British Journal of Pharmacology (1997) 120, 165–176

Effects of (−)-RO363 at human atrial β-adrenoceptor subtypes, the human cloned β_3-adrenoceptor and rodent intestinal β_3-adrenoceptors

[1]Peter Molenaar, Doreen Sarsero, *Jonathan R.S. Arch, *John Kelly, *Sian M. Henson & †Alberto J. Kaumann

Department of Pharmacology, University of Melbourne, Parkville 3052, Australia; *SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR and †Human Pharmacology Laboratory, The Babraham Institute, Cambridge CB2 4AT

1 Chronic treatment of patients with β-blockers causes atrial inotropic hyperresponsiveness through β_2-adrenoceptors, 5-HT_4 receptors and H_2-receptors but apparently not through β_1-adrenoceptors despite data claiming an increased β_1-adrenoceptor density from homogenate binding studies. We have addressed the question of β_1-adrenoceptor sensitivity by determining the inotropic potency and intrinsic activity of the β_1-adrenoceptor selective partial agonist (−)-RO363 and by carrying out both homogenate binding and quantitative β-adrenoceptor autoradiography in atria obtained from patients treated or not treated with β-blockers. In the course of the experiments it became apparent that (−)-RO363 also may cause agonistic effects through the third atrial β-adrenoceptor. To assess whether (−)-RO363 also caused agonistic effects through β_3-adrenoceptors we studied its relaxant effects in rat colon and guinea-pig ileum, as well as receptor binding and adenylyl cyclase stimulation of chinese hamster ovary (CHO) cells expressing human β_3-adrenoceptors.

2 β-Adrenoceptors were labelled with (−)-[^{125}I]-cyanopindolol. The density of both β_1- and β_2-adrenoceptors was unchanged in the 2 groups, as assessed with both quantitative receptor autoradiography and homogenate binding. The affinities of (−)-RO363 for β_1-adrenoceptors ($pK_i = 8.0 - 7.7$) and β_2-adrenoceptors ($pK_i = 6.1 - 5.8$) were not significantly different in the two groups.

3 (−)-RO363 increased atrial force with a pEC_{50} of 8.2 (β-blocker treated) and 8.0 (non-β-blocker treated) and intrinsic activity with respect to (−)-isoprenaline of 0.80 (β-blocker treated) and 0.54 (non-β-blocker treated) ($P < 0.001$) and with respect to Ca^{2+} (7 mM) of 0.65 (β-blocker treated) and 0.45 (non-β-blocker treated) ($P < 0.01$). The effects of (−)-RO363 were resistant to antagonism by the β_2-adrenoceptor antagonist, ICI 118,551 (50 nM). The effects of 0.3–10 nM (−)-RO363 were antagonized by 3–10 nM of the β_1-adrenoceptor selective antagonist CGP 20712A. The effects of 20–1000 nM (−)-RO363 were partially resistant to antagonism by 30–300 nM CGP 20712A.

4 (−)-RO363 relaxed the rat colon, partially precontracted by 30 mM KCl, with an intrinsic activity of 0.97 compared to (−)-isoprenaline. The concentration-effect curve to (−)-RO363 revealed 2 components, one antagonized by (−)-propranolol (200 nM) with $pEC_{50} = 8.5$ and fraction 0.66, the other resistant to (−)-propranolol (200 nM) with $pEC_{50} = 5.6$ and fraction 0.34 of maximal relaxation.

5 (−)-RO363 relaxed the longitudinal muscle of guinea-pig ileum, precontracted by 0.5 μM histamine, with intrinsic activity of 1.0 compared to (−)-isoprenaline and through 2 components, one antagonized by (−)-propranolol (200 nM) with $pEC_{50} = 8.7$ and fraction 0.67, the other resistant to (−)-propranolol with $pEC_{50} = 4.9$ and fraction 0.33 of maximal relaxation.

6 (−)-RO363 stimulated the adenylyl cyclase of CHO cells expressing human β_3-adrenoceptors with $pEC_{50} = 5.5$ and intrinsic activity 0.74 with respect to (−)-isoprenaline ($pEC_{50} = 5.9$). (−)-RO363 competed for binding with [^{125}I]-cyanopindolol at human β_3-adrenoceptors transfected into CHO cells with $pK_i = 4.5$. (−)-Isoprenaline ($pK_i = 5.2$) and (−)-CGP 12177A ($pK_i = 6.1$) also competed for binding at human β_3-adrenoceptors.

7 We conclude that under conditions used in this study, (−)-RO363 is a potent partial agonist for human β_1- and β_3-adrenoceptors and appears also to activate the third human atrial β-adrenoceptor. (−)-RO363 relaxes mammalian gut through both β_1- and β_3-adrenoceptors. (−)-RO363, used as a β_1-adrenoceptor selective tool, confirms previous findings with (−)-noradrenaline that β_1-adrenoceptor-mediated atrial effects are only slightly enhanced by chronic treatment of patients with β-blockers. Chronic treatment with β_1-adrenoceptor-selective blockers does not significantly increase the density of human atrial β_1- and β_2-adrenoceptors.

Keywords: β_3- and atypical β-adrenoceptors; cloned human β_3-adrenoceptors; (−)-RO363; human atrium; guinea-pig ileum; rat colon; chronic β_1-adrenoceptor blockade

Introduction

Chronic treatment of patients with selective β_1-adrenoceptor blockers causes *in vitro* atrial inotropic hyperresponsiveness to (−)-adrenaline and (−)-noradrenaline mediated through β_2-adrenoceptors but hardly affects responses mediated through β_1-adrenoceptors (Kaumann *et al*., 1989a; Hall *et al*., 1990; Motomura *et al*., 1990; Kaumann, 1991). Chronic treatment with β blockers also increases atrial responses to salbutamol (Hall *et al*., 1990) and procaterol (Motomura *et al*., 1990) mediated through β_2-adrenoceptors. The β_2-adrenoceptor mediated hyperresponsiveness to salbutamol induced by chronic β blocker treatment observed *in vitro* (Hall *et al*., 1990) has been confirmed *in vivo* by Hall *et al*. (1991) by showing that salbutamol-evoked tachycardia was also potentiated compared

[1] Author for correspondence.

to that observed in patients not treated with β blockers. Interestingly, *in vitro* atrial positive inotropic responses to 5-hydroxytryptamine and histamine, mediated through the Gs protein-coupled 5-HT_4-receptors and H_2-receptors are also increased after chronic treatment of patients with β blockers (Sanders *et al*., 1995; 1996).

In contrast to the apparent lack of potentiation of the positive inotropic effects of (−)-noradrenaline, chronic β blocker treatment of patients enhances the incidence of (−)-noradrenaline-evoked arrhythmias through β_1-adrenoceptors in isolated atrial preparations (Kaumann & Sanders, 1993). Experimental atrial arrhythmias mediated through β_2-adrenoceptors (Kaumann & Sanders, 1993), 5-HT_4-receptors (Kaumann & Sanders, 1994) and H_2-receptors (Sanders *et al*., 1996) are also increased by chronic treatment of patients with β blockers.

The atrial hyperresponsiveness mediated through Gs protein-coupled receptors induced by chronic β blocker treatment is not understood. Early suggestions that chronic β blocker treatment enhances β_1-adrenoceptor inotropic responsiveness through an increase in their density, thereby improving patients with heart failure (Michel *et al*., 1988) have not been verified. The density of β_1-adrenoceptors in atrial myocytes is not significantly increased, as assessed by quantitative receptor autoradiography (Kaumann *et al*., 1995) and the atrial positive inotropic responses to (−)-noradrenaline (Kaumann *et al*., 1989a; Hall *et al*., 1990; Motomura *et al*., 1990) and (−)-adrenaline (Kaumann, 1991) mediated through β_1-adrenoceptors are not significantly enhanced by chronic β blocker treatment. The atrial β_2-adrenoceptor hyperresponsiveness cannot be explained by changes in their density because it remains unchanged after chronic treatment with β blockers in both tissues (Michel *et al*., 1988) and myocytes (Kaumann *et al*., 1995), while causing marked potentiation of atrial β_2-adrenoceptor-mediated positive inotropic effects (Kaumann *et al*., 1989a; Hall *et al*., 1990; Motomura *et al*., 1990) and arrhythmias (Kaumann & Sanders, 1993; Kaumann *et al*., 1995). A comparison of densities of 5-HT_4-receptors and H_2-receptors in atria from β blocker-treated (βB) and non-β-blocker-treated (non-βB) patients is still pending.

Chronic β blocker treatment could facilitate the coupling to effectors of Gs protein-coupled receptors because the positive inotropic effects of dibutyryl adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (atrial tissues, Hall *et al*., 1990) and forskolin (atrial myocytes, Sanders *et al*., 1995) are not augmented after chronic treatment of patients with β blockers, thus excluding changes of the cyclic AMP pathway downstream of adenylyl cyclase. For example 5-hydroxytryptamine (5-HT) causes greater increases of atrial cyclic AMP levels through 5-HT_4-receptors in atria obtained from βB patients than in atria from non-βB patients (Sanders *et al*., 1995). The lack of β_1-adrenoceptor-mediated inotropic hyperresponsiveness is out of line with the induction of a general improved coupling of all Gs protein-coupled receptors to effects after chronic β blocker treatment.

(−)-Noradrenaline is a full agonist for positive inotropic responses caused by activation of β-adrenoceptors in human right atrial trabeculae (Gille *et al*., 1985; Lemoine *et al*., 1988). Theorectically, a selective β_1 partial agonist should reveal more effectively changes in β_1-adrenoceptor-mediated responsiveness and its coupling mechanism, as has been described for inotropic responses to the β_2 partial agonist salbutamol (Hall *et al*., 1990). In that study β_2-adrenoceptor hyperresponsiveness was characterized by an increased intrinsic activity of salbutamol. (−)-RO363 (structure in Figure 1) is a β_1-selective partial agonist which has previously been shown to have high intrinsic activity compared to (−)-isoprenaline in human cardiac tissue (Buxton *et al*., 1987). Previous studies in guinea-pig atrium and uterus had shown (±)-RO363 to be highly selective for the β_1-adrenoceptor on the basis of affinity (63 fold) and efficacy (over 100 fold; McPherson *et al*., 1984) and therefore may be a sensitive tool to assess β_1-adrenoceptor responsiveness.

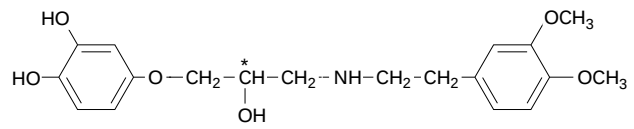

Figure 1 Chemical structure of (−)-RO363.

The intial aim of this study was to verify the β_1-selectivity of (−)-RO363 in human right atrial myocardium *in vitro* and then to use this catecholamine to determine whether supersensitivity of the β_1-adrenoceptor occurs for positive inotropic responses in tissues taken from patients pretreated with selective β_1 blockers. During the course of experiments with the β_1-selective antagonist CGP 20712A we noticed, unexpectedly, a component of the positive inotropic response to (−)-RO363 that was partially resistant to antagonism by CGP 20712A and therefore suspected that in addition to its β_1-adrenoceptor activity, (−)-RO363 might also activate the third cardiac β-adrenoceptor (Kaumann, 1989), shown recently to exist in human atrium (Kaumann, 1996). Also (−)-RO363 has structural similarities with the β_3-adrenoceptor selective agonists ICI 201651, ICI 198157 and ICI D7114 (reviewed in Arch & Kaumann, 1993). We therefore studied the effects of (−)-RO363 on two intestinal models of β_3-adrenoceptors, the rat colon (McLaughlin & MacDonald, 1990; Kaumann & Molenaar, 1996) and guinea-pig ileum (Bond & Clarke, 1988) as well as on the human cloned (Emorine *et al*., 1989) and transfected β_3-adrenoceptor.

The results demonstrate that (−)-RO363 reveals a small increase of β_1-adrenoceptor mediated inotropic responses in atria obtained from βB compared to non-βB patients. In addition (−)-RO363 is a potent and efficacious β_3-adrenoceptor partial agonist of β_3-adrenoceptors and also appears to stimulate the third human cardiac β-adrenoceptor.

Methods

Patients

Human right atrial appendages were amputed carefully to avoid damage to muscle immediately before the institution of cardiopulmonary bypass from patients undergoing coronary artery bypass grafting, aortic or mitral valve replacement or combined aortic valve replacement-coronary artery bypass grafting at The Royal Melbourne Hospital (Hospital Ethics Approval No BOMR Project 10/94, University of Melbourne Human Research Ethics Committee Registration 534-021 P-95-1647). Premedication included ranitidine 150 mg orally on the night before surgery and ranitidine 150 mg, papaveretum/scopolamine (15/0.3–20/0.4 mg) s.c., heparin 5000 i.u. s.c. and diazepam (5–10 mg) orally approximately 2 h before surgery. Anaesthesia was induced with fentanyl 20 $\mu g\ kg^{-1}$ supplemented with medazalam, propofol or isofluorane. Patients were subdivided into two groups according to whether they were treated chronically with selective β_1-adrenoceptor blockers or not before surgery. Those receiving β-blockers were treated with either atenolol (range 25 mg–100 mg daily) or metoprolol (range 50–200 mg daily). Patients who were receiving anti-asthma medication were not prescribed β blockers. However, drug therapy for both groups included the use of hypolipidemics, hypoglycemics, diuretics, angiotensin converting enzyme inhibitors, nitrates and calcium antagonists. Right atrial appendages were used for either muscle contraction, quantitative receptor autoradiography or homogenate radioligand binding experiments. Table 1 provides a summary of patient age, sex, surgical procedure and drug administration before surgery.

Radioligand binding studies in human right atrial myocardium

Homogenate radioligand binding experiments Homogenate radioligand binding studies were performed to determine the

Table 1 Summary of patient histories

	Tissue bath experiments		*Autoradiography*		*Homogenate binding*	
	βB	*non-βB*	*βB*	*non-βB*	*βB*	*non-βB*
n	24	15	14	10	5	6
Sex	5F,19M	6F,9M	3F,11M	5F,5M	1F,4M	1F,5M
Age	61±2	63±1	61±3	64±2	59±3	52±4
Surgical procedure						
AVR*	1	1	1	1	0	0
CABG*	22	14	12	9	5	5
AVR/CABG	1	0	1	0	0	0
MVR*	0	0	0	0	0	1
Drugs						
Atenolol	15	0	9	0	3	0
Metoprolol	9	0	5	0	2	0
ACE I	7	8	5	4	0	0
Diuretic	3	6	3	4	1	0
Hypolipidaemic	5	3	2	3	1	2
Nitrates	19	9	13	6	2	4
Ca^{2+} antagonist	10	12	7	7	2	2
Hypoglycaemic	3	6	2	4	1	1
Salbutamol Inhaler	0	3	0	3	0	0

*AVR-aortic valve replacement; *CABG-coronary artery bypass graft; *MVR-mitral valve replacement. βB-β-blocker treated; non-βB-not β-blocker treated.

density of β_1- and β_2-adrenoceptors in βB and non-βB myocardium, the proportions of β_1 and β_2-adrenoceptors by use of CGP 20712A and (−)-RO363 and the affinity of CGP 20712A and (−)-RO363 for human right atrial myocardial β_1- and β_2-adrenoceptors. Table 1 shows a summary of patient information from which five βB and six non-βB tissues were obtained for homogenate radioligand binding.

Right atrial appendages were placed immediately into ice-cold pre-oxygenated modified Krebs solution (mM: Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3- 32, HPO_4^{2-} 1, EDTA 0.04, Gille *et al*., 1985) and transferred to the laboratory. Atrial myocardium was immediately and carefully dissected free from epicardium, connective tissue and adipose tissue in continuously oxygenated (95% O_2/5% CO_2) ice-cold Krebs solution, weighed, snap frozen in liquid nitrogen and stored at −70°C until use.

Atrial myocardium was homogenized in Krebs-phosphate buffer (mM: Na^+ 138.7, K^+ 5, Ca^{2+} 1.9, Mg^{2+} 1.2, Cl^- 127.6, SO_4^{2-} 1.2, HPO_4^{2-} 1.3, PO_4^{3-} 8.7, pH 7.4; McPherson *et al*., 1984), the homogenate filtered through a nylon filter (210 μm) and centrifuged for 15 min at 50,000 *g* (4°C), after which the pellet was resuspended in Krebs-phosphate buffer. The homogenate was centrifuged again and the final pellet resuspended in 40 volumes of Krebs-phosphate buffer and stored on ice until use. A further 2 volumes was added to homogenates used for saturation experiments.

Ascorbic acid (1 mM), GTP (0.1 mM) and EDTA (0.1 mM) were added to incubation tubes. For saturation experiments, membrane aliquots were incubated with (−)-[^{125}I]-cyanopindolol (CYP) (1–200 pM). Competition experiments used one concentration of (−)-[^{125}I]-CYP (50 pM). The concentrations of (−)-[^{125}I]-CYP used in these studies were designed to label β_1- and β_2-adrenoceptor binding sites whilst higher concentrations are typically used to label non β_1 and non β_2 binding sites (see below). Twenty concentrations (50 pM–100 μM) of the selective β_1-adrenoceptor antagonist, CGP 20712A or 17 concentrations (500 pM–100 μM) of (−)-RO363 were used in competition binding experiments. Non-specific binding was determined in the presence of (−)-propranolol (1 μM). The final volume was 250 μl. The tubes were incubated for 2 h at 37°C and the assay terminated by the addition of 8 ml ice-cold Krebs-phosphate buffer followed by rapid filtration through Whatman GF/B filters. The radioactivity retained on the filters was counted with a Packard gamma counter.

Data from radioligand binding experiments were initially processed by EBDA (McPherson, 1983) and finally by LIGAND (Munson & Rodbard, 1980).

Protein was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Quantitative receptor autoradiography At the completion of dissection of right atrial trabeculae for tissue bath experiments, those not used were rapidly frozen in liquid N_2 and stored at −70°C until use. Sections (10 μm) were cut on a Reichert-Jung cryostat at −22.5°C, mounted onto gelatine chromic potassium sulphate coated microscope slides and stored at −70°C until further use. Tissue sections were used for quantitative receptor autoradiography, protein determination (Miller *et al*., 1988; Molenaar *et al*., 1990a) or haematoxylin and eosin staining.

Thirty one tissue blocks from 14 right atrial appendages from patients chronically treated with β_1-blockers and 22 tissue blocks from 10 right atrial appendages from patients not treated with β_1-blockers were mounted onto each slide to eliminate any possible inter-slide variation.

Incubation of cardiac tissue sections Slide mounted tissue sections were labelled with the β-adrenoceptor radioligand (−)-[^{125}I] CYP (59 pM) in the absence or presence of the β_1 selective antagonist CGP 20712A (100 nM) or (−)-propranolol (1 μM), to define non-specific binding, for 150 min at 25°C as described in detail previously for guinea-pig (Molenaar *et al*., 1987; 1990a, b) and human cardiac tissue sections (Buxton *et al*., 1987; Elnatan *et al*., 1994).

X-ray film autoradiography Dry labelled sections were apposed to X-ray film (Kodak Ektascan EC-1) and exposed for 28 days. The film was then developed and fixed.

Quantification of autoradiographs Film images were quantified by use of the MCID system (Imaging Research, Ontario, Canada) by comparisons with a standard curve generated from calibrated radioactive (^{125}I) 10 μm rat heart paste sections. Labelled human atrial tissue sections were co-exposed with calibrated tissue sections.

Calculations The density of β_1- and β_2-adrenoceptors in the myocardium of right atrial trabeculae was calculated from the extent of inhibition of (−)-[^{125}I]-CYP binding by the selective

β_1 antagonist CGP 20712A (100 nM). The inhibition of binding of a selective competing agent was described by the equation developed by Neve *et al.* (1986) and later used for autoradiography (Murphree & Saffitz, 1988):

$$B = (B_{max1}.L)/(L + K_{D1}.[1 + I/K_{I1}]) + (B_{max2}.L)/(L + K_{D2}.[1 + I/K_{I2}] \quad (1)$$

where B is the amount of radioligand bound, B_{max1} and B_{max2} are the densities of β_1- and β_2-adrenoceptors, L is the concentration of radioligand (−)-[^{125}I]-CYP, K_{D1} and K_{D2} are equilibrium dissociation constants of the radioligand at β_1- and β_2-adrenoceptors and K_{I1} and K_{I2} are equilibrium dissociation constants of competing agent (CGP 20712A) at β_1- and β_2-adrenoceptors and I is the concentration of competing agent.

In the absence of competitors this equation reduces to:

$$B = (B_{max1}.L)/(L + K_{D1}) + (B_{max2}.L)/(L + K_{D2}) \quad (2)$$

Equations 1 and 2 were solved simultaneously by use of the computer programme SIMUL (Williams & Summers, 1990) which is part of the REAP package (Gamma Research Systems, Knoxfield, Australia). Values used for dissociation constants of CGP 20712A (β_1 1.80 nM, β_2 2.40 μM) and (−)-[^{125}I]-CYP (β_1, β_2 24.8 pM) were obtained in another autoradiography study using slide mounted tissue sections of human right ventricular free wall (unpublished data). The proportion of β_1- and β_2-adrenoceptors occupied by (−)-[^{125}I]-CYP was determined by the relationship between the concentration of radioligand used and its K_D (concentration of radioligand which occupies 50% of receptors) at β_1- and β_2-adrenoceptors. This information together with the protein density were used to determine the maximal density of β-adrenoceptors as described in detail previously (Molenaar *et al.*, 1990a; b).

Tissue bath experiments

Preparation of human right atrial trabeculae Human right atrial appendages were prepared as previously described in detail (Gille *et al.*, 1985; Hall *et al.*, 1990) with minor variations. Atrial appendages were placed immediately into ice-cold pre-oxygenated modified Krebs solution (mM: Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 32, HPO_4^{2-} 1 and EDTA 0.04) and transferred to the laboratory. Dissection of strips containing intact trabeculae (<1 mm in diameter to facilitate oxygen and drug diffusion) under continuous oxygenation (95% O_2/5% CO_2) commenced within 5–10 min of surgical removal. Each right atrial appendage yielded 1–7 strips (average 4) which were mounted into 50 ml tissue baths (Blinks, 1965) containing Krebs solution at 37°C. Strips were attached to strain-gauge transducers and driven with square wave pulses (1 Hz, 5 ms duration, just over threshold voltage). A length tension curve was constructed to determine the length at which maximal contractions occurred (L_{max}) and the tension adjusted to 50% L_{max} to decrease reductions in basal tension (Gille *et al.*, 1985; Hall *et al.*, 1990). Tension of atrial strips was recorded on an 8 channel Watanabe recorder.

Responses to (−)-RO363, (−)-isoprenaline and Ca^{2+} of human right atrial appendage The incubation medium was exchanged with modified Krebs solution containing in addition (mM: Na^+ 15, fumarate 5, pyruvate 5, L-glutamate 5 and glucose 10) together with phenoxybenzamine (5 μM) to antagonize irreversibly α-adrenoceptors and inhibit neuronal and extraneuronal uptake of catecholamines (Gille *et al.*, 1985). In some experiments, the β_1-antagonist, CGP 20712A (Dooley *et al.*, 1986; Kaumann, 1986; Kaumann & Lemoine, 1987) or the β_2-antagonist ICI 118,551 (Bilski *et al.*, 1983; Lemoine *et al.*, 1985) were added to some tissue baths. After 90 min the incubation solution was exchanged to remove unbound phenoxybenzamine and supplemented with ascorbic acid (0.2 mM) to prevent catecholamine oxidation and as before with fumarate, pyruvate, L-glutamate and glucose with or without β-adrenoceptor antagonists. When tissues had stabilized a cumulative concentration-effect curve was constructed to (−)-RO363 using ½ log increments up to 60 μM or lower concentrations if a maximal response was obtained. (−)-Isoprenaline (200 μM) was then added to determine the maximal β-adrenoceptor-mediated response of the tissue followed by Ca^{2+} (7 mM).

Rat colon Experiments were carried out on colon from Sprague-Dawley rats of either sex (220–280 g). Rats were stunned by a blow on the head, rapidly exsanguinated and the colon (a 6 cm segment, with the proximal end cut 3 cm distal from the ileal-ceacal junction) dissection and placed in oxygenated modified Krebs solution (composition above) at room temperature.

A partially depolarized colon preparation as described previously (McLaughlin & MacDonald, 1990) with modifications (Kaumann & Molenaar, 1996) was used. Briefly, two 'whole' 3 cm segments were mounted in separate organ baths (to enable time-matched experiments to be performed) containing modified Krebs solution (above) at 37°C with 30 μM corticosterone (to block extraneuronal uptake of catecholamines), 3 μM cocaine (to block neuronal uptake of catecholamines) and 1 μM phentolamine (to block α-adrenoceptors). Tissues were allowed to equilibrate for 30 min, some in the presence of β-adrenceptor antagonists before the addition of KCl 30 mM. Tone was maintained by KCl for 30 min, the tissues washed twice and allowed to equilibrate for a further 60 min before re-addition of KCl 30 mM. β-Antagonists were re-added following each wash and had incubated with tissue for at least 2 h before the addition of agonist to the organ bath. Cumulative concentration-effect curves to agonist ((−)-RO363 or (−)-isoprenaline) in the presence of ascorbic acid (0.2 mM) using ½ log increments in concentration was commenced following stabilisation of KCl-induced tone. Concentration-effect curves to (−)-RO363 were followed by a β-adrenoceptor saturating concentration of (−)-isoprenaline (200 μM).

Guinea-pig ileum Experiments were carried out on distal ileum taken from male guinea-pigs (350–500 g) pretreated with reserpine (5 mg kg^{-1}, i.p. for 18 h) as described by Bond & Clarke (1988) using modified Krebs solution (above) containing 30 μM corticosterone, 3 μM cocaine, 1 μM phentolamine and 1 μM atropine. Tissues were allowed to equilibrate for 90 min, some in the presence of β-adrenoceptor antagonists before the addition of histamine (0.5 μM). The organ bath solution was exchanged 5 times before re-addition of histamine (0.5 μM). Tissues were exposed to histamine for 7 min intervals, every 20 min. The first response to histamine was discarded (Bond & Clarke, 1988) and the second and third were set at 100% against which responses in the presence of β-adrenoceptor agonists were assessed. (−)-RO363 or (−)-isoprenaline were added to some tissues 5 min before the addition of histamine. Other tissues were used as time controls. Ascorbic acid (0.2 mM) was added to all tissues. One concentration-effect curve using 1 log increments in concentration was established in each tissue. Histamine contractions in the presence of the β-agonists were expressed as a percentage of contraction in the absence of agonists and corrected for desensitization, as assessed with time controls.

Experimental design and analysis

Right atrial trabeculae Changes in tension above basal were calculated. Where 2 or more strips from one patient were used, mean changes in tension above basal values were calculated for each concentration. For (−)-RO363, pEC_{50} and maximal responses, expressed as a percentage of the response to (−)-isoprenaline (200 μM) or Ca^{2+} (7 mM) were measured. The (−)-isoprenaline (200 μM) response was also expressed as a percentage of the Ca^{2+} (7 mM) response.

A mean concentration-effect curve to (−)-RO363 was then obtained for experiments performed in trabeculae from βB and non-βB patients.

pEC_{50} and maximal response values for (−)-RO363 in the presence of increasing concentrations of CGP 20712A in βB and non-βB tissues were measured. Schild-plots (Arunlakshana & Schild, 1959) were constructed. Schild-plots were not linear and therefore were not used to obtain CGP 20712A pA_2 estimates.

In another set of experiments, maximal response values and pEC_{50} values for (−)-RO363 in the absence and presence of ICI 118,551 (50 nM) were compared to determine whether responses to (−)-RO363 were in part caused by activation of β_2-adrenoceptors.

Rat colon and guinea-pig ileum Cumulative concentration-response curves to (−)-RO363 in the absence of antagonists were visually biphasic and were therefore analysed by non-linear regression by use of the equation,

$$\text{Response} = f_{\beta 1AR}\{[RO363]/([RO363] + K_{RO363_{\beta 1AR}})\} + f_{\beta 3AR}\{[RO363]/([RO363] + K_{RO363_{\beta 3AR}})\} \quad (3)$$

where

f is the fraction of the response at β_1- and β_3-adrenoceptors (AR)

[RO363] is the concentration of (−)-RO363

K is the concentration of (−)-RO363 causing half maximal response at β_1- and β_3-AR. For a partial agonist, K will be a measure of affinity.

The error of the agonist concentration-ratio (CR), caused by an antagonist B, was estimated by using log forms ($-\log EC_{50} = pD_2$, $-\log EC_{50}$ in the presence of antagonist $= pD_{2,B}$) as by Kaumann (1990):

$$pD_2 - pD_{2,B} \pm (\text{s.e.mean}^2 pD_2 + \text{s.e.mean}^2 pD_{2,B})^{\frac{1}{2}} \quad (4)$$

EC_{50} values of agonists were estimated in log form.

Human cloned β_3-adrenoceptors

β_3-adrenoceptor expression and cell culture The human β_3-adrenoceptor cDNA (Liggett, 1992) was subcloned into a dihydrofolate reductase (dhrf) amplifiable expression vector CNOD before transfection into CHO dhfr− cells by use of calcium phosphate-precipitation methodology (Kaufman & Sharp, 1982). Transformants were grown in selection media, cloned in microtitre plates and screened for β_3-adrenoceptor expression by measuring stimulation of adenylyl cyclase activity with (−)-isoprenaline. All clones expressed β_3-adrenoceptors stably over a minimum of 30 population doublings.

Membrane preparation Cells were washed four times with 10 ml ice-cold phosphate-buffered saline (pH 7.4) then scraped into ice-cold lysis buffer (10 ml Tris HCl, 2 mM EDTA, 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} benzamidine and 10 μg ml^{-1} soybean trypsin inhibitor, pH 7.4 at 4°C). Lysed cells were washed twice by centrifugation at 40,000 *g* followed by resuspension in lysis buffer. The final resuspension was dispensed into aliquots, snap frozen on dry ice and stored in liquid nitrogen until assayed. The protein content of each batch was determined by the use of commercially available protein assay reagent (Pierce, Rockford, IL, U.S.A.).

Adenylyl cyclase activity The method of Chambers *et al.* (1994) was used. Briefly, 10–40 μg membrane protein obtained from cells expressing 400 fmol mg^{-1} β_3-adrenoceptors was incubated in the presence of 27 mM Tris-base, 1.8 mM EDTA, 2.5 mM $MgCl_2$, 0.2 mM adenosine 5′-triphosphate (ATP), 1 μM guanosine 5′-triphosphate (GTP), 1 mg ml^{-1} creatine phosphokinase, 22 mM phosphocreatine, 0.1 mg ml^{-1} bovine serum albumin, 0.1 mM (−)-ascorbate, 10 mM theophylline, 20–40 μCi ml^{-1} of [α^{32}P]-ATP and varying concentrations of agonist at 30°C in a final volume of 0.1 ml. Incubations were started by addition of membrane and terminated after 20 min by addition of 1 ml of 175 μM [^{3}H]-adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (≈5 nCi ml^{-1}), 10 mM Tris, 2 mM EDTA. Cyclic AMP was purified by double chromotography on Dowex cation exchange resin and aluminium oxide.

Binding to cloned and transfected β_3-adrenoceptors β_3-Adrenoceptors were labelled with [^{125}I]-CYP. Membranes were incubated in assay buffer (50 mM Tris, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4 at 37°C) at 37°C for 60 min in tubes pretreated with Sigmacote (Sigma Chemical, Poole, U.K.). For binding inhibition assays, membranes from cells expressing 2400 fmol ml^{-1} β_3-adrenoceptors were incubated with a concentration (0.62–0.70 nM) of [^{125}I]-CYP around its K_D value (0.61 ± 0.05 nM, determined in separate assays) and nine concentrations (from 10 nM to 1 mM) of competing ligand. Bound radioligand was separated from free ligand by rapid filtration through GF/C filters (Whatman, Maidstone, U.K.). Binding inhibition data were analysed by non-linear regression by GRAFIT (Leatherbarrow, 1992, Software U.K.) and K_i values (mean ± 95% confidence interval) calculated.

Statistics

Comparisons of groups of data were performed by Student's *t* test (unpaired). Values are expressed as means ± s.e.mean.

Drugs used

The drugs used were: (−)-CGP 12177A ((−)-4-(3-tertiarybutylamino-2-hydroxypropoxy)benzimidazol-2-one) from Dr Lee Beeley (SmithKline Beecham Pharmaceuticals, Epsom, U.K.); SR 59230A (3-(2-ethylphenoxy)-1[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol oxalate) from Dr Luciano Manara (Sanofi, Milan, Italy), CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl) 1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulphonate) from Alexandra Sedlacek (Ciba-Geigy, AG, Basal, Switzerland); phenoxybenzamine from Dr P Treagust, (SmithKline Beecham Pharmaceuticals, Worthing, U.K.), ICI 118,551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol) (Zeneca, Wilmslow, Cheshire, U.K.), (−)-RO363 ((−)-1-(3,4-dimethoxyphenethylamino)-3-(3,4-dihydroxy)-2-propanol)oxalate) (Institute of Drug Technology, Boronia, Australia), (−)-propranolol hydrochloride (Sigma, St Louis, U.S.A. & Zeneca, Wilmslow, Cheshire, U.K.); (−)-isoprenaline bitartrate, histamine dihydrochloride (Sigma, St. Louis, U.S.A.), (−)-[^{125}I]-cyanopindolol, [α^{32}P]-ATP (Amersham International, Bucks, U.K.); guanosine triphosphate (Boehringer Mannheim, Australia).

Results

Radioligand binding studies in human right atrial myocardium

Homogenate binding Homogenate radioligand binding experiments were performed on the myocardium of right atrial appendages from patients which had, or had not previously been treated with β blockers. In βB and non-βB tissues, (−)-[^{125}I]-CYP binding was saturable and not statistically significantly different in the two groups (Figure 2, Table 2). The competition binding curves between CGP 20712A and (−)-[^{125}I]-CYP were visually biphasic (Figure 2) and could be resolved into two binding sites corresponding to β_1- and β_2-adrenoceptor binding sites (Table 3). There was no difference in the proportions or densities of β_1-adrenoceptor binding sites in

βB and non-βB tissues ($P>0.05$). The competition binding curves between (−)-RO363 and (−)-[^{125}I]-CYP could also be resolved into competition at β_1- and β_2-adrenoceptor binding sites. Table 3 shows affinity values for (−)-RO363 at β_1- and β_2-adrenoceptor binding sites and the proportions of each site. There was no difference in the proportions of β_1- and β_2-adrenoceptors when determined by either CGP 20712A or (−)-RO363 ($P>0.05$). The selectivity of (−)-RO363 for β_1- versus β_2-adrenoceptor binding sites on the basis of affinity was approximately 33–166 fold in these tissues. There was no difference in the proportions or densities of β_1- and β_2-adrenoceptor binding sites determined by (−)-RO363 ($P>0.05$).

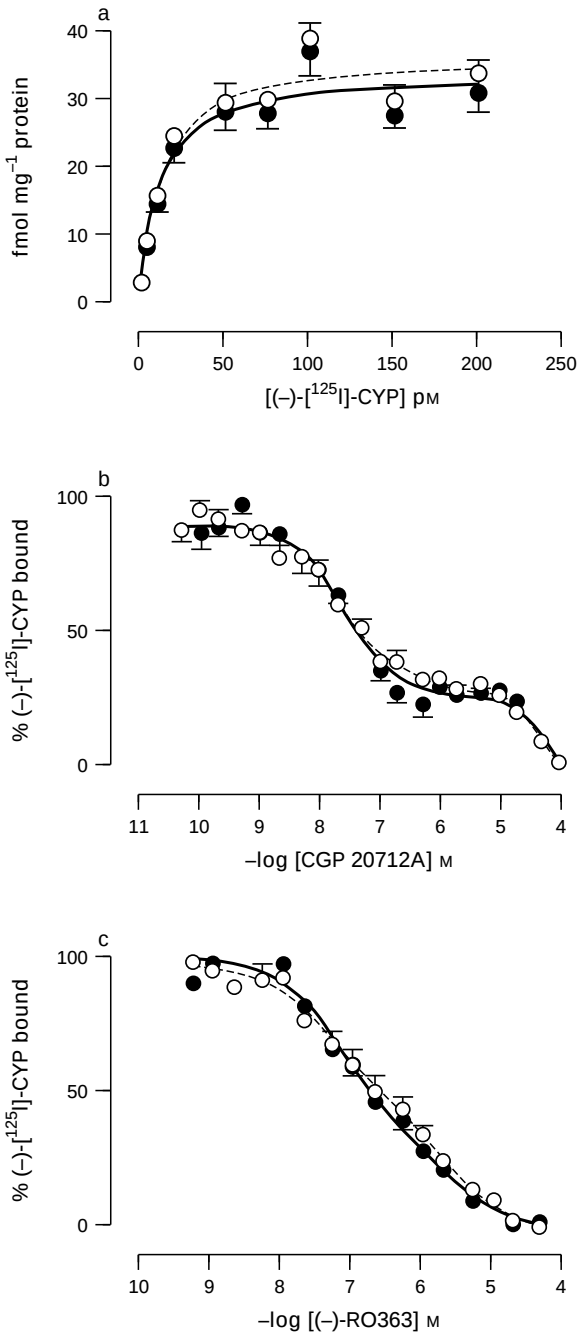

Figure 2 Mean data from (a) saturation binding experiments for (−)-[^{125}I]-cyanopindolol (CYP) and competition binding experiments between (−)-[^{125}I]-CYP and (b) CGP 20712A and (c) (−)-RO363 on right atrial myocardial homogenates from patients treated with β blockers (βB) (○, $n=5$) and not treated with β blockers (non-βB) (●, $n=6$). Competition binding curves for CGP 20712A and (−)-RO363 could be resolved into a high affinity component corresponding to β_1-adrenoceptors and a low affinity component corresponding to β_2-adrenoceptors.

Quantitative receptor autoradiography Quantitative receptor autoradiography was used to determine the densities of β_1- and β_2-adrenoceptors in right atrial trabeculae. Figure 3 presents photographs of X-ray film images showing the distribution of β-adrenoceptors. Right atrial trabeculae used for autoradiography were from appendages also used for tissue bath experiments. There was no difference in the density of β_1-adrenoceptor binding sites (βB 59.8 ± 6.4, $n=14$; non-βB 44.8 ± 5.5 fmol mg^{-1} protein, $n=10$, $P=0.11$) or β_2-adrenoceptor binding sites (βB 21.6 ± 1.1, $n=14$; non-βB 20.7 ± 1.3 fmol mg^{-1} protein, $n=10$, $P=0.62$).

Tissue bath experiments

Human right atrial appendage (−)-RO363 caused concentration-dependent positive inotropic responses in strips of trabeculae obtained from human right atrial appendages.

The maximal response to (−)-RO363 was greater in tissues taken from βB than non-βB patients in terms of both Ca^{2+} (7 mM) and (−)-isoprenaline (200 μM) responses (Figure 4, Table 4). There was no difference in the maximal response to (−)-isoprenaline (200 μM) (Table 4). There was also no relationship between basal tension and maximal evoked tension caused by (−)-RO363 determined by linear regression analysis of basal tension vs maxima for both βB and non-βB ($r^2=0.069$, $n=66$ strips from 24 βB + 15 non-βB). There was also no difference between pEC_{50} values between concentration effect curves to (−)-RO363 in βB and non-βB patients (Table 4).

(−)-RO363 caused concentration-dependent positive inotropic responses which were shifted to the right by CGP 20712A in βB and non-βB tissues (Figure 5). However the resultant Schild plots were not linear over the entire range of concentrations used, possibly suggesting the presence of multiple receptor interactions.

To determine whether part of the response to (−)-RO363 was caused by activation of β_2-adrenoceptors, positive inotropic responses to (−)-RO363 were determined in the presence of ICI 118,551 (50 nM) in trabeculae from 5 patients who had previously received β blockers (Figure 6). The β_2-adreno-

Table 2 Summary of (−)-[^{125}I]-CYP saturation binding data in human right atrial myocardium

	*βB**	*non-βB**
n_H	0.86 ± 0.04	0.82 ± 0.05
pK_D	11.0 ± 0.1	11.2 ± 0.1
B_{max} (fmol mg^{-1} protein)	36.8 ± 1.4	42.0 ± 8.2

*Data from 5β-blocker treated (βB) and 6 non-βB patients.

Table 3 Summary of data from homogenate radioligand competition binding studies in human right atrial myocardium

Patient	*Ligand*	n_H	$pK_1\beta_1AR$	$pK_1\beta_2AR$	$\%\beta_1AR$	$\%\beta_2AR$
βB*	CGP 20712A	0.32 ± 0.01	8.5 ± 0.2	5.5 ± 0.1	61.0 ± 1.8	39.0 ± 1.8
	(−)-RO363	0.63 ± 0.06	7.7 ± 0.2	6.1 ± 0.1	66.1 ± 4.1	33.9 ± 4.1
non-βB*	CGP 20712A	0.36 ± 0.05	8.5 ± 0.1	5.4 ± 0.1	67.1 ± 3.1	32.9 ± 3.1
	(−)-RO363	0.68 ± 0.02	8.0 ± 0.1	5.8 ± 0.3	64.2 ± 6.7	35.8 ± 6.7

*Data from 5β-blocker treated (βB) and 6 non-βB patients.

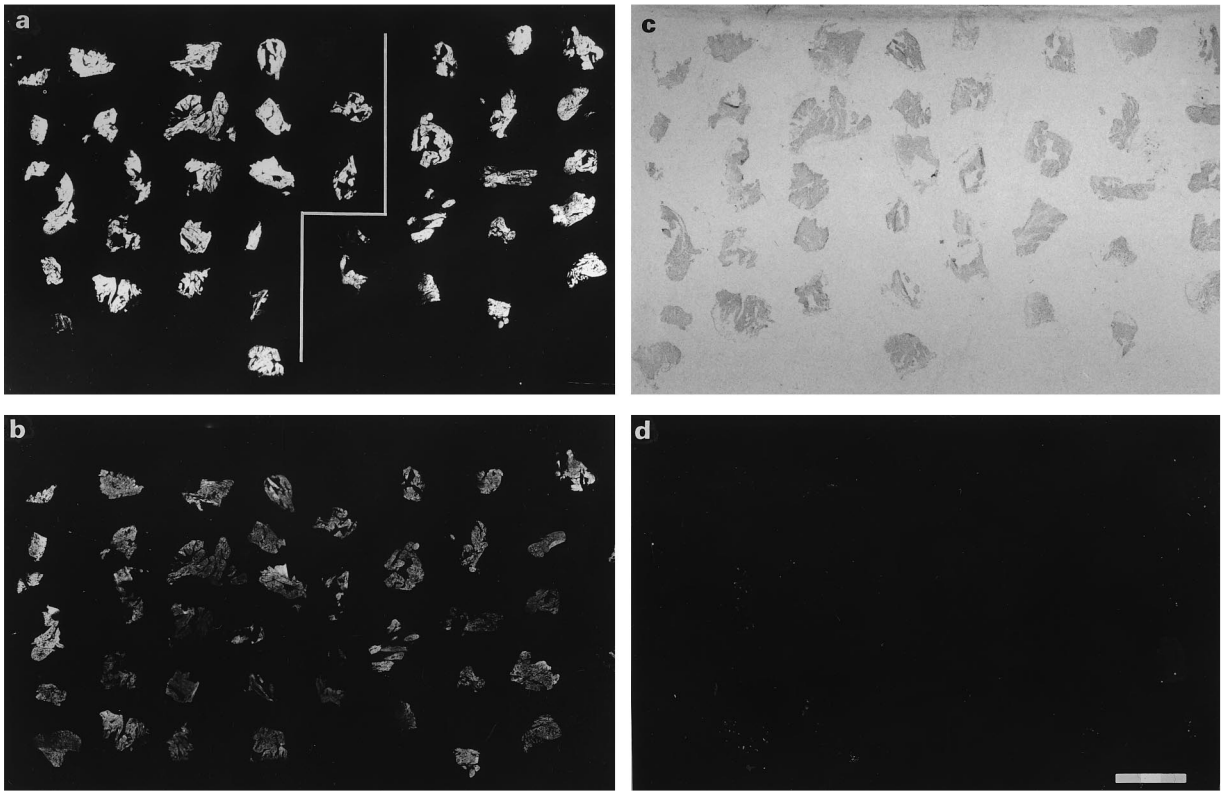

Figure 3 Film images of (−)-[^{125}I]-cyanopindolol (CYP) binding to human right atrial trabeculae in the absence (a) or presence of CGP 20712A (100 nM, b) to show β_2-adrenoceptor binding sites or (−)-propranolol (1 μM, d) to show non-specific binding. (c) Photomicrograph of a haematoxylin and eosin-stained section of trabeculae. Trabeculae were from patients treated with β blockers (right of vertical line in (a)) and without β blockers. Bar = 5 mm.

Table 4 Comparison of inotropic potency and intrinsic activity (%) of (−)-RO363 in atria from β-blocker treated (βB) and non-βB patients

	βB	*non-βB*	P-*value**
n	24	15	
pEC_{50}	8.2±0.1	8.0±0.1	0.09
(−)-RO363/Ca^{2+}	65.2±4.0	45.0±5.3	0.009
(−)-RO363/(−)-Isoprenaline	79.6±4.1	54.0±5.5	0.0006
(−)-Isoprenaline/Ca^{2+}	81.9±2.7	82.5±3.5	0.90

*Student's *t*-test.

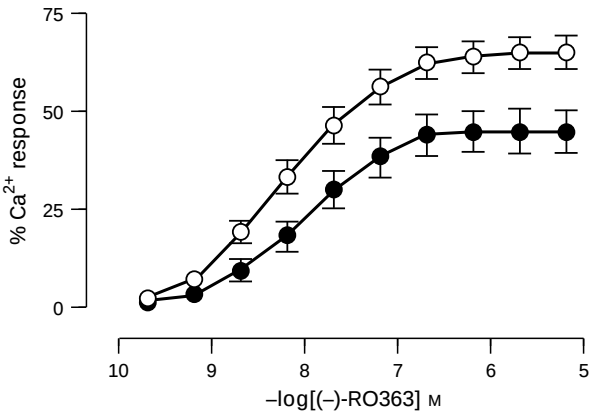

Figure 4 Comparison of the positive inotropic effects of (−)-RO363 in human right atrial trabeculae from patients treated with β blockers (atenolol or metroprolol, ○, n = 24 patients) and from patients not treated with β blockers (●, n = 15 patients). Inotropic responses caused by (−)-RO363 were normalized to Ca^{2+} 7 mM. Vertical lines show s.e.mean.

ceptor antagonist had no effect on responses to (−)-RO363 (pEC_{50} 8.2±0.1, n = 5, P = 0.75 compared to (−)-RO363 in the absence of ICI 118,551), nor was there any difference in the maximal effect of (−)-RO363, expressed in terms of the Ca^{2+} (7 mM) response (61.9±8.5, n = 5, P = 0.86 compared to (−)-RO363 in the absence of ICI 118,551).

Rat colon (−)-RO363 caused relaxation of the KCl 30 mM precontracted rat colon. The concentration-effect curve was visually biphasic, covered 6 log units (Figure 7a) and could be described by an interaction with β_1- (pEC_{50} = 8.5) and β_3-adrenoceptors (pEC_{50} = 5.6) (Figure 7, Table 5). The proportion of the response caused by β_3-adrenoceptors determined by (−)-RO363 was 34.4%. (−)-Propranolol 200 nM caused a preferential shift of lower concentrations of (−)-RO363 to cause a steepening of the concentration-effect curve (Figure 7a). The concentration-effect curve to (−)-RO363 was unaffected by the β_2-antagonist ICI 118,551 50 nM. However, the β_1 antagonist CGP 20712A 100 nM caused a preferential shift of lower concentrations of (−)-RO363 (Figure 7b). Thus, lower concentrations of (−)-RO363 caused relaxation by activation of β_1-adrenoceptors. In the presence of 200 nM (−)-propranolol, the β_3 antagonist SR 59230A 3.3 μM did not modify the effects of low concentrations of (−)-RO363 but caused a reduction in the maximum response (95.0±0.85%, n = 10 vs 79.5±3.0, n = 4, P < 0.01). The resistance of the high concentrations of (−)-RO363 to antagonism by (−)-propranolol, ICI 118,551 and CGP 20712A together with antagonism by SR 59230A strongly suggests an interaction with β_3-adrenoceptors. (−)-Propranolol 200 nM caused a small rightward shift in the concentration-effect curve to (−)-isoprenaline (log concentration-ratio 0.75±0.14, Figure 7d).

Guinea-pig ileum (−)-RO363 caused inhibition of the contractile effects of histamine (0.5 μM). The concentration-effect curve was also biphasic, covering 6 log units and could easily be described by an interaction with β_1- ($pEC_{50}=8.7$) and β_3-adrenoceptors ($pEC_{50}=4.9$) (Figure 8a, Table 5). The proportion of the response caused by β_3-adrenoceptors was 33.0%. (−)-Propranolol 200 nM and 2 μM caused a steepening of the concentration-effect curve by causing a preferential shift of the lower concentrations of (−)-RO363 (Figure 8a). The effects of (−)-RO363 were also shifted to the right by the β_1-adrenoceptor antagonist (−)-CGP 20712A 100 nM but only marginally by the β_2-adrenoceptor antagonist ICI 118,551 50 nM (Figure 8b). We also tested the effects of (−)-RO363 in the presence of 200 nM (−)-propranolol + 3.3 μM SR 59230A; however, SR 59230A appeared to inhibit the contractile effect of histamine (not shown). (−)-Propranolol 200 nM caused a 1.4 ± 0.1 log unit and 2 μM caused a 2.0 ± 0.1 log unit rightward shift of the concentration-effect curve to (−)-isoprenaline (Figure 8c) which was less than expected for a simple interaction with β_1-adrenoceptors (Gille *et al*., 1985).

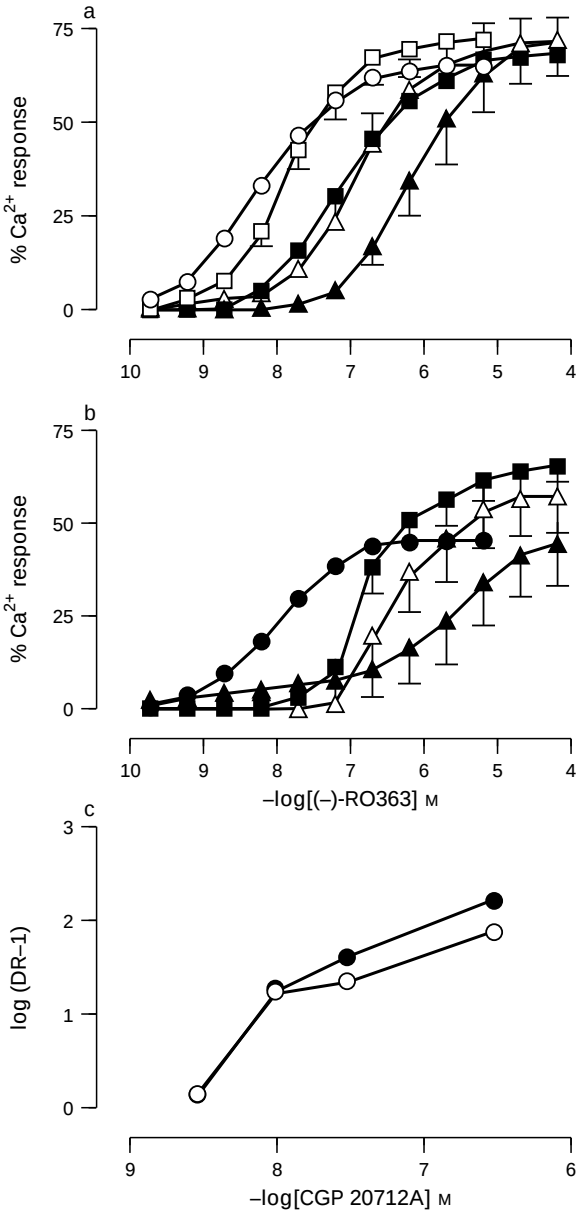

Figure 5 Antagonism of the positive inotropic effects of (−)-RO363 by CGP 20712A. Shown are mean concentration-effect curves for (−)-RO363 in trabeculae from patients treated with (a) or without a β blocker (b) in the absence (a, ○, b, ●) or presence of CGP 20712A 3 nM (□), 10 nM (■), 30 nM (△) or 300 nM (▲). Note the lack of concentration-dependent antagonism between CGP 20712A 10 and 30 nM. Data were transformed into a Schild plot for responses in tissues from β-blocker treated (βB; ○) and non βB patients (●, c). Slope values were 0.45 ± 0.06 for βB (○) and 0.64 ± 0.02 for non-βB (●, c) over the concentration range 10–300 nM which were less than unity ($P<0.05$). Shown are mean values from 4–24 patients together with s.e.mean (vertical lines).

Human cloned β_3-adrenoceptors

Adenylyl cyclase assays (−)-RO363 also caused stimulation of adenylyl cyclase in membranes expressing human β_3-adrenoceptors. Its pEC_{50} value was 5.47 ± 0.05 and it was a strong partial agonist with respect to (−)-isoprenaline with an intrinsic activity of 0.74 ± 0.08 ($n=4$, Figure 9, Table 5). (−)-Isoprenaline caused stimulation of adenylyl cyclase in the human cloned β_3-adrenoceptors with a pEC_{50} value of 5.88 ± 0.12 but was without effect in non-tranfected cells. The intrinsic activity and potency of (−)-RO363 were greater than those of (−)-CGP 12177A (0.33 ± 0.05, $n=4$, Figure 9).

Binding of agonist to β_3-adrenoceptors (−)-Isoprenaline, (−)-RO363 and (−)-CGP 12177A competed for binding with [^{125}I]-CYP with K_i (μM) values (geometric means with 95% confidence interval of 4 to 5 independent assays for each agonist) of 5.8 (3.0–11.4), 30.8 (15.5–61.7) and 0.88 (0.40–1.36), respectively. There was no specific binding of [^{125}I]-CYP in non-transfected cells.

Discussion

Four results of our work require discussion: (i) we did not detect significant changes in β_1-adrenoceptor density in atria obtained from βB compared to atria from non-βB patients, (ii) we observed a significant but small increase in the inotropic efficacy of (−)-RO363 in atria obtained from βB compared to atria from non-βB patients, (iii) we detected a CGP 20712A-resistant component of the positive inotropic effects of (−)-RO363 which we attributed to an interaction with the third cardiac β-adrenoceptor, and (iv) we found that (−)-RO363 is a partial agonist on the cloned human β_3-adrenoceptor and on intestinal β_3-receptors of both rat and guinea-pig.

Lack of β_1-adrenoceptor upregulation after chronic β_1-adrenoceptor antagonism

Two independent techniques, homogenate binding and quantitative receptor autoradiography failed to detect dif-

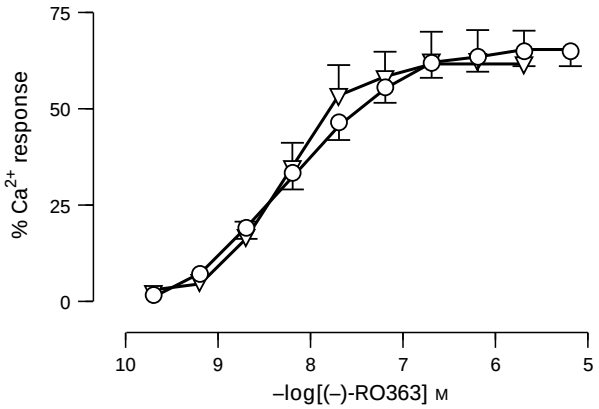

Figure 6 Lack of effect of β_2-adrenoceptor antagonism on responses to (−)-RO363. Shown are mean concentration-effect curves for (−)-RO363 in human right atrial trabeculae from patients treated with a β blocker in the absence (○) or in the presence (▽) of 50 nM ICI 118,551. Mean values are from 5–24 patients together with s.e.mean (vertical lines).

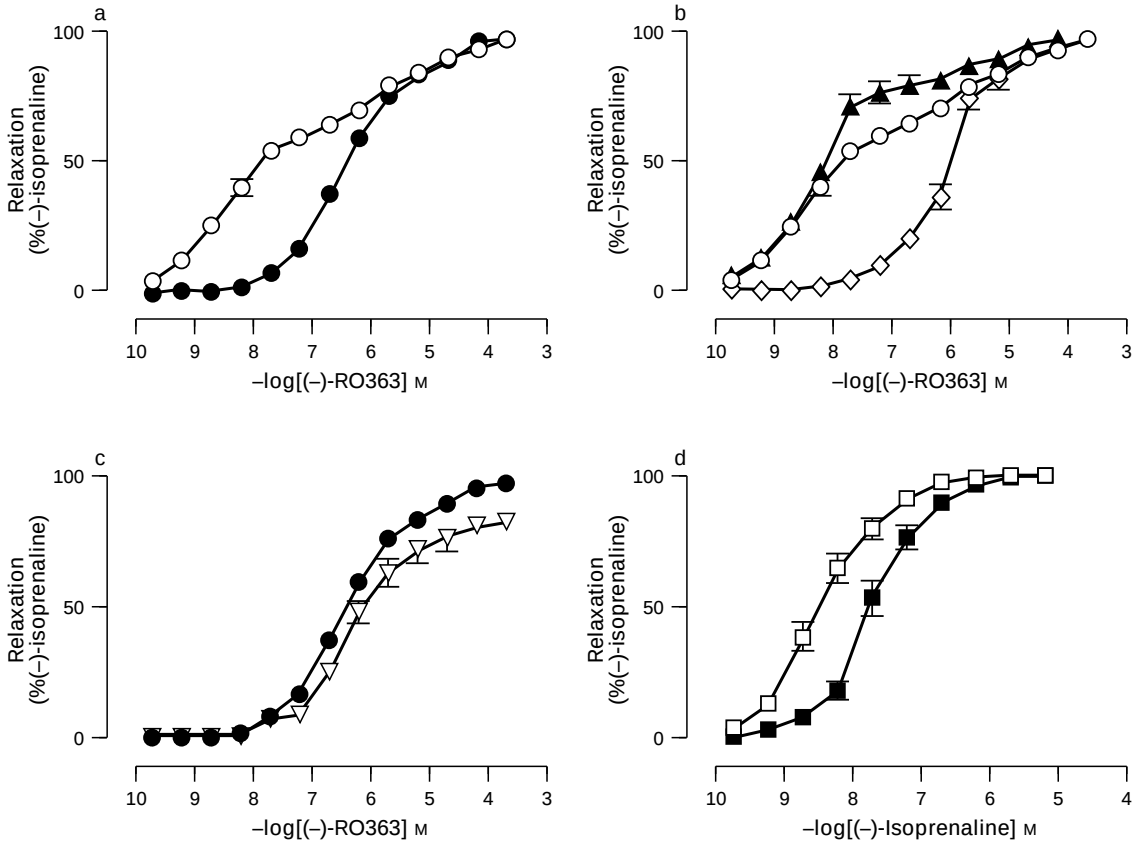

Figure 7 Relaxant effects of (−)-RO363 and (−)-isoprenaline in rat colon pre-contracted with KCl 30 mM. Responses are to (−)-RO363 in (a) the absence (○) or presence (●) of 200 nM (−)-propranolol, (b) in the absence (○) or presence of 50 nM ICI 118,551 (▲) or 100 nM CGP 20712A (◇) and (c) in the presence of 200 nM (−)-propranolol without (●) or with 3.3 μM SR 59230A (▽). In (d) responses are to (−)-isoprenaline in the absence (□) or presence of 200 nM (−)-propranolol (■). (−)-RO363 caused stimulation of β_1-adrenoceptors at lower concentrations and β_3-adrenoceptors at higher concentrations. Values are mean together with s.e.mean from 4–6 experiments.

ferences in the density of β_1-adrenoceptors (and β_2) between atria from βB and non-βB patients. These results are consistent with those of a previous study with quantitative receptor autoradiography (Kaumann *et al*., 1995). For unknown reasons, others have found using homogenate binding an approximately 30% increase of atrial β_1-adrenoceptor density in atria from βB compared to atria from non-βB (Michel *et al*., 1988), which is inconsistent with our own results. The ultimate answer as to whether or not β_1-receptors can be upregulated in atria from βB patients can only be provided from prospective studies.

Small β_1-adrenoceptor hyperresponsiveness in atria from βB patients

The positive inotropic responses to (−)-RO363 were more efficacious in atria from βB than in atria from non-βB patients, regardless of whether the effects were expressed as a percentage of the response to (−)-isoprenaline (200 μM) or the response to Ca^{2+} (7 mM). We have previously shown that the absolute responses to (−)-isoprenaline and Ca^{2+} are not significantly different between atria from βB and non-βB patients (Sanders *et al*., 1995). The β_1-adrenoceptor hyperresponsiveness detected with (−)-RO363, could be mediated through β_1-adrenoceptors of the third cardiac β-adrenoceptor or both. It has previously been shown that neither the inotropic potency nor efficacy of the non-conventional partial agonist (−)-CGP 12177A differs between groups of small numbers of atria obtained from βB and non-βB patients (Kaumann, 1996). It appears unlikely, therefore, that an interaction of (−)-RO363 with the third cardiac β-adrenoceptor (see below) could be enhanced in atria from βB patients, suggesting instead a small hyperresponsiveness of β_1-adrenoceptors. Although a slight increase in inotropic potency of (−)-noradrenaline was initially seen in atria from βB patients compared to atria from non-βB patients (Kaumann *et al*., 1989a), it was probably due at least in part to mediation through β_2-adrenoceptors (Hall *et al*., 1990). The greater β_1 selectivity with respect to β_2-adrenoceptors of (−)-RO363 (two orders of magnitude in this study) compared to that of (−)-noradrenaline (one order of magnitude, Kaumann *et al*., 1989b) and partial agonistic activity of (−)-RO363 probably facilitated the visualisation of the small β_1-adrenoceptor hyperresponsiveness.

Table 5 Potency and intrinsic activity of (−)-RO363 on β_3-adrenoceptors

	Cloned human β_3AR	*Rat colon*	*Guinea-pig ileum*
$-\log EC_{50}$ (M)	5.47 ± 0.05	5.63	4.85
Intrinsic activity	⩾0.74 ± 0.08	0.34	0.33

How does the β_1-adrenoceptor hyperresponsiveness come about? As mentioned in the Introduction, the marked β_2-adrenoceptor hyperresponsiveness in atria from βB patients is unlikely to be the result of a change at some biochemical system downstream from adenylyl cyclase, but more likely due to enhanced coupling of β_2-adrenoceptors to effectors, including adenylyl cyclase. The same argument applies to the hyperresponsiveness of other Gs protein-coupled receptors, such as 5-HT_4-receptors (Sanders *et al*., 1995) and H_2-receptors (Sanders *et al*., 1996), including β_1-receptors. Interestingly, however, the β_2-adrenoceptor hyperresponsiveness is considerably greater than that of β_1-adrenoceptors, with those of 5-HT_4-receptors and H_2-receptors being inbetween the two β-adrenoceptor subpopula-

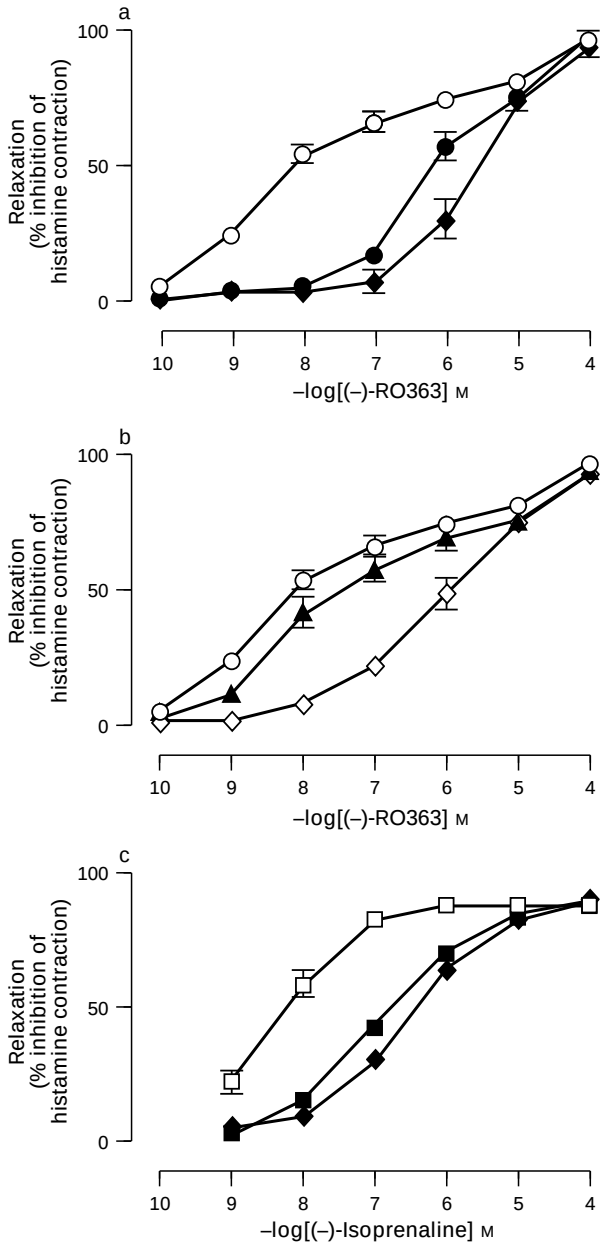

Figure 8 Relaxant effects of (−)-RO363 and (−)-isoprenaline in guinea-pig ileum pre-contracted with histamine 0.5 μM. Responses are to (−)-RO363 (a) in the absence (○) or presence of 200 nM (●) or 2 μM (◆) (−)-propranolol, (b) in the absence (○) or presence of 50 nM ICI 118,551 (▲) or 100 nM CGP 20712A (◇). In (c) responses are to (−)-isoprenaline in the absence (□) or presence of 200 nM (■) or 2 μM (◆) (−)-propranolol. Stimulation of β_1-adrenoceptors became apparent at low (−)-RO363 concentrations whilst stimulation of β_3-adrenoceptors occurred at higher (−)-RO363 concentrations. Values are mean together with s.e.mean from 4–6 experiments.

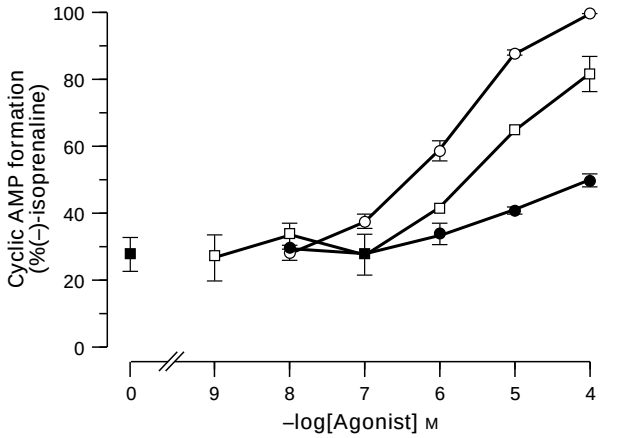

Figure 9 Effect of β-adrenoceptor agonists on adenylyl cyclase activity in CHO cell membranes transfected with human β_3-adrenoceptor. Shown is basal activity (■, $n=16$) and mean ($n=4$ for each agonist) concentration-effect curves to (−)-RO363 (□), (−)-CGP 12177A (●) and (−)-isoprenaline (○). Vertical lines show s.e.mean.

tions. It appears that the chronic activation of β_1-adrenoceptors by noradrenaline has a greater inhibitory effect upon the transduction of β_2-mediated effects than of β_1-mediated effects.

Possible activation of the third cardiac β-adrenoceptor by (−)-RO363

Our initial implicit assumption that (−)-RO363 is a highly selective β_1-adrenoceptor partial agonist was not fulfilled in human atrium. If the effects of (−)-RO363 were solely mediated through β_1-adrenoceptors, the β_1-selective antagonist CGP 20712A should have caused simple competitive antagonism, as reflected by a Schild-plot with slope of unity; this was not observed. Although 3 nM and 10 nM CGP 20712A antagonized the effects of (−)-RO363 with the nanomolar affinity expected for the β_1-adrenoceptor CGP 20712A complex (Kaumann & Lemoine, 1987), higher CGP 20712A concentrations did not, as manifested by the flattening of the slope of the Schild plot between 10–300 nM CGP 20712A (Figure 5). The Schild-plots were similar and non-linear with data from both βB and non-βB patients, in line with the assumption that CGP 20712A-resistant effects of (−)-RO363 are similar in the two groups.

What is the nature of the CGP 20712A-resistant positive inotropic effects of (−)-RO363? We ruled out β_2-adrenoceptors as possible mediators because the effects of (−)-RO363 were completely resistant to antagonism by the β_2-adrenoceptor selective antagonist ICI 118,551 (Figure 6) and because the binding affinity of (−)-RO363 for β_2-adrenoceptors (Table 3) is considerably lower than its CGP 20712A-resistant inotropic potency (Figure 5). By exclusion we propose that (−)-RO363 may interact with the third cardiac β-adrenoceptor, recently identified in human atrium (Kaumann, 1996). The proposal is based on the similarity, but not identity, of the third cardiac β-adrenoceptor with the β_3-subtype (Kaumann & Molenaar, 1996) and on the structural similarity of (−)-RO363 with certain β_3-adrenoceptor selective agonists (see Introduction).

(−)-RO363 is a β_3-adrenoceptor partial agonist

Our early suspicion that (−)-RO363 in addition to activating β_1-adrenoceptors may also activate β_3-adrenoceptors, based on its CGP 20712A-resistant effects and structural similarity to other β_3 agonists (see Introduction), was confirmed for intestinal β_3-adrenoceptors of rat and guinea-pig where low concentrations caused relaxation by activating β_1-adrenoceptors and higher concentrations activated β_3-adrenoceptors, and verified for human β_3-adrenoceptors expressed in CHO cells. On recombinant human β_3-adrenoceptors (−)-RO363 was only 3 times less potent than (−)-isoprenaline as an agonist, in agreement with its 5 times lower affinity. On the human cloned β_3-adrenoceptor (−)-RO363 was considerably more efficacious than the non-conventional partial agonist (−)-CGP 12177A, a finding requiring verification with human native β_3-adrenoceptors, including those of adipocytes. The affinity and intrinsic activity of (−)-RO363, estimated for intestinal β_3-adrenoceptors, were lower in guinea-pig ileum than in rat colon, perhaps reflecting differences in β_3-adrenoceptor homologues between the two species.

Conclusions

The synthetic catecholamine (−)-RO363 has agonist activity at multiple β-adrenoceptor subtypes. In human atrial myocardium it causes positive inotropic effects by activation of β_1- and presumably of the third cardiac β-adrenoceptor. (−)-RO363 also causes relaxation of rodent intestinal preparations by stimulation of β_1-adrenoceptors and at higher concentrations by stimulation of β_3-adrenoceptors. (−)-RO363 is a selective β_1-adrenoceptor agonist with greater affinity for human β_1- than β_2- or β_3-adrenoceptors. (−)-RO363 revealed a small inotropic β_1-adrenoceptor hyperresponsiveness in atria from βB patients compared to atria from non-βB patients, despite unchanged β_1-adrenoceptor densities in either group.

A.J.K. is grateful to Professor James Angus (Department of Pharmacology, University of Melbourne) for his hospitality and was supported by a Visiting Research Scholars Award from the University of Melbourne. The authors wish to thank the cardiac surgeons at the Royal Melbourne Hospital, Jim Tatoulis, John Goldblatt, Michael Mullerworth, Peter Skillington, Robin Brown & Alistair Royce who carefully dissected right atrial appendages and took an interest in this study and the many theatre staff who coordinated the collection of right atrial appendages. This work was supported by the NH&MRC of Australia. P.M. is an NH&MRC Research Fellow.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.*, **14,** 48 – 58.

ARCH, J.R.S. & KAUMANN, A.J. (1993). β_3-Adrenoceptors and atypical β-adrenoceptors. *Med. Res. Rev.*, **48,** 663 – 729.

BILSKI, A.J., HALLIDAY, S.E., FITZGERALD, J.D. & WALE, J.L. (1983). The pharmacology of a β_2-selective adrenoceptor antagonist (ICI 118,551). *J. Cardiovasc. Pharmacol.*, **5,** 430 – 437.

BLINKS, J.R. (1965). Convenient apparatus for recording contractions of isolated muscle. *J. Appl. Physiol.*, **20,** 755 – 757.

BOND, R.A. & CLARKE, D.E. (1988). Agoinst and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95,** 723 – 734.

BUXTON, B.F., JONES, C.R., MOLENAAR, P. & SUMMERS, R.J. (1987). Characterization and autoradiographic localization of β-adrenoceptor subtypes in human cardiac tissues. *Br. J. Pharmacol.*, **92,** 299 – 310.

CHAMBERS, J., PARK, J., CRONK, D., CHAPMAN, C., KENNEDY, F.R., WILSON, S. & MILLIGAN, G. (1994). β_3-Adrenoceptor agonist-induced down-regulation of $G_s\alpha$ and functional desensitization in a Chinese hamster ovary cell line expressing a β_3-adrenoceptor refractory to down-regulation. *Biochem. J.*, **303,** 973 – 978.

DOOLEY, D.J., BITTIGER, H. & REYMANN, N.C. (1986). CGP 20712A: a useful tool for quantitating $beta_1$ and $beta_2$-adrenoceptors. *Eur. J. Pharmacol.*, **130,** 137 – 139.

ELNATAN, J., MOLENAAR, P., ROSENFELDT, F.L. & SUMMERS, R.J. (1994). Autoradiographic localization and quantitation of β_1 and β_2-adrenoceptors in the human atrioventricular conducting system: A comparison of patients with idiopathic dilated cardiomyopathy and ischemic heart disease. *J. Mol. Cell. Cardiol.*, **26,** 313 – 323.

EMORINE, L.J., MARULLO, S., BRIEND-SUTREN, M.M., PATEY, G., TATE, K., DELAVIER-KLUTCHKO, C. & STROSBERG, A.D. (1989). Molecular characterization of the human β_3-adrenoceptor. *Science*, **245,** 1118 – 1121.

GILLE, E., LEMOINE, H., EHLE, B. & KAUMANN, A.J. (1985). The affinity of (−)-propranolol for β_1- and β_2-adrenoceptors of human heart. Differential antagonism of the positive inotropic effects and adenylate cyclase stimulation by (−)-noradrenaline and (−)-adrenaline. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **331,** 60 – 70.

HALL, J.A., KAUMANN, A.J. & BROWN, M.J. (1990). Selective β_1-adrenoceptor blockade enhances a positive inotropic response to endogenous catecholamines mediated through β_2-adrenoceptors in human atrial myocardium. *Circ. Res.*, **66,** 1610 – 1623.

HALL, J.A., PETCH, M.C. & BROWN, M.J. (1991). In vivo demonstration of cardiac β_2-adrenoceptor sensitization by β_1-antagonist treatment. *Circ. Res.*, **69,** 959 – 964.

KAUFMAN, R.J. & SHARP, P.A. (1982). Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary DNA gene. *J. Mol. Biol.*, **159,** 601 – 621.

KAUMANN, A.J. (1986). The β_1-adrenoceptor antagonist CGP 20712A unmasks β_2-adrenoceptors activated by (−)-adrenaline in rat sinoatrial node. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **332,** 406 – 409.

KAUMANN, A.J. (1989). Is there a third heart β-adrenoceptor? *Trends Pharmacol. Sci.*, **10,** 316 – 320.

KAUMANN, A.J. (1990). Piglet sinoatrial 5-HT receptors resemble human atrial 5-HT_4-like receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **342,** 619 – 622.

KAUMANN, A.J. (1991). Some aspects of heart beta adrenoceptor function. *Cardiovasc. Drugs Ther.*, **5,** 549 – 560.

KAUMANN, A.J. (1996). (−)-CGP 12177-induced increase of human atrial contraction through a putative third β-adrenoceptor. *Br. J. Pharmacol.*, **117,** 93 – 98.

KAUMANN, A.J., HALL, J.A., MURRAY, K.J., WELLS, F.C. & BROWN, M.J. (1989a). A comparison of the effects of adrenaline and noradrenaline on human heart: the role of β_1- and β_2-adrenoceptors in the stimulation of adenylate cyclase and contractile force. *Eur. Heart J.*, **10,** (Supplement B) 29 – 37.

KAUMANN, A.J. & LEMOINE, H. (1987). β_2-Adrenoceptor-mediated positive inotropic effects of adrenaline in human ventricular myocardium. Quantitative discrepancies with binding and adenylate cyclase stimulation. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **335,** 403 – 411.

KAUMANN, A.J., LEMOINE, H., SCHWEDERSKI-MENKE, U. & EHLE, B. (1989b). Relations between β-adrenoceptor occupancy and increases of contractile force and adenylate cyclase activity induced by catecholamines in human ventricular myocardium. Acute desensitization and comparison with feline ventricle. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **339,** 99 – 112.

KAUMANN, A.J., LYNHAM, J.A., SANDERS, L., BROWN, A.M. & MOLENAAR, P. (1995). Contribution of differential efficacy to the pharmacology of human β_1- & β_2-adrenoceptors. *Pharmacol. Commun.*, **6,** 215 – 222.

KAUMANN, A.J., & MOLENAAR, P. (1996). The third cardiac β-adrenoceptor is different from the colonic β_3-adrenoceptor in the rat. *Br. J. Pharmacol.*, **118,** 2085 – 2098.

KAUMANN, A.J. & SANDERS, L. (1993). Both β_1- and β_2-adrenoceptors mediate catecholamine-evoked arrhythmias in isolated human right atrium. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **348,** 536 – 540.

KAUMANN, A.J. & SANDERS, L. (1994). 5-Hydroxytryptamine causes rate-dependent arrhythmias through 5-HT_4 receptors in human atrium: facilitation by chronic β-adrenoceptor blockade. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **349,** 331 – 337.

LEATHERBARROW, R.J. (1992). *GRAFIT VERSION 3.0*. Staines, U.K: Erithacus Software Ltd.

LEMOINE, H., EHLE, B. & KAUMANN, A.J. (1985). Direct labelling of β_2-adrenoceptors: Comparison of binding potency of ^{3}H-ICI 118,551 and blocking potency of ICI 118,551. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **331,** 40 – 51.

LEMOINE, H., SCHOENELL, H. & KAUMANN, A.J. (1988). Contribution of β_1- and β_2-adrenoceptors of human atrium and ventricle to the effects of noradrenaline and adrenaline as assessed with (−)-atenolol. *Br. J. Pharmacol.*, **95,** 55 – 66.

LIGGETT, S.B. (1992). Functional properties of the rat and human β_3-adrenergic receptors: differential agonist activation of recombinant receptors in chinese hamster ovary cells. *Mol. Pharmacol.*, **42,** 634 – 637.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193,** 265 – 275.

MCLAUGHLIN, D.P. & MACDONALD, A. (1990). Evidence for the existence of 'atypical' β-adrenoceptors (β_3-adrenoceptor) mediating relaxation in the rat distal colon *in vitro*. *Br. J. Pharmacol.*, **101,** 569 – 574.

MCPHERSON, G.A. (1983). A practical computer-based approach to the analysis of radioligand binding experiments. *Comp. Prog. Biomed.*, **17,** 107 – 114.

MCPHERSON, G.A., MALTA, E., MOLENAAR, P. & RAPER, C. (1984). The affinity and efficacy of the selective β_1-adrenoceptor stimulant RO363 at β_1- and β_2-adrenoceptor sites. *Br. J. Pharmacol.*, **82,** 897–904.

MICHEL, M.C., PINGSMANN, A., BECKERINGH, J.J., ZERKOWSKI, H.R., DOETSCH, N. & BRODDE, O-E. (1988). Selective regulation of β_1- and β_2-adrenoceptors in the human heart by chronic β-adrenoceptor antagonist treatment. *Br. J. Pharmacol.*, **94,** 685–692.

MILLER, J.A., CURELLA, P. & ZAHNISER, N.R. (1988). A new densitometric procedure to measure protein levels in tissue slices in quantitative autoradiography. *Brain Res.*, **447,** 60–66.

MOLENAAR, P., CANALE, E. & SUMMERS, R.J. (1987). Autoradiographic localization of beta-1 and beta-2 adrenoceptors in guinea pig atrium and regions of the conducting system. *J. Pharmacol. Exp. Ther.*, **241,** 1048–1064.

MOLENAAR, P., RUSSELL, F.D., SHIMADA, T. & SUMMERS, R.J. (1990a). Densitometric analysis of β_1- and β_2-adrenoceptors in guinea-pig atrioventricular conducting system. *J. Mol. Cell. Cardiol.*, **22,** 483–495.

MOLENAAR, P., SMOLICH, J.J., RUSSELL, F.D., MCMARTIN, L.R. & SUMMERS, R.J. (1990b). Differential regulation of beta-1 and beta-2 adrenoceptors in guinea-pig atrioventricular conducting system after chronic (−)-isoprenaline infusion. *J. Pharmacol. Exp. Ther.*, **255,** 393–400.

MOTOMURA, S., DEIGHTON, N.M., ZERKOWSKI, H.-R., DOETSCH, N., MICHEL, M.C. & BRODDE, O.-E. (1990). Chronic β_1-adrenoceptor antagonist treatment sensitizes β_2-adrenoceptors, but desensitizes M_2-muscarinic receptors in the human right atrium. *Br. J. Pharmacol.*, **101,** 363–369.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: A versatile computerised approach for the characterisation of ligand binding systems. *Anal. Biochem.*, **107,** 220–239.

MURPHREE, S.S. & SAFFITZ, J.E. (1988). Delineation of the distribution of β-adrenergic receptor subtypes in canine myocardium. *Circ. Res.*, **63,** 117–125.

NEVE, K.A., MCGONIGLE, P. & MOLINOFF, P.B. (1986). Quantitative analysis of the selectivity of radioligands for subtypes of beta adrenergic receptors. *J. Pharmacol. Exp. Ther.*, **238,** 46–53.

SANDERS, L., LYNHAM, J.A., BOND, B., DEL MONTE, F., HARDING, S.E. & KAUMANN, A.J. (1995). Sensitization of human atrial 5-HT_4 receptors by chronic β blocker treatment. *Circulation*, **92,** 2526–2539.

SANDERS, L., LYNHAM, J.A. & KAUMANN, A.J. (1996). Chronic β_1-adrenoceptor blockade sensitises the H_1 and H_2 receptor systems in human atrium: role of cyclic nucleotides. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353,** 661–670.

WILLIAMS, D.W. & SUMMERS, R.J. (1990). SIMUL: an accurate method for the determination of receptor subtype proportions using a personal computer. *Comput. Meth. Prog. Biomed.*, **32,** 137–139.

(Received July 15, 1996
Accepted September 16, 1996)