THE INFLUENCE OF THE ENDOTHELIUM ON THE RESPONSE OF VASCULAR SMOOTH MUSCLE

A thesis presented for the degree of
Doctor of Philosophy

by

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DECLARATION & PUBLICATIONS

The experimental work and other research which is contained within this thesis was undertaken by myself with the exception of the experiments resulting in Figures 18, 20a,b; 21a,b; 22a,b: which were carried out by Ian Dainty as part of a joint project on the influence of the initial tension on EDRF-mediated responses. Some of the results have been published as detailed below.

DALY,C.J., McGRATH,J.C., MUIR,A.G.B. & O'BRIEN,J.W. (1985) Ca^{++}- dependence of noradrenaline-induced contraction in rat aortic smooth muscle. Br.J.Pharmac., 86 : 723P

McGRATH,J.C. & MUIR,A. (1986) . Effect of EDRF on contraction of rat aorta by noradrenaline and interaction with an α_2-antagonist and a Ca^{++} activator. Br.J.Pharmac., 89 : 687P.

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TEMPLETON,A.G.B., DREW,G.M. & McGRATH,J.C. (1987) The effect of diabetes and hypertension on contraction to NA and relaxation to Ach and sodium nitroprusside in rat isolated aortic rings. Br.J.Pharmac., 92 : 766P.

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.Pharmac., 92 : 619P.

SUMMARY

This project was carried out to examine the influence of the vascular endothelium on the responses of the underlying smooth muscle. I have examined the influence of the endothelium on sensitivity of the smooth muscle to calcium and the interaction of a Ca^{2+}-channel activator and blocker. A further study was carried out to examine how the initial tone affects the responses to both contractile and relaxatory agents and how the size of the induced tone affects the ability of Ach to cause relaxation. I studied the changes, if any, in this relationship in certain cardiovascular diseases. These effects were studied in an isolated aortic ring from the rat and also a complete vascular bed, the perfused rat tail. The following is a summary of the results:

1) Removal of the vascular endothelium from isolated aortic rings affects the sensitivity of the tissue to various agonists. The influence on the responses is greater for some agonists than for others. The agonists influenced to the greatest extent had both the pD_2 and the maximum response significantly changed by removal of the endothelium: these agonists were either α_1-adrenoceptor partial agonists or α_2-adrenoceptor agonists in other tissues. The agonist affected to the least extent, the thromboxane mimetic drug U46619 showed no significant change on removal of the endothelium in either the maximum response or the pD_2 value.

2) The presence of the vascular endothelium had no influence on the actions at α_1-adrenoceptors, of the selective antagonists prazosin and corynanthine, or the α_2-adrenoceptor antagonist, Wyeth 26703. The results using Wyeth 26703 did not support the possibility of α_2-adrenoceptor stimulation releasing EDRF, since it had the same effect on responses to NA in tissues with both an intact and disrupted endothelium.

3) Increasing the initial length in aortic ring segments caused a length-dependent increase in the resting tension. The absence of the endothelium had no significant effect on the resting tensions compared with intact tissues. The size of the contraction to Phe (1μM) and relaxation to Ach (1μM) (tone induced by Phe) were dependent on the initial length of the preparation. The optimum stretch to demonstrate the proportionate effect of Ach (1μM) did not coincide with the optimum for Phe-induced contractions.

4) The size of the induced tone against which Ach produced relaxation affected the sensitivity to this agent. The greater the size of the induced tone the less sensitive the tissue was to relaxation. Atropine did not affect the size of the tone induced by NA but inhibited the relaxation to Ach. BAY-K 8644, a calcium channel facilitator, increased the tone induced by NA and inhibited the relaxation to Ach to a greater extent than would be expected by increase in tone alone. The α_2-antagonist drug Wyeth 26703, at concentrations where it acts selectively at α_2-adrenoceptors (in other tissues), had no effect on NA-induced tone or on Ach-induced relaxation.

5) Using a maximal concentration of NA (3μM) in rat aortic rings, with either an intact or disrupted endothelium, in both a buffered and unbuffered calcium system, the contractile response was shown to be dependent on the concentration of extracellular calcium. At concentrations of calcium greater than 1.25mM the response started to fall off. This response was not potentiated using either system in the presence of BAY-K 8644 (1μM) but was significantly inhibited in the presence of nifedipine (1μM).

6) Using a submaximal concentration of NA (30nM) in the rat aortic rings either intact or disrupted, in an unbuffered system, again the response was shown to be dependent on the concentration of extracellular calcium. In this system the

responses to NA (30nM) were potentiated by BAY-K 8644 (1-100nM). The potentiation was similar regardless of the presence of the endothelium and reached a maximum at BAY-K (10nM). In the presence of this drug the drop in maximum response at high concentrations of calcium was still evident.

7) In the presence of nifedipine (0.1-1μM) the responses to NA (30nM) were inhibited to a similar extent in both intact and disrupted rings. With this drug the drop in the maximum response at high calcium concentrations was not seen.

8a) There was no significant difference in either the NA pD_2 or the maximum response developed in aortic rings with a disrupted endothelium from diabetic, renal and Doca/salt hypertensive rats when compared with control. This implied that the contractile response of the smooth muscle to adrenoceptor stimulation was not affected by these disease states.

b) In Doca/salt hypertensive rats there was no difference in pD_2 values and maximum response developed to NA between intact and disrupted tissues. In both control and renal hypertensive rats the pD_2 values to NA were significantly higher in aortic rings with an intact endothelium compared with endothelium disrupted but the maximum responses developed were not significantly different. However, in diabetic rats there was a significant increase in the pD_2 and decrease in the maximum response developed in tissues with an intact compared with disrupted endothelium.

9) Responses to Ach were not affected by either diabetes or hypertension in both the pD_2 and the maximum relaxation obtained.

10) Relaxation to sodium nitroprusside was not affected by the presence or absence of the endothelium in all groups with the exception of the diabetic group. However relaxation to sodium nitroprusside was less effective in both hypertensive groups

when compared with controls. This was true for tissues with either an intact or disrupted endothelium.

11) In the rat isolated perfused tail the sensitivity to Phe was increased in the diabetic rats and decreased in the tails from renal hypertensive rats compared with controls.

12) In the rat tail artery Ach produced a dose-dependent relaxation which was reproducible and was markedly inhibited by pretreatment with atropine (0.1μM) or Hb (10μM). Sensitivity to relaxation by Ach was similar in the tails from diabetic and untreated groups. In both hypertensive groups the relaxation to Ach was less sensitive but not significantly so at the EC_{20} level.

13) Only in tails from Doca/salt hypertensive rats was there a diminished relaxation-response to sodium nitroprusside, but the effect was not as marked as in the rat aorta. A similar effect was obtained in tails from renal hypertensive rats but it was not significant.

14) In the perfused rat tail we could obtain no contraction to the α_2-adrenoceptor agonists UK14304 or BHT-920. However, when the tone was raised by including either Phe or 5-HT in the perfusate, UK14304 produced a contraction. This contraction was not inhibited by high concentrations of both α_1 antagonists prazosin or corynanthine, but was blocked by the α_2-antagonist rauwolscine or the prostaglandin inhibitor flurbiprofen.

INTRODUCTION

The importance of the various roles of the vascular endothelium has become better understood over the years as more information has been found about its properties and possible function. Up to the mid-sixties it was thought simply to be a semi-permeable membrane barrier between the circulating blood and the interstitial space. By the use of endothelial cell culture techniques the various metabolic and functional activities by which the endothelium may influence the homeostasis of the vessel wall became apparent (Jaffe , 1973). As well as capillary transport, regulation of plasma lipids and participation in the control of hemostasis, it modulates the reactivity of the vascular smooth muscle. This role involves various mechanisms. In addition to being a physical barrier the endothelium can extract or metabolize vasoactive substances including noradrenaline (NA), serotonin (5HT) and kinins and by this method effectively reduce their activity on the smooth muscle. It can convert precursors, such as angiotensinogen, into their active form. The endothelium can synthesise and release prostacyclin, which is a vasodilator, and can also release a factor or factors which relax the arterial smooth muscle known as the endothelial derived relaxant factor(EDRF).

Furchgott and co-workers studied the contrasting finding that acetylcholine (Ach) generally was noted to cause vasodilation in vivo but not in isolated blood vessels, and they discovered that the relaxation by Ach of isolated preparations of arteries, which had been precontracted by addition of an exogenous stimulating agent, NA, was strictly dependent on the presence of the endothelial cells (Furchgott, et al., 1981; Furchgott, 1981; Furchgott & Zawadzki, 1980a;b). Their initial studies were carried out on the descending thoracic aorta of the rabbit but subsequently acetylcholine has been shown to

produce endothelium-dependent relaxations in most isolated mammalian pulmonary and systemic arteries (Chand & Altura,1981 a&b; Cohen et al., 1983; Davies & Williams, 1984; De Mey & Vanhoutte, 1981; Furchgott & Zawadzki, 1980 a&b; Lee, 1982).

Using the rabbit aorta Furchgott demonstrated that the endothelium dependent relaxation elicited by Ach was mediated via a high-affinity muscarinic receptor on the endothelial cells which is blocked by atropine (Kb 0.35nM). The endothelium-dependent inhibitory effect of Ach is much less manifest in isolated venous preparations (De Mey & Vanhoutte, 1982). Most studies have been carried out on large arteries, but experiments carried out in smaller arteries or in vascular beds indicate that Ach may induce endothelium-mediated relaxations in resistance vessels as well (Owen & Bevan, 1985). Several types of experiments have been carried out that demonstrate that a chemical factor is released from the endothelium. These have included 'sandwich' or layered preparations as well as bioassay.

In the 'sandwich' experiments vascular strips having a disrupted endothelium were attached to an isometric transducer in the normal manner. Strips having an intact or disrupted endothelium were then wrapped around the tissue. Only when the tissue in contact with the strip attached to the transducer had an intact endothelium did the strip regain its ability to relax to Ach (Furchgott & Zawadzki, 1980; Furchgott et al., 1981). In bioassay experiments the perfusate from an Ach-stimulated rabbit thoracic aorta with an intact endothelium produced dilatation of a de-endothelialized preconstricted rabbit coronary artery (Griffith et al., 1984). By varying the length of the perfusion tubing between the aortic and coronary preparations, the half-life of the EDRF released could be determined. The results obtained varied from 6 to 80 secs depending on the experimental

conditions and the species used (Forstermann et al., 1985; Rubanyi et al., 1985; Rubanyi & Vanhoutte, 1985a; Rapoport & Murad , 1983a).

As well as Ach there are many other agents which require the presence of the endothelium for all or part of their relaxant effects :

1) The calcium Ionophore A23187 has been shown to be wholly dependent on the endothelium for its relaxant action in arterial preparations from rabbit, (Furchgott, 1981; Zawadzki et al., 1980; Singer & Peach , 1982) dog and human (Furchgott et al., 1983), rat (Rapoport & Murad, 1983a) and pig (Gordon & Martin , 1983).

2) Both ATP and ADP produce concentration-dependent relaxations (1-100uM), which are significantly reduced in the absence of the endothelium in the rabbit aorta (Furchgott, 1981; Furchgott et al ., 1983; Furchgott & Zawadzki, 1980) and in the dog femoral artery (De Mey & Vanhoutte, 1981).

3) Substance P is a very potent vasodilator in greyhound and mongrel dog and pig coronary arteries but only in the presence of the endothelium (Cocks & Angus, 1983; Zawadzki et al., 1981). Substance P(3nM) relaxed rings precontracted by 5-HT, NA or U46619 by 90-100% but those to K^+ by only 30-50%.

4) Bradykinin can also cause relaxation which is often endothelium dependent. This is determined however, by the species and tissue studied. It causes relaxation in many different canine and human arteries (Cherry et al., 1982 ; Altura & Chand, 1981) and because it is blocked by indomethacin the effect is thought to be mediated by prostaglandins. However in the canine pulmonary artery (Chand & Altura, 1981) the relaxation is not blocked by the cyclo-oxygenase inhibitors, but both bradykinin- and acetylcholine-induced

relaxation is reduced by high [K^+]. In contrast the relaxation to bradykinin in renal or rabbit artery is not dependent on the presence of the endothelium.

5) Histamine causes relaxation in the rat aorta only if the endothelium is present (Van de Voorde & Leusen 1983). Relaxation to histamine in isolated arteries of the dog is also endothelium dependent. (Toda 1984).

6) In the dog femoral artery (De Mey & Vanhoutte., 1983) thrombin relaxation is dependent on the endothelium.

7) In the rabbit aorta arachidonic acid causes endothelial dependent relaxation (Chand & Altura, 1981; Furchgott et al., 1982,1983; Singer & Peach., 1983) which is not blocked by indomethacin but is blocked by 5,8,11,14-Eicosatetraynoic Acid (ETYA) and Nordihydroguaiaretic Acid (NDGA) which are both lipoxygenase inhibitors. This would seem to suggest that prostaglandins are not involved in the endothelial dependent relaxation.

8) In the dog and pig coronary artery Cocks & Angus, (1983) have shown endothelial dependent relaxations to NA, adrenaline and 5HT. Relaxations to the first two agonists are blocked by phentolamine and RX781094 (Idazoxan) and would suggest an action at α_2-adrenoceptors.

9) Spokas et al, (1983) showed that the relaxation to hydralazine is dependent on the endothelium in the rabbit aorta.

The action of EDRF on vascular smooth muscle:

The relaxations produced by Ach are thought to be caused by an increase in the production of cyclic guanosine monophosphate (cGMP) in the aortae of the rabbit and rat and in bovine coronary arteries. Removing the endothelium abolishes not only the relaxation but also the accumulation of cGMP caused by Ach , but does not affect the relaxant effects of sodium nitroprusside (Rapoport & Murad., 1983b). Compounds which are known to inhibit guanylate cyclase will also block relaxation to Ach in tissues with an intact endothelium (Holzmann, 1982; Mulsch, et al 1985). Also (M&B 22,948) an inhibitor of cGMP phosphodiesterase which breaks down cGMP has been shown to enhance the response to Ach (Holzmann, 1982). Therefore, it would seem that the relaxatory effect of EDRF, released by Ach, is mediated via stimulation of guanylate cyclase which leads to the production of cGMP and the activation of cGMP-dependent protein kinase in vascular smooth muscle. Rapoport & Murad (1984) have demonstrated, in the rat aorta with an intact endothelium, that the pattern of protein phosphorylation induced by Ach was the same as that caused (irrespective of the presence of the endothelium) by sodium nitroprusside. This process could be mimicked by cGMP analogues e.g. 8-bromo-cyclic GMP but not by adenosine mono phosphate (AMP) analogues. The authors' conclusions were that the relaxation induced by both endothelial stimulation and nitro-vasodilators might be mediated through cGMP-dependent protein phosphorylations and through dephosphorylation of the myosin light chain, which may lead to relaxation.

To test whether EDRF-mediated cGMP increases involved direct guanylate cyclase activation Furchgott & Zawadzki (1980a:b), looked at the effect of EDRF on the catalytic activity of a soluble guanylate cyclase preparation which was introduced into the lumen of several arteries along with GMP. By putting

the mixture in tissues having an intact endothelium there was a significant increase in guanylate cyclase activity in comparison to that in a test tube. The addition of Ach produced a marked stimulation of the activity of the soluble guanylate cyclase. The absence of the endothelium abolished this increase.

The finding that guanylate cyclase activity increased, even in the absence of Ach but in the presence of the endothelium, indicates that there is a continuous basal release of EDRF (Furchgott & Zawadzki., 1980 a;b). In bioassay experiments some antioxidants do not affect the release of endothelium-derived relaxing factor, but inactivate it in transit (Griffith et al., 1984). This chemical interaction with EDRF explains the inhibitory effect that haemoglobin (but not methaemoglobin) has on the endothelial dependent relaxations (Rubanyi et al., 1985). Haemoglobin is a large protein which is unable to pass through the cell membrane and so must interact with the EDRF during its presence in the extracellular space (Furchgott et al, 1984). This suggests that the factor is required to be in an oxidised form to be biologically active

Chemical nature of EDRF:

Early studies ruled out adenosine and adenosine monophosphate as possible mediators of endothelium-dependent relaxations (Furchgott & Zawadzki, 1980). Arachidonic acid and its metabolites have been the possible candidates most studied. Phospholipase A_2 is the calcium-sensitive enzyme which produces arachidonic acid from the membrane phospholipids. Quinacrine, an inhibitor of this enzyme, prevents EDRF-mediated relaxations in a variety of blood vessels from different species including canine and human arteries (Cherry et al., 1982; De Mey et al., 1982; Rapoport & Murad, 1983; Rubanyi & Vanhoutte, 1985; Singer & Peach, 1983; Van de Voorde & Leusen, 1983; Furchgott & Zawadzki, 1980; Furchgott et al., 1981). The relaxation is also

blocked by ETYA which is an inhibitor of lipoxygenase. This seemed to suggest that the relaxation produced by Ach may activate a reaction sequence where arachidonic acid or an unsaturated fatty acid is released from the phosphatides and is then oxidised by lipoxygenase in the endothelial cells to a product which is the relaxing factor (Furchgott & Zawadzki, 1980). It was also speculated that EDRF may be a labile hydroperoxide or free radical intermediate product (Furchgott et al., 1981). This seemed to be supported by the findings that Ach relaxation could be blocked by hydroquinone (a free radical scavenger), NDGA (a lipoxygenase inhibitor) and BPB (α-p-Dibromoacetophenone [p-Bromophenacyl Bromide] which inhibits phospholipase A_2). Although this might seem conclusive, these compounds could be producing blockade by a completely different mechanism. This speculation assumes a causal relationship between increases in cGMP levels and relaxation in vascular smooth muscle. Evidence which supports such a relationship has been reported and includes the relaxation of isolated bovine coronary artery by nitric oxide and nitric oxide yielding vasodilators (Kukovetz et al., 1979; Ignarro et al., 1981; Gruetter et al., 1981).

Rapoport and Murad (1983) used spiral strips of rat thoracic aorta incubated in Krebs Bicarbonate solution to study the influence of the endothelium on cGMP levels. Endothelium-intact or disrupted strips were in contact with either NA alone, or in combination with Ach, A23187, or histamine for varying times before freezing in liquid nitrogen. In tissues having only NA present , those with an intact endothelium showed slightly higher levels of cGMP than those without endothelium. However, in the presence of any of the relaxant agents, in tissues with the endothelium intact, cGMP levels increased 20-40 fold compared with those from disrupted tissues. Sodium nitroprusside produced an approximately 100-fold increase in both rubbed and unrubbed preparations. Ach did not produce

any change in levels of cAMP.

If EDRF is a metabolite of arachidonic acid its production is likely to be reduced by inhibition of cyclo-oxygenase, however this is not consistant with the observation that several different inhibitors of this enzyme have no effect on EDRF-mediated relaxation. If phospholipase activation, followed by the release and metabolism of arachidonic acid, produces EDRF then the relaxation produced should be dependent on extracellular calcium. The calcium ionophore A23187 in rabbit aorta is 10-30 times more potent than Ach at producing relaxation. Like Ach, however, its effect is not inhibited by cyclo-oxygenase inhibitors. Release of a factor from the endothelium by this compound has been demonstrated by replacement of Ach by the ionophore in the previously outlined 'sandwich' experiment. However unlike that to Ach, the relaxation to A23187 is not inhibited by quinacrine (Furchgott & Zawadzki, 1980; Singer & Peach, 1983). It has been postulated that quinacrine may interfere with Ach relaxation not by enzyme inhibition directly but possibly by an interference with the ion fluxes or Ca^{++} coupling which are induced by an action on the muscarinic receptor (Furchgott et al., 1981; Singer & Peach , 1983). Because of these results Furchgott hypothesised that an increase in calcium ions in the vicinity of a Ca^{++} activated enzyme may be an early step in the sequence of reactions bringing about the release of EDRF by both Ach and A23187.

A study by Singer and Peach (1982) has shown a critical role for calcium. Eliminating Ca^{++} from the incubation medium inhibited methacholine-induced relaxation (of phenylephrine-induced contractions) by 67% and A23187-induced relaxation by 92%. They attribute the smaller attenuation of receptor-induced relaxation to a greater access to a 'Ca^{++}-pool' than has 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^{++} across the membrane directly rather than via the calcium channels. They speculate that it may reflect direct interaction between the blockers and A23187.

Rubanyi & Vanhoutte, (1985b) have demonstrated in canine coronary artery that both hypoxia and anoxia cause release of a vasoconstrictor substance from endothelial cells. It is possibly release of this substance which accounts for the inhibition of the endothelium-dependent relaxation and may explain why no relaxation to Ach is seen in this tissue.

Most recent ideas on the nature of EDRF have suggested that it is in fact nitric oxide (NO), Furchgott, R.F. (1987). A series of experiments by Palmer et al, 1987, have shown that EDRF and NO have very similar properties. They demonstrated their similarities using EDRF obtained from cultured porcine aortic endothelial cells and solutions of NO obtained from NO gas. They measured the activity of both compounds via a bioassay cascade system as described earlier using rabbit aorta as the bioassay tissue. They found that the activity of both EDRF and NO deteriorated at the same rate as it passed down the system. The relaxation of the bioassay tissues was the same whether EDRF or NO was used. Also both relaxations were blocked by haemoglobin and enhanced by superoxide dismutase. Due to these various similarities the authors suggest that EDRF and NO are identical, i.e. EDRF is NO . In another recent review Paul Vanhoutte, (1987) also suggests that EDRF is NO. However, he puts forward the possibility that there may in fact be more than one EDRF, and proposes as a likely candidate ammonia (NH_3). This is because endothelial cells possess several metabolic pathways that can lead to its formation and also it is a potent relaxant of vascular smooth muscle.

Although much of the work on EDRF has been carried out on large arterial

vessels, it was important to find out whether EDRF could be released from endothelial cells on veins and the small resistance vessels. If EDRF has any physiological effect then it should also be present in these small vessels which are important in the control of overall blood pressure. De Mey & Vanhoutte, (1982) showed that in the isolated rings of various canine veins very little or no relaxation to Ach was produced. In the pulmonary vein Ach produced contraction rather than relaxation. In the splenic vein the relaxations seemed to be independent of the endothelium. An explanation of the small relaxatory responses could be the masking by strong contractile effects. However the inability to cause relaxation is also seen with bradykinin and A23187 and therefore the reason is unclear. In a preparation of the perfused isolated arterial vasculature of the rabbit mesentery, (a modified preparation by Blumberg et al., 1977) preconstricted by continuous perfusion of NA, Carvalho & Furchgott (1981) found that vasodilation was produced by additions of either Ach or A23187. They also found that the relaxation was still present even when a cyclo-oxygenase inhibitor was included in the perfusate. They then passed collagenase through the bed in the perfusate to remove the endothelium and found that they lost the response to Ach but never eliminated the vasodilator response to A23187. Byfied et al, (1986) used the perfused rat mesentery to demonstrate dose-dependent relaxation to Ach. They showed that it was endothelium dependent by perfusing with sodium deoxycholate which removed the endothelial cells and the response to Ach but not those of sodium nitroprusside.

The influence of the endothelium on contractile responses of vascular smooth muscle:

The presence of the endothelium has been shown to reduce the sensitivity of the rat aorta and many other arterial preparations to both Phe and NA but not to

alter significantly the maximum response to these agents (Martin et al., 1986). These two compounds are strong agonists for the α_1-adrenoceptor. In tissues having a disrupted endothelium, clonidine, an α_1- adrenoceptor partial agonist, had an increase in sensitivity and maximum response when compared to their intact counterparts, (Allan et al.,1983; Egleme et al., 1984; Godfraind et al., 1985; Lues & Schumann., 1984; Carrier & White., 1985). This inhibitory effect of the endothelium is thought to be due to the relaxatory action of EDRF (Furchgott & Zawadzki.,1980; Furchgott., 1983). Haemoglobin (Hb), which will block the actions of EDRF (Martin et al ., 1985 a&b), potentiated the responses of all the agonists in endothelium-intact preparations to that of their disrupted couterparts (Martin et al 1986). Since the difference in size of the response between disrupted and intact preparations is greater when clonidine is the agonist used it has been proposed by Egleme et al (1984) that clonidine may be acting on α_2-adrenoceptors on the endothelial cells to release EDRF : Cocks & Angus (1983) had demonstrated this in canine and porcine coronary arteries. Miller et al (1984) also demonstrated a large potentiation of the contractile response on removal of endothelium using the selective α_2-agonist BHT-920. However evidence against this hypothesis has been found in the rat aorta by Martin et al (1986) and Godfraind et al (1985), who have both failed to obtain endothelium-dependent-relaxation to α_2-agonists. Also Lues & Schumann (1984) showed that the selective α_2-antagonist rauwolscine did not mimic the effects of removing the endothelium on the ability of the tissues to contract to an agonist.

A common property among the α-adrenoceptor-agonists whose maximum contraction is significantly reduced by the endothelium is that they are all partial agonists in the rat aorta relative to the full agonists noradrenaline (NA) and phenylephrine (Phe) (Ruffolo et al ., 1979; Digges & Summers., 1983; Lues &

Schumann., 1984). A partial agonist has a low intrinsic efficacy compared to a strong agonist: using the methods developed by Furchgott & Bursztyn (1967), Ruffolo et al (1979) were able to obtain the estimate that clonidine had only 2% of the intrinsic activity of Phe. This meant that clonidine would have to occupy fifty times as many α_1-receptors as Phe to produce an equivalent response.

Martin et al (1986) used dibenamine to inactivate a proportion of the α_1-receptors and thereby reduce the relative efficacy of Phe to a similar level to that of clonidine: now removal of the endothelium showed a significant potentiation of the responses to Phe. More evidence from cGMP measurements was found by Martin et al (1986) against the idea that α_2-adrenoceptor agonists stimulate release of EDRF. They confirm the report by Rapoport & Murad (1983) that Ach caused a rise in cGMP content of aortic rings and showed that neither Phe nor clonidine caused a rise. However Miller et al (1984) & Biguad et al (1984) found that clonidine, BHT-920 and methoxamine produced small increases in the cGMP content of rat aorta. The significance of the disparate observations remains unclear. If stimulated release of EDRF by the agonist is not responsible for the depression of its responses in the presence of the endothelium then another possibility is that it could be due to the basal release of EDRF. This was first discovered in experiments using the rabbit aorta where Hb potentiated contractions to agonists only when the endothelium was present (Martin et al 1985,a&b). It has now been shown in the rat where the effect of Hb was even more pronounced: Whether the sensitivity of this vessel to EDRF is greater or whether there is more of it released is still unclear.

Possible role in Hemostasis:

There is evidence that substances generated during the process of haemostasis can result in endothelium-dependent relaxations. Thrombin, produced during coagulation, causes endothelium-dependent relaxations in basilar, coronary, femoral, saphenous, splenic and pulmonary arteries of the dog and in aorta of the rat (De Mey et al., 1982; Katusic et al., 1984; Ku., 1982; Rapoport et al., 1984). The relaxation to thrombin can be prevented by heparin but not by inhibitors of cyclo-oxygenase It can however be counteracted in many tissues by a direct contractile action on the smooth muscle by a factor released from the endothelium (Ku., 1982; De Mey et al., 1982). Platelets carry nearly all the serotonin in blood and this is released during aggregation. Most blood vessels will contract to serotonin but in the canine coronary artery both in vivo and in vitro the contraction is diminished in the presence of the endothelium (Brum et al 1984, Cohen et al 1983). As well as serotonin, platelets release large quantities of adenosine di- and triphosphate (ADP and ATP) during aggregation. These substances can induce endothelium-dependent relaxations in arterial tissues such as canine femoral and coronary arteries, pig and rabbit aorta (De Mey et al., 1982; De Mey & Vanhoutte, 1981;1983; Gordon & Martin 1985; Furchgott, 1981). Apyrase, an enzyme which breaks down ADP and ATP inhibits their relaxations, and will almost abolish relaxations in response to platelets

NA and certain 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 & Angus, 1983; Miller & Vanhoutte, 1984). Also in the canine coronary artery the β-relaxatory effect is reduced after removal of the endothelium (Rubanyi & Vanhoutte, 1985b). Whether this

effect on β-receptors is due to the basal release of EDRF or β stimulated release on the endothelial cells is still unknown. The existence of relaxation produced via α_2- receptor and β-receptor stimulation may possibly explain why catecholamines produce vasodilatation in certain vascular beds. 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. It follows that contractions to agonists which are not selective, between α_1 and α_2-adrenoceptors, especially NA, will be increased in tissues without endothelium. Many workers have looked at the effect of removal of the endothelium on the responses of a variety of agonists selective for both α_1 and α_2 adrenoceptors. In all cases removing the endothelium caused an increase in sensitivity to the agonists with , in some cases, especially with the α_2 agonists, great increases in maximum responses (Carrier & White, 1985; Egleme et al 1984; Bullock et al 1985; Martin et al 1986).

Endothelial Derived Contractile Factor (EDCF):

Busse et al (1983) showed that in intact perfused coronary arterial segments, smooth muscle relaxation elicited by an intraluminal hypoxia is completely abolished after removal of the endothelium. They came to the conclusion that the dilatation to hypoxia was produced by a specific endothelial mechanism. They also found that pretreatment with indomethacin significantly reduced the hypoxic-induced dilatation. This led them to suggest that PGI_2 from the endothelium was the possible mediator. A further study was carried out by Busse et al (1984) on the tail artery of the rat, canine femoral artery and epicardial canine coronary arteries. They found, in all vessels having an intact endothelium, at least a two fold increase

in 6-keto $PGF_{1\alpha}$ release during hypoxia. Concentration-effect curves showed large differences in the sensitivity of the different vessels to the prostaglandins. These differences were explained to some extent by species differences, along with regional differences in sensitivity to prostaglandins in the same animal (Forstermann et al 1984). They concluded that the hypoxic dilatation was endothelium dependent and may be produced via release of vasodilatory prostaglandins from endothelial cells.

In another study by Rubanyi & Vanhoutte (1985), they carried out experiments to determine the role of the endothelium in the facilitation by anoxia of contractile responses of isolated coronary arteries. They found that coronary arteries without endothelium could be contracted by hypoxia or anoxia when layered with femoral arteries and veins with endothelium . This experiment also demonstrated that the anoxic facilitation was not due to release of a vasoactive substance from components of the blood vessel wall other than the endothelium. However they found that indomethacin did not inhibit these responses to anoxia and hypoxia ruling out the production of endothelial vasodilatory prostanoids. They also found that inhibitors of the production or action of endothelial derived relaxant factor did not affect the anoxic augmentation. They concluded therefore that anoxia and hypoxia caused the release of a vasoconstrictor substance from the endothelial cells.

Functional importance

How the endothelium may be involved in particular disease states which involve malfunction of vascular blood vessels is an important consideration. The condition of hypertension is thought to be associated with an increase in the vascular resistance of most organ systems. However, whether the main bulk of the resistance occurs in the larger blood vessels or the smaller vessels is of some importance .i.e. does an isolated or overall vascular problem result in the

development and continuation of hypertension? There are many different ways of looking at the possible changes which may occur e.g. any structural changes in the vessel walls. Also in vivo work has been carried out in most major vascular beds examining any changes in vasoconstriction. Alterations in responsiveness to various endogenous vasodilators and constrictors are found in hypertension. These changes have been the subject of many studies using different models of experimental hypertension in many species. Vascular hyperreactivity to various pressor agents have been reported in renal (Phelan, 1966; McGregor & Smirk, 1970), desoxycorticosterone (DOCA)/sodium chloride (Sturtevant, 1956; Beilin & Wade, 1970) and in genetically hypertensive rats (Laverty, 1961; Haeusler & Haefly 1970). However, in contrast with these results several other reports have shown that aortae from hypertensive rats do not show any supersensitivity to NA, (Redleaf & Tobian, 1958; Mallov, 1959; Clindeschmidt et al., 1970).

Most workers in the past have looked solely at the effects of hypertension on constrictor agents when the importance of the endothelium had not been fully elucidated. More recent studies have examined the effects of hypertension on the response to relaxatory agents, (Cohen & Berkowitz, 1976; Konishi & Su, 1983; Shibata & Cheng, 1978; Triner et al., 1975). However these reports paid little attention to the effects of hypertension on the vascular endothelium and the intima and how they may alter the function of the smooth muscle cells.

Another disease which results in vascular malfunction is diabetes. Nearly 80% of all deaths in diabetic patients can be accounted for by cardiovascular diseases (Kessler, 1971), which are two to three times greater than in the normal population. In experimentally induced diabetes in animal models it has been identified that there are specific myocardial lesions. Several studies have been carried out using various preparations comparing diabetic hearts to control hearts, e.g. isolated rat heart

(Lopaschuck et al., 1983b; Vadlamudi & McNeill, 1984) papillary muscles (Ramanadham & Tenner, 1983), which show decreasing heart rate, cardiac output and pressure development in the intact heart with depressed rates of shortening and relaxation in cardiac muscle. Other studies looking at what causes the cardiac dysfunctions of diabetes have shown depressed contractile protein activity (ATP-ase) (Rubinstein et al., 1984; Fein et al., 1981; Malhorta et al., 1981), and also reduced levels of sarcolemmal and sarcoplasmic reticulum membrane activity for transportation of calcium (Lopaschuk et al., 1983a; Pierce et al., 1983).

One of the cardiovascular diseases linked to diabetes mellitus is atherosclerosis. Work by Ruderman & Haudenschild, 1984 has shown that the origins of the condition lie not only in changes in the histology of the endothelial cell but also in changes in its functional ability. Electron microscope studies in vessels which are subjected to the action of permeability factors show that in normal animals the interendothelial spaces are easily found but they are rare in diabetic animals (Garcia Leme et al., 1974). All these results would seem to suggest changes in the properties of the endothelial cells in diabetic animals compared with control animals. How these changes affect the reactivity of particular vessels can be seen through the varying results obtained by different groups. Changes in both constrictor and relaxatory agents have been seen. Fortes et al (1983), found that using the thoracic aorta from alloxan-diabetic rats, in preparations where the endothelium was disrupted similar results were obtained from normal and diabetic animals. In the diabetic aortae which had their endothelium intact the pD_2 values were markedly increased in comparison with normal tissues i.e. the tissues were less sensitive. They also found that the relaxatory response to Ach and histamine were equally effective in control and diabetic tissues providing the endothelium remained intact. This report contrasts with that from Oyama et al 1986, who using streptozotocin treatment , which gave a similar level of hyperglycemia found a

decrease in sensitivity to Ach in the diabetic animals with a similar decrease seen using histamine. They also used the endothelial independent relaxatory agent sodium nitroprusside and found no change in sensitivity between the control and diabetic groups using this agent.

The aim of this thesis was to examine as fully as possible the effects of the presence of the endothelium on sensitivity to contractile agents in the isolated rat thoracic aorta. I also wanted to examine how the initial tension placed on the tissues affected their responses to agonists and whether the endothelium had any influence on this length-tension relationship. Following from these observations I was interested in how the size of the induced tone and the effects of drugs which can interfere with it can alter the sensitivity of the tissue to relaxation by Ach. Since I knew calcium was essential for both contractions produced by agonists and also release of EDRF I examined sensitivity of contractile agents to calcium using both a buffered and unbuffered system. How the endothelium affected this relationship was also examined. The influences of both a calcium facilitating drug (BAY-K 8644) and a calcium channel antagonist (nifedipine) were looked at. The final series of experiments were carried out at Glaxo Group Research . The purpose of these experiments was to examine the effects of various induced cardiovascular diseases (hypertension and diabetes) on the sensitivity of intact and disrupted preparations to both contractile and relaxatory agents. If as described previously EDRF is considered important in the regulation of blood flow and the maintainance of blood pressure it should have an effect in small blood vessels like arterioles and capillaries. We therefore examined the effects of cardiovascular disease of responsiveness of the isolated perfused rat tail to both contractile and relaxatory agents.

MATERIALS and METHODS

General

In all experiments (except those carried carried out at Glaxo Group Research) male Wistar rats (240-280 grammes) were used. The rats were killed by stunning and cervical dislocation , with care taken not to stretch the aorta.. A large length of the descending thoracic aorta was then removed and placed in cold Krebs Bicarbonate solution where any remaining connective tissue could be cleared away. Rings of length 2-3mm were then cut. In experiments where the endothelium was intended to be disrupted this was done by placing the ring on the end of one prong of a roughened pair of forceps and gently rotating it round the tip several times, to ensure as much of the endothelium was removed as possible. In some cases histology was carried out to determine the extent of removal . The tissues were then mounted in a 30ml organ bath containing a Krebs Bicarbonate solution of the following composition (mM: NaCl 119, KCl 4.7, $NaHCO_3$ 24 .8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 11.1, EDTA 0.023 which was maintained at 37°C and bubbled with a gas mixture 95% O_2/5% CO_2. The tissues were positioned on two stainless steel hooks, one of which was attached to a platform fixed in position, the other attatched to a piece of thread which was tied firmly to an isotonic Grass FTO3 transducer for force measurements and recorded on a Linseis 6-channel recorder. Initially all tissues were placed under 1.5 gram tension, (except in the tension studies) from which most tissues relaxed to some extent. However, resting tension was readjusted to 1.5 grams before experimentation. After being left for one hour and having been subjected to three washes the tension on the tissues was normally between 1.3 and 1.7 grams. This was the basic initial method used for setting up the tissues. The protocols followed after this point depended on the experiment being carried out and are described in the relevant chapters.

Drugs

The drugs used and their sources were: Noradrenaline bitartrate, acetylcholine chloride, sodium nitroprusside, haemoglobin (bovine), (-) phenylephrine hydrochloride, clonidine hydrochloride, 5-hydroxytryptamine, $PGF_{2\alpha}$, angiotensin II (Acetate salt), ethylene glycol Bis-B Aminoethyl ether N,N,N',M'- tetraacetic acid (EGTA), nitrilotriacetic acid (NTA), (Sigma Chemicals): atropine sulphate, ethylene diaminetetra-acetic acid (disodium salt) (EDTA), (BDH Laboratories): Ficoll 70 (Pharmacie Fine Chemicals), Wyeth 26703 (Wyeth), prazosin HCl (Pfizer), corynanthine (Phase separation labs.), rauwolscine HCl (Roth), BAY-K 8644, nifedipine, xylazine HCl (Bayer), UK14304 (Pfizer), BHT 920 (Boehringer Ingelheim), cocaine HCl (McCarthy's), U46619(Gift from Mike Drew, Glaxo Group Research).

Drugs were dissolved in distilled water with the following exceptions: Noradrenaline bitartrate was dissolved in 23μM EDTA in distilled water; both nifedipine and BAY-K 8644 were dissolved first in a small volume of absolute alcohol then made up with distilled water. $PGF_{2\alpha}$: a stock of 3mM solution i.e. 1mg/ml was made: for every 1mg of solid, 100μl ethanol was added; to every 100μl ethanol, 900μl of molar Na_2CO_2 was added. After dilution a grain of solid Na_2CO_2 was added. This was frozen and stored.

CHAPTER 1

THE INFLUENCE OF THE ENDOTHELIUM ON RESPONSES TO SELECTIVE AGONISTS AND ANTAGONISTS

INTRODUCTION

It has long been recognised that the presence of the endothelium has a modulatory role in the responsiveness of different tissues to various contractile agents. The significance of this role is dependent upon the agonist being studied, the tissue and also the species. The rat aorta has been studied by many workers using various different selective α_1 and α_2- adrenoceptor agonists. The results obtained from most workers (Godfraind et al 1985; Martin et al 1986; Bullock et al 1986; Carrier & White 1985; Egleme et al 1984), pointed to α_2-adrenoceptor agonist responses being potentiated to a far greater extent than those to α_1-adrenoceptor agonists on removal of the endothelium. This led Egleme et al (1984) to suggest that, similarly to the results of Cocks & Angus (1983) in the coronary arteries, there were α_2-adrenoceptors on the endothelium which stimulated release of EDRF. Martin et al (1986) however, suggested that partial agonists were better inhibited by spontaneously released EDRF than full agonists due to their poorer efficacy at the α_1-adrenoceptor. As well as affecting the responses to agonists, the presence of the endothelium has been suggested to be the reason for the differential effects (against NA) of prazosin, a selective α_1-adrenoceptor antagonist, in the rat aorta . Alosachie & Godfraind, (1986) found that prazosin acted as a non-competitive antagonist when the endothelium was present but competitively when the endothelium was absent. The following study was carried out to examine the influence of the endothelium on responses to both selective agonists and antagonists.

MATERIALS AND METHODS

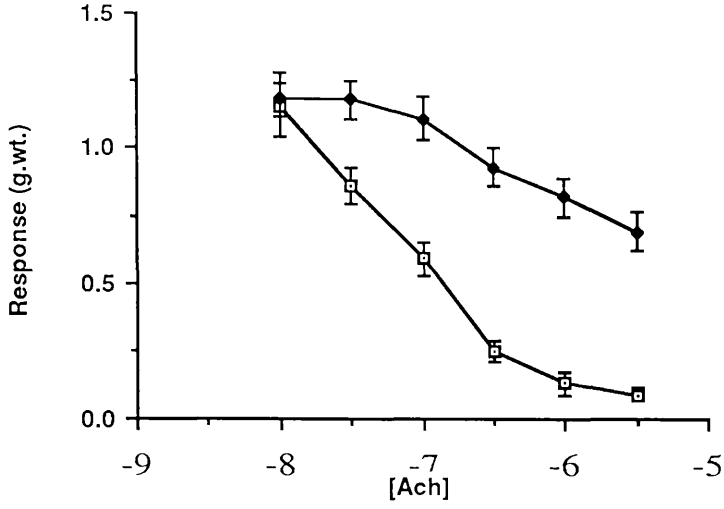
Agonist studies

Tissues were set up as described previously and allowed to equilibrate for approximately one hour. In all cases except when using U46619 (a thromboxane mimetic) the first concentration-response curve in the tissue was obtained to NA (3nM-10μM). The tissues were then washed three times and allowed to rest for 30mins . Then a concentration of NA which had previously been shown to contract the tissues by approximately 80% was added until the contraction was well maintained. ACh , (3μM) was then added to the bath to test the integrity of the endothelium: this was recognised by the presence of a relaxatory response to the ACh. In some tissues in which the endothelium had been removed Ach caused a further contraction . The tissues were again washed and equilibrated for a further 30 minutes after which a cumulative-concentration response curve to the second agonist was carried out. When the agonist used was U46619 it was the first and only agonist that each tissue was exposed to. Endothelial integrity was again tested but using U44619 as the contractile agent instead of NA. In general ACh relaxation was less effective when the contractile agent was U46619 (Figure 1). The effect of Hb(10^{-5}M) was examined, which is known to inhibit the action of EDRF, on responses to NA, Phe and clonidine. A cumulative- concentration-response curve was constructed to the agonist then repeated it in the presence of Hb at a concentration which inhibited relaxation to ACh (3μM).

Antagonist studies

An initial set of experiments were carried out to determine if the

Figure 1



This graph shows how relaxation to Ach is significantly reduced when U46619 is used as the contractile agent instead of NA. The first point on the curve represents the tone induced by the agonist. The results are expressed in g.wt. Values are given as mean ± s.e.m. (n=8). NA-□ U46619-◆

responsiveness to NA changed with time in both rubbed and unrubbed preparations. These results showed no significant changes in pD_2 values, and only a slight but non-significant increase in the maximum response. Therefore in subsequent experiments, progressively increasing concentrations of the antagonist were added to each preparation which thus acted as its own internal control.

Protocol

A control concentration-response curve to NA was carried out in each tissue. It was then washed three times and allowed to rest for one hour. The antagonist was then added and left in contact with the tissue for thirty minutes after which the agonist concentration-response-curve was repeated. This process was repeated twice using two progressively higher antagonist concentrations.

Cocaine was not routinely added to the bathing fluid as preliminary studies had shown that the presence of cocaine (3μM), a concentration which blocks neuronal uptake of NA did not potentiate the effect of NA on this tissue (Figures 2a,b) . Propanolol (1μM) was used in the antagonist studies involving prazosin and corynanthine to prevent NA β-adrenoceptor activation especially at higher concentrations. The effect of the β-adrenergic agonist isoprenaline in both rubbed and unrubbed preparations was studied. The tone was raised with 30nM NA until the response reached a maximum, at which point the isoprenaline was added cumulatively.

Figure 2 a

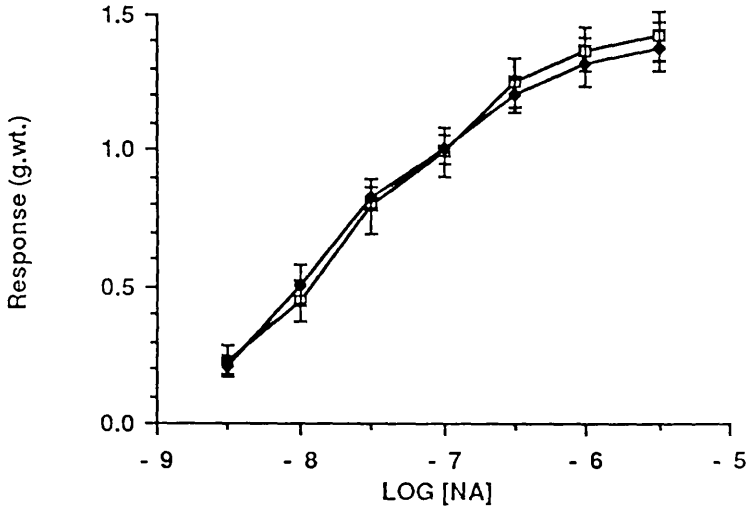
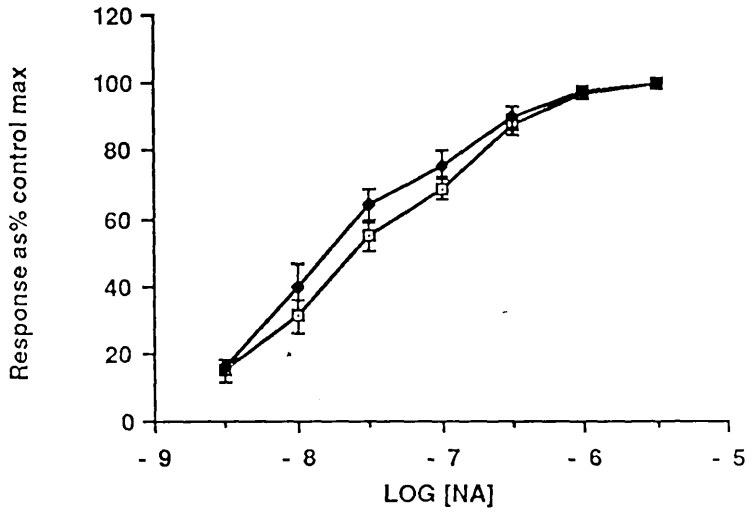


Figure 2b



Both graphs illustrate the effects of cocaine (3μM), on responses to NA in rat aortic rings with an intact endothelium . Results are expressed in both (g.wt.) (Figure 2a) and as % of the maximum (Figure 2b). Values are given as mean + s.e.m. (n=6). Control-⊡ +(3μM) cocaine-◆

STATISTICAL ANALYSIS

Agonist studies

Results obtained from intact and disrupted aoric rings were compared with each other for each agonist studied at each concentration of drug. Maximum responses and pD_2 values from all agonists were compared with their corresponding values with NA. Statistical significance was determined using the paired t-test with values as follows *P<0.05 **0.01<P<0.001 ***P<0.001.

Antagonist studies

Results obtained in the presence of the antagonists were compared with control in the same tissue. Statisitical significance was determined using the paired t-test with values as above.

RESULTS

Agonist Studies

Table 1 shows the pD_2 values of the agonists used in both tissues with an intact (+E) and disrupted (-E) endothelium (paired tissues). Paired tissues were compared rather than mounting both tissues initially with the endothelium intact then taking them down and removing the endothelium from one, this saved time and possible damage to the tissues through being handled too much. The table also shows the maximum response developed by the tissues to each agonist (g.wt.). The results are also illustrated in Figures 3-9 as both a percentage of the maximum response to NA in each tissue and also in absolute terms (g.wt.). From the results it can be seen that the endothelium plays a more significant role in the regulation of the responses for some agonists than for others. At the extremes, for clonidine the difference was greatest and for U46619 there was no significant difference between the responses in endothelium intact and disrupted tissues. Phe and NA responses were affected to a similar extent by the presence of the endothelium, although Phe was slightly less potent and produced a slightly smaller maximum response. The responses to 5-HT were also affected to a similar extent as NA but the sensitivity was some 100 times less. The responses to both clonidine and UK14304 were greatly increased on removing the endothelium but did not reach a similar maximum to that of NA. However, no response could be obtained to the other α_2- agonists xylazine and BHT920 in the tissues from the six animals on which they were tested. $PGF_{2\alpha}$ produced a small contraction which was significantly increased on removal of the endothelium but with the highest response being only 40% of that to NA. However its true maximum was not attained within the range tested. When angiotensin II was added

Figure 3

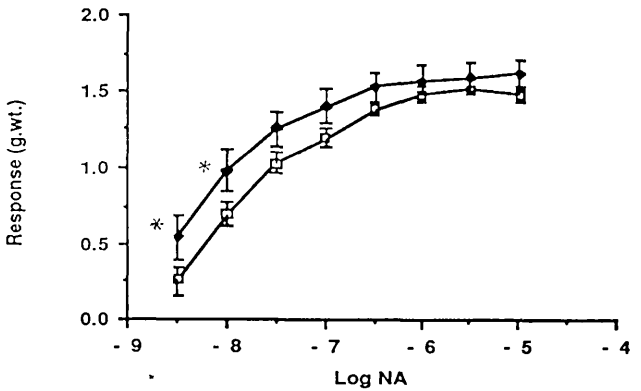


Figure 4a

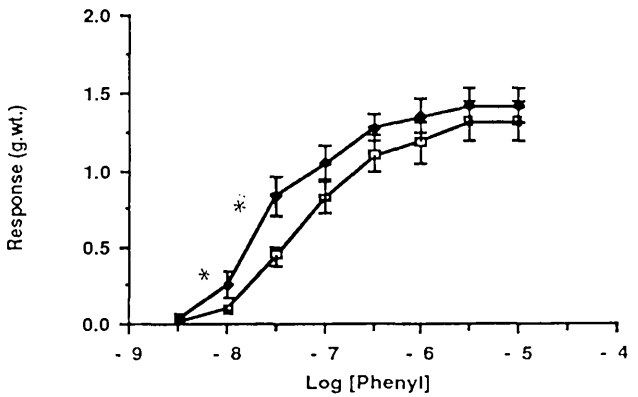


Figure 4b

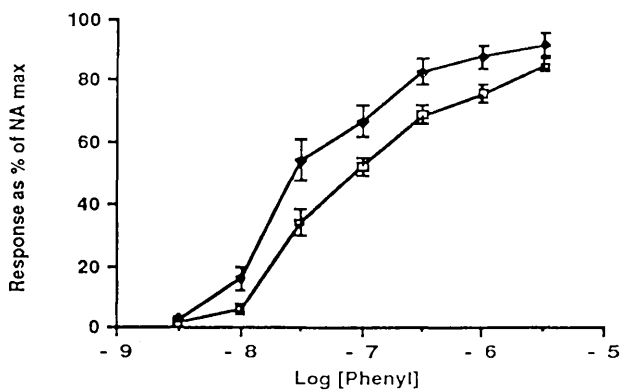


Figure 3 shows the effect of removal of the endothelium on responses to NA . Figure 4a shows the effect of removal of the endothelium on responses to Phe. Results in both figures are expressed in g.wt. Values are given as mean + s.e.m. (n= 8 &10 respectively). Statistical analysis was carried out comparing values from rings with endothelium disrupted to rings with endothelium intact. Results to Phe are also expressed as a % of the maximum response to NA in the tissue (Figure 4b). +E - □ -E- ◆

(Statistical significance was determined as follows: * $P<0.05$ ** $0.01>P>0.001$ *** $P<0.001$)

Figure 5 a

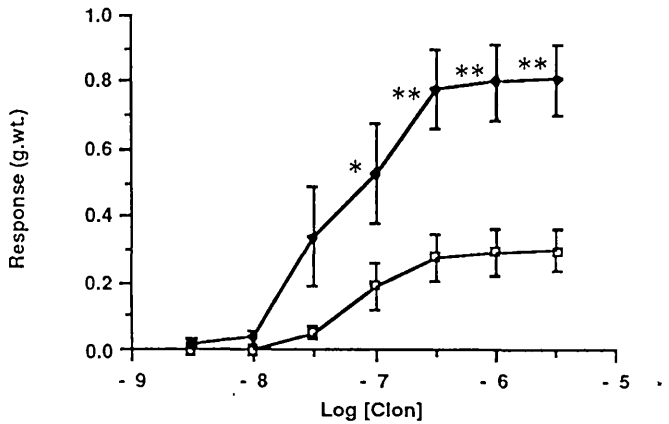


Figure 5 b

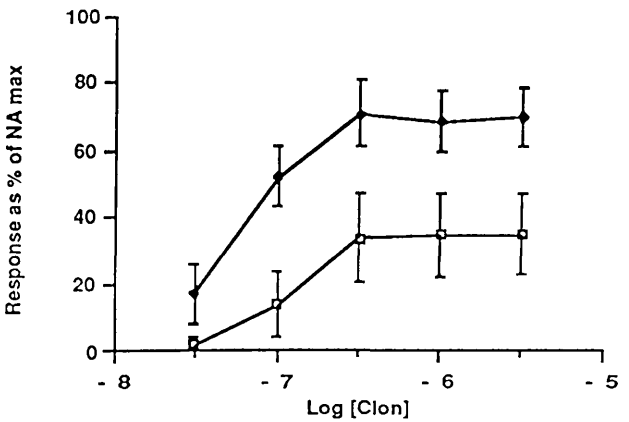


Figure 6a

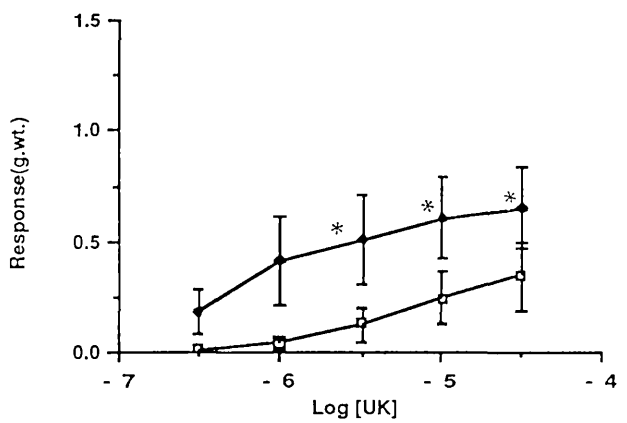
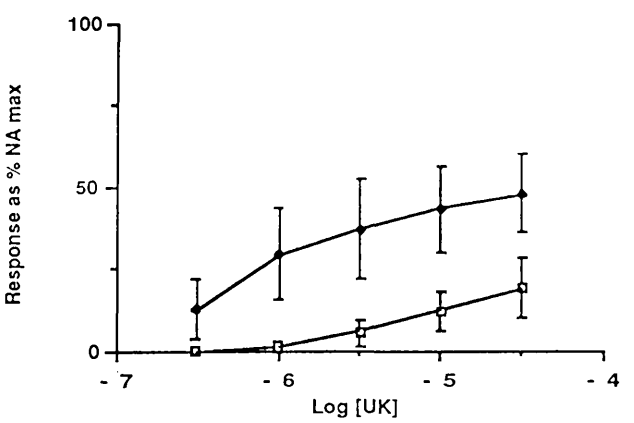


Figure 6 b



These figures show the effect of removal of the endothelium on responses to clonidine and UK14304. Results are expressed in g.wt. (Figures 5a, 6a). Statistical analysis was carried out comparing values from rings with endothelium disrupted to rings with endothelium intact. Results are also expressed as a % of the maximum response to NA in the tissue (Figures 5b 6b). Values are given as mean ± s.e.m. (n=8 & 6 respectively).
+E-□ -E-◆ Statistical significance was determined as follows: *P<0.05 **0.01<P<0.001 ***P<0.001.

Figure 7a

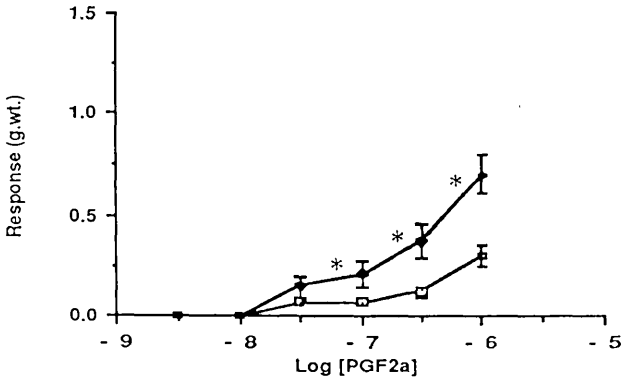


Figure 7 b

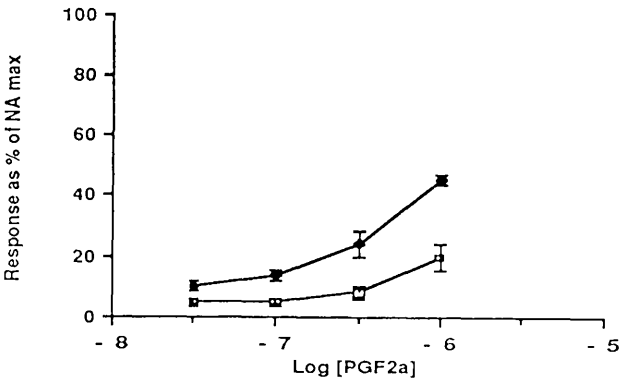
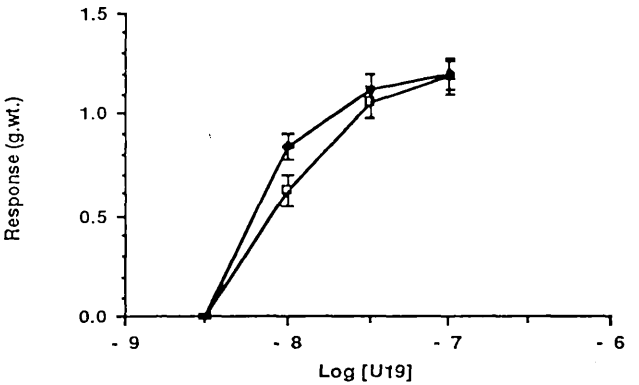


Figure 8a



These figures show the effect of removal of the endothelium on responses to PGF2a and U44619. Results are expressed in g.wt. (Figures 7a,8a). Results were also expressed as a percentage of the maximum response to NA in the tissue for PGF2a only (Figure 7b). This was impossible for U46619 since it was the only agonist used on the tissue.Values are given as the mean ± s.e.m. (n=4 & 8 respectively). Statistical analysis was carried out comparing tissues with endothelium disrupted with endothelium intact. +E- □ -E- ◆
(Statistical significance was determined as follows: * P<0.05 ** 0.01>P>0.001 *** P<0.001)

Figure 9a

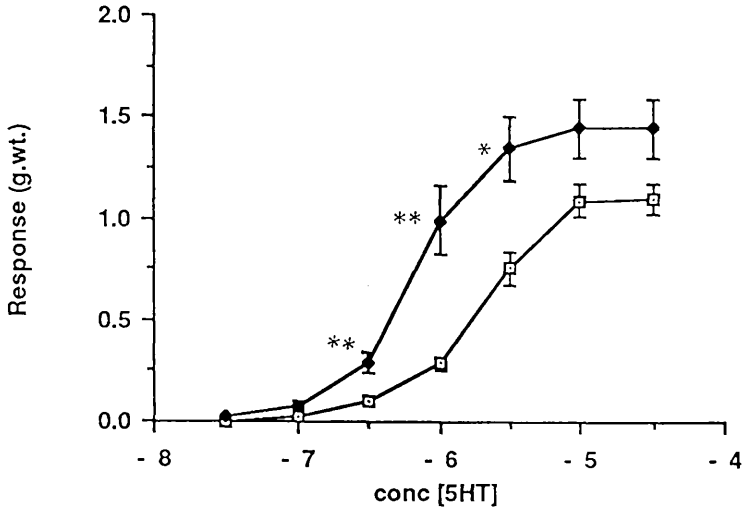
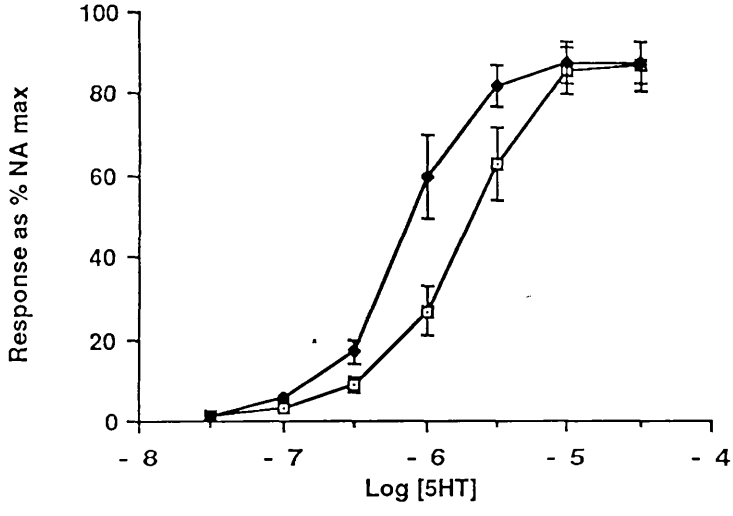


Figure 9b



These figures show the effect of removal of the endothelium on responses to 5HT. Results are expressed in g.wt. (Figure 9a). Results are also expressed as a % of the maximum response to NA in the tissue (Figure 9b). Values are given as the mean ± s.e.m. Statistical analysis was carried out comparing tissues with endothelium disrupted with endothelium intact. +E-◆ -E-⊡

(Statistical significance was determined as follows: * P<0.05 ** 0.01>P>0.001 *** P<0.001)

TABLE 1

Agonist	pD_2 Value + end	pD_2 Value - end	Max Response(g.wt.) + end	Max Response(g.wt.) - end	
Noradrenaline	7.92±0.03 *P	8.29±0.02	1.56±0.03	1.64±0.03	(n=58)
Phenylephrine	7.28±0.07 *P *S	7.60±0.07 *S	1.31±0.14	1.42±0.10	(n=10)
Clonidine	6.90±0.12 *P *S	7.30±0.15 *S	0.30±0.06 *P *S	0.81±0.11 *S	(n=8)
UK14304	5.21±0.16 *P *S	5.90±0.20 *S	0.34±0.16 *P *S	0.65±0.18 *S	(n=6)
Xylazine	no response(0.1-100uM)		no response(0.1-100uM)		(n=6)
BHT-920	no response(0.1-100uM)		no response(0.1-100uM)		(n=4)
5-HT	5.70±0.07 *P *S	6.20±0.08 *S	1.10±0.08 *P *S	1.46±0.15	(n=6)
PGF_{2a}	6.48±0.06 *S	6.60±0.10 *S	0.30±0.05 *P *S	0.70±0.10 *S	(n=4)
U44169	8.07±0.05	8.28±0.08	1.18±0.08 *S	1.20±0.08 *S	(n=8)
Angiotensin II	tachyphylaxis		tachyphylaxis		(n=6)

This table shows the pD_2 values for various agonists in the rat aorta with either an intact or disrupted endothelium. It also shows the maximum responses to each agonist with either an intact or disrupted endothelium. Results are expressed in g.wt. Values are given as mean ± s.e.m. (n values in brackets). Statistical analysis was carried out by comparing both pD_2 values and maximum responses beween tissues with an intact and disrupted endothelium and also with the various agonists compared to NA. Statistical significance was determined as follows:

*P- The value is significantly different from the intact tissue contracted with the same agonist.

*S- The value is significantly different from the corresponding tissue contracted to NA.

All values are $P<0.05$.

cumulatively the responses developed tachyphylaxis regardless of whether the endothelium was intact or disrupted and so this was not examined further ; some workers have found that initial depolarization with high concentrations of potassium or a threshold concentration of NA (Bullock et al 1986) prevents this tachyphylaxis from occurring. The thromboxane mimetic drug U46619 showed no difference in the potency of response in tissues with an intact or disrupted endothelium. Hb(10^{-5}M) potentiated the responses to all three agonists significantly only when the endothelium was present . When the endothelium was disrupted there were slight increases in the responses but they were not significant. Hb potentiated the responses to NA to a greater extent than those to either Phe or clonidine (Figures 10-12). The smaller potentiation of responses to clonidine which contrasts with that of Martin et al 1985 emphasises the relative inability at least under these conditions of clonidine to cause contraction regardless of an intact or disrupted endothelium. We also were able to obtain small contractile responses to Hb alone in the absence of any tone. This effect was seen in both intact and disrupted tissues but was greater when the endothelium was intact (Figure 12c). This figure also shows how Hb blocks responses to Ach, and how it can produce a further contraction on top of tone induced by NA. This contraction was greatest when the induced tone was smaller.

Antagonist Studies

From the results of the time control curves to NA (Figures 13a,b) it can be seen that there was a slight increase in maximum response with time in both intact and disrupted preparations which was not significant : intact 1.24g±0.12 rising by the fourth curve to 1.48g±0.09; disrupted 1.27g±0.13 rising to 1.31g± 0.06. There was also no significant change with time in the pD_2 values for both groups intact 7.85/8 ; disrupted 8.5/8.3 .

Figure 10 a

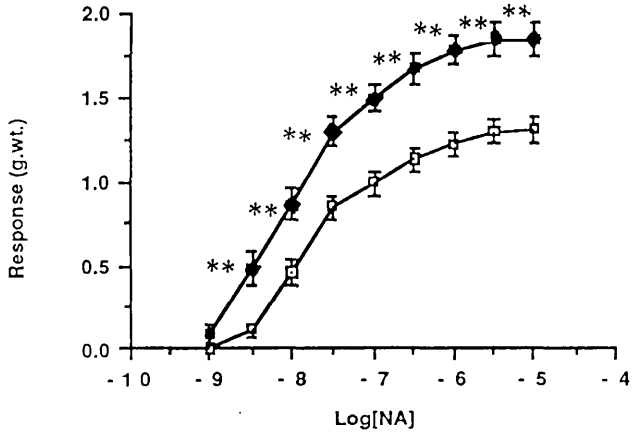


Figure 10 b

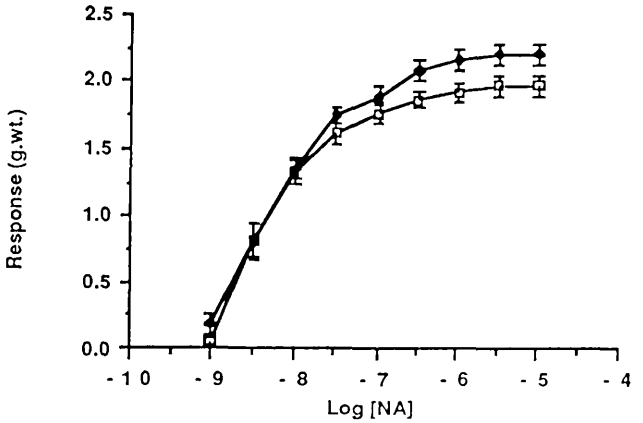


Figure 11 a

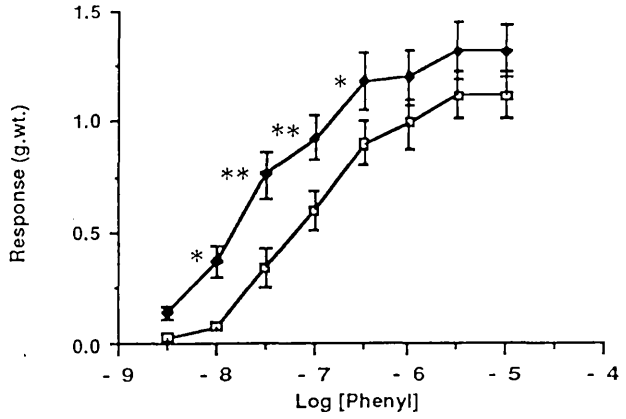
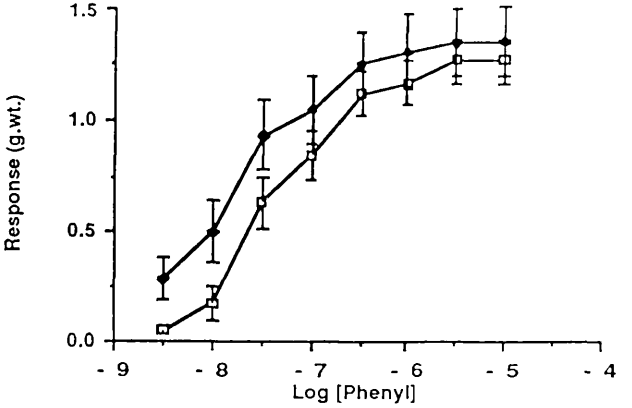


Figure 11 b



These figures show the effect of Hb on responses to NA and Phe in aortic rings having either an intact (Figures 10a,11a) or disrupted endothelium (Figures 10b,11b). Responses expressed in g.wt. Values are given as mean + s.e.m. (n=6). Statistical analysis was carried out comparing the responses in the presence of Hb and in its -absence. Hb-□ +Hb-◆
Statistical analysis was determined as follows: *P<0.05 **0.01<P<0.001 ***P<0.001.

Figure 12 a

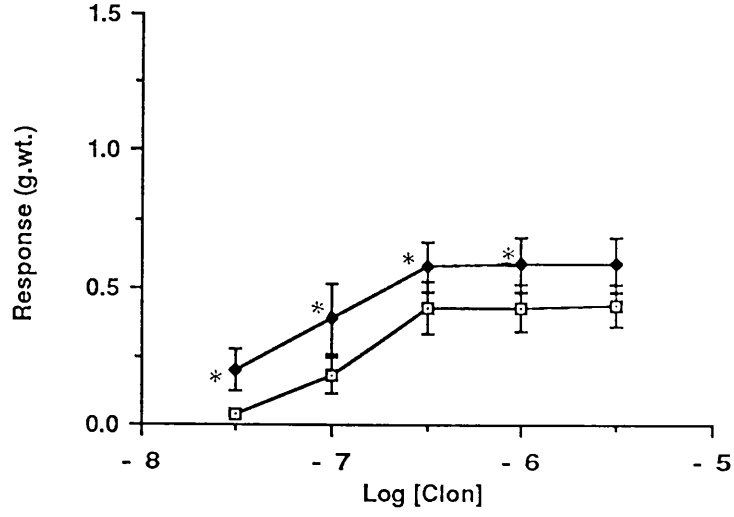
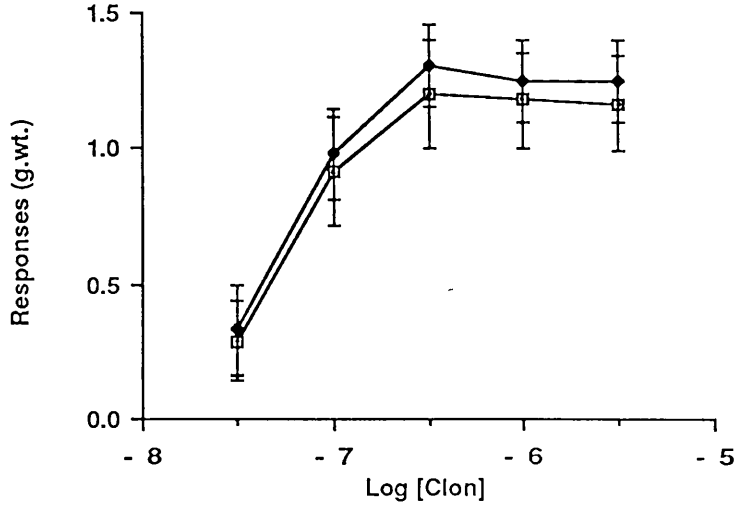


Figure 12 b



These figures show the effect of Hb on responses to clonidine with an intact (Figure 12a) and disrupted (Figure12b) endothelium. Responses are expressed in g.wt. Values are given as mean ± s.e.m. (n=6) Hb-⊡ +Hb-◆

(Statistical significance was determined as follows: * P<0.05 ** 0.01>P>0.001 *** P<0.001)

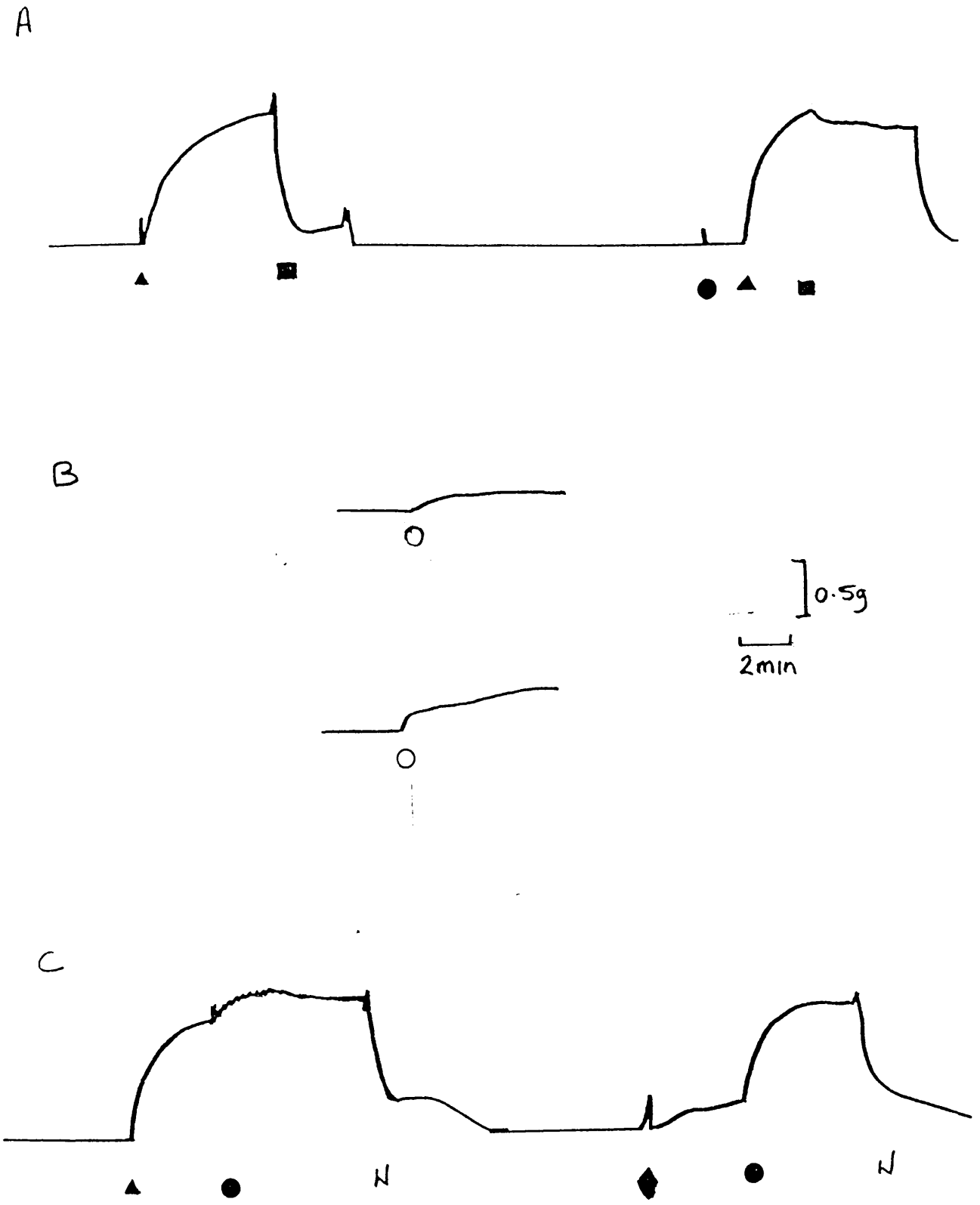

A
B
O
0.5g
2min
O
C
N
N

Figure 13 a

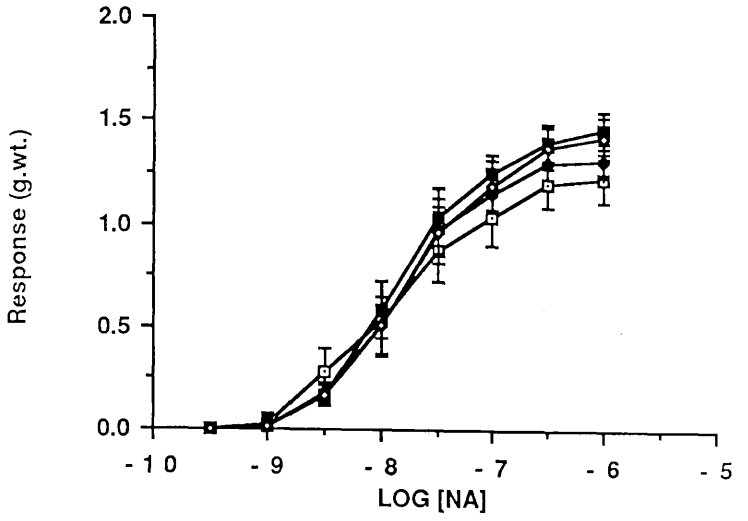
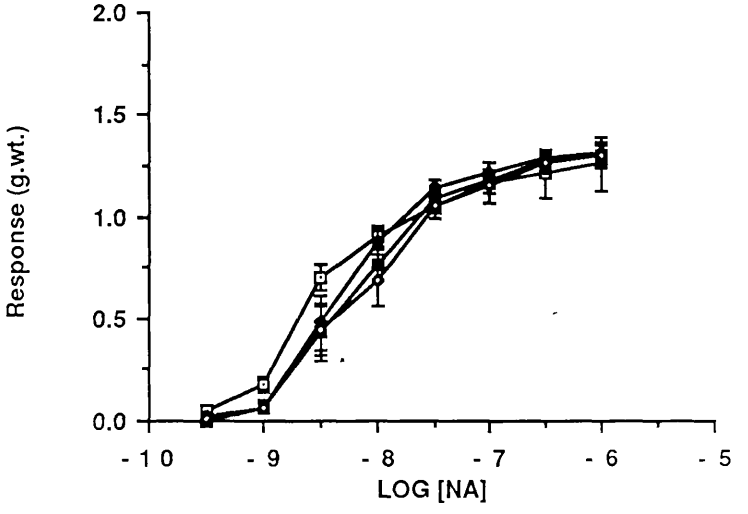


Figure 13 b



These figures show the effect of repeated concentration response curves to NA in rat aorta with an intact (Figure 13a) or disrupted endothelium (Figure 13b). Results expressed in g.wt. Values are given as mean ± s.e.m. Statistical analysis was carried out comparing the responses of curves 2,3 &4 with the 1st curve at all concentrations control-⊡ 2nd curve-◇ 3rd curve- ■ 4th curve-◆

There was no statistically significant differences obtained between the responses at any given concentration of NA in the different curves in either endothelium intact or disrupted tissues.

cumulatively the responses developed tachyphylaxis regardless of whether the endothelium was intact or disrupted and so this was not examined further ; some workers have found that initial depolarization with high concentrations of potassium or a threshold concentration of NA (Bullock et al 1986) prevents this tachyphylaxis from occurring. Danthuluri & Deth (1986) found that desensitization to angiotensin 11 did not occurr in the presence of an agonist. They postulate that this is due to agonist-induced diacylglycerol production which maintains the contraction. The thromboxane mimetic drug U46619 showed no difference in the potency of response in tissues with an intact or disrupted endothelium. Hb(10^{-5}M) potentiated the responses to all three agonists significantly only when the endothelium was present . When the endothelium was disrupted there were slight increases in the responses but they were not significant. Hb potentiated the responses to NA to a greater extent than those to either Phe or clonidine (Figures 10-12). The smaller potentiation of responses to clonidine which contrasts with that of Martin et al 1985 emphasises the relative inability at least under these conditions of clonidine to cause contraction regardless of an intact or disrupted endothelium. We also were able to obtain small contractile responses to Hb alone in the absence of any tone. This effect was seen in both intact and disrupted tissues but was greater when the endothelium was intact (Figure 12c). This figure also shows how Hb blocks responses to Ach, and how it can produce a further contraction on top of tone induced by NA. This contraction was greatest when the induced tone was smaller.

Antagonist Studies

From the results of the time control curves to NA (Figures 13a,b) it can be seen that there was a slight increase in maximum response with time in both intact and disrupted preparations which was not significant : intact 1.24g±0.12 rising by the fourth curve to 1.48g±0.09; disrupted 1.27g±0.13 rising to 1.31g± 0.06. There was also no significant change with time in the pD_2 values for both groups intact 7.85/8 ; disrupted 8.5/8.3 .

TABLE 2

Treatment	pD_2 Value +E	pD_2 Value -E	Max Response(g.wt.) +E	Max Response(g.wt.) -E
Control	7.96±0.07	*P 8.33±0.03	1.81±0.15	1.70±0.30
+0.01nM Prazosin	7.76±0.03	*P 8.10±0.06	1.83±0.13	1.71±0.17
+0.1nM Prazosin	7.22±0.09 *S	*P 7.57±0.12 *S	1.71±0.20	1.72±0.18
+1nM Prazosin	5.88±0.10 *S	*P 6.28±0.05 *S	1.41±0.19	1.54±0.13
Control	7.98±0.08	*P 8.42±0.05	1.62±0.19	1.75±0.18
+0.1uM Corynan	7.50±0.11 *S	*P 7.78±0.10 *S	1.82±0.18	2.00±0.18
+1uM Corynan	6.20±0.09 *S	*P 6.60±0.05 *S	1.96±0.18	2.12±0.23
+10uM Corynan	5.50±0.07 *S	*P 5.75±0.02 *S	2.08±0.20	2.08±0.22
Control	7.87±0.09	*P 8.22±0.13	1.23±0.14	1.38±0.13
+0.03uM Wyeth	7.76±0.05	*P 8.20±0.13	1.25±0.15	1.47±0.12
+0.1uM Wyeth	7.75±0.13	*P 8.10±0.12	1.34±0.20	1.48±0.11
+0.3uM Wyeth	7.31±0.06 *S	*P 7.66±0.08 *S	1.23±0.17	1.46±0.10
+1uM Wyeth	7.05±0.10	7.14±0.07	1.06±0.14	1.46±0.06

This table shows the pD_2 values and maximum responses obtained in the presence of three different antagonists. The results are given for tissues with both an intact (+E) and disrupted (-E) endothelium and expressed as mean +/- s.e.m. (n=6). Statistical analysis was carried out between tissues in the presence of the antagonist and with its own control and also between intact and disrupted tissues in the same tissue. Wyeth (Wyeth 26703). pA_2 values are shown in Table 3.

*P- value is statistically significantly different from the corresponding tissue with an intact endothelium.
*S- value is statistically significant from that of the corresponding control.

Figure 14 a

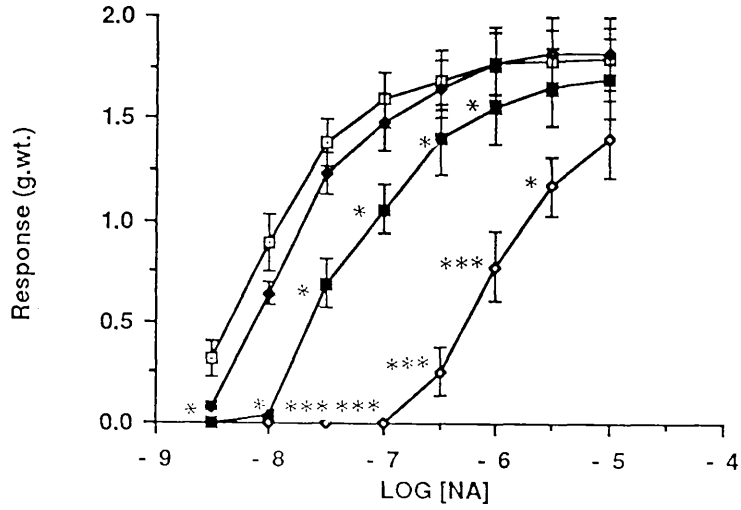
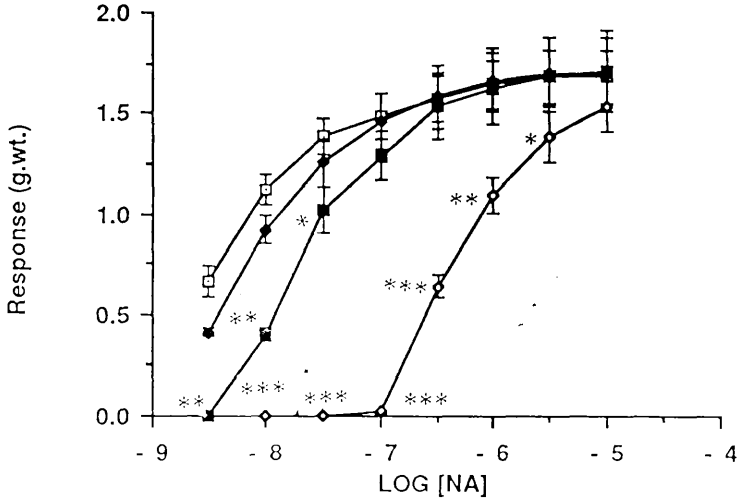


Figure 14 b



These figures show the effect of prazosin on concentration response curves to NA in rat aortic rings with intact (Figure14a) and disrupted endothelium (Figure 14b) . Results are expressed in g.wt. Values are expressed as mean ± s.e.m. (n=6). Statistical analysis was carried out comparing the response in the presence of each concentration of antagonist with that of the control. control-⊡ +0.01nM praz-◆ +0.1nM praz-■ +1nM praz-◇

(Statistical significance was determined as follows: * P<0.05 ** 0.01>P>0.001 *** P<0.001)

Figure 15 a

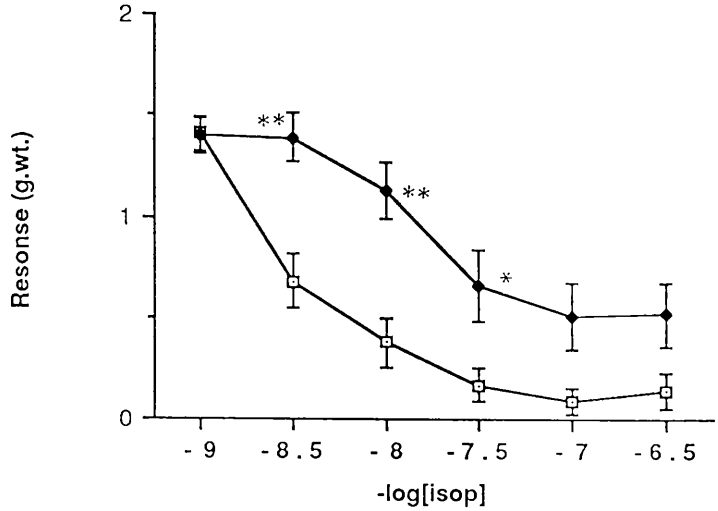
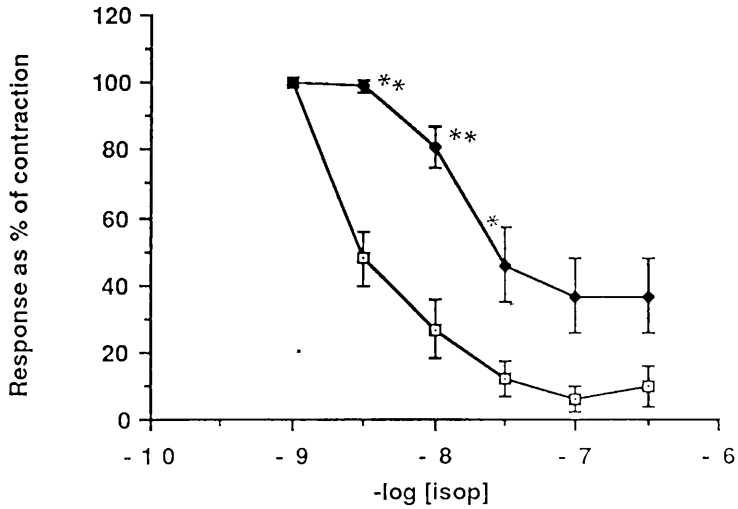


Figure 15 b



These figures show the effect of removal of the endothelium on relaxation to isoprenaline on rat aortic rings precontracted with 30nM NA. Results are expressed in g.wt.(Figure 15a) and as a percentage of the induced tone (Figure 15b). First point on the curve represents the tone induced by NA. Values are given as mean ± s.e.m. (n=8). Statistical analysis was carried out comparing the responses with endothelium disrupted to intact. +E- ⊡ -E- ◆
(Statistical significance was determined as follows: * P<0.05 ** 0.01>P>0.001 *** P<0.001)

Figure 16 a

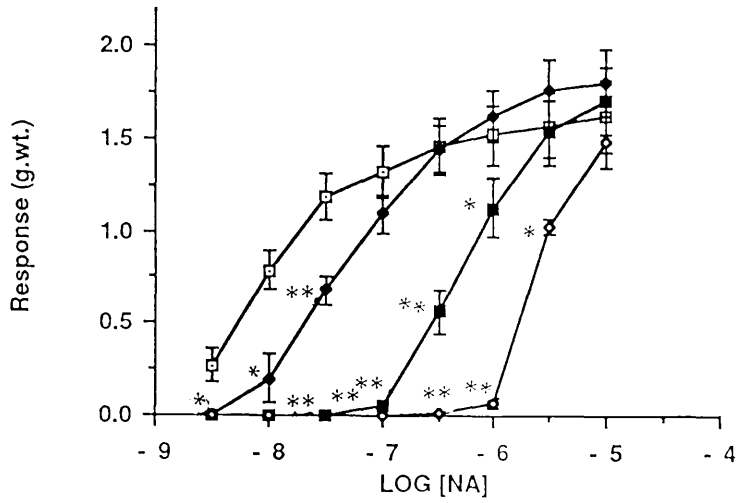
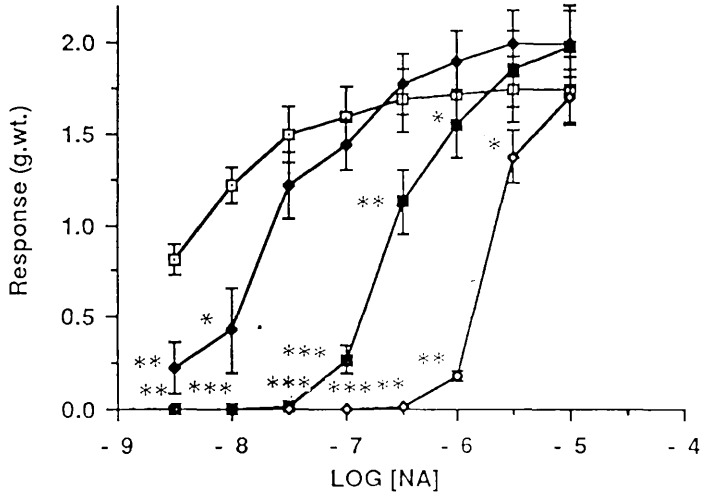


Figure 16 b



These figures show the effect of corynanthine on concentration response curves to NA in rat aorta with an intact (Figure 16a) and disrupted endothelium(Figure 16b). Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6). Statistical analysis was carried out comparing the response in the presence of each concentration of the antagonist with that of the control. control- ⊡ +10nM coryn- ◆ +100nM coryn- ■ +1uM coryn- ◇

(Statistical significance was determined as follows: * P<0.05 ** 0.01>P>0.001 *** P<0.001)

Figure 14 c

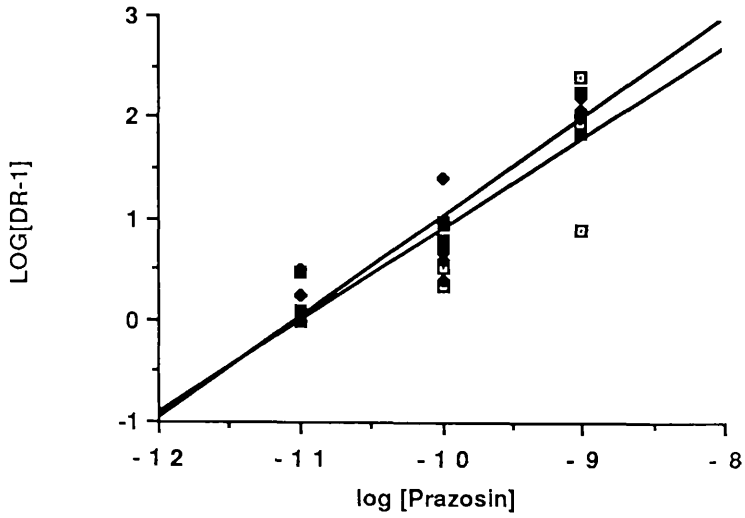


Figure 16 c

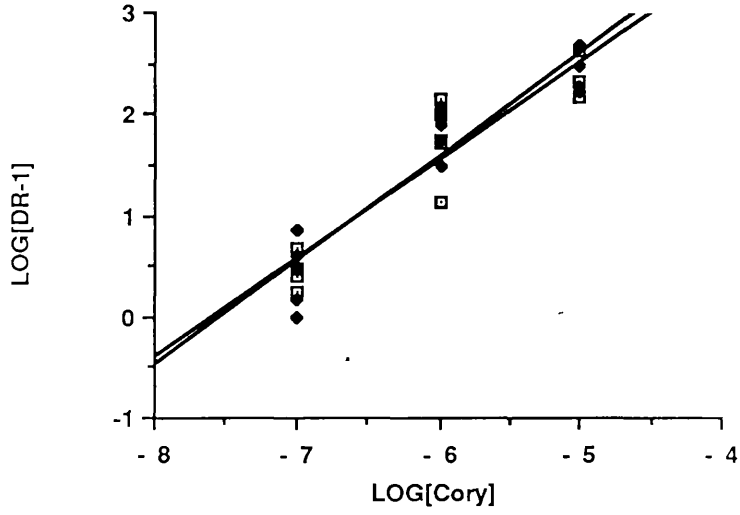


Figure 14 c depicts the Schild plot for both intact and disrupted aortic rings for prazosin against NA contraction. The slopes for both groups are not significantly different from 1 +E- 0.94(0.74-1.14) -E-0.99(0.8-1.18). Fig 16 c depicts the Schild plot for both intact and disrupted aortic rings for corynanthine against NA contraction. The slopes for both groups are not significantly different from 1+E-0.97(0.73-1.22) -E-1.03(0.81-1.25).

+E- ⊡ -E- ◆

tissues. At higher concentrations (0.3μM-1μM) the pD_2 to NA was increased significantly (Figure 17a,b). Lattimer et al (1984) showed that Wyeth 26703 was more potent than yohimbine at α_2 than α_1 adrenoceptors. They used the vas deferens(α_2) and obtained a pA_2 value of 8.16(7.99-8.40) and the anococcygeus(α_1) and obtained a pA_2 of 6.49(6.37-6.63). The calculation of the pA_2 value for this antagonist in the rat aorta showed a similar value for endothelium intact 7.33(7.06-7.6) and disrupted 7.31(7.03-7.59) tissues (Table 3) this is intermediate between that of either α_1 or α_2. The slope of the Schild plot for this antagonist was 0.59(0.41-0.76) in tissues with endothelium intact and 0.66(0.45-0.86) endothelium disrupted. (Figure 17c). This value was significantly different from 1 which indicates that the drug was not acting as a competitive antagonist in this preparation. This probably explains why the pA_2 value obtained is neither α_1 or α_2.

Figure 17 a

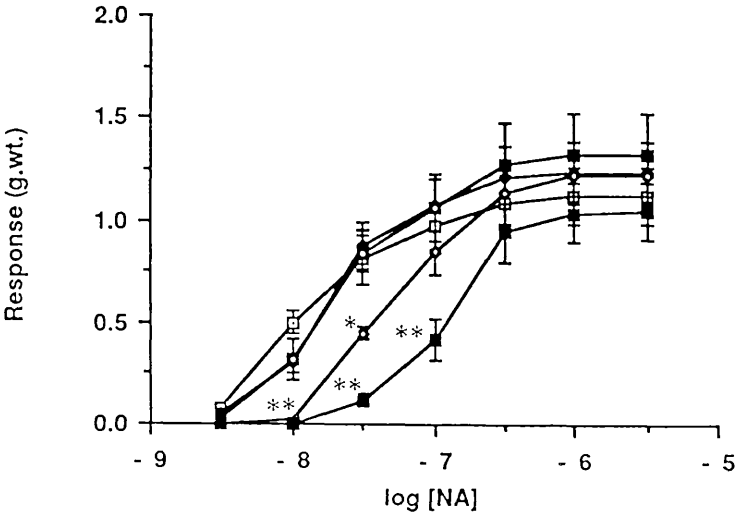
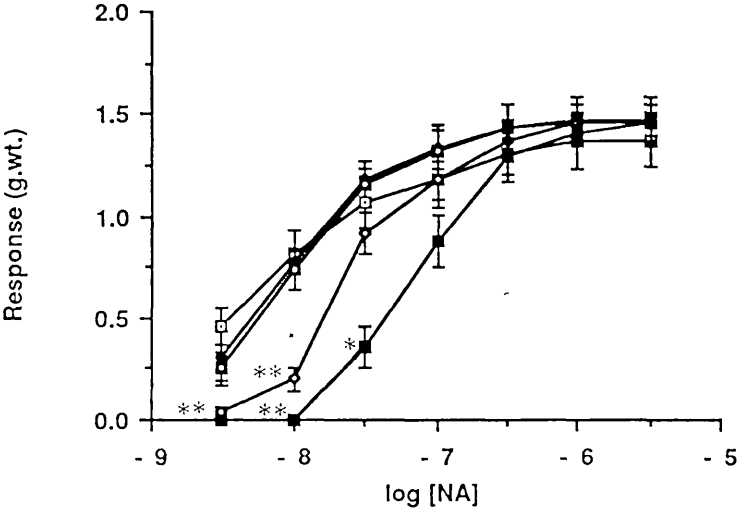


Figure 17 b



These figures show the effect of Wyeth 26703 on concentration response curves to NA in aortic rings with an intact (Figure 17a) and disrupted endothelium (Figure 17b). Responses are expressed in g.wt. Values are given as mean ± s.e.m. Statistical analysis was carried out comparing the responses in the presence of all concentrations of the antagonist with that of the control. control-⊡ +30nM Wyeth-◆ +100nM Wyeth-■ +0.3uM Wyeth-◇ +1uM Wyeth-■

(Statistical significance was determined as follows: (* $P<0.05$ ** $0.01>P>0.001$ *** $P<0.001$).

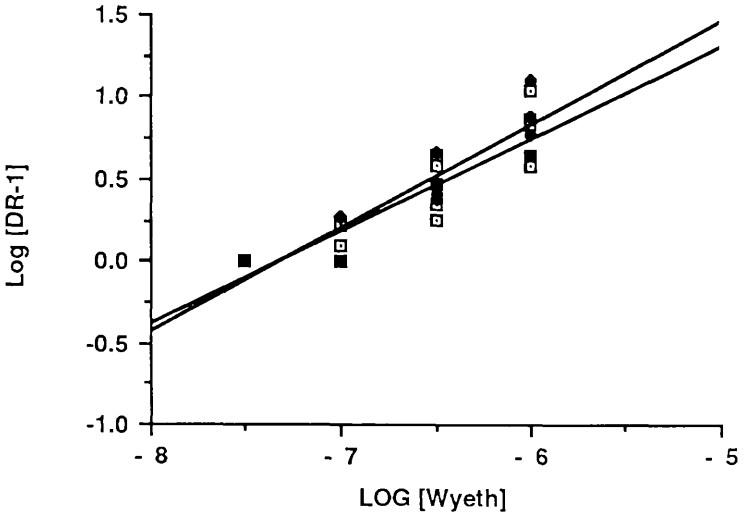


Fig 17c depicts the Schild plot for both intact or disrupted aortic rings for Wyeth 26703. The slopes for both groups are 0.59(0.41-0.76) and 0.66(0.45-0.86) respectively making them significantly different from 1 and therefore a non-competitive antagonist. Intercept 7.33(7.06-7.6) and 7.31(7.03-7.59) respectively.+ E-□ -E-◆

TABLE 3

Antagonist	pA_2		Slope	
	+E	-E	+E	-E
Prazosin	10.77 (10.34-11.29)	10.97 (10.78-11.29)	0.94 (0.74-1.14)	0.99 (0.8-1.18)
Corynanthine	7.60 (7.17-8.03)	7.55 (7.2-7.9)	0.97 (0.73-1.22)	1.03 (0.81-1.25)
Wyeth 26703	7.33 (7.06-7.61)	7.31 (7.03-7.59)	0.59 (0.41-0.76)	0.66 (0.45-0.86)

This table shows the values obtained for the pA_2 of three different antagonists in the presence and the absence of the endothelium. It also shows the slope of the lines for the Schild plots. There was no significant difference in the values obtained between tissues with an intact or disrupted endothelium.

DISCUSSION

From the results, which are similar to those of other workers (Martin et al 1986), the responses to partial α_1-adrenoceptor agonists were better potentiated than those to strong α_1- adrenoceptor agonists, when the endothelium was removed. However the responses to BHT-920 and xylazine were very poor or non-existent and did not increase on removal of the endothelium. The results with Phe and NA were similar to those of Martin et al, (1986) but contrast with those from Godfraind et al, (1985) and Carrier and White, (1985) both of whom found a large potentiation of the maximum response to Phe and in the study by Carrier and White a similar increase in the maximum to NA. Carrier and White also found a large potentiation to responses of the selective α_1-adrenoceptor agonist methoxamine They have suggested that it may be due to α_2-adrenoceptor activation on the endothelial cells to release EDRF and so inhibit the contractile effect (although how this explains the potentiation of responses to methoxamine is not clear). Angus et al (1986) found in canine large arteries that NA and UK14304 caused relaxation only when the endothelium was intact. Including Idazoxan in the baths shifted both curves to the right which was consistent with an action at α_2-adrenoceptors. However Miller et al , (1984) and Martin et al, (1986) were unable to reveal a relaxant effect to BHT-920 or clonidine in KCl or $PGF_{2\alpha}$ contracted rat aortic strips respectively, even though the α_1-adrenoceptor contractile effects of the α-adrenoceptor agonists were prevented by pretreatment with prazosin. Further evidence presented by Lues & Schumann, (1984) using rauwolscine, a moderately selective α_2-antagonist, and the results in our study using the more selective α_2-antagonist Wyeth 26703 showed that α_2-adrenoceptor blockade could not mimic the effect of removing the endothelial cells (i.e. increase in response to contractile agonists as would be expected if the agonists were holding

back their own contractile responses by releasing an inhibitory factor from the endothelium through α_2-adrenoceptors).

Further evidence against α-adrenoceptor agonists stimulating release of EDRF was obtained from experiments measuring levels of cGMP which are known to increase during relaxation to EDRF stimulated by Ach. Rapoport & Murad, (1983a) and Martin et al, (1986) saw no increase in cGMP levels in the presence of clonidine. Bullock et al, (1986) showed that the resting basal concentrations of cGMP were significantly lower in the absence than in the presence of the endothelium, and saw an increase when Ach was added. This would suggest that relaxation by EDRF was accompanied by increases in the levels of cGMP and the fact that clonidine did not increase them suggested that it was not acting to release EDRF. However Biguad et al, (1984) and Miller et al, (1984) found that clonidine, BHT 920 and methoxamine (a selective α_1-adrenoceptor agonist) all produced small increases in the cGMP content of the rat aorta.

A common property of the agonists where removal of the endothelium had such a marked effect is that they were all partial agonists at the α_1-adrenoceptors in the rat aorta. (Ruffolo et al., 1979; Digges & Summers, 1983; Lues & Schumann, 1984). Martin et al, (1985) used the α_1-adrenoceptor irreversible antagonist dibenamine to reduce the efficacy of the full α_1- receptor agonist Phe to a similar level to that of clonidine. The results they obtained were then similar for both Phe and clonidine, i.e. a highly significant increase in response between intact and disrupted preparations demonstrating that the endothelium, possibly acting through spontaneous release of EDRF, can depress contractions to a greater extent to agonists which have a low efficacy for the α_1- adrenoceptor.

The results with Hb were similar to those of Martin et al, (1986) when either NA or Phe were the agonists used. Hb at a concentration which inhibits relaxation to Ach (3μM), and therefore inhibits stimulated release of EDRF, also potentiated the responses to NA, Phe and clonidine in tissues which had an intact endothelium, but had no significant effect on responses in tissues having the endothelium removed. Our inability to obtain a significantly greater increase to clonidine in the presence of Hb compared with NA does not fit in with the hypothesis that partial agonists are affected to a greater extent by the spontaneous release of EDRF. However the fact that we can obtain significant increase in the size of the contraction on removal of the endothelium does support the theory. Why we were unable to obtain a large increase in response to clonidine with Hb is not clear. That the Hb did not destroy the endothelial cells was apparent as the inhibition by Hb of relaxation to Ach was completely reversible on washing out. It has been postulated that the inhibitory action of Hb may be to bind to the EDRF preventing interaction with the ferrous heme group of the soluble guanylate cyclase (Craven & De Rubertes, 1979; Gerzer et al ., 1981 a,b), which is important in the action of the enzyme. That the ability of the smooth muscle to relax to stimulation of guanylate cyclase was not hampered is shown by the results with sodium nitroprusside, where there was no significant difference in sensitivity between intact and disrupted tissues (results in chapter 4).

Using the α_1-adrenoceptor antagonists my results revealed no significant difference in their potency to inhibit responses to NA between endothelium intact and disrupted preparations. Unlike Alosachie & Godfraind, (1986) we did not find a non-competitive antagonism to prazosin when the endothelium was intact. In my experiments prazosin showed a competitive antagonism at the concentrations I used regardless of whether the endothelium was intact or disrupted. I also obtained a similar pA_2 value for the antagonist regardless of the

presence or absence of the endothelium. I however, did not use such high concentrations of prazosin as this group and it is possible that this effect might take place. This is unlikely as at the highest concentration which I use (1nM) they already show the maximum response to be reduced to 75% of control. Another difference between the experimental protocols is the inclusion of propranolol (1μM) in my experiments to prevent any β-receptor stimulation at high concentrations of NA. I have found in experiments using the β– receptor agonist isoprenaline that in the endothelium intact preparations this agonist was significantly more potent at producing relaxation than in preparations where the endothelium was rubbed, indicating a more potent β-receptor influence when the endothelium was present. It could also be seen in some preparations, where the endothelium was intact, that at high concentrations of NA the responses would decrease from the maximum acheived at lower NA concentration when propranolol was absent. Using corynanthine I again found that the responses to NA were similarly shifted in tissues with either an intact or disrupted endothelium. This shift was competitive in nature similar to prazosin. These results with corynanthine taken together with those of prazosin suggest that the endothelium has no effect on the inhibitory responses of selective α_1-adrenoceptor antagonists. Although the conclusion does not agree with those of Alosachie & Godfraind (1986) I have at least used propranolol to block any relaxatory involvement from the stimulation of β-adrenoceptors. I have also used two antagonists and found the results to be the same for each one therefore what these workers are postulating is a selective action of prazosin on tissues with an intact endothelium, it would be interesting to know what their results would be using corynanthine.

My results using the selective α_2-adrenoceptor antagonist Wyeth 26703 agree with those of Lues & Schumann (1984) in that I found no potentiation of responses to NA in tissues with an intact endothelium in the presence of this drug.

At concentrations where it acts on α_2-adrenoceptors(10nM-100nM) the responses to NA in tissues with either an intact or disrupted endothelium were not affected. Only at higher concentrations where it loses its selectivity(0.3μM-10μM) (Lattimer et al 1984) were curves to NA from both groups shifted significantly to the right. This again supports our overall pattern of results for antagonists, i.e. that the presence of the endothelium has no effect. These last results also do not support α_2-adrenoceptor mediated relaxation and are in agreement with most other workers (Martin et al, 1985; Lues & Schumann, 1984; Miller et al 1984).

In conclusion, from our results we can find no evidence for the existence of an α_2-adrenoceptor on the endothelium which, when activated, stimulates the release of EDRF. This is supported by findings from other workers. It is possible that our approach was not subtle enough. However others have found using the rat aorta that they were unable to obtain relaxation to a_2-adrenoceptor agonists. Our results most resemble those of Martin et al,1985. However we did not find such an increase in response to clonidine with Hb in tissues having an intact endothelium. We also found that Hb could increase tone in the absence of any existing tone. This was seen best when the endothelium was absent. We were able to obtain small responses to Hb in the absence of the endothelium : Martin et al 1985 comment on this observation only in the later stages of their experiments but offer no explanation. It seems possible that the potentiating effect of Hb is at least partly due to a direct effect on smooth muscle which is facilitated by removal of the countervailing inhibitory influence of spontaneously released EDRF. Other experiments in Ch 2 show that removing the endothelium does not uncover spontaneous tone of the preparation so there is support from this for the concept that Hb can uncover spontaneous tone by blocking the spontaneous release of EDRF. We conclude therefore, that the spontaneous release of EDRF modulating responses of smooth muscle is most

likely to be present. It would then have the greatest effect when the muscle is being stimulated by an agonist since the potentiations seen in their presence are greater than when Hb is given alone. This indicates that although Hb does have an effect alone, this effect is potentiated in the presence of an agonist.

CHAPTER 2

INFLUENCE OF BASAL TENSION, INDUCED TONE AND VARIOUS DRUGS ON THE RELAXATION TO ACETYLCHOLINE

INTRODUCTION

Variation in results obtained between different groups when demonstrating the effects of basal and stimulated release of EDRF can be marked. Egleme et al, (1984) showed that removing the endothelium greatly increased responses to the mixed α_1- and α_2- adrenoceptor agonist clonidine whereas the same 25-fold increase was not seen when using the selective α_1-adrenoceptor agonists Phe or NA, but there was a similar large increase when the selective α_2-adrenoceptor agonist UK14304 was used. They attributed this difference to the release of EDRF by clonidine and UK14304 acting via α_2-adrenoceptors on the endothelium, which would therefore inhibit the concomitant contractile response to stimulation of α_1-adrenoceptors. Carrier & White (1985) found that responses were increased to the greatest extent on removal of the endothelium when the agonists were highly selective for either α_1- or α_2-adrenoceptors. They try to explain the results by indicating that potentiation is seen to the greatest extent with agonists which are highly selective for a particular receptor regardless of which one. An alternative explanation was postulated by Martin et al, (1986) who suggested there was no need to invoke a specific release of EDRF by the agonist but that spontaneous release may occur and have a more potent inhibitory effect on responses to partial rather than 'full'agonists at the α_1-adrenoceptor.

Looking at various agonists I have carried out similar experiments to the groups above examining the differential effects of removing the endothelium on their responses. However, I have been unable to reproduce the huge increases in maximum response to both clonidine or UK14304 shown by Godfraind et al, (1985). Using the same protocol as with clonidine I obtained, similar to this group, a small significant increase in the sensitivity but no significant increase in the maximum response to NA. Also the results of Carrier & White, (1985) show a

marked potentiation in the size of the responses in aortic rings with an intact endothelium compared to that with disrupted, to both Phe and methoxamine but not to NA. The results obtained by Martin et al, (1986) were similar to those of my own regardless of the agonist used. The problem was therefore why different groups looking at the same agonists on the same tissue were obtaining qualitatively different results. There are various factors in experimental protocol which may be different and so have to be considered: Krebs solution, temperature, composition of gasses, strain of rat used, age and weight of the rats and initial tension placed on the tissue. Since the first three conditions are normally fairly constant and in most groups compared the strain of rats were the same, the age and weight of the rats and initial tone placed on the tissue may be an important factor.

That age may be an important factor was demonstrated by Godfraind (personal communication) when he bubbled distilled water through the aortas of old rats (>350gms) and found that the relaxation response to Ach was completely inhibited. This was not so when the water was bubbled through aortas from the younger rats (<250gms).

How the initial tension may affect the stimulated release of EDRF is also an important consideration and again may explain differences between groups in sensitivity to Ach relaxation. Since Ach is known to act on muscarinic receptors to release EDRF the effect of atropine on this concentration dependent release was examined. Another important factor which may influence the stimulated release of EDRF is the induced tone by the agonist, against which the relaxation is measured. Several other factors may also be involved in altering stimulated release of EDRF. Since calcium is known to play a part in EDRF release as demonstrated by Singer & Peach, (1986), the effect of the calcium-channel activator Bay-K

8644 was examined. If as suggested by Godfraind et al (1985), α_2-adrenoceptors are involved in the release of EDRF then blockade of these receptors might be expected to produce an effect on its release. For this reason the effects of Wyeth 26703 were examined on relaxation to Ach.

This study was carried out to examine how the initial stretch of the smooth muscle and the degree of tone induced by an agonist, influence the quantitative demonstration of the effects of basal or stimulated release of EDRF. Also examined was how various drugs may interfere with the Ach stimulated release of EDRF.

MATERIALS AND METHODS

Effects of Resting Length (Tension)

For this length-tension study paired aortic ring segments were used. The endothelium of one segment of each pair was disrupted as previously described. This study was carried out by Ian Dainty using Phe to induce tone. I feel that although a different α-adrenoceptor agonist was used for this particular study, the observations made are complimentary to those made by myself (see below) and have therefore been included in this thesis. Whilst there is a difference in sensitivity of the rat aorta to NA and Phe (Figures 3,4) both activate α_1-adrenoceptors and therefore would expect similar results using 'full' agonists (α_2-adrenoceptors are not known to be present in this tissue). The equipment used allowed for accurate measurement, using a vernier scale, of the distance between lower and upper tissue holders The measurements of "stretch" (length) quoted in the study take into account the thickness of both tissue holders (500μm) and indicate the distance between the upper and lower edges of the top and bottom tissue holders. The holders were initially separated by a distance of 1500μm. From this point each tissue was stretched (the length between holders was increased) by increasing the separation of the holders in 300μm steps to a final separation of 2700μm. After each change in length, the rings were given 15 minutes equilibration before administration of Phe (1μM) to produce 90-95% maximal contraction. When the response had reached a plateau a concentration of ACh (1μM) known to produce near maximal relaxation in the tissue was added to the baths and relaxation, if any, observed. 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. The resting tension at various degrees of stretch was recorded and also the contractile

Figure 18
Legend: This figure shows a summary of the influence of initial stretch on the optimum conditions for the demonstration of both contraction and relaxation.

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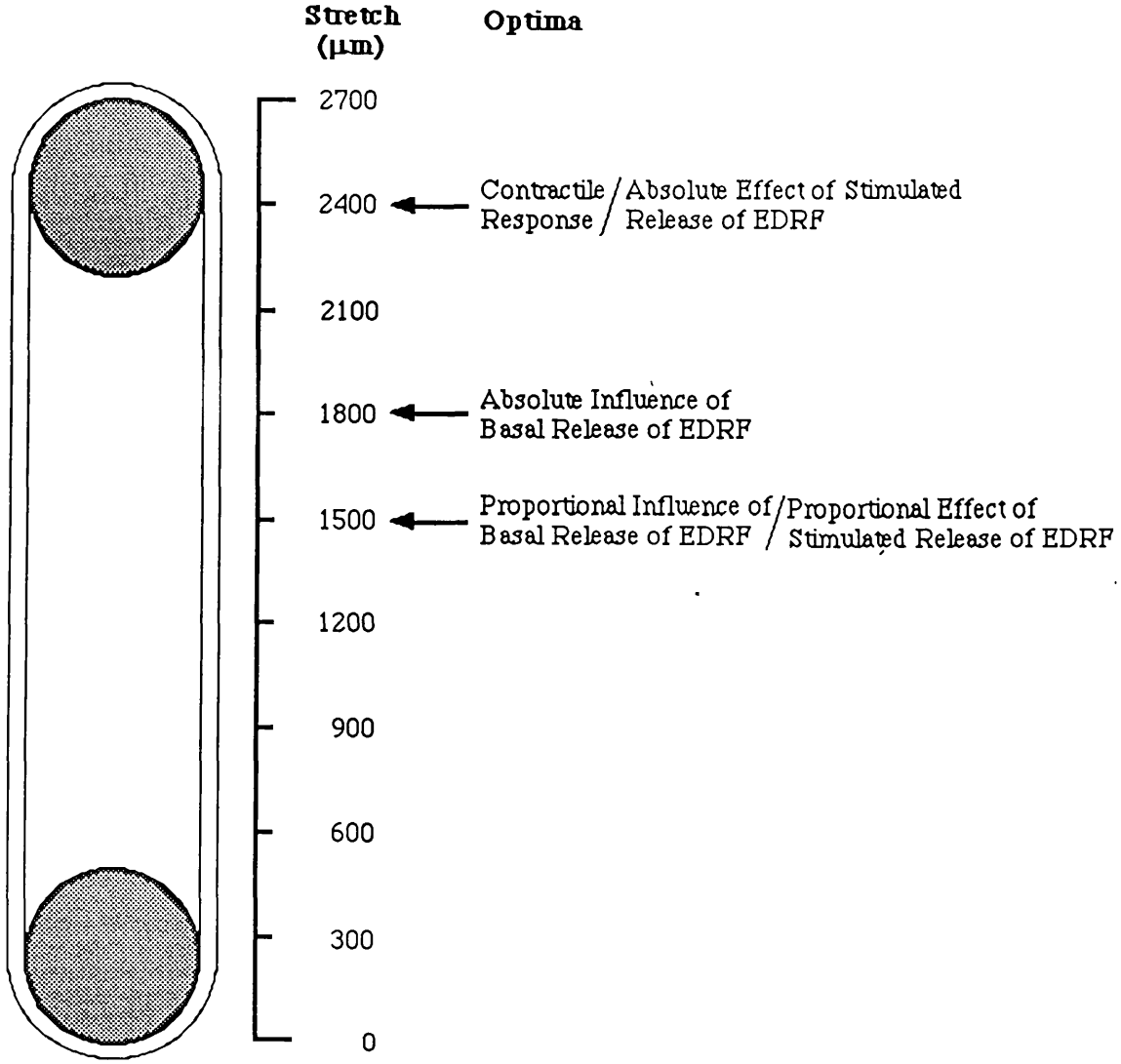
Figure 18

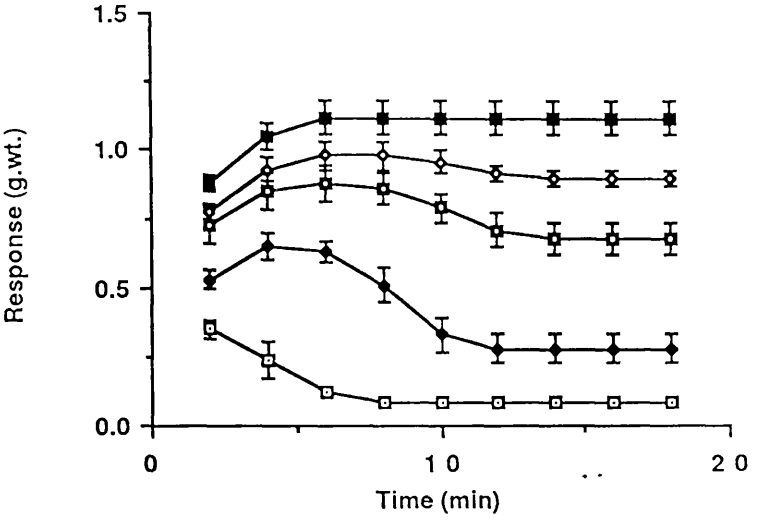

Stretch (µm)
Optima
2700
2400
2100
1800
1500
1200
900
600
300
0
Contractile / Absolute Effect of Stimulated Response / Release of EDRF
Absolute Influence of Basal Release of EDRF
Proportional Influence of / Proportional Effect of Basal Release of EDRF / Stimulated Release of EDRF

response to Phe (1μM), and relaxation to ACh (1μM). Results are summarised in Figure 18.

Effects of Induced Tone

Only aortic rings having an intact endothelium were used for this part of the study. Each ring segment was set at the same initial tension of 1500mg. The preparations were washed twice by exchanging the bathing Krebs' solution and left for at least sixty 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 fifteen minute period and allowed to equilibrate for a further 30 minutes. Muscle tone was then raised by adding a fixed amount of NA. Only concentrations of NA were used which would maintain a good contraction for at least 20 minutes (figure 19): at lower concentrations the responses were not as well maintained therefore making it impossible to determine the actual relaxation to Ach from deterioration of the contraction . After the contractile response had reached a plateau, Ach was added cumulatively (0.03-10μM). This was repeated for three different concentrations of NA in each ring segment or in the case of BAY-K 8644 and atropine , three different drug concentrations. With Wyeth 26703 only two different drug concentrations were used. In the case of both BAY-K 8644 and Wyeth 26703 the concentration of NA used to induce tone was 30nM. When the effects of atropine were examined the concentration of NA used was 100nM. Although this concentration was approximately three times greater than previously, in this series of experiments the responses to NA were smaller than normal therefore a higher concentration of NA had to be used to enable the relaxation to Ach to be measured with any accuracy. However this did not affect the control relationship of relaxation varying with NA-

Figure 19



This figure shows how responses develop with time in rat aortic rings with an intact endothelium, using different concentrations of NA. Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6) +3nM-⊡ +10nM-◆ 30nM-□ 0.1uM-◇ 0.3uM-■

induced tone when it was expressed as a percentage of the initial maximum response to NA. The sensitivity of the preparations to Ach-induced release of EDRF in the absence of drugs was estimated by the concentration of Ach producing 50% maximal relaxation (IC_{50}) and expressed as its negative log10 i.e. the pIC_{50}. Results were also calculated as % of induced tone to detect any change in the maximum relaxation. In the presence of drugs the sensitivity of the preparations to Ach-induced release of EDRF was estimated by the concentration of Ach producing 25% relaxation from the induced tone (IC_{25}) since in the case of BAY-K 8644 relaxation did not reach 50%. Control values for the IC_{25} were also obtained and used in the figures.

STATISTICAL ANALYSIS

Results from experiments looking at the changes to Ach relaxation were compared with their own control values. Statistical significance was determined using the Students paired t-test since each tissue acted as its own control. Significance was determined as follows *$P<0.05$ **$0.01<P<0.001$ ***$P<0.001$. The experiments which relate the % induced tone to Ach relaxation used cross correlation of two variables to determine the relationship. In the presence of certain drugs this relationship was significantly altered. Significance was determined if the relationship was not within the control limits.

RESULTS

In rat aortic ring segments having an intact endothelium Ach (1μM) produced a marked reduction in Phe-induced tone (1μM) which was rapid in onset and well maintained. In preparations where the endothelium was previously disrupted, Ach had little effect but the responses to Phe were usually greater than that seen in the intact vessel (figure 20b).

Effect of Resting Length (Tension)

In ring segments with and without an intact endothelium, increases in the initial length (1500-2700μm) produced a length dependent increase in the resting tension of aortic rings which was linear between 2100 and 2700μm. Absence of the endothelium had no significant effect on the resting tensions of the preparations compared with paired intact controls (figure 20a).

Addition of Phe (1μM) produced a marked contractile response which was well sustained. The size of this contractile response was also dependent on the initial length of the preparation. The contractile response in both rubbed and unrubbed preparations increased as the resting length was increased reaching a maximum at 2400μm, after which no further increase in the response to Phe was seen. At all resting lengths the response to Phe was significantly greater in vessels with a disrupted endothelium except at 2100μm (1.5g tension) where P=0.075 (figure 20b). The absolute difference in contractile response in the rubbed and unrubbed preparation was dependent on the initial resting tension of the preparation, being greatest at 1800μm and similar at the other lengths tested (figure 21a). The proportionate difference in contractile response to Phe (expressed as a percentage of the response in the intact vessel), was also

Figure 20a

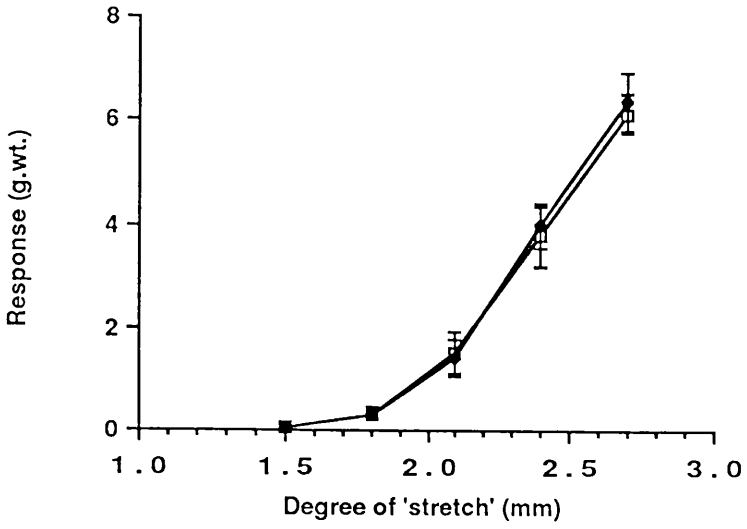


Figure 20b

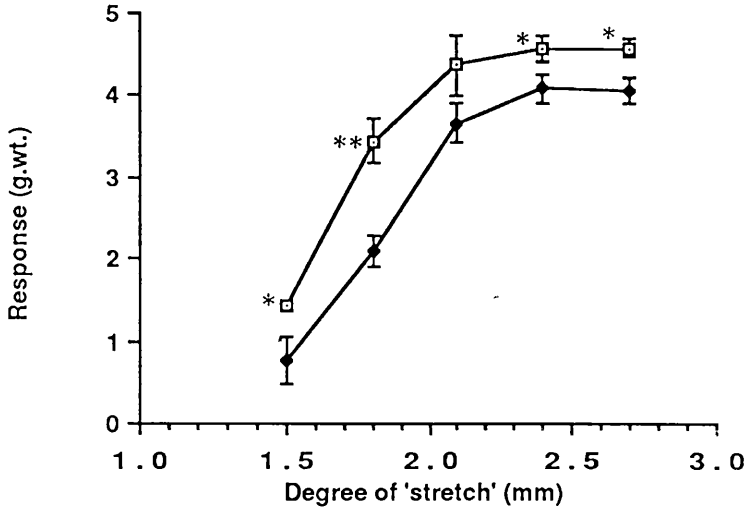


Figure 20a shows how the degree of 'stretch' (mm) affects the resting tension of the tissues. Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=7) +E-◆ -E-⊡

Figure 20b shows how the degree of 'stretch' affects the size of the response to 1μM Phe. Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=7) +E-◆ -E-⊡ Statistical analysis was carried by comparing the response in the absence of the endothelium with that of intact.Statistical significance was determined as follows:
* P<0.05 **0.01<P<0.001 ***P<0.001

Figure 21a

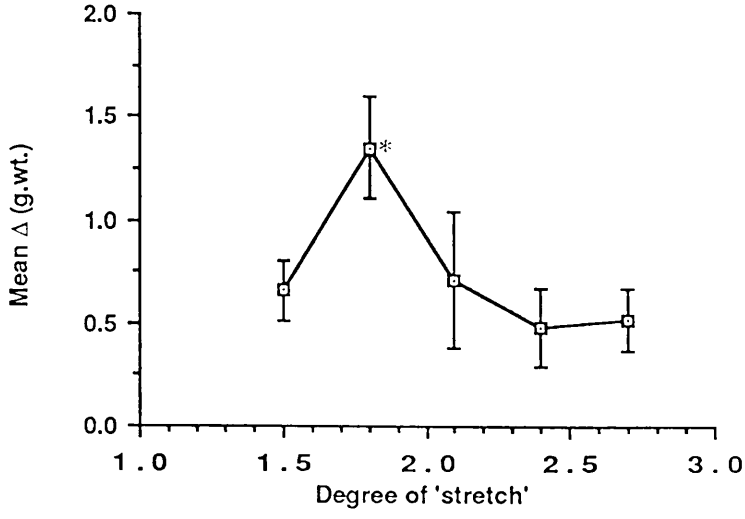
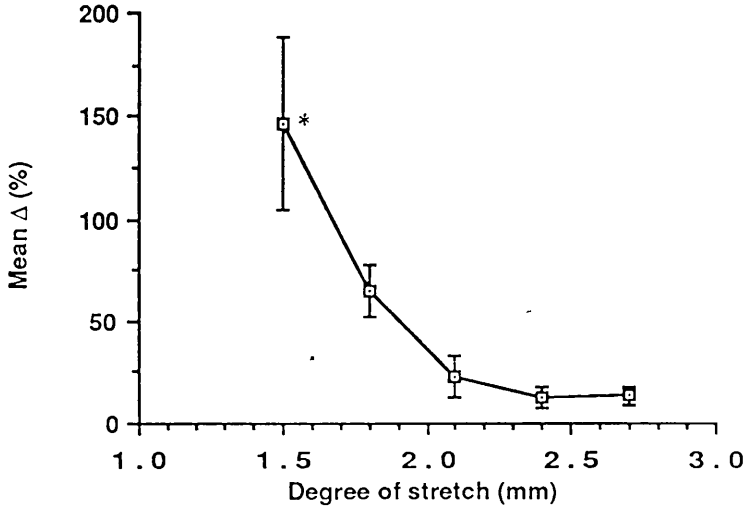


Figure 21b



Both figures show how the degree of 'stretch'(mm) affects the difference in the size of the response between intact and disrupted tissues to 1μM Phe, expressed as the absolute change in tone. Results are expressed in g.wt. (Figure 21a); and also as a percentage of the maximum when the endothelium is intact (Figure 21b). Statistical analysis was carried out to determine whether or not the optimum response for this effect was significantly different from the response at the optimum degree of stretch for contraction per se (2.4mm). Values are given as mean ± s.e.m. (n=7)

dependent on the initial length of the preparation being greatest at the lowest degree of stretch (1500μm) declining rapidly until 2100μm after which there was little change (figure 21b).

In rings with an intact endothelium, precontracted with Phe (1μM), Ach (1μM) induced a rapid, well maintained relaxation. The Ach-induced relaxation was dependent on the initial length of the preparation. The absolute relaxation of induced tone increased with increasing length reaching an optimal relaxation at 2400μm with a slight decrease at 2700μm (figure 22a). 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. At 2400μm and 2700μm the proportionate relaxation to Ach was slightly greater than that at 2100μm and similar to that seen at 1800μm (figure 22b).

Effect of Induced Tone

In tissues with an intact endothelium, Ach (0.03μM-10μM), produced a concentration-dependent relaxation of NA-induced tone. The sensitivity of the intact vessels to Ach showed no significant change with time (figure 23a;b). When the tissues were contracted with various concentrations of NA (0.01-0.3μM) to produce different degrees of tone (65-105% of initial maximum contraction to NA), the IC_{50} for Ach-induced relaxations varied with the initial degree of induced tone. (The maximum contraction to NA commonly increases in the course of such experiments without a significant change in sensitivity (figure 24)). When the concentration of NA used to produce the contraction was low, the apparent sensitivity of the tissues to Ach was high. On increasing the concentration of NA used and so the degree of tone, the tissues were apparently

Figure 22a

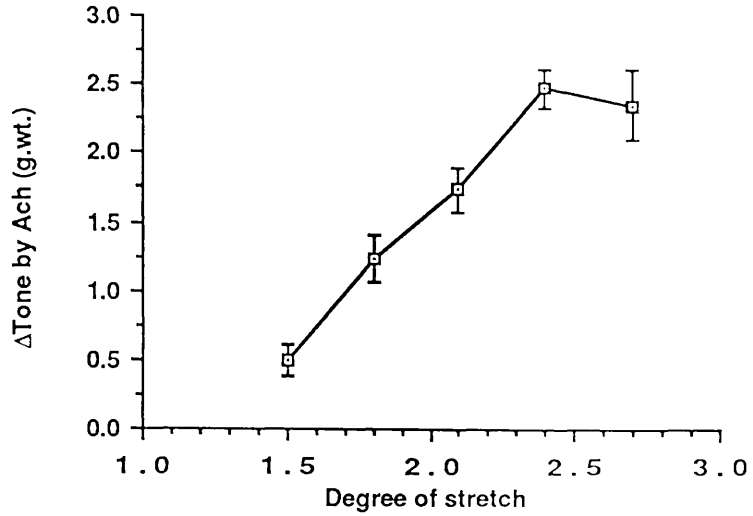
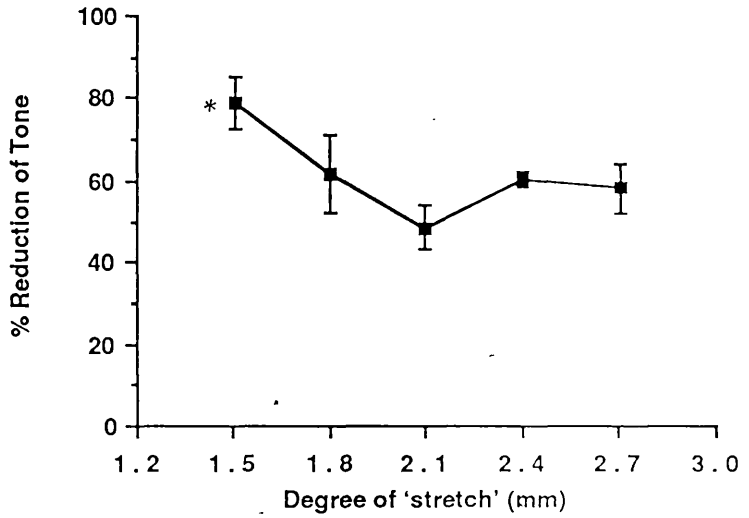


Figure 22 b



Both figures show how the degree of 'stretch'(mm) affects the relaxation to 1μM Ach on tissues with an intact endothelium, precontracted with 1μM Phe expressed as both an absolute change in tone in g.wt. (Figure 22a) and also as a percentage of the induced tone (Figure 22b). Statistical analysis was carried out to determine whether or not the optimum response for this effect was significantly different from the response at the optimum degree of stretch for contraction *per se* (2.4mm). Values are given as mean ± s.e.m. (n=6).

Figure 23 a

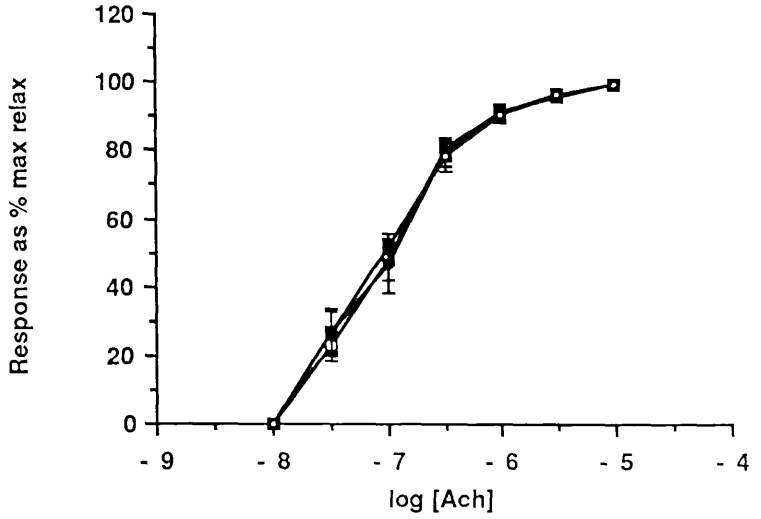
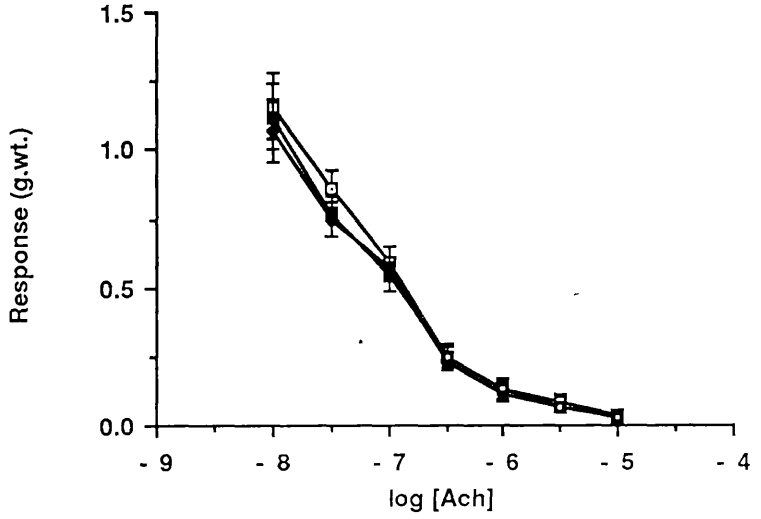


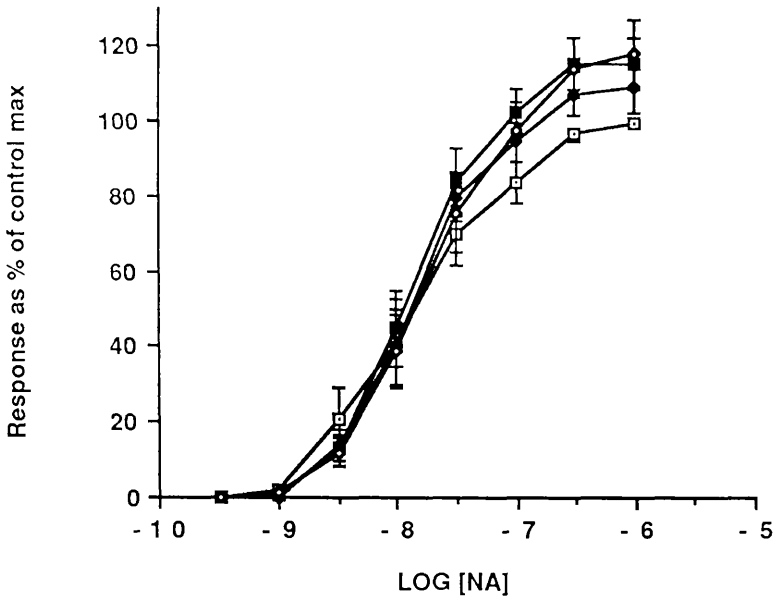
Figure 23 b



The effect of time on relaxation response curves to Ach. Results are expressed as a percentage of the maximum relaxation (Figure 23a) and also in (g.wt.) (Figure 23b). Values are given as mean ± s.e.m. (n=6). The first point on the curves represents the tone induced by NA. There was no significant change in the response with time.

1st curve- ⊡ 2nd curve- ◆ 3rd curve- ■

Figure 24



The effect of time on concentration response curves to NA in tissues with an intact endothelium. Results are expressed as a maximum of each curve. This graph is similar to that of 13a where the results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6). 1st curve-⊡ 2nd curve-◆ 3rd curve-■ 4th curve-◇

less sensitive to Ach with a greater concentration required to elicit a similar percentage relaxation (figure 25). 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 ($P>0.001$) (figure 26).

A similar correlation between the tissue sensitivity for 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, expressed in terms of the maximum relaxation of induced tone, showed no statistically significant correlation (figure 27) at all concentrations of NA used to induce that tone.

Effect of Drugs on Relaxation to Acetylcholine

ATROPINE

The tissues were contracted with NA (0.1μM) which produced approximately 85-90% of the initial maximum contraction: a relaxation-response curve to Ach (0.03-10μM) was carried out in the presence of three increasing concentrations of atropine (1ηM-100ηM) (figure 28a). Previous experiments (figures 23a,b) showed that responses to Ach did not change with time. Atropine at all concentrations used had no effect on the size of the contraction to NA. Shifts in the relaxation response curve to Ach were not parallel but all reached the same maximum relaxation with the exception of the curve in the presence of the highest concentration of atropine. The maximum relaxation to Ach(10μM) with 100nM atropine was 45% of the contraction compared with an average of 12% in the controls and 11% and 15% in the presence of 1nM and 10nM atropine. From a Schild analysis having a slope of 0.6(0.57-0.62) a pA_2 value for atropine of 9.59(9.53-9.63) (figure 28b) was obtained. This value was similar to that

Figure 25 a

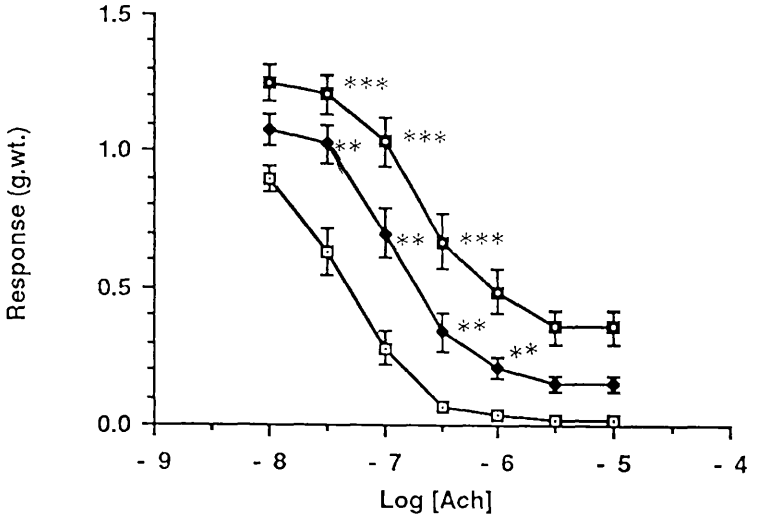
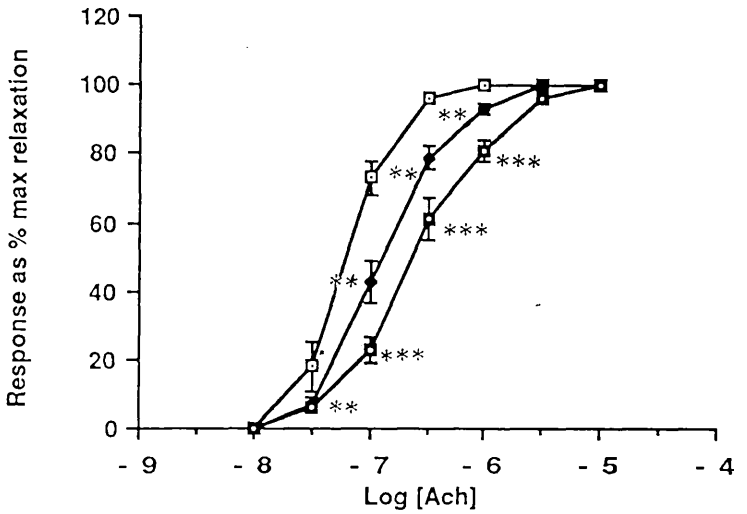


Figure 25 b



The effect of changing the tone by increasing [NA] on the ability of Ach to produce relaxation. Results are expressed as a percentage of the maximum relaxation (Figure 25a) and also in (g.wt.) (Figure 25b). Values are given as mean ± s.e.m. (n=6). The first point on each curve indicates the contraction to NA. Statistical significance was determined by comparing the responses with those obtained with the lowest concentration of NA (30nM) to induce tone. 30nM NA- ⊡ 0.1μM NA- ◆ 0.3μM NA- ■
(Statistical significance was determined as follows: (* $P<0.05$ ** $0.01>P>0.001$ *** $P<0.001$).

Figure 26

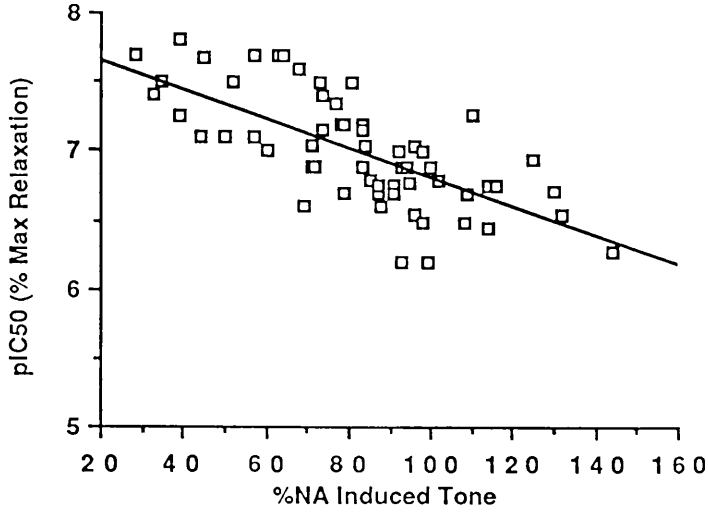


Figure 27

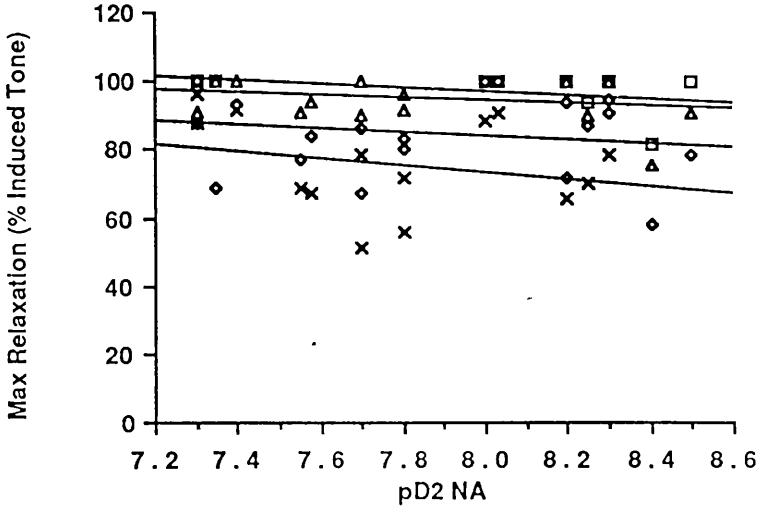


Figure 26 shows how the pIC50 to Ach varies depending on the % of induced tone to NA. Results are plotted as % induced tone against the pIC50 to Ach. A relationship was obtained between the two variables by cross correlation .

Figure 27 shows how the maximum relaxation to Ach with various NA induced tone does not depend on the initial pD2 value to NA. No relationship was obtained between the two variables by cross correlation.

Figure 28a

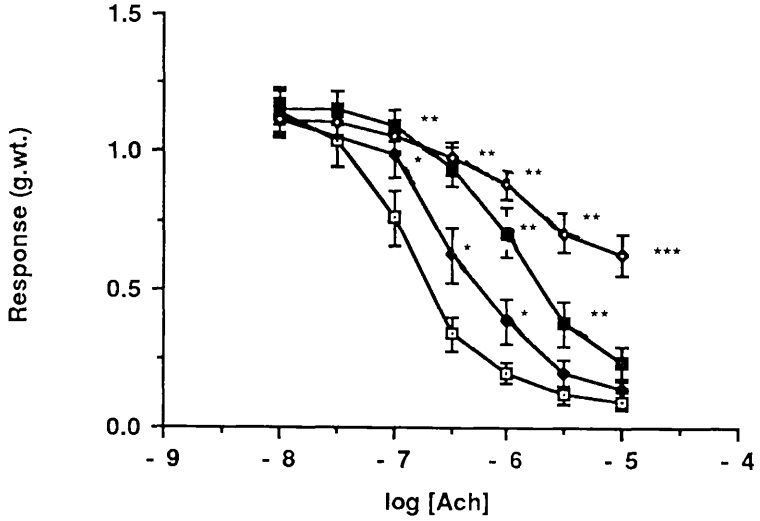
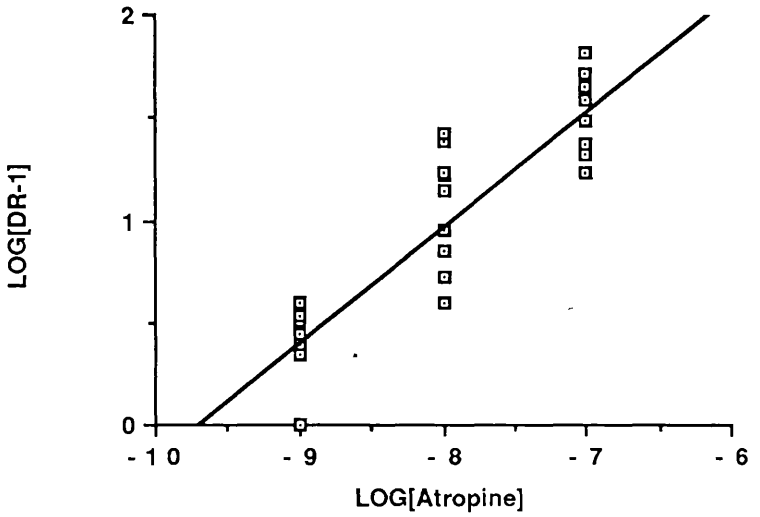


Figure 28b



The effect of increasing concentrations of atropine on relaxation to Ach. The results are expressed in g.wt.(Figure 28a). Values are given as mean ± s.e.m. (n=8). Statistical analysis was determined by comparing the response in the presence of the antagonist with that of control. control- ⊡ +1nM atrop- ◆ +10nM atrop- ■ +100nM atrop- ◇ (Statistical significance was determined as follows: * $P<0.05$ ** $0.01>P>0.001$ *** $P<0.001$)

Figure 28b shows the Schild plot for atropine against Ach. The slope of this line is 0.6(0.57-0.62). pA_2 value of 9.59(9.53-9.63).

obtained by Furchgott (1980) in the rabbit aorta where it acted as a competitive antagonist. The -log value for the concentration of Ach which produced 25% relaxation of the induced tone was plotted for controls and in the presence of atropine and shown in figure 30a. This was done to enable comparison with data obtained using BAY-K 8644.

BAY-K 8644

When the calcium facilitating drug BAY-K 8644 was used (0.03- 0.3μM) a lower concentration of NA was used to induce tone (0.03μM) 75-80% of initial maximum response. When this drug was present the relaxatory responses to Ach were significantly decreased (figures 29a,b). Analysis is complicated because the contractile responses to NA were increased for each concentration of BAY-K 8644 (hence the reason for using a lower initial concentration of NA to induce contraction). However, even accounting for this increase in response to NA by comparing the data with the normal control relationship between initial contraction and sensitivity to Ach, the effect of BAY-K 8644 was still significant, as tested by cross correlation of two variables. The -log value for the concentration of Ach which produced 25% relaxation of the induced tone was plotted and shown in figure 30b. This was done to enable comparison of responses with control since in the presence of BAY-K 8644 the relaxation did not always fall as far as 50% of the induced tone making it impossible to use the -log 50% values as used in figure 24. In figure 28 all the values for Ach relaxation obtained in the presence of BAY-K 8644 were significantly less potent than would be expected for a given size of contraction and lie outwith the line obtained linking % induced tone with IC_{25}. When the relaxation to Ach was expressed as a percentage of the maximum relaxation reached for each concentration of BAY-K 8644 the sensitivity to Ach appeared to be only slightly decreased but this was not significant (Figure 29c). This was because the maximum relaxation

Figure 29a

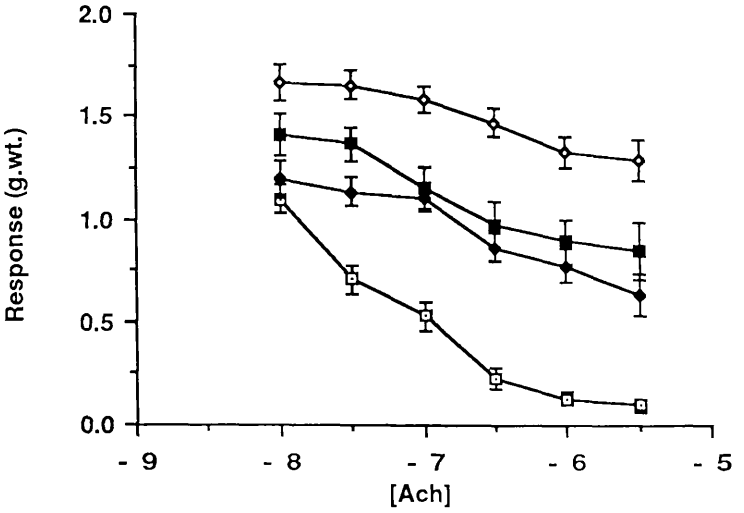
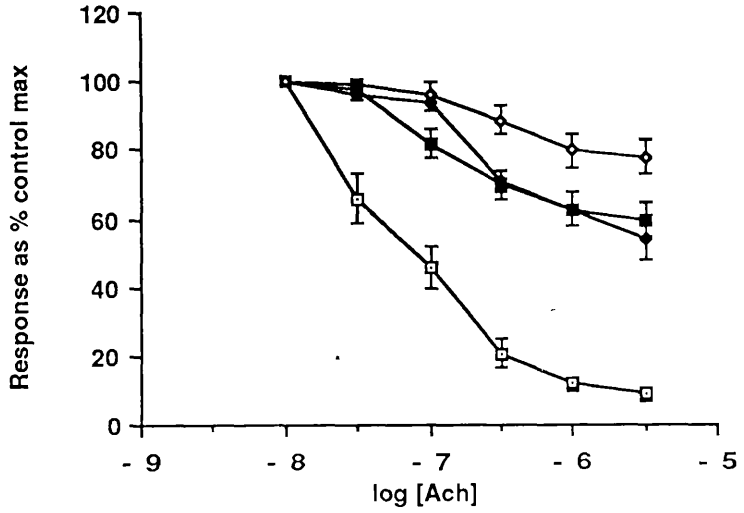
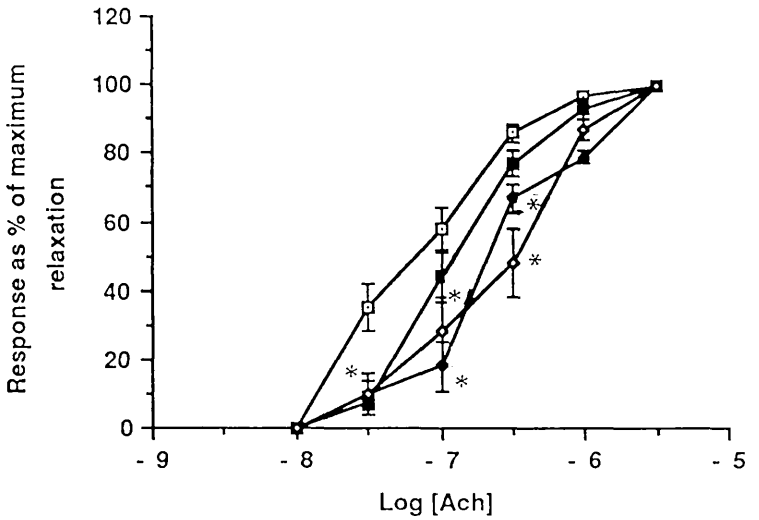


Figure 29b



The effect of increasing concentrations of BAY-K 8644 on relaxation to Ach. The results are expressed as g.wt. (Figure 29a) also as a percentage of the induced tone (Figure 29b). Values are given as mean ± s.e.m. (n=6). First point on each curve represents the tone induced by NA. Statistical analysis was carried out comparing values in the presence of the drug to the control. control-⊡ +30nM BAY-K-◆ +0.1uM BAY-K-■ +0.3uM BAY-K-◇
All points in the presence of BAY-K 8644 were significantly different from controls $P<0.05$.

Figure 29c



This figure shows the effect of BAY-K 8644 on relaxation to Ach against NA induced tone. The results are expressed as a percentage of the maximum relaxation for each curve. The first point on each curve represents the contraction to NA. Responses are expressed as mean ± s.e.m. (n=6). Statistical analysis was carried out by comparing the responses in the presence of the drug with those of control. control- ⊡
30nM BAY-K- ■ 100nM BAY-K- ◇ 0.3uM BAY-K- ◆

Statistical significance was determined as follows : *P<0.05 **0.05<P<0.01 ***P<0.001.

Figure 30a

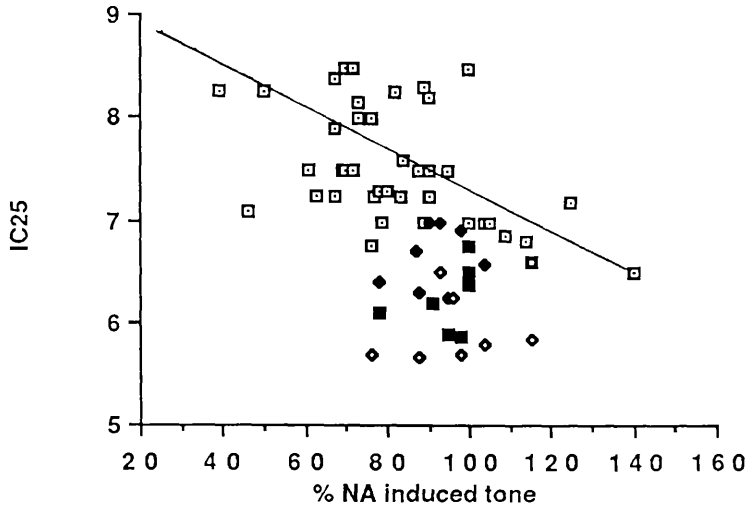
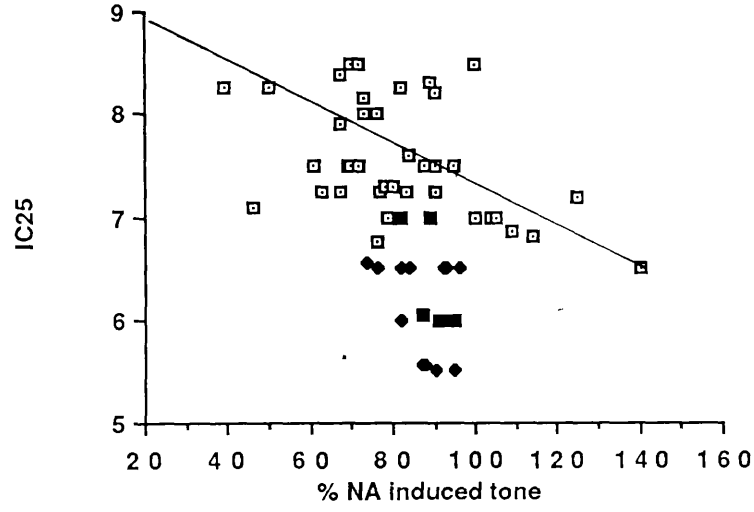


Figure 30b



This figure shows the relationship between the % of NA induced tone against the pIC_{25} to Ach in the presence of Atropine (Figure 30a) control- ⊡ 0.1nM atrop- ◆ 1nM atrop- ■ 10nM atrop- ◇
or BAY-K 8644 (Figure 30b) control- ⊡ 0.01uM BAY-K- ■ 0.1uM BAY-K- ◆

The values in the presence of the drugs are significantly different from the control by cross correlation of two variables.

was significantly reduced from that of the controls and so was not acting as a competitive antagonist, but rather as a non-competitive one. Using BAY-K 8644 at the lower concentrations (3nM-10ηM) did not significantly, when tested by cross- correlation of two variables, alter the relaxation to Ach .

WYETH 26703

When the α_2-adrenoceptor drug Wyeth 26703 was present there was no significant effect on relaxation to Ach (Figure 31a,b) regardless of how the results were expressed. The -log value for the concentration of Ach which produced 25% relaxation of the induced tone was plotted against % of NA induced tone (Figure 32). This was done to enable comparisson of responses with BAY-K 8644 and atropine since the responses to BAY-K 8644 did not always reach 50%.

Figure 31a

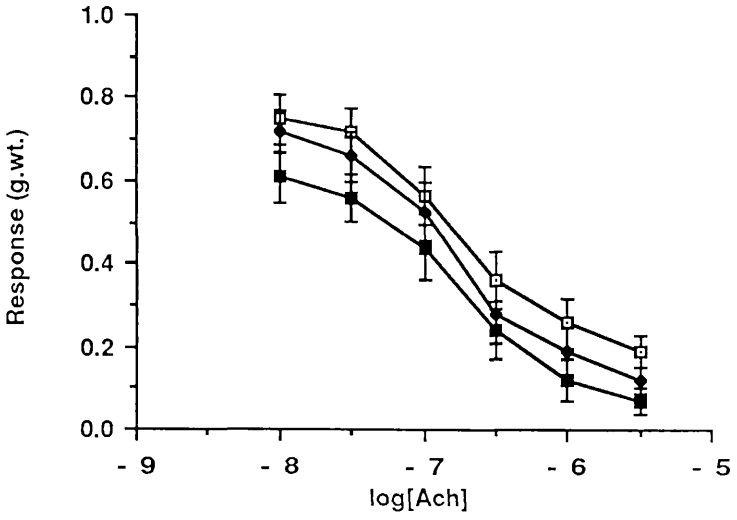
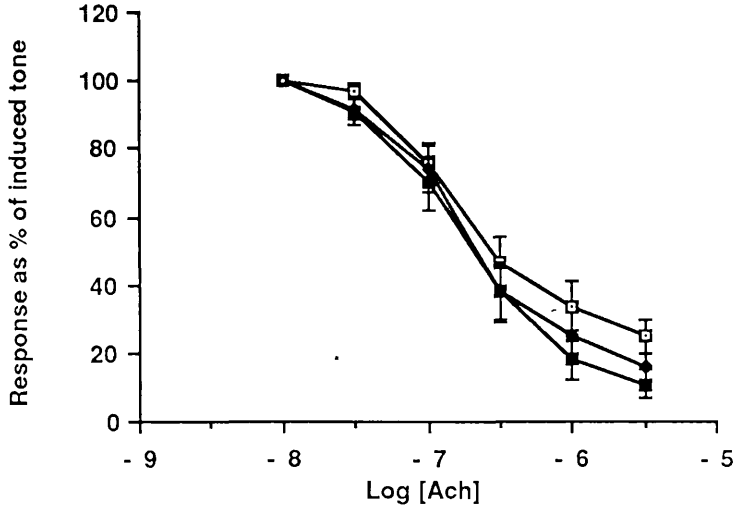


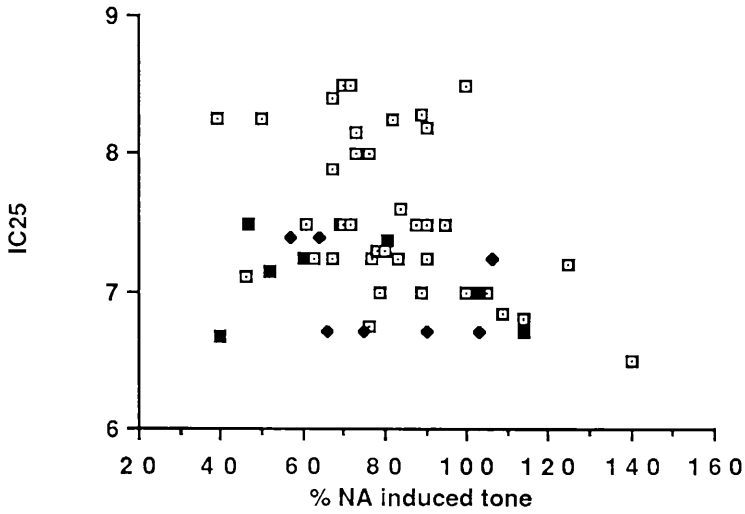
Figure 31b



The effects of Wyeth 26703 on relaxation to Ach. Results are expressed in g.wt. (Figure 31a) and as % of the induced tone (Figure 31b). The first point in each curve represents the tone induced by NA. Values are given as mean ± s.e.m. (n=6).
control- ⊡ +10nM Wyeth- ◆ +0.1uM Wyeth- ■

There was no significant difference in the values from the control.

Figure 32



This shows the relationship between the % of NA induced tone against the pIC_{25} to Ach in the presence of Wyeth 26703.

control-⊡ +10nM Wyeth-◆ +0.1uM Wyeth-■

This relationship is not significantly different from that of control when compared by cross correlation of two variables..

DISCUSSION

This study was carried out to examine the influence of some of the basic elements in the protocol of all experiments designed to demonstrate quantitatively the effects of basal or stimulated release of EDRF. Using the descending thoracic aorta of the rat we examined a) the effect of the resting degree of stretch (and hence tension) of the aortic ring segments with and without an intact endothelium, also the effect of the degree of induced tone on endothelium-dependent Ach-induced relaxations; b) the effects of BAY-K 8644 which at certain concentrations increased the responses to NA and found it had more of an inhibitory effect on relaxation than would be expected from increased tone alone; c) the effects of Wyeth 26703 which acting as an α_2-adrenoceptor antagonist may alter the relationship between NA pD_2 and Ach pIC_{50}, if α_2-adrenoceptors are involved in release of EDRF from the endothelium. The results showed that, despite a slight blocking of the responses to NA, there was little evidence of a disturbance in the normal relationship between contraction and relaxation; d) The antagonism of atropine on Ach relaxation and its relationship to contraction size was examined and compared to the effects of BAY-K 8644.

The method which we used to cause functional disruption of the endothelium of the aortic ring segments was clearly effective since the 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 where the endothelium had been disrupted possibly indicating the removal of a basal release of EDRF but little damage to the underlying smooth muscle layer.

As previously described in section 1 and by others, (Martin et al., 1986;Singer & Peach, 1985; Bullock et al., 1986), there is evidence that the basal release of EDRF modulates the responses of contractile agents on vascular smooth muscle. This hypothesis is based mainly on increased active responses to agonists on removal of the endothelium. This basal release might decrease the resting tone of isolated blood vessels in such a way that tissues with endothelium would have less tension at any given length than in the absence of endothelium. Thus tissues with endothelium would need to be stretched further to achieve the same resting tension as an equivalent tissue without endothelium. This factor could then contribute to variations in the reported differences in the contractile responses between tissues with and without endothelium since the tissues with an intact endothelium would have initially been set up at a different length, if both had been set up at the same tension. 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 indicates 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) is similar. This therefore, will not play a significant part in the difference seen in the responses to contractile agents in paired intact/disrupted preparations. This is perhaps to be expected in rat aorta, which has little, if any, intrinsic active tone, but might not remain true in other vascular muscle preparations which have intrinsic tone. Another corollary of this lack of influence of the endothelium on resting tone is that the pharmacological blockade of EDRF by Hb should not, on its own, increase the vessel tone. Therefore the contractions seen to Hb cannot be due solely to the loss of the effect of EDRF as has been suggested by Martin et al, 1986. The results which we obtained using Hb indicate that its action on tissues which have no tone present is not limited to endothelium intact tissues (Figure 12c). At a later point in his paper Martin reports that as the experiment progresses they do obtain a contraction to Hb

in tissues which have no endothelium, in the absence of any induced tone. However they were unable at that time to offer any explanation for this observation which agrees with our own and may indicate a direct action of Hb on the smooth muscle itself, as well as an inhibitory action on basal release of EDRF.

Whilst the responses to Phe (1μM) increased with increasing stretch, reaching a plateau at the same point in both intact and disrupted preparations, the response seen in the absence of the endothelium was consistantly greater than that in the intact vessel at all degrees of stretch measured. This demonstrated that the presence of the vascular endothelium in the rat aorta depressed the contractile responses to α_1-adrenoceptor activation but did not affect the optimal conditions (in terms of initial tension) for demonstration of the contractile effects of the α_1-adrenoceptor agonist Phe. However, although the initial length appeared to have no differential effect on the resting tension between intact and disrupted preparations, the increase in contractility 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 depending on how the increase was expressed. The optimum length for demonstration of the absolute difference in contractility to Phe (in terms of g.wt) 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. Regardless of the method used to express the difference in contractility, the optimum length for demonstration of this phenomenom did not coincide with the optimum stretch for demonstration of the contractile response.

Demonstration of the relaxation produced by Ach (1μM), was also dependent on the initial length of the preparation. As with the demonstration of the influence of the endothelium on the 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(1500μm) for demonstration of the proportionate effect of Ach did not coincide with the optimum for Phe-induced contractions(2400μm). However, the optimum stretch for demonstration of the absolute relaxation (2400μm) did coincide with the optimum for Phe-induced contractions. This is possibly because at this length there is a greater absolute amount of tone that can be inhibited. It is common practice to express Ach-induced relaxations as a percentage of the induced tone, but this is not optimised by use of the conditions optimal for contraction. When the sensitivity 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) 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.

The sensitivity of rat aortic ring segments to Ach relaxation 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 (as expressed as its pIC_{50}) decreased. This relationship was further supported by the significant inverse correlation seen for the tissue sensitivity to Ach against the level of induced tone produced by NA. In the presence of the muscarinic receptor antagonist atropine this relationship was significantly altered. Increasing concentrations of atropine had no effect on the size of the contraction to NA therefore the pIC_{50} values obtained for Ach in the presence of this drug which were significantly different from the control values indicate that the drug was having a real effect on the relaxation to Ach. Since the Schild plot is significantly

different from 1 the antagonism could not be considered as competitive. However since atropine does have a blocking effect indicates that the receptor Ach is acting on is muscarinic. The results obtained in the presence of BAY-K 8644 were not those expected when compared to control values even accounting for the increase in the size of the contraction to NA. That we were only able to obtain pIC_{25} values to Ach indicates the poor relaxation in the presence of BAY-K 8644. When compared to the results with atropine, the pIC_{25} values obtained for Ach in the presence of BAY-K 8644 were similar in that they appeared below the regression line for control values. However in the presence of any concentration of BAY-K 8644 relaxation to Ach did not reach a maximum unlike that in the presence of atropine and possibly means a different action. How BAY-K 8644 brings about this inhibition of relaxation to Ach is unclear and may in part be explained by the increased size of contraction that it produced. It may be that the increase it produced was less able to relax to Ach as it may have been a supra maximal contraction . Although Singer & Peach (1986), demonstrated that calcium was essential for release of EDRF, it may however be an effect of calcium overload which could act to inhibit release of EDRF. In an investigation to determine whether Ca^{2+}-channel activation by BAY-K 8644 would cause release of EDRF, Spedding et al (1987) found that in the rat aorta following partial K^{+}- depolarisation, BAY-K 8644 contracted preparations directly. This effect was most sensitive when the endothelium was disrupted. However they did not find an increase in cGMP levels (which is a function of EDRF release) in the presence of the endothelium. Although our results suggest the inhibition of EDRF release by BAY-K 8644 these observations at least demonstrate that this activator has no potentiating effect on EDRF release. In Chapter 3 (Figures 39a,b) concentration-response-curves to NA in the rat aorta with inatct or disrupted endothelium show a shift to the left in the presence of BAY-K 8644. From these results it would seem that the drug has a greater effect on tissues with an intact endothelium (possibly by inhibiting

spontaneously released EDRF) and would support our observations with Ach stimulated release. Before any conclusions can be made measurements of release of EDRF, as determined by an increase in cGMP activity, or alternatively using a superfusion cascade, would have to be made in the presence of BAY-K 8644 to determine whether it was acting to decrease release of EDRF of else having some effect on its action on smooth muscle.

When Wyeth 26703 (10nM) was used, a concentration expected to antagonise α_2-adrenoceptors, relaxation to Ach was not altered significantly from the control values. If the concentration of Wyeth 26703 was increased further (100nM) it had a slight inhibitory effect on the size of the contraction induced by NA. This action was compatible with an action on α_1-adrenoceptors (Lattimer et al, 1984). That Wyeth 26703 had no inhibitory action on relaxation to Ach suggests that α_2-adrenoceptors have no involvement in the stimulated release of EDRF, at least in this preparation and under these conditions. However, other workers have looked at α_2-adrenoceptor agonists and their relaxatory effects in the presence of α_1-adrenoceptor antagonists and have found them to produce good relaxations which are blocked by α_2-adrenoceptor antagonists (Cocks & Angus 1983, Angus et al 1985). However they were using a different arterial preparation, coronary arteries from dog and pig and it may be that this process is only present in certain arteries. These results taken with the effects of Wyeth 26703 on CRC to NA in intact and disrupted endothelium is some evidence to eliminate the role of α_2-adrenoceptor-mediated relaxation in this tissue. Our approach was, however, more indirect and may have been unable to detect the small effect of NA on α_2-adrenoceptors producing relaxation especially since the more dominant α_1-adrenoceptor effect had not been blocked by an antagonist. However as described in the discussion of chapter 1, other workers using the rat aorta have been unable to obtain relaxation to α_2-adrenoceptor agonists using more direct methods. They have also been unable to detect any changes in the levels of cGMP in the presence of selective α_2-agonists

which is essential for release of EDRF (Martin et al 1986) (Ach can increase cGMP levels 3.6 fold). However other workers have detected changes in cGMP levels but this has not been confined to selective α_2-agonists and therefore cannot support the arguement (Bullock et al ,1986).

In a separate study, (Dainty, unpublished observations) aortic ring segments were set up where the endothelium was intentionally damaged to various degrees such that the responsiveness of the tissue to Ach-induced relaxations varied. In these tissues the sensitivity to contractile agents showed an inverse, statistically significant correlation with the responsiveness to Ach-induced relaxations. The lack of a significant correlation therefore, between the sensitivities of the tissues to NA-induced contractions and subsequent Ach-induced relaxations indicated that the basal release of EDRF (and hence the integrity of the endothelium) in the preparations used in this study was consistent and therefore played no significant role in the difference in apparent sensitivity observed at different levels of induced tone.

In conclusion, we have presented evidence to suggest that the differences seen in the quantitative demonstration of both the basal and stimulated release of EDRF between laboratories and within studies may be, at least in part, a result of the experimental protocol used to examine these phenomena. We have also shown evidence for a lack of involvement of α_2-adrenoceptors in EDRF release, at least under the conditions described. Difficult to explain are the findings with BAY-K 8644 which blocked Ach-induced relaxation similarly to atropine but not in a competitive manner, but it may have been due to an effect of calcium overload in the smooth muscle.

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 the method in which the data is expressed. In addition, the degree of induced tone plays a critical role when used to examine the effects of the stimulated release of EDRF. The apparent sensitivity of the tissue was altered when different levels of induced tone were employed. This sensitivity could also be altered by the presence of drugs like atropine and BAY-K 8644. These factors may account for the variations in interpretation of the role of EDRF between different vessels, pre-treatments and laboratories. The conditions used therefore, to compare vessels from different species or in different patho-physiological states, should be chosen carefully in order to reduce mis-interpretation of data obtained and ensure optimum demonstration of the effects of basal and stimulated release of EDRF. There is no simple solution for this. It is recommended however, that the protocol for each vessel is optimised for the particular effect under study.

CHAPTER 3

THE IMPORTANCE OF CALCIUM FOR THE CONTRACTILE RESPONSE OF SMOOTH MUSCLE

INTRODUCTION

Smooth muscle structure and activation

Vascular smooth muscle is made up of small fibres which have a single nucleus in a central position of the cell. Unlike skeletal muscle there is no striated pattern of thick (myosin) and thin (actin) filaments. Instead there is a random overlap of thick and thin filaments. However, at high magnification cross bridges can be seen from the thick filaments to the thin filaments. The actin (thin) is attached to dense bodies which transmit the tension developed by the cross bridges to the cell periphery. Also two proteins, troponin and tropomyosin, which play a vital role in the contraction of skeletal muscle, have been isolated from smooth muscle.

The initial step in smooth muscle contraction is thought to be brought about by the activation of the myosin light chain kinase enzyme via the Ca^{2+}-calmodulin complex which results in phosphorylation of myosin (Sobieszak, 1977). The phosphorylation of the 20,000 Dalton unit of myosin light chain results in rapid shortening of the muscle. Dephosphorylation of myosin by myosin light chain phosphatase in the presence of calcium, is a much slower process and results in a slower but maintained contractile state of the muscle. Removal of calcium causes the cross-bridge filaments to return to their resting state.

Since it is important that the intracellular calcium levels be kept as constant as possible, two membranal systems are responsible for the control and the rapid fluctuation of cytoplasmic calcium concentrations: the sarcolemma and the sarcoplasmic reticulum. Both systems function in calcium delivery which, due to the favourable electro-chemical gradient, will enter through channels; these two

systems are also involved in calcium removal which requires ATP-dependent pumps; Van Breemen et al, (1979) showed that the inward movement of calcium was more dependent on levels of cellular ATP than on the electrochemical gradient of the calcium ion, and that when ATP was depleted cellular calcium concentration increased. Removal was also dependent to a lesser extent on Na^{+}-Ca^{2+} exchange pumps. Burton & Godfraind, (1974), demonstrated that the amount of exchangeable calcium increased when the intracellular sodium concentration increased, and therefore may play a part in the control of calcium movement.

Release of Intracellular Calcium

Intracellular calcium is mainly released via the sarcoplasmic reticulum although a small component may be obtained from the inner plasmalemma surface (Daniel, 1985; Saida, & Van Breemen, 1983). Leijten & Van Breemen (1984) measured the caffeine-induced $^{45}Ca^{2+}$ release in the rabbit aorta and found that the value obtained was similar to that for the caffeine-releasable Ca^{2+} from the sarcoplasmic reticulum. This seemed to favour the hypothesis that the sarcoplasmic reticulum was the main intracellular store of releasable calcium. The role of intracellular calcium stores in excitation-contraction coupling has been determined from the observation that agonists can induce a transient contraction in tissues which were present in Ca^{2+}-free solution or alternatively in normal Krebs solution where Ca^{2+} entry is blocked by lanthanum. This transient response was accompanied by a phasic increase in $^{45}Ca^{2+}$ efflux which indicates release of calcium from an intracellular store (Deth & Van Breemen, 1977; Droogmans et al, 1977). The degree to which agonist-induced release of intracellular calcium is involved in the activation of vascular smooth muscle is dependent on the species and the artery examined. For example in the rat aorta (Godfraind & Kaba, 1969) agonist-induced calcium release plays a major role in its activation, but not so in the dog

basilar artery (Allen et al, 1976; Allen & Banghart, 1979). Bohr, (1973), found that α-adrenoceptor stimulation depended on two calcium pools; an intracellular one which was responsible for the phasic contraction and an extracellular pool responsible for the tonic contraction.

Calcium entry via Channels

Van Breemen et al, 1979 and Bolton 1979 postulated the existence of two types of Ca^{2+} channels.

1) Receptor operated channels (ROC)

2) Potential operated channels (POC)

Later work by Van Breemen et al (1986), using $^{45}Ca^{2+}$ influx measurements, found that calcium entered the cell in the absence of any excitation either chemical or electrical in nature, i.e. leakage of Ca^{2+} into the cell. Evidence that the ROC and POC are distinct from each other was that the influxes of $^{45}Ca^{2+}$ they induce are additive. Conversely when different agonists are used their apparent influx of Ca^{2+} is similar as are their sensitivities to blockade by calcium antagonists, suggesting calcium entry via a common mechanism, (Van Breemen et al 1985).

Effect of Calcium Entry Blocking Drugs

The activity of a calcium-entry blocking drug is usually assessed by placing the preparation in a calcium-free Krebs solution which is then depolarised with a high concentration of KCl. The calcium is then slowly re-introduced into the bathing solution. This process can also be used to examine the calcium-dependency of transmitter or hormone induced contractions. If the process is repeated in the presence of a calcium-channel blocking agent the contractile responses are depressed in a dose-dependent manner. However in any given blood vessel the contractions of the smooth muscle cells produced by various vasoconstrictors can be differentially affected by calcium-entry blockers. For arteries in a calcium-free medium, NA (10μM) produces a fast non-maintained contraction.

However on the reintroduction of calcium a slow sustained contraction develops for review see Godfraind et al (1986). Calcium entry blockers can also block to a different extent the contractions induced by α_1 or α_2-adrenoceptor agonists, e.g. verapamil is significantly more effective in inhibiting contractile responses to selective α_1 agonists , e.g. methoxamine rather than those evoked by the more selective α_2 agonist clonidine (Vanhoutte,1982). This suggests that responses produced in vascular smooth muscle by α_1-adrenergic agonists are more dependent on extracellular calcium than those brought about by α_2-adrenoceptor stimulation. (McGrath, 1985) using the rat anococcygeus found that when calcium was omitted from the bathing solution (leaving a contamination level of approximately 10μM) that responses induced by NA, Phe and amidephrine were rapid contractions (20-30 sec to peak) followed by a slow contraction (peak 2-3 min) which declined to baseline within 5-10 min. Methoxamine, xylazine and indanidine all produced slower contractions (peak 2-5 min) which declined to baseline within 5-10 min. The slow component of each these agonists responses was blocked by the calcium channel blocking agent nifedipine. This suggests the component was due to entry of extracellular calcium present due to contamination and that both groups of agonists were inhibited to a similar extent with no selectivity between α_1 and α_2-adrenoceptor agonists. Also, work carried out by Alabaster (1984) demonstrated that α_1 and α_2-adrenoceptor activation, especially by phenylethanolamine derivatives were equally dependent on extracellular calcium influx. Janssens & Verhage (1984) came to a similar conclusion using the dog saphenous vein. They found that α_1- and α_2-adrenoceptors caused both an influx of Ca^{2+} ions and released intracellular calcium. They concluded that the source of intracellular calcium from activation by either of the receptors was the same and that the process of influx by the two receptors was likely to be similar . Another factor which may play an important role in the influx or efflux of calcium is the vascular endothelium. It has been well documented as mentioned in

ring and the isolated perfused rat tail to examine the influence of the endothelium. We have also studied the effect in various disease states and how the relationship alters if any from the control. The conclusions from this study would have to be that under certain circumstances and depending on the tissue being studied that the endothelium does play a role in the modulation of responses both excitatory and inhibitory. However in several cardiovascular disease states which we studied the relationship between endothelium intact and disrupted tissues remains fairly similar with only some subtle differences.

MATERIALS AND METHODS

The rat aortic rings were set up as described in the general methods.

Buffered experimental Protocol

Paired tissues, endothelium intact and disrupted, were set up in normal Krebs for one hour. Tissues were then contracted with NA (30nM) until a plateau was reached. Then, Ach (3μM) was added to test the integrity of the endothelium which was indicated by 85-100% relaxation. The bathing fluid in which the tissues were placed was replaced with Buffer 6 [Ca^{2+}] (1μM) three times and then left to stabilise for twenty minutes. A concentration of NA was then added and the response, if any, was allowed to develop for ten minutes. Buffer 6 was then exchanged with to buffer 5 [Ca^{2+}] (3μM) which contained the same concentration of NA but a slightly higher calcium concentration. This process was repeated until the buffer having the highest concentration of calcium was present. Thereafter calcium chloride was added directly to the bath to give final calcium concentrations of 1.25mM, 2.5mM and 5mM.

Unbuffered experimental Protocol

In this series of experiments the initial testing of the integrity of the endothelium was carried out similarly to that in the buffered experiments. The bathing fluid was then exchanged with 1.25mM calcium Krebs solution three times and allowed to equilibrate for twenty minutes. A further three exchanges of bathing fluid were carried out into a 'calcium-free' Krebs solution and the tissues were again left for

twenty minutes to equilibrate. A concentration of NA was then added and a response allowed to develop after which calcium was re-introduced directly to the bath in steps of 0.3 log units (at each step the response was allowed to reach a plateau) until a final bath concentration of 5mM calcium was obtained.

Initial studies used NA (3μM) as the contractile agent and tested possible facilitation by BAY-K 8644 (1μM) . These experiments showed no evidence for facilitation, unlike the anococcygeus (McGrath et al 1985), with these concentrations of the drugs. Thereafter a series of experiments was carried out to determine the calcium sensitivity to different concentrations of NA. Four different concentrations of NA were examined (0.003-3μM) in tissues with either an intact or disrupted endothelium using the unbuffered calcium re-addition protocol. The purpose of this was to find a concentration of NA which would be suitable for looking at both facilitation and blockade.

In some studies the buffered protocol was carried out once, then followed by the unbuffered protocol . In other studies this procedure was reversed with the unbuffered protocol first followed by the buffered.

Concentration response curves (CRC) to NA were also carried out in both the intact and disrupted preparations in the presence of various concentrations of BAY-K 8644 (0.01-0.1μM). In these experiments three CRC were carried out, with the first acting as the control and the other two in the presence of increasing concentrations of BAY-K 8644.

Buffer mixtures

The problem of lack of a suitable Ca^{2+} buffer for the 10μM to 100μM range

(near neutral pH) is partially circumvented with an EGTA and NTA mixture (Miller & Smith 1984).

EGTA (Ethylene glycol bis- (B- aminoethyl ether) N,N,N'N' - tetra acetic acid).

EGTA is a tetravalent ion which buffers near 1μM Ca^{2+} at physiological pH.

NTA (Nitrotriacetic acid) (N,N-bis[Carboxymethyl] glycine)

NTA is a trivalent ion which buffers near 0.1mM Ca^{2+}

This combination of buffers allows us to accurately determine the free Ca^{2+} when the buffers are present. Certain conditions must be taken into account in determining these calculations:

(1) Ionic strength.

(2) pH of the medium. It is important that this is kept constant, because this will shift the equilibrium between the different forms of EGTA to emerge and therefore affect its buffering capacity and the [Ca^{2+}].

Buffer Recipe

Buffer 1 (mM: NaCl 112 , KCl 4.7 , $NaHCO_3$ 24.8 , $CaCl_2$ 4.98, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 11.1, NaOH 7.5 , EGTA 2.5, NTA 2.5,)

Buffer 6 is the same with the exception of calcium which is 2.48mM. The remaining buffers 2 to 5 were produced by mixing these two buffers in different proportions to obtain intermediate concentrations of 'free-calcium'. These proportions and concentrations being as follows: amounts are given in ml to make up to 250mls total.

	Buffer 1	Buffer 6	Log [Ca]
Buffer 2	167.2	82.8	-4.0
Buffer 3	82.8	167.2	-4.5
Buffer 4	36.64	213.36	-5.0
Buffer 5	13.36	236.64	-5.5

STATISTICAL ANALYSIS

Results of the effects of BAY-K 8644 and nifedipine on the sensitivity to calcium were compared with those of controls at each calcium concentration. Results were compared using the Students paired t-test since the same tissue was used for each concentration. Statistical significance was determined as follows * $P<0.05$ **$0.01<P<0.001$ ***$P<0.001$.

RESULTS

The initial experiments tested the sensitivity to calcium in the rat aorta and followed a similar protocol to that already established in the laboratory using the rat anococcygeus (McGrath et al, 1985). This involved using NA (3μM) (which in the aorta is a maximal concentration) to examine the sensitivity of the tissues to calcium using a buffered and unbuffered protocol. The buffered protocol was carried out first followed by the unbuffered protocol. Figure 33a shows the effect of removal of the endothelium on the responses using the buffered protocol. Figure 33b shows the effect again but using the unbuffered protocol. Figures 34a,b compare the responses in individual tissues with either intact or disrupted endothelium between the buffered and unbuffered protocols. These graphs show that at the lower concentrations of calcium the buffered system allows responses to NA to be accurately measured but at the higher concentrations of calcium the responses are significantly depressed when compared to the unbuffered protocol. It is interesting to note that there was no significant difference in the size of the response at any of the calcium concentrations, between the intact and disrupted tissues. This is as expected, however, as the concentration of NA used is at a point on the concentration response curve where there is no significant difference in the size of response between intact and disrupted tissues. This lack of difference could also be shown by the similar pD_2 values to calcium of tissues with intact and disrupted endothelium. However these values were different depending on whether the buffered or unbuffered protocol was used. When the buffered protocol was used the calcium pD_2 values were: intact/disrupted [4.98/4.92] . In contrast, when the unbuffered protocol was used the pD_2 values were [3.8/3.68]. Although the pD_2 values are different when the graphs are examined the buffered calcium curve is less steep when compared to the unbuffered calcium curve. This is due to both the lower concentrations of calcium being accurately determined in the

Figure 33a

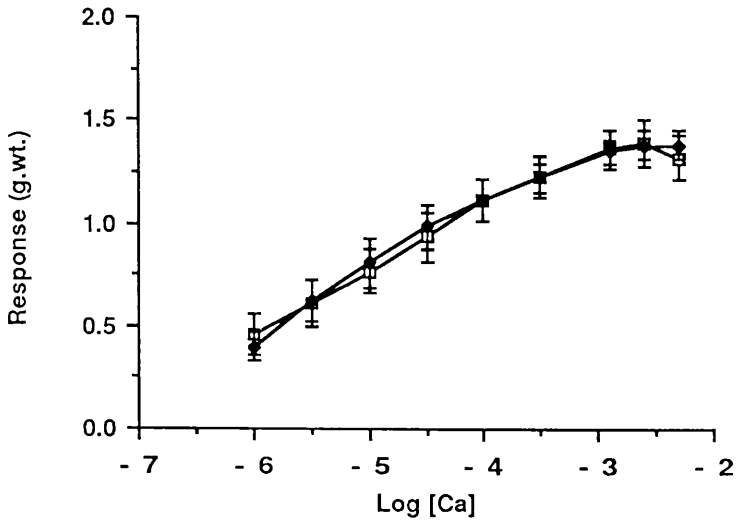


Figure 33b

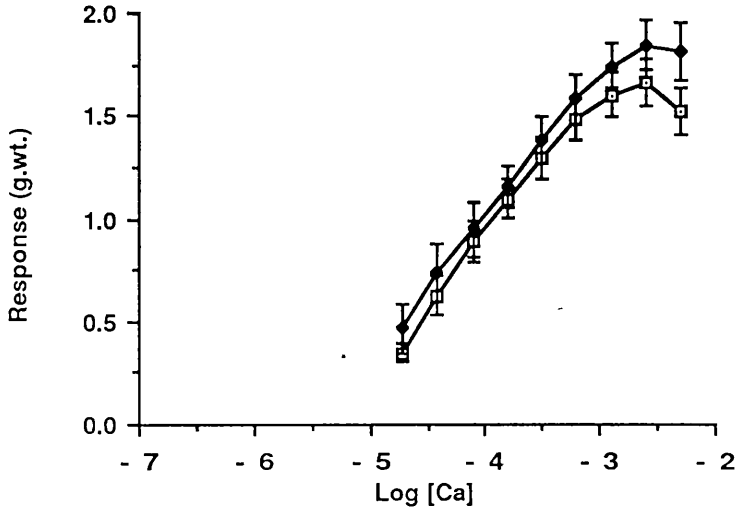


Figure 33a shows the effect of removal of the endothelium on the sensitivity of the rat aorta to calcium in a buffered system using 3μM NA as the contractile agent. +E-⊡ -E-◆

Figure 33b shows the effect again but using an unbuffered calcium system.+E-⊡ -E-◆

Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6).

Figure 34a

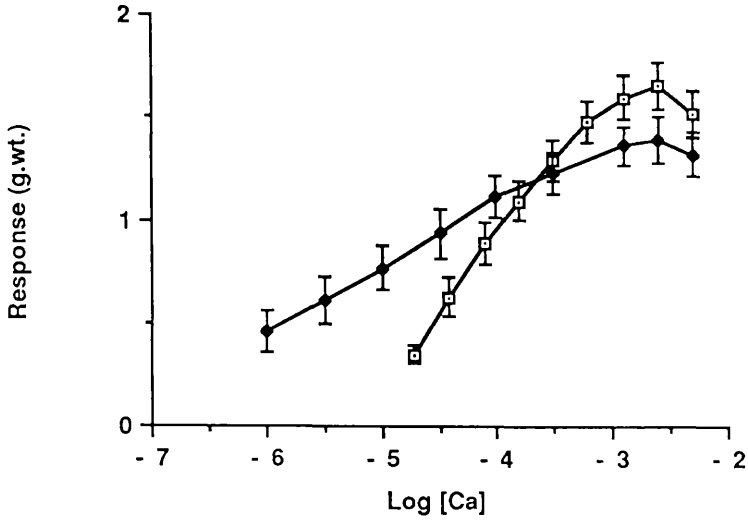
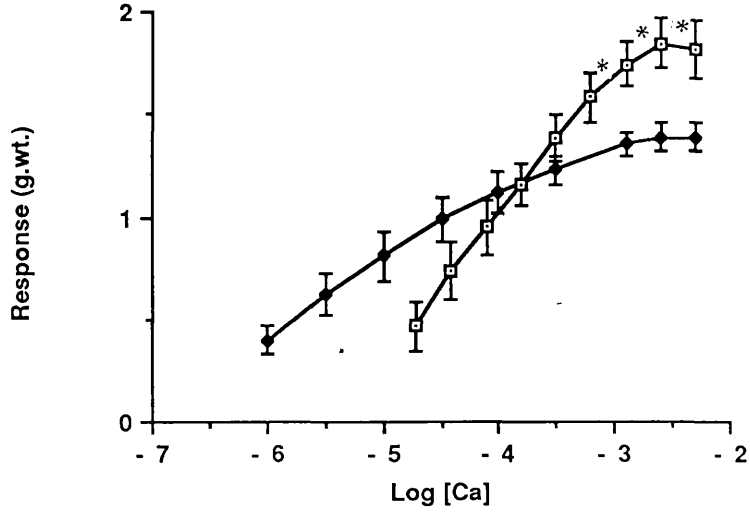


Figure 34b



The effect of buffering on sensitivity to calcium using NA 3uM in aortic rings with an intact (Figure 34a) and disrupted (Figure 34b) endothelium. Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6). Buffered- ◆ Unbuffered- ⊡

Statistical analysis was carried out by comparing curves in the buffer with those from the unbuffered. Statistical significance was determined as follows *P<0.05 **0.01<P<0.001 ***P<0.001.

buffered system and also the depressing effect on the responses to NA at the high concentrations of calcium when compared with the unbuffered system. The difference in the size of the maximum response using the buffered protocol may have been due to the order of testing i.e. buffered system carried out before the unbuffered system. However I tested this by carrying out the systems in the reverse order with results shown in Figures 35 & 36. These results demonstrate no significant difference between buffered curves carried out either before or after unbuffered curves. This was also shown for unbuffered curves carried out either before or after buffered curves. These experiments were all carried out in tissues with an intact endothelium.

BAY-K 8644 (1μM), a concentration which potentiates the responses to NA in the rat anococcygeus muscle (McGrath et al 1985), was used to see if the effect was similar in the rat aorta. Figure 37a shows the results of using BAY-K 8644 on the buffered protocol. Only at high calcium concentrations was the mean response increased, however this was not significant. Similarly using BAY-K 8644 on the unbuffered protocol there was no difference at any calcium concentration, figure 37b. These experiments with BAY-K 8644 involved carrying out the buffered protocol followed by the unbuffered protocol, with the endothelium intact on all tissues. The tissues were paired, with one having the drug present, the other acting as the control. This was done to minimise the number of calcium curves carried out in a single tissue. The effect of nifedipine on the calcium sensitivity of the response to NA using only the unbuffered protocol showed the responses to be significantly inhibited (Figure 38).

Since BAY-K 8644 showed no potentiation of the sensitivity to calcium when NA (1μM) was used as the contractile agent, I looked at the effect of the drug on a complete CRC to NA in tissues with either disrupted or intact endothelium. I also

Figure 35

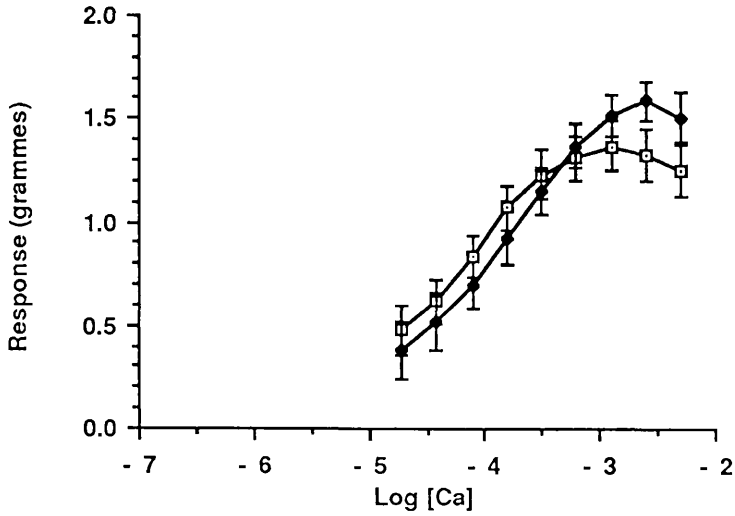


Figure 36

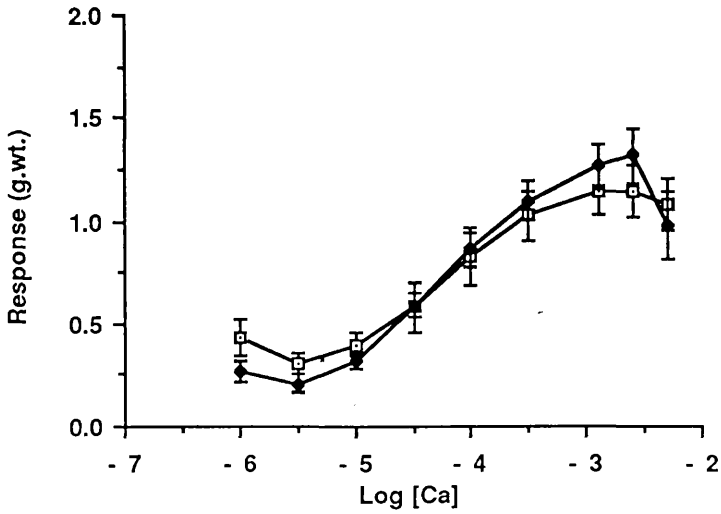


Figure 35 shows the effect of testing Ca++ sensitivity in the unbuffered system before or after testing in the buffered system in tissues with an intact endothelium. before-⊡ after-◆

Figure 36 shows the effect of testing the buffered system before or after the unbuffered system. before-⊡ after-◆
The tissues all had an intact endothelium. Results are expressed in g.wt.. Values given as mean ± s.e.m. (n=6).

Figure 37a

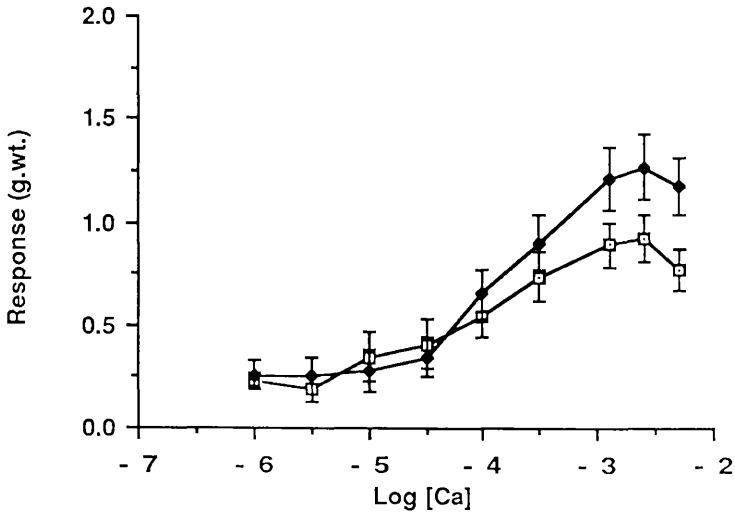
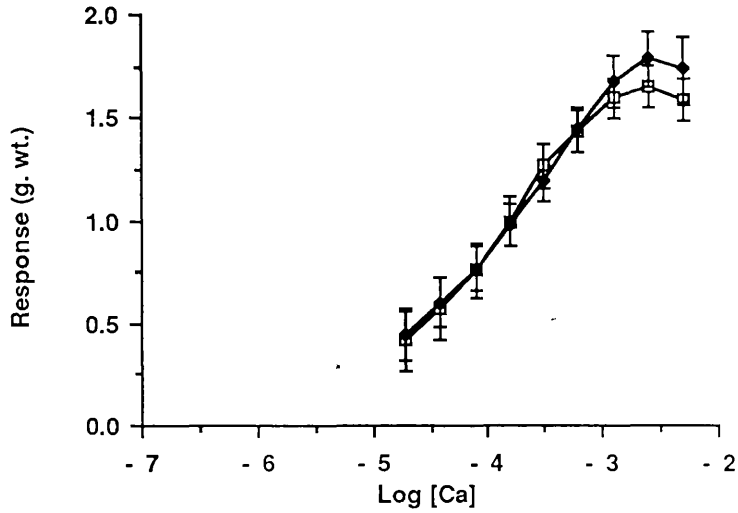


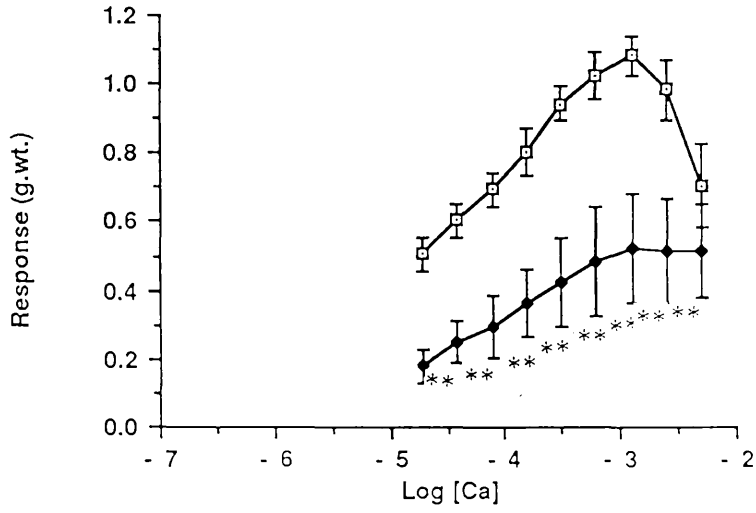
Figure 37b



Each graph shows the effects of BAY-K 8644 (1μM), on the sensitivity to calcium usin 3uM NA with a buffered (Figure 37a) and unbuffered (Figure 37b) calcium system. Responses were obtained from the first curve in paired tissues which had an intact endothelium . Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6).control- ⊡ +BAY-K -◆

There was no significant difference at any calcium concentration.

Figure 38



This figure shows the effects of nifedipine (1uM) on calcium sensitivity to NA 3uM in an unbuffered system using rat aortic rings with an intact endothelium. Results are expressed in g.wt. Values are given as mean ± s.e.m. Statistical analysis was carried out by comparing values in the presence of nifedipine with those of control. control-⊡ nif-◆ Statistical significance was determined as follows. *P<0.05 **0.01<P<0.001 ***P<0.001.

used lower concentrations of BAY-K 8644 (10-100nM) since it was possible that the 1μM may have been too high and acting in an antagonistic fashion : which it has been shown to do (Schramm et al 1983). Using 10nM BAY-K in tissues which had an intact endothelium the CRC was shifted significantly to the left. Using the higher concentration of BAY-K 8644 (100nM) the curves were not shifted further. At both concentrations of drug the maximum response was not increased significantly (Figure 39a). Using 10nM BAY-K 8644 in tissues with a disrupted endothelium the CRC was not shifted as far to the left as in tissues with endothelium intact. This was tested by comparing the shift in pD_2 values between intact and disrupted tissues. There was a significant shift in pD_2 value to calcium in the presence of 10nM BAY-K 8644 only when the endothelium was intact. Again the higher concentration of BAY-K 8644 (100nM) produced no further leftward shift in the curve, also no increase in the maximum response at both concentrations (Figure 39b). Since there was potentiation with BAY-K 8644, but only at lower NA concentrations, we decided to test the compound on the calcium sensitivity at a lower concentration of NA. This may also explain why we obtained no potentiation when using the high concentration of NA.

First, responses to different concentrations of NA and their sensitivity to calcium using the unbuffered protocol only were examined (figures 40 a,b). Responses were not increased further going from 0.3μM NA to 3μM NA. NA(0.03μM) produced a submaximal response in both intact and disrupted tissues. It was also the only concentration (from those examined) where there was a significant difference in the size of the response at the various calcium concentrations between intact and disrupted tissues. Due to these results this concentration of NA was thus chosen to examine the effects of BAY-K 8644 and nifedipine.

BAY-K 8644 (1-100nM) was examined using the unbuffered protocol. Due to results from early experiments I decided to carry out either the unbuffered or

Figure 39a

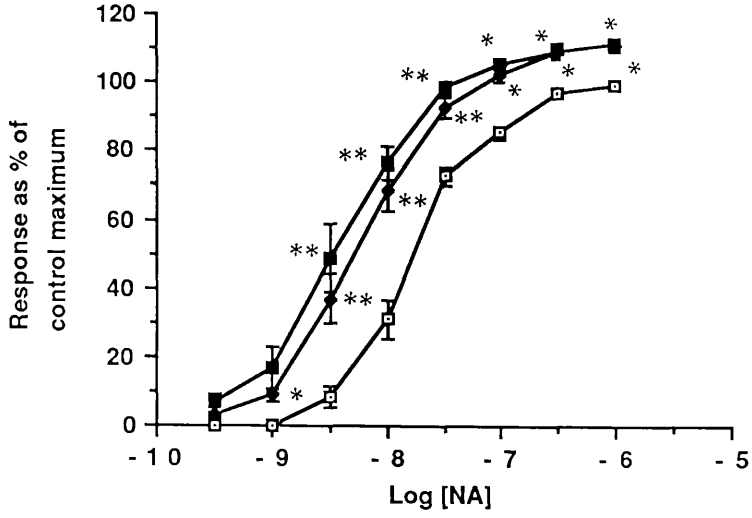
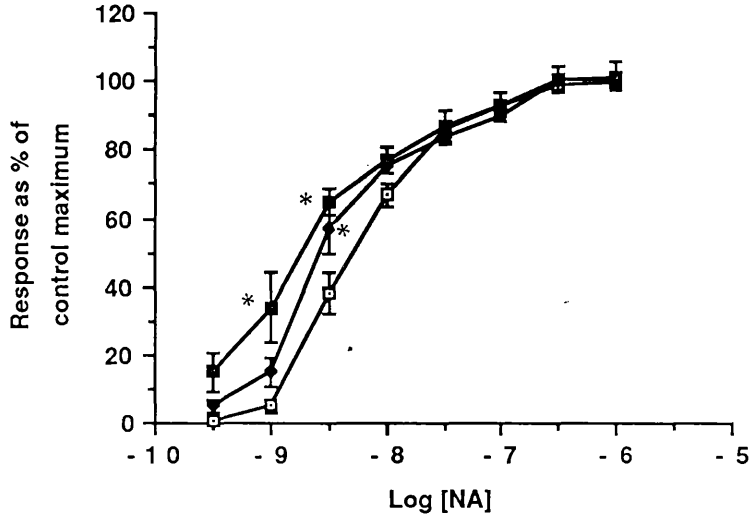


Figure 39b



The effects of BAY-K 8644 (10nM-100nM) on concentration response curves to NA in rat aortic rings with an intact (Figure 39a) and disrupted (Figure 39b) endothelium. Results are given as % of control maximum. Values are given as mean ± s.e.m. (n=6). Statistical analysis carried out by comparing responses in the presence of the drug with the control carried out in the same tissue. Control-□ +10nM BAY-K-◆ +100nM BAY-K-■ (Statistical significance was determined as follows: *P<0.05 **0.01>P>0.001 ***P<0.001.)

Figure 40a

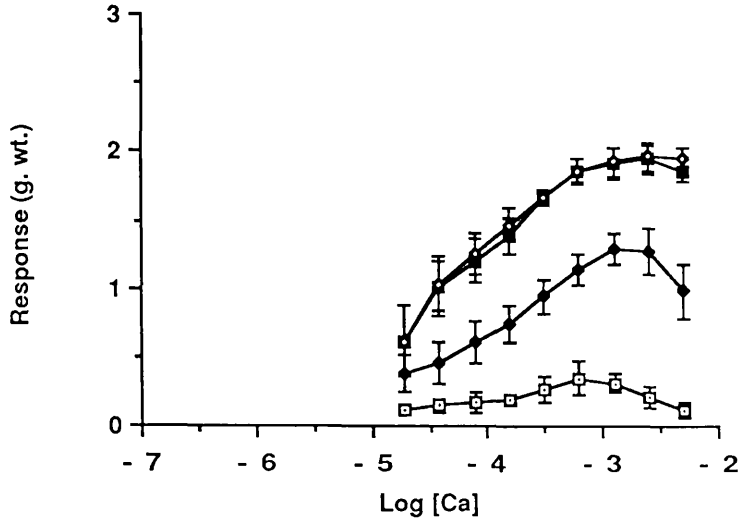
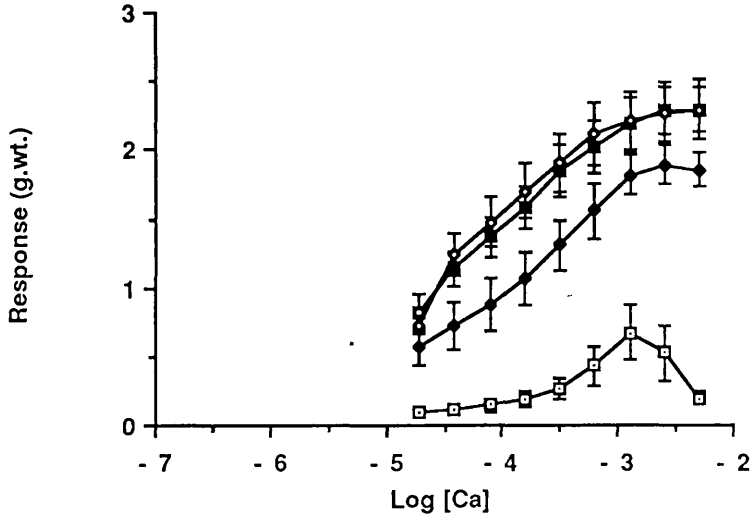


Figure 40 b



The effect of increasing the concentration of NA on the sensitivity of the contraction to calcium in an unbuffered system with an intact (Figure 40a) or disrupted endothelium (Figure 40b). Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6).
3nM NA-⊡ 30nM NA-◆ 0.3uM NA-■ 3uM NA-◇

buffered protocol on each tissue, but not both on the same one. In the presence of BAY-K 8644 the size of the responses to NA were significantly potentiated in both intact and disrupted tissues (Figures 41 a,b). The pD_2 values for calcium with this concentration of NA were also shifted significantly to the left. This effect of BAY-K 8644 was maximum at 10nM, with the response in the presence of 100nM not being potentiated any further; indeed they were no greater than those using 1nM BAY-K 8644. The shifts in the calcium curve with BAY-K 8644 were similar in both intact and disrupted preparations; intact tissues pD_2 values increased from 3.6 in control to 4.2 in the presence of 10nM BAY-K 8644 ; disrupted tissues pD_2 values increased from 3.55 in control to 4.1 in the presence of 10nM BAY-K 8644. There was also a significant increase in the maximum response in the presence of BAY-K 8644; in intact tissues the responses increased from 0.86±0.08 in controls to 1.2±0.1 in the presence of 10nM BAY-K 8644 , in tissues with a disrupted endothelium the responses increased from 0.94±0.1 in controls to 1.38±0.08 in the presence of 10nM BAY-K 8644.

Nifedipine (0.1-1μM) was examined using the unbuffered protocol. The size of the responses were significantly inhibited with also a significant decreases in the pD_2 value to calcium (Figure 42 a,b). In tissues with an intact endothelium the pD_2 to calcium decreased from 3.8 in control to 2.9 in the presence of 1μM nifedipine, in disrupted tissues pD_2 values decreased from 3.75 in controls to 2.7 in the presence of 1μM nifedipine . The size of the maximum responses in intact tissues decreased from 1.27± 0.08 in controls to 0.71± 0.06 in the presence of 1μM nifedipine, in disrupted tissues responses decreased from 1.44±0.08 in controls to 0.86±0.07 in the presence of 1μM nifedipine. There is a choice of taking the pD_2 value using only the control maximum response, or using for each curve its own maximum. There are problems with both methods. If the control maximum is used then we may see a false change in sensitivity to calcium if the

Figure 41 a

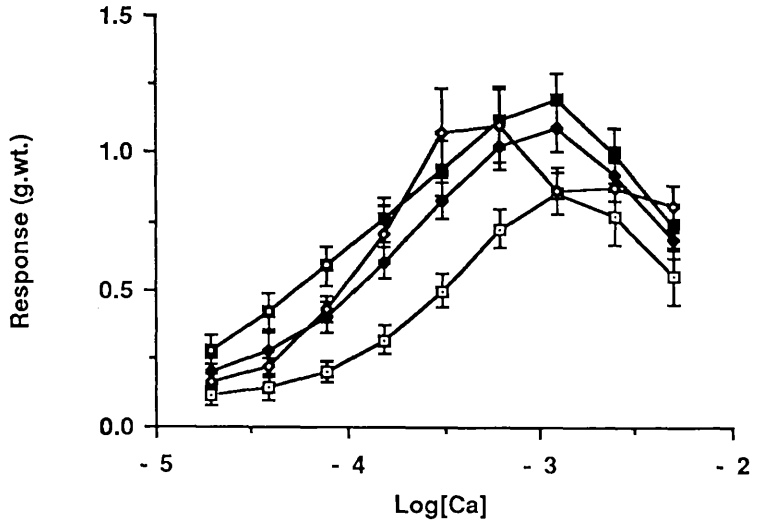
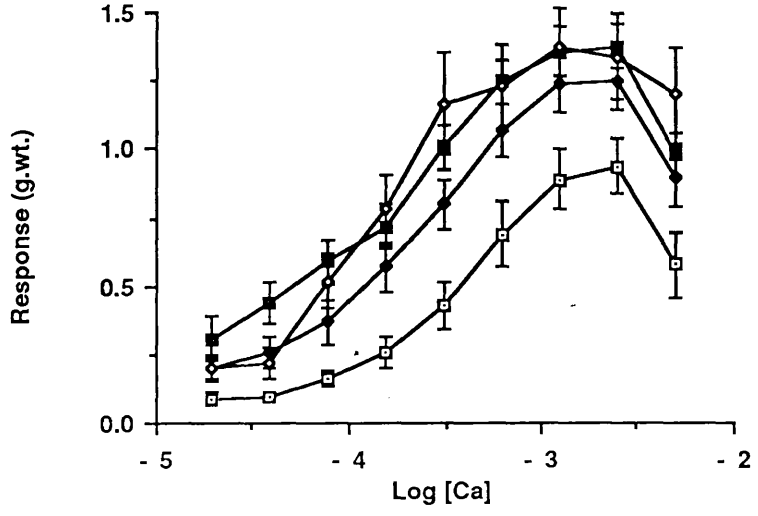


Figure 41 b



The effect of increasing concentrations of BAY-K 8644 (1nM-100nM) on calcium sensitivity in an unbuffered system to 30nM NA with an intact (Figure 41a) or disrupted endothelium (Figure 41b). Results are expressed in g.wt. Values are given as mean ± s.e.m (n=6). Statistical analysis was carried out by comparing the values obtained in the presence of the drug with those of the controls from the same tissue. control- ⊡ +1nM BAY-K- ◆ +10nM BAY-K- ■ +100nM BAY-K- ◇
The responses in the presence of BAY-K 8644 are all significantly different from control values with the exception of a few points at calcium concentrations of greater than 1.25mM. In all other cases $P<0.05$ asterisks have been omitted for clarity.

Figure 42 a

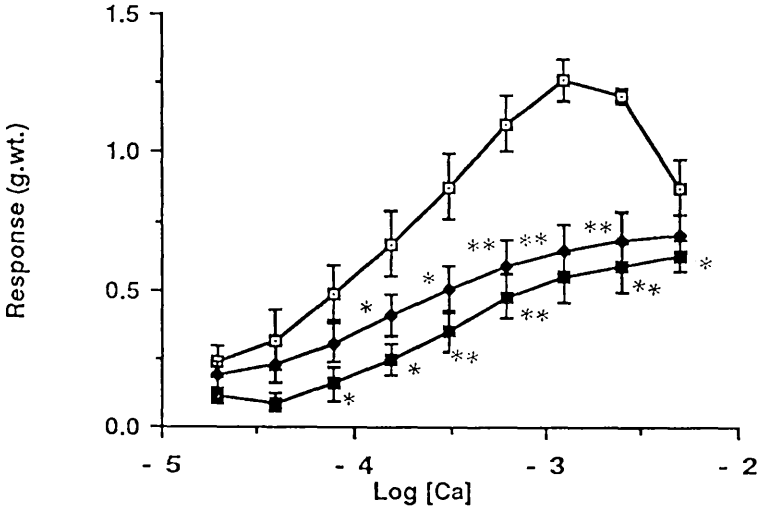
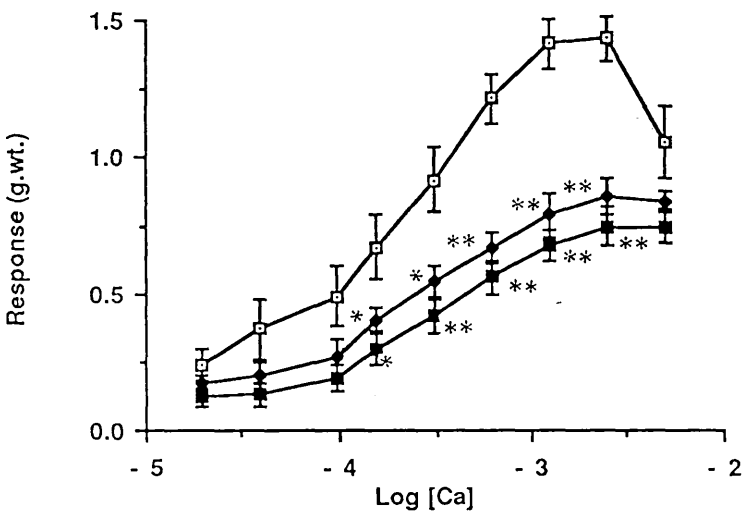


Figure 42 b



The effect of increasing concentrations of nifedipine (0.1uM-1uM) on calcium sensitivity in an unbuffered system to 30nM NA with an intact (Figure 42a) or disrupted endothelium (Figure 42b). Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=6). Statistical analysis was carried out by comparing the values obtained in the presence of the drug with those of controls from the same tissue. control-⊡ +0.1uM nif-◆ +1uM nif-■
(Statistical significance was determined as follows: * P<0.05 ** 0.01>P>0.001 *** P<0.001)

maximum in the presence of the drug had been significantly changed. However if the maximum from each curve is used we would have obtained the sensitivity to calcium but would not have taken into account the actual change in the size of the response. Tables 4 & 5 show results expressed with each method.

The difference in the absolute size of the control responses between these two groups of experiments in both intact and disrupted tissues is quite considerable. However, since each tissue acts as its own internal control the changes seen in the presence of the drugs can be taken as real effects. If the curves are expressed as a percentage of their maximum they give the same values for the pD_2 for calcium and therefore both groups of controls have a similar sensitivity to calcium but differ only in the absolute size of response. Graphs 43a,b show the results expressed as a percentage of the control maximum. Only a single concentration of the calcium agonist or antagonist is included for clarity. Figure 43a shows the effect of both drugs in tissues with an endothelium . Figure 43b shows the effect in tissues with disrupted endothelium. The graphs show clearly the shift to the right and depression of the maximum response by nifedipine and the shift to the left and elevation of maximum by BAY-K 8644.

The shift seen when 1μM nifedipine was present was not significantly different from that of the lower concentration (0.1μM) suggesting a nifedipine insensitive component to the response. Looking at the shape of the control calcium re-addition curve it can be seen that the responses reached a maximum at 1.25mM calcium and then decreased when the calcium was increased to 2.5mM (normal) with a further decrease on increasing the calcium to 5mM. A similar decrease from the maximum response was also seen when BAY-K 8644 was used. This decrease was most profound in tissues which had an intact endothelium; the fall occurring only after 2.5 mM calcium in tissues with a disrupted endothelium. This decline

TABLE 4

	CONTROL	+BAY-K 8644		
(a)	(30nM) pD_2	+1nM	+10nM pD_2	+100nM
+E	3.70±0.07	4.10±0.10**	4.35±0.07**	4.02±0.13*
-E	3.53±0.09	4.08±0.14**	4.31±0.10**	4.11±0.09**
(b)	MAXIMUM RESPONSE (g.wt.)		MAXIMUM RESPONSE(g.wt.)	
+E	0.86±0.08	1.10±0.09*	1.20±0.10**	1.11±0.14*
-E	0.94±0.11	1.25±0.10*	1.38±0.08**	1.38±0.14**
(c)	pD_2			pD_2
+E	3.70±0.07	3.89±0.10	4.10±0.08*	3.97±0.12
-E	3.53±0.09	3.72±0.10	3.90±0.07*	3.92±0.13*

This table shows the effects of BAY-K 8644 on the calcium pD_2 values(a) & (c) and maximum response (b) using 30nM NA as the contractile agent. (a) pD_2 values in the presence of BAY-K 8644 were calculated based on the control curve as 100% maximum. (c) pD_2 values in the presence of BAY-K 8644 were calculated with each curve acting as its own maximum. Responses are expressed as mean ± s.e.m.(n=6)
Statistical Analysis *P<0.05 **0.01<P<0.001 ***P<0.001

TABLE 5

	CONTROL	+NIFEDIPINE	
(a)	(30nM NA) pD_2	(0.1uM) pD_2	(1uM)
+E	3.89±0.11	2.80±0.23**	2.28±0.03**
-E	3.77±0.12	2.90±0.21**	2.54±0.17**
(b)	Maximum Response(g.wt.)	Maximum Response(g.wt.)	
+E	1.27±0.08	0.71±0.07*	0.63±0.06**
-E	1.44±0.08	0.86±0.07*	0.75±0.06**
(c)	pD_2	pD_2	
+E	3.89±0.11	4.00±0.20	3.60±0.05
-E	3.77±0.12	3.70±0.20	3.55±0.17

This table shows the effects of nifedipine on the calcium pD_2 values (a) & (c) and maximum response (b) using 30nM NA as the contractile agent. (a) pD_2 values obtained in the presence of nifedipine were calculated based on the control curve as being 100% maximum. (c) pD_2 values in the presence of nifedipine were calculated with each curve acting as its own maximum. Responses are expressed as mean ± s.e.m (n=6). Statistical analysis was carried out by comparing the values in the presence of nifedipine with their corresponding controls.
Statistical significance was determined as follows: *P<0.05 **0.01<P<0.001 ***P<0.001.

Figure 43a

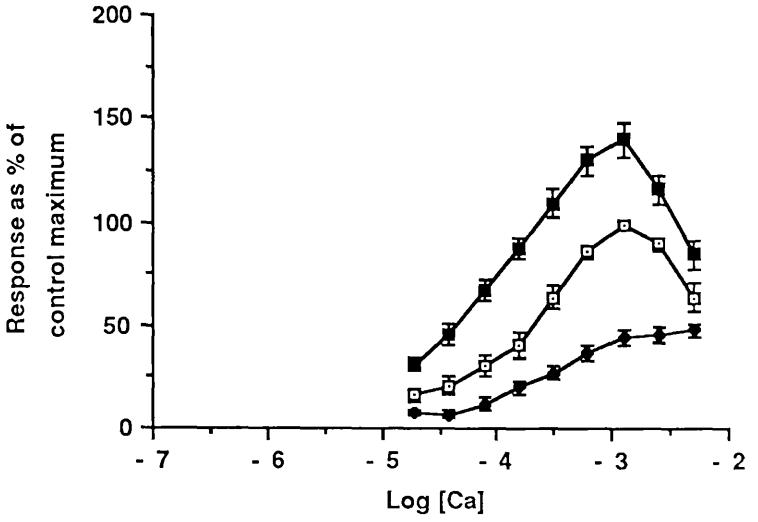


Figure 43b

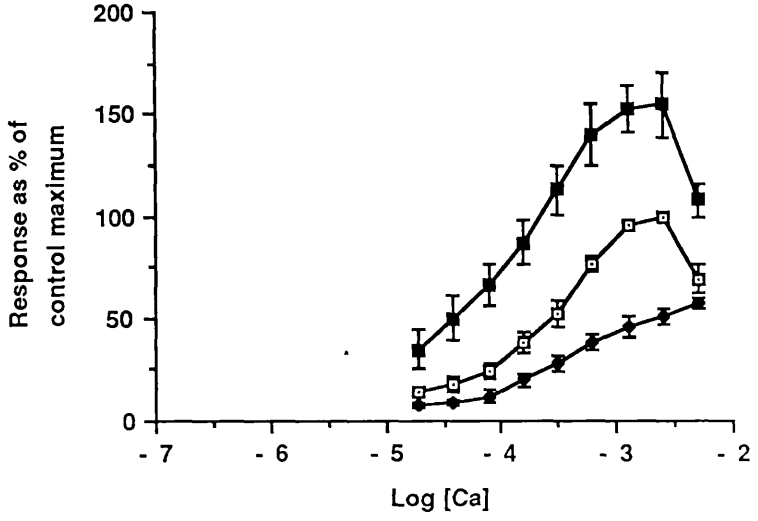
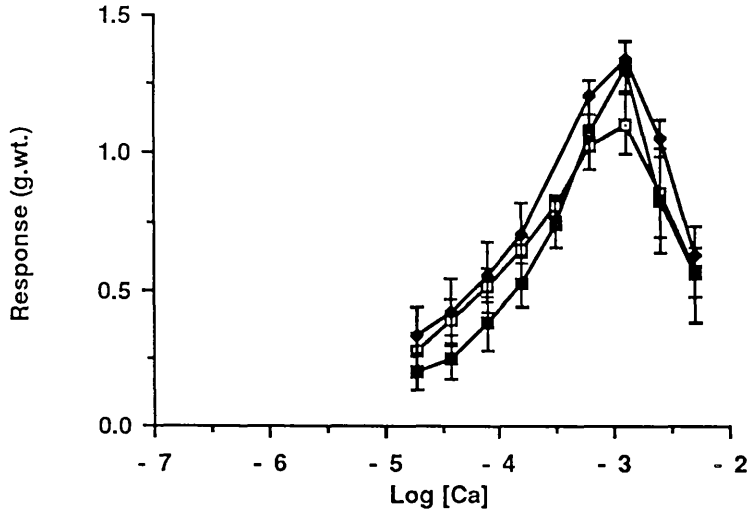


Figure 43a shows the effect of maximally effective concentrations of both BAY-K 8644 and nifedipine on the CRC to calcium using 30nM NA in tissues with an intact endothelium. Figure 43b shows the same effect except in tissues with the endothelium disrupted. Only maximal concentrations of the drugs were used for clarity. Results are expressed as a percentage of the control maximum ± s.e.m. (n=6). Control- ⊡
1μM Nif- ◆ 10nM BAY-K 8644- ■

from the maximum response was not seen in either intact or disrupted tissues when the calcium entry blocking drug nifedipine was present; the response continued to rise, reaching a maximum at 5mM calcium. Time control curves were carried out to calcium re-addition using 0.03μM NA, which showed no change in pD_2 value with time and a slight but non-significant increase in the maximum response (Figure 43c) . (This is a similar effect to repeated CRC to NA).

Figure 43c



The effect of repeating the calcium sensitivity curves to 30nM NA using the unbuffered system in tissues with an intact endothelium.Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=4) curve 1-⊡ curve 2-◆ curve 3-■

There was no significant difference in the responses with time.

DISCUSSION

The purpose of this series of experiments was to examine the sensitivity to calcium of the rat aortic ring using NA as the contractile agent, and to establish the effect of the endothelium on this sensitivity . Two drugs which are active at calcium channels, BAY-K 8644 (calcium facilitator), and nifedipine (calcium blocker), and their effects on calcium sensitivity to NA were studied.

The protocol for the experiments in the initial part of the study was based on experience obtained in our laboratory using the rat anococcygeus muscle. These initial conditions chosen may not have been those most suitable for studying calcium sensitivity in the rat aorta largely because the aorta is more sensitive than is the anococcygeus, to NA, so that the concentration of NA chosen to examine this effect was supra-maximal. However these results did enable us to conclude that with maximal concentrations of NA, at least, there was no significant difference in the sensitivity to calcium in tissues with an intact or disrupted endothelium. Under these conditions the results show that BAY-K 8644 (1μM), had no potentiating effect on either the buffered or unbuffered calcium sensitivity in tissues with either an intact or disrupted endothelium. This is possibly because at this maximal concentration of NA, the calcium channels are already fully facilitated, therefore the presence of a facilitating drug will have no potentiating effect. The observation that nifedipine acted to inhibit the responses successfully suggests the involvement of calcium channels in the production of the contraction.

As a result of these findings we examined the effect of BAY-K 8644 on the responses in the CRC to NA with either an intact or disrupted endothelium. The potentiating effect of the drug was most apparent at the lower concentrations of NA. The effect of BAY-K 8644 was more effective in tissues with an intact

endothelium. This was demonstrated by the increase in the pD_2 value of NA in the presence of BAY-K 8644 (0.01μM) giving a similar value to that when the endothelium was disrupted with NA present alone. Therefore in tissues with an intact endothelium BAY-K 8644 may be acting in two ways to potentiate the response to NA; a) increase the entry of calcium b) inhibit spontaneous release of EDRF. That it increases calcium entry is shown by the fact that it can potentiate responses to NA in the tissues with the endothelium disrupted. However, since in earlier experiments we demonstrated the inhibition of endothelium-dependent relaxation to Ach, by BAY-K 8644 the second possibility would also be likely to contribute to the potentiation seen. Before this can be stated conclusively measurements of cGMP levels in the presence and absence of BAY-K 8644 would have to be made.

These results suggested therefore that using a lower concentration of NA to study calcium sensitivity may enable the effects of BAY-K 8644 to be seen. Studies were then carried out to examine the effects of calcium re-addition on various concentrations of NA. The higher the concentration of NA used, the more sensitive the response was to calcium since the pD_2 values were higher. This was true whether tissues had an intact or disrupted endothelium. This could explain why BAY-K 8644 had little facilitating effect at high [NA]: since the tissues were already very sensitive to calcium it is possible that the curve could not be shifted any further over to the left i.e. maximum facilitation of Ca^{2+} channels. A concentration of NA (30nM) was then chosen, which was neither maximal nor near threshold in either intact or disrupted tissues in order to observe the effects, if any, of both BAY-K 8644 and nifedipine.

BAY-K 8644 caused a significant potentiation at all concentrations tested in both intact and disrupted tissues. The shift in the CRC to calcium using NA with

1nM BAY-K 8644 was significant at most calcium concentrations. Using 10nM the shift in calcium sensitivity was even greater, being significant at all calcium concentrations, along with a significant increase in the maximum response with both concentrations. In the presence of 100nM BAY-K 8644 no further potentiation of the responses was seen; instead a slight decrease in the maximum and a rightward shift (when compared with the shift in the presence of 10nM) of the pD_2 value was observed. If this trend continued, when 1μM BAY-K was used, as in our initial experiments, there may be little or no potentiation , which would help to explain our earlier results. This depression is seen in the rat portal vein (Fasehun et al 1987) when BAY-K increases above 100nM. There was a similar pattern of leftward shift of the curve in tissues with either an intact or disrupted endothelium. The only difference between the two groups was that at most calcium concentrations the responses in the disrupted tissues were significantly greater. This was to be expected since this concentration (30nM) is on the steep part of the CRC to NA where the differences between the responses from intact and disrupted tissues are the greatest. These results of potentiation with BAY-K 8644 now agree with experiments carried out in our laboratory using the anococcygeus muscle. That the BAY-K 8644 has no greater potentiating effect in tissues with a disrupted endothelium when compared to intact supports our original observation of both groups having a similar sensitivity to calcium and that the endothelium does not appear to selectively block calcium entry.

The effects using nifedipine were similar to those obtained in the initial experiments where a higher concentration of NA was used. The responses were significantly depressed at all concentrations of calcium in the presence of nifedipine 0.1μM. Increasing the concentration of nifedipine to 1μM decreased the responses slightly from the previous concentration but in both cases the maximum responses were the same. This would seem to suggest the existence of a nifedipine-insensitive component of the response to NA. This agrees with the results obtained by

Godfraind et al (1982) who also found that a sizeable percentage of the contraction to NA was retained in the presence of high concentrations of nifedipine. This also agrees with Chui et al (1986) who found that calcium entry blocking drugs were most effective on agonists which were only partial agonists for the α_1-adrenoceptor, with responses to NA being affected to the least extent. However, the results from the study by Godfraind et al, (1985) suggest that the percentage of the contraction which was resistant to the calcium-channel inhibitors was greater when the endothelium was disrupted than when the endothelium was intact. Unlike us they have only used maximum concentrations of NA and therefore have no indication of what happens to the sensitivity at lower concentrations of the agonist. Even taking this into account we have still been unable to observe selective inhibition in the presence of the endothelium by nifedipine at any concentration of NA.

If the shape of the calcium re-addition response is examined in the control situation it can be seen that it is a bell shaped curve. This means that when the tissues were exposed to calcium levels of 2.5mM and above the responses were depressed. This phenomenon is also seen in tissues with a disrupted endothelium but was more pronounced in those tissues having an intact endothelium. This same depression of the response at high calcium concentrations was also seen when the calcium facilitating drug BAY-K 8644 was present. However, in the presence of nifedipine the responses had almost reached a plateau at 5mM calcium. This would seem to suggest that the amount of calcium entering the cell affects the response by putting the tissue into 'calcium overload'. This 'overload' could possibly be due to a negative feedback effect by the high free calcium in the cells bringing about closure of the calcium channels thereby reducing the amount of calcium entering the cells. It could also be acting to drive the sodium-calcium exchange in reverse and therefore inhibiting calcium entry. This effect does not happen with nifedipine

probably because the levels of calcium inside the cell never become high enough to cause 'overload' or to produce a reversal of the calcium-sodium pump. This phenomenon has also been seen with other smooth muscle tissues used in this laboratory e.g. rat anococcygeus, rat portal vein and the isolated perfused tail artery.

These results when taken together indicate that the endothelium has little or no effect on the stimulated entry of calcium into the cells which induces contraction. This was shown by the similar Ca^{2+} pD_2 values in tissues with either an intact or disrupted endothelium for all concentrations of NA used to induce the contraction. Spedding et al (1986), found that BAY-K 8644 itself caused a contraction in the rat aorta if the tissue was depolarised first with a low concentration of KCl. This contraction was shown to be most effective in the absence of the endothelium. They suggested therefore that the endothelium inhibits calcium entry into the smooth muscle cell. Their results with measurements of cGMP also suggest an increase in calcium entry in the absence of the endothelium. My results suggest no such increase in calcium entry as determined by sensitivity to calcium readdition on the response to NA. My results concur with those of Malta et al (1986), who measured the basal and α-adrenoceptor stimulated fluxes of calcium in rat aorta and the effect that the endothelium had on these fluxes. They found that basal stimulation of ^{45}Ca was reduced (when compared to tissues with a disrupted endothelium) in the first 7 minutes of observation, when the endothelium was intact. However, when the ^{45}Ca accumulation was stimulated with Phe, the increase in uptake was not significantly different in the presence or absence of the endothelium, even although the size of the contractile responses were markedly different. When they used BHT-920 as the agonist (10μM), exposure time 5 minutes, stimulated Ca^{2+} uptake was reduced in the presence of the endothelium. Collins et al 1986 found the converse of the above results in the rabbit aorta where the unstimulated ^{45}Ca influx

was greater in the presence of the endothelium than in its absence. This is therefore the opposite to that found by Spedding et al (1986).

These results would seem to suggest that the endothelium does have some influence in calcium metabolism and the contractile activity of the rat aorta. How the endothelium influences calcium metabolism or entry must be a complex process, but entry reduction into smooth muscle cells is probably not the method by which it modulates contraction as shown by the similar pD_2 values to calcium between intact and disrupted tissues. The only difference seen between the two groups was in the CRC to NA in the presence of BAY-K 8644. Here the endothelium did seem to have an effect and it is possible that as well as inhibiting Ach-induced EDRF release it can also inhibit spontaneous EDRF release. This would therefore potentiate the response to NA in tissues with an intact endothelium to similar to those with endothelium disrupted i.e. the effect of BAY-K 8644 is not additive to that of endothelium removal.

CHAPTER 4

EFFECT OF HYPERTENSION AND DIABETES ON RESPONSIVENESS OF VASCULAR SMOOTH MUSCLE

INTRODUCTION

Acetylcholine(Ach)-induced relaxation of several arterial preparations is totally dependent on the presence of endothelial cells on the intimal surface of the smooth muscle (Furchgott & Zawadzki,1980). This relaxation is thought to be brought about by release of an endothelial derived relaxant factor (EDRF) which is able to act on smooth muscle directly. Superfusion experiments have corroborated this (Griffith et al 1984) and have also established that the half life of the factor is between 6 and 30 seconds, depending on experimental conditions. This discovery led to the finding that many endogenous agents, which are potent vasodilators, are dependent for their action on the presence of the endothelium whereas other substances act directly on the smooth muscle e.g. sodium nitroprusside .

In the present study, we have used both an isolated artery (rat aortic ring) and an intact vascular bed (perfused rat tail) to examine whether different cardiovascular disease states (hypertension and diabetes) modify the endothelium-dependent, Ach-induced vascular relaxation. In an attempt to establish the mechanism involved in any such changes, we have compared the effect of Ach with that of sodium nitroprusside which, like the EDRF released by Ach, stimulates the soluble guanylate cyclase activity in the vascular smooth muscle (Ignarro and Kadowitz, 1985) but which does not, itself, require the presence of the vascular endothelium.

MATERIALS AND METHODS

The rats used in the initial part of this study were male Sprague-Dawley (Glaxo Strain) (80-120g) age six weeks at the start of the experiment. At this stage the various conditions were instigated. The control rats were age matched. Experiments were carried out at least two months after the induction of the conditions. The mean weights of the animals in the various groups at the time of experimentation were as follows: i) aortic ring experiments; control, 314±4g n=6; diabetic, 113± 7g n=5; renal hypertensive,363±28g n=5; DOCA/salt hypertensive, 358±18g n=5. From this group of experiments only the diabetic rat weights were significantly different from controls. ii) perfused tail experiments; control, 382±17g n=6; diabetic, 139±7g n=4; renal hypertensive, 482±16g n=4; DOCA/salt hypertensive, 456±16g n=4 again diabetic rat weights were significantly different from controls as were those from the other two groups.

The rats in the second part of the study were male Wistar 314±7g; or Spotaneously Hypertensive rats 317±10g n=6.

ANIMAL TREATMENTS

Diabetes

This condition was induced by an intraperitoneal injection of streptozotocin 80mg/kg. To determine the degree of diabetes initially urine samples were tested using clini-stick and then more accurately by taking a blood sample immediately after the animal had been stunned and exsanguinated. Glucose levels were measured using the Glucose Rapid Test (Roche). Samples were taken from rats in all other groups and in all cases the blood glucose levels were significantly lower (range of means ± s.e.m) 4.16±0.43 to 5.12±0.58 mM n=30)

than in the diabetic rats (21.96±0.27mM n=9).

HYPERTENSIVE RATS

Renal Hypertensive

This type of hypertension was induced using the Goldblatt technique (Goldblatt et al 1934). Rats were first anaesthetised with pentobarbital sodium 60mg/kg and an incision was made on the left flank to allow access to the left renal artery, on to which a solid silver clip (0.25mm gap width) was applied as close as possible to the kidney. The other artery remained intact. Hypertension produced by this technique is dependent upon an increased level of renin in the plasma which converts angiotensinogen to angiotensin I, and in turn converted to angiotensin II which can act either directly on vascular smooth muscle to give an increase in tone or else via increased levels of aldosterone causing an increase in plasma sodium. At the time of use the mean systolic blood pressure in these rats ranged between 204±4mmHg (aortic ring experiments) and 229±7mmHg (perfused tail experiments). Control rat blood pressure normally being 130mmHg.

Doca Hypertensive

Among mammals the rat is particularly prone to desoxycorticosterone acetate (DOCA) salt induced hypertension (Selye et al, 1943). A 75mg DOCA pellet was implanted subcutaneously in the back of the neck, as described by Conway et al (1985) and the rats were also maintained on drinking water containing 1% NaCl, 0.2% KCl. This type of hypertension is thought to be dependent on the increase in plasma fluid volume and recent evidence also points

to an involvement of vasopressin (Crofton et al 1980). In contrast to renal hypertension, renin levels are low in DOCA/salt hypertension. At the time of use, the mean systolic blood pressure in these rats ranged between 206 ± 6mmHg (aortic ring experiments) and 231 ± 9mmHg (perfused tail experiments).

Spontaneously Hypertensive rats

These rats were obtained from the MRC Blood Pressure Unit (Glasgow Western Infirmary). This type of rat has been genetically bred to have hypertension (3-4 months old) . The blood pressure of this group of rats was measured by a similar tail cuff method as described below, at the MRC Blood Pressure Unit and was 230±5mmHg.

Blood Pressure Measurements

Experiments using the hypertensive animals were carried out at least 2-3months after the condition was induced. The animals were selected on the basis of their systolic blood pressures (B.P.). This was measured in conscious animals by use of the tail cuff method. This method involves warming the animals (40°C atmosphere for 30 mins) to ensure a good blood flow through the tail. The tail artery was then occluded with a pressure calibrated occlusion cuff so that blood flow ceased temporarily. A sensitive piezo-electric transducer was placed distal to the cuff, which was allowed to deflate slowly. When the pressure in the tail artery was equal to or greater than the pressure of the occluding cuff, blood flow returned to the tail and the arterial pulse was detected by the transducer. The pressure in the cuff at which the pulse was first detectable was taken as systolic B.P. This technique for indirect measurement incorporates the use of a W+W electronic (type BP5002e) B.P. recorder which registers the pulse.

Rat aortic ring measurements

Rats were stunned and bled and the thoracic aorta quickly removed and placed in Krebs Bicarbonate solution. Two rings (3-4mm) were cut from each vessel, and the endothelium on one was deliberately disrupted by mechanical rubbing. Rings were then mounted in a 10ml organ bath containing Krebs Bicarbonate solution (mmol/L) NaCl,119; $NaHCO_3$,24.8; KCl,4.7; $CaCl_2$,2.5; $MgSO_4$,1.2; KH_2PO_4,1.2; Glucose,11.1; Ascorbic Acid,0.1; at 37°C through which a mixture of 95%/5% O_2/CO_2 was bubbled. Rings were mounted under an initial tension of 1.5 g and were allowed to eqilibrate for at least an hour and were washed twice before the addition of drugs. Tension was measured isometrically using Dynamometer UFI /2oz strain guages and recorded on an eight channel Lectromed recorder. In the second part of the study using Wistar and Spontaneously Hypertensive rats, aortic rings were set up similarly to the procedure outlined. However, the Krebs solution contained EDTA 23μM in place of Ascorbic Acid. The tissues were placed in 30ml organ baths and measurements were made isometrically using Grass FTO3 transducers, recorded on a six channel Linseis recorder.

Protocol

A cumulative concentration-response curve to NA range (0.1nM-1μM) was first carried out on each ring until a maximum response was obtained. A concentration (30nM) was then selected which, in the majority of tissues, elicited approximately 80% of the initial maximum response. This was then added to the bath and a sustained contraction was allowed to develop. Against this contraction, relaxation produced by Ach (10nM-3μM) added cumulatively was first established. After a period of thirty minutes during which the tissues were washed with drug free Krebs solution, the NA standard concentration was given again and a relaxation

concentration-response curve to sodium nitroprusside (0.1nM-10nM) was then established. In all experiments aortic rings from diabetic, Doca hypertensive and renal hypertensive rats were examined in parallel with age matched control rats.

In the second series of experiments on the rat aortic rings using spontaneously hypertensive rats we examined the possible existence of α_2-adrenoceptors on the smooth muscle as suggested by Medgett et al (1984) in the rat tail artery. A cumulative concentration-curve to NA (0.1nM-10nM) was first carried out on each ring until a maximum response was obtained. A concentration (30nM) was selected which produced approximately 80% of the initial maximum response. A maximal relaxatory concentration of Ach(3μM) was then added to test for a relaxation. The bathing fluid was then exchanged twice and the tissues allowed to rest for thirty minutes before a CRC to UK14304 (0.1μM-10μM) was carried out. Experiments were carried out in parallel using age matched Normotensive Wistar rats.

Isolated rat tail perfusion pressure measurements

For this preparation rats were injected intraperitoneally with pentobarbital 60mg/kg. The ventral surface of the tail was exposed and any surface fur was removed. An incision was made in the skin close to the base of the tail and the artery was exposed. Care was taken to cut a small hole in the artery and a specially made polyethylene cannula, filled with heparinised saline, was inserted in the direction of blood flow and tied firmly in place. Having done this, blood filled the cannula and the system was carefully flushed through with heparinised saline. The tail was then amputated by cutting through an intervertebral space. Both tail and cannula were rapidly weighed and placed carefully in a heated water jacket. The arterial cannula was then attached to a perfusion circuit and the tail vasculature was perfused with modified Krebs solution at a constant rate of 0.5ml/min. A diagram

of the apparatus used is shown in figure 44. The pressure was high initially but soon declined and stabilised, generally at about 45-55mmHg. This value was similar for all groups of animals studied. After attaining a constant resting pressure, at least twenty minutes were allowed for equilibration before further experimentation.

Perfusion medium

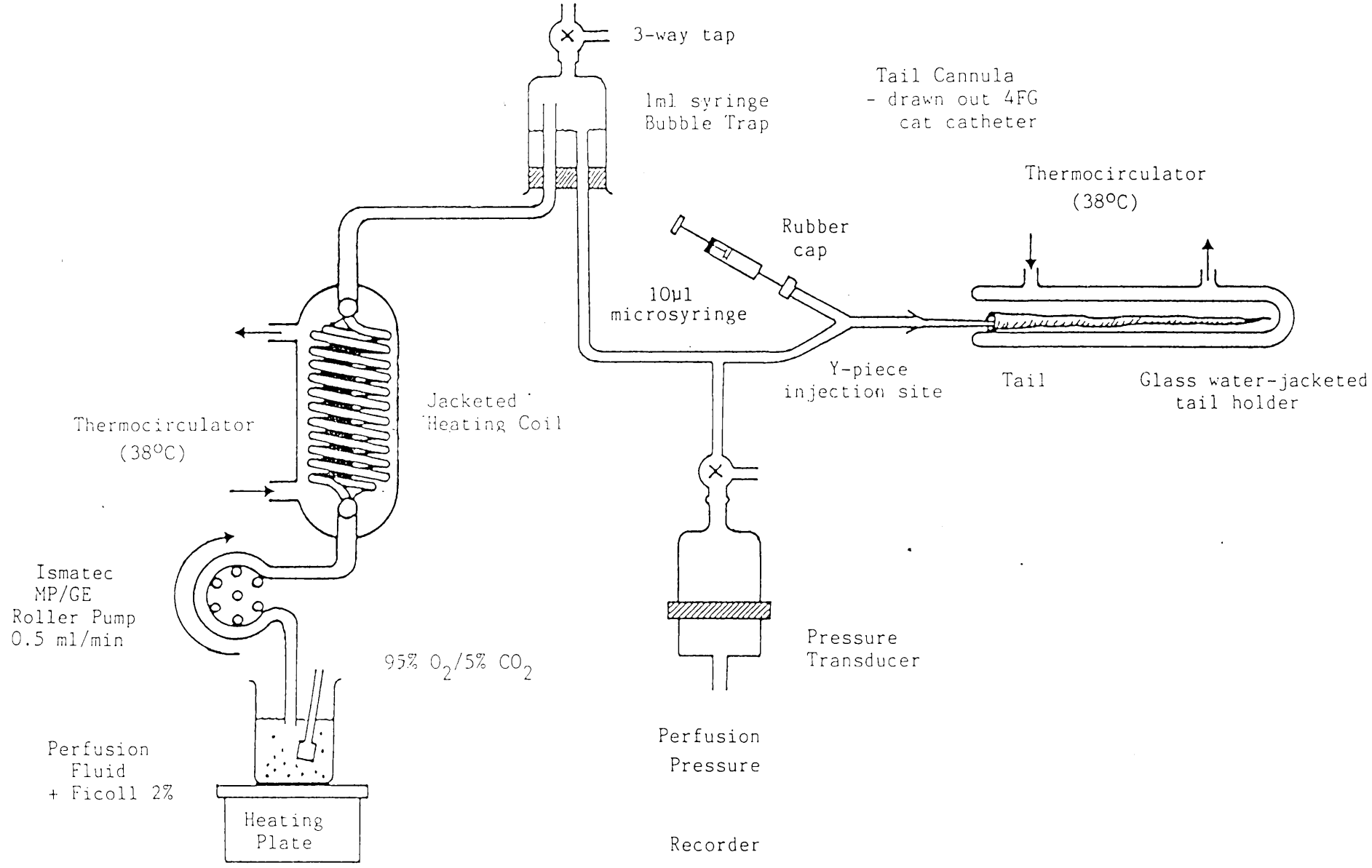
The perfusion medium was a modified Krebs-Bicarbonate solution similar to that used to bathe the isolated aortic rings, with the omission of Ascorbic Acid and inclusion of 2% Ficoll (mol.wt. 70,000) to prevent excessive water retention by the tail. Its temperature was 28°C which is close to that (26°C) normally found in the proximal part of the tail artery in anaesthetised rats (Wade & Beilin, 1970). Drugs were injected into the perfusate via a Y-piece fitted with a rubber cap close to the tail, either in 10µl volumes, or else as part of the perfusion medium from the reservoir, except for haemoglobin which was dissolved in Krebs and infused via the Y-piece at 0.1ml/min with the perfusion rate of the Krebs from the reservoir reduced to 0.4ml/min, to maintain the control rate of 0.5ml/min.

Protocol

In the initial experiments bolus doses of Phe (0.05-5 nano moles) were injected first in order to obtain a measure of the sensitivity of the tail vasculature. Then, a suitable concentration of Phe (1-5 micro moles/ml/min), depending on initial sensitivity of the preparation, was included in the perfusion medium in order to obtain a steady maintained increase in perfusion pressure of between 70 to 120mmHg. Bolus doses of Ach (0.055-5.5 nano moles intra arterial (i.a.)) were

Figure 44

This figure illustrates the apparatus used in the rat isolated perfused tail experiments.


3-way tap
1ml syringe
Bubble Trap
Tail Cannula
- drawn out 4FG
cat catheter
Thermocirculator
(38°C)
Rubber
cap
10µl
microsyringe
Y-piece
injection site
Tail
Glass water-jacketed
tail holder
Thermocirculator
(38°C)
Jacketed
Heating Coil
Ismatec
MP/GE
Roller Pump
0.5 ml/min
95% O_2/5% CO_2
Pressure
Transducer
Perfusion
Fluid
+ Ficoll 2%
Heating
Plate
Perfusion
Pressure
Recorder

injected to produce a vasodilator dose response curve. This was followed in some experiments by repeated doses of Ach or, in others, by bolus doses of sodium nitroprusside (0.03-3 nano moles i.a.).

In a second series of experiments the effects of α_2–adrenoceptor agonists were compared to those of a relatively selective α_1-adrenoceptor agonist Phe, and changes, if any, on removal of the endothelium with sodium deoxycholate. The α_2-adrenoceptor agonists used were BHT-920 and UK14304 . The methods for preparing and perfusing sodium deoxycholate were the same as that described for the perfused mesenteric bed (Byfield et al 1986). The responses after sodium deoxycholate perfusion were then compared. In some experiments the responses to Phe and UK14304 before and during perfusion with Hb were compared.

Developing from these results the effects of prazosin (30nM) on bolus doses of Phe and 5HT were examined. Repeated bolus dose-responses were carried out to ensure changes were not simply time related.

The final series of experiments examined the possibility of α_2-adrenoceptor mediated endothelial-dependent relaxation as described by Cocks & Angus (1983) for the pig coronary artery. In these experiments tone was raised by 5HT (30 nano moles/ml/min i.a.) to avoid the possibilty of loss of tone in the presence of prazosin or corynanthine, given to block any α_1-adrenoceptor mediated effect. Bolus injections of Ach were given to determine if the endothelium was present and functional. Responses were then obtained to bolus injections of UK14304.

STATISTICAL ANALYSIS

Aortic rings (disease experiments)

Results obtained from endothelium-intact rings were compared with those from endothelium-disrupted rings from the same animals. Analyses of varience were performed on maximum responses to NA, EC_{50} values to NA, Ach and sodium nitroprusside. Statistical significance was assessed for each group of rats using the t-statistic. Homogeneity of variance was confirmed using Bartletts test.

Results from endothelium intact were also analysed independently from those from disrupted rings using separate analysis of variance. Comparisons were made between data from control rats and those from diabetic, renal and DOCA/salt hypertensive rats. Statistical significance was assessed using the t-statistic.

Perfused rat tails (disease experiemnts)

Bartletts test showed that the variability of data obtained from the different groups of animals did not differ significantly, and so data between groups were subjected to analysis of variance.

Results from the remaining experiments were compared using the t-statistic.

Rat Aortic Rings

The effects of NA in endothelium-intact and disrupted aortic rings from untreated rats and from hypertensive and diabetic rats are shown in Figures 45(a-d) and summarised in Table 6. Comparison between groups showed that there were no significant differences between the maximum responses obtained to NA in preparations in which the endothelium had been disrupted. Nor were there any significant differences in the maximum responses to NA in endothelium-intact preparations from the different groups. However, it seemed that the maximum contractile responses to NA in the endothelium-disrupted tissues tended to be greater than those obtained in the corresponding intact preparation, but this effect was statistically significant only in tissues from diabetic rats.

CRC to NA were normalised with respect to the maximum response obtained in each preparation: thus the response to each concentration of NA was expressed as a percentage of the maximum. Comparison of data expressed in this way reveals that, although NA CRC from endothelium-disrupted tissues from all groups were almost superimposable, curves obtained from the corresponding intact preparations were displaced rightwards. This effect was statistically significant at the pD_2 value in all groups except the DOCA/salt hypertensive group (Figures 46a,b).

Ach (0.01-3μM) relaxed aortic rings contracted by NA (30nM) only if the endothelium was not damaged (Figures 47 a,b); it produced a contraction in some tissues in which the endothelium had been disrupted. There was no significant difference in the potency of Ach in endothelium-intact aortic rings between any of the groups of animals at either the pD_2 or in the maximum relaxation obtained.

Figure 45a

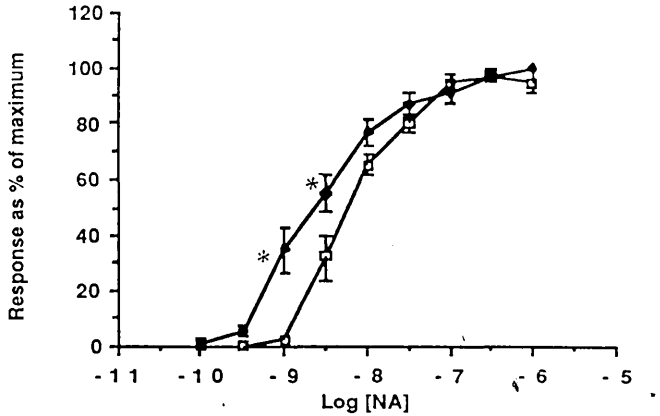


Figure 45b

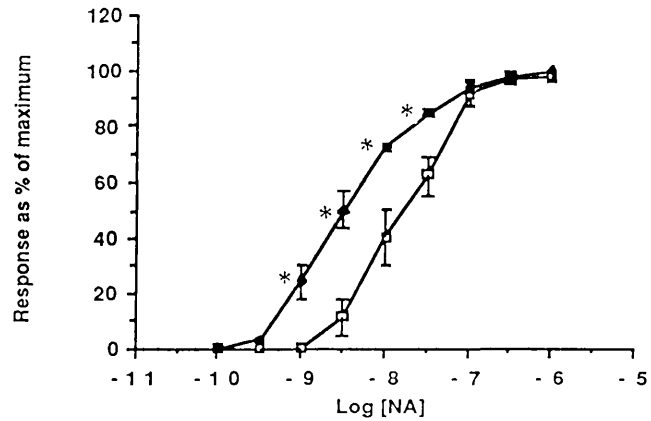


Figure 45c

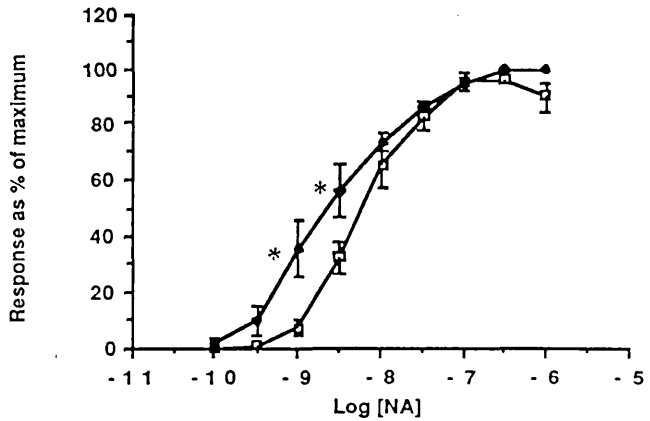
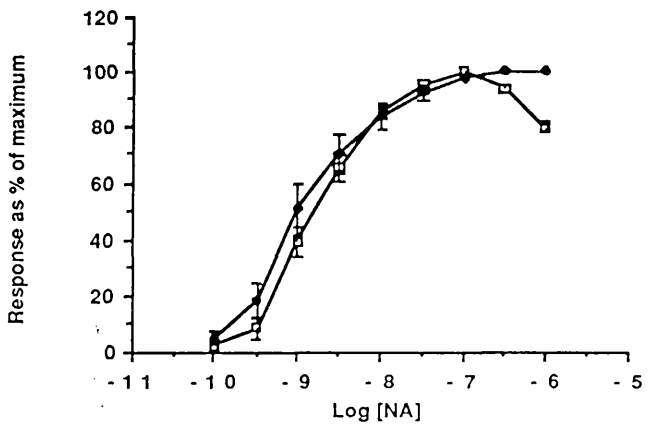


Figure 45d



The effect of different disease states on sensitivity to NA, in tissues with an intact and disrupted endothelium. a)control b)diabetic c)Renal Hypertensive d)Doca/salt Hypertensive. Results are expressed as a percentage of the maximum response. Values are given as mean + s.e.m. (n=6). Statistical analysis was carried out comparing values from rings with endothelium disrupted to intact.

+E-□ -E-◆ Statistical significance was determined as follows: *P<0.05 ** 0.01>P>0.001 ***P<0.001

Figure 46a

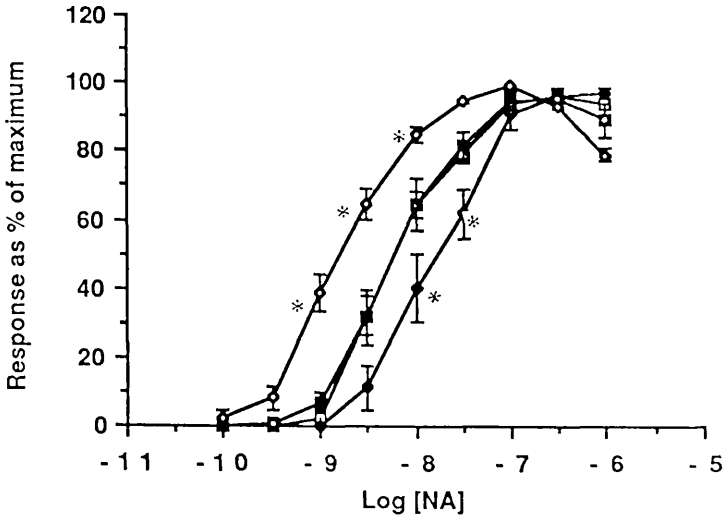
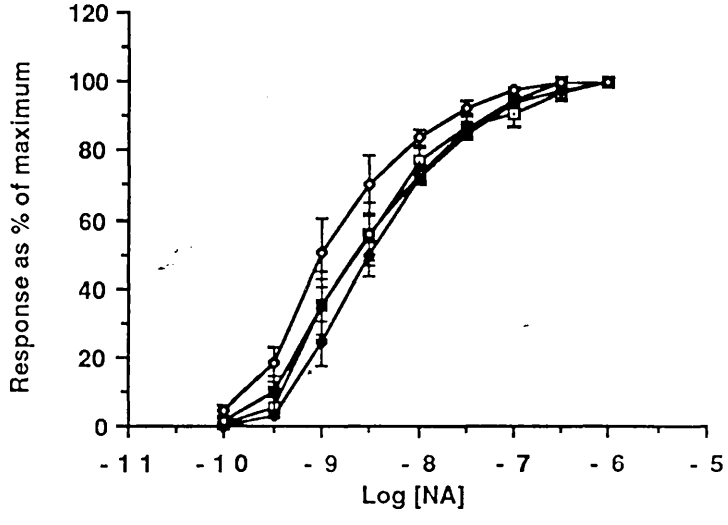


Figure 46b

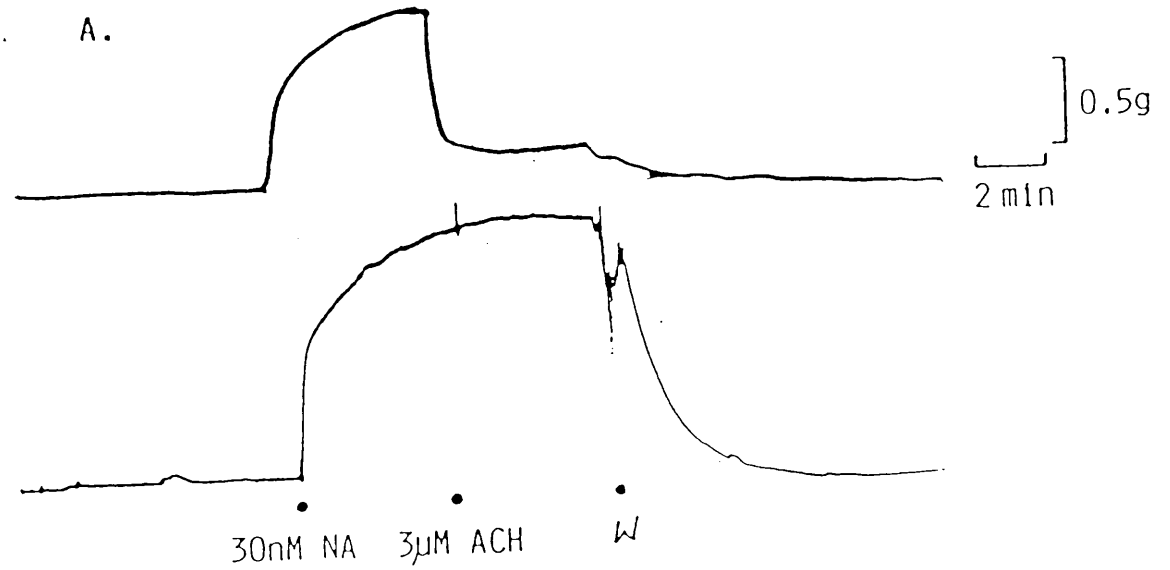


The effect of different disease states on sensitivity to NA in tissues with an intact (Figure 46a) and disrupted endothelium (Figure 46b). Responses are expressed as a percentage of their own maximum. Values are given as mean ± s.e.m. Statistical analysis was carried out comparing the results from the disease states with the corresponding controls. control-⊡ diabetic-◆ renal-■ doca/salt-◇

(Statistical significance was determined as follows: * $P<0.05$ ** $0.01>P>0.001$ *** $P<0.001$)

Figure 47a

This figure illustrates the effects of single concentrations of Ach (A) in both intact (upper trace) and disrupted (lower trace) aortic rings. B shows the effect of a complete CRC to Ach in endothelium intact (upper trace) and disrupted (lower trace) aortic rings.


A.
0.5g
2 min
30nM NA
3μM ACH
W

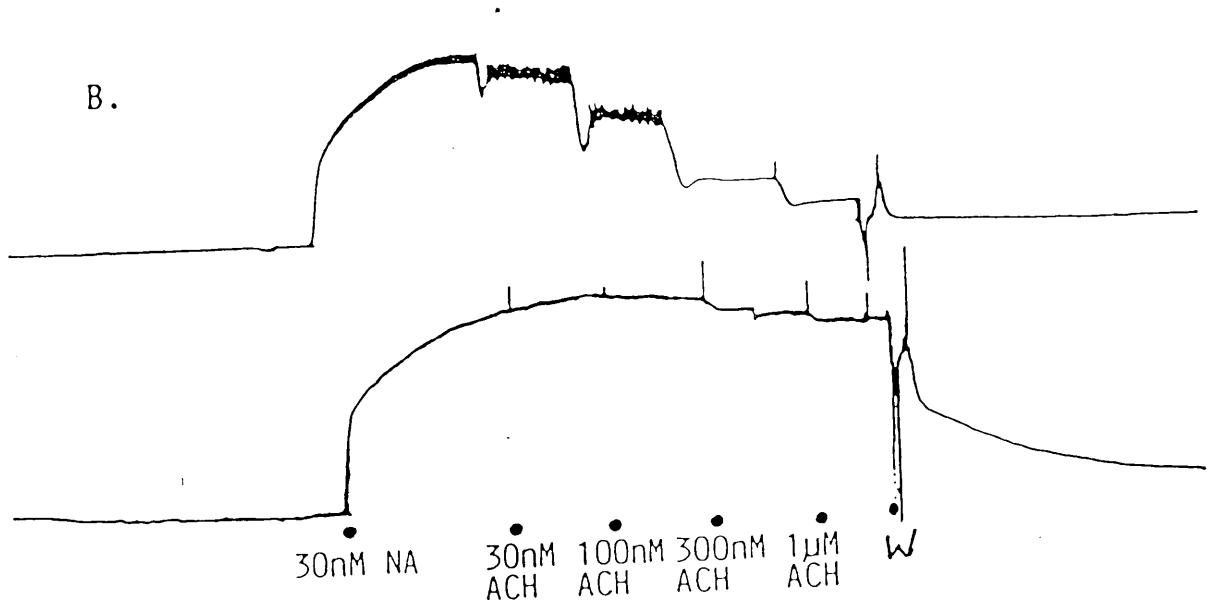
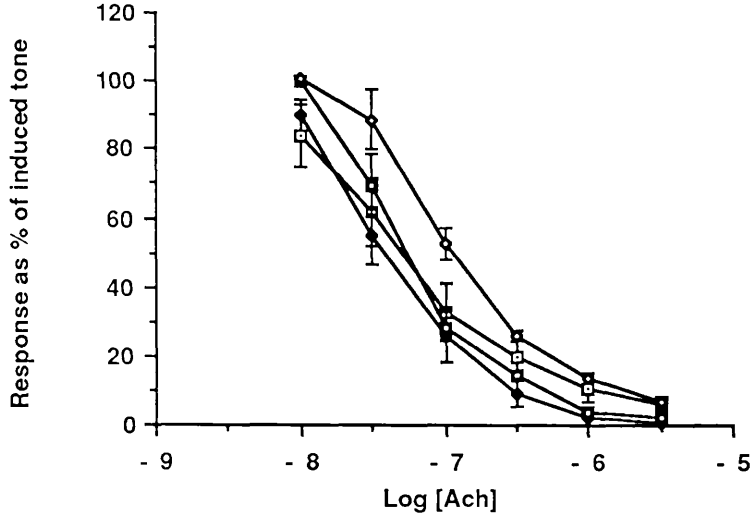

B.
30nM NA
30nM ACH
100nM ACH
300nM ACH
1μM ACH
W

Figure 47a

Figure 47b



The effects of different disease states on relaxation to Ach (Figure 47b). Results are expressed as a % of the induced tone. Values are given as mean ± s.e.m. (n=4-6). There was no significant difference between groups at any concentration.
control-⊡ diabetic-◆ renal-■ doca/salt-◇

Sodium nitroprusside also relaxed aortic rings; it was approximately 10-100 times more potent than Ach and exerted its effect in both the presence and absence of the vascular endothelium (Figures 48 a,b). Sodium nitroprusside was similar in potency in endothelium-intact aortic rings from untreated and diabetic rats. Its effectiveness was not significantly influenced by the removal of the endothelium from tissues from untreated rats, but was reduced from diabetic rats. The sensitivity of endothelium-intact aortic rings from renal and DOCA/salt hypertensive rats, to sodium nitroprusside, was significantly less than that of control and diabetic animals, but was not influenced by removal of the endothelium. Results are shown in Figures 48 c-f.

Perfused Rat Tail

The purpose of these experiments was to evaluate the role of the endothelium in a complete vascular bed rather than in a single isolated blood vessel. Tails (including cannula) were weighed before and after experimentation. Any tail which increased in weight by more than 10% was excluded from the results. Data from the remaining experiments is summarised in Table 7. Figure 49 shows how bolus doses of Phe caused a dose-dependent increase in perfusion pressure (basal perfusion pressure was the same in all groups and ranged between 45 and 55mmHg). Tails from untreated and DOCA/salt hypertensive rats were similar in sensitivity to Phe. In contrast, tails from diabetic rats tended to be more sensitive (significantly so at doses required to increase perfusion pressure by 20 and 60mmHg), whereas those from renal hypertensive rats were invariably much less sensitive. The continuous infusion of Phe (1 micro mole/ml/min) into tails from untreated, diabetic and DOCA/salt hypertensive rats led to a sustained increase in perfusion pressure that ranged from 70 to 120mmHg in different preparations. A

Figure 48a

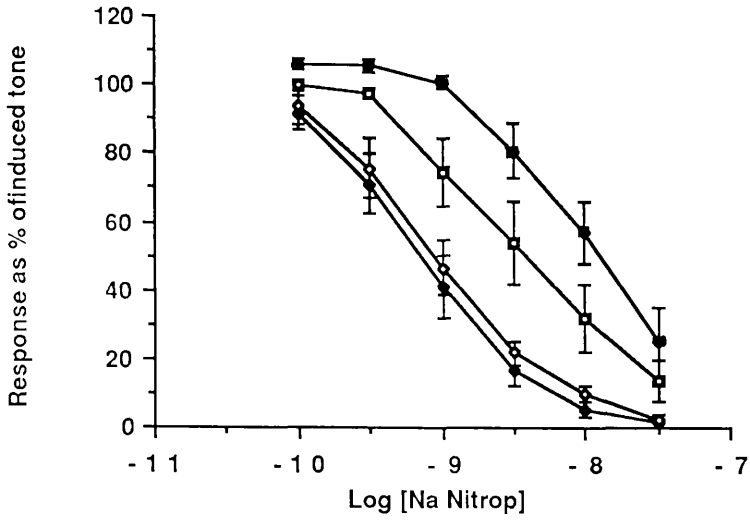
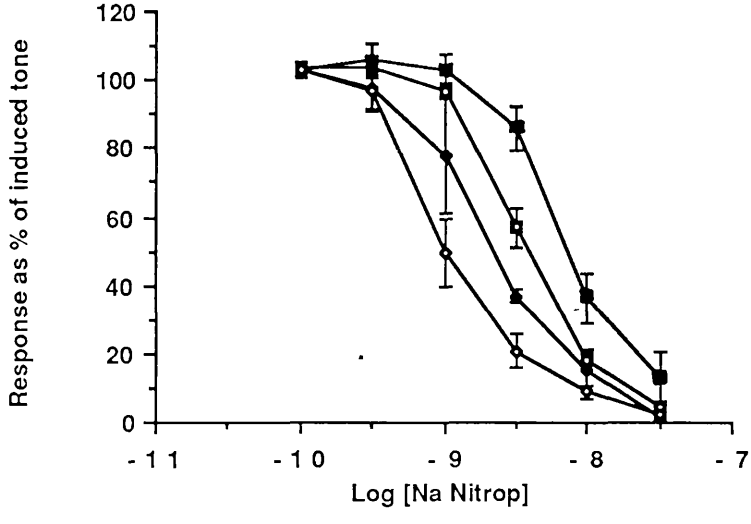


Figure 48b



The effects of different disease states on relaxation to sodium nitroprusside in tissues with an intact (Figure 48a) or disrupted endothelium (Figure 48b). Results are expressed as a % of the induced tone. Values are given as mean ± s.e.m. (n=4-6). Statistical analysis was carried out by comparing the values at the pD_2 level in the disease states with those from control. Results are given in Table 6. control-◇ diabetic-◆ renal-□ doca/salt-■

Figure 48c

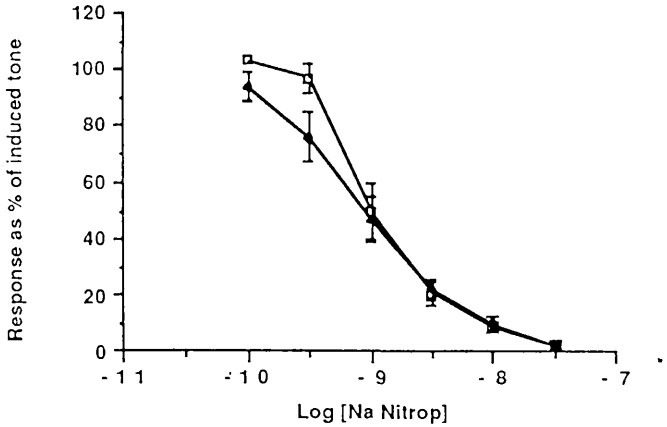


Figure 48d

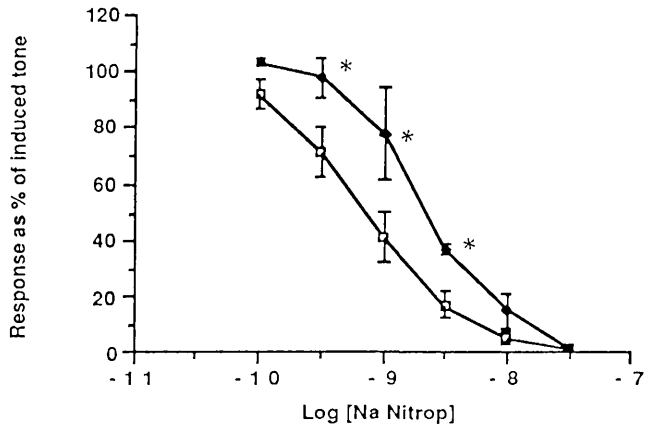


Figure 48 e

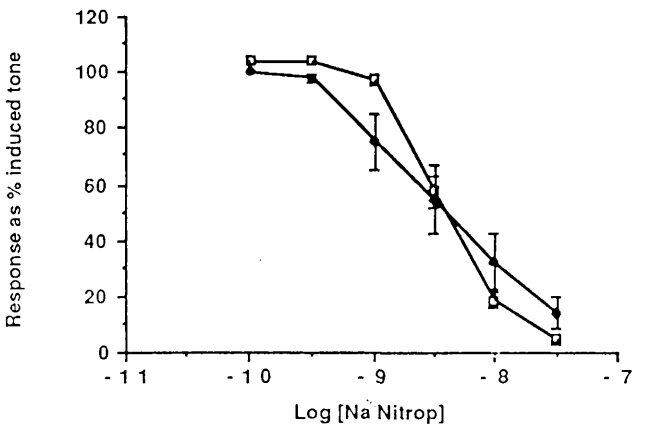
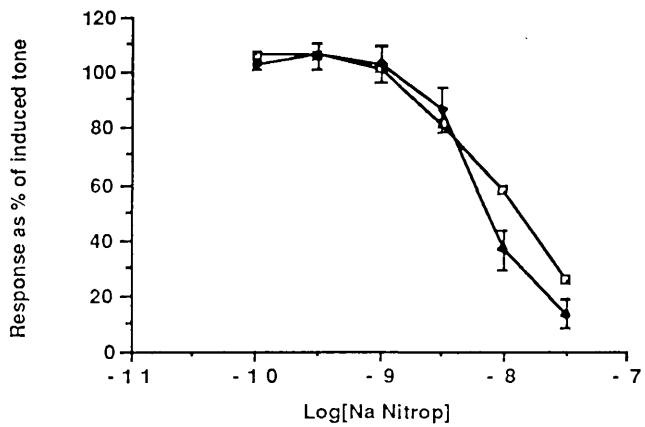


Figure 48 f



The effect of removal of the endothelium on relaxation to sodium nitroprusside in various disease conditions. Results are expressed as % of induced tone. Values are given as mean ± s.e.m.(n=4-6). Statistical analysis was carried out by comparing the values for endothelium disrupted with their corresponding intact tissue. c) control d) diabetic e) renal hypertensive f) DOCA/salt hypertensive +E-□ -E-◆

Statistical significance was determined as follows: *P<0.05 **0.01<P<0.001 ***P<0.001.

TABLE 6

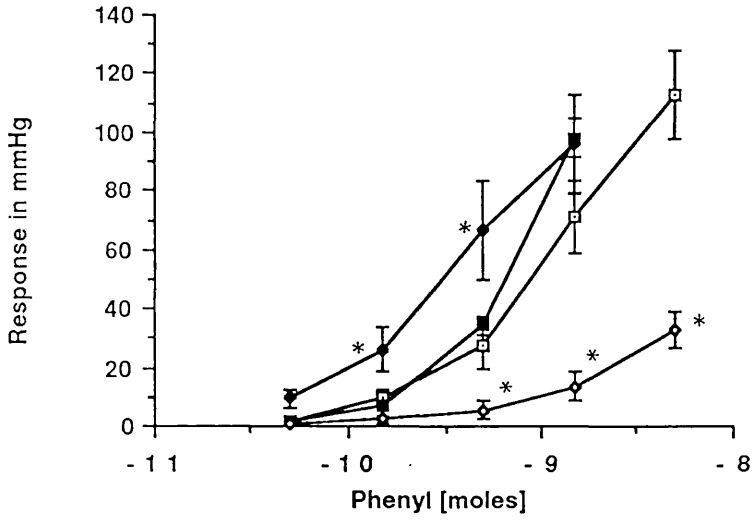
Group	Controls	Diabetic	Renal	DOCA/salt
Noradrenaline maximum				
+E	1.45+0.21	1.18+0.22	1.91+0.15	1.49+0.22
	NS	P<0.05	NS	NS
-E	1.81+0.26	1.89+0.22	2.07+0.30	1.61+0.12
Noradrenaline pD_2				
+E	8.26+0.09	7.82+0.16	8.16+0.09	8.79+0.08
	P<0.05	P<0.05	P<0.05	NS
-E	8.65+0.15	8.48+0.13	8.67+0.02	8.95+0.16
Acetylcholine pD_2				
+E	7.32+0.19	7.42+0.14	7.26+0.16	7.01+0.17
Sodium nitroprusside pD_2				
+E	9.22+0.13	9.19+0.17	8.21+0.20	7.92+0.17
	NS	P<0.05	NS	NS
-E	8.95+0.09	8.62+0.21	8.43+0.06	8.05+0.09

This table shows the effects of different disease states on the sensitivity of the rat aortic ring to NA, Ach and sodium nitroprusside, with an intact(+E) and disrupted(-E) endothelium. The pD_2 to all agents are shown as well as the maximum response to NA in g.wt.

NS - not significantly different from each other within the treatment group.

P - significantly different from corresponding value within the treatment group.

Figure 49



The effect of different disease states on responses to bolus injections of Phe in perfused rat tail (Figure 49). Responses are expressed in mmHg. Values are given as mean ± s.e.m. (n=4-6). Statistical analysis was carried out by comparing values in disease states from control. control- ⊡ diabetic- ◆ renal- ◇ doca/salt- ■

(Statistical significance was determined as follows: * $P<0.05$ ** $0.01>P>0.001$ *** $P<0.001$)

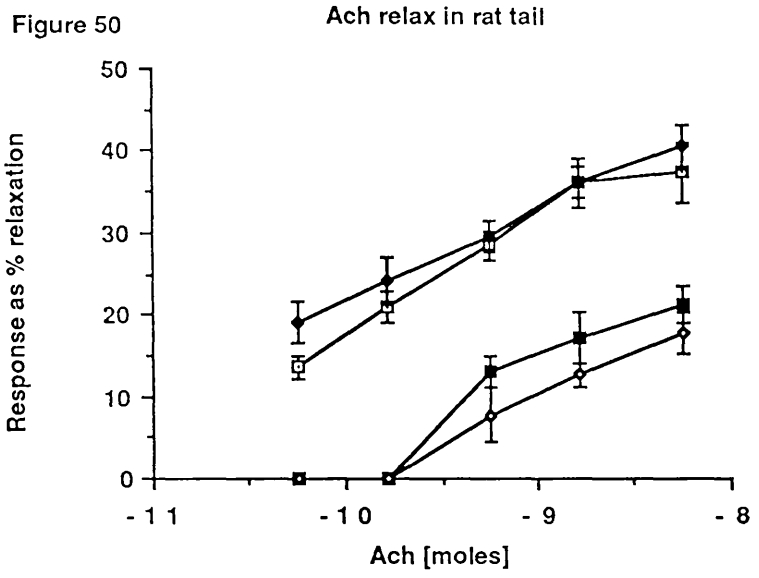
five fold higher concentration of Phe was required to elicit a similar sized response in tails from renal hypertensive rats (Table 7).

In tails from untreated rats, the subsequent bolus injections of Ach (0.055-5.5nano moles i.a.) elicited a dose-dependent vasodilitation. Repeated injection of Ach into tails from untreated rats elicited highly reproduceable responses (Figure 50). In separate experiments it was found that inclusion of atropine (1μM) or haemoglobin (10μM) in the perfusate produced about a 100-fold rightwards displacement of the second dose-response curve to Ach (Figure 50), suggesting that the effect of Ach could be attributed to a muscarinic receptor-mediated release of EDRF. Tails from diabetic rats were similar to those from untreated rats in their responsiveness to Ach (Figure 51a). Those from DOCA/salt hypertensive rats were less so (Figure 51b). However, there were no significant differences in the doses of Ach required to reduce perfusion pressure by 20% in any of the groups (comparisons were made at this level of response in order to accomodate data from all experiments). In contrast to results obtained in the aortic rings, in which sodium nitroprusside was always more potent than Ach, sodium nitroprusside was, at best, only equipotent with Ach (diabetic rats) or less potent (other groups) in the perfused tail (Figure 51b).

The purpose of the second group of experiments was a) to examine in rings of aorta from spontaneously hypertensive rats the effects of NA, Ach and α_2-adrenoceptor agonists and compare with controls b) in the rat perfused tail to determine whether there were α_2-adrenoceptors either mediating contraction or else producing endothelial-dependent relaxations.

a) The results from the spontaneously hypertensive rat (SHR) aortic rings showed that the contractile responses were significantly depressed from control responses in both intact and disrupted aortic rings (Figures 52, 53). The results obtained from

Figure 50



Consecutive relaxation-response curves to bolus injections of Ach in perfused rat tail with Phe-induced tone. Effects of atropine (100nM) and Hb(10uM) on the second curves. Results are expressed in mm Hg. Values are given as mean ± s.e.m. (n=4-6).
control-⊡ 2nd curve-◆ +Atrop-■ +Hb-◇

Figure 51a

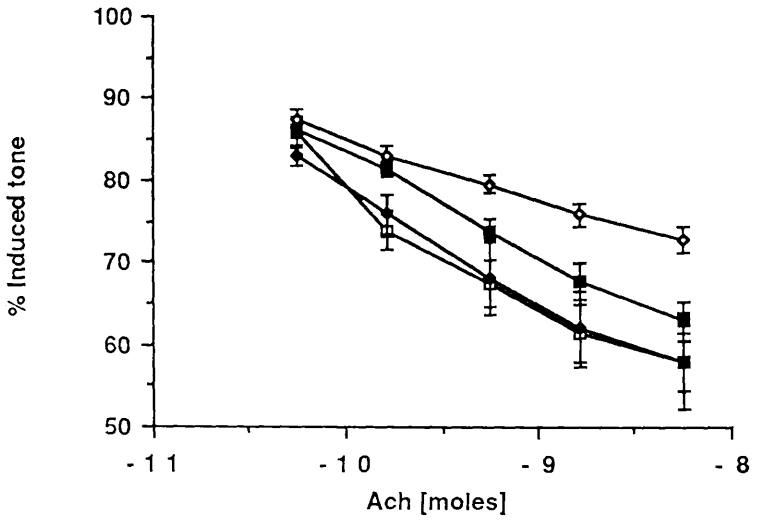
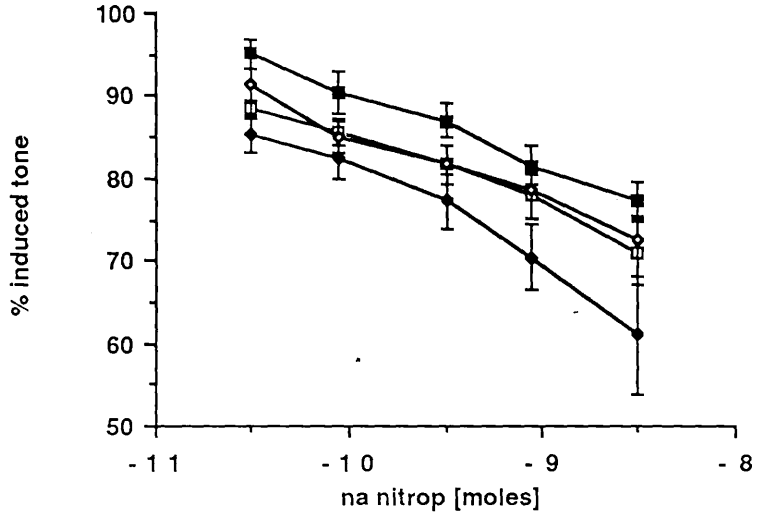


Figure 51b



The effect of different disease states on relaxation to Ach (Figure 51a) and on relaxation to sodium nitroprusside in rat perfused tail (Figure 51b).Results are expressed as a % of induced tone. Values are given as mean ± s.e.m. (n=4-6). Statistical analysis was carried out by comparing values at the -log ED_{20} level , of disease states with those of control. Results are given in table 7. control-□ diabetic-◆ renal-◇ doca/salt-■

TABLE 7

Group	Controls	Diabetic	Renal	DOCA/salt
Phenylephrine				
-log ED_{30}	9.17+0.18	10.03+0.13*	8.62+0.15**	9.3+0.18
-log ED_{60}	8.89+0.15	9.70+0.12*	- ***	9.08+0.14
Increase in perfusion pressure to Phe (mmHg)	88.0+7.9	103+13	82+10	88+16
Acetylcholine				
-log ED_{20}	9.81+0.08	9.85+0.22	9.57+0.11	9.73+0.33
Sodium Nitroprusside				
-log ED_{20}	9.39+0.04	9.76+0.24	9.00+0.18*	8.86+0.15**

This table shows the effect of various disease states on responses to phenylephrine, acetylcholine and sodium nitroprusside in the isolated perfused rat tail. The results are expressed as -log moles of the drug + s.e.m. (n=4-6). This was done because drugs were added as bolus doses therefore concentrations were not available. -log $ED_{30,60}$ for Phe were the values in moles which gave rises of 30 or 60 mmHg. For the two relaxing agents Ach and sodium nitroprusside the -log ED_{20} values are those which reduced the maintained tone by 20%.Statistical analysis was carried out comparing the values obtained in the disease states with those of controls. Statistical significance was determined as follows: *$P<0.05$ **$0.01<P<0.001$ ***$P<0.001$.

Figure 52

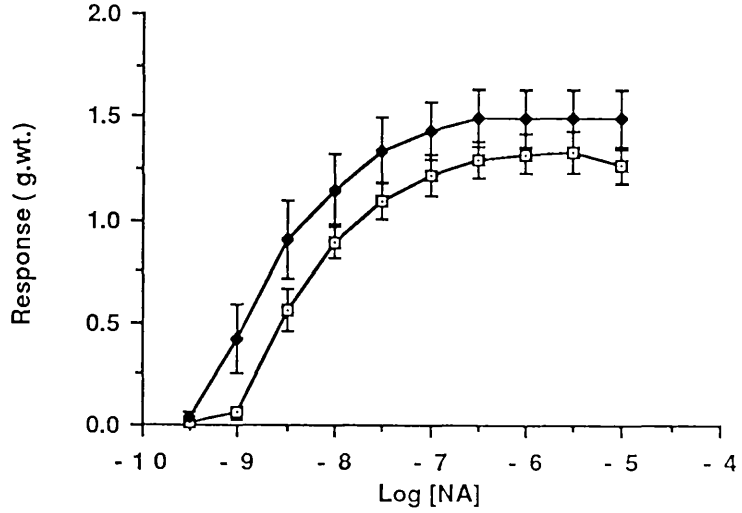
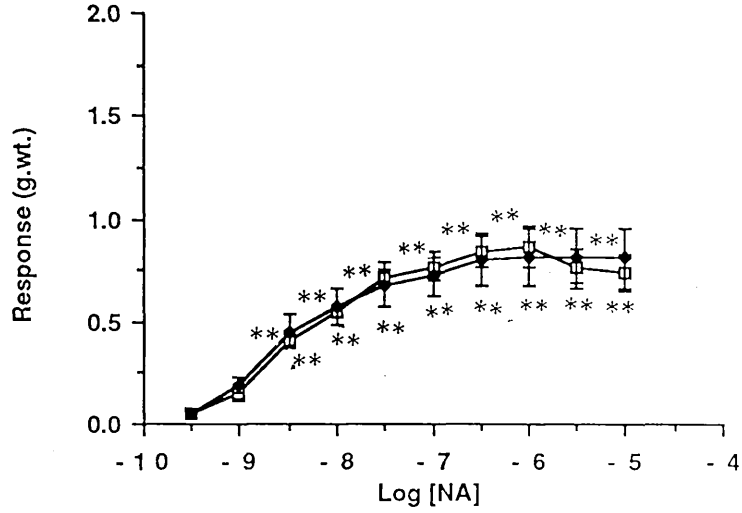


Figure 53



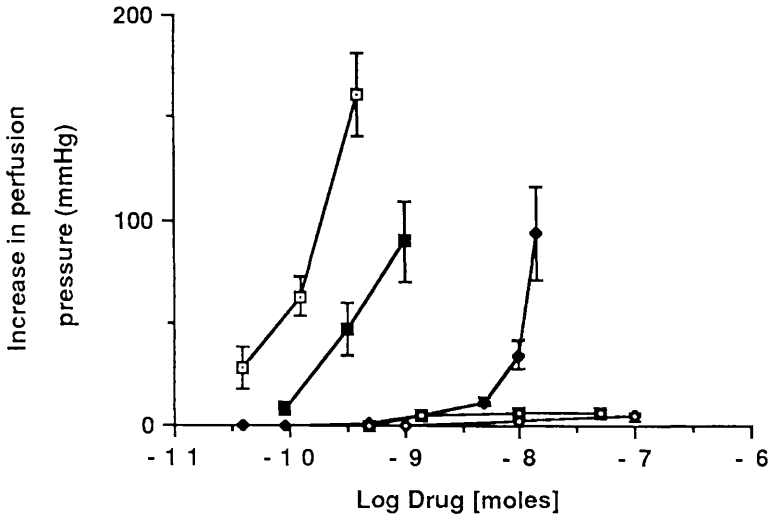
The effect of removal of the endothelium on responses to NA in the isolated rat aorta in control (Figure 52) and SHR (Figure 53). Results are expressed in g.wt. Values are given as mean ± s.e.m. (n=4). Statistical analysis was carried out and the values obtained in the SHR were compared with the corresponding values in control. +E-□ -E-◆

Statistical significance was determined as follows: *P<0.05 **0.01>P>0.001 ***P<0.001

the control animals were carried out at the same time as the SHR and are distinct from the control responses shown in Figure 3. Although control responses at the maximal contraction were not significantly different between intact and disrupted tissues, at the pD_2 value however there was a significant shift to the left of the response in the absence of the endothelium. Comparing the results from the intact and disrupted tissues from SHR there was no significant difference at any concentration of NA. In the four SHR animals used in this study UK14304 did not produce a contraction up to 10μM. Also, tissues with an intact endothelium showed very poor relaxation to a maximal concentration of Ach (3μM), with only an average of 25%± 9% (n=4) relaxation i.e. 75% of the induced tone remained. However the rings of aorta were obtained from the same rat which supplied the tail and for this reason the animals were injected with pentobarbital before being bled (this was due to the lack of numbers of these animals available for experimentation). Nevertheless experiments carried out in normal rats which had first been injected with sodium pentobarbital showed no change in sensitivity to relaxation to Ach so this is probably not the reason for the decrease in sensitivity in the SHR. Our main purpose of these experiments was to determine the presence or absence of α_2-adrenoceptors in the tail preparations and so no further work was carried out on Ach effects. This latter study also was limited by the small number of animals available.

b) Initial experiments using four different agonists Phe, 5HT , BHT-920, and UK14304 show definitely that the order of potency for agonists was 5HT >NA>Phe>UK14303=BHT 920 (fig 54). This did not support the existence of α_2-adrenoceptors on the smooth muscle. The results using sodium deoxycholate to remove the endothelium were very erratic. A typical trace is shown in figure 55. Perfusing with sodium deoxycholate did not reduce the size of the relaxatory response to Ach as would be expected if the endothelium had been successfully removed. Sometimes it made the shape of the perfusion pressure response very

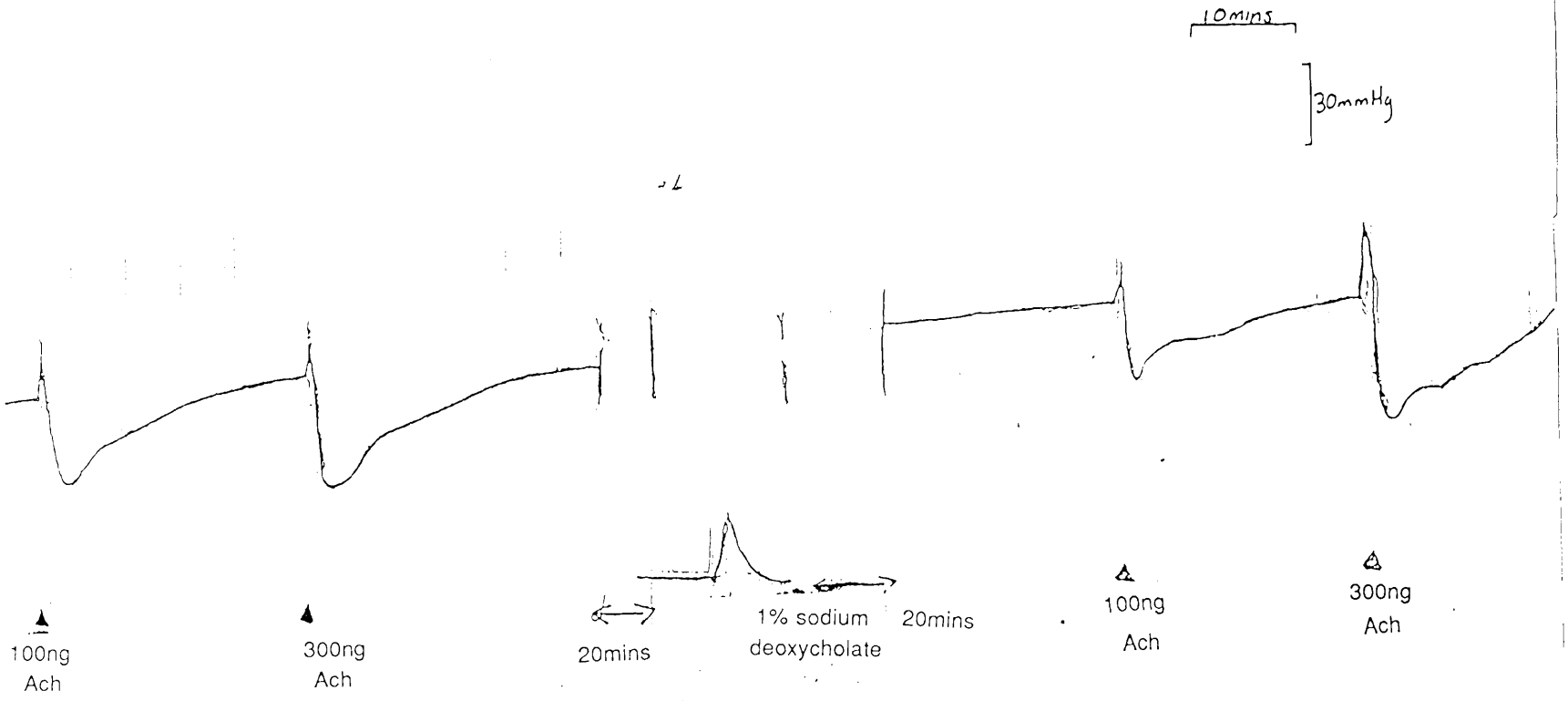
Figure 54



This figure shows the increase in perfusion pressure obtained by injection of bolus doses of various agonists in perfused rat tails. Results are expressed in mmHg. Values are given as mean ± s.e.m. (n=3-6). 5-HT-□ NA-■ Phe-◆ UK14304-◘ BHT-920-◇

Figure 55

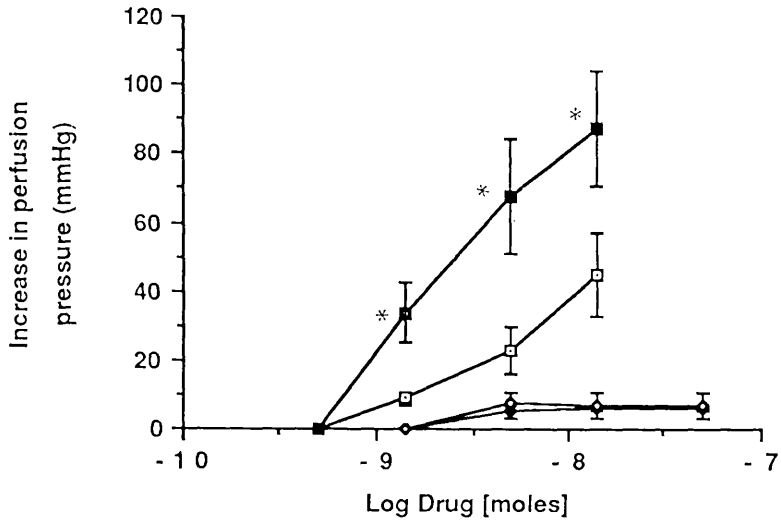
This figure illustrates the effects of perfusion of 1% sodium deoxycholate in the perfused rat tail on relaxation responses to Ach. After a 20 minute period, during which sensitivity to Ach was tested, the Phe was withdrawn from the perfusate and the response droped to baseline where the sodium deoxycholate aws added. In the second 20 minute period Phe was re-introduced and tone again increased.


10mins
30mmHg
100ng
Ach
300ng
Ach
20mins
1% sodium
deoxycholate
20mins
100ng
Ach
300ng
Ach

variable. Even adapting the methods by increasing the flow rate (this compound is thought to act by sloughing off the cells) the relaxation to Ach was still not successfully lost. Therefore we were unable to determine the response without the influence of the endothelium. It is possible that sodium deoxycholate would act to destroy the intact vascular bed since the capillaries are composed only of endothelial cells and this may explain the disappointing results. However, the other method of eliminating the influence of the endothelium a constant perfusion of Hb yeilded more positive results. Responses to Phe were increased significantly with the very small responses to UK14304 remaining the same (Figure 56). Hb itself caused a small increase in the basal perfusion pressure.

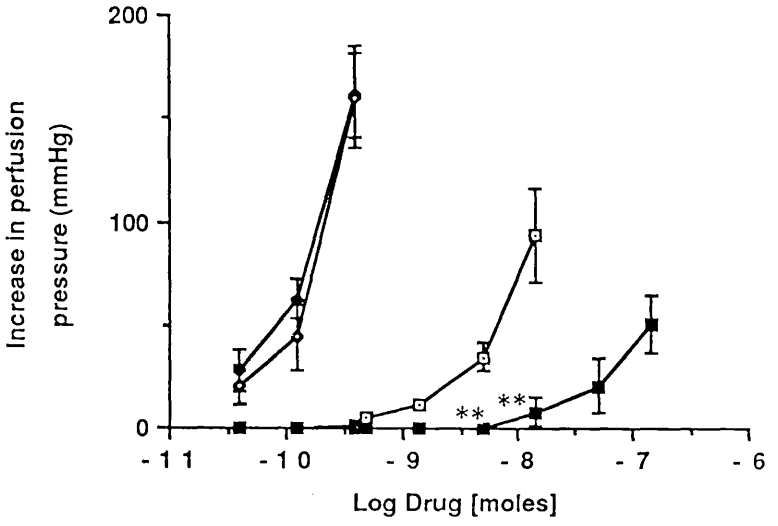
Since there seemed to be no evidence for α_2-adrenoceptors producing contraction, we attempted to design experiments to determine whether there were any α_2-adrenoceptors producing relaxation. First experiments were carried out comparing the effects of prazosin on increases in perfusion pressure produced by Phe and 5HT since this antagonist would have to be present to block any α_1-adrenoceptor action that UK14304 may possess. The curve to 5HT was not shifted at all with this concentration of prazosin but that to Phe was shifted significantly to the right (Fig 57). Tone was increased with 5HT (0.1nano moles/ml/min). The presence of the endothelium was determined by relaxation to Ach. Tone was maintained for a further ten minutes before a dose of UK14304 was added as a bolus injection. The dose was injected again after twenty minutes to ensure the response was reproducible. From Figure 58 the response to UK14304 was not relaxation but was a contraction.When prazosin (10nM) was included in the perfusion medium it had no effect on the contractile response to UK14304. However in the presence of rauwolscine (100nM) the response to UK14304 was completely blocked (Figure 59a). Also further experiments included flurbiprofen in the perfusion medium and in three out of four tails used it also blocked the contraction caused by UK14304 (Figure 59b). It is interesting to note that in four

Figure 56



This figure shows the effects of Hb on responses to Phe and UK14304 in the perfused rat tail. The Hb wasperfused along with the normal perfusate. Results are expressed in mmHg. The values are given as mean ± s.e.m. (n=4). Statistical analysis was carried out by comparing the values in the presence of Hb to the corresponding controls.
Phe-⊡ Phe+Hb-■ UK14304-◇ UK14304+Hb-◆
Statistical significance was determined as follows: *P<0.05 **0.01<P<0.001.

Figure 57



This graph shows the effect of prazosin (10nM) on responses to bolus injections of 5-HT and Phe in the rat perfused tail. Results are expressed in mmHg. Values are given as mean ± s.e.m. (n=4). Statistical analysis was carried out by comparing the values obtained in the presence of the antagonist with its corresponding control. 5-HT- ◆ 5-HT+praz-◇ Phe- □ Phe+praz- ■
Statististical significance was determined as follows: *P<0.05 **0.01<P<0.001 ***P<0.001.

Figure 58
Legend

This figure shows a typical trace of contraction to UK14304 in the perfused rat tail only when tone is present.

1.6UK-1.6 nano moles 4.5UK- 4.5 nano moles 16UK- 16 nano moles

▲ - tone raised by constant perfusion with Phe.

2.5 Ach- 2.5 nanomoles 7.5Ach- 7.5 nano moles.

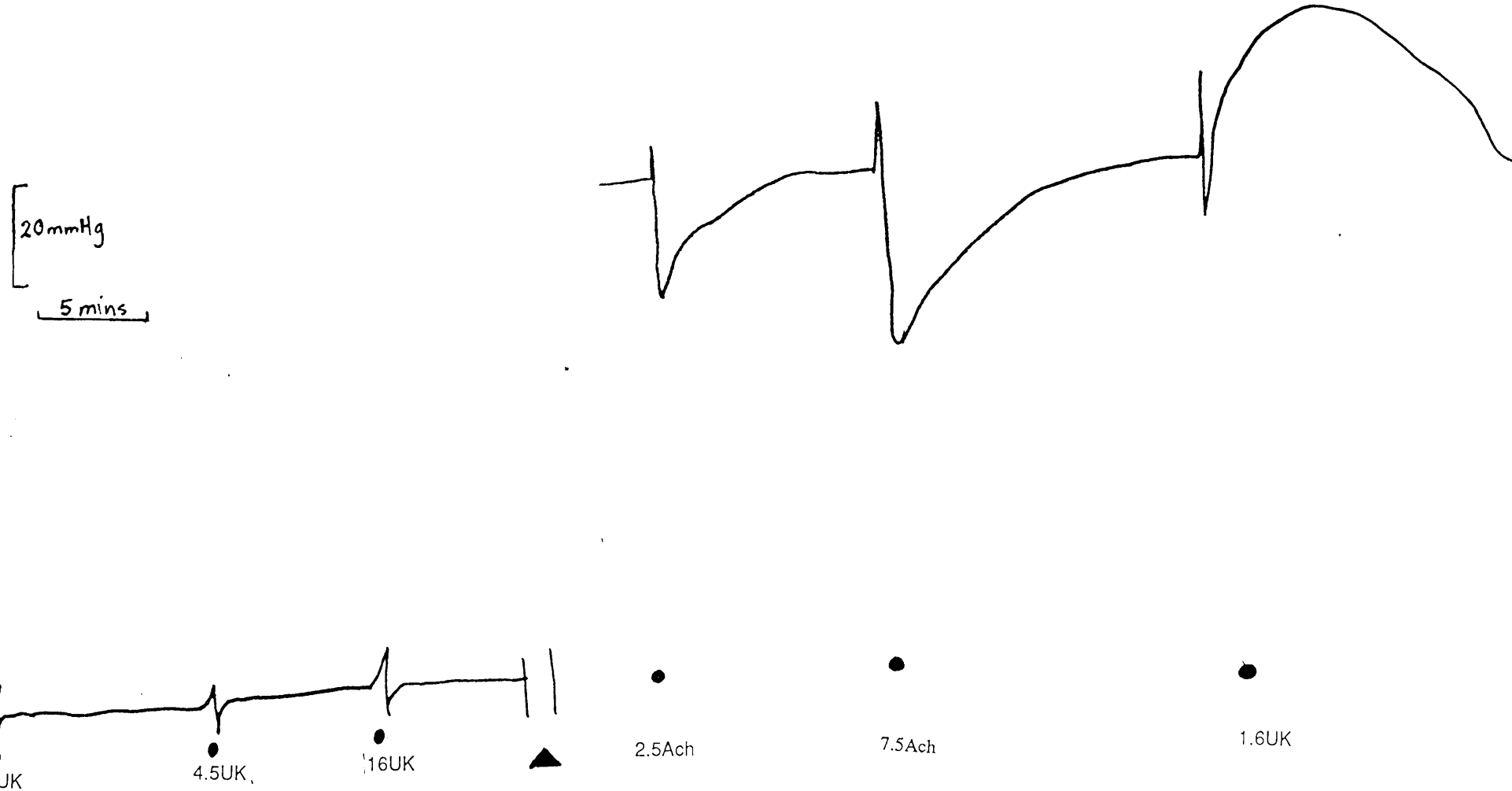
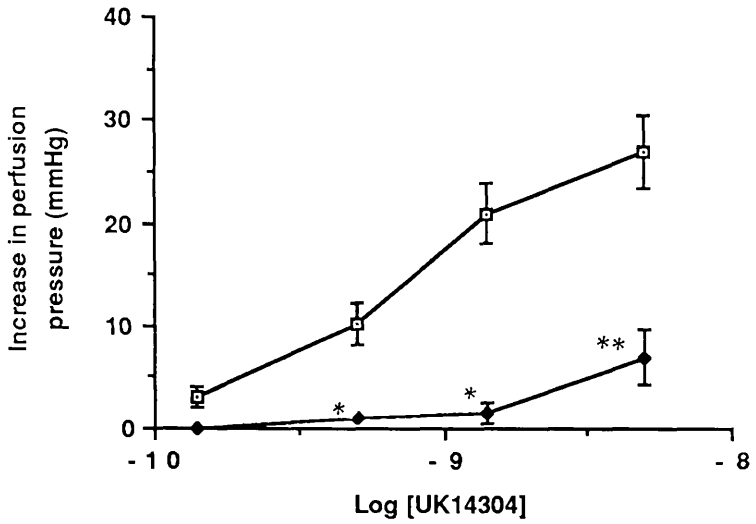
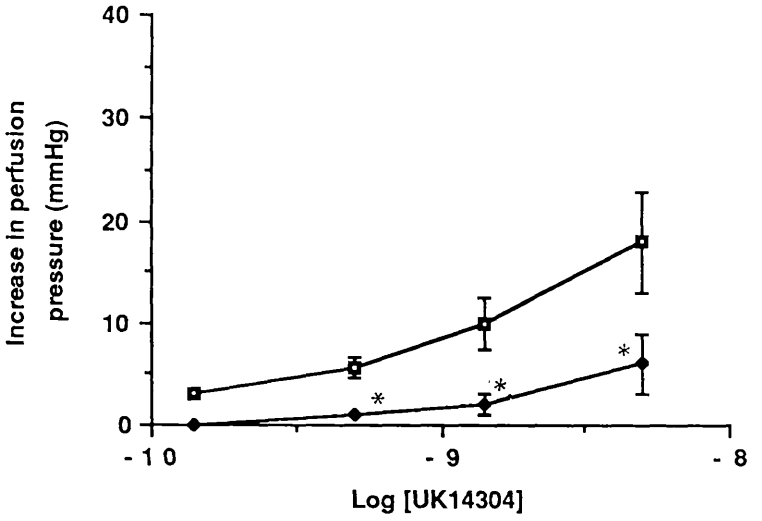

20mmHg
5 mins
1.6UK
4.5UK
16UK
2.5Ach
7.5Ach
1.6UK

Figure 59a



This figure shows the effect of rauwolscine (100nM) on the contractile responses to UK14304 which were obtained when the tone was raised with a constant perfusion of 5-HT. Responses are given as increases in perfusion pressure in mmHg as mean ± s.e.m. (n=4). Statistical analysis was carried out by comparing the responses before and after addition of rauwolscine. Statistical significance was determined as follows *P<0.05 **0.01<P<0.001 ***P<0.001. control-⊡ +rauwol-◆

Figure 59b



This figure shows the effect of flurbiprofen (1μM) on the contractile responses to UK14304 which were obtained when tone was raised with a constant perfusion of 5-HT. Responses are given as increases in perfusion pressure in mmHg as mean ± s.e.m. (n=4). Statistical analysis was carried out by comparing the responses before and after addition of flurbiprofen. Statistical significance was determined as follows *P<0.05 **0.01<P<0.001. control-□ +flur-◆

out of thirty tails examined that Ach did not produce relaxation. In these tails also UK14304 produced little or no contraction. It is possible that in these tails the endothelium was damaged therefore stimulation of α_2-adrenoceptors can no longer release prostaglandins.

DISCUSSION

In the rat isolated aortic ring, preparations from most, but not all, of the groups of rats tested, the sensitivity to the contractile agent, NA, was influenced in a manner dependent upon the presence or absence of the endothelium. Several points were clear.

First, there was no significant difference in the NA pD_2 or maximum tension developed in endothelium-disrupted aortic rings from diabetic, renal and DOCA/salt hypertensive rats, compared with those from control rats. Thus, the contractile response of the muscle to adrenoceptor stimulation was not affected by these disease states.

Secondly, in DOCA/salt hypertensive rats the maximum response to NA, and the pD_2 values were not different in endothelium-intact rings, when compared with the corresponding values in disrupted rings.

Thirdly, in control and renal hypertensive rats, pD_2 values were significantly higher than in corresponding disrupted rings. Despite this reduced sensitivity to NA in the presence of the endothelium, there was no significant difference in the maximum response to NA in endothelium-intact rings in these groups compared with their disrupted counterparts.

Fourthly, in diabetic rats there was a marked reduction in the potency of NA in endothelium-intact, compared with disrupted, aortic rings in terms of both the pD_2 and in the maximum tension developed.

Fifthly, in rings from SHR disruption of endothelium affected neither NA pD_2 nor maximum: although these are not directly comparable with regard to experimental protocol and population the results are essentially similar to the DOCA/salt hypertensives.

As described previously in Chapter 1 there is a spontaneous release of EDRF

(Allan et al., 1983; Egleme et al., 1984; Martin et al., 1986) which modulates the response to agonists when the endothelium is intact. The present results show that this modulatory influence is most marked in diabetes, with the result that the maximum response, as well as the overall sensitivity to NA was depressed. In contrast, the endothelium exerted no such influence in DOCA/salt hypertensive animals or in SHR. This effect is unlikely to be attributable to the high blood pressure of these animals per se, because the influence of the endothelium in renal hypertensive rats, in which blood pressure was equally as high, was the same as that seen in control animals.

In contrast to the influence of the spontaneous release of EDRF the Ach-induced relaxation of NA-contracted rings was not significantly influenced by any of the disease states. Thus, Ach-induced release of EDRF seemed to be unimpaired. However, it is interesting to note that there was a tendency for Ach to be least effective in aortic rings from DOCA/salt hypertensive rats, in which the spontaneous influence of the endothelium was absent, and to be most effective in rings from diabetic rats, in which the spontaneous influence was greatest.

Unexpectedly the effect of sodium nitroprusside varied considerably, depending upon disease state. Responses to sodium nitroprusside should reflect the degree to which soluble guanylate cyclase can be activated by the formation of S-nitrosothiols (Ignarro & Kadowitz, 1985) and/or the responsiveness of the smooth muscle to the cGMP so generated. Because the activity of sodium nitroprusside is believed to be independent of the endothelium, differences in the relationship between the relaxant responses to Ach and sodium nitroprusside in any particular group should reflect changes in endothelial function secondary to the disease state. There were no significant differences in the responsiveness to sodium nitroprusside between corresponding endothelium-intact and disrupted rings within

the control, renal and DOCA/salt hypertensive groups. In diabetic rats the greater potency of sodium nitroprusside in endothelium-intact, compared with disrupted rings may be attributable to the greater influence of spontaneously released EDRF in this group. More difficult to explain is why sensitivity to sodium nitroprusside should be lower in rings from hypertensive animals (endothelium-intact and disrupted) compared with their control and diabetic counterparts. Perhaps the high blood pressure in these animals, and the well known secondary morphological changes that result from it (e.g. intimal thickening) somehow impair the ability of sodium nitroprusside to stimulate guanylate cyclase and generate cGMP; alternatively, it may decrease the sensitivity of the smooth muscle to whatever cGMP is formed, or enhance the breakdown of the cGMP, although these would have been expected to reduce sensitivity to spontaneous or activated release of EDRF. Whatever the explanation, if responsiveness to sodium nitroprusside is regarded as an index, or marker, of the capacity of the vascular smooth muscle to respond to activation of soluble guanylate cyclase, through which Ach ultimately exerts its relaxant effects, then the present findings would need to be reinterpreted as providing evidence for an enhanced influence of EDRF which overcomes the insensitivity to it of the smooth muscle in hypertensive rats. This would need to be either due to an enhanced production of EDRF, by Ach in endothelial cells from aortae (spontaneous and activated) or a sensitisation of the processes in smooth muscle which respond to it.

The responses obtained to NA from the aortic rings from the spontaneously hypertensive rats showed a similar pattern to DOCA/salt hypertensive rats when comparing responses between intact and disrupted rings. Like the DOCA rats there was no difference between intact and disrupted rings at either the pD_2 value or the maximum response. The absolute sizes of the responses in the SHR were significantly smaller than those from age matched control animals. However, there was a difference in the method of preparation of the rings from the SHR in that they

were injected with pentobarbital first. Control rats which had been injected with this anaesthetic showed no decrease in sensitivity to Ach, so if this was to be an explanation we would have to believe that the pentobarbital had a selective inhibitory effect on the SHR relaxation.

It is interesting to compare our results with those reported recently by others who have examined endothelial function in the same disease states. A reduced sensitivity to NA in endothelium-intact (compared with disrupted) aortic rings from alloxan-diabetic (Fortes et al., 1983) and streptozotocin diabetic (Oyama et al., 1986) rats has been reported, and our results confirm this. Our data also confirm the observation (Fortes et al., 1983) that Ach-induced relaxation is unimpaired in diabetes. This finding is in contrast to that of Oyama et al (1986) who found it to be greatly impaired, despite the fact that, as in our experiments, sodium nitroprusside-induced relaxation was not affected. We cannot explain this difference but they did use a concentration of NA(1μM) to induce tone which is near maximal and may explain why even in control animals the relaxation to Ach was much less potent than we (or most other workers) find.

Lockette et al (1986) and Van de Voorde & Leusen (1986) reported a marked reduction in the sensitivity and a decrease in the maximum response to Ach in aortic rings from DOCA/salt hypertensive rats, compared with untreated rats. Both groups also noted a decrease in sensitivity (though not the maximum response) to sodium nitroprusside, the effect being more evident in the report by Lockette et al. Although no explanation was advanced for the latter finding, Van de Voorde and Leusen showed by bioassay of EDRF that the reduced effect of Ach was not attributable to a decreased production by Ach, but seemed to reflect an impaired coupling mechanism between EDRF and the vascular smooth muscle. However the fact that sodium nitroprusside is unimpaired suggests that the smooth muscle in these

animals is still able to respond normally to a direct relaxant agent. Also since Ach stimulated release of EDRF is not reduced in any way this seems to suggest a selective effect of the disease on its action or half life. If in fact EDRF is nitric oxide as has been postulated recently (Palmer et al 1987) it would seem to be unlikely that it would be selectively inhibited. These results therefore do not seem to be compatible. Although in our experiments Ach was not significantly less potent in relaxing aortic rings from DOCA/salt hypertensive rats, compared with control rats, there was clearly a tendency for this to be so. Finally our failure to observe any significant change in responsiveness to Ach in aortae from renal hypertensive rats, despite a decrease in sensitivity to sodium nitroprusside similar to that seen in DOCA/salt hypertensive rats, contrasts sharply with that reported by Van de Voorde & Leusen (1986), who found that, as in DOCA/salt hypertensive rats, responsiveness to Ach was severely impaired.

The points of disagreement between our results and these other reports could be attributable to differences in the experimental protocol. From our results in Chapter 2 we demonstrated that the initial tone placed on the tissue was a vital factor in the sensitivity of the rat aorta to both contractile and relaxatory agents. If the initial tone on the preparations from all disease states is the same, this does not take into account any possible differences in the length-tension relationship that develop in disease conditions. In this respect our experiments may give misleading results since we have made no compensation for this. Nevertheless, the central problem in comparing our results with those of others is that our responses to NA and to Ach are remarkably consistent between groups so that if we are masking effects inadvertently then we are managing this very precisely. Van de Voorde and Leusen report that they carried out length-tension studies, although the details they give on how these studies were carried out were not very clear. They cover only four different tensions over a range of 0.5 to 15 grammes therefore making it not a very

accurate study. From our results (Figures 20a,b) differences in the size of response to Phe(1μM) were great even over only 0 to 6 grammes resting tension. It is not clear whether they have set the tension to be optimal for contraction (to NA) or relaxation (to Ach) in the results that they present. Essentially their hypertensive aortic rings showed maximal relaxation to Ach which was considerably different from that of control tissues. The initial tension on their control tissues was 0.5g which from our results is optimal for the observation of relaxation to Ach in control tissues with an intact endothelium. However the initial tension of 1.5g which was used by myself was at the point at which least sensitivity to Ach was seen (see Figure 22b) yet the hypertensives were still sensitive in my study. Lockette et al (1986), used strips of aorta and set the tissues up for maximal response to contractile agents and do not mention optimisation for relaxatory agents which is the factor to which they were most interested. Unfortunately we carried out the comprehensive length-tension studies after these experiments had been completed and so further interpretation of this factor remains speculative.

The results obtained in the perfused tails were somewhat different from those obtained in the aorta. Phe, rather than NA, was used as the constrictor agent in the tails for several reasons. First, neuronal uptake of catecholamines is more marked in the tail vascular bed than in the aorta (from which it is almost absent) and Phe is less subject than NA to this process. Furthermore, Phe is less potent than NA as a β-adrenoceptor agonist. Finally, Phe is more selective than NA for α_1-adrenoceptors . Although it is not known whether α_2-adrenoceptors, influencing EDRF release, are present on endothelial cells in the vasculature of the rat tail, Phe would be less likely than NA to stimulate them if they do exist. From our results however with UK14304 there does seem to be α_2-adrenoceptors present in this preparation mediating contraction (at least when there is tone already present) although whether they are present soley on the endothelium or not has not been elucidated. For all these reasons, responsiveness

of the tail vascular bed to Phe is likely to reflect its action primarily, if not exclusively, at vascular α_1-adrenoceptors.

The sensitivity of the vascular bed of tails from untreated and DOCA/salt hypertensive rats to Phe was similar. This was surprising since in DOCA/salt hypertensive rats increased sensitivity to NA has been found in perfused tails Hinke (1965) and Beilin and Wade (1970), mesentery (Finch, 1971) and hindquarters (Finch and Haeusler, 1971). Perhaps the difference lies in their use of NA rather than Phe. Even more difficult to explain is the reduced sensitivity, to Phe, of the vasculature of the tails from renal hypertensive rats. Although we can find no reports on vascular reactivity in the caudal artery of renal hypertensive rats, reactivity is increased in the femoral artery (Holloway and Bohr,1973) and mesenteric vascular bed (McGregor and Smirk, 1968). Our findings of reduced reactivity in the caudal artery remain unexplained.The increased sensitivity, to Phe, of the tail vasculature from diabetic rats is in keeping with the findings of Ramanadham et al (1984), although Hart et al (1984) found no change in sensitivity to NA.

Ach induced a dose-dependent reduction in the sustained increase in perfusion pressure in tails from rats from each group. In untreated rats the effect of Ach was markedly inhibited by pretreatment with atropine or by Hb, which strongly suggests an action at the muscarinic receptor and involvement of EDRF, respectively. Despite the fact that Phe produced a larger sustained increase in perfusion pressure in tails from diabetic rats, the responses to Ach in these preparations were superimposable with those obtained in untreated rats. In contrast, tails from DOCA/ salt hypertensive rats were on average approximately three times less sensitive to Ach than was found in untreated rats.Although this effect was not significantly different when evaluated in terms of the doses required to reduce

perfusion pressure by 20%, it follows the same pattern as that seen in the aorta. Responses to Ach were less marked in tails from renal hypertensive rats. Although this was not statistically significant at the ED_{20} dose level, the whole dose-response curve to Ach was flatter in tails from this group than in the others. We cannot say whether this is a consequence of the 5-fold higher concentration of Phe that had to be used to elevate the perfusion pressure in these preparations or whether it was a true decrease in sensitivity.

The overall pattern of sensitivity to sodium nitroprusside in the perfused tails was also slightly different from that seen in the aorta. Only in tails from DOCA/salt hypertensive rats was the response to sodium nitroprusside significantly diminished, and the effect was not as marked as in the aorta. Although a similar trend was apparent in tails from renal hypertensive rats, the effect was not significant. The most outstanding difference between the aorta and the tail vasculature, however, was that, although sodium nitroprusside was invariably more potent than Ach in the aorta, it was, at best, only equipotent with Ach in the tail vascular bed of diabetic rats and in other groups was less potent . A number of explanations for this is possible: i) the difference in temperature of the Krebs solution used in the aortic rings (37°C) and tails (28°C), ii) the inclusion of Ficoll in the tail experiments, iii) the use, in the tail, of bolus doses of drugs which may not have had sufficient time to elicit their full effects before being washed out, iv) a true difference in the ways in which Ach and sodium nitroprusside stimulate guanylate cyclase. It is unlikely that (i) or (ii) can account for this finding, however, because Gerkens (1987) has reported very similar potencies for Ach and sodium nitroprusside in isolated caudal arteries, perfused with Ficoll-free Krebs solution at 37°C, and constricted by NA. Whatever the explanation, it is clear that the relative effects of the drugs which mediate vasodilatation by stimulating guanylate cyclase activity, either directly or indirectly, can differ widely under different experimental

conditions, and effects obtained in the isolated aorta (the most commonly used preparation) may not be representative of those in other tissues. Despite these contradictions one message from these experiments seems clear: the ability of Ach to produce vasodilatation, an effect believed to be mediated via release of EDRF, is only minimally affected by diabetes or hypertension.

Using the perfused tail the effects of two α_2-adrenoceptor agonists were examined to determine the presence, if any, of an α_2-mediated contraction. The two agonists UK14304 and BHT-920 however produced no contraction even at very high concentrations. Since we could not successfully show loss of relaxation to Ach after perfusing with sodium deoxycholate this prevented use of this method to examine the responses to selective α_1 and α_2-adrenoceptor agonists without the influence of the endothelium and therefore possibly uncover α_2-adrenoceptors. However we did use Hb which, when perfused, increased the responses obtained to Phe but not those to UK14304. This suggested that the endothelium did interfere with responses to the selective α_1-adrenoceptor agonist but there was no evidence that it was responsible for suppressing any putative response to the α_2-adrenoceptor agonist UK14304. These results are not the same as those obtained in the rat aorta where the responses to both α_1 and α_2 adrenoceptor agonists are increased in the presence of Hb (Martin et al 1985) although there is no evidence in that tissue for the involvement of α_2-adrenoceptors, only α_1-adrenoceptors activated by all of the agonists. (See also results in chapter two). However the response to UK14304 in these conditions were fairly trivial and could not be definately ascribed as mediated by any particular receptor. Since there was no evidence for α_2-adrenoceptor mediated contraction in the tail we examined possible α_2-mediated relaxatory effects when tone was increased with either Phe or 5HT (as demonstrated in the dog & pig coronary artery by Cocks & Angus ,1983). However in the presence of tone α_2-agonists produced no relaxation but instead a dose- dependent contraction. This contraction was not blocked by high

doses of prazosin or corynanthine but was blocked by high doses of rauwolscine. Due to the lack of time available when the preparation was viable only single doses of each antagonist were used but the results were clear cut.. It was possible that the contraction was mediated via release of prostaglandins which are known to be released under certain conditions (Rubanyi & Vanhoutte 1986). We used flurbiprofen to test this possibility and found the responses to be significantly inhibited in three out of four tails. In conclusion we have found evidence to suggest that there may be α_2-adrenoceptors on the smooth muscle of the rat tail. However these receptors only become evident if tone is already present, brought about by either Phe or 5HT or possibly any other contractile agent. That this contraction is inhibited by flurbiprofen suggests that prostaglandins are involved. It is likely that prostacyclin is released and is responsible for the contraction since it is known to be present in endothelial cells. More work will have to be carried out before this can be stated conclusively.

GENERAL DISCUSSION

The results presented in this thesis demonstrate the influence or lack of influence of the vascular endothelium on smooth muscle responsiveness under various conditions. Similarly to many other workers I have found that the presence of the endothelium can have a profound effect on the responsiveness of the tissue to certain agonists. I have found this effect to be more apparent with the partial agonists at the α_1-adrenoceptor rather than those agonists producing a similar maximum to NA. There are a few exceptions to this e.g. U46619, $PGF_{2\alpha}$. We found however, that the presence of the endothelium had no effect on the ability of a variety of α-adrenoceptor antagonists to inhibit the contractile response to NA. This could possibly be due to the inclusion of propanolol in the Krebs since we have demonstrated that β-relaxation is more potent in tissues with an intact endothelium. This β receptor stimulation would contribute more in the overall response to NA at the high concentrations required to overcome the α_1-adrenoceptor antagonism. The effects on the NA CRC of Wyeth 26703 a specific α_2-adrenoceptor antagonist would also seem to discount the possibility that stimulation of α_2-adrenoceptors on the endothelium to release EDRF may be responsible for the diminution of the contractile response seen when the endothelium is present. This is supported by the lack of effect of Wyeth 26703 on relaxation to Ach. If α_2-adrenoceptors were involved, then their blockade might be expected to have some inhibitory effect on relaxation of NA-induced tone to Ach-stimulated release. I did not carry out experiments using α_2-adrenoceptor agonists to see if they would cause relaxation directly. However some other workers (Martin et al 1986) have been unable to obtain α_2-adrenoceptor relaxation to clonidine in the rat aorta and observed only further contraction which was blocked by an α_1-adrenoceptor antagonist.

The studies with calcium demonstrate that the decrease in sensitivity to agonists with an intact endothelium cannot be due to a decrease in calcium entry into the cells at least when NA is the contractile agent. I found no difference in the sensitivity between intact or disrupted to calcium in either a buffered or unbuffered calcium system. However the buffered system was more effective at determining the responses in very low concentrations of free calcium but had a blocking effect at the higher levels of calcium when compared to the unbuffered system. This would suggest that the endothelium does not act as a "physiological" barrier to calcium entry. BAY-K 8644 has a similar potentiating effect on the calcium CRC to NA (30nM) between intact and disrupted tissues. Similarly nifedipine has a similar blocking effect in both intact and disrupted tissues. However BAY-K 8644 potentiated the responses to a CRC to NA in 2.5mM calcium to a greater extent in tissues with an intact compared to dirupted endothelium. This could be due to blockade of spontaneous release of EDRF since from other experiments I have shown that BAY-K 8644 can block Ach-stimulated release of EDRF.

The difference in sensitivity to some agonists between tissues with an intact endothelium compared to disrupted could have been due to an influence of the initial tension that the preparations were set up at. This initial tension is also important when considering the results from animals which have cardiovascular diseases e.g. hypertension and diabetes. It was unfortunate that the length-tension studies were carried out at a later date to the disease study, however they do not help entirely to explain the results we obtained. In our study we set out to determine changes if any in disease states to both contractile (NA) and relaxatory (Ach and sodium nitroprusside) agents. The results of the length-tension study carried out in healthy,untreated rats, summarised in Figure 18 shows that the initial tension which is optimal to show the absolute influence of basal release of EDRF (i.e. the greatest difference in the size of responses between intact and disrupted tissues to Phe 1μM)

did not coincide with that for the release of EDRF by Ach. Therefore in the disease studies there would have been two possible initial tensions at which the tissues could be set depending on the optima desired. This is further complicated in the disease state where this relationship may not be the same. The fact that we set the initial tension in all four preparations at the same level at least gives us a constant factor to work from. Before results can be compared with each other and between different groups of workers with any great accuracy a complete length-tension study would have to be carried out in hypertensive and diabetic aortic rings as had been done in untreated animals. Only after this had been carried out can the results obtained be directly comparable.

Our results using the rat aorta support the general view that there exists a homogeneous population of α_1-adrenoceptors on the smooth muscle. Using the isolated perfused rat tail we have found evidence which may indicate the presence of α_2-adrenoceptors somewhere in the vasculature. These receptors were not apparent until there was tone already present. This is a similar situation with Angiotensin II which requires depolarisation of the smooth muscle of the rat aorta before a contraction is obtained. More work could be carried out to further characterise these receptors using more selective agonists and antagonists. It would also be interesting to see if the responses were still apparent after removal of the endothelium. Our experience with sodium deoxycholate (which acts to disrupt the endothelium) have been unsuccessful in this preparation and may indicate that it is not possible to use this agent in a complete vascular bed like the tail. Similarly methylene blue used in the rat tail experiments caused the tone in the preparations to rise uncontrollably and so could not be used for further experimentation. It may be worth trying Triton-X 100 (0.1%) which has been successful in removing the endothelium in the dog basilar artery (Connor et al in press).

To conclude therefore we have carried out an extensive study using the rat aortic

ring and the isolated perfused rat tail to examine the influence of the endothelium. We have also studied the effect in various disease states and how the relationship alters if any from the control. The conclusions from this study would have to be that under certain circumstances and depending on the tissue being studied that the endothelium does play a role in the modulation of responses both excitatory and inhibitory. However in several cardiovascular disease states which we studied the relationship between endothelium intact and disrupted tissues remains fairly similar with only some subtle differences.

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