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Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta

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1 The relaxant effects of isoprenaline may result from activation of another β-adrenoceptor subtype in addition to β_1 and β_2. This study evaluated the role of a third β-adrenoceptor subtype, β_3, in β-adrenoceptor-induced relaxation of rat thoracic aorta by isoprenaline.

2 Isoprenaline produced a concentration-dependent relaxation of phenylephrine pre-contracted rings of the thoracic aorta ($pD_2 = 7.46 \pm 0.15$; $E_{max} = 85.9 \pm 3.4\%$), which was partially attenuated by endothelium removal ($E_{max} = 66.5 \pm 6.3\%$) and administration of the nitric oxide (NO) synthase inhibitor, L-N^G-monomethyl arginine (L-NMMA) ($E_{max} = 61.3 \pm 7.9\%$).

3 In the presence of nadolol, a β_1- and β_2-adrenoceptor antagonist, isoprenaline-induced relaxation persisted ($E_{max} = 55.6 \pm 5.3\%$), but occurred at higher concentrations ($pD_2 = 6.71 \pm 0.10$) than in the absence of nadolol and lasted longer.

4 Similar relaxant effects were obtained with two β_3-adrenoceptor agonists: SR 58611 (a preferential β_3-adrenoceptor agonist), and CGP 12177 (a partial β_3-adrenoceptor with β_1- and β_2-adrenoceptor antagonistic properties). SR 58611 caused concentration-dependent relaxation ($pD_2 = 5.24 \pm 0.07$; $E_{max} = 59.5 \pm 3.7\%$), which was not modified by pre-treatment with nadolol but antagonized by SR 59230A, a β_3-adrenoceptor antagonist. The relaxation induced by SR 58611 was associated with a 1.7 fold increase in tissue cyclic GMP content.

5 Both relaxation and the cyclic GMP increase induced by SR 58611 were greatly reduced by endothelium removal and in the presence of L-NMMA.

6 We conclude that in the rat thoracic aorta, β_3-adrenoceptors are mainly located on endothelial cells, and act in conjuction with β_1- and β_2-adrenoceptors to mediate relaxation through activation of an NO synthase pathway and subsequent increase in cyclic GMP levels.

Keywords: Rat thoracic aorta; relaxation; β_3-adrenoceptor; endothelium; nitric oxide; cyclic GMP

Abbreviations: E_{max}, maximal relaxant response; CGP 12177, 4-[3-*t*-butylamino-2-hydroxypropoxy]benzimadazol-2-one; IBMX, 3-isobutyl-1-methylxanthine; L-NMMA, L-N^G-monomethyl-arginine; SR 58611, (RS)-*N*-[(25)-7-ethoxycarbonyl-methoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2R)-2-(3-chlorophenyl)-2 hydroethanamine hydrochloride; SR 59230A, 3-(2-ethylphenoxy)-1-[(1S)1,2,3,4-tetrahydronaphth-1-ylaminol]-(2S)-2-propanol oxalate

Introduction

The cloning of a third β-adrenoceptor subtype (β_3) in 1989 (Emorine *et al*., 1989) has allowed an explanation of some of the effects of catecholamines which are not related to simultaneous or concomitant activation of β_1- and β_2-adrenoceptors. β_3-adrenoceptors were subsequently found to mediate lipolysis in adipose tissues (for review, see Lafontan, 1994) and the relaxation of gastrointestinal (for review, see Manara *et al*., 1995b) and airway (for review, see Martin & Advenier, 1995) smooth muscle. More recently, β_3-adrenoceptors have been characterized in human heart, activation of which induces a negative inotropic effect (Gauthier *et al*., 1996).

In vascular smooth muscle, β-adrenoceptors were initially classified as β_2-adrenoceptors (Lands *et al*., 1967). Later studies using more selective agonists and antagonists showed that vascular relaxation could result from activation of either β_1- or β_2-adrenoceptor subtypes and that the involvement of each subtype depended on the vascular bed and the species studied. In most vessels, the relaxation induced by isoprenaline is mediated essentially through the activation of β_2-adrenoceptors, with little contribution from β_1-adrenoceptors. The involvement of a third β-adrenoceptor subtype in β-adrenoceptor agonist-induced vasorelaxation has been suggested in several studies. Pindolol, a non-specific β-adrenoceptor antagonist with significant intrinsic sympathomimetic activity, induced the relaxation of canine isolated perfused mesenteric vessels (Clark & Bertholet, 1983) and rat aorta (Doggrell, 1990). In rat aorta, propranolol, a non-selective β-adrenoceptor antagonist, inhibited the vasorelaxant effect of isoprenaline, a non-selective β-adrenoceptor antagonist, inhibited the vasorelaxant effect of isoprenaline at higher concentrations than those expected solely from activation of β_1- and β_2-adrenoceptors. This effect was attributed to catecholamine-induced stimulation of atypical β-adrenoceptors (Oriowo, 1995) as well as β_2-adrenoceptors and a small population of β_1-adrenoceptors (O'Donnell & Wanstall, 1985). The possibility of an atypical β-adrenoceptor in vessels was strengthened by the use of preferential β_3-adrenoceptor agonists (BRL 37344, CL 316243). In *in vivo* studies, these agents produced vasodilatation in dogs (Tavernier *et al*., 1992;

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Shen *et al*., 1994; 1996) and to a lesser extent in rats (Shen *et al*., 1996).

The main purpose of the present study was to determine the role of β_3-adrenoceptors in relaxation of the rat thoracic aorta induced by β-adrenoceptor agonists. The effects of two β_3-adrenoceptor agonists, SR 58611 (a preferential agonist) and CGP 12177 (a partial agonist which also possesses β_1- and β_2-adrenoceptor antagonistic properties (Blin *et al*., 1993)), were compared with those of isoprenaline in the absence and presence of a β_1- and β_2-adrenoceptor-blocking drug, nadolol (Lee *et al*., 1975). A second purpose of this study was to characterize the cellular coupling pathway involved after stimulation of vascular β_3-adrenoceptors, especially the role of the endothelium and cyclic GMP-dependent and/or -independent NO pathways.

Methods

Tissue preparation and tension studies in rat aortic rings

Adult male Wistar rats (250–300 g) were anaesthetized with pentobarbital (30 mg kg^{-1} i.p.). Descending thoracic aortae were isolated, cleared of fat and connective tissue and cut into 3 mm rings. In some rings, the endothelium was removed by gentle rubbing of the intimal surface with a fine pair of small forceps. Rings were suspended on stainless-steel wires in a 20 ml organ bath containing Krebs solution composed as follows (mM): NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; EDTA (ethylenediaminetetraacetic acid), 0.016; glucose, 11.1; and $CaCl_2$, 2.5 (pH 7.4). Bath temperature was maintained at 37°C, and the Krebs solution was continuously oxygenated with a 95% O_2, 5% CO_2 gas mixture. Rings were progressively stretched to a resting tension of 2 g. Isometric tension was recorded by a force displacement transducer (IT2, EMKA Technologies, Paris, France) and displayed on a computer (IOX1.5.7 software, EMKA Technologies). Data were analysed using Datanalyst software (EMKA Technologies).

Functional endothelium was checked by the presence of at least 70% relaxation in response to acetylcholine (1 μM) in rings pre-contracted with phenylephrine (0.3 μM). In denuded vascular rings, endothelium removal was confirmed by the absence of acetylcholine-induced relaxation. Some rings were equilibrated in Krebs containing nadolol (a β_1- and β_2-adrenoceptor antagonist), SR 59230A (a β_3-adrenoceptor antagonist), or the NO synthase inhibitor, N^G-monomethyl-L-arginine monoacetate (L-NMMA), for 30 min. Control rings were not treated during this period. Aortic rings were contracted again with phenylephrine to obtain a similar magnitude of sustained tension as in control rings. A cumulative concentration-response curve to either isoprenaline or a β_3-adrenoceptor agonist was then constructed. The concentration of phenylephrine (0.1–1 μM) was adjusted to produce a similar level of tone for each experimental condition. Relaxation produced by each concentration of β-adrenoceptor agonist was measured after steady-state was reached. Values are expressed as the percentage change in the maximal tension of vessel rings after addition of phenylephrine.

As SR 58611 and CGP 12177 induced long-lasting relaxation, spontaneous time-dependent relaxation was evaluated in control vessels. This phenomenon was taken into account by systematic subtraction of the corresponding spontaneous relaxation obtained in control vessels from the relaxation produced by the β_3-adrenoceptor agonist.

Cyclic GMP assay

Frozen aortic rings were individually ground in a pestle and mortar with ice-cold 6% v v^{-1} trichloroacetic acid plus 100 μM 3-isobutyl-1-methylxanthine (IBMX). After centrifugation at $1500 \times g$ for 10 min at 4°C, trichloroacetic acid was extracted by washing supernatants three times with water-saturated ether (five volumes of ether to one volume of supernatant). The remaining ether was evaporated by heating the samples to 70°C for 5 min. Cyclic GMP contents were measured using an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.). Absorbance was read on a spectrophotometer at 405 nm. A standard curve was drawn for each assay. The mean value was calculated from duplicate measurements of each sample and normalized to total cell protein content as determined by the method of Lowry *et al*. (1951).

Drugs

L-phenylephrine hydrochloride, acetylcholine chloride, (−)-isoprenaline, nadolol, IBMX and prostaglandin $F_{2\alpha}$ Tris salt ($PGF_{2\alpha}$) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-NMMA was purchased from Calbiochem (La Jolla, CA, U.S.A.) and CGP 12177 (4-[3-*t*-butylamino-2-hydroxypropoxy]benzimidazol-2-one) from RBI (Natick, MA, U.S.A.). SR 58611 [(RS)-*N*-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2R)-2-(3-chlorophenyl)-2 hydroethanamine hydrochloride] and SR 59230A (3-(2-ethylphenoxy)-1-[(1S)1,2,3,4-tetrahydronaphth-1-ylaminol]-(2S)-2-propanol oxalate) were a generous gift from Sanofi Recherche (Montpellier, France). All drugs were prepared as stock solutions in distilled water, with the exception of nadolol which was dissolved in hydrochloric acid before being neutralized to pH 7.4, and SR 59230 which was dissolved in dimethylsulphoxide (DMSO; Sigma Chemical) such that the final concentration of the solvent in the organ bath was less than 0.1% v v^{-1}. At this concentration, the solvent alone had no effect on the tissue.

Data and statistical analysis

Results are expressed as the mean ± s.e.mean of n experiments. The statistical significance of a drug effect was assessed using one-way analysis of variance (ANOVA) followed by a Dunnett's test. Comparison of the different concentration response curves was performed by two-way ANOVA. To determine agonist potencies from concentration response curves, concentrations producing 50% of maximum effect (EC_{50}) were calculated by fitting curves with the Boltzmann equation. pD_2 values were then determined according to the equation $pD_2 = -\log(\text{molar } EC_{50})$ and compared using Student's t-test ($P < 0.05$ being considered as significant).

Results

Phenylephrine (0.3 μM) increased the resting tone of rat aortic rings when endothelium was intact. As the contractile response to 0.3 μM phenylephrine was more marked when endothelium was removed or L-NMMA was present, the concentration of phenylephrine was decreased to 0.1 μM to obtain a tone level equivalent to that in control rings. In this way, the contractile tone caused by phenylephrine was similar in the different experimental conditions shown in Table 1. When the endothelium was intact, acetylcholine (1 μM) induced a significant relaxation (up to 70%), whereas

no relaxant effect was observed in endothelium-denuded rings (data not shown).

Isoprenaline-induced relaxation

As shown in Figures 1A and 2, isoprenaline (0.001–3 μM) induced concentration–dependent relaxation in control aortic rings (with an intact endothelium) pre-contracted with phenylephrine (pD_2 value of 7.46±0.15 ($n=10$) and an E_{max} of 85.9±3.4% ($n=10$)). This effect was produced rapidly, reaching its maximum level within about 4 min after isoprenaline administration. In rings without endothelium, the concentration-relaxation curve for isoprenaline was shifted to the right ($pD_2=6.50\pm0.11$), and the maximal relaxant effect was reduced ($E_{max}=66.5\pm6.3\%$; $n=7$; $P<0.05$ *versus* isoprenaline in intact rings; Figure 2).

Effect of nadolol on isoprenaline-induced relaxation

In another set of experiments, the effect of isoprenaline was tested after administration of nadolol for 30 min. Nadolol (10 μM) alone failed to produce any intrinsic effect on phenylephrine-induced concentration of aortic rings. The relaxant effect of isoprenaline was not abolished in the presence of nadolol. However, the effect occurred at higher concentrations of isoprenaline (up to 0.03 μM), and relaxation was both delayed and long-lasting (Figure 1B). The maximal relaxation for a given concentration was observed within 10–

Table 1 Effect of endothelium-removal, L-NMMA and nadolol on contractile responses induced by phenylephrine in rat thoracic aortic rings

	Tension (g)	*Number of experiments*
Control rings	3.93±0.14	19
Denuded rings	4.29±0.11	14
L-NMMA (100 μM)*	4.19±0.17	18
Nadolol (10 μM)*	4.03±0.17	16

Data are expressed as changes in isometric tension (g) and shown as mean±s.e.mean for n experiments. *Changes in contractile responses induced by phenylephrine was measured 30 min after administration of L-NMMA or nadolol.

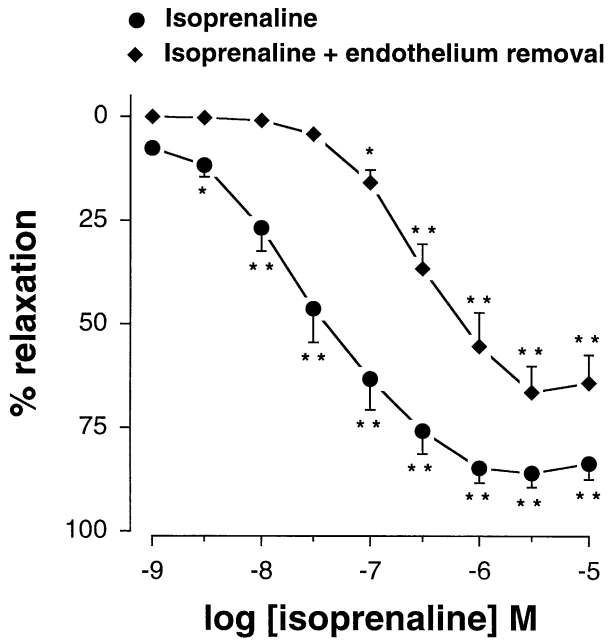

Figure 2 Concentration-response curves to isoprenaline in rat thoracic aortic rings with ($n=10$) or without endothelium ($n=7$). Results are expressed as the percentage of relaxation from the maximal level of contraction induced by phenylephrine. Each point is the mean of n experiments, and vertical lines show the s.e.mean. When no error bar is shown, the error is smaller than the symbol. *$P<0.05$ and **$P<0.01$ indicate significant differences from control.

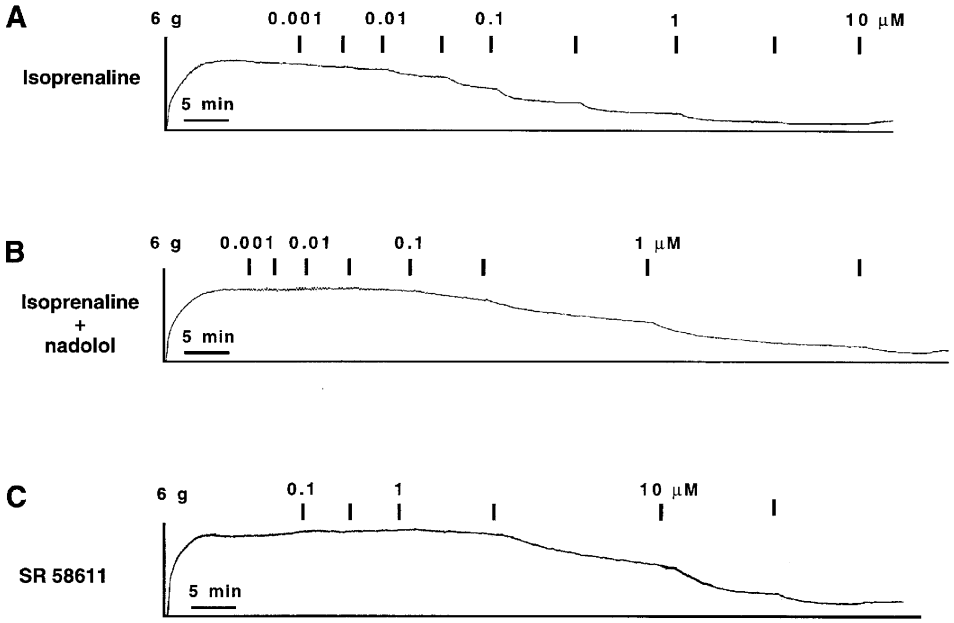

Figure 1 Typical recordings of relaxant effects of β-adrenoceptor agonists in rat thoracic aortic rings constricted with phenylephrine (0.3 μM). (A) isoprenaline induces a rapid and potent relaxant effect at concentrations from 0.001–1 μM. (B) In the presence of 10 μM nadolol, a β_1- and β_2-adrenoceptor antagonist, isoprenaline still induces relaxation (characterized by its long duration) at concentrations up to 0.03 μM. (C) SR 58611, a β_3-adrenoceptor agonist, induces a concentration-dependent relaxant effect similar to that exhibited by isoprenaline in the presence of nadolol.

12 min after administration of isoprenaline. This long-lasting effect led us to evaluate spontaneous time-dependent relaxation of vessels in control rings pre-treated with nadolol (10 μM), which was found to be responsible for 17.6±1.1% of relaxant effect at the end of the experiment (about 90 min after administration of phenylephrine; $n=5$). The corresponding spontaneous relaxation of control vessels was then subtracted from that exhibited by isoprenaline plus nadolol (10 μM). In these conditions, the concentration response curve to isoprenaline was shifted to the right in the presence of nadolol (10 μM) ($pD_2=6.71\pm0.10$; $n=9$; $P<0.01$ *versus* isoprenaline alone), and the maximal relaxant effect of isoprenaline was significantly reduced ($E_{max}=55.6\pm5.3\%$; $n=9$; $P<0.01$ *versus* isoprenaline alone; Figure 3).

Effects of L-NMMA on isoprenaline-induced relaxation

To characterize the involvement of a NO synthase pathway in the relaxant effect of β-adrenoceptor stimulation, the ability of the NOS inhibitor, L-NMMA to alter the response to isoprenaline was determined. L-NMMA shifted the relaxant response to isoprenaline to the right ($pD_2=6.57\pm0.10$; $n=9$; $P<0.01$ *versus* isoprenaline alone) and decreased the maximal level of relaxation from 85.9±3.4% to 61.3±7.9% ($n=9$; $P<0.01$ *versus* isoprenaline alone; Figure 4). The concentration-relaxant response curve to isoprenaline was shifted toward higher concentrations in the presence of L-NMMA (100 μM) plus nadolol (10 μM) ($pD_2=5.81\pm0.20$; $n=5$; $P<0.01$ *versus* isoprenaline alone and $P<0.05$ *versus* isoprenaline plus nadolol) than in the presence of either agent alone. In these conditions, the maximal relaxant effect of isoprenaline

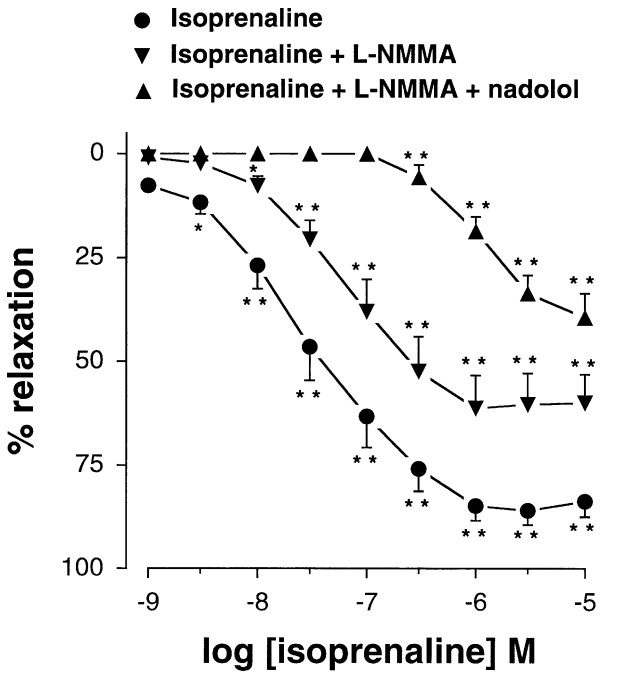

Figure 4 Concentration-response curves for isoprenaline in the absence ($n=10$) and presence of L-NMMA (100 μM; $n=9$) and in the presence of nadolol (10 μM) + L-NMMA ($n=5$) in rat thoracic aortic rings pre-contracted with phenylephrine. Results are expressed as the percentage of relaxation from the maximal contraction level induced by phenylephrine. Each point is the mean of n experiments, and vertical lines show the s.e.mean. When no error bar is shown, the error is smaller than the symbol. *$P<0.05$ and **$P<0.01$ indicate significant differences from control.

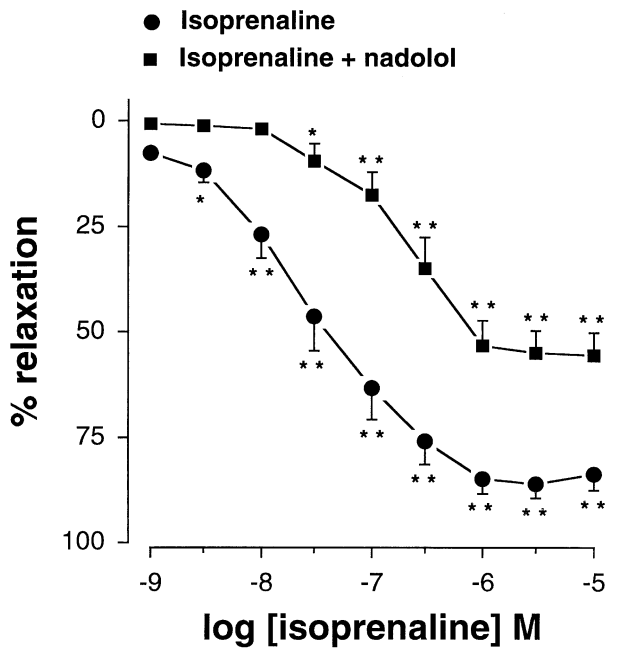

Figure 3 Concentration-response curves for isoprenaline in the absence ($n=10$) and presence of nadolol (10 μM; $n=9$) in rat thoracic aortic rings pre-contracted with phenylephrine. As the relaxant effect of isoprenaline in the presence of nadolol was slow in reaching steady-state, spontaneous relaxation of control vessels was subtracted from the relaxation exhibited by isoprenaline in the presence of nadolol. Results are expressed as the percentage of relaxation from the maximal contraction level induced by phenylephrine. Each point is the mean of n experiments, and vertical lines show the s.e.mean. When no error bar is shown, the error is smaller than the symbol. *$P<0.05$ and **$P<0.01$ indicate significant differences from control.

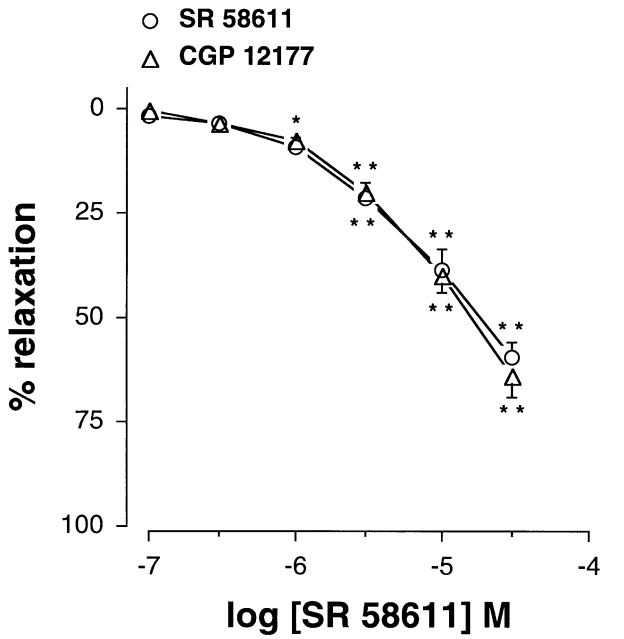

Figure 5 Concentration-response curves for SR 58611, a preferential β_3-adrenoceptor agonist ($n=11$), and CGP 12177, a partial β_3-adrenoceptor agonist ($n=6$), in rat thoracic aortic rings constricted with phenylephrine. The mean curves resulting from subtraction of the spontaneous relaxation of control vessels are shown. Results are expressed as the percentage of relaxation from the maximal contraction level induced by phenylephrine. Each point is the mean of n experiments, and vertical lines show the s.e.mean. When no error bar is shown, the error is smaller than the symbol. *$P<0.05$ and **$P<0.01$ indicate significant differences from control.

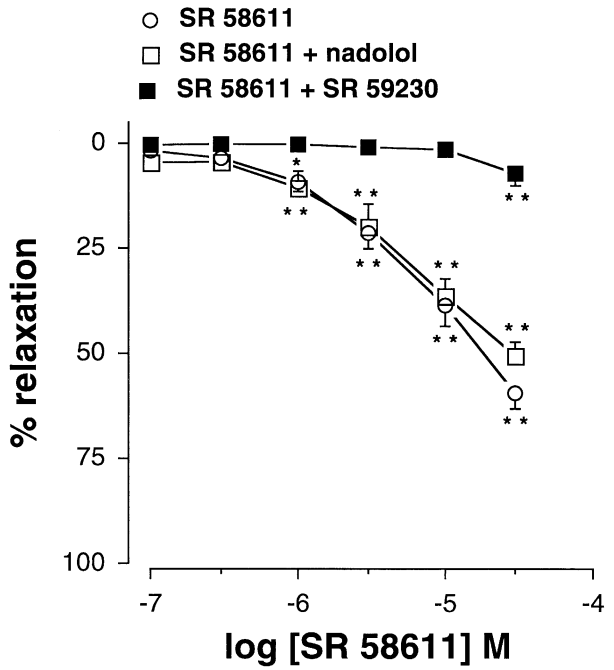

Figure 6 Concentration-response curves for SR 58611 in the absence ($n=11$) and presence of nadolol (10 μM; $n=7$) or SR 59230A (10 μM; $n=6$) in rat thoracic aortic rings constricted with phenylephrine. The mean curves resulting from subtraction of the spontaneous relaxation of control vessels pre-treated or not with nadolol or SR 59230A are shown. Results are expressed as the percentage of relaxation from the maximal contraction level induced by phenylephrine. Each point is the mean of n experiments, and vertical lines show the s.e.mean. When no error bar is shown, the error is smaller than the symbol. $^*P<0.05$ and $^{**}P<0.01$ indicate significant differences from control.

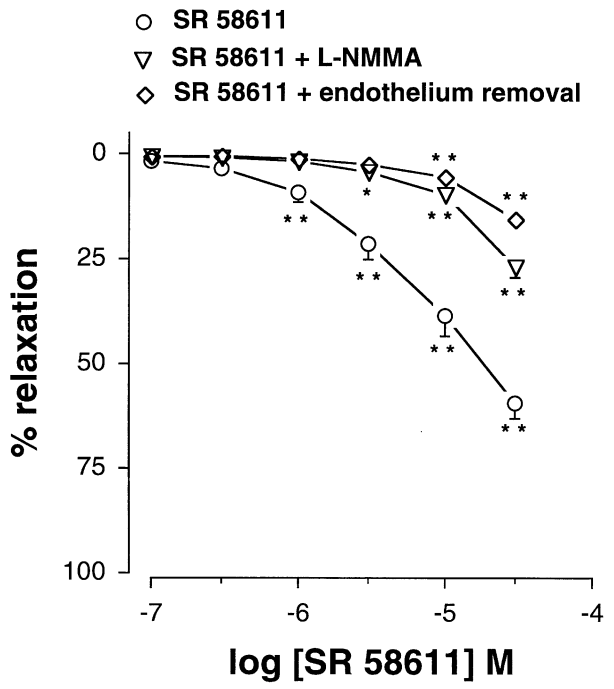

Figure 7 Concentration-response curves for SR 58611 in rat thoracic aortic rings with ($n=11$) or without endothelium ($n=7$) and after pre-treatment with L-NMMA (100 μM; $n=9$). Results are expressed as the percentage of relaxation from the maximal contraction level induced by phenylephrine. Each point is the mean of n experiments, and vertical lines show the s.e.mean. When no error bar is shown, the error is smaller than the symbol. $^*P<0.05$ and $^{**}P<0.01$ indicate significant differences from control.

Table 2 Intracellular cyclic GMP levels measured in rat thoracic aorta under different experimental conditions

	cyclic GMP (pmol mg^{-1} protein)	*Number of experiments*
Phenylephrine (0.3 μM)	9.98 ± 1.49	12
Acetylcholine (1 μM)	21.38 ± 2.81**	10
SR 58611 (30 μM)	16.94 ± 2.13**	12
L-NMMA (100 μM)	8.81 ± 1.48	5
LNMMA + SR 58611	8.91 ± 1.01	9
Denuded rings	6.40 ± 1.79	5
Denuded rings + SR 58611	11.95 ± 2.23	5

For each experimental condition, aortic rings were pre-contracted with phenylephrine in the presence or absence of L-NMMA or endothelium. In some rings, acetylcholine or cumulative concentrations of SR 58611 were added. At the end of the experiments, rings were frozen in order to measure intracellular cyclic GMP levels. Results show mean ± s.e.mean. $^{**}P<0.01$ *versus* phenylephrine.

was also reduced (39.3 ± 5.8% at 10 μM isoprenaline; $P<0.01$; Figure 4).

Relaxant effects of the β_3-adrenoceptor agonists, SR 58611, and CGP 12177

As shown in Figures 1C and 5, SR 58611 (0.1 – 30 μM) induced concentration-dependent relaxation of rat aortic rings contracted with phenylephrine (0.3 μM). This effect was similar to that observed with isoprenaline in the presence of nadolol. The relaxant effect of SR 58611 was initially slow, and maximal relevant response was achieved within 10 – 12 min (Figure 1C). As for the experiments in which isoprenaline was administrated after nadolol, the spontaneous relaxation obtained in control rings (18.1 ± 5.2% relaxant effect at the end of the experiment; $n=8$) was subtracted from the corresponding relaxant effect of the β_3-adrenoceptor agonist. In these conditions, the pD_2 value was 5.24 ± 0.07 ($n=11$) and the E_{max} value 59.5 ± 3.7% ($n=11$) at a concentration of 30 μM SR 58611 (Figure 5). Similar results were obtained with another contractile agent, $PGF_{2\alpha}$ (3 μM) (data not shown). CGP 12177, a partial β_3-adrenoceptor agonist which also possesses β_1- and β_2-adrenoceptor antagonistic properties (Blin *et al.*, 1993), produced similar concentration-dependent relaxation. The pD_2 value was 5.15 ± 0.03 ($n=6$) and the E_{max} value 63.9 ± 5.2% ($n=6$) at a concentration of 30 μM CGP 12177. To analyse whether SR 58611 produced relaxant effects through activation of β_3-adrenoceptors alone, concentration response curves for this agonist were also determined in the presence of the β-adrenoceptor antagonists, nadolol and SR 59230A (a β_3-adrenoceptor antagonist). The concentration-response curve to SR 58611 was not modified by 30 min pre-treatment with 10 μM nadolol ($pD_2=5.37\pm0.14$; $E_{max}=50.6\pm3.3\%$; $n=7$) (Figure 6), whereas the effects of SR 58611 were abolished in the presence of SR 59230A (10 μM; $n=6$; $P<0.01$ *versus* SR 58611 alone; Figure 6).

To characterize the involvement of the endothelium and of the NO synthase pathway in the vasorelaxant effect of β_3-adrenoceptor stimulation, the effect of endothelium removal and L-NMMA on the ability to modify contractile response to SR 58611 was determined. The relaxant effect of SR 58611 was shifted to the right after endothelium removal ($E_{max}=15.9\pm1.9\%$; $pD_2=4.88\pm0.03$; $n=7$; $P<0.01$ *versus* SR 58611 alone; Figure 7). The relaxant effect of SR 58611 was also markedly attenuated by 30 min pre-treatment with 100 μM L-NMMA (11.5 ± 1.8% at 10 μM SR 58611; $n=9$;

$P<0.01$ *versus* SR 58611 alone); the pD_2 value was 4.89 ± 0.02 ($n=9$). In these conditions, a relaxant effect of SR 58611 was only observed at the higher concentration of 30 μM ($27.0 \pm 2.5\%$; $n=9$) (Figure 7).

Intracellular cyclic GMP content

Intracellular cyclic GMP levels were measured in rat thoracic aorta rings at the end of the relaxation experiments in control and denuded rings pre-contracted with phenylephrine. As shown in Table 2, basal cyclic GMP levels were slightly lower in denuded rings and after 30 min pre-treatment with L-NMMA than in control rings. In rings with endothelium, cyclic GMP levels were increased 2 and 1.7 fold in the presence of acetylcholine (1 μM) and SR 58611 (30 μM) respectively. The SR 58611-induced increase in cyclic GMP levels was reduced by pre-treatment of aortic rings with L-NMMA (100 μM) or after removal of the endothelium.

Discussion

Several lines of evidence in this study show that activation of β_3-adrenoceptors significantly contributes to the relaxant effect of isoprenaline in rat thoracic aorta through activation of an endothelium-dependent NO synthase pathway. The relaxant effect of isoprenaline was still apparent after blockade of both β_1- and β_2-adrenoceptors by nadolol, and was similar to that exhibited both by the preferential β_3-adrenoceptor agonist, SR 58611, and by the partial β_3-adrenoceptor agonist, CGP 12177. The relaxant effect of SR 58611 was abolished by SR 59230A (a β_3-adrenoceptor antagonist) and greatly reduced by endothelium removal or by previous administration of L-NMMA. Finally, the increase in intracellular cyclic GMP levels observed in the same preparations after addition of SR 58611 was also abolished after previous administration of L-NMMA or endothelium removal.

Isoprenaline, a non-selective β-adrenoceptor agonist, induced rapid, concentration-dependent relaxation of rat thoracic aorta. Surprisingly, this effect persisted in the presence of nadolol, a potent β_1- and β_2-adrenoceptor antagonist with low affinity for native and recombinant β_3-adrenoceptors (Bond & Clarke, 1987; Emorine *et al*., 1989; Galitzky *et al*., 1993), but was shifted to the right with a reduction in the maximum. It is unlikely that this response was related to the competitive antagonism by nadolol, since the relaxation in the presence of nadolol was quite different from that obtained with isoprenaline alone. Thus, the relaxation developed more slowly and took a longer time to reach a steady-state than with isoprenaline alone. Thus, this relaxant effect may have been due to the stimulation of a third β-adrenoceptor subtype, possibly β_3, as suggested by our experiments conducted with two β_3-adrenoceptor agonists, SR 58611 and CGP 12177. The relaxant effect of these two agents was similar to the slowly developing relaxations induced by another β_3-adrenoceptor agonist, BRL 37344, in isolated common carotid arteries of the rat (Oriowo, 1994) or smooth muscle of the gastrointestinal tract (McLaughlin & MacDonald, 1990; 1991). The potency of SR 58611 and CGP 12177 in rat thoracic aorta was similar to that of BRL 37344 and CGP 12177 in rat isolated carotid arteries (Oriowo, 1994), but much lower than previous values reported for the rat gastrointestinal tract (MacLaughlin & MacDonald, 1991; De Boer *et al*., 1993). It is still unclear why β_3-adrenoceptor agonists have a poorer potency in vessels than in the gastrointestinal tract. In our study, the most convincing pharmacological evidence for the presence of β_3-adrenoceptors in rat thoracic aorta was provided by β-adrenoceptor antagonists. The relaxant effect of SR 58611 was not modified by pre-treatment with nadolol, indicating that this effect was not mediated by β_1- or β_2-adrenoceptors. This was confirmed by the relaxant effects achieved with CGP 12177, another β_1- or β_2-adrenoceptor antagonist. Conversely, SR 59230A, a β_3-adrenoceptor antagonist (Manara *et al*., 1995a; 1996), abolished the relaxant effect of SR 58611. To exclude a non selective effect of SR 59230 at the relatively high concentration used in our study, such as an activation of β_3-adrenoceptors (Strosberg & Pietri-Rouxel, 1996), we have performed some preliminary experiments with a lower concentration (1 μM) of this antagonist. In these experiments, the inhibition of SR 58611-induced relaxation by 1 μM SR 59230 persisted but was not as important as at 10 μM SR 59230 (data not shown). In rat pulmonary vessels, several β_3-adrenoceptor agonists (SR 58611, SR 59119 and SR 59104) have produced relaxant effects. However, only the effect of SR 59104 was antagonized by SR 59230A (Dumas *et al*., 1998), which suggests that the pharmacology of vascular atypical β-adrenoceptors is complex.

The involvement of β_3-adrenoceptors in the relaxation of rat thoracic aorta and carotid arteries is consistent with the effects of β_3-adrenoceptor stimulation reported *in vivo* in dogs and rats. β_3-adrenoceptor stimulation produced peripheral vasodilatation, primarily in skin and adipose tissues in unanaesthetized dogs (Berlan *et al*., 1994; Shen *et al*., 1994), and to a lesser extent in rats (Shen *et al*., 1996). In dogs, studies using radioactive microspheres showed a different pattern of regional blood flow distribution after β_3-adrenoceptor stimulation as compared with isoprenaline-induced β_1- and β_2-adrenoceptor-mediated peripheral vasodilatation. BRL 37344, a β_3-adrenoceptor agonist administered in the presence of a β_1- and β_2-adrenoceptor antagonist, selectively increased blood flows in skin and adipose tissues (Shen *et al*., 1994). Vasodilatation has also been observed in brown adipose tissue of anaesthetized rats after administration of BRL 26830A, another β_3-adrenoceptor agonist, in the presence of arotinolol, a mixed α-, β_1- and β_2-adrenoceptor antagonist (Takahashi *et al*., 1992). However, β_3-adrenoceptor agonists failed to induce vasodilatation in non-human primates (Shen *et al*., 1996).

In rat thoracic aortic rings, isoprenaline-induced relaxation persisted after endothelium removal, but only at higher concentrations and with a reduced maximal effect (Figure 2). In these conditions, addition of nadolol (10 μM) abolished the relaxant effect of isoprenaline (data not shown). Similarly, β_3-adrenoceptor-induced relaxation was strongly reduced by endothelium removal, which suggests that β_3-adrenoceptors are mainly located on endothelial cells. However, in isolated rat carotid arteries, the relaxation induced by two other β_3-adrenoceptor agonists (BRL 37344 and CGP 12177) was found to be endothelium-independent (Oriowo, 1994). Several factors could account for this discrepancy: (1) the vascular bed investigated; as previously described in the involvement of endothelium in β_1- and/or β_2-adrenoceptor mediated-relaxation (Gardiner *et al*., 1991; Gray & Marshall, 1992; Graves & Poston, 1993; Béa *et al*., 1994); (2) the contractile agent used, i.e. norepinephrine in experiments with carotid arteries (Oriowo, 1994) and phenylephrine in the present study; and (3) the action of β_3-adrenoceptor agonists on β_1- and/or β_2-adrenoceptors. BRL 37344 activates β_1- and β_2-adrenoceptors, as well as β_3-adrenoceptors, at high concentrations (Muzzin *et al*., 1992; Ida *et al*., 1996), and CGP 12177 may also stimulate the putative β_4-adrenoceptors previously described in the heart (Kaumann, 1996; 1997) and adipose tissue (Galitzky *et al*., 1997). Unfortunately, no monoclonal antibody selectively

directed against β_3-adrenoceptors is currently available to confirm the location of these receptors on endothelial cells.

Pre-treatment of rat thoracic aorta with L-NMMA partially inhibited isoprenaline-induced relaxation, and the addition of nadolol produced a further rightward shift in the concentration-response curve of the drug. SR 58611 also produced a slight relaxation in the presence of L-NMMA. These results strongly suggest that β_3-adrenoceptors mediate vascular relaxation through the activation of an NO synthase-dependent pathway. Conversely, both β_1 and β_2-adrenoceptors relax rat thoracic aorta through activation of an NO-dependent (Gray & Marshall, 1992; Wang *et al*., 1993) and/or -independent (Eckly *et al*., 1994) pathway. β_3-adrenoceptor-induced relaxation was associated with an increase in intracellular cyclic GMP levels, which was abolished by pre-treatment with L-NMMA. Thus, the activation of vascular β_3-adrenoceptors stimulated the NO synthase pathway, leading to an increase in intracellular cyclic GMP levels. An NO synthase pathway has also been described in human ventricle recently (Gauthier *et al*., 1998) in which β_3-adrenoceptor stimulation induced a negative inotropic effect secondary to increases in both NO production and cyclic GMP levels. These effects were blunted after NO synthase inhibition (Gauthier *et al*., 1998). Thus, the signalling pathway of β_3-adrenoceptors in both heart and vessels seems to be different from that described in adipose tissues where β_3-adrenoceptors, like β_1- and β_2-adrenoceptors, stimulate a cyclic AMP pathway (for review, see Strosberg, 1997).

The characterization of a β_3-adrenoceptor subtype in vessels, in addition to β_1 and β_2, raises the question of the role of these β_3-adrenoceptors in vasorelaxation. As the stimulation of these receptors produces vasorelaxation, as in the case with β_2-adrenoceptor stimulation, β_3-adrenoceptors could play a redundant function in vessels in which β_2- and β_3-adrenoceptors are co-expressed. It has been shown that catecholamines stimulate β_3-adrenoceptors in rat and dog fat cells at higher concentrations than those required to recruit β_1- and β_2-adrenoceptors (Granneman, 1992; Galitzky *et al*., 1993). Furthermore, β_3-adrenoceptors, unlike β_1- and β_2-subtypes, lack regulatory phosphorylation sites for G protein receptor kinases (Liggett *et al*., 1993) and could be relatively resistant to agonist-induced desensitization. Thus, β_3-adrenoceptors may be involved when the sympathetic nervous system is overstimulated.

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