Br. J. Pharmacol. (1992), **107**, 684–690

Novel signal transduction pathway mediating endothelium-dependent β-adrenoceptor vasorelaxation in rat thoracic aorta

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1 Isoprenaline (3×10^{-8}–10^{-5} M), salbutamol (3×10^{-7}–10^{-4} M) and forskolin (3×10^{-9}–3×10^{-7} M) relaxed rat isolated thoracic aortic rings contracted with noradrenaline (10^{-7} M). Removal of the endothelium from the aortic rings abolished the effect of acetylcholine (10^{-6} M) and completely prevented the vascular relaxation induced by isoprenaline, salbutamol or forskolin.

2 The isoprenaline concentration-relaxation curve was shifted in parallel to the right about 10 fold by propranolol (3×10^{-7} M) with no change in the maximum response, showing that the relaxation was mediated by a β-adrenoceptor.

3 The inhibitor of nitric oxide synthesis, N^G-nitro-L-arginine (L-NOARG; 10^{-5} M), shifted the isoprenaline relaxation curve to the right and reduced the maximum response.

4 Isoprenaline (10^{-6} M) relaxed noradrenaline-induced tone by approximately 95% and at the same time increased levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) 4 fold and guanosine 3':5'-cyclic monophosphate (cyclic GMP) 12 fold in the aortic rings. Sodium nitroprusside (3×10^{-8} M) relaxed noradrenaline-evoked tone by 82% without changing levels of cyclic AMP but raised cyclic GMP 19 fold.

5 Forskolin (10^{-7} M) relaxed noradrenaline-induced tone by approximately 41% and, like isoprenaline, increased levels of cyclic AMP (2.5 fold) and cyclic GMP (12 fold) in the aortic rings.

6 Removal of the endothelium abolished the relaxant effects of isoprenaline (10^{-6} M) and also the associated accumulation of cyclic AMP and cyclic GMP.

7 L-NOARG (10^{-5} M) inhibited the relaxant responses and accumulation of cyclic GMP induced by isoprenaline (10^{-6} M) and forskolin (10^{-7} M) without affecting the associated cyclic AMP accumulation.

8 It is concluded that, in the rat aorta, isoprenaline acts through a β-adrenoceptor on the endothelium to raise cyclic AMP and that this may, directly or indirectly, release nitric oxide to evoke vascular relaxation via the increase in cyclic GMP. The importance of this novel transduction pathway for cardiovascular regulation remains to be determined.

Keywords: Isoprenaline; forskolin; cyclic GMP; cyclic AMP; nitric oxide; endothelium-dependent vascular relaxation; N^G-nitro-L-arginine (L-NOARG); β-adrenoceptor

Introduction

The role of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in vascular relaxation is currently considered to be confined to activation of protein kinase A and subsequent myosin light chain kinase activation within the smooth muscle. Thus, for example, isoprenaline and other β-adrenoceptor agonists are thought to mediate their vascular relaxant effects by activation of adenylate cyclase within the smooth muscle (Kukovetz *et al.*, 1981). Such a mechanism of vascular relaxation is not dependent on the presence of an intact endothelium and would not involve nitric oxide as an endothelium-derived relaxant factor.

However, in the rat aorta the relaxations to isoprenaline are inhibited by methylene blue and haemoglobin (Grace *et al.*, 1988), agents known to affect the action of nitric oxide (Martin *et al.*, 1985). Therefore, it is possible that in this tissue there is a nitric oxide component to the relaxant response to isoprenaline. This could be mediated by endothelial β-adrenoceptors which are known to be present on endothelial cells (Stephenson & Summers, 1987; Molenaar *et al.*, 1988).

In this study we investigated the role of the endothelium and the signal transduction pathway mediating β-adrenoceptor-induced vasorelaxation in the rat thoracic aorta.

A preliminary account of these findings has been presented to the British Pharmacological Society (Gray & Marshall, 1991).

Methods

Male Sprague-Dawley rats (350–450 g) were stunned and killed by cervical dislocation. The thoracic aorta was removed, cleared of fat and connective tissue, and cut into rings of approximately 3 mm length. The endothelium was removed in some experiments by gently abrading the intimal surface of the aortic rings with fine wires. The rings were suspended on tungsten wires (diameter 0.125 mm) under 0.5 g resting tension and allowed to equilibrate for 75 min in Krebs solution containing (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 11 at 37°C and oxygenated with 95% O_2/5% CO_2. Tension was recorded with Grass FT.03 isometric transducers connected to a Grass polygraph.

Aortic rings were sub-maximally contracted with noradrenaline (10^{-7} M) and the contraction assessed for stability over a period of 15 min. Then the tissues were contracted and checked for the presence of endothelium by confirming at least 80% relaxation to acetylcholine (10^{-6} M). Tissues showing less than this level of relaxation were discarded as having partially damaged endothelium. The presence of endothelium was confirmed histologically in some tissues by

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en face silver staining (Griffith *et al.*, 1984). This technique was also used to confirm the loss of endothelium from rings which had been rubbed and which showed no relaxation by acetylcholine (10^{-6} M) of noradrenaline (10^{-7} M)–evoked tone. Some tissues were equilibrated in either propranolol or N^G-nitro-L-arginine (L-NOARG, which inhibits nitric oxide production; Ishii *et al.*, 1990; Moore *et al.*, 1990) for 15 min, control tissues receiving no treatment in this period. The aortic rings were contracted again before a cumulative concentration-effect curve to isoprenaline or salbutamol or forskolin was constructed.

For the cyclic nucleotide studies, aortic rings were prepared as described above with either the endothelium remaining intact or being removed. The state of the endothelium in all tissues was checked by use of acetylcholine and, sometimes, additionally by silver staining (see above). A single concentration of isoprenaline (10^{-6} M), or forskolin (10^{-6} M) or sodium nitroprusside (3×10^{-8} M) was added, the tissues then being removed into liquid nitrogen at various time-points for cyclic nucleotide determination. Some rings were incubated for 15 min with L-NOARG (10^{-5} M) before being contracted with noradrenaline and relaxed with a single concentration of either isoprenaline or forskolin. These tissues were removed into liquid nitrogen to determine the effect of L-NOARG on cyclic nucleotide accumulation.

Cyclic nucleotide determination

Frozen tissues were individually ground in 95% ethanol (pH 3.0) in a mortar and pestle and left overnight for extraction of the cyclic nucleotides. The samples were centrifuged to pellet the residual tissue fragments. The supernatant was decanted and evaporated to dryness under nitrogen. The sample was then resuspended in sodium acetate (50 mM at pH 5.0) and split into two aliquots for simultaneous measurement of both cyclic AMP and guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels by scintillation proximity assay (Amersham) using the acetylation protocol.

The tissue residue was dissolved in sodium hydroxide solution (0.5 M) and the protein content determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Chemicals

N^G-nitro-L-arginine (Sigma) was prepared in 1.0 M hydrochloric acid before being neutralized to pH 7.0 and diluted to form a 10^{-3} M stock solution. Forskolin (Sigma) was prepared as a 10^{-2} M stock in 70% ethanol. Salbutamol was obtained from Glaxo and prepared daily in Krebs solution. All other chemicals were obtained from Sigma; noradrenaline bitartrate, acetylcholine chloride, isoprenaline hemisulphate, (±)-propranolol hydrochloride and sodium nitroprusside and prepared daily in Krebs solution.

Statistics

Results are expressed as mean ± s.e.mean. Analysis of variance and Students unpaired *t* test were used where appropriate to assess the significance of differences between means and $P \leqslant 0.05$ was taken as statistically significant.

Results

Noradrenaline (10^{-7} M) evoked an increase in tone of the rat aortic rings with intact endothelium to 1.2 ± 0.1 g ($n = 4$). In endothelium-denuded rings the contractor response to the same concentration of noradrenaline was significantly increased (2.0 ± 0.2 g, $n = 4$). Intact rings relaxed to acetylcholine (10^{-6} M) by greater than 80%, while endothelium-denuded rings displayed no relaxant (or constrictor) response.

Endothelium-dependent relaxation

Isoprenaline (3×10^{-8}–10^{-5} M) concentration-dependent relaxation was only seen in rings with an intact endothelium (Figure 1) and began within 15 s, the effect of a given concentration reaching a maximum within about 180 s after administration. Salbutamol (3×10^{-7}–10^{-4} M) and forskolin (3×10^{-9}–3×10^{-7} M) also elicited concentration-dependent relaxations only in preparations where an intact endothelium was present (Figures 2 and 3). The pD_2 values (the negative log of the molar concentration of the drug giving 50% of the maximal relaxation for that drug) were 6.8 ± 0.1, 5.3 ± 0.3 and 7.6 ± 0.3 for isoprenaline, salbutamol and forskolin, respectively. All the vasodilators gave maximum relaxations of approximately 100% of the contraction induced by noradrenaline (10^{-7} M).

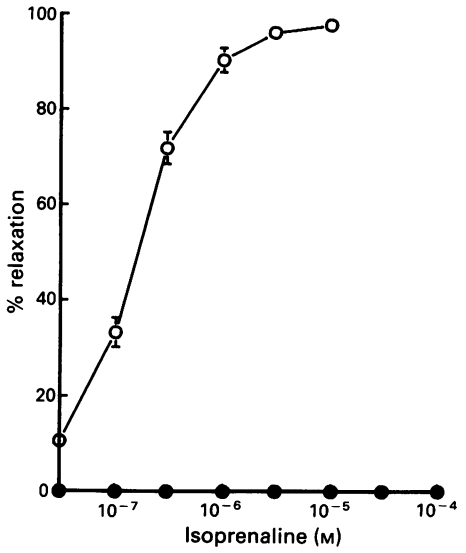

Figure 1 The effect of removal of endothelium on vasorelaxation induced by isoprenaline in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the intact rings and (●) represents rings from which the endothelium has been removed. Points represent mean (± s.e.mean, vertical bars) of 4 separate experiments.

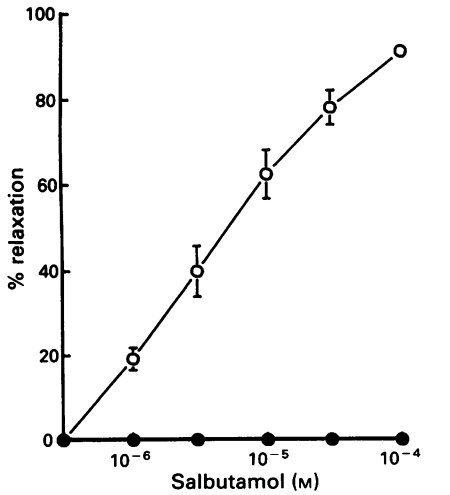

Figure 2 The effect of removal of endothelium on vasorelaxation induced by salbutamol in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the intact rings and (●) represents rings from which the endothelium has been removed. Points represent mean (± s.e.mean, vertical bars) of 4 separate experiments.

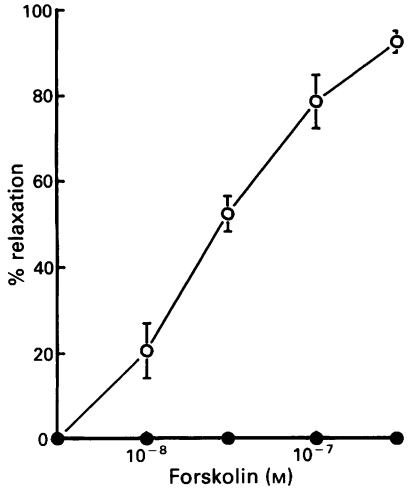

Figure 3 The effect of removal of endothelium on vasorelaxation induced by forskolin in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the intact rings and (●) represents rings from which the endothelium has been removed. Points represent mean (± s.e.mean, vertical bars) of 4 separate experiments.

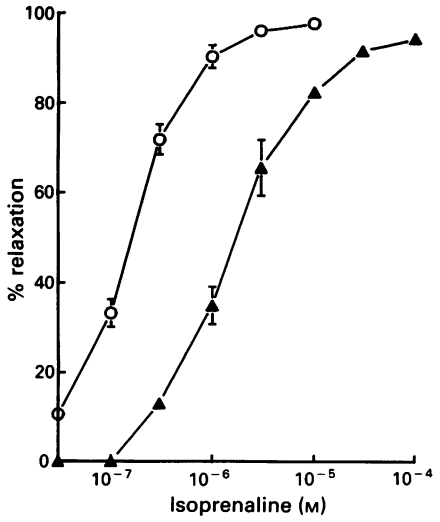

Figure 4 The effect of propranolol (3×10^{-7} M) on isoprenaline-induced vasorelaxation in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the control and (▲) represents the propranolol pretreated values. Points represent the mean (± s.e.mean, vertical bars) of 4 separate experiments.

Endothelium-independent relaxation

The nitrovasodilator, sodium nitroprusside (3×10^{-9}–10^{-6} M), relaxed rat aortic rings independently of the presence of endothelium (the pD_2 being 7.1 ± 0.1 and 7.1 ± 0.1 in the presence and absence of the endothelium respectively) with the maximum relaxation being 100% of the noradrenaline (10^{-7} M)-induced contraction.

Effect of propranolol on isoprenaline endothelium-dependent relaxations

Propranolol (3×10^{-7} M) had no significant effect on the noradrenaline (10^{-7} M)-induced contraction. The concentration-effect curve to isoprenaline was shifted by propranolol (3×10^{-7} M) to the right in a parallel fashion (Figure 4). Preliminary experiments with higher concentrations of propranolol gave larger shifts (propranolol, 3×10^{-6} M, giving approximately a 100 fold rightward parallel shift with no decrease in the maximum response), indicating that propranolol is a competitive antagonist at this site (calculated K_B value 3.2×10^{-8} M). The degree of shift is consistent with the known mechanisms of action of both isoprenaline and propranolol, indicating that this endothelium-dependent relaxant effect of isoprenaline is being mediated via a β-adrenoceptor.

Effect of L-NOARG on isoprenaline-induced endothelium-dependent relaxation

The nitric oxide synthase inhibitor, L-NOARG (10^{-5} M), significantly augmented the tone induced by noradrenaline (10^{-7} M) to 1.9 ± 0.2 g ($n = 4$) in endothelium-intact rings of rat aorta. L-NOARG (10^{-5} M) shifted the relaxant response to isoprenaline to the right while decreasing the maximum response from 98% to 48% of the tone induced by noradrenaline (10^{-7} M) (Figure 5) but did not affect the relaxations induced by the endothelium-independent vasodilator, sodium nitroprusside (Gray & Marshall, 1992a).

Time course for cyclic nucleotide accumulation

Cyclic AMP and cyclic GMP control levels in rat thoracic aortic rings with intact endothelium constricted with noradrenaline (10^{-7} M) were 760 ± 114 fmol mg^{-1} protein and 52 ± 9 fmol mg^{-1} protein, respectively. Removal of the endothelium did not significantly alter the levels of cyclic AMP (941 ± 122 fmol mg^{-1} protein), but significantly reduced the levels of cyclic GMP (22 ± 4 fmol mg^{-1} protein).

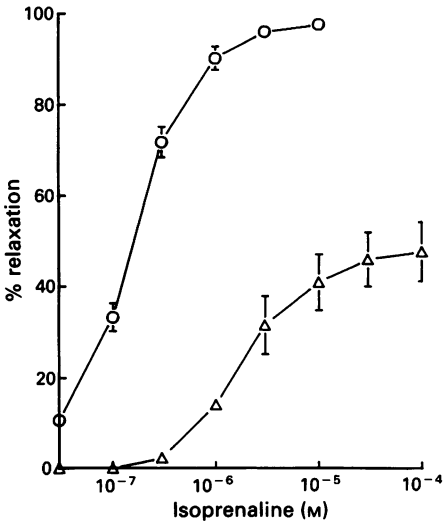

Figure 5 The effect of N^G-nitro-L-arginine (L-NOARG, 10^{-5} M) on relaxation induced by isoprenaline in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the control and (▲) represents the L-NOARG pretreated values. Points represent the mean (± s.e.mean, vertical bars) of 4 separate experiments.

The nitrovasodilator, sodium nitroprusside (3×10^{-8} M), which acts within the smooth muscle to increase guanylate cyclase activity directly, caused a maximum relaxation of 82 ± 2% which developed over 180 s. While this vasorelaxation was developing there was no significant increase in levels of cyclic AMP but, as expected, cyclic GMP levels were elevated (19 fold, to 998 ± 209 fmol mg^{-1} protein; Figure 6).

Isoprenaline (10^{-6} M) caused 95 ± 5% relaxation at 60 s in rings with intact endothelium. Cyclic GMP levels were elevated 12 fold (621 ± 105 fmol mg^{-1} protein) above basal levels with almost a 4 fold rise in cyclic AMP (2751 ± 151 fmol mg^{-1} protein) at the optimum time point for cyclic nucleotide accumulations of 30 s (Figure 7).

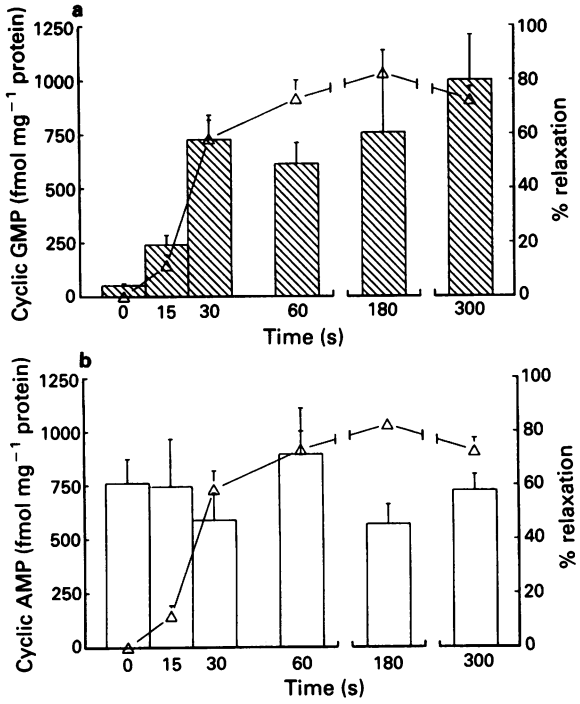

Figure 6 The effect of sodium nitroprusside (3×10^{-8} M) on cyclic GMP and cyclic AMP levels and on vascular tone induced by noradrenaline (10^{-7} M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic GMP (a) and cyclic AMP (b) levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (△) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7} M) in the same tissues. Columns and triangles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

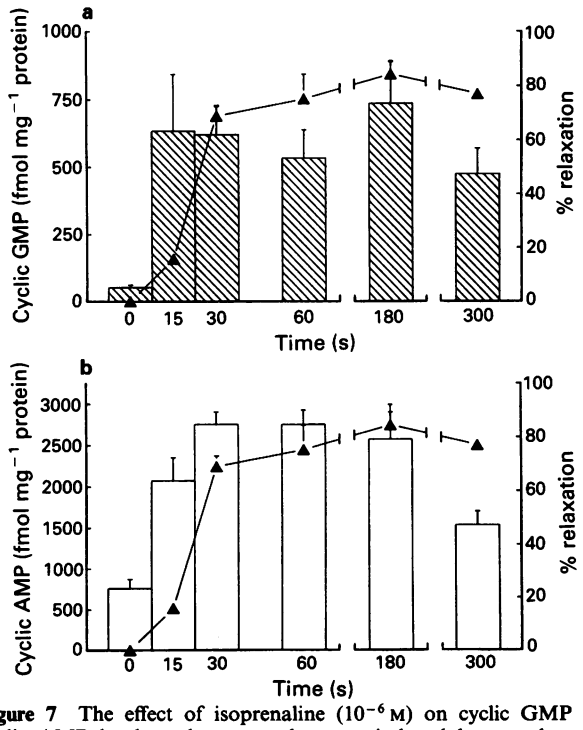

Figure 7 The effect of isoprenaline (10^{-6} M) on cyclic GMP and cyclic AMP levels and on vascular tone induced by noradrenaline (10^{-7} M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic GMP (a) and cyclic AMP (b) levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (▲) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7} M) in the same tissues. Columns and triangles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

Effect of endothelium removal on cyclic nucleotide accumulation induced by isoprenaline

In rings denuded of endothelium, isoprenaline (10^{-6} M) evoked no relaxant response in noradrenaline (10^{-7} M)-constricted rings. Further, it elicited no alterations in levels of either cyclic AMP (control, 941 ± 122 fmol mg^{-1} protein; with isoprenaline, 924 ± 78 fmol mg^{-1} protein) or cyclic GMP (control, 22 ± 4 fmol mg^{-1} protein; with isoprenaline, 19 ± 6 fmol mg^{-1} protein; Figure 8).

Effect of L-NOARG on relaxation and second messenger accumulation induced by isoprenaline and forskolin

After 60 s exposure, isoprenaline (10^{-6} M) caused a relaxant response of 69 ± 4% ($n = 10$). Preincubation with L-NOARG (10^{-5} M) significantly reduced this response to 13 ± 6% ($n = 7$). Levels of cyclic GMP were also reduced from 534 ± 105 fmol mg^{-1} protein where isoprenaline (10^{-6} M) alone was present to 38 ± 15 fmol mg^{-1} protein where L-NOARG (10^{-5} M) was also present (Figure 9). Levels of

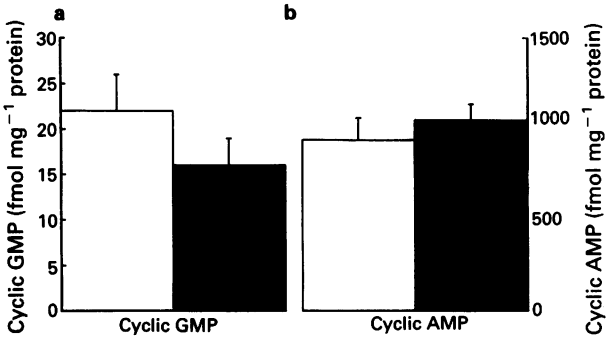

Figure 8 The effect of isoprenaline on cyclic GMP and cyclic AMP levels in rat thoracic aortic rings denuded of endothelium and preconstricted with noradrenaline (10^{-7} M). Open columns represent basal levels of cyclic nucleotides and solid columns represent the levels of cyclic nucleotides after 60 s exposure to isoprenaline (10^{-6} M). Levels of cyclic GMP (a), and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

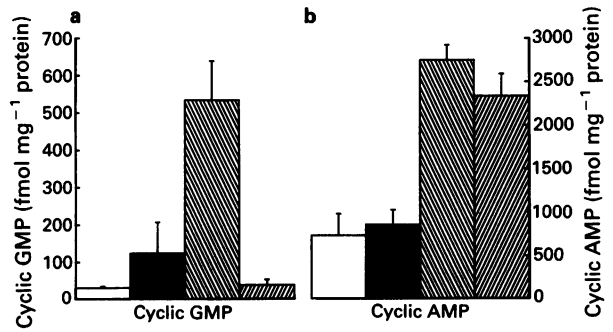

Figure 9 The effect of N^G-nitro-L-arginine (L-NOARG) on cyclic GMP and cyclic AMP levels induced by isoprenaline in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7} M): □ represents basal levels of cyclic nucleotides in rings denuded of endothelium; ■ represents basal levels of cyclic nucleotides in rings with intact endothelium; ▨ represents levels of cyclic nucleotides in endothelium intact rings after 60 s exposure to isoprenaline (10^{-6} M) and ▨ represents levels of cyclic nucleotides after 60 s exposure to isoprenaline (10^{-6} M) in endothelium intact rings preincubated with L-NOARG (10^{-5} M). Levels of cyclic GMP (a) and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 5 and 10 separate experiments.

cyclic AMP induced by isoprenaline (10^{-6} M) were not significantly affected by pretreatment with L-NOARG (10^{-5} M), the levels being 2750 ± 174 fmol mg^{-1} protein and 2336 ± 253 fmol mg^{-1} protein for control and L-NOARG pretreated tissues respectively.

Forskolin (10^{-7} M, 60 s) caused a relaxant response of $41 \pm 5\%$ which was completely abolished by preincubation with L-NOARG (10^{-5} M). Levels of cyclic GMP were reduced from 775 ± 170 fmol mg^{-1} protein where forskolin alone was present to 52 ± 9 fmol mg^{-1} protein where L-NOARG was also present (Figure 10). The raised levels of cyclic AMP induced by forskolin (10^{-7} M) were not significantly affected by pretreatment with L-NOARG (10^{-5} M), the levels being 1808 ± 140 fmol mg^{-1} protein and 1935 ± 31 fmol mg^{-1} protein for control and L-NOARG pretreated tissues, respectively.

Discussion

There are two main groups of endothelial-independent vasodilators. There are those, like sodium nitroprusside, which act via cyclic GMP and there is another group which are thought to act through cyclic AMP. In this latter group isoprenaline and other β-adrenoceptor agonists have been regarded as 'archetypal' endothelium-independent vasodilators mediating their effects by increasing cyclic AMP in the smooth muscle (Furchgott & Martin, 1985; Furchgott & Vanhoutte, 1989). Recent reports, however, have suggested that at least part of the response to a number of classical endothelium-independent vasodilator drugs known to activate adenylate cylcase is endothelium-dependent. These include:- (1) the inhibition of isoprenaline vasorelaxations in the rat aorta *in vitro* by haemoglobin and methylene blue (Grace *et al.*, 1988); (2) the inhibition of prostacyclin and forskolin vasorelaxations in the pig coronary artery *in vitro* by haemoglobin and methylene blue (Shimokawa *et al.*, 1988); and (3) the inhibition of salbutamol- and adrenaline-induced vasorelaxations *in vivo* in the rat by the nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester (Gardiner *et al.*, 1991a,b).

In this paper it has been shown that isoprenaline causes relaxations in the rat thoracic aorta which are totally dependent on the presence of an intact endothelium.

The receptors mediating this response appear to be β-adrenoceptors since salbutamol also has an endothelium-dependent mechanism of inducing relaxation, but is between

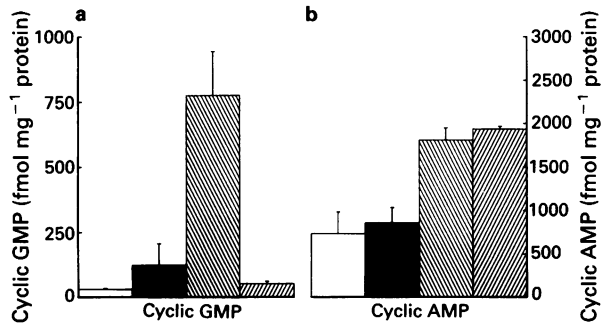

Figure 10 The effect of N^G-nitro-L-arginine (L-NOARG) on cyclic GMP and cyclic AMP levels induced by forskolin in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7} M): ☐ represents basal levels of cyclic nucleotides in rings denuded of endothelium; ■ represents basal levels of cyclic nucleotides in rings with intact endothelium; ▨ represents levels of cyclic nucleotdies in endothelium intact rings after 60 s exposure to forskolin (10^{-7} M) and ▨ represents levels of cyclic nucleotides after 60 s exposure to forskolin (10^{-7} M) in endothelium intact rings preincubated with L-NOARG (10^{-5} M). Levels of cyclic GMP (a) and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 5 and 10 separate experiments.

10 and 30 fold less potent than isoprenaline. In addition, propranolol competitively inhibits the relaxant response to isprenaline with a potency characteristic of a typical β-adrenoceptor.

Since the β-adrenoceptor is known to be linked to adenylate cyclase (Nahorski *et al.*, 1975) via the guanine nucleotide binding protein, G_s (Gilman, 1986) it appears likely that the first stage in the signal transduction pathway mediating the endothelium-dependent vasorelaxant response to isoprenaline in the rat thoracic aorta is an increase in cyclic AMP within the endothelium. This hypothesis is supported by the lack of vasorelaxation or increase in either cyclic AMP or cyclic GMP in tissues denuded of endothelium when exposed to isoprenaline.

The role of the rise in cyclic AMP appears to be activation of nitric oxide synthase, either directly or indirectly. This is apparent as forskolin, which activates adenylate cyclase directly, causes endothelium-dependent relaxations in this tissue. Further, both isoprenaline and forskolin-induced vasorelaxations and the associated elevations in cyclic GMP levels but not the rises in cyclic AMP levels in the rat aorta can be inhibited by the nitric oxide synthase inhibitor, L-NOARG.

The signal transduction pathway mediating the endothelium-dependent relaxant effects of the β-adrenoceptor agonists and forskolin in the rat thoracic aorta appears to share a number of characteristics with that postulated to mediate human α-calcitonin gene-related peptide (α-CGRP) relaxations in the same tissue (Gray & Marshall, 1992b). Firstly, all these vasodilators require the presence of the endothelium to exert their relaxant effects. Secondly, the vasorelaxations in this tissue induced by the β-adrenoceptor agonists and forskolin are associated with rises in cyclic AMP and cyclic GMP. Thirdly, removal of the endothelium, as well as abolishing vasorelaxations to these agents, also abolishes the increases in cyclic AMP and cyclic GMP. Finally, in endothelium-intact rings of rat thoracic aorta, the relaxant effects of these vasodilators are inhibited by L-NOARG which also selectively inhibits the accumulation of cyclic GMP without altering the increases in cyclic AMP.

In view of these similarities, a common signal transduction mechanism may mediate the endothelium-dependent relaxant responses to the β-adrenoceptor agonists, forskolin and human α-CGRP. This would entail activation of adenylate cyclase either directly or through a receptor on the endothelium, consequent activation of nitric oxide synthase and vasorelaxation by activation of guanylate cyclase within the smooth muscle (Figure 11).

Whether other vasodilators can activate this signal transduction pathway remains open to question. However, there is some circumstantial evidence suggesting that both prostacyclin and vasoactive intestinal polypeptide (VIP) may exert some of their relaxant effects via this mechanism. Both are known to act on receptors that are linked to activation of adenylate cyclase (Tateson *et al.*, 1977; Gorman *et al.*, 1977; Huang & Rorstad, 1983; Itoh *et al.*, 1985) and have at least a component of their relaxant effect which is endothelium-dependent (Davies & Williams, 1983; Thom *et al.*, 1986; Shimokawa *et al.*, 1988).

Although the results presented above appear to indicate that isoprenaline-induced relaxation is endothelium-dependent and mediated by nitric oxide in the rat thoracic aorta, a number of studies have reported that relaxations to this vasodilator occur independently of the endothelium in this tissue (Grace *et al.*, 1988; Kamata *et al.*, 1989; Dainty *et al.*, 1990; Weir *et al.*, 1991). Although different strains of rat have been used in these studies, preliminary experiments indicated that this could not account for the differences observed since, in addition to the Sprague-Dawley rats, aorta from Wistar strain rats (male, 350–450 g) also had endothelium-dependent relaxations to isoprenaline (3×10^{-8}–10^{-5} M) (unpublished observations). Clearly there must be some fundamental variation in the protocol used above and in those from the reported studies.

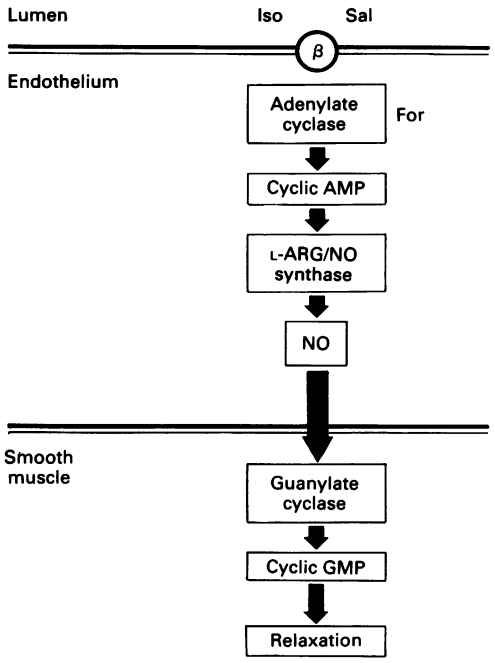

Figure 11 Novel transduction pathway mediating isoprenaline (Iso), salbutamol (Sal) and forskolin (For) endothelium-dependent relaxations in rat aorta. Isoprenaline or salbutamol act on β-adrenoceptors which appear to be present only on the endothelium. These endothelial receptors are linked to the activation of adenylate cyclase, elevating cyclic AMP levels in the endothelium. Forskolin directly activates adenylate cyclase. The cyclic AMP, either directly or indirectly, activates the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation in the smooth muscle.

One explanation could be that in previous experiments the endothelium has not been removed sufficiently to abolish the relaxant effects of isoprenaline. There are a number of factors suggesting this may be the case.

Firstly, a number of studies have shown that 'complete' removal of the endothelium (shown by lack of response to muscarinic agonists) alters the relaxant response to isoprenaline in the rat aorta, shifting the concentration-effect curve to the right and decreasing the maximum response (Grace *et al.*, 1988; Kamata *et al.*, 1989; Dainty *et al.*, 1990). Although varying levels of tone were induced in the above studies, there is general agreement that in endothelium-intact rings of rat aorta, isoprenaline induces a relaxant response with EC_{50} and maximum response similar to that found in the present study. However, in supposedly completely endothelium-denuded rings of rat aorta, the maximum relaxation varies widely from approximately 30% (Grace *et al.*, 1988) to 80% of the spasmogen-induced tone (Weir *et al.*, 1991) with virtually every intermediate value being represented (Martin *et al.*, 1986; Kamata *et al.*, 1989; Dainty *et al.*, 1990). This variability of the isoprenaline vasorelaxant response in the rat aorta has also been found in individual studies with maximum responses varying between 30 and 80% of the spasmogen-induced tone in one report (Maurice *et al.*, 1991). This observation is difficult to reconcile with an endothelium-independent mechanism of relaxation but is consistent with an endothelium-dependent relaxant effect where varying proportions of the endothelium have been removed.

Secondly, it has been reported that isoprenaline-induced relaxations in the rat aorta can be inhibited by methylene blue (3×10^{-5} M) and haemoglobin (10^{-5} M) (Grace *et al.*, 1988), compounds known to inhibit the action of nitric oxide. This is in marked contrast to the rabbit aorta (where our preliminary studies indicated that isoprenaline-induced relaxations (3×10^{-8}–10^{-5} M) were endothelium-independent (female, New Zealand White, 1.5–2 kg), unpublished observations), where methylene blue and haemoglobin at similar concentrations were without effect on isoprenaline-induced vasorelaxation (Martin *et al.*, 1985).

The criterion used to confirm absence of endothelium in the literature was lack of relaxation to a muscarinic agonist, normally acetylcholine. The use of a muscarinic agonist to confirm loss of endothelium relies on the assumption that removal of the entire endothelium is required to abolish the endothelium-dependent relaxant response. This has been demonstrated in the rabbit aorta (Furchgott & Zawadzki, 1980) (where isoprenaline induces endothelium-independent vasorelaxation). However, in the rat it has been shown that relaxations to acetylcholine are abolished when some portion of the endothelium is intact, as shown by a doubling of cyclic GMP levels (Grace *et al.*, 1987). The basic assumption i.e. that removal of the entire endothelium is required to abolish the endothelium-dependent relaxant effects of muscarinic agonists, is invalid in the rat aorta where the degree of endothelium removal is best assessed histologically. Therefore, maintenance of vascular relaxation after loss of acetylcholine vasorelaxation does not prove there is an endothelial-independent component in the action of a vasodilator.

In summary, the results presented in this paper support a link between cyclic AMP elevation within the endothelium and the consequent activation of nitric oxide synthase. This represents another endothelium-dependent mechanism by which vasorelaxation can occur. There are some observations *in vivo* with adrenaline and salbutamol which support a potential physiological role for this mechanism (Gardiner *et al.*, 1991a,b). The transduction mechanism might also be found in other situations where nitric oxide is generated as a biological mediator.

D.W.G. was supported by an MRC studentship.

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(Received March 18, 1992
Revised June 25, 1992
Accepted July 3, 1992)