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Validity of (−)-[^{3}H]-CGP 12177A as a radioligand for the 'putative β_4-adrenoceptor' in rat atrium

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1 We have recently suggested the existence in the heart of a 'putative β_4-adrenoceptor' based on the cardiostimulant effects of non-conventional partial agonists, compounds that cause cardiostimulant effects at greater concentrations than those required to block β_1- and β_2-adrenoceptors. We sought to obtain further evidence by establishing and validating a radioligand binding assay for this receptor with (−)-[^{3}H]-CGP 12177A ((−)-4-(3-tertiarybutylamino-2-hydroxypropoxy) benzimidazol-2-one) in rat atrium. We investigated (−)-[^{3}H]-CGP 12177A for this purpose for two reasons, because it is a non-conventional partial agonist and also because it is a hydrophilic radioligand.

2 Increasing concentrations of (−)-[^{3}H]-CGP 12177A, in the absence or presence of 20 μM (−)-CGP 12177A to define non-specific binding, resulted in a biphasic saturation isotherm. Low concentrations bound to β_1- and β_2-adrenoceptors (pK_D 9.4±0.1, B_{max} 26.9±3.1 fmol mg^{-1} protein) and higher concentrations bound to the 'putative β_4-adrenoceptor' (pK_D 7.5±0.1, B_{max} 47.7±4.9 fmol mg^{-1} protein). In other experiments designed to exclude β_1- and β_2-adrenoceptors, (−)-[^{3}H]-CGP 12177A (1–200 nM) binding in the presence of 500 nM (−)-propranolol was also saturable (pK_D 7.6±0.1, B_{max} 50.8±7.4 fmol mg^{-1} protein).

3 The non-conventional partial agonists (−)-CGP 12177A (pK_i 7.3±0.2), (±)-cyanopindolol (pK_i 7.6±0.2), (−)-pindolol (pK_i 6.6±0.1) and (±)-carazolol (pK_i 7.2±0.2) and the antagonist (−)-bupranolol (pK_i 6.6±0.2), all competed for (−)-[^{3}H]-CGP 12177A binding in the presence of 500 nM (−)-propranolol at the 'putative β_4-adrenoceptor', with affinities closely similar to potencies and affinities determined in organ bath studies.

4 The catecholamines competed with (−)-[^{3}H]-CGP 12177A at the 'putative β_4-adrenoceptor' in a stereoselective manner, (−)-noradrenaline (pK_{iH} 6.3±0.3, pK_{iL} 3.5±0.1), (−)-adrenaline (pK_{iH} 6.5±0.2, pK_{iL} 2.9±0.1), (−)-isoprenaline (pK_{iH} 6.2±0.5, pK_{iL} 3.4±0.1), (+)-isoprenaline (pK_i < 1.7), (−)-RO363 ((−)-(1-(3,4-dimethoxyphenethylamino)-3-(3,4-dihydroxyphenoxy)-2-propranol)oxalate, pK_i 5.5±0.1).

5 The inclusion of guanosine 5-triphosphate (GTP 0.1 mM) had no effect on binding of (−)-CGP 12177A or (−)-isoprenaline to the 'putative β_4-adrenoceptor'. In competition binding studies, (−)-CGP 12177A competed with (−)-[^{3}H]-CGP 12177A for one receptor state in the absence (pK_i 7.3±0.2) or presence of GTP (pK_i 7.3±0.2). (−)-Isoprenaline competed with (−)-[^{3}H]-CGP 12177A for two states in the absence (pK_{iH} 6.6±0.3, pK_{iL} 3.5±0.1; % H 25±7) or presence of GTP (pK_{iH} 6.2±0.5, pK_{iL} 3.4±0.1; % H 37±6). In contrast, at β_1-adrenoceptors, GTP stabilized the low affinity state of the receptor for (−)-isoprenaline.

6 The specificity of binding to the 'putative β_4-adrenoceptor' was tested with compounds active at other receptors. High concentrations of the β_3-adrenoceptor agonists, BRL 37344 ((**RR**+**SS**)[4-[2-[[2-(3-chlorophenyl)-2-hydroxy-ethyl]amino]propyl]phenoxy]acetic acid, 6 μM), SR 58611A (ethyl{(7**S**)-7-[(2**R**)-2-(3-chlorophenyl)-2-hydroxyethylamino]-5,6,7,8-tetrahydronaphtyl2-yloxy} acetate hydrochloride, 6 μM), ZD 2079 ((±)-1-phenyl-2-(2-4-carboxymethylphenoxy)-ethylamino)-ethan-1-ol, 60 μM), CL 316243 (disodium (**R**,**R**)-5-[2-[2-(3-chlorophenyl)-2-hydroxyethyl-amino]propyl]- 1,3-benzodioxole-2,2-dicarboxylate, 60 μM) and antagonist SR 59230A (3-(2-ethylphenoxy)-1-[(1**S**)-1,2,3,4-tetrahydronaphth-1-ylamino]-2**S**-2-propanol oxalate, 6 μM) caused less than 22% inhibition of (−)-[^{3}H]-CGP 12177A binding in the presence of 500 nM (−)-propranolol. Histamine (1 mM), atropine (1 μM), phentolamine (10 μM), 5-HT (100 μM) and the 5-HT$_4$ receptor antagonist SB 207710 ((1-butyl-4-piperidinyl)-methyl 8-amino-7-iodo-1,4-benzodioxan-5-carboxylate, 10 nM) caused less than 26% inhibition of binding.

7 Non-conventional partial agonists, the antagonist (−)-bupranolol and catecholamines all competed for (−)-[^{3}H]-CGP 12177A binding in the absence of (−)-propranolol at β_1-adrenoceptors, with affinities (pK_i) ranging from 1.6–3.6 log orders greater than at the 'putative β_4-adrenoceptor'.

8 We have established and validated a radioligand binding assay in rat atrium for the 'putative β_4-adrenoceptor' which is distinct from β_1-, β_2- and β_3-adrenoceptors. The stereoselective interaction with the catecholamines provides further support for the classification of the receptor as 'putative β_4-adrenoceptor'.

Keywords: (−)-[^{3}H]-CGP 12177A; (−)-CGP 12177A; 'putative β_4-adrenoceptor'; atypical β-adrenoceptors; β_1-adrenoceptor; non-conventional partial agonists; catecholamines; (−)-RO363; rat atrium

Introduction

Carlsson *et al*. (1972) showed that both β_1- and β_2-adrenoceptors mediate positive chronotropic effects in cat sino-atrial node and were the first to propose the co-existence of β-adrenoceptors. Since then, the concept of co-existence of β_1- and β_2-adrenoceptors has been confirmed in various cardiac regions in many species including man (Elnatan *et al*., 1994; Kaumann, 1997; Kaumann & Molenaar, 1997). Recently, Gauthier *et al*. (1996) provided evidence for a β_3-adrenoceptor in endomyocardial ventricular biopsies from transplanted hearts and from patients

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undergoing open heart surgery. Interestingly, unlike β_1- and β_2-adrenoceptors which mediate cardiostimulant effects (Kaumann & Molenaar, 1997), stimulation of the β_3-adrenoceptor caused cardiodepressant effects (Gauthier *et al*., 1996). However, cardiodepressant effects mediated by stimulation of β_3-adrenoceptors have not been confirmed in human right ventricular trabeculae, from explanted hearts in terminal failure, or in right ventricular septal strips, from young children with congenital defects (Kaumann & Molenaar, 1997).

For well over 20 years now, evidence has been slowly accumulating for the existence of a cardiac β-adrenoceptor which is pharmacologically distinct from the β_1-, β_2- and β_3-adrenoceptor subtypes. First described in 1973 (Kaumann, 1973), some compounds which had been classified as β_1- and β_2-adrenoceptor antagonists, caused cardiostimulant effects at much higher concentrations than those that caused antagonism. These compounds were termed non-conventional partial agonists. It was not until later (Kaumann, 1989) that it was hypothesized that non-conventional partial agonists may mediate their effects through a β-adrenoceptor distinct from β_1- and β_2-adrenoceptors. Non-conventional partial agonists also cause agonist effects on β_3-adrenoceptors of adipocytes (for review see Arch & Kaumann, 1993), leading on occasion, to the assumption that the cardiac atypical β-adrenoceptor is a β_3-adrenoceptor (Bond & Lefkowitz, 1996). However, more recently, Malinowska & Schlicker (1996) and Kaumann & Molenaar (1996) showed that the non-conventional partial agonists, CGP 12177A and cyanopindolol caused cardiostimulant effects in rat heart through a receptor that was pharmacologically distinct from β_3-adrenoceptors. Furthermore, Kaumann & Molenaar (1996) showed that the β_3-adrenoceptor agonists, BRL 37344, CL 316243, ZD 2079, SR 58611A at concentrations up to 60 μM, concentrations considerably higher than those required to relax rat colon through β_3-adrenoceptors, did not have any effect in rat left or right atrium, nor did they have any effect on cardiostimulant responses to (−)-CGP 12177A. The cardiostimulant effects of (−)-CGP 12177A were unaffected by the β_1- and β_2-adrenoceptor antagonist (−)-propranolol (200 nM), only marginally blocked by the β_3-adrenoceptor selective antagonist SR 59230A ($pK_B = 5.1-5.4$ compared to 6.3–7.5 at rat colonic β_3-adrenoceptors) and were blocked with moderate affinity by (−)-bupranolol ($pK_B = 6.4-6.8$) and CGP 20712A ($pK_B = 6.3-6.4$) (Kaumann & Molenaar, 1996). There is now evidence for coupling of the receptor to the Gs protein-adenylyl cyclase pathway. Positive inotropic and chronotropic effects of (−)-CGP 12177A were potentiated by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Kaumann & Lynham, 1997). Furthermore (−)-CGP 12177A enhances cyclic AMP levels (Kaumann *et al*., 1997) and causes increased activity of cyclic AMP-dependent protein kinase in left and right atrium (Kaumann & Lynham, 1997).

Until now a radioligand binding assay for the receptor that mediates cardiostimulant effects of non-conventional partial agonists has not been available. In this study we have developed and validated a radioligand binding assay in rat atrium with (−)-[^{3}H]-CGP 12177A. In addition we have provided evidence for binding to the receptor of the endogenous catecholamines, (−)-noradrenaline and (−)-adrenaline and synthetic catecholamines, (−)-isoprenaline and (−)-RO363. We now propose that non-conventional partial agonists and catecholamines interact with the cardiac atypical β-adrenoceptor which we designate 'putative β_4-adrenoceptor'.

A preliminary account of this work was presented at the American Society for Pharmacology and Experimental Therapeutics meeting, San Diego, March, 1997 (Sarsero *et al*., 1997).

Methods

Membrane preparation

Sprague-Dawley rats of either sex (250–300 g) were stunned by a blow on the head, exsanguinated and left and right atrium dissected. Atria were homogenized in ice-cold Tris/Mg^{2+} assay buffer (composition in mM: Tris-HCl 50, EGTA 5, EDTA 1, $MgCl_2$ 4, ascorbic acid 1 and phenylmethylsulphonyl fluoride 0.5; pH 7.4), then centrifuged for 5 min at $2{,}250 \times g$ (4°C). The supernatant was centrifuged at $50{,}000 \times g$ (4°C) for 15 min and the pellet resuspended in 15 volumes of ice-cold assay buffer.

Experiments were performed in assay buffer with or without guanosine 5′-triphosphate (GTP; 0.1 mM).

Association and dissociation experiments

The association of (−)-[^{3}H]-CGP 12177A (specific activity 44.5 Ci $mmol^{-1}$) to 'putative β_4-adrenoceptor' binding sites in rat atrium was determined at time points ranging from 0–15 min.

The dissociation of (−)-[^{3}H]-CGP 12177A from binding sites previously incubated with (−)-[^{3}H]-CGP 12177A for 20 min at 37°C was determined for time points ranging from 0–60 min after the addition of 20 μM (−)-CGP 12177A. K_1 was determined from the equation

$$B_t = B_{eq} \cdot e^{-K_{-1} \cdot t} \quad (1)$$

where B_t is specific binding at time t and B_{eq} is specific binding at equilibrium.

Saturation experiments

For binding to β_1-, β_2- and 'putative β_4-adrenoceptor' binding sites 0.01–200 nM (−)-[^{3}H]-CGP 12177A was used in the absence or presence of 20 μM (−)-CGP 12177A to define non-specific binding.

Saturation binding experiments were analysed for two binding sites by non-linear curve fitting with the equation

$$B_{eq} = (B_{max_{\beta_1 AR+\beta_2 AR}} \cdot [(-)\text{-}[^3H]\text{-CGP 12177A}])/(K_{D_{\beta_1 AR+\beta_2 AR}} + [(-)\text{-}[^3H]\text{-CGP 12177A}]) + (B_{max_{\beta_4 AR}} \cdot [(-)\text{-}[^3H]\text{-CGP 12177A}])/(K_{D_{\beta_4 AR}} + [(-)\text{-}[^3H]\text{-CGP 12177A}]) \quad (2)$$

where $B_{max_{\beta_1 AR+\beta_2 AR}}$ is the maximal density of β_1- + β_2-adrenoceptors, $B_{max_{\beta_4 AR}}$ is the maximal density of 'putative β_4-adrenoceptors', $K_{D_{\beta_1 AR+\beta_2 AR}}$ is the dissociation constant of (−)-[^{3}H]-CGP 12177A for β_1- and β_2-adrenoceptors and $K_{D_{\beta_4 AR}}$ is the dissociation constant of (−)-[^{3}H]-CGP 12177A at the 'putative β_4-adrenoceptor'.

Alternatively, for binding to β_1- and β_2-adrenoceptor binding sites only, 0.01–20 nM (−)-[^{3}H]-CGP 12177A was used in the absence or presence of 500 nM (−)-propranolol to define non-specific binding. For binding to 'putative β_4-adrenoceptor' binding sites only, 1–200 nM (−)-[^{3}H]-CGP 12177A was used in the presence of 500 nM (−)-propranolol with 20 μM (−)-CGP 12177A to define non-specific binding.

Saturation binding experiments were analysed for one binding site by non-linear curve fitting.

Assays were conducted at 37°C for 120 min.

Competition binding studies

For binding to β_1-adrenoceptor binding sites a single concentration of (−)-[^{3}H]-CGP 12177A (1.0 − 3.8 nM) was used in the presence of the β_2-adrenoceptor selective antagonist ICI 118,551 50 nM, a concentration which is at least 100 times greater than its affinity for β_2-adrenoceptors (Bilski *et al*., 1983; Lemoine *et al*., 1985). The concentrations of (−)-[^{3}H]-CGP 12177A used were higher than its pK_D value at rat atrial β_1- or β_2-adrenoceptor binding sites (pK_D 9.4, this study) and were used to obtain a clear signal between total and non-specific binding. A higher concentration (47 − 57 nM) was used to label 'putative β_4-adrenoceptor' binding sites in the presence of 500 nM (−)-propranolol. Non-specific binding was defined as above. Assays were conducted at 37°C for 120 min (β_1- and β_2-adrenoceptors) or 60 min ('putative β_4-adrenoceptors').

Competition binding experiments were analysed according to the equation:

$$B_{eq} = B_{nsb} + (B_{sb} - B_{nsb})/(1 + 10^{([competitor] - logIC_{50})}) \quad (3)$$

where B_{sb} = specifically bound (−)-[^{3}H]-CGP 12177A in the absence of competitor, B_{nsb} = non-specifically bound (−)-[^{3}H]-CGP 12177A and IC_{50} = the concentration of competitor causing 50% inhibition.

The pK_i for the competitor was then calculated from the Cheng and Prussoff equation (Cheng & Prussoff, 1973).

Competition binding experiments were analysed for two sites according to the equation:

$$B_{eq} = B_{nsb} + f_H(B_{sb} - B_{nsb})/(1 + 10^{([competitor] - logIC_{50H})}) + f_L(B_{sb} - B_{nsb})/(1 + 10^{([competitor] - logIC_{50L})}) \quad (4)$$

where f_H is the fraction of high affinity binding sites, f_L is the fraction of low affinity binding sites, IC_{50H} and IC_{50L} are concentrations of competitor causing 50% inhibition at high and low affinity binding sites, respectively. pK_{iH} and pK_{iL} were calculated as above.

Assays were terminated by addition of 5 ml ice-cold Tris wash buffer (Tris-HCl, 50 mM, pH 7.4) followed by rapid filtration (Brandel M-30R cell harvester) over GF/B filters. Radioactivity retained on filter paper was counted in a Packard beta-counter (Model Tri-Carb 460 CD).

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

Kinetic, saturation and competition binding data were analysed by non-linear regression by PRISM (GraphPad Software, Inc.).

Drugs used

(−)-CGP 12177A, BRL 37344, SB 207710 (SmithKline Beecham Pharmaceuticals, Welwyn, U.K.), CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl) 1H-imidazole-2-yl)-phenoxy) propyl) amino) ethoxy)-benzamide monomethane sulfphonate) (Ciba-Geigy AG, Basel, Switzerland), (±)-carazolol (Boehringer Mannheim GmbH, Mannheim, Germany), SR 58611A (Sanofi, Montpellier, France), SR 59230A (Sanofi, Milan, Italy), CL 316243 (Wyeth-Ayerst Research Princeton, NJ, USA), ZD 2079 (Zeneca Pharmaceuticals, Macclesfield, U.K.), ICI 118,551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) (Zeneca, Wilmslow, Cheshire, U.K.), (±)-cyanopindolol, (−)-pindolol (Sandoz, Basle, Switzerland), (−)-bupranolol (Sanol, Monheim, Germany), (+)-isoprenaline (+)-bitartrate (Sterling-Winthrop Research Institute, Rensselaer, N.Y., USA), guanosine 5′-triphosphate (Boehringer Mannheim, Australia), (−)-RO363 (Institute of Drug Technology, Boronia, Australia), (−)-noradrenaline bitartrate (Research Biochemicals International, Natick, MA, USA); (−)-propranolol hydrochloride, (±)-propranolol hydrochloride, histamine dihydrochloride, (−)-adrenaline bitartrate; (−)-isoprenaline bitartrate; atropine sulphate; 5-hydroxytryptamine hydrochloride; phentolamine methanesulphonate; phenylmethysulfonyl fluoride; bovine serum albumin, (Sigma, St Louis, MO, USA); (−)-[^{3}H]-CGP 12177A (Dupont, Boston, MA, U.S.A.).

Results

Kinetic binding experiments

Specific binding of 20 nM − 84 nM (−)-[^{3}H]-CGP 12177A in the presence of 500 nM (−)-propranolol to 'putative β_4-adrenoceptors' in rat atrial homogenates was time-dependent and reached over 50% of equilibrium by 15 s, our first sample point (n = 6, Figure 1). Therefore, we were not able to obtain an accurate estimate of the observed association rate constant. Binding remained stable for up to 60 min (n = 3). The addition of excess (−)-CGP 12177A (20 μM) caused dissociation of (−)-[^{3}H]-CGP 12177A with a dissociation rate constant, K_1 $5.9 \pm 2.2 \times 10^{-2}$ min^{-1}, n = 5 (Figure 1).

Saturation binding experiments

Specific binding of increasing concentrations (0.01 − 200 nM) of (−)-[^{3}H]-CGP 12177A (defined with 20 μM (−)-CGP 12177A) to rat atrial homogenates was biphasic with a low Hill slope (0.77 ± 0.05, n = 6). Non-specific binding of (−)-[^{3}H]-CGP 12177A increased linearly with concentration (not shown). Specific binding was 56 ± 4% of total binding, 72 ± 14 d.p.m. at 60 pM; 86 ± 1%, 311 ± 36 dpm at 500 pM; 81 ± 1%, 830 ± 68 d.p.m. at 5 nM and 50 ± 2%, 1499 ± 79 d.p.m. at 40 nM. The curve could be resolved into two sites, one with high affinity (pK_D 9.4 ± 0.1, B_{max} 26.9 ± 3.1 fmol mg^{-1} protein) and another site with lower affinity (pK_D 7.5 ± 0.1, B_{max} 47.7 ± 4.9 fmol mg^{-1} protein, n = 6). Figure 2 shows a representative saturation binding experiment.

In other saturation binding experiments, binding of low concentrations of (−)-[^{3}H]-CGP 12177A (0.01 − 20 nM) in the absence or presence of 500 nM (−)-propranolol to rat atrial membrane homogenates was saturable (B_{max} 30.1 ± 1.6 fmol mg^{-1} protein, n = 6) and of high affinity (pK_D 9.4 ± 0.1, n = 6) (Figure 2). This value is similar to that observed for binding to β_1- and β_2-adrenoceptor binding sites in rat ventricle by Nanoff *et al*. (1987). The presence of β_1- and β_2-adrenoceptor binding sites was confirmed by competition binding studies with the selective β_1-adrenoceptor antagonist CGP 20712A, which revealed binding to β_1-(pK_i 8.2 ± 0.2, % β_1 78 ± 1, n = 6) and β_2-adrenoceptor binding sites (pK_i 6.1 ± 0.1, % β_2 22 ± 1, n = 6) (Figure 3). (−)-Propranolol competed with (−)-[^{3}H]-CGP 12177A with high affinity (pK_i 8.4 ± 0.1, n_H 0.91 ± 0.15, n = 6) (Figure 3) and was therefore used at 500 nM to block binding of (−)-[^{3}H]-CGP 12177A to β_1- and β_2-adrenoceptor binding sites.

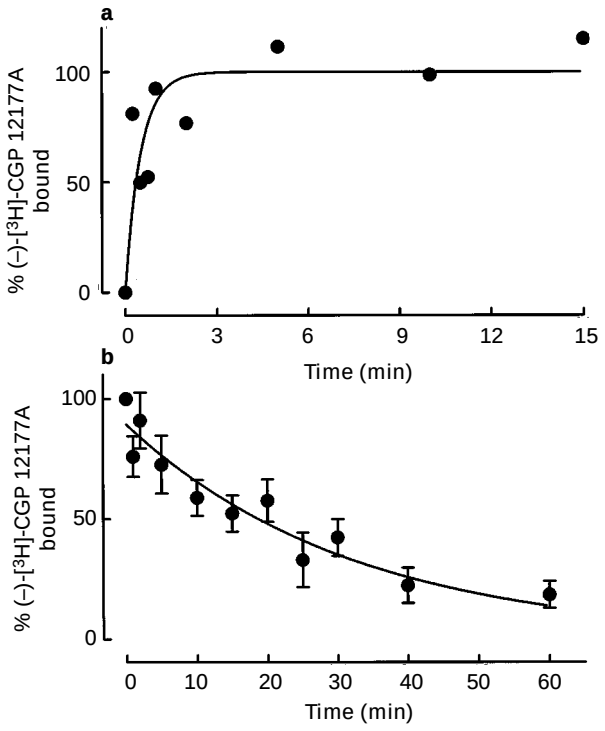

Figure 1 Kinetic analysis of (−)-[^{3}H]-CGP 12177A binding to 'putative β_4-adrenoceptor' binding sites in rat atrial membranes. (−)-[^{3}H]-CGP 12177A binding was performed in the presence of 500 nM (−)-propranolol to block β_1- and β_2-adrenoceptors. Non-specific binding was determined with 20 μM (−)-CGP 12177A. Representative association curve (a) shows specific binding of (−)-[^{3}H]-CGP 12177A (84 nM) at 37°C against time. Mean dissociation curve (b) shows specific binding at various times after addition of 20 μM (−)-CGP 12177A. Each point is expressed as a percentage of the binding occurring at equilibrium in association experiments and at time zero in dissociation experiments. Vertical lines in (b) show s.e.mean from 5 separate experiments.

(−)-[^{3}H]-CGP 12177A (1–200 nM) binding was saturable in the presence of 500 nM (−)-propranolol (B_{max} 50.8±7.4 fmol mg^{-1} protein, $n=6$) and of lower affinity (pK_D 7.6±0.1, $n=6$) (Figure 2).

The results of the separate saturation binding experiments for (−)-[^{3}H]-CGP 12177A together with competition binding experiments with CGP 20712A are consistent with the presence of high affinity β_1- and β_2 -adrenoceptor binding sites, together with a lower affinity binding site, i.e. the 'putative β_4-adrenoceptor'.

Competition binding experiments at the 'putative β_4-adrenoceptor'

The non-conventional partial agonists (−)-CGP 12177A, (±)-cyanopindolol, (−)-pindolol and (±)-carazolol and the antagonist (−)-bupranolol all competed for binding at the 'putative β_4-adrenoceptor' (Table 1, Figure 4 and 5). High concentrations of the selective β_3-adrenoceptor agonists, 6 μM BRL 37344, 60 μM ZD 2079, 60 μM CL 316243, 6 μM SR 58611A and the β_3-adrenoceptor antagonist 6 μM SR 59230A caused less than 22% inhibition (Table 2). Drugs from other classes of receptors, 1 mM histamine, 1 μM atropine, 10 μM phentolamine, 100 μM 5-HT and the 5-HT$_4$ antagonist 10 nM SB 207710 did not compete for (−)-[^{3}H]-CGP 12177A binding (Table 2).

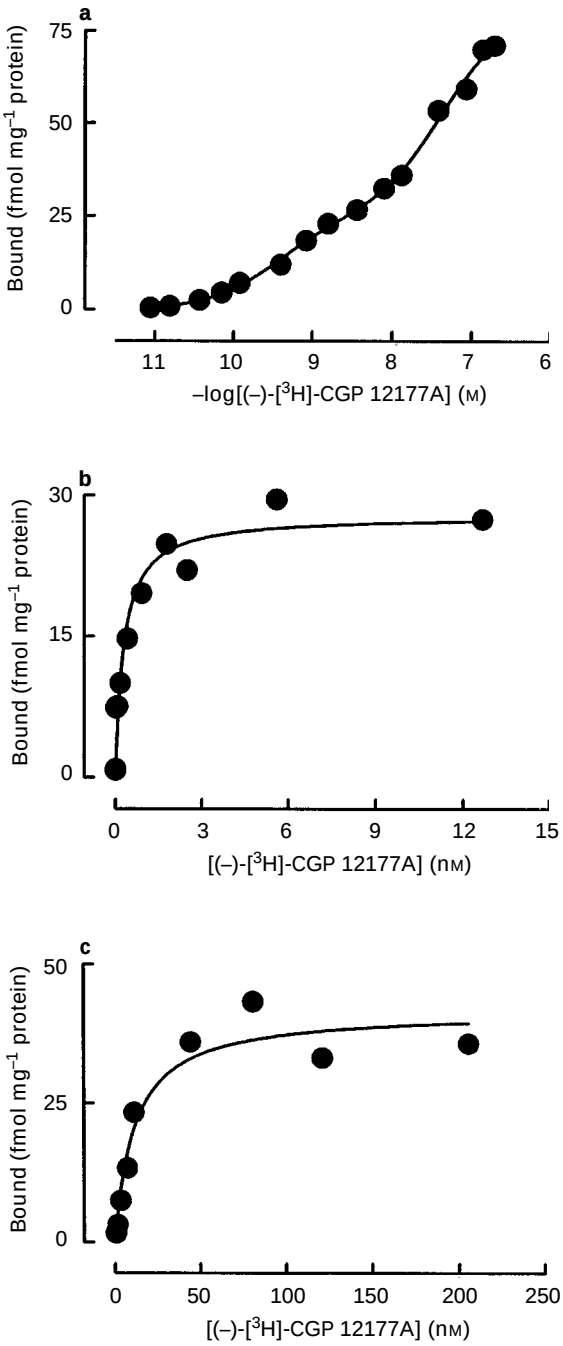

Figure 2 Individual saturation binding experiments showing specific binding in rat atrial membranes for (a) (−)-[^{3}H]-CGP 12177A (0.01–200 nM) binding to β_1-, β_2- and 'putative β_4-adrenoceptors' with non-specific binding determined by 20 μM (−)-CGP 12177A, (b) (−)-[^{3}H]-CGP 12177A (0.01–20 nM) binding to β_1- and β_2- adrenoceptors with non-specific binding determined with 500 nM (−)-propranolol and (c) (−)-[^{3}H]-CGP 12177A (1–200 nM) binding to 'putative β_4-adrenoceptors' in the presence of 500 nM (−)-propranolol, with non-specific binding determined with 20 μM (−)-CGP 12177A. In (a) the saturation binding isotherm was biphasic with one component corresponding to binding at β_1- and β_2- adrenoceptors and the other component corresponding to binding at the 'putative β_4-adrenoceptor'. Note, experiments shown in (a), (b) and (c) were performed on different batches of atrial membranes.

The catecholamines (−)-noradrenaline, (−)-adrenaline, (−)-isoprenaline, (+)-isoprenaline and (−)-RO363 competed for binding in a stereoselective manner (Table 1, Figure 6).

Competition binding experiments at β_1-adrenoceptors

Competition binding experiments were also performed at β_1-adrenoceptors. The non-conventional partial agonists and the antagonist (−)-bupranolol competed for (−)-[^{3}H]-CGP 12177A binding at β_1-adrenoceptors (Figures 4 and 5, Table 1). The catecholamines, (−)-noradrenaline, (−)-adrenaline, (−)-isoprenaline, (+)-isoprenaline and (−)-RO363 also competed at the β_1-adrenoceptor in a stereoselective manner (Figure 6, Table 1). The difference of log affinity estimates between β_1- and 'putative β_4-adrenoceptor' binding sites (log pK_i β_1-adrenoceptor-log pK_i 'putative β_4-adrenoceptor') were not consistent and ranged between 1.6 and 3.6 log units (Table 1).

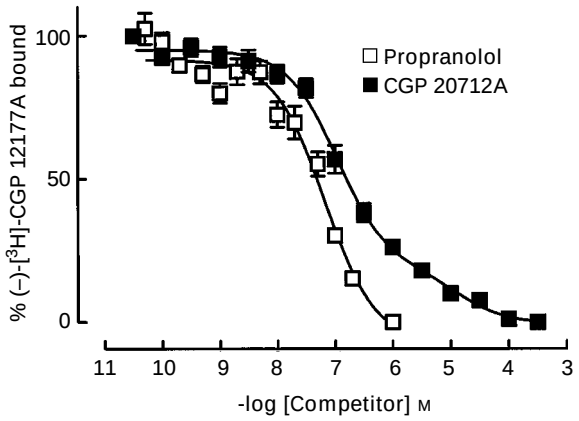

Figure 3 Mean competition binding curves between (−)-[^{3}H]-CGP 12177A and (−)-propranolol and CGP 20712A at β_1 and β_2-adrenoceptor binding sites. Competition binding curves for CGP 20712A could be resolved into a high affinity component corresponding to β_1-adrenoceptors and a low affinity component corresponding to β_2-adrenoceptors. Vertical lines show s.e.mean.

Effect of GTP on radioligand binding at β_1- and 'putative β_4-adrenoceptor' binding sites

GTP (0.1 mM) stabilized the low affinity binding site for (−)-isoprenaline at β_1-adrenoceptors. In the absence of GTP, two binding sites (pK_{iH} 8.47, pK_{iL} 6.79, % H 35.1) were identified. However, in the presence of GTP, one site only with pK_i 7.1 was detected (Figure 7, Table 1).

At 'putative β_4-adrenoceptor' binding sites, inclusion of GTP (0.1 mM) had no effect on competition binding experiments with (−)-CGP 12177A or (−)-isoprenaline (Figure 7). (−)-CGP 12177A competed with (−)-[^{3}H]-CGP 12177A for one receptor state in the absence or presence of GTP with affinity pK_i 7.3 (Figure 7, Table 1). (−)-Isoprenaline competed with (−)-[^{3}H]-CGP 12177A at two states of the receptor in the absence (pK_{iH} 6.6, pK_{iL} 3.5; % H 25.0) and presence of GTP (pK_{iH} 6.2, pK_{iL} 3.4; % H 37.5) Figure 7, Table 1).

Discussion

(−)-[^{3}H]-CGP 12177A was used to label β_1-, β_2- and 'putative β_4- adrenoceptor' binding sites in rat atrium. The design of the 'putative β_4-adrenoceptor binding assay was based on functional studies in which (−)-CGP 12177A stimulated an atypical β-adrenoceptor resistant to blockade of β_1- and β_2-adrenoceptors by (−)-propranolol in rat right and left atrium (Kaumann & Molenaar, 1996; 1997; Kaumann, 1997), which we now call the 'putative β_4-adrenoceptor'. We considered (−)-[^{3}H]-CGP 12177A to be a suitable candidate to investigate as a radioligand for the 'putative β_4-adrenoceptor' in preference to other non-conventional partial agonists, such as iodocyanopindolol or carazolol because of its low lipophilicity (Partition Dulbecco's phosphate buffer solution: octanol ratio [^{3}H]-carazolol 1:23; [^{125}I]-iodocyanopindolol 1:18; [^{3}H]-CGP 12177A 3.1:1; Staehlin *et al*., 1983). We also considered, as did Staehlin *et al*., (1983), that (−)-[^{3}H]-CGP 12177A was less likely than [^{3}H]-carazolol

Table 1 Comparison of competition binding data between (−)-[^{3}H]-CGP 12177A and catecholamines, non-conventional partial agonists and the antagonist (−)-bupranolol at β_1-adrenoceptors and the putative 'β_4-adrenoceptor' in the presence or absence of guanosine triphosphate (GTP, 0.1 mM).

	*β_1-adrenoceptors**				*Putative β_4-adrenoceptors***			
	n	pK_i	%	n_H	n	pK_i	%	n_H	ΔpK_i
+GTP (0.1 mM)									
(−)-Noradrenaline					5	6.30±0.30(H)	20.5±3.6(H)	0.58±0.07	
	4	5.89±0.12(L)	100	0.82±0.02		3.46±0.05(L)	79.5±3.6(L)		2.43
(−)-Adrenaline					5	6.51±0.19(H)	24.4±3.4(H)	0.31±0.03	
	4	5.66±0.07(L)	100	0.83±0.06		2.92±0.11(L)	75.6±3.4(L)		2.74
(−)-Isoprenaline					4	6.23±0.46(H)	37.5±6.3(H)	0.43±0.04	
	7	7.06±0.04(L)	100	0.99±0.05		3.44±0.12(L)	62.5±6.3(L)		3.62
(+)-Isoprenaline	4	3.71±0.13		0.74±0.11	6	<1.7		–	–
(−)-RO363	3	7.45±0.06		0.99±0.12	6	5.46±0.13		0.81±0.09	1.99
(−)-CGP 12177A	–				4	7.34±0.18		0.75±0.06	–
(±)-Cyanopindolol	4	10.75±0.05		0.98±0.05	6	7.56±0.17		0.65±0.09	3.19
(−)-Pindolol	4	8.69±0.04		0.91±0.09	6	6.57±0.14		0.77±0.16	2.12
(±)-Carazolol	4	9.96±0.10		1.24±0.14	6	7.23±0.22		0.85±0.21	2.73
(−)-Bupranolol	4	8.19±0.02		1.01±0.05	10	6.63±0.20		0.83±0.06	1.56
−GTP									
(−)-Isoprenaline	3	8.47±0.31(H)	35.1±7.9(H)	0.74±0.02	4	6.56±0.35(H)	25.0±6.6(H)	0.49±0.05	1.91
		6.79±0.20(L)	64.9±7.9(L)			3.50±0.07(L)	75.0±9.6(L)		3.29
(−)-CGP 12177A	–	–		–	4	7.32±0.19		0.82±0.13	–

* β_1-Adrenoceptor binding performed in the presence of the β_2-adrenoceptor antagonist ICI 118,551 50 nM.
** 'Putative β_4-adrenoceptor' binding performed in the presence of (−)-propranolol 500 nM. Affinity (pK_i) and pseudo Hill coefficient values (n_H) were obtained from *n* individual experiments and are shown as mean±s.e.mean. ΔpK_i = pK_i (β_1-adrenoceptor) − pK_i ('putative β_4-adrenoceptor'). H: high affinity binding state; L: low affinity binding state; %: percentage of receptor in high or low affinity binding state.

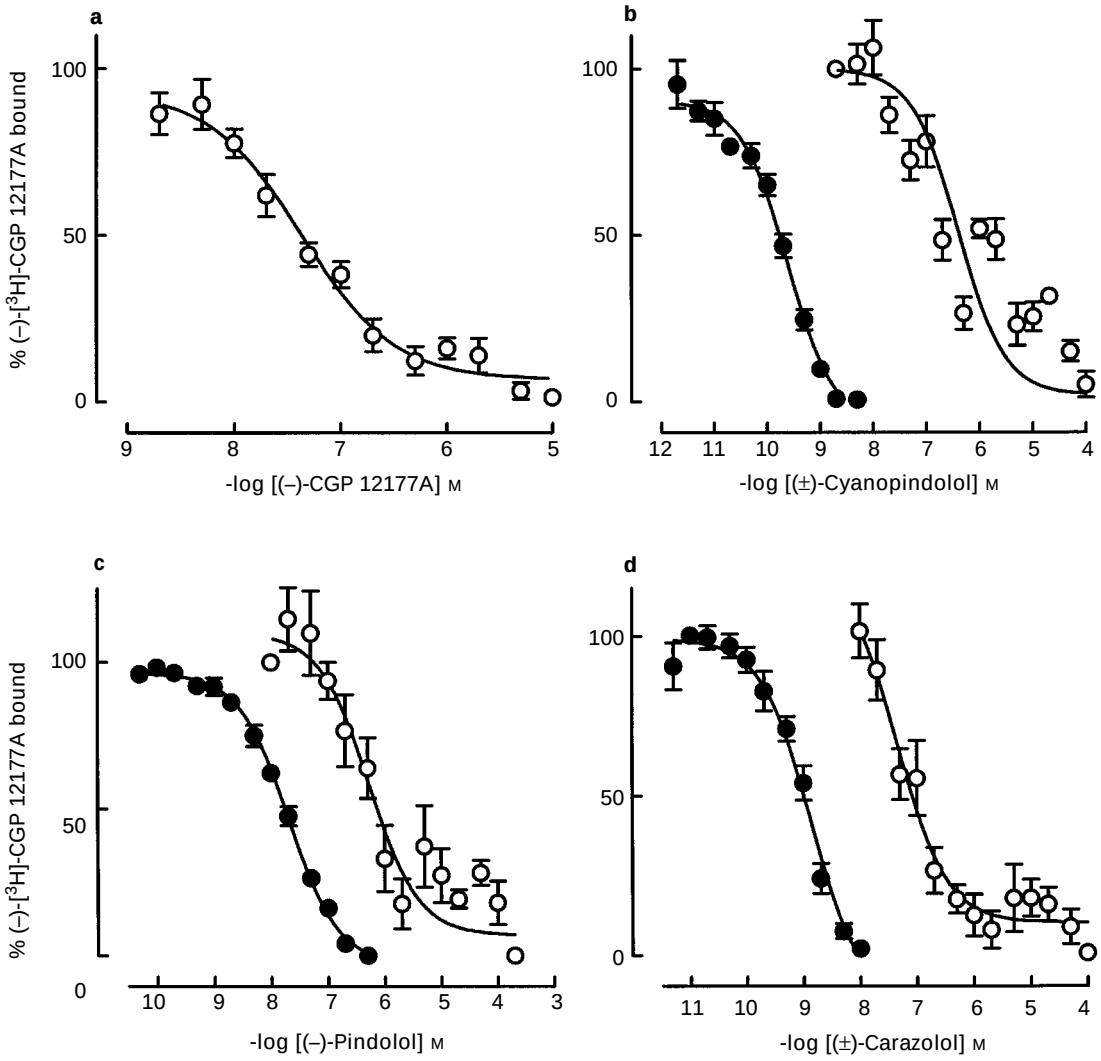

Figure 4 Mean competition binding curves between (-)-[^{3}H]-CGP 12177A and non-conventional partial agonists ((a)(−)-CGP 12177A, (b) (±)-cyanopindolol, (c) (−)-pindolol and (d) (±)-carazolol) at β_1-adrenoceptor binding sites in the presence of the selective β_2-adrenoceptor antagonist 50 nM ICI 118,551 (solid circles) and 'putative β_4-adrenoceptor' binding sites (open circles). Vertical lines show s.e.mean.

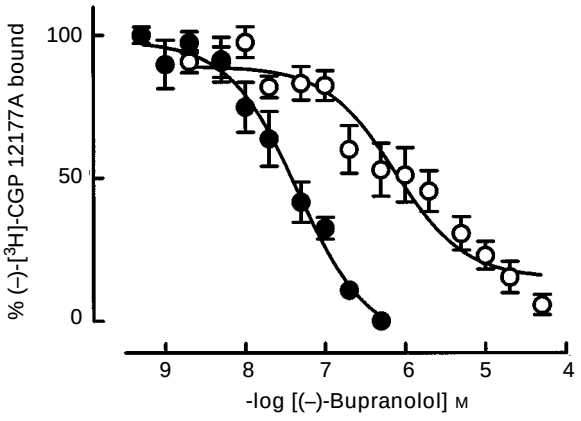

Figure 5 Mean competition binding curves between (-)-[^{3}H]-CGP 12177A and the antagonist (-)-bupranolol at β_1-adrenoceptor binding sites in the presence of the selective β_2-adrenoceptor antagonist 50 nM ICI 118,551 (solid symbols) and 'putative β_4-adrenoceptor' binding sites (open symbols). Vertical lines show s.e.mean.

or [^{125}I]-iodocyanopindolol to bind to non-specific, (−)-propranolol-resistant binding sites (Molenaar *et al.*, 1992).

The continuous saturation binding experiment with (−)-[^{3}H]-CGP 12177A in the absence or presence of 20 μM CGP 12177A was biphasic, suggesting the presence of at least two distinct receptor populations. The higher affinity site was to β_1- and β_2-adrenoceptor binding sites which we did not distinguish between. Earlier radioligand binding studies showed evidence for β_1- and β_2-adrenoceptor binding sites in rat whole heart (Minneman *et al.*, 1979a;b), ventricle (Nanoff *et al.*, 1987; Sarsero & Molenaar, 1995) and in separate (Juberg *et al.*, 1985) or combined left and right atrium, (this study). In rat ventricle, Nanoff *et al.* (1987) showed that (−)-[^{3}H]-CGP 12177A had a slightly higher affinity for β_1- (pK_i 9.5) than β_2-adrenoceptor binding sites (pK_i 9.0) which was similar to our 'combined' β_1- and β_2-adrenoceptor value. In spontaneously beating rat right atrium β_1-adrenoceptors mediate powerful chronotropic effects, but it is also possible to detect β_2-adrenoceptor-mediated chonotropic effects with either the selective β_2-adrenoceptor agonist procaterol (O'Donnell & Wanstall, 1985)

Table 2 Summary of competition binding data at the 'putative β_4-adrenoceptor' * between (−)-[^{3}H]-CGP 12177A and single concentrations of compounds active at β_3-adrenoceptors, histamine, muscarinic, α-adrenoceptors and 5-HT receptors

	n	*Inhibition %*
BRL 37344 6 μM	6	6.7±3.7
SR 58611A 6 μM	6	22.2±5.3
ZD 2079 60 μM	6	12.0±6.7
CL 316243 60 μM	6	11.3±3.3
SR 59230A 6 μM	6	16.3±4.5
Histamine 1 mM	6	25.1±4.9
Atropine 1 μM	8	25.5±9.0
Phentolamine 10 μM	6	10.0±3.9
5-HT 100 μM	6	7.4±4.2
SB 207710 10 nM	6	17.9±11.1

*'Putative β_4-adrenoceptor' binding performed in the presence of (−)-propranolol 500 nM. Shown are percentage inhibition values obtained from *n* individual experiments expressed as mean±s.e.mean.

or with (−)-adrenaline using the selective β_1-adrenoceptor antagonist CGP 20712A (Kaumann, 1986).

We then set up separate saturation radioligand binding assays, one for β_1- and β_2-adrenoceptors and another for the low affinity receptor population. For β_1- and β_2-adrenoceptor binding sites, non specific binding was determined with 500 nM (−)-propranolol. There was good agreement between the two methods for the determination of β_1- and β_2-adrenoceptor density (26.9 and 30.1 fmol mg^{-1} protein) and affinity of (−)-[^{3}H]-CGP 12177A (pK_D 9.4 both assays). For the low affinity binding site, a saturation binding assay was designed with 500 nM (−)-propranolol to block β_1- and β_2-adrenoceptors and 20 μM (−)-CGP 12177A to define non-specific binding. Again there was good agreement between B_{max} and pK_D values (B_{max}, 47.7 and 50.8 fmol mg^{-1} protein; pK_D 7.5 and 7.6). These studies show the presence of a low affinity site in addition to β_1- and β_2-adrenoceptor binding sites.

We then determined whether the low affinity binding site was the 'putative β_4-adrenoceptor' binding site.

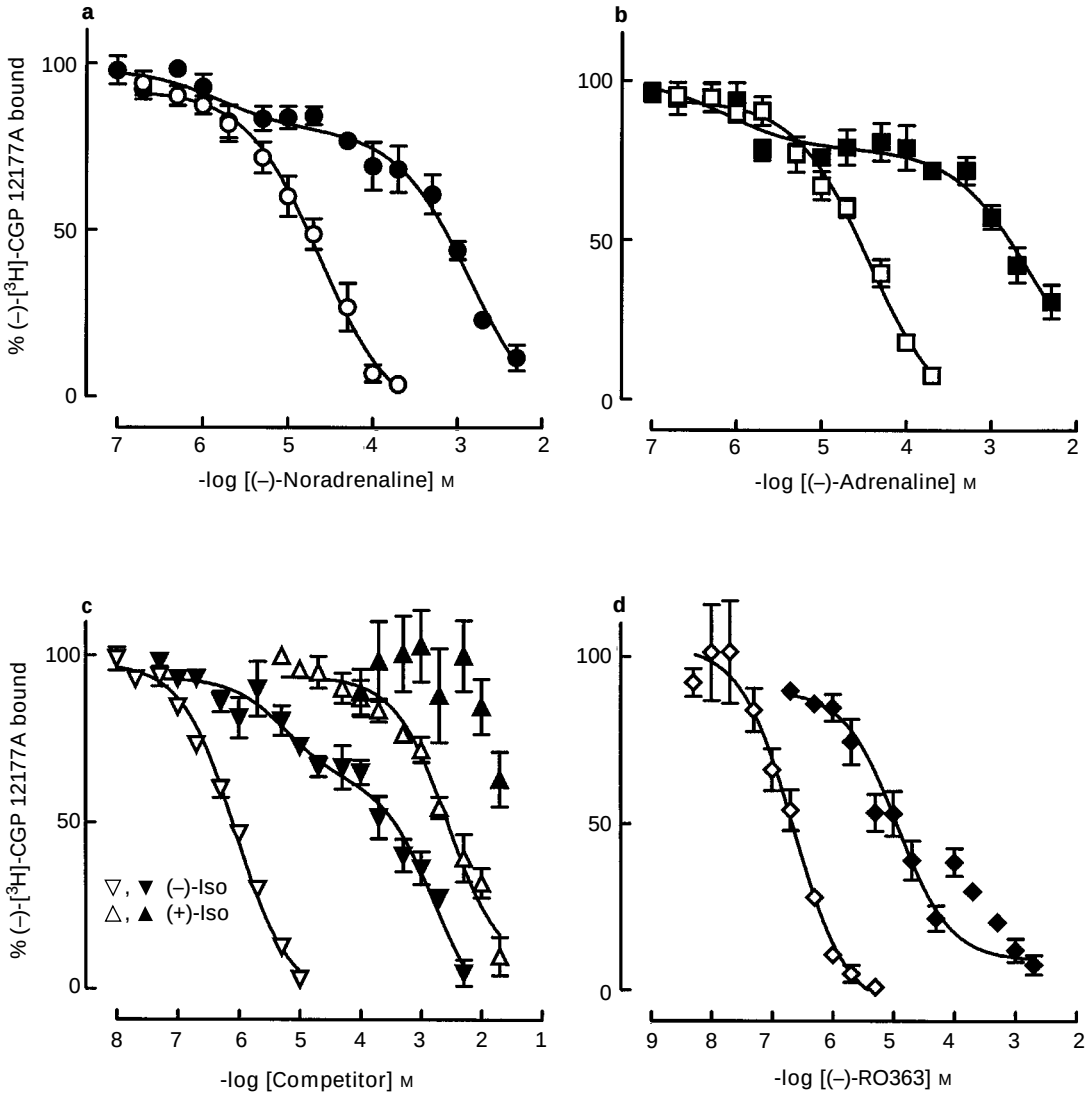

Figure 6 Mean competition binding curves between (−)-[^{3}H]-CGP 12177A and the catecholamines, (a) (−)-noradrenaline, (b) (−)-adrenaline, (c) (−)-isoprenaline ((−)-Iso) and (+)-isoprenaline ((+)-Iso) and (d) (−)-RO363 at β_1-adrenoceptor binding sites in the presence of the selective β_2-adrenoceptor antagonist 50 nM ICI 118,551 (open symbols) and 'putative β_4-adrenoceptor' binding sites (solid symbols). Vertical lines show s.e. mean.

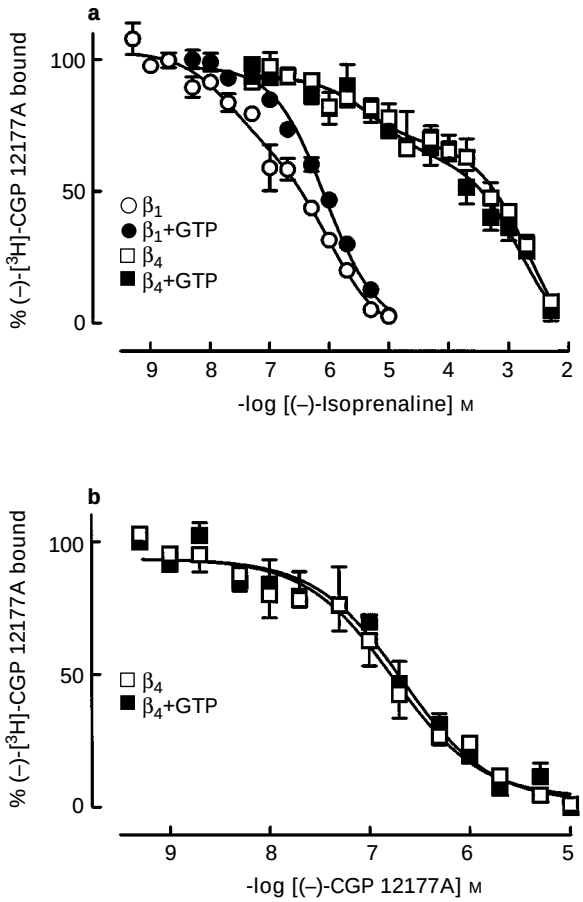

Figure 7 Effect of GTP on binding at β_1-adrenoceptor binding sites (β_1, in the presence of the selective β_2-adrenoceptor antagonist 50 nM ICI 118,551) and at the 'putative β_4-adrenoceptor' (β_4). Competition binding experiments were performed between (−)-[^{3}H]-CGP 12177A and (−)-isoprenaline (a) and (−)-CGP 12177A (b) in the absence or presence of GTP, 0.1 mM. Competition binding isotherms for (−)-isoprenaline in the absence of GTP at β_1-adrenoceptors, and for (−)-isoprenaline in the absence and presence of GTP at 'putative β_4-adrenoceptors' were fitted to a two state affinity model.

Non-conventional partial agonists

The affinities of non-conventional partial agonists were closely similar to potencies determined from organ bath studies. The affinity of (−)-CGP 12177A determined from saturation (7.5–7.6) and competition binding experiments (7.3) was similar to its pEC_{50} value in rat left (7.5) and right (7.3) atrium (Kaumann & Molenaar, 1996). The affinity of (±)-cyanopindolol (7.6) was also similar to its pEC_{50} value in rat left (7.1) and right (7.7) atrium (Kaumann & Molenaar, 1996). Similar comparisons are also valid for (−)-pindolol (pK_i 6.6, pEC_{50} guinea-pig right atrium 7.0, Walter *et al*., 1984) and (±)-carazolol (pK_i 7.2, pEC_{50} rat right atrium 7.0, Kaumann *et al*., 1979). The affinity of (−)-bupranolol (6.6) was also similar to its affinity in rat atrium (pK_B 6.4–6.8, Kaumann & Molenaar, 1996). These results indicate similarity of the functional receptor and binding site.

Lack of activity of ligands from other receptor classes

(−)-[^{3}H]-CGP 12177A has also been used as a radioligand for β_3-adrenoceptor binding sites in murine 3T3-F442A adipocytes and human β_3-adrenoceptors expressed in Chinese hamster ovary cells (Feve *et al*., 1991), rat brown (D'Allaire *et al*., 1995) and white adipocytes (Germack *et al*., 1997). In these studies the affinities of (−)-[^{3}H]-CGP 12177A ranged from 23–80 nM. Therefore we determined whether the β_3-adrenoceptor agonists, BRL 37344 (6 μM), ZD 2079 (60 μM) SR 58611A (6 μM) and CL 316243 (60 μM) and the antagonist SR 59230A (6 μM) competed for (−)-[^{3}H]-CGP 12177A binding in rat atrium. The affinities (pK_D) of BRL 37344 range from 6.6–7.1 on the rat cloned β_3-adrenoceptor (Muzzin *et al*., 1991; Liggett, 1992; Dolan *et al*., 1994), CL 316243, 6.0 on rat cloned β_3-adrenoceptor (Dolan *et al*., 1994) and SR 58611A 5.2 at the human and 5.9 at the murine cloned β_3-adrenoceptor (Blin *et al*., 1994). These agonists did not compete for binding with (−)-[^{3}H]-CGP 12177A in rat atrium. The antagonist SR 59230A, has an affinity (pK_B) of 6.3–7.5 on rat colonic β_3-adrenoceptors (Kaumann & Molenaar, 1996) but also did not compete for (−)-[^{3}H]-CGP 12177A binding in rat atrium. These studies are in line with our previous functional studies (Kaumann & Molenaar, 1996), in which we showed that the β_3-adrenoceptor agonists did not affect basal contractile activity (left atrium) or sinoatrial rate (right atrium) and nor did they modify the cardiostimulant effects of (−)-CGP 12177A. We also determined the affinity of SR 59230A to be (pK_B) 5.1–5.4 for the 'putative β_4-adrenoceptor' in rat right and left atrium which is lower than at β_3-adrenoceptors (Kaumann & Molenaar, 1996). Our radioligand binding studies support the conclusion made from previous studies (Kaumann & Molenaar, 1996; Malinowska & Schlicker, 1996) that the receptor mediating cardiostimulant effects of CGP 12177A in rat is not a β_3-adrenoceptor.

Further specificity of (−)-[^{3}H]-CGP 12177A binding was tested with compounds which are active at histamine, muscarinic, α-adrenoceptors and 5-HT receptors. The affinity values of histamine for H_1- and H_2-receptors in guinea-pig left atrium, determined from competition binding experiments with [^{3}H]-mepyramine (H_1-) were (pK_i) 4.8 and [^{3}H]-tiotidine (H_2-) 5.1, respectively (Hattori *et al*., 1991). Atropine has an affinity (pK_i) of 8.9 for rat atrial M_2-receptors, determined in competition binding experiments with [^{3}H]-N-methyl scopolamine (Doods *et al*., 1987). Phentolamine has affinities (pK_i) of 8.2, 7.2 and 7.5 for α_{1a}- α_{1b}- and α_{1d}-receptors (Michel *et al*., 1995). 5-Hydroxytryptamine (5-HT) has an affinity (pK_i) of 6.6 and SB 207710 (9.5) for piglet right atrial 5-HT_4 receptors (Kaumann *et al*., 1995a) determined in radioligand binding experiments with [^{125}I]-SB 207710. Our studies showed that (−)-[^{3}H]-CGP 12177A does not label histamine, muscarinic, α-adrenoceptors or 5-HT receptors in rat atrium.

Interaction of catecholamines with the 'putative β_4-adrenoceptor'

We had previously observed that positive inotropic effects of the catecholamine (−)-RO363 in human right atrium *in vitro* were partially resistant to blockade by (−)-CGP 20712A (Molenaar *et al*., 1997). We ruled out stimulation of co-existing β_2-adrenoceptors and suggested that it also stimulated the 'putative β_4-adrenoceptor' (Molenaar *et al*., 1997). Wheeldon *et al*. (1993) also provided evidence that (−)-isoprenaline could cause inotropic and lusitropic effects in human myocardium *in vivo* by stimulation of an atypical β-adrenoceptor. The atypical β-adrenoceptor effects of (−)-isoprenaline were observed in the presence of atenolol 25 mg (p.o.), a concentration that attenuates (−)-isoprenaline-induced increases in heart rate (β_1-adrenoceptor) and nadolol 5, 20 and 80 mg (p.o), concentrations, that attenuate (−)-isoprenaline-mediated increases in heart rate (β_1-adrenoceptor) and postural finger tremor (β_2-adrenoceptor). Wheeldon *et al*. (1993) described the receptor as a β_3-adrenoceptor, but this is

not the same receptor which causes cardiodepression in ventricular endomyocardial biopsies of cardiac transplant patients or patients undergoing open heart surgery (Gauthier *et al*., 1996). Wheeldon *et al*., (1993) provided further evidence for a cardiostimulant atypical β-adrenoceptor which we think corresponds to the 'putative β_4-adrenoceptor'.

We have shown that the endogenous catecholamines (−)-noradrenaline, (−)-adrenaline and synthetic catecholamines, (−)-RO363, (−)-and (+)-isoprenaline bind to the 'putative β_4-adrenoceptor' in a stereoselective manner. Two affinity states of the receptor were observed for (−)-noradrenaline, (−)-adrenaline and (−)-isoprenaline. The high affinity state comprised 20–38% of the total population of receptors. These catecholamines had similar affinities for the high affinity state (pK_{iH} 6.2–6.5) and also for the low affinity state of the 'putative β_4-adrenoceptors' (pK_{iL} 2.9–3.5). The affinities of catecholamines at the low affinity state of the receptor were slightly higher than those at native β_3- adrenoceptors of rat adipocytes (Germack *et al*., 1997). Furthermore, the overall affinity of (−)-RO363 was higher at the rat atrial 'putative β_4-adrenoceptor' (pK_i 5.5) than at the human cloned β_3-adrenoceptor (pK_i 4.5, Molenaar *et al*., 1997). These small but consistent differences further appear to differentiate the 'putative β_4-adrenoceptor' from the β_3-adrenoceptor. The functional significance of the high and low affinity binding sites remains to be determined.

Comparison between binding at 'putative β_4-adrenoceptor' and β_1-adrenoceptor binding sites

We included 500 nM (−)-propranolol in the 'putative β_4-adrenoceptor' binding assay to block co-existing β_1- and β_2-adrenoceptor sites. In order to validate further binding to the 'putative β_4-adrenoceptor' and to ensure that it did not represent binding to β_1-adrenoceptor binding sites, we also estimated the affinities of non-conventional partial agonists and catecholamines at β_1-adrenoceptors. Affinities for all compounds tested were higher at the β_1-adrenoceptor than at the 'putative β_4-adrenoceptor'. However, the difference between pK_i values at β_1- and 'putative β_4- adrenoceptor' (pK_i β_1- − pK_i 'putative β_4-adrenoceptor') were not consistent and ranged from 1.6–3.6 log units (Table 1). This indicates that 'putative β_4-adrenoceptor' binding cannot be interpreted as β_1-adrenoceptor binding shifted to the right by (−)-propranolol. If this were the case, one would expect the same difference between (pK_i β_1- − pK_i 'putative β_4-adrenoceptor'), regardless of ligand affinity.

Agonist binding at β_1- and β_2-adrenoceptor subtypes is sensitive to guanine nucleotides. In the absence of guanine nucleotides, the receptor binding site exists in high and low affinity states and in their presence, the high affinity state is converted to the low affinity state (Lefkowitz *et al*., 1976; 1981; Maguire *et al*., 1976; Kaumann *et al*., 1995b). We also showed that inclusion of GTP stabilized the low affinity state of the β_1-adrenoceptor for the agonist (−)-isoprenaline. In this assay at β_1-adrenoceptors, (−)-[^{3}H]-CGP 12177A is an antagonist. At 'putative β_4-adrenoceptors', GTP had no effect on agonist binding with (−)-isoprenaline and (−)-CGP 12177A. It is important to realize that the radioligand, (−)-[^{3}H]-CGP 12177A is an agonist at the 'putative β_4-adrenoceptor' and we do not know whether a high or low affinity receptor state is stabilized at the concentrations used for competition binding with cold ligands. Therefore, the effects of GTP on agonist binding cannot be strictly compared between β_1-adrenoceptors and the 'putative β_4-adrenoceptor'. Further clarification of the effects of guanine nucleotides on agonist binding at the 'putative β_4-adrenoceptor' will be possible with the development of an antagonist radioligand.

Conclusions and future prospects

This study has established and validated a radioligand binding assay for the cardiac atypical β-adrenoceptor. The stereoselective catecholamine binding profile and difference from β_1-, β_2- and β_3-adrenoceptors indicate that the atypical β-adrenoceptor is novel. We designate this receptor provisionally as 'putative β_4-adrenoceptor'. However, we await the cloning of the receptor for a formal classification of it. We are currently using this radioligand binding assay in human right atrium, where we have also obtained functional evidence for the existence of the 'putative β_4-adrenoceptor' (Kaumann, 1996; 1997; Kaumann & Molenaar, 1996; Sarsero *et al*., 1996a;b; Kaumann *et al*., 1997; Molenaar *et al*., 1997). We are also planning quantitative receptor autoradiography studies to determine the density and distribution of 'putative β_4-adrenoceptors' in discrete regions such as rat and human sinoatrial node.

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