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Comparison of the affinity of β-blockers for two states of the β_1-adrenoceptor in ferret ventricular myocardium

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1 We compared the potency of 11 clinically available β-blockers as antagonists of the positive inotropic effects of (−)-isoprenaline and CGP12177 on ferret ventricular myocardium.

2 (−)-CGP12177, (−)-pindolol and (−)-alprenolol were non-conventional partial agonists with intrinsic activity of 0.7, 0.2 and 0.1 respectively.

3 All β-blockers antagonized in a concentration-dependent and surmountable manner the positive inotropic effects of both (−)-isoprenaline and CGP12177. The potency of each β-blocker was consistently higher against (−)-isoprenaline than against CGP12177. Two groups of β-blockers were identified. In one group the difference between the pK_B values of blockade against (−)-isoprenaline and CGP12177 was 1.1–1.6 log units ((−)-alprenolol, (−)-pindolol, (−)-bupranolol, nadolol and carvedilol). In the other group the pK_B difference was of 2.1–3.0 log units ((−)-atenolol, metoprolol, bisoprolol, sotalol, (−)-propranolol and (−)-timolol).

4 The β-blockers competed with (−)-[^{125}I]-cyanopindolol for binding to ventricular β_1-adrenoceptors. The binding affinities correlated with the corresponding blocking potencies against (−)-isoprenaline. On average the pK_i values were 0.5 log units smaller than the pK_B values against (−)-isoprenaline but 1.6 log units greater than the pK_B values against CGP12177.

5 In ferret ventricle the effects of (−)-isoprenaline appear to be antagonized by β-blockers through the state of the β_1-adrenoceptor for which (−)-[^{125}I]-cyanopindolol and β-blockers have high affinity. The cardiostimulant effects of CGP12177 appear to be mediated through a low-affinity state of the β_1-adrenoceptor for which β-blockers have low affinity.

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Abbreviations: CGP12177, (±)-4(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one; CGP20712A, (±)-[2-(3-aminocarbomoyl-4hydroxyphenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol hydrochloride; (−)-[^{125}I]-CYP, (−)-[^{125}I]-iodocyanopindolol; ICI 118,551, D-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol

Introduction

Non-conventional partial agonists are β-adrenoceptor blocking agents (β-blockers) that produce cardiostimulant effects at concentrations considerably greater than those which antagonize the effects of catecholamines (Kaumann, 1973). CGP12177 is a non-conventional partial agonist that has nanomolar affinity for β_1- and β_2-adrenoceptors (Staehelin *et al*., 1983; Nanoff *et al*., 1987) and micromolar cardiostimulant potency (Kaumann, 1983). CGP12177 increases contractile force in a variety of species (Kaumann, 1983; Kaumann & Molenaar, 1996; Kaumann *et al*., 1998; Lowe *et al*., 1998; 1999; Sarsero *et al*., 1999) including man (Kaumann, 1996), shortens ventricular action potential (Lowe *et al*., 1998) and elicits ventricular and atrial arrhythmias in intact hearts (Lowe *et al*., 1998) and myocytes (Sarsero *et al*., 1999; Freestone *et al*., 1999). The cardiostimulant effects of CGP12177 and other non-conventional partial agonists were initially interpreted to be mediated through a third cardiostimulant β-adrenoceptor that resembled the β_3-adrenoceptor (Kaumann, 1989). Indeed, CGP12177 has been shown to be active at β_3-adrenoceptors (Konkar *et al*., 2000a). The participation of the β_3-adrenoceptor was ruled out, however, by the persistency of cardiostimulant effects of CGP12177 in β_3-adrenoceptor knockout mice (Kaumann *et al*., 1998) and accordingly mediation through a putative β_4-adrenoceptor was proposed (Kaumann, 1997).

We and others have argued recently, however, that the cardiostimulant effects of CGP12177 could be mediated through a low-affinity state of the β_1-adrenoceptor, differing from a high-affinity state through which the effects of catecholamines are blocked (Lowe *et al*., 1999; Sarsero *et al*., 1999; Freestone *et al*., 1999; Kompa & Summers, 1999). The argument is supported by evidence that CGP12177 stimulates adenylyl cyclase in murine and human recombinant β_1-adrenoceptors (Pak & Fishman, 1996; Konkar *et al*., 2000a, b). CGP12177 can also stimulate adenylyl cyclase through recombinant β_2-adrenoceptors (Pak & Fishman, 1996). Experiments with mice lacking expression of β_2-adrenoceptors (β_2 knockout, Chruscinski *et al*., 1999) or both β_1- and β_2-adrenoceptors (β_1/β_2-double knockout, Rohrer *et al*., 1999) have provided further insight as to which receptors are involved

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in the cardiostimulant effects of CGP12177. (−)-CGP12177 does not cause cardiostimulant effects in double β_1/β_2-adrenoceptor knockout mice, but responses are still present in β_2-adrenoceptor knockout mice, proving that the β_1-adrenoceptor is obligatory for these effects (Kaumann *et al*., 2001).

Another property of non-conventional partial agonists is that their cardiostimulant effects are relatively resistant to blockade by (−)-propranolol but antagonized with moderate potency by (−)-bupranolol, a β-adrenoceptor antagonist that also blocks β_1- and β_2-adrenoceptors with high affinity (Kaumann, 1989). These findings suggest that although both β-blockers interact with a high affinity state of the β_1-adrenoceptor associated with blockade of catecholamine effects, only (−)-bupranolol has sufficient affinity for the low-affinity receptor state to achieve stabilization associated with blockade of the effects of non-conventional partial agonists such as CGP12177. To what extent do clinically used β-blockers discern between the two β_1-adrenoceptor states? To answer this question we compared the blocking potency of β-blockers against the cardiostimulant effects of (−)-isoprenaline and CGP12177 in ventricular preparations of ferret, a species that shows robust cardiostimulant effects of CGP12177 (Lowe *et al*., 1998; 1999). To obtain an independent estimate for the high-affinity state, we also labelled ferret ventricular β_1-adrenoceptors with (−)-[^{125}I]-CYP and used β-blockers to compete for binding.

Methods

Isolated myocardial preparations

Experiments were undertaken in accordance with Home Office regulations (Project License PPL 80/1035). Male ferrets, 6–10 months of age, were anaesthetized with sodium pentobarbital (250 mg kg^{-1} i.p.). The hearts were removed and placed into an ice-cold oxygenated (95% O_2/CO_2 5%) solution containing (mM): NaCl 119, $NaHCO_3$ 25, KCl 4, KH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.8, Glucose 10 and Na-pyruvate 2. Right and left atria, as well as right and left ventricular papillary muscles and trabeculae, were dissected from each heart and mounted in pairs in an apparatus with a 50-ml organ bath (Blinks, 1965) and attached to strain-gauge transducers as described (Kaumann, 1972). Experiments were carried out at 37°C. Left atria, papillary muscles and trabeculae were driven with square wave pulses (1 Hz, 5-ms duration, just over threshold voltage). Spontaneously beating right atria were set up with just enough tension (range 0.2–0.5 mN) to enable contractions to be counted on a polygraph. For paced tissues a length–tension curve was constructed to determine the length at which maximal contraction occurred (L_{max}) and set at 50% L_{max} for left atria and maintained at L_{max} for ventricular tissues (Kaumann, 1972). The tissues were allowed to stabilize for 1 h. To inhibit extra-neuronal uptake of (−)-isoprenaline (Kaumann, 1972) the tissues were incubated with 30 μM corticosterone and left in the bath for the remainder of the experiment. To exclude a small contribution from ferret β_2-adrenoceptors (Lowe *et al*., 1998; and this work, Figure 1), experiments with β-blockers and (−)-isoprenaline were carried out in the presence of ICI 118,551 (50 nM; Lemoine *et al*., 1985). To block α-adrenoceptors and both neuronal and extraneuronal uptake of these catecholamines, experiments with (−)-adrenaline and (−)-noradrenaline were carried out after exposing the tissues to phenoxybenzamine (6 μM) for 90 min followed by washout (Gille *et al*., 1985). A single concentration-effect curve for CGP12177 or a catecholamine was carried out on each tissue, either in the absence or in the presence of indicated β-blocker concentrations. β-blockers were incubated at least 60 min before starting a concentration-effect curve for an agonist. After an equilibrium effect to the highest used concentration of CGP12177 or catecholamine was obtained, (−)-isoprenaline was administered at a β-adrenoceptor-saturating concentration (0.2–0.6 mM). All experiments on ventricular and left atrial tissues were terminated by raising the $CaCl_2$ concentration to 6.7 mM.

Membrane preparation

After excision of the ferret heart, the left ventricle was cut away and placed into ice-cold solution (see above). All further procedures were on ice. After being washed free of blood, the ventricle was carefully dissected and freed of valves, chordae tendinae, blood vessels and fat, cut into pieces, blotted, quickly weighed and freeze-clamped in liquid nitrogen for storage at −80°C. Ventricular tissue was reduced to powder under liquid N_2 and stored at −80°C. Crude membrane particles were prepared from a homogenate of ventricular powder. Approximately 100 mg of powder was suspended in 5 ml of binding buffer containing (mM): EGTA 5, EDTA 1, $MgCl_2$ 4, ascorbic acid 1, phenyl methyl sulphonyl fluoride 0.5 and Tris.HCl 50, pH 7.4. Homogenization was with 3 × 10 s bursts of a 7 mm Polytron probe at setting number 8, 4°C. The homogenate was further diluted with 4 volumes of binding buffer and centrifuged for 10 min at 200 × *g*, 4°C. The resultant supernatant was filtered through nylon mesh (300 microns) and centrifuged for 30 min at 17,000 × *g*, 4°C. The final pellet was resuspended in binding buffer using a short burst of the Polytron and then diluted to a final protein concentration of ∼50 μg ml^{-1} ((−)-[^{125}I]-CYP binding), as measured by the bicinchoninic acid method (Pierce, Rockford, Ill, U.S.A.) against bovine serum albumin as standard.

Binding assays

Saturation binding assays were performed in a total volume of 500 μl of binding buffer with 1–300 pM (−)-[^{125}I]-CYP (2000 Ci.mmole^{-1}). Non-specific binding was defined as the binding observed in the presence of 200 μM (−)-isoprenaline. Samples were incubated for 2 h at 37°C, bound radioactivity was retained on Whatman GF/B filters using a Brandel Harvester and 3 × 5 ml washes with ice-cold 50 mM Tris.HCl, pH 7.4. Competition binding assays were carried out in binding buffer containing ICI 118,551 (50 nM), usually GTP (1 mM), with ∼40 pM (−)-[^{125}I]-CYP and using 12 concentrations, one every half log unit, of competing β-blocker. All assays were carried out in duplicate. Assays were carried out for each condition on membranes from 3–7 hearts. Radioactivity was measured in a γ-spectrophotometer.

Statistics

The blocking potency of the β-blockers against CGP12177 and (−)-isoprenaline was estimated by analysis with agonist EC_{50} concentration-ratios (CR; Arunlakshana & Schild,

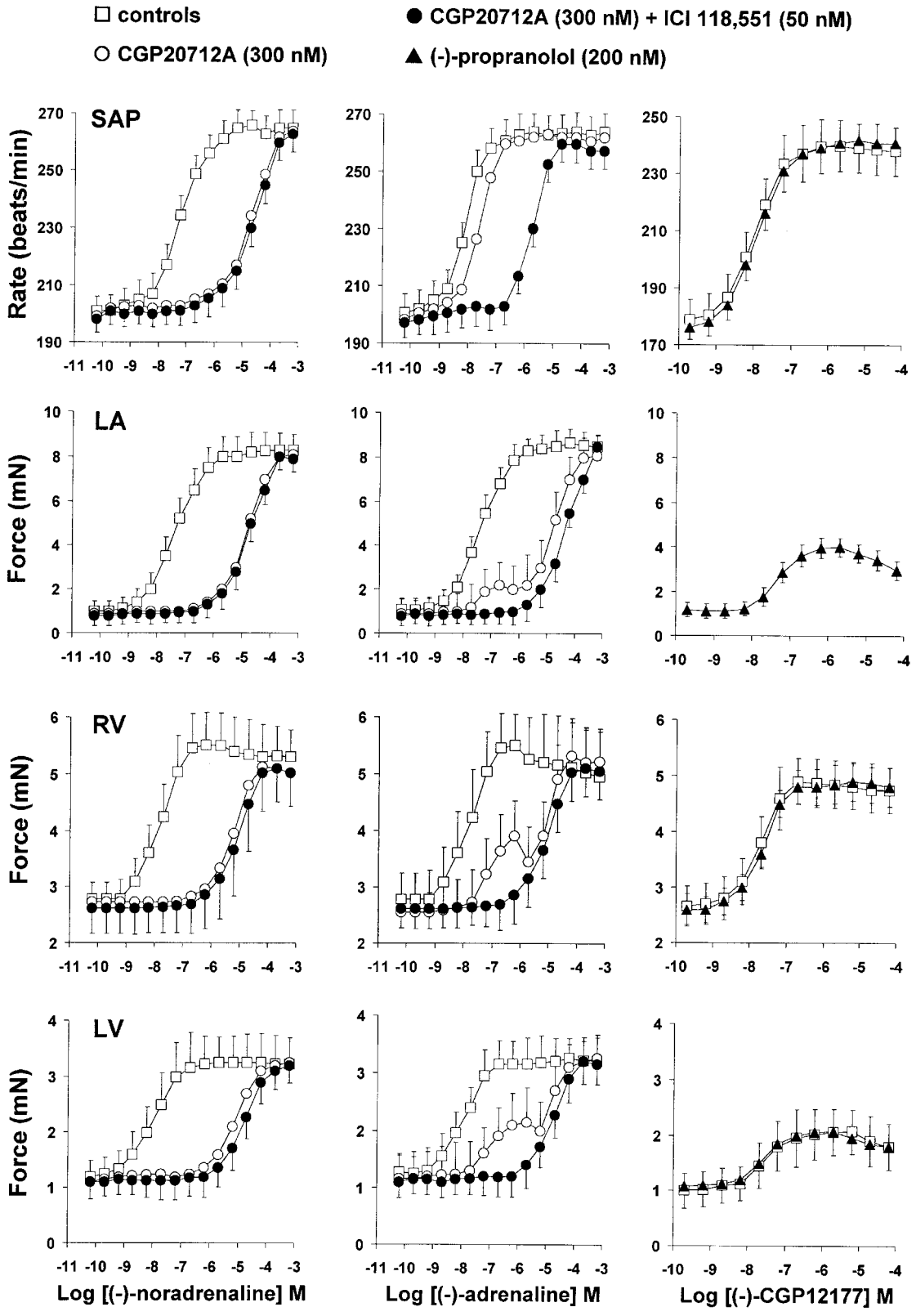

Figure 1 Comparison of the cardiostimulant effects of (−)-noradrenaline, (−)-adrenaline and (−)-CGP12177 in different regions of ferret heart: sino-atrial pacemaker (SAP), left atrium (LA), right ventricle (RV) and left ventricle (LV). Concentration-effect curves for (−)-noradrenaline and (−)-adrenaline are shown in the left-hand and middle panels in the absence and presence of CGP20712A or CGP20712A plus ICI 118,551. Curves for (−)-CGP12177 in the absence and presence of propranolol are shown on right-hand panels. For left atrial tissues all curves for (−)-CGP12177 were carried out in the presence of 200 nM (−)-propranolol. Data for each curve are from 4–6 ferrets.

1959). The error of CR was estimated by using log forms ($-\log EC_{50} = pD_2$; $-\log EC_{50}$ in the presence of β-blocker $= pD_{2\beta B}$; Kaumann, 1990):

$$pD_2 - pD_{2\beta B} \pm (\text{s.e.m. } pD_2{}^2 + \text{s.e.m. } pD_{2\beta B}{}^2)^{1/2}. \quad (1)$$

pK_B values of β-blockers were calculated assuming a Schild-plot slope of one.

Data are expressed as mean ± s.e.m. Differences were considered significant at the $P<0.05$ level. All numbers ($n \geqslant 3$) refer to number of ferrets.

Data from saturation binding and inhibition of binding by competing ligands were analysed by non-linear regression using GRAFIT (Leatherbarrow, 1992).

Drugs

CGP12177 and CGP20712A were gifts of Novartis (Basle, Switzerland). (−)-Noradrenaline, (−)-adrenaline, (−)-isoprenaline hydrochloride, (−)-alprenolol, (−)-atenolol, metoprolol, nadolol, (−)-pindolol, (−)-propranolol, (−)-timolol and corticosterone were purchased from Sigma (Poole, U.K.). Bisoprolol, sotalol and ICI 118,551 were purchased from Tocris (Bristol, U.K.). Carvedilol and (−)-CGP12177 were gifts of GlaxoSmithKline (Philadelphia, PA, U.S.A.). (−)-Bupranolol was a gift of Schwarz Pharmaka (Monheim, Rheinland, Germany).

Results

Positive chronotropic effects of (−)-adrenaline, (−)-noradrenaline and CGP12177

(−)-Adrenaline, (−)-noradrenaline and CGP12177 increased sinoatrial rate as full agonists compared to (−)-isoprenaline (Figure 1). The rank order of chronotropic potency was (−)-CGP12177 > (−)-adrenaline > (−)-noradrenaline (Figure 1). The concentration-effect curve of (−)-noradrenaline was shifted by CGP20712A in right atrium and right ventricle by 2.5 and 2.8 log units respectively (Figure 1), at a concentration (300 nM) that selectively blocks β_1-adrenoceptors (Kaumann, 1986). The antagonism was consistent with K_B values of 1 nM and 0.5 nM, similar to K_B values observed for β_1-adrenoceptors of other species (rat, Kaumann, 1986; man, Kaumann & Lemoine, 1987; cat, Lemoine & Kaumann, 1991), indicating interaction with β_1-adrenoceptors. Blockade of β_2-adrenoceptors with 50 nM ICI 118,551 in the presence of CGP20712A did not cause additional antagonism of the effects of (−)-noradrenaline, consistent with a sole interaction with β_1-adrenoceptors.

In contrast to (−)-noradrenaline, CGP20712A (300 nM) only caused a 0.7 log shift of the curve for the positive chronotropic effects of (−)-adrenaline, inconsistent with sole interaction with β_1-adrenoceptors (Figure 1). ICI 118,551 at a concentration that selectively blocks β_2-adrenoceptors (50 nM) (Lemoine *et al*., 1985; Lemoine & Kaumann, 1991), produced an additional 2 log shift of the curve for (−)-adrenaline (Figure 1), indicating an important involvement of β_2-adrenoceptors in sinoatrial node.

The positive chronotropic effects of (−)-CGP12177 were resistant to blockade of both β_1- and β_2-adrenoceptors by 200 nM (−)-propranolol (Figure 1).

The rank order of potency of (−)-CGP12177 (in the presence of 200 nM (−)-propranolol – effect mediated through the low affinity-state of the β_1-adrenoceptor), (−)-adrenaline (in the presence of 300 nM CGP20712A – effect mediated through β_2-adrenoceptors) and (−)-noradrenaline (in the presence of 50 nM ICI 118,551 – effect mediated through the high-affinity state of the β_1-adrenoceptor) were: (−)-CGP12177 ⩾ (−)-adrenaline > (−)-noradrenaline (Table 1).

Positive intropic effects of (−)-adrenaline, (−)-noradrenaline and CGP12177

The atrial and ventricular effects of (−)-noradrenaline (Figure 1, Table 1) were antagonized by CGP20712A, without additional blockade by ICI 118,551, as expected from an exclusive involvement of β_1-adrenoceptors. In contrast, a small and variable component of the effects of (−)-adrenaline (Table 1) was resistant to blockade by CGP20712A, but blocked by ICI 118,551 (Figure 1). Thus, the left atrial and ventricular inotropic effects of adrenaline are mainly mediated through β_1-adrenoceptors and to a minor and variable extent through β_2-adrenoceptors. In contrast, the positive inotropic ventricular effects of CGP12177 were resistant to blockade of β_1- and β_2-adrenoceptors with 200 nM (−)-propranolol (Figure 1, Table 1). CGP12177 was a partial agonist with intrinsic activity (with respect to (−)-isoprenaline) of 0.73 ± 0.02, 0.52 ± 0.04 and 0.41 ± 0.03 on right ventricle, left atrium and left ventricle respectively (Figure 1, Table 1). A representative experiment, which compares the ventricular effects of CGP12177 and (−)-isoprenaline, is shown on Figure 2.

Cardiostimulant effects of (−)-pindolol and (−)-alprenolol

(−)-Pindolol increased sinoatrial rate and right ventricular force with intrinsic activity of 0.41 ± 0.08 and 0.22 ± 0.09 respectively (Figure 3). Because the concentration-effect curves were non-cumulative only approximate $-\log EC_{50}$ values could be estimated for (−)-pindolol (7.2 for sinoatrial node, 7.4 for ventricle). The positive inotropic effects of (−)-pindolol and the antagonism of the positive inotropic effects of CGP12177 by (−)-pindolol on right ventricle are shown in the representative experiment of Figure 2.

(−)-Alprenolol had no effects up to 100 nM and only increased significantly ($P<0.05$) sinoatrial rate and ventricular force at 600 nM by 11 ± 3% and 9 ± 2% of the corresponding effects of (−)-isoprenaline (not shown).

Antagonism of the ventricular effects of (−)-isoprenaline and CGP12177

We only used concentrations of β-blockers that did not produce significant cardiodepressant effects (Figures 4 and 5). All β-blockers consistently antagonized more the effects of (−)-isoprenaline than the effects of CGP12177 (Figures 4 and 5; Table 2). Antagonism was always surmountable and slopes of Schild-plots did not significantly differ from a slope of one so that pK_B values were estimated (Table 2). To avoid cardiodepressant effects, the concentration-range used with some β-blocker vs CGP12177 was small. However, it was possible to estimate log potency ratios of (−)-isoprenaline/

Table 1 Agonist potencies ($-\log EC_{50}$) and intrinsic activities (as a fraction of (−)-isoprenaline, between parentheses) of adrenergic ligands in different regions of ferret myocardium

	$\beta_{1\text{-}H}$ *mediated** (−)-Noradrenaline	β_2 *mediated*# (−)-Adrenaline	$\beta_{1\text{-}L}$ *mediated*** (−)-CGP12177
Sinoatrial pacemaker	7.32 (0.96)	7.81 (0.95)	8.12 (0.94)
	±0.21 (±0.04)	±0.22 (±0.05)	±0.15 (±0.03)
Left artium	7.40 (0.93)	7.45 (0.26)	7.37 (0.52)
	±0.22 (±0.05)	±0.28 (±0.10)	±0.20 (±0.04)
Right ventricle	7.84 (0.94)	6.94 (0.46)	7.81 (0.73)
	±0.17 (±0.03)	±0.21 (±0.07)	±0.14 (±0.02)
Left ventricle	7.68 (0.91)	7.23 (0.43)	7.64 (0.41)
	±0.25 (±0.06)	±0.27 (±0.08)	±0.17 (±0.03)

Data from 4–6 ferrets for each agonist and tissue. *$\beta_{1\text{-H}}$ mediated: high affinity state of the β_1-adrenoceptor. Experiments performed in the presence of 50 nM ICI 118551. **$\beta_{1\text{-L}}$ mediated: low affinity state of the β_1-adrenoceptor. Experiments performed in the presence of 200 nM (−)- propranolol. #Experiments performed in the presence of 300 nM CGP 20712A.

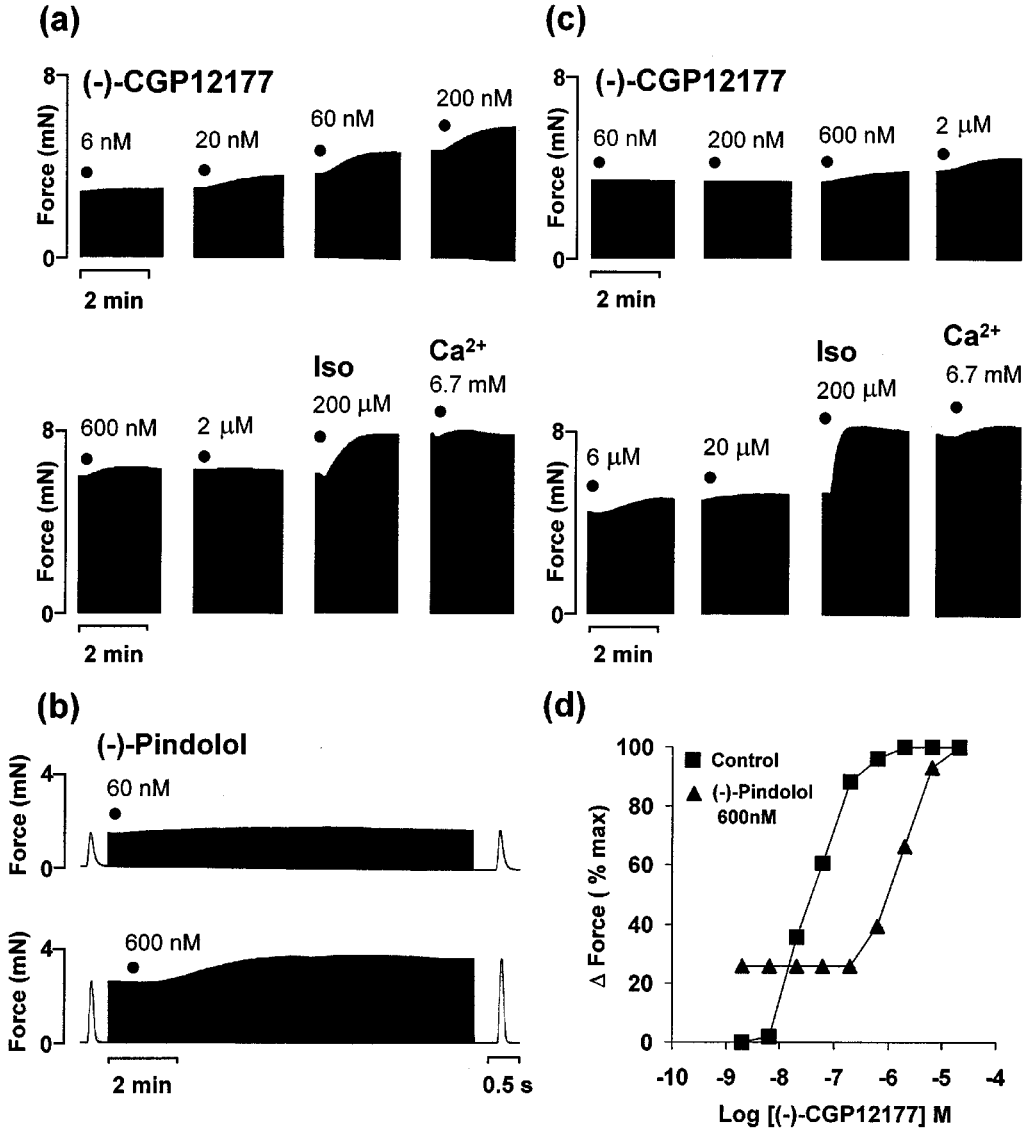

Figure 2 Representative experiment with right ventricular tissues from the same ferret heart. (a) Positive inotropic effects of (−)-CGP12177 compared with the effects of 200 μM (−)-isoprenaline and 6.7 mM calcium. (b) Positive inotropic effects of 60 nM and 600 nM (−)-pindolol. (c) Concentration-effect curve for (−)-CGP12177 in the presence of 600 nM (−)-pindolol on the same tissues as (b). (d) Increases in right ventricular developed force as a percentage of maximum for (−)-CGP12177 in the absence and presence of 600 nM (−)-pindolol.

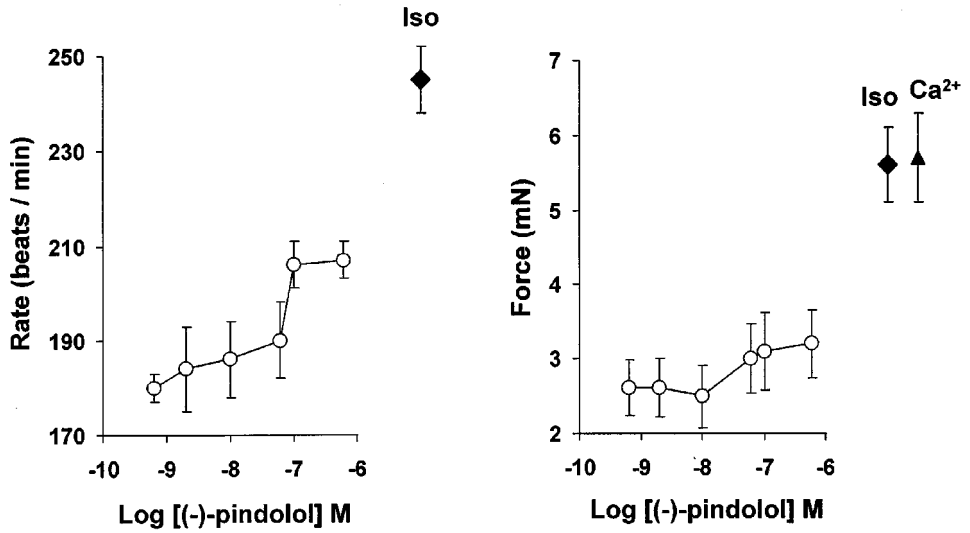

Figure 3 Concentration-effect curves for (−)-pindolol on sino-atrial pacemaker (left) and right ventricle (right). The effects of 200 μM (−)-isoprenaline (Iso) and 6.7 mM $CaCl_2$ (Ca^{2+}) are shown for comparison.

Table 2 Schild-plots and pK_B values for each β-blocker as an antagcnist of the positive inotropic effects of (−)-isoprenaline and CPG12177 in ferret right ventricle

		Shild-plot slopes		*pK_B values*		
	n	*(−)-Isoprenaline*	*CGP12177*	*(−)-Isoprenaline*	*CGP12177*	*pK_B difference*
(−)-Atenolol	4	0.97±0.12	0.92±0.13	7.0±0.2	4.2±0.3	2.8
(±)-Bisoprolol	4	1.01±0.08	1.00±0.11	8.1±0.2	5.1±0.2	3.0
(±)-Metoprolol	3	1.12±0.10	1.10±0.12	7.8±0.2	5.3±0.3	2.5
(−)-Propranolol	5	0.94±0.07	0.96±0.06	9.0±0.1	6.2±0.1	2.8
(±)-Sotalol	3	0.85±0.16	1.01±0.13	5.6±0.3	3.5±0.3	2.1
(−)-Timolol	4	0.90±0.10	0.90±0.11	9.1±0.2	6.3±0.2	2.8
(−)-Alprenolol	5	1.06±0.07	0.89±0.10	8.3±0.2	6.8±0.2	1.5
(−)-Bupranolol	4	1.00±0.07	1.02±0.08	9.1±0.2	7.5±0.2	1.6
(±)-Carvedilol[a]	6	0.98±0.06	1.02±0.07	8.1±0.3	6.8±0.1	1.3
(±)-Nadolol	4	1.07±0.10	0.99±0.11	7.2±0.2	5.7±0.2	1.5
(−)-Pindolol	5	1.03±0.08	0.89±0.09	9.1±0.2	7.7±0.3	1.4

n = number of ferrets. [a]Data from Lowe *et al.* (1999).

(−)-CGP12177 for each β-blocker. Two groups of β-blockers were identified. In the first group the pK_B difference against (−)-isoprenaline and CGP12177 was between 2.1 and 3.0 log units (Figure 4 and top half of Table 2). In the second group the difference between these pK_B values was 1.3 to 1.6 log units (Figure 5 and bottom half of Table 2). β-blockers of the first and second group are shown in Figure 6 as symbols joined by solid and broken lines, respectively.

Labelling of β_1-adrenoceptors

(−)-[^{125}I]-CYP (0.3–100 pM) bound to 57.5±4.1 fmol mg^{-1} $\beta_1+\beta_2$ adrenoceptors with K_D of 20±5 pM ($n=7$) in ferret left ventricular membranes. Inhibition of [^{125}I]-CYP binding by ICI 118,551 ($n=4$) and CGP20712A ($n=4$) yielded a ratio of β_1/β_2-adrenoceptors of 88/12±4% (Experiments not shown). K_i values of ICI 118,551 were 0.6±0.2 nM for β_2-adrenoceptors and 240±26 nM for β_1-adrenoceptors. This 400-fold β_2-adrenoceptor selectivity is similar in other species (guinea-pig, Lemoine *et al.*, 1985; man, Kaumann & Lemoine, 1987; cat, Lemoine & Kaumann, 1991). K_i values of CGP20712A were 9.4±2.4 nM for β_1-adrenoceptors and 16±11 μM for β_2-adrenoceptors. The 1700 fold β_1-adrenoceptor-selectivity, compared to β_2-adrenoceptors, is in line with similar selectivities estimated in other species (man, Kaumann & Lemoine, 1987; cat, Lemoine & Kaumann, 1991).

Binding of β-blockers to the high-affinity state of the β_1-adrenoceptor

The β-blockers competed with (−)-[^{125}I]-cyanopindolol for binding to β_1-adrenoceptors. The equilibrium dissociation constants K_i, calculated using a K_D of 20 pM for (−)-[^{125}I]-CYP, are listed in Table 3.

It has been reported that the affinity of carvedilol for β-adrenoceptors in ventricular membranes of human failing heart is decreased by a GTP analogue, a property shared with catecholamine binding (Bristow *et al.*, 1992; Yoshikawa *et al.*, 1996). In contrast, the binding of other β-blockers (Propra-

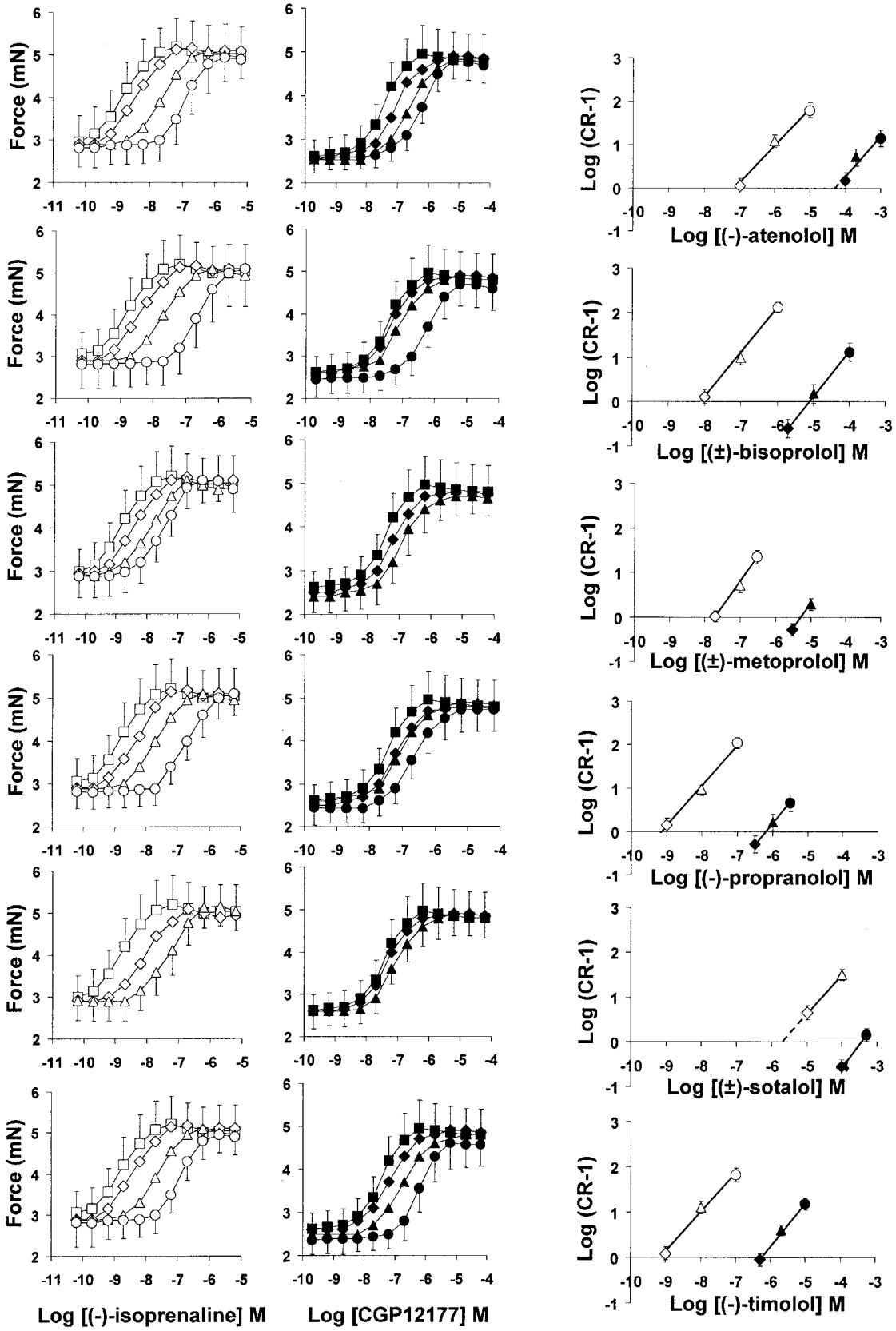

Figure 4 Comparison of the antagonism by six β-blockers of the effects of (−)-isoprenaline and CGP12177 in ferret right ventricle. Concentration-effect curves for (−)-isoprenaline (left hand panel) and CGP12177 (middle panel), in the absence (squares) and presence of individual β-blockers. Schild-plots for β-blockers at varying concentrations shown in right hand panel. The symbols of the Schild-plots correspond to the antagonist concentrations used in the concentration-effect curves of the left-hand and middle panels. For slopes of Schild-plots and number of experiments see Table 2.

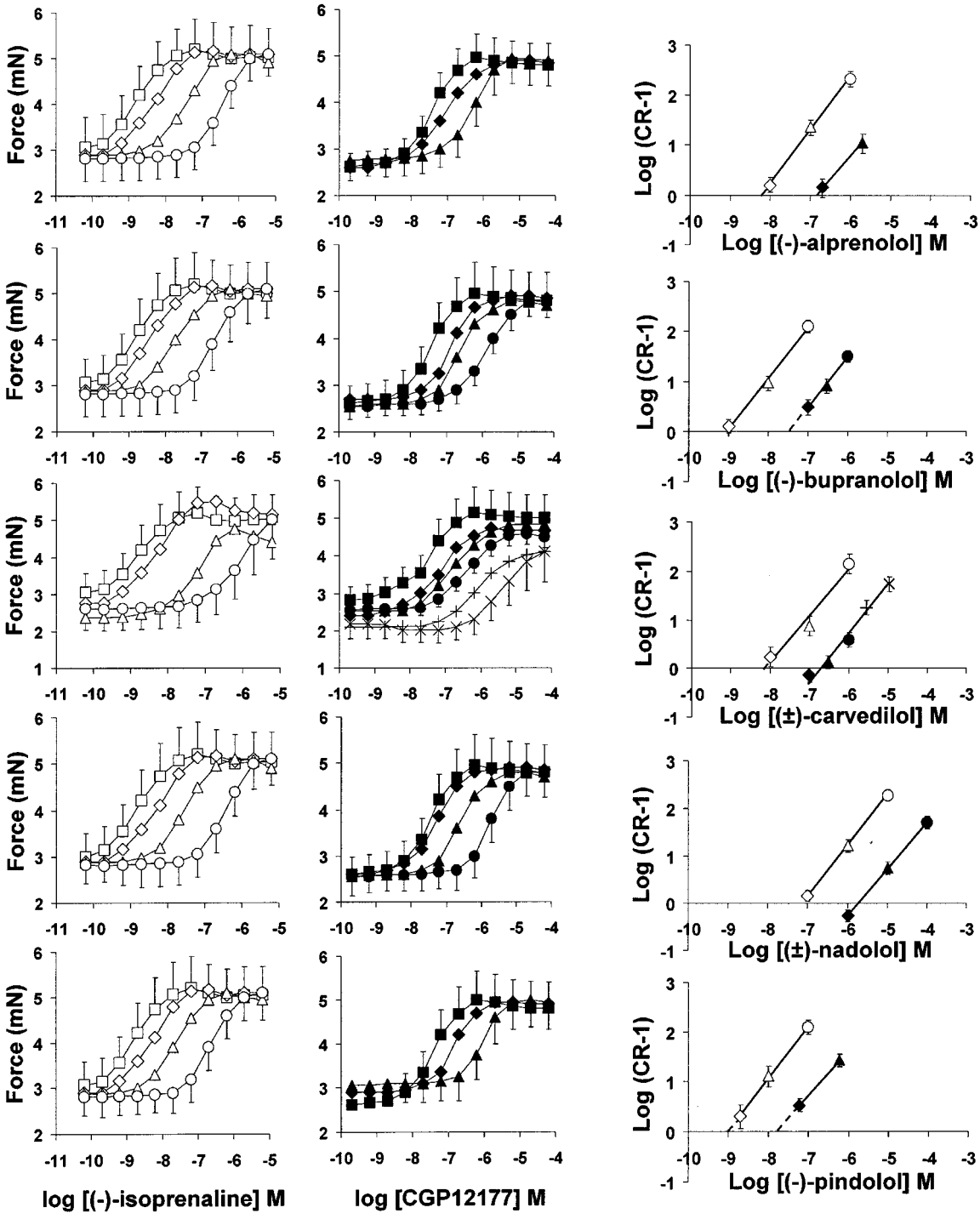

Figure 5 Comparison of the antagonism by five β-blockers of the effects of (−)-isoprenaline and CGP12177 in ferret right ventricle. Details as in Figure 4.

nolol, bisoprolol – Bristow *et al*., 1992; metoprolol – Yoshikawa *et al*., 1996) is unaffected by a GTP analogue. Because carvedilol is a relatively potent antagonist of the effects of CGP12177, we hypothesized that this property could be related to its agonist binding behaviour. We therefore investigated the inhibition of (−)-[^{125}I]-CYP binding by carvedilol in the presence and absence of GTP. The affinity estimates of carvedilol in the absence of GTP ($K_i = 1.8 \pm 0.3$ nM, $n = 3$) and presence of GTP ($K_i = 1.6 \pm 0.3$ nM, $n = 6$) (not shown), suggesting that GTP does not influence carvedilol binding to β_1-adrenoceptors in the non-failing heart of ferret.

Discussion

Using (−)-noradrenaline and (−)-adrenaline, we have obtained a regional assessment of the relative functional

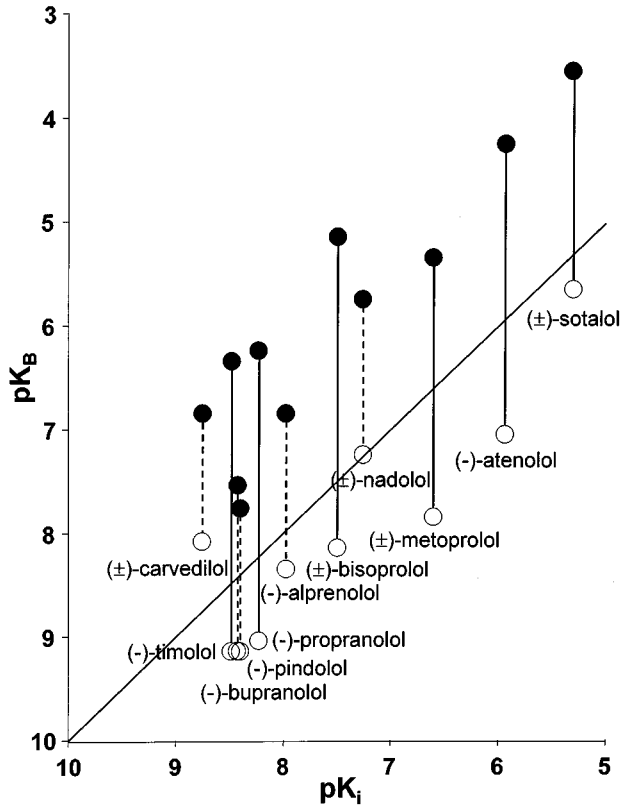

Figure 6 Comparison of blocking constants (pK_B) and binding constants (pK_i) of β-blockers on ferret ventricle. pK_B values against (−)-isoprenaline and CGP12177 are represented by open and closed circles respectively. Solid lines and broken lines joining circles represent β-blockers with high and moderate preference for the high affinity state of the β_1-adrenoceptor respectively.

Table 3 Binding inhibition constants ($-\log K_i = pK_i$, nM) of β_1-blockers for ventricular β_1-adrenoceptors, labelled with (−)-[^{125}I]-cyanopindolol

	pK_i		pK_i
(−)-Atenolol	5.94±0.06	(−)-Alprenolol	8.00±0.12
(±)-Bisoprolol	7.51±0.05	(−)-Bupranolol	8.45±0.12
(±)-Metoprolol	6.63±0.12	(±)-Carvedilol	8.90±0.08
(−)-Propranolol	8.29±0.08	(±)-Nadolol	7.27±0.06
(±)-Sotalol	5.31±0.07	(−)-Pindolol	8.48±0.12
(−)-Timolol	8.48±0.14		

Data are from 3–5 independent assays.

importance of β_1- and β_2-adrenoceptors in the ferret heart. With CGP12177 as a non-conventional partial agonist, we have also established the functional and regional role of the low-affinity state of the β_1-adrenoceptor. We then proceeded to compare the affinity of 11 clinically used β-blockers for the high-affinity and low-affinity states of the β_1-adrenoceptor.

Regional function of β_1- and β_2-adrenoceptors in the ferret heart

β_1-adrenoceptors appear exclusively to mediate the effects of (−)-noradrenaline in all regions of ferret heart investigated, as demonstrated by CGP20712A-sensitivity and the absence of ICI 118,551-sensitive effects. This is in contrast to some other species, in which β_2-adrenoceptors can mediate sinoatrial tachycardia, elicited by high (−)-noradrenaline concentrations (cat, Lemoine & Kaumann, 1991), or increases in contractile force (cat left atrium, Lemoine & Kaumann, 1991; human ventricle, Kaumann & Lemoine, 1987).

Sinoatrial tachycardia by (−)-adrenaline in the ferret appears to be mediated to a major extent through β_2-adrenoceptors, while positive inotropic effects in ventricle and left atrium are mediated mainly through β_1-adrenoceptors, a situation similar to the feline heart (Lemoine & Kaumann, 1991). The effects of (−)-adrenaline mediated through β_2-adrenoceptors were small and variable in left atrium and left and right ventricle as seen by the large errors of the effects in the presence of β_1-adrenoceptor blockade by CGP20712A (Figure 1). The small and variable function of ventricular β_2-adrenoceptors is consistent with the small and variable β_2-adrenoceptor density (12±4% of the total β-adrenoceptor population) assessed from (−)-[^{125}I]-CYP binding and competition with subtype-selective antagonists. The function of β_2-adrenoceptors is considerably more important in human atrium (Lemoine *et al*., 1988; Kaumann *et al*., 1989; 1996; Hall *et al*., 1990) and ventricle (Kaumann *et al*., 1999; Molenaar *et al*., 2000) than in ferret heart. Unlike ferret heart, in human myocardium (−)-adrenaline can be inotropically as efficacious through β_2-adrenoceptors as (−)-noradrenaline through β_1-adrenoceptors (atrium, Lemoine *et al*., 1988; ventricle, Molenaar *et al*., 2000). The species differences may be due to the lower β_2-adrenoceptor density in ferret compared to human ventricular myocardium (Kaumann & Lemoine, 1987; Molenaar *et al*., 2000) and to tighter coupling of human β_2-adrenoceptors to G_S protein compared to human β_1-adrenoceptors (Kaumann & Lemoine, 1987; Molenaar *et al*., 2000).

Functional evidence for a (−)-propranolol resistant state of the β_1-adrenoceptor in ferret heart

The existence of β_1-adrenoceptors is obligatory for the mediation of cardiostimulant effects of CGP12177 because the effects are abolished in β_1-/β_2 adrenoceptor double knockout but not in β_2-adrenoceptor knockout mice (Kaumann *et al*., 2001). As seen in other species, the cardiostimulant effects of CGP12177 in ferret heart are resistant to blockade by (−)-propranolol, 200 nM, consistent with mediation through an atypical state of the β_1-adrenoceptor (see Introduction). The cardiostimulant effects of CGP12177 were more pronounced than those of (−)-adrenaline, mediated through β_2-adrenoceptors, in all investigated regions of the ferret heart, presumably due to the marked predominance of β_1-adrenoceptors over β_2-adrenoceptors. Thus, the ferret heart provides an excellent model to study the atypical state of the β_1-adrenoceptor. Although feline (Kaumann, 1983) and murine (Kaumann *et al*., 1998; 2001) hearts, as well as rat hearts (Kaumann & Molenaar, 1996; Sarsero *et al*., 1999; Kompa & Summers, 1999), have provided useful models for studies with CGP12177, ferret ventricle appears to be particularly robust. Unlike ferret ventricle, responses of human ventricle to (−)-CGP12177 are weak and require inhibition of phosphodiesterases to become prominent (Kaumann & Molenaar, 1997).

β-blockers and the low-affinity β_1-adrenoceptor state

All β-blockers antagonized the positive inotropic effects of CGP12177 considerably less than the effects of (−)-isoprenaline. However, the kinetics of blockade appeared competitive against both agonists, suggesting that the ligands form high and low affinity receptor complexes that follow the law of mass action. It has been proposed that evidence with phenoxypropanolamines from recombinant β_1-adrenoceptors supports the existence of two active receptor states (Granneman, 2001). Our evidence in ferret heart agrees with this suggestion, but shows that a phenethanolamine, sotalol, also has detectable affinity for the two states. It is possible that the phenethanolamines isoprenaline, adrenaline and noradrenaline interact with the low-affinity state but only evidence from binding assays in rat atrial membranes has been reported, showing that catecholamines bind with millimolar affinity (Sarsero *et al*., 1998). The functional relevance of this binding remains unknown.

Two groups of β-blockers were identified, one with high preference (2.1 – 3.0 log units) for the high affinity state (activated by (−)-isoprenaline) and another with moderate (1.3 – 1.6 log units) preference, compared to the low affinity state (activated by (−)-CGP12177) (Table 2, Figure 6). CGP12177 is a remarkably hydrophilic ligand (Staehelin *et al*., 1983), but the difference in the high-affinity preference seems to be unrelated to hydrophilicity because hydrophilic β-blockers ((−)-atenolol, (±)-sotalol, (±)-nadolol) and lipophilic β-blockers ((−)-propranolol, (−)-timolol, (±)-carvedilol) are found in both groups. The data with β-blockers may provide chemical leads for the synthesis of a high affinity antagonist of the effects of CGP12177, as well as highly selective antagonists of the effects of catecholamines, thereby unmasking the effects of non-conventional antagonists.

We assume that the cardiostimulant effects of (−)-pindolol and (−)-alprenolol are mediated through the low-affinity state. This is supported by the considerably lower cardiostimulant potency of these non-conventional partial agonists compared to their corresponding affinity and blocking potency against (−)-isoprenaline. The degree of electronic activation of the phenyl ring by substituents of phenoxypropranolamines is greater by a pyrrol ((−)-pindolol) than by an allyl ((−)-alprenolol) (Fernandez & Kaumann, 1975). Because of the presence of two nitrogen atoms in the heterocycle, the electronic density of CGP12177 is likely to be greater than that of pindolol. The ability to stabilize a conformational state of the low-affinity β_1-adrenoceptor site may therefore be related to the electron density of the ring of the β-blocker.

It has been suggested that (−)-pindolol may cause tachycardia through the atypical cardiac β-adrenoceptor (Kaumann, 1989), now recognized as a low-affinity state of the β_1-adrenoceptor. Although (−)-pindolol may cause some beneficial tachycardia in patients with orthostatic hypotension (Man'Int Veld & Schalekamp, 1981) or neurocardiogenic syncope (Iskos *et al*., 1998), β-blockers with cardiostimulant effects, including non-conventional partial agonists, could potentially be harmful. A meta-analysis of β-blocker treatment following myocardial infarction has shown a significant reduction in efficacy of those antagonists with cardiostimulant effects compared to those without (Yusuf *et al*., 1985), and in several studies these β-blockers have even been shown to shorten survival (Soriano *et al*., 1997). These findings may relate to the triggering of arrhythmias by these agents mediated through an atypical state of the β_1-adrenoceptor (Lowe *et al*., 1998; Sarsero *et al*., 1999; Freestone *et al*., 1999).

We conclude that 11 clinically used β-blockers antagonize cardiostimulant effects of (−)-isoprenaline with considerably higher potency than those of CGP12177 in ferret heart. The results are consistent with interaction of β-blockers with two conformations of the β_1-adrenoceptor. Through one conformation with high affinity for (−)-[^{125}I]-CYP and other β-blockers, (−)-isoprenaline elicits cardiostimulation. Through another conformation with low affinity for β-blockers, non-conventional partial agonists, such as (−)-CGP12177, (−)-pindolol and (−)-alprenolol, cause cardiostimulation.

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