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Role of endothelium/nitric oxide in atypical β-adrenoceptor-mediated relaxation in rat isolated aorta

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Abstract

The role of endothelium in the modulation of classical and atypical β-adrenoceptor-mediated vasorelaxation was investigated in ring preparations of rat isolated thoracic aorta. Rings were pre-constricted with a sub-maximal concentration of noradrenaline (1 μM) and relaxant responses to cumulative concentrations of β-adrenoceptor agonists obtained. Endothelium removal or pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) or 1*H*-[1,2,4] oxadiazolol[4,3,-*a*] quinoxalin-1-one (ODQ, 10 μM) significantly reduced the relaxant effects of isoprenaline, but had less effect on relaxant responses to the atypical β-adrenoceptor agonist, (±)-4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one hydrochloride (CGP 12177A). Sodium nitroprusside (3 nM) shifted the isoprenaline concentration–response curve to the left and restored the attenuated responses in the presence of L-NAME back to control levels. Sodium nitroprusside had little effect on the CGP 12177A concentration–response curve. The results show that the endothelium/nitric oxide (NO) pathway modulates β-adrenoceptor-mediated vasorelaxation in rat aorta and that classical β-adrenoceptors are modulated to a greater extent than atypical β-adrenoceptors.

Keywords: β-Adrenoceptor; β-Adrenoceptor, atypical; Endothelium; Nitric oxide (NO); Guanylyl cyclase; Smooth muscle, vascular

1. Introduction

Stimulation of β-adrenoceptors in smooth muscle is widely held to involve activation of adenylyl cyclase with a subsequent increase in intracellular cAMP concentration (Kukovetz et al., 1981). Although β-adrenoceptor-mediated vasodilatation has been regarded as endothelium-independent in accordance with this mechanism (Furchgott and Vanhoutte, 1989), there are conflicting reports regarding the role of endothelium. For example, in support of an endothelium-independent effect of isoprenaline, removal of endothelium was found to have to have no inhibitory effect on isoprenaline-induced relaxation in rat aorta (Konishi and Su, 1983; Moncada et al., 1991; Satake et al., 1996), canine coronary arteries (Cohen et al., 1983, 1984; White et al., 1986; Macdonald et al., 1987), rat carotid artery (Oriowo, 1994) or human internal mammary artery (Molenaar et al., 1988). In contrast, other studies found that removal of endothelium reduced the relaxations induced by isoprenaline in rat thoracic aorta (Grace et al., 1988; Kamata et al., 1989; Dainty et al., 1990; Gray and Marshall, 1992; Delpy et al., 1996; Toyoshima et al., 1998; Trochu et al., 1999), canine coronary arteries (Rubanyi and Vanhoutte, 1985) and human umbilical vein (Ferro et al., 1999). Additional evidence supporting a role for endothelium came from studies showing that relaxations to isoprenaline in rat aorta were inhibited by methylene blue and by haemoglobin (Grace et al., 1988), previously shown to block endothelium-dependent relaxation (Martin et al., 1985). Nitric oxide (NO) synthase inhibitors have also been shown to inhibit isoprenaline-induced relaxations in endothelium-intact rat aorta (Gray and Marshall, 1992; Delpy et al., 1996; Toyoshima et al., 1998; Trochu et al., 1999), rat mesenteric arteries (Graves and Poston, 1993), rat pulmonary arteries (Priest et al., 1997), rat carotid artery (MacDonald et al., 1999) and human umbilical vein (Ferro et al., 1999).

It seems therefore that the presence of endothelium can, in some situations at least, affect the response to isoprenaline. β-adrenoceptors have been identified in cultured endothelial cells by radioligand binding (Steinberg et al., 1984) and the presence of endothelial β-adrenoceptors in some blood vessels has also been shown by autoradiography (Stephenson and Summers, 1987; Molenaar et al.,

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1988) raising the possibility that endothelial β-adrenoceptors may release NO. However, Grace et al. (1988) found that, in aortic rings with or without endothelium, isoprenaline elevated cAMP levels but had no effect on cGMP levels. They concluded that isoprenaline did not release NO, but that the presence of endothelium amplified the response to isoprenaline via a synergistic interaction between cAMP and cGMP. This conclusion is supported by Delpy et al. (1996) who suggested that cGMP may potentiate cAMP-dependent relaxation by inhibition of cGMP-inhibited phosphodiesterase (phosphodiesterase 3). In contrast, Gray and Marshall (1992) and others (Iranami et al., 1996) found that isoprenaline increased both cAMP and cGMP levels in endothelium-intact aortic rings and that removal of endothelium abolished these increases. Gray and Marshall (1992) proposed that isoprenaline produces relaxation by stimulating endothelial β-adrenoceptors to raise cAMP and thus release NO, which then evokes relaxation via an increase in smooth muscle cGMP. Direct support for an ability of β_2-adrenoceptors to activate NO synthase via cAMP elevation in human umbilical vein endothelial cells has recently been provided (Ferro et al., 1999).

Thus, endothelium may have a role in modulation of β-adrenoceptor-mediated vasorelaxation although the mechanism is unclear. Vascular β-adrenoceptors were originally classified as β_2- (Lands et al., 1967), and this appears to be the predominant type in rat aorta although β_1-adrenoceptors are also present (O'Donnell and Wanstall, 1984; Brawley et al., 2000a). In addition, recent studies have provided evidence for the presence of atypical β-adrenoceptors, not conforming to the β_1-/β_2-classification, in rat aorta (Oriowo, 1995; Sooch and Marshall, 1997; Brawley et al., 2000a). The nature of the atypical β-adrenoceptor is not fully understood, although our previous results suggest that the atypical β-adrenoceptor in rat aorta does not correspond to the β_3-adrenoceptor, but is similar to the putative β_4-adrenoceptor (Brawley et al., 2000a). The present study was carried out to compare the role of endothelium in modulating classical and atypical β-adrenoceptor-mediated effects. Preliminary accounts of some of these results have been presented in abstract form (Brawley et al., 1997, 1998).

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (150–300 g), were stunned and killed by cervical dislocation followed by exsanguination. The thoracic aorta was isolated, removed carefully to prevent endothelium damage and cleared of fat and connective tissue. The thoracic aorta was cut into 3-mm ring seg-

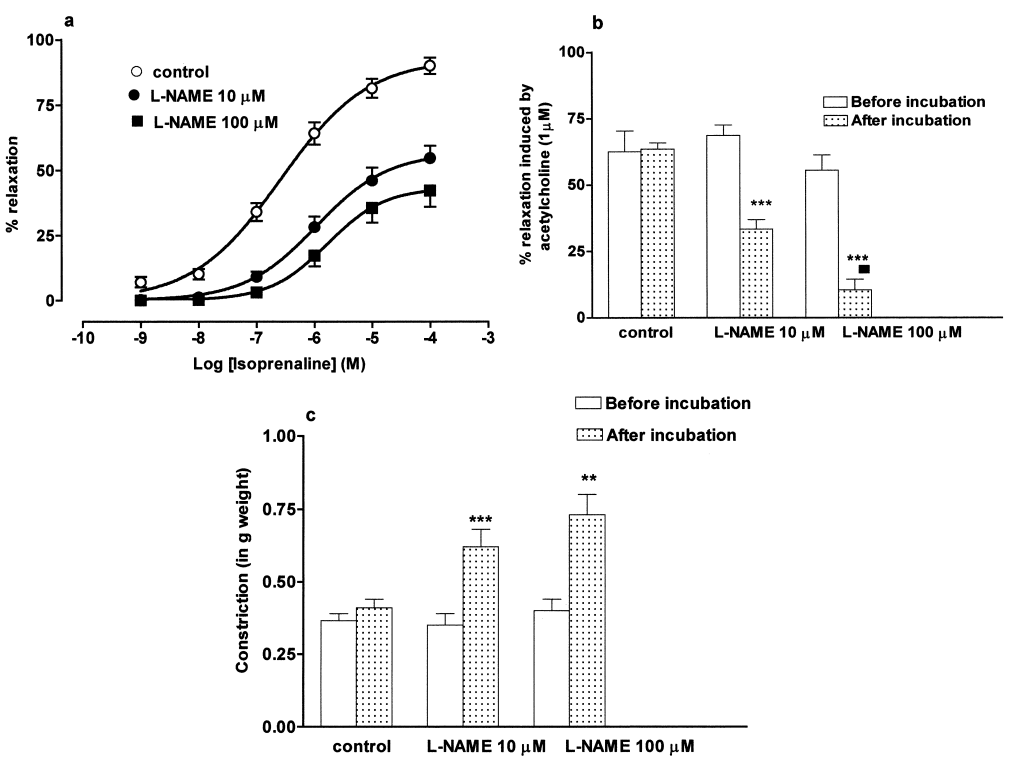

Fig. 1. Effects of L-NAME in rat thoracic aortic rings. Values are mean ± S.E. of 8–17 observations. In (a) and (b), results are expressed as percentage relaxation of tone induced by noradrenaline (1 μM). (a) Effect of L-NAME on relaxation induced by isoprenaline. (b) Effect of L-NAME on acetylcholine-induced (1 μM) relaxation. $^{***}P < 0.001$ vs. before L-NAME treatment. ■ $P < 0.01$ vs. after L-NAME (10 μM). (c) Effect of L-NAME incubation on noradrenaline pre-constriction . Results are expressed in g weight. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. before L-NAME.

ments, which were mounted on stainless-steel wires in 20-ml organ baths containing Krebs' medium with the following composition (mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; D-glucose, 11.1. The Krebs' medium also contained ethylene diamine tetra-acetic acid (30 μM) and ascorbic acid (30 μM) to prevent oxidation of catecholamines. The medium was maintained at 37°C and gassed continuously with 95% O_2 and 5% CO_2. Each tissue was placed under an initial resting tension of 1 g weight and allowed to equilibrate for 60 min prior to the execution of experimental protocols. Muscle tension was recorded with Grass transducers and displayed on a Goerz Servogor 400 oscillograph.

2.2. Concentration–response curves

After the equilibration period, the artery rings were constricted with a sub-maximal concentration of noradrenaline (1 μM) and the contraction was allowed to stabilise over a period of 10 min. In some preparations, the endothelium was removed mechanically by gently abrading the intimal surface of the aortic rings with the tip of small stainless forceps. The integrity of the endothelium was tested with acetylcholine (1 and 10 μM). Preparations with intact endothelium produced greater than 50% relaxation while successful endothelial denudation was confirmed by lack of acetylcholine-induced relaxation. After washout, some tissues were incubated with an appropriate treatment for 30 min with control tissues receiving no treatment. The rings were then contracted again with noradrenaline (1 μM) and cumulative concentration–response curves to agonists were conducted. After washing, tissues were contracted with noradrenaline for the third time before challenging with acetylcholine (1 and 10 μM) to monitor endothelium function.

2.3. Drugs used

The following were dissolved in distilled water, with the exception of 1*H*-[1,2,4] oxadiazolol[4,3,-*a*] quinoxalin-1-one (ODQ), which was dissolved in dimethyl sulphoxide (DMSO): (±)-noradrenaline bitartrate (Sigma), acetylcholine chloride (Sigma), (−)-isoprenaline bitartrate (Sigma), N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), (±)-propranolol hydrochloride (Sigma), (±)-4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one hydrochloride (CGP 12177A hydrochloride, gift from Novartis Pharma), (±)-cyanopindolol hemifumarate (Tocris Cookson), (*R*)-*N*-(2-[4-(carboxymethyl)phenoxy]ethyl)-*N*-(β-hydroxyphenethyl)ammonium chloride (ZD 2079, gift from Zeneca), (*S*)-4-(2-hydroxy-3-phenoxypropylamino-ethoxy)-*N*-(2-methoxyethyl)phenoxyaceticacid (ZM 215001, gift from Zeneca), sodium nitroprusside (Sigma) and ODQ (Tocris Cookson).

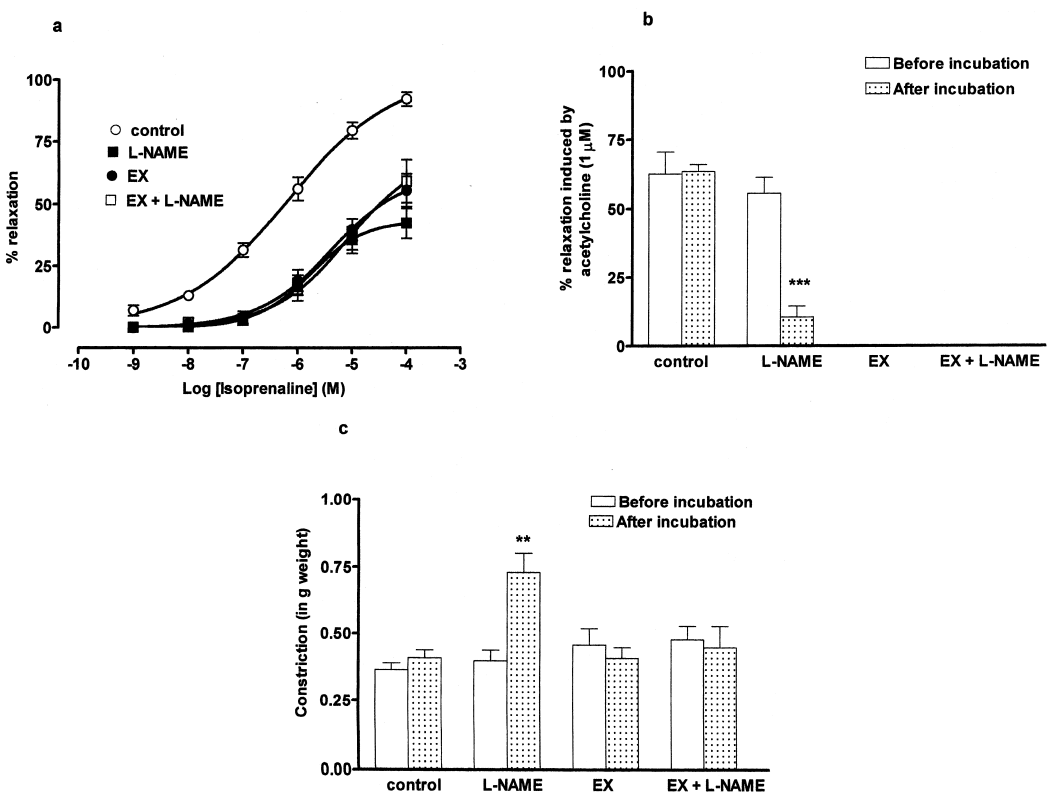

Fig. 2. Effect of L-NAME (100 μM) and endothelium removal (EX) in rat thoracic aortic rings. Values are mean ± S.E. of 8–12 observations. In (a) and (b), results are expressed as percentage relaxation of tone induced by noradrenaline (1 μM). (a) Effect of L-NAME and EX on relaxation induced by isoprenaline. (b) Effect of L-NAME and EX on acetylcholine-induced (1 μM) relaxation. $^{***}P < 0.001$ vs. before L-NAME treatment. (c) Effect of L-NAME and EX on noradrenaline pre-constriction. Results are expressed in g weight. $^{**}P < 0.01$ vs. before L-NAME treatment.

2.4. Calculations and statistical analysis

Responses to agonists were calculated as % inhibition of the noradrenaline-induced contraction and expressed as mean ± S.E. Mean concentration response curves to agonists were analysed by fitting to a four-parameter logistic equation using non-linear regression (Graph Pad Prism). Maximum responses, Hill slopes and $-\log EC_{50}$ values were obtained where EC_{50} is the concentration (M) of agonist that produces 50% of its maximum response. Agonist concentration ratios (CR) were determined from EC_{50} values and where appropriate estimates of pA_2 obtained from the equation, $pA_2 = \log(\text{agonist CR} - 1) - \log[\text{antagonist}]$.

Statistical analyses were performed using Student's or paired *t* tests to compare two groups and one-way analysis of variance followed by the Bonferroni multiple comparison test for comparison of three or more groups.

3. Results

3.1. Isoprenaline and L-NAME

Isoprenaline produced a concentration-dependent relaxation of noradrenaline constricted rings ($-\log EC_{50}$, 6.59 ± 0.09; % maximum relaxation, 93 ± 3, $n = 17$) (Fig. 1a). Incubation with L-NAME (10 and 100 μM) significantly reduced the % maximum relaxation induced by isoprenaline to 57 ± 1 ($P < 0.001$, $n = 13$) and 43 ± 0.5, ($P < 0.001$, $n = 8$), respectively, and shifted the relaxant responses rightward ($-\log EC_{50}$ values: L-NAME (10 μM), 5.97 ± 0.05, $P < 0.001$; L-NAME (100 μM), 5.78 ± 0.02, $P < 0.001$) (Fig. 1a). The effect of 100 μM L-NAME was significantly greater than that of 10 μM on the maximum response ($P < 0.05$), but not on the $-\log EC_{50}$ ($P > 0.05$). L-NAME (10 and 100 μM) significantly reduced the relaxant response evoked by acetylcholine with 100 μM producing greater inhibition than 10 μM (Fig. 1b). There was also a significant increase in the size of noradrenaline constriction following L-NAME (10 and 100 μM), although there was no significant difference between the 10 and 100 μM treatments (Fig. 1c).

3.2. Isoprenaline and removal of endothelium

Removal of endothelium significantly reduced the relaxant response induced by isoprenaline (Fig. 2a). The isoprenaline concentration–response curve in endothelium-denuded preparations was similar to that in endothelium-intact preparations in the presence of L-NAME (100 μM) (Fig. 2a). The combination of endothelium-removal and L-NAME had no additional effect on isoprenaline relaxation to either treatment alone (Fig. 2a). Endothelium removal completely abolished the inhibitory effect of acetylcholine (1 and 10 μM) (Fig. 2b), but had no significant effect on noradrenaline pre-constriction (Fig. 2c). The combination of endothelium-removal and L-NAME (100 μM) had no effect on noradrenaline-constriction even

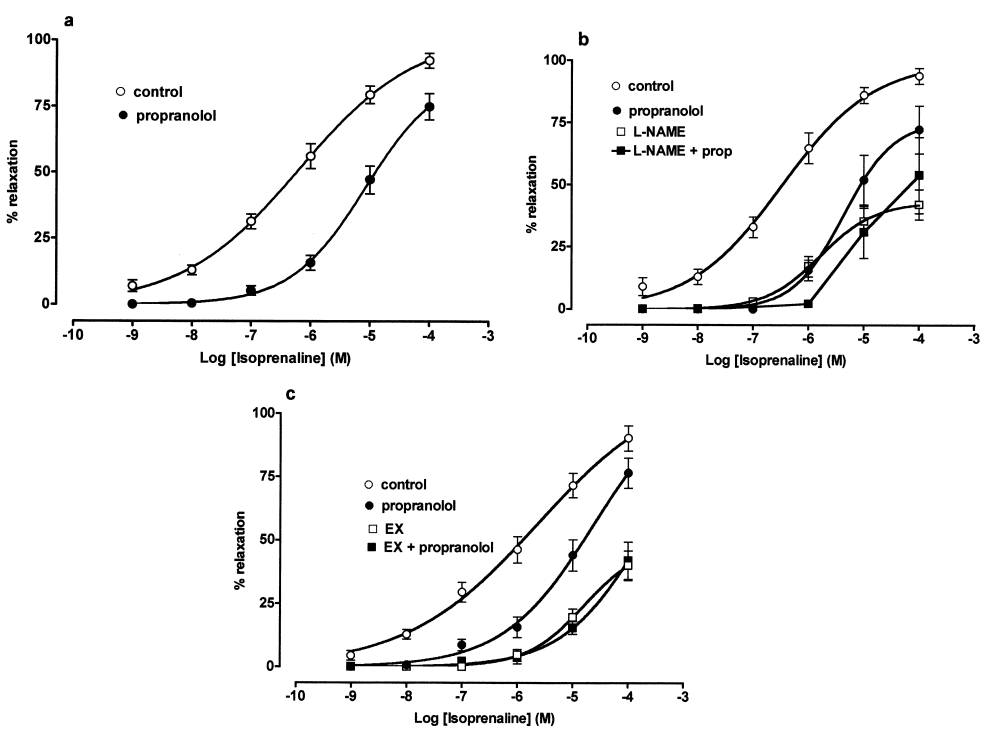

Fig. 3. The effect of L-NAME (100 μM), endothelium removal (EX) and propranolol (0.3 μM) on isoprenaline-induced relaxation in rat thoracic aorta. Results are expressed as percentage relaxation of tone induced by noradrenaline (1 μM). Values are mean ± S.E. of *n* observations. (a) Effect of propranolol ($n = 13$–15). (b) Effect of L-NAME and propranolol ($n = 5$–8). (c) Effect of endothelium removal (EX) and propranolol ($n = 6$–8).

though L-NAME treatment singly produced a significant potentiation (Fig. 2c).

3.3. Propranolol in the presence of L-NAME or endothelium removal

Propranolol (0.3 μM) shifted the isoprenaline concentration–response curve to the right ($-\log EC_{50}$ values: control, 6.20 ± 0.02, $n = 15$; propranolol, 5.07 ± 0.06, $n = 13$, $P < 0.001$) with an increase in the slope (Hill slopes: control, 0.45 ± 0.02; propranolol, 0.69 ± 0.04, $P < 0.001$) (Fig. 3a). After L-NAME pretreatment, propranolol (0.3 μM) had little additional effect on the isoprenaline concentration–response curve — only the response to 1 μM isoprenaline was significantly attenuated ($P < 0.05$) (Fig. 3b). Similarly, propranolol had no effect on the isoprenaline concentration–response curve in endothelium-denuded preparations (Fig. 3c).

3.4. Endothelium and atypical β-adrenoceptor agonists

CGP 12177A produced a concentration-dependent relaxation of the noradrenaline-constricted rings ($-\log EC_{50}$, 4.40 ± 0.1; % maximum relaxation, 108 ± 8, $n = 19$). In the presence of L-NAME (100 μM), the CGP 12177 concentration–response curve was shifted to the right by 3.2-fold with no apparent reduction in the maximum response ($-\log EC_{50}$ after L-NAME: 3.90 ± 0.02, $n = 8$, $P < 0.01$). Removal of endothelium had a similar effect to that of L-NAME ($-\log EC_{50}$: 3.85 ± 0.05, $n = 9$, $P < 0.01$) (Fig. 4a). The efficacy of these treatments was confirmed by their effects on acetylcholine-induced relaxation (e.g. % relaxation to 1 μM acetylcholine, mean ± S.E. mean: (a) control, 64 ± 7, $n = 19$; (b) L-NAME, 10 ± 4, $P < 0.001$, $n = 8$; (c) endothelium removal, 0 ± 0, $P < 0.001$, $n = 9$). Removal of endothelium and pretreatment with L-NAME increased noradrenaline-induced constriction (constriction (g weight) to 1 μM noradrenaline: (a) control, 0.64 ± 0.06, $n = 19$; (b) L-NAME, 0.73 ± 0.09, $P < 0.01$, $n = 8$); (c) endothelium removal, 1.10 ± 0.18, $P < 0.01$, $n = 9$).

L-NAME (100 μM) also shifted the cyanopindolol concentration–response curve to the right, 3.8-fold ($-\log EC_{50}$s: control, 5.59 ± 0.02, $n = 8$; L-NAME, 5.01 ± 0.02, $n = 5$, $P < 0.001$) with no reduction in maximum response (% maximum relaxation: control, 100 ± 1; L-NAME, 103 ± 1) (Fig. 4b). Relaxations to ZD 2079 and ZM 215001

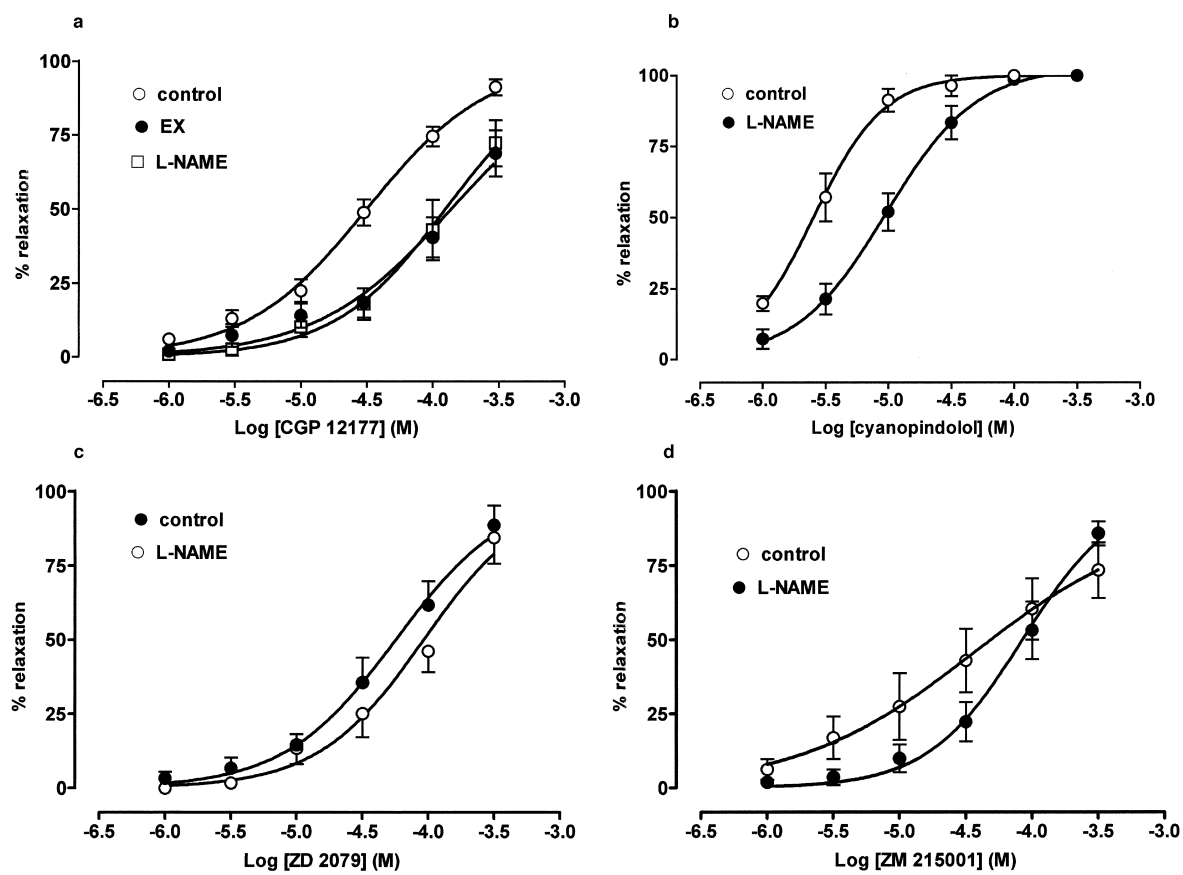

Fig. 4. The effect of L-NAME (100 μM) and endothelium removal (EX) on atypical β-adrenoceptor agonist-induced relaxation in rat thoracic aorta. Results are expressed as percentage relaxation of tone induced by noradrenaline (1 μM). Values are mean ± S.E. of n observations. (a) Effect of L-NAME and EX on relaxation induced by CGP 12177A ($n = 8$–19). (b) Effect of L-NAME (100 μM) on relaxation induced by cyanopindolol ($n = 5$–8). (c) Effect of L-NAME on relaxation induced by ZD 2079 ($n = 6$–7). (d) Effect of L-NAME on relaxation induced by ZM 215001 ($n = 4$–6).

were unaltered by L-NAME ($-\log EC_{50}$s before and after L-NAME (100 μM): ZD 2079 -4.24 ± 0.03, $n = 7$, 4.03 ± 0.05, $n = 5$, $P > 0.05$; ZM 215001 -4.43 ± 0.1, $n = 4$, 4.07 ± 0.03, $n = 6$, $P > 0.05$) (Fig. 4c and d). The effectiveness of L-NAME on acetylcholine-induced relaxation was confirmed in all of these experiments (results not shown).

3.5. ODQ

ODQ (10 μM) reduced the maximum response to isoprenaline (% maximum relaxation: control, 98 ± 4; ODQ, 60 ± 3, $P < 0.001$) and shifted the isoprenaline concentration–response curve 4.2-fold to the right ($-\log EC_{50}$s: control, 6.39 ± 0.13, $n = 4$; ODQ, 5.77 ± 0.12, $n = 5$, $P < 0.01$) (Fig. 5a). In the presence of ODQ, propranolol (0.3 μM) produced a 6.2-fold shift of the isoprenaline concentration–response curve ($-\log EC_{50}$: 4.98 ± 0.02) (Fig. 5a).

In the presence of propranolol (0.3 μM), ODQ reduced the maximum response to isoprenaline (% maximum relaxation: control, 93 ± 7; ODQ, 68 ± 1, $P < 0.01$) and shifted the isoprenaline concentration–response curve twofold to the right ($-\log EC_{50}$s: propranolol, 5.29 ± 0.13, $n = 5$; propranolol + ODQ, 4.98 ± 0.02, $n = 5$, $P < 0.001$) (Fig. 5b).

ODQ (10 μM) incubation did not affect CGP 12177-induced relaxation ($-\log EC_{50}$s: control, 4.60 ± 0.04, $n = 5$; ODQ, 4.59 ± 0.04, $n = 5$, $P > 0.05$ (Fig. 5c).

ODQ (10 μM) significantly reduced the relaxant response evoked by acetylcholine (Fig. 6a). There was also a significant increase in the size of noradrenaline constriction following ODQ (10 μM) pretreatment (Fig. 6b).

3.6. Sodium nitroprusside, propranolol and L-NAME

Sodium nitroprusside on its own produced complete relaxation of the noradrenaline-constricted rings ($-\log EC_{50}$, 7.7, $n = 2$). A concentration of 3 nM, which produced approximately 23% relaxation was chosen for further studies.

Sodium nitroprusside (3 nM) shifted the isoprenaline concentration–response curve 12.3-fold leftward ($-\log EC_{50}$s: control, 6.19 ± 0.09, $n = 9$; sodium nitroprusside, 7.28 ± 0.05, $n = 6$, $P < 0.001$) with no effect on the maximum response (Fig. 7a). In the presence of propranolol (0.3 μM), sodium nitroprusside failed to significantly potentiate isoprenaline-induced relaxant responses producing only a 1.6-fold shift leftward of the control concentration–response curve (propranolol alone) ($-\log EC_{50}$ values: propranolol, 5.12 ± 0.02, $n = 5$; sodium nitroprusside + propranolol, 5.32 ± 0.02, $n = 5$, $P > 0.05$) (Fig. 7a).

In the presence of L-NAME (100 μM), sodium nitroprusside restored the attenuated relaxation back to control levels (% maximum relaxation: L-NAME, 33 ± 1, $n = 4$; sodium nitroprusside + L-NAME, 101 ± 3, $n = 4$, $P < 0.001$) (Fig. 7b).

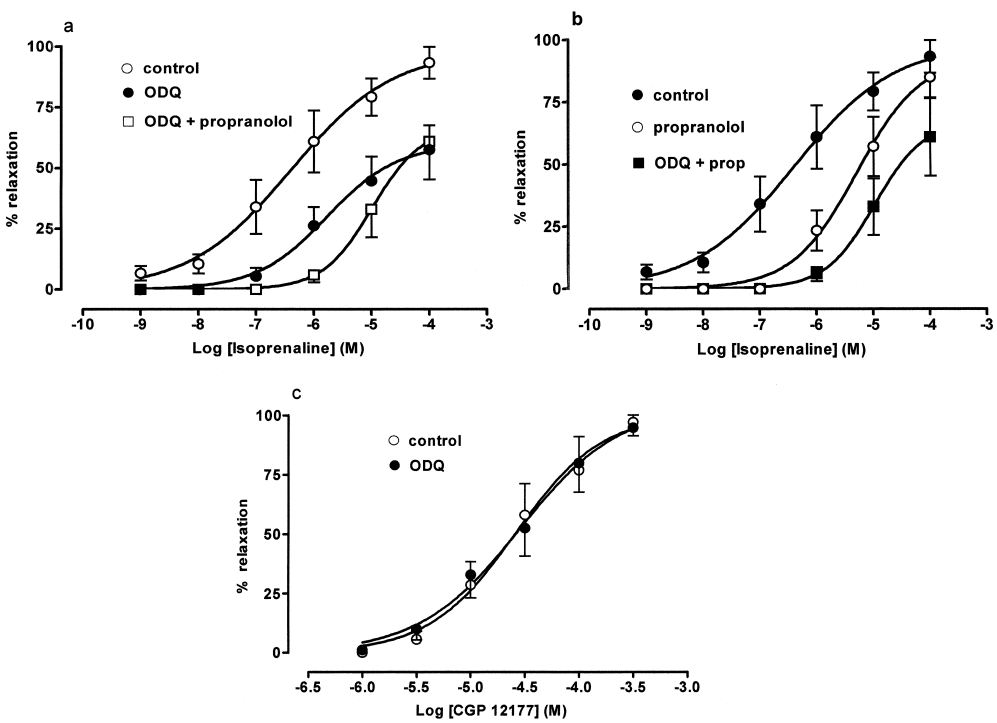

Fig. 5. The effect of ODQ (10 μM) and propranolol (0.3 μM) on β-adrenoceptor agonist-induced relaxation in rat thoracic aorta. Results are expressed as percentage relaxation of tone induced by noradrenaline (1 μM). Values are mean ± S.E. of n observations. (a and b) Effect of ODQ and propranolol on relaxation induced by isoprenaline ($n = 4$–5). (c) Effect of ODQ on relaxation induced by CGP 12177A ($n = 5$).

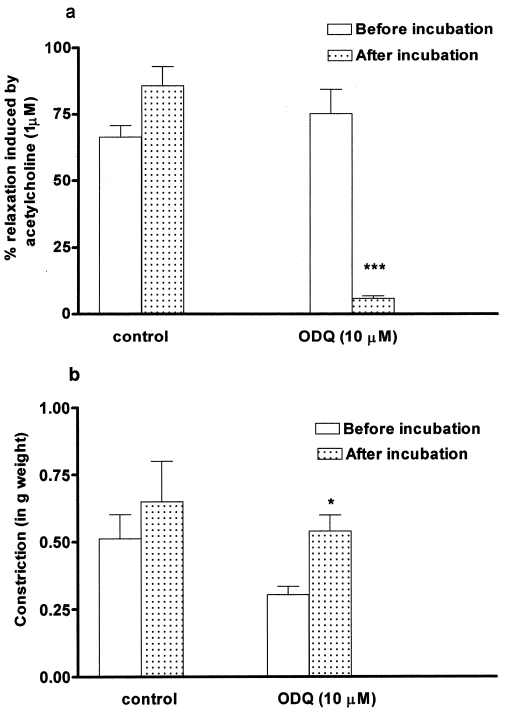

Fig. 6. The effect of ODQ (10 μM) on acetylcholine (1 μM) relaxation and noradrenaline (1 μM) contraction in rat thoracic aortic rings. Values are mean ± S.E. of n observations. (a) Effect of ODQ on relaxation induced by acetylcholine (1 μM) ($n = 4$–5). $^{***}P < 0.001$ vs. before ODQ (10 μM) treatment. (b) Effect of ODQ (10 μM) incubation on noradrenaline preconstriction. Results are expressed in g weight ($n = 4$–5). $^{*}P < 0.05$ vs. before ODQ (10 μM).

Sodium nitroprusside (3 nM) shifted the CGP 12177A concentration–response curve 1.4-fold to the left ($-\log EC_{50}$s: control, 4.66 ± 0.07, $n = 10$; sodium nitroprusside, 4.80 ± 0.05, $n = 4$, $P < 0.05$) with no effect on the maximum response (% maximum relaxation: control, 100 ± 1; sodium nitroprusside, 105 ± 4, $P > 0.05$) (Fig. 7c). In the presence of L-NAME (100 μM), sodium nitroprusside shifted the CGP 12177A concentration–response curve fourfold to the left ($-\log EC_{50}$s: L-NAME, 4.30 ± 0.06, $n = 4$; sodium nitroprusside + L-NAME, 4.90 ± 0.02, $n = 3$, $P < 0.01$) (Fig. 7d). Sodium nitroprusside incubation significantly reduced noradrenaline constriction and potentiated acetylcholine-induced relaxation (Fig. 8a and b).

4. Discussion

In the present study, acetylcholine-induced relaxations of the pre-constricted rat aortic rings were greatly reduced following L-NAME pretreatment or completely abolished by endothelium removal confirming the role of endothelium-derived NO in acetylcholine-induced vasodilatation (Furchgott, 1988; Furchgott and Zawadzki, 1980; Ignarro et al., 1988). Endothelium removal was more effective in reducing acetylcholine-induced relaxant responses than inhibition of endothelial nitric oxide synthase (eNOS) with L-NAME. This could be due to incomplete inhibition of eNOS by L-NAME or may be attributed to the release of other endothelium-derived factors by acetylcholine such as a diffusible endothelium-derived hyperpolarising factor (EDHF) (Feletou and Vanhoutte, 1988).

Incubation with L-NAME, at concentrations that substantially reduced acetylcholine-induced relaxation, was found to inhibit isoprenaline-induced relaxant responses. A similar inhibition of isoprenaline-induced relaxation was found with endothelium removal and, furthermore, the combination of endothelium removal and L-NAME treatment had no additional effect compared to either treatment alone. This suggests that the effect of endothelium removal can be entirely attributed to loss of NO. These results therefore support previous findings that endothelium removal (Grace et al., 1988; Kamata et al., 1989; Dainty et al., 1990; Gray and Marshall, 1992; Delpy et al., 1996; Iranami et al., 1996; Toyoshima et al., 1998; Trochu et al., 1999) or inhibition of NO synthase (Gray and Marshall, 1992; Delpy et al., 1996; Iranami et al., 1996; Toyoshima et al., 1998; Trochu et al., 1999) can inhibit isoprenaline-induced relaxation in rat aorta. It is not clear why some other studies found no effect of endothelium removal on isoprenaline-induced relaxation in the same preparation (Konishi and Su, 1983; Moncada et al., 1991; Satake et al., 1996), but one controversial suggestion is that any endothelium-independent relaxation to isoprenaline is due to incomplete removal of endothelium (Gray and Marshall, 1992). These authors found that removal of endothelium completely abolished the isoprenaline-induced relaxation and proposed that isoprenaline produces relaxation entirely by stimulating endothelial β-adrenoceptors and thus releasing NO. The possibility that incomplete removal of endothelium accounts for the remaining isoprenaline relaxation in the present study would seem unlikely for several reasons : (i) endothelium removal completely abolished the relaxation to acetylcholine; (ii) L-NAME produced a similar inhibition of the isoprenaline concentration–response curve to endothelium removal; and (iii) treatment of endothelium-denuded preparations with L-NAME produced no further inhibition of the isoprenaline-induced relaxation. Thus, there appear to be endothelium-dependent and endothelium-independent components of the response to isoprenaline. The endothelium-dependent component of the isoprenaline relaxation in the present study could be explained by stimulation of NO release by endothelial β-adrenoceptors (Gray and Marshall, 1992) or by potentiation of smooth muscle β-adrenoceptors by basal release of NO (Grace et al., 1988; Delpy et al., 1996). Radioligand binding studies have identified β-adrenoceptors both in vascular smooth muscle (Asano et al., 1991) and on cultured endothelial cells (Steinberg et al., 1984). The presence of β-adrenoceptors in smooth muscle and in endothelium has also been shown by autoradiography in some blood vessels (Stephenson and Summers, 1987; Molenaar et al., 1988). However, in the latter study, although β_2-

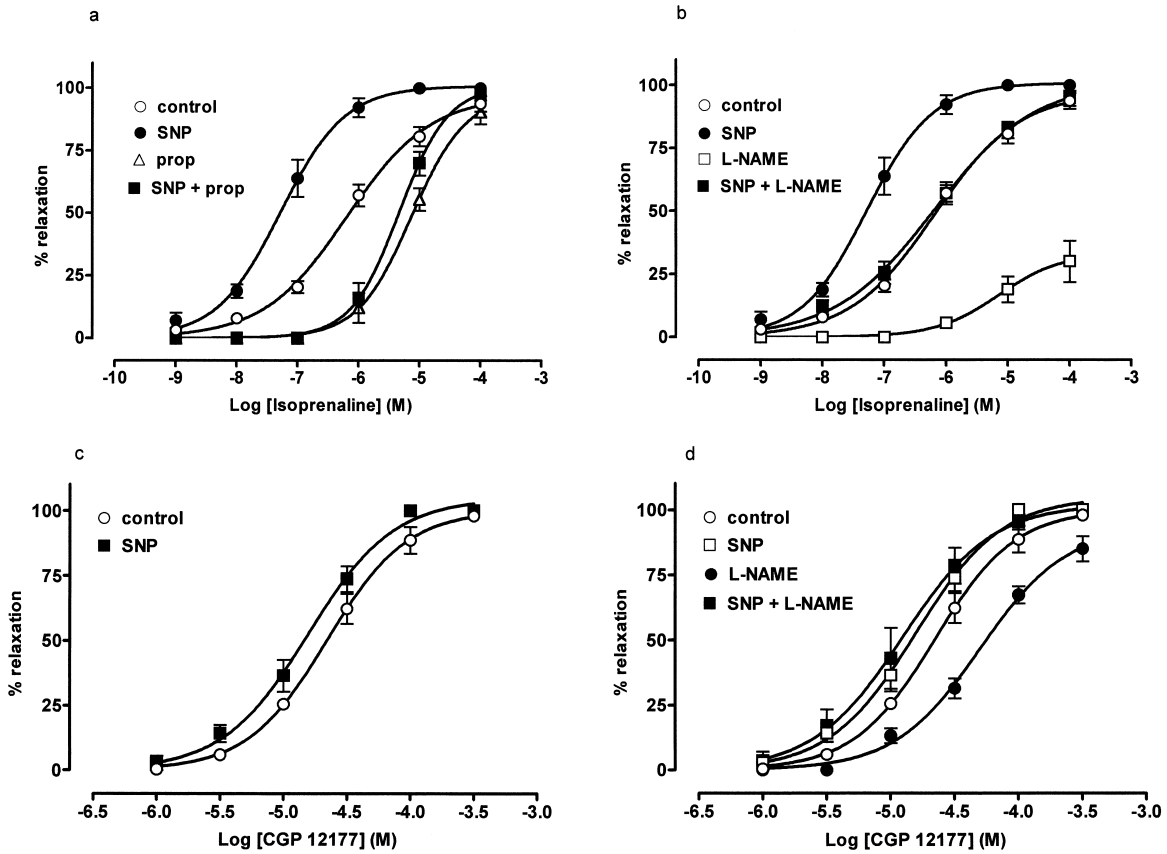

Fig. 7. The effect of sodium nitroprusside (3 nM), L-NAME (100 μM) and propranolol (0.3 μM) on β-adrenoceptor agonist-induced relaxation in rat thoracic aorta. Results are expressed as percentage relaxation of tone induced by noradrenaline (1 μM). Values are mean ± S.E. of n observations. (a) Effect of sodium nitroprusside and propranolol on relaxation induced by isoprenaline ($n = 5–9$). (b) Effect of sodium nitroprusside and L-NAME on relaxation induced by isoprenaline ($n = 4–9$). (c) Effect of sodium nitroprusside on relaxation induced by CGP 12177A ($n = 4–10$). (d) Effect of sodium nitroprusside and L-NAME on relaxation induced by CGP 12177A ($n = 3–10$).

adrenoceptors were shown to be present in endothelium they did not contribute to vasorelaxation and presumably mediate other functions.

One possible explanation for the reduction in response to isoprenaline after L-NAME incubation and endothelium removal is that the contraction to noradrenaline is greater due to removal of basal NO release (Eckly et al., 1994). However, if relaxation is plotted in g tension, the difference in the effect of isoprenaline between control and L-NAME-treated preparations is still apparent (results not shown). In addition, although responses to noradrenaline were increased after L-NAME incubation, endothelium removal did not consistently increase constriction induced by noradrenaline. Therefore, the reduction in response to isoprenaline in the present study is not simply explained by an increased contraction to noradrenaline. A recent study (Delpy et al., 1996) also failed to note an increase in phenylephrine constriction after endothelium removal although the constriction was increased after L-NAME. The failure to consistently obtain an increase in noradrenaline constriction after endothelium removal may be due to the fact that endothelial cells can release contracting factors such as superoxide anions, endothelin, vasoconstrictor prostanoids and angiotensin II (reviewed by Lüscher and Barton, 1997), although damage to the vascular smooth muscle cannot be ruled out.

Propranolol shifted the isoprenaline concentration–response curve to the right, supporting the presence of classical (β_1-/β_2-) β-adrenoceptors. However, the estimated pA_2 of 7.6 is low for an action of isoprenaline only at classical β-adrenoceptors, suggesting the presence of atypical β-adrenoceptors. In addition, the non-parallel shift of the isoprenaline concentration–response curve suggests propranolol-sensitive (low concentrations of isoprenaline) and -insensitive (high concentrations of isoprenaline) components of the response, corresponding to classical and atypical β-adrenoceptors, respectively. These results support previous findings suggesting that atypical β-adrenoceptors co-exist with classical β-adrenoceptors in rat aorta (Oriowo, 1995; Sooch and Marshall, 1997; Brawley et al., 2000a).

After L-NAME or endothelium removal, propranolol had little or no additional effect on the isoprenaline concentration–response curve suggesting that the classical β-adrenoceptor-mediated component is mainly endothelium-dependent and that the remaining response is largely

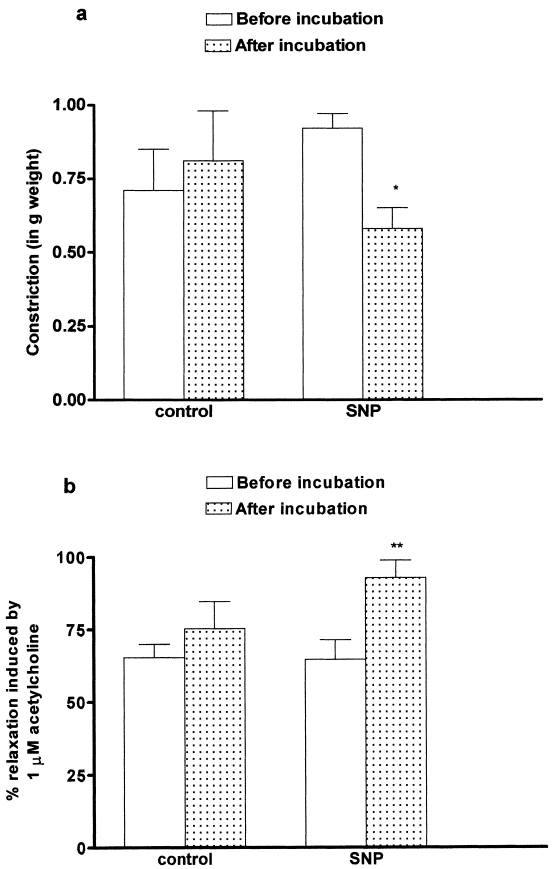

Fig. 8. The effect of sodium nitroprusside (3 nM) on noradrenaline (1 μM) contraction and acetylcholine (1 μM) relaxation in rat thoracic aortic rings. Values are mean ± S.E. of *n* observations. (a) Effect of sodium nitroprusside incubation on noradrenaline preconstriction. Results are expressed in g weight ($n = 6–9$). $^{*}P < 0.05$ vs. before sodium nitroprusside. (b) Effect of sodium nitroprusside on relaxation induced by acetylcholine (1 μM) ($n = 6–9$). Results are expressed as percentage relaxation of tone induced by noradrenaline (1 μM). $^{**}P < 0.01$ vs. before sodium nitroprusside treatment.

mediated through atypical β-adrenoceptors. Shafiei and Mahmoudian (1999) found a greater effect of propranolol in endothelium-denuded preparations than reported here, although the antagonism was non-competitive, agreeing with the presence of atypical β-adrenoceptors on the vascular smooth muscle. Similarly, Trochu et al. (1999) found a nadolol-resistant response in the presence of the NO synthase inhibitor, L-NMMA.

The atypical β-adrenoceptor-mediated component of the response to isoprenaline appears to be partially endothelium-dependent since L-NAME or endothelium removal attenuated isoprenaline relaxation in the presence of propranolol.

Relaxation was also produced by the atypical β-adrenoceptor agonists CGP 12177A (Mohell and Dicker, 1989), cyanopindolol (Engel et al., 1981), ZD 2079 (Grant et al., 1994) and ZM 215001 (Tesfariam and Allen, 1994) lending further support to the presence of atypical β-adrenoceptors. Incubation with L-NAME or endothelium removal inhibited responses to CGP 12177A although the reduction in relaxation was less than obtained with isoprenaline. Similarly, L-NAME reduced responses to cyanopindolol to a lesser extent than to isoprenaline. L-NAME had no effect on the selective β_3-adrenoceptor agonists ZD 2079 and ZML 215001. These results further suggest that endothelium-derived NO plays less of a role in atypical compared with classical β-adrenoceptor-mediated relaxation. The reason for the complete lack of effect of L-NAME on the selective β_3-adrenoceptor agonists is not clear, particularly as Trochu et al. (1999) have shown that L-NMMA attenuates the response to the selective β_3-adrenoceptor agonist SR 58611 and further work is necessary to clarify this difference.

ODQ is a novel compound that selectively inhibits soluble guanylyl cyclase in tissues (Garthwaite et al., 1995) including rat aorta (Moro et al., 1996). Incubation with ODQ reduced isoprenaline-induced relaxations in a similar way to L-NAME pretreatment and endothelium removal. In the presence of ODQ, propranolol had less of

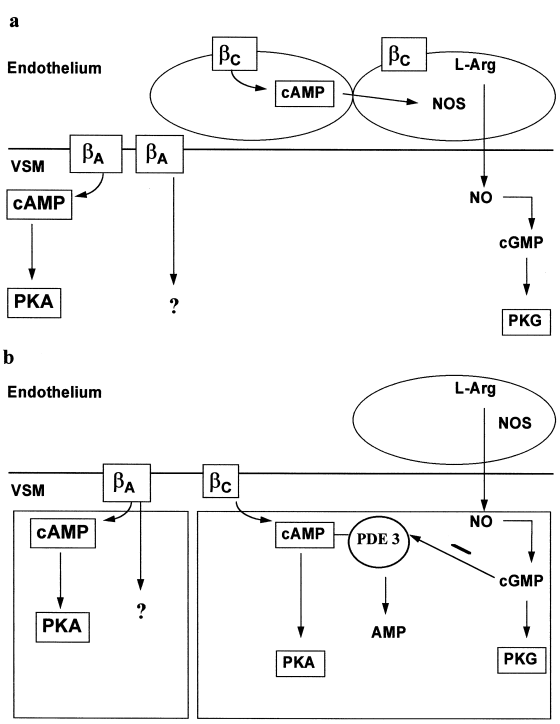

Fig. 9. Possible models to explain differential modulation by endothelium of classical and atypical β-adrenoceptor-mediated vasorelaxation. (a) Different distribution of β-adrenoceptor subtypes on the vascular endothelium and smooth muscle (VSM). Classical β-adrenoceptors (β_C) are located predominantly on endothelium and mediate nitric oxide release. Atypical β-adrenoceptors (β_A) are located predominantly on the vascular smooth muscle and mediate relaxation via cAMP-dependent or -independent mechanisms. (b) Both classical (β_C) and atypical (β_A) β-adrenoceptors are located on vascular smooth muscle (VSM) and endothelial modulation is due to basal release of NO via inhibition of phosphodiesterase 3 by cGMP. Atypical β-adrenoceptor-mediated increase in cAMP is not affected by cGMP due to functional compartmentalisation of cAMP pools. Alternatively the atypical β-adrenoceptor-mediated response is via another signalling pathway.

an effect on the isoprenaline concentration–response curve, producing only a sixfold shift (16-fold in the absence of ODQ). This further supports the conclusion that the classical β-adrenoceptor-mediated component is largely dependent on the endothelium/NO pathway and that the response remaining after inhibition of this pathway is mediated mainly through atypical β-adrenoceptors.

In the presence of propranolol to block classical β-adrenoceptors, ODQ produced some attenuation of the isoprenaline relaxation. The effects of ODQ in the presence of propranolol were similar to those of L-NAME and endothelium removal (see above), lending weight to the suggestion that the atypical β-adrenoceptor-mediated component may be partially endothelium-dependent. Relaxant responses to CGP 12177A, however, were unaffected by ODQ, although as discussed earlier, L-NAME and endothelium removal did have an effect. Thus, there is not complete agreement between the effects of guanylyl cyclase inhibition by ODQ and the effects of L-NAME and endothelium removal although qualitatively the results are similar, with less effect of ODQ on atypical compared with classical β-adrenoceptor-mediated relaxation.

Sodium nitroprusside, at a concentration, which by itself produced a relaxation of approximately 23%, potentiated the relaxation to isoprenaline, shifting the isoprenaline concentration–response curve 12-fold to the left. A synergistic effect of sodium nitroprusside and isoprenaline has previously been reported (Maurice and Haslam, 1990). Sodium nitroprusside had little or no effect on the relaxation to isoprenaline in the presence of propranolol or on the relaxation to CGP 12177A, suggesting that exogenously applied NO interacts with the classical β-adrenoceptor signalling pathway to a greater extent than with the atypical β-adrenoceptor pathway. In the presence of L-NAME, sodium nitroprusside enhanced relaxation to both isoprenaline and CGP 12177A, in each case offsetting the attenuation produced by L-NAME. Again, the contribution of NO to the atypical β-adrenoceptor-mediated response is less than to the classical β-adrenoceptor-mediated relaxation.

The results of this study show that various treatments affecting the stimulation of soluble guanylyl cyclase in vascular smooth muscle (NOS inhibition, endothelium removal, guanylyl cyclase inhibition or stimulation) affect β-adrenoceptor-mediated relaxation. The guanylyl cyclase-dependent component of the isoprenaline relaxation in the present study could be explained by the presence of endothelial β-adrenoceptors mediating stimulation of NOS (Gray and Marshall, 1992) or by potentiation of the smooth muscle β-adrenoceptor-mediated response by basal release of NO and subsequent inhibition of phosphodiesterase 3 by cGMP (Grace et al., 1988; Delpy et al., 1996). The guanylyl cyclase-dependent component of the response is greater in classical (β_1-/β_2-) than in atypical β-adrenoceptor-mediated relaxation. There could be several explanations for this. Firstly, if endothelial β-adrenoceptors do mediate relaxation through release of NO (Gray and Marshall, 1992), it is possible that there may a different distribution of classical and atypical β-adrenoceptors, with classical β-adrenoceptors being located predominantly on the endothelium and atypical β-adrenoceptors predominantly on smooth muscle (Fig. 9a). Secondly, if both classical and atypical β-adrenoceptors are located on the smooth muscle and endothelial modulation is due to basal release of NO (Grace et al., 1988; Delpy et al., 1996), then differential modulation may be explained by compartmentalisation of cAMP production (Houslay and Milligan, 1997), with the classical β-adrenoceptor-mediated increases in cAMP potentiated to a greater extent by cGMP (Fig. 9b). A third possibility is that there are cAMP-dependent and cAMP-independent components of the response to β-adrenoceptor stimulation, with the response to classical β-adrenoceptors dependent on cAMP and endothelium (by either of the possible mechanisms described above) and responses to atypical β-adrenoceptor-mediated stimulation mediated through other signalling pathways not modulated by endothelium. Our preliminary results provide some evidence for the latter (Brawley et al., 2000b). These putative mechanisms for differential modulation by endothelium of classical and atypical β-adrenoceptor-mediated relaxation are not mutually exclusive and may all contribute to the circumstances in vascular tissue. Further work is required to establish the relative contributions of endothelial and smooth muscle β-adrenoceptors, cAMP compartmentalisation and cAMP-independent pathways in classical and atypical β-adrenoceptor-mediated vasorelaxation.

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