascular endothelium on the effect of stimulation of α-adrenoceptors, 5-HT receptors and muscarinic receptors in the isolated rat thoracic aorta. The work has been divided into five main areas, i) the dependence on extracellular Ca^{2+} of the contractions to the relatively selective α_1-adrenoceptor agonist phenylephrine and to 5-hydroxytryptamine and also the effect of the presence of the endothelium on their contractile responses, ii) the role of the initial tension placed on the tissues in the subsequent responses of the tissues to agonists and influence of the endothelium on this 'length-tension' relationship, iii) the effect of the endothelium on activation mediated by 5-HT receptors, iv) classification of the muscarinic receptor mediating endothelium-dependent relaxations in this preparation and v) the effect of a lipid metabolite of ischaemia (palmitoyl carnitine) which may have a modulatory role on the effect of the endothelium in ischaemic conditions. The major findings of the study are briefly summarised below.

1. When rat aortic rings are incubated in PSS with no added Ca^{2+}_o, addition of $CaCl_2$ in the presence of PhE but not 5-HT caused a contraction of the preparation. The contractions to $CaCl_2$ in the presence of 5-HT were much smaller than those observed when 5-HT was added in the presence of 2.5mM $[Ca^{2+}]_o$. In contrast, the responses to PhE were consistent at each concentration of Ca^{2+}_o irrespective of whether the agonist was added before or after Ca^{2+}.

2. Pre-exposure of the preparations to 5-HT but not PhE appeared to attenuate the subsequent contractile response to $CaCl_2$ in the presence of the same agonist.

3. Pre-incubation with BAY K 8644 caused a significant increase in the contraction to $CaCl_2$ in the presence of both 5-HT and PhE. The magnitude of this increase was greatest for '5-HT/Ca^{2+}-re-addition'.

4. Addition of BAY K 8644 at the end of a cumulative concentration-response curve (CCRC) to $CaCl_2$ produced a marked contraction in the presence of PhE and also 5-HT.

5. Mild depolarisation with KCl and hyperpolarisation with BRL 34915 caused similar degrees of potentiation and attenuation of the concentration-response curves to 5-HT and PhE.

6. Chronic pretreatment of the rats with LiCl caused attenuation of the response of the aorta to both PhE and 5-HT. This effect was greatest for PhE and in the presence of the endothelium.

7. The optimum conditions for resting tension (hence length) of the tissue for demonstration of contraction *per se* and the basal release of EDRF (depression of contractility) or stimulated release of EDRF (relaxation) did not necessarily coincide and were dependent upon how the data was expressed.

8. The degree of induced tone influenced the apparent sensitivity of the tissue to endothelium-dependent relaxations such that the higher the degree of induced tone then the lower the subsequent sensitivity to endothelium-dependent relaxations.

9. Removal of the endothelium increased the sensitivity and maximum contractile response of the preparations to both 5-HT and PhE.

10. The depression of the maximum contractile response by the presence of the endothelium was inversely related to the intrinsic activity of a series of 5-HT_2-receptor agonists.

11. There was no evidence for an endothelium dependent relaxation to 5-HT receptor stimulation though an endothelium-independent relaxation at high concentravions of 5-HT and 5-CT was revealed.

12. Using selective antagonists, the muscarinic receptor mediating endothelium-dependent relaxations was found to be of the M_2 muscarinic receptor subtype.

13. The receptor subtype was different from the M_2 subtype found in the atria but similar to that found in non vascular smooth muscle.

14. In contrast to the muscarinic receptor found in non-vascular smooth muscle preparations, the endothelium-dependent relaxations to muscarinic agonists of different efficacies were unaffected by chronic pre-treatment of the animals with LiCl.

15. In addition to reversing the relaxation induced by ACh, palmitoyl carnitine caused concentration-dependent inhibition of the endothelium-dependent relaxations to ACh, ATP and A23187 but had no significant effect on the endothelium-independent relaxations to sodium nitroprusside and did not augment the agonist-induced tone of the preparation.

16. BAY K 8644 reversed and inhibited the ACh-induced relaxations. This effect was accompanied by an increase in the agonist-induced tone of the preparation.

17. Using a simple overflow techniquethe inhibitory action of palmitoyl carnitine was found be at a site at the endothelium to inhibit release/synthesis of EDRF.

General Introduction

The purpose of this introduction is to provide a general overview of aspects of my work which are presented later in this thesis. The introduction comprises: i) a very brief account of the Ca^{2+} requirement for contraction of smooth muscle and the mechanisms by which this Ca^{2+} requirement can be met; and ii) a more comprehensive overview of the discovery and evaluation of nature and effect of endothelium-derived relaxing factor(s) (EDRF).

Each chapter of the thesis has a separate introduction which is intended to give a more precise indication of the topic area covered by that particular section of work and the reasons for carrying out that work.

Ca^{2+} Requirement for Contraction of Smooth Muscle

In common with a number of cellular functions (including cellular secretion), smooth muscle contraction is controlled by fluctuations in the free Ca^{2+} ion concentration in the cytosol. Using chemically skinned smooth muscle preparations which are exquisitely sensitive to the Ca^{2+} concentration of the bathing PSS, it has been shown that activation of the contractile proteins in vascular smooth muscle to produce contraction depends on an increase in the concentration of free Ca^{2+} ions in the cytosol (Filo *et al.*, 1965; Endoh *et al.*, 1977; Gordon, 1978). These studies have shown that the threshold for contraction of the vascular smooth muscle is of the order 0.1μM free Ca^{2+} with a maximal contraction being obtained at around 10μM free Ca^{2+}.

Source of Intracellular Free Ca^{2+}

Studies using photoproteins have shown that the concentration of extracellular free Ca^{2+} ($[Ca^{2+}]_o$) in smooth muscle is in excess of 1000μM (Daniel *et al.*, 1983) whilst the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is around 0.1μM (Defoe and Morgan, 1985; Willaims and Fay, 1986). There exists therefore, a large, inwardly directed

concentration gradient for Ca^{2+} ions of the order of 10 000 fold. This concentration gradient is maintained principally by the low permeability of the sarcolemma under resting conditions, an efficient Ca^{2+} pumping ATPase (an active extrusion pump) and uptake of Ca^{2+} by intracellular organelles such as the sarcoplasmic reticulum and mitochondria. Thus there are both intra- and extracellular pools of Ca^{2+} available for utilisation in the contractile process in smooth muscle and contractions of vascular smooth muscle can be initiated by influx of extracellular Ca^{2+} and/or mobilisation of intracellular Ca^{2+} stores.

Influx of $Ca^{2+}{}_o$

Extracellular Ca^{2+} enters the cell via calcium channels that are anchored in the plasma membrane. Whilst there is a small influx of Ca^{2+} under resting conditions through channels termed 'leak' channels (shown by a small ^{45}Ca influx in the absence of excitation and unaffected by Ca^{2+} entry blockers: Godfraind, 1983; Schramm *et al.*, 1985) the principal route of entry of Ca^{2+} is via two separate types of excitable Ca^{2+} channels. In 1979, both Bolton and van Breemen and co-workers independently postulated the existence of two distinct excitable Ca^{2+} channels in smooth muscle: receptor operated channels (ROC) activated by agonists in a number of preparations without a change in the membrane potential and insensitive to blockade by Ca^{2+} entry blockers; and voltage-operated channels (VOC) activated by a change in membrane potential (see Reuter, 1983; Schramm and Towart, 1985). The separate identity of the ROC and VOC was confirmed by the observation that simultaneous stimulation with 80mM K^+ and NA produced a unidirectional ^{45}Ca influx, which was additive, indicating that the influx was not via a common mechanism (Meisheri *et al.*, 1981). Thus, by receptor activation and/or membrane depolarisation Ca^{2+} channels open in the cell membrane and this results in the influx of Ca^{2+} ions into the cell and subsequent activation of the contractile proteins.

Release of Ca^{2+}_i

As mentioned above another source of Ca^{2+}_i arises from the release of Ca^{2+} from intracellular stores. This release can be seen by a phasic (transient) contraction in the absence of extracellular Ca^{2+} which is accompanied by a transient efflux of ^{45}Ca (Deth and Casteels, 1977; Deth and Van Breemen, 1977; Droogmans *et al.*, 1977). Under these conditions, the tonic component of contraction which is due to influx of Ca^{2+}_o (Godfraind and Kaba, 1972; Yamashita *et al.*, 1977; Godfraind *et al.*, 1982) is abolished indicating that the remaining transient contraction is dependent on the release of Ca^{2+} from intracellular sites. These stores may be located on the inner surface of the sarcolemma and in the mitochondria but it is thought that the stores are principally located in the sarcoplasmic reticulum (Somlyo, 1984).

In 1975, Michell proposed that the mobilisation of Ca^{2+} may arise from agonist-induced breakdown of cell membrane phosphatidylinositol 4,5-bisphosphate (PIP_2). Receptor-stimulated hydrolysis of PIP_2 by agonist-induced activation of phospholipase C generates two second messengers: 1,2 diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3) which modulate the release of intracellular Ca^{2+} and possibly influx of extracellular Ca^{2+} (Berridge, 1984; Berridge, 1987; Taylor, 1987; Penner *et al.*, 1988). DG activates protein kinase C (Nishizuka, 1984) whilst IP_3 can release Ca^{2+} from intracellular pools and inhibit Ca^{2+} extrusion ATPase (Berridge, 1984; Popescu, *et al.*, 1986) producing an increase in the concentration of intracellular free Ca^{2+} which consequently causes contraction of vascular smooth muscle (Suetmatsu *et al.*, 1984; Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986; Popescu, *et al.*, 1986).

Although DG and IP_3 are products of the same substrate and each has separate actions it is likely that these two second messengers act synergistically to produce the physiological effect seen. This has been suggested from studies involving stimulation of each signal pathway by phorbol esters (activating protein kinase C and thereby mimicking the effect of DG) and calcium ionophores such as A23187 (to raise $[Ca^{2+}]_i$

and thereby mimic the effects of IP_3); these produced a maximal response only when both pathways were activated simultaneously (see Rasmussen, 1986).

Thus, the contraction of vascular smooth muscle can be initiated by the influx of Ca^{2+}_o or release of Ca^{2+}_i. Whilst activation by both processes can occur independently, under normal circumstances it is likely that contraction of smooth muscle is a combination of both the release of Ca^{2+}_i and the influx of Ca^{2+}_o though the relative size of these two components will vary between species, preparations and type of stimulus. For example, release of Ca^{2+}_i plays a major role in the activation of rat and rabbit aorta (Godfraind and Kaba, 1972; Deth and Van Breemen, 1977) but not dog basilar or coronary artery (Allen and Banghart, 1979; Van Breemen and Seigl, 1980).

The Vascular Endothelium

The vascular endothelium is the layer of squamous epithelial cells that is in direct contact with the blood. Until the last twenty years this endothelial cell layer was considered to be a nucleated amorphous membrane which functioned as a metabolically inert, non-thrombogenic, semi-permeable barrier between the circulating blood and the interstitial space (Florey, 1966). In subsequent years, following development in techniques such as cell culturing (Jaffe *et al.*, 1973), the possible ability of the endothelium to alter the smooth muscle tone became apparent. The endothelial cells have been shown to be able to inactivate bradykinin, activate angiotensin I to produce angiotensin II, synthesise prostacyclin, and to degrade adenine nucleotides and biogenic amines such as 5-HT and noradrenaline (see Cryer, 1983 and Ryan *et al.*, 1984 for references). In the past eight years a great deal of work has been carried out to evaluate the effects and nature of a vasoactive substance(s) which is released from the vascular endothelium to induce vasodilatation. The remainder of this general introduction will describe the discovery and investigation of this so called endothelium-derived relaxant factor (EDRF).

Endothelium Derived Relaxant Factors(s)

Historical Viewpoint

Prior to a report by Jelliffe (1962) which demonstrated ACh-induced relaxations of rabbit descending thoracic aorta the response to ACh in this preparation was contractile in nature (Furchgott and Bhadrakom, 1953; Furchgott, 1955). These ACh-induced relaxations to ACh, but not other non-muscarinic relaxing agents such as glyceryl trinitrate and isoprenaline, were later shown to be abolished in preparations in which the endothelial cell layer had been disrupted by rubbing the intimal layer of the vascular preparations (Figure I.1; Furchgott and Zawadski, 1980). This partly explained the paradox that whilst ACh induced a depressor response *in vivo*, ACh-induced vasodilatation could not be demonstrated *in vitro*.

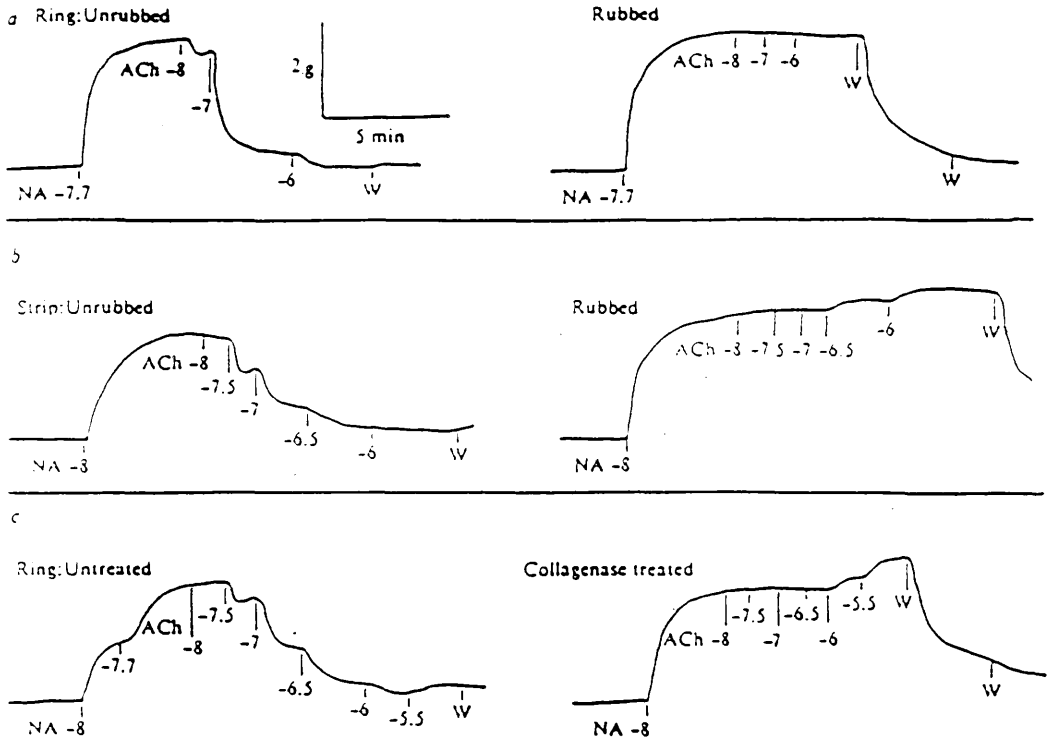


Figure I.1

Loss of relaxant response of preparations of rabbit aorta to ACh after rubbing the intimal surface of the preparations or exposure to collagenase in order to remove the vascular endothelium.

Figure is taken from Furchgott and Zawadzki, 1980.

In this initial study, Furchgott and Zawadski demonstrated that the endothelium-dependent relaxations to ACh were mediated by muscarinic receptors which are assumed to be located on the endothelial cells. In addition, by using a simple 'sandwich' technique, these workers showed that activation of muscarinic receptors in endothelium-intact preparations produced a diffusible substance (or substances) which mediates the observed vasodilatation. In these experiments, ACh-induced relaxations of endothelium-disrupted strips only occurred when an endothelium-intact strip was placed in contact with the rubbed preparation with the intimal surfaces touching. These original observations have been confirmed in a number of studies. Whilst the location and subtype of the muscarinic receptor may be questioned (see discussion, Chapter Four) the release of a vasoactive substance(s) from the endothelium of vascular tissue mediating relaxation is now without doubt. The release of this so called endothelium derived relaxing factor(s) (EDRF; Cherry *et al.*, 1982) has been confirmed by bioassay experiments in which the perfusate flowing through endothelium-intact blood vessels or over cultured endothelial cells is passed over or through pre-contracted, endothelium-disrupted preparations to produce relaxation (Cocks *et al.*, 1983; Gryglewski *et al.*, 1986; Griffith *et al.*, 1984; Rubanyi *et al.*, 1985).

Stimulated release of EDRF

Since the original study by Furchgott and Zawadski, the actions of a variety of substances have been shown to be dependent on the presence of an intact endothelium. These endothelium-dependent relaxations have been shown to occur in most mammalian isolated blood vessels studied including preparations from rat, rabbit, cat, dog, monkey and man and to be induced by a number of vasodilators including acetylcholine, histamine, hydralazine, thrombin, the calcium ionophore A23187, ATP, ADP and also substance P (Table I.1). In addition to these agonist-induced relaxations, endothelium dependent relaxations are also produced by other stimuli such as aggregating platelets, hypoxia and increased blood flow (Table I.1).

Stimulus	Preparation
Acetylcholine	rabbit thoracic aorta[a]; dog femoral artery[b]; human coronary artery[c]; endothelial/smooth cell cultures[d]
A23187	human coronary artery[c]; rabbit aorta[e]; pig aorta[f]; rat aorta[g]; dog femoral artery[b]
Angiotensin II	dog renal and mesenteric arteries[h],
ATP, ADP	dog femoral artery[b]; endothelial/smooth cell cultures[d]; rat aorta[g]; dog coronary arteries[i]
Bradykinin	pig aorta[f]; dog coronary arteries[j]; dog renal and pulmonary arteries[k]
Histamine	rat aorta[g,l], dog renal and mesenteric arteries[h], guinea-pig pulmonary artery[m]
Hydralazine	rat caudal artery[n]; rabbit aorta[o]
5-HT	dog coronary arteries[i,p]; pig coronary arteries[p]
Noradrenaline	pig coronary arteries[p]; dog coronary artery [q]; rat thoracic aorta[r,s]
Substance P	dog coronary arteries[p]; pig coronary arteries[p]; guinea pig mesenteric artery[t]
Thrombin	dog femoral artery[b]; dog coronary artery[u]
Aggregating Platelets	dog coronary artery[v, w]
Hypoxia	dog coronary arteries[x]; rabbit femoral artery[y]
Increased Flow	dog coronary arteries[z]; dog femoral artery[aa]

Furchgott and Zawadski, 1980[a]; De Mey and Vanhoutte, 1981[b]; Ginsberg and Zera 1984[c]; Loeb *et al.*, 1985[d]; Furchgott, 1983[e]; Gordon and Martin, 1983[f]; Toda, 1983[h]; Cohen *et al.*, 1983b[i]; Cherry *et al.*, 1982[j]; Van de Voorde and Leusen, 1983[l]; Satoh and Inui, 1984[m]; Spokas *et al.*, 1983[n]; Spokas *et al.*, 1984[o]; Cocks and Angus, 1983[p]; Matsuda *et al.*, 1985[q]; Lues and Schumann, 1984[r]; Miller and Vanhoutte[s]; Bolton and Clapp, 1986[t]; Cohen *et al.*, 1984[u]; Cohen *et al.*, 1983c[v]; Houston *et al.*, 1985[w]; Busse *et al.*, 1984[x]; Busse *et al.*, 1985[y]; Holtz *et al.*, 1984[z]; Rubanyi *et al.*, 1986[aa]

Table I.1

Examples of stimuli inducing endothelium-dependent relaxations.

Basal Release of EDRF

The presence of the endothelium has been shown to reduce the sensitivity of a number of arterial preparations to a number of agents including 5-HT, angiotensin, $PGF_{2\alpha}$, K^+, BAY K 8644, and α-adrenoceptor agonists (Allan *et al.*,1983; Egléme *et al.*, 1984a,b; Godfraind *et al.*, 1985; Lues and Schumann, 1984; Miller and Stoclet, 1985; Spedding *et al.*, 1987; Carrier and White, 1985). This inhibitory effect of the endothelium is thought to be due to the relaxatory action of EDRF. Both mechanical disruption of the endothelium and haemoglobin, which blocks the actions of EDRF (Martin *et al.*, 1985a, b), potentiate the responses to 5-HT and α-adrenoceptor agonists in endothelium-intact preparations compared with those in endothelium-disrupted preparations (Martin *et al.* 1986a). In the latter study, Martin and co-workers, by reducing the receptor reserve for a full α-adrenoceptor agonist using an irreversible antagonist, showed that agonists with a low relative efficacy were affected to a greater extent than 'full' agonists.

In addition to its vasodilator activity EDRF inhibits platelet aggregation (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Moncada *et al.*, 1987). When platelets do aggregate, the substances released (e.g., 5-HT, ADP, ATP) can induce endothelium-dependent relaxations or contractions of the underlying smooth muscle. However, contractions induced by products of platelet aggregation, e.g. thromboxane, are greater in the absence of endothelium (Cohen *et al.*, 1983b,c; Houston *et al.*, 1985) .

Substances generated during the process of hemostasis can result in endothelium-dependent relaxations. For example thrombin causes endothelium-dependent relaxations in dog femoral, basilar and coronary arteries (De Mey *et al.*, 1982; De Mey and Vanhoutte, 1982; Katusic *et al.*, 1984; Ku., 1982) though this relaxation can be counteracted in many tissues by a direct contractile action of thrombin on the smooth muscle (Ku., 1982; De Mey *et al.*, 1982).

Noradrenaline and selective α_2-adrenoceptor agonists can cause relaxation of canine and porcine coronary and systemic arteries and canine pulmonary arteries and veins if the endothelium is present (Cocks and Angus, 1983; Miller and Vanhoutte, 1984). Therefore, absence or malfunction of the endothelium would favour vasoconstriction due to the action of catecholamines on the α-adrenoceptors of the vascular smooth muscle.

Thus the endothelial cell layer exerts a profound depressent effect on the ability of vasoconstrictor substances in a variety of preparations. This may indicate that EDRF is an important regulator of vascular tone. Moreover, the effect of haemoglobin on this basal release may be of some importance in the pathology of vasospasm particularly in cerebral vessels (Defeudis, 1985).

Mechanism of Action

Following the observation of endothelium-dependent relaxations, the mechanism by which EDRF relaxes vascular smooth muscle was soon elucidated. Endothelium-dependent relaxations to the stimulated release of EDRF are thought to be mediated by an increase in the levels of smooth muscle guanosine 3':5'-cyclic monophosphate (cGMP). EDRF stimulates soluble guanylate cyclase activity in the smooth muscle and increases intracellular cGMP (Holzmann, 1982; Furchgott and Jothianandan, 1983; Rapoport and Murad,1983; Miller *et al.*, 1984; Griffith *et al.*, 1985; Forstermann *et al.*, 1986). This leads to a lowering of the concentration of $Ca^{2+}{}_o$ possibly by stimulation of the plasmalemmal Ca^{2+}-extrusion ATPase and sequestration of free Ca^{2+} by the sarcoplasmic reticulum or by reducing the phosphorylation of myosin light chain (Popescu *et al.*, 1985; Twort and Van Breemen, 1988; Rapoport *et al.*, 1983).

In a similar manner, the presence of a basal release of EDRF was further confirmed by the observation that the smooth muscle cGMP content was greater in endothelium-intact preparations than in preparations with a disrupted endothelium (Holzmann, 1982; Rapoport and Murad, 1983; Furchgott and Jothianandan, 1983; Miller *et al.*,

1984). Moreover, in the rat aorta (in which the tonic influence of EDRF is marked; Martin *et al.*, 1986a) phosphodiesterase inhibitors induce endothelium-dependent relaxations (Martin *et al.*, 1986b).

Recent studies have indicated that some agents that produce endothelium-dependent relaxations also generate endothelium-dependent hyperpolarisations (Bolton and Clapp, 1986; Beny *et al.*, 1986). This transient hyperpolarisation may be independent of the stimulation of guanylate cyclase by EDRF and appears to be due to activation of smooth muscle Na^+/K^+-ATPase or K^+ channels (Feletou and Vanhoutte, 1988; Taylor *et al.*, 1988) by a substance other than nitric oxide (Vanhoutte, 1987; see below for nature of EDRF). One substance which has been shown to activate K^+ channels has been termed endothelium-dependent hyperpolarising factor (EDHF; Taylor and Weston, 1988). The significance of EDHF(s) is dependent upon the species and preparation used (Taylor and Weston, 1988).

Nature of EDRF

The chemical nature of EDRF(s), in contrast to its mechanism of action, was somewhat more difficult to determine. Even now, after eight years of research in a great number of laboratories the precise nature of all EDRF(s) is not certain though recent work has provided evidence for the identity of at least one distinct EDRF.

Part of the problem in the isolation and identification of EDRF was the very labile nature of the substance(s) itself. Using a bioassay technique where a pre-contracted rabbit coronary artery was relaxed by the effluent from an ACh-stimulated endothelium-intact rabbit aorta, Griffith *et al.* (1984), showed that the half life of EDRF was 6.3 seconds. In contrast, using another bioassay technique, Forstermann and co-workers (1985), showed that the half lives of EDRF from rabbit aorta and dog femoral artery were different (24 and 49 seconds respectively) and greater than that reported by Griffith and co-workers. This suggested that there might exist a heterogeneity in the nature of EDRF possibly indicating the existence of more than one EDRF.

Early work by Furchgott and Zawadski (1980), eliminated the possibility that EDRF was bradykinin, prostacyclin, cAMP or cGMP since these substances applied exogenously to the rabbit aorta produced little relaxation. Other cyclo-oxygenase products were eliminated as mediators of endothelium-dependent relaxations since cyclo-oxygenase inhibitors such as indomethacin and aspirin had little effect on ACh-induced relaxations. Since arachidonic acid had been shown to induce the release of EDRF attention was focussed on the possibilty that EDRF might be a non-cyclo-oxygenase product of arachidonic acid.

Phospholipase A_2 (the Ca^{2+} sensitive enzyme producing arachidonic acid from membrane phospholipids) is inhibited by quinacrine. Quinacrine prevents and reverses endothelium-dependent relaxations in a variety of blood vessels from different species including dog femoral artery (De Mey *et al.*, 1982); dog coronary arteries (Rubanyi and Vanhoutte, 1985); rabbit aorta (Furchgott and Zawadzki, 1980; Singer and Peach, 1983); and rat aorta (Van de Voorde and Leusen, 1983). The site of action of quinacrine has been determined by bioassay techniques as being at the endothelium and not via an action on EDRF in transit or at the smooth muscle (Griffith et al., 1984) though this inhibition may be due to an action other than inhibition of phospholipase A_2. Endothelium-dependent relaxations to ACh are also blocked by ETYA (5,8,11,14 Eicosatetraynoic acid) which is an inhibitor of both cycloxygenase and lipoxygenase and also NDGA (nordihydroguiracetic acid) which is an antioxidant that blocks lipoxygenase (see Furchgott et al., 1984). In addition, free radical scavengers such as hydroquinone have also been shown to significantly attenuate endothelium dependent relaxations in the rat aorta (Van de Voorde and Leuson, 1983). These observations prompted Furchgott to speculate that ACh, and other stimulants of EDRF, may activate a reaction sequence where arachidonic acid or an unsaturated fatty acid is released from the membrane phospholipids and is then oxidised by lipoxygenase in the endothelial cells to produce a labile hydroperoxide or free radical intermediate product (Furchgott, 1981).

Whilst the use of the inhibitors described above support this proposal of Furchgott, subsequent studies provided new findings which were in contradiction to this hypothesis. Evidence against the proposal by Furchgott was provided by Furchgott himself (see Cherry *et al.*, 1983; Fuchgott *et al.*, 1984). Using arteries from both rabbit and dog in the presence or absence of cyclooxygenase inhibitirs, Furchgott and co-workers showed that other unsaturated fatty acids such as *cis*-4,7,10,13,16,19-docosohexanoic, oleic, eladeic and *cis*-vaccenic produced endothelium-dependent relaxations. Even stearic acid (a saturated fatty acid) produces graded endothelium-dependent relaxations (Fuchgott *et al.*, 1984). Thus it is unlikely that arachidonic acid acts as a precursor of EDRF but is more likely to interact with the endothelium to induce the release of production of EDRF possibly by increasing membrane fluidity (Cherry *et al.*, 1983).

The possibility that EDRF is a free radical was disputed by Griffith *et al.* (1984). On the basis of the very short half life of EDRF (6.3 secs) and the lack of effect of spin trap reagents and specific oxygen centered radical scavengers these workers suggested that EDRF was neither a lipoxygenase derivative or free radical but an unstable compound with a carbonyl group at or near its active site.

There is a marked parallelism between the effects of EDRF and nitrovasodilators (which are known to be mediated by nitric oxide; NO). Similarities are found in the transient nature of the vasoactive substances, inhibitors of their action including methylene blue which inhibit guanylate cyclase, ferrous hemoproteins and haemoglobin which bind and inactivate these substances (Martin *et al.*, 1985a, 1985b, 1986c) and in their mechanism of relaxation i.e., increasing cGMP levels (Holzmann, 1982; Furchgott and Jothianandan, 1983; Rapoport and Murad,1983; Miller *et al.*, 1984; Griffith *et al.*, 1985; Forstermann *et al.*, 1986). Moreover, the relaxing effects of both EDRF and NO are markedly prolonged by superoxide dismutase (Gryglewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986; Palmer *et al.*, 1987). These similarities prompted Furchgott to suggest that EDRF was in fact NO. More recently, Moncada

and co-workers have confirmed that at least some relaxations induced by EDRF are mediated by the production of NO.

Palmer and co-workers (1987), demonstrated the similarity in properties of NO obtained from NO gas and EDRF obtained from cultured porcine aortic endothelial cells. The comparative pharmacology clearly demonstrated that both agents were indistinguishable. Relaxations to both EDRF and NO were qualitatively similar with a similar half life in oxygenated PSS and down a bioassay cascade (Palmer et. al., 1987). Also both relaxations were blocked by haemoglobin and Fe^{2+} (binding and inactivating the vasoactive moiety), hydroquinone and pyrogallol (generating superoxide ions; O_2^-) and enhanced by superoxide dismutase (SOD) (Hutchinson *et al.*, 1987). In a similar manner the anti-aggregating action of both compounds in platelets was the same (Radomski *et al.*, 1987). Confirmation of the identity of EDRF in these studies was provided by the measurement of NO released by stimulation of cultured endothelial cells with bradykinin using chemoluminescent detection of the product of NO and ozone (Palmer *et. al.*, 1987). This group have also recently presented evidence using cultured endothelial cells that EDRF (NO) is formed as a product of the metabolism of l-arginine (Palmer *et al.*, 1988).

Although the identity of one EDRF has now been confirmed the heterogeneity of the effect of inhibitors of EDRF, species variation in the effects of EDRF and possible release of different EDRFs by various agonists suggests that there are more than one EDRF. Moreover, NO and endogenous EDRF have been shown to have a differential selectivity for different smooth muscles: EDRF relaxes vascular smooth muscles but not respiratory, gastro-intestinal or reproductive smooth muscles whilst NO can relax vascular and gastro-intestinal but not respiratory or reproductive smooth muscles (Shikano and Berkowitz, 1987; Shikano *et al.*, 1987).

Vanhoutte (1987), has suggested that in addition to the EDRF which stimulates guanylate cyclase (NO) another substance (possibly NH_3) released by the endothelium

can produce hyperpolarisation of the vascular smooth muscle. Recently, Taylor and Weston (1988), have suggested the existence of an endothelium-dependent substance which induces hyperpolarisation by activation of K^+ channels which they have named endothelium-dependent hyperpolarising factor (EDHF; see Mechanism of Action (above) for more detailed references on endothelium-dependent hyperpolarisations).

Role of Ca^{2+} in release of EDRF

The presence of extracellular calcium is a pre-requisite for the production of endothelium-dependent relaxations since exclusion of Ca^{2+}_o attenuates endothelium-dependent relaxations (Singer and Peach, 1982; Griffith *et al.* 1984; Long and Stone, 1985; Winquist *et al.*, 1985). Moreover, the requirement of the endothelium for relaxations to the calcium ionophore A23187 in rabbit aorta and its relatively high potency (10-30 times greater than ACh) supports the suggestion that Ca^{2+} plays an important role in producing endothelium-dependent relaxations. In the study by Singer and Peach (1982), eliminating Ca^{2+}_o from the PSS inhibited methacholine-induced relaxation of rabbit aortic rings pre-contracted with phenylephrine. The relaxation to methacholine was inhibited to a lesser degree than that to the ionophore (67% and 97% inhibition of maximal response respectively). This was attributed to the receptor-induced relaxation having greater access to a 'Ca^{2+}-pool' than the ionophore. Calcium channel blockers, verapamil and nifedipine, inhibited the maximum relaxation to both the methacholine and A23187 to the same extent (40-45%). This was unexpected as the ionophore is believed to transfer Ca^{2+} across the membrane directly rather than via the calcium channels. They speculated that this effect might reflect direct interaction between the blockers and A23187.

Rubanyi *et al.* (1985), using the Ca^{2+} channel activators BAY K 8644 and (+) 202, 791, have shown that, in dog femoral arteries, activation of Ca^{2+} channels by subcontractile concentrations of these agents can stimulate the release and augment the action of EDRF. In contrast however, in the rat aorta, BAY K 8644 does not increase

the release of EDRF although the basal release of EDRF depresses the contractile response to the Ca^{2+} channel activator possibly indicating modulation of Ca^{2+} channel function by EDRF (Spedding *et al.*, 1987). Moreover, in contrast to the study by Singer and Peach, some studies have reported a lack of effect of calcium antagonists (Miller *et al.*, 1985; Winquist *et al.*, 1985; Jayakody *et al.*, 1987). Thus although Ca^{2+} is a requirement for the production/release of EDRF the precise involvement of Ca^{2+} channels is still under question.

Endothelium-Dependent Contractile Factor

In addition to the production and release of endothelium-dependent relaxant factors, endothelium-dependent contractions have also been demonstrated.

In a study by Rubanyi and Vanhoutte (1985), the role of the endothelium in the facilitation by anoxia of contractile responses of isolated coronary arteries was examined. They found that coronary arteries without endothelium could be contracted by hypoxia when layered with femoral arteries and veins with endothelium due to the release of a vasoactive substance from the endothelium. Inhibitors of cyclo-oxygenase, lipoxygenase and phospholipase A_2 did not inhibit these responses to hypoxia or the augmentation of noradrenaline-induced contractions by anoxia and hypoxia. In addition, inhibitors of EDRF such as quinacrine and methylene blue did not affect the anoxic augmentation. Moreover, the release of substances inducing endothelium-dependent contractions occurs in spontaneously hypertensive rats (Luscher and Vanhoutte, 1986), stretch in dog basilar arteries (Katusic *et al.*, 1987) and cultured bovine aortic endothelial cells (Gillespie *et al.*, 1985; Hickey *et al.*, 1985). These endothelium-dependent contractions have been attributed to prostaglandins or substances whose identity has not yet been unequivocally established. Recent studies have indicated that the endothelium is capable of releasing a highly potent vasoconstictor substance which has been termed Endothelin (Yanagisawa *et al.*, 1988). In addition, Vanhoutte (1988) has suggested that the superoxide anion ($.O_2^-$) may be

responsible for endothelium-dependent contractions in tissues such as the dog basilar artery.

Thus, although the endothelial cell layer does function as a non-thrombogenic, semi-permeable barrier between the circulating blood and the interstitial space the ability of the endothelium to alter the smooth muscle tone by release of vasodilator substances and also vasoconstrictor substances makes it a highly physiologically significant structure that has become a target for pharmacological manipulation in disease states.

Aims of the Study

The work presented in this thesis examines the influence of the vascular endothelium on the effect of stimulation of α-adrenoceptors, 5-HT receptors and muscarinic receptors in the isolated rat thoracic aorta. Since the depressant effects of the basal release of EDRF may be partly due to modulation of Ca^{2+} channel activity, I have examined the dependence on extracellular Ca^{2+} of the contractions to the relatively selective α_1-adrenoceptor agonist phenylephrine and to 5-hydroxytryptamine and also the effect of the presence of the endothelium on their contractile responses. As a joint study with Dr A.G.B. Templeton, we have examined how the initial tension placed on the tissues affected the responses of the tissues to agonists and whether the endothelium had any influence on this length-tension relationship. The presence of the endothelium was found to depress the contractions to both PhE and 5-HT. This depression of the response to α-adrenoceptor activation has been well characterised. Therefore I decided to examine more closely the effect of the endothelium on activation mediated by 5-HT receptors. There is some doubt as to the type of muscarinic receptor mediating the release of EDRF. Thus, I have used specific antagonists in order to attempt a definitive classification of the muscarinic receptor mediating endothelium-dependent relaxations in this preparation. Finally, I have made a brief study of the effect of a lipid metabolite (palmitoyl carnitine) which may have a modulatory role on the effect of the endothelium in ischaemic conditions.

General Methods

General Methods

Throughout the study male Wistar or Sprague Dawley rats (270-340 g. wt.) were used. The rats were killed by a blow to the back of the head followed by exsanguination or decapitation taking care to avoid stretching the aorta at any stage. The descending thoracic aorta was exposed by removal of the heart and lungs, and cut just above the diaphragm and just below the aortic arch. Whilst *in situ* the aorta was cleared of all connective tissue, removed from the animal and placed in a petri dish containing warm Krebs' physiological salt solution (PSS). In early studies the excised aorta was then cut into 2-3mm long ring segments measured by eye. In later studies however, ring segments precisely 3mm long were cut using a device consisting of a series of dissection blades set at a separation of 3mm by perspex spacers.

When required, endothelium was mechanically removed from ring segments by one of two methods; i) inserting the bent tip of a pair of forceps into the lumen and gently rubbing the intima whilst rolling the ring segment back and forth, ii) gently rolling the ring between thumb and forefinger rubbing the intima of the ring against the opposite intimal wall. In the majority of studies the latter method was used since the use of forceps sometimes caused damage to the vascular smooth muscle as seen by a depressed contractile response to phenylephrine (PhE) compared to that seen in a paired intact vessel (Figure G.1). The presence or absence of a functional endothelium was later demonstrated by the ability or inability of acetylcholine (ACh) to relax the vessel pre-contracted with PhE. In addition, in some cases the presence or absence of an intact endothelium was determined histologically (figure G.2).

Each ring segment was suspended between an isometric force transducer and a wire support in a 10ml isolated organ bath containing PSS. The rings were attached to the transducers by either cotton thread (forming a 'stirrup' holding the ring) or a rigid wire tissue holder (figure G.3). The latter method was preferred since the resultant agonist

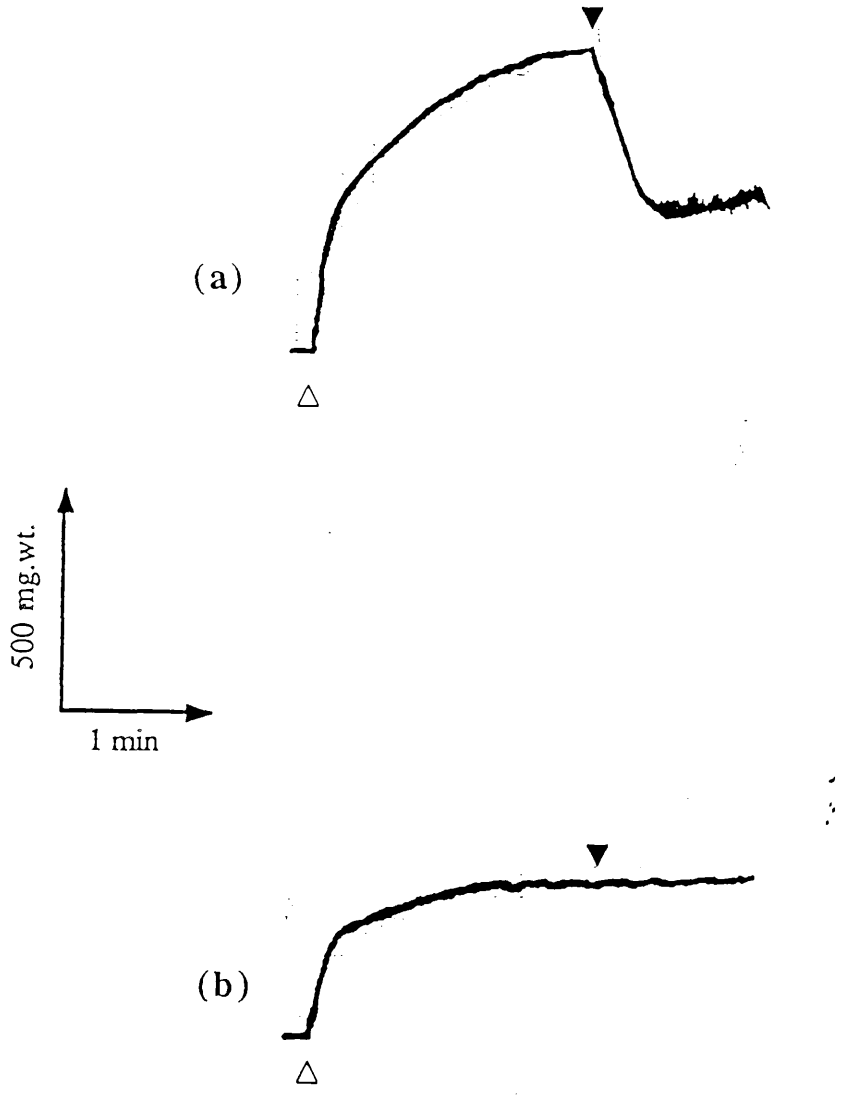
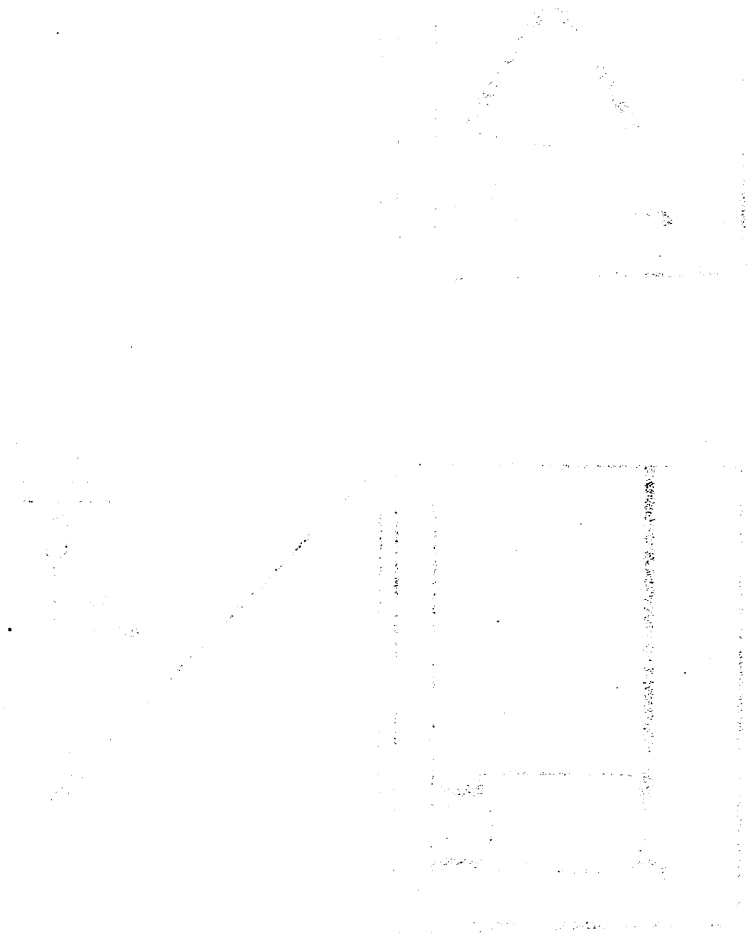


Figure G.1

Response of the rat aortic to 1μM PhE (△) and 1μM ACh (▼) in preparations with (a) an intact endothelium and (b) an endothelium disrupted by rubbing the intimal surface of the ring segment with a pair of curved forceps. PhE produced contraction of the aorta which was smaller in the rubbed preparation. In the unrubbed preparation only, ACh produced relaxation of induced tone indicating the presence of functional endothelium.

The depressed contraction in the rubbed preparation indicates that the vascular smooth muscle has been damaged by over-zealous rubbing of the intima whilst attempting to remove the endothelium since removal of the basal release of EDRF by rubbing would normally increase contractile responses (see Chapter 1).



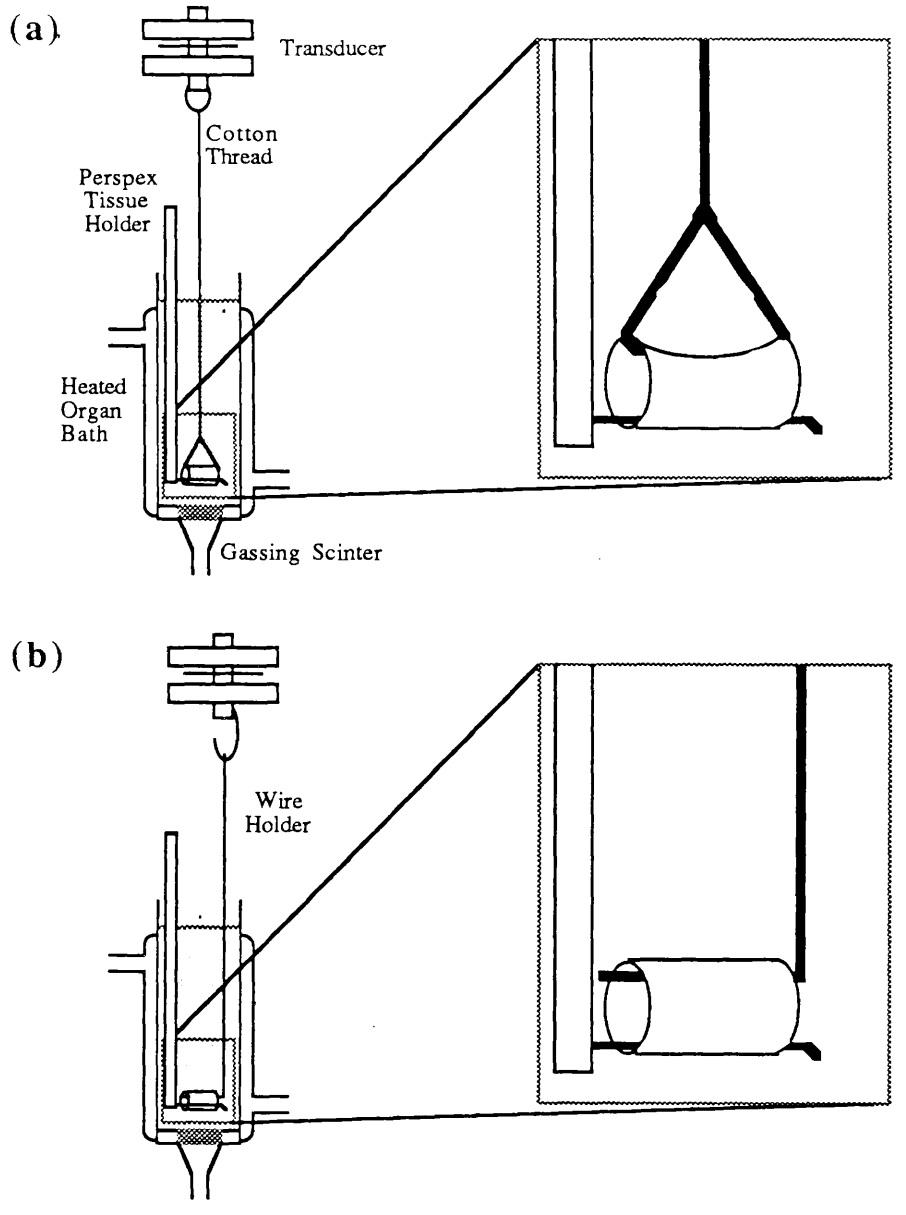


Figure G.3

Diagram representing the method of suspension of a rat aortic ring segment in an isolated organ bath between an isometric tension transducer and lower wire tissue holder by (a) cotton thread and (b) a wire tissue holder. As can be seen in (a) the use of a cotton 'stirrup' tended to lead to a 'bowing' of the aortic ring segment. When a wire tissue holder was used (b) the upper and lower edges of the aortic ring are kept parallel. This bowing of the ring segment may be responsible for the depressed size of contractions of the preparation when used.

induced contractions were significantly greater than those seen using the former method presumably due to the bowing of the tissue on the 'stirrup'.

Unless otherwise stated the PSS used had the following composition (mM): NaCl 118.4, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, Glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2.6H_2O$ 2.5 and was aerated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37°C .

Unless otherwise stated, after suspension in the organ baths the ring segments were washed at 5 minute intervals for 15 minutes by twice exchanging the PSS in the bath. This schedule was used as the standard wash cycle throughout the study. During this initial procedure, the resting tension of the rings was re-adjusted to 1000mg. wt. following each wash at 5 minute intervals, and then left unchanged throughout the remainder of the experiment. The tissues were allowed to equilibrate for a further 15 minutes after which time each was exposed to PhE (1-10μM). A sufficient time for the contractile response to reach a plateau was allowed and then ACh (1-10μM) was added to the organ bath and any relaxation observed. After any relaxant response to ACh had reached equilibrium the tissues were again washed according to the above schedule. Unless stated otherwise each preparation was left for 45 minutes after washing before any further treatment was given. When required, cumulative concentration response curves (CCRC) were obtained by cumulative addition of agonist in 0.5 log Molar increments (Van Rossum, 1963).

Isometric tension changes (mg. wt.) were measured using either Harvard, Grass FTO3 or H.S.E. K30 force transducers and recorded on either a Linseis six channel or a Watanabe WB4101 chart recorder. All data was stored on a BBC microcomputer using a program system developed by myself (DATAFILE - see Appendix A). In addition, when using the Linseis chart recorder, data was collected directly from the recorder using DATAFILE and later 'measured' on a BBC microcomputer. The stored data was then processed on the BBC microcomputer (calculation of percentage

response values, EC_{50} values etc) before transfer to an Apple Macintosh microcomputer for further processing, statistical analysis and plotting.

Statistical Analysis

Unless stated otherwise values given are the arithmetic mean with the ± standard error of the mean. All statistical analysis was carried out on the Apple Macintosh microcomputer using the software application StatWorks™. When necessary, statistical comparison of group means was made using Student's *t* test for paired or unpaired data where appropriate. Statistical analysis of correlations was carried out for two dependent variables and slopes of Schild plots were determined using linear regression analysis.

A probability level of $P<0.05$ was taken as being statistically significant from e.g. control values. Probability levels are denoted as follows:

* $P<0.05$

** $P<0.01$

*** $P<0.005$

Drugs

Compound	Source	Abbreviation
Acetylcholine chloride	Sigma	ACh
Atropine sulphate	BDH	Atropine
BAY-K 8644	Bayer	
Bethanecol	Sigma	
BRL 34195	Beecham	
5-carboxamidotryptamine	Syntex	5-CT
4DAMP	Syntex	
Himbacine	Syntex	
5-Hydroxytryptamine creatinine sulphate	Sigma	5-HT
Imiloxan	Syntex	
Indomethacin	Sigma	
Methoctramine	Syntex	
5-methyltryptamine hydrochloride	Sigma	5-MeT
N-methyltryptamine	Sigma	N-MeT
Noradrenaline	Sigma	NA
Palnitoyl-*dl*-carnitine	Sigma	PC
Panuramine hydrochloride	Wyeth	Panuramine
l-Phenylephrine hydrochloride	Sigma	PhE
Pilocarpine	Sigma	
Pindolol	Sandoz	
Pirenzepine	Syntex	
Sodium nitroprusside	Sigma	SNP
U46619	Upjohn	

Drugs were dissolved in distilled water with the following exceptions: Nifedipine and BAY-K 8644 were dissolved in absolute alcohol. Approximately 7mg of panuramine was dissolved in di-methyl formamide (200µl). Concentrated hydrochloric acid (40µl) was added to this solution and then the final concentration (1mM) was made by addition of distilled water. This was then added when required to the PSS to produce a concentration of 0.1µM.

Chapter One

Contractions of the rat aorta to phenylephrine and 5-hydroxytryptamine: dependence on extracellular Ca^{2+} and effect of lithium pre-treatment.

Introduction

The smooth muscle of rat aorta can be contracted by adrenoceptor agonists or 5-HT (Krishnamurty, 1971) by activation of α-adrenoceptors or 5-HT-receptors respectively. Whilst the subtype of α-adrenoceptors in the rat aorta has been questioned it is generally accepted that the predominant subtype present is the α_1-adrenoceptor (Ruffolo *et al.*, 1980; Randriantsoa *et al.*, 1981; Godfraind *et al.*, 1982; Digges and Summers, 1983; Downing *et al.*, 1983). Contractions to 5-HT are mediated by a distinct receptor (Downing *et al.*, 1983; Doggrell, 1987) which has been classified as a 5-HT_2 subtype (Cohen *et al.*, 1981).

The common factor involved in agonist-induced contraction is an increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$). This effect can occur via an increase in the influx of extracellular Ca^{2+} ($Ca^{2+}{}_o$) and/or the release of intracellular Ca^{2+} ($Ca^{2+}{}_i$) from intracellular binding sites. In the rat aorta, activation of both α_1-adrenoceptors and 5-HT_2- receptors can cause the release of $Ca^{2+}{}_i$ and influx of $Ca^{2+}{}_o$ as seen by a two component contractile response: the second, tonic phase (due to the influx of $Ca^{2+}{}_o$) in contrast to the first phasic component (due to the release of $Ca^{2+}{}_i$) is abolished in the absence of extracellular Ca^{2+} (Godfraind and Kaba, 1972; Yamashita *et al.*, 1977; Godfraind *et al.*, 1982; Downing *et al.*, 1983; Hughes and Doggrell, 1985; Chiu *et al.*, 1985; Doggrell, 1987).

The influx of $Ca^{2+}{}_o$ into the cytosol can occur via voltage operated Ca^{2+} channels (VOCs) and receptor operated Ca^{2+} channels (ROCs) located in the outer cell membrane (Bolton, 1979; Zschauer *et al.*, 1988; Van Breemen *et al.*, 1979; Meisheri, *et al.*, 1981). Receptor linked hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) leads to the formation of inositol 1,4,5-trisphosphate (IP_3) This produces an increase in the free $[Ca^{2+}]_i$ by releasing $Ca^{2+}{}_i$ from intracellular stores and also by inhibiting Ca^{2+} extrusion ATPase (Berridge, 1984; Popescu, *et al.*, 1986) which consequently causes contraction of vascular smooth muscle (Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986;

Popescu, *et al.*, 1986). In the rat aorta activation of both α_1-adrenoceptors (Legan *et al.*, 1985; Chiu *et al.*, 1987) and 5-HT_2 receptors (Roth *et al.*, 1984; Nakaki *et al.*, 1985; Doyle *et al.*, 1986) can activate phospholipase C hydrolysis of PIP_2 to produce IP_3 and release intracellularly stored Ca^{2+}.

In this part of the study I have compared the requirement for extracellular Ca^{2+} of the responses to 5-HT and the α_1-adrenoceptor agonist PhE. Using lithium (which leads to a disruption of the hydrolysis of inositol phospholipids; Sherman *et al.*, 1986) I have attempted to examine indirectly the possible involvement of receptor mediated hydrolysis of inositol phospholipids in contractions to these agonists. Preliminary studies indicated that PhE and 5-HT had different requirements for extracellular Ca^{2+}. Since the attenuation of agonist-induced contractions by basal release of EDRF may be due, at least in part, to alteration of Ca^{2+} fluxes (Malta *et al.*, 1986) I have also examined the effect of the presence of the vascular endothelium on contractions to PhE and 5-HT.

Methods

Male Wistar rats (290-340g) were killed by stunning followed by decapitation. Ring segments (2-3mm) of aorta were prepared as described in General Methods. When required, the endothelial cell layer was disrupted by gently rolling the ring between thumb and forefinger rubbing the intima of the ring against the opposite intimal wall. For all other rings care was taken to maintain an intact endothelium and the ring segments were suspended in 10ml isolated organ baths in PSS maintained at 37°C for isometric tension recordings.

For Ca^{2+} dependency experiments a modified PSS was used with a low concentration of PO_4^- (KH_2PO_4, 0.12mM) in order to allow the use of a high concentration of Ca^{2+}_o: for all other experiments a normal PSS was used (see General Methods).

Ca^{2+} Dependency of PhE and 5-HT

Ring segments with a disrupted endothelium were used in this part of the study. The effect of re-addition of Ca^{2+}_o in the presence of PhE or 5-HT was examined. Preparations were first tested for responsiveness to PhE or 5-HT in 2.5mM Ca^{2+}_o and removal of the endothelium demonstrated by the inability of the contracted tissue to relax to 1μM ACh. PhE (0.1μM) and 5-HT (3μM) were selected as equipotent and equieffective concentrations, i.e., in 2.5mM Ca^{2+}_o these concentrations produced similar sized contractions which were approximately 50 percent of the maximum response to that agonist. For Ca^{2+} re-addition experiments the following protocol was used: the $[Ca^{2+}]_o$ was reduced to 'nominal zero' by 'washing' the contracted tissue with PSS containing no added Ca^{2+} and left to equilibrate in this solution for 30 minutes. Either PhE or 5-HT was then added and 3 minutes later $CaCl_2$ was added cumulatively (0.01-20mM) over a 15-20 minute period. In some preparations, following cumulative addition of $CaCl_2$ in the presence of 3μM 5-HT to produce a final concentration of 3mM Ca^{2+}_o, PhE (0.1μM) was added to the bath before washing the preparations and any contraction observed.

The effect of adding PhE and 5-HT in the presence of 'fixed' concentrations of Ca^{2+}_o was examined. Ring preparations were contracted with PhE (0.1μM) or 5-HT (3μM) in PSS containing 2.5mM Ca^{2+}_o. The contracted tissues were 'washed' with 2.5mM Ca^{2+} PSS, the bathing solution was reduced to the appropriate $[Ca^{2+}]_o$ and left for 15 minutes to equilibrate before the agonist was added. This was repeated for the whole range of $[Ca^{2+}]_o$ examined (2.5-0.16mM).

The effect of pre-exposure to PhE or 5-HT on subsequent contractions to Ca^{2+} re-addition in the presence of the agonist was also examined. Four ring preparations from each rat were used for this part of the study. Two of the preparations were maximally contracted with 10μM PhE and two with 30μM 5-HT. The absence of a functional endothelium was demonstrated by addition of 1μM ACh and the preparations were prepared for Ca^{2+} re-addition in the manner described above. Ca^{2+} re-addition was then carried out in the presence of PhE in tissues which had been maximally contracted previously with PhE or 5-HT. In a similar manner, Ca^{2+} re-addition was then carried out in the presence of 5-HT in tissues which had been maximally contracted previously with PhE or 5-HT.

Effects of BAY K 8644

In some experiments 1μM BAY K 8644 (a concentration shown to produce maximal contractions in partially depolarised rat tail artery; Su *et al.*, 1984) was added 10 minutes prior to addition of 5-HT or PhE and then re-addition of Ca^{2+}. The effect of addition of 1μM BAY K 8644 at the end of a CCRC to re-addition of Ca^{2+} in the presence of 5-HT or PhE was also examined.

Effects of Potassium and BRL 34915

Since experiments using BAY K 8644 showed a differential effect on the responses to re-addition of Ca^{2+} in the presence of 5-HT and PhE the effects of procedures designed to alter the smooth muscle membrane potential (and therefore VOC function)

on contraction to 5-HT and PhE in 2.5mM Ca^{2+} PSS was examined. Ring segments with a disrupted endothelium were used in this part of the study. Four ring segments from each rat were used: two were kept as controls whilst the others were partially depolarised by increasing the $[K^+]_o$ with addition of KCl to the PSS to create a final, subcontractile concentration of 20mM. Following 45 minutes equilibration in high K^+ PSS or control PSS, CCRCs to PhE and 5-HT were obtained as described in the General Methods in the presence or absence of 20mM KCl.

In a separate group of preparations the K^+ channel activator BRL 34915 was used to produce partial hyperpolarisation (Southerton *et al.*, 1987) of the rat aortic smooth muscle. Five preparations from each rat were used: one preparation was used as a control and the other four preparations were incubated in PSS containing 0.01μM, 0.1μM, 1μM or 10μM BRL 34915 for 20 minutes before obtaining a CCRC to 5-HT. The preparations were washed and left to equilibrate for 30 minutes before repeating the procedure with PhE as the agonist.

For the remainder of the study, PSS with a normal composition (see General Methods) and 2.5mM $[Ca^{2+}]_o$ was used.

Effect of Endothelium

The effect of the presence of a functional endothelium on contractions to PhE, 5-HT and the thromboxane-mimetic U46619 (11α, 9α, -epoxymethano-prostaglandin H_2; Coleman *et al.*, 1981) was examined. Paired rubbed/unrubbed ring preparations were used. After demonstration of the presence of a functional endothelium CCRCs to 5-HT and then PhE were obtained in the same preparation with a 45 minutes equilibration period between CCRCs. When U44619 was used only one CCRC was obtained since it is difficult to 'wash out' this agonist. The responses in endothelium-intact and disrupted preparations were compared.

Effects of Lithium

In order to examine if there is a differential utilisation of receptor-mediated phosphatidylinositol hydrolysis in the contractions induced by 5-HT and PhE, the effect of chronic pre-treatment of rats with lithium on subsequent agonist-induced contractions of the aorta with intact and disrupted endothelium was examined. Four aortic ring segments from rats chronically pretreated with LiCl ('treated' preparations) or rats receiving no pre-treatment ('control' preparations) were prepared as normal. The endothelium of two of these preparations was disrupted by rubbing whilst care was taken to avoid disruption of the endothelium in the remaining rings. After the initial setting up procedure and demonstration of the presence of a functional endothelium, CCRCs to 5-HT followed by PhE were obtained in control and treated preparations with a 45 minutes equilibration period between each CCRC. The responses in control and treated preparations to each agonist in the presence and absence of a functional endothelium were compared.

Animal Pretreatment

The protocol used for pre-treatment of animals was that described by Eglen *et al.*, (1987). Animals were given 6.8 mmol kg^{-1} LiCl dissolved in distilled water by intra-peritoneal injection at the same time each day for four consecutive days (control animals received no pretreatment). The dose was given by two injections of a relatively dilute LiCl solution (3.4 mmol ml^{-1} in a volume of 1 ml kg^{-1} body weight), one on each side of the abdomen. Eighteen hours following the last injection the rat was killed as described above, blood collected for determination of the plasma lithium concentration and the aorta excised in the normal manner. Pre-treatment of the rats with LiCl in this manner causes natriuresis and therefore the rats were allowed access to water and 0.9% NaCl (%$_v$) throughout the treatment period. In experiments where the rats were pre-treated with myoinositol, the myoinositol was administered by addition to the drinking water at a concentration of 30mM. For practical reasons (limitations of availability of additional animal housing which would have allowed a

more comprehensive control protocol), these rats were allowed access to 0.45% NaCl ($^{w}/_{v}$) only (containing myoinositol for treated rats and no myoinositol for the control rats).

The sensitivity of the preparation to agonist-induced contraction was estimated as the concentration of agonist producing 50 percent of the agonist's own maximum contraction (EC_{50}) and expressed as its negative $\log_{10}$, i.e. the pEC_{50}. Differences in the sensitivity of the agonist between stated treatments or conditions were expressed as $\Delta\ pEC_{50}$ where:

$$\Delta\ pEC_{50} = pEC_{50\ (control)} - pEC_{50\ (test)}$$

Results

Ca^{2+} Dependency of PhE and 5-HT

In aortic ring segments with a disrupted endothelium incubated in PSS containing nominally zero $[Ca^{2+}]_o$, addition of 0.1μM PhE or 3μM 5-HT produced a small transient contraction (Figure 1.1). Re-addition of Ca^{2+} (0.01-3mM) in the presence of PhE caused a concentration dependent contraction of the preparations. Further addition of $CaCl_2$ to produce higher concentrations of Ca^{2+}_o (10-20mM) resulted in a decrease in the Ca^{2+}-induced tone. In marked contrast, whilst addition of 3μM 5-HT in 2.5mM Ca^{2+} produced a relatively well maintained contraction, re-addition of Ca^{2+} (0.01-20mM) in the presence of this concentration of 5-HT produced little increase in smooth muscle tension (Figure 1.1). At all concentrations of added $CaCl_2$ the contraction induced in the presence of PhE was significantly greater ($P<0.05$; Student's unpaired *t test*) than that seen in the presence of 5-HT (Figure 1.2). Addition of 0.1μM PhE after obtaining a CCRC to $CaCl_2$ in the presence of 5-HT resulted in a rapid contraction of the ring preparation even though there was no marked contraction seen to the re-addition of Ca^{2+} itself (Figure 1.3).

When 5-HT or PhE were added to PSS containing a 'fixed' concentration of Ca^{2+}, both agonists produced a contraction the size of which increased with increasing Ca^{2+}_o concentration (Figure 1.4). The size of the contractions to PhE were not significantly different from those to 5-HT ($P>0.05$; Student's unpaired *t test*) except at the lowest concentrations of Ca^{2+} used (Figure 1.4). Thus, when Ca^{2+} was 're-added' in the presence of 5-HT but not PhE, the tissue produced unexpectedly small responses at sub-physiological and physiological concentrations of Ca^{2+}_o. These responses were considerably smaller than the responses seen with PhE present and much smaller than that observed when 5-HT was added in the presence of 2.5mM Ca^{2+} (Figure 1.5). In contrast, the responses to PhE were consistent at each concentration of Ca^{2+}_o irrespective of whether the agonist was added before or after Ca^{2+} (Figure 1.5).

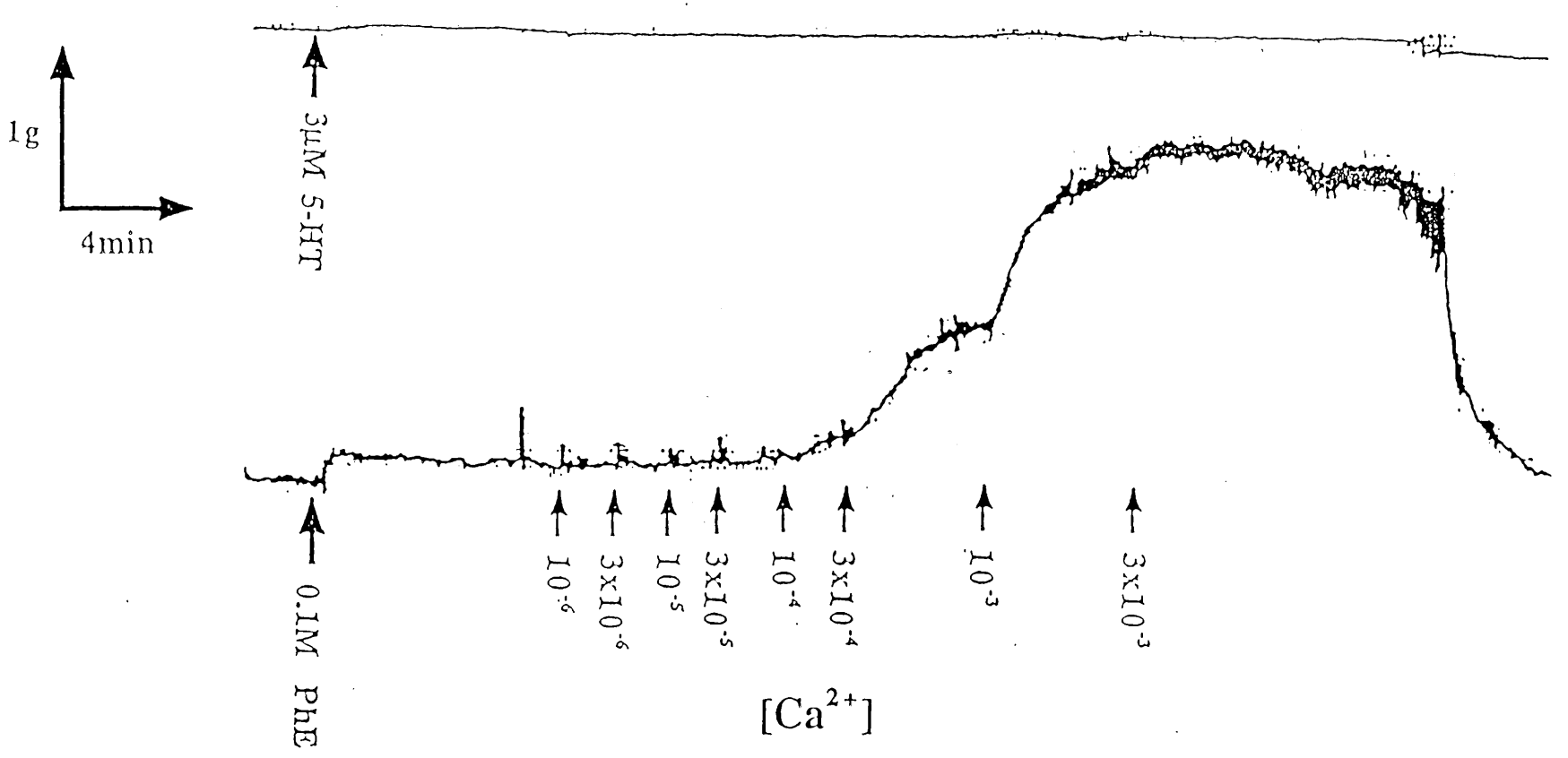


Figure 1.1

Contractile response of rat aortic rings to re-addition of extracellular Ca^{2+}, in the presence of 3μM 5-HT or 0.1μM PhE. $CaCl_2$ (0.001-3mM) was added cumulatively in 0.5 $\log_{10}$ molar increments to paired aortic rings.

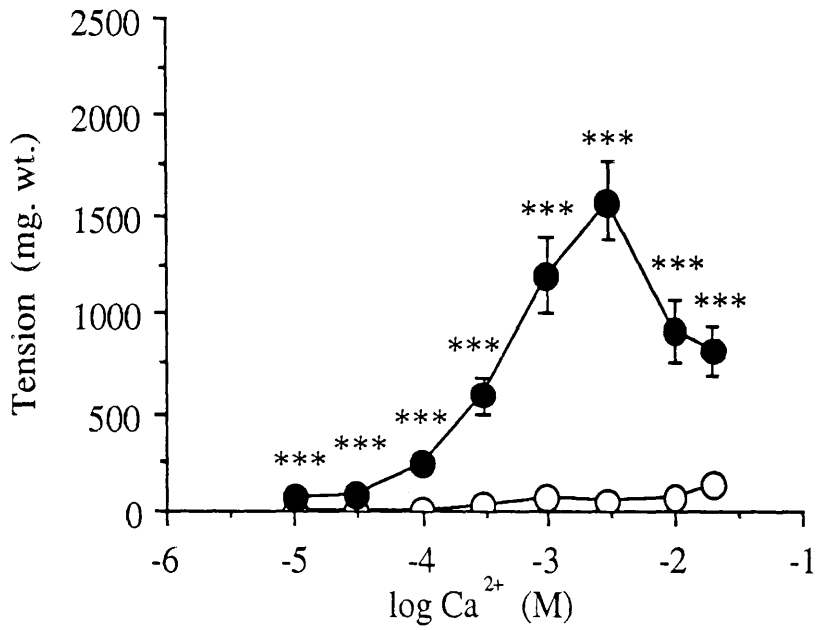


Figure 1.2

Effect of re-addition of $[Ca^{2+}]_o$ in the presence of 0.1μM PhE (●) or 3μM 5-HT (○) in endothelium-denuded aortic ring preparations previously contracted in 2.5mM $[Ca^{2+}]_o$ with 10μM PhE or 30μM 5-HT.

Points shown are mean ± s.e.m. (n=9). Statistically significant differences between $[Ca^{2+}]_o$ re-addition response in the presence of PhE and 5-HT using unpaired Student's *t test*; *** $P<0.005$

Figure 1.3

Representative trace showing contractile response to 0.1μM PhE (△) following re-addition of Ca^{2+} in the presence of 3μM 5-HT (▲). For clarity, the cumulative addition of Ca^{2+} which caused no contraction of the tissue has not been shown.

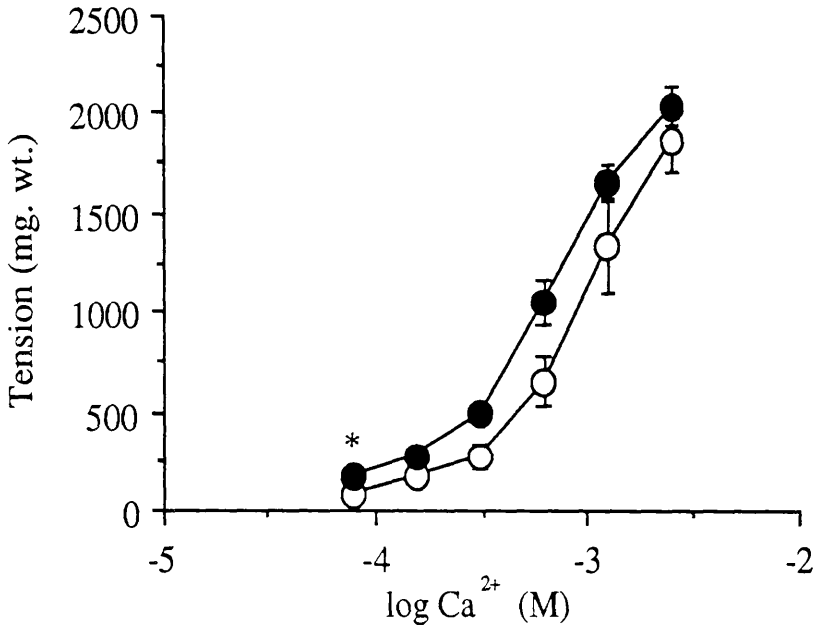


Figure 1.4

Contractions of endothelium-disrupted rat aortic rings to 0.1μM PhE (●) and 3μM 5-HT (○) in PSS containing a fixed concentration of $[Ca^{2+}]_o$.

Points shown are mean ± s.e.m. (n=9). Statistically significant differences between contractions to PhE and 5-HT using unpaired Student's *t test*; * $P<0.05$

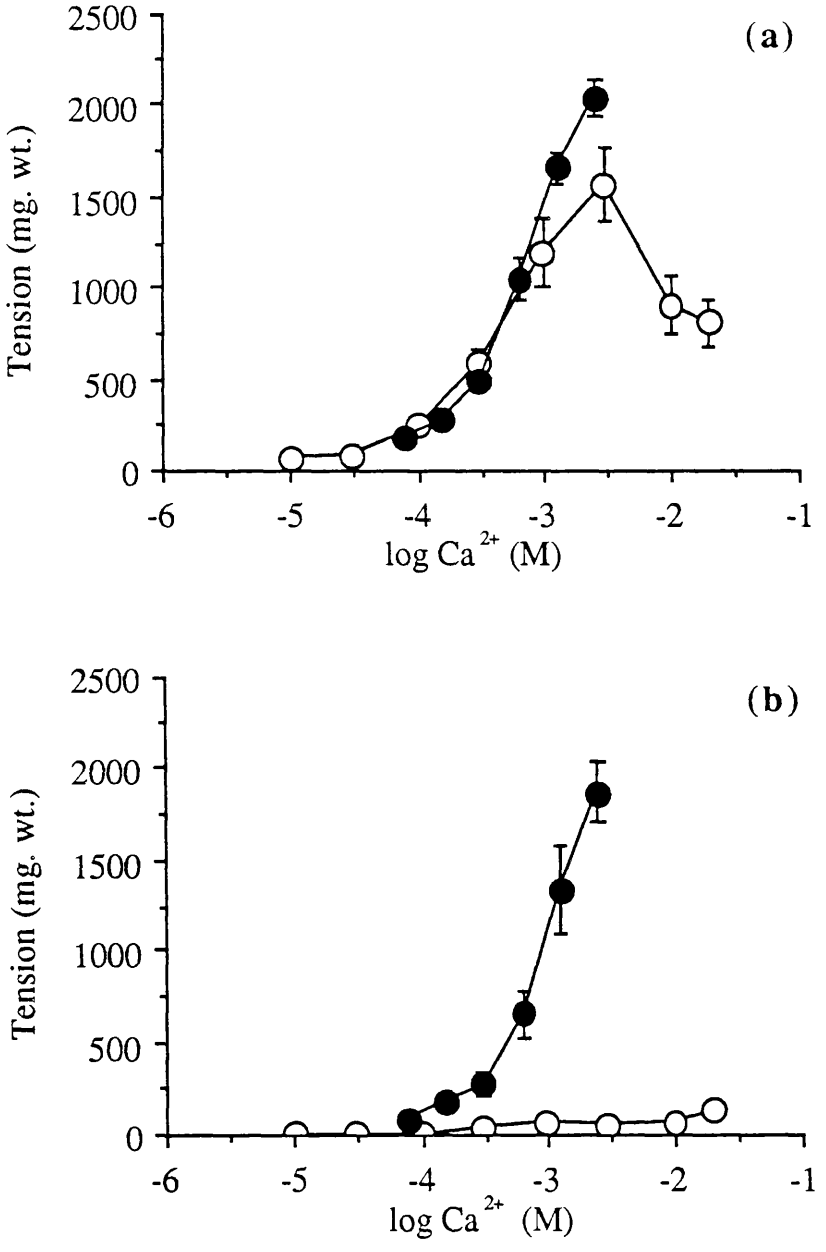


Figure 1.5

Contractions of endothelium-disrupted rat aortic rings to (a) PhE and (b) 5-HT. Data represents the contraction to re-addition of $[Ca^{2+}]_o$ in the presence of the agonist (○) and also addition of the agonist in a fixed concentration of $[Ca^{2+}]_o$ (●).

Points shown are mean ± s.e.m. (n=6-9).

There was no significant difference (P>0.05; Students unpaired *t test*) in the response to Ca^{2+} re-addition at each concentration of Ca^{2+}_o tested in the presence of PhE whether the preparations had been previously exposed to PhE or 5-HT (Figure 1.6). However, the response to re-addition of Ca^{2+} (0.3-10mM) in the presence of 5-HT was significantly greater in tissues which had been previously contracted with PhE but not 5-HT (Figure 1.6).

Effects of BAY K 8644

Incubation of ring preparations with 1μM BAY K 8644 for 20 minutes prior to re-addition of Ca^{2+} had no effect on the tension of the tissue *per se* but caused a significant increase (P<0.05; Student's unpaired *t test*) in the contractile response seen in the presence of both PhE and 5-HT (Figure 1.7). This effect was significantly greater for 5-HT than for PhE (Figure 1.7). Moreover, addition of BAY K 8644 at the end of a CCRC to $CaCl_2$ produced a marked contraction in the presence of 5-HT and a reversal (contraction) of the decreased tension induced by high concentrations of Ca^{2+}_o in the presence of PhE (Figure 1.8).

Effects of Potassium and BRL 34915

Raising the $[K^+]_o$ of the bathing PSS to 20 mM had no effect on the resting tension of ring preparations. This partial depolarisation with KCl caused a significant increase in both PhE- and 5-HT-induced contractions (Figure 1.9). The maximum responses to both PhE and 5-HT were not significantly different (P>0.05; Student's unpaired *t test*) in either normal or high K^+ PSS (Figure 1.10, Table 1.1). In a similar manner, the sensitivity of the preparation (pEC_{50}) to both agonists was significantly greater in high K^+ PSS (P<0.05; Student's unpaired *t test*) compared with that in normal PSS (Figure 1.10, Table 1.1). However, the change in sensitivity (i.e., Δ pEC_{50}) for PhE and 5-HT was similar (Figure 1.11, Table 1.1). Thus, the potentiation of the contractile response to both 5-HT and PhE in terms of increased maximum response and increased sensitivity of the preparations to the agonists was similar.

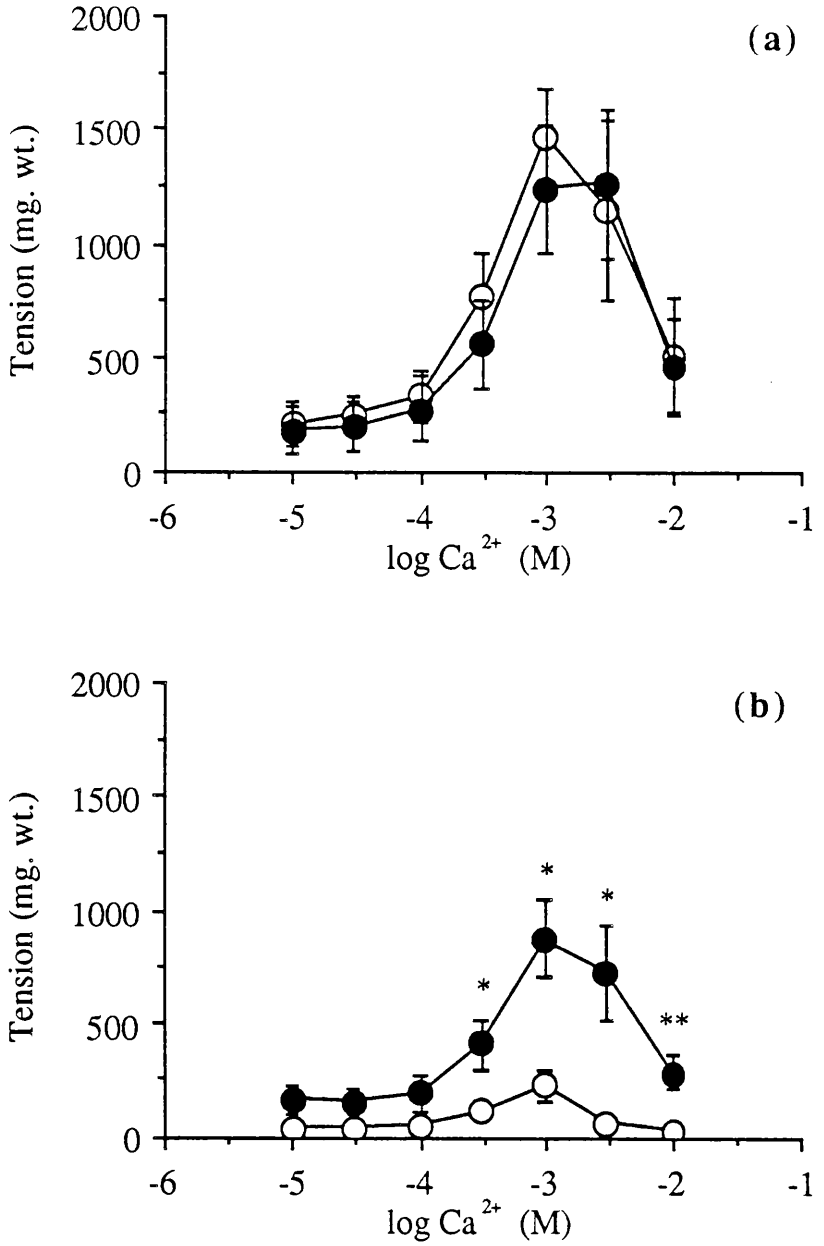


Figure 1.6

Effect of re-addition of $[Ca^{2+}]_o$ in the presence of (a) 0.1μM PhE or (b) 3μM 5-HT in tissues previously contracted in PSS containing 2.5mM $[Ca^{2+}]_o$ with 30μM 5-HT (○) or 10μM PhE (●).

Points shown are mean ± s.e.m. (n=4). Statistically significant differences between tissues previously exposed to PhE and 5HT using Student's unpaired *t test*; * $P<0.05$, ** $P<0.01$

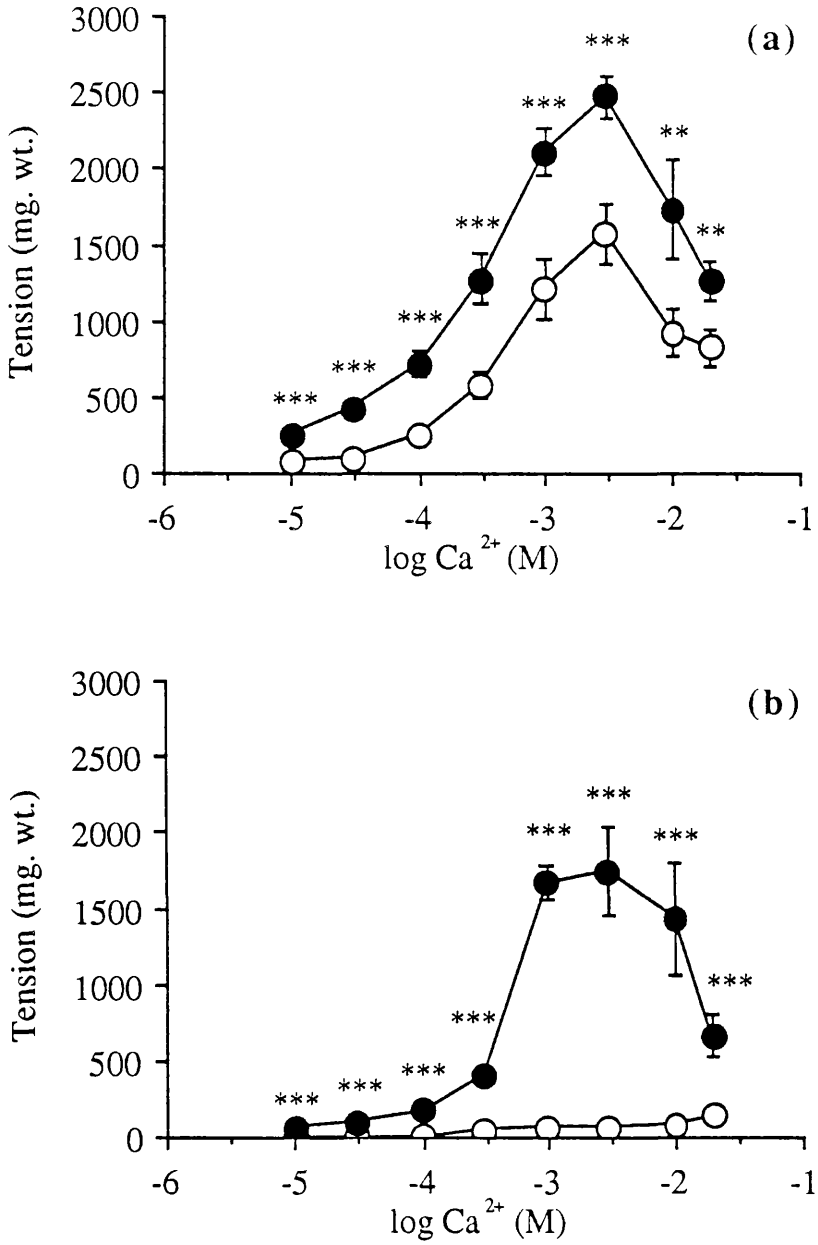


Figure 1.7

Contractile response of rat aortic rings to re-addition of $[Ca^{2+}]_o$ in the presence of (a) 0.1μM PhE or (b) 3μM 5-HT in control preparations (○) and preparations pre-incubated with 1μM BAY K 8644 (●).

Points shown are mean ± s.e.m. (n=6-9). Statistically significant differences between contractile responses in the presence and absence of BAY K 8644 using Student's unpaired *t test* ; ** P<0.01, *** P<0.005

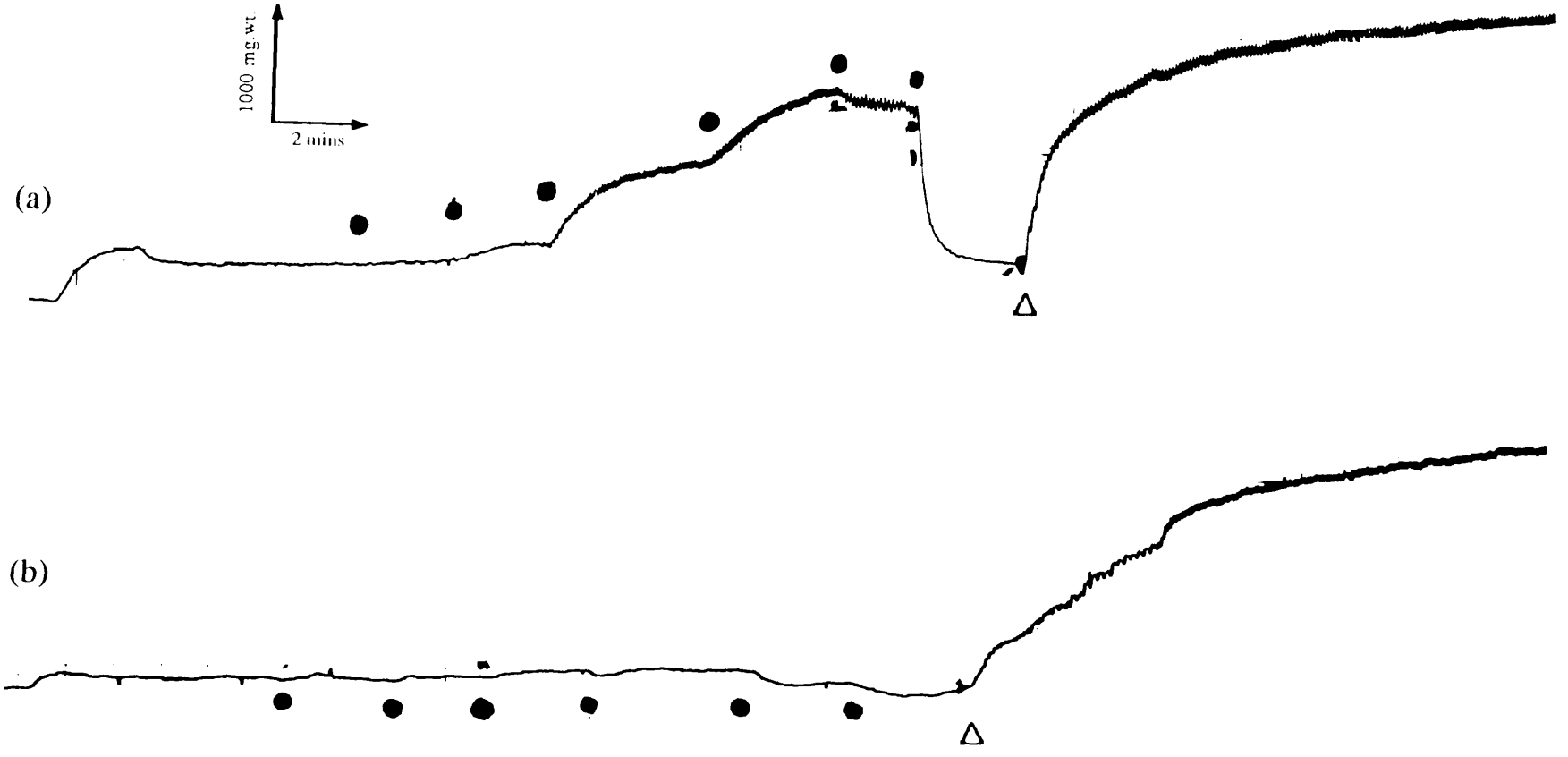


Figure 1.8

The effect of BAY K 8644 (Δ) following re-addition of Ca^{2+} in the presence of (a) PhE or (b) 5-HT. Ca^{2+} (0.03-10mM) was added cumulatively in 0.5 $\log_{10}$ molar increments indicated by the closed circles (●).

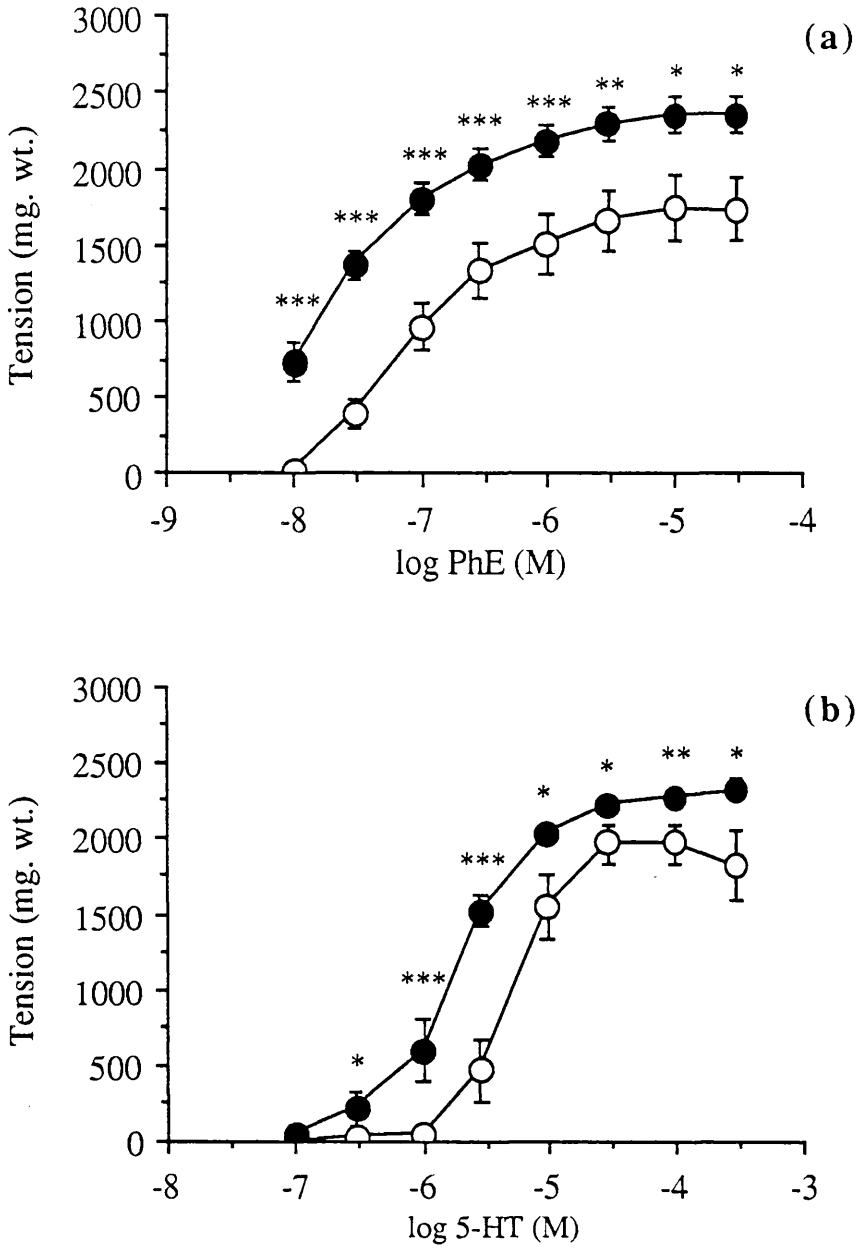


Figure 1.9

Contractions of rat aortic rings to (a) PhE and (b) 5-HT in 2.5 mM $[Ca^{2+}]_o$ PSS in the presence (●) or absence (○) of a subcontractile concentration (20mM) of KCl.

Points shown are mean ± s.e.m. (n=9-10). Statistically significant differences between contractile responses in the presence and absence of KCl using unpaired Student's *t test*; * P<0.05, ** P<0.01, *** P<0.005

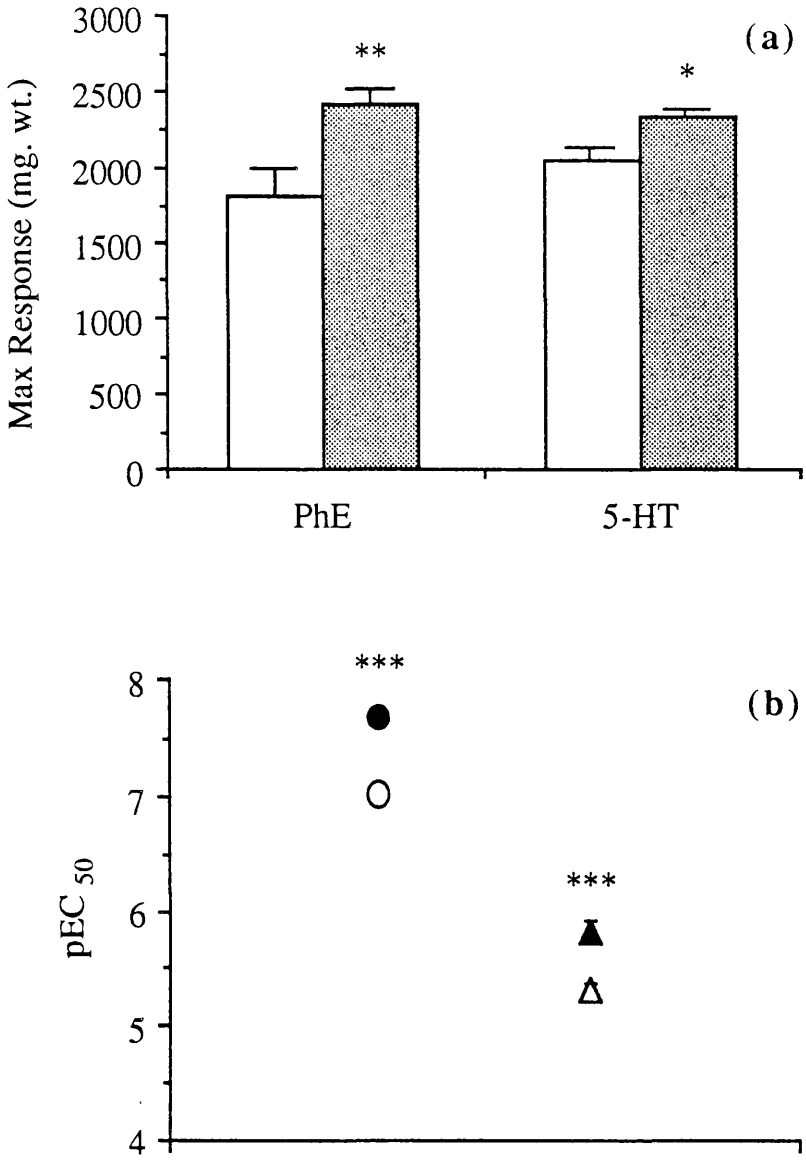


Figure 1.10

Effect of a subcontractile concentration (20mM) of KCl on (a) the maximum contraction and (b) the sensitivity (pEC_{50}) of endothelium-disrupted rat aortic rings to PhE (○/●) and 5-HT (△/▲). Open bars and symbols indicate values in control PSS and closed bars and symbols indicate values in 20mM KCl PSS.

Data shown represents mean ± s.e.m. (n=9-10). There was no statistically significant difference (P>0.05) between the contractile response to PhE and 5-HT in control and 20mM KCl. Statistically significant differences for each agonist between contractile responses in the presence and absence of KCl using unpaired Student's *t test*; * P<0.05, ** P<0.01, *** P<0.005

	Control	20mM $[K^+]_o$	
	Maximum Response (mg.wt.)		
PhE	1812.3 ± 189.0	2427.7 ± 105.7 **	
5-HT	2043.6 ± 122.4	2339.9 ± 50.3 *	
	Tissue Sensitivity (pEC_{50})		*ΔpEC_{50}*
PhE	7.01 ± 0.07	7.70 ± 0.07 ***	0.68 ± 0.10
5-HT	5.31 ± 0.07	5.82 ± 0.09 ***	0.57 ± 0.08

Table 1.1

Effect of incubation in 20mM $[K^+]_o$ PSS of rat aortic ring segments on the maximum contractile response (expressed in mg.wt.) and sensitivity (expressed as the pEC_{50}) to PhE and 5-HT.

Differences between control and 20mM $[K^+]_o$ preparations are shown as Δ pEC_{50} for potency (i.e. $pEC_{50\,(20mM\,KCl)}$ - $pEC_{50\,(control)}$). The values shown are the means ± s.e.mean for n=9-10.

There was no significant difference either between the maximum contractile responses to PhE and 5-HT in both control and 20mM $[K^+]_o$ PSS or the difference in sensitivity (Δ pEC_{50}) for each agonist in control and 20mM $[K^+]_o$ PSS. Significant differences from control preparation using Student's unpaired *t-test*: * $P<0.05$, *** $P<0.005$

In PSS containing 2.5mM Ca^{2+}, 0.01-10μM BRL 34915, which has been shown to produce hyperpolarisation of the smooth muscle of the rat aorta in the concentration range used, produced a concentration-dependent unsurmountable inhibition of the contractions to both PhE and 5-HT (Figure 1.12). The maximum response to both agonists was similar at each concentration of BRL 34915 used indicating a similar degree of depression of contractility of each agonist by BRL 34915.

Effect of Endothelium

Contractions induced by both PhE and 5-HT in rings with a disrupted endothelium were significantly greater than in rings with a functional endothelium (Figure 1.14). The maximum response of PhE was similar to that of 5-HT in both rubbed and unrubbed rings. Thr maximum responses were significantly greater for each agonist in rubbed rings ($P<0.05$; Student's unpaired *t test*) compared with the response in the unrubbed ring (Figure 1.15, Table 1.2). In a similar manner, the sensitivity of the preparation (pEC_{50}) to both agonists was significantly greater in endothelium-disrupted preparations ($P<0.05$; Student's unpaired *t test*) compared with intact rings (Figure 1.15, Table 1.2). However, the change in sensitivity (Δ pEC_{50}) for both PhE and 5-HT was similar (Figure 1.16, Table 1.2). Thus, the increase in the contractile response to both 5-HT and PhE by disruption of the endothelium in terms of increased maximum response and increased sensitivity of the preparations to the agonists was similar.

At low concentration of U46619, the contractions induced in rubbed preparations were significantly greater ($P<0.05$; Student's unpaired *t test*) than in unrubbed preparations. However, at higher concentations the converse was seen, i.e. the response in the presence of a functional endothelium was greater than that in the rubbed preparations (Figure 1.17). The maximum contractile response to U46619 was significantly less ($P<0.05$; Student's unpaired *t test*) in the endothelium-disrupted rings compared with the contractions in intact preparations (Figure 1.17, Table 1.2). In

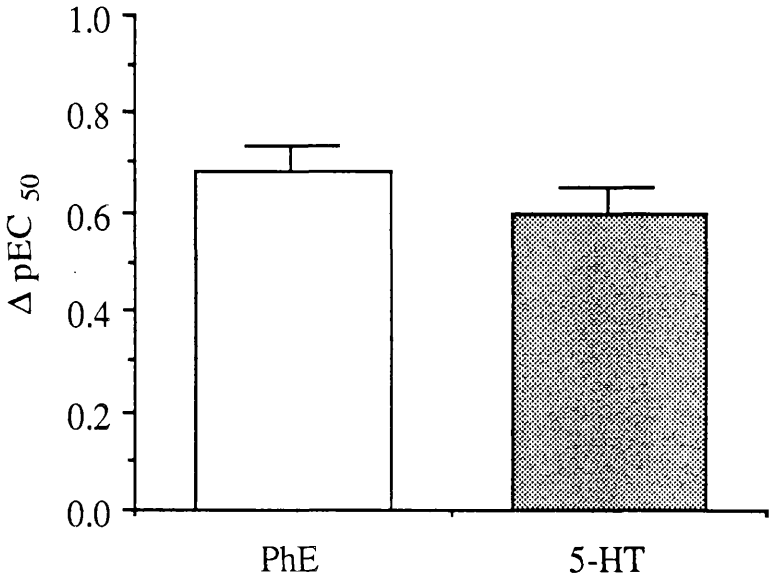


Figure 1.11

Difference in sensitivity of endothelium-disrupted rings (Δ pEC_{50}) to PhE (open bars) and 5-HT (filled bars) between responses in control PSS and PSS containing 20mM KCl.

Data shown represents mean Δ pEC_{50} values $\pm$ s.e.m. (n=9-10). There was no statistically significant difference ($P>0.05$) between Δ pEC_{50} for PhE and 5-HT using unpaired Student's *t test*.

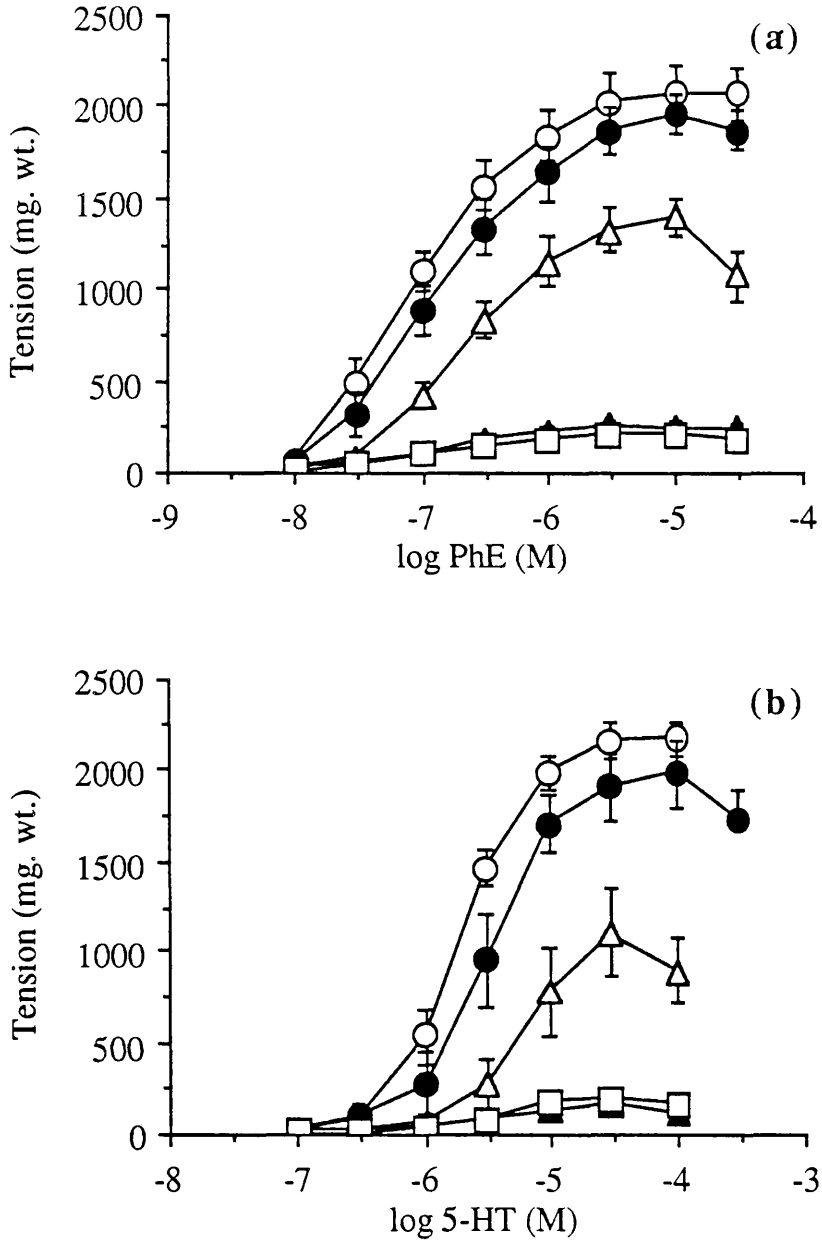


Figure 1.12

Contractions of endothelium-disrupted rat aortic rings to (a) PhE and (b) 5-HT in 2.5 mM $[Ca^{2+}]_o$ PSS in the presence of BRL 34195; control (○), 0.01μM (●), 0.1μM (△), 1μM (▲), and 10μM (□).

Points shown are mean ± s.e.m. (n=6).

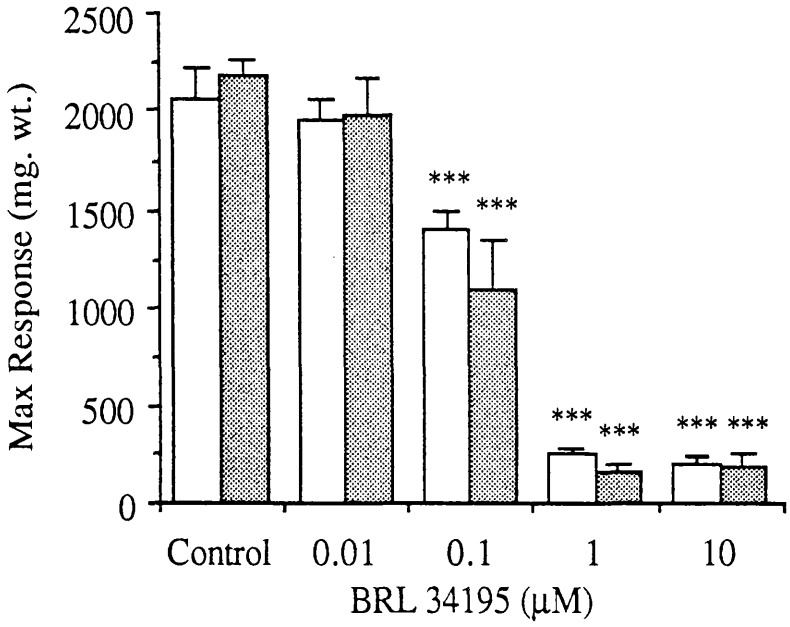


Figure 1.13

Effect of increasing concentrations of BRL 34195 on the maximum contraction of endothelium-disrupted rat aortic rings to PhE (open bars) and 5-HT (filled bars).

Points shown are mean ± s.e.m. (n=6). There was no statistically significant difference (P>0.05) between the maximum contractile response to PhE and 5-HT in control and 20mM KCl. Statistically significant differences between contractile responses for each agonist in the absence and presence of BRL 34195 using unpaired Student's *t test*; *** P<0.005

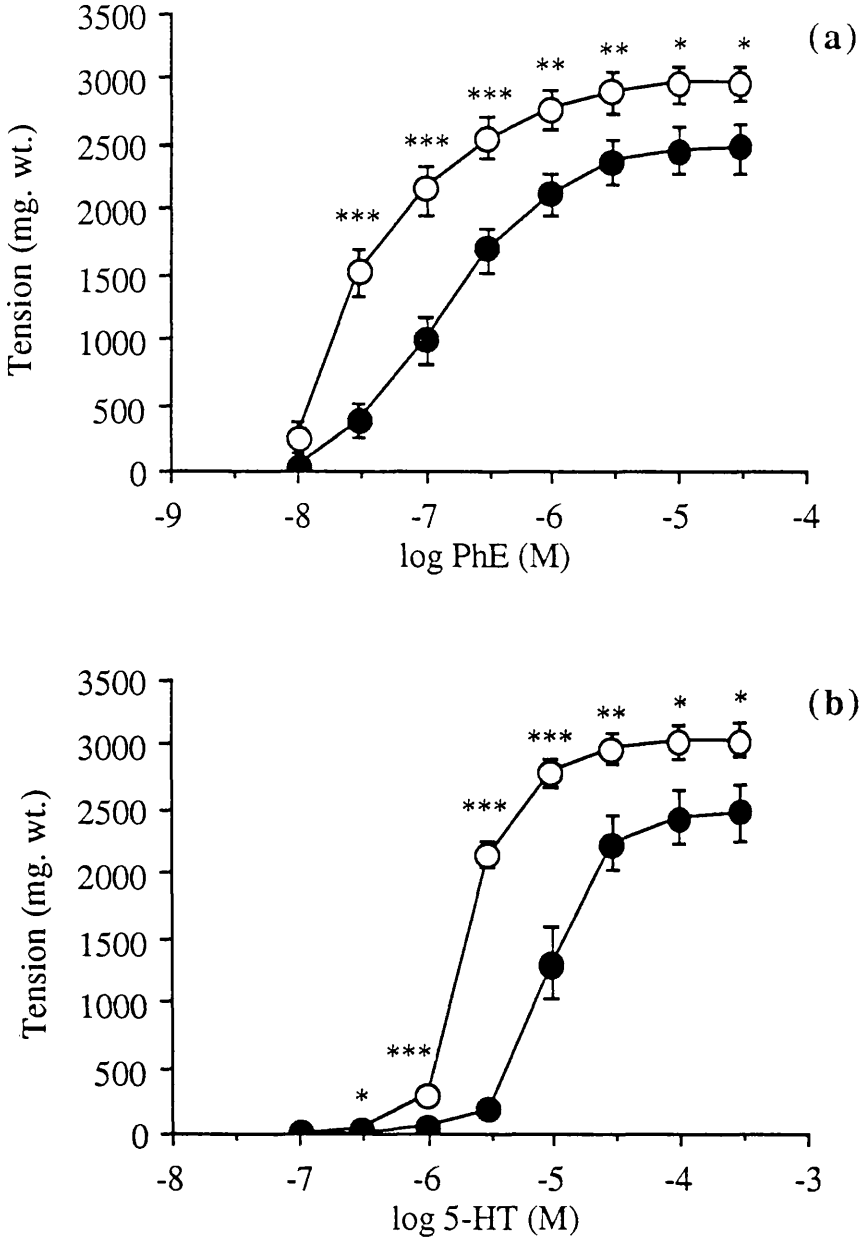


Figure 1.14

Effect of disruption of the endothelium on contractions of rat aortic rings to (a) PhE and (b) 5-HT.

Points shown represent mean contraction ± s.e.m. (n=9-10). Statistically significant differences between contractile responses in the absence (○) and presence (●) of a functional endothelium using unpaired Student's *t test*; * $P<0.05$, ** $P<0.01$, *** $P<0.005$

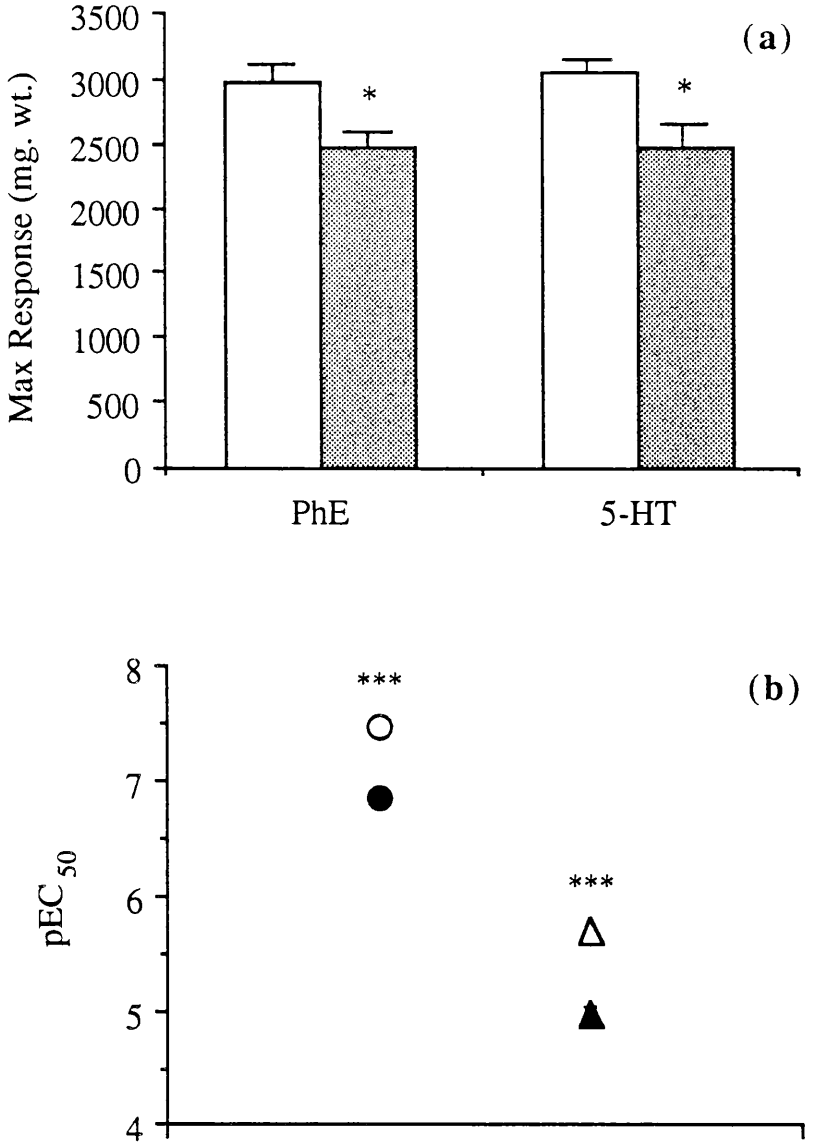


Figure 1.15

Effect of disruption of the endothelium on (a) the maximum contraction and (b) the sensitivity (pEC_{50}) of rat aortic rings to PhE (○/●) and 5-HT (△/▲). Open bars and symbols indicate values in rubbed preparations and closed bars and symbols indicate values in unrubbed preparations.

Data represents mean ± s.e.m. (n=9-10). Statistically significant differences between contractile responses for each agonist in the absence and presence of a functional endothelium using unpaired Student's *t test*; * $P<0.05$

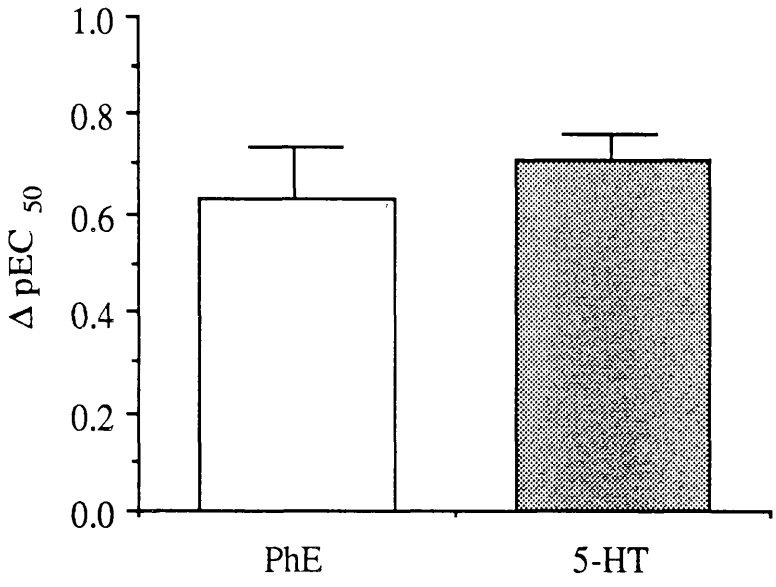


Figure 1.16

Difference in sensitivity of endothelium-disrupted rings (Δ pEC_{50}) to PhE (open bars) and 5-HT (filled bars) between responses in the absence and presence of an intact endothelium.

Data shown represents mean Δ pEC_{50} values $\pm$ s.e.m. (n=9-10). There was no statistically significant difference ($P>0.05$) between Δ pEC_{50} for PhE and 5-HT using unpaired Student's *t test*.

	Unrubbed	Rubbed	
	Maximum Response (mg.wt.)		
PhE	2964.2± 142.6	2469.2 ± 191.3 *	
5-HT	3038.5 ± 122.4	2464.8 ± 122.0 *	
	Tissue Sensitivity (pEC_{50})		*ΔpEC_{50}*
PhE	7.48 ± 0.07	6.85 ± 0.09 ***	0.63 ± 0.10
5-HT	5.69 ± 0.02	4.97 ± 0.07 ***	0.71 ± 0.05

Table 1.2

Effect of removal of the endothelial cell layer of rat aortic ring segments on the maximum contractile response (expressed in mg.wt.) and sensitivity (expressed as its pEC_{50}) to PhE and 5-HT.

Differences between rubbed and unrubbed preparations are shown as Δ pEC_{50} for potency (i.e. $pEC_{50\ (rubbed)}$ - $pEC_{50\ (unrubbed)}$). The values shown for the unrubbed and rubbed vessel are the means ± s.e.mean for n=9-10.

There was no significant difference between the two agonists either in the maximum contractile responses (rubbed or unrubbed) or in the difference in sensitivity (Δ pEC_{50}) caused by rubbing. Significant differences from unrubbed preparation using student's unpaired *t*-test: * $P<0.05$, *** $P<0.005$

contrast, the sensitivity of the rat aortic rings to this agonist was significantly greater in rubbed preparations compared with that in unrubbed preparations (Figure 1.17, Table 1.2).

Effects of Lithium

In endothelium-denuded rings the contractile response to PhE was significantly smaller in LiCl pre-treated rats (treated preparations) compared with untreated rats (control preparations). In the same preparations however, in general, the contractile response to 5-HT in treated rings was the same as that in control rings (Figure 1.18). In a similar manner the sensitivity to PhE but not 5-HT in rubbed preparations was significantly greater in control rings compared with treated rings (Figure 1.19).

In the presence of an intact endothelium the responses to PhE and high concentrations of 5-HT were significantly less in treated preparations than in control preparations (Figure 1.20). Whilst the maximum response to each agonist was depressed in unrubbed rings the sensitivity to PhE but not 5-HT was significantly greater in control rings compared with treated rings (Figure 1.21).

The contractile responses to both PhE and 5-HT in endothelium-disrupted rings from rats pretreated with myoinositol and LiCl (myo/LiCl preparations) were not significantly different ($P>0.05$; Student's unpaired *t test*) from the responses in rings from rats pre-treated with LiCl alone (Figure 1.22). In contrast whilst the contractile response to PhE in unrubbed myoi/LiCl preparations was not significantly different from the response in unrubbed rings from rats treated with LiCl alone, the contractile response to high concentrations of 5-HT in the same preparations were significantly greater ($P<0.05$; Student's unpaired *t test*) in the myoinositol/lithium treated rings compared with the LiCl treated rings (Figure 1.23). Thus, concomitant pre-treatment of rats with LiCl and myoinositol had no effect on agonist-induced contractions except in the case of 5-HT where the contractions in the presence of the endothelium were significantly greater compared with those in LiCl pre-treated rings.

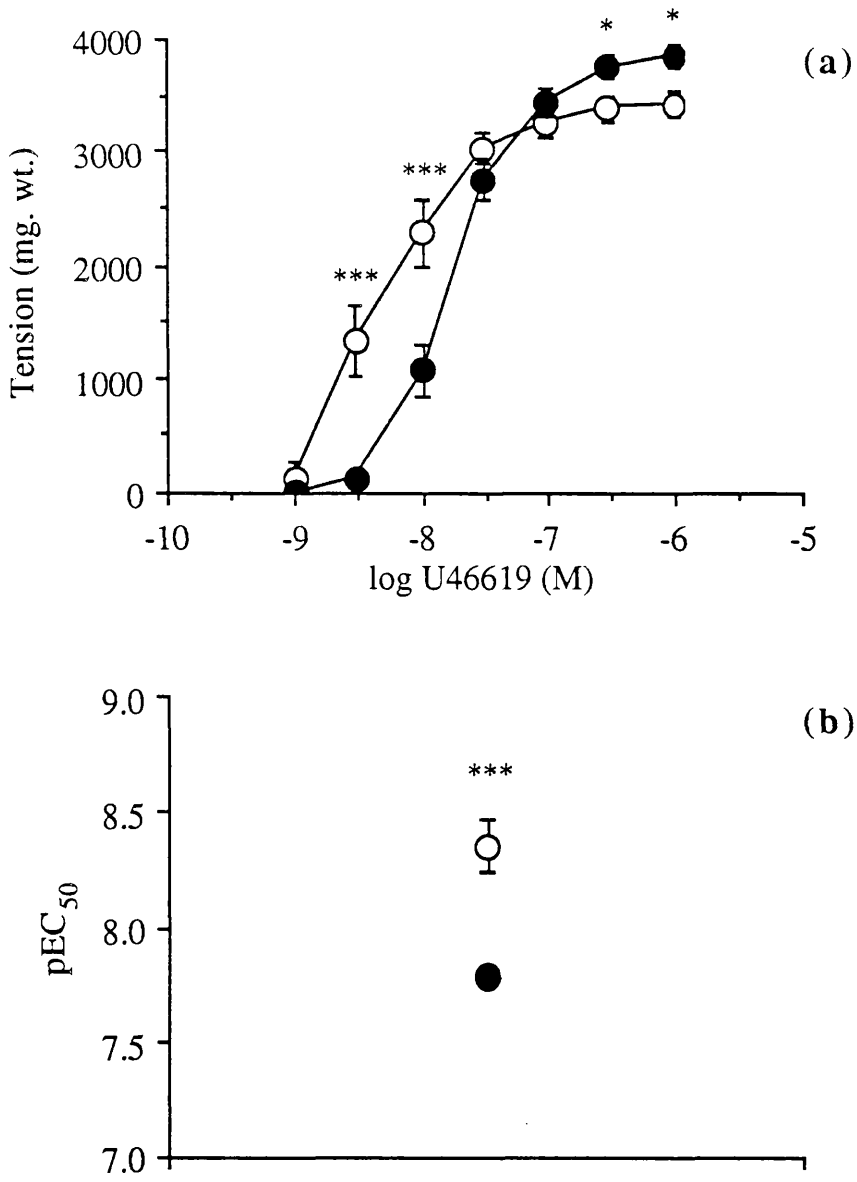


Figure 1.17

Effect of disruption of the endothelium on (a) the contractile response and (b) the sensitivity (pEC_{50}) of rat aortic rings to U46619.

Data represents mean ± s.e.m. (n=9-10). Values shown are from rubbed (○) and unrubbed (●) preparations. Statistically significant differences between contractile responses in the absence and presence of a functional endothelium using unpaired Student's *t test*; * $P<0.05$, *** $P<0.005$

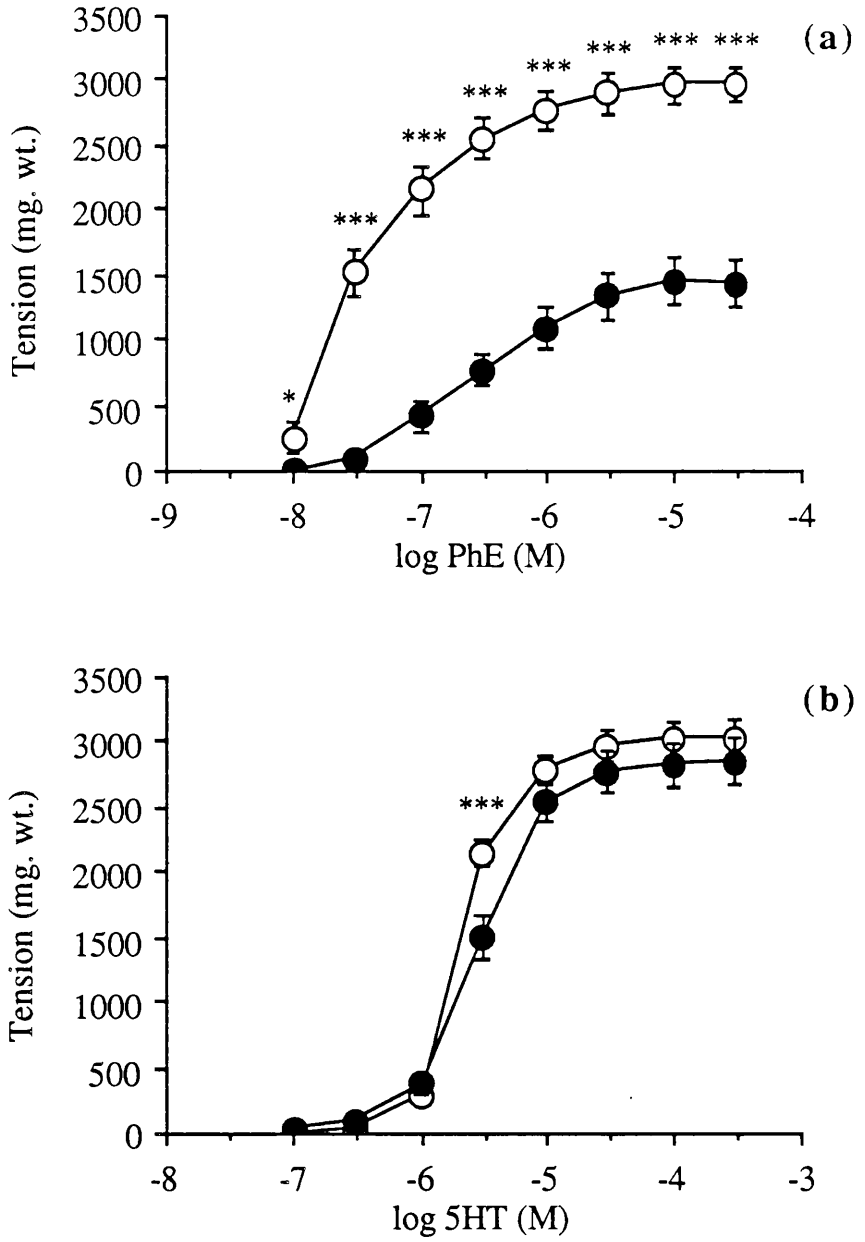


Figure 1.18

Contractions of aortic rings with a disrupted endothelium to (a) PhE and (b) 5-HT from control rats (○) and rats chronically pre-treated with LiCl (●).

Points shown are mean ± s.e.m. (n=6). Statistically significant differences between contractile responses in control and treated preparations using unpaired Student's *t test*; * $P<0.05$, *** $P<0.005$

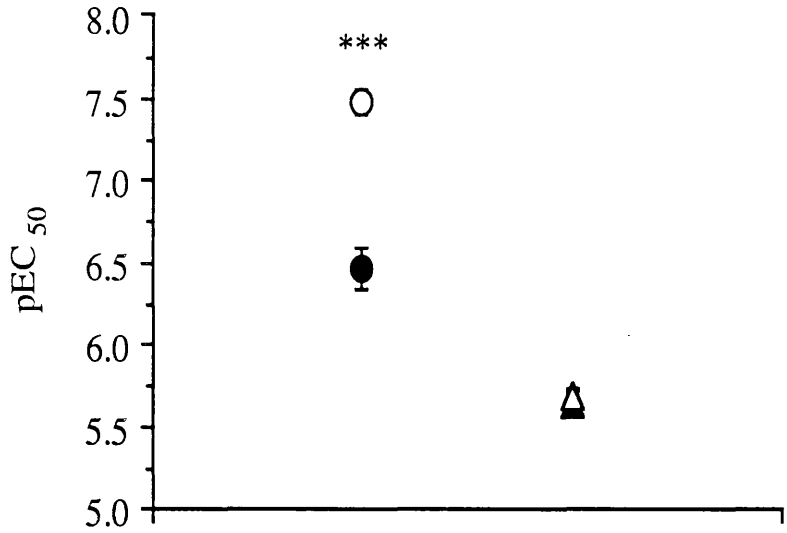


Figure 1.19

Sensitivity (pEC_{50}) to PhE (○ and ●) and 5-HT (△ and ▲) in endothelium-disrupted aortic rings from control rats (open symbols) and rats chronically pre-treated with LiCl (closed symbols).

Data shown represents mean pEC_{50} values ± s.e.m. (n=9-10). There was no statistically significant difference ($P>0.05$) in pEC_{50} for 5-HT between control and treated preparations using unpaired Student's *t test*. Statistically significant differences in pEC_{50} for PhE between control and treated preparations using unpaired Student's *t test*; *** $P<0.005$

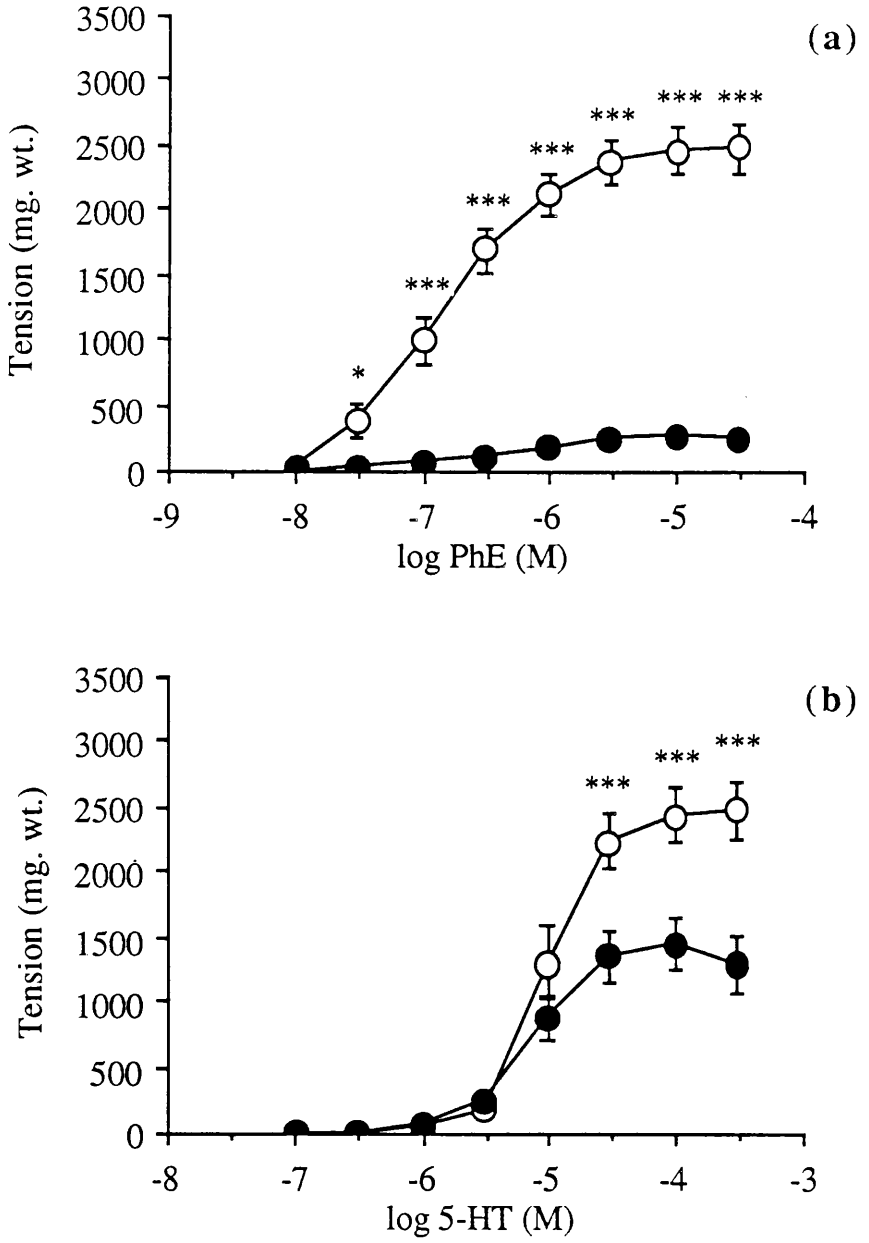


Figure 1.20

Contractions of aortic rings with a functional endothelium to (a) PhE and (b) 5-HT from control rats (○) and rats chronically pre-treated with LiCl (●).

Points shown are mean ± s.e.m. (n=6). Statistically significant differences between contractile responses in control and treated preparations using unpaired Student's *t test*; * $P< 0.05$, *** $P<0.005$

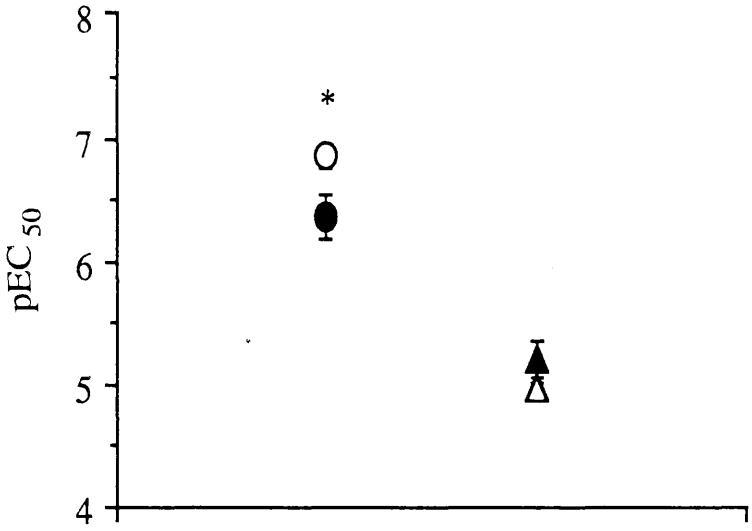


Figure 1.21

Sensitivity (pEC_{50}) to PhE (○ and ●) and 5-HT (△ and ▲) in endothelium-intact aortic rings from control rats (open symbols) and rats chronically pre-treated with LiCl (closed symbols).

Data shown represents mean pEC_{50} values ± s.e.m. (n=9-10). There was no statistically significant difference ($P>0.05$) in pEC_{50} for 5-HT between control and treated preparations using unpaired Student's *t test*. Statistically significant differences in pEC_{50} for PhE between control and treated preparations using unpaired Student's *t test*; * $P<0.05$

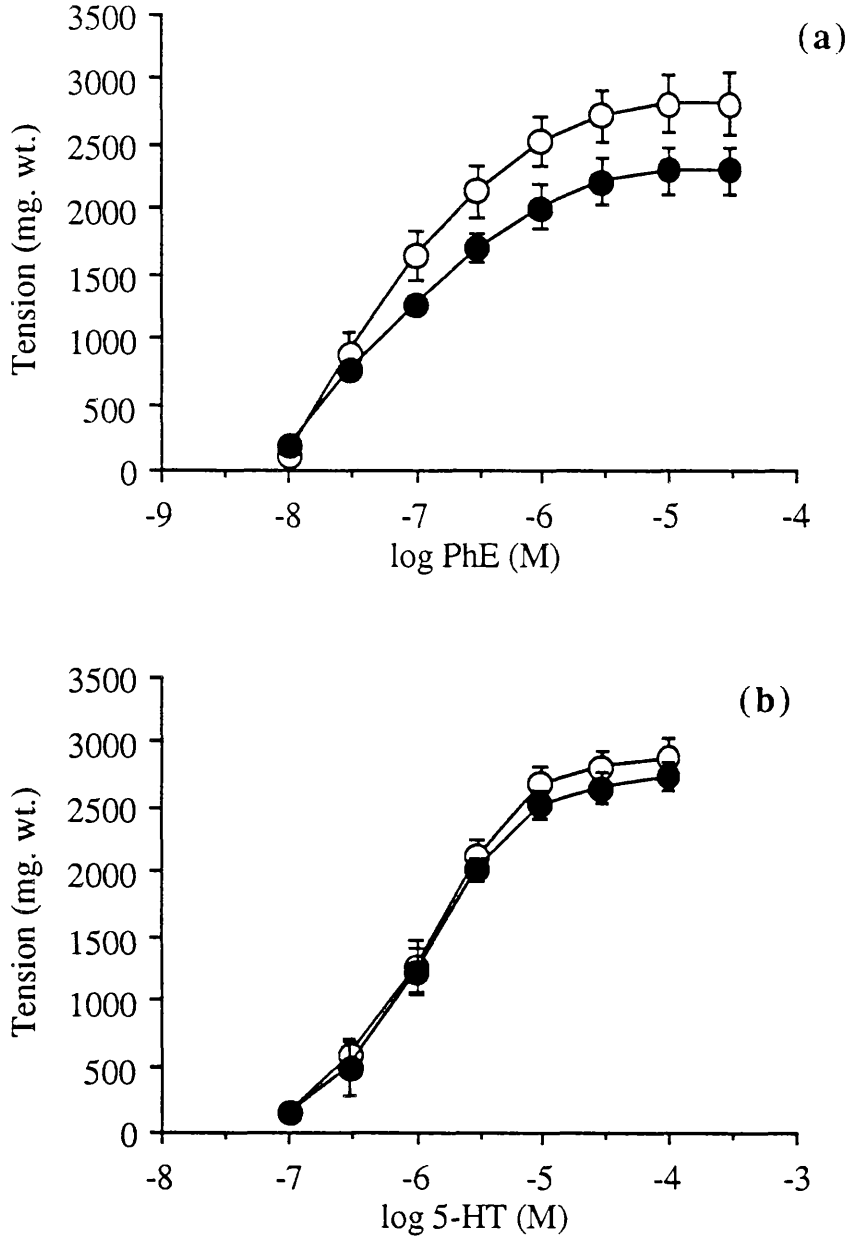


Figure 1.22

Contractions of aortic rings with a disrupted endothelium to (a) PhE and (b) 5-HT from rats chronically pre-treated with LiCl (○) and rats chronically pre-treated with a combination of LiCl and myoinositol (●).

Points shown are mean ± s.e.m. (n=4-5). There was no statistically significant difference (P>0.05) in responses to PhE and 5-HT between both pre-treatments using unpaired Student's *t test.*

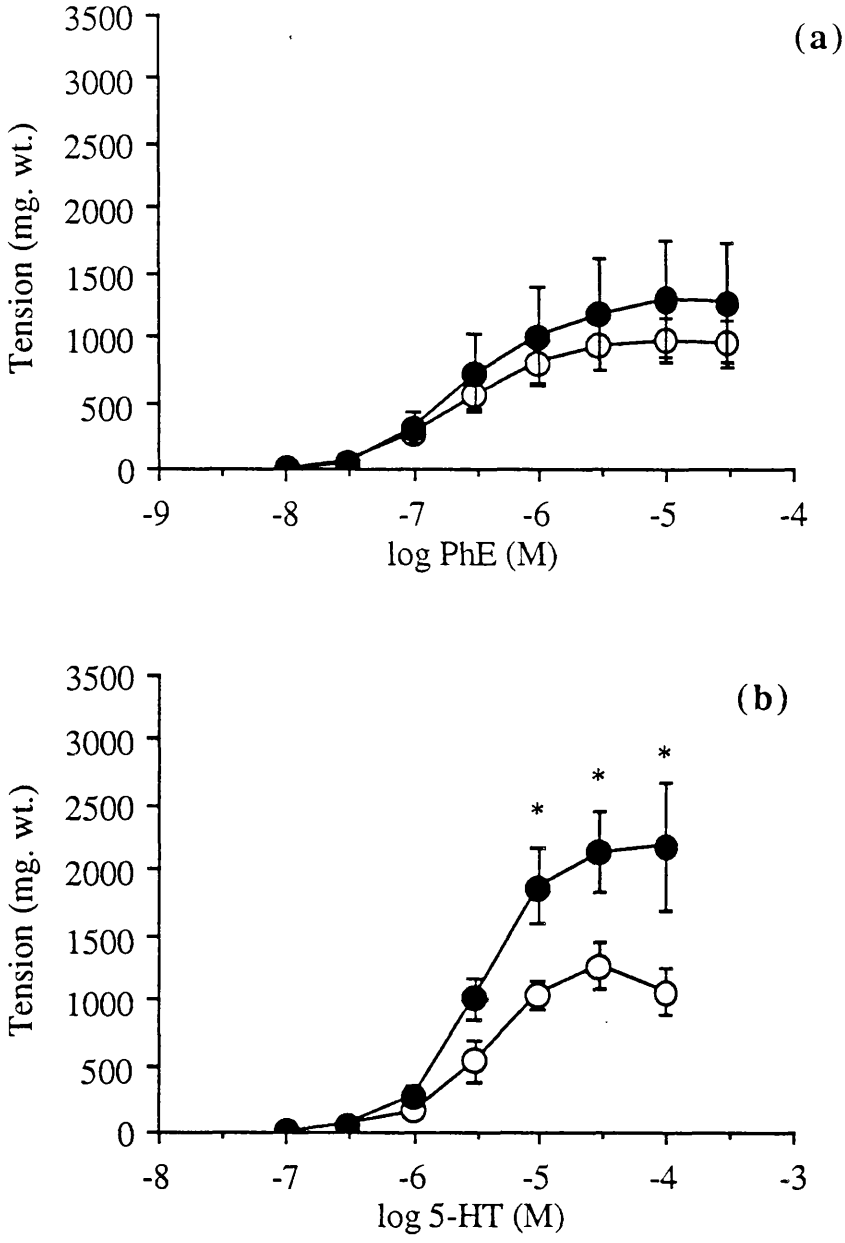


Figure 1.23

Contractions of aortic rings with a functional endothelium to (a) PhE and (b) 5-HT from rats chronically pre-treated with LiCl (○) and rats chronically pre-treated with a combination of LiCl and myoinositol (●).

Points shown are mean ± s.e.m. (n=4-5). There was no statistically significant difference ($P>0.05$) in responses to PhE between both pre-treatments using unpaired Student's *t test*. Statistically significant differences in responses to PhE between both pre-treatments using unpaired Student's *t test*; * $P<0.05$

It should be noted that the effects of chronic pre-treatment with LiCl on the responses of the rat aorta to endothelium-dependent relaxations has been examined and is discussed in detail in Chapter Four of this thesis.

Discussion

In the first part of this study I have compared the requirement for extracellular Ca^{2+} of the responses to 5-HT and the α_1-adrenoceptor agonist PhE in rat aortic rings. Agonists were added in the presence of a) nominally 'zero' $[Ca^{2+}]_o$, i.e. the absence of 'added' Ca^{2+} and b) various 'fixed' concentrations of $Ca^{2+}{}_o$. Endothelium-disrupted rings were used because the responses of the rat aorta, and other vascular smooth muscles, to a variety of contractile agents are depressed in the presence of a functional endothelium due to a basal release of EDRF (Allan *et al.*, 1983; Fortes *et al.*, 1983; Egleme *et al.*, 1984b; Carrier and White, 1985; Furchgott *et al.*, 1985; Miller and Stoclet, 1985). Thus, disruption of the endothelium excludes any possible variation in the contractile response to the agonists due to changes in the basal release of EDRF which might contribute to any differences seen in the response to Ca^{2+} re-addition in the presence of either PhE or 5-HT.

The results show that when rat aortic rings are incubated in PSS with no added Ca^{2+}, re-addition of Ca^{2+} in the presence of 0.1μM PhE but not 3μM 5-HT results in a contraction of the circular smooth muscle of the preparations. This is in marked contrast to the effect seen when 0.1μM PhE or 3μM 5-HT are added in the presence of a concentration of $Ca^{2+}{}_o$ which allows the development of an agonist-induced contractions. Under these conditions the responses to both PhE and 5-HT are similar except at the lowest concentration of $Ca^{2+}{}_o$ when the response to 5-HT is significantly less than the response to PhE: this observation is consistent with the lack of contractile response to Ca^{2+} re-addition in the presence of 5-HT when the agonist is added in nominally zero Ca^{2+} PSS. The contraction of vascular smooth muscle to 5-HT and PhE has been shown to be mediated by both release of intracellular Ca^{2+} and influx of $Ca^{2+}{}_o$. A possible explanation for the lack of effect of '5-HT/Ca^{2+} re-addition' might have been that 5-HT, in contrast to PhE is wholly dependent on the release of intracellular Ca^{2+} for production of its contractile response and is not able to open Ca^{2+} channels to allow the influx of extracellular Ca^{2+}. This is unlikely to be the case

however, since the major part of the response is proportional to the concentration of Ca^{2+}_o. Furthermore, BAY K 8644 inhibited and reversed the densensitisation or depression of contraction seen with 5-HT/Ca^{2+} re-addition. The Ca^{2+} channel activation produced by this dihydropyridine requires the pre-activation of the vascular smooth muscle and presumably therefore the opening of voltage operated Ca^{2+} channels (Schramm *et al.*, 1984, Su *et al.*, 1984). Thus, the effect of BAY K 8644 on 5-HT/Ca^{2+} re-addition indicates that there is some activation of VOCs by 5-HT in the absence of added Ca^{2+}.

Another explanation for the absence of contraction to 5-HT/Ca^{2+} re-addition could be that pre-incubation of the rat aorta in PSS containing nominally zero $[Ca^{2+}]_o$ in some way densensitises the smooth muscle to subsequent agonist-induced contraction. Whilst this is not the case for 5-HT-induced contractions in 2.5mM Ca^{2+} (no marked attenuation is seen in consecutive CCRCs to 5-HT), 5-HT-induced desensitisation of subsequent contractions to 5-HT/Ca^{2+} re-addition appears to occur since pre-exposure of the preparations to PhE results in contractions to subsequent 5-HT/Ca^{2+} re-addition which are significantly greater than in preparations pre-exposed to 5-HT. This 'desensitisation' is specific for 5-HT/Ca^{2+} re-addition in that contractions to 'PhE/Ca^{2+} re-addition' in preparations pre-exposed to 5-HT are not significantly different from those in preparations pre-exposed to PhE alone. Moreover, this desensitisation does not prevent contraction to addition of PhE at the end of a CCRC to $CaCl_2$ in the presence of 5-HT indicating that the contractile processes in the smooth muscle have not been disrupted.

Contractions of vascular smooth muscle have been shown to be modulated by the presence of 5-HT. In addition to its direct contractile effect on vascular smooth muscle, 5-HT can enhance the contractile response to a number of agonists possibly by enhancing the influx of extracellular Ca^{2+} (Van Nueten *et al.*, 1982; Medgett *et al.*, 1983; Su and Uruno, 1985; Felix *et al.*, 1986; Meehan *et al.*, 1988). Also, 5-HT can attenuate responses to contractile agents of vascular smooth muscle including the dog

mesenteric artery and rabbit ear artery even after it has been washed from the tissue (Bohr and Elliot, 1962; Rapaport and Bevan, 1982). Moreland *et al.* (1985), have suggested that in the dog mesenteric artery, 5-HT, acting via ketanserin-resistant receptors (i.e. not 5-HT_2 receptors) attenuates contractile responses as a result of an increased sodium influx that stimulates the electrogenic sodium pump to cause membrane hyperpolarisation and decreased stimulated calcium influx. In contrast to the present study however, Moreland demonstrated that in addition to attenuating its own response, 5-HT attenuated the responses to a wide range of agonists including noradrenaline, methoxamine, prostaglandin $F_{2\alpha}$ and non-receptor mediated contractions to KCl. Thus, it is unlikely that the attenuation by 5-HT in the present study is brought about by the same mechanism as that seen in the dog mesenteric artery.

In the present study, BAY K 8644 inhibited and reversed the densensitisation or depression of contraction seen with 5-HT/Ca^{2+} re-addition indicating that 5-HT under the conditions used can activate dihydropyridine sensitive Ca^{2+} channels. In addition, BAY K 8644 potentiated the responses to PhE Ca^{2+} re-addition as would be expected of this agent which prolongs mean open times of Ca^{2+} channel (Hess *et al.*, 1984; Triggle, 1984). However, BAY K 8644 produced a much greater potentiation of the response to 5-HT/Ca^{2+} re-addition than to PhE/Ca^{2+} re-addition. This might simply indicate that the opening of VOCs by PhE is near the maximum possible for this agonist whereas there is a much greater scope for increasing the number of channels for a given concentration of 5-HT.

Another possible explanation for the differential effect of BAY K 8644 is that PhE and 5-HT induce contraction of the vascular smooth muscle of the aorta by recruiting both receptor operated Ca^{2+} channels (ROCs) and VOCs (Bolton, 1979; Van Breemen *et al.*, 1979; Meisheri, *et al.*, 1981) but in different proportions. For example, the Ca^{2+} channels involved in the response to PhE may be predominantly ROCs whilst those involved in the response to 5-HT may be VOCs and require some extracellular Ca^{2+} for their opening. This would explain the apparently differential effect of BAY K 8644 on

the responses to 5-HT/Ca^{2+} re-addition. In order to test this hypothesis I examined the effects of changing the membrane potential on the responses to PhE and 5-HT since the function of VOCs can depend on the membrane potential of the smooth muscle.

Partial depolarisation of the aortic smooth muscle with a subcontractile concentration of KCl (20mM) caused a potentiation of the response to 5-HT as would be expected since such treatment would predispose to the opening of more VOCs thus producing a greater contraction by agonists acting at least in part by activation of VOCs. However, the response to PhE was also potentiated by incubation of the ring preparations in partially depolarising PSS. Moreover, the degrees by which responses to the two agonists were potentiated were not significantly different ($P>0.05$; Students unpaired *t test*). Therefore it is unlikely that contractions to the agonists are mediated by different proportions of VOC and ROC since, if this were the case, partial depolarisation would be expected to have a preferentially greater effect on the contractions of the agonist which recruits the greater number of VOCs for its contraction. This suggestion is supported by the observation that the concentration-dependent contractions to both PhE and 5-HT were inhibited to a similar extent by hyperpolarisation of the rat smooth muscle by the K^+ channel activator BRL 34915 (Southerton *et al.*, 1987). In a similar manner to depolarisation with KCl, hyperpolarisation would be expected to differentially inhibit the contractions to agonists which act mainly via activation of VOC since mild hyperpolarisation would inhibit the opening of VOCs.

Thus the preferentially greater effect of BAY K 8644 versus 5-HT/Ca^{2+} re-addition is not simply due to a clear difference in the activation processes utilised by 5-HT and PhE under 'normal' conditions. This effect is more likely to be due to a greater scope by which 5-HT/Ca^{2+} re-addition can be potentiated since alteration of the membrane potential by partial depolarisation with KCl or hyperpolarisation with BRL 34915 did not differentially affect the contractions to 5-HT and PhE in 2.5mM Ca^{2+}_o.

To summarise so far: when 5-HT is added in a low concentration of extracellular Ca^{2+} (thereby preventing production of a significant contractile response), activation of receptors changes the excitation-contraction coupling process in such a way that a subsequent increase in $[Ca^{2+}]_o$ is no longer effective at producing contraction, possibly due to an alteration of Ca^{2+} channel function. This does not, however, occur with activation of the α_1-adrenoceptor indicating a difference in the excitation-contraction coupling process between activation of 5-HT_2 receptors and α_1-adrenoceptors in this tissue. The effect does not cause a disruption of the contractile processes in the smooth muscle or even of Ca^{2+} channel function in general since addition of PhE at the end of a CCRC to $CaCl_2$ in the presence of 5-HT induced a rapid contraction. This interesting phenomenon does not appear to be a simple function of a differential activation by PhE and 5-HT of VOC and ROC since depolarisation and hyperpolarisation of the smooth muscle in 2.5mM $[Ca^{2+}]_o$ had no differential effect on the contraction induced by these agonists.

The basal release of EDRF attenuates the response to a number of contractile agents in vascular smooth muscle and its action may be due at least in part to alteration of Ca^{2+} fluxes (Malta *et al.*, 1986). Since there is a difference in the excitation-contraction coupling process between activation of 5-HT_2 receptors and α_1-adrenoceptors in the rat aorta (with respect to the requirement for extracellular Ca^{2+} of responses to 5-HT and PhE), in the second part of this study I tested to see if EDRF has a differential effect on the contractions to PhE and 5-HT. In addition I investigated the possible involvement of receptor-mediated inositol phospholipid hydrolysis in contractions to PhE and 5-HT by chronic pre-treatment of rats with LiCl to inhibit receptor-mediated increases in inositol phospholipid hydolysis: this was done both in the presence and absence of the endothelium.

In ring preparations with a disrupted or intact endothelium both PhE and 5-HT caused concentration-dependent contractions. The response to both PhE and 5-HT was greater in the absence of the endothelium presumably due to the basal release of

EDRF directly opposing the contractile response to the agonists. The difference in maximum response and sensitivity for both agonists between rubbed and unrubbed preparations was the same. This clearly indicates that EDRF does not have a differential effect on the contractions mediated by 5-HT_2-receptors or α_1-adrenoceptors in this tissue even though these agonists appear to have a subtly different excitation-contraction coupling mechanism which is only revealed in nominally zero $[Ca^{2+}]_o$ PSS.

For comparative purposes, I also examined the effect of disruption of the endothelium on responses of the rat aorta to the thromboxane-mimetic U46619 (Coleman *et al.*, 1981). Contractions of the rabbit jugular vein and rat aorta to U46619 have been reported as being unaffected by the presence of the vascular endothelium (Leff *et al.*, 1987; A.G.B. Templeton, PhD thesis, 1988). The results obtained in this study do not confirm these observations. In contrast to these studies, the presence of the endothelium had a marked and somewhat surprising effect on the contractions to U46619. Although the sensitivity of the preparation to U46619 was greater in rings with a disrupted endothelium, the maximum contraction produced by this agonist was greatest in the presence of a functional endothelium. A similar effect was noted in a study by Bullock *et al.*, (1986) though these workers simply reported the observation and made no attempt to explain it. The most likely explanation of this phenomenon is that at high concentrations, U46619 is able to release an endothelium-dependent substance that can augment the contractile response to the agonist without inducing a contraction *per se* (an effect seen with anoxia and neuropeptide Y; De Mey and Vanhoutte, 1982, 1983; Daly and Hieble, 1987). Alternatively, U46619 may produce an endothelium-dependent substance that can contract the smooth muscle of the aorta, as occurs in spontaneously hypertensive rats (Luscher and Vanhoutte, 1986), hypoxic conditions in dog coronary artery (Rubanyi and Vanhoutte, 1985), stretch in dog basilar arteries (Katusic *et al.*, 1987) and cultured bovine aortic endothelial cells (Gillespie *et al.*, 1985; Hickey *et al.*, 1985). These endothelium-dependent contractions have been attributed to prostaglandins or substances whose identity has

not yet been unequivocally established. Recent studies have indicated that the endothelium is capable of releasing a highly potent vasoconstictor substance which has been termed Endothelin (Yanagisawa *et al.*, 1988). This substance may be of some physiological significance and therefore this effect of the endothelium on contractions of the rat aorta to U46619 and possibly other vascular preparations warrants further investigation. Furthermore, the possible involvement of cycloxygenase products in the endothelium-dependent augmenation of U46619-induced contractions needs to be evaluated.

In addition to the differential effect of Ca^{2+} re-addition in the presence of 5-HT and PhE, chronic pre-treatment of rats with LiCl revealed differences in the contractile response to 5-HT and PhE. Receptor mediated increases in inositol phospholipid hydrolysis are thought to be important in the agonist-induced release of intracellular Ca^{2+}. In addition to this direct release of intracellular Ca^{2+}, inositol phospholipid metabolites, specifically diacylglycerol (DG), can activate protein kinase C by a Ca^{2+} dependent process (see Berridge, 1987) and hence indirectly modify the function of VOCs (Harris *et al.*, 1986; Osugi *et al.*, 1986; Wakade *et al.*, 1986). Lithium has been shown to inhibit myo-inositol-l-phosphate phosphatase activity and therefore prevent the metabolism of the metabolites of inositol phospholipids: this decreases receptor-mediated inositol phospholipid hydrolysis possibly by decreasing membrane polyphosphoinositide levels (Figure 1.24; Allison and Stewart, 1971; Berridge *et al.*, 1982; Drummond and Raeburn, 1984; Sherman *et al.*, 1986; Kendall and Nahorski, 1986; Menkes *et al.*, 1986). Thus if 5-HT or PhE has a relatively large degree of action via the hydrolysis of inositol phospholipids then this pretreatment will have a preferential effect on that agonist.

In rings from rats pre-treated with LiCl (treated preparations) the contractile response of the vascular smooth muscle to PhE in the absence and presence of a functional endothelium were markedly depressed in terms of both a decreased maximum response and a decreased sensitivity of the preparation to PhE. In contrast however, the

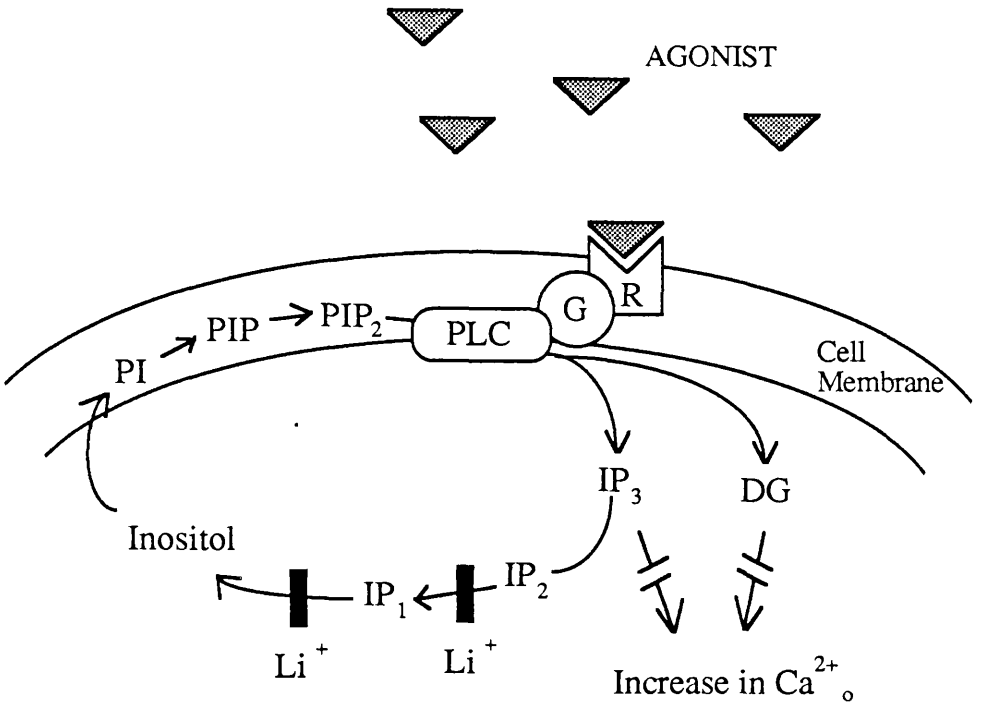


Figure 1.24

Simplified diagram representing the proposed pathway for agonist-induced turnover of membrane inositol phospholipids. Agonists bind to the membrane receptor (R) and, via a regulator protein (G), stimulate the hydrolysis phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). This process generates two second messengers: inositol 1,4,5-triphosphate (IP_3) and 1,2 diacylglycerol (DG) which can directly or indirectly modulate $[Ca^{2+}]_i$. Chronic pre-treatment with LiCl blocks the metabolism of IP_3 and its metabolites such that a build up of inositol monophosphate isomers and a decrease in cellular inositol occurs. Ultimately the levels of membrane PIP_2 will become so low that agonists are unable to produce sufficient amounts of second messengers to ilicit a cellular response.

responses to 5-HT in treated preparations where depressed only at high concentrations in unrubbed rings and there was no difference in sensitivity to 5-HT. These results suggest that at least part of the response to PhE is mediated by α_1-adrenoceptor inositol phospholipid hydrolysis. A similar effect of LiCl on α_1-adrenoceptor mediated responses has been demonstrated by Mantelli *et al.*, (1988). These workers showed that acute administration of LiCl attenuated the inotropic effects of α- but not ß-adrenoceptors in the guinea-pig heart.

The greater depression of PhE-induced contractions in the presence of a functional endothelium by LiCl pre-treatment is consistent with the effect of EDRF being greater on agonists which have a lower intrinsic efficacy (Martin *et al.*, 1985). Disruption of the receptor mediated hydrolysis of inositol phospholipids would necessarily reduce the efficiency of the receptor-response coupling for α_1-adrenoceptors in the smooth muscle of the aorta. This would effectively reduce the receptor reserve for PhE-induced responses and hence lower its intrinsic efficacy.

The effect of LiCl pre-treatment on the responses to 5-HT is more difficult to explain. The absence of effect of LiCl in the rubbed preparation suggests no role for the hydrolysis of inositol phospholipids in 5-HT_2-receptor mediated contractions or that this is not a rate limiting step. However in the presence of the endothelium the contractions to high concentrations of 5-HT are depressed in treated preparations. This might indicate that the effective receptor reserve for 5-HT_2-induced contractions of the rat aorta is much greater than that for PhE and consequently an effect of LiCl can be seen only when the intrinsic efficacy is lowered by the influence of a basal release of EDRF directly opposing the contractile response to 5-HT. However, this is unlikely to be the case since reductions in the relative efficacy of an agonist (e.g., by reducing receptor reserve with irreversible receptor antagonists) should, theoretically, cause a change in the sensitivity to the agonist particularly when the depression of the maximum contraction is as great as that seen in the present study (see Kenakin, 1982). An alternative explanation for this effect of LiCl in unrubbed preparations

might be that, at high concentrations, 5-HT acts via activation of α_1-adrenoceptors and it is this activation that is preferentially blocked in treated preparations. This is also unlikely since it is well established that, in the rat aorta, 5-HT acts via activation of 5-HT_2-receptors (Cohen *et al.*, 1981; Downing *et al.*, 1983; Doggrell, 1987). Moreover this requires an explanation of why the action of 5-HT should be mediated by activation of α_1-adrenoceptors only in the presence of the endothelium. Possibly explanation of this effect is that at high concentrations, 5-HT induces endothelium dependent contractions which are highly sensitive to LiCl either in the mechanism of contraction *per se* or the mechanism of production of the mediators of this contraction. To my knowledge, such an effect at high concentrations of 5-HT has not been reported although the existence of such endothelium-dependent contractile factors has been shown in a number of studies (Rubanyi and Vanhoutte, 1985; Luscher and Vanhoutte, 1986; Katusic *et al.*, 1987; Vanhoutte, 1988) and this may explain the results obtained in the present study.

Chronic pre-treatment with myoinositol has been shown to prevent the inhibitory effects of LiCl when both substances are administered together (Eglen *et al.*, 1987; Mantelli *et al.*, 1988) presumably by circumventing the blockade of metabolism of inositol phosphates. In the present study, myoinositol (when given in drinking water as a supplement to the LiCl pretreatment) caused partial inhibition of the effect of LiCl on 5-HT-induced contractions in unrubbed preparations. This would suggest an involvement in inositol phosphilipid turnover in this apparently endothelium-dependent effect of lithium pre-treatment on the responses of the rat aorta to 5-HT. The effect on 5-HT at high concentrations may be highly sensitive to lithium and therefore would be more susceptible to reversal by low concentrations of myoinositol. Alternatively, the effect of myoinositol may be a function of the ability of the endothelium to more easily utilise extracellular inositol than the smooth muscle.

In contrast to the effect seen with 5-HT-induced contractions in preparations with an intact endothelium, myoinositol had no effect on the attenuation of the contractile

response to PhE in treated preparations. This would suggest that the effect of LiCl on PhE-induced contractions in the present study is not at the level of phosphoinositol lipid turnover. An alternative mode of action at the level of G-protein interactions for LiCl has recently been suggested (Avissar *et al.*, 1988) and it may be at this level that lithium exerts its effects on the contractions to α-adrenoceptor activation. Whilst this may be the case a more likely explanation for the lack of effect of myoinositol in this study is that the route of administration used may lead to variability in the dose of myoinositol administered which, in any case, was not sufficient to overcome the effect of lithium on α-adrenoceptor mediated responses. The apparent lack of effect may also be due to differences in the effect of lithium on responses of the aorta in both groups of animals, i.e. the rats used in the initial comparison between control and LiCl treatment and the second group of rats used for comparison of responses in LiCl and LiCl/myoinositol treated rats: the effect of lithium pre-treatment on PhE-induced contractions in the second group of rats was less marked than in the first group and therefore it would be expected that the responses in the second group would not be affected to a large extent by concomitant pre-treatment with myoinositol. Cells can obtain inositol by *de novo* synthesis, active uptake and passive diffusion (Downes and Stone, 1986). Different cell types show different requirements for extracellular inositol and *de novo* synthesis of inositol (Drummond, 1987). The smooth muscle of the rat aorta may be unable to utilise extracelluar inositol and depend almost entirely on *de novo* synthesis of inositol for any effects mediated by this system. If this were so then it would explain the marked effect seen with lithium treatment on PhE-induced contractions and also the lack of effect of myoinositol on the lithium-induced inhibitions of those contractions.

To conclude: the responses of the rat aorta to PhE and 5-HT can be differentiated by both their utilisation of extracellular Ca^{2+} in Ca^{2+} re-addition experiments and also by pre-treatment of lithium (presumably by blocking receptor-mediated inositol phospholipid hydrolysis). The difference in the effect of Ca^{2+} re-addition in the

presence of either agonist is dependent on pre-exposure to 5-HT and not due to activation of different proportions of ROCs and VOCs despite the observation that BAY K 8644 appears to have a preferential effect in the presence of 5-HT. The densensitisation seen with 5-HT only in low $[Ca^{2+}]_o$ is difficult to explain. It may indicate that the 5-HT_2-receptor induced activation of Ca^{2+} channels (at least to a level at which Ca^{2+} influx is possible) is somehow highly dependent on extracellular Ca^{2+}. Either the opening of the channels themselves, or the binding of 5-HT to a binding site which results in the opening of the channels, may require Ca^{2+} ions. These suggestions are purely speculative and further more detailed experimentation is required to define precisely the mechanism of this phenomenon.

Finally, using lithium pre-treated rats I have demonstrated indirectly that the contractile response to PhE in contrast to that of 5-HT is likely to be dependent, at least in part , on the hydrolysis of membrane inositol phospholipids. This does not exclude the possibility that this system may play a minor role in the effect of 5-HT but it would appear that the contractions of the aortic smooth to 5-HT in 2.5mM $[Ca^{2+}]$ are on the whole, independent of receptor mediated hydrolysis of membrane inositol phospholipids. It would be interesting to see if pre-treatment with LiCl would block the effect of BAY K 8644 on reversal/inhibition of 5-HT/Ca^{2+} re-addition since the partial opening of VOCs (as indicated by the effect of BAY K 8644 in these experiments) might result from modulation by 5-HT of the VOC by some metabolite of inositol phospholipid hydrolysis.

Despite the possible differences in utilisation of Ca^{2+} to induce their contractions, the presence of the vascular endothelium appears to have no differential effect between the responses to PhE and 5-HT.

Chapter Two

The influence of the initial stretch and the agonist induced tone on the effect of basal and stimulated release of EDRF.

Introduction

Variation between different laboratories with respect to the demonstration of both the basal and the stimulated release of EDRF can be marked. For example there has been some debate concerning a possible differential effect of vascular endothelium on contractile responses to different α-adrenoceptor agonists. Eglème *et al.* (1984) showed that the contractile effect of the mixed α_1- and α_2-adrenoceptor agonist clonidine was markedly greater in rat aortic ring segments with a disrupted endothelium yet this was not seen with the relatively more selective α_1-adrenoceptor agonist phenylephrine. It was suggested that clonidine released EDRF via α_2-adrenoceptors and this normally offset contraction mediated via stimulation of α_1-adrenoceptors. In another study Martin *et al.* (1986) found qualitatively similar but quantitatively different phenomena. These workers suggested an alternative explanation that clonidine did not actually release EDRF but that its responses were somehow more susceptible because the spontaneous release of EDRF has a greater effect on the contractile response to agonists of lower efficacy than on that to 'full' agonists.

In studies carried out within the Institute of Physiology, Glasgow University we were unable to reproduce, to the same extent, the potentiation of the response to clonidine by removal of the vascular endothelium but did find potentiation of responses to phenylephrine (Templeton, PhD thesis 1988). This suggested that variations in experimental protocol might affect the perceived influence of the endothelium on contractile responses of the smooth muscle. Consequently we carried out the present study to examine the influence of the initial stretch of the smooth muscle and the agonist-induced tone in experiments designed to demonstrate quantitatively the effects of basal or stimulated release of EDRF. The results indicate that both factors can have a marked influence.

Methods

General

Aortic ring segments from male Wistar rats (250-300g) were prepared as indicated earlier (see General Methods). For the length-tension study the rats were age and weight matched (10 weeks and 265g) in order to reduce any variation that may occur due to possible differences in responseiveness with age and ring size. When required, endothelium was mechanically removed from ring segments by inserting the bent tip of a pair of forceps into the lumen and gently rubbing the intima whilst rolling the ring segment back and forth. Each 2-3 mm long ring segment was suspended between an isometric force transducer and a wire support in a 10 or 30ml isolated organ bath containing Krebs' solution.

The Krebs' solution used throughout the study had the composition outlined earlier (see General Methods). In addition, when noradrenaline (NA) was used, the Krebs' solution also contained 23μM ethylene diaminetetra-acetic acid (EDTA) in order to prevent degradation of the NA. Isometric tension changes were measured using either Harvard or Grass force transducers and recorded on a Linseis four channel chart recorders.

Effect of Resting Length (Tension)

Paired, rubbed/unrubbed aortic ring segments were used. In a first group of experiments the effects of endothelial disuption on resting tension at different degrees of stretch, the contractile response to 1μM PhE, and subsequent relaxation to 1μM ACh were assessed. The apparatus used allowed accurate measurement of the distance between lower and upper tissue holders using a vernier scale (Figure 2.1). The measurements of 'stretch' (length) allow for the thickness of the upper and lower tissue holders (500μm) and indicate the distance between the upper and lower edges of the top and bottom tissue holders respectively (see Figure 2.1). The tissue holders

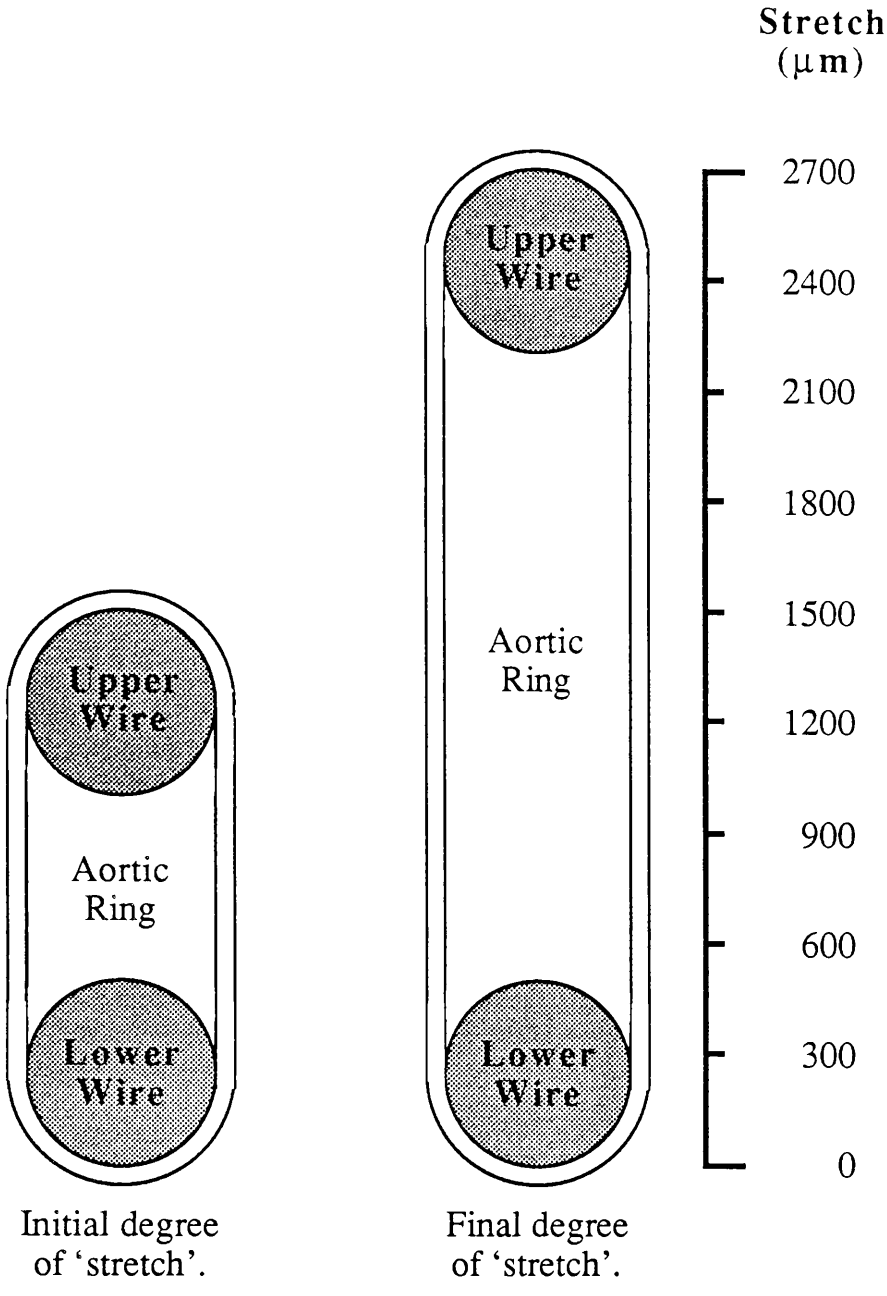


Figure 2.1

Diagram representing a 'side-on' view of a rat aortic ring segment suspended between an upper and lower wire tissue holder for measurement of isometric tension at different degrees of 'stretch'. From the initial degree of 'stretch' (1500μm) tissues were stretched in 300μm increments to the final degree of 'stretch' (2700μm). At each degree of 'stretch' examined resting tension, response to 1μM PhE and any subsequent relaxation to 1μM ACh were measured. Values of 'stretch' indicate wire separation with 'zero' indicating the position where the upper and lower edge of the upper and lower wire holders theoretically meet.

were separated by an initial distance of 1500μm. From this point each tissue was 'stretched' (i.e. its resting length increased) by increasing the separation of the tissue holders in steps of 300μm to a final separation of 2700μm. After each alteration of length, the ring segments were allowed 15 minutes to equilibrate before administering 1μM PhE to produce 90-95% maximal contraction. When the contractile response had reached a plateau a concentration of acetylcholine 1μM ACh (a concentration producing near maximal relaxation) was added to the baths and any relaxation monitored. The tissues were then washed by exchanging the bathing Krebs' solution three times over a 15 minute period before further increasing the degree of stretch of the tissue.

In a separate group of tissues the effects of initial passive tension (hence stretch) on the quantitative assessment of sensitivity to PhE and ACh were examined. The contractile response to cumulative addition of PhE (0.01-30μM) was examined over the range of resting tensions corresponding to those seen at each degree of 'stretch' used in the first set of experiments (see above). Relaxations of 1μM PhE-induced tone by cumulative addition of ACh (0.01-10μM) in endothelium-intact preparations at various resting tensions were then obtained. The sensitivity of the preparation to PhE-induced contraction was estimated by interpolation of the concentration of PhE producing 50% maximal contraction (EC_{50}) and expressed as its negative $\log_{10}$ i.e. the pEC_{50} or pD_2. The sensitivity of the preparation to ACh-induced release of EDRF was estimated as the concentration of ACh producing 50% maximal relaxation (IC_{50}) and expressed as its negative $\log_{10}$ i.e. the pIC_{50}.

Effect of Agonist-Induced Tone

The effect on sensitivity to ACh-induced relaxations of inducing tone with different concentrations of NA and PhE was examined.

Noradrenaline

Aortic ring segments with an intact endothelium were used. Each ring segment was set at the same initial tension of 1500 mg. wt. The preparations were washed twice by exchanging the bathing Krebs' solution and left for 60 minutes to equilibrate before a cumulative-concentration response curve to NA (0.003-10μM) was obtained. After reaching maximum contraction, the tissues were washed by exchanging the bathing Krebs' solution three times over a 15 minute period and allowed to equilibrate for a further 30 minutes. Muscle tone was then raised by adding a fixed amount of NA. After the contractile response had reached a plateau, ACh was added cumulatively (0.03-10μM). This was repeated for three different concentrations of NA (3, 10, and 30 nM) in each ring segment added in a randomised order. NA-induced tone was expressed as a percentage of the initial maximum response to NA. The sensitivity of the preparations to ACh-induced relaxation was expressed as pIC_{50} values.

Phenylephrine

From each rat, five aortic ring segments with an intact endothelium were used. In this case each ring segment was set at the same initial tension of 1000 mg. wt. The preparations were washed by exchanging the bathing Krebs' solution three times at five minute intervals (re-adjusting the resting tension to 1000 mg. wt. after each wash) and left for 15 minutes to equilibrate. The presence of a functional endothelium was demonstrated as described in General Methods. Muscle tone was then raised by adding a concentration of PhE to each of the preparations (0.1, 0.3, 1, 3, or 10μM). After the contractile response had reached a plateau, ACh was added cumulatively (0.01-10μM) to all preparations thus avoiding any variation in ACh sensitivity that might occur with time. The sensitivity of the preparations to the ACh-induced relaxations was expressed as pIC_{50} values.

Results

In rat aortic ring segments with an intact endothelial cell layer 1μM ACh produced a rapid, well maintained reduction in 1μM PhE-induced tone. With the endothelium disrupted, ACh had little effect but the responses to PhE were greater than those seen with intact endothelium (Figures 2.2 and 2.4).

Effect of Resting Length (Tension)

With or without an intact endothelium, there was a length dependent increase in the resting tension of the aortic rings. Disruption of the endothelium had no significant effect ($P>0.05$) on the resting tension of the preparations compared with paired intact controls at all degrees of 'stretch' examined (Figure 2.3).

1μM PhE produced a well maintained contractile response in both rubbed and unrubbed praparations (Figure 2.2). The size of this contractile response was dependent on the initial length of the preparation. In both rubbed and unrubbed vessels the contractile response increased with increasing resting length, reaching a maximum at 2400μm, after which no further increase in the response to PhE was seen. The response to PhE was significantly greater (unpaired Student's *t* test; $P<0.05$) at all resting lengths with a disrupted endothelium than with an intact endothelium (Figure 2.4). The 'absolute' difference in contractile response between the rubbed and unrubbed preparation (expressed in mg. wt.) was greatest at 1800μm but similar at the other lengths tested (Figure 2.5a). The 'absolute' difference at 1800μm was significantly greater ($P<0.05$) than at 2400μm i.e. at the optimum degree of stretch for contraction *per se*. The 'proportionate' difference in contractile response to PhE (expressed as the percentage of the response in the intact, paired vessel), was dependent on the initial length of the preparation. This was greatest at the lowest degree of stretch (1500μm) declining rapidly until 2100μm after which the difference was constant Figure 2.5b). The 'proportionate' difference at 1500μm was significantly greater ($P<0.05$) than at 2400μm.

Figure 2.2

Response of the rat aorta to 1μM PhE (△) and 1μM ACh (▼) in preparations with (a) an intact endothelium and (b) a disrupted endothelium. The endothelium was disrupted by gently rubbing the intimal surface of the ring segment with a pair of curved forceps. PhE produces a well maintained contraction of the aorta which is greatest in the preparation with a disrupted endothelium (a) due to basal release of EDRF directly opposing the contraction. Only in the unrubbed preparation (b) did ACh produce a well maintained relaxation of induced tone.

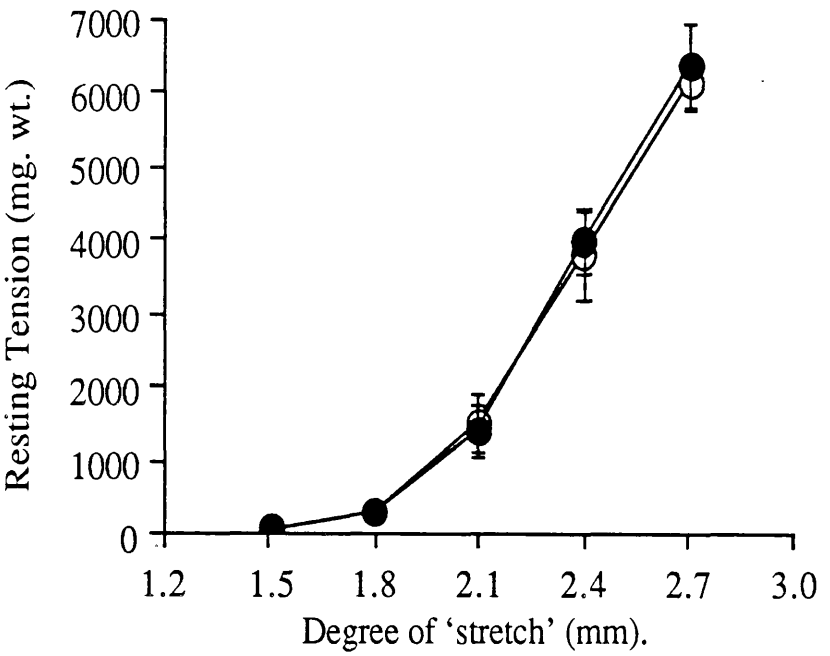


Figure 2.3

Effect of increasing the degree of 'stretch' on the resting tension of rat aortic rings in the presence (●) and absence (○) of a functional endothelium.

Points shown are mean ± s.e.m. (n=7). No statistically significant difference in resting tension between rubbed and unrubbed preparations was seen using unpaired Student's *t* test at all degrees of 'stretch' examined.

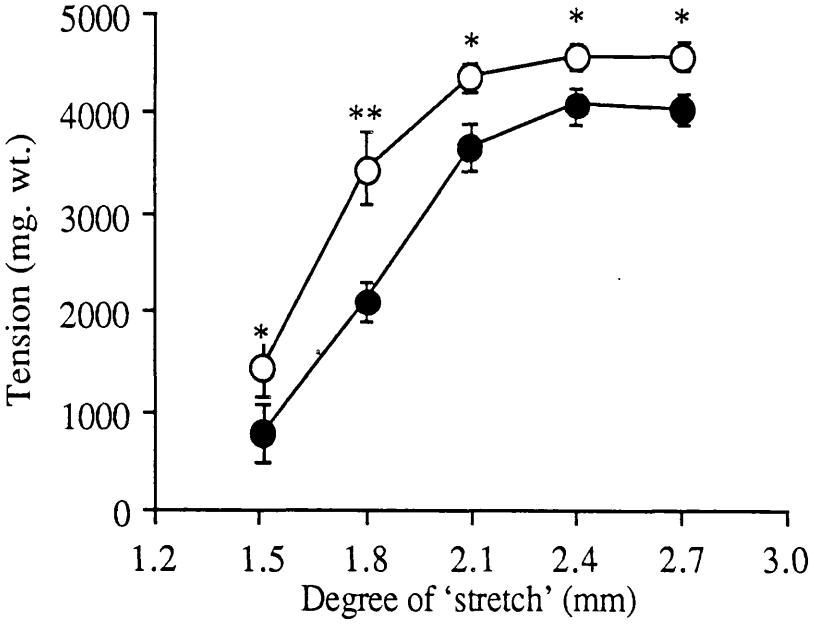


Figure 2.4

Effect of increasing the degree of 'stretch', hence resting tension, on contractile response of rat aortic rings to 1μM PhE in the presence (●) and absence (○) of a functional endothelium.

Points shown are mean ± s.e.m. (n=7). Statistically significant differences between contractile responses in rubbed and unrubbed preparations; * $P<0.05$, ** $P<0.01$, using unpaired Student't *t* test.

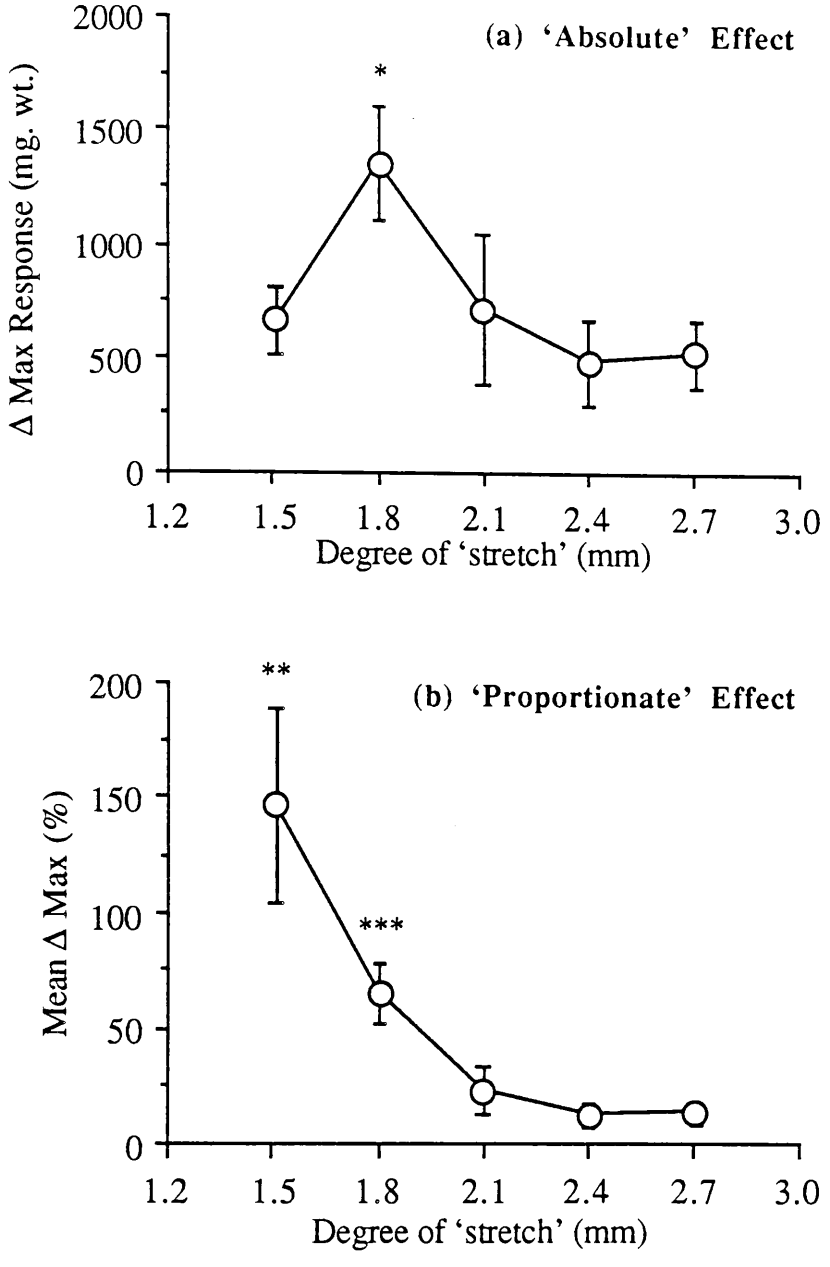


Figure 2.5

Effect of increasing the degree of 'stretch', on the difference in contractile response of rat aortic rings to 1μM PhE in the presence and absence of a functional endothelium. The difference is expressed as (a) the 'absolute' effect (i.e. in terms of mg. wt.) and (b) the 'proportionate' effect (i.e. as a percentage of the contraction in the intact vessel).

Points shown are mean ± s.e.m. (n=7). Statistically significant difference between effect at 2.4 mm and other degrees of 'stretch' examined; * $P<0.05$, ** $P<0.01$, *** $P<0.005$, using unpaired Student's *t* test.

In rings with an intact endothelium, pre-contracted with 1μM PhE, ACh (1μM) induced a rapid, well-maintained relaxation (Figure 2.2b). The ACh-induced relaxation was dependent on the initial length of the preparation. The 'absolute' relaxation of induced tone (i.e. in terms of mg. wt.) increased with increasing length reaching an optimal relaxation at 2400μm (Figure 2.6a). The 'proportionate' relaxation (i.e. the relaxation expressed as the percentage of the induced tone) was also length-dependent being optimal at 1500μm and decreasing with increasing length to a minimal response at 2100μm. The 'proportionate' relaxation at 1500μm was significantly greater ($P<0.05$) than the response at 2400μm (the optimum degree of 'stretch' for contraction *per se*). At 2400μm and 2700μm the 'proportionate' relaxation to ACh was slightly greater than that at 2100μm and similar to that seen at 1800μm (Figure 2.6b).

In the second set of tissues, length was not monitored but the rings were set up at initial resting tensions corresponding to those seen at each degree of 'stretch' used in the first set of tissues (see Figure 2.1). Cumulative concentration-response curves to PhE were obtained at each of the five increasing tensions. Paired time controls were run at a fixed resting tension of 1000 mg. wt. to determine any possible variation with time. This time control showed a small, time-dependent variation in sensitivity to PhE (Figure 2.7) possibly complicating demonstration of any change in sensitivity of the preparations to PhE-induced contractions which might occur with increasing initial resting tension of the preparation. When account was taken of this, the initial resting tension had no effect on sensitivity to PhE (as indicated by the pD_2 values; $P>0.05$) in either endothelium-intact (Figure 2.7a) or endothelium-denuded (Figure 2.7b) preparations. The sensitivity to PhE-induced contractions in the endothelium-denuded preparations was consistently greater than in the endothelium-intact vessel whether the resting tension of the preparation was increased or fixed (Figure 2.8). The difference in sensitivity between rubbed and unrubbed preparations varied with time to a similar degree both in preparation with fixed and increasing resting tensions. This difference

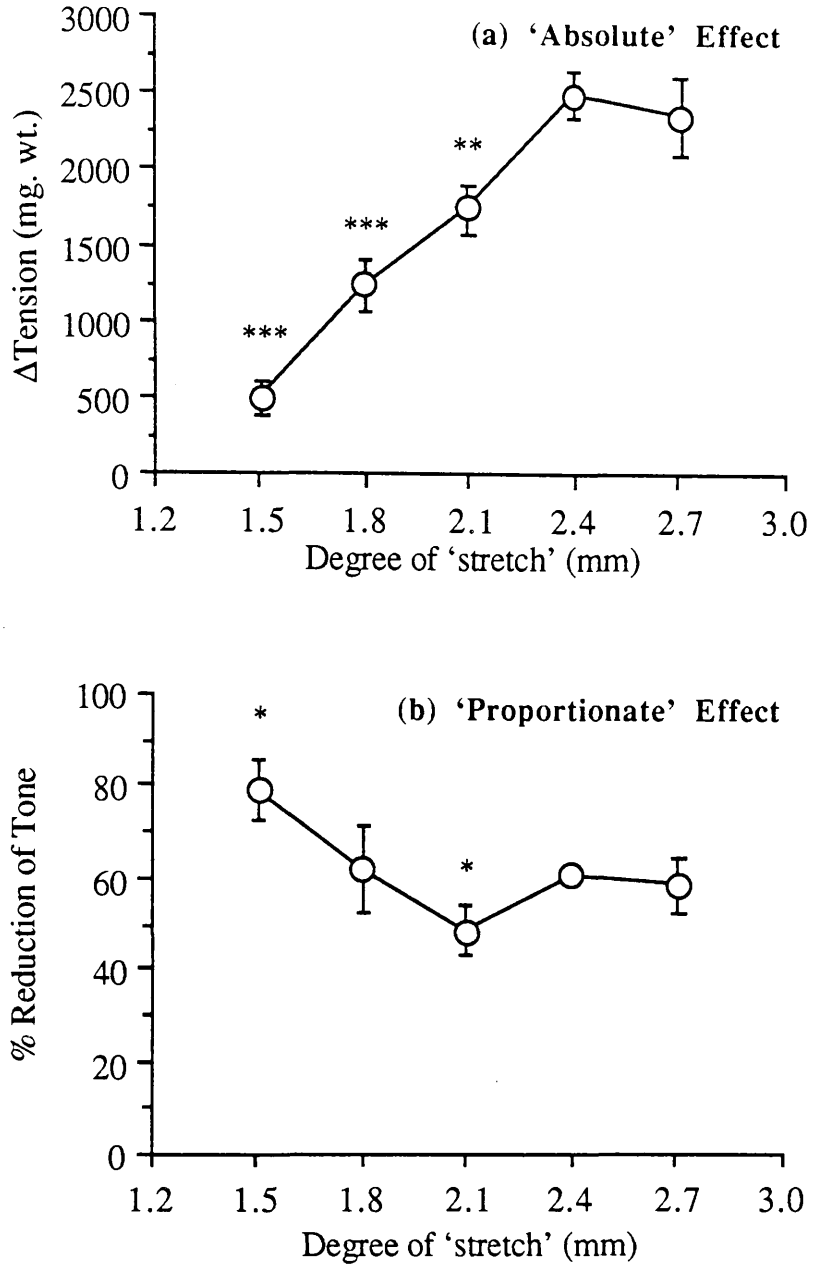


Figure 2.6

Effect of increasing the degree of 'stretch' on ACh-induced relaxations of rat aortic rings precontracted with 1μM PhE. The response is expressed as (a) the 'absolute effect (i.e. in terms of mg. wt.) and (b) the 'proportionate' effect (i.e. as a percentage reduction of the induced tone).

Points shown are mean ± s.e.m. (n=7). Statistically significant difference between effect at 2.4 mm and other degrees of 'stretch' examined; * $P<0.05$, ** $P<0.01$, *** $P<0.005$, using paired Student's *t* test.

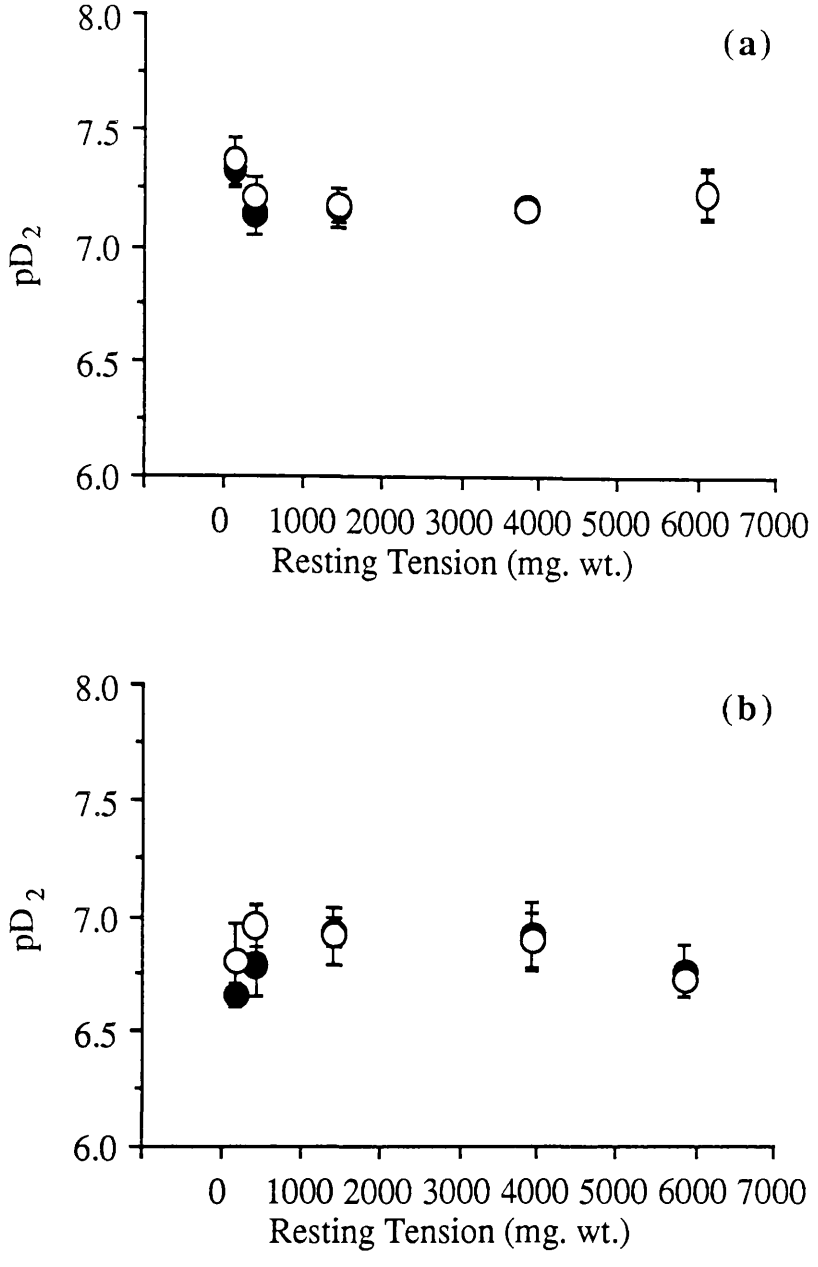


Figure 2.7

Effect of increasing the resting tension, hence degree of 'stretch', on sensitivity of rat aortic rings to PhE-induced contractions expressed as the pD_2 for PhE. Data shown represents the pD_2 values in (a) the absence and (b) the presence of a functional endothelium for time controls (○) and with increasing initial resting tension (●).

Points shown are mean ± s.e.m. (n=6). There was no significant difference ($P>0.05$) in pD_2 values between time controls and increasing initial resting tension in the absence and presence of endothelium using unpaired Student's *t* test

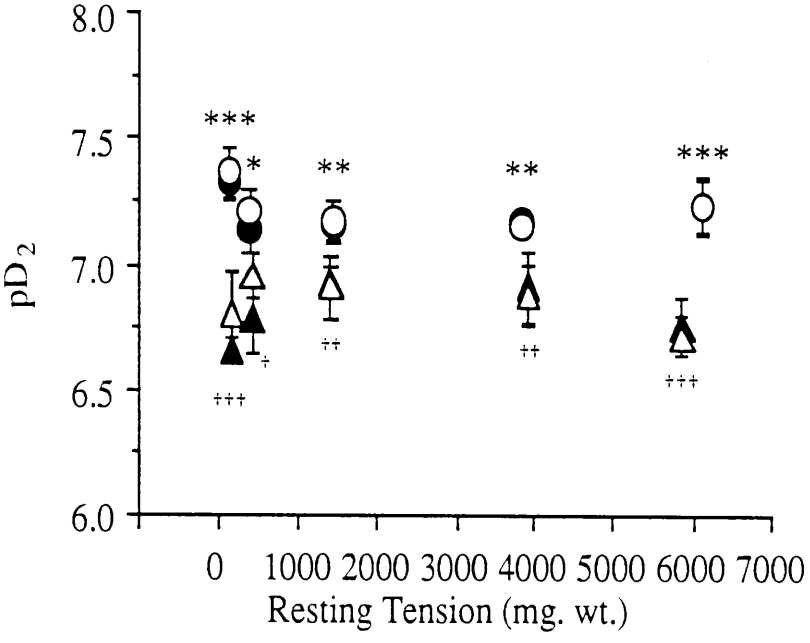


Figure 2.8

Effect of increasing the resting tension, hence degree of 'stretch', on sensitivity of rat aortic rings to PhE-induced contractions expressed as the pD_2 for PhE. Data shown represents the pD_2 values for time controls in the absence (○) and presence (△) of a functional endothelium and the pD_2 values with increasing initial resting tension in the absence (●) and presence (▲) of a functional endothelium.

Points shown are mean ± s.e.m. (n=6). Statistically significant differences between pD_2 values for time controls and pD_2 values with increasing initial resting tension in the absence and presence of a functional endothelium using unpaired Student's *t* test; * $P<0.05$, ** $P<0.01$, *** $P<0.005$ for comparison of time controls and † $P<0.05$, †† $P<0.01$, ††† $P<0.005$ for comparison of preparations with increasing tension.

in sensitivity appeared to be greater for the first and last CCRC (Figure 2.9). Statistical analysis however showed that the difference was not statistically greater (Student's unpaired *t* test; P>0.05) for either the first or last CCRC compared to the second, third and fourth CCRC.

The sensitivity to ACh-induced relaxations of 1μM PhE-induced tone in endothelium-intact aortic rings decreased slightly with time in this set of experiments (Figure 2.10a). This again complicated demonstration of any change in sensitivity to ACh-induced relaxations which might occur with increasing the initial resting tension of the preparation (Figure 2.10b). Although the size of the PhE-induced contraction increased with increasing initial resting tension, when account was taken of the time dependent change in sensitiity to ACh, the initial resting tension of the tissue had no effect on sensitivity to ACh-induced relaxations (as indicated by the pIC_{50} values, P>0.05) in the presence of an intact endothelium (Figure 2.11).

Effect of Agonist Induced Tone

Noradrenaline

In tissues with an intact endothelium, ACh produced a concentration-dependent inhibition (relaxation) of 0.3 μM NA-induced tone. In this set of experiments the sensitivity of the intact vessels to ACh-induced relaxations showed no significant change with time (Figure 2.12). When the tissues were contracted with various concentrations of NA (0.01-3 μM) to produce different degrees of tone (35-145% of initial maximum contraction to NA: the maximum contraction to NA commonly increases in the course of such experiments without a significant change in sensitivity (Figure 2.13)), the relaxations to ACh varied with the degree of induced tone (Figure 2.14). When the concentration of NA used was low (0.03μM), the sensitivity of the tissue was high (pIC_{50} = 7.36 ± 0.07). When the degree of NA-induced tone was increased by increasing the concentration of NA used to induce that tone (0.1 and 0.3μM), the tissues were less sensitive to ACh (pIC_{50} = 6.89 ± 0.06 and 6.57 ± 0.07,

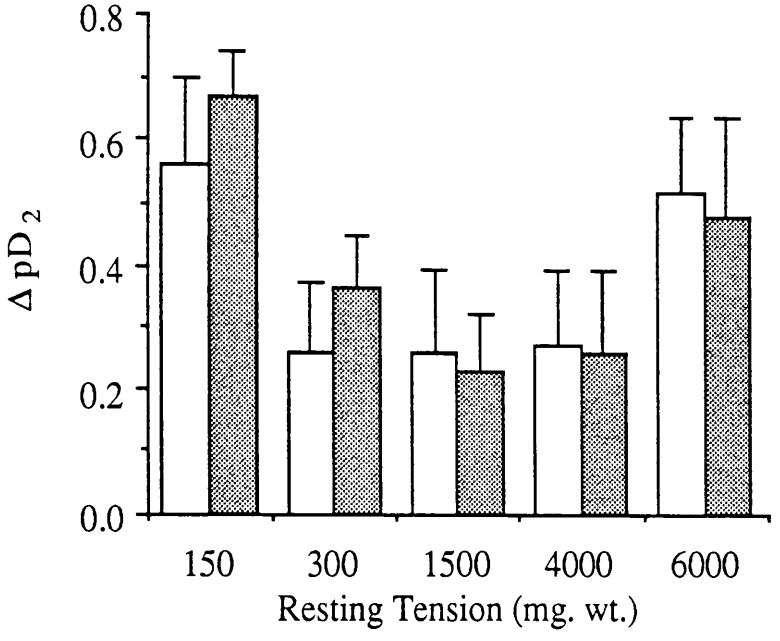


Figure 2.9

The increase in sensitivity to PhE-induced contractions due to removal of the vascular endothelium of rat aortic rings in preparations with increasing resting tension (filled bars) and in concomitant time controls (open bars) set at a resting tension of 1000 mg. wt.

Points shown are mean ± s.e.m. (n=6).

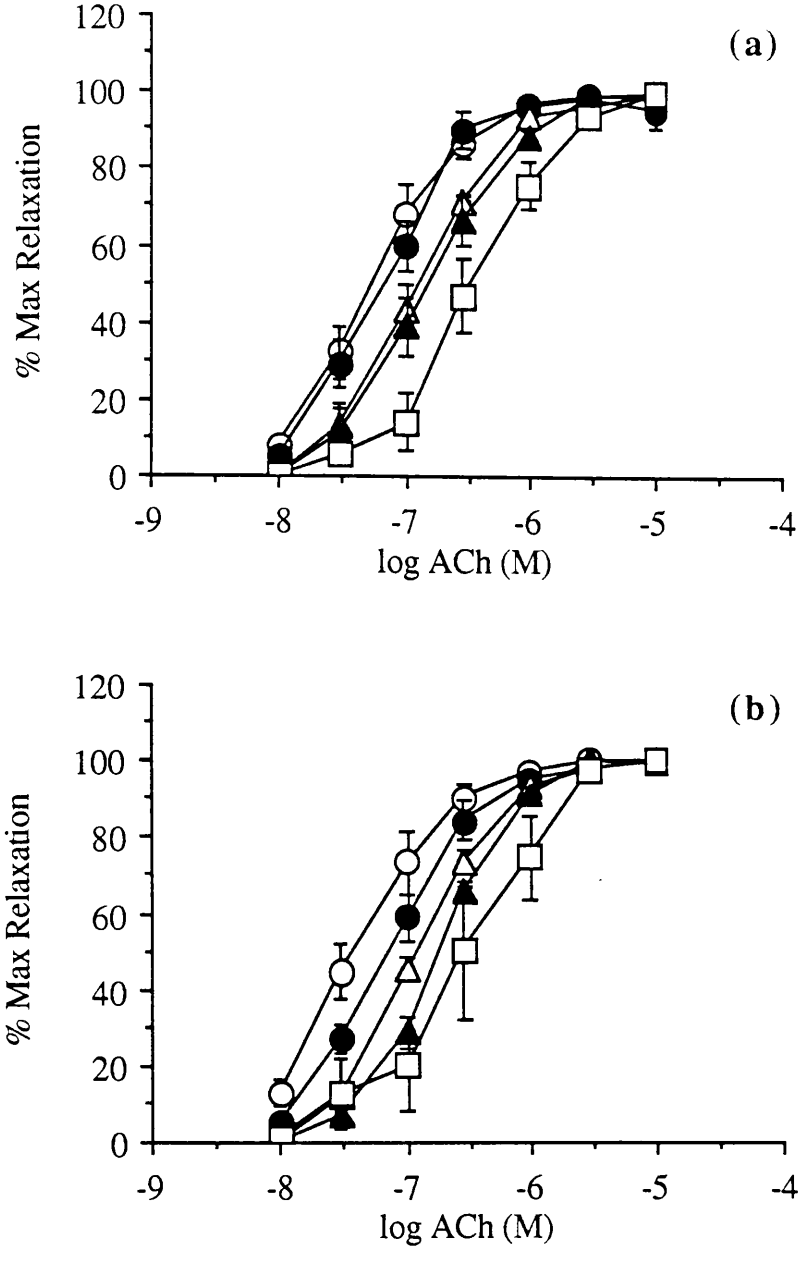


Figure 2.10

Relaxations of intact rat aortic rings by successive cumulative concentration-response curves for ACh in terms of the percentage maximum response. Effect of (a) time; first (○), second (●), third (△), fourth (▲) and fifth (□) successive CCRC for ACh, and (b) increasing initial resting tension; approximately (○) 153 ± 35, (●) 347 ± 52, (△) 1466 ± 56, (▲) 3377 ± 138, and (□) 6036 ± 176 mg.wt..

Points shown are mean ± s.e.m. (n=8).

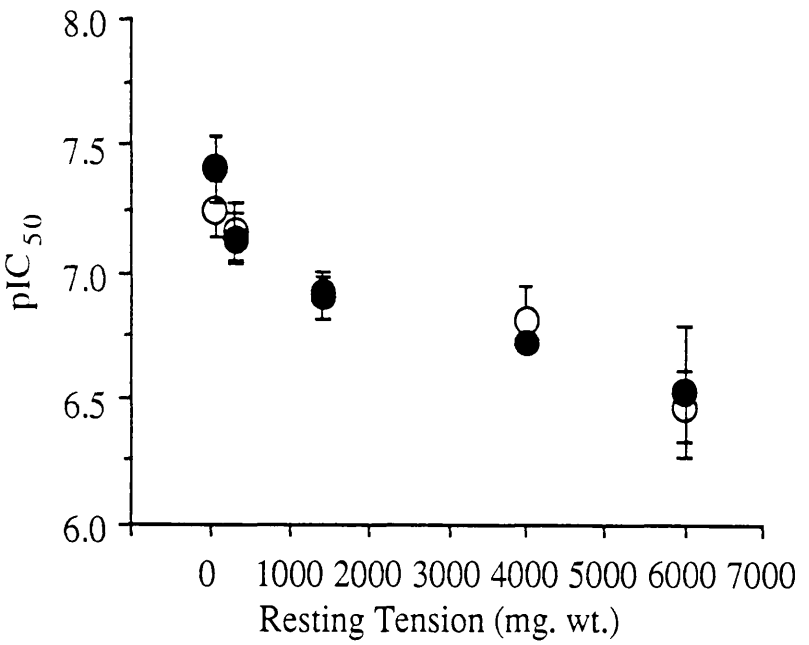


Figure 2.11

Effect of increasing the resting tension, hence degree of 'stretch', on sensitivity of rat aortic rings to ACh-induced relaxations of 1μM PhE-induced tone expressed as the pIC_{50} for ACh (i.e. the negative $\log_{10}$ of the concentration of ACh producing 50% maximal relaxation). Data shown represents the pIC_{50} values for time controls (○) and the pIC_{50} values with increasing initial resting tension (●).

Points shown are mean ± s.e.m. (n=6). pIC_{50} values for relaxations at increasing initial resting tensions were not significantly different from pIC_{50} values in time controls; $P>0.05$ using unpaired Student't *t* test.

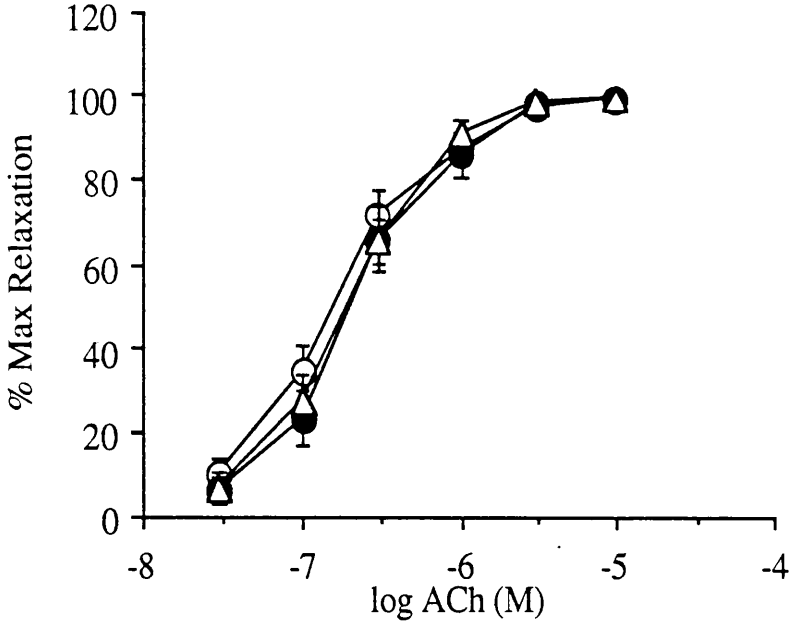


Figure 2.12

Effect of time on relaxations of intact rat aortic rings to successive cumulative concentration-response curves for ACh. Tone was induced by addition of 0.3μM NA. Data shown represents first (○), second (●), and third (△) successive CCRC for ACh in terms of the percentage maximum response.

Points shown are mean ± s.e.m. (n=13).

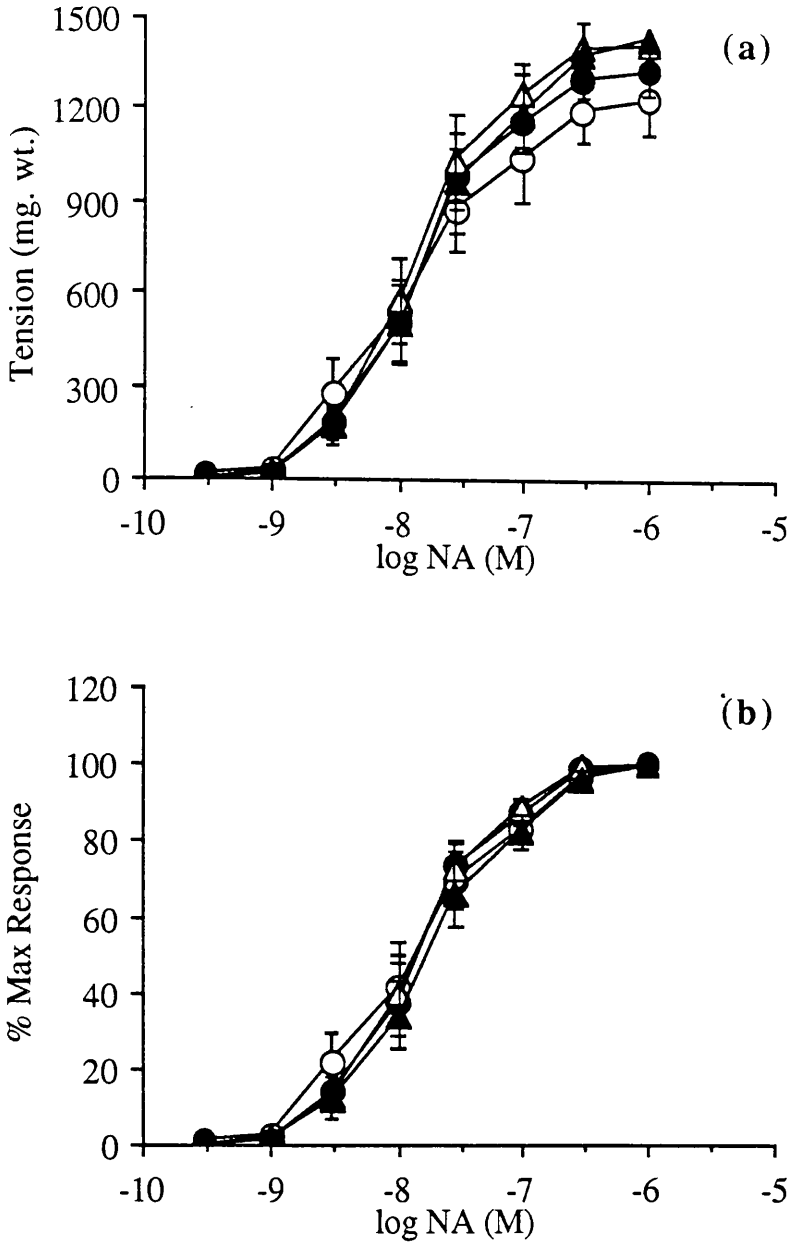


Figure 2.13

Effect of time on contraction of intact rat aortic rings to successive cumulative concentration-response curves for NA. Data shown represents first (○), second (●), third (△), and fourth (▲) successive CCRC for NA in terms of (a) the absolute response (i.e. mg. wt.) and (b) the proportionate response (i.e. percentage maximum response).

Points shown are mean ± s.e.m. (n=13).

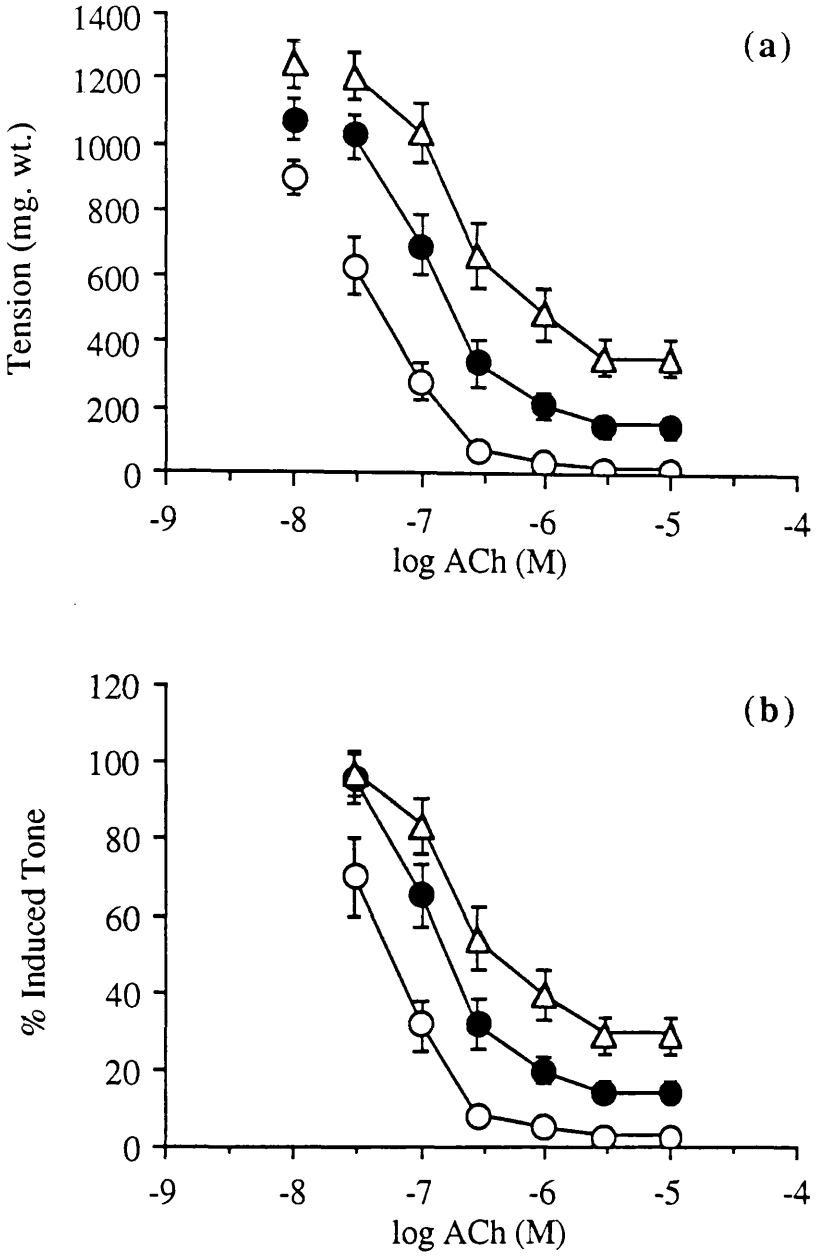


Figure 2.14

Effect of increasing the degree of NA-induced tone (by increasing the concentration of NA used to induce that tone) on ACh-induced relaxations of intact aortic rings. Relaxations are expressed as (a) the 'absolute' effect (i.e. in terms of mg. wt.) and (b) the 'proportionate' effect (i.e. as a percentage reduction of the induced tone). The concentrations of NA used to induce tone were; 0.03μM (○), 0.1μM (●) and 0.3μM (△). Single symbols in (a) represent the degree of tone induced by 0.03μM (○), 0.1μM (●) and 0.3μM (△) NA before addition of ACh

Points shown are mean ± s.e.m. (n=13).

respectively) since a greater concentration was required to elicit a similar percentage relaxation (Figure 2.15). The sensitivity of the tissues for ACh-induced relaxations of 0.03 and 0.1μM NA-induced tone was significantly greater (Student's paired *t* test; $P<0.05$) than that for ACh-induced relaxations of 0.3μM NA-induced tone.

Analysis of correlation for two dependent variables (namely induced tone (expressed as a percentage of the initial maximum contractile response to NA) and subsequent pIC_{50} values for ACh-induced relaxation) using pooled data showed an inverse correlation which was statistically significant ($P<0.0005$) (Figure 2.16a). A similar analysis of correlation for the same data but substituting the $\log_{10}$ molar concentration of NA for values of induced tone, again showed an inverse correlation which was statistically significant ($P<0.0005$) (Figure 2.16b). These correlations indicate an apparent decrease in tissue sensitivity to ACh with increasing initial tone or with an increase in the concentration of NA causing that tone. Correlations of the data obtained for individual tissues showed a similar effect but the slopes of the correlation lines were most similar when the $\log_{10}$ molar concentration of NA was used (Figure 2.17b) instead of the percentage of the control maximum contractile response (Figure 2.17a) (data for slopes not shown for clarity). An attempt was made to correlate tissue sensitivity to NA expressed as its pD_2 (i.e. the negative $\log_{10}$ of the concentration of NA producing 50% maximal contraction) and the subsequent responsiveness of the tissue to ACh-induced relaxation. Responsiveness to ACh-induced relaxation was expressed in terms of the maximum relaxation of induced tone and as the sensitivity (pIC_{50}) of the tissue to ACh. At each of the concentrations of NA used in the study to induce tone there was no statistically significant correlation between NA sensitivity and ACh responsiveness (Figure 2.18).

Phenylephrine

In this set of experiments the protocol used eliminated any possible variation in sensitivity of the intact vessels to ACh-induced relaxations with time. In tissues with

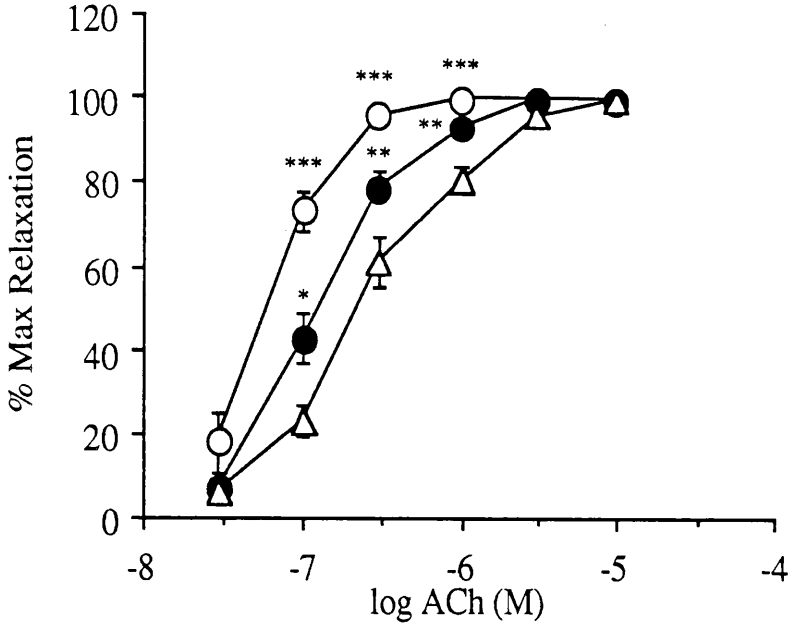


Figure 2.15

Effect of increasing the degree of NA-induced tone on ACh-induced relaxations of rat aortic rings. Tone was induced with 0.03μM (○), 0.1μM (●) and 0.3μM (△) NA.

Points shown are mean ± s.e.m. (n=13). Statistically significant differences between relaxant responses in the presence of 0.3μM NA and 0.1 or 0.03μM NA; * $P<0.05$, ** $P<0.01$, *** $P<0.005$ using paired Student't *t* test.

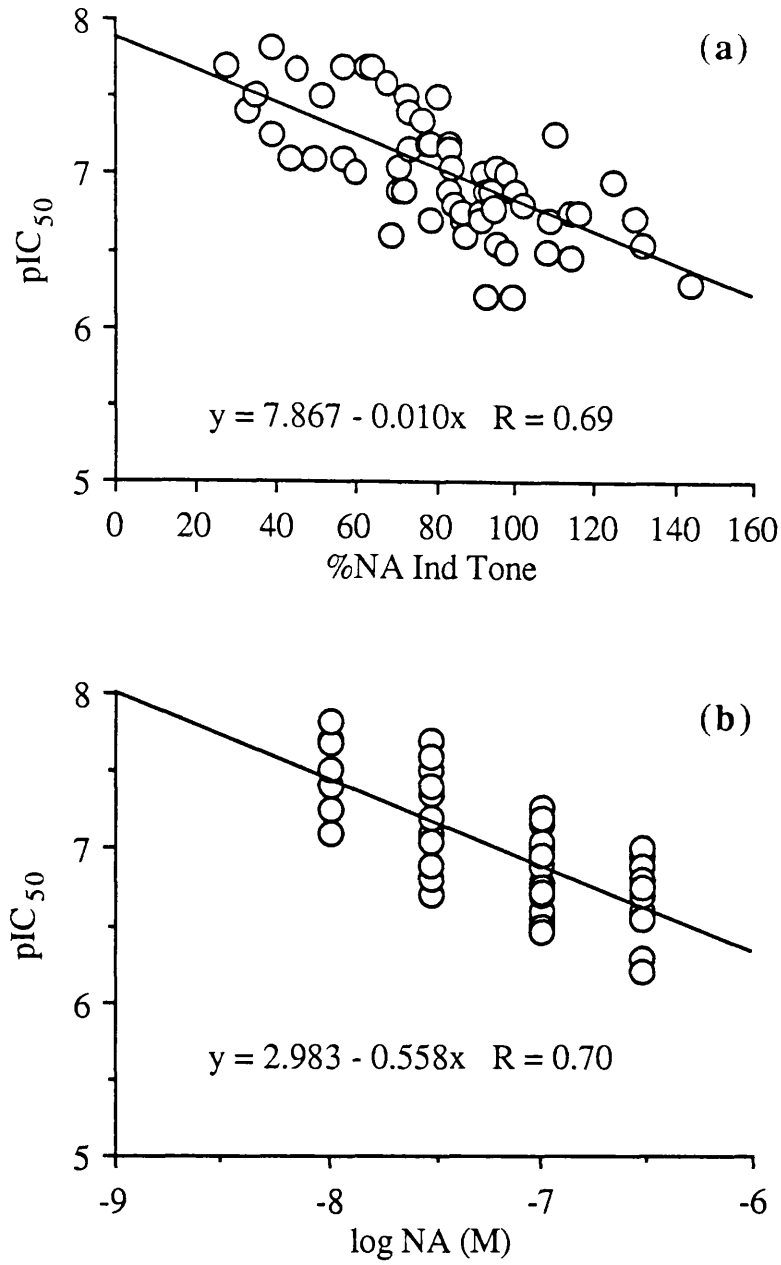


Figure 2.16

Correlation of sensitivity of intact aortic rings to ACh-induced relaxations of NA-induced tone expressed as the pIC_{50} for ACh (i.e. the negative $\log_{10}$ of the concentration of ACh producing 50% maximal relaxation) and the degree of induced tone expressed in terms of (a) a percentage of the initial control maximum response to NA and (b) the $\log_{10}$ molar concentration of NA used to induce the tone.

Points shown represent pooled data for n=13. Correlations in both (a) and (b) are statistically significant ($P<0.01$) for correlation of two dependent variables.

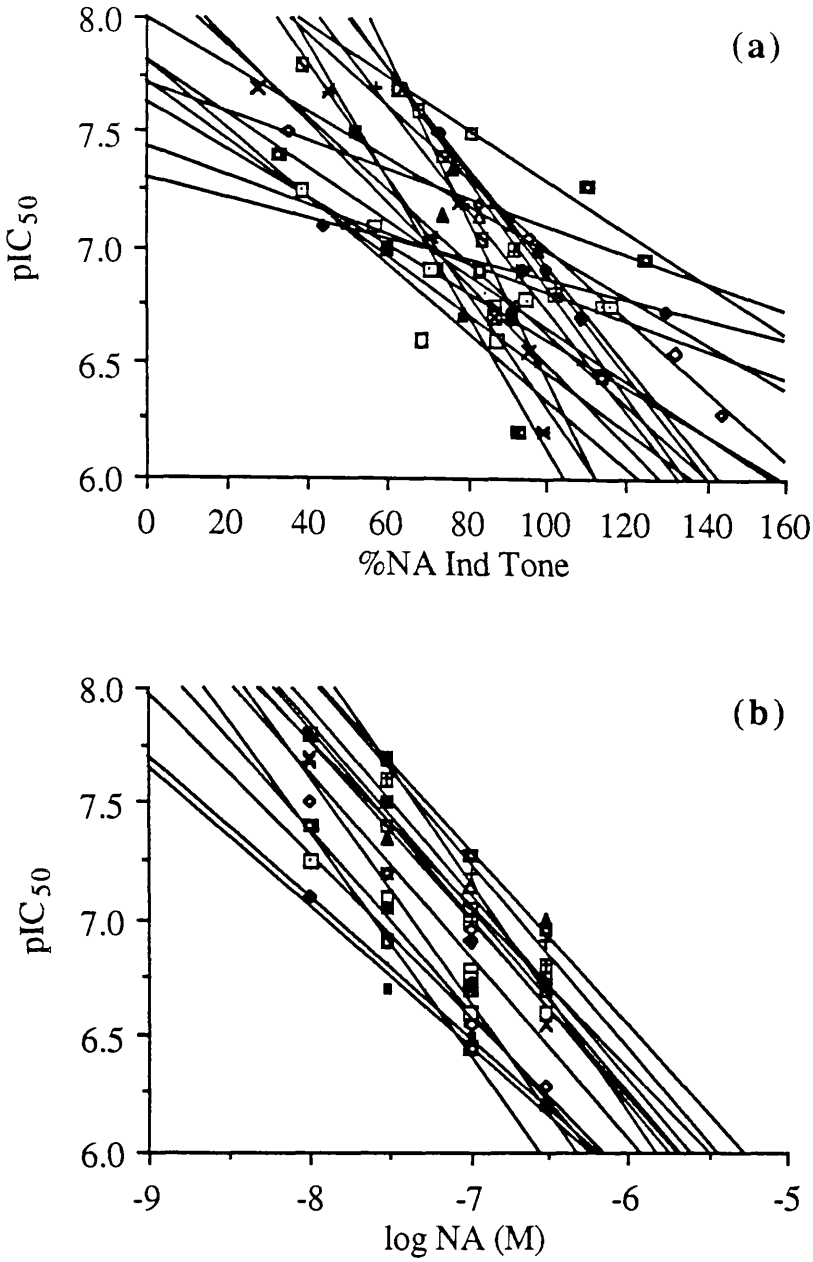


Figure 2.17

Correlation of sensitivity of intact aortic rings to ACh-induced relaxations of NA-induced tone expressed as the pIC_{50} for ACh (i.e. the negative $\log_{10}$ of the concentration of ACh producing 50% maximal relaxation) and the degree of induced tone expressed in terms of (a) a percentage of the initial control maximum response to NA and (b) the $\log_{10}$ molar concentration of NA used to induce the tone.

Points shown represent data for individual preparations (n=20).

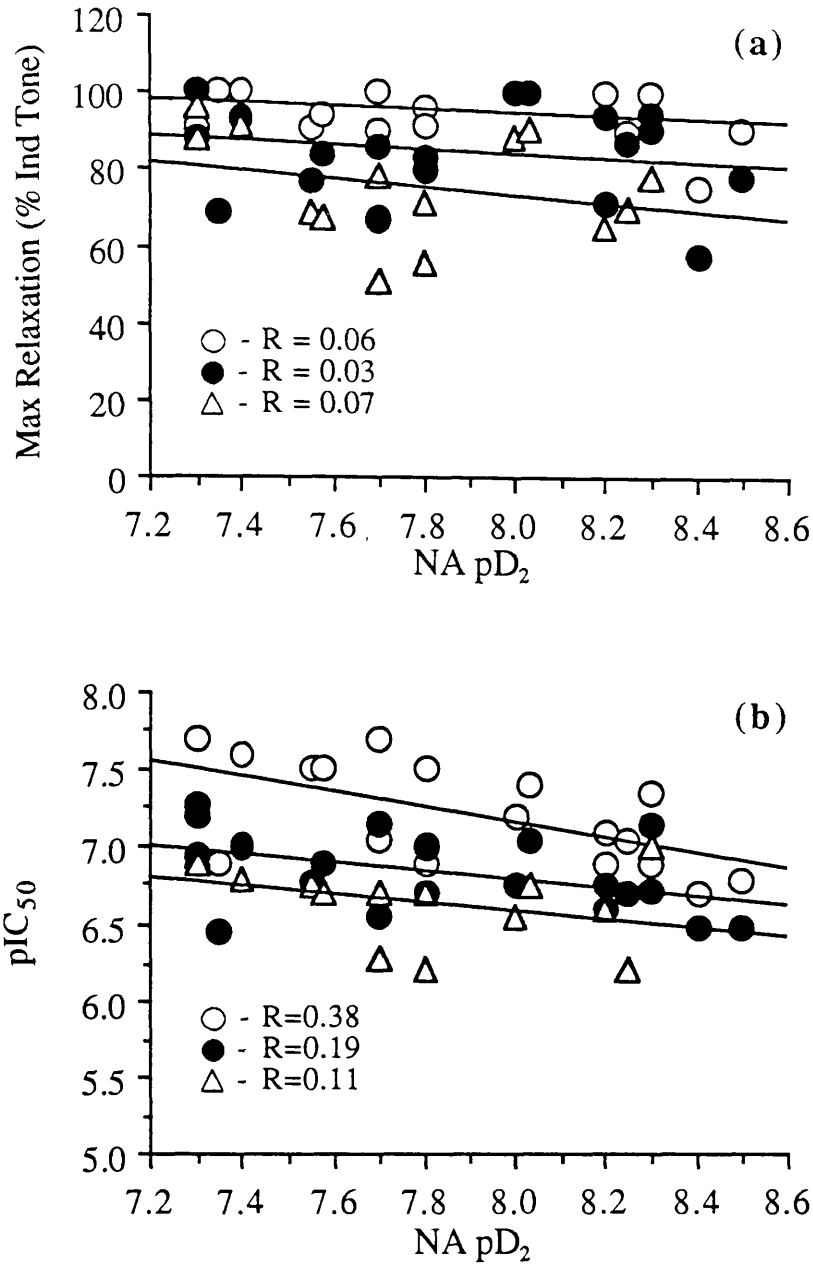


Figure 2.18

Correlation of tissue sensitivity to the contractile response of NA expressed as its pD_2 and the subsequent responsiveness of the tissue to ACh-induced relaxation of tone induced by 0.03μM (○), 0.1μM (●) and 0.3μM (△) NA expressed in terms of (a) the maximum relaxation of induced tone and (b) the sensitivity of the preparations to ACh (pIC_{50}).

Points shown are mean ± s.e.m. (n=13). There was no statistically significant correlation between sensitivity to NA and responsiveness to ACh at any of the concentrations of NA used to induce tone. Values of the correlation coefficient (R) are shown above.

an intact endothelium, ACh produced a concentration-dependent inhibition (relaxation) of PhE-induced tone. When the tissues were pre-contracted with various concentrations of PhE (0.1, 0.3, 1, 3, or 10μM) to produce different degrees of tone, the relaxations to ACh varied with the degree of induced tone (Figure 2.19). When the concentration of PhE used was low (0.1μM), the sensitivity of the tissue to ACh was high (pIC_{50} = 7.27 ± 0.04). When the degree of PhE-induced tone was increased by increasing the concentration of PhE used to induce that tone (0.3, 1, 3 and 10μM), the tissues were less sensitive to ACh (pIC_{50} = 7.09 ± 0.08, 6.76 ± 0.08, 6.83 ± 0.14 and 6.80 ± 0.04 respectively) since a greater concentration was required to elicit a similar percentage relaxation (Figure 2.20). The sensitivity of the tissues to ACh-induced relaxation of 1μM and 3μM PhE-induced tone was not significantly different from that for relaxation of tone induced by 10μM PhE. However, the sensitivity to ACh-induced relaxation of 0.1 and 0.3 μM PhE-induced tone was significantly greater than that for relaxation of 10μM PhE-induced tone (Student's paired *t* test; $P<0.05$)

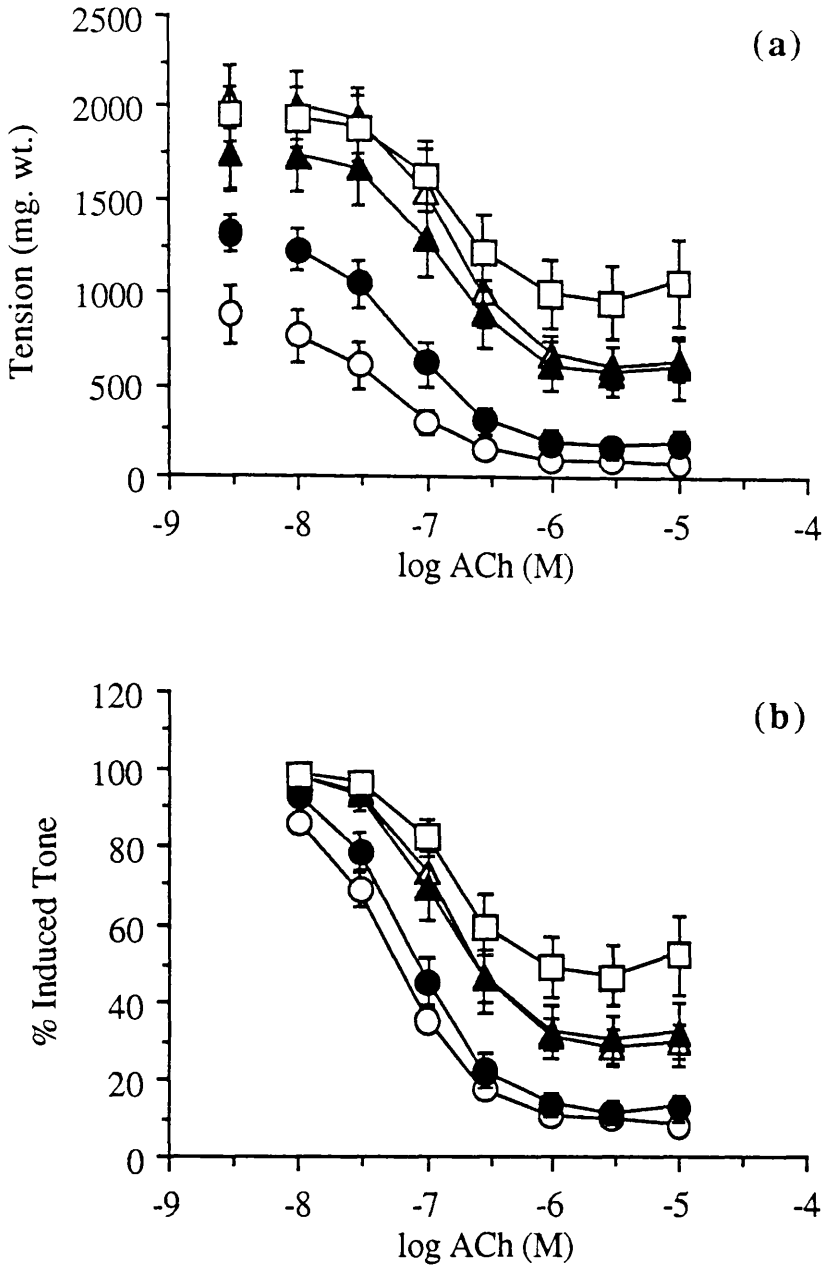


Figure 2.19

Effect of increasing the degree of PhE-induced tone (by increasing the concentration of PhE used to induce that tone) on ACh-induced relaxations of intact aortic rings. Relaxations are expressed as (a) the 'absolute' effect (i.e. in terms of mg. wt.) and (b) the 'proportionate' effect (i.e. as a percentage reduction of the induced tone). The concentrations of PhE used to induce tone were; 0.1μM (○), 0.3μM (●), 1μM (△) 3μM (▲) and 10μM (□). Single symbols in (a) represent the degree of tone induced by 0.1μM (○), 0.3μM (●), 1μM (△) 3μM (▲) and 10μM (□) PhE before addition of ACh

Points shown are mean ± s.e.m. (n=6).

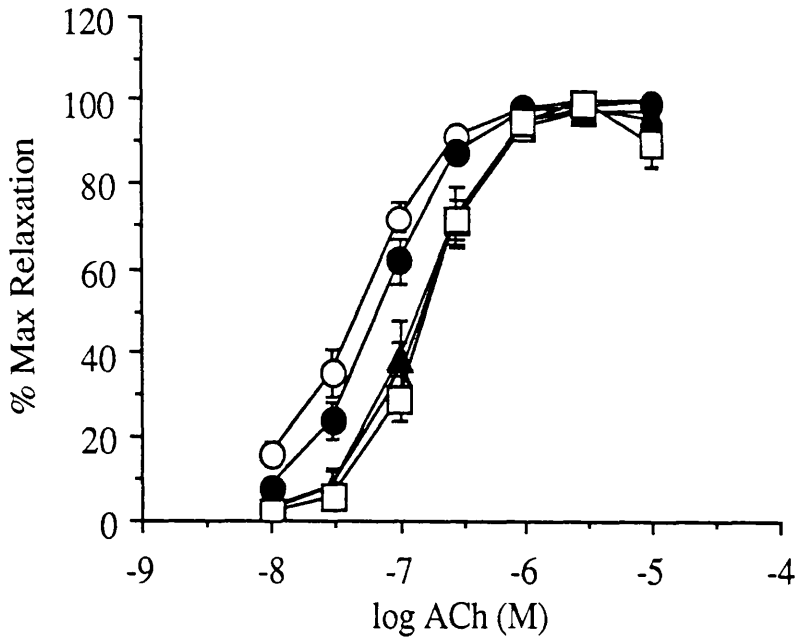


Figure 2.20

Effect of increasing the degree of PhE-induced tone (by increasing the concentration of PhE used to induce that tone) on sensitivity to ACh-induced relaxations of intact aortic rings. Relaxations are expressed as a percentage of the maximum relaxation. The concentrations of PhE used to induce tone were; 0.1μM (○), 0.3μM (●), 1μM (△) 3μM (▲) and 10μM (□).

Points shown are mean ± s.e.m. (n=7).

Discussion

The method which we employed to cause functional disruption of the endothelium of the aortic ring segments was clearly effective since vessels in which the vascular endothelium had been disrupted showed no relaxation to ACh. In addition, the responses to contractile agents such as PhE were greater in preparations with a disrupted endothelium presumably indicating the removal of a basal release of EDRF with little damage to the underlying smooth muscle layer.

The basal release of EDRF has been shown to modulate the response of vascular smooth muscle to contractile agents (Martin *et al.*, 1986; Bullock *et al.*, 1986). Removal of this basal release of EDRF might change the resting tone of isolated blood vessels such that tissues with and without endothelium would be stretched to different degrees to achieve the same resting tension. This might contribute to differences in contractile response between tissues with and without endothelium. We found, however, that over a wide range of lengths, removal of the endothelium had no significant effect on the resting tension of the preparations. This is not surprising since the failure of sodium nitroprusside (10μM) to relax the preparations under resting conditions (data not shown) indicates that they have no resting tone under the conditions used in this study. However, this does indicate that in experiments where paired ring segments with and without endothelium are set at the same resting tension, the degree of stretch (and hence length) in each tissue is similar. This therefore, will not play a significant part in the difference seen in the responses to contractile agents in paired rubbed/unrubbed preparations of rat aorta. This might not remain true in other vascular smooth muscle preparations particularly if they have intrinsic tone or myogenic activity e.g. portal vein. A corollary of the lack of influence of the endothelium on resting tone is that pharmacological blockade of EDRF, like mechanical disruption, should not increase vessel tone *per se*. Thus contractions to haemoglobin (Martin *et al.*, 1986) cannot be due solely to the loss of the effect of EDRF and may be at least partly attributable to an action of the haemoglobin leading to

activation of the quiescent muscle. This may lead to errors in interpretation of the effects of haemoglobin since it has recently been shown that in preparations such as the dog portal vein (Furuta *et al.*,, 1988) and the whole perfused rat tail (Templeton *et al.*, 1988) activation of the smooth muscle uncovers a previously 'unseen' population of functional α_2-adrenoceptors. Activation of these receptors might contribute to the increase in contractility of the preparations which might be attributed wholly to the blockade of the basal release of EDRF by haemoglobin.

The response to 1μM PhE increased with increasing stretch and reached a maximum at the same degree of stretch in both rubbed and unrubbed preparations. Therefore the presence of a functional endothelium (and hence the basal release of EDRF) does not affect the optimal conditions (in terms of initial tension) for demonstration of the contractile effects of the α_1-adrenoceptor agonist PhE. Thus, when paired rubbed and unrubbed preparations of rat aorta are set at the optimum resting tension for contraction of the rubbed preparation then the conditions (in terms of resting tension) will be equivalent in the unrubbed preparation or vice versa.

The contractile response to 1μM PhE seen in the absence of endothelium was consistently greater than in the intact vessel. This was observed at all degrees of stretch measured. This clearly demonstrates that in the rat aorta the presence of the vascular endothelium depresses the contractile response to α_1-adrenoceptor activation by PhE. This inhibition of contractility is presumably due to the basal release of EDRF which directly opposes the contractile response to PhE. However, whilst the initial length appeared to have no differential effect on the resting tension between rubbed and unrubbed preparations, the increase in contractility which was seen in the absence of endothelium was dependent on the initial tension of the preparation. In addition, the optimum stretch for demonstration of this difference varied with the manner in which the increase was expressed. The optimum length for demonstration of the 'absolute' difference in contractility to PhE (i.e. in terms of milligrams) was 2400μm whilst the optimum length for demonstration of the 'proportionate' difference in contractility to

PhE (i.e. as a percentage increase from the response seen in the intact vessel) was 1500μm. Irrespective of the method used to express the increased contractility seen in the absence of the endothelium, the optimum length for demonstration of this phenomenon does not coincide with the optimum stretch for demonstration of the contractile response *per se* (summarised in Figure 2.21). Clearly, the higher the initial resting tone, and hence stretch, the less will be the apparent effect of the endothelium on the PhE-induced contractions particularly when the increase in response in the absence of a basal release of EDRF is expressed as a proportion of the response in the intact vessel. This may account, at least in part, for conflicting reports of the effect of the vascular endothelium on the contractility of vascular smooth muscle. The basis of this is not clear but it could be due to recruitment by stretch of cells resistant to EDRF, e.g. cells near the adventitia where the effect of EDRF are less apparent due a greater diffusion distance between the endothelium and the contracting smooth muscle cells. This effect however, is more probably due stretching causing an increased efficiency of excitation-contraction coupling for agonist induced contraction thereby increasing the degree of contraction which the EDRF must overcome.

Demonstration of the relaxation produced by 1μM ACh in the intact vessel was dependent on the initial length of the preparation. As with the demonstration of the influence of the vascular endothelium on contraction to PhE, the optimal conditions for demonstration of the 'absolute' and 'proportionate' relaxation (i.e. percentage relaxation of induced tone) to ACh did not coincide. The optimum stretch for demonstration of the 'proportionate' effect of ACh (1500μm) did not coincide with the optimum for PhE-induced contractions. However, the optimum stretch for demonstration of the 'absolute' relaxation (2400μm) did coincide with the optimum for PhE-induced contractions. This is presumably because at this length there is a greater absolute amount of tone that can be inhibited. However, it is common practice to express ACh-induced relaxations as a percentage of the induced tone and this is not optimised by the use of conditions optimal for contraction. This has several important

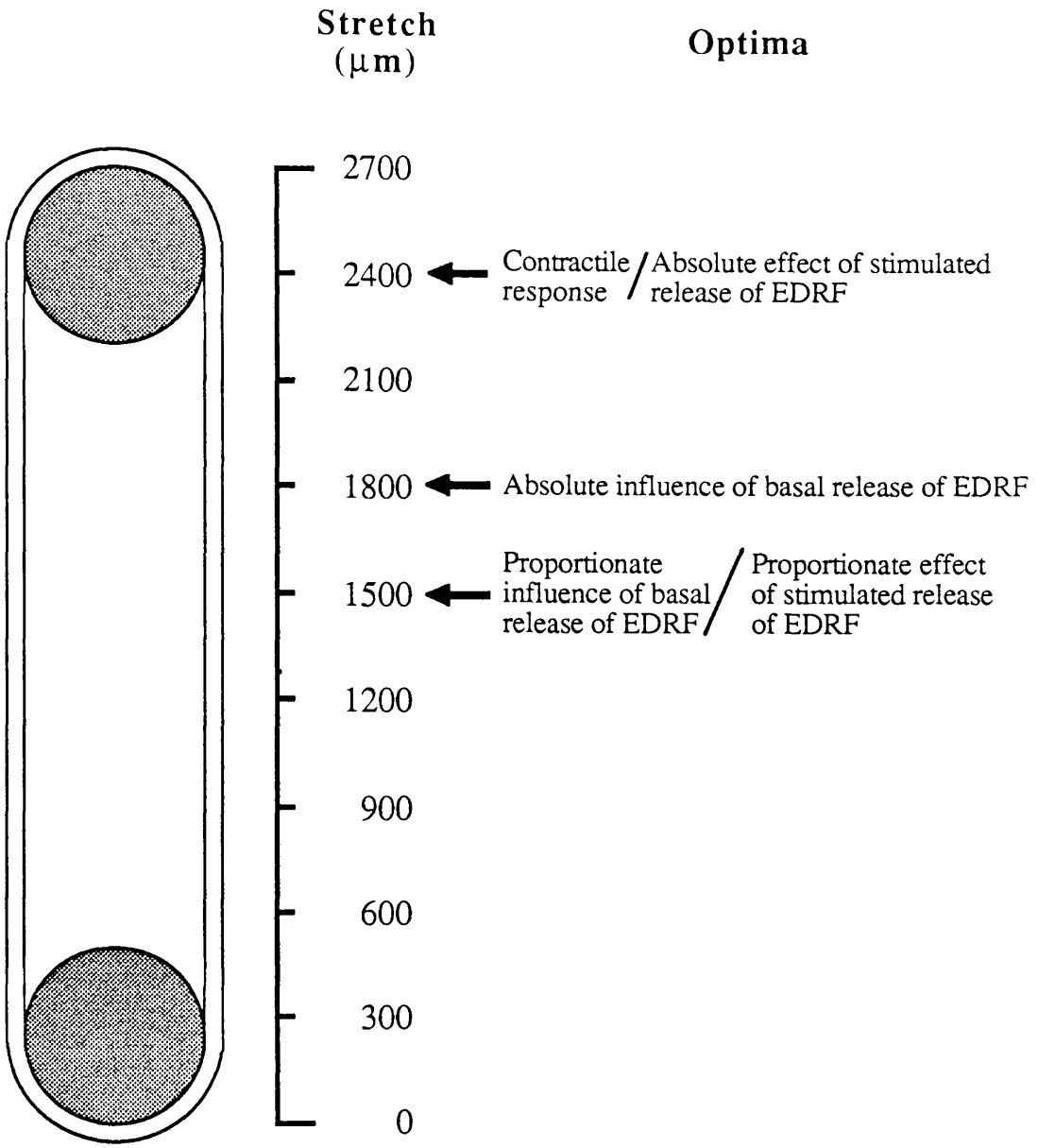


Figure 2.21

Summary of study examining the effect of varying the initial degree of stretch, hence length, of rat aortic ring segments on the contractile response to 1μM PhE and the subsequent relaxant response to 1μM ACh in the presence of an intact endothelium. Diagram indicates the optimum degree of stretch for demonstration of contraction *per se* and the proportionate and absolute influence of both basal and stimulated release of EDRF.

implications. When the responsiveness of different vessels to EDRF is examined either from different anatomical sites, from different species or from different patho-physiological states, the resting tensions (hence length) at which the vessels are set is usually that at which the response to the contractile agent is at a maximum (Collins *et al.*, 1986; Christie and Lewis, 1987) or is entirely arbitrary, based on evolved practice. Clearly, since this resting length may not be ideal for ACh-induced relaxations, it may be unrealistic to compare differences in responsiveness to ACh-induced release of EDRF without evaluating the influence of the initial stretch or induced tone on relaxations.

Changes in the distension of small arteries of the rat causes a change in the sensitivity of vascular preparations to contractile agents (Nilsson and Sjöblom, 1985). If this were so in the case of the rat aorta then the differences in responsiveness to single concentrations of PhE and ACh with stretch might be due to a change in sensitivity of the preparation to PhE-induced contractions or ACh-induced relaxations. In addition, there may be a change with time of the effect of basal release of EDRF which could contribute to the apparent differences seen with increasing stretch. In the case of PhE however this is not so since the sensitivity of neither endothelium-intact nor disrupted vessels varied with resting tension or with time. Moreover, the effect of basal release of EDRF, when expressed in terms of an increase in sensitivity (pD_2) to PhE in the rubbed preparation did not significantly change with time or increasing resting tension. Whilst the sensitivity of endothelium-intact preparations to ACh-induced relaxations did decrease slightly with time, there was no evidence for an influence of resting tension. It is unlikely that the decrease in sensitivity with time contributed a great deal to the differences in response to 1μM ACh at each degree of stretch examined since the degree of relaxation to this near maximal concentration of ACh seen in the time controls varied only a small amount.

The sensitivity of rat aortic ring segments to ACh was also found to be dependent on the level of induced tone. We found that with increasing levels of tone (produced by

increasing concentrations of NA) the sensitivity of the tissue to ACh-induced relaxations (expressed as its pIC_{50}) decreased. This relationship was validated by the significant inverse correlations seen between the tissue sensitivity to ACh and either the level of induced tone produced by NA or the $\log_{10}$ molar concentration of NA used to induce that tone. By extrapolation to zero % of the correlation between the tissue sensitivity to ACh-induced relaxations and the level of induced tone produced by NA, (Figure 2.16a), it is possible to estimate the maximum possible sensitivity of the tissue to ACh ($pIC_{50(max)} = 7.87$) i.e. the theoretical sensitivity of the tissue with no induced tone. It is interesting to note that slopes of the correlation lines between ACh sensitivity and degree of induced tone were most similar when the $\log_{10}$ molar concentration of NA was used (indicating the degree of 'activation ' of the tissue) than when the percentage of the control maximum contractile response was used (indicating the degree of 'responsiveness' of the tissue). This shows a better 'inter tissue' correlation between ACh sensitivity and tissue 'activation' than tissue 'responsiveness', suggesting that, with respect to induced tone the degree of 'activation' (best represented by the concentration of agonist used assuming no change in agonist sensitivity) and not the degree of 'responsiveness' (i.e. absolute size of response) is more important in determining the subsequent sensitivity to ACh-induced relaxations. This suggestion is supported by the observation that whilst the size of contraction to 1μM PhE increases with increasing resting tension there is no concomitant decrease in sensitivity to ACh-induced relaxations (see below). Thus whilst the degree of 'activation' of the tissue remains the same i.e. the same concentration of PhE is used to induce tone (assuming no change in sensitivity) despite an increase in 'responsiveness' there is no change in sensitivity to the stimulated release of EDRF.

In order to exclude the possibility that this phenomenon of increasing sensitivity with decreasing tone was due to the use of NA, similar experiments were carried out using another, more specific α_1-adrenoceptor agonist namely PhE. Since experiments

carried out to examine the effect of resting tension on ACh sensitivity revealed a small variation in sensitivity with time the protocol used eliminated any time-dependent changes in sensitivity to ACh-induced relaxations of PhE-induced tone or PhE induced contractions. This again revealed a decrease in sensitivity to ACh-induced relaxations with increasing induced tone confirming the observations seen when NA was used to induce tone. However, there was a limit to the decrease in ACh sensitivity seen with increasing activation of the tissue by PhE (no further decrease when tone was induced by >1μM PhE). This lack of effect of increasing the degree of activation on sensitivity to ACh-induced relaxations is somewhat surprising particularly since the maximum relaxation of the induced tone as a percentage of that tone decreased with the higher concentrations of PhE. This might suggest that the sudden increase in sensitivity with decreasing concentration of contractile agonist is at least partly due to the ACh-induced relaxations reaching a maximum 'response' (i.e. complete loss of the EDRF sensitive part of the induced tone) although the agonist induced release of EDRF is not itself yet at a maximum: thus there is merely an illusion of change in sensitivity. The observation that ACh is unable to produce complete relaxation even when the concentration of agonist used to induce a sustained contraction is very low supports this view. The 'EDRF resistant' component of agonist induced contraction may be due to an inability of EDRF to reach the outer contracted layers of the blood vessel. Since EDRF is a highly labile substance (half life 6-80 seconds; see General Introduction) there would presumably be a limit to the distance that released EDRF could diffuse through the smooth muscle cells of the blood vessel wall from the intimal layers, closely associated with EDRF's site of production, to the outer adventitial layers. This resistance of the outer layers of a blood vessel to the influence of EDRF might produce a differential effect of basal release of EDRF on contractile agents if the agent causes a preferential activation of the adventitial rather than intimal layers of the vessel wall. In addition it is reasonable to expect vessels with a thinner wall to be more susceptible to both the stimulated and basal release of EDRF.

In a study described later in this thesis, (see Chapter 3), where the endothelium of aortic ring segments was intentionally damaged to varied degrees such that the responsiveness of ring segments to ACh-induced relaxations varied, the sensitivity of the tissue to contractile agents showed an inverse, statistically significant correlation with the responsiveness to ACh-induced relaxations. In the present study the lack of a significant correlation between the sensitivities of the tissues to NA-induced contractions and subsequent ACh-induced relaxations indicates that the basal release of EDRF (and hence the integrity of the endothelium) in the preparations used in this study is consistent and therefore this plays no significant role in the difference in sensitivity observed at different levels of induced tone. There was, therefore, no evidence that variation in the spontaneous release of EDRF was a major factor determining the sensitivity to NA.

In conclusion, differences seen in the quantitative demonstration of both the basal and stimulated release of EDRF may be, at least in part, a result of the experimental protocol used to examine these phenomena. The resting tension of the tissue (hence length) is an important determinant in the demonstration of the effects of spontaneous or induced release of EDRF. Even within the same tissue the optimum conditions for demonstration of these different relaxant effects or of contractile effects do not necessarily coincide and are dependent upon how the data is expressed. In addition, the degree of induced tone can influence the perceived effects of stimulated release of EDRF. The apparent sensitivity of the tissue is altered when different levels of induced tone are employed and this may obscure any real changes in sensitivity to agents releasing EDRF. This apparent change in sensitivity may be due, at least in part, to the relaxation to agonist-induced release of EDRF appearing to reach a maximum at a concentration of agonist lower than that needed to produce a maximal release of EDRF *per se*. This may occur even if the maximum relaxation induced is less than 100% of the induced tone since there is likely to be a component of the induced tone that is resistant to endothelium-dependent relaxation.

These factors may account for the variations in interpretation of the role of EDRF between different vessels, pre-treatments and laboratories. The conditions used to compare vessels from different species or in different patho-physiological states, should be carefully chosen to ensure optimum demonstration of the effects of basal, and stimulated, release of EDRF. There is no simple prescription for this. The protocol may need to vary in order to optimise the particular phenomenon under analysis. Alternatively, and perhaps more satisfactorily, the vessels to be compared may be suspended under conditions where the transmural pressure in each vessel is the same or at a physiological level for each tissue being examined (Mulvaney & Halpern, 1986; Cocks & Angus, 1986). With respect to the level of induced tone used to examine the effects of stimulated release of EDRF, it seems advisable to use a concentration of agonist which produces a sufficient level of tone such that there would be no decrease in the sensitivity to agonist induced release of EDRF if a higher concentration of contractile agent were used. This would ensure that the apparent sensitivity to EDRF release is not affected by a component of the induced tone resistant to released EDRF.

Chapter Three

The influence of the endothelium on
5HT-induced responses of the rat aorta.

Introduction

An intact endothelial cell layer has been shown to have a marked depressant effect on the response of a variety of vascular smooth muscle to spasmogens (Criscione *et al.*, 1984; Carrier and White, 1985; Angus *et al.*, 1986; Calvette *et al.*, 1984). Eglème *et al.* (1984) showed that in the absence of the endothelium the response of the rat aorta to the α_2-adrenoceptor agonist clonidine was enhanced much more markedly than was the response to the relatively selective α_1-adrenoceptor agonist PhE. This suggested that in the presence of endothelium, an α_2-adrenoceptor stimulated release of EDRF (a phenomenon seen in canine and coronary arteries; Cocks and Angus, 1983), directly opposed the contractile effects of the agonist. This suggestion was somewhat controversial since the authors showed no evidence for clonidine-stimulated release of EDRF in the absence of its contractile effects. Furthermore there was evidence indicating that α_2-adrenoceptors mediating release of EDRF were not present in the rat aorta since the relatively specific α_2-adrenoceptor antagonist rauwolscine did not produce the potentiation of α-adrenoceptor mediated contractile responses seen following disruption of the endothelium (Lues and Schümann, 1984).

Studies examining the effect of removal of vascular endothelium on smooth muscle cGMP levels (Rapaport and Murad, 1983; Bigaud *et al.*, 1984), superfusion experiments (Griffith *et al.*, 1984) and the effect of haemoglobin (Martin *et al.*, 1985a and b) provided evidence for the existence of a tonic, basal release of EDRF which, in preparations with an intact endothelium, produced an elevation of tissue cGMP levels and depression of contractile responses (effects which could be blocked by inhibitors of EDRF). Subsequently, Martin *et al.*, (1986) suggested that this difference was more probably due to spontaneously released EDRF depressing the contractions induced by the agonist of lower efficacy, namely clonidine, to a greater extent than the contractions to PhE. Despite the observations of Lues and Schümann (1984), Bullock *et al.*, (1986) did find an α_2-adrenoceptor mediated release of EDRF in this tissue but

suggested that the spontaneous release of EDRF accounts for most of the inhibitory effect of the endothelium on depression of contractile agents.

Thus there are two ways in which EDRF may reduce the response to contractile stimuli. Firstly, the basal release of EDRF may directly depress agonist induced contractions. For example the Ca^{2+} channel activator Bay K 8644 does not induce endothelium-dependent relaxations but Bay K 8644-induced contractions are inhibited by basal release of EDRF (Spedding *et al.*, 1987). Secondly, in addition to the basal release of EDRF depressing agonist induced contractions, the agonist itself may evoke release of EDRF which will directly oppose the contractile effects of the agonist. For example α_2-adrenoceptor agonists such as UK 14304 stimulate the release of EDRF even though the basal release of EDRF plays a major role in the depression of the contractions induced by these agonists (Bullock *et al.*, 1986; Spedding, *et al.*, 1987) .

5-Hydroxytryptamine (5-HT), has been shown to produce contractions of a variety of vascular smooth muscle preparations including; rat mesenteric bed (Criscione *et al.*, 1984); dog saphenous vein (Feniuk *et al.*, 1985); bovine coronary arteries (Kaumann and Frenken, 1985); aorta from the rabbit (Apperley *et al.*, 1976) and rat (Krishnamurty, 1971; Forster and Whalley, 1982); and human basilar artery (Forster and Whalley, 1982). In many instances these contractions have been shown to be mediated via the 5-HT_2 receptor subtype (Cohen *et al.*, 1981 and 1983). In addition, 5-HT can produce both endothelial dependent and independent relaxations of vascular smooth muscle (Cocks and Angus, 1983; Cohen *et al.*, 1983; Feniuk *et al.*, 1983; Mecca and Webb, 1984; Trevethick *et al.*, 1984). In some preparations (e.g. the rat mesenteric bed) under appropriate conditions both the relaxant and contractile response to 5-HT can be demonstrated (MacLennan and Taylor, 1984). In other tissues both relaxant and contractile responses can be shown to be mediated by different 5-HT receptor subtypes (Houston and Vanhoutte, 1988).

In the rat aorta I have found that 5-HT-induced contractions of the vascular smooth muscle are depressed by the presence of an intact endothelial cell layer (see Chapter One). To determine if the spontaneous release of EDRF is the main cause of the inhibitory effect of the endothelium I have examined the relative effects of the presence of a functional endothelium on contractions induced by 5-methyltryptamine (5-MeT), N-methyl-tryptamine (N-MeT), and 5-carboxamidotryptamine (5-CT). These 5-HT receptor agonists have a lower intrinsic activity than 5-HT and have been reported as having a lower relative efficacy at 5-HT_2-receptors (Clancy and Maayani, 1985; Cory *et al.*, 1986).

In order to determine whether a concomitant, stimulated release of EDRF directly opposing the contractile response contributes to the depression of 5-HT receptor-induced contractions I decided to compare the relaxant effects of 5-HT and 5-CT in the rat aorta. In addition to its contractile effect, 5-CT is a potent agonist at 5-HT_1-like receptors which mediate relaxation of vascular smooth muscle (Feniuk et al, 1984; Trevethick et al, 1986) and therefore it is possible that any depression of 5-CT-induced contractions may be at least partly due to a receptor mediated, concomitant release of EDRF which directly opposes the contractile effects of the agonist.

The results obtained indicate that the depression of 5-HT-induced contractions are wholly dependent on the basal relesae of EDRF since in this tissue, endothelium dependent relaxations are not induced by 5-HT or 5-CT.

Methods

Aortic ring segments (2-3mm) were prepared from male Wistar rats (250-300 g. wt.) as described earlier (see General Methods). When required the endothelial cell layer of the preparations was removed by inserting the bent tip of a pair of forceps into the lumen and gently rubbing the intima whilst rolling the ring segment back and forth over a finger moistened with Krebs' solution. The presence or absence of a functional endothelium was later demonstrated by the ability or inability of 1μM acetylcholine (ACh) to relax the vessel pre-contracted with 1μM PhE.

Surgical thread was carefully passed through the lumen of the ring segments forming a stirrup. This was then used to suspend the preparation between an isometric force transducer and a wire support in a 10ml isolated organ bath containing Krebs' solution maintained at 37°C (see General Methods).

The Krebs' solution used throughout this study had the composition described in the General Methods. The solution was continuously aerated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37°C . In order to increase the potency of the 5-HT-receptor agonists the Krebs' solution used also contained 0.1μM Panuramine (a neuronal 5-HT-uptake blocker; Blurton *et al.*, 1984). Preliminary studies showed that 0.1-1μM desmethylimipramine (to block extra-neuronal uptake), 10-100μM iproniazid (to block mono-amine oxidase), or a combination of both agents had no facilitatory effect on the contractions to 5-HT. However, panuramine at the optimal concentration of 0.1μM, produced a small (4-fold) leftward shift in the concentration-response curve to 5-HT without producing a contraction *per se*.

Paired ring segments were used in all experiments. One ring of each pair was left intact whilst the endothelial cell layer of the other was disrupted by rubbing.

Contractile Effects

Cumulative concentration-response curves (CCRC) to either 5-HT, N-MeT, 5-MeT, or 5-CT were obtained. Each tissue was again washed and left for a further 45 minutes prior to obtaining a CCRC to 5-HT.

Relaxant Effects

For relaxation studies, after the demonstration of either the absence or presence of an endothelium-dependent relaxation to ACh and therefore the functional state of the endothelium, tissues were incubated in Krebs' solution containing ketanserin (1μM) for the remainder of the experiment. This was done in order to prevent any contraction mediated by 5-HT_2 receptor activation. After a period of 45 minutes the tissues were precontracted to approximately 1000mg. wt. tension using 10μM PhE (a high concentration of PhE was needed since 1μM ketanserin also blocks α-adrenoceptors) prior to cumulative addition of either 5-HT or 5-CT and relaxations measured. All tissues were then washed according to the above schedule and allowed to equilibrate for a further 45 minutes in the presence of either 1μM methysergide (to block any 5-HT receptors (5-HT_1-like) remaining after blockade by ketanserin), 10μM pindolol (to block any relaxations induced by β-adrenoceptors or 5-HT_1-like receptors; Middlemiss *et al.*, 1977), 3μM indomethacin (to block any involvement of cyclo-oxygenase products), or 1μM imiloxan (to block any relaxations due to α_2-adrenoceptor stimulation; Cocks and Angus, 1985) before repeating the cumulative concentration response curve to the agonist previously used in that preparation.

Analysis of Data

The potencies of the 5-HT agonists were estimated as the pEC_{50} value for each agonist, i.e. the negative $\log_{10}$ Molar concentration of the agonist producing 50% of its maximum response. For analysis of the effect of removal of the vascular endothelium, differences in agonist potency between rubbed and unrubbed preparations were expressed as Δ pEC_{50} values where:

$$\Delta\ pEC_{50} = pEC_{50\ (rubbed)} - pEC_{50\ (unrubbed)}$$

Differences in the maximum response of each agonist between rubbed and unrubbed preparations were expressed as Δ Max Response (%) values, i.e. the difference in maximum response expressed as a percentage of the maximum response in the unrubbed preparation:

$$\Delta\ \text{Max Response (\%)} = ((Max_{(unrubbed)} - Max_{(rubbed)}) / Max_{(rubbed)}) \times 100$$

Results

Contractile Effects

In rat aortic ring segments with an intact endothelial cell layer 1μM ACh produced a 57 ± 3.6% (n=28) reduction of tone induced by 1μM PhE. In preparations where the endothelium had previously been disrupted by rubbing, identical treatment caused 0.9 ± 0.5% (n=28) reduction in induced tone (Figure 3.1).

5-HT (0.01-300μM), N-MeT (1-300μM), 5-MeT (1-1000μM), and 5-CT (1-1000μM) each caused concentration-dependent contractions of rat aortic ring segments both in the absence, and presence, of an intact endothelial cell layer, i.e. in rubbed and unrubbed preparations (Figures 3.2 and 3.3). Each agonist, with the exception of N-MeT which was examined only up to 300μM, caused a depression of the contractile response at high concentrations both in rubbed and unrubbed preparations. This depression of contractile response was particularly evident for 5-CT-induced contractions but was seen for the response to N-MeT only in the unrubbed preparation (Figures 3.2b and 3.3b). In the unrubbed preparations the intrinsic activity for contraction of each agonist used relative to 5-HT (i.e. maximum response to agonist divided by maximum response to 5-HT) was; **5-HT** (1.00) > **N-MeT** (0.70 ± 0.04) > **5-MeT** (0.67 ± 0.03) > **5-CT** (0.44 ± 0.08), (Figure 3.4 and Table 3.1).

The potency of the agonists to induce contractions in aortic ring segments, as indicated by the pEC_{50} i.e. the negative $\log_{10}$ of the concentration of agonist producing 50% of its maximum response, was significantly greater (Student's unpaired *t* test; $P<0.05$) in the rubbed preparations for all agonists tested when compared to the potency in the unrubbed preparation (Figure 3.5 and Table 3.1). In terms of differences in agonist potency (pEC_{50}) between the rubbed and unrubbed preparations (Δ pEC_{50}), the effect of removal of the endothelium on the responses to the agonist tested was in the order **5-MeT** (0.75 ± 0.09) > **5-CT** (0.59 ± 0.12) > **5-HT** (0.48 ±

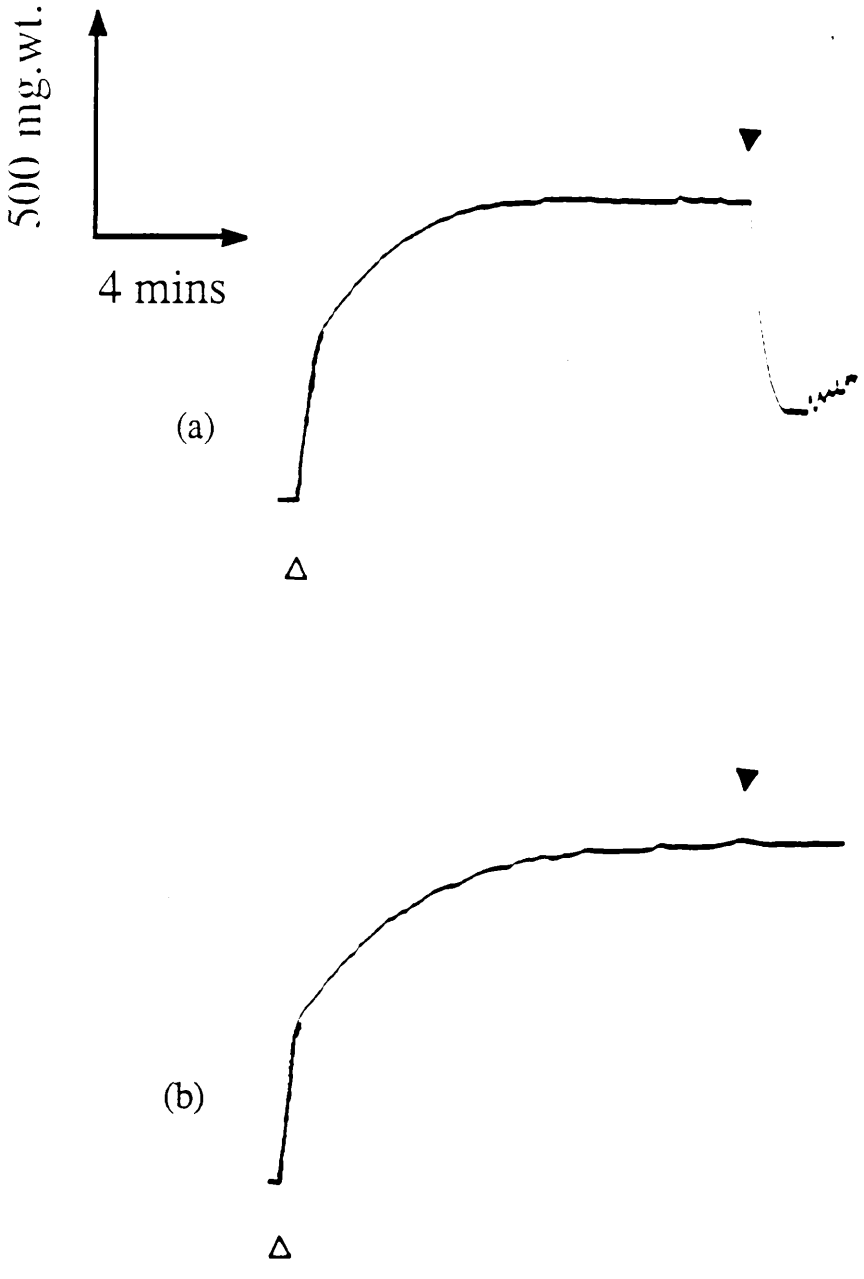


Figure 3.1

Representative trace showing contractile response to 1μM PhE (△) and the relaxant response to 1μM ACh (▼) in aortic ring preparations with (a) an intact endothelium and (b) a disrupted endothelium.

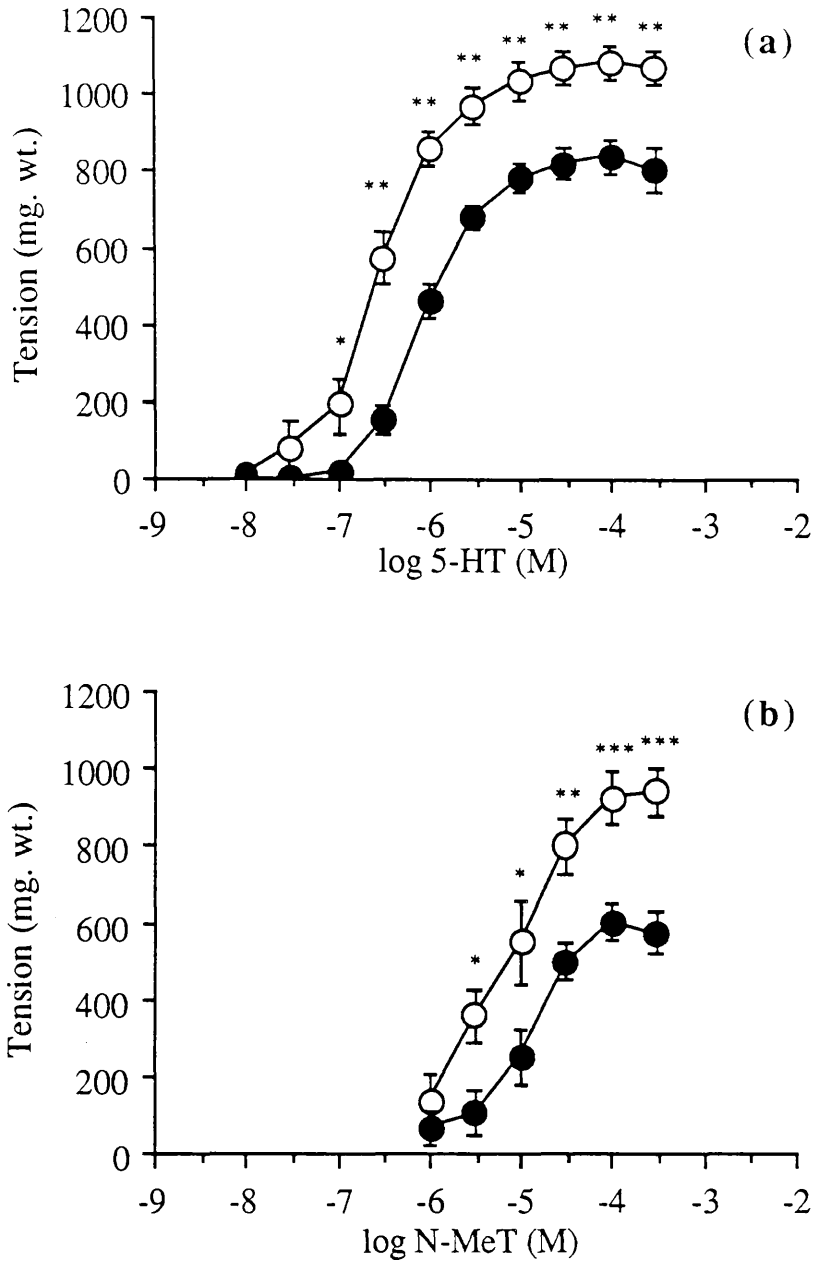


Figure 3.2

Effect of increasing concentrations of (a) 5-HT and (b) N-MeT on the rat aorta. Points shown are the mean contractile response (mg. wt.) ± s.e.m. (n=6-15) in paired preparations with an intact endothelium (●) and after disruption of the endothelium by gentle rubbing (○).

Statistically significant difference between contractile response in the rubbed and unrubbed preparation; * $P<0.05$, ** $P<0.01$, *** $P<0.005$, using unpaired Student's *t* test.

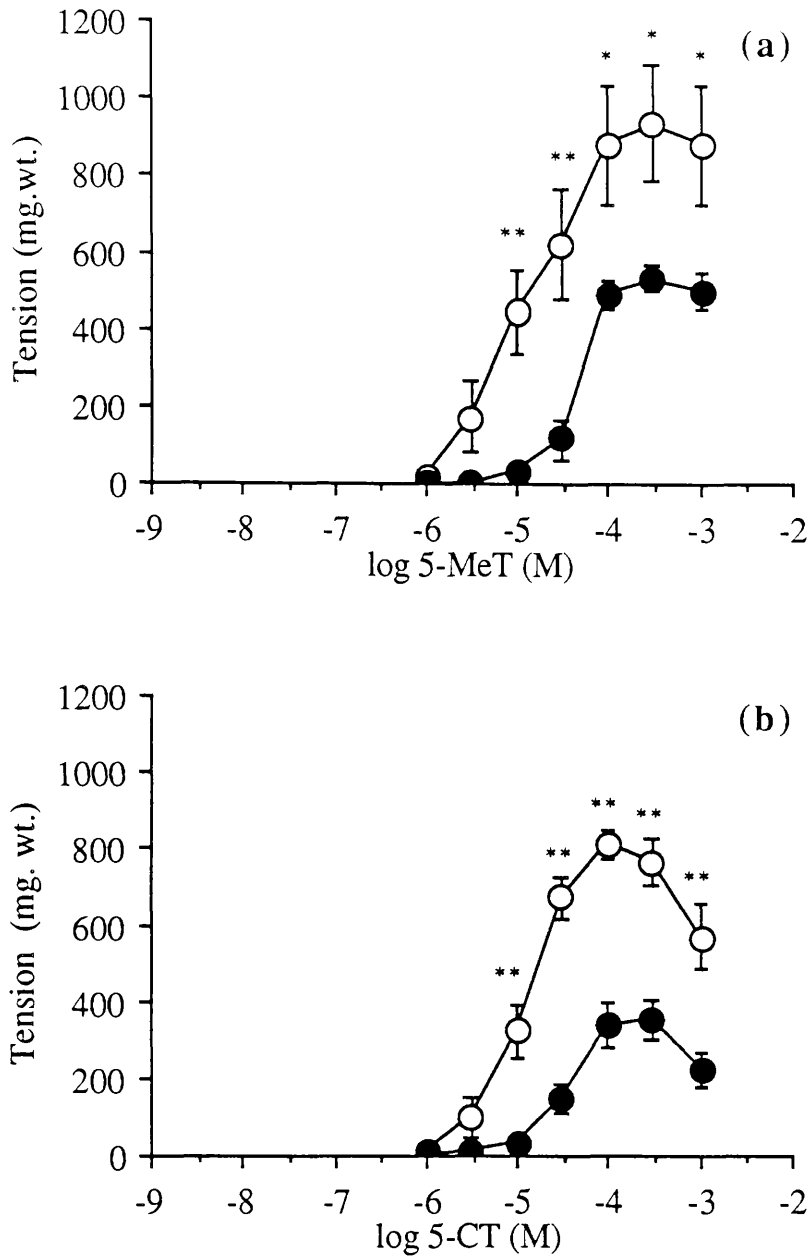


Figure 3.3

Effect of increasing concentrations of (a) 5-MeT and (b) 5-CT on the rat aorta. Points shown are the mean contractile response (mg. wt.) ± s.e.m. (n=6-15) in paired preparations with an intact endothelium (●) and after disruption of the endothelium by gentle rubbing (○).

Statistically significant difference between contractile response in the rubbed and unrubbed preparation; * $P<0.05$, ** $P<0.01$, *** $P<0.005$, using unpaired Student's *t* test.

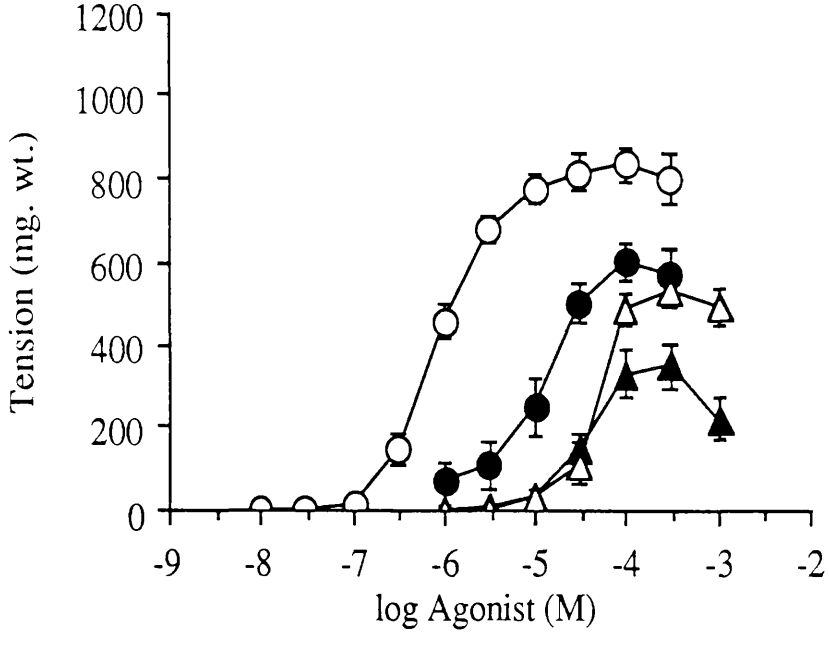


Figure 3.4

Effect of increasing concentrations of 5-HT (○), N-MeT (●), 5-MeT (△) and 5-CT (▲) on preparations of the rat aorta with an intact endothelium.

Points shown are the mean contractile response (mg. wt.) ± s.e.m. (n=6-15).

	Unrubbed	Rubbed			
Agonist	*Potency (pEC_{50})*			*ΔpEC_{50}*	*Intrinsic Activity*
5-HT	6.13 ± 0.07	6.60 ± 0.10	***	0.48 ± 0.11	1.00
5-CT	4.36 ± 0.06	4.95 ± 0.09	*	0.59 ± 0.12	0.44 ± 0.08 †††
5-MeT	4.29 ± 0.05	5.03 ± 0.12	*	0.75 ± 0.09	0.67 ± 0.03 †††
N-MeT	4.90 ± 0.09	5.35 ± 0.12	**	0.45 ± 0.09	0.70 ± 0.04 †††
	Max Response (mg)			*Δ Max (%)*	*Intrinsic Activity*
5-HT	845.5 ± 41.4	1086.3 ± 45.2	***	33.7 ± 9.3	1.00
5-CT	455.2 ± 53.7	837.4 ± 49.8	***	141.4 ± 24.3 ††	0.44 ± 0.08 †††
5-MeT	550.3 ± 40.7	934.7 ± 150.8	*	70.6 ± 14.7 †	0.67 ± 0.03 †††
N-MeT	605.0 ± 48.7	940.7 ± 60.6	***	59.0 ± 13.0 †	0.70 ± 0.04 †††

Table 3.1

Effect of removal of the endothelial cell layer of rat aortic ring segments on the potency of each 5HT receptor agonist (expressed as its pEC_{50}) and maximum contractile response (expressed in mg.wt.).

Differences between rubbed and unrubbed preparations are shown as Δ pEC_{50} for potency (i.e. $pEC_{50\,(rubbed)}$ - $pEC_{50\,(unrubbed)}$) and as Δ Max (%) for the maximum response (i.e. (($Max_{(unrubbed)}$ - $Max_{(rubbed)}$) / $Max_{(rubbed)}$) x 100).The values shown for the unrubbed and rubbed vessel are the means ± s.e.mean for n=6-15.

Significant differences from unrubbed preparation using Student's unpaired *t*-test: * $P<0.05$, ** $P<0.01$, *** $P<0.005$; and for differences from corresponding value for 5-HT, † $P<0.05$, †† $P<0.01$, ††† $P<0.005$

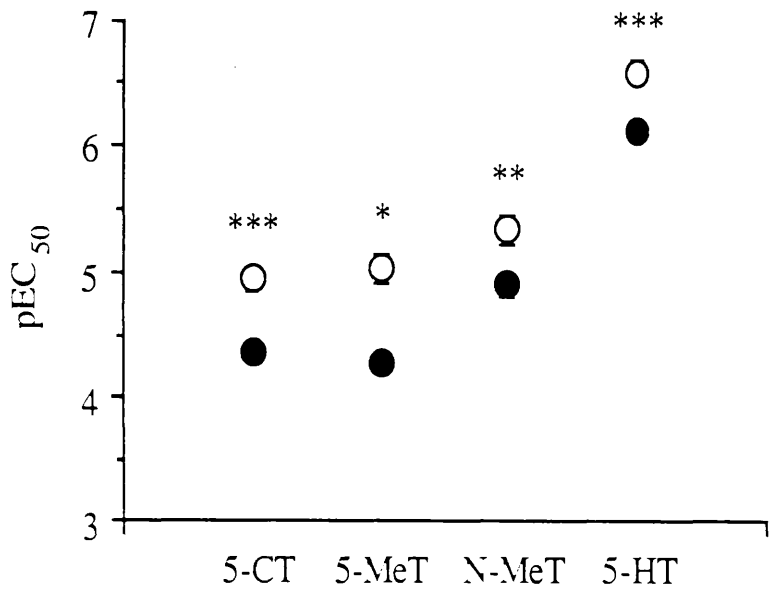


Figure 3.5

The effect of removal of the endothelium of rat aortic rings on the potency (pEC_{50}) of 5-CT, 5-MeT, N-MeT and 5-HT.

Data shown represents mean pEC_{50} values ± s.e.m. (n=6-15) in preparations with an intact (●) and disrupted (○) endothelium. Statistically significant differences between potency in rubbed and unrubbed preparations using unpaired Student's *t* test; * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

0.11) > **N-MeT** (0.45 ± 0.09). The difference in potency for 5-MeT, 5-CT and N-MeT was not significantly different from that for 5-HT ($P>0.05$; Figure 3.6). Moreover, there was no statistically significant difference between the values of Δ pEC_{50} for 5-CT, 5-MeT or N-MeT. An attempt to correlate the Δ pEC_{50} values and relative intrinsic activity for each agonist tested using analysis of correlation for two dependent variables showed no statistically significant correlation ($P>0.05$; Figure 3.7).

In addition to the changes in agonist potency between rubbed and unrubbed preparations, the maximum response of the rat aorta to all the agonists tested was significantly greater in the preparation with a disrupted endothelium than the intact preparation (Student's unpaired *t* test; $P<0.05$; Figures 3.8 and Table 3.1). In the absence of the endothelium the maximum responses to N-MeT, 5-MeT, and 5-CT were similar though still smaller than that to 5-HT itself (Figure 3.9). With respect to the difference in maximum response between rubbed and unrubbed preparations (expressed as a percentage of the maximum response in the unrubbed preparation, i.e. Δ Max (%)) the effect of removal of the endothelium on the responses to the agonists tested was in the order; **5-CT** (141.4 ± 24.3) > **5-MeT** (70.6 ± 14.7) > **N-MeT** (59.0 ± 13.0) > **5-HT** (33.7 ± 9.3). The value of Δ Max (%) for N-MeT, 5-MeT and 5-CT was significantly greater than that for 5-HT ($P<0.05$; Figure 3.10). In addition the value of Δ Max (%) for 5-CT was significantly greater than that for N-MeT and 5 MeT though there was no statistically significant difference between the values of Δ Max Response (%) for N-MeT and 5-MeT. An analysis of correlation between Δ Max Response (%) and relative intrinsic activity for each agonist tested) showed a statistically significant correlation (Student's unpaired *t test*; $P<0.001$; Figure 3.11).

Analysis of correlation between percentage reduction of 1μM PhE-induced tone by 1μM ACh (i.e. the 'marker' of a functional endothelium) and subsequent potency (pEC_{50}) of 5-HT-receptor agonists in the same preparation) showed statistically significant correlations (using Student's paired *t* test) for 5-HT ($0.0025>P>0.001$) and

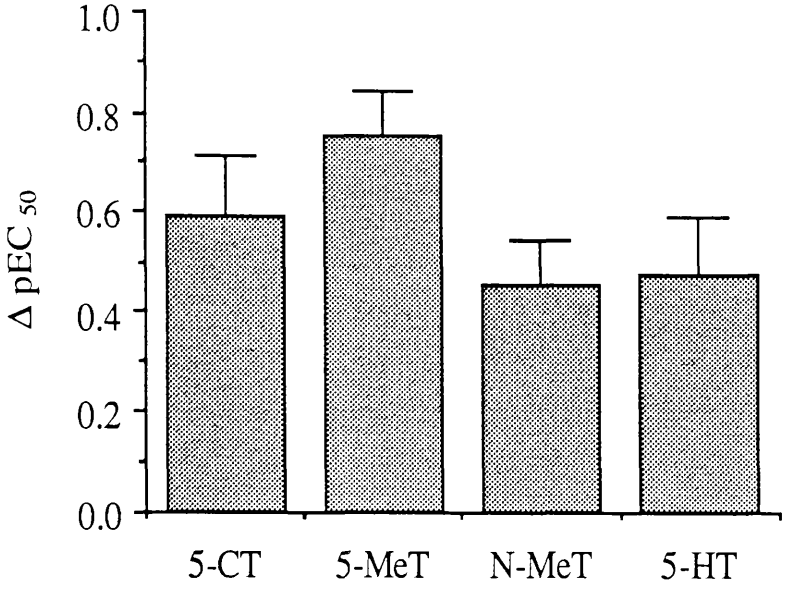


Figure 3.6

The difference in potency between rubbed and unrubbed preparations expressed as values of Δ pEC_{50} for 5-CT, 5-MeT, N-MeT, and 5-HT.

Data shown represents mean Δ pEC_{50} values $\pm$ s.e.m. (n=6-15). There was no statistically significant difference between values of Δ pEC_{50} for 5-CT, 5-MeT and N-MeT compared to that for 5-HT using unpaired Student's *t* test ($P>0.05$).

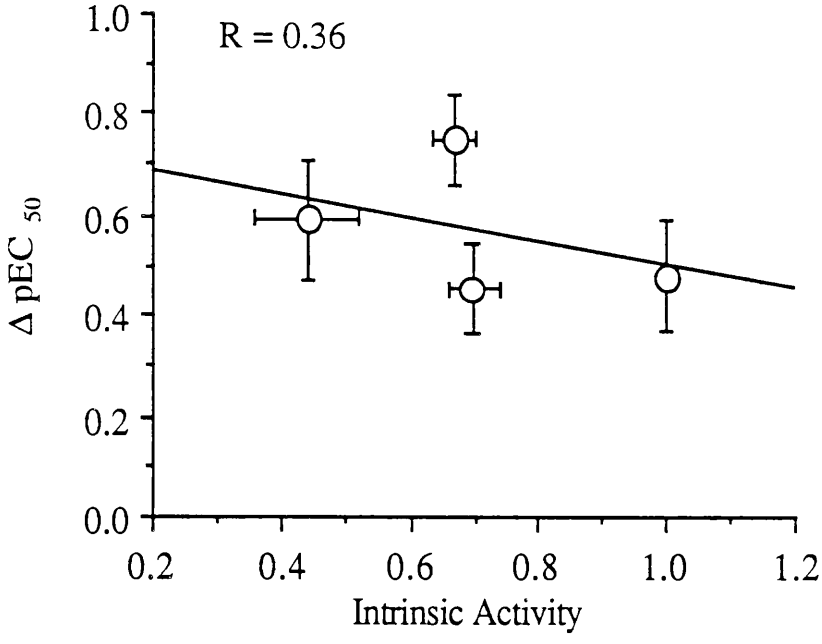


Figure 3.7

Correlation of difference in potency (ΔpEC_{50}) between rubbed and unrubbed preparations of rat aorta for 5-CT, 5-MeT, N-MeT and 5-HT and the intrinsic activity of each agonist relative to 5-HT.

Points shown represent mean data for n=6-15. There was no statistically significant correlation for correlation of two dependent variables ($P>0.05$; Student's unpaired *t test*). Correlation coefficient (R) is shown above.

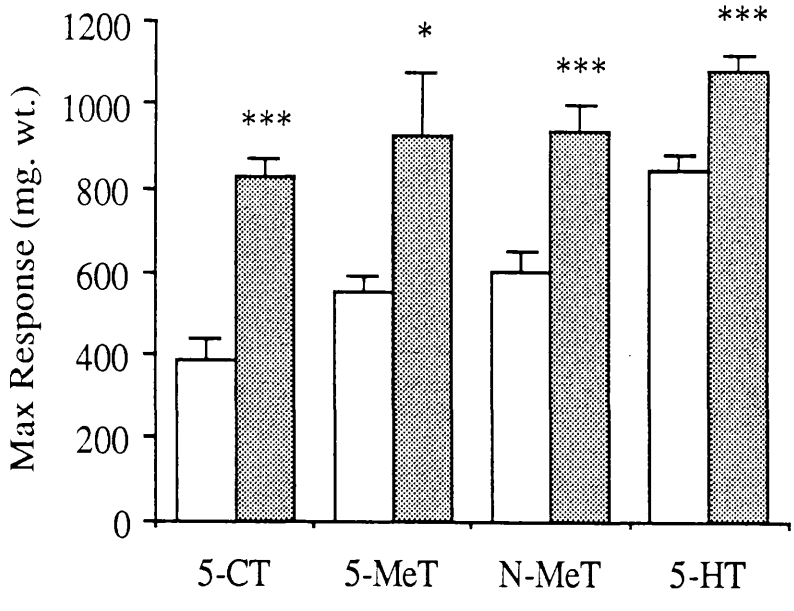


Figure 3.8

The effect of disruption of the endothelium of rat aortic rings on the potency (pEC_{50}) of 5-CT, 5-MeT, N-MeT and 5-HT.

Data shown represent mean maximum response (mg. wt.) ± s.e.m. (n=6-15) in preparations with an intact (open bars) and disrupted (filled bars) endothelium. Statistically significant differences between maximum response in rubbed and unrubbed preparations using unpaired Student's *t* test; * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

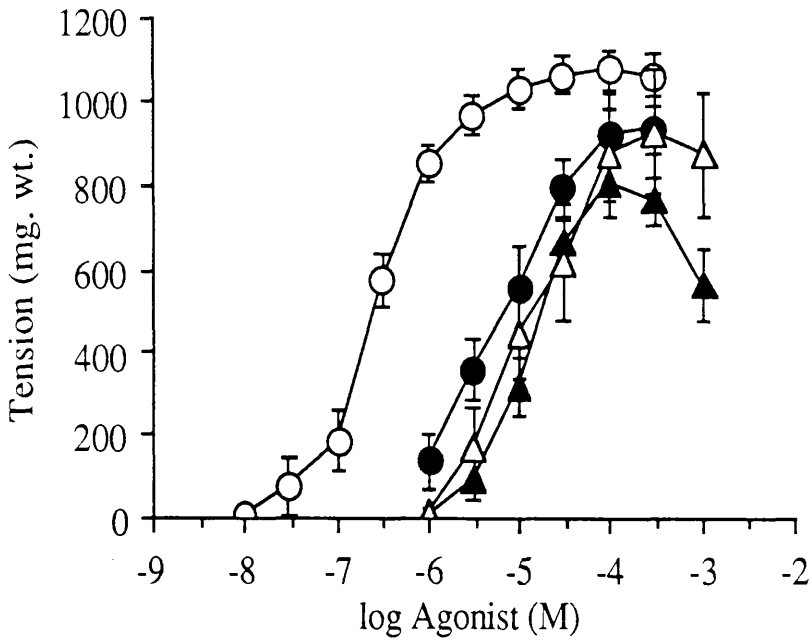


Figure 3.9

Effect of increasing concentrations of 5-HT (○), N-MeT (●), 5-MeT (△) and 5-CT (▲) on preparations of the rat aorta following disruption of the endothelium by gentle rubbing.

Points shown are the mean contractile response (mg. wt.) ± s.e.m. (n=6-15).

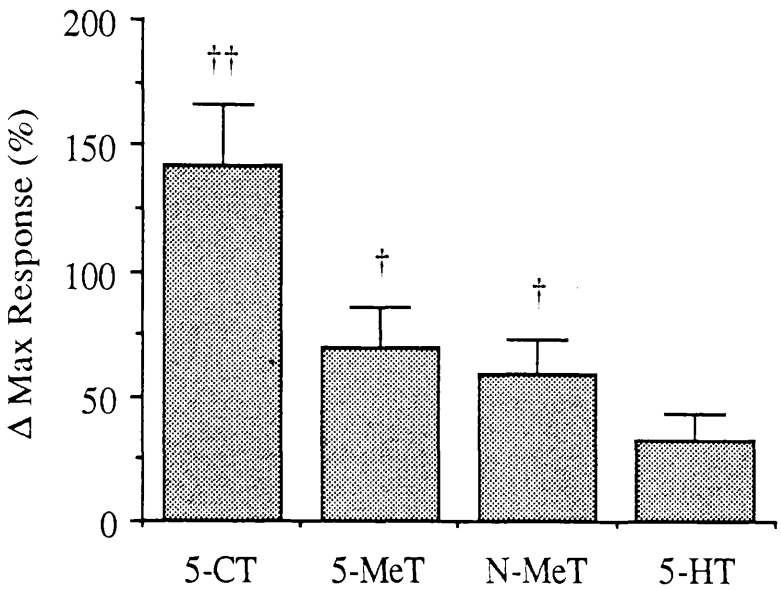


Figure 3.10

The difference in maximum contractile response to 5-CT, 5-MeT, N-MeT, and 5-HT between rubbed and unrubbed preparations expressed as a percentage of the maximum contraction in the unrubbed preparation (Δ Max Response (%)).

Data shown represents mean Δ Max Response (%) ± s.e.m. (n=6-15). Statistically significant differences between values of Δ Max Response (%) for 5-CT, 5-MeT an N-MeT compared to that for 5-HT; † $P<0.05$, †† $P<0.01$, ††† $P<0.005$ using unpaired Student's *t* test.

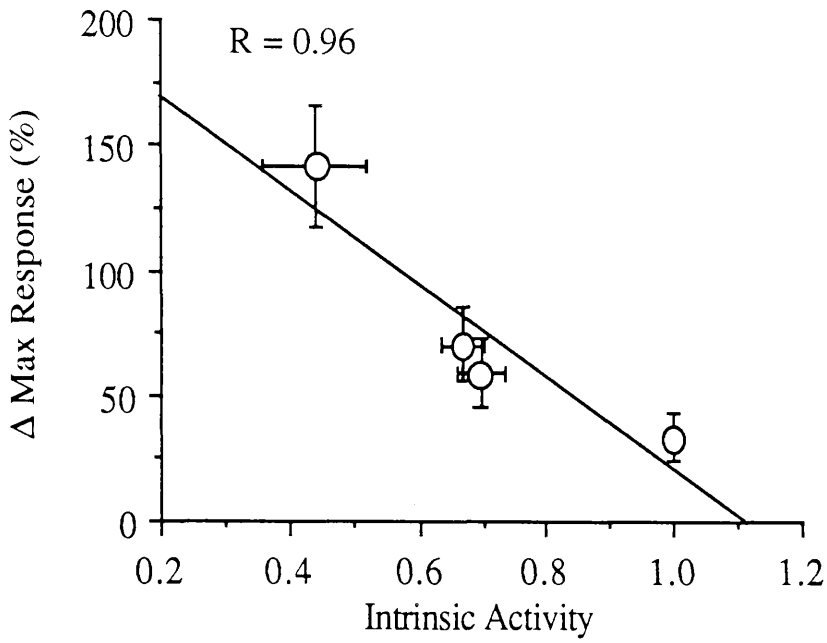


Figure 3.11

Correlation of difference in maximum response (Δ Max Response (%)) between rubbed and unrubbed preparations of rat aorta for 5-CT, 5-MeT, N-MeT and 5-HT and the intrinsic activity of each agonist relative to 5-HT.

Points shown represent mean data for n=6-15. Correlation was statistically significant for correlation of two dependent variables ($P<0.01$; Student's unpaired *t test*). Correlation coefficient (R) is shown above.

5-CT ($P<0.0005$). The analysis showed an inverse correlation indicating that with an increase in the degree of ACh-induced relaxation there was a decrease in the subsequent potency of both 5-HT and 5-CT (Figure 3.12)

Relaxant Effects

In the presence of 1μM ketanserin, high concentrations of 5-HT and 5-CT relaxed PhE-induced contractions of the rat aorta. These relaxations were similar in both intact and endothelium-disrupted rings (Figure 3.13) and were unaffected by the presence of 1μM methysergide, 10μM pindolol, 3μM indomethacin, or 1μM imiloxan (Figure 3.14).

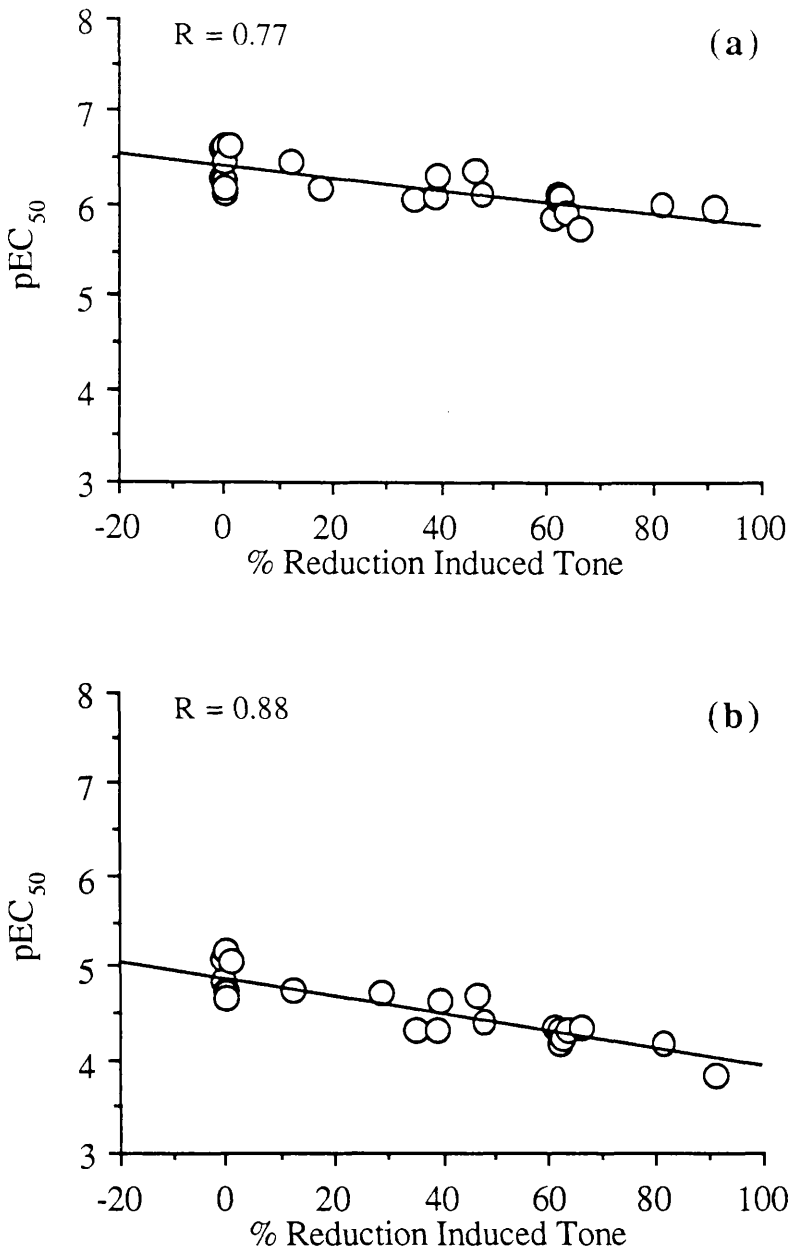


Figure 3.12

Correlation of percentage reduction of 1μM PhE-induced tone by 1μM ACh and subsequent potency (pEC_{50}) for (a) 5-HT and (b) 5-CT in the same preparation.

Points shown represent data for n=24. Correlations in both (a) and (b) are statistically significant ($P<<0.01$) for correlation of two dependent variables. Correlation coefficients (R) are shown above.

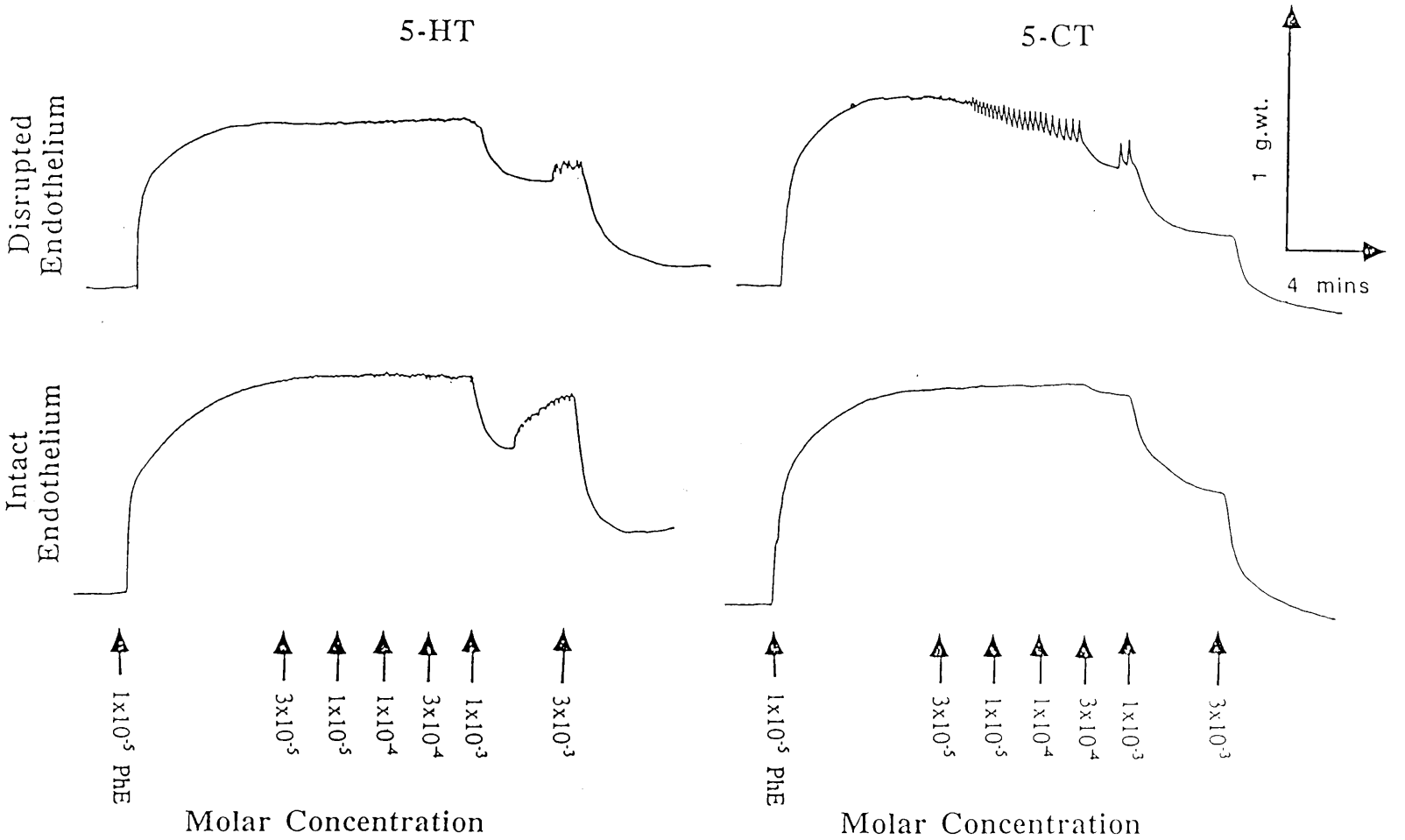


Figure 3.13

Relaxations of rat aortic rings in the presence and absence of an intact endothelium to 5-HT and 5-CT.

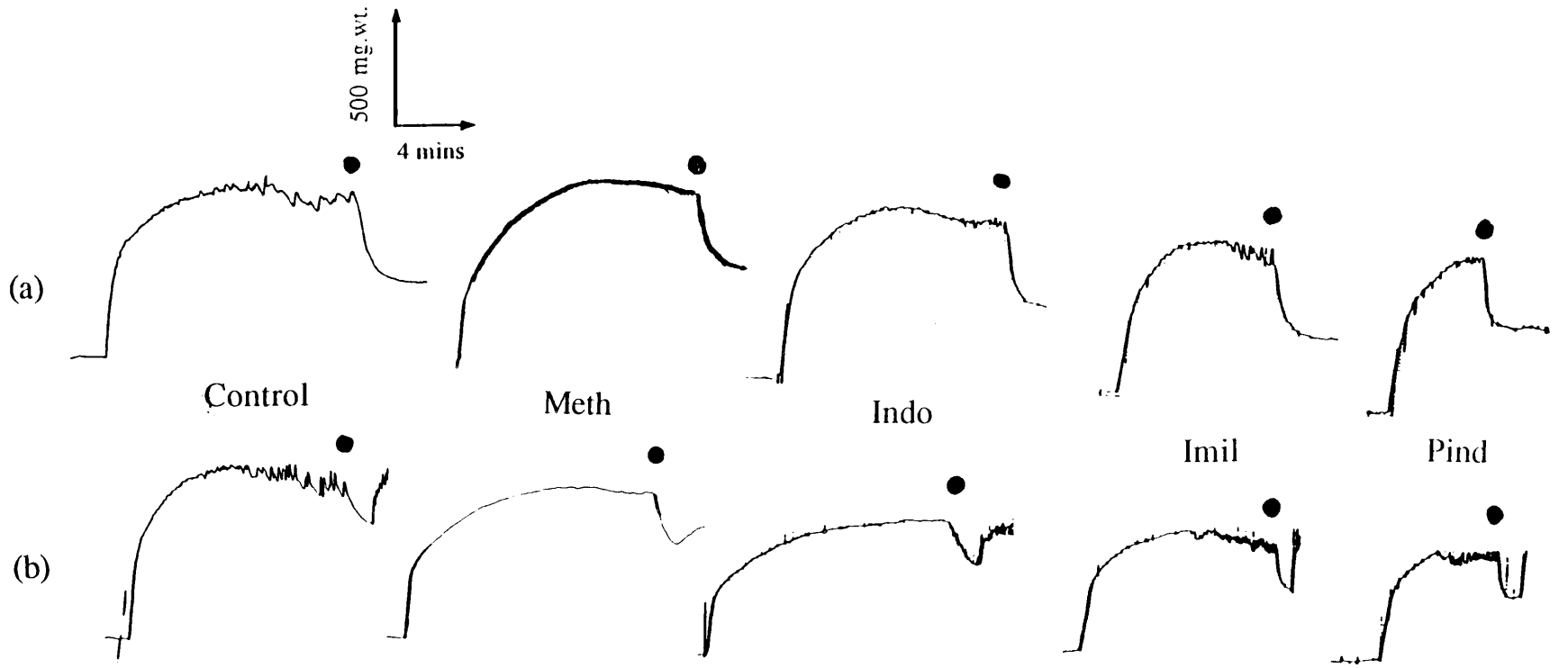


Figure 3.14

Effect of indomethacin (Indo), methysergide (Meth), imiloxan (Imil) and pindolol (Pind) on relaxations of aortic rings to (a) 1mM 5-CT and (b) 1mM 5-HT (●) in absence of an intact endothelium. Tone was induced with 10μM PhE in the presence of 0.1μM ketanserin.

Discussion

The aim of this study was to examine the effects of the vascular endothelium on the responses of the rat aorta to 5-HT-receptor agonists. In order to do this I have compared the relative effects of removal of the endothelium on contractions induced by 5-HT receptor agonists which were known to have different relative efficacies (Clancy and Maayani, 1985; Cory *et al.*, 1986).

In the unrubbed preparation, examination of the different maximum contractions produced indicates that the agonists used had low intrinsic activities relative to 5-HT since the order of magnitude of maximum response was **5-HT > N-MeT > 5-MeT > 5-CT**. Therefore, assuming 5-HT to be a 'full' agonist with an intrinsic activity of one, 5-CT, 5-MeT, and N-MeT were found to be 'partial' agonists using the criteria of relative maximum responses (see Ruffolo, 1982). This is, however, likely to be an over simplification since each agonist had a bell shaped CCRC suggesting that they caused a depression of their own contraction at high concentrations: this was particularly evident for 5-CT. Therefore, it is possible that if this relaxation was not present or could be blocked by some pharmacological intervention each of the 'partial' agonists would be able to produce a maximum response similar to 5-HT, and would therefore be 'full' agonists of low potency. In other words the agonists may appear to be 'partial' due to depression of the maximum response by an unknown mechanism which is particularly evident for agonists of low potency since the depression occurs at a concentration at which the agonist has not yet produced its maximum possible contractile response. In addition to errors in the estimation of intrinsic activity this depression of response at high concentration would greatly affect the measure of potency of the agonists in the tissue since a true EC_{50} could not be obtained.

5-HT and 5-CT are known to produce receptor-mediated relaxations in other examples of vascular smooth muscle by activation of $5HT_1$-like receptors (Feniuk et al, 1983; Trevethick et al, 1984). In this tissue, however, both 5-HT and 5-CT produced

endothelium-independent which were not mediated by $5HT_1$-like receptors, prostaglandins or α_2-adrenoceptor relaxations at high concentrations and it would seem reasonable to assume that this effect is responsible for the depression of response for each 5-HT receptor agonist described above. A similar phenomenon has recently been reported by Houston and Vanhoutte (1988) in the canine coronary artery. It is unlikely that the relaxant effects of 5-HT and 5-CT in the rat aorta and canine coronary artery are comparable since a much higher concentration of agonist is needed in the rat tissue to produce similar sized responses. However, the relaxant responses in the canine coronary artery were highly sensitive to methiothepin and therefore the use of this antagonist in the rat aorta would clarify the situation. Since the effect in the rat aorta could not be blocked by the antagonists which I tested and therefore, I was unable to examine the contractile response of each agonist outside the influence of this relaxatory effect, the estimation of the intrinsic activities of the agonists are the best available.

The change in agonist potency that occurs due to removal of the endothelium (Δ pEC_{50}) appears to bear no relationship to either the potency or the intrinsic activity of the agonist in the unrubbed preparation. With respect to changes in agonist potency the rank order of the effect of removal of the endothelium was

5-MeT > 5-CT > 5-HT > N-MeT,

whilst the rank orders of agonist potency and relative intrinsic activity in the intact preparation were

5-MeT < 5-CT < N-MeT < 5-HT

and

5-CT < 5-MeT < N-MeT< 5-HT

respectively. Moreover, there was no statistically significant difference between values of Δ pEC_{50} for the agonists tested compared to 5-HT or each other ($P>0.05$). Also, analysis of correlation between Δ pEC_{50} values and relative intrinsic activity of each agonist was not statistically significant ($P>0.05$; Student's unpaired *t test*).

The difference in the maximum response between rubbed and unrubbed preparations to 5-HT, N-MeT, 5-MeT, and 5-CT due to removal of the endothelium is inversely related to the intrinsic activity of the agonist used. When this difference was expressed as Δ Max Response (%), i.e. $((Max_{(unrubbed)} - Max_{(rubbed)}) / Max_{(rubbed)}) \times 100)$ the rank order of the effect of removal of the endothelium was

5-CT > 5-MeT > N-MeT > 5-HT

This corresponds to the rank order of the agonist relative intrinsic activity in the intact vessel. Moreover there was a statistically significant inverse correlation between values of Δ Max Response (%) and the relative intrinsic activity for each agonist. Therefore, the lower is the intrinsic activity of the agonist (i.e. the greater degree of 'partiality' that it exhibits), then the more marked is the effect of the removal of the endothelium on its maximum response.

Assuming that the magnitude of the ACh-induced relaxation of tone is an indicator of the amount of functional endothelium present then it would be reasonable to expect that the greater the degree of this relaxation then the greater will be the amount of basally released EDRF. Since the removal of a basal release of EDRF induces a significant increase in the potency of contractile agents, preparations showing a poor relaxant response to ACh would be expected to show a relatively greater sensitivity to contractile agonists than preparations were there was a good relaxatory response to ACh. We have found this to be the case in this study. There was a statistically significant correlation between the relaxation of 1μM PhE-induced tone by 1μM ACh at the start of each experiment (used as an indicator of the presence of a functional endothelium) and the subsequent potency of both 5-HT and 5-CT. Thus, if the conditions are standardised the magnitude of relaxation of PhE-induced tone by ACh (a measure of the viability of the endothelium) can be used as an indicator of the subsequent potency of contractile agonists and conversely the less sensitive the tissue is to contractile agonists the more viable the endothelium.

In conclusion, I have found: 1) no evidence for a 5-HT receptor-mediated release of EDRF in the rat aorta. The depression of the contractile response to these 5-HT receptor agonists in the presence of the endothelium can be accounted for solely by the influence of a basal release of EDRF. Thus, there is no concomitant, 5-HT receptor-stimulated release of EDRF which could oppose the contractile effects of the agonist; 2) The response of the pre-contracted aortic ring segments to ACh can, in certain circumstances, give a good indication of the amount of functional endothelium present in the vessel and might allow prediction of the sensitivity of that vessel to an agonist subsequently administered; 3) Although the removal of the endothelium caused an increase in the sensitivity of this vascular preparation to all of the 5-HT receptor agonists tested, the magnitude of this increase bore no relationship to the sensitivity of the intact vessel to that agonist nor to the intrinsic activity of that agonist. In contrast however, I found that the inhibitory effect of the endothelium on maximum contractions induced by 5-HT receptor stimulation was inversely related to the intrinsic activity of the agonists used, i.e. the greatest effect was seen with agonists of lower efficacy.

Thus, it appears that as in the case of α-adrenoceptor stimulated contractions, spontaneous release of EDRF largely accounts for the inhibitory effect of the endothelium on contractions induced by agonists acting at 5-HT receptors and the magnitude of the depression of contractile reponses is inversely dependent on the intrinsic activity of the agonist itself in the intact vessel.

Chapter Four

Classification of the muscarinic receptor subtype mediating endothelium-dependent relaxations of the rat aorta.

Introduction

Prior to a report by Jelliffe (1962), which demonstrated ACh-induced relaxations of rabbit descending thoracic aorta, the response to ACh in this preparation was contractile in nature (Furchgott and Bhadrakom, 1953; Furchgott, 1955). In 1980 Furchgott and Zawadzki showed that these ACh-induced relaxations were abolished in preparations in which the endothelial cell layer had been disrupted by rubbing the intimal layer of the vascular preparations. This partly explained the paradox that whilst ACh induced a depressor response *in vivo*, ACh-induced vasodilatation could not be demonstrated *in vitro* and implied the presence of cholinoceptors on the endothelium mediating the relaxant response to ACh in the intact vessel. The estimated K_B value for the cholinoceptor antagonist atropine (0.35 ± 0.04nM) and the relative potencies of ACh, methacholine and carbachol to inhibit or induce these relaxations indicated that the cholinoceptor mediating endothelium-dependent relaxations is of the muscarinic subtype. Since this time ACh-induced, endothelium-dependent relaxation of a number of vascular preparations has been reported (see General Introduction).

The muscarinic receptor has been proposed to exist in a number of subtypes (Hirschowitz *et al.*, 1984). The muscarinic receptor antagonist pirenzepine has been used to discriminate between two muscarinic receptor sub-classes in both functional and binding studies: high affinity for receptors in the cerebral cortex and sympathetic ganglia, M_1, and low affinity for receptors in the atria, ileum and other smooth muscles, M_2, (Goyal and Rattan, 1978; Brown *et al.*, 1980 and 1984; Hammer *et al.*, 1980; Hammer and Giachetti, 1982; Watson *et al.*, 1983; Eglen and Whiting, 1986). In addition, the M_2-receptor subtype can be further subdivided into atrial and smooth muscle receptors on the basis of differential antagonist affinities in functional studies. Thus, antagonist (ligand) affinities suggest that muscarinic cholinoceptors can be subdivided into at least three subtypes (Birdsall and Hulme, 1983).

Muscarinic receptors may also be differentiated according to the effector system to which the receptor is coupled. Harden *et al.* (1986) have suggested that the M_1 muscarinic receptor may be coupled to inositol phospholipid hydrolysis via a regulatory N_p protein whilst the M_2-receptor may be coupled to inhibition of adenylate cyclase by the regulatory N_i protein. This proposed receptor-effector linkage is likely to be an over simplification since there have been reports that the M_1- and M_2-receptors may be coupled to both effector systems (Kelly *et al.*, 1985; Brown *et al.*, 1985). Indeed, Eglen *et al.*, (1987), have shown that the M_2-receptor in guinea-pig isolated preparations, which is subdivided into atrial and ileal receptors on the basis of antagonist affinities, may also be differentiated by virtue of the effector system to which they are coupled. This study showed that responses of the atrial but not the ileal M_2-receptor were attenuated by pretreatment of the animals with pertussis toxin which ADP-ribosylates the N_i regulatory protein coupling the muscarinic receptor to adenylate cyclase and therefore functionally inactivating the receptor (Ui, 1984; Endoh *et al.*, 1985; Sorota *et al.*, 1985; Gilman, 1986; Boyer *et al.*, 1986). Conversely, responses of the ileal but not the atrial M_2-receptor were attenuated by pretreatment of the animals with lithium inhibiting myo-inositol-l-phosphate phosphatase activity (Allison and Stewart, 1971) thereby decreasing receptor-mediated inositol phospholipid hydrolysis.

The aim of the present study was to assess the receptor profile of the muscarinic receptors mediating endothelium-dependent relaxations of the rat aorta. In order to confirm that the receptor resembles the M_2 receptor subtype present on non-vascular smooth muscle I have used pirenzepine, 4DAMP and the atrial selective muscarinic receptor antagonists, himbacine and methoctramine (Gilani and Cobbin, 1986; Melchiorre *et al.*,1987). In addition I have examined the effects of lithium on the relaxant response of the rat aorta to ACh and the partial muscarinic agonists bethanecol and pilocarpine in order to determine if the muscarinic receptor mediating endothelium-

dependent relaxations is linked to phospholipid hydrolysis as in the case of the ileal M_2 muscarinic receptor (Eglen *et al.*, 1987).

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Methods

Male Sprague-Dawley or Wistar rats (290-340g) were killed by stunning followed by decapitation. 2-3mm long ring segments of aorta were prepared as described in General Methods. Care was taken to maintain an intact endothelium and the ring segments were suspended in 10ml isolated organ baths in Krebs' solution maintained at 37°C for isometric tension recordings.

Antagonist Studies

The presence of a vascular endothelium was first established for each tissue by the ability of 1μM ACh to produce relaxation of 1μM PhE-induced tone. After this preliminary procedure the effect of atropine (0.01-1μM), pirenzepine (0.3-10μM), 4DAMP (0.01-1μM), methoctramine (0.3-100μM) and himbacine 0.3-10μM) on ACh-induced relaxations of agonist-induced tone was examined.

Four ring segments from each rat were used. One ring segment was used as a time control, i.e. was not exposed to any antagonist, whilst each of the other tissues was incubated with a different concentration of antagonist. Successive CCRCs for ACh-induced relaxations showed an appreciable decrease in sensitivity to ACh in the fourth CCRC only (Figure 4.1). Therefore, with the exception of experiments using methoctramine, a maximum of three CCRC to ACh were obtained from each preparation: one control CCRC followed by two CCRCs in the presence of successively greater concentrations of antagonist.

In most cases tone (approx 1000 mg. wt.) was induced in the preparations by addition of PhE (1μM). At high concentrations methoctramine acts as a weak α_1-adrenoceptor antagonist with a pK_B of 5.2 (Melchiorre *et al.*, 1987). Preliminary experiments have shown that PhE-induced contractions are attenuated by the antagonist (data not shown). Therefore, for experiments using methoctramine, the stable thromboxane-mimetic U46619 (11α, 9α, -epoxymethano-prostaglandin H_2; Coleman

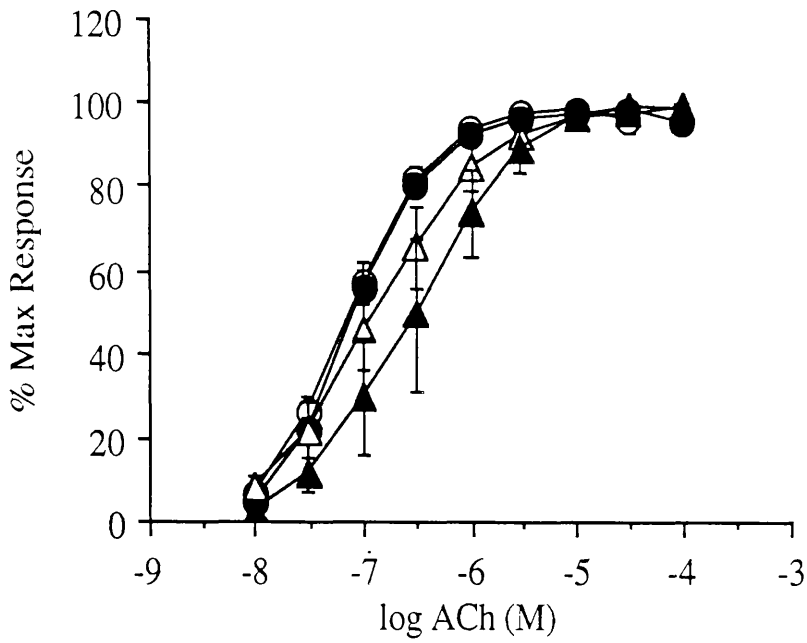

Figure 4.1

Effect of time on relaxations of intact rat aortic rings to successive cumulative concentration-response curves for ACh. Tone was induced by addition of 10μM PhE. Data shown represents first (○), second (●), third (△) and fourth (▲) successive CCRC for ACh in terms of the percentage maximum response.

Points shown are mean ± s.e.m. (n=28).

et al., 1981) (2.5 - 25 nM) was used to induce tone since atttenuation of PhE-induced tone could result in changes in sensitivity to ACh-induced relaxations which reduce the effects of muscarinic receptor blockade: α_1-adrenoceptor blockade would reduce the degree of PhE-induced 'activation' of the preparation and would result in an increase in sensitivity to ACh (see Chapter Two) and therefore an unexpectedly small antagonist-induced shift in the ACh CCRC. When contractions to either PhE or U46619 had reached a stable plateau, CCRC for endothelium-dependent relaxations were obtained by addition of ACh (0.01-3000μM) in 0.5 $\log_{10}$ molar increments. When used, antagonists were left in contact with the tissues for one hour prior to obtaining another CCRC for ACh (see Figure 4.2). It is difficult to completely wash out U46619 from these preparations and therefore when methoctramine was used only one CCRC to ACh was obtained per preparation: from the four preparations removed from the animal one was kept as control and a different concentration of methoctramine used in each of the remaining preparations (see Figure 4.2).

The sensitivity of the preparation to ACh-induced relaxation was estimated as the concentration of ACh producing 50% maximal relaxation (IC_{50}) and expressed as its negative $\log_{10}$, i.e. the pIC_{50}. Antagonist affinities were estimated by the method of Arunlakshana and Schild (1959) using 3-4 concentrations of each antagonist. Except in experiments using methoctramine, concentration-ratios in the absence and presence of antagonist were determined by dividing the IC_{50} values for ACh in the presence of the antagonist by the IC_{50} value for ACh in the absence of antagonist in the same preparation. Any shifts in the concentration-ratio related to the length of time in the baths or tissue fatigue were eliminated by dividing the observed concentration-ratio in the presence of the antagonist by the relevant time control concentration-ratio to give the corrected concentration-ratio. When methoctramine was used, concentration-ratios in the absence and presence of antagonist were determined by dividing the IC_{50} values for ACh in the presence of the antagonist by the IC_{50} value for ACh in the absence of

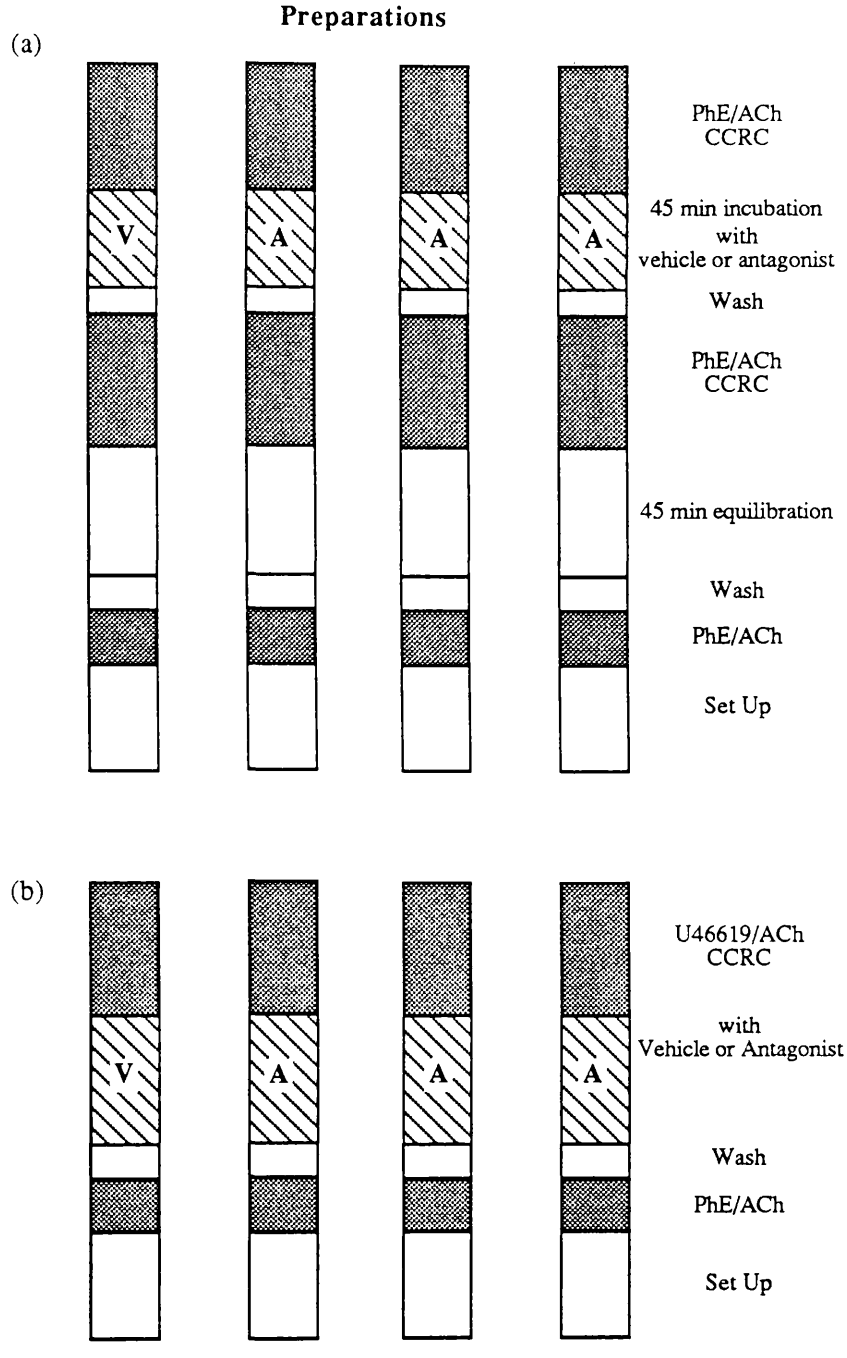

Figure 4.2

Schematic showing the experimental protocols used in determination of pA_2 values for muscarinic receptor antagonists using four ring preparations of rat aorta. Panel a) indicates the protocol for 'paired' (control/antagonist) curves when PhE was used as the contractile agents. When U46619 was used single curves to ACh were obtained in the presence of different concentrations of antagonist. For more detailed description see Methods and General Methods.

antagonist in the concomitant control preparation. Linear regression analysis was used to give values for pA_2 and slope of the Schild plot with 95% confidence intervals.

Effect of Lithium

The effect of lithium on muscarinic agonist-induced relaxations of rat aorta was examined. Three aortic ring segments from rats chronically pretreated with LiCl ('treated' preparations) or rats receiving no pre-treatment ('control' preparations) were prepared as normal. Care was taken to avoid disruption of the endothelium. After the initial setting up procedure (see above) the rings were exposed to 10μM PhE followed by 1μM ACh to demonstrate the presence of a functional endothelium.

The contractile response to PhE is markedly attenuated in animals pretreated with LiCl such that a well maintained contractile response is difficult to obtain (Figure 4.3; see Chapter One). Contractions of the rat aorta to the thromboxane mimetic U46619 are also attenuated but to a much smaller degree and therefore this agonist was used to induce tone when examining the relaxations induced by ACh, bethanecol and pilocarpine. Tone was induced in control and treated preparations by addition of equi-potent concentrations of U46619 (0.03μM and 0.3μM respectively; see Chapter One). After the U46619-induced contraction had reached a stable plateau (up to 15 minutes due to the very slowly developing nature of the response) a CCRC for relaxations to either ACh (0.01-10μM), bethanecol (1-1000μM), or pilocarpine (10-3000μM) was obtained. Since U46619 was used to induce tone, only one muscarinic agonist per preparation was obtained for the reasons mentioned above. When the maximum relaxations to bethanecol and pilocarpine had been obtained, 10μM ACh was added to the organ baths in order to give some indication of the relative size of the maximum response of the former two agonists to ACh.

The relaxatory response to the muscarinic agonists was expressed in terms of the percentage of the induced tone, absolute tension (mg. wt.) and as a percentage of each agonist's own maximum response. The intrinsic activity of bethanecol was estimated

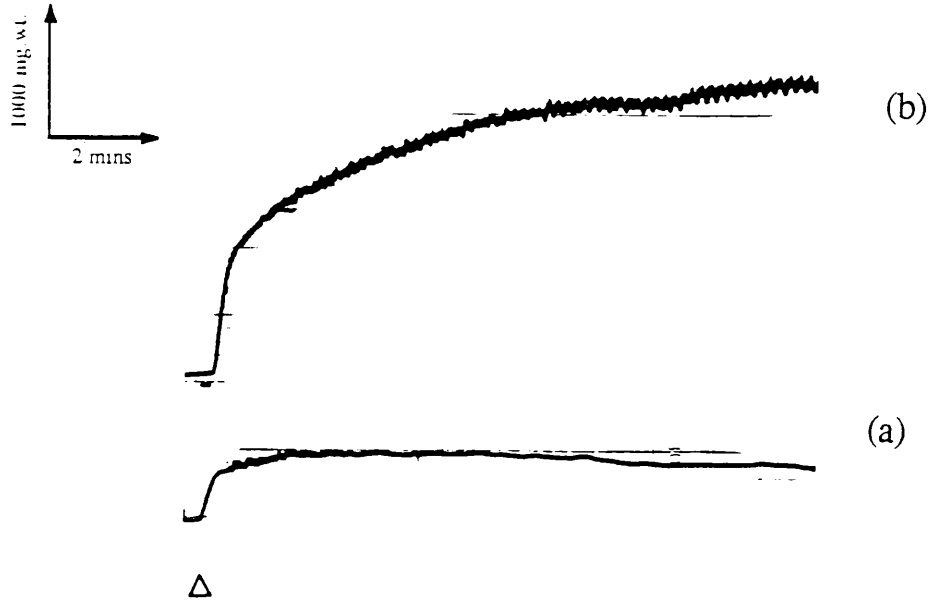

Figure 4.3

Representative trace showing contractile response depressed response to 1μM PhE (Δ) in aortic rings from LiCl pre-treated rats (a) compared to preparations from control rats (b).

as maximum response to bethanecol relative to the maximum response to ACh in preparations from the same rat, i.e.

$$\text{Intrinsic Activity} = \text{Max Response}_{(\text{bethanecol})}/\text{Max Response}_{(\text{ACh})}$$

In order to eliminate any variation that might occur due to differences in absolute induced tone between preparations the maximum relaxant response for this determination was taken as the maximum percentage reduction of induced tone by the agonist.

With the exception of pilocarpine the potency of the muscarinic agonists was estimated as the concentration of agonist producing 50% maximal relaxation (IC_{50}) and expressed as its negative $\log_{10}$ i.e. the pIC_{50}. Pilocarpine induced relaxations at high concentrations and it was not possible to raise the concentration of pilocarpine in the organ bath to a level sufficient to produce a true 'maximum' response. Therefore, for determination of the intrinsic activity and potency of pilocarpine the response to 3mM pilocarpine was used as its 'maximum' response.

Animal Pretreatment

The protocol used for pretreatment of animals was that described by Eglen *et al.* (1987). Animals were given 6.8 mmol kg^{-1} LiCl (dissolved in distilled water) by intra-peritoneal injection at the same time each day for four consecutive days (control animals received no pretreatment). The necessary dose was given by two injections of a relatively dilute solution of LiCl, one on each side of the abdomen (see Chapter One for further details). Eighteen hours following the last injection the rat was killed as described above, blood collected for determination of the plasma lithium concentration and the aorta excised in the normal manner.

Results

Antagonist Studies

ACh induced concentration-dependent relaxations of rat aortic ring segments pre-contracted with either PhE (pIC_{50} = 7.05 ± 0.05) or U46619 (pIC_{50} = 7.10 ± 0.10). The sensitivity to ACh-induced relaxation of PhE-induced tone was not significantly different from that for relaxation of U46619-induced tone ($P<0.05$; Student's unpaired *t* test). Atropine, pirenzepine, 4DAMP, and himbacine each produced parallel, concentration-dependent rightward shifts in the CCRC to ACh (Figures 4.4, 4.5, 4.6 and 4.7). At high concentrations, methoctramine produced non-parallel rightward shifts in the ACh CCRC (Figure 4.8). Analysis of the effects of each antagonist using the method of Arunlakshana and Schild (1959) indicated a competitive antagonism by atropine, pirenzepine and 4DAMP, since the Schild slopes were not significantly different from unity ($p>0.05$) (Figures 4.4, 4.5, and 4.6). The pA_2 values for these antagonists were 8.72 (8.43-9.01), 7.21 (6.91-7.49) and 8.87 (8.35-9.39) respectively (Table 4.1). In contrast, himbacine and methoctramine acted in a non-competitive manner exhibiting Schild slopes less than unity (0.79 (0.62-0.97) and 0.74 (0.59-0.88) respectively) (Figures 4.7 and 4.8). The pA_2 values for these antagonists were 6.92 (6.57-7.28) and 5.87 (5.62-6.12) respectively (Table 4.1).

Effect of Lithium

ACh, bethanecol, and pilocarpine produced concentration dependent inhibition (relaxation) of U46619-induced tone in control and treated preparations (Figure 4.9). The intrinsic activity of bethanecol and pilocarpine in both control and treated preparations was significantly less than that for ACh in the corresponding preparation ($P>0.05$; Student's paired *t* test). However, in control and treated preparations the intrinsic activity for bethanecol was not significantly different from that for pilocarpine ($P<0.05$; Student's paired *t* test). There was no significant difference ($P<0.05$;

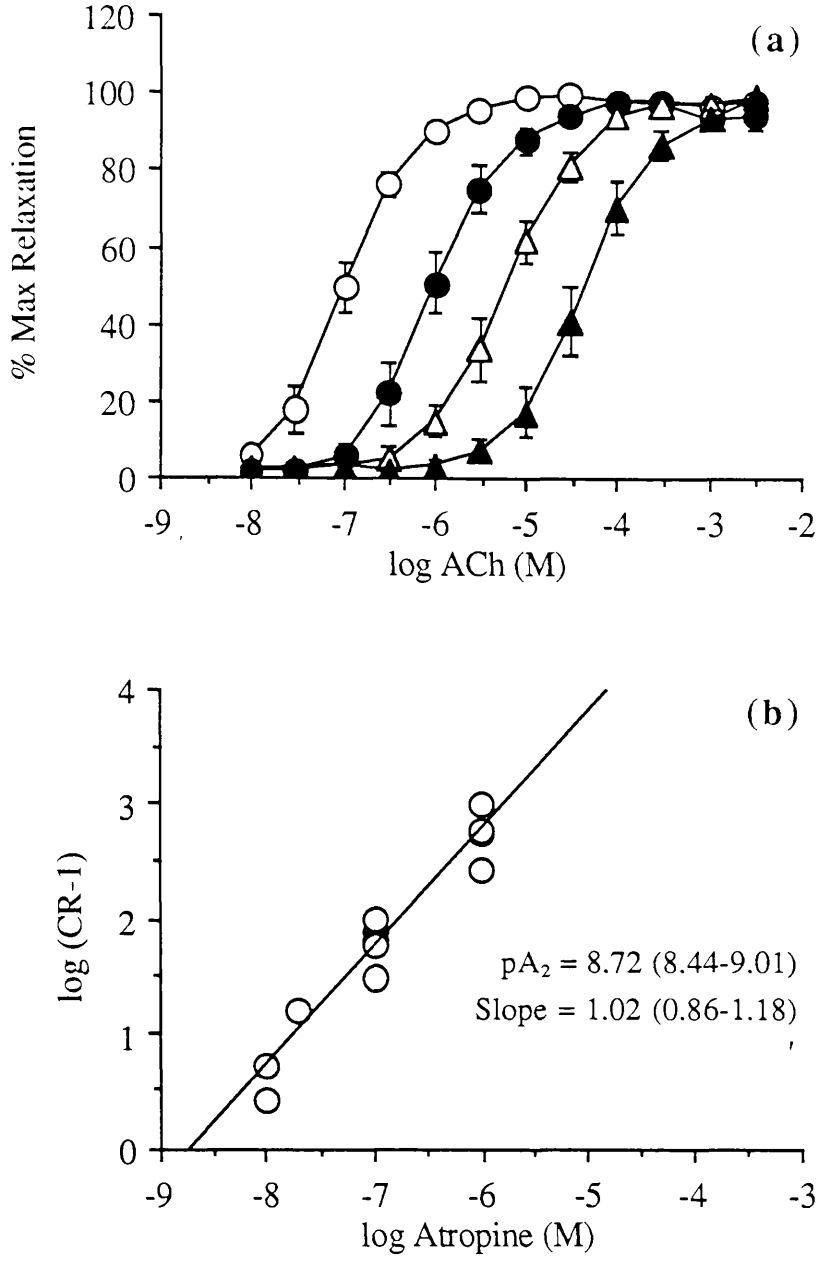

Figure 4.4

Effect of atropine on ACh-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. ACh-induced relaxations are expressed as; a) a percentage of the maximum response in the absence (○) and presence of 0.01μM (●), 0.1μM (△) and 1μM (▲) atropine; b) the Arunlakshana-Schild analysis of the inhibition by atropine of these responses.

Points shown in a) are mean ± s.e.m. (n=4-6).

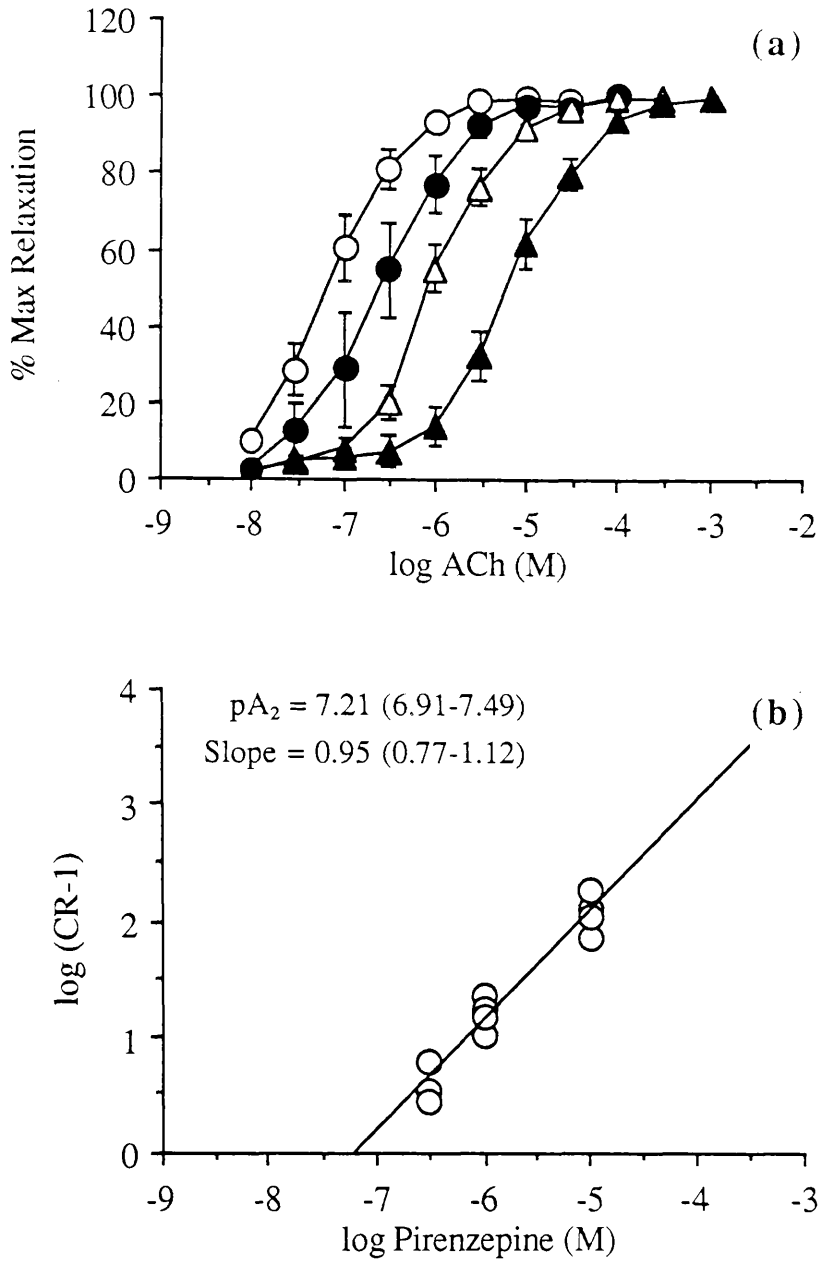

Figure 4.5

Effect of pirenzepine on ACh-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. ACh-induced relaxations are expressed as; a) a percentage of the maximum response in the absence (○) and presence of 0.3μM (●), 1μM (△) and 10μM (▲) pirenzepine; b) the Arunlakshana-Schild analysis of the inhibition by pirenzepine of these responses.

Points shown in a) are mean ± s.e.m. (n=4-6).

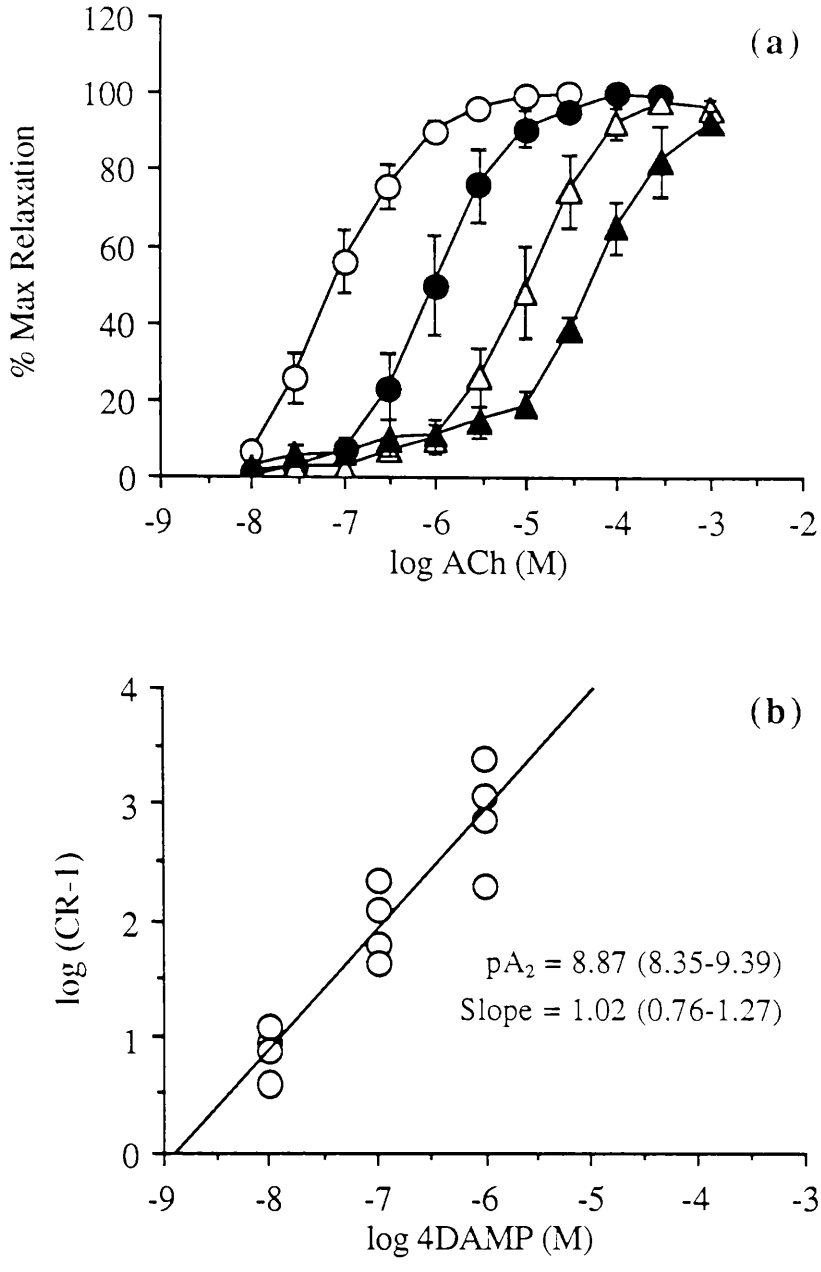

Figure 4.6

Effect of 4DAMP on ACh-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. ACh-induced relaxations are expressed as; a) a percentage of the maximum response in the absence (○) and presence of 0.01μM (●), 0.1μM (△) and 1μM (▲) 4DAMP; b) the Arunlakshana-Schild analysis of the inhibition by 4DAMP of these responses.

Points shown in a) are mean ± s.e.m. (n=4-6).

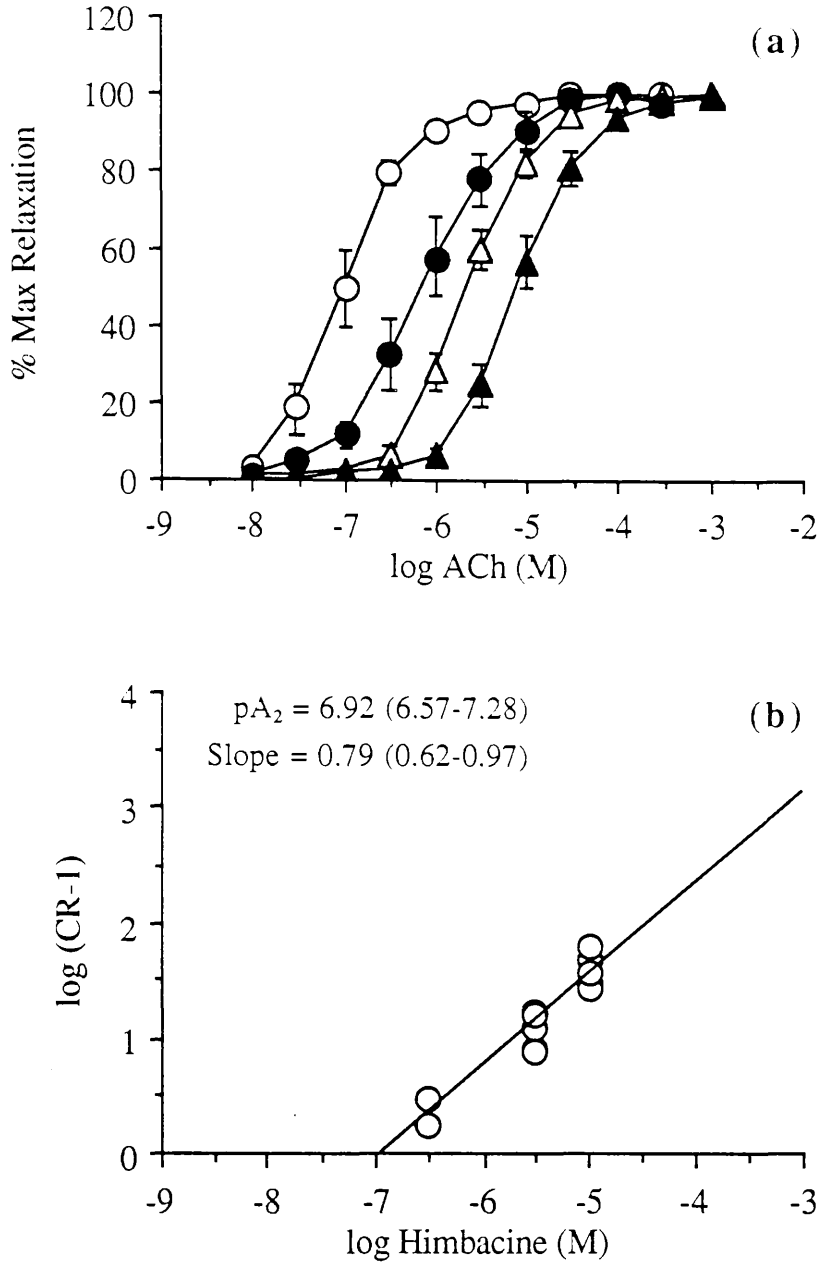

Figure 4.7

Effect of himbacine on ACh-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. ACh-induced relaxations are expressed as; a) a percentage of the maximum response in the absence (○) and presence of 0.3μM (●), 3μM (△) and 10μM (▲) himbacine; b) the Arunlakshana-Schild analysis of the inhibition by himbacine of these responses.

Points shown in a) are mean ± s.e.m. (n=4-6).

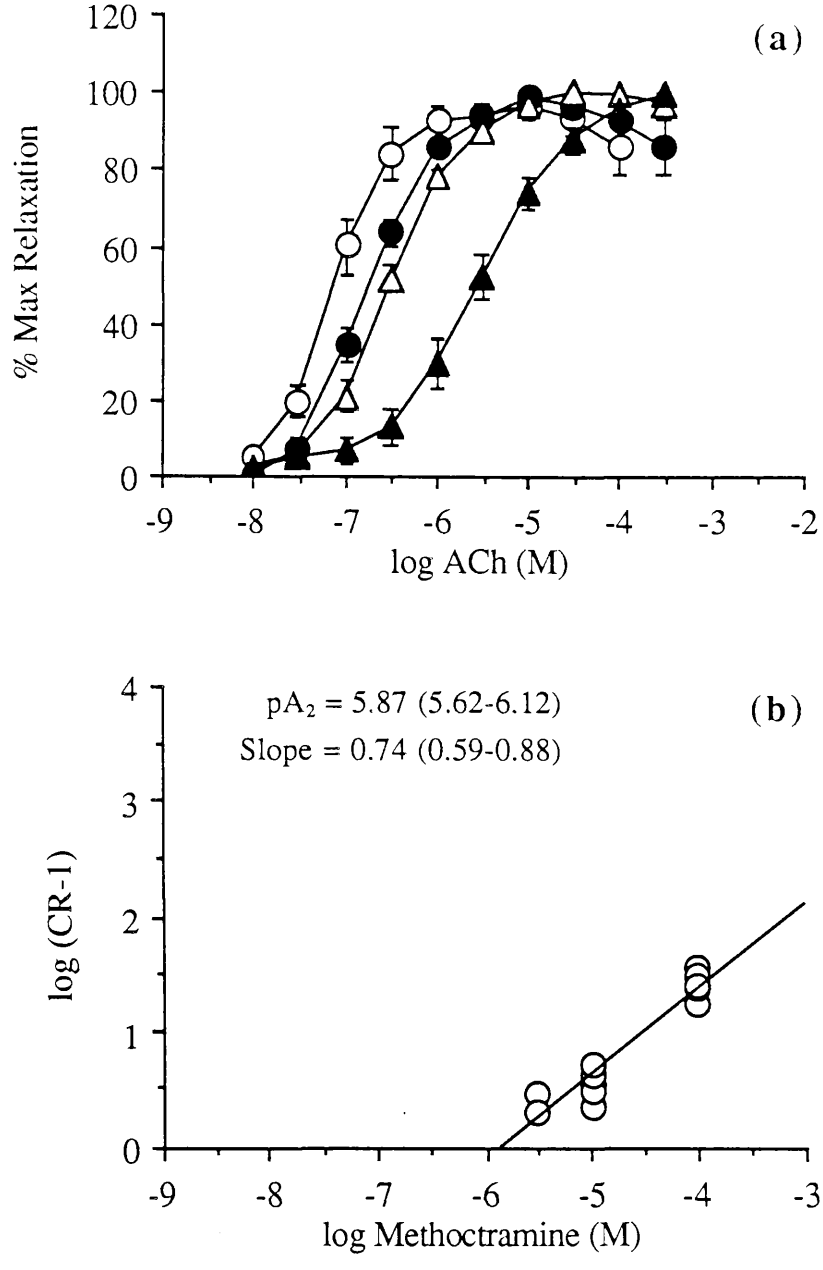

Figure 4.8

Effect of methoctramine on ACh-induced relaxations of rat aortic rings pre-contracted with 2.5 - 25 nM U46619. ACh-induced relaxations are expressed as; a) a percentage of the maximum response in the absence (○) and presence of 0.3μM (●), 10μM (△) and 100μM (▲) methoctramine; b) the Arunlakshana-Schild analysis of the inhibition by methoctramine of these responses.

Points shown in a) are mean ± s.e.m. (n=5).

Antagonist	pA_2	Slope
Atropine	8.72 (8.44-9.01)	1.02 (0.86-1.18)
Pirenzepine	7.21 (6.91-7.49)	0.95 (0.77-1.12)
4DAMP	8.87 (8.35-9.39)	1.02 (0.76-1.27)
Methoctramine	5.87 (5.62-6.12)	0.74 (0.59-0.88)
Himbacine	6.92 (6.57-7.28)	0.79 (0.62-0.97)

Table 4.1

Effect of atropine, pirenzepine, 4DAMP, methoctramine and himbacine on acetylcholine-induced relaxations of rat aortic ring segments pre-contracted with phenylephrine or U46619.

Values shown for pA_2 and slope of Schild plot are mean with 95% confidence intervals derived from 4-6 animals.

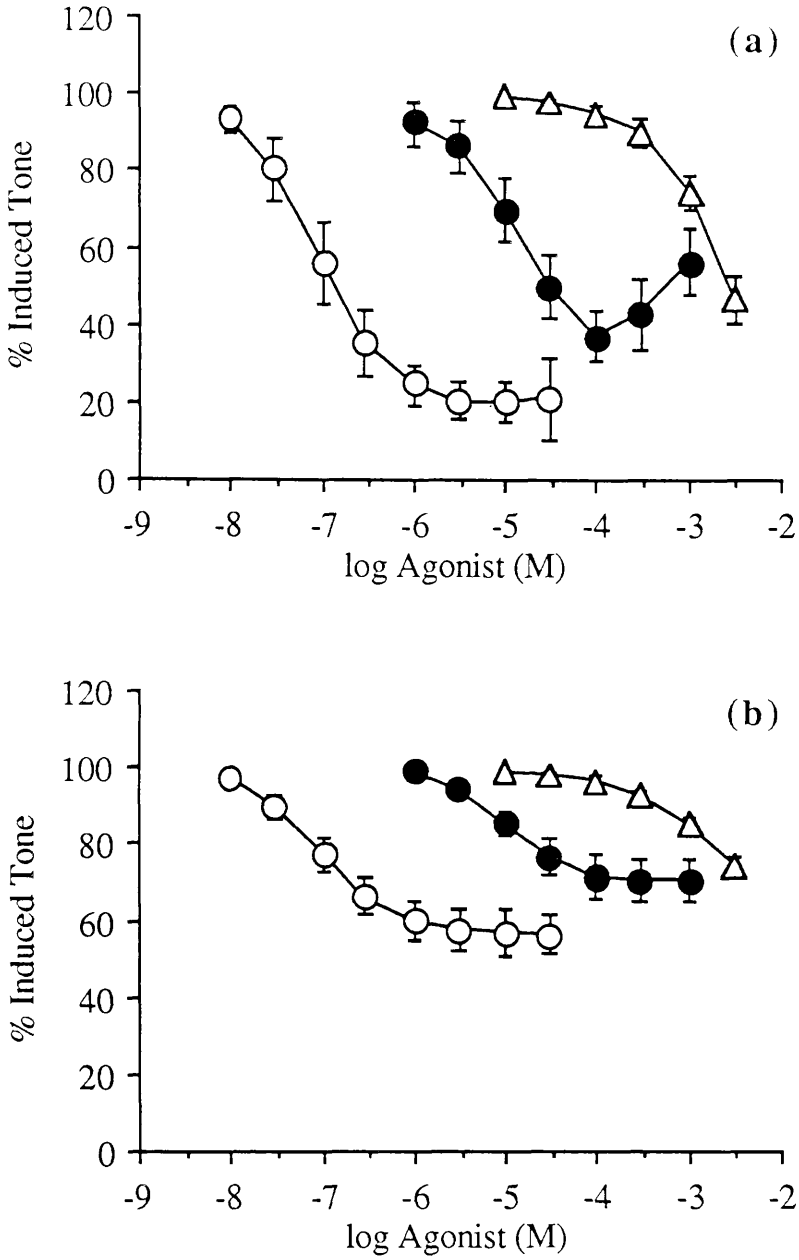

Figure 4.9

Effect of ACh (○), bethanecol (●) and pilocarpine (△) on tone induced by a) 0.03μM U46619 in control preparations and b) 0.3μM U46619 in preparations from rats pre-treated with LiCl.

Data shown mean reaponse (expressed as a percentage of the induced tone) ± s.e.m. (n=6-9).

Student's unpaired *t* test) between the intrinsic activity of each agonist in control and treated preparations (Table 4.2 and Figure 4.10).

The percentage reduction of induced tone produced by ACh, bethanecol and pilocarpine was greater in control preparations than in treated preparations and was particularly pronounced at the higher concentrations of agonist used (Figure 4.11). When the effect of each agonist was expressed in terms of absolute tension (mg. wt.) it was found that the initial tone was consistently greater in the treated preparations than in the control preparations though this difference was not statistically significant ($P>0.05$; Student's unpaired *t* test). The difference in tension between control and treated preparations at the higher concentrations of muscarinic agonist was greater than the difference in induced tone before addition of the agonist (Figure 4.12). The maximum relaxation to bethanecol and pilocarpine in both control and treated preparations was significantly less than that for ACh in the corresponding preparation ($P>0.05$; Student's paired *t* test). In addition, the maximum relaxant response to each agonist was significantly greater ($P<0.05$; Student's unpaired *t* test) in the control preparation compared to that in the treated preparation (Table 4.2 and Figure 4.13).

The potency of the agonists tested in this study was of the order **ACh > bethanecol > pilocarpine** in both control and treated preparations (Table 4.2 and Figure 4.14). However, there was no significant difference ($P>0.05$; Student's unpaired *t* test) between the potency of each agonist in control and treated preparations (Table 4.2 and Figure 4.15).

	Control	Lithium
	Intrinsic Activity	
ACh	1.00	1.00
Bethanecol	0.67 ± 0.08 *	0.73 ± 0.06 ***
Pilocarpine	0.57 ± 0.05 ***	0.54 ± 0.08 ***
	Agonist Potency (pIC_{50})	
ACh	7.07 ± 0.15	6.98 ± 0.08
Bethanecol	5.01 ± 0.18 ***	5.15 ± 0.12 ***
Pilocarpine	3.02 ± 0.06 ***	3.19 ± 0.13 ***
	Max Response (% reduction induced tone)	
ACh	86.2 ± 4.6	48.7 ± 5.8 †††
Bethanecol	57.7 ± 7.9 *	36.1 ± 5.1 *** †
Pilocarpine	49.9 ± 6.8 ***	24.6 ± 2.6 *** †††

Table 4.2

Effect of chronic pre-treatment of rats with LiCl on the potency of ACh, bethanecol and pilocarpine to induce relaxations of precontracted preparations (expressed as its pEC_{50}) and their maximum relaxant responses (expressed as % reduction of induced tone).

The values shown for the unrubbed and rubbed vessel are the means ± s.e.mean for n=6-9.

Significant differences between corresponding value for ACh using Student's unpaired *t*-test: * $P<0.05$, *** $P<0.005$; and between control and treated preparations , † $P<0.05$, ††† $P<0.005$

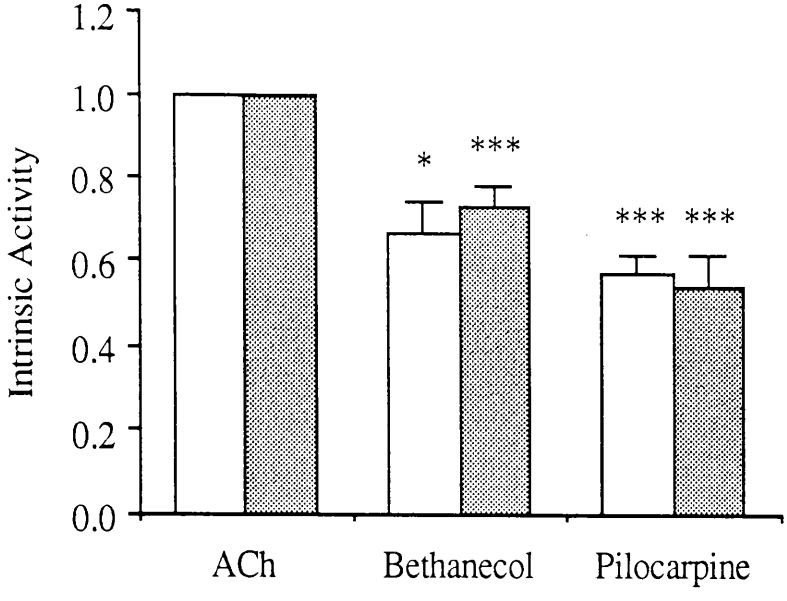

Figure 4.10

The intrinsic activity for endothelial-dependent relaxations by ACh, bethanecol and pilocarpine in control and treated preparations.

Data shown represents mean intrinsic activity values ± s.e.m. (n=6-9) in preparations from control animals (open bars) and animals chronically pre-treated with LiCl (filled bars). Statistically significant differences in control and treated preparations using paired Student's *t* test between the intrinsic activity of bethanecol and pilocarpine compared to ACh in the corresponding preparation; * $P<0.05$ and ** $P<0.005$.

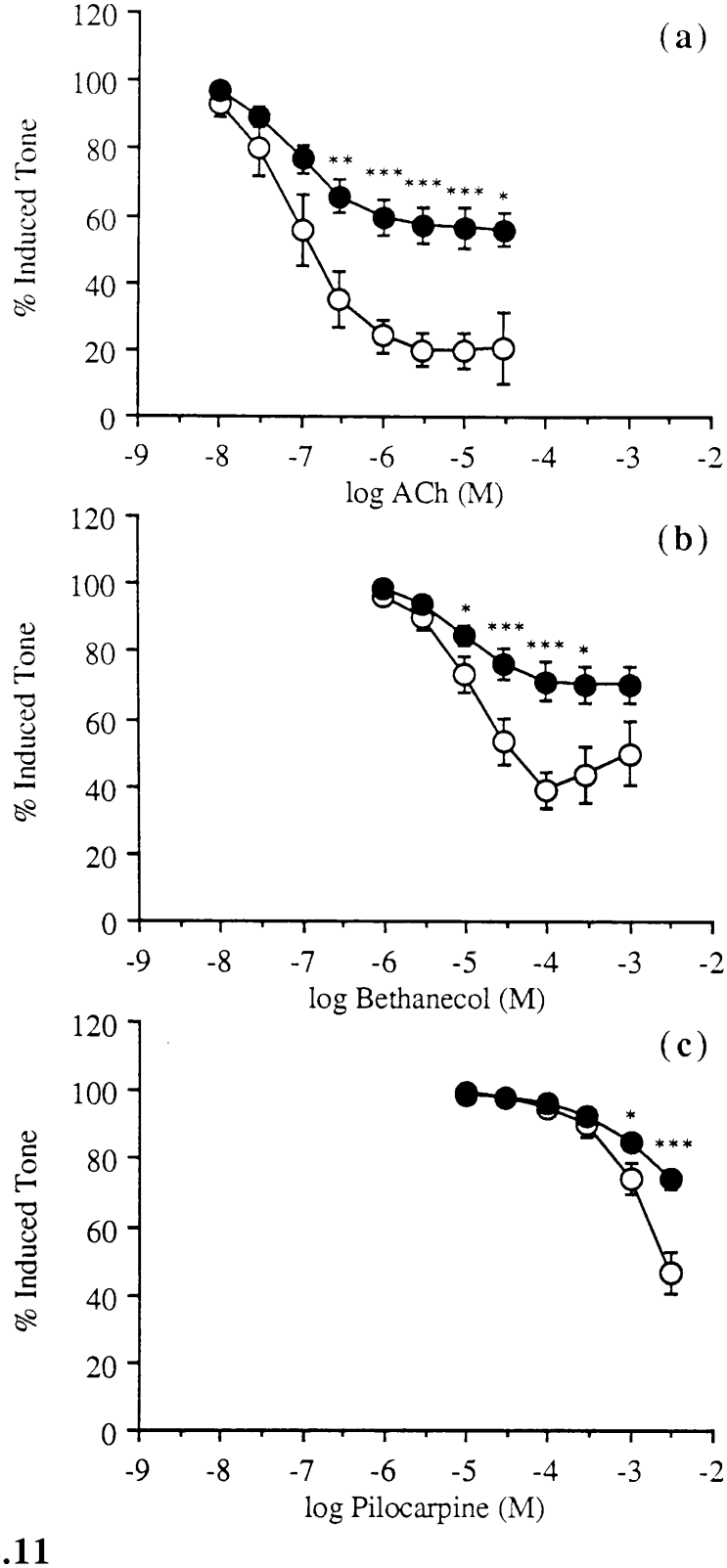

Figure 4.11

Relaxation (expressed as a percentage of the induced tone) of intact rat aortic rings by a) ACh, b) bethanecol and c) pilocarpine. Tone was induced by U46619 in preparations from control (○) and LiCl pre-treated (●) rats.

Points shown in are mean ± s.e.m. (n=4-6).

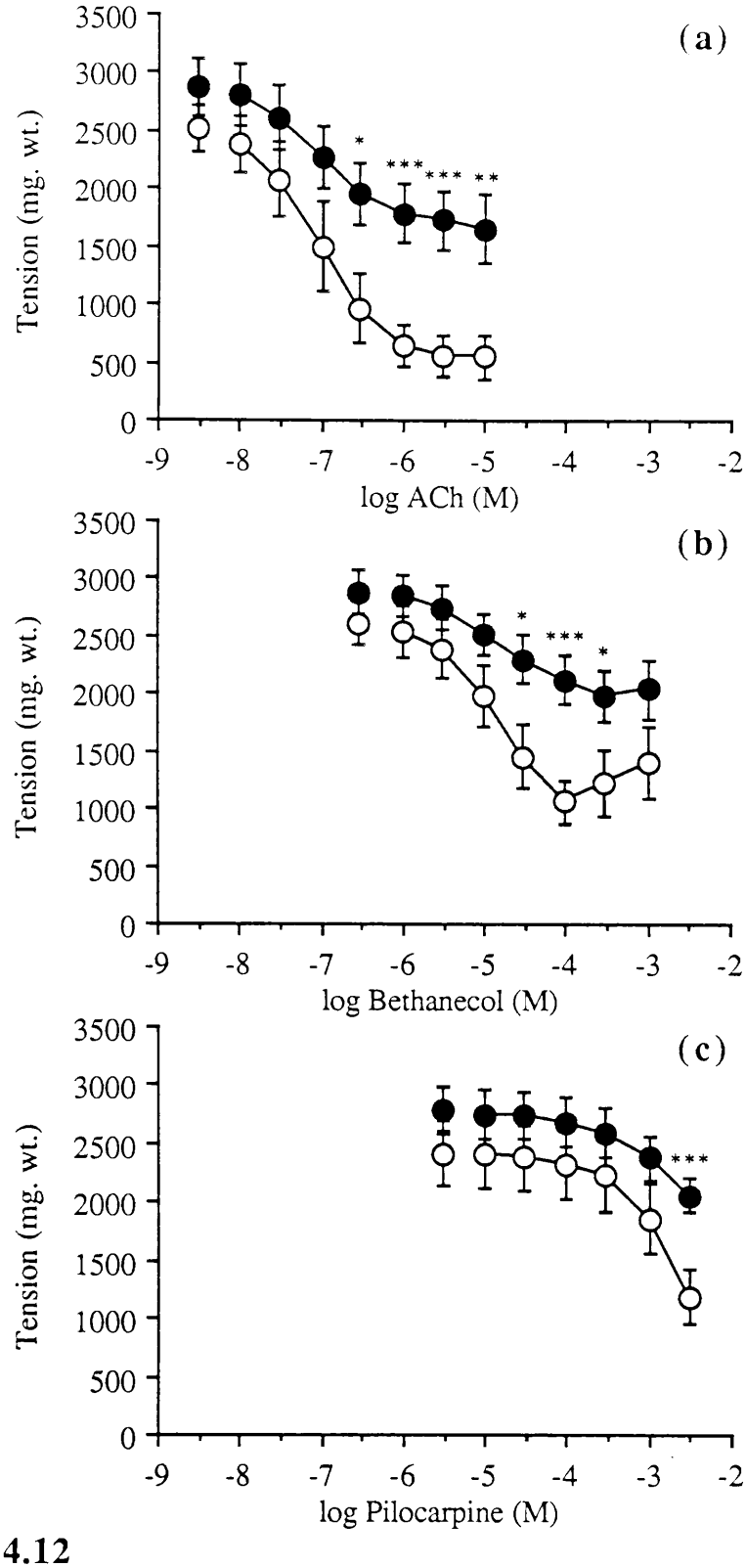

Figure 4.12

Relaxation (expressed as mg. wt.) of intact rat aortic rings by a) ACh, b) bethanecol and c) pilocarpine. Tone (represented by single symbols) was induced by U46619 in preparations from control (○) and LiCl pre-treated (●) rats.

Points shown in are mean ± s.e.m. (n=6-9).

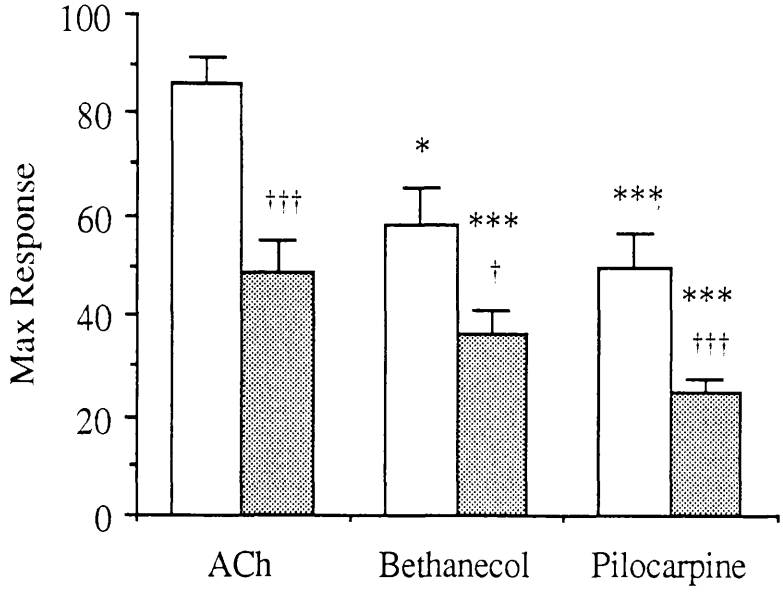

Figure 4.13

The maximum relaxation of induced tone in rat aortic rings (expressed as a percentage of the induced tone) to ACh, bethanecol and pilocarpine in control and treated preparations.

Data shown represents mean maximum response ± s.e.m. (n=6-9) in preparations from control animals (open bars) and animals chronically pre-treated with LiCl (filled bars). Statistically significant differences in control and treated preparations using paired Student's *t* test between the maximum response of bethanecol and pilocarpine compared to ACh in the corresponding preparation; * $P<0.05$ and ** $P<0.005$. Statistically significant differences using unpaired Student's *t* test between the maximum response of ACh, bethanecol and pilocarpine control and treated preparations; † $P<0.05$ and †† $P<0.005$.

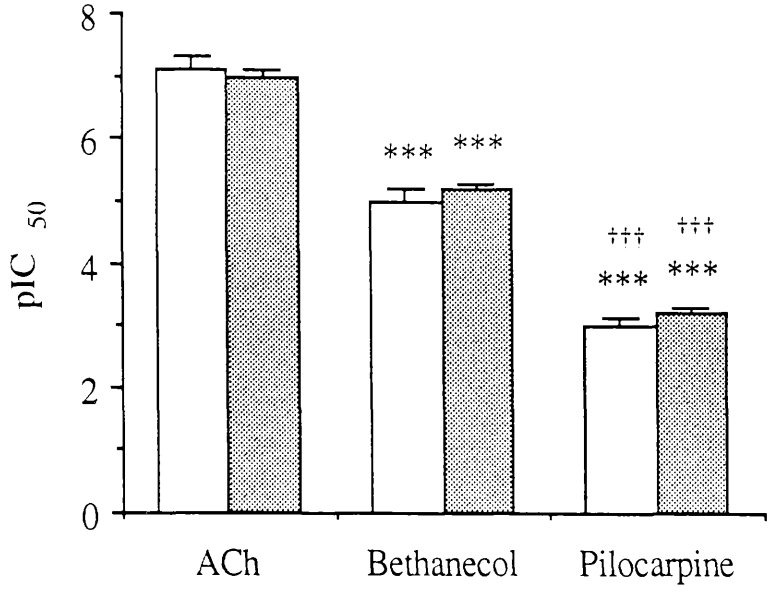

Figure 4.14

The effect of LiCl pre-treatment on the potency (pIC_{50}) of ACh, bethanecol and pilocarpine in rat aortic rings.

Data shown represents mean pIC_{50} values ± s.e.m. (n=6-9) in preparations from control animals (open bars) and animals chronically pre-treated with LiCl (filled bars). Statistically significant differences in control and treated preparations using Student's paired *t* test between (i) the potencies of bethanecol and pilocarpine compared to ACh in the corresponding preparation; *** $P<0.005$ and (ii) the potency of bethanecol compared to pilocarpine in the corresponding preparation; ††† $P<0.005$.

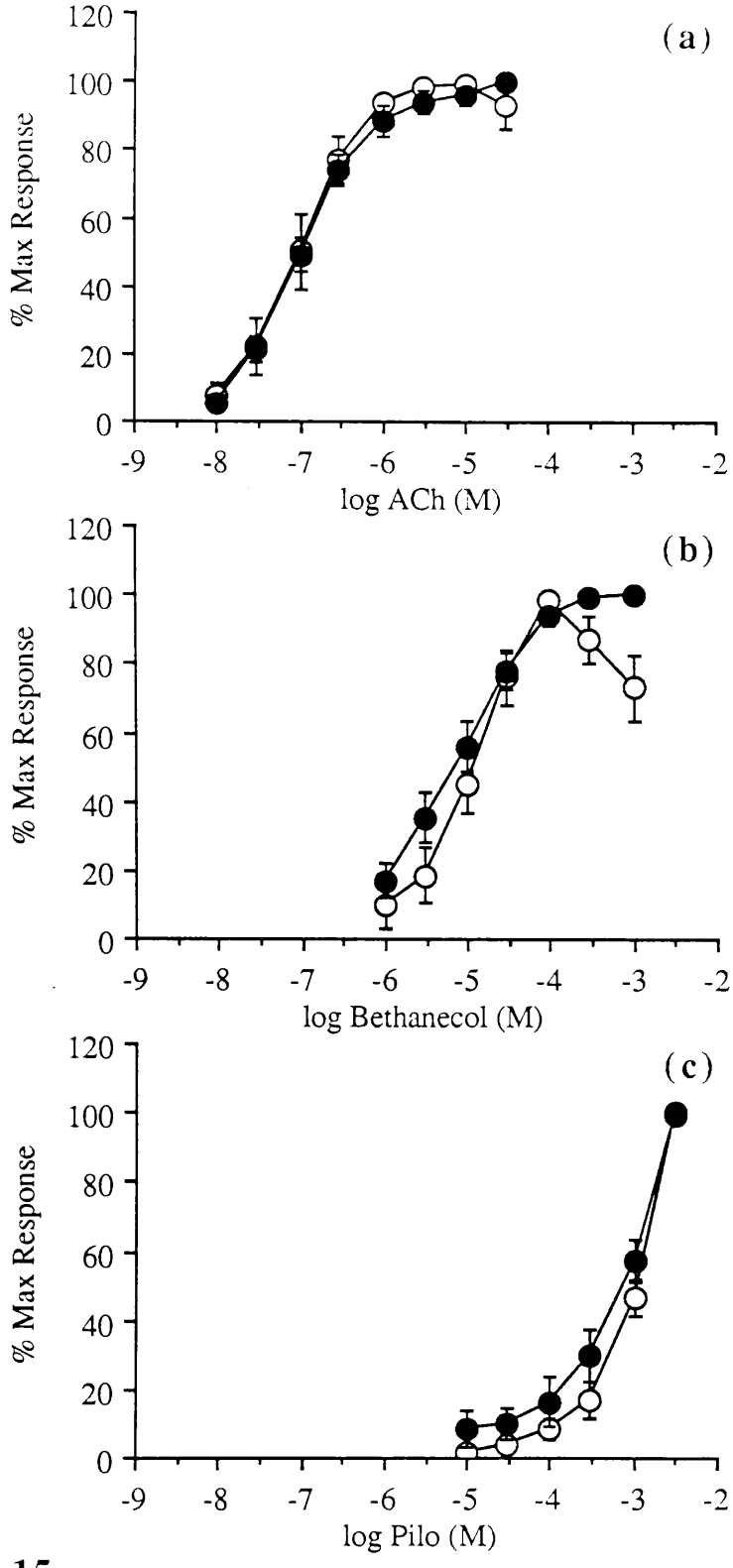

Figure 4.15

Relaxation (expressed as a percentage of the agonist maximum response) of intact rat aortic rings by a) ACh, b) bethanecol and c) pilocarpine. Tone was induced by U46619 in preparations from control (○) and LiCl pre-treated (●) rats.

Points shown in are mean ± s.e.m. (n=4-6).

Discussion

Although the use of selective antagonists such as 4DAMP suggests that the muscarinic receptor mediating endothelium-dependent relaxations of the rabbit aorta resembles the M_2 receptor found on non-vascular smooth muscle (Eglen and Whiting, 1985; Choo *et al.*, 1986) there is still some controversy as to the affinity of pirenzepine for these receptors. Eglen and Whiting (1985) reported a pA_2 value for pirenzepine (7.6) which was intermediate between that for the M_1 and M_2 subtypes (8.4 and 6.8 respectively). In contrast other workers have reported pA_2 values for pirenzepine that are similar to reported values at M_2 receptors (Choo *et al.*, 1986). Therefore, in the light of this controversy I have attempted to classify definitively the muscarinic receptor subtype which mediates endothelium-dependent relaxations of the rat aorta.

The pA_2 value obtained in this study for atropine against ACh-induced relaxations of the rat aorta is consistent with an interaction with muscarinic receptors and is similar to that reported for the action of atropine against endothelial dependent relaxations in other preparations such as the dog femoral artery, rabbit ear artery and rabbit aorta (Rubanyi *et al.*, 1986; Hynes *et al.*, 1986; Eglen and Whiting, 1985).

The affinity of pirenzepine obtained in this study is higher than that previously reported for M_2 muscarinic receptors in gastrointestinal smooth muscle (Brown *et al.*, 1980; Barlow *et al.*, 1981) and intermediate between the values reported by Eglen and Whiting (1985) and Choo *et al.*, (1986). The reasons for this disparity are unclear but may be due to a combination of factors: (i) use of superfusion techniques (Eglen and Whiting, 1985) as opposed to 'classical' organ bath experiments, (e.g. this study; Rubanyi *et al.*, 1986), (ii) differences in antagonist equilibration time, (e.g. 60 mins used in this study and 30 mins used by Hynes *et al.*, 1986), (iii) species variation in the affinity of pirenzepine (Yamanaki *et al.*, 1986). The use of superfusion techniques in classification of the muscarinic receptor mediating endothelium-dependent relaxations is further complicated by the observation that in the perfused dog femoral

artery ACh induces biphasic release of EDRF with a differential potency of pirenzepine for the phases (Rubanyi *et al.*, 1986) suggesting a possible heterogeneity in muscarinic receptors or the release of more than one EDRF. Although not completely eliminating any controversy concerning the affinity of pirenzepine at the muscarinic receptor mediating endothelial dependent relaxations, the affinity of pirenzepine obtained in this study is clearly distinct from that observed at M_1 muscarinic receptors (Brown *et al.*, 1980). Thus, the muscarinic receptor mediating ACh-induced relaxations of the rat aorta is of the M_2 type cholinoceptor similar to that found in gastrointestinal smooth muscle.

Whilst neither atropine nor pirenzepine discriminates between subtypes (atrial and ileal) of M_2 muscarinic receptors (Mitchelson, 1984) the antagonist 4DAMP exhibits a 10 fold selectivity for the ileal muscarinic receptor (Brown et al., 1980). In addition, himbacine and methoctramine show 10 and 270 fold selectivity for the atrial muscarinic receptor (Gilani and Cobbin, 1986; Melchiorre et al.,1987). Therefore, 4DAMP, himbacine and methoctramine provide tools for the definitive classification of the muscarinic receptor subtype mediating ACh-induced relaxation of the rat aorta.

As can be seen in Figure 4.16, the pA_2 value obtained using 4 DAMP was similar to that reported for the muscarinic M_2 receptors in smooth muscle (9.0) but different from that reported for these receptors in the atria (7.9; Eglen and Whiting, 1986). The similarity to the smooth muscle M_2 muscarinic receptor is supported by the values obtained using methoctramine and himbacine, which were dissimilar to those observed at atrial muscarinic receptors (8.0 and 8.2 respectively) but similar to those observed at smooth muscle receptors (5.9 and 7.1 respectively, Melchiorre *et al.*,1987; Gilani and Cobbin, 1986). Thus, on the basis of selective antagonist studies the muscarinic receptor mediating endothelium-dependent relaxations of the rat aorta is similar to M_2 receptors in non-vascular smooth muscle but dissimilar to M_2 receptors in the atria.

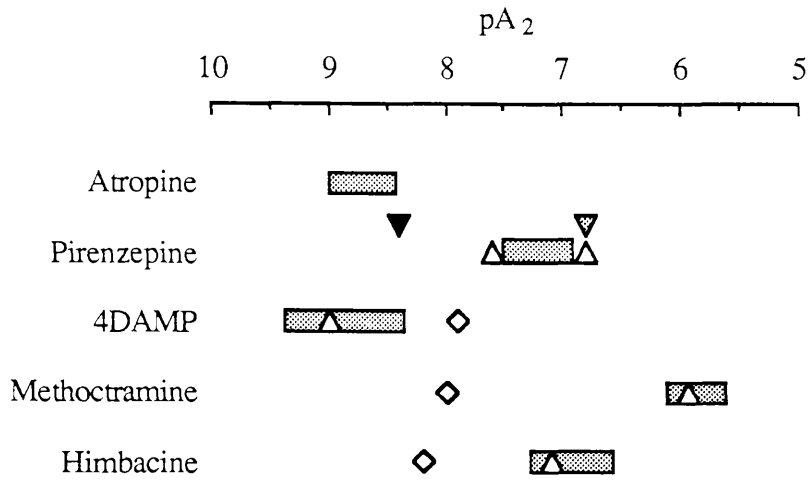

Figure 4.16

Summary of pA_2 values for muscarinic antagonists inhibiting acetylcholine-induced relaxations of rat aortic rings.

▒ Indicates mean pA_2 (midpoint) with 95% confidence intervals (limits of box) for data obtained in this study.

Data from other studies (see Eglen and Whiting, 1986):

▼ & ▽ Indicate affinity for pirenzepine at, ▼ - M_1 and, ▽ - M_2 receptors.

◇ Indicates pA_2 for antagonists at atrial receptors.

Δ Indicates pA_2 for antagonists at smooth muscle receptors.

Lithium has been shown to inhibit myo-inositol-l-phosphate phosphatase activity and thereby decrease receptor-mediated inositol phospholipid hydrolysis (Allison and Stewart, 1971; Sherman *et al.*, 1986; see Chapter One). Eglen *et al.*, (1987), demonstrated that pretreatment of animals with lithium selectively inhibited muscarinic receptor function in the ileum (M_2) from the guinea-pig. Therefore, if this subtype of M_2 muscarinic receptor is universally coupled to inositol phospholipid hydrolysis and muscarinic receptors can be practically defined according to their effector systems (Harden *et al.*, 1986) then the ACh-induced, M_2 receptor mediated relaxations of rat aortic rings would be selectively attenuated by pre-treatment with lithium.

In their study, Eglen *et al.*, (1987), could not show an effect of lithium on the sensitivity or maximum response of carbachol-induced contractions in the guinea-pig ileum (mediated by M_2 muscarinic receptors) despite a significant attenuation of the effect of bethanecol and pilocarpine. This could indicate that carbachol activates different receptors than bethanecol and pilocarpine or it may indicate that whilst the agonists bind to the same receptor, carbachol, through an unknown mechanism, produces contraction via activation of a different second messenger system than bethanecol and pilocarpine. Alternatively, carbachol may have such a high relative efficacy that lithium pre-treatment has no apparent effect whilst the responses to the agonists with lower relative efficacy are markedly attenuated. Preliminary experiments suggested that lithium pre-treatment had little if any effect on ACh-induced relaxations of the rat aorta. I therefore decided to examine the effect of lithium pretreatment on ACh-, bethanecol- and pilocarpine-induced relaxations of rat aortic rings, i.e. relaxations induced by agonists which have been shown to be 'full' and 'partial' (Furchgott and Cherry, 1984).

For this part of the study the preparations were pre-contracted with U46619 since lithium treatment significantly attenuated contractions to PhE (see Chapter One). There was no significant difference in the potency of ACh to relax U46619-induced contractions compared to PhE-induced contractions. This was somewhat surprising

since work carried out previously (Templeton, PhD thesis 1988) indicated that ACh-induced relaxations of U46619-induced tone in the rat aorta were smaller than relaxations of PhE-induced tone. However, the results from the work carried out by Templeton may be atypical since the pA_2 for atropine indicated a non-competitive interaction with the muscarinic receptor mediating endothelium-dependent relaxation (slope of Schild plot = 0.6) and an unusually high pA_2 (9.6) . Lithium also depresses the contractile response to U46619 (data not shown) and therefore care was taken to try to ensure that equieffective concentrations of U46619 were used: differences in activation of the rat aorta can produce changes in the sensitivity of the preparations to ACh-induced relaxations (see Chapter Two).

In this study bethanecol and pilocarpine were shown to be partial agonists since both agonists exhibited a lower intrinsic activity than ACh in paired preparations (i.e., the maximum relaxant response obtained to these agonists was significantly smaller than that obtained for ACh). Bethanecol and pilocarpine were found to be partial agonists in both control and treated preparations. Antagonist studies were not performed to demonstrate conclusively that ACh, bethanecol and pilocarpine all act at the same receptor to cause relaxation. However, 10μM ACh added in the presence of maximal relaxation to bethanecol and pilocarpine did not induce further, substantial relaxations of the preparation despite the maximum response to the test agonist being significantly less than that for ACh in the paired preparation. This provides indirect evidence that bethanecol and pilocarpine are acting at the same receptor as ACh: bethanecol and pilocarpine would act as antagonists of the action of a full agonist at high concentrations and therefore if ACh were to act through different receptors then it would be expected to induce further relaxations of the rat aorta.

At high concentrations agonist-induced relaxations of U46619-induced tone (in terms of the percentage reduction of that tone) by ACh, bethanecol and pilocarpine were significantly smaller in the lithium pre-treated preparations compared to the control preparations. However, there were no significant differences in the potencies (pIC_{50})

of the agonists between control and treated preparations. Furthermore, the lithium-induced depression of maximum relaxant response of the partial agonists was not significantly greater than that for ACh. Therefore it would appear that whilst lithium appears to attenuate muscarinic receptor mediated relaxations of the rat aorta, this effect is not preferentially greater for agonists of lower intrinsic activiy. The lack of difference in potency of the agonists between control and treated preparations is surprising particularly since this treatment did produce a depression of the maximum relaxation. This would suggest that in contrast to the response of the guinea-pig ileum (Eglen and Whiting, 1987) which is mediated by muscarinic M_2 receptors the M_2 receptor mediating relaxation of rat aortic rings is not selectively inhibited by lithium.

The apparent depression by lithium of agonist-induced relaxation may at least partly be explained by differences in the degree of U46619-induced activation of the smooth muscle. Although care was taken to try to ensure that the concentration of U46619 used to induce tone was equieffective in treated and control preparations, when the effects of ACh, bethanecol and pilocapine were expressed as absolute tension (mg. wt.) the tone induced in the treated preparation was consistently greater than in the control preparations. Increases in the degree of activation used to examine endothelium-dependent relaxations can result in attenuation of the size of relaxation without a concomitent decrease in potency of the relaxant agonist (see Chapter Two). Whilst the relaxant responses to the higher concentrations of ACh, bethanecol and pilocarpine were significantly greater in control compared to lithium pre-treated preparations, the difference in the absolute size of induced tone between control and treated tissues was not statistically significant. Therefore it is unlikely that the apparent depression of maximum response to the muscarinic agonists is due wholly to differences in induced tone. A significant reduction in maximum relaxation without a change in sensitivity to the relaxing agent can also be observed when the resting tension of the preparation is varied (see Chapter Two). In this study the resting tension in control and treated preparations was equal. However, treatment with lithium

may have had some effect on the rat aorta such that the optimum degree of stretch for demonstration of endothelium-dependent relaxations is different in both preparations and this may play a major role in the differences in the maximum relaxant response to muscarinic receptor activation seen in the present study between treated and control preparations.

Although removal of the vascular endothelium leads to loss of *in vitro* vasodilatation to ACh (Furchgott and Zawadzki, 1980), and thereby implies the presence of cholinoceptors on the endothelium mediating these relaxant responses, this has not been definitively shown. The published evidence appears to indicate that the muscarinic receptor is not located on the endothelium. Studies using cultured endothelial cells have failed to show a muscarinic-receptor mediated increase in ^{86}Rb efflux or release of EDRF (Gordon and Martin, 1983; Loeb *et al.*, 1985; Cocks *et al.*, 1985; Gryglewski *et al.*, 1986). In addition, radioligand binding studies using [^{3}H]QNB as the muscarinic receptor ligand have failed to detect binding in endothelial cell cultures (Peach *et al.*, 1985). This may be attributed to the loss of muscarinic receptors on culturing (a phenomenon not seen with bradykinin receptors; Cocks *et al.*, 1985). The lack of functional responses to muscarinic receptor agonists in endothelial cell cultures may also be due to the highly unphysiological nature of the technique since there is evidence for differences in the response to agonists in cell culture and intact preparations (Chamley-Campbell *et al.*, 1981) and also a decline in cultured endothelial cell responsiveness with time (Ager and Martin, 1983; Needham *et al.*, 1987). In addition, autoradiographic studies have shown that in a variety of arterial preparations from different species there is a high density of muscarinic receptors present in the adventitial layers of the vascular smooth muscle but not on the endothelium (Stephenson and Summers, 1987). This is somewhat surprising since in preparations such as the rat aorta, the functional response to ACh (particularly at low concentrations) appears to be mediated via an intact endothelium. Hynes *et al.*, (1986) using organ bath and radioligand binding techniques demonstrated that pirenzepine had

the same affinity at the receptors mediating ACh-induced relaxation of the rabbit ear artery and the underlying smooth muscle. Moreover, these workers showed that removal of the endothelium did not reduce the density of the muscarinic receptors in the preparation but rather increased it. This might suggest that muscarinic receptors were present only on the vascular smooth muscle and not on the endothelium.

Therefore the evidence to-date suggests that muscarinic receptors present on the smooth muscle of vascular preparations may release some unknown substance which induces or promotes the release of EDRF from the endothelium (Stephenson and Summers, 1987). This hypothesis is supported by the study of Loeb *et al.*, (1985) who showed that an increase in cell cGMP content (a marker of EDRF-induced vasodilatation; Rapaport and Murad, 1983) could be produced by activation of muscarinic receptors in cultured endothelial cells only when the endothelial cells were co-cultured with smooth muscle cells: stimulation of single cell-type cultures of smooth muscle or endothelial cells with the muscarinic receptor agonist methacholine produced no release of relaxant substance(s). Since EDRF induces an increase in smooth muscle cGMP (Rapaport and Murad, 1983) then it is perhaps to be expected that single cultures of endothelial cells or smooth muscle cells would not show an increase in cGMP levels in response to muscarinic receptor stimulation. However, Martin *et al.*, (1988) did show that levels of cGMP are increased in endothelial cell cultures by stimulation of EDRF production with agents such as A23187 and bradykinin but not ACh. Therefore if muscarinic receptors mediating EDRF release were present on the cultured endothelial cells and the smooth muscle cells play no part in ACh-induced release of EDRF then activation of muscarinic receptors in co-cultures of smooth muscle and endothelial cells (Loeb *et al.*, 1985) would not be expected to induce an increase in levels of cGMP.

To conclude, whilst the present study does not investigate the intriguing question of the location of muscarinic receptors mediating endothelium-dependent relaxations of the rat aorta I have classified this receptor on the basis of differential antagonist

affinities as being an M_2 muscarinic receptor similar to that found in smooth muscle preparations such as the guinea-pig ileum and trachea. This M_2 muscarinic receptor, in contrast to that found in the ileum, is insensitive to lithium pre-treatment of the donor animal and is therefore probably not coupled to inositol phospholipid hydrolysis. The location and coupling mechanism of the receptor remain to be evaluated.

Chapter Five

The effect of palmitoyl carnitine on endothelium-dependent relaxations of the rat aorta.

Introduction

In myocardial cells, ischaemic conditions lead to the breakdown of mitochondrial β-oxidation of fatty acids resulting in the accumulation of acyl-CoA in mitochondria and acyl carnitines in the cytosol (Liedtke *et al.*, 1978; Neely and Feuvray, 1981; Knabb *et al.*, 1986). Under these conditions the level of cytosolic acyl carnitines can reach 75-200μM (Corr *et al.*, 1984) and it has been suggested that the accumulation of these lipid metabolites contributes to cellular damage and electrophysiological derangements associated with cardiac ischaemia (Neely and Feuvray, 1981; Knabb *et al.*, 1986).

Palmitoyl carnitine is a long chain acyl carnitine which accumulates in ischaemic conditions, rapidly partitions into the sarcolemma and under prolonged ischaemic conditions can leak into the extra-cellular space (Shug *et al.*, 1978; Knabb *et al.*, 1986). In cardiac myocytes, hypoxia causes a 70 fold increase in sarcolemmal acyl carnitines (Knabb *et al.*, 1986). In myocardium palmitoyl carnitine may increase contractile force by increasing membrane permeability to calcium (Inoue and Pappano, 1983) and can directly activate Ca^{2+} channels in smooth muscle (Mir and Spedding, 1986). Palmitoyl carnitine has been shown to resemble the dihydropyridine Ca^{2+} channel activator BAY K 8644 (Schramm *et al.*, 1983) in a variety of interactions at voltage-operated Ca^{2+} channels and has been suggested to be an endogenous ligand at voltage operated calcium channels (Spedding and Mir, 1987).

Recent work in vascular smooth muscle has shown that hypoxia augments K^+-induced contractility of rat portal vein and that this augmentation is attenuated by phenylalkyloxirane carboxylic acid (POCA), an inhibitor of carnitine acyltransferase (Fasehun *et al.*, 1987). In addition, palmitoyl carnitine can increase K^+-induced contractions in the rat tail artery by increasing sensitivity to Ca^{2+} (Ugwu *et al.*, 1987). These experiments suggest that, as with cardiac muscle, acyl carnitine accumulates in vascular smooth muscle under ischaemic conditions and can act as an endogenous Ca^{2+} channel activator in the vascular smooth muscle. During ischaemia this would limit the

blood supply further by potentiating the effect of any basal tone in the vascular smooth muscle due to circulating and neuronally released hormones or autocoids from aggregating platelets. This would then lead to further vasoconstriction and hence decreases in blood flow to the affected area possibly exacerbating the ischaemic damage.

Preliminary experiments have shown that BAY K 8644-induced contractions in ring preparations of rat aorta partially depolarised with 12mM KCl are potentiated by disruption of the endothelial cell layer (Spedding *et al.*, 1987). This was attributed to removal of the basal release of EDRF which directly opposed the contractile response to BAY K 8644. However, under similar conditions the contractions to cumulative addition of palmitoyl carnitine are unaffected by removal of the endothelium. Thus, contractions to palmitoyl carnitine are resistant to basal release of EDRF possibly indicating activation of layers of vascular smooth muscle or some contractile mechanism which is not activated by BAY K 8644 and which is resistant to EDRF. Alternatively, palmitoyl carnitine may inhibit the release or action of EDRF and therefore removal of the endothelium has no net effect on palmitoyl carnitine-induced contractions. In ring segments of rat aorta precontracted with 1μM PhE, palmitoyl carnitine inhibits, and reverses, the endothelium-dependent relaxation to 1μM acetylcholine (Bigaud and Spedding, 1986). Therefore it would appear that palmitoyl carnitine inhibits the release or action of EDRF. This could be of some physiological significance since in ischaemic conditions when blood supply may be limited due to the Ca^{2+} channel activating action of palmitoyl carnitine this would limit the blood supply even further by removing the effect of basal release of EDRF which directly opposes the effect of contractile agents on the vascular smooth muscle (see Chapter Three for references).

In this study I have further investigated the inhibition of endothelium-dependent relaxations by palmitoyl carnitine in this tissue and made a comparison with the effect of BAY K 8644 on endothelium-dependent and independent relaxations.

Methods

Male Wistar rats (250-300g) were killed by stunning followed by exsanguination. Ring segments of descending thoracic aorta (2-3mm) were prepared as described in General Methods. When necessary, the endothelial cell layer was disrupted by gently rubbing the intimal layer of the ring with curved forceps. When this procedure was not carried out care was taken to maintain an intact endothelium.

Agonist-Induced Relaxations

Ring segments were suspended between rigid wire tissue holders in 10ml isolated organ baths containing PSS maintained at 37°C for isometric tension recordings. The presence of an intact endothelium was demonstrated by the ability of 1μM ACh to relax 1μM PhE-induced tone as described in General Methods. After this preliminary procedure the effect of palmitoyl carnitine (3-100μM) on agonist-induced relaxations was examined.

Four endothelium-intact ring preparations from each rat were used. One ring was kept as control, i.e. unexposed to palmitoyl carnitine, and the other three used to examine the effect of various concentrations of palmitoyl carnitine on agonist induced relaxations of PhE-induced tone.

When required, one concentration of palmitoyl carnitine (3-100μM) was added to the organ baths 10 minutes prior to the addition of 1μM PhE. This concentration of PhE induces 90-95% maximum contraction (approx 1000 mg. wt.). When the contraction to PhE had reached a stable plateau, a CCRC for agonist-induced relaxations was obtained by addition of ACh (0.01-100μM), ATP (10-1000μM), the calcium ionophore A23187 (0.01-10μM) or sodium nitroprusside (SNP; 0.001-3μM) in 0.5 $\log_{10}$ molar increments. Preliminary experiments had already shown that SNP caused endothelium-independent. The sensitivity of intact aortic rings to ACh-induced relaxations can vary with time (see Chapter Four). Therefore, in order to avoid any

possible variation in tissue sensitivity with time only one CCRC for the agonist tested was obtained in each preparation.

The effect of repeated washing in palmitoyl carnitine-free saline on the relaxant response to ACh in preparations pre-contracted with PhE and previously exposed to palmitoyl carnitine was examined. In these experiments two endothelium-intact preparations from each rat were used: one preparation to be used as control (i.e. not exposed to palmitoyl carnitine) and the other preparation to be treated with palmitoyl carnitine. After the preparations were set up and the presence of a functional endothelium had been demonstrated as described previously, the preparations were pre-contracted with 1μM PhE. A relaxant response to a maximal concentration of ACh (10μM) was obtained. The preparations were washed as described in General Methods and allowed to equilibrate for 30 mins. Palmitoyl carnitine (30μM) was added to the organ bath of one ring segment 10 minutes prior to inducing tone with 1μM PhE. The other ring segment was used as a vehicle control: 10 μl 20% ethanol. When the contractions to PhE had reached a stable plateau in both preparations, ACh (10μM) was added to both organ baths and any relaxation observed. When relaxation had reached a plateau the preparations were washed according to the schedule described in General Methods with palmitoyl carnitine-free saline and left for 30 minutes before once more obtaining 10μM ACh-induced relaxation of 1μM PhE-induced tone in the absence of palmitoyl carnitine. This procedure was repeated four times. Relaxations in the preparation exposed to palmitoyl carnitine were compared to those in the control preparation which showed any deviation in ACh-induced relaxations due to time.

Comparative Effect of BAY K 8644

The effect of BAY K 8644 and palmitoyl carnitine on PhE-induced tone and ACh-induced relaxation of that tone was examined. Paired ring preparations with an intact and disrupted endothelium were used. In preparations pre-contracted with 1μM PhE

the effect of cumulative addition of palmitoyl carnitine (0.01-100μM) or BAY K8644 (0.001-3μM) in 0.5 $\log_{10}$ molar increments was examined. In some intact preparations tone was induced by addition of 1μM PhE 10 minutes after addition of palmitoyl carnitine (30μM) or BAY K 8644 (3μM). The effect of this pre-treatment on ACh-induced relaxations was then examined by cumulative addition of ACh after the PhE-induced contraction had reached a plateau. In addition the effect of palmitoyl carnitine (30μM) or BAY K 8644 (3μM) on an established relaxation of 1μM PhE-induced tone by 1μM ACh or 0.1μM SNP was examined.

Bioassay of EDRF

The endothelium of some rings obtained in the manner described above was disrupted by gently rubbing the intimal layers with forceps. These rings were cut longitudinally to form strips to be used as 'recipients' for EDRF. Ring preparations with an intact endothelium were also cut longitudinally with care taken to minimise disruption of the endothelium. These were used as 'donors' of EDRF for superfused 'recipient' aortic strips. Recipient aortic strips were suspended in a perspex gutter for isometric tension recording of the circular smooth muscle (Figure 5.1). A 'donor' strip was placed over the 'recipient' strip such that the circular smooth muscle of the 'donor' and 'recipient' strips were at right angles in order to prevent mechanical interference from agonist-induced contraction of the 'donor' strip. This arrangement was then superfused with PSS at a rate of 6 ml min^{-1}.

After 45 minutes equilibration, the strips were superfused with 0.1μM PhE in order to contract the rubbed 'recipient' strip. When the recorded tension in the 'recipient' strip had reached a plateau the strips were superfused with 10μM carbachol and any relaxations of the 'recipient' strip recorded. The effect of palmitoyl carnitine on carbachol-induced relaxations was examined by 20 minutes pre-incubation of the 'donor' or the 'recipient' preparation with 30μM palmitoyl carnitine prior to superfusion with carbachol.

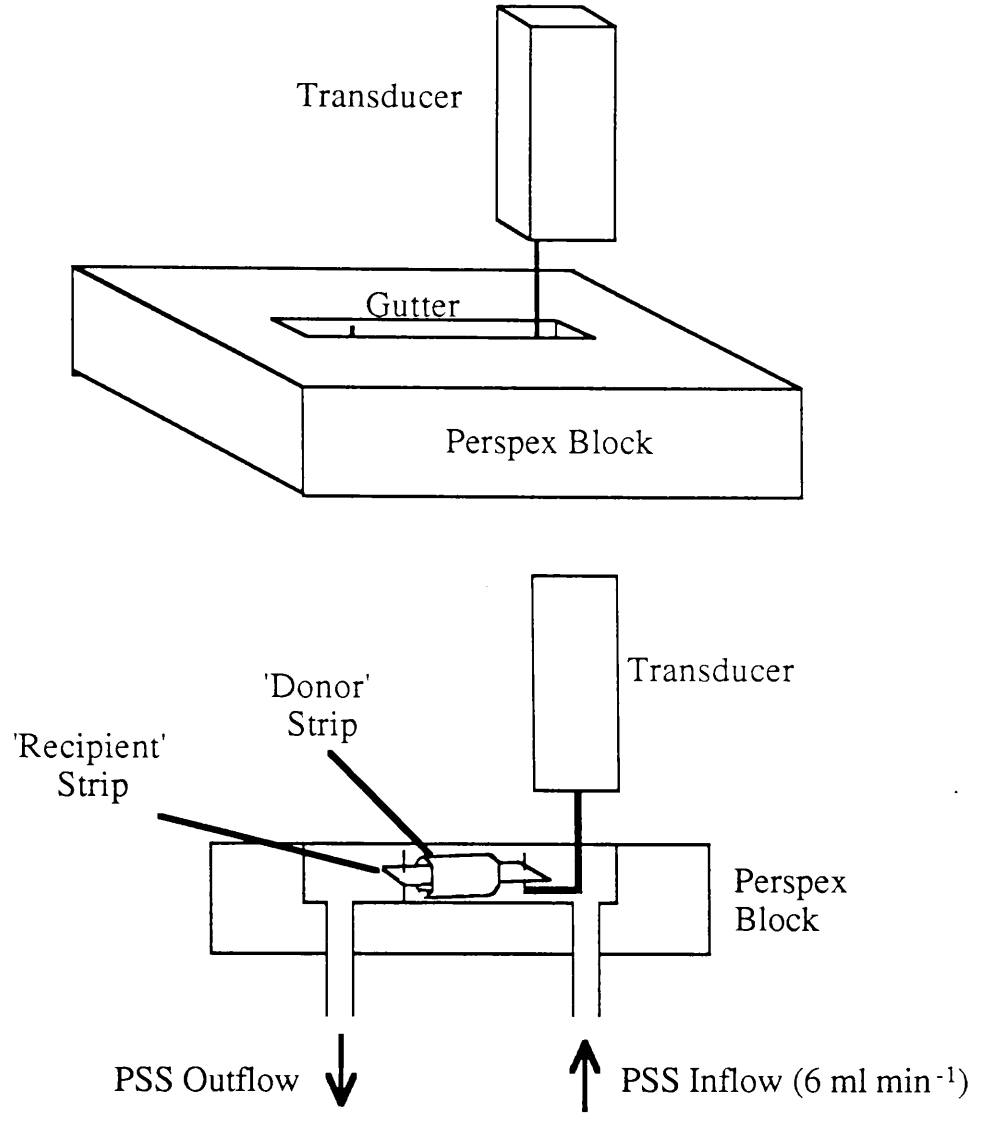

Figure 5.1

Diagram of apparatus used for bioassay of EDRF. An endothelium-disrupted ('recipient') strip of rat aorta is suspended in a channel in a perspex block between a fixed wire support and an isometric tension transducer. The strip is superfused with PSS (6ml min^{-1}) and an endothelium intact ring, cut to form a strip, placed over the 'recipient' with its intimal layer in contact with the smooth muscle of the 'recipient' to act as a 'donor' of EDRF.

All agonist-induced relaxation responses were expressed as a percentage of the induced tone. When necessary statistical analysis was carried out using Student's unpaired *t test*. The potency for the inhibitory action of palmitoyl carnitine against agonist-induced relaxation of PhE-induced tone was estimated as the molar concentration of palmitoyl carnitine required to inhibit the maximum relaxation by 50% (IC_{50}) and expressed as the negative $\log_{10}(IC_{50})$, i.e. the pIC_{50}.

Results

Agonist-Induced Relaxations

In ring preparations of rat aorta with an intact endothelium, ACh, ATP and A23187 induced concentration-dependent relaxations of 1μM PhE-induced tone (Figure 5.2a). The order of potency for agonist-induced relaxations was **ACh = A23187 > ATP** (Figure 5.2b). The maximum relaxation (as a percentage of induced tone) to ACh, ATP and A23187 was 62.0 ± 6.7, 47.2 ± 5.2 and 34.4 ± 7.2 respectively (Figure 5.2a). In addition to these endothelium-dependent relaxations, SNP induced concentration-dependent relaxations of pre-contracted preparations with a maximum relaxation of 97.3 ± 7.1 % of the induced tone (Figure 5.3).

At concentrations greater than 1μM, A23187 produced contraction of the ring preparation which directly opposed its relaxant effect (Figure 5.2b). When added prior to inducing tone with PhE, palmitoyl carnitine (3-100μM) had no effect on the resting tension of the ring preparations. Palmitoyl carnitine (3-30μM) had little effect on the contraction produced by 1μM PhE when compared to the contraction in the control preparation (i.e. in the absence of palmitoyl carnitine). However, at a higher concentration (100μM), palmitoyl carnitine caused a slight depression in the PhE-induced contraction compared to that in the control preparation (Figure 5.4). In ring segments pre-contracted with 1μM PhE, palmitoyl carnitine caused concentration-dependent inhibition of the ACh- and ATP-induced relaxations (Figures 5.5 and 5.6). A23187-induced relaxations were not inhibited by concentrations of palmitoyl carnitine < 30μM but were completely abolished by 30μM palmitoyl carnitine. However, contractions induced by high concentrations of A23187 were augmented by palmitoyl carnitine in a concentration-dependent manner (Figure 5.7). Examination of the effect of palmitoyl carnitine on relaxation to a single, submaximal concentration of ACh (3μM), ATP (100μM) or A23187 (1μM) clearly illustrates the concentration-dependent inhibition of agonist induced relaxation (Figure 5.8). The potencies (pIC_{50}) for the

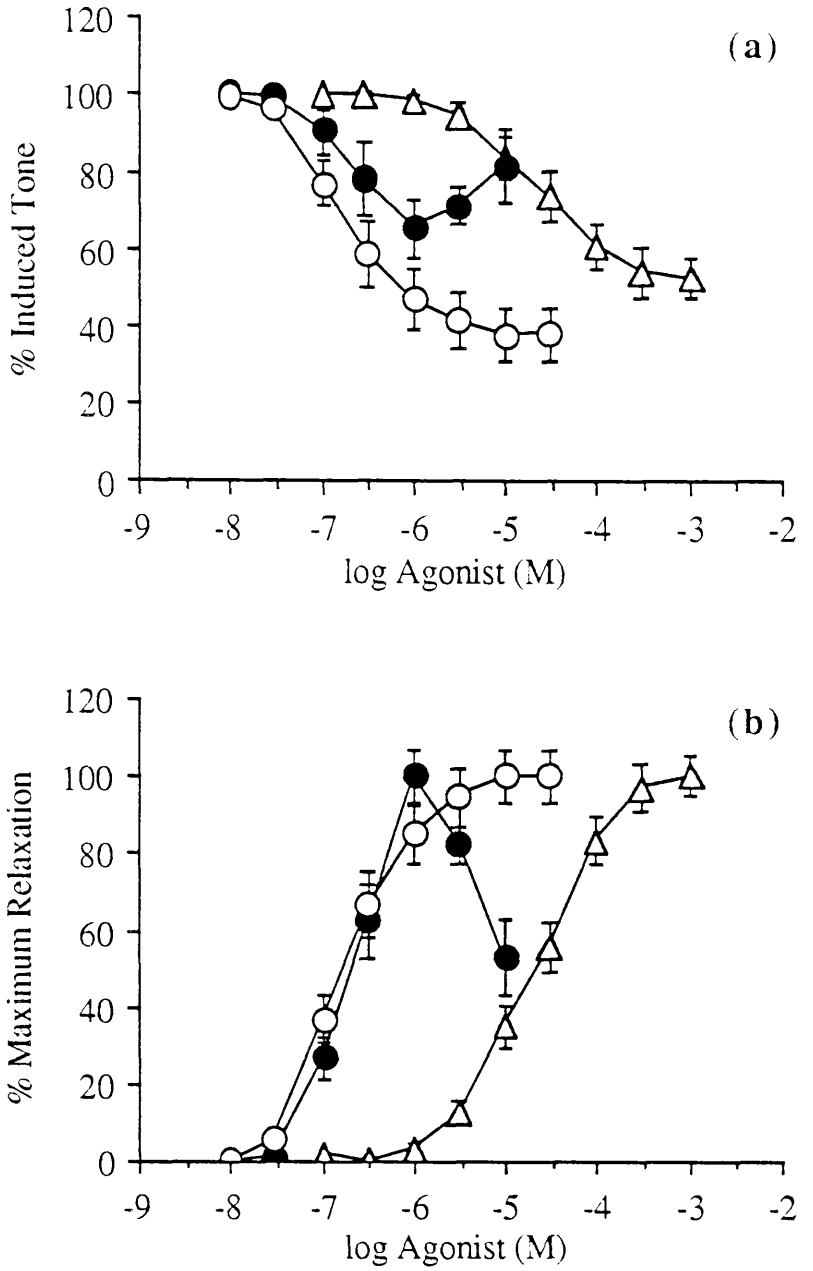

Figure 5.2

Relaxations of 1μM PhE-induced tone in intact rat aortic rings to cumulative addition of ACh (○), A23187 (●), and ATP (△). Data shown represents relaxations expressed as (a) the percentage reduction of induced tone and (b) the percentage maximum relaxation to that agonist.

Points shown are mean ± s.e.m. (n=4-6).

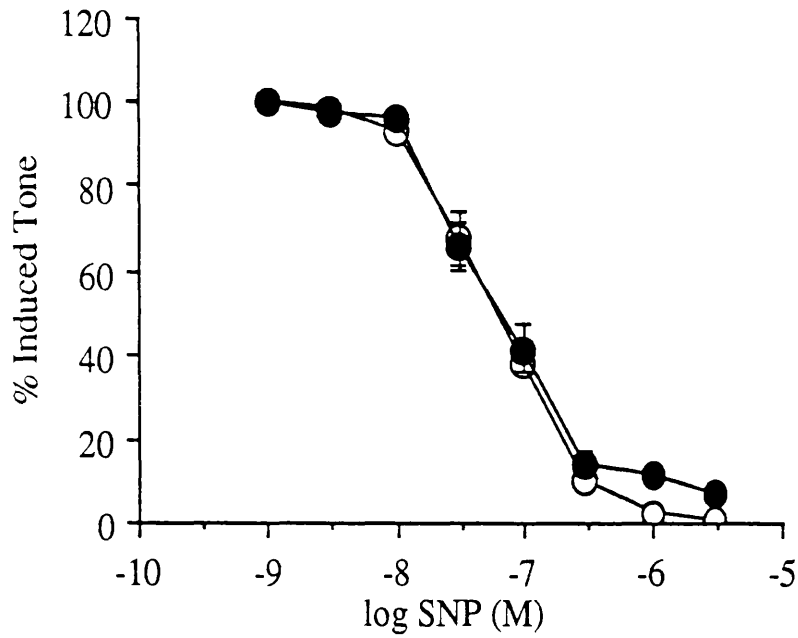

Figure 5.3

The effect of palmitoyl-carnitine on SNP-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. SNP-induced relaxations are expressed as a percentage of the induced tone in the absence (○) and presence of 30μM palmitoyl-carnitine.

Points shown are mean ± s.e.m. (n=6).

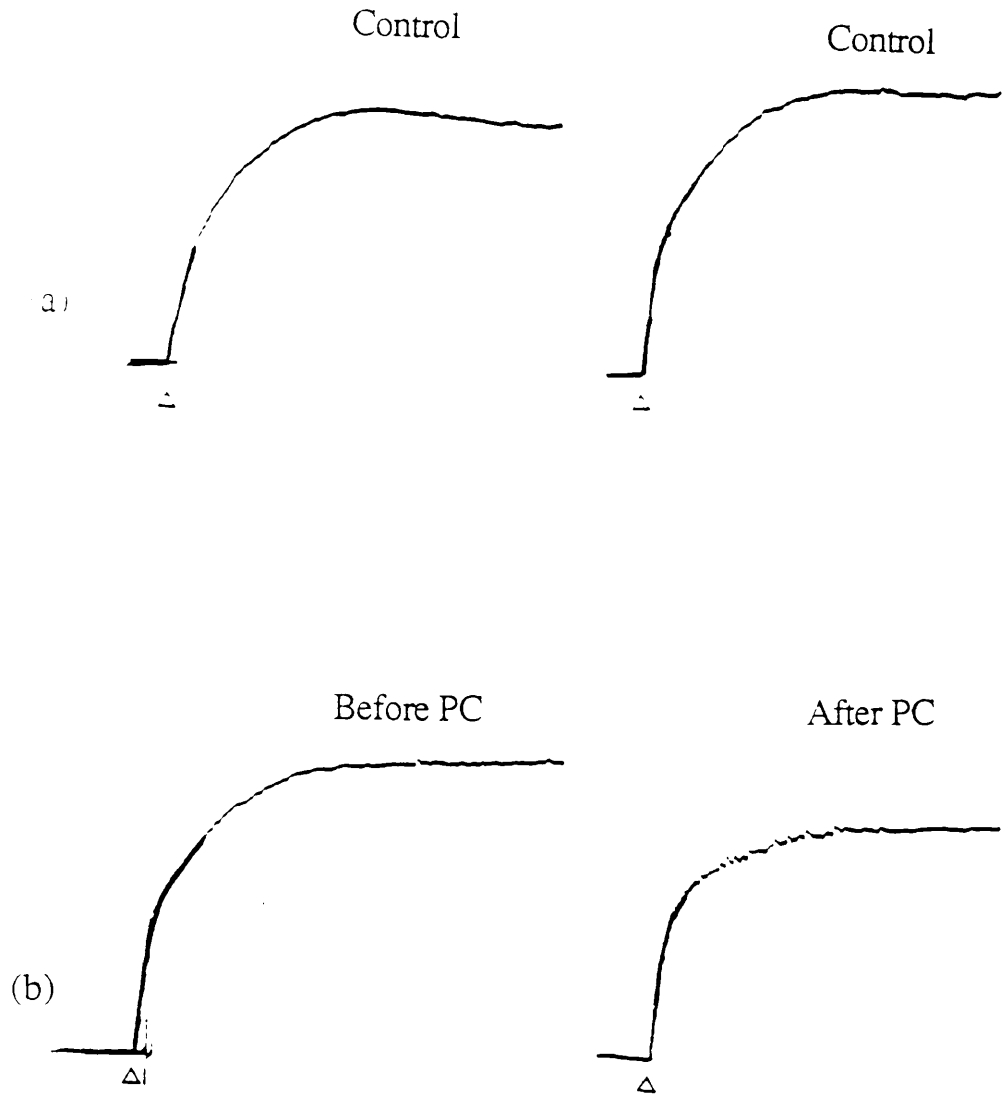

Figure 5.4

Response of the rat aorta to repeated administration of 1μM PhE (Δ) in preparations with an intact endothelium. Responses shown are (a) time control and (b) the response before and after incubation with 100μM palmitoyl carnitine 10 minutes before addition of PhE.

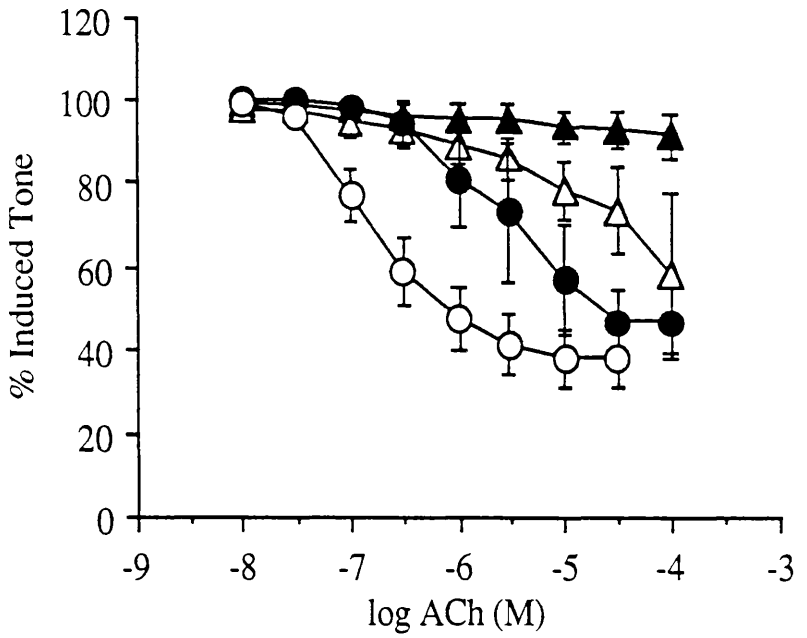

Figure 5.5

The effect of palmitoyl-carnitine on ACh-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. ACh-induced relaxations are expressed as a percentage of the induced tone in the absence (○) and presence of 10μM (●), 30μM (△) and 100μM (▲) palmitoyl-carnitine.

Points shown are mean ± s.e.m. (n=4-6).

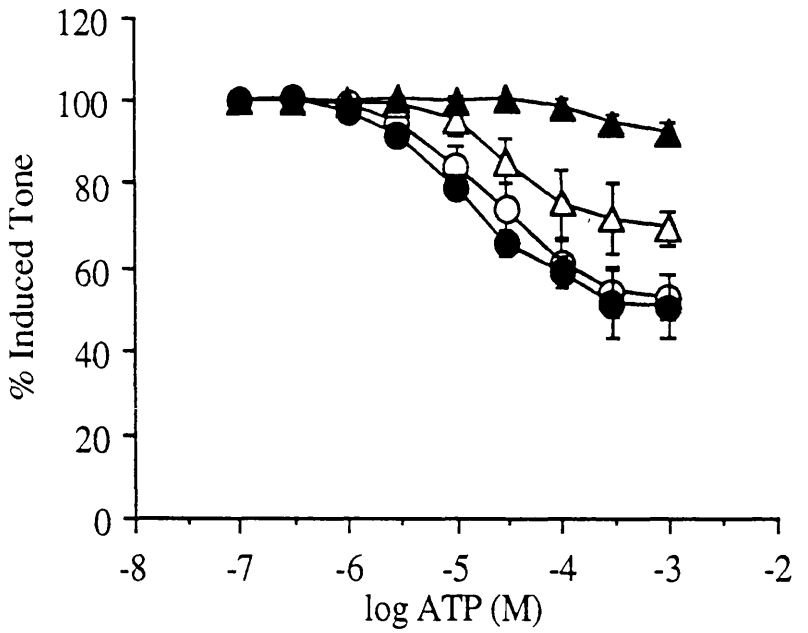

Figure 5.6

The effect of palmitoyl-carnitine on ATP-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. ATP-induced relaxations are expressed as a percentage of the induced tone in the absence (○) and presence of 3μM (●), 10μM (△) and 30μM (▲) palmitoyl-carnitine.

Points shown are mean ± s.e.m. (n=4-6).

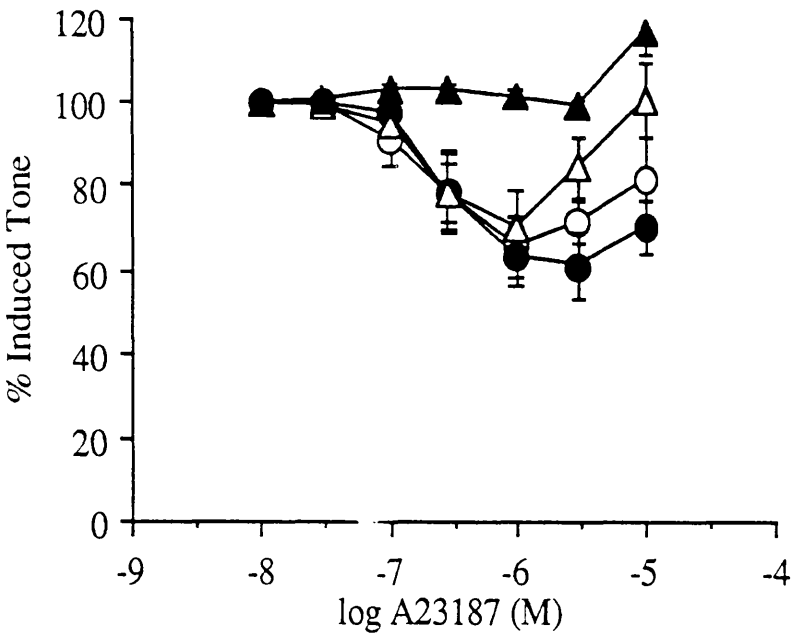

Figure 5.7

The effect of palmitoyl-carnitine on A23187-induced relaxations of rat aortic rings pre-contracted with 1μM PhE. A23187-induced relaxations are expressed as a percentage of the induced tone in the absence (○) and presence of 3μM (●), 10μM (△) and 30μM (▲) palmitoyl-carnitine.

Points shown are mean ± s.e.m. (n=4-6).

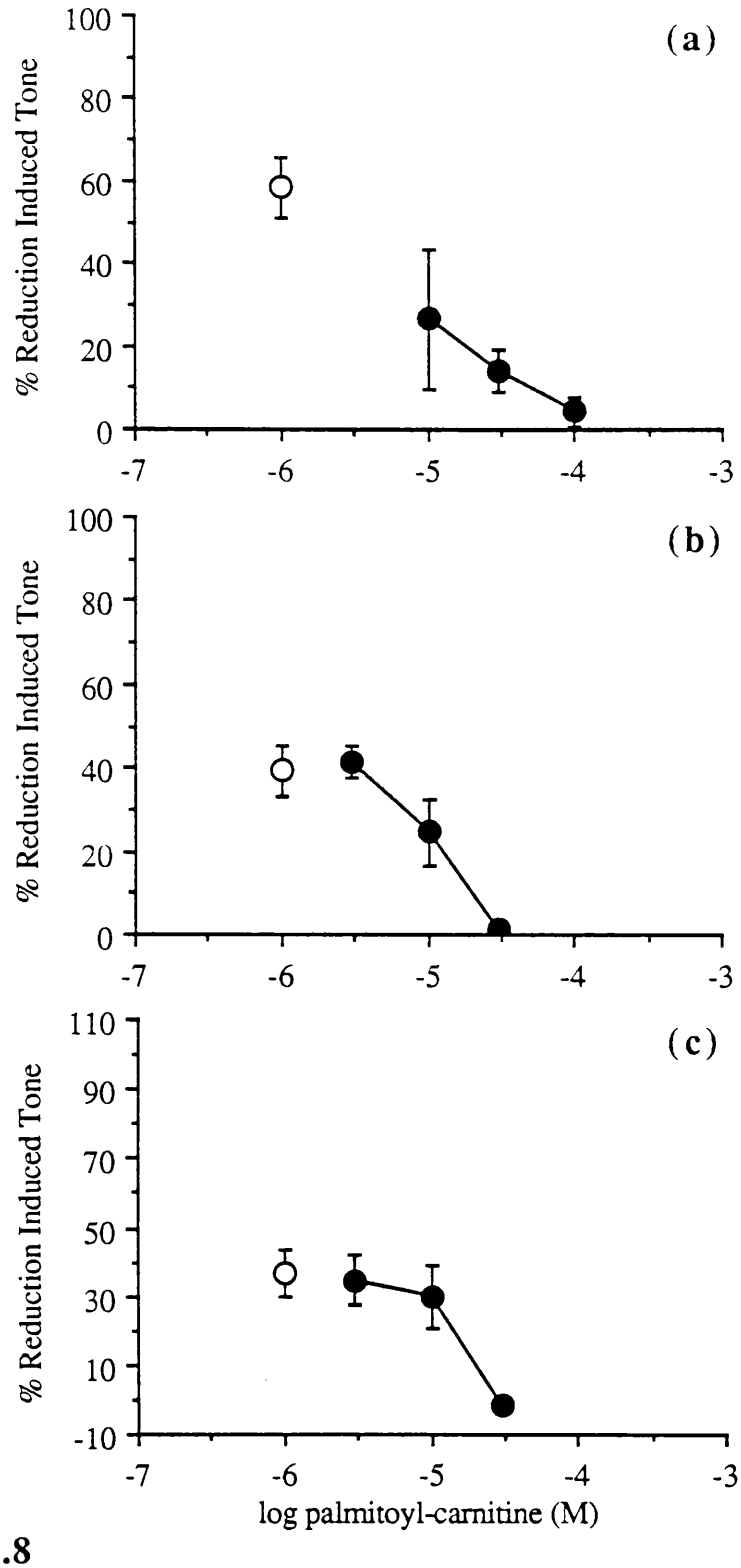

Figure 5.8

The effect of palmitoyl carnitine on relaxation of PhE-induced tone by a) 3μM ACh, b) 100μM ATP and c) 1μM A23187. Data shown is mean percentage reduction of induced tone ± s.e.m. (n=4-6) in the presence of palmitoyl carnitine (●).

Please note: ○ represents the mean response in the absence of palmitoyl carnitine.

inhibitory action of palmitoyl carnitine against ACh-, ATP- and A23187-induced relaxation of PhE-induced tone were estimated as 5.12, 4.90 and 4.83 respectively. palmitoyl carnitine, at a concentration which markedly inhibited ACh-induced relaxations (30μM) had no significant effect on the endothelium-independent relaxation induced by SNP (Figure 5.3).

In ring segments with an intact endothelium 10μM ACh induced a rapid, well maintained relaxation of tone induced by 1μM PhE which was markedly attenuated by incubation with 30μM palmitoyl carnitine. The relaxation to 10μM ACh in the preparation previously exposed to palmitoyl carnitine was restored to levels comparable to those seen in the paired control preparation after four wash cycles (Figure 5.9). Histological examination of the intima by electron microscopy showed that pre-incubation with 30μM palmitoyl carnitine had no visible effect on the endothelial cell layer compared to control preparations (Figure 5.10).

Comparative Effect of BAY K 8644

In paired preparations of rat aorta pre-contracted with 1μM PhE, cumulative addition of palmitoyl carnitine or BAY K8644 had no significant relaxant effect in either the presence or absence of an intact endothelium. BAY K 8644 but not palmitoyl carnitine caused further contraction of the preparations (Figure 5.11). In endothelium-intact preparations, incubation with palmitoyl carnitine (30μM) or BAY K 8644 (3μM) 10 minutes before addition of 1μM PhE attenuated subsequent ACh-induced relaxations. However, BAY K 8644 caused a marked potentiation of the contraction to PhE; this phenomenon was not seen with palmitoyl carnitine (Figure 5.12).

Palmitoyl carnitine (30μM) or BAY K 8644 (3μM) caused reversal (contraction) of 1μM ACh-induced relaxation in preparations with an intact endothelium which had been pre-contracted with PhE. The magnitude of the contraction due to reversal of relaxation (contraction) by BAY K 8644 was greater than would be expected in the absence of ACh-induced relaxation. However, the magnitude of the contraction

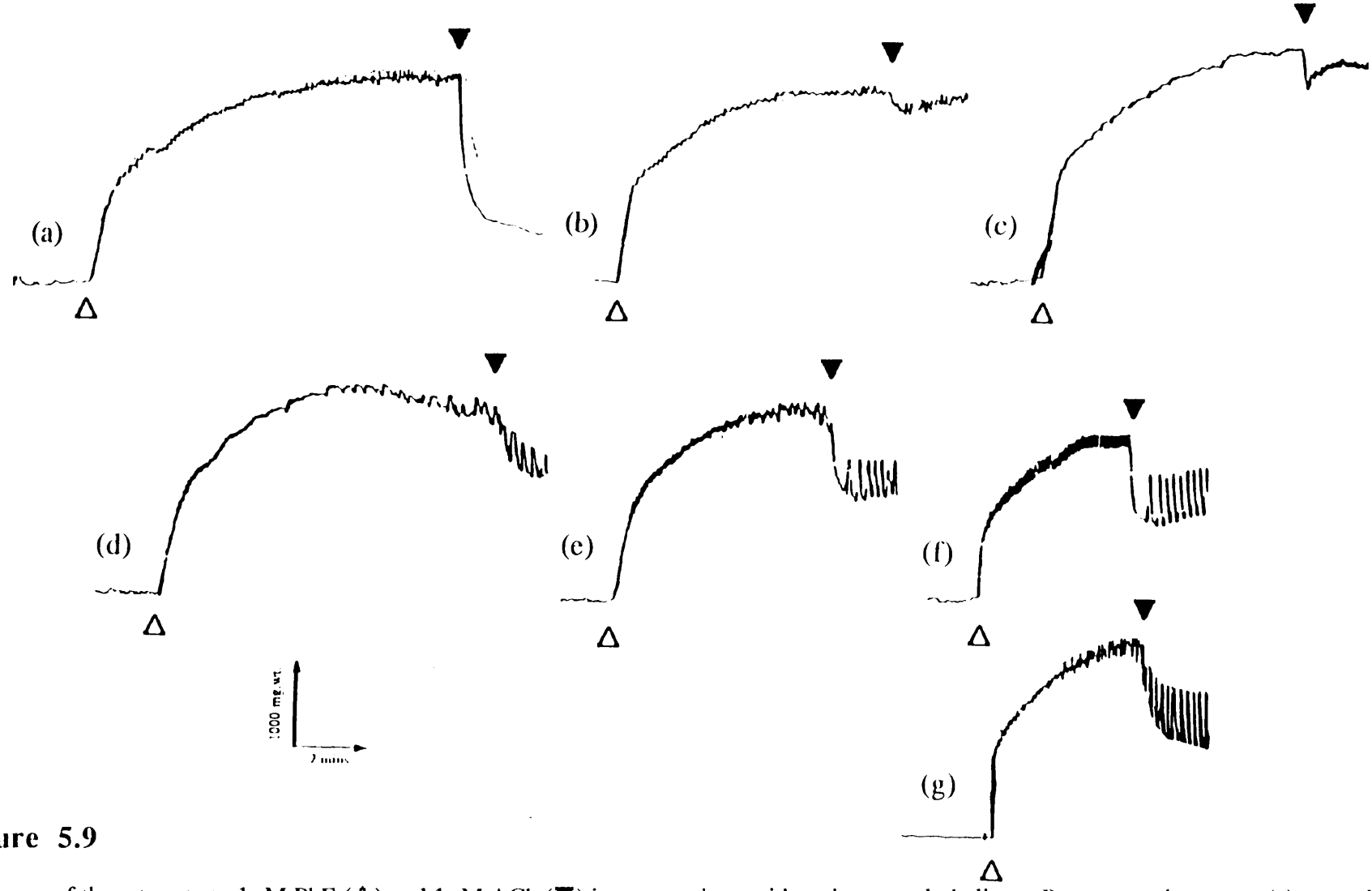

Figure 5.9

Response of the rat aorta to 1μM PhE (Δ) and 1μM ACh (▼) in preparations with an intact endothelium. Responses shown are (a) control and (b), (c), (d), (e) and (f) responses after incubation with 30μM palmitoyl carnitine. The preparation was washed between repeated administration of PhE and ACh with palmitoyl carnitine-free PSS after the first exposure to PC. Response (g) is the response of the concomitant time control.

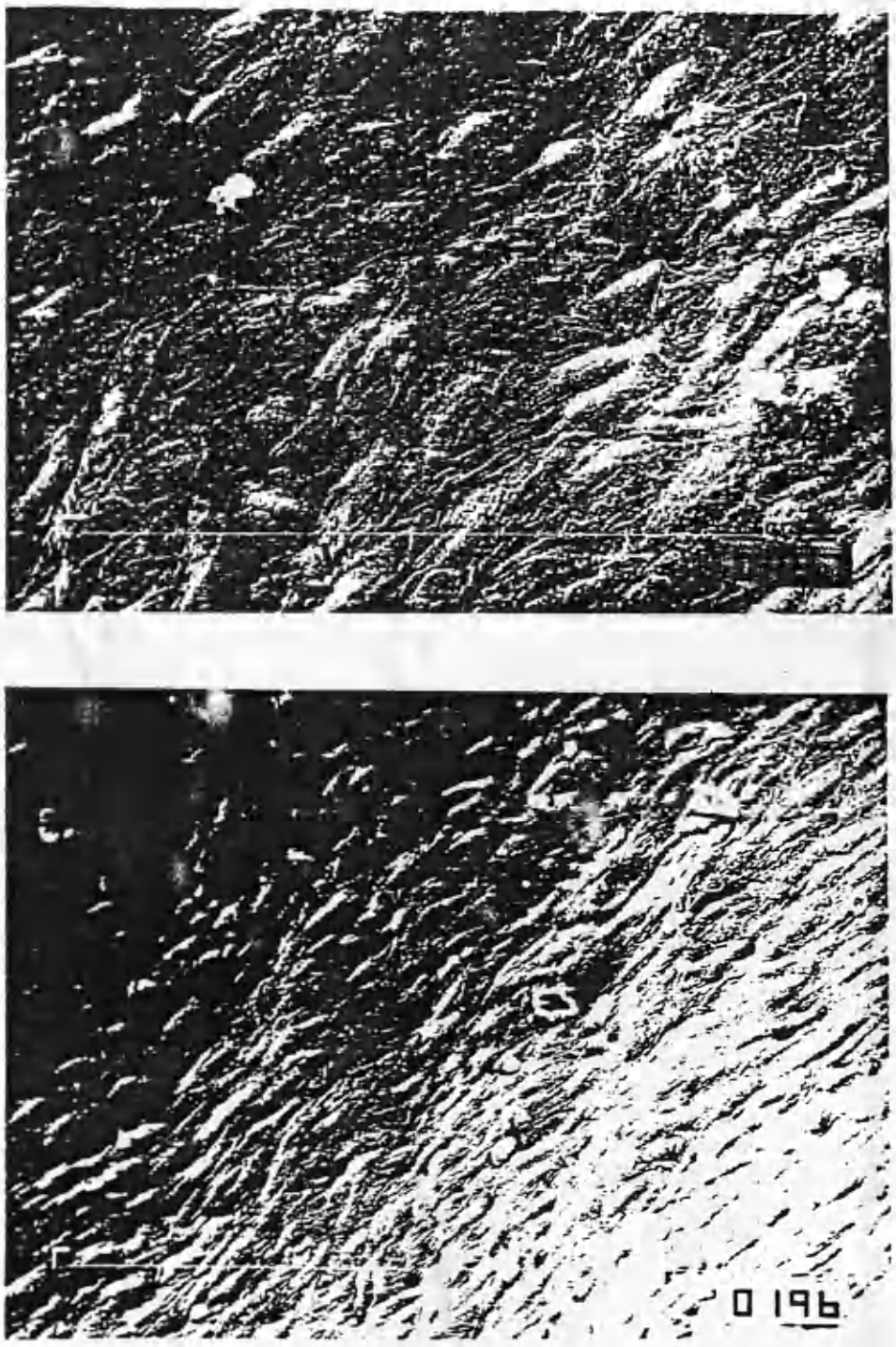

Figure 5.10

Scanning electron micrograph of rat aortic ring with an intact endothelium after incubation for 20 minutes with 100μM palmitoyl carnitine. Upper and lower panels show the intimal surface of the aortic ring at magnifications of x500 and x200 respectively.

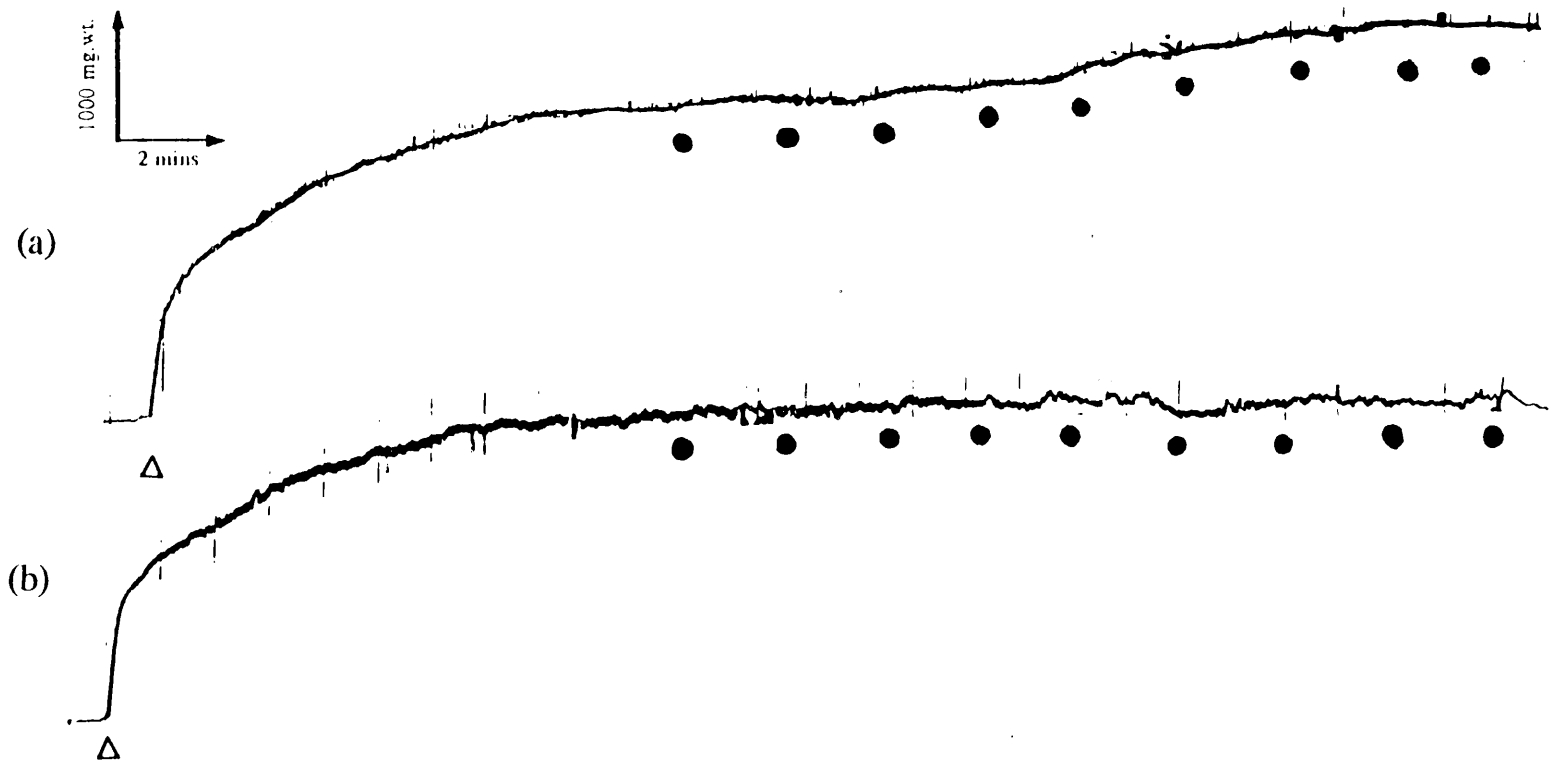

Figure 5.11

The effect of cumulative addition of (a) BAY K 8644 and (b) palmitoyl-carnitine on endothelium-intact rat aortic rings pre-contracted with 1μM PhE (Δ). BAY K 8644 (0.001-10μM) and Palmitoyl-carnitine (0.01-100μM) are cumulatively added in 0.5 $\log_{10}$ molar increments indicated by the closed circles (●).

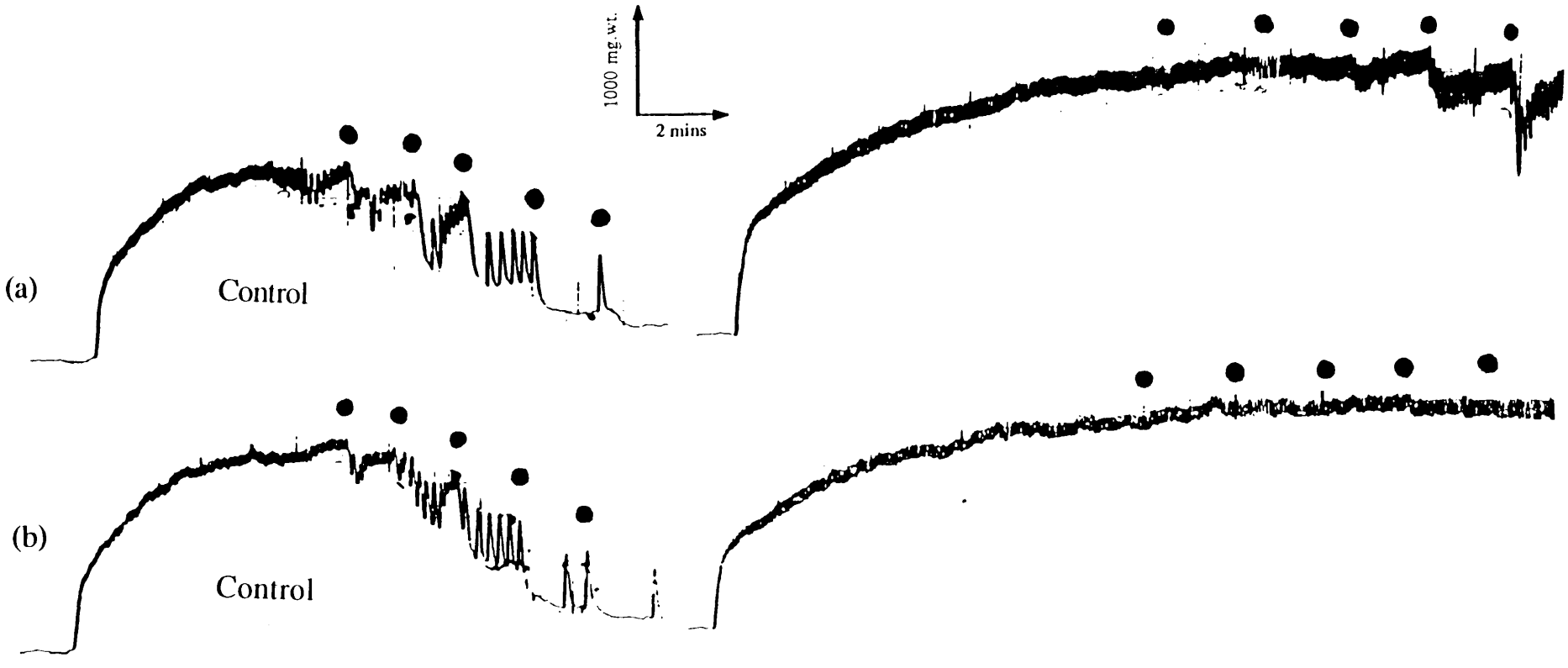

Figure 5.12

Potentiation of the contraction of endothelium-intact rat aortic rings to 1μM PhE (Δ) by pre-incubation with (a) BAY K 8644 (3μM) and (b) palmitoyl-carnitine (30μM). Also shown is the inhibition of ACh-induced relaxation (added cumulatively (0.01-1μM) added in 0.5 $\log_{10}$ molar increments indicated by the closed circles (●)) by both palmitoyl-carnitine and BAY K 8644.

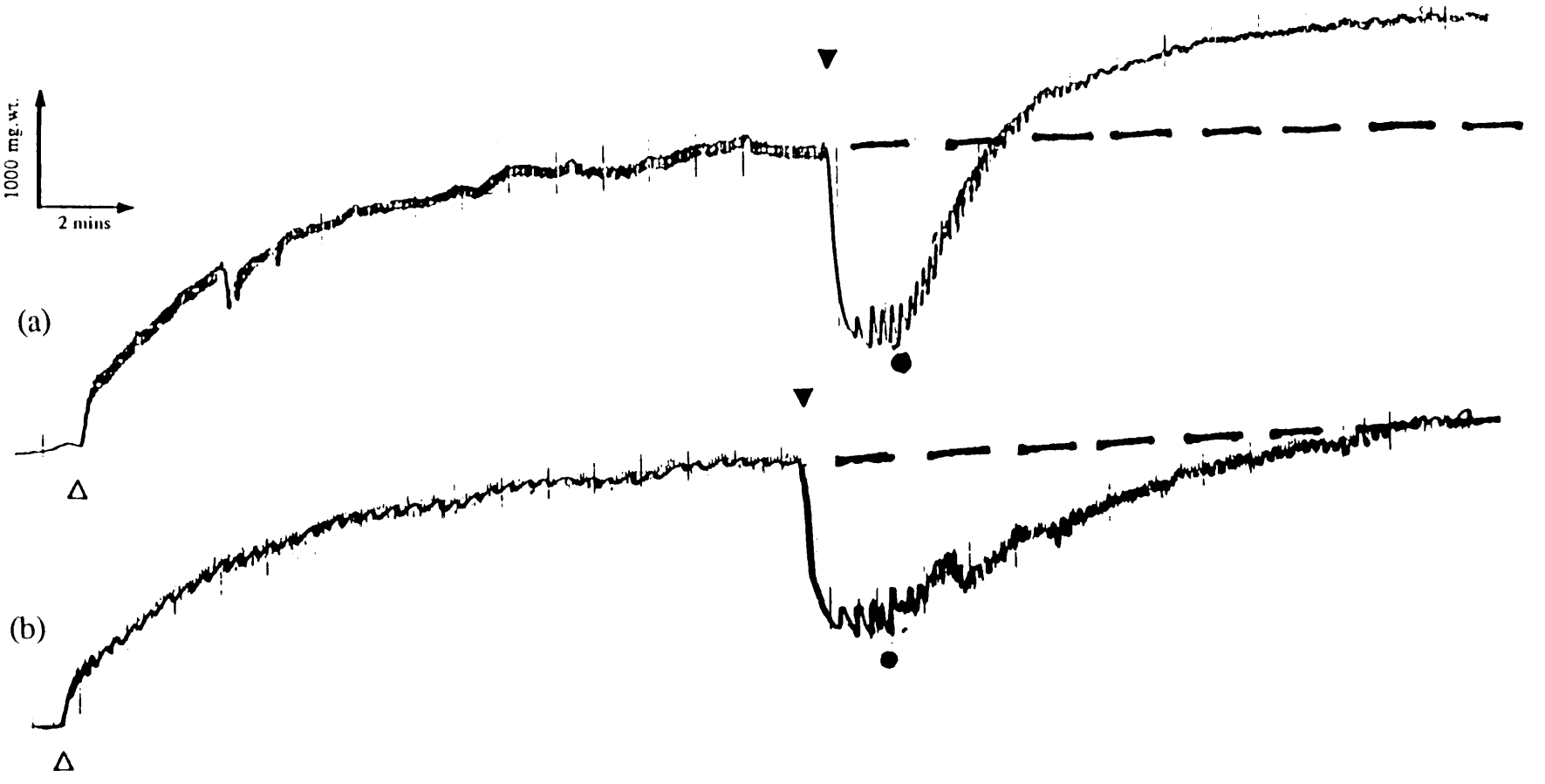

Figure 5.13

Reversal of the relaxation of to 1μM ACh (▼) in rat aortic rings pre-contracted with 1μM PhE-induced tone (Δ) by addition of (a) BAY K 8644 and (b) palmitoyl-carnitine (●). Dotted line represents the degree of PhE-induced tone expected before addition of ACh and palmitoyl-carnitine or BAY K 8644.

resulting from the reversal of relaxation (contraction) by palmitoyl carnitine was comparable to that which would be expected in the absence of ACh-induced relaxation (Figure 5.13). Similarly, BAY K 8644 (3μM) caused reversal (contraction) of 0.1μM SNP-induced relaxation in preparations with an intact endothelium which had been pre-contracted with PhE: this contraction, as seen with reversal of ACh-induced relaxations was greater than would be expected in the absence of SNP-induced relaxation. In contrast palmitoyl carnitine (30μM) had no effect on relaxations induced by 0.1μM SNP (Figure 5.14).

Bioassay of EDRF

In superfused recipient strips of rat aorta with a disrupted endothelium PhE produced a well maintained contraction. Superfusion with 10μM carbachol caused a rapid well maintained relaxation of this PhE-induced tone (35.7 ± 5.3, n=4, percentage reduction of induced tone) only when 'donor' strips with an intact endothelium were placed over them. When the 'donor' segment was removed from the superfusion gutter the carbachol-induced relaxation in the 'recipient' strip was rapidly reversed and the PhE-induced tone returned to a level which would be expected in the absence of carbachol-induced relaxation (Figure 5.15).

The relaxation, by carbachol, in the presence of an intact 'donor' ring, was significantly reduced ($P<0.05$; Student's unpaired *t test* to 11.7 ± 2.9, n=4, percentage reduction of induced tone) if the 'donor' ring was pre-incubated with palmitoyl carnitine. No significant change in response to carbachol was observed if the recipient strip alone was pre-incubated with palmitoyl carnitine (35.3 ± 8.7 percentage reduction of induced tone; Figure 5.16).

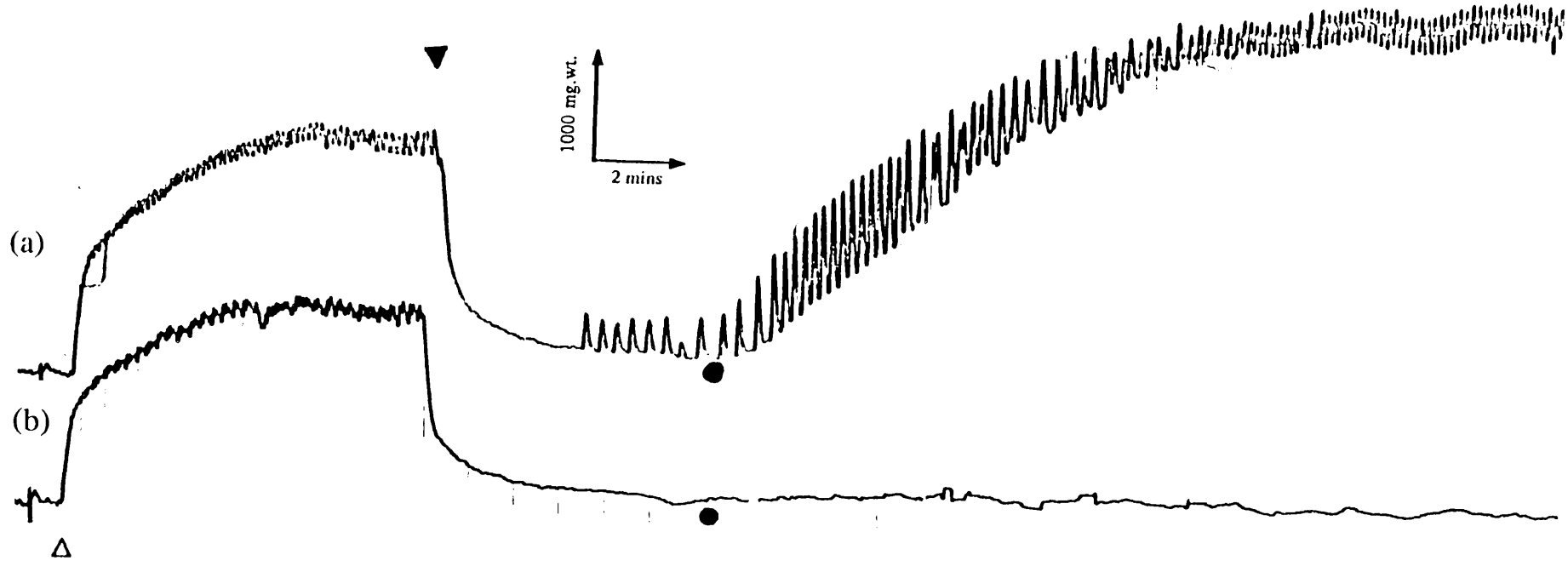

Figure 5.14

Reversal of the relaxation of to 0.1μM SNP (▼) in rat aortic rings pre-contracted with 1μM PhE-induced tone (Δ) by addition of (a) BAY K 8644 and (b) palmitoyl-carnitine. ●

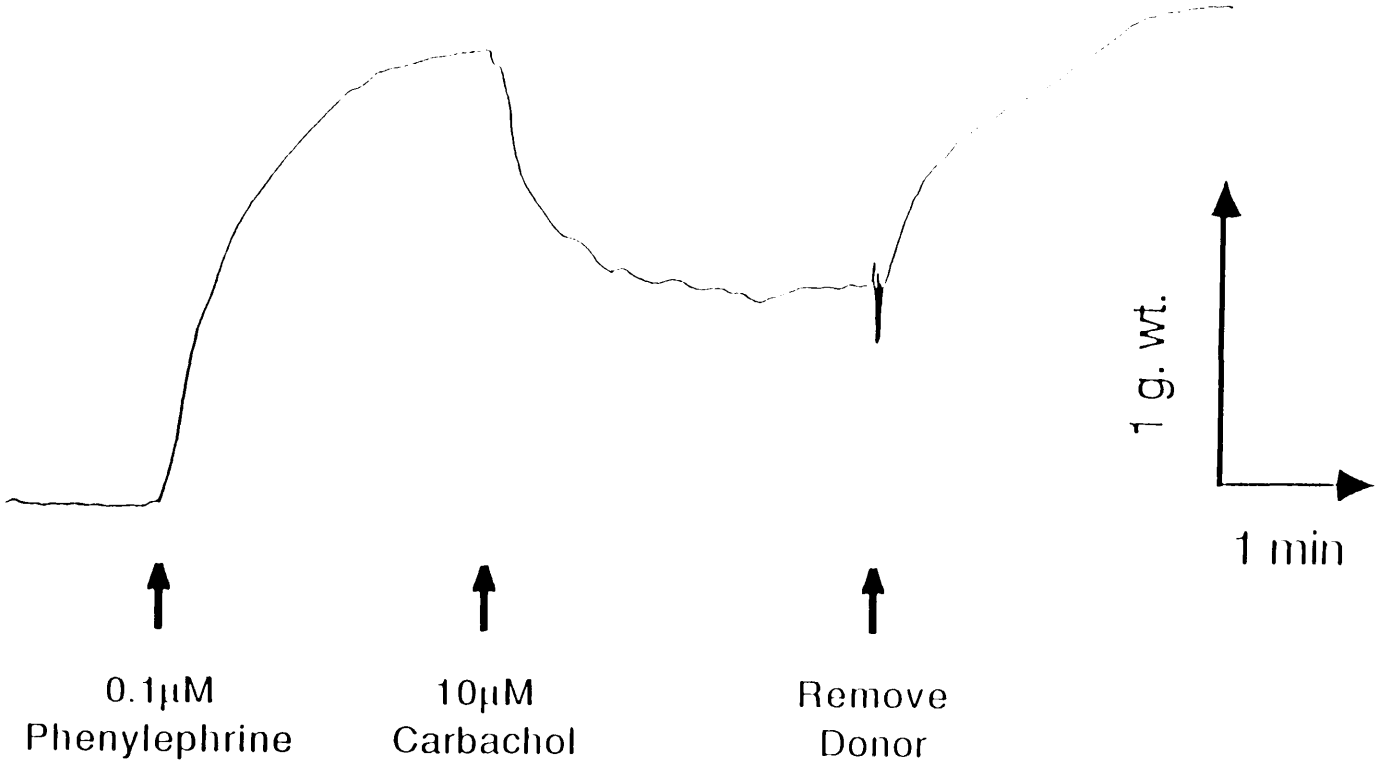

Figure 5.15

Relaxation, in the presence of a 'donor' tissue, by superfusion with 10μM carbachol, of a rat aortic strip with disrupted endothelium. Tone was induced by superfusion with 0.1μM phenylephrine. Removal of the 'donor' tissue rapidly reversed the carbachol-induced relaxation of the 'recipient' tissue.

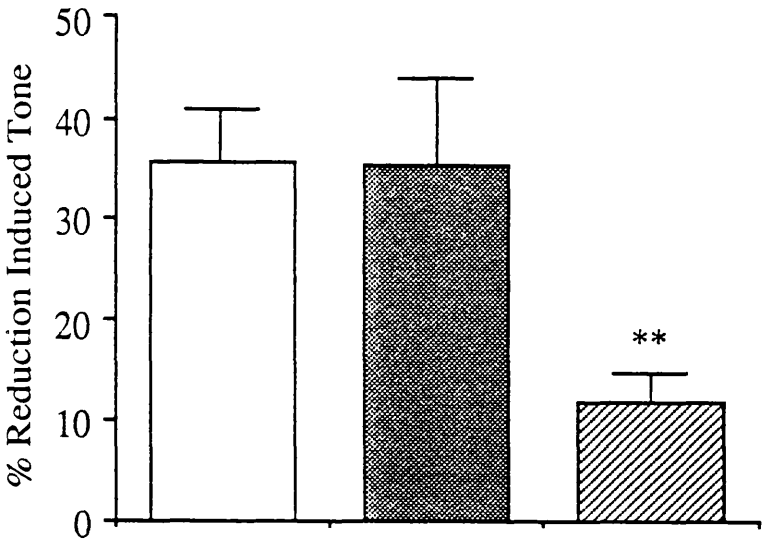

Figure 5.16

Relaxation of PhE-induced tone (expressed as a percentage reduction of the induced tone) in superfused 'recipient' aortic strips with a disrupted endothelium. Relaxations are due to carbachol-induced release of EDRF from a 'donor' preparation placed over the 'recipient'.

Data shown represents mean relaxations ± s.e.m. (n=4) in control preparations (open bar), after incubation with palmitoyl-carnitine (30μM) of 'recipient' strips alone (filled bar) and after incubation with palmitoyl-carnitine of 'donor' strips alone (hatched bar). Statistically significant difference from control preparations using unpaired Student's *t* test; ** $P<0.005$.

Discussion

The contractile effects of the dihydropyridine Ca^{2+} channel activator, BAY K 8644, in partially depolarised rat aorta are reduced in the presence of the endothelium by a tonic release of EDRF (Spedding *et al.*, 1987). However, the contractile effects of the lipid metabolite palmitoyl carnitine (Corr *et al.*, 1984) which also acts as a Ca^{2+} channel activator (Spedding and Mir, 1987) under similar conditions of partial depolarisation were unaffected by the presence of a functional endothelium (Bigaud and Spedding, 1986). This suggested that palmitoyl carnitine, unlike BAY K 8644, depressed the tonic release or action of EDRF such that palmitoyl carnitine-induced contractions were unaffected by the endothelium. Evidence for the blocking action of palmitoyl carnitine on the release or effect of EDRF was provided by the observation that palmitoyl carnitine (10-100μM) but not BAY K 8644 (1μM) attenuated and reversed ACh-induced relaxations of pre-contracted rat aortic rings.

The results of the present study confirm the inhibitory effect of palmitoyl carnitine on ACh-induced relaxations of the rat aorta (Bigaud and Spedding, 1986). A possible interaction of palmitoyl carnitine with the muscarinic receptor mediating endothelium-dependent relaxations has been excluded since relaxations to both ATP and the calcium ionophore A23187 (Pressman, 1976) were also inhibited by palmitoyl carnitine. The inhibitory action of palmitoyl carnitine is exclusive to endothelium-dependent relaxations since endothelium-independent relaxations to sodium nitroprusside were unaffected by a high concentration of palmitoyl carnitine which markedly inhibited the relaxations to ACh. Therefore, palmitoyl carnitine appears to block the release or effects of EDRF and this would explain the lack of effect of removal of the endothelium on palmitoyl carnitine-induced contractions of the rat aorta.

Whilst relaxations to ACh and ATP were inhibited in a concentration-dependent manner the relaxations to A23187 were blocked only by 30μM palmitoyl carnitine. This might have indicated a preferential blockade by palmitoyl carnitine of ACh- and

ATP-induced relaxations over relaxations induced by A23187. A similar preferential effect has been observed for the phospholipase A_2 inhibitor monoalide (Long *et al.*, 1987). Thus, A23187 could release EDRF via a different cellular mechanism or release a different EDRF since there is evidence for species differences in EDRF or its metabolism (Forstermann *et al.*, 1985) and there is now strong evidence for the existence of more than one endothelium-dependent relaxing factor (Vanhoutte, 1987; Komori and Suzuki, 1987; Feletou and Vanhoutte, 1988; Taylor *et al.*, 1988). A simpler, and perhaps more likely, explanation is that A23187, at concentrations greater than 1μM, induced a contraction which masked the stimulated release of EDRF. This hypothesis is supported by the fact that the estimated potency for palmitoyl carnitine inhibition of A23187-induced relaxation was not markedly different from that for ATP and ACh. In addition, palmitoyl carnitine induced a concentration-dependent augmentation of the A23187-induced contractions: an observation consistent with inhibition of the A23187 mediated release of EDRF which in the absence of palmitoyl carnitine would attenuate its contractile effects.

In the study of Bigaud and Spedding, (1986), BAY K 8644 (1μM) had no inhibitory action against ACh-induced relaxations of the rat aorta. In contrast, the results of the present study indicate an inhibitory effect of BAY K 8644 (3μM). This difference in the observed effects of BAY K 8644 is difficult to explain but may be partly due to the different concentrations of BAY K 8644 used. However, this cannot be the full explanation since in two experiments using the lower concentration of BAY K 8644 (data not shown) I have observed marked attenuation of the relaxation to ACh though less than that seen with the higher concentration used in this study (3μM). Thus, both BAY K 8644 and palmitoyl carnitine inhibit endothelium dependent relaxations.

The inhibitory action of BAY K 8644 is perhaps surprising since it has been shown that in dog femoral arteries Ca^{2+} channel activators stimulate the release and augment the action of EDRF by activation of voltage operated Ca^{2+} channels (Rubanyi *et al.*, 1985) though this effect may be species or tissue dependent since in the rat aorta, BAY

K 8644 does not increase the release of EDRF (Spedding *et al.*, 1987). The explanation of this discrepancy is probably that the concentration range for BAY K 8644 used in the present study (0.001-3μM) is such that the facilitation of contractility caused by the activator masks any stimulated release of EDRF (seen also in the study of Rubanyi *et al.*). Since increasing the degree of activation of vascular smooth muscle attenuates subsequent endothelium-dependent relaxations (see Chapter Two) the inhibitory action of BAY K 8644 may not be due to a specific interaction with EDRF but secondary to its smooth muscle activation. This suggestion is supported by the observation that when added cumulatively to a pre-contracted preparation BAY K 8644 did not induce any measurable relaxation in rubbed or unrubbed preparations indicating no release of EDRF. Moreover, the apparent reversal (contraction) of an established ACh-induced relaxation by BAY K 8644 produced a final tone which was greater than would be expected in the absence of ACh-induced relaxation and BAY K 8644. In a similar manner, BAY K 8644 reverses the endothelium-independent relaxation to SNP to a similar extent probably indicating that the inhibition of relaxation by BAY K 8644 is due to activation of the vascular smooth muscle and not to an interaction with the mediators of endothelium-dependent relaxation. This is consistent with the observation that removal of the basal release of EDRF potentiates BAY K 8644-induced contractions (Spedding *et al.*, 1987)

A comparison between the effects of palmitoyl carnitine and BAY K 8644 indicates that their actions differ. Although both are Ca^{2+} channel activators in smooth muscle (Spedding and Mir, 1987) the action of palmitoyl carnitine in contrast to that of BAY K 8644 is not due to augmentation of smooth muscle tone. In contrast to BAY K 8644, concentrations of palmitoyl carnitine which inhibited endothelium-dependent relaxations did not potentiate the tone induced by PhE whether added before or after establishment of the PhE-induced contraction. In addition, in the presence of an established ACh-induced relaxation, palmitoyl carnitine caused a contraction which could be accounted for wholly by reversal of the relaxation to ACh. Moreover, in

contrast to BAY K 8644, palmitoyl carnitine had no effect on an established endothelium-independent relaxation to SNP. Thus palmitoyl carnitine inhibits the relaxations of the rat aorta only by agents whose action is mediated by release of endothelium dependent relaxing factor and this effect is not simply due to augmentation of smooth muscle tone.

The presence of extracellular calcium is a pre-requisite for the production of endothelium-dependent relaxations since exclusion of Ca^{2+}_o attenuates endothelium-dependent relaxations (Singer and Peach, 1982; Long and Stone, 1985; Winquist *et al.*, 1985) although the precise involvement of Ca^{2+} channels is still under question since some studies have reported a lack of effect of calcium antagonists (Winquist *et al.*, 1985; Jayakody *et al.*, 1987). Thus activation of Ca^{2+} channels of the endothelium by palmitoyl carnitine might be expected to induce the release of EDRF in a manner similar to that seen by Rubanyi *et al.*, (1985). Indeed, Criddle *et al.*, (1987) have reported endothelium-dependent relaxations of pre-contracted rat aortic rings by palmitoyl carnitine. If this activation resulted in a maximal release of EDRF then this would obviously inhibit any further stimulated (or basal) release of EDRF. This is unlikely to be the mechanism of the inhibitory action of palmitoyl carnitine in the present study since addition of palmitoyl carnitine to a pre-contracted ring preparation did not produce relaxations in the presence or absence of functional endothelium. Moreover, palmitoyl carnitine reversed relaxations induced by a near maximal concentration of ACh, an effect which would not be seen if palmitoyl carnitine also stimulated the release of EDRF at the concentration used.

The discrepancy in the effects of palmitoyl carnitine on pre-contracted tissues between this study and that of Criddle *et al.*, (1987) is difficult to explain since the same preparation was used and the concentration ranges overlap. The influence of the basal release of EDRF in the rat aorta has been shown to be particularly marked (Collins *et al.*, 1986). One explanation of the effects reported by Criddle *et al.*, (1987) could be that palmitoyl carnitine at the concentrations used did not induce the release of

EDRF but rather potentiated the effect of EDRF released under basal conditions. The tonic and stimulated release of EDRF activates guanylate cyclase elevating cytosolic cGMP levels (Rapaport and Murad, 1983; Griffiths *et al.*, 1985). This leads to lowering of the cytosolic concentration of Ca^{2+} (Kobayashi *et al.*, 1985) due to stimulation of the plasmalemmal Ca^{2+}-extrusion ATPase (Popescu *et al.*, 1985) and sequestration of Ca^{2+} into the sarcoplasmic reticulum (Twort and van Breemen, 1988). Low concentrations of palmitoyl carnitine can stimulate Ca^{2+} ATPase and sequestration of Ca^{2+} into the sarcoplasmic reticulum (Adams *et al.*, 1979) and it is possible that this might potentiate the effects of tonicly released EDRF and may produce an apparently endothelium-dependent relaxation. This effect, therefore might explain the results obtained with low concentrations of palmitoyl carnitine by Criddle *et al.*, (1987).

Using a simple bioassay technique we have demonstrated that carbachol can induce relaxations of an endothelium denuded 'recipient' strip of aortic smooth muscle only when an endothelium intact 'donor' strip is placed on the recipient such that the intima of the donor is in contact with the smooth muscle of the 'recipient'. This clearly demonstrates the diffusion of EDRF from 'donor' to 'recipient' (Furchgott & Zawadzki, 1980). The relaxation of endothelium denuded 'recipient' strips by EDRF released from intact 'donor' rings was inhibited if the 'donor' strip was pre-incubated with palmitoyl carnitine but not if the assay strip itself was pre-incubated with palmitoyl carnitine. This suggests that the inhibitory effect is not due to an inhibitory action of palmitoyl carnitine at the smooth muscle and is unlikely to be due to 'deactivation' of EDRF. Thus the inhibitory action of palmitoyl carnitine is likely to be on the endothelium to inhibit release or synthesis of EDRF, and not on the vascular smooth muscle itself.

The present study provides evidence for the site of action of palmitoyl carnitine for inhibition of stimulated and basal release of EDRF. The precise mechanism of this effect has not been found though an action at Ca^{2+} channels to facilitate contraction and hence attenuate relaxation has been excluded. In addition to being a possible

endogenous Ca^{2+} channel activator (Spedding and Mir, 1987), palmitoyl carnitine also acts as a detergent at relatively high concentrations (Idell-Wenger *et al.*, 1978; Neely and Feuvray, 1981; Spedding and Mir, 1987) and this could have accounted for the loss of endothelium-dependent relaxation due to removal or irreversible damage of the endothelial cell layer. This is probably not the case however, since histological examination of ring preparations incubated in 30μM palmitoyl carnitine showed the presence of an intact endothelial cell layer. Moreover, in preparations where the relaxant response to ACh had been abolished by pre-treatment with 30μM palmitoyl carnitine, repeated washes in palmitoyl carnitine-free PSS resulted in almost complete restoration of the relaxant response to ACh. Thus, whilst the effect of palmitoyl carnitine was somewhat difficult to 'wash out', the eventual restoration of most of the relaxant response to ACh indicates that palmitoyl carnitine does not irreversibly damage the endothelium suggesting that the detergent properties of palmitoyl carnitine are not the major factor in its inhibitory action on EDRF.

Hypoxia, and incubation with low concentrations of palmitoyl carnitine (1μM) for 10 minutes, leads to an increase in the number of α_1-adrenoceptors in dog myocytes (Heathers *et al.*, 1987). An increase in the number of α_1-adrenoceptors would presumably lead to a greater degree of activation by a submaximal concentration of PhE and therefore would cause attenuation of the effects of agonist-induced release of EDRF (see Chapter Two). In a similar manner to the Ca^{2+}-channel activating action of palmitoyl carnitine, this effect cannot explain the inhibition of endothelium-dependent relaxations since there is no palmitoyl carnitine-induced increase in the contraction to PhE.

In addition to the actions described above, palmitoyl carnitine can also cause inhibition of Na^+/K^+-ATPase (McMillan-Wood *et al.*, 1977; Lamers and Hulsmann, 1977; Adams *et al.* 1979). Recent studies have indicated that some agents that produce endothelium-dependent relaxations also generate endothelium-dependent hyperpolarisations (Bolton and Clapp, 1986; Beny *et al.*, 1986). This transient

hyperpolarisation is independent of the stimulation of guanylate cyclase by EDRF and appears to be due to activation of smooth muscle Na^+/K^+-ATPase or K^+ channels (Feletou and Vanhoutte, 1988; Taylor et al., 1988) by a substance other than nitric oxide (Vanhoutte, 1987). One substance which has been shown to activate K^+ channels has been termed endothelium-dependent hyperpolarising factor (EDHF; Taylor and Weston, 1988). The significance of EDHF(s) is dependent upon the species and preparation used (Taylor and Weston, 1988) but the action of palmitoyl carnitine on Na^+/K^+-ATPase may play an important role in its inhibition of endothelium-dependent relaxations. However, if this inhibition of Na^+/K^+-ATPase is to account for the inhibition of endothelium-dependent relaxations by palmitoyl carnitine then the action must be at the endothelial Na^+/K^+-ATPase since there is no inhibitory action of palmitoyl carnitine at the smooth muscle. This is consistent with the observation by Feletou and Vanhoutte (1988) that the relaxations and membrane hyperpolarisation of 'recipient' dog coronary artery by ACh-induced release of EDRF(s) from 'donor' dog femoral artery was abolished by ouabain. Although these workers suggested that this effect may be due to a pharmacological action of ouabain not involving Na^+/K^+-ATPase this may give an indication of the mechanism of action of palmitoyl carnitine in inhibiting endothelium-dependent relaxations of the rat aorta.

To conclude: we have provided evidence for the inhibition of endothelium-dependent relaxation of the rat aorta by a lipid metabolite that accumulates under ischaemic conditions: palmitoyl carnitine. The mechanism of action of the inhibition is unknown but it appears not to be associated with the Ca^{2+} channel activating action of palmitoyl carnitine since the inhibition is dissimilar to that seen with the dihydropyridine Ca^{2+} channel activator, BAY K 8644. The site of action of palmitoyl carnitine appears to be at the endothelium but is unlikely to be due to a detergent action of palmitoyl carnitine on the endothelial cell layer. Further work is required to determine (1) the precise mechanism of action of palmitoyl carnitine in inhibiting endothelium-dependent relaxation, (2) any possible involvement of palmitoyl carnitine

in anoxic augmentation and hypoxic contraction and (3) whether the build up of palmitoyl carnitine during ischaemia and possible blockade of release of EDRF occurs and is of pathological significance.

General Discussion

Using the descending thoracic aorta of the rat, I have examined several aspects of the influence of the vascular endothelium on the effect of stimulation of α-adrenoceptors, 5-HT receptors and muscarinic receptors in a vascular smooth muscle preparation. The basal release of EDRF may modulate agonist activity by affecting Ca^{2+} fluxes of the smooth muscle cells. In order to rule out the possibility that any differential effect of removal of the endothelium on the responses to α-adrenoceptor and 5-HT receptor activation may have been due to differences in their Ca^{2+} dependency I first examined the responses to agonists at these receptors. This part of the study revealed an interesting phenomenon in that whilst contractions induced by 'PhE/Ca^{2+}-re-addition' were comparable with PhE-induced contractions in 'fixed' $[Ca^{2+}]_o$, '5-HT/Ca^{2+}-re-addition' produced unexpectedly smaller contractions than those seen with addition of 5-HT in 'fixed' $[Ca^{2+}]_o$. No definitive explanation of this was discovered but differential activation of VOCs by the agonists was ruled out. However, a possible differential effect of the agonists on phosphotidyl-inositide hydrolysis was indicated by the differential effect of LiCl pre-treatment on contractions to PhE and 5-HT. Despite this marked difference between the Ca^{2+} re-addition response in the presence of 5-HT and PhE, removal of the endothelium (and therefore the basal release of EDRF) potentiated the responses to both agonists to a similar degree suggesting that, in this case, the depressant effect of the endothelium on the agonist-induced contractions was not mediated by an effect on Ca^{2+} fluxes.

Since the first reports concerning EDRF there has been a great deal of work carried out to characterise the nature and action of the factor(s). It is noticeable that the reports of the effects of both the basal and stimulated release of EDRF can vary markedly between laboratories and/or workers. In addition to the usual factors affecting experimental results (PSS composition, temperature etc) I decided (as a joint study with Alison Templeton) to investigate the possibility that the tissue resting tension and degree of agonist-induced tone used to examine the effects of EDRF might contribute to the variation seen in the published data. The results of the study show that when the

initial resting tension of paired rubbed/unrubbed rat aortic rings is the same then the degree of stretch in both tissues is comparable. Thus, there can be no contribution from different degrees of stretch of the muscle to the differences seen between the tissues. However, the optimum degree of stretch for demonstration of contraction of the tissue *per se* was, in most cases, different from the optimum for demonstration of the basal or stimulated release of EDRF. Therefore, suspending a smooth muscle preparation at the optimum tension for contraction (as is often the case in published studies) might mask the effect of EDRF which is being examined leading to errors in interpretation of experimental data. This is important in comparing preparations of different vessels or from vessels in different pathological states where the relationship between stretch and the optima for different responses may be different.

In addition to the influence of resting tension, the degree of agonist induced tone was found to affect the sensitivity to endothelium-dependent relaxations such that increases in tone produced decreases in the sensitivity of the preparation. This decrease in sensitivity to endothelium-dependent relaxations did however reach a point beyond which further increases in tone had no further effect on the tissue sensitivity. Thus the conditions under which the effect of basal and stimulated release of EDRF are examined can markedly alter the observed effects and this must be taken into consideration when designing experiments and interpreting experimental results. For the remainder of the study, standard conditions of resting tension and agonist-induced tone (when examining stimulated release of EDRF) were used.

The potentiation of agonist-induced contractions (in particular those mediated by α-adrenoceptors) by removal of the endothelium has been characterised by a number of researchers. The apparently greater degree of potentiation of responses to α_2-adrenoceptor agonists compared to those of α_1-adrenoceptor agonists was first attributed to removal of a concomitant α_2-adrenoceptor mediated stimulation of EDRF release which directly opposed the contractile effects of the agonists. The effect was later shown to be mainly due to the low efficacy of the 'α_2-adrenoceptor' agonists at

α_1-adrenocepttors through which they cause contraction (the basal release of EDRF having a greater depressant effect on the contractions to 'partial' rather than 'full' agonists). Since the earlier work in the present study indicated that, in addition to the depression of α-adrenoceptor mediated contractions, responses to 5-HT in the rat aorta were also depressed I decided to examine if 5- HT receptor agonists of lower efficacy (i.e. N-MeT, 5-MeT and 5-CT) would be depressed to a greater degree than the 'full' agonist (5-HT). The study indicated that this was indeed the case. Despite the fact that in a number of preparations 5-HT and its analogues have been shown to induce endothelium-dependent relaxations, in the rat aorta no evidence for such an effect could be obtained. Thus in this preparation, the potentiation of 5-HT-induced contractions seen on disruption of the endothelium is wholly attributable to removal of a basal and not a stimulated release of EDRF.

Having assessed the influence of the basal release of EDRF on agonist-induced contraction I decided to examine the stimulated release of EDRF. Two aspects of the stimulated release of EDRF were examined. The first aspect was the classification of the muscarinic receptor mediating the endothelium-dependent relaxations of the preparation. There has been some debate as to the precise subtype of muscarinic receptor mediating endothelium-dependent relaxations. Using selective antagonists I have now characterised this receptor as being of the M_2 subtype. Interestingly, although the antagonists studied indicate that the receptor is similar to that found in non vascular smooth muscle preparations such as the guinea-pig ileum and trachea, the receptor would appear to be linked to a different second messenger system. This is indicated by the observation that chronic pre-treatment with LiCl (a treatment attenuating the responses to muscarinic agonists in the ileum) had no effect on the sensitivity to several muscarinic agonists of various efficacies. Further work is needed to characterise the second messenger system of this receptor population since pertussis toxin (a substance which inactivates the atrial type M_2 receptor) has no effect on ACh-induced relaxations in this preparation (R.M. Eglen, personal communication).

The second and final aspect of the stimulated release of EDRF examined was the effect of a lipid metabolite of ischaemia (palmitoyl carnitine) on endothelium-dependent relaxations. This substance may have a modulatory role on the effect of the endothelium in ischaemic conditions. I found that palmitoyl carnitine caused a concentration-dependent inhibition of endothelium-dependent relaxations to a number of agonists without affecting endothelium-independent relaxations to sodium nitroprusside. This inhibition did not appear to be related to the Ca^{2+} channel facilitation that occurs with this substance since the inhibition of endothelium-dependent relaxations by BAY K 8644 was qualitatively different. Whilst the site of action of palmitoyl carnitine appears to be at the endothelium the precise mechanism of action was not determined. However, since the extent to which palmitoyl carnitine plays a role in the damage found in ischaemic tissues and the role of EDRF under such conditions is largely unknown, further work in this area could lead to a therapeutically useful target for drug development.

In conclusion, using a readily available preparation I have carried out (in part in collaboration with colleagues) an extensive study into the effects of the endothelium on responsiveness of vascular smooth muscle. I have characterised the Ca^{2+} requirement for activation of the preparation by α-adrenoceptor and 5-HT receptor activation. The study has illustrated that varying the initial experimental conditions can, in some cases, influence the interpretation of the experimental data. I have confirmed and expanded observations made by others on the depression of contractile responses by the basal release of EDRF and have made a definitive classification of the muscarinic receptor subtype mediating endothelium-dependent relaxations. Finally, I have examined the effect of a possible mediator of ischaemic damage on the production and/or release of EDRF.

Whilst the experimental work which I have presented in this thesis has answered a number of questions, the nature of pharmacological research is such that more questions have been asked. In the future I hope to be able to answer some of these questions.

References

AGER, A. & MARTIN, W., (1983). Loss of receptor mediated ^{86}Rb efflux from pig aortic endothelial cells in culture. *Br. J. Pharmacol.*, **80**, 5-6

ALLAN, G., BROOK, C.D., CAMBRIDGE, D., HLADKIWISKYJ, K, (1983). Enhanced responsiveness of vascular smooth muscle to vasoconstrictor agents after removal of endothelial cells. *Br. J. Pharmacol.*, **79**, 334P

ALLEN, G.S. & BANGHART, S.B., (1979). Cerebral arteriospasm, part 9. In vitro effects of nifedipine on serotonin, phenylephrine, and potassium-induced contractions of canine basilar and femoral arteries. *Neurosurgery*, **4**, 37-42

ALLISON, J.H. & STEWART, M.A., (1971). Reduced brain inositol in lithium-treated rat cerebral cortex. *Nature*, **233**, 267-268

ALTURA, B.M. & CHAND, N., (1981). Bradykinin-induced relaxation of renal and pulmonary arteries is dependent upon intact endothelial cells. *Br. J. Pharmacol.*, **74**, 10-11

ANGUS, J.A., COCKS, T.M., & SATOH, K., (1986). α_2-Adrenoceptor and endothelium-dependent relaxation in canine coronary large arteries. *Br. J. Pharmacol.*, **88**, 767-777

APPERLEY, E., HUMPHREY, P.P.A. & LEVY, G.P., (1976). Receptors for 5-hydroxytryptamine and noradrenaline in rabbit isolated ear artery and aorta. *Br. J. Pharmacol.*, **58**, 211-221

ARUNLAKSHANA, O & SCHILD, H.O., (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48-58

AVISSAR, S., SCHREIBERT, G., DANON, A. & BELMAKER, R.H., (1988). Lithium inhibits adrenergic and cholinergic increases in GTP binding in rat cortex. *Nature*, **331**, 440-442

BARLOW R.B., & SHEPHERD M.K., (1985). A search for selective antagonists at M_2 muscarinic receptors. *Br. J. Pharmacol.*, **85**, 427-435

BERRIDGE, M. J., (1984). Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.*, **212**, 849-858

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

BERRIDGE, M.J., DOWNES, C.P. & HANLEY, M.R., (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary gland. *Biochem. J.*, **206**, 587-595

BIGAUD, M., SCHOEFFTER, P., STOCLET, J.C. & MILLER, R.C., (1984). Dissociation between endothelium mediated increases in tissue cGMP levels and modulation of aortic contractile responses. *Naunyn-Schmiedebergs Arch. Pharmac.*, **328**, 221-223

BIGAUD, M. & SPEDDING, M., (1986). Inhibition of the effects of endothelial-derived relaxant factor (EDRF) in aorta by palmitoyl carnitine. *Br. J. Pharmacol.*, **89**, 540P

BIRDSALL, N.J.M., & HULME, E.C., (1983). Muscarinic receptor subclasses. *TIPS*, **4**, 459-463

BLURTON, P.A., BROADHURST, A.M., CROSS, J.A., ENNIS, C., WOOD, M.D. & WYLLIE, M.G., (1984). Panuramine, a selective inhibitor of uptake of 5-hydroxytryptamine in the brain of the rat. *Neuropharmacology*, **23**, 1049-1052

BOLTON, T.B., (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **3**, 606-718

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

BOYER, J.L., MARTINEZ-CARCAMO, M., MONROY-SANCHEZ, A., JUAREZ-AYALA, J., PASTELIN, G., POSADAS, C. & GARCIA-SAINZ, J.A., (1986). Effect of pertussis toxin on the heart muscarinic-cholinergic receptors and their function. *Life Sciences*, **39**, 603-610

BROWN D.A., (1984). Muscarinic excitation of sympathetic and central neurones. *TIPS, Suppl 5*, 32-34

BROWN, D.A., FORWARD, A. & MARSH, S., (1980). Antagonist discrimination between ganglionic and ileal muscarinic receptors. *Br. J. Pharmacol.*, **71**, 362-364

BROWN, J.H., GOLDSTEIN, D. & MASTERS, S.B., (1985). The putative M_1 receptor does not regulate phosphoinositide hydrolysis: Studies with pirenzepine and McN-A-343 in chick heart and astrocytoma cells. *Mol. Pharmac.*, **27**, 525-531

BULLOCK, G.R., TAYLOR, S.G., & WESTON A.H., (1986). Influence of the vascular endothelium on agonist-induced contractions and relaxations in rat aorta. *Br. J. Pharmacol.*, **89**, 819-830

BUSSE, R., FORSTERMANN, U., MATSUDA, H., & POHL, U., (1984). The role of prostaglandins in the endothelium-mediated vasodilatory response to hypoxia. *Pflugers Archiv*, **401**, 77-83

BUSSE, R., TROGISCH, G & BASSENGE, E., (1985). The role of endothelium in the control of vascular tone. *Basic Res. in Cardiol.*, **80**, 475- 490.

CALVETTE, J.A., HAYES, R.J., OATES, N.S., SEVER, P.S. & THOM, S., (1984). α_1- and α_2-adrenceptor responses in human isolated arteries. *Br. J. Pharmacol.*, **83**, 364P

CARRIER, G.O. & WHITE, R.E., (1985). Enhancement of α-$_1$ and α-$_2$ adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. *J. Pharmac. Exp. Ther.*, **232 (3)**, 682-687

CHAMLEY-CAMPBELL, J., CAMPBELL, G., (1981). What controls smooth muscle phenotype? *Atherosclerosis*, **40**, 347-357

CHERRY, P.D., FURCHGOTT, R.F., ZAWADSKI, J.V. & JOTHIANANDAN, D., (1982). The role of endothelial cells in the relaxation of isolated arteries by bradykinin. *Proc. Natl. Acad. Sci.* USA, *72*, 2106-2110

CHIU, A.T., BOZARTH, J.M. & TIMMERMANS, P.B.M.W.M., (1987). Relationship between phosphatidylinositol turnover and Ca^{++} mobilization induced by α-$_1$ adrenoceptor stimulation in the rat aorta. *J. Pharmac. Exp. Ther.*, **240 (1)**, 123-127

CHIU, A.T., MCCALL, D.E., THOOLEN, M.J.M. & TIMMERMANS, P.B.M.W.M., (1986). Ca^{++} utilisation in the constriction of rat aorta to full and partial α_1-adrenoceptor agonists. *J. Exp. Pharmac. Ther.*, **238 (1)**, 224-231

CHOO, L.K., MALTA, E. & MITCHELSON, F., (1986). The affinity of some selective muscarinic receptor antagonists for the muscarinic receptor mediating endothelial-dependent relaxation in the rabbit and rat thoracic aorta. J. *Pharm. Pharmacol.*, **38**, 843-845

CHRISTIE, M.I., & LEWIS, M.J., (1987). The pig coronary artery is more responsive to endothelium-derived relaxing factor (EDRF) than the rabbit aorta. *Br. J. Pharmacol.*, **91**, 318P

CLANCY, B.M. & MAAYANI, S. (1985). 5-Hydroxytryptamine receptor in isolated rabbit aorta: characterisation with tryptamine analogs. *J. Pharmac. Exp. Ther.*, **233 (3)**, 761-769

COCKS, T.M., & ANGUS, J.A., (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627-630

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

COHEN, M.L., FULLER, R.W. & WILEY, S.K., (1981). Evidence for $5HT_2$ receptors mediating contraction in vascular smooth muscle. *J. Pharmac. Exp. Ther.*, **218**, 421-425

COHEN, M.L., MASON, N., WILEY, S.K. & FULLER, R.W., (1983a). Further evidence that vascular serotonin receptors are of the $5HT_2$ type. *Biochem. Pharmacol.*, **32**, 567-570

COHEN, R.A., SHEPHERD, J.T. & VANHOUTTE, P.M., (1983b). 5-Hydroxy-tryptamine can mediate endothelium-dependent relaxation of coronary arteries. *Am. J. Physiol.*, **247**, H1077-H1080

COHEN, R.A., SHEPHERD, J.T. & VANHOUTTE, P.M., (1983c). Inhibitory role of the endothelium the response of isolated coronary arteries to platelets. *Science*, **221**, 273-274

COHEN, R.A., SHEPHERD, J.T. & VANHOUTTE, P.M., (1984). Endothelium and asymmetrical responses of the coronary arterial wall. *Am. J. Physiol.*, **247**, 403-408

COLEMAN, R.A., HUMPHREY, P.P.A., KENNEDY, I., LEVY, G.P. & LUMLEY, P. (1981). Comparison of the actions of U46619, a prostaglandin H_2-analogue, with those of prostaglandin H_2 and thromboxane A_2 on some isolated smooth muscle preparations. *Br. J. Pharmacol.*, **73**, 773-778

COLLINS, P., CHAPPELL, S.P., GRIFFITH, T.M., LEWIS, M.J., & HENDERSON, A.H., (1986). Differences in basal endothelium-derived relaxing factor activity in different artery types. *J. Cardiovasc. Pharmacol.*, **8**, 1158-1162

CORR, P.B., GROSS, R.W. & SOBEL, B.E., (1984). Amphipathic metabolites and membrane dysfunction in ischemic myocardium. *Circ. Res.*, **55 (2)**, 135-154

CORY, R.N., OSMAN, R. & MAAYANI, S. (1986). Kinetic definition of agonist efficacy at a 5-hydroxytryptamine ($5\text{-}HT_2$) receptor in the isolated rabbit aorta. *J. Pharmac. Exp. Ther.*, **233 (3)**, 761-769

CRIDDLE, D.N., HIGGINS, A.J. & WOODWARD, B., (1987). Effects of palmitoyl carnitine on the isolated rat heart and thoracic aorta. *J. Physiol.*,

CRISCIONE, L., MÜLLER, K. & PRESCOTT, M.F., (1984). Endothelial cell loss enhances the pressor response in resistance vessels. J. *Hypertension*, **2 (suppl 3)**, 441-444

CRYER, A., (ed.), (1983). Biochemical interactions at the endothelium. Elsevier, Amsterdam New York Oxford.

DALY, R.N. & HIEBLE, J.P., (1987). Neuropeptide Y modulates adrenergic neurotransmission by an endothelium-dependent mechanism. *Eur. J. Pharmac.*, **138**, 445-446

DANIEL, E.E., GROVER, A.K. & KWAN, C.Y., (1983). Calcium. In : Biochemistry of smooth muscle. Vol III. N.L. Stephens (ed.). CRC Press, 1-89

DE MEY, J.G., CLAEYS, M. & VANHOUTTE, P.M., (1982). Endothelium-dependent inhibitory effects of acetylcholine, adenosine triphosphate, thrombin and arachidonic acid in the canine femoral artery. *J. Pharmac. Exp. Ther.*, **222**, 166-173

DE MEY, J.G. & VANHOUTTE, P.M., (1982). Anoxia and endothelium-dependent reactivity of the canine femoral artery. *J. Physiol.*, **335**, 65-74

DE MEY, J.G. & VANHOUTTE, P.M., (1983). Heterogeneous behaviour of the canine arterial and venous wall. *Circ. Res.*, **51**, 439-477

DE MEY, J.G. & VANHOUTTE, P.M., P.M., (1981a). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J. Physiol.*, **316**, 347-355

DE MEY, J.G. & VANHOUTTE, P.M., (1981b). Role of the intima in the relaxation of the canine femoral artery caused by thrombin. *Arch. Int. Pharmacodyn. Ther.*, **250**, 314

DEFEUDIS, F.V., (1985). New studies with EDRF and hemoglobin - are we approaching the mechanism of cerebral vasospasm. *TIPS*, **April**, 151-152

DETH, R. & CASTEELS, R., (1977). A study of the releasable Ca fraction in smooth muscle of the rabbit aorta. *J. Gen. Physiol.*, **69**, 401-416

DETH, R. & VAN BREEMEN, C., (1977). Agonist induced release of intracellular Ca in the rabbit aorta. *J. Membrane Biol.*, **30**, 363-381

DIGGES, K.G. & SUMMERS, R.J., (1983). Characterisation of postsynaptic α-adrenoceptors in rat aortic strips and portal veins. *Br. J. Pharmacol.*, **79**, 655-665

DOGGRELL, S.A., (1987). Differentilal antagonism of the initial fast and secondary slow contractile responses of the rat isolated aorta to 5-hydroxytryptamine by mianserin and ketanserin. *J. Auton. Pharmac.*, **7**, 157-164

DOWNING, O.A., WILSON, K.A. & WILSON, V.G., (1983). Non-competitive antagonism of the α-adrenoceptor-mediated fast component of contraction of rat aorta, by doxazosin and prazosin. *Br. J. Pharmacol.*, **80**, 315-322

DOYLE, V.M., CREBA, J.A., RUEGG, U.T. & HOYER, D., (1986). Serotonin increases the production of inositol phosphates and mobilises calcium via the 5-HT_2 receptor in A_7r_5 smooth muscle cells. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **333**, 98-103

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

DRUMMOND, A.H., (1987). Lithium and inositol lipid-linked signalling mechanisms. *TIPS*, **8**, 129-133

DRUMMOND, A.H., (1988). Lithium affects G-protein receptor coupling. *Nature*, **331**, 388

DRUMMOND, A.H. & RAEBURN, C.A., (1984). The interaction of Lithium with thyrotropin-releasing hormone-stimulated lipid metabolism in GH_3 pituitary tumour cells. *Biochem. J.*, **224**, 129-136

EGLÉME, C., GODFRAIND, T. & MILLER, R.C., (1984a). Enhanced responsiveness of rat isolated aorta to clonidine after removal of the endothelial cells. *Br. J. Pharmacol.*, **81**, 016-018

EGLÉME, C., GODFRAIND, T. & MILLER, R.C., (1984b). Role of endothelium in the rat aorta response to vasoconstrictors. *Arch. Int Pharmacodyn.*, **268**, 116-

EGLEN, R.M., HUFF, M.M., MONTGOMERY, W.W. & WHITING, R.L., (1987). Differential effects of pertussis toxin and lithium on muscarinic responses in the atria and ileum: evidence for receptor heterogeneity. *Br. J. Pharmacol.*, **91**, 6-8

EGLEN, R.M. & WHITING, R.L., (1985). Determination of the muscarinic receptor subtype mediating vasodilatation. *Br. J. Pharmacol.*, **84**, 3-5

EGLEN, R.M. & WHITING, R.L., (1986). Muscarinic receptor subtypes: a critique of the current classification and a proposal for a working nomenclature. *J. Auton. Pharmac.*, **5**, 323-346

ENDOH, M., KITAZAWA, T., YAGI, S., IINO, M. & KAKUTA, Y., (1977). Some properties of of chemically skinned smooth muscle fibres. In: Excitation-contraction coupling in smooth muscle. R. Casteels, T. Godfraind and J. Rüegg (eds.). Amsterdam: Elsevier, 199-209

ENDOH, M., MARUYAMA, M. & IIJIMA, T., (1985). Attenuation of muscarinic cholinergic inhibition by islet-activating protein in the heart. *Am. J. Physiol.*, **249**, H309-H320

FASEHUN, O., JENNETT, S.M., MCGRATH, J.C. & SPEDDING, M.J., (1987). Effects of calcium modulators on the hypoxia-resistant response of the isolated rat portal vein to potassium chloride. *Br. J. Pharmacol.*, **92**, 553P

FELETOU, M. & VANHOUTTE, P.M., (1988). Endothelium-derived hyper-polarisation of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93**, 515-524

FENIUK, W., HUMPHREY, P.P.A., PERREN, M.J. & WATTS, A.D., (1985). A comparison of 5-HT receptors mediating contraction in rabbit aorta and dog saphenous vein: evidence for different receptor types obtained by use of selective agonists and antagonists. *Br. J. Pharmacol.*, **86**, 697-704

FENIUK, W., HUMPHREY, P.P.A. & WATTS, A.D., (1983). 5-Hydroxytryptamine-induced relaxation of isolated mammalian smooth muscle. *Eur. J. Pharmac.*, **96**, 71-78

FENIUK, W., HUMPHREY, P.P.A. & WATTS, A.D., (1984). 5-Carboxamido-tryptamine - a potent agonist at 5-hydroxytryptamine receptors mediating relaxation. *Br. J. Pharmacol.*, **82**, 209P3

FILO, R.S., BOHR, D.F. & RÜEGG, J.C., (1965). Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. *Science*, **147**, 1581-1583

FLOREY, H.W., (1966). The endothelial cell. *Br. Med. J.*, **ii**, 487-489

FORSTER, C. & WHALLEY, E.T., (1982). Analysis of the 5-hydroxytryptamine induced contraction of the human basilar arterial strip compared with the rat aortic strip in vitro. *Naunyn-Schmiedebergs Arch. Pharmac.*, **319**, 12-17

FORSTERMANN, U., MULSCH, A., BOHME, E. & BUSSE, R., (1986). Stimulation of soluble Guanylate Cyclase by an Acetylcholine-induced endothelium-derived factor from rabbit and canine arteries. *Circ. Res.*, **58**, 531-538.

FORSTERMANN, U., TROGISCH, G. & BUSSE, R., (1985). Species-dependent differences in the nature of endothelium-derived vascular relaxing factor. *Eur. J. Pharmacol.*, **106**, 639-643

FORTES, Z. B., GARCIA LEME, J. & SCIVOLETTO, R., (1983). Vascular reactivity in diabetes mellitus: role of the endothelial cell. *Br. J. Pharmacol.*, **79**, 771-7811

FURCHGOTT, R.F., (1955). Pharmacology of vascular smooth muscle. *Pharmac. Rev.*, **7**, 183-265

FURCHGOTT, R.F., (1981). The requirement for endothelial cells in the relaxation of arteries by acetylcholine and some other vasodilators. *TIPS*, 2, 373-376

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

FURCHGOTT, R.F. & BHADRAKOM, S., (1953). Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J. Pharmac. Exp. Ther.*, **108**, 129-143

FURCHGOTT, R.F., & CHERRY, P.D., (1984). The muscarinic receptor of vascular endothelium that subserves vasodilation. *TIPS*, **5**, 45-48

FURCHGOTT R.F., CHERRY P.D., ZAWADZKI J.V. & JOTHIANANDAN D., (1984). Endothelial cells as mediators of vasodilation of arteries. *J. Card. Pharmacol.*, **6**, S336-S343

FURCHGOTT, R.F. & JOTHIANANDAN, D., (1983). Relation of cyclic GMP levels to endothelium-dependent relaxation by acetylcholine in rabbit aorta. *Fed. Proc.*, **29**, 619

FURCHGOTT, R.F., JOTHIANDAN, D., MARTIN, W. & VILLANI, G.M., (1985). Depression of contractile responses by spontaneously released EDRF in rat aorta. *Br. J. Pharmacol.*, **86**, 565P

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

FURUTA, T., (1988). Precontraction-induced contractile response of isolated canine portal vein to alpha$_2$-adrenoceptor agonists. *Naunyn-Schmeidebergs Arch. Pharmac.*, **337**, 525-530

GILANI, S.A.H. & COBBIN, L.B., (1986). The cardio-selectivity of himbacine: a muscarinic receptor antagonist. *Naunyn-Schmeidebergs Arch. Pharmac.*, **332**, 16-20

GILLESPIE M.N., OWASOYO J.O., MCMURTRY, I.F. & O'BRIEN R.F., (1985). Sustained coronary vasoconstriction provoked by peptidergic substance released from endothelial cells in culture. *J. Pharmac. Exp. Ther.*, **236 (2)**, 339-343

GINSBERG, R. & ZERA, P.H., (1984). Endothelial relaxant factor in the human epicardial coronary artery. Circulation, 60 (Suppl II), 122

GODFRAIND, T., (1983). Actions of nifedipine on calcium fluxes and contractions in isolated rat arteries. J. Pharmac. Exp. Ther., 224, 443-450

GODFRAIND, T., EGLÉME, C. & OSACHIE, I.A., (1985). Role of endothelium in the contractile response of rat aorta to α-adrenoceptors agonists. *Clin. Sci.*, **68 (Suppl. 10)**, 65s-71s

GODFRAIND, T. & KABA, A., (1972). The role of calcium in the action of drugs on vascular smooth. *Archs. Int. Pharmacodyn.*, **196**, 35-49 (Supplement)

GODFRAIND, T., MILLER, R.C. & SOCRATES LIMA, J., (1982). Selective α_1- and α_2-adrenoceptor agonist-induced contractions and ^{45}Ca fluxes in the rat isolated aorta. *Br. J. Pharmacol.*, **77**, 597-604

GORDON, A.R., (1978). Contraction of detergent treated smooth muscle. Proc. Natl. Acad. Sci. U.S.A., 75, 3527-3530

GORDON, J.L. & MARTIN, W., (1983). Endothelium-dependent relaxation of the pig aorta: relationship to stimulation of ^{86}Rb efflux from isolated endothelial cells. *Br. J. Pharmacol.*, **79**, 531

GOYAL, R.K. & RATTAN, S., (1978). Neurohumoral and drug receptors for the lower oesophageal sphincter. *Gastroenterology*, **74**, 598-619

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J. & HENDERSON, A.H., (1985). Evidence that cyclic guanosine monophsophate (cGMP) mediates endothelium-dependent relaxation. *Eur. J. Pharmac.*, **112**, 195-202

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON, A.H., (1984). The nature of endothelium-derived relaxant factor. *Nature*, **308 (12)**, 645-647.

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

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

HAMMER, R., BERRIE, C.P., BIRDSALL, N.J.M., BURGER, A.S.V. & HULME, E.C., (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature*, **283**, 90-92

HAMMER, R. & GIACHETTI, A., (1982). Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterisation. *Life Sci.*, **31**, 2991-2998

HAMMER, R., & GIACHETTI, A., (1984). Selective muscarinic receptor antagonists. *TIPS*, **JAN**, 18-20

HARDEN, T.K., TANNER, L.I., MARTIN, M.W., NAKAHATA, N., HUGHES, A.R., HEPLER, J.R., EVANS, T., MASTERS, S.B. & BROWN, J.H., (1986). Characteristics of two biochemical responses to stimulation of muscarinic cholinergic receptors. *TIPS*, (**suppl 7**), 14-18

HARRIS, K.M., KONGSAMUT, S. & MILLER, R.J., (1986). Protein kinase C mediated regulation of calcium channels in PC-12 pheochromocytoma cells. *Biochem. biophys. Res. Comm.*, **134**, 1298-1305

HASHIMOTO, T., HIRATA, M., ITOH, T., KANMURA, Y. & KURIYAMA, H., (1986). Inositol 1,4,5-triphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. *J. Physiol.*, **370**, 605-618

HEATHERS, G.P., YAMADA, K.A., KANTER, E.M. & CORR, P.B., (1987). Long-chain acyle carnitines mediate the hypoxia-induced increase in α_1-adrenergic receptors on adult canine myocytes. *Circ. Res.*, **61**, 735-746

HESS, P., LANSMAN, J.B. & TSIEN, R.W., (1984). Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonist and antagonists. *Nature*, **311**, 538-544

HICKEY, K.A., RUBANYI, G., PAUL, R.J. & HIGHSMITH, R.F., (1985). Characterisation of a coronary vasoconstrictor produced by cultured endothelial cells. *Am. J. Physiol.*, **248**, C550-C556

HIRSCHOWITZ, B.I., HAMMER, R., GIACHETTI, A., KEIRNS, J., & LEVINE, R., (1984). Symposium preface, *TIPS*, **5**, Symposium supplement

HOLTZ, J., FORSTERMANN, U., POHL, U., GEISLER, M. & BASSENGE, E., (1984). Flow-dependent, endothelium-mediated dilation of cardial coronary arteries in conscious dogs: effects of cyclooxygenase inhibition. *Cardiovascular Pharmacology*, 1161-1169

HOLZMANN, S., (1982). Endothelium-dependent relaxation by acetylcholine associated with larger rises in cyclic GMP in coronary arterial strips. *J. Cyclic Nucleotide Res.*, **8**, 409-419

HOUSTON, D.S. & VANHOUTTE, P.M., (1988). Comparison of serotonergic receptor subtypes on the smooth muscle and endothelium of the canine coronary artery. *J. Pharmac. Exp. Ther.*, **244 (1)**, 1-10

HOUSTON, D.S., SHEPHERD, J.T. & VANHOUTTE, P.M., (1985). Adenine nucleotides, serotonin, and endothelium dependent relaxations to platelets. *Am. J. Physiol.*, **248**, H389-H395

HUGHES, E.W. & DOGGRELL, S.A., (1985). The effects of amosalulol, a novel combined α- and ß-adrenoceptor antagonist on the rat isolated right ventricle and aorta. *J. Auton. Pharmacol.*, **5**, 169-176

HYNES, M.R., BANNER, W., YAMAMURA, H.I. & DUCKLES, S.P., (1986). Characterisation of muscarinic receptors of the rabbit ear artery smooth muscle and endothelium. *J. Pharmac. Exp. Ther.*, **238 (1)**, 100-105

IDELL-WENGER, J.A., GROTYOHANN, L.W. & NEELY, J.R., (1978). Coenzyme A and carnitine distribution in normal and ischaemic heart. *J. Biol. Chem.*, **253**, 4310-4318

JAFFE, E.A., NACHMAN, R.L., BECKER, C.G. & MINICK, C.R., (1973). Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *J. Clin. Invest.*, **52**, 2745-2756

KATUSIC, Z.S., SHEPHERD, J.T. & VANHOUTTE, P.M., (1986). Oxytocin causes endothelium-dependent relaxations of canine basilar arteries by activating V1-vasopressinergic receptors. *J. Pharmac. Exp. Ther*, **236 (1)**: 166-170.

KATUSIC, Z.S., SHEPHERD, J.T. & VANHOUTTE, P.M., (1986). Vasopressin causes endothelium-dependent relaxations of canine basilar artery. *Circ. Res.*, **55**, 575-579

KAUMANN, A.J. & FRENKEN, M., (1985). A paradox: the $5HT_2$-receptor antagonist ketanserin restores 5HT-induced contraction depressed by methysergide in large coronary arteries of calf. Allosteric regulation of $5HT_2$-receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **328**, 295-300

KELLY, E., ROONEY, T.A. & NAHORSKI, S.R., (1985). Pertussis toxin separates two muscarinic receptor effector mechanisms in the striatom. *Eur. J. Pharmacol.*, **119**, 129-130

KENAKIN, T., (1984). The classification of drugs and drug receptors in isolated tissues. *Pharmac. Rev.*, **36**, 3, 165-222

KENDALL, D.A. & NAHORSKI, S.R., (1986). Effects of lithium on agonist-stimulated inositol phospholipid hydrolysis in rat brain slices. *Br. J. Pharmacol.*, **88**, 255P

KNABB, M.T., SAFFITZ, J.E., CORR, P.B. & SOBEL, E.B., (1986). The dependence of electrophysiological derangements on accumulation of endogenous long-chain acyle carnitine in hypoxic neonatal rat myocytes. *Circ. Res.*, **58**, 230-240

KOBAYASHI, S., KANAIDE, H. & NAKAMURA, M., (1984). Cytosolic-free calcium transients in cultured vascular smooth muscle cells: microfluorometric. *Science*, **773**, 83-90

KOMORI, K. & SUZUKI, H., (1987). Heterogeneous distribution of muscarinic receptors in the rabbit saphenous artery. *Br. J. Pharmacol.*, **92**, 657-664

KRISHNAMURTY, V.S.R., (1971). Receptors for sympathomimetic amines and 5-HT in the rat aorta. *Arch. Int. Pharmacodyn.*, **189**, 90-99

KU, D.D., (1982). Coronary vascular reactivity after acute myocardial ischemia. *Science*, **218**, 576-578

LEFF, P., MARTIN, G.R. & MORSE, J.M., (1987). Differential classification of vascular smooth muscle and endothelial cell 5-HT receptors by use of tryptamine analogues. *Br. J. Pharmacol.*, **91**, 321-331

LEGAN, E., CHERNOW, B., PARRILO, J. & ROTH, B.L., (1985). Activation of phosphatidylinositol turnover in rat aorta by α_1-adrenergic stimulation. *Eur. J. Pharmacol.*, **110**, 389-

LIEDTKE, A.J., NELLIS, S. & NEELY, J.R., (1978). Effects of excess free fatty acids on mechanical and metabolic function in normal and ischemic myocardium in swine. *Cir. Res.*, **9**, 652-661

LOEB, A.L., OWENS, G.K., & PEACH, M.J, (1985). Evidence for endothelium-derived relaxing factor in cultured cell. *Hypertension*, Rap. Comm. 804-807

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

LONG, C.J. & STONE, T.W., (1985). The release of endothelium-derived relaxant factor is calcium dependent. *Blood Vessels*, **22**, 205-208

LUES, I. & SCHÜMANN, H.-H., (1984). Effect of removing the endothelial cells on the reactivity of rat aortic segments to different a-adrenoceptor agonists. *Naunyn. Schmiedebergs. Arch. Phamac.*, **325**, 160-163

LUSCHER, T.F. & VANHOUTTE, P.M., (1986). Endothelium-dependant contractions to ach in the aorta of the SHR. *Hypertension*, **8**, 344-348

MALTA, E., SCHINI, V. & MILLER, R.C., (1986). Effect of endothelium on basal and α-adrenoceptor stimulated calcium fluxes in rat aorta. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **334**, 63-70

MALTA, E., SCHINI, V., MILLER, R.C., (1986). Role of efficacy in the assessment of the action of α-adrenoceptor agonists in rat aorta with endothelium. *J. Pharm. Pharmac.*, **38**, 209-213

MANTELLI, L., CAPANNI, L., CORTI, C., BENNARDINI, F., MATUCCI, R. & LEDDA, F., (1988). Influence of lithium on the positive inotropic effect of phenylephrine and isoprenaline in guinea-pig heart. *Eur. J. Pharmacol.*, **150**, 123-129

MARTIN, W., FURCHGOTT, R.F., VILLANI, G.M., & JOTHIANANDAN, D., (1986a). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmac. Exp. Ther.*, **237** (2), 529-538

MARTIN, W., FURCHGOTT, R.F., VILLANI, G.M., & JOTHIANANDAN, D., (1986b). Phosphodiesterase inhibitors induce endothelium-dependent relaxations of rat and rabbit aorta by potentiating the effects of spontaneously released EDRF. J. Pharmac. Exp. Ther., **237** (2), 539-547

MARTIN, W., SMITH, J. A. & WHITE, D.G., (1986c). The mechanisms by which haemoglobin inhibits the relaxation of rabbit aorta induced by nitro vasodilators, nitric oxide, or bovine retractor penis inhibitory factor. *Br. J. Pharmacol.*, **89**, 563-571

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F., (1985a). Blockade of endothelium dependent and glyceryl trinitrate induced relaxation of rabbit aorta by ferrous hemoproteins. *J. Pharm. Exp. Ther.*, **233**, 679-685

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F., (1985b). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharm. Exp. Ther.*, **232** (3), 708-715

MARTIN, W., WHITE, D.G. & HENDERSON, A.H., (1988). Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br. J. Pharmacol.*, **93**, 229-239

MATSUDA, H., KUON, E., HOLTZ, J. & BUSSE, R., (1985). Endothelium-mediated dilations contribute to the polarity of the arterial wall in vasomotion induced by α_2-adrenergic agonists. *J. Card. Pharmac.*, **7**, 680-688

MCLENNAN, P.L. & TAYLOR, D.A., (1984). Antagonsim by ketanserin of 5-HT-induced vasoconstriction unmasks a 5-HT-induced vasodilation. *Eur. J. Pharmacol.*, **104**, 313-318

MECCA, T.E., & WEBB, R.C. (1984). Serotonin and vasodilatation. *Biblthca Cardiol.*, **38**, 81-90

MEDGETT, I.C., FEARN, H.J. & RAND, M.J., (1983). Serotonin enhances sympathetic vasoconstrictor responses in rat isolated perfused tail artery by activation of postjunctional 5-HT_2-receptors. *Clin. Exp. Pharmacol. Physiol.*, **11**, 343-346

MEISHERI, K.D., HWANG, O. & VAN BREEMEN, C., (1981). Evidence for two separate Ca^{2+} pathways in smooth muscle plasmalemma. *J. Membr. Biol.*, **59**, 19-25

MELCHIORRE, C., CASSINELLI, A. & QUAGLIA, W., (1987). Differential blockade of muscarinic receptor subtypes by polymethylene tetraamines. Novel class of selective antagonists of cardiac M_2 muscarinic receptors., **30**, 201-2044

MENKES, H.A., BARABAN, J.M., FREED, A.N. & SNYDER, S.H., (1986). Lithium dampens neurotransmitter response in smooth muscle: relevance to action in affective illness. *Proc. Natl. Acad. Sci.*, **83**, 5727-5730

MICHELL, R.H., (1975). Inositol phospholipid and cell surface receptor function. *Biochem. Biophys. Acta.*, **415**, 81-147

MIDDLEMISS, D.M., BLAKEBOROUGH, L. & LEATHER, S.R., (1977). Direct evidence for an interaction of ß-adrenergic blockers with the 5-HT receptor. *Nature*, 267, 289-290

MILLER, R.C., MONY, M., SCHINI, V., SCHOEFFTER, P., & STOCLET J.C., (1984). Endothelial mediated inhibition of contraction and increase in cGMP levels evoked by the a-adrenoceptor agonist B-HT 920 in rat isolated aorta. *Br. J. Pharmacol.*, **83**, 903-908

MILLER, R.C. & STOCLET, J.C., (1985). Modulation by endothelium of contractile responses in rat aorta in absence and presence of flunarizine. *Br. J. Pharmacol.*, **86**, 655-661

MILLER, R.C., SCHOEFFTER, P. & STOCLET, J.C., (1985). Insensitivity of calcium-dependent endothelial stimulation in rat isolated aorta to the calcium entry blocker flunarizine. *Br. J. Pharmacol.*, **85**, 481-487

MITCHELSON, F., (1984). Heterogeneity in muscarinic receptors: evidence from pharmacologic studies with antagonists. *TIPS*, **Symposium Supplement**, 12-16

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

MIR, A.K. & SPEDDING, M., (1986). Palmitoyl carnitine, a lipid metabolite produced in ischaemia, activates Ca^{2+} channels in smooth muscle. *Br. J. Pharmacol.*, **88**, 381P

MORELAND, R.S., VAN BREEMEN, C. & BOHR, D.F., (1985). Mechanism by which serotonin attenuates contractile response of canine mesenteric arterial smooth muscle. *J. Pharmac. Exp. Ther.*, **232** (2), 322-329

MULVANY, M. J., & HALPERN, W., (1976). Contractile properties of small arterial resistence vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19-26

NAKAKI, T., ROTH, B. L., CHUANG, D. & COSTA, E., (1985). Phasic and tonic components in 5-HT_2 receptor-mediated rat aorta contraction: Participation of Ca^{2+} channels and phospholipase C. *J. Pharmac. Exp. Ther.*, **234** (2), 442-446

NEEDHAM, L., CUSACK, N.J., PEARSON, J.D. & GORDON, J.L., (1987). Characteristics of the P_2 purinoceptor that mediates prostacyclin production by pig aortic endothelial cells. *Eur. J. Pharmacol.*, **134**, 199-209

NEELY, J.R. & FEUVRAY, D., (1981). Metabolic products and myocardial ischemia. *Am. J. Pathol.*, **102**, 282-291

NILSSON, H. & SJÖBLOM, N., (1985). Distension-dependent changes in noradrenaline sensitivity in small arteries from the rat. *Acta Physiol Scand*, **125**, 429-435

NISHIZUKA, Y., (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693-698

OSUGI, T., IMAIZUMI, T., MIZUSHIMA, A., UCHIDA, S. & YOSHIDA, H., (1986). 1-Oleoyl-2-acetyl-glycerol and phorbol diester stimulate Ca^{2+} influx through Ca^{2+} channels in neuroblastoma x glioma hybrid NG 108-15 cells. *Eur. J. Pharmacol.*, **126**, 47-51

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S., (1988). Vascular endothelial cells synthesize nitric oxide from l-arginine. *Nature*, **333**, 665-666

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

PENNER, R., MATTHEWS, G. & NEHER, E., (1988). Regulation of calcium influx by second messengers in cat mast cells. *Nature*, **334**, 499-504

POPESCU, L.M., HINESCU, M.E., MUSAT, S., IONESCU, M. & PISTRITZU, F., (1986). Inositol triphosphate and the contraction of vascular smooth muscle cells. *Eur. J. Pharmacol.*, **123**, 167-169

POPESCU, L. M., PANOIU, C., HINESCU, M., NUTU, O., (1984). The mechanism of cGMP-induced relaxations in vascular smooth muscle. *Eur. J. Pharmacol.* **107**, 393-394

PRESSMAN, B.C., (1976). Biological applications of ionophores. *Ann. Rev. Biochem.*, **45**, 501

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S., (1987). Comparitive pharmacology of EDRF, nitric oxide and prostacylin in plateletes. *Br. J. Pharmacol.*, 92, 181-187

RANDRIANTSOA, A., HEITZ, C. & STOCLET, J., (1981). Functional characterisation of postjunctional a-arenoceptors in rat aorta. *Eur. J. Pharmacol.*, **75**, 57-60

RAPAPORT, R.M. & BEVAN, J.A., (1982). Increased contractility induced in rabbit ear artery by prior exposure to serotonin masks concomitant desensitisation to norepinephrine. *J. Pharmacol. Exp. Ther.*, **222**, 338-343

RAPOPORT, R.M., DRAZNIN, M.B. & MURAD, F., (1983). Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation, *Nature*, **306**, 174

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

RASMUSSEN, H., (1986). The calcium messenger system. *New Eng. J. Med.*, **314**, 1164-1170

REUTER, H., (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature*, **301**, 569-574.

ROTH, B.L., NAKAKI, T., CHUANG, D.M. & COSTA, E., (1984). Aortic recognition sites for serotonin (5HT) are coupled to phospholipase C and modulate phosphatidylinositol turnover. *Neuropharmacology*, **23**, 1223-

RUBANYI, G.M., LORENZ, R.R. & VANHOUTTE, P.M., (1985). Bioassay of endothelium-derived relaxing factor(s). Inactivation by catecholamines. *Am. J. Physiol.*, **249**, H95-H101

RUBANYI, G.M., MCKINNEY, M. & VANHOUTTE, P. M., (1986). Biphasic release of endothelium-derived relaxing factor(s) by acetylcholine from perfused canine femoral arteries. Characterisation of muscarinic receptors. *J. Pharmac. Exp. Ther.*, **240** (3), 802-808

RUBANYI, G.M., ROMERO, J.C., M. & VANHOUTTE, P. M., (1986). Flow-induced release of endothelium-derived relaxing factors. *Am. J. Physiol.*, **250**, H1145-H1149

RUBANYI, G.M., SCHWARTZ, A. & VANHOUTTE, P.M., (1985). The calcium agonists BAY K 8644 and (+)202,791 stimulate the release of endothelial relaxing factor from canine femoral arteries. *Eur. J. Pharmacol.*, **117**, 143-144

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

RUBANYI, G.M. & VANHOUTTE, P.M., (1986). Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle. *Am. J. Physiol.*, **250**, H815-H821

RUFFOLO, R.R., (1982). Important concepts of receptor theory. *J. Auton. Pharmac.*, **213**, 557-

RUFFOLO, R.R., YADEN, E.L. & WADDELL, J.E., (1980). Receptor interactions of imidazolines. V. Clonidine differentiates post-synaptic alpha adrenergic receptor subtypes in tissues from the rat. *J. Pharmacol. Exp. Ther.*, **213**, 557-

RYAN, U.S., & RYAN, J.W., (1984). Cell biology of pulmonary endothelium. *Circulation*, **70**, (Suppl 11)46-62

SATOH, H., & INUI, J., (1984). Endothelial cell-dependent relaxation and contraction induced by histamine in the isolated guinea-pig pulmonary artery. *Eur. J. Pharmacol.*, **97**, 321-324

SCHRAMM, M., THOMAS, G., TOWART, R. & FRANCKOWIAK, G., (1983). Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature*, **303**, 535-537

SCHRAMM, M. & TOWART, R., (1985). Modulation of calcium channel function by drugs. *Life Sci.*, **37**, 1843-1860.

SCHRAMM, M., TOWART, R., LAMP & THOMAS, G., (1985). Modulation of calcium influx by the 1,4 dihydropyridines, nifedipine and BAY K 8644. *J. Cardiovasc. Pharmacol.*, **7**, 493-496.

SHERMAN, W.R., GISH, B.G., HONCHAR, M.P. & MUNSELL, L.Y., (1986). Effects of lithium on phosphoinositide metabolism in vivo. *Federation Proc.*, **45 (11)**, 2639-2646

SHUG, A.L., THOMSEN, J.H., FOLTS, J.D., BITTAR, N., KLEIN, M.I., KOKE, J.R. & HUTH, P.J., (1978). Changes in the tissue levels of carnitine and the other metabolites during myocardial ischaemia and anoxia. *Arch. Biochem. Biophys.*, **187**, 25-33

SINGER, H.A. & PEACH, M.J., (1982). Calcium and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension*, **4, Suppl II**, II.19-II.25

SINGER, H.A. & PEACH, M.J., (1983). Endothelium-dependant relaxation of rabbit aorta.11.inhibition of relaxation stimulated by methacholine and A23187 with antagonists of arachidonic acid metabolism. *J. Pharmac. Exp. Ther.*, **226**, 796-801

SOMLYO, A.P., (1984). Cellular site of calcium regulation. *Nature*, **309**, 516-517

SOMLYO, A.V., BOND, M., SOMLYO, A.P. & SCARPA, A., (1985). Inositol triphosphate-induced calcium release and contraction in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 5231-

SOROTA, S., TSUJI, Y., TAJIMA, T. & PAPPANO, A.J., (1985). Pertussis toxin treatment blocks hyperpolarisation by muscarinic agonists in chick atrium. *Circ. Res.*, **57**, 748-758

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

SPEDDING, M. & MIR, A.K., (1987). Direct activation of Ca^{2+} channels by palmitoyl carnitine, a putative endogenous ligand. *Br. J. Pharmacol.*, **92**, 457-468

SPEDDING, M., SCHINI, V., SCHOEFFTER, P. & MILLER, R.C., (1987). Calcium channel activation does not increase release of endothelial-derived relaxant factor (EDRF) although tonic release of EDRF may modulate calcium channel activity in smooth muscle. *J. Cardiovas. Pharmac.*, **8**, 1130-1137

SPOKAS, E.G. & FOLCO, G., (1984). Intima-related vasodilatation of the perfused rat caudal artery. *Eur. J. Pharmacol.*, 211-217

SPOKAS, E.G., FOLCO, G., QUILLEY, J., CHANDER, P. & MCGIFF, J.C., (1983). Endothelial mechanism in the vascular action of hydralazine. *Hypertension*, **5**, 107-111

STEPHENSON, J.A. & SUMMERS, R.J., (1987). Autoradiographic analysis of receptors on vascular endothelium

SU, C.M., SWAMY, V.C., & TRIGGLE, D.J., (1984). Calcium channel activation in vascular smooth muscle by BAY K 8644. *Can. J. Physiol. Pharmacol.*, **62**, 1401-1410

SU, C. & URUNO, T., (1985). Excitatory and inhibitory effects of 5-hydroxytryptamine in mesenteric arteries of spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **106**, 283-290

SUETMATSU, E., HIRATA, M., HASHIMOTO, T. & KURIYAMA, H., (1984). Inositol 1,4,5,-triphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. *Biochem. Biophys. Res. Comm.*, **120**, 481-485

TAYLOR, C.W., (1987). Receptor regulation of calcium entry.*TIPS*, **8** (3), 79-80

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

TAYLOR, S.G. & WESTON, A.H., (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular andothelium. *TIPS*, **9**, 272-274

TEMPLETON, A.G.B, (1988). The influence of the endothelium on the response of vascular smooth muscle. Ph.D. Thesis, University

TEMPLETON, A.G.B, MACMILLAN, J., MCGRATH, J.C., STOREY, N.D. & WILSON, V.G., (1988). Evidence for a population of prazosin-resistant, rauwolscine-sensitive alpha-adrenoceptors mediating contractions in the isolated vascular bed of the rat tail. *Br. J. Pharmacol.*, submitted October 1988

TODA, N., (1983). Endothelium-dependent relaxation induced by angiotensin II and histamine in isolated arteries of the dog. *Br. J. Pharmacol.*, **81**, 301-307

TREVETHICK, M.A., FENIUK, W. & HUMPHREY, P.P.A., (1984). 5-Hydroxytryptamine-induced relaxation of neonatal porcine vena cava in vitro. *Life Sci*, **35**, 477-486

TREVETHICK, M.A., FENIUK, W. & HUMPHREY, P.P.A., (1986). 5-Carboxamidotryptamine: a potent agonist mediating relaxation and elevation of cyclic AMP in isolated neonatal porcine vena cava in vitro. *Life Sci.*, (in press).

TRIGGLE, D.J., (1984). Ca^{2+}-channels revisited; problems and promises. *TIPS*, **53**, 4-5

TWORT, C.H.C. & VAN BREEMEN, C., (1988). Cyclic guanosine monophosphate-enhanced sequestration of Ca^{2+} by sarcoplasmic reticulum in vascular smooth muscle. *Circ. Res.*, **62**, 961-964

UGWU, A.C., MCGRATH, J.C. & SPEDDING, M., (1987). Comparisons of the effects of palmitoyl carnitine and Bay K 8644 on the calcium sensitivity of the rat tail artery. *Br. J. Pharmacol.*, **92**, 552P

UI, M., (1984). Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. *TIPS*. **5**, 277-279

VAN BREEMEN, C., AARONSON P. & LOUTZENHISER, R., (1979). Na^{+}-Ca^{2+} interactions in mammalian smooth muscle. *Pharmacol. Rev.*, **30**, 167-208

VAN BREEMEN, C. & SEIGL, B., (1980). Mechanisms of α-adrenergic activation of the dog coronary artery. *Circ. Res.*, **46**, 426-429

VAN DE VOORDE, J. & LEUSEN, I., (1983). Role of the endothelium in the vasodilator response of rat thoracic aorta to histamine. Eur. J. Pharmacol., **87**, 113-120

VAN ROSSUM, J.M., (1963). Cumulative dose-response curves. II. technique for the making of dose response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn. Ther.*, **143**, 229-330

VANHOUTTE, P.M., (1976). Effects of anoxia and glucose depletion on isolated veins of the dog. *Am. J. Physiol.*, **230**, 1261-1268

VANHOUTTE, P.M., (1987). The end of the quest? *Nature*, **327**, 459-460

VANHOUTTE, P.M. & KATUSIC, Z.S., (1988). Endothelium-derived contracting factor: endothelin and/or superoxide anion. *TIPS*, **9**, 229-230

WAKADE, A.R., MALHROTA, R.K. & WAKADE, T.D., (1986). Phorbol ester facilitates ^{45}Ca accumulation and catechlamine secretion by nicotine and excess K^+ bu not by muscarine in rat adrenal medulla. *Nature*, **321**, 698-700

WATSON, M. & YAMAMURA, H.I., (1983). A unique regulatory profile and regional distribution of [3H] pirenzepine binding in the rat provide evidence for distinct M_1 and M_2 receptor subtypes. *Life Sci.*, **32**, 3001-3011

WINQUIST, R.J., BUNTING, P.B. & SCHOFIELD, T.L., (1985). Blockade of endothelium-dependent relaxation by the amiloride analog dichlorobenzamil: possible role of Na^+/Ca^{2+} exchange in the release of endothelium-derived relaxing factor. *J. Pharmac. Exp. Ther.*, **235**, 644-650

YAMANAKI, K., MASHIMOTO, S. & MURAMATSU, L., (1986). Species differences in the muscarinic receptor of the thoracic aorta. *J. Pharm. Exp. Ther.*, **237**, 980-986

YAMASHITA, K., TAKAGI, T. & HOTTA, K., (1977). Mobilisation of cellular Ca^{++} and contraction-relaxation of vascular smooth muscle. *Jap. J. Physiol.*, **27**, 551-564

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

ZAWADSKI, J.V., FURCHGOTT, R.F. & CHERRY, P.D., (1981). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by substance P. *[Abstract]. Fed Proc.*, **40**, 689

ZSCHAUER, A., VAN BREEMEN, C., BÜHLER, F.R. & NELSON, M.T., (1988). Calcium channels in thrombin-activated human platelet membrane. *Nature*, **334**, 703-705

Appendix

Computerised system for
acquisition and analysis of data.

The analysis of experimental data by measuring traces recorded on chart recorders is often time consuming and tedious. This is particularly the case if a number of different parameters are to be measured from one trace e.g. tension at set times, tension at plateau of response, and time between events.

In order to reduce the time spent on this task, I designed a system for the BBC microcomputer which allows the direct sampling of data recorded on a Linseis chart recorder. As with the majority of serious software applications for the BBC microcomputer, the system is 'soft key driven' i.e. it utilises the programmable 'soft keys' of the computer, and is therefore simple to use. The sampled data (or data entered independently of the chart sampling facility) is stored and can then be retrieved repeatedly for analysis by a variety of methods.

With maximum flexibility of the system in mind, in addition to the data collection, storage, retrieval, and analysis functions of the system, it is also possible to 'program' a series of instructions for the recorder to follow. For example the recorder can be switched on and off and the speed can be altered at set times for various lengths of time. This is particularly useful when physiological responses change slowly over a long period and there is a need to examine more closely the response at set intervals (by speeding up the trace) e.g. twitch response of vas deferens.

In the following pages is a description of this data retrieval and storage system which I have called **DATAFILE.**

DATAFILE has been designed as a simple to use, 'user-friendly', menu driven, data storage system principally for the storage and analysis of concentration-response curves and can be used by people with a minimum of 'computer know-how'. Within DATAFILE there is a limited scope for analysis of the stored data e.g. expression of the tissue response as; a percentage of its own maximum or another entered value, actual changes in tension, absolute tension. DATAFILE can be used to plot stored data using an Epson printer. Stored data can also be converted to a 'BEEMAC' format which may be transferred to an Apple Macintosh Microcomputer, a powerful facility which allows further, more sophisticated data analysis and plotting and gives an indirect method of transferring data to an IBM PC.

DATAFILE was designed to have several advantages over 'manual' data storage systems:

i) standardised print-out of data thus making reading of the data much easier and avoiding complications of individual handwriting.

ii) storage of raw data thus enabling greater ease of varied analysis at a later date.

iii) rapid effortless plotting of data gives a very quick indication of the results of the current experiment.

iv) disc storage (in particular of more than one copy kept in different locations) offers greater security against loss of valuable experimental data.

DATAFILE was written primarily on and for the BBC model B microcomputer but will function equally as well with a BBC B+ and/or in conjunction with a second processor. Use of a BBC Master 128K microcomputer allows data captured from a Linseis chart recorder to be analysed practically. In these systems with expanded capacity the programs run faster and it is possible to enter a greater number of data items, e.g. curves, preparations, concentrations.

Disc and Disc Drive

DATAFILE cannot be used with a single-sided, single disc drive. The minimum requirement for the system is a single sided, double disc drive. However, due to the limitations of the BBC disc filing system (i.e. a maximum of 31 files per side of diskette) a double sided, double disc drive is preferable thus reducing the number of data discs necessary. Although it is possible to store files on side 2 of the program discs this is not advisable since it necessitates a comprehensive knowledge of the DATAFILE programs, an error in which may result in the loss of data which has taken some time to enter.

The Datafile System

The program disc has been designed with an 'AUTO BOOT' and therefore after placing the program disc into drive 0, pressing <SHIFT-BREAK> will give access to the system.

DATAFILE is a menu-driven system and thus once within it the user may move within the system via certain menus.

In order to maximise the speed of operation and the amount of data that can be entered DATAFILE utilises the 'shadow' memory of the BBC (see User Manual). If the user's computer does not support shadow memory DATAFILE will compensate for this and prevent a system crash. After a brief title page the system then displays the first menu, i.e. MAIN MENU.

MAIN MENU

A FOR DATA INPUT	Entering of data for future analysis
B TO PROCESS FILE	Manipulation of stored files and further analysis
C LINCHART	Allows data collection from Linseis chart recorder.
D FOR VOLADD	Volume of liquid needed to give a concentration
E FOR AMMADD	Amount of solid needed to give a set concentration
F SCHILD ANALYSIS	Allows Schild Analysis on entered $\log_{10}$ (CR-1) or EC_{50} values.
G KERMIT	Allows access to communications program (if Kermit ROM is installed in the BBC micro) for transfer of data to the Macintosh.

A FOR DATA INPUT

Within this option various 'screens' are used to input data. Errors made whilst entering data can be rectified at the time of entering or at a later date using the EDIT facility available in option B. Below is a brief summary of option A.

Enter a) date of experiment, b) preparation, c) agonist, d) antagonist, and e) conditions used e.g.

14/2/86,

RAT A RINGS,

5-HT,

RITANSERIN,

KREBS' 5/95% CARBOGEN 37'C pH 7.4

Enter Number of concentrations used, number of preparations used, number of curves generated

Enter Concentrations used - entries made in exponential format, i.e. 1E-6 = 1μM, 1x10-6 M. The red function keys are programmed to aid concentration entry, i.e. f1 = 1E-, f2 = 2E-, f3 = 3E- etc.

Enter Baseline tensions - important if there is a need to know the absolute tension (response + resting tension) of the tissue.

Enter Treatment - of each preparation for each curve, e.g. 5HT. This is printed when a printout of a file is obtained.

Enter Response - to each concentration of each preparation for each curve constructed. It is recommended that these values be entered as mg.wt. rather than g.wt. for reasons of formatting.

Enter Comments - the comments are (if necessary) entered as two entries each of 238 characters.

After entry of any relevant comments (which should include any observations made throughout the experiment) DATAFILE asks the user to declare the drive containing the data disc followed by a suitable filename. The user is not allowed to store the data that has been entered using the name of a file which already exists on the declared drive since this will erase that file. When DATAFILE saves a datafile it 'locks' the file thus preventing accidental erasure whilst outside the DATAFILE system. When files are converted by options in other menus (see later discussion) and again stored the datafile are again 'locked'. DATAFILE unlocks and re-locks files automatically when necessary.

On completion of data input DATAFILE returns to MAIN MENU.

B TO PROCESS FILE

This option allows access to the PROCESS FILE menu which will be discussed later.

C LINCHART

The method of capturing data from a Linseis chart recorder was developed as a separate function from the data-storage and retrieval function of DATAFILE and incorporated into the system at a later date. The LINCHART option gives access to both data capture from the Linseis chart recorder and also a means by which to 'program' a series of instructions for the recorder to follow. For example the recorder can be switched on and off and the speed can be altered at set times for various lengths of time. This is particularly useful when physiological responses change slowly over a long period and there is a need to examine more closely the response at set intervals (by speeding up the trace) e.g. twitch response of vas deferens. LINCHART is operated using the red function keys of the BBC.

Sampling Data

Data sampling from the Linseis recorder is accomplished by continuous measurement of the position of the recorder pens. The user enters a conversion factor (which will be used to convert the captured data into a more useful format (e.g. mg.wt.). The maximum number of available data samples will depend on the number of channels being used for a particular set of curves. The number of samples required (in combination with the sampling interval) will limit the duration of sampling. The interval between sampling determines the resolution of the sampling. If the sampled responses are slowly developing, a five second sampling interval will be sufficient to give a clear resolution when later measuring the recorded data. The minimum sampling interval is 0.5 seconds.

The user uses the BBC's red function keys to 'mark' an event e.g. the addition of a drug. Other function keys are used to begin, pause, and end continuous sampling after which the captured data is stored on disc. Individual files are created for the data obtained from all channels at a particular time. For example if three concentration response curves are obtained from six channels then three files, each containing the data from all six channels, will be created.

Programming the Linseis Chart Recorder

Sequences of instructions may be entered and saved for the chart recorder to follow at a later stage. The user enters a sequence of instructions that are to be followed, again using function keys assigned a particular instruction.

Available Instructions:

Pen Up	Lifts pens if pens already in the down position.
Pen Down	Lowers pens if pens already in the raised position.
Start Chart	Starts chart: this instruction is followed by a time parameter which is the length of time (in seconds) that the chart is to run at the present speed and direction.
Stop Chart	Stops chart: this instruction is followed by a time parameter which is the length of time (in seconds) that the chart is to be stopped for. This can be used as a method of pausing the sequence of instructions.
Move Chart	Moves the chart in a declared direction (forward or reverse) for a set distance
MZK Sync	This instruction has a variety of functions and is dependent on the parameter which is entered. (For further details see DATAFILE manual).

D FOR VOLADD

This option is a utility program which calculates the volume (in mls) of liquid that must be added to an entered weight (in mgs), of a compound of entered molecular weight (in grammes), to give an entered molar concentration.

E FOR AMADD

Another utility program which calculates the amount (in mgs), of a compound of entered molecular weight (in grammes), to be added to an entered volume of liquid (in mls), to give an entered molar concentration.

F SCHILD ANALYSIS

This option allows the calculation of pA_2 values and slope of Schild plots ($y = \log_{10}$ (Concentration ratio - 1); x = Antagonist Concentration) giving mean values ± standard deviation for both pA_2 and slope. The Schild analysis can be plotted and the entered data saved.

PROCESS FILE MENU

This menu is accessed via option B of MAIN MENU and allows the manipulation of whole datafiles and to give access to further analysis of the data contained within.

A EDIT DATAFILE	Editing of stored datafiles.
B PROCESS DATAFILE	Manipulation and further analysis of stored data
C RENAME DATAFILE	Renaming of stored datafiles.
D DELETE DATAFILE	Deletion of stored datafiles.
E CATALOGUE	Catalogue of files stored on disc in declared drive.

A EDIT DATAFILE

Within this option the stored data can be edited or simply examined. Each of the 'input' screens used in option A of the MAIN MENU are shown and any changes can be made. The edited file can be saved using the old filename, a new filename, or simply aborted.

B PROCESS DATAFILE

This option allows access to the PROCESS DATAFILE menu discussed below.

C RENAME DATAFILE

This option allows the re-naming of stored files. If the new filename already exists the computer will inform the user. Renaming of files to existing file names is not allowed.

D DELETE DATAFILE

This option gives the means by which datafiles can be deleted. Deletion then takes place following a prompt from the user.

E CATALOGUE

This option provides a list of the files stored on the disc in a declared drive.

PROCESS DATAFILE MENU

This menu is accessed via option B of the PROCESS FILE menu. The menu allows manipulation and access to further analysis of the data contained within a stored datafile.

A PRINT DATAFILE	Print-out of stored datafile using standard format.
B DATAFILE CALCS	Manipulation of data contained in datafiles.
C PLOT DATAFILE	Plots data from stored datafiles.
D BEEMAC FORMAT	Converts stored datafile to BEEMAC usable format.
E LINCHART-DATFILE	Conversion of data captured using LINCHART into a datafile.
F CATALOGUE	Catalogue of files stored on datadisc in declared drive.

A PRINT DATAFILE

This option produces a print-out in standardised, easy-to-read format from a datafile stored on a declared disc drive. The print-out can be made as a 'rough' or a 'store' copy the latter having a superior presentation. This provides a 'hard copy' of the disc data.

B DATAFILE CALCS

This option allows access to the DATAFILE CALCS menu discussed below.

C PLOT DATAFILE

This option allows the plotting of data (or transformed data) from stored datafiles via a screen dump facility. The quality of the plots are not of report standard but are intended to be used as a quick indication of the results obtained from an experiment and any simple analysis that has taken place. More sophisticated plotting can be made using the Macintosh via DATAFILE's BEEMAC option.

After certain preliminaries are completed DATAFILE produces a screen plot of the data which may then be screen dumped to an Epson printer with or without the addition of a key. The user can then replot data from the same file, another file, or return to the PROCESS DATAFILE menu.

D BEEMAC FORMAT

This option allows the conversion of stored files to a format which can be sent to a Macintosh microcomputer for more complicated statistical analysis or plotting.

Files are converted to a text format and stored on disc. The converted files can then be transferred, as the text file, to a Macintosh by use of the communications program KERMIT (available on ROM for the BBC and software for the Macintosh) or any communications package which allows transfer of text files. The transferred text file

can be read using a word processor or read directly into the spreadsheet package EXCEL.

E LINCHART-DATFILE

This option allows the measurement of data previously captured from a Linseis chart recorder using the LINCHART option of MAIN MENU. Data captured using LINCHART is stored as individual files. The user enters the number of files to be used (equivalent to the number of curves entered during manual data input), the treatments for each curve of each preparation and any comments. The filename of the LINCHART files to be analysed are then entered and each recalled to produce an on-screen 'plot' of the data contained within it. The range of concentrations used is displayed at the top of the screen and the response for each measured using a horizontal measuring line (positioned at the point of measurement using the cursor keys. The values measured are converted to mg. wt. or any other unit (depending upon the scaling factor entered in the LINCHART option). When all of the desired LINCHART files has been measured it is saved on a declared drive as a DATAFILE file which can then be treated as any other datafile.

F CATALOGUE

This option has the same function as in other menus.

DATAFILE CALCS MENU

This menu is accessed via option B of the PROCESS DATAFILE menu. The menu allows manipulation and analysis of the data contained within stored datafiles.

A	CALC ABSOLUTES	Calculates absolute size of stored responses.
B	CALC PERCENTAGES	Converts stored data into percentage values.
C	CALC SUBTRACTS	Subtracts an entered value from stored data.
D	EC-Response	Calculates $EC_{Response}$ values.
E	EC-Percentage	Calculates $EC_{Percentage}$ values.
F	SCALING FACTOR	Multiplies stored data by an entered factor.

A CALC ABSOLUTES

Sometimes it is useful to know the absolute tension of the tissue, i.e. baseline tension + change in tension due to treatment. For example the same concentration of a drug in two tissues may produce very different responses in terms of the changes in tension which the drug produces. This may be due to several things, one of them being that the resting tension in each tissue is different. Expression of the results in this manner will give some indication as to whether or not this plays a role in such differences in responses.

DATAFILE retains a note of the fact that a file has been transformed and this is noted on any printout (including printed plots) that might be obtained. This transformed data can be further manipulated or analysed in the usual manner via options contained within DATAFILE.

B CALC PERCENTAGES

This option allows the stored data of a datafile to be expressed in terms of percentages. The data for each individual curve in a datafile may be expressed automatically as a percentage of the maximum response of that particular curve.

Alternatively a single value to be used as the 100% value for all the curves contained within the datafile or individual values for each curve separately may be entered. As with option A, this transformed data can be further manipulated or analysed in the usual manner via options contained within DATAFILE.

C CALC SUBTRACTS

This option allows the responses contained as data within a datafile to be subtracted from any value. In a similar manner to option B above the value used may be the maximum for each curve (automatically determined by DATAFILE) or a single entered value for all curves or individual values entered for each curve. This option is designed primarily for analysis of relaxation response curves where the response to increasing concentrations is a decrease in developed tension but it is desirable to express the observed response as an increase (in relaxation) with increasing drug concentration. This can be seen more clearly in Figure A.1 below.

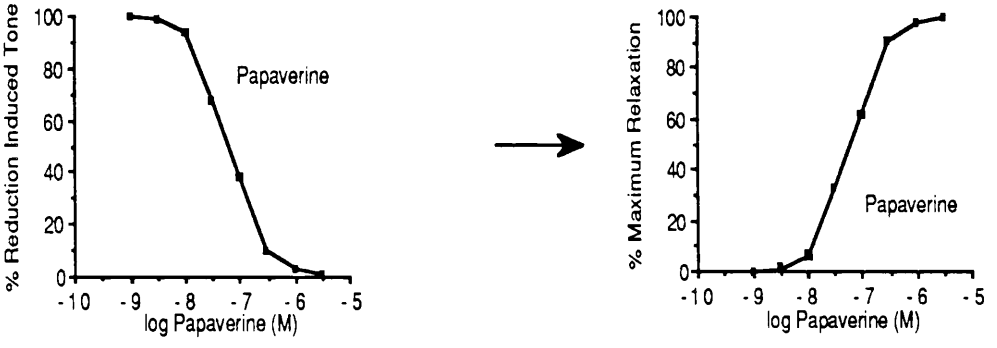

Figure A.1: Use of the CALC SUBTRACTS option to 'invert data'.

As with options A and B, this transformed data can be further manipulated or analysed in the usual manner via options contained within DATAFILE.

D EC-Response

This option is used to calculate multiple EC-response values i.e. the effective concentration of agonists inducing a particular response, e.g. 500 mg.wt. increase in tone (EC_{500} mg.wt.). EC-Response values are calculated by finding the responses above and below the entered value, 'drawing' a straight line between them and the

extrapolating downwards to determine the actual concentration (see Figure A 2). In order to determine IC-Responses for relaxation responses the data must be first 'inverted' using the CALC SUBTRACTS option above. The calculated EC- Response values may also be expressed as $-\log_{10}$ EC- Response values i.e. pEC-Response. These EC-Response/pEC-Response values may be printed or saved on disc for direct incorporation into options such as SCHILD ANALYSIS and BEEMAC FORMAT.

E EC-Percentage

This option is used to calculate multiple EC-Percentage values, i.e. the effective concentration of agonists inducing a particular percentage response e.g. 50%. increase in tone (EC_{50}). Calculation of EC-Percentage values is similar to that for EC-Responses and is calculated by finding the percentage responses above and below the entered value, 'drawing' a straight line between them and extrapolating downwards to determine the actual concentration (see Figure A.2). To determine IC-Percentages for relaxation responses the data must be first 'inverted' using the CALC SUBTRACTS option above. The calculated EC-Percentage values may also be expressed as $-\log_{10}$ EC-Percentage values i.e. p EC-Percentage. These EC-Response/pEC-Response values may be printed or saved on disc for direct incorporation into options such as SCHILD ANALYSIS and BEEMAC FORMAT.

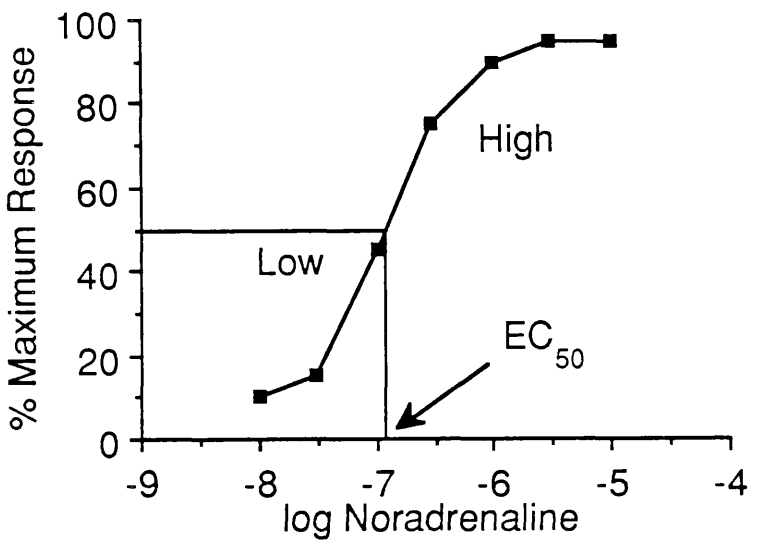

Figure A.2: Method of calculation of EC- values.

F SCALING FACTOR

This option is designed to aid the user who is entering data into DATAFILE manually, i.e. not via the use of the LINCHART and LINCHART-DATFILE options. This simply allows data measured in arbitrary units, such as mm or cm, to be converted to more meaningful values (e.g. mg.wt.) by simply entering a conversion factor. The converted data may then be saved as a normal DATAFILE file.