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Evidence for two atypical conformations of beta-adrenoceptors and their interaction with Gi proteins

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Abstract

In this study, we investigated whether the responses of right atria from sinoaortic denervated rats to CGP12177 (4(3-*t*-butylamino-2-hydroxypropoxy benzidimidazole-2 one, hydrochloride)), isoprenaline and norepinephrine desensitized in parallel and whether CGP12177 interacted with distinct conformations of β-adrenoceptors. Right atria from rats 48 h after sinoaortic denervation were subsensitive to isoprenaline, norepinephrine and CGP12177. One week after sinoaortic denervation, the sensitivity to CGP12177 had recovered whereas the responses to isoprenaline and norepinephrine were still subsensitive, suggesting that the binding sites for these molecules showed independent behavior. In atria from 48 h sinoaortic-denervated rats, propranolol or 3 μM CGP20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((methyl-4-trifluormethyl)1H imidazole-2-yl)-phenoxypropyl) amino) ethoxy)-benzamide monomethane sulphonate)) blocked the responses to 10 nM–1 μM CGP12177 and steepened the curves. The concentration–response curves to CGP12177 in the presence of ICI118,551 (*erythro*-DL-1(-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol) were biphasic, suggesting that CGP12177 interacted with at least two conformations of β-adrenoceptors that showed negative cooperativism, one acting through β_2-adrenoceptor-Gi and the other via β_1-adrenoceptor-Gs. This hypothesis was confirmed in right atria from sinoaortic-denervated rats treated with pertussis toxin.

Keywords: β_1-Adrenoceptor; β_4-Adrenoceptor; Sinoaortic denervation; CGP12177

1. Introduction

Cardiac tissues contain β_1- and β_2-adrenoceptors (Carlson et al., 1972). In rats, the chronotropic and inotropic responses to neurally released and circulating norepinephrine are mediated by the β_1-adrenoceptor subtype (Juberg et al., 1985) while the responses to high concentrations of epinephrine are also mediated by β_2-adrenoceptors (Kaumann, 1986).

Kaumann (1989) proposed that some compounds, termed "non-conventional agonists", may mediate their effects in cardiac tissue through a β-adrenoceptor distinct from β_1- and β_2-adrenoceptors, and suggested that this receptor should be identified as a "putative β_4-adrenoceptor". The cardiostimulant effect of CGP12177A (4(3-*t*-butylamino-2-hydroxypropoxy benzidimidazole-2 one, hydrochloride)), a non-conventional agonist, is unaffected by the β_1- and β_2-adrenoceptor antagonist (−)-propranolol (200 nM) but is blocked with moderate affinity by bupranolol and CGP20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((methyl-4-trifluormethyl)1H imidazole-2-yl)-phenoxypropyl) amino) ethoxy)-benzamide monomethane sulphonate)) (Kaumann and Molenaar, 1996, 1997). Additionally, Kaumann and Lynham (1997) have suggested that the putative β_4-adrenoceptor is coupled to adenylate cyclase, by a Gs-protein.

Despite evidence from functional and binding studies indicating the existence of a putative β_4-adrenoceptor, the responses originally attributed to putative β_4-adrenoceptors may also be produced by β_1-adrenoceptors (Lowe et al., 1999). An alternative hypothesis is that the β_1-adrenoceptor and the putative β_4-adrenoceptor may use the same signal-

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ling pathway (Kompa and Summers, 1999). Since a β_4-adrenoceptor gene has not yet been identified, and since a cardiac response to CGP12177 has not been demonstrated in β_1/β_2-adrenoceptor knockout mice (Kaumann et al., 2001), the response to CGP12177 seems to be definitely attributable to a low affinity site located in the β_1-adrenoceptor (Arch, 2002).

We have previously shown that right atria from stressed rats have a lower sensitivity to the chronotropic effects of norepinephrine (Marcondes et al., 1996) and a decreased affinity for β_1-adrenoceptor selective antagonists (Santos, I.N., Marcondes, F.K., Spadari-Bratfisch, R.C., unpublished data). This subsensitivity is accompanied by an increase in the sensitivity to non-selective β-adrenoceptor agonists (Bassani and De Moraes, 1987; Marcondes et al., 1996; Vanderlei et al., 1996). Moreover, right atria from stressed but not from control rats responded to nanomolar concentrations of TA2005 (8-hydroxy-5-[(1R)-1-hydroxy-2-[*N*-[(1R)-2-(*p*-methoxy-phenyl)-1-methylethyl] amino] ethyl] carbostyril hydrochloride), a β_2-adrenoceptor selective agonist (Voss et al., 1994), and these responses were abolished by ICI118,551 (50 nM).

Based on these data, we suggested that foot-shock stress induces a β_2-adrenoceptor subtype-mediated response in rat right atria simultaneously with a decrease in the response mediated by β_1-adrenoceptors (Spadari-Bratfisch et al., 1999), whereas the response to CGP12177 was unaffected (Santos and Spadari-Bratfisch, 2001; Santos et al., 2003). Myslivecek et al. (2003) have shown that the densities of the β_1-adrenoceptor and the propranolol-resistant [^{3}H]-CGP12177 binding sites are differentially regulated. We also proposed that the putative β_4-adrenoceptor is different from a low-affinity state of β_1-adrenoceptors, unless it is assumed that the two proposed conformations show independent behavior (Santos and Spadari-Bratfisch, 2001). Recently, atypical β-adrenoceptors and β_2-adrenoceptors were shown to relax the rat mesenteric artery, with the atypical β-adrenoceptor being different from the low affinity state of the β_1-adrenoceptor (Koslowska et al., 2003), suggesting that the β_1-adrenoceptor may present polymorphism. Moreover, human β_1-adrenoceptors may show polymorphism that is associated with pathological conditions (Sarsero et al., 2003).

Sinoaortic denervation (SAD) disrupts the baroreceptor-mediated regulation of heart rate and blood pressure (Krieger, 1964), causing tachycardia and labile hypertension (Vasquez and Krieger, 1980). Although blood pressure lability persists for months, tachycardia is transient and usually returns to near control rates within 2 weeks after SAD. During this period, right atria are subsensitive to β-adrenoceptor agonists because of the down-regulation of β_1-adrenoceptors (Zanesco et al., 1997).

In this study, we examined the chronotropic response to CGP12177A in right atria from sinoaortic-denervated rats to determine whether the response to this non-conventional agonist was modified in parallel with the down-regulation of β_1-adrenoceptors and whether in this pathological condition CGP12177 interacted with distinct active conformations or states of the β_1-adrenoceptor.

2. Methods

2.1. Animals

Male Wistar rats (*Rattus norvergicus*, 300–350 g) were housed in standard cages in a temperature-controlled room (22 °C), on a 12 h light/dark cycle with the lights on at 6:30 a.m. Standard laboratory chow and tap water were available ad libitum. During the experiments, the animals were cared for in accordance with the principles reported by Olfert et al. (1993) and the experimental protocols were approved by the Animal Care Committee (CEEA) of the Institute of Biology, UNICAMP.

2.2. Surgical procedures

All surgical procedures were done under aseptic conditions following anesthesia with ketamine (50 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.). Bilateral SAD was done as described by Krieger (1964). Briefly, after the induction of anesthesia, the external and internal branches of the carotid arteries were exposed. The vagas nerve, the sympathetic trunk and surrounding connective tissue were gently dissected away from the vessels, the superior laryngeal nerve was resected and a section of the sympathetic trunk removed. Sham surgery consisted of the same procedures used to expose and free the arteries, but without denervation.

After SAD or sham surgery, catheters containing sterile saline were placed in the left femoral vein and artery for the subsequent administration of drugs and the recording of blood pressure and heart rate. The vein and artery were catheterized using sterile PE-50 and PE-10 tubing, respectively, and the catheters were exteriorized in the dorsal neck region. Following surgery, all rats were treated with benzathine penicillin (100,000 U, i.m.) to prevent infection.

Twenty-four hours before being killed, the efficacy of baroceptor deafferentation was evaluated. The rats received an intravenous injection of phenylephrine (1.5–3.0 μg/kg) to elicit at least a 50 mm Hg increase in arterial pressure and were considered to have adequate baroreceptor denervation if the subsequent maximal decrease in heart rate was less than 30 beats/min. The bradycardia typically observed in sham or control rats was 60–120 beats/min after the same dose of phenylephrine.

2.3. Treatment with pertussis toxin

Carbachol-induced cardiodepression is mediated by Gi protein-coupled M_2 receptors (Caulfied and Birdsall, 1998)

and, since pertussis toxin (PTX) inactivates Gi, treatment with this toxin would be expected to abolish carbachol-induced cardiodepression (Heubach et al., 2002). To examine the involvement of Gi proteins in the responses to CGP12177, rats were treated with pertussis toxin (PTX; 10 μg/kg, i.p.) for 3 days, with the first dose given 24 h before sinoaortic denervation in the SAD group. Twenty-four hours after the third dose of PTX, the rats were sacrificed and cumulative concentration–response curves to CGP12177 were obtained in isolated right atria, as described below. To assess the effectiveness of the treatment with PTX, right atria were incubated with 20 μM carbachol for 5 min shortly after mounting, followed by washing and equilibration for 90 min.

2.4. Organ-bath studies

The rats were killed by a blow to the back of the head and exsanguinated. The hearts were immediately removed and the right atria isolated and suspended in 20 ml organ baths containing Krebs–Henseleit solution of the following composition (in mM): NaCl 115.0, KCl 4.6, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 2.5, $NaHCO_3$ 25.0, glucose 11.0 and ascorbic acid 0.1. The solution was warmed (36.5 ± 0.1 °C) and continuously gassed with 95% O_2–5% CO_2 (pH 7.2–7.4). The atria were attached to isometric force transducers (Narco F-60, Narco Biosystems, Houston, TX, USA), under a resting tension of 5 mN. The tissues were allowed to stabilize for 1 h during which the bathing medium was changed at 15 min intervals.

Complete cumulative concentration–response curves (Van Rossum, 1963) to isoprenaline, norepinephrine and (±)-CGP12177A (4(3-*t*-butylamino-2-hydroxypropoxy benzidimidazole-2 one, hydrochloride)) were obtained by stepwise 0.5 log unit increases in the agonist concentration, in the absence of any antagonist. After this curve, the preparation was washed with Krebs–Henseleit solution to remove the agonist and to allow recovery of the initial beating rate. Antagonist was then added and left in contact with the tissue for 2 h before another concentration–response curve was obtained, using the same agonist, in the presence of antagonist. CGP20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((methyl-4-trifluormethyl)1H imidazole-2-yl)-phenoxypropyl) amino) ethoxy)-benzamide monomethane sulphonate), at concentrations of 1 and 3 μM, was used to antagonize the effect of (±)-CGP12177A (Kaumann and Molenaar, 1996). Propranolol (200 nM) or CGP20712A (10 nM) plus ICI118,551 (50 nM; *erythro*-DL-1(-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol) were used to antagonize β_1- and β_2-adrenoceptor-mediated responses (Kaumann, 1996; Kaumann and Molenaar, 1996; Kompa and Summers, 1999). A maximum response was reached when a 0.5 log unit increase in the agonist concentration produced no additional increase in the frequency of atrial beating. The experiments ended with the administration of a saturating concentration of isoprenaline (400 μM).

2.5. Analysis of the concentration–response curves

Changes in the sensitivity to agonists were evaluated by determining the concentration that produced a response that was 50% of the maximum response (EC_{50}). This calculation was done using the software Graph Pad Prism (GraphPad Software, San Diego, CA, version 2.0). The data are presented as the mean negative logarithm of the EC_{50} (pD_2) ± S.E.M.

The concentrations of agonist producing a half-maximal response in the absence [A] and presence [A'] of antagonist were estimated (Arunlakshana and Schild, 1959) as follows:

$$\log(CR-1) = n\log[B] - \log K_B$$

where CR (concentration ratio) is [A']/[A], n is the slope, [B] is the concentration of the antagonist, and $-\log K_B$ is the estimated antagonist dissociation constant when n is not different from unity. The apparent molar equilibrium dissociation constant for the interaction of the antagonist with the receptor, K_B, was determined using the equation:

$$K_B = [B]/(CR-1).$$

The estimated dissociation constants are given as pK_B values, i.e., $-\log K_B$.

2.6. Pretreatment of atria in vitro

Concentration–response curves were obtained after incubating isolated right atria with phenoxybenzamine (10 μM) for 15 min to block α-adrenoceptors (Besse and Furchgott, 1976), extraneuronal uptake (Iversen and Wilson, 1972) and muscarinic receptors (Furchgott and Bursztyn, 1967). This period was followed by 45 min of thorough washing. After recovery of the spontaneous rate, corticosterone (30 μM) and desipramine (0.1 μM) were added and maintained in the organ-bath throughout the experiment to inhibit the extraneuronal (Iversen and Salt, 1970) and neuronal (Salt, 1972) uptake of agonist, respectively.

2.7. Statistical analysis

The results were expressed as the mean ± S.E.M. of the number of experiments indicated. The significance of differences was assessed using Student's *t*-test for unpaired samples when comparing two groups (control versus sinoaortic-denervated) and Student's paired *t*-test when comparing pD_2 values or maximum responses for the agonist in the absence and presence of antagonist. For multiple comparisons, analysis of variance (ANOVA) followed by the Tukey test was used (Zar, 1984). Differences were considered significant for $P<0.05$.

3. Results

The concentration–response parameters for isoprenaline, norepinephrine and CGP12177 in isolated right atria from control rats and from rats with SAD are summarized in Table 1. The spontaneous beating frequency of right atria from rats sacrificed 1 week after SAD ($P<0.05$) was lower than the controls, as previously reported (Zanesco et al., 1997). Right atria obtained from sham-operated rats 48 h or 7 days after surgery showed pD_2 values for isoprenaline (8.9 ± 0.17 and 8.72 ± 0.10, respectively), norepinephrine (7.95 ± 0.09 and 7.42 ± 0.08, respectively) and (±)-CGP12177 (6.85 ± 0.10) that were similar to the controls (Table 1). The potencies, but not the maximum responses, of isoprenaline and norepinephrine were significantly lower in right atria from 48 h and 1 week SAD rats when compared to the controls (Table 1).

The concentration–response curves for isoprenaline and norepinephrine are shown in Fig. 1A and B, respectively. The Hill slopes of the isoprenaline concentration–response curves were not significantly altered in right atria from 48 h or 1 week SAD rats (Table 1). However, there was an increase in the Hill slope of the norepinephrine concentration–response curve in right atria from 48 h SAD rats. CGP12177 produced a positive, concentration-dependent, chronotropic effect in right atria from control rats (Fig. 1C), with a maximum response that was around 53% of the maximum response to isoprenaline or norepinephrine (Table 1). The maximum response to CGP12177 and the Hill slopes of the respective concentration–response curves were lower in right atria from rats sacrificed 48 h after SAD compared to the control or 1 week SAD rats (Table 1; Fig. 1C). The potency of CGP12177A was lower in right atria from rats sacrificed 48 h after SAD compared to the control or 1 week SAD group.

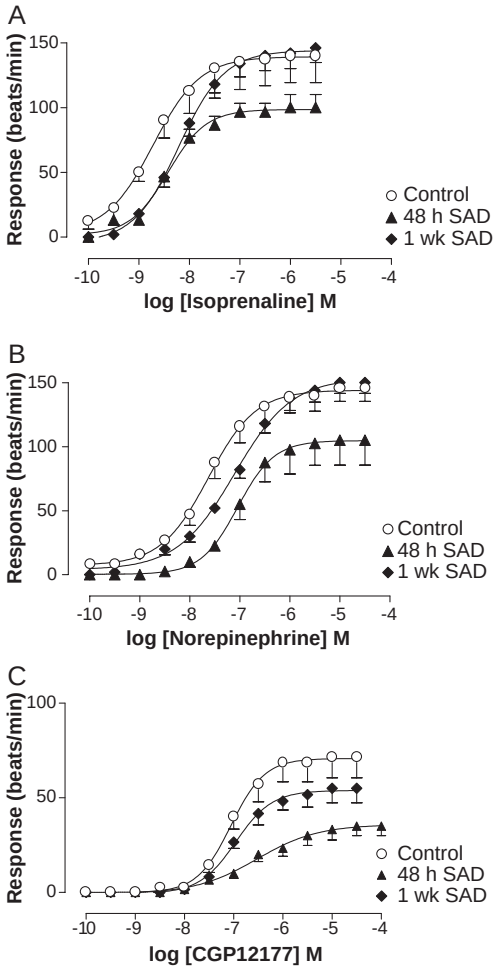

Fig. 1. Concentration–response curves for isoprenaline (A), norepinephrine (B) and (±)-CGP12177A (C) in right atria isolated from control rats and from rats sacrificed 48 h or 1 week after sinoaortic denervation (SAD). The points are the mean ± S.E.M. of the number of rats indicated in Table 1.

Table 1
Spontaneous beating rate (BR), pD_2 values for isoprenaline, norepinephrine and (±)-CGP12177, maximum response (MR) and the Hill slope of the concentration–response curves obtained in right atria from control and sinoaortic denervated (SAD) rats

	n	BR (beats/min)	pD_2	MR	Hill slope
Isoprenaline					
Control	4	283 ± 8	8.76 ± 0.09	140 ± 21	0.93 ± 0.07
48 h SAD	5	240 ± 17	8.40 ± 0.04[a]	122 ± 15	1.02 ± 0.09
1 week SAD	5	232 ± 7[a]	8.18 ± 0.07[a]	146 ± 11	1.07 ± 0.05
Norepinephrine					
Control	7	290 ± 6	7.62 ± 0.15	146 ± 10	0.81 ± 0.11
48 h SAD	4	270 ± 11	7.05 ± 0.03[a]	105 ± 19	1.18 ± 0.09[a,b]
1 week SAD	5	220 ± 6[a]	7.15 ± 0.08[a]	150 ± 8	0.70 ± 0.06
CGP12177					
Control	7	260 ± 7	7.04 ± 0.05	71 ± 11	1.31 ± 0.09
48 h SAD	6	235 ± 9	6.33 ± 0.07[a,b]	35 ± 5[a,b]	0.63 ± 0.11[a,b]
1 week SAD	6	210 ± 11[a]	6.95 ± 0.08	55 ± 8	1.39 ± 0.23

The values are the mean ± S.E.M. of the number of experiments (*n*) indicated.
[a] $P<0.05$ compared to the corresponding control group for each treatment.
[b] $P<0.05$ compared to the 1 week SAD group (ANOVA plus the Tukey test).

Propranolol did not alter the spontaneous beating rate of right atria from control rats (Table 2 versus Table 1), but decreased the spontaneous beating rate of right atria from 48 h SAD rats ($P<0.05$). In the presence of 200 nM propranolol, the concentration–response curve to isoprenaline shifted to the right in right atria from control and 48 h SAD rats (Fig. 2). The concentration–response curve to CGP12177 was resistant to blockade by 200 nM propranolol in right atria from control rats (Fig. 3A, Table 2). Moreover, the response to CGP12177 in the concentration range of 10 nM to 1 μM was completely blocked by 200 nM propranolol (Fig. 3B). The maximum response to CGP12177 (Table 2, Fig. 3B) returned to the same level as that seen in the control group in the absence of antagonist (Table 1). These combined effects resulted in a steeper concentration–response curve (Hill slope: 1.73 ± 0.12) for

Table 2
Spontaneous beating rate (BR), pD_2 values, maximum response (MR) and the Hill slope of concentration–response curves for isoprenaline and (±)-CGP12177 in the presence of 200 nM propranolol obtained in right atria from control and sinoaortic denervated (SAD) rats

	n	BR (beats/min)	pD_2	MR	Hill slope
Isoprenaline					
Control	3	243 ± 23	7.53 ± 0.23	203 ± 13	0.94 ± 0.27
48 h SAD	7	171 ± 12[a,b]	6.80 ± 0.27[a]	174 ± 13	0.92 ± 0.08
(±)-CGP12177					
Control	3	280 ± 6	6.87 ± 0.05	70 ± 17	1.08 ± 0.07
48 h SAD	4	158 ± 5[a,b]	5.28 ± 0.04[a,b]	73 ± 9	1.73 ± 0.12[a,b]

The values are the mean ± S.E.M. of the number of experiments (*n*) indicated.
[a] $P<0.05$ compared to the concentration–response curve for the corresponding β-AR agonist in the absence of antagonist (Table 1; Student's *t*-test).
[b] $P<0.05$ compared to the corresponding control group (Student's *t*-test).

CGP12177 that was shifted to the right 11-fold compared to the curve obtained in the absence of antagonist (Hill slope: 0.66 ± 0.06; Table 1).

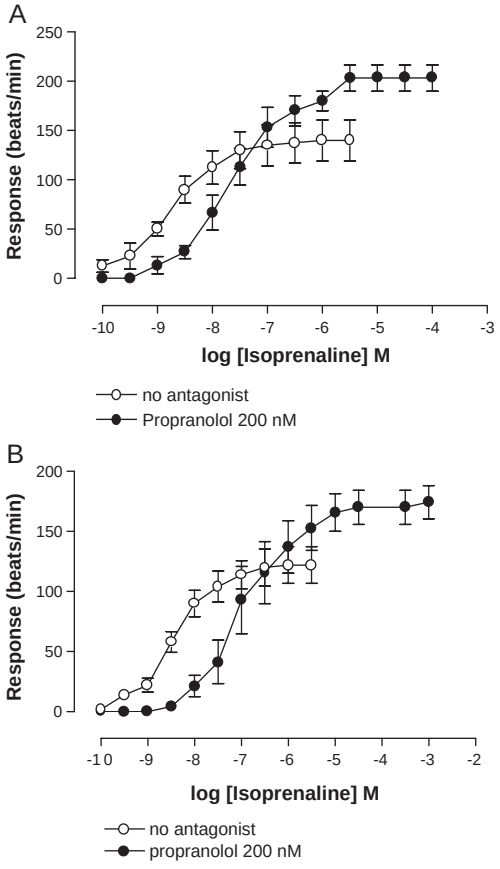

Fig. 2. Concentration–response curves for isoprenaline in the absence (○) or presence (●) of 200 nM propranolol in right atria isolated from control rats (A) and from rats sacrificed 48 h after sinoaortic denervation (SAD) (B). The points are the mean ± S.E.M. of the number of rats indicated in Table 2.

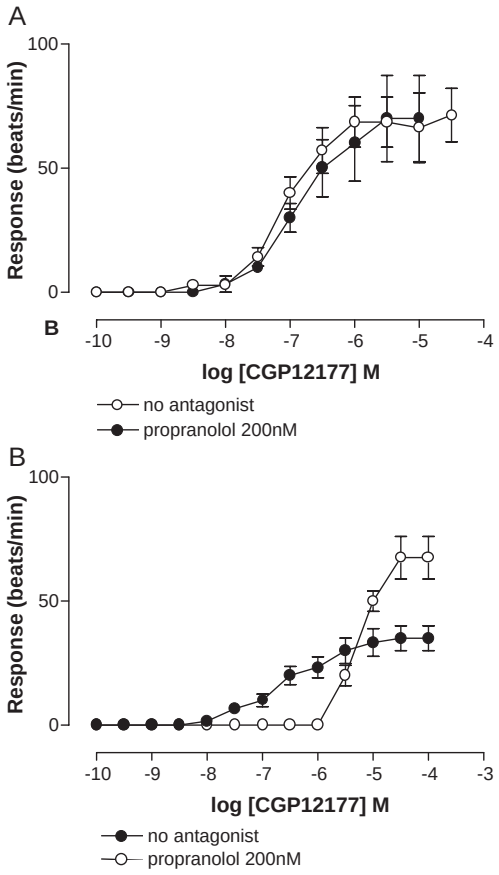

Fig. 3. Concentration–response curves for (±)-CGP12177 in right atria isolated from control rats (A) and from rats sacrificed 48 h after sinoaortic denervation (SAD) (B) in the absence (○) or presence (●) of 200 nM propranolol. The points are the mean ± S.E.M. of the number of rats indicated in Table 2.

Fig. 4 shows that, in right atria from control rats, CGP20712A caused a concentration-dependent shift to the right in the concentration–response curve to CGP12177. In right atria from 48 h SAD rats, the effect of 3 μM CGP20712A was similar to that of propranolol, i.e., blockade of the effect of CGP12177 in the concentration range of 10 nM to 1 μM, restoration of the maximum response to CGP12177 to control levels, and a 9.0-fold shift to the right in the concentration–response curve (Fig. 4D). Moreover, in this group, the estimated pK_B value for CGP20712A was dependent on the antagonist concentration (Table 3).

Concentration–response curves to (±)-CGP12177A were also obtained in the presence of 10 nM CGP20712A plus 50 nM ICI118,551 (Table 4 and Fig. 5). At these concentrations, the compounds antagonized β_1- and β_2-adrenoceptors, respectively (Dooley et al., 1986; O'Donnell and Wanstall, 1985). The double blockade did not affect the pD_2 value of CGP12177 in right atria from control rats (Table 4 versus Table 1), but caused a 13-fold shift to the left in the concentration–response curve to CGP12177 in right atria from 48 h SAD rats and restored the maximum response to control levels (Fig. 5B).

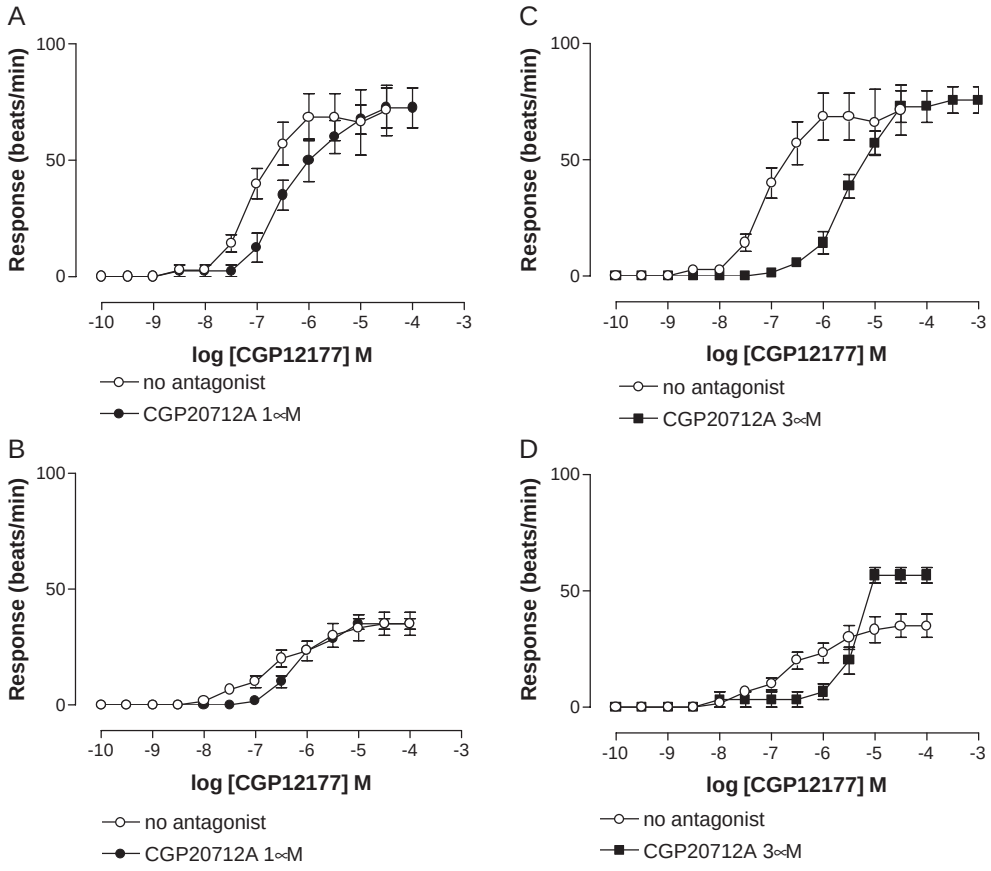

Fig. 4. Concentration–response curves for (±)-CGP12177A in right atria isolated from control rats (A and C) and from rats sacrificed 48 h after sinoaortic denervation (SAD) (B and D) in the absence (○) or presence of 1 μM (●) or 3 μM (■) CGP20712. The points are the mean ± S.E.M. of the number of rats indicated in Table 3.

The results shown in Figs. 4 and 5 suggested that the response to (±)-CGP12177 in right atria from 48 h SAD rats had at least two components: one that was blocked by CGP20712 and another that was sensitive to ICI115,881. Moreover, there could be negative cooperativism between the two β-adrenoceptor conformations or subtypes activated by (±)-CGP12177 in right atria from 48 h SAD rats. The blockade of one of these two components by 5 nM ICI118,551 yielded a concentration–response curve for (±)-CGP12177 that was clearly biphasic (Table 5 and Fig. 6B).

To confirm the involvement of a β_2-adrenoceptor-mediated response that is antagonized by ICI118,551 and that would act via Gi-protein, right atria were isolated from 48 h SAD treated with PTX. In these tissues, the responsiveness

Table 3
Spontaneous beating rate (BR), pD_2 values, maximum response (MR) and the Hill slope of concentration–response curves for (±)-CGP12177 in the presence of CGP20712A and apparent pK_B values for this β-adrenoceptor antagonist in right atria from control and sinoaortic denervated (SAD) rats

	n	BR (beats/min)	pD_2	MR	pK_B	Hill slope
1 μM CGP20712A						
Control	4	240 ± 6	6.38 ± 0.12[a]	73 ± 9	6.63 ± 0.14	1.01 ± 0.11
48 h SAD	6	217 ± 10	6.21 ± 0.06	35 ± 2[b]	5.97 ± 0.11[b]	1.08 ± 0.06
3 μM CGP20712A						
Control	7	223 ± 8[a]	5.48 ± 0.07[a]	76 ± 6	7.10 ± 0.07	1.22 ± 0.08
48 h SAD	3	203 ± 3	5.39 ± 0.09[a]	57 ± 3	6.40 ± 0.09[b]	2.88 ± 0.69[b]

The values are the mean ± S.E.M. of the number of experiments (*n*) indicated.
[a] $P<0.05$ compared to the concentration–response curve for CGP12177 in the absence of antagonist (Table 1; Student's *t*-test).
[b] $P<0.05$ compared to the corresponding control group (Student's *t*-test).

Table 4
Spontaneous beating rate (BR), pD_2 values, maximum response (MR) and the Hill slope of concentration–response curves for (±)-CGP12177 obtained in the presence of 10 nM CGP20712A plus 50 nM ICI118,551 in right atria from control and sinoaortic denervated (SAD) rats

	n	BR (beats/min)	pD_2	MR	Hill slope
Control	4	208 ± 8[a]	7.32 ± 0.04	80 ± 17	0.75 ± 0.04
48 h SAD	4	200 ± 4[a]	7.47 ± 0.09[a]	75 ± 5[a]	1.98 ± 0.23[a,b]

The values are the mean ± S.E.M. of the number of experiments (*n*) indicated.
[a] $P<0.05$ compared to the concentration–response curve for CGP12177 in the absence of antagonist (Table 1; Student's *t*-test).
[b] $P<0.05$ compared to the control group (Student's *t*-test).

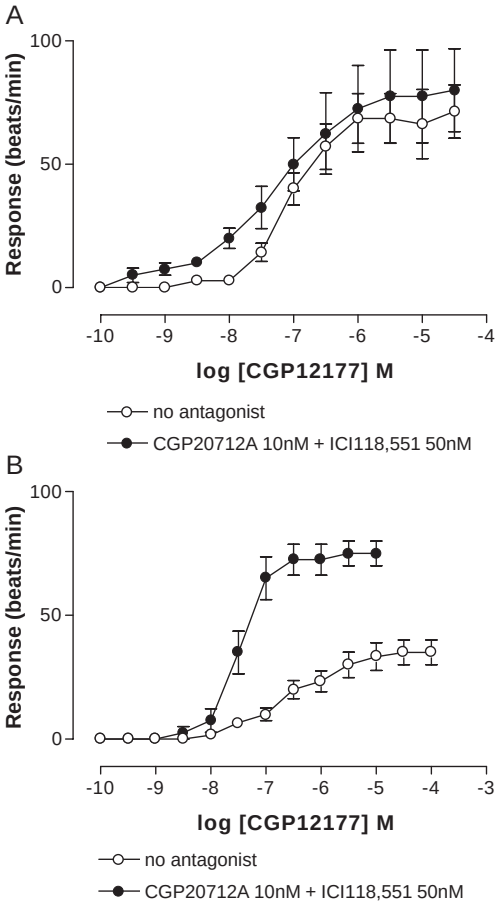

Fig. 5. Concentration–response curves for (±)-CGP12177A in right atria isolated from control (A), and 48 h sinoaortic denervation (SAD) (B) rats in the absence (○) or presence (●) of 10 nM CGP20712 + 50 nM ICI118,551. The points are the mean ± S.E.M. of four rats.

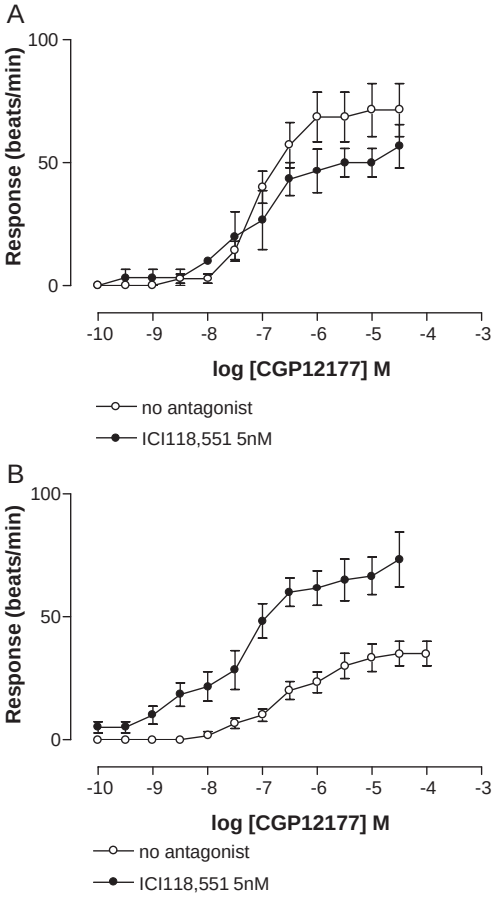

Fig. 6. Concentration–response curves for (±)-CGP12177A in right atria isolated from control (A), and 48 h sinoaortic denervation (SAD) (B) rats in the absence (○) or presence (●) of 5 nM ICI118,551. The points are the mean ± S.E.M. of the number of rats indicated in Table 5.

and sensitivity to CGP12177 were restored to the same levels as those seen in the control group (Table 6 and Fig. 7).

4. Discussion

As shown here, right atria isolated from 48 h SAD rats were subsensitive to the full agonists isoprenaline and norepinephrine, and also to the non-conventional partial agonist CGP12177. The subsensitivity to CGP12177 was more labile than that to the classic β-adrenoceptor agonists since it was not present 1 week after SAD, when the atria were still subsensitive to isoprenaline and norepinephrine because of β_1-adrenoceptor down-regulation (Zanesco et al., 1997). Non-conventional agonists function as antagonists with classic β_1- and β_2-adrenoceptors and as partial agonists with the putative β_4-adrenoceptor, which is now known to be a low affinity site on the β_1-adrenoceptor (for review, see Sarsero et al., 2003).

The interaction between CGP12177 and the putative "low affinity site of the β_1-adrenoceptor" is expected to be

Table 5
Spontaneous beating rate (BR), pD_2 values, maximum response (MR) and the Hill slope of concentration–response curves for (±)-CGP12177 obtained in the presence of 5 nM ICI118,551 in right atria from control and sinoaortic denervated (SAD) rats

	n	BR (beats/min)	pD_2	MR	Hill slope
Control	3	227 ± 23	7.07 ± 0.33	57 ± 9	1.35 ± 0.40
48 h SAD	6	203 ± 8	7.35 ± 0.21[a,b]	72 ± 12[a]	1.21 ± 0.66[a]

The values are the mean ± S.E.M. of the number of experiments (*n*) indicated.
[a] $P<0.05$ compared to the concentration–response curve for CGP12177 in the absence of antagonist (Table 1; Student's *t*-test).
[b] $P<0.05$ compared to the control group (Student's *t*-test).

Table 6
Spontaneous beating rate (BR), pD_2 values, maximum response (MR) and the Hill slope of concentration–response curves for (±)-CGP12177 pD_2 values obtained in right atria from control and sinoaortic denervated (SAD) rats treated with pertussis toxin (10 μg/kg i.p.) for 3 days prior to sacrifice

	n	BR (beats/min)	pD_2	RM (beats/min)	Hill slope
Control	4	253 ± 11	6.64 ± 0.11	88 ± 10	1.45 ± 0.50
48 h SAD	3	198 ± 6	6.69 ± 0.06	60 ± 4[a]	1.26 ± 0.21

The values are the mean ± S.E.M. of the number of experiments (*n*) indicated.
[a] $P<0.05$ compared to the control group (Table 1; Student's *t*-test).

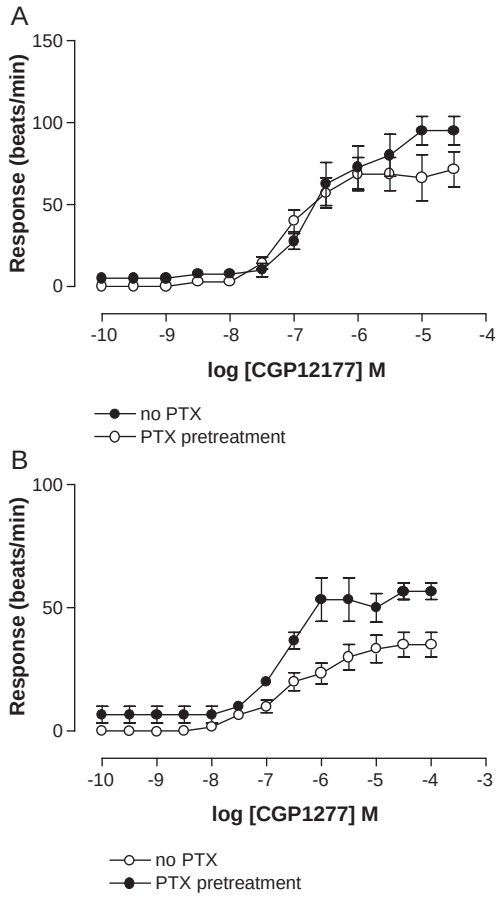

Fig. 7. Concentration–response curves for (±)-CGP12177A in right atria isolated from control (A), and 48 h sinoaortic denervation (SAD) (B) rats treated (○) or not treated (●) with pertussis toxin (10 μg/kg, i.p.) for 3 days prior to sacrifice. The points are the mean ± S.E.M. of the number of rats indicated in Table 6.

resistant to blockade by propranolol and CGP20712A at concentrations that block the classic β_1-adrenoceptor (Kaumann and Molenaar, 1996, 1997). Accordingly, in right atria from control rats there was a typical competitive antagonism between propranolol and isoprenaline, and the interaction of CGP12177 with the β-adrenoceptor population was resistant to blockade by 200 nM propranolol or 10 nM CGP20712 plus 50 nM ICI118,551. However, in right atria from 48 h SAD rats, propranolol caused significant rightward shifts in the concentration–response curves to isoprenaline and to CGP12177.

The apparent pK_B value for propranolol was 8.3 ± 0.27 when isoprenaline was the agonist. This pK_B value is consistent with the interaction of propranolol with β_1-adrenoceptor (Gille et al., 1985). However, when CGP12177 was the agonist, the apparent pK_B for propranolol was 7.70 ± 0.04. This value was higher than that reported for the interaction of propranolol with the putative "β_4"-adrenoceptor ($pK_B < 5.7$; Kaumann and Molenaar, 1996), and was also higher than the value obtained by Kompa and Summers (1999) in right atria from rats with myocardial infarction ($pK_B = 6.8 \pm 0.1$). On the other hand, the pK_B for propranolol from 48 h sinoaortic-denervated rats was situated between the pK_B for propranolol interacting with the classic β_1-adrenoceptor conformation ($pK_B = 8.5$; Gille et al., 1985) and with the novel β_1-adrenoceptor state proposed by Konkar et al. (2000) for CHO cells expressing only β_1-adrenoceptors ($pK_B = 7.2$).

Some compounds apparently interact predominantly with one of at least two forms of the β_3-adrenoceptor, with one conformation predominating in whole cells and the other being more common in membranes (Arch, 2002). The different pK_B values for propranolol obtained using a variety of approaches and experimental models suggest that a similar behavior may be attributed to the β_1-adrenoceptor or that in this model propranolol interacts with more than one subtype or conformation of β-adrenoceptors. In right atria from 48 h SAD rats, propranolol or 3 μM CGP20712 completely blocked the response to CGP12177 (10 nM–1 μM). Increasing the agonist concentration overcame the blockade by propranolol and restored the agonist maximum response to control levels. These results suggested a possible interaction of the agonist with two β-adrenoceptor subtypes, one sensitive and the other resistant to propranolol. A similar behavior has been suggested by Sarsero et al. (1999) to explain the interaction of cyanopindolol with β-adrenoceptors in rat ventricular papillary muscle. These authors subsequently proposed that β_1-adrenoceptor polymorphisms were associated with physiological differences that guaranteed the survival of patients with cardiac pathologies (Sarsero et al., 2003).

An atypical β-adrenoceptor different from the low affinity state of β_1-adrenoceptor has also been considered to be present in rat isolated mesenteric artery since the concentration–response curves to isoprenaline and fenoterol following the administration of propranolol were steeper than those obtained in the absence of the antagonist because the shift was greater at lower concentrations of agonist (Koslowska et al., 2003). In right atria from 48 h SAD rats, the maximum effect of CGP12177 was higher in the presence of propranolol or 3 μM CGP20712, indicating a negative cooperativism between the two β-adrenoceptor conformations. This observations suggests that one of the β-adrenoceptor conformations activated by CGP12177 could interact with Gi proteins, thereby reducing the chronotropic effect caused by the interaction of CGP12177 with the other conformation that would interact only with Gs. In this case, blockade of the Gi-linked conformation would restore the maximum response to CGP12177 via the other conformation.

In the presence of ICI118,551 (5 nM), the CGP12177 concentration–response curve was biphasic, with 38% of the effect of CGP12177 corresponding to the activation of one β-adrenoceptor conformation, probably the β_1-adrenoceptor low affinity conformation that is sensitive to propranolol (Fig. 3B). Blockade with ICI118,551 (5 nM) or with ICI118,551 (50 nM) plus CGP20712A (10 nM) restored the maximum response to CGP12177 to control levels and

shifted the concentration–response curve to CGP12177 to the left, suggesting the activation of Gi by β_2-adrenoceptors. To test this hypothesis, 48 h SAD rats were treated with PTX. The inactivation of Gi restored the sensitivity and the maximum response to CGP12177 to control levels. These results indicated that in this experimental model, CGP12177 activated β_2-adrenoceptors that interacted with Gi, thereby reducing the chronotropic effect caused by interaction of the same agonist with the low affinity site of β_1-adrenoceptors that interacts only with Gs.

The interaction of CGP12177 with β_2-adrenoceptors has already been proposed to occur (Pak and Fishman, 1996; Baker et al., 2002). The coupling of β_2-adrenoceptors to Gi proteins may play a cardioprotective role against Gs-protein-mediated cardiac overstimulation, especially in patients with heart failure (Xiao, 2000). The main function of this pathway may be to activate anti-apoptosis mechanisms to protect cells against the damage induced by chronic or repeated β_1-adrenoceptor activation during stress since β_2-adrenoceptor-Gi coupling has only a small negative effect on the beating rate of myocytes (Devic et al., 2001). The simultaneous coupling of a receptor to more that one G-protein is frequently more evident in high-density recombinant receptor models (Eason et al., 1992; Kenakin, 1995). The results presented here provide a demonstration of this mechanism operating in a pathological condition in wild-type rats rather than in cultured myocytes or genetically modified mice. Our findings also indicate that this system is transitory since it is not present in right atria from rats sacrificed 1 week after surgery, when β_1-adrenoceptors have already been down-regulated.

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