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

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1 The effects of initial stretch and degree of agonist-induced tone on acetylcholine-induced relaxations were examined in rings of rat isolated aorta. The relaxation to acetylcholine was antagonized by atropine and almost completely abolished by haemoglobin. Relaxation to sodium nitroprusside was similar in rings with an intact or disrupted endothelium but that to isoprenaline was greater in intact preparations.

2 In preparations with either an intact or disrupted endothelium there was a similar length-dependent increase in the resting tension of the aortic rings. The size of the contractile response to phenylephrine (1 μM) was dependent on the initial length (and hence degree of stretch) of the preparation in both rubbed and unrubbed tissues. The absolute difference in contractile response between rubbed and unrubbed was greatest at 1.8 mm and less at the other lengths tested, including the optimum degree of stretch for contraction i.e. 2.4 mm.

3 The absolute acetylcholine-induced relaxation (only seen in rings with an intact endothelium) was dependent on the initial length (and hence degree of stretch) of the preparation and was maximum at 2.4 mm. The proportionate relaxation (i.e. expressed as a percentage of induced tone) was also length-dependent, being optimal at 1.5 mm.

4 The sensitivity of the vessels to acetylcholine varied depending on the level of agonist-induced tone. When tone was low, acetylcholine sensitivity was high (at [NA] 0.03 μM: $pIC_{50} = 7.36 \pm 0.07$), when the concentration of noradrenaline was increased the tone increased and the acetylcholine sensitivity was low (at [NA] 0.3 μM: $pIC_{50} = 6.57 \pm 0.07$).

5 The absolute sensitivities and maximum relaxations induced by acetylcholine are discussed in relation to the initial degree of stretch (and hence length of the preparation) or the degree of agonist-induced tone.

Introduction

Endothelium-derived relaxant factor (EDRF) (Cherry *et al.*, 1982), now thought to be nitric oxide in several cases (Palmer *et al.*, 1987; Vanhoutte, 1987) mediates the action of several vasodilators including acetylcholine (Furchgott & Zawadski, 1980).

Considerable quantitative variation between different laboratories exists with regard to the demonstration of the vaso-inhibitory influence of spontaneous basal release and agonist-stimulated release of EDRF. For example, in rat aorta contractile responses to some α-adrenoceptor agonists, particularly partial agonists such as clonidine, are sometimes found to be markedly potentiated by disruption of the endothelium, while other agonists, particularly those with greater intrinsic activity such as phenylephrine, are relatively unaffected (e.g. Egleme *et al.*, 1984). However, in an essentially similar study, Martin *et al.* (1986) found qualitatively similar but quantitatively different results wherein endothelial removal had less effect versus clonidine but more versus phenylephrine. On this basis the two groups of workers suggested quite different explanations, the former based on adrenoceptor-mediated release of EDRF, the latter on the continuous, spontaneous, basal release of EDRF.

In an attempt to resolve this we carried out similar experiments. On producing yet another set of results (with potentiation of both clonidine and phenylephrine) we wondered whether variations in experimental protocol might affect the perceived influence of the endothelium on contractile responses sufficiently to lead to spurious conclusions. Thus the present study sought to examine the effect of two of the experimental variables most likely to differ between laboratories, viz. the initial stretch of the smooth muscle and the extent of agonist-induced tone from which inhibition is judged. These were tested against (i) the effects of basal release of EDRF judged by the increase of contractile stimuli caused by endothelial disruption and (ii) the effects of EDRF released by acetylcholine.

There is also some physiological relevance to this, i.e. does EDRF influence vascular tone to the same extent over the wide range of passive wall tensions engendered by different arterial pressures *in vivo* and is this modified by active vascular smooth muscle tone?

Methods

Male Wistar rats (250–300 g) were killed by a blow to the back of the head followed by exsanguination. The descending thoracic aorta was exposed by removal of the heart and lungs, and cut just above the diaphragm and just below the aortic arch. The aorta was cleared of all connective tissue, removed from the animal and placed in a petri dish containing Krebs solution and cut into 2–3 mm long ring segments.

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

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

In addition, when noradrenaline was used, the Krebs solution also contained 23 μM ethylene diaminetetra-acetic acid (EDTA) in order to reduce degradation of the noradrenaline.

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Isometric tension changes were measured with either Harvard or Grass force transducers and recorded on a Linseis four channel chart recorder.

Effect of resting length (tension)

Paired, rubbed/unrubbed aortic ring segments were used. In a first group of experiments the effects of endothelial disruption were assessed on the resting tension at different degrees of stretch, the contractile response to 1 μM phenylephrine, and the subsequent relaxation to 1 μM acetylcholine. The apparatus used allowed accurate measurement of the distance between lower and upper tissue holders on a vernier scale (Figure 1). The measurements of 'stretch' (length) allow for the thickness of the upper and lower tissue holders (0.5 mm) and indicate the distance between the upper and lower edges of the top and bottom tissue holders respectively (see Figure 1). The tissue holders were separated by an initial distance of 1.5 mm. From this point each tissue was 'stretched' (i.e. its resting length increased) by increasing the separation of the tissue holders in steps of 0.3 mm to a final separation of 2.7 mm. After each alteration of length, the ring segments were allowed 15 min to equilibrate before adminstration of 1 μM phenylephrine which produced 90–95% maximal contraction at all lengths examined. When the contractile response had reached a plateau, 1 μM acetylcholine (a concentration that produced near maximal relaxation at all lengths of unrubbed segments: maximal relaxation is normally obtained at 3 μM acetylcholine) was added to the baths and the relaxation if any was measured. The tissues were then washed by exchanging the bathing Krebs solution three times over a 15 min period before further increasing the degree of stretch of the tissue.

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

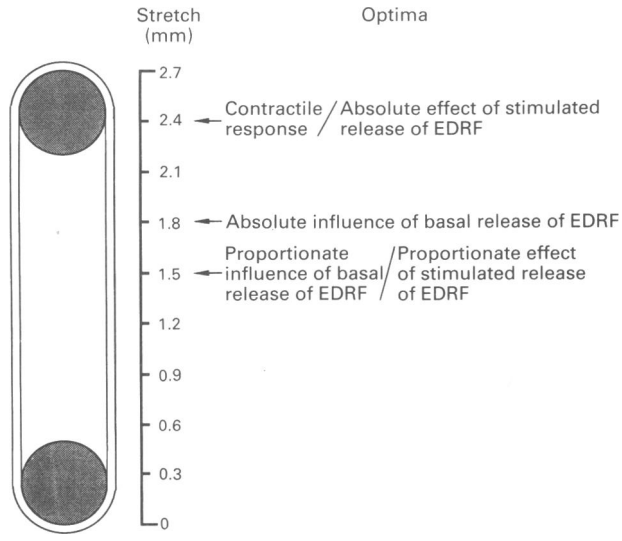

Figure 1 A diagram representing a 'side-on' view of a rat aortic ring segment suspended between an upper and lower wire tissue holder for measurement of isometric tension at different degrees of 'stretch'. From the initial degree of stretch of 1.5 mm, tissues were stretched in 0.3 mm increments to the final degree of stretch of 2.7 mm. The diagram also summarizes the optimum degree of stretch for demonstration of contraction *per se* and the proportionate and absolute influence of both basal and stimulated release of endothelium-derived relaxing factor (EDRF), based on the results shown in the subsequent figures.

Effect of agonist-induced tone

The effect on sensitivity to acetylcholine-induced relaxations of inducing tone with different concentrations of noradrenaline and phenylephrine was examined. Each group of experiments (noradrenaline or phenylephrine) was carried out by different workers, in different laboratories, using different colonies of rats and different protocols. We do not, therefore, make any quantitative comparisons between the two groups of data.

Noradrenaline Aortic ring segments with an intact endothelium were used. Each ring segment was set at the same initial tension of 1.5 g wt. The preparations were washed twice by exchanging the bathing Krebs solution and left for 60 min to equilibrate before a cumulative-concentration response curve to noradrenaline (0.003–10 μM) was obtained. After reaching maximum contraction, the tissues were washed by exchanging the bathing Krebs solution three times over a 15 min period and allowed to equilibrate for a further 30 min. Muscle tone was then raised by adding a fixed amount of noradrenaline. After the contractile response had reached a plateau, acetylcholine was added cumulatively (0.03–10 μM). This was repeated for three different concentrations of noradrenaline (0.03, 0.1, and 0.3 μM) in each ring segment added in a randomised order with three washouts at 5 min intervals followed by 45 min rest before the next noradrenaline addition. Noradrenaline-induced tone was expressed as a percentage of the initial maximum response to noradrenaline. The sensitivity of the preparations to acetylcholine-induced relaxation was expressed as pIC_{50} values. When carrying out relaxation response curves to increasing concentrations of both nitroprusside and isoprenaline only 0.03 μM noradrenaline was used to induce the contraction.

Phenylephrine From each rat, five aortic ring segments with an intact endothelium were used. In this case each ring segment was set at the same initial tension of 1 g wt. The preparations were washed by exchanging the bathing Krebs solution three times at 5 min intervals (re-adjusting the resting tension to 1 g wt. after each wash) and left for 15 min to equilibrate. The presence of a functional endothelium was demonstrated by the presence or absence of relaxation to acetylcholine (3 μM) on top of a contraction to phenylephrine (1 μM). Muscle tone was then raised by adding a single concentration of phenylephrine to each of the preparations (0.1, 0.3, 1, 3, or 10 μM). After the contractile response had reached a plateau, acetylcholine was added cumulatively (0.01–10 μM) to all preparations. The sensitivity of the preparations to the acetylcholine-induced relaxations was expressed as pIC_{50} values. When assessing the antagonism by atropine only 3 μM phenylephrine was used to induce tone. Atropine (10, 30, 100 nM) was added 40 min before the addition of phenylephrine and left in contact for the duration of the acetylcholine exposure (Results in Figure 2b illustrate the effect of 10 nM).

Drugs

Acetylcholine chloride, atropine sulphate, haemoglobin-(bovine), isoprenaline bitartrate, noradrenaline bitartrate, (−)-phenylephrine hydrochloride and sodium nitroprusside were used. All drugs were obtained from Sigma.

Statistical analysis

Unless otherwise stated, values given are the arithmetic mean ± s.e.mean. All statistical analysis was carried out with an Apple MacIntosh microcomputer using the software application Statworks™. When necessary, statistical comparison of group means was made by Student's t test for paired or unpaired data as appropriate. A probability value of $P < 0.05$ was taken as showing significant difference.

Results

In rat aortic ring segments with an intact endothelial cell layer, acetylcholine (30 nM–10 μM) produced a concentration-dependent reduction in phenylephrine- or noradrenaline-induced tone (Figure 2a,b). With the endothelium disrupted, acetylcholine had little effect (Figure 2a) but the contractions to both noradrenaline and phenylephrine were greater than those seen with intact endothelium (Figure 3b phenylephrine only). These responses were competitively antagonized by atropine (pA_2 8.72: 8.43–9.01: 95% confidence limits, slope 1.02) (Figure 2b) and virtually eliminated by haemoglobin (10 μM) (Figure 2c). Removal of the endothelium had little effect on relaxations to nitroprusside (Figure 2d) but considerably attenuated the relaxations to isoprenaline (Figure 2e).

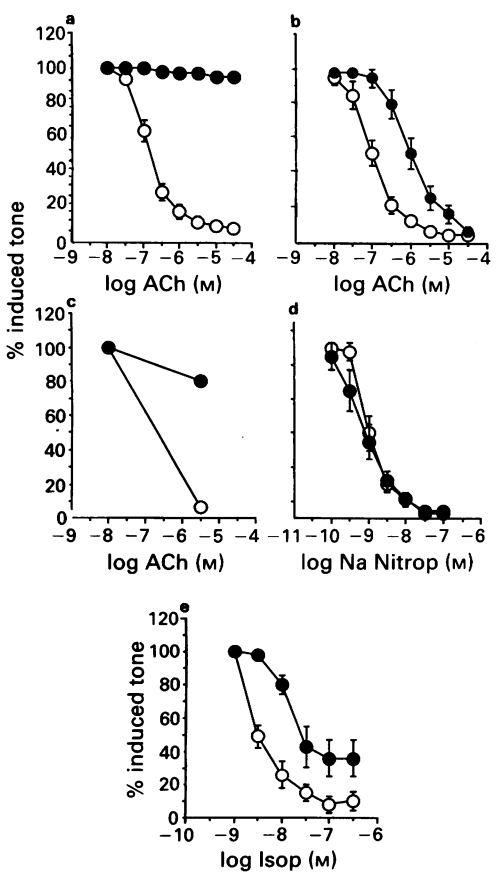

Figure 2 A comparison of the effects of blocking drugs on relaxant responses to acetylcholine (ACh) on rat aortic rings precontracted with 0.1 μM noradrenaline (NA) or for (b) only, phenylephrine 3 μM. Also the effect of several vasodilators on tissues with (○) or without (●) endothelium: (a) acetylcholine with (○), without endothelium (●); (b) acetylcholine, control (○), + atropine 10 μM (●); (c) acetylcholine control (○), + haemoglobin (10^{-5} M) (●); (d) sodium nitroprusside; (e) isoprenaline, both with (○) and without endothelium (●). Points are shown as mean of 6 observations and vertical lines denote the s.e.mean.

Effect of resting length (tension)

With or without an intact endothelium, there was a length-dependent increase in the resting tension of the aortic rings. Disruption of the endothelium had no significant effect ($P > 0.05$) on the resting tension of the preparations compared with paired intact controls at all degrees of 'stretch' examined (Figure 3a).

Phenylephrine (1 μM) produced a well maintained contractile response in both rubbed and unrubbed preparations. The size of this contractile response was dependent on the resting length of the preparation. In both rubbed and unrubbed vessels the contractile response increased with increasing resting length, reaching a maximum at 2.4 mm, after which no further increase in the response to phenylephrine was seen. The response to phenylephrine was significantly greater (unpaired Student's t test; $P < 0.05$) at all resting lengths with a disrupted endothelium than with an intact endothelium (Figure 3b). The 'absolute' difference in contractile response between the rubbed and unrubbed preparation (expressed in g wt.) was greatest at 1.8 mm but similar at the other lengths tested (Figure 3c). The 'absolute' difference at 1.8 mm was significantly greater ($P < 0.05$) than at 2.4 mm, i.e. at the optimum degree of stretch for contraction *per se*. The 'proportionate' difference in contractile response to phenylephrine (expressed as the percentage of the response in the intact, paired vessel), was dependent on the resting length of the preparation at the time of drug addition. This was greatest at the lowest degree of stretch (1.5 mm) declining rapidly until 2.1 mm after which the difference was constant (Figure 3d). The 'proportionate' difference at 1.5 mm was significantly greater ($P < 0.05$) than at 2.4 mm.

In rings with an intact endothelium, pre-contracted with 1 μM phenylephrine, acetylcholine (1 μM) induced a rapid, well-maintained relaxation. The acetylcholine-induced relaxation was dependent on the resting length of the preparation. The

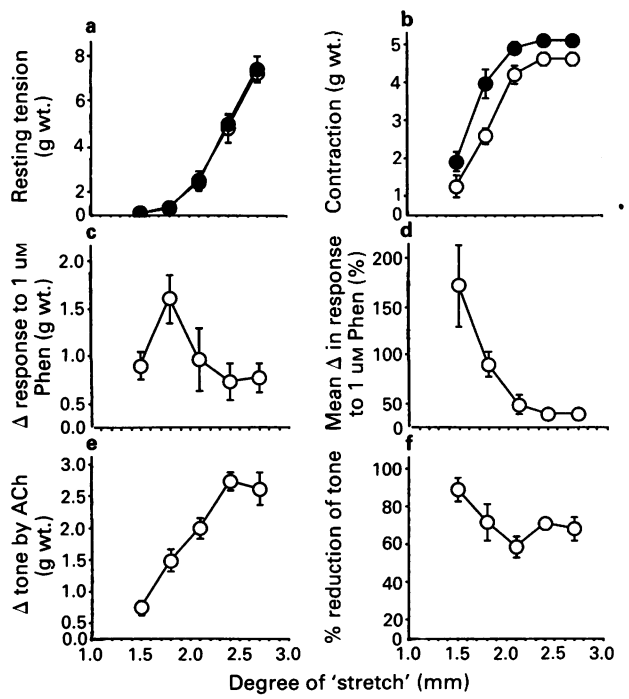

Figure 3 Effect of increasing the degree of 'stretch' and hence length of the rat aorta unrubbed (○) or rubbed (●) with regard to (a) the resting tension of the tissue, (b) contractions to 1 μM phenylephrine (Phen), and (c) absolute difference in the size of contractions to 1 μM Phen. Also (d) % difference in response to 1 μM Phen between rubbed and unrubbed aortae; (e) absolute decrease in Phen (1 μM)-induced tone with 1 μM ACh (in g.wt.) and (f) percentage change in tone to 1 μM ACh in Phen (1 μM)-induced tone. The values shown are the mean of 7 observations and the vertical lines denote the s.e.mean.

absolute relaxation of induced tone (i.e. in terms of g wt.) increased with increasing length reaching an optimal relaxation at 2.4 mm (Figure 3e). The 'proportionate' relaxation (i.e. the relaxation expressed as a percentage of the induced tone) was also length-dependent, being optimal at 1.5 mm and decreasing with increasing length to a minimal response at 2.1 mm. The 'proportionate' relaxation at 1.5 mm was significantly greater ($P < 0.05$) than the response at 2.4 mm (the optimum degree of 'stretch' for contraction *per se* (Figure 3e)). At 2.4 mm and 2.7 mm the 'proportionate' relaxation to acetylcholine was greater but not significantly, than that at 2.1 mm and similar to that seen at 1.8 mm (Figure 3f). This difference, i.e. a mean at 50% or at 80% might be large enough to be of spurious note in, for example, different blood vessels.

In a second set of tissues, length was not monitored but the rings were set up at resting tensions corresponding to those seen at each degree of 'stretch' used in the first set of tissues (i.e. 0.15, 0.3, 1.5, 4 and 6 g) (see Figure 3a). Cumulative concentration-response curves to phenylephrine were obtained at each of the five increasing tensions. Paired time controls were run at a fixed resting tension of 1 g wt. to determine any possible variation with time. This time control showed a small, time-dependent variation in sensitivity to phenylephrine. When account was taken of this, the initial resting tension had no effect on sensitivity to phenylephrine (as indicated by the pD_2 values; $P > 0.05$) in either endothelium-intact or endothelium-denuded preparations. The sensitivity to phenylephrine-induced contractions in the endothelium-denuded preparations was significantly greater than in the endothelium-intact vessels at all resting tensions tested. Although the mean difference in sensitivity between rubbed and unrubbed preparations varied through the experiment, this was not significant and occurred as much in the time controls as with varying tension (Figure 4a).

The sensitivity to acetylcholine-induced relaxations of 1 μM phenylephrine-induced tone in endothelium-intact aortic rings decreased with time in this set of experiments. Taking this into account the resting tension of the tissue had no effect on sensitivity to acetylcholine-induced relaxations (as indicated by the pIC_{50} values, $P < 0.05$) in the presence of an intact endothelium (Figure 4b). However the fall in sensitivity with time indicates the importance of time controls in this type of study.

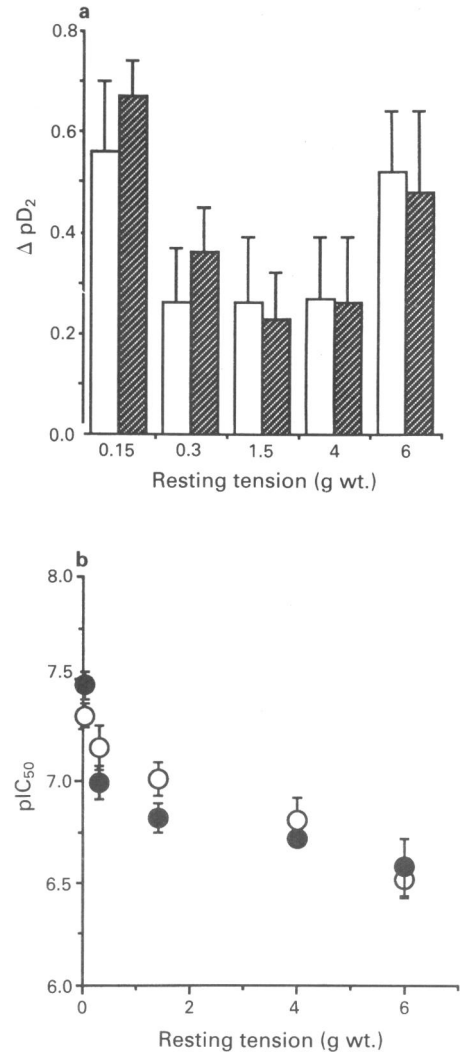

Figure 4 (a) The effect of resting tension on the increase in sensitivity to phenylephrine (Phen)-induced contractions on removal of the vascular endothelium of aortic rings. In (a) hatched columns represent increasing resting tension and open columns concomitant time controls set at a resting tension of 1.0 g wt. (b) The effect of resting tension on sensitivity of aortic rings to acetylcholine (ACh)-induced relaxations of 1 μM Phen-induced tone expressed as pIC_{50}. Data represent pIC_{50} values for time controls (○) and with increasing initial resting tension (●). All values shown are the mean of 6 observations and the vertical lines denote the s.e.mean.

Effect of agonist-induced tone

Noradrenaline In tissues with an intact endothelium, acetylcholine produced a concentration-dependent inhibition (relaxation) of 0.03 μM noradrenaline-induced tone. In this set of experiments the sensitivity of the intact vessels to acetylcholine-induced relaxations showed no significant change with time (Figure 5a). When the tissues were contracted with various concentrations of noradrenaline (0.01–0.3 μM) to produce different degrees of tone (35–145% of initial maximum contraction to noradrenaline, the maximum contraction to noradrenaline commonly increases in the course of such experiments without a significant change in sensitivity), the relaxations to acetylcholine varied depending on the degree of induced tone (Figure 5b). When the concentration of noradrenaline used was low (0.03 μM), the sensitivity of the tissue was high ($pIC_{50} = 7.36 \pm 0.07$). When the degree of induced tone was increased by increasing the concentration of noradrenaline used to induce that tone (0.1 and 0.3 μM), the tissues were less sensitive to acetylcholine ($pIC_{50} = 6.89 \pm 0.06$ and 6.57 ± 0.07, respectively) since a greater concentration was required to elicit a similar percentage relaxation (Figure 5c). The sensitivity of the tissues for acetylcholine-induced relaxations of 0.03 and 0.1 μM noradrenaline-induced tone was significantly greater (Student's paired t test; $P < 0.05$) than that for acetylcholine-induced relaxations of 0.3 μM noradrenaline-induced tone.

Phenylephrine In this set of experiments the protocol eliminated the variation in sensitivity of the intact vessels to acetylcholine-induced relaxations with time by testing each tissue only once. In tissues with an intact endothelium, acetylcholine produced a concentration-dependent inhibition (relaxation) of phenylephrine-induced tone. When the tissues were precontracted with various concentrations of phenylephrine (0.1, 0.3, 1, 3, or 10 μM) to produce different degrees of tone, the relaxations to acetylcholine varied with the degree of induced tone (Figure 6a,b). When the concentration of phenylephrine used was low (0.1 μM), the sensitivity of the tissue to acetylcholine was high ($pIC_{50} = 7.27 \pm 0.04$). When the degree of phenylephrine-induced tone was increased by increasing the concentration of phenylephrine used to induce that tone (0.3, 1, 3 and 10 μM), the tissues were less sensitive to acetylcholine ($pIC_{50} = 7.09 \pm 0.08$, 6.76 ± 0.08, 6.83 ± 0.14 and 6.80 ± 0.04 respectively) since a greater concentration was required to elicit a similar percentage relaxation (Figure 6c). The sensitivity of the tissues to acetylcholine-induced relaxation of 1 μM and 3 μM phenylephrine-induced tone was not significantly different from that for relaxation of tone induced by 10 μM phenylephrine. However, the sensitivity to acetylcholine-induced relaxation of 0.1 and 0.3 μM phenylephrine-induced tone was significantly greater than that

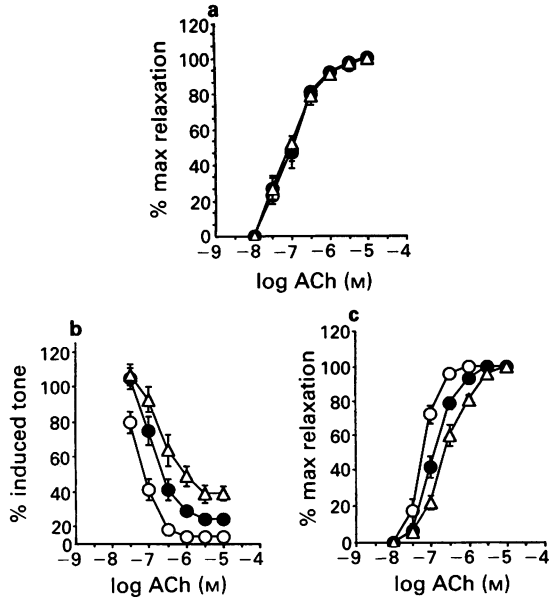

Figure 5 (a) The effect of time on relaxations of intact rat aortic rings to successive cumulative concentration-response curves (CCRC) for acetylcholine (ACh). Tone was induced by addition of 0.3 μM noradrenaline. Initial tension on all preparations was 1.5 g wt. Data represent first (○), second (●), and third (△) successive CCRC for acetylcholine in terms of percentage maximum response. (b and c) The effect of increasing the degree of noradrenaline-induced tone on relaxation to acetylcholine expressed as (b) % of the induced tone and (c) as % of the maximum relaxation for each curve. Significant differences were seen between relaxant responses in the presence of 0.3 μM (△) noradrenaline and 0.1 (○) or 0.03 μM (●) noradrenaline. The values shown are the mean of 6 observations and the vertical lines denote the s.e.mean.

for relaxation of 10 μM phenylephrine-induced tone (Student's paired t test; $P < 0.05$).

Discussion

These results show two clear positive effects. First, the relaxant effect of agonist-induced release of EDRF is markedly reduced by increasing the strength of the active contractile stimulus that opposes it, particularly where this involves increasing the concentration of excitatory agonist: where the contraction is increased by moving to a more favourable point on the smooth muscle length-tension curve, but keeping the excitatory stimulus constant, the relaxant effect of agonist-induced EDRF is relatively constant. Thus there can be an effective physiological antagonism between EDRF-releasing factors and direct vasoconstrictor factors. This shows that a quantitative relationship can be provided for this phenomenon previously described qualitatively (see Furchgott, 1983).

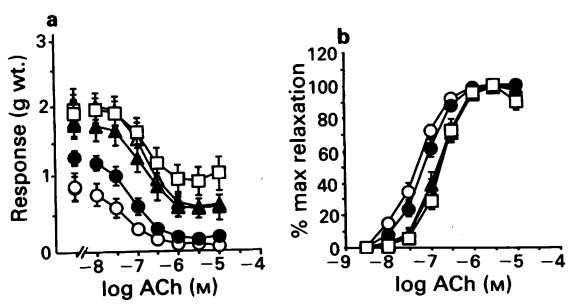

Figure 6 Effect of increasing the degree of phenylephrine-induced tone on acetylcholine (ACh)-induced relaxations of intact aortic rings. Initial tension on all preparations was 1 g wt. Relaxations are expressed as (a) absolute effect (g wt.) and (b) as % reduction of maximum relaxation for each curve. The concentrations of phenylephrine used to induce tone were 0.1 μM (○), 0.3 μM (●), 1 μM (▲), 3 μM (△) and 10 μM (○). The values shown are the mean of 6 observations and the vertical lines denote the s.e.mean. The extreme left-hand points in (a) represent the initial pre-acetylcholine tension.

Secondly, the effect of spontaneous basal release of EDRF on the size of contraction to a given excitatory stimulus is markedly altered by the starting point on the length tension curve. It could be argued that this second effect is the more important physiologically: the on-going interaction between locally determined continuous release of EDRF and varying levels of other physiological contractile and relaxant factors (blood borne or neural) will determine vascular tone. In the case of the non-innervated rat aorta, presumably the interplay of blood-borne catecholamines and EDRF is a major determinant of vascular compliance. If the present results are any guide, this phenomenon should help ensure that compliance decreases with increasing arterial blood pressure. By analogy, in resistance vessels increased pressure should favour vasoconstriction and assist autoregulation of blood flow. These results will in fact underestimate the role of the endothelium in restricting the vasoconstrictor influence of catecholamines *in vivo* since β-adrenoceptors will be stimulated by adrenaline. Noradrenaline produces marked β-adrenoceptor-mediated vasodilatation in this preparation but only at concentrations $\geqslant 0.3\,\mu$M (data not shown). Figure 2e shows how powerfully the β-adrenoceptor-mediated vasodilator action can be potentiated by the presence of the endothelium.

Several observations validate the technical methods used. Functional disruption of the endothelium of the aortic ring segments was clearly effective since vessels in which the vascular endothelium had been disrupted showed no relaxation to acetylcholine. In addition, the responses to contractile agents were greater in preparations with a disrupted endothelium presumably indicating the removal of a basal release of EDRF with little damage to the underlying smooth muscle layer. The absolute sizes of responses to phenylephrine increased with increasing stretch and reached a maximum at the same degree of stretch in both rubbed and unrubbed preparations. Therefore the presence of a functional endothelium (and hence of the basal release of EDRF) does not affect the optimal conditions (in terms of initial tension) for demonstration of the contractile effects of this α_1-adrenoceptor agonist. Thus, when paired rubbed and unrubbed preparations of rat aorta are being compared, optimal length/tension conditions for contractile stimuli are similar for each, eliminating this particular variable.

Our results are all consistent with the hypothesis that the basal release of EDRF modulates the response of vascular smooth muscle to contractile agents (Martin *et al.*, 1986; Bullock *et al.*, 1986). We found, however, that over a wide range of lengths, removal of the endothelium had no significant effect on the resting tension of the preparations. This is not surprising since the failure of sodium nitroprusside (10 μM) to relax the preparations under resting conditions (data not shown) indicates that they have no resting tone under the conditions used in this study. However, this does indicate that in experiments where paired ring segments with and without endothelium are set at the same resting tension, the degree of stretch (and hence length) in each tissue is similar. This therefore, will not play a significant part in the difference seen in the responses to contractile agents in paired rubbed/unrubbed preparations of rat aorta. This may not remain true in other vascular smooth muscle preparations which have intrinsic tone or myogenic activity.

A corollary of the lack of influence of the endothelium on resting tone is that pharmacological blockade of EDRF, like mechanical disruption, should not increase vessel tone *per se*. Thus contractions to haemoglobin (Martin *et al.*, 1986) cannot be due solely to the loss of the effect of EDRF and must be at least partly attributable to another action of the haemoglobin leading to activation of the quiescent muscle. This may lead to errors in interpretation of the effects of haemoglobin since it has recently been shown in the dog portal vein (Furuta *et al.*, 1988) and the whole perfused rat tail (Templeton *et al.*, 1989) that activation of the smooth muscle uncovers a previously

'unseen' population of functional α_2-adrenoceptors. Activation of these receptors might contribute to the increase in contractility of the preparations which might be attributed wholly to the blockade of the basal release of EDRF by haemoglobin.

The perceived potentiation of contractile responses by endothelial removal clearly depended on the position of the tissue on the length/tension curve. Thus, while the passive length/tension relationship was unaffected by the presence of the endothelium, the active length/tension relationship was modified in a way that showed a greater proportionate effect at low degrees of stretch. Thus, the optimum position for demonstration of the endothelial influence varied with the manner in which the increase was expressed. The optimum length for demonstration of the 'absolute' difference in contractility to phenylephrine (i.e. in terms of grams) was 2.4 mm whilst the optimum length for demonstration of the 'proportionate' difference in contractility to phenylephrine (i.e. as a percentage increase from the response seen in the intact vessel) was 1.5 mm. Furthermore, irrespective of the method used to express the increased contractility seen in the absence of the endothelium, the optimum length for demonstration of this phenomenon does not coincide with the optimum stretch for demonstration of the contractile response *per se* (summarised in Figure 1). Clearly, the higher the initial resting tone, and hence stretch, the less will be the apparent effect of the endothelium on the phenylephrine-induced contractions, particularly when the increase in response in the absence of a basal release of EDRF is expressed as a proportion of the response in the intact vessel. This may account, at least in part, for conflicting reports of the effect of the vascular endothelium on the contractility of vascular smooth muscle. The cellular basis of this is not clear but it could be due to recruitment, by stretch, of cells resistant to EDRF, e.g. cells near the adventitia where the effects of EDRF are less apparent, due to a greater diffusion distance between the endothelium and the contracting smooth muscle cells, or it may be due to stretching causing an increased efficiency of excitation-contraction coupling, thereby increasing the degree of activation which the EDRF must overcome.

Demonstration of the relaxation produced by 1 μM acetylcholine in the intact vessel was dependent on the initial length of the preparation, although this was not so striking, quantitatively, as the equivalent effect of basal release on agonist-induced contraction. As for the effect on contraction to phenylephrine, the optimal conditions for demonstration of the 'absolute' and 'proportionate' relaxation (i.e. percentage relaxation of induced tone) to acetylcholine did not coincide. The optimum stretch for demonstration of the 'proportionate' effect of acetylcholine (1.5 mm) did not coincide with the optimum for phenylephrine-induced contractions. However, the optimum stretch for demonstration of the 'absolute' relaxation (2.4 mm) did coincide with the optimum for phenylephrine-induced contractions. This is presumably because at this length there is a greater absolute amount of tone that can be inhibited. However, it is common practice to express acetylcholine-induced relaxations as a percentage of the induced tone and this is not optimised by the use of conditions optimal for contraction. This has several implications. When the responsiveness of different vessels to EDRF is examined either from different anatomical sites, from different species or from different patho-physiological states, the resting tensions (hence length) at which the vessels are set is usually that at which the response to the contractile agent is at a maximum (Collins *et al.*, 1986; Christie & Lewis, 1987) or is entirely arbitrary, based on evolved practice. In order to determine, for each preparation, the optimum point on the resting wall tension/internal circumference curve, a normalization procedure to find the point $L_1 = 0.9\ L_{100}$ as described more fully by Mulvany & Halpern (1976) can be carried out. By use of the values obtained from our experiments (and also unpublished observations from S.J. MacLennan) we have calculated the tensions required to produce maximum activation and those likely to be present under physiological pressures in the aorta i.e. 120/80 mmHg. The results are approximations since the exact length of vessel segments was not known, but the assumption was made that they were 2.5 mm in length which is the average we aimed for. The values obtained for L_1 correspond to 56 ± 3 mmHg or 2.9 ± 0.28 g ($n = 6$). The derived values for force under physiological pressures (120/80 mmHg) were $6.05 \pm 0.2/4.12 \pm 0.21$ g ($n = 6$). This indicates that the range of tensions employed in our study encompassed the tension for maximum activation and the physiological range. However, since these resting lengths may not be ideal for acetylcholine-induced relaxations, it may be unrealistic to compare differences in responsiveness to acetylcholine-induced release of EDRF without evaluating the influence of the initial stretch or induced tone on relaxations. For example, the tensions of considerably less than 3 g which are commonly employed with this preparation will show up relatively high sensitivity to EDRF but will correspond to unphysiologically low pressures.

Changes in the distension of small arteries of the rat cause changes in their sensitivity to contractile agents (Nilsson & Sjöblom, 1985). In the present experiments using phenylephrine however, this is not so since the sensitivity of neither endothelium-intact nor disrupted vessels varied with resting tension or with time. Moreover, the effect of basal release of EDRF, when expressed in terms of an increase in sensitivity (pD_2) to phenylephrine in the rubbed preparation did not change significantly with time or increasing resting tension. Whilst the sensitivity of endothelium-intact preparations to acetylcholine-induced relaxations did decrease slightly with time, there was no evidence for an influence of resting tension. It is unlikely that the decrease in sensitivity with time contributed a great deal to the differences in response to 1 μM acetylcholine at each degree of stretch examined since the degree of relaxation to this near maximal concentration of acetylcholine seen in the time controls varied by only a small amount.

The effect of vasoconstrictor agonist concentration and hence 'induced-tone' on sensitivity to acetylcholine was marked. Increasing tone (to increasing concentrations of noradrenaline) decreased sensitivity to acetylcholine-induced relaxations (expressed as pIC_{50}). This suggests that with respect to induced tone the degree of 'activation' by a particular concentration of agonist is important in the determination of sensitivity to acetylcholine-induced relaxations. This suggestion is supported by the observation that whilst the size of contraction to 1 μM phenylephrine increases with increasing resting tension, there is no concomitant decrease in sensitivity to acetylcholine-induced relaxations (see below). Therefore the degree of 'activation' of the tissue remains the same, i.e. if the same concentration of phenylephrine is used to induce tone (assuming no change in sensitivity) despite an increase in 'responsiveness' (absolute size of contraction) there is no change in sensitivity to the stimulated release of EDRF.

We have used both noradrenaline and phenylephrine as activators in this study, sometimes examining one or more thoroughly than the other but have not reported all of the repeated data. However in order to exclude the specific possibility that increasing sensitivity to acetylcholine with decreasing tone was due to the use of noradrenaline, similar experiments were carried out with phenylephrine: it is not relevant that this drug is more selective than is noradrenaline for α_1-adrenoceptor agonists, since in this tissue there is no evidence for α_2-adrenoceptor-mediated responses. Since experiments on the effect of resting tension on acetylcholine sensitivity had revealed a small variation in sensitivity with time, the protocol employed time-controls. These experiments confirmed the decrease in sensitivity to acetylcholine with increasing induced tone but this time a limit was found to the decrease in acetylcholine sensitivity seen with increasing activation of the tissue by phenylephrine (no further decrease when tone was induced by > 1 μM phenylephrine).

In conclusion, differences seen in the quantitative demonstration of both the basal and stimulated release of EDRF can be produced by varying the experimental protocol. The

resting tension of the tissue (hence length) can influence, in particular, the effects of spontaneous basal release of EDRF and, to a lesser degree, of the induced release of EDRF. Even within the same tissue the optimum conditions for demonstration of these different relaxant effects or contractile effects do not necessarily coincide and are dependent upon how the data are expressed. In addition, the degree of induced tone can influence the perceived effects of stimulated release of EDRF. The apparent sensitivity of the tissue is altered when different levels of induced tone are employed and this may obscure any real changes in sensitivity to agents releasing EDRF. This apparent change in sensitivity may be due, at least in part, to the relaxation to agonist-induced release of EDRF appearing to reach a maximum at a concentration of agonist lower than that needed to produce a maximal release of EDRF *per se* i.e. the apparent receptor reserve is increased. This may occur even if the maximum relaxation induced is less than 100% of the induced tone since there is likely to be a component of the induced tone that is resistant to endothelium-dependent relaxation. These factors may account for the variations in interpretation of the role of EDRF between different vessels, pretreatments and laboratories. The conditions used to compare vessels from different species, or in different pathophysiological states, should be chosen to ensure optimum demonstration of the effects of basal, and stimulated, release of EDRF. There is no simple prescription for this. The protocol may need to vary in order to optimize the particular phenomenon under analysis. Alternatively, and perhaps more satisfactorily, the vessels to be compared may be suspended under conditions where the transmural pressure in each vessel is the same or is at a physiological level for each tissue being examined (Angus *et al.*, 1986; Mulvany & Halpern, 1976). With respect to the level of induced tone used to examine the effects of stimulated release of EDRF, it seems advisable to use a concentration of agonist which produces a sufficient level of tone such that there would be no decrease in the sensitivity to agonist-induced release of EDRF if a higher concentration of contractile agent were used. This would ensure that the apparent sensitivity to EDRF release is not affected by a component of the induced tone resistant to released EDRF. Finally, the results suggest that both spontaneous release of EDRF and induced release of EDRF are more effective against vasopressor tone at low degrees of passive stretch: a corollary is that in intact vessels this should also apply at lower intraluminal blood pressures.

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