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Characterization of β_3-adrenoceptor-mediated relaxation in rat abdominal aorta smooth muscle

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Abstract

The present study was carried out to characterize β-adrenoceptor subtypes mediating relaxation of rat abdominal aorta smooth muscle. (−)-Isoprenaline and a nonconventional β_3-adrenoceptor agonist, (±)-[4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one] hydrochloride ((±)-CGP12177A), induced concentration-dependent relaxation of (−)-phenylephrine (0.3 μM) preconstricted spiral preparations. Pretreatment with a combination of (±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate (CGP20712A, a selective β_1-adrenoceptor antagonist) and (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride (ICI-118,5511, a selective β_2-adrenoceptor antagonist) (0.1 μM for each) produced a 14-fold rightward shift of the concentration–response curve for (−)-isoprenaline; however, the relaxation in response to (±)-CGP12177A was unaffected by the blockade of β_1- and β_2-adrenoceptors. In the presence of CGP20712A and ICI-118,551 (0.1 μM for each), the concentration–response curves for (−)-isoprenaline and (±)-CGP12177A were shifted to the right by a nonselective β_1-, β_2- and β_3-adrenoceptor antagonist, (±)-bupranolol (3 and 10 μM). These results clearly suggest that β_3-adrenoceptors are involved in β-adrenoceptor-mediated relaxation of rat abdominal aorta smooth muscle.
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Keywords: β_3-Adrenoceptor; β-Adrenoceptor; (±)-Bupranolol; (±)-CGP12177A; Abdominal aorta, rat; Smooth muscle relaxation

1. Introduction

Adrenoceptors were originally classified into α- and β-adrenoceptors by Ahlquist (1948). Subsequently, β-adrenoceptors were subclassified into β_1- and β_2-adrenoceptors based on the relative potency of a series of sympathomimetic amines in various organ systems (Lands et al., 1967a,b). The authors proposed that vasodilatation in response to the stimulation of β-adrenoceptors was basically mediated through β_2-adrenoceptors (Lands et al., 1967a,b). Thus, β_2-adrenoceptors in a number of vascular smooth muscles are considered to play a predominant role in the regulation of vascular tone. However, later studies using more selective agonists and antagonists indicated that β_1-adrenoceptors mainly participate in the relaxation of coronary artery (O'Donnell and Wanstall, 1984a) and cerebral artery (Edvinsson and Owman, 1974), and that rat thoracic aorta and rat pulmonary artery contain a mixed population of β_1- and β_2-adrenoceptors, both mediating relaxation (O'Donnell and Wanstall, 1981, 1984b).

Recently, pharmacological studies and molecular cloning techniques have revealed that atypical β-adrenoceptors including β_3-adrenoceptors, which are different from classical β_1- and β_2-adrenoceptors, are the predominant β-adrenoceptors mediating both lipolysis in adipocytes (Hollenga and Zaagsma, 1989) and intestinal relaxation (Koike and Takayanagi, 1998; Manara et al., 1995; Roberts et al., 1999). In vascular smooth muscles, likewise, the involvement of such a ''third β-adrenoceptor subtype'' in β-adrenoceptor-mediated relaxation was suggested by the following results obtained in several tissues. Pindolol, which acts as a potent β_1- and β_2-adrenoceptor antagonist with β_3-adrenoceptor agonistic activity (Arch and Kau-

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mann, 1993; Horinouchi and Koike, 2001a,b), elicited relaxation of canine mesenteric vessel (Clark and Bertholet, 1983) and rat aorta (Doggrell, 1990), and these relaxant responses to pindolol were not significantly antagonized by propranolol, a nonselective β_1- and β_2-adrenoceptor antagonist. Thus, the presence of further β-adrenoceptor subtypes different from conventional β_1- and β_2-adrenoceptors was predictable from their pharmacological characteristics. Following this finding, propranolol-resistant relaxations in response to the stimulation of putative β_3- and/or atypical β-adrenoceptors with (−)-isoprenaline and a nonconventional partial β_3-adrenoceptor agonist, (±)-CGP12177A, were observed in a few vascular smooth muscles, including rat carotid artery (MacDonald et al., 1999; Oriowo, 1994, 1995) and rat thoracic aorta (Brawley et al., 2000a,b; Oriowo, 1995). In this way, evidence has now accumulated for the presence of β_3- and/or atypical β-adrenoceptors in the vascular system.

The present study was therefore carried out to characterize β-adrenoceptors mediating relaxation to (−)-isoprenaline and (±)-CGP12177A in rat abdominal aorta smooth muscle, with a special attention being paid to the possible contribution of β_3-adrenoceptors. Our present findings demonstrate that the activation of β_3-adrenoceptors produces potent relaxation in rat abdominal aorta and their characteristics in vascular smooth muscle appears to differ from those of well-characterized β_3-adrenoceptors in gastrointestinal smooth muscles.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 150–200 g (Murai Experimental Animals, Saitama, Japan) were used in the present study. Animals were housed under a 12-h light/dark cycle (lights on 8:00 a.m.; lights off 8:00 p.m.) and had free access to standard laboratory food and tap water. The room temperature and relative air humidity were strictly regulated at 22–24 °C and 50–60%, respectively. The present study was performed according to the Guideline for the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan), and the protocol of the present study was approved by the Institutional Animal Care and Use Committee.

2.2. Tissue preparation

Rats were stunned and killed by cervical dislocation. The abdominal aorta was isolated immediately and carefully cleaned of fat and connective tissues. The abdominal aorta was spirally cut into sections approximately 15 mm in length and 1 mm in width. The endothelium was removed by gentle rubbing of the intimal surface with a cotton swab. Endothelium removal was functionally confirmed by the absence of relaxation in response to acetylcholine (10 μM) in spiral preparations precontracted with (−)-phenylephrine (0.3 μM). Two spiral preparations were obtained from each aorta. These preparations were mounted vertically under an initial resting tension of 10 mN in a 20-ml organ bath containing Krebs solution composed as follows (mM): NaCl, 118; KCl, 4.75; $CaCl_2 \cdot 2H_2O$, 2.54; $MgSO_4$, 1.20; KH_2PO_4, 1.19; $NaHCO_3$, 25.0 and D-(+)-glucose, 11.0. Krebs solution was oxygenated continuously with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C (pH = 7.4). The following drugs were present in the bath solution throughout the experiments: indomethacin (10 μM, a cyclo-oxygenase inhibitor) and L-ascorbic acid (10 μM, an antioxidant for catecholamines).

2.3. Measurement of tension changes

Isometric tension changes of the muscle preparation were recorded with a force–displacement transducer (TB-612T, Nihon Kohden, Tokyo, Japan) connected to a carrier amplifier (AP-621G, Nihon Kohden). Preparations were allowed to equilibrate for 30 min before the start of experimental procedures.

2.4. Assessment of the effect of phentolamine against vasocontraction induced by (−)-phenylephrine

After equilibration for 30 min, (−)-phenylephrine was added cumulatively until a maximal contractile response was observed. The contraction induced by (−)-phenylephrine is expressed as a percentage of the maximal contraction produced by (−)-phenylephrine (0.3 μM). To assess the antagonistic effect of phentolamine, the antagonist was added to the bath 30 min before the addition of (−)-phenylephrine. The concentration–response curves for (−)-phenylephrine were then obtained in the presence of phentolamine.

2.5. Assessment of vasorelaxation induced by (−)-isoprenaline and (±)-CGP12177A

After equilibration of smooth muscle preparations for 30 min, each preparation was initially contracted with (−)-phenylephrine (0.3 μM) to test its contractile capacity, followed by addition of acetylcholine (10 μM) to test the lack of functional endothelium. Then drugs were washed out 10 times with Krebs solution. This protocol was carried out two times and baseline tone was readjusted to 10 mN when necessary.

After equilibration of smooth muscle preparations for 30 min, each preparation was initially contracted with (−)-phenylephrine (0.3 μM). A sustained plateau phase was observed approximately 30 min after the addition of (−)-

phenylephrine. Then the concentration–response relationship (0–10 μM) for the relaxation induced by (−)-isoprenaline was established twice. Drugs were washed out, and 30 min later, preparations were contracted again with (−)-phenylephrine (0.3 μM), and each of the β-adrenoceptor agonists ((−)-isoprenaline and (±)-CGP12177A) was cumulatively added to the bath medium in half-log increments until a maximum relaxant response was obtained. Then preparations were washed with Ringer solution 10 times. After the preparations were allowed to equilibrate for 30 min, the protocol for the next concentration–response curve was started.

2.6. Assessment of the effects of β-adrenoceptor antagonists on relaxation in response to (−)-isoprenaline and (±)-CGP12177A

To assess the contribution of β-adrenoceptor subtypes involved in the relaxant response to (−)-isoprenaline and (±)-CGP12177A, the preparation was incubated for 60 min (30 min before and 30 min during stabilization of the contractile response induced by (−)-phenylephrine) with the following β-adrenoceptor antagonists either alone or in combination: CGP20712A (a selective β_1-adrenoceptor antagonist), ICI-118,551 (a selective β_2-adrenoceptor antagonist), (±)-propranolol (a nonselective β_1- and β_2-adrenoceptor antagonist) and (±)-bupranolol (a nonselective β_1-, β_2- and β_3-adrenoceptor antagonist).

2.7. Drugs

The following drugs were used in the present study: (−)-isoprenaline hydrochloride, (−)-phenylephrine hydrochloride, (±)-propranolol hydrochloride, indomethacin, L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA); CGP20712A ((±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate), ICI-118,5511 ((±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), (±)-CGP12177A ((±)-[4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one] hydrochloride) (Research Biochemicals International, Natick, MA, USA), phentolamine mesylate (Novartis, Basel, Switzerland), acetylcholine chloride (Daiichi Pharmaceutical, Tokyo, Japan) and (±)-bupranolol hydrochloride (Kaken Pharmaceutical, Tokyo, Japan). The other chemicals used were of analytical grade. CGP20712A was dissolved in dimethylsulfoxide to form a stock solution of 20 mM, and further diluted in distilled water. Indomethacin was dissolved in 2% Na_2CO_3 to form a stock solution of 20 mM, and further diluted in distilled water. Final dimethylsulfoxide and Na_2CO_3 concentrations in the bath solution did not affect muscle responses (data not shown). All other drugs were dissolved in distilled water.

2.8. Data analysis

The results are expressed as means ± S.E.M. or mean values with 95% confidence intervals (CL) and *n* refers to the number of experiments. The contractile potency of (−)-phenylephrine and the relaxant potency of β-adrenoceptor agonists ((−)-isoprenaline and (±)-CGP12177A) are expressed as pEC_{50} values (negative logarithm of the effective drug concentration that produces a 50% response of the maximum response). The relaxant effect (Relaxation (%) in figures and E_{max} in tables) of each drug was calculated with respect to basal tension (100% relaxation) and steady-state contraction in response to contractile drugs (0% relaxation). The antagonistic potency of phentolamine, (±)-propranolol and (±)-bupranolol is expressed as a pA_2 value. It was calculated according to the method of Arunlakshana and Schild (1959).

Data were plotted as a function of drug concentration and fitted to the equation:

$$E = E_{max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H})$$

where E is % response at a given drug concentration, E_{max} is the maximum response, A is the concentration of drug, n_H is the slope function and EC_{50} is the effective drug concentration that produces a 50% response. Curve-fitting was carried out using GraphPad Prism™ (version 2.01, GraphPad Software, San Diego, CA, USA). The significance of the difference between mean values was evaluated with GraphPad Prism™ by paired or unpaired *t* test, or with unpaired *t* test with Welch's correction when necessary. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was also used. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Assessment of the effect of (−)-phenylephrine

(−)-Phenylephrine, an α_1-adrenoceptor agonist, is often used as a vasoconstrictor drug in order to observe β-adrenoceptor-mediated relaxation clearly. In the first place, the effect of (−)-phenylephrine was determined to confirm its contractile action in abdominal aorta smooth muscle of rat. As shown in Fig. 1, (−)-phenylephrine induced a concentration-dependent contraction in spiral preparations of rat abdominal aorta, with a pEC_{50} of 8.51 ± 0.03 (n = 3). The contractile response to (−)-phenylephrine was antagonized by phentolamine (0.01–0.1 μM, a nonselective α-adrenoceptor antagonist) in a concentration-dependent manner. Schild plot analysis for phentolamine against the contraction in response to (−)-phenylephrine gave a pA_2 value of 8.20 (95% CL: 8.06–

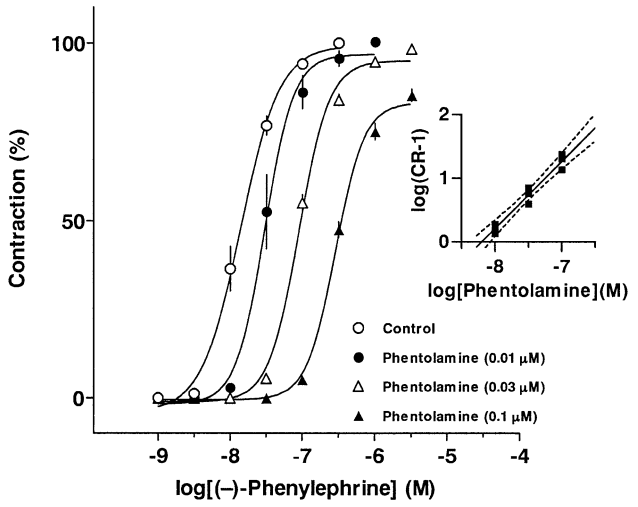

Fig. 1. Effects of phentolamine on concentration–response curves for (−)-phenylephrine in rat abdominal aorta smooth muscle. (−)-Phenylephrine was applied cumulatively to the bath solution in the absence (○) or presence of phentolamine (0.01 μM, ●; 0.03 μM, △; 0.1 μM, ▲). Ordinate: contraction (%), expressed as a percentage of the maximum contraction induced by (−)-phenylephrine (0.3 μM) in the absence of phentolamine; abscissa: the logarithm concentration (M) of (−)-phenylephrine. Each point represents the mean ± S.E.M. of three experiments. The inset shows the corresponding Schild plot.

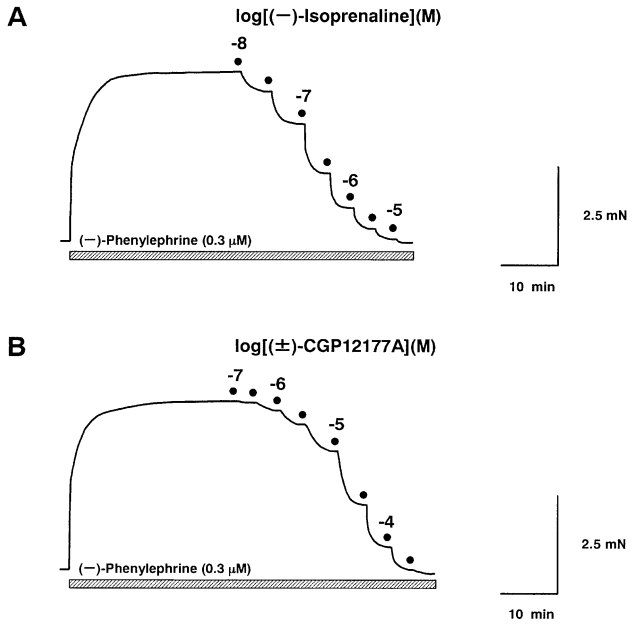

Fig. 2. Typical traces showing the relaxant effect of (−)-isoprenaline (A) and (±)-CGP12177A (B) in rat abdominal aorta spiral preparations precontracted with (−)-phenylephrine (0.3 μM). Closed circles indicate where (−)-isoprenaline or (±)-CGP12177A was added. Half-log unit increments in drug concentration were added.

8.35, $n=3$), and the Schild plot slope of 1.06 (95% CL: 0.86–1.26, $n=3$) was not significantly different from unity. These data indicate that (−)-phenylephrine activates mainly, if not exclusively, α-adrenoceptors in rat abdominal aorta, and can be used to induce reproducible contractions.

3.2. Relaxant response to (−)-isoprenaline and (±)-CGP12177A in rat abdominal aorta

Fig. 2A illustrates a typical trace of the relaxant response to (−)-isoprenaline in rat abdominal aorta spiral preparations precontracted by the stimulation of α_1-adrenoceptors with (−)-phenylephrine (0.3 μM). (−)-Isoprenaline produced a concentration-dependent relaxation with a pEC_{50} value of 7.26 ± 0.07 ($n=16$) and a maximum relaxation induced by 10 μM of 101.2 ± 1.0% ($n=16$) (Table 1).

As shown in Fig. 2B, (±)-CGP12177A, a nonconventional partial β_3-adrenoceptor agonist, also produced a concentration-dependent relaxation with a pEC_{50} value of 5.14 ± 0.06 ($n=18$) and a maximum relaxation induced by 300 μM of 101.8 ± 1.1% ($n=18$) (Table 1).

3.3. Resistance to desensitization of β_3-adrenoceptors by (±)-CGP12177A

It was noted that desensitization of β_3-adrenoceptors was observed with CGP12177A and BRL37344 (a selective β_3-adrenoceptor agonist) in cardiac tissues (Kaumann, 1996) and gastrointestinal smooth muscles (Oriowo, 1995), respectively, when concentration–response curves were repeated in the same tissues (see Summers et al., 1997, for desensitization mechanisms). In order to clarify whether (±)-CGP12177A evokes desensitization of β_3-adrenoceptors after its repeated application to rat abdominal aorta smooth muscle pretreated with a combination of CGP20712A and ICI-118,551 (0.1 μM for each), further successive cumulative concentration–response curves were established after

Table 1
Effects of different types of β-adrenoceptor antagonists on relaxant responses to (−)-isoprenaline in spiral preparations of rat abdominal aorta smooth muscle

	n	pEC_{50}	E_{max} (%)
(−)-Isoprenaline (β_1-, β_2-, β_3-stimulation)			
Control	16	7.26 ± 0.07	101.2 ± 1.0
CGP20712A (0.1 μM) + ICI-118,551 (0.1 μM) (β_1-, β_2-block)	11	6.11 ± 0.05[a,b]	94.2 ± 0.9[a]
CGP20712A (0.1 μM) + ICI-118,551 (0.1 μM) + (±)-bupranolol (3 μM) (β_1-, β_2-, β_3-block)	5	5.42 ± 0.08[b]	91.4 ± 2.5
CGP20712A (0.1 μM) + ICI-118,551 (0.1 μM) + (±)-bupranolol (10 μM) (β_1-, β_2-, β_3-block)	5	4.95 ± 0.07[b]	87.4 ± 3.1

Values are expressed as means ± S.E.M. of the number (n) of experiments.
[a] $P<0.05$, compared with control values (paired t test).
[b] $P<0.05$, significant differences between three values (ANOVA followed by Tukey's multiple comparison post test for three groups).

the control concentration–response curve was determined. The differences in the pEC_{50} value and the maximum relaxant response between control (1st application) and 2nd application were not significant ($P>0.05$), while a slight reduction in sensitivity was observed for the 3rd and 4th concentration–response curves ($P<0.05$) (Fig. 3, Table 2). Hence, two concentration–response curves for (±)-CGP12177A per tissue were measured in the following studies.

3.4. Effects of (±)-propranolol, a nonselective β_1- and β_2-adrenoceptor antagonist, on vasorelaxation elicited by (−)-isoprenaline and (±)-CGP12177A

(±)-Propranolol (0.01–0.1 μM), a nonselective β_1- and β_2-adrenoceptor antagonist, competitively antagonized the relaxant response to (−)-isoprenaline (Fig. 4A). The Schild plot analysis revealed the pA_2 value for (±)-propranolol against (−)-isoprenaline to be 8.58 (95% CL: 8.42–8.80, $n=7$). The slope of 0.99 (95% CL: 0.83–1.15, $n=7$) was not significantly different from unity. However, further addition of (±)-propranolol in a concentration ranging from 0.3 to 3 μM produced little or no further shift of the concentration–response curve for (−)-isoprenaline (Fig. 4A).

The relaxant response to (±)-CGP12177A was unaffected by a high concentration of (±)-propranolol (1 μM) ($P>0.05$) (Fig. 4B). The pEC_{50} value and the maximum relaxation elicited by (±)-CGP12177A in the presence of (±)-propranolol (1 μM) were 4.96 ± 0.06 ($n=4$) and 99.9 ± 0.07 ($n=4$), respectively.

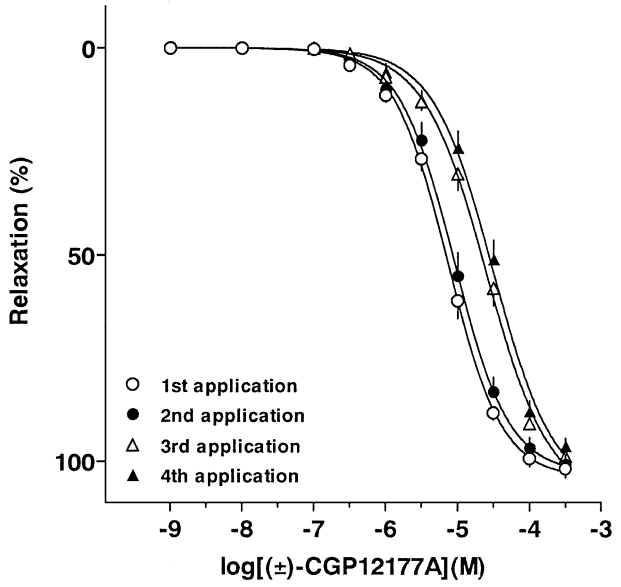

Fig. 3. Effects of repeated administration of (±)-CGP12177A to rat abdominal aorta spiral preparations pretreated with a combination of CGP20712A and ICI-118,551 (0.1 μM for each). Percentage of smooth muscle relaxation is calculated with respect to basal tension before application of contracting agents (100% relaxation) and (−)-phenylephrine (0.3 μM) steady-state contractile responses (0% relaxation). Data represent means ± S.E.M. of six experiments. When no error bar is shown, the error is smaller than the symbol.

Table 2
Effects of different types of β-adrenoceptor antagonists on relaxant responses to (±)-CGP12177A in spiral preparations of rat abdominal aorta smooth muscle

	n	pEC_{50}	E_{max} (%)
(±)-CGP12177A (β_3-stimulation, β_1-, β_2-block)			
Control			
1st application	18	5.14 ± 0.06	101.8 ± 1.1
2nd application	6	5.05 ± 0.08	100.7 ± 2.3
3rd application	6	4.66 ± 0.06[a]	98.8 ± 1.0
4th application	6	4.53 ± 0.06[a]	96.2 ± 1.7[a]
CGP20712A (0.1 μM)+ ICI-118,551 (0.1 μM) (β_1-, β_2-block)	32	5.07 ± 0.04[b]	101.0 ± 0.5
CGP20712A (0.1 μM)+ ICI-118,551 (0.1 μM)+(±)-bupranolol (3 μM) (β_1-, β_2-, β_3-block)	7	4.62 ± 0.04[b]	100.5 ± 0.8
CGP20712A (0.1 μM)+ ICI-118,551 (0.1 μM)+(±)-bupranolol (10 μM) (β_1-, β_2-, β_3-block)	9	4.19 ± 0.06[b]	103.4 ± 0.9

Values are expressed as means ± S.E.M. of the number (n) of experiments.
[a] $P<0.05$, compared with control (1st application).
[b] $P<0.05$, significant differences between three values (ANOVA followed by Tukey's multiple comparison post test for three groups).

3.5. Effects of selective β_1- and β_2-adrenoceptor antagonists on vasorelaxation elicited by (−)-isoprenaline and (±)-CGP12177A

CGP20712A (a selective β_1-adrenoceptor antagonist) and ICI-118,551 (a selective β_2-adrenoceptor antagonist) were used to clarify the β-adrenoceptor subtypes involved in (±)-propranolol-sensitive relaxation in response to (−)-isoprenaline in rat abdominal aorta smooth muscle. These antagonists at concentrations ranging from 0.01 to 0.3 μM produced small or no concentration-dependent rightward shifts of the concentration–response curve for (−)-isoprenaline (Fig. 5A,B). Schild plot analyses for these data yielded regression lines with slopes of 0.08 (95% CL: −0.03–0.20, $n=6$) for CGP20712A and 0.53 (95% CL: 0.40–0.67, $n=6$) for ICI–118,551, respectively (Fig. 5A,B), and all slopes were significantly different from unity.

Pretreatment with a combination of CGP20712A and ICI-118,551 (0.1 μM for each) produced a rightward shift of 14-fold in the concentration–response curve for (−)-isoprenaline with a slight reduction in the maximum response (Table 1). In contrast, the relaxant response to (±)-CGP12177A was unaffected by a combination of CGP20712A and ICI-118,551 (0.1 μM for each) (Table 2). The pEC_{50} value and maximum relaxation (E_{max}) are summarized in Tables 1 and 2.

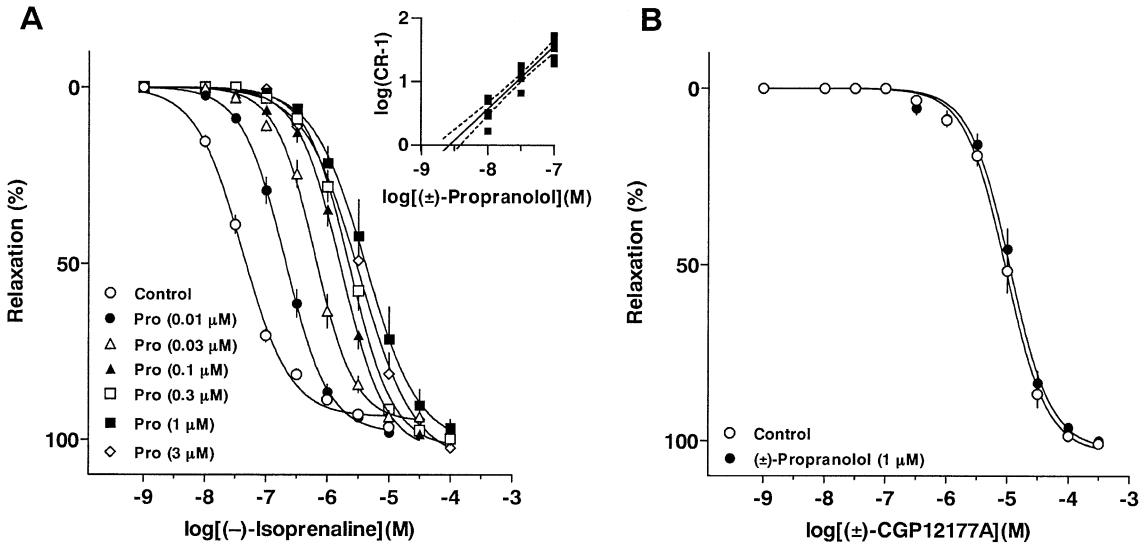

Fig. 4. Effects of (±)-propranolol on concentration–response curves for (−)-isoprenaline (A) and (±)-CGP12177A (B) in rat abdominal aorta spiral preparations precontracted with (−)-phenylephrine (0.3 μM). (−)-Isoprenaline (A) and (±)-CGP12177A (B) were applied cumulatively to the bath solution in the absence (○) or presence of (±)-propranolol (A: 0.01 μM, ●; 0.03 μM, △; 0.1 μM, ▲; 0.3 μM, □; 1 μM, ■; 3 μM, ◇; B: 1 μM, ●). Percentage of smooth muscle relaxation is calculated with respect to basal tension before application of contracting agents (100% relaxation) and (−)-phenylephrine (0.3 μM) steady-state contractile responses (0% relaxation). Data represent means ± S.E.M. of seven experiments. When no error bar is shown, the error is smaller than the symbol. The inset shows the corresponding Schild plot.

3.6. β_3-Adrenoceptor antagonistic effects of (±)-bupranolol in the presence of selective β_1- and β_2-adrenoceptor antagonists

In the presence of CGP20712A and ICI-118,551 (0.1 μM for each), the concentration–response curves for (−)-isoprenaline and (±)-CGP12177A were shifted to the right by (±)-bupranolol (3 and 10 μM) without a decrease in the maximum response (Fig. 6A,B; Tables 1 and 2). The Schild plot analyses gave the pA_2 values for the effects of (±)-bupranolol against these agonists to be 6.10 (95% CL: 5.90–6.50, (−)-isoprenaline) and 5.78 (95% CL: 5.66–5.97, (±)-CGP12177A). The slopes of 1.00 (95% CL: 0.70–1.31, (−)-isoprenaline) and 1.11 (95% CL: 0.81–1.40,

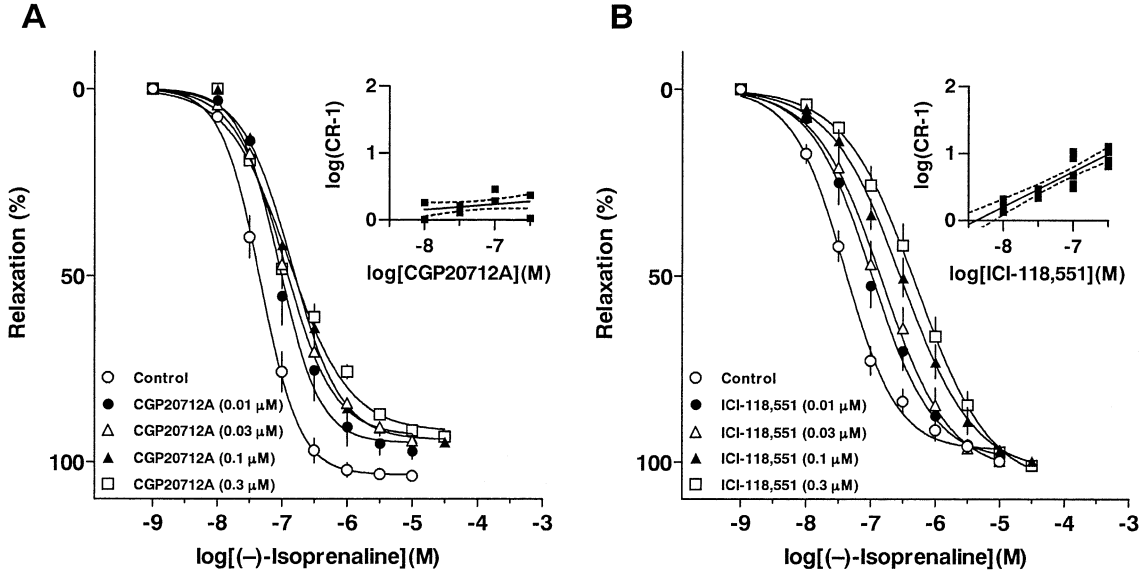

Fig. 5. Effects of CGP20712A (A) and ICI-118,551 (B) on concentration–response curves for (−)-isoprenaline in rat abdominal aorta spiral preparations precontracted with (−)-phenylephrine (0.3 μM). (−)-Isoprenaline was applied cumulatively to the bath solution in the absence (○) or presence of CGP20712A (A) or ICI-118,551 (B) (0.01 μM, ●; 0.03 μM, △; 0.1 μM, ▲; 0.3 μM, □). Percentage of smooth muscle relaxation is calculated with respect to basal tension before application of contracting agents (100% relaxation) and (−)-phenylephrine (0.3 μM) steady-state contractile responses (0% relaxation). Data represent means ± S.E.M. of six experiments. When no error bar is shown, the error is smaller than the symbol. The insets show the corresponding Schild plots.

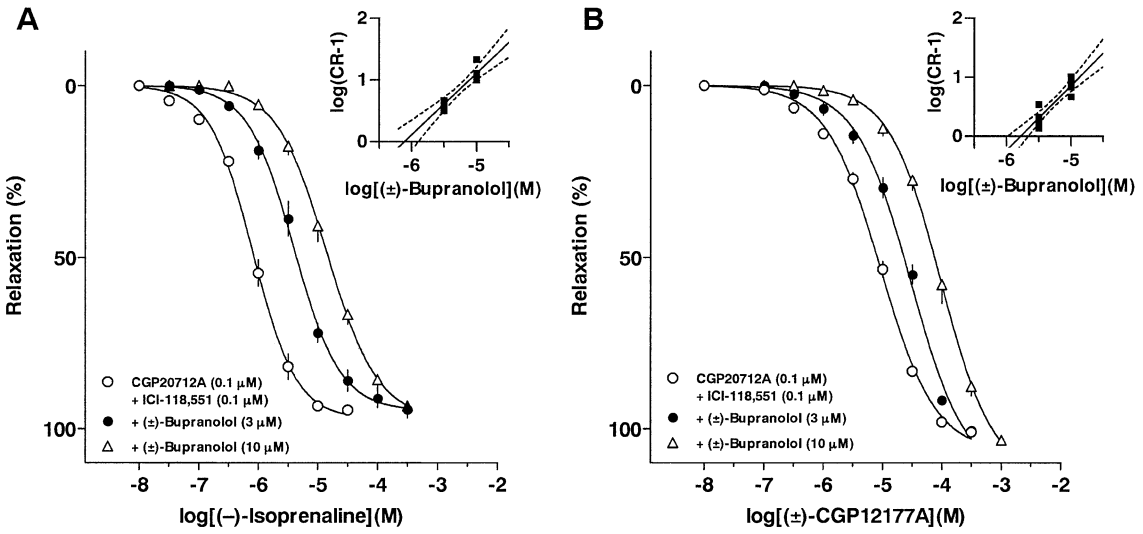

Fig. 6. Effects of (±)-bupranolol on concentration–response curves for (−)-isoprenaline (A) and (±)-CGP12177A (B) in rat abdominal aorta spiral preparations pretreated with a combination of CGP20712A and ICI-118,551 (0.1 μM for each). Each β-adrenoceptor agonist was applied cumulatively to the bath solution in the absence (○) or presence of (±)-bupranolol (3 μM, ●; 10 μM, △). Percentage of smooth muscle relaxation is calculated with respect to basal tension before application of contracting agents (100% relaxation) and (−)-phenylephrine steady-state contractile responses (0% relaxation). Data represent means ± S.E.M. of *n* experiments. The *n* values and the results are shown in Tables 1 and 2. When no error bar is shown, the error is smaller than the symbol. The insets show the corresponding Schild plot for effects of (±)-bupranolol against (−)-isoprenaline and (±)-CGP12177A.

(±)-CGP12177A) were not significantly different from unity (P>0.05) (Fig. 6A,B). Since higher concentrations of (−)-isoprenaline and (±)-CGP12177A were needed, we could not use (±)-bupranolol (30 μM) in the present study.

4. Discussion

The relaxation of vascular smooth muscles in response to the stimulation of β-adrenoceptors with (−)-isoprenaline is known to be mediated mainly through β_2-adrenoceptors. In the present study, the vasorelaxation induced by (−)-isoprenaline (a nonselective β-adrenoceptor agonist) or (±)-CGP12177A (a nonconventional partial β_3-adrenoceptor agonist) was examined by using several types of β-adrenoceptor antagonists in order to determine the contributions of different subtypes to the relaxation of rat abdominal aorta endothelium-denuded preparation.

4.1. Characterization of smooth muscle relaxation induced by (−)-isoprenaline and (±)-CGP12177A in rat abdominal aorta

(−)-Isoprenaline induced concentration-dependent relaxation of rat abdominal aorta smooth muscle precontracted by the activation of α_1-adrenoceptors with (−)-phenylephrine (0.3 μM). The relaxant response to (−)-isoprenaline was competitively antagonized by (±)-propranolol (0.01–0.1 μM), a nonselective β_1- and β_2-adrenoceptor antagonist. The estimated pA_2 value of 8.58 (95% CL: 8.42–8.80) for (±)-propranolol is in good agreement with the affinity values (as pA_2 or pK_i values) reported earlier, 8.6 at β_1-adrenoceptors and 8.3 at β_2-adrenoceptors (Arch and Kaumann, 1993). These findings suggest that β_1- and/or β_2-adrenoceptors are involved in (±)-propranolol-sensitive relaxation to (−)-isoprenaline in rat abdominal aorta smooth muscle. A further increase in the concentration of (±)-propranolol (0.3–3 μM) evoked little or no further antagonism of responses, suggesting the possible contribution of other β-adrenoceptor subtypes ((±)-propranolol-insensitive β-adrenoceptors) except β_1- and/or β_2-adrenoceptors, e.g., β_3-adrenoceptors, in the relaxation in response to (−)-isoprenaline of rat abdominal aorta.

(±)-CGP12177A acts as a β_3-adrenoceptor agonist with potent β_1- and β_2-adrenoceptor antagonistic activities (Arch and Kaumann, 1993; Horinouchi and Koike, 2001a). In rat abdominal aorta smooth muscle, (±)-CGP12177A also produced potent vasorelaxation of (−)-phenylephrine-precontracted preparations, but the relaxant response was unaffected by the pretreatment with (±)-propranolol (1 μM). The failure to inhibit the β_1- and β_2-adrenoceptor antagonistic effects with (±)-propranolol indicates that β_1- and β_2-adrenoceptors are not involved in (±)-CGP12177A-induced relaxation in this preparation. Moreover, the results strongly support the functional presence of β_3-adrenoceptors in rat abdominal aorta smooth muscle.

Such (±)-propranolol-sensitive and -insensitive smooth muscle relaxation was also observed in endothelium-intact ring preparations of rat thoracic aorta (Brawley et al., 2000b). Functional studies of rat thoracic aorta have shown that (±)-propranolol-sensitive (β_1- and/or β_2-) β-adrenoceptors are predominately present in endothelium compared with smooth muscle (Brawley et al., 2000b), and vascular smooth muscle relaxation induced by the activation of

endothelial β-adrenoceptors is mediated via nitric oxide release (Brawley et al., 2000b; Gray and Marshall, 1992). (−)-Isoprenaline and (±)-CGP12177A produced relaxation by stimulating mainly (±)-propranolol-insensitive (non β_1-, β_2-) atypical β-adrenoceptors in endothelium-denuded preparations of rat thoracic aorta (Brawley et al., 2000b). Hence, it is possible that, in contrast to rat abdominal aorta in the present study, (±)-propranolol-sensitive β-adrenoceptors are rarely present in smooth muscle of rat thoracic aorta.

4.2. (±)-Propranolol-sensitive vasorelaxation in response to (−)-isoprenaline in rat abdominal aorta smooth muscle

The results described above provided functional evidence that the vasorelaxation in response to (−)-isoprenaline was elicited due to the activation of (±)-propranolol-sensitive β-adrenoceptors and β_3-adrenoceptors ((±)-propranolol-insensitive) in rat abdominal aorta. In addition, simple competitive antagonism of (−)-isoprenaline by (±)-propranolol suggests the predictable involvement of β_1- and/or β_2-adrenoceptors in the (±)-propranolol-sensitive relaxant response to (−)-isoprenaline. Therefore, detailed studies with highly selective β_1- and β_2-adrenoceptor antagonists were performed to characterize the (±)-propranolol-sensitive component. CGP20712A (Dooley et al., 1986) and ICI-118,551 (Bilski et al., 1983) were used as tools to selectively block β_1- and β_2-adrenoceptors, respectively. Their affinity values (as pA_2 or pK_i values) at β_1-, β_2- and β_3-adrenoceptors are as follows: 8.5–9.3 (β_1-subtype), 5.4 (β_2-subtype) and <5.5 (β_3-subtype) for CGP20712A, and 7.2 (β_1-subtype), 8.3–9.2 (β_2-subtype) and <5.5 (β_3-subtype) for ICI-118,551 (Alexander et al., 2001; Arch and Kaumann, 1993; Kaumann and Molenaar, 1996). In rat abdominal aorta smooth muscle, CGP20712A and ICI-118,551 at concentrations (0.01–0.3 μM) enough to block β_1- and β_2-adrenoceptors, respectively, failed to cause concentration-dependent rightward shifts of concentration–response curves for (−)-isoprenaline. The Schild slopes of regression lines in Fig. 5A and B were significantly less than unity. According to the theory of Arunlakshana and Schild (1959), the Schild equation predicts a linear regression line with a slope of unity when a simple competitive antagonist binds to a homogeneous population of receptors. In terms of this theory, the present results obtained for rat abdominal aorta smooth muscles suggest that neither β_1- nor β_2-adrenoceptors are involved in the vasorelaxation in response to (−)-isoprenaline. However, two possible explanations for this phenomenon are as follows: (i) mixed relaxations mediated by both β_1- and β_2-adrenoceptors were apparently antagonized by (±)-propranolol in a concentration-dependent manner, and (ii) a new type of (±)-propranolol-sensitive β-adrenoceptor differing pharmacologically from conventional β_1-, β_2- and β_3-adrenoceptors may exist in rat abdominal aorta smooth muscles.

4.3. Functional dissimilarity between vascular β_3-adrenoceptors and gastrointestinal β_3-adrenoceptors

Cross-desensitization is a general phenomenon in several tissues pretreated with either CGP12177A or a selective β_3-adrenoceptor agonist, BRL37344 (Kaumann, 1996; Oriowo, 1995). For example, pretreatment of rat distal colon with BRL37344 (100 μM) for 30 min led to approximately 400-fold inhibition of isoprenaline-induced relaxation without a decrease in the maximum response (Oriowo, 1995). In the present study, however, β_3-adrenoceptors in rat abdominal aorta smooth muscle were resistant to desensitization by prolonged exposure to (−)-isoprenaline and (±)-CGP12177A, since both the potency and the maximum relaxation induced by the three agonists were unaltered when cumulative addition over 30 min was repeated twice in the same tissues. Thus, β_3-adrenoceptors of vascular smooth muscles mediating relaxation elicited by (−)-isoprenaline and (±)-CGP12177A are not identical to the well-characterized β_3-adrenoceptors of gastrointestinal smooth muscles. These results would suggest that the relative rate of desensitization of β_3-adrenoceptors induced by β-adrenoceptor agonists is essentially different between vascular and non-vascular smooth muscles.

4.4. Functional evidence for the presence of β_3-adrenoceptors in rat abdominal aorta smooth muscle

Thus, β_3-adrenoceptors are thought to be involved in the vasorelaxation in response to (−)-isoprenaline and (±)-CGP12177A. However, there is also a possibility that the relaxation in response to β_3-adrenoceptor agonists or non-conventional partial agonists is due to nonspecific blockade of α_1-adrenoceptors or interference with their signaling pathway (Brahmadevara et al., 2003). Therefore, it is necessary to clarify the site of action of (−)-isoprenaline and (±)-CGP12177A in the relaxation induced in the rat abdominal aorta.

To determine β_3-adrenoceptor-mediated responses, both the (−)-isomer and the racemate of bupranolol have frequently been used in cardiovascular tissues (Kaumann, 1996) and gastrointestinal smooth muscles (Horinouchi and Koike, 1999a,b, 2000; Koike and Takayanagi, 1998). Bupranolol at nanomolar concentrations blocks β_1- and β_2-adrenoceptors with high affinity (Arch and Kaumann, 1993), and high concentrations (μM order) of bupranolol also have sufficient affinity for atypical β-adrenoceptors associated with blockade of the pharmacological response to (±)-CGP12177A: e.g., β_3-adrenoceptors in guinea-pig gastrointestinal smooth muscles (Horinouchi and Koike, 1999a,b, 2000) and atypical β-adrenoceptors in rat thoracic aorta (Brawley et al., 2000a,b). In rat abdominal aorta pretreated with CGP20712A and ICI-118,551 (0.1 μM for each) in combination, the vasorelaxation in response to (−)-isoprenaline and (±)-CGP12177A was antagonized by (±)-bupranolol (3 and 10 μM) in a concentration-dependent manner, indicating the

possible contribution of β_3-adrenoceptors to the relaxation. Estimated pA_2 values of 6.10 ((−)-isoprenaline) and 5.78 ((±)-CGP12177A) for (±)-bupranolol in the present study are comparable to those reported earlier: 6.02–6.08 against (−)-isoprenaline and 5.70–5.80 against (±)-CGP12177A in β_3-adrenoceptors of guinea-pig gastrointestinal smooth muscles (Horinouchi and Koike, 1999a,b, 2000). Furthermore, the slopes of Schild plots were not different from unity. Thus, the present observation clearly suggests that, in the presence of β_1- and β_2-adrenoceptor antagonists, relaxant responses to (−)-isoprenaline and (±)-CGP12177A in rat abdominal aorta smooth muscle are mediated by β_3-adrenoceptors and not by nonspecific blockade of α_1-adrenoceptors.

In rat thoracic aorta, the (−)-isomer of bupranolol inhibited the relaxation in response to (−)-isoprenaline in a different manner (Brawley et al., 2000a). (−)-Bupranolol (5–30 μM) shifted the concentration–response curve for (−)-isoprenaline and reduced the maximum response induced by 100 μM in rat thoracic aorta treated with (±)-propranolol (0.3 μM) (Brawley et al., 2000a). Moreover, apparent pA_2 values of 5.0–5.2 for (−)-bupranolol (the pA_2 values can be converted to "4.7–4.9" for (±)-bupranolol) in rat thoracic aorta (Brawley et al., 2000a) were smaller than pA_2 values for (±)-bupranolol obtained in rat abdominal aorta (6.10 against (−)-isoprenaline; 5.78 against (±)-CGP12177A; in the present study). The different antagonism exerted by bupranolol in thoracic and abdominal aorta in rat suggests the possibility that each β_3-adrenoceptor (atypical β-adrenoceptors) is essentially heterogeneous.

4.5. Conclusion

In summary, the present study with (±)-bupranolol as competitive antagonist provided functional evidence that the relaxation of rat abdominal aorta in response to (−)-isoprenaline and (±)-CGP12177A is mediated through the activation of β_3-adrenoceptors.

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