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(−)-CGP 12177 Causes Cardiostimulation and Binds to Cardiac Putative β_4-Adrenoceptors in Both Wild-Type and β_3-Adrenoceptor Knockout Mice

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ABSTRACT

Some blockers of β_1- and β_2-adrenoceptors cause cardiostimulant effects through an atypical β-adrenoceptor (putative β_4-adrenoceptor) that resembles the β_3-adrenoceptor. It is likely but not proven that the putative β_4-adrenoceptor is genetically distinct from the β_3-adrenoceptor. We therefore investigated whether or not the cardiac atypical β-adrenoceptor could mediate agonist effects in mice lacking a functional β_3-adrenoceptor gene (β_3KO). (−)-CGP 12177, a β_1- and β_2-adrenoceptor blocker that causes agonist effects through both β_3-adrenoceptors and cardiac putative β_4-adrenoceptors, caused cardiostimulant effects that were not different in atria from wild-type (WT) mice and β_3KO mice. The effects of (−)-CGP 12177 were resistant to blockade by (−)-propranolol (200 nM) but were blocked by (−)-bupranolol (1 μM) with an equilibrium dissociation constant of 15 nM in WT and 17 nM in β_3KO. (−)-[^{3}H]CGP 12177 labeled a similar density of the putative β_4-adrenoceptor in ventricular membranes from the hearts of both WT (B_{max} = 52 fmol/mg protein) and β_3KO (B_{max} = 53 fmol/mg protein) mice. The affinity of (−)-[^{3}H]CGP 12177 for the cardiac putative β_4-adrenoceptor was not different between WT (K_d = 46 nM) and β_3KO (K_d= 40 nM). These results provide definitive evidence that the cardiac putative β_4-adrenoceptor is distinct from the β_3-adrenoceptor.

The existence of a third cardiostimulatory β-adrenoceptor, in addition to coexisting cardiac β_1- and β_2-adrenoceptors, was proposed in 1989 (Kaumann, 1989). The receptor has been found in the hearts of all mammalian species investigated so far, including mouse and man (Kaumann, 1997). The receptor mediates increases in heart rate and force caused by nonconventional partial agonists, which are compounds that are also high affinity blockers of β_1- and β_2-adrenoceptors (Kaumann, 1989). The cardiostimulant effects of nonconventional partial agonists are relatively resistant to blockade by the β_1/β_2-adrenoceptor blocker (−)-propranolol but are antagonized by the β_1/β_2-adrenoceptor blocker (−)-bupranolol, albeit with lower affinity than for β_1- and β_2-adrenoceptors (Walter *et al.*, 1984; Kaumann, 1989, 1996; Kaumann and Molenaar, 1996).

Certain properties of the third cardiostimulant β-adrenoceptor resemble those of β_3-adrenoceptors (Kaumann, 1989) but differences have also been pointed out recently (Kaumann, 1997; Kaumann and Molenaar, 1997). For example, after it was published that (−)-bupranolol blocked the effects of the third cardiostimulant β-adrenoceptor (Kaumann, 1989), reports started to appear showing that bupranolol also blocks the lipolytic effects of β_3-adrenoceptor-selective agonists mediated through native β_3-adrenoceptors in adipocyte tissues (Langin *et al.*, 1991; Galitzky *et al.*, 1997) and adenylyl cyclase stimulation in cells transfected with the β_3-adrenoceptor (Blin *et al.*, 1994; Strosberg and Pietri-Rouxel, 1996). Furthermore, nonconventional partial agonists have agonist effects, mediated through native (Granneman *et al.*, 1991; Langin *et al.*, 1991; Lönnqvist *et al.*, 1993; Kaumann and Molenaar, 1996) and recombinant (Granneman *et al.*, 1991, 1993; Blin *et al.*, 1994; Molenaar *et al.*, 1997a) β_3-adrenoceptors.

The introduction of β_3-adrenoceptor-selective agonists [see Arch and Kaumann (1993) for review] has provided a tool to test the hypothesis of whether or not the cardiostimulatory atypical β-adrenoceptor is a β_3-adrenoceptor. β_3-adrenoceptor-selective agonists do not cause tachycardia in rat hearts *in vivo* through the atypical β-adrenoceptor (Malinowska and

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ABBREVIATIONS: (−)-CGP 12177, (−)-4–3(tertiarybutylamino-2-hydroxypropoxy)benzimidazol-2-one hydrochloride; BAT, brown adipose tissue; β_3KO, β_3-adrenoceptor knockout; IBMX, 3-isobutyl-1-methylxanthine; kb, kilobase pair(s); WT, wild-type; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Schlicker, 1996) or are completely devoid of stimulant or depressant chronotropic effects *in vitro* (Kaumann and Molenaar, 1996). Moreover, β_3-adrenoceptor-selective agonists fail to cause positive inotropic effects and do not block the cardiostimulation evoked by the nonconventional partial agonist (−)-CGP 12177 in rat (Kaumann and Molenaar, 1996) and human (Sarsero *et al.*, 1996; Molenaar *et al.*, 1997b) cardiac preparations. A β_3-adrenoceptor-selective antagonist, SR 59230A, caused only marginal blockade of the cardiostimulant effects of (−)-CGP 12177 (Kaumann and Molenaar, 1996).

The failure of β_3-adrenoceptor-selective ligands to affect cardiostimulant responses to nonconventional partial agonists suggested that β_3-adrenoceptors were not involved in these effects (Kaumann and Molenaar, 1996; Malinowska and Schlicker, 1996). It has therefore been proposed that a putative β_4-adrenoceptor mediates the cardiostimulant effects of nonconventional partial agonists and other ligands (Kaumann, 1997; Kaumann and Molenaar, 1997). A binding assay for the cardiac putative β_4-adrenoceptor has recently been introduced using (−)-[^{3}H]CGP 12177 (Molenaar *et al.*, 1997b; Sarsero *et al.*, 1997, 1998a). As expected, nonconventional partial agonists and (−)-bupranolol compete for binding with affinities expected from their stimulant and blocking potencies obtained from functional studies. There is evidence that the catecholamine (−)-isoproterenol causes cardiostimulant effects, presumably through the putative β_4-adrenoceptor. *In vivo,* Wheeldon *et al.* (1993) showed in man that (−)-isoproterenol could cause positive inotropic and lusitropic effects by stimulation of a β-adrenoceptor other than a β_1- or β_2-adrenoceptor, which we also argued was the putative β_4-adrenoceptor (Molenaar *et al.*, 1997b; Sarsero *et al.*, 1998). Functional evidence with the endogenous catecholamines is still pending. However, as expected for a β-adrenoceptor, the catecholamines (−)-norepinephrine and (−)-epinephrine bind to the rat atrial putative β_4-adrenoceptor with affinity similar to that of (−)-isoproterenol in a stereoselective manner (Sarsero *et al.*, 1998). The putative β_4-adrenoceptor does not bind 5-hydroxytryptamine, histamine, atropine, or the α-adrenoceptor antagonist phentolamine (Sarsero *et al.*, 1998a) at concentrations of these drugs that saturate their corresponding receptors. In agreement with functional studies, β_3-adrenoceptor-selective agonists and antagonists fail to compete for binding.

Cardiodepressant effects of β_3-adrenoceptor-selective agonists have been reported in human right interventricular septal biopsies from transplanted patients (Gauthier *et al.*, 1996). The effects of one of these agonists, BRL 37344, was blocked by (−)-bupranolol (Gauthier *et al.*, 1996). It has been claimed that nanomolar concentrations of β_3-adrenoceptor-selective agonists decrease contractile force and shorten action potential duration, and that the cardiodepression is blunted by pertussis toxin, suggesting coupling of the β_3-adrenoceptor to G_i protein (Gauthier *et al.*, 1996). Further support was obtained by the detection of β_3-adrenoceptor mRNA in human ventricular myocytes (Gauthier *et al.* 1996), as found previously in human heart tissues (Krief *et al.*, 1993; Berkowitz *et al.*, 1995). Gauthier *et al.* (1996) suggested that β_3-adrenoceptors may worsen heart failure by mediating cardiodepressant effects of norepinephrine (Gauthier *et al.*, 1996). However, others have failed to observe significant cardio-depressant effects using micromolar concentrations of β_3-adrenoceptor-selective agonists in human ventricular trabeculae (Kaumann and Molenaar, 1997; Molenaar *et al.*, 1997b) and cardiomyocytes (Harding, 1997). In contrast to the claimed coupling of human ventricular β_3-adrenoceptors to G_i protein (Gauthier *et al.*, 1996), evidence suggests that the cardiac putative β_4-adrenoceptor is coupled to the G_s protein/adenylyl cyclase pathway (Kaumann, 1997; Kaumann and Lynham, 1997; Kaumann and Molenaar, 1997; Kaumann *et al.*, 1997). As expected from receptor coupling to G_s protein, activation of the putative β_4-adrenoceptor increases cardiac cAMP levels and stimulates cAMP-dependent protein kinase in cardiac preparations of rat (Kaumann and Lynham, 1997; Kaumann *et al.*, 1997) and human atrium (Sarsero *et al.*, 1998b).

Taken together, the above evidence suggests that the proposed cardiac putative β_4-adrenoceptor differs from β_3-adrenoceptors. However, an alternative explanation has been forwarded. It has been suggested (Arch, 1997) that the cardiac atypical β-adrenoceptor is a β_3-adrenoceptor that adopts a conformation or associates with G proteins differently from transfected cells (Kenakin, 1995), so that it is stimulated by nonconventional partial agonists but not by β_3-adrenoceptor-selective agonists. To obtain an unambiguous answer to the question of whether the atypical cardiostimulant β-adrenoceptor is merely a β_3-adrenoceptor or a novel putative β_4-adrenoceptor, we decided to use cardiac tissues from a mouse with targeted disruption of its β_3-adrenoceptor [i.e., β_3KO (Susulic *et al.*, 1995; Revelli *et al.*, 1997)]. We studied the positive chronotropic and inotropic effects of the nonconventional partial agonist (−)-CGP 12177 on right and left atria from WT mice and investigated whether or not these effects could be produced in atria from β_3KO mice. We measured atypical β-adrenoceptor binding sites, labeled with (−)-[^{3}H]CGP 12177 in ventricular membranes of the two groups of mice. We also used Northern blots to assess whether β_3-adrenoceptor mRNA could be detected in mouse heart. All results are consistent with the existence of a cardiac putative β_4-adrenoceptor that is distinct from the β_3-adrenoceptor.

Materials and Methods

β_3KO mice. The targeted disruption (i.e., cloning) of the 129 Sv mouse β_3-adrenoceptor gene, the construction of the targeting plasmid, the electroporation, the injection of the ES cell line carrying the disrupted β_3-adrenoceptor into C57BL/6J blastocysts, and the screening of the progeny have been described in detail elsewhere (Revelli *et al.*, 1997). Five- to seven-week-old homozygous WT and β_3KO and female mice were obtained from established colonies.

Southern blot genotyping. The identification of the WT or homozygous β_3KO mice was performed by Southern blot analysis on *Bgl*II-digested tail DNA using our *Sfi*I-*Bgl*II external probe. The *Bgl*II fragment of the recombinant allele encompasses the neomycin resistance gene driven by a PGK promoter (PGK-NEO) and is therefore larger than the WT fragment (4.7 and 3.4 kb, respectively) (Revelli *et al.*, 1997).

Northern blots. Experiments were performed on tissues from female mice housed in groups of six at a temperature of 24° and fed *ad libitum* a standard laboratory chow diet. Interscapular BAT and heart ventricles were dissected and carefully trimmed from contaminating white adipose tissue. Total RNA was isolated using the Trizol technique (GIBCO BRL, New York, NY) and poly(A)$^+$ RNA using an mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). Twelve micrograms of total RNA or 10 μg of poly(A)$^+$ RNA was electrophoresed in a 1.2% agarose gel containing formaldehyde, as

described by Lehrach *et al.* (1977), and transferred to Electran Nylon Blotting membranes (BDH Laboratory Supplies, Poole, UK) by vacuum blotting. The β_3-adrenoceptor probe used was that previously described (Revelli *et al.*, 1992). It was labeled by random priming with [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK) to a specific radioactivity of approximately 1×10^9 dpm/μg DNA. Northern blots were hybridized for 2 hr at 65° in QuickHyb solution (Stratagene, La Jolla, CA), and washed in a solution of 2× standard saline citrate (2× = 300 mM NaCl, 30 mM sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate at 50° twice for 5 min and in 0.1× standard saline citrate/0.1% sodium dodecyl sulfate at 50° for 5 min. Blots were exposed to Hyperfilm electrochemiluminescence films (Amersham, Buckinghamshire, UK) at −80° with intensifying screens. Size estimates for the RNA species were established by comparison with an RNA Ladder (GIBCO BRL).

Isolated atria. Female mice aged 5–7 weeks (weight 17–23 g) were killed by dislocation of the neck in accordance with Home Office (UK) procedures and the hearts immediately taken out and placed in oxygenated solution at room temperature containing 106 mM NaCl, 5 mM KCl, 2.25 mM $CaCl_2$, 0.5mM $MgSO_4$, 1 mM Na_2HPO_4, 34 mM $NaHCO_3$, 5 mM fumarate, 5 mM pyruvate, 5 mM glutamate, 10 mM glucose, and 0.04 mM EDTA, equilibrated with 95% O_2 and 5% CO_2; the water was deionized and double distilled. Right atria and left atria were carefully dissected at room temperature. After cutting away valves and great vessels, the ventricles were freeze-clamped in liquid nitrogen. The atria were set up in pairs at 37° in a 50-ml organ bath (Blinks, 1965) containing the above solution; one tissue was from a WT mouse and the other from a β_3KO mouse. The tissues were attached to Swema 4–45 strain gauge transducers and force was recorded on a Watanabe polygraph. Spontaneously beating right atria were stretched enough to count rate from fast-speed tracings. Left atria were paced at 2 Hz with square-wave pulses of 5 msec duration and of just over threshold voltage. After determination of a length-tension curve, the length of each strip was set to obtain 50% of the resting tension associated with maximum developed force. A single cumulative concentration-effect curve to (−)-CGP 12177 was determined on right atria in the absence or presence of (−)-propranolol (200 nM) or (−)-bupranolol (1 μM). These antagonists were present for at least 60 min before a curve was begun. Positive inotropic responses to (−)-CGP 12177 in mouse left atria are smaller than positive chronotropic responses in mouse right atria. We have previously observed that IBMX potentiates the responses to (−)-CGP 12177 on rat atria, in line with a cAMP-dependent pathway (Kaumann and Lynham 1997), so to obtain robust positive inotropic responses to (−)-CGP 12177, we incubated left atria with IBMX (6 μM). When an equilibrium response to IBMX was observed, a single concentration of (−)-CGP 12177 was added as shown in the representative experiment of Fig. 2. The experiments were concluded by the administration of a β-adrenoceptor-saturating concentration of (−)-isoproterenol (400 μM) and in the case of left atria, after an equilibrium response to (−)-isoproterenol was established, by raising the $CaCl_2$ concentration to 6.7 mM. −Log EC_{50} values were estimated from each concentration-effect curve of (−)-CGP 12177 on right atrium. The equilibrium dissociation constant K_B for the (−)-bupranolol-β_4-adrenoceptor complex was estimated from the concentration-ratio of (−)-CGP 12177 using EC_{50} values in the presence and absence of (−)-bupranolol. The error of the concentration-ratio was estimated as described (Kaumann, 1990).

Binding assay. Binding was carried out as described (Sarsero *et al.*, 1998). During dissection of the hearts of WT and β_3KO mice, the ventricles were cleaned of pericardium, valves, and blood vessels and quickly freeze-clamped and stored at −70° until use. The ventricles were homogenized in ice-cold Tris/Mg^{2+} assay buffer containing 50 mM Tris·HCl, 5 mM EGTA, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM ascorbic acid, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4, centrifuged for 5 min at 175 × g at 4°. The supernatant was centrifuged for 15 min at 50,000 × g at 4° and the pellet resuspended in 15 volumes of ice-cold assay buffer. For binding to putative β_4-adrenoceptors, 1–200 nM (−)-[^{3}H]CGP 12177 (specific activity 44.5 Ci/mmole) was used in the presence of 500 nM (−)-propranolol and 100 μM GTP with 20 μM (−)-CGP 12177 to define nonspecific binding. Assays were carried out at 37° for 120 min. Protein was determined using bovine serum albumin as standard (Lowry *et al.*, 1951). The results were analyzed by assuming a single population of sites by nonlinear curve fitting using PRISM (GraphPAD Software, San Diego, CA).

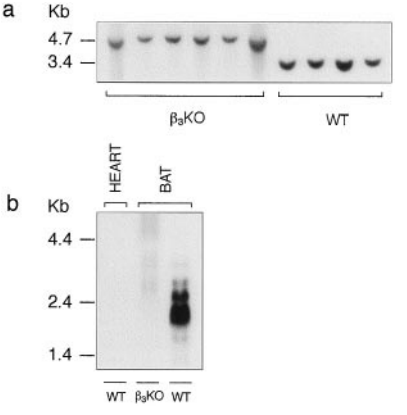

Fig. 1. a, Southern blot of WT or homozygous β_3KO mouse tail genomic DNA digested with *Bgl*II and hybridized with the *Sfi*I-*Bgl*II external probe, showing the 3.4 kb WT and the 4.7 kb recombinant allele. *Lanes,* pools of the DNAs obtained from two mice of the same genotype. b, Northern blot detection of the β_3-adrenoceptor mRNA in ventricle (HEART) total RNAs of WT mice. Expression of β_3-adrenoceptor mRNA is also shown in BAT total RNAs of β_3KO or WT mice. The radiogram was exposed 8 hr. The positions of the molecular size markers are indicated in kilobase pair(s).

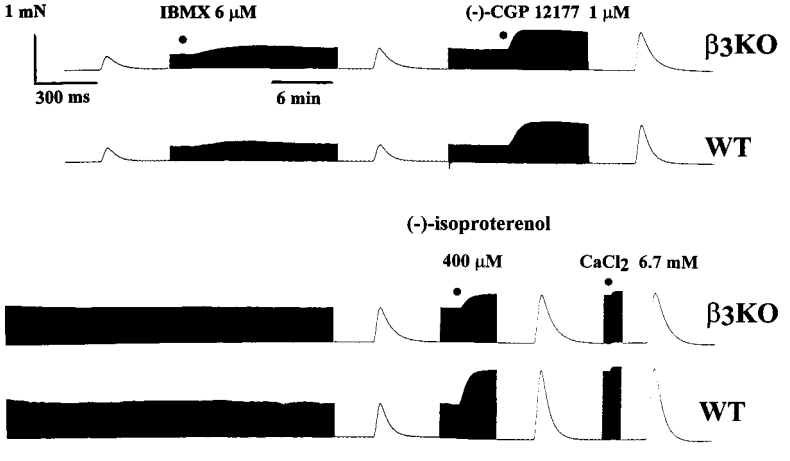

Fig. 2. Comparison of the positive inotropic effects of (−)-CGP 12177 on a left atrium from a WT mouse and a β_3KO mouse set up in the same organ bath. Fast and slow speed tracings are shown. Experiments were carried out in the presence of (−)-propranolol (200 nM). *Top*, The effects of IBMX and (−)-CGP 12177 in the presence of BMX. *Bottom*, The time course of the effects of (−)-CGP 12177 followed by the effects of (−)-isoproterenol added 40 min after the administration of (−)-CGP 12177. The experiment was terminated by raising the $CaCl_2$ concentration to 6.7 mM.

Drugs. (−)-CGP 12177 was a gift from SmithKline Beecham (Harlow, Essex, UK) and (−)-bupranolol was a gift from Sanol (Monheim, Germany). (−)-[^{3}H]CGP 12177 was purchased from Dupont (Boston, MA) and (−)-propranolol and (−)-isoproterenol were purchased from Sigma (St Louis, MO).

Statistics. All data are expressed as mean ± standard error. Significance between differences was assessed with Student's t test at $p < 0.05$.

Results and Discussion

The genotypes of mice used in this study were validated in the Southern blots of Fig. 1a. The potential expression of the β_3-adrenoceptor gene on WT mouse heart ventricle was assessed by Northern blot analysis using total or poly(A)$^+$ RNA. As shown in Fig. 1b, no β_3-adrenoceptor signal could be detected in total RNA of heart ventricles of WT mice, even on a 3-day-exposed autoradiogram. To increase the sensitivity of detection of β_3-adrenoceptor mRNA, Northern blot analysis was performed on 10 μg of poly(A)$^+$ of WT heart ventricle. No signal could be seen after an 8-hr exposure of the film (data not shown). However, on a 3-day-exposed autoradiogram, a faint band appeared (not shown). This band was estimated to represent no more than 1/1000 of that found in BAT. This finding suggests two interpretations: the ventricles express β_3-adrenoceptors at very low level or, alternatively, were contaminated with adipocytes. In any case, heart ventricles of β_3KO provide a model of a tissue devoid of β_3-adrenoceptors. Fig. 1b demonstrates that the BAT of β_3KO mice does not express β_3-adrenoceptor mRNA whereas BAT of WT does at the sizes of the major transcripts (i.e., 2.3, 2.7 and 3.1 kb), confirming previous results (Revelli *et al.*, 1997).

Human and rat β_3-adrenoceptor genes have been shown to contain at least three and two exons, respectively (Giacobino, 1995). The first exon encodes the major part of the β_3-adrenoceptor coding sequence, and it has been postulated that, in rodent as in man, alternative splicing of the β_3-adrenoceptor primary transcripts could generate isoforms with different carboxyl termini (Lelias *et al.*, 1993; Giacobino, 1995). In our targeting plasmid, the β_3-adrenoceptor was interrupted by the PGK-NEO cassette in the first exon *Xho*I site, which is far upstream of the splice signal. Therefore, in our targeted disruption of the β_3-adrenoceptor by homologous recombination (Revelli *et al.*, 1997), there is no possibility of a splice variant bypassing the interruption of the gene.

In contrast to the reported lack of β_3-adrenoceptor-mediated effects in β_3KO mice (Susulic *et al.*, 1995; Revelli *et al.*, 1997), marked cardiostimulation by (−)-CGP 12177 was observed in atria from both WT and β_3KO mice. Basal contractile force was similar in atria from WT (0.52 ± 0.18 mN, n = 7) and β_3KO mice (0.54 ± 0.12 mN, n = 11). The effects of (−)-CGP 12177 were investigated in the presence of 200 nM (−)-propranolol and 6 μM IBMX. IBMX caused a small increase in contractile force that was different in WT (24 ± 8%) and β_3KO mice (17 ± 6%) but statistically insignificant. A representative experiment is shown in Fig. 2. (−)-CGP 12177 (1 μM) increased contractile force further in atria from both WT and β_3KO mice. The effects of (−)-CGP 12177 (0.1 and 1 μM, respectively) did not differ significantly between the two groups of mice (Figs. 3). The effects of (−)-CGP 12177 were prevented by 1 μM (−)-bupranolol in atria from three β_3KO mice (Fig. 3) and from two WT mice (not shown).

(−)-CGP 12177 increased beating rate in right atria of both WT and β_3KO mice to a similar extent (Fig. 4). Neither the potency (Table 1) nor the maximal effects (Figs. 4–6) of (−)-CGP 12177 differed significantly in atria from both groups of mice. (−)-Propranolol (200 nM) did not significantly affect the potency (Table 1) or maximal effects of (−)-CGP 12177 on atria from WT and β_3KO mice (Fig. 5). In contrast, (−)-bupranolol (1 μM) blocked surmountably the effects of (−)-CGP 12177 and shifted its concentration-effect curve to a similar extent in atria from WT and β_3KO mice (Table 1, Fig. 6). A pK_B of around 7.8 was estimated for (−)-bupranolol in the right atria of both groups of mice (Table 1).

The unperturbed cardiostimulation by (−)-CGP 12177 in β_3KO, compared with WT, suggests that the density of the putative β_4-adrenoceptor population was the same in the two groups of mice. Fig. 7 shows that this was the case. (−)-[^{3}H]CGP 12177-labeled saturable binding sites in ventricular membranes in the presence of (−)-propranolol (500 nM) and GTP (100 μM) with B_{max} values of 51.6 ± 8.9 fmol/mg protein

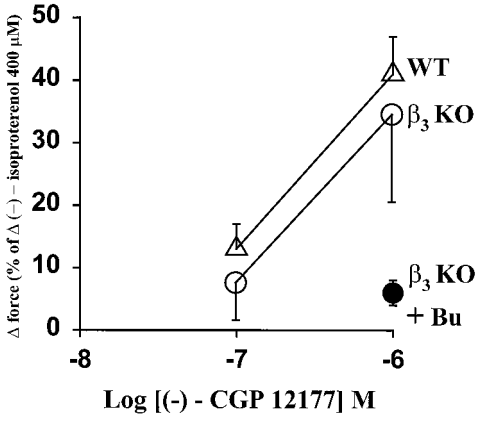

Fig. 3. Comparison of the positive inotropic effects of (−)-CGP 12177 and blockade by (−)-bupranolol (1 μM, BU, ●, n = 3) on left atria from WT (△, n = 6) and β_3KO (○, n = 6) mice. Experiments carried out in the presence of (−)-propranolol (200 nM) and IBMX (6 μM).

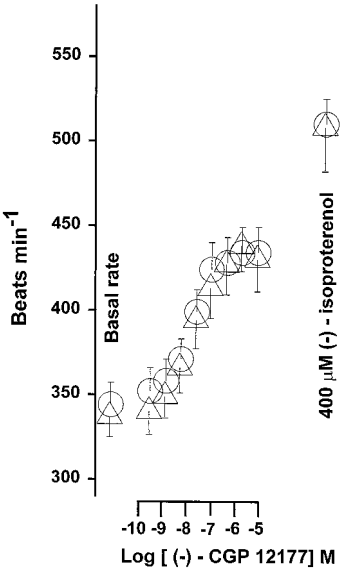

Fig. 4. Comparison of the positive chronotropic effects of (−)-CGP 12177 and (−)-isoproterenol on spontaneously beating right atria from WT (○, n = 4) and β_3KO (△, n = 4) mice.

(pseudo Hill coefficient = 1.16 ± 0.13, n = 5) in WT and 53.3 ± 6.4 fmol/mg protein (pseudo Hill coefficient = 0.98 ± 0.03, n = 3) in β_3KO. The affinity of (−)-[^{3}H]CGP 12177 did not differ between WT (pK_D = 7.34 ± 0.04) and β_3KO (pK_D = 7.40 ± 0.02). These affinity estimates are slightly lower than the potency estimates for the positive chronotropic effects (Table 1) but slightly larger than the potency estimates for the inotropic effects (Fig. 1) of (−)-CGP 12177. The results suggest the existence of some spare receptor capacity for this agonist in sinoatrial node but not in left atrium. Binding of (−)-[^{3}H]CGP 12177 (55–57 nM) to ventricular putative β_4-adrenoceptors in the presence of (−)-propranolol (500 nM) and GTP (100 μM) was inhibited by competing ligands to the same extent in WT and β_3KO. The percentage binding inhibition in WT and β_3KO, respectively, was as follows (n = 3–6): with 200 nM (−)-CGP 12177, 60.6 ± 7.6 and 79.0 ± 9.7; with 200 μM (−)-isoproterenol, 63.7 ± 5.6 and 52.6 ± 10.8; and with 1 μM (−)-bupranolol, 75.5 ± 7.2 and 57.0 ± 9.7. The differences between WT and β_3KO were not statistically significant. These data are consistent with the β_4-adrenoceptor nature of the saturable (−)-[^{3}H]CGP 12177 binding site. These binding inhibition data agree with previous affinity estimates for the putative β_4-adrenoceptor, obtained in rat atria from the corresponding binding inhibition curves of the three competing ligands (Sarsero *et al.*, 1998).

TABLE 1

Comparison of chronotropic potency of (−)-CGP 12177 and blocking potency of (−)-bupranolol, lack of blockade by (−)-propranolol.

The number of tissues is shown in parentheses. Potency values for (−)-CGP 12177 in the absence or presence of antagonist are given as −log EC_{50}, M.

	Wild-type	β_3 knockout
Control	7.76 ± 0.10 (6)	7.88 ± 0.08 (6)
(−)-propranolol 200 nM	7.92 ± 0.10 (5)	7.83 ± 0.17 (3)
(−)-bupranolol 1 μM	5.97 ± 0.06 (5)	6.05 ± 0.03 (3)
Log CR(−)-CGP 12177	1.79 ± 0.12	1.83 ± 0.09
pK(−)-bupranolol	7.78 ± 0.12	7.82 ± 0.09

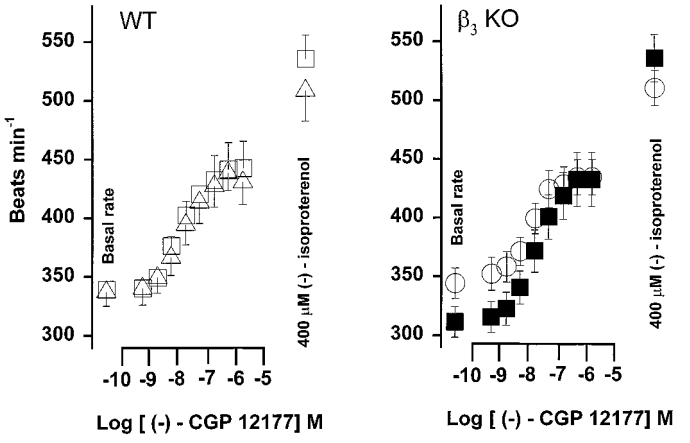

Fig. 5. Lack of blockade by (−)-propranolol (200 nM, open and closed squares) of the positive chronotropic effects of (−)-CGP 12177 on spontaneously beating right atria from WT (△, *left*) and β_3KO (○, *right*). n = 4 atria for each curve.

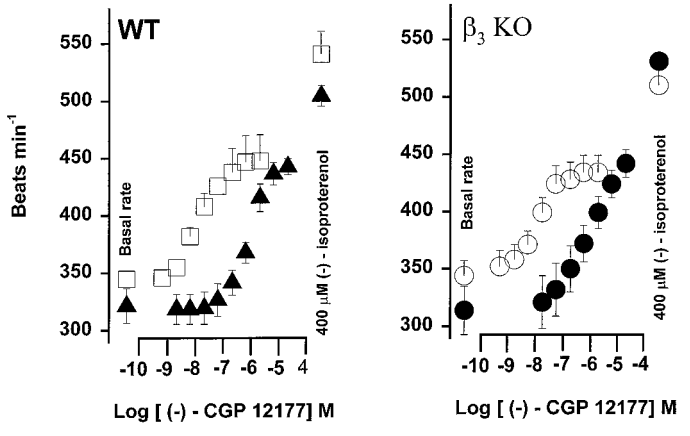

Fig. 6. Blockade by (−)-bupranolol (1 μM, closed symbols) of the positive chronotropic effects of (−)-CGP 12177 on spontaneously beating right atria from WT (□, *left*) and β_3KO (○, *right*) mice. n = 4 atria for each curve.

Expression of the putative β_4-adrenoceptor may not be restricted to cardiac tissues. The β_4-adrenoceptor may coexist and cofunction with β_3-adrenoceptors. This may be the case for lipolysis in rat adipocytes (Galitzky *et al.*, 1997) and relaxation in rat colon (Kaumann and Molenaar, 1996). In both systems the agonist effects of CGP 12177 were only blocked with low potency, whereas the effects of β_3-adrenoceptor agonists were blocked with high potency by the β_3-adrenoceptor-selective antagonist SR 59230A. The SR 59230A-resistant component of the CGP 12177 responses that are blocked by bupranolol in both adipocytes and colon could be mediated through the putative β_4-adrenoceptor.

We conclude that (−)-CGP 12177 causes similar cardiostimulant effects in atria from both WT and β_3KO mice. The ventricular putative β_4-adrenoceptors are expressed at the same density and possess the same affinity for (−)-[^{3}H]CGP 12177 in WT and β_3KO. Our results demonstrate that the

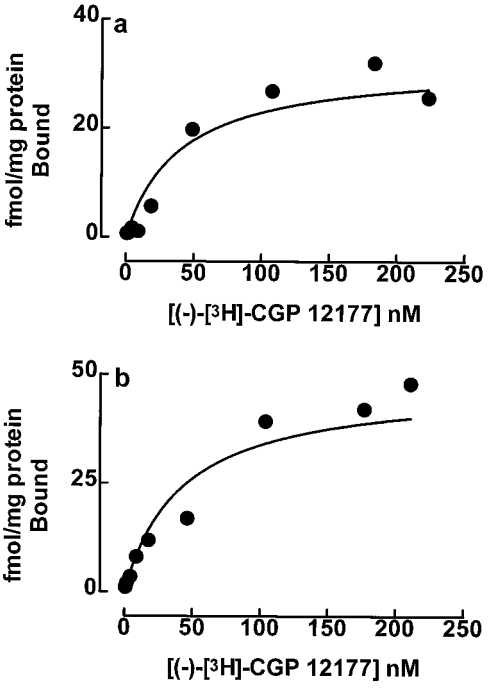

Fig. 7. Saturability of the putative β_4-adrenoceptor. Individual saturation binding experiments show specific binding of (−)-[^{3}H]CGP 12177 (1–200 nM) to mouse ventricular membranes: a, Membranes prepared from β_3KO. b, Membranes prepared from WT. Binding of (−)-[^{3}H]CGP 12177 was performed in the presence of 500 nM (−)-propranolol to block β_1- and β_2-adrenoceptors and nonspecific binding was determined with 20 μM (−)-CGP 12177.

cardiostimulant effects of (−)-CGP 12177 are mediated through the putative β_4-adrenoceptor, which is not a splice variant of the β_3-adrenoceptor and hence may be encoded by a distinct gene. Our results rule out the invoked possibility of tissue-dependent differences of G protein-coupling of β_3-adrenoceptor to account for the cardiostimulant effects of (−)-CGP 12177 and support mediation through a different receptor (i.e., the putative β_4-adrenoceptor). However, both the β_3-adrenoceptor and putative β_4-adrenoceptor mediate agonist effects of (−)-CGP 12177 that are resistant to blockade by (−)-propranolol but blocked by (−)-bupranolol.

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