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RESEARCH PAPER

The selectivity of β-adrenoceptor agonists at human β_1-, β_2- and β_3-adrenoceptors

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Background and purpose: There are two important properties of receptor–ligand interactions: affinity (the ability of the ligand to bind to the receptor) and efficacy (the ability of the receptor–ligand complex to induce a response). Ligands are classified as agonists or antagonists depending on whether or not they have efficacy. In theory, it is possible to develop selective agonists based on selective affinity, selective intrinsic efficacy or both. This study examined the affinity and intrinsic efficacy of 31 β-adrenoceptor agonists at the three human β-adrenoceptors to determine whether the current agonists are subtype selective because of affinity or intrinsic efficacy.
Experimental approach: Stable clonal CHO-K1 cell lines, transfected with either the human β_1, β_2 or β_3-adrenoceptor, were used, and whole-cell [^{3}H]-CGP 12177 radioligand binding and [^{3}H]-cAMP accumulation were measured.
Key results: Several agonists were found to be highly subtype selective because of selective affinity (e.g. salmeterol and formoterol, for the β_2-adrenoceptor over the β_1 or β_3), while others (e.g. isoprenaline) had little affinity–selectivity. However, the intrinsic efficacy of salmeterol, formoterol and isoprenaline was similar across all three receptor subtypes. Other ligands (e.g. denopamine for β_1; clenbuterol, AZ 40140d, salbutamol for β_2) were found to have subtype-selective intrinsic efficacy. Several ligands appeared to activate two agonist conformations of the β_1- and β_3-adrenoceptors.
Conclusions and implications: There are agonists with subtype selectivity based upon both selective affinity and selective intrinsic efficacy. Therefore, there is scope to develop better selective agonists based upon both selective affinity and selective intrinsic efficacy.
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Keywords: β-adrenoceptor; β-blocker; β-agonist; β-antagonist; cAMP; drug selectivity; whole-cell binding; subtype selectivity; efficacy; intrinsic efficacy; GPCR

Abbreviations: AZ 40140d, (*R*)-*N*-[5-[2-[2-(9H-carbazole-2-iloxy)ethylamino]-1-hydroxyethyl]-2-hydroxyphenyl] methanesulphonamide; BAAM, bromoacetylalprenololmenthane; BRL 35135A, (*R**,*R**)-[4-2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]-acetic acid methyl ester; BRL 37344, (*R**,*R**)- (±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid; CGP 12177, (–)-4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one; CGP 20712A, 2-hydroxy-5-(2-[{hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy)propyl}amino]ethoxy)benzamide; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; ICI 118551 (–)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol; L 748337, *N*-[[3-[(2*S*)-2-Hydroxy-3-[[2-[4-[(phenylsulphonyl) amino]phenyl]ethyl]amino]propoxy]phenyl]methyl]-acetamide; L 755507, 4-[[(hexylamino)carbonyl]amino]-*N*-[4-[2-[[(2*S*)-2-hydroxy-3-(4-hydroxyphenoxy)propyl]amino]ethyl]phenyl]-benzenesulphonamide; SDZ 21009, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1H-indole-2-carboxylic acid, 1-methylethyl ester; SR59230A, 1-(2-ethylphenoxy)-3-[[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2*S*)-2-propanol; TAK 677, [3-[(2*R*)-[[(2*R*)-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1*H*-indol-7-yloxy]-acetic acid; ZD 2079, 4-[2-[[(2*R*)-2-hydroxy-2-phenylethyl]amino]ethoxy]-benzeneacetic acid hydrochloride; ZD 7114, (*S*)-4-[2-hydroxy-3-phenoxypropylaminoethoxy]-*N*-(2-methoxyethyl)phenoxyacetamide hydrochloride

Introduction

There are two important properties of receptor–ligand interactions: affinity (the ability of the ligand to bind to the receptor) and efficacy (the ability of the receptor–ligand complex to induce a response; Strange, 2008). Ligands are classified as

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agonists or antagonists depending on whether or not they have efficacy (Clarke and Bond, 1998; Strange, 2008). Thus, a neutral antagonist binds to a given receptor but does not stimulate a response (i.e. it has affinity, but no efficacy). The affinity of antagonist ligands can be measured directly from radioligand binding or from antagonism of agonist responses in functional assays (Strange, 2008). Thus, the selectivity of antagonist drugs, either between receptors or between receptor subtypes, is relatively easily determined (e.g. Büscher *et al*., 1996; Smith and Teitler, 1999; Hoffmann *et al*., 2004; Baker, 2005a). Subtype-selective antagonist drugs have been sought for many clinical areas with the expected benefit that a high target receptor selectivity increases clinical effectiveness while reducing clinical side effects (Clarke and Bond, 1998).

In reality, many ligands considered as antagonists actually do stimulate responses, either by reducing the basal response (inverse agonist in systems with constitutive activity) or by stimulating a small response on their own (partial agonist). Truly neutral antagonists are few and far between, and a great many 'antagonist' drugs in clinical use are either inverse agonists or very weak partial agonists (e.g. Baker *et al*., 2003a,b; Kenakin, 2004 and examples in this study).

Measurements of agonist selectivity are more complex because agonists bind (and so have affinity), as well as being able to induce a response (efficacy; Kenakin, 1999a). Thus, the selectivity for agonist ligands between receptors or receptor subtypes depends upon these two variables, and it is theoretically possible to have subtype-selective agonists by different mechanisms. A ligand with higher affinity for a given receptor subtype would appear selective even if its efficacy was similar across different subtypes. Similarly, a ligand would appear as a subtype-selective agonist if its efficacy at a certain receptor subtype was significantly greater even if its affinity were the same across the other subtypes.

Agonist affinity can be measured in the same manner as that for antagonist ligands (e.g. binding studies); however, the response an agonist induces depends upon at least six factors: affinity, efficacy, number of receptors present, efficiency of the receptor–effector coupling, effector response measured and any desensitization that occurs within the time-frame of the measurement (Kenakin, 1999a; 2002). Many of these factors alter between different cells, tissues and organisms, and between responses measured within the same tissue. Ligand affinity and ligand efficacy are, however, considered to depend upon the chemical interaction between the ligand and receptor, and are thus considered to be independent of the cells/tissues/organisms under study. Therefore, this chemical ability of a certain ligand to induce a response has been termed 'intrinsic efficacy', and is a measure of molecular efficacy for a given ligand at a given receptor, rather than at a tissue or organism level (Furchgott, 1966; Clarke and Bond, 1998).

Intrinsic efficacy is therefore an important property, and although direct measurements are difficult, relative intrinsic efficacies can be compared across different agonists if the other variables are controlled for. For example, using a stable cell line or single tissue preparation, and measuring a single response remove any tissue variables (including receptor expression level), and thus the differences in the response seen are due to those of ligand affinity and intrinsic efficacy. Thus, in a single system, partial agonists can be ranked in order of efficacy by the proportion of the maximal response they stimulate (Kenakin, 1999a; Strange, 2008). For full agonists, the intrinsic efficacy of ligands in the same system can be compared by means of an 'efficacy ratio' (K_D/EC_{50}; Furchgott, 1966; Kenakin, 1982; 1999b; Strange, 2008). For example, a ligand with an affinity (K_D) of 100 nM, but an agonist response EC_{50} value of 1 nM has an intrinsic efficacy ratio of 100. This ligand would have a higher intrinsic efficacy than a ligand with a K_D of 100 nM and an EC_{50} value of 10 nM (ratio of 10), provided both ligands were measured in the same system.

This study examines these two variables (affinity and intrinsic efficacy) for a range of ligands to determine whether the agonist ligands currently available are selective because of selective affinity, selective intrinsic efficacy or both. The three human β-adrenoceptors (Alexander *et al*., 2008) have been chosen because they are all G_s-coupled GPCRs, and therefore identical responses (cAMP) can be measured, and there is a large range of β-adrenoceptor agonist ligands available.

Methods

Materials

Fetal calf serum was obtained from PAA Laboratories (Teddington, Middlesex, UK). White-sided view plates were from Matrix Laboratories (supplied by Thermo Fisher Scientific, Basingstoke, UK), and Microscint 20 scintillation fluid was from PerkinElmer (Shelton, CT, USA). [^{3}H]-CGP 12177 was from Amersham International (Buckinghamshire, UK). BRL 37344, cimaterol, formoterol, isoprenaline, L 755507, procaterol, SDZ 21009, SR 59230A, ZD 2079 and ZD 7114 were from Tocris Life Sciences (Avonmouth, UK). Carazolol was a gift from Dr Chris Tate (LMB, Cambridge, UK); nebivolol and butoxamine were a gift from Stefano Evangelista (Menarini Ricerche Spa, Florence, Italy); zinterol was a gift from Dr Torsten Christ (Department of Pharmacology and Toxicology, Dresden University of Technology, Dresden, Germany.); L 748337, TAK677 and (*R*)-*N*-[5-[2-[2-(9H-carbazole-2-iloxy) ethylamino]-1-hydroxyethyl]-2-hydroxyphenyl]methanesulphonamide were gifts from Dr Kerry af Forselles, Pfizer, Sandwich, UK. (*R*)-*N*-[5-[2-[2-(9H - carbazole - 2 - iloxy) ethylamino] - 1 - hydroxyethyl] - 2-hydroxyphenyl]methane sulphonamide is compound d from US2003/0018061A1, which is believed to be AZ40140 (SB418790) and shall thus be referred to as AZ40140d throughout this paper. Christophe Fromont (Centre for Biomolecular Sciences, University of Nottingham) kindly performed mass spectrometry, which confirmed that it was (*R*)-*N*-[5-[2-[2-(9H-carbazole-2-iloxy) ethylamino]-1-hydroxyethyl]-2-hydroxyphenyl]methanesulphonamide). All other reagents were from Sigma Chemicals (Poole, Dorset, UK). The source, catalogue numbers, CAS RN number and isomeric status of the agonists used are given in Supporting Information Table S1.

Cell culture

CHO-K1 stably expressing either the human β_1 (1146 fmol·mg^{-1} protein), the human β_2 (466 fmol·mg^{-1}

protein) or the human β_3-adrenoceptor (790 fmol·mg^{-1} protein) were used throughout this study (Baker, 2005a). Parent CHO cells were also used (i.e. CHO cells without the transfected receptor). Cells were grown in Dulbecco's modified Eagle's medium nutrient mix F12 (DMEM/F12) containing 10% fetal calf serum and 2 mM L-glutamine in a 37°C humidified 5% CO_2 : 95% air atmosphere.

[^{3}H]-CGP 12177 whole-cell binding

Cells were grown to confluence in white-sided, clear-bottomed 96-well view plates. [^{3}H]-CGP 12177 whole-cell binding was carried out the following day in a manner identical to that described in Baker (2005a). Briefly, the medium was removed from each well, and 100 µL of serum-free medium containing the competing ligand at twice the final required concentration was added to each well followed immediately by a fixed concentration of [^{3}H]-CGP 12177. The cells were incubated for 2 h at 37°C, 5% CO_2. The cells were washed twice by the addition and removal of 200 µL 4°C phosphate-buffered saline; 100 µL Microscint 20 was added to each well, a white sticky base applied to the bottom of the plate and a sealant top applied to the top of the plate. The plates were left at room temperature overnight in the dark, and the plates were counted on a Topcount at 21°C for 2 min per well.

[^{3}H]-cAMP accumulation

Cells were grown to confluence in 24-well plates. The medium was removed and the cells were pre-labelled with [^{3}H]-adenine by incubation for 2 h with 2 µCi·mL^{-1} [^{3}H]-adenine in serum-free medium (0.5 mL per well). The [^{3}H]-adenine was removed, and each well was washed by the addition and removal of 1 mL serum-free medium. Then, 1 mL serum-free medium containing 1 mM IBMX was added to each well, and the cells were incubated for 15 min. Agonist (in 10 µL serum-free medium) was added to each well, and the plates were incubated for 10 min–5 h. The reaction was terminated by the addition of 50 µL concentrated HCl per well. The plates were then frozen, thawed and [^{3}H]-cAMP separated from other ^{3}H-nucleotides by sequential Dowex and alumina column chromatography, as previously described (Donaldson *et al*., 1988).

Data analysis

Whole-cell binding. All data points on each binding curve were performed in triplicate, and each 96-well plate also contained three to six determinations of total and non-specific binding. Non-specific binding was determined in the presence of 1 µM CGP 20712A or 1–10 µM propranolol for the β_1-adrenoceptor, 1 µM ICI 118551 or 1–10 µM propranolol for the β_2-adrenoceptor and 10 µM propranolol for the β_3-adrenoceptor. In all cases where a K_D value is stated, the competing ligand completely inhibited the specific binding of [^{3}H]-CGP 12177.

One-site competition binding. A one-site sigmoidal binding curve (Equation 1) was then fitted to the data using Graphpad Prism 2.01, and the IC_{50} was then determined as the concentration required to inhibit 50% of the specific binding.

$$\%\ \text{Uninhibited binding} = 100 - \frac{(100 \times A)}{(A + IC_{50})} + NS \quad (1)$$

where A is the concentration of the competing ligand, IC_{50} is the concentration at which half of the specific binding of [^{3}H]-CGP 12177 has been inhibited and NS is the non-specific binding.

From the IC_{50} value and the known concentration of radioligand ([^{3}H]-CGP 12177 conc), a K_D (concentration at which half the receptors are bound by the competing ligand) value was calculated using the equation:

$$K_D = \frac{IC_{50}}{(1 + [^3\text{H}]\text{-CGP 12177 conc}/K_D\ [^3\text{H}]\text{-CGP 12177})} \quad (2)$$

Two-site competition binding. Several of the responses were best fitted to a two-site competition curve (e.g. Figure 2C). In these cases, Equation 3 was used:

$$\%\ \text{Inhibition of specific binding} = \frac{[A] \times N}{([A] + IC1_{50})} + \frac{[A] \times (100 - N)}{([A] + IC2_{50})} \quad (3)$$

where N is the percentage of site 1, [A] is the concentration of agonist and $IC1_{50}$ and $IC2_{50}$ are the respective IC_{50} values for the two agonist sites.

[^{3}H]-cAMP accumulation. Most agonist responses were best described by a one-site sigmoidal concentration–response curve (Equation 4):

$$\text{Response} = \frac{E_{max} \times [A]}{EC_{50} + [A]} \quad (4)$$

where E_{max} is the maximum response, [A] is the agonist concentration and EC_{50} is the concentration of agonist that produces 50% of the maximal response.

Several of the responses were, however, best fitted to a two-site concentration response – Equation 5 (e.g. Figure 5A):

$$\%\ \text{Maximal stimulation} = \frac{[A] \times N}{([A] + EC1_{50})} + \frac{[A] \times (100 - N)}{([A] + EC2_{50})} \quad (5)$$

where N is the percentage of site 1, [A] is the concentration of agonist and $EC1_{50}$ and $EC2_{50}$ are the respective EC_{50} values for the two agonist sites.

Isoprenaline (10 µM) was included in all experiments, and therefore all maximal responses are expressed as a percentage of this maximum.

Efficacy ratios. For comparative purposes, efficacy ratios were calculated by dividing the K_D value by the EC_{50} value for each ligand as per method of Furchgott (1966).

Results

[^{3}H]-CGP 12177 whole-cell binding

The K_D values for [^{3}H]-CGP 12177 have previously been determined in these cell lines as 0.42 nM for the β_1-adrenoceptor, 0.17 nM for the β_2-adrenoceptor and 109.2 nM for the β_3-adrenoceptor (Baker, 2005a).

Table 1 Log K_D values obtained from [3H]-CGP 12177 whole-cell binding studies in CHO cells stably expressing either the human β1-, β2- or β3-adrenoceptors

	β_1	n	β_2	n	β_3	n
Nebivolol	–9.06 ± 0.03	8	–7.92 ± 0.04	8	–7.04 ± 0.14	17
Noradrenaline	–5.74 ± 0.03	5	–5.41 ± 0.07	5	–5.53 ± 0.10	8
Denopamine	–6.12 ± 0.03	7	–5.83 ± 0.04	7	–5.30 ± 0.03	6
ZD 7114	–7.58 ± 0.09	8	–7.31 ± 0.03	9	–6.78 ± 0.07	12
ZD 2079	–4.21 ± 0.05	5	>–4	9	–4.59 ± 0.07	8
TAK 677	–7.44 ± 0.03	9	–7.32 ± 0.05	9	Site 1 –8.59 ± 0.07 Site 2 –5.65 ± 0.14 57.4 ± 3.2% site 1	10
Ractopamine	–6.97 ± 0.02	7	–6.93 ± 0.02	7	–5.82 ± 0.07	7
Octopamine	–3.91 ± 0.03	6	–4.03 ± 0.02	9	$IC_{50} \geq -3$	6
Dopamine	–3.57 ± 0.02	6	–3.93 ± 0.11	6	–3.10 ± 0.03	9
Methoxyphenamine	–3.94 ± 0.02	11	–4.32 ± 0.04	7	–3.96 ± 0.03	7
AZ 40140d	–6.69 ± 0.05	8	–7.14 ± 0.09	10	Site 1 –8.94 ± 0.23 Site 2 –6.11 ± 0.23 47.4 ± 3.7% site 1	7
Isoprenaline	–6.06 ± 0.08	9	–6.64 ± 0.09	11	–5.52 ± 0.08	9
Metaproterenol	–4.71 ± 0.02	6	–5.30 ± 0.02	7	–4.06 ± 0.07	6
L 755507	–6.23 ± 0.03	20	–6.83 ± 0.04	21	Site 1 –8.60 ± 0.12 Site 2 –6.20 ± 0.07 57.7 ± 2.4% site 1	19
Bamethane	–4.30 ± 0.03	7	–4.83 ± 0.03	7	–4.42 ± 0.09	9
Dobutamine	–5.23 ± 0.06	15	–5.84 ± 0.05	14	–5.09 ± 0.08	8
Pindolol	–8.57 ± 0.03	8	–9.23 ± 0.03	8	–7.08 ± 0.08	7
Bucindolol	–9.31 ± 0.03	6	–9.99 ± 0.07	6	–8.04 ± 0.08	5
Cimaterol	–6.57 ± 0.06	11	–7.26 ± 0.09	9	–5.28 ± 0.06	6
S-cyanopindolol	–10.39 ± 0.04	7	–11.09 ± 0.08	7	–8.36 ± 0.10	7
Carazolol	–9.69 ± 0.06	7	–10.49 ± 0.07	7	–8.35 ± 0.10	7
SR 59230A	–7.54 ± 0.05	9	–8.45 ± 0.04	8	–7.37 ± 0.09	8
BAAM	–7.65 ± 0.07	7	–8.57 ± 0.09	9	–6.40 ± 0.09	7
Adrenaline	–5.15 ± 0.06	9	–6.13 ± 0.05	9	–4.70 ± 0.08	8
Oxprenolol	–7.96 ± 0.03	8	–8.97 ± 0.04	8	–6.25 ± 0.11	9
L 748337	–5.44 ± 0.06	16	–6.47 ± 0.04	16	Site 1 –8.04 ± 0.11 Site 2 –5.46 ± 0.17 54.2 ± 3.7% site 1	16
Isoxsuprine	–4.87 ± 0.06	7	–5.93 ± 0.09	7	–5.29 ± 0.08	7
Tulobuterol	–5.62 ± 0.04	7	–6.83 ± 0.09	7	–4.72 ± 0.08	9
Clenbuterol	–6.62 ± 0.03	5	–7.90 ± 0.05	5	–5.35 ± 0.07	7
BRL 35135A	–6.08 ± 0.03	7	–7.38 ± 0.04	7	–6.73 ± 0.09	7
BRL 37344	–5.19 ± 0.07	7	–6.51 ± 0.06	5	–6.45 ± 0.06	7
Ritodrine	–4.48 ± 0.02	7	–5.81 ± 0.07	7	–4.73 ± 0.11	7
Salbutamol	–4.68 ± 0.03	12	–6.01 ± 0.03	10	–3.98 ± 0.06	9
SDZ 21009	–8.94 ± 0.05	11	–10.28 ± 0.08	11	–7.10 ± 0.10	9
Butoxamine	–4.85 ± 0.02	10	–6.23 ± 0.07	6	>–4	6
Terbutaline	–3.90 ± 0.03	12	–5.51 ± 0.04	12	–3.68 ± 0.06	12
Fenoterol	–5.04 ± 0.03	5	–7.03 ± 0.06	5	–5.39 ± 0.07	8
Zinterol	–5.96 ± 0.04	9	–8.04 ± 0.10	9	–6.27 ± 0.10	8
Procaterol	–4.81 ± 0.02	8	–7.11 ± 0.04	8	–3.99 ± 0.11	7
Formoterol	–6.11 ± 0.05	8	–8.63 ± 0.02	8	–5.82 ± 0.05	8
Salmeterol	–5.73 ± 0.03	11	–9.26 ± 0.06	10	–6.33 ± 0.10	8

Values represent mean ± SEM of *n* separate experiments. The binding of four ligands to the human β_3-adrenoceptor was best described by a two-component curve. In these instances, the log K_D values are given for each component along with the percentage of the total inhibition that was represented by the first component. Ligands are arranged in order of β_1 versus β_2 selectivity.

^{3}H-CGP 12177 at concentrations from 0.34 to 4.65 nM was used for experiments involving the β_1- and β_2-adrenoceptors, and from 5.18 to 32.53 nM for the β_3-adrenoceptor.

The affinity of ligands for the human β-adrenoceptors is presented in Table 1, arranged in order of selectivity for the β_1- over the β_2-adrenoceptor (Tables 1 and 2). Thus, several compounds originally considered as antagonists were found to have high affinity for the receptors, while as expected most ligands generally considered as agonists had relatively low affinity. Table 2 shows the selectivity of these ligands for the three adrenoceptors based on binding alone. Nebivolol was found to be the most selective ligand for the human β_1-adrenoceptor in this current set of ligands (Table 2); however, the neutral antagonist CGP 20712A has previously been shown to have 501 times higher affinity for the β_1- than for the β_2-adrenoceptor in this system (Baker, 2005a). Salmeterol and formoterol were the most selective β_2-compounds (Figure 1). There were several β_3-selective compounds (e.g. AZ 40140d, L 755507, L 748337 and TAK 677); however, careful examination of their [^{3}H]-CGP 12177 inhibitory responses yielded an inhibition that was best described by a two-component curve (Figure 2).

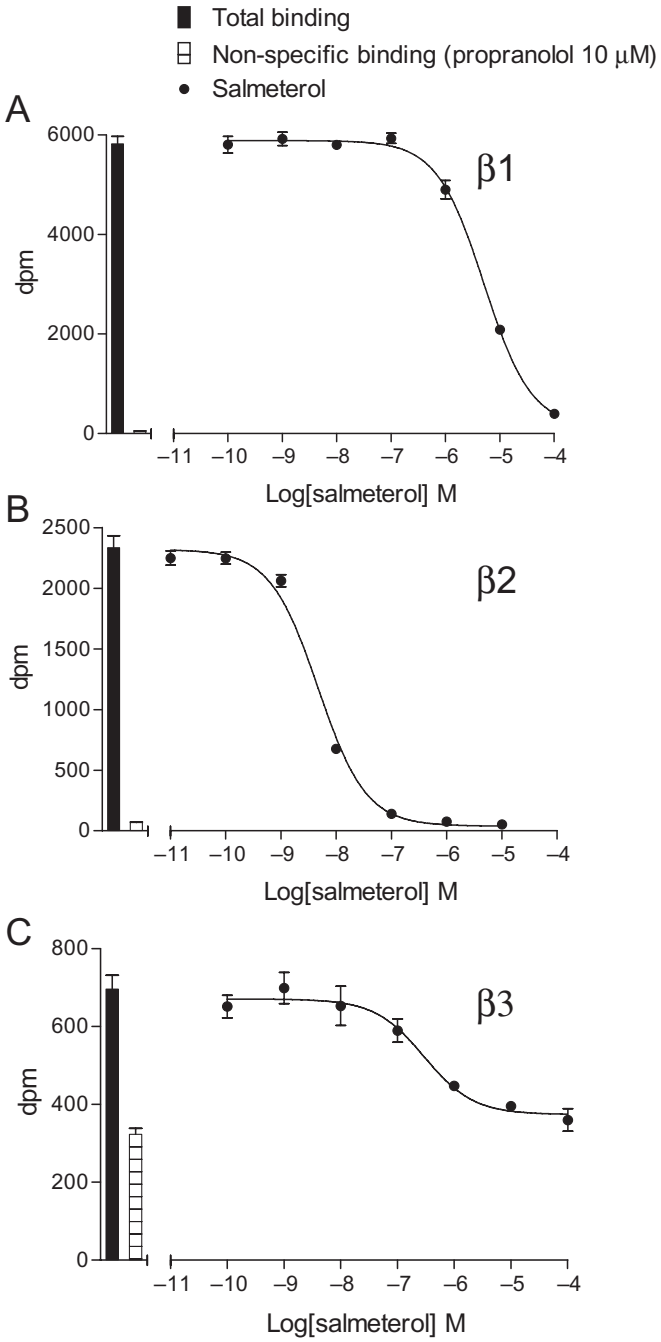

Figure 1 Inhibition of [^{3}H]-CGP 12177 binding to whole cells by salmeterol in (A) CHO β_1 cells, (B) CHO β_2 cells and (C) CHO β_3 cells. The columns represent total [^{3}H]-CGP 12177 binding and non-specific binding determined in the presence of 10 μM propranolol. The concentrations of [^{3}H]-CGP 12177 present in each case are (A) 0.90 nM, (B) 1.39 nM and (C) 17.9 nM. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of (A) 11, (B) 10 and (C) 8 separate experiments.

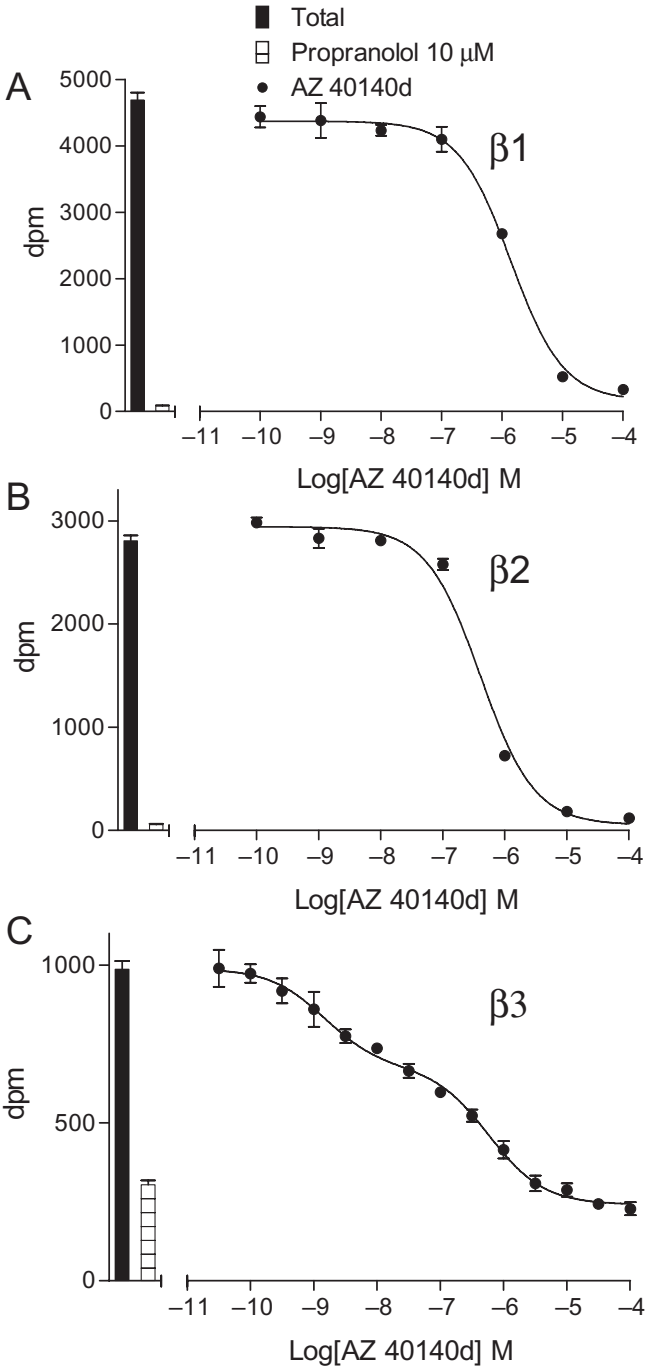

Figure 2 Inhibition of [^{3}H]-CGP 12177 binding to whole cells by AZ 40140d in (A) CHO β_1 cells, (B) CHO β_2 cells and (C) CHO β_3 cells. The columns represent total [^{3}H]-CGP 12177 binding and non-specific binding determined in the presence of 10 μM propranolol. The concentrations of [^{3}H]-CGP 12177 present in each case are (A) 1.41 nM, (B) 1.29 nM and (C) 19.31 nM. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of (A) 8, (B) 10 and (C) 7 separate experiments.

[^{3}H]-cAMP accumulation

To investigate whether the length of agonist incubation time had any effect on the log EC_{50} value or the percentage maximum response obtained, several ligands (full and partial) were incubated for 10 and 30 min, and 5 h before HCl was added to terminate the assay (Supporting Information Tables S2–S4). Isoprenaline stimulated [^{3}H]-cAMP accumulation responses that were 13-, 27- and 12-fold over basal at 10 min; 45-, 69- and 28-fold at 30 min; and 92-, 154- and 34-fold at 5 h for the β_1-, β_2- and β_3-adrenoceptors respectively. The log EC_{50} values and percent maximum responses

Table 2 Selectivity ratios of the ligands for human β_1-, β_2- and β_3-adrenoceptors, where a ratio of 1 demonstrates no selectivity (based on binding) for a given receptor subtype over another

		Selectivity ratios (based on whole-cell binding)								
		β_1	*versus*	β_2	β_2	*versus*	β_3	β_1	*versus*	β_3
Nebivolol		13.8			7.6			104.7		
Noradrenaline		2.1					1.3	1.6		
Denopamine		1.9			3.4			6.6		
ZD 7114		1.9			3.4			6.3		
ZD 2079		>1.6					>3.9			2.4
TAK 677	Component 1	1.3					18.6			14.1
Ractopamine		1.1			12.9			14.1		
Octopamine				1.3						
Dopamine				2.3	6.8			2.9		
Methoxyphenamine				2.4	2.3					1.0
AZ 40140d	Component 1			2.8			63.1			177.8
Isoprenaline				3.8	13.2			3.5		
Metaproterenol				3.9	17.4			4.57		
L 755507	Component 1			4.0			58.9			234.4
Bamethane				4.0	2.6					1.3
Dobutamine				4.1	5.6			1.4		
Pindolol				4.6	141.3			30.9		
Bucindolol				4.8	89.1			18.6		
Cimaterol				4.9	95.5			19.5		
Cyanopindolol				5.0	537.0			107.2		
Carazolol				6.3	138.0			21.9		
SR 59230A				8.1	12.0			1.5		
BAAM				8.3	147.9			17.8		
Adrenaline				9.6	26.9			2.8		
Oxprenolol				10.2	524.8			51.3		
L 748337	Component 1			10.7			37.2			398.1
Isoxsuprine				11.5	4.4					2.6
Tulobuterol				16.2	128.8			7.9		
Clenbuterol				19.5	354.8			18.6		
BRL 35135A				19.9	4.5					4.5
BRL 37344				20.9	1.2					18.2
Ritodrine				21.4	12.0					1.8
Salbutamol				21.4	107.2			5.0		
SDZ 21009				21.9	1513.6			69.2		
Butoxamine				24.0	>169.8			>7.1		
Terbutaline				40.7	67.6			1.7		
Fenoterol				97.7	43.7					2.2
Zinterol				120.2	58.9					2.0
Procaterol				199.5	1318.36			6.6		
Formoterol				331.1	645.7			2.0		
Salmeterol				3388.4	851.1					4.0

Where the inhibition of [^{3}H]-CGP 12177 binding curve was best described by two components (Table 1), the selectivity ratio for the first component only is given.

remained constant over time for the great majority of ligands tested including full and partial agonists (Supporting Information Tables S2–S4; Figure 3) as found previously (Baker *et al.*, 2004). However, for some ligands, the log EC_{50} values differed between 10 and 30 min (Figure 4). For some ligands [e.g. salmeterol, formoterol and CGP 12177 (β_2)], this is most probably because of the slow onset of these ligands at this receptor (Lötvall, 2001; Baker *et al.*, 2002), and indeed the degree of change reflects the known relative order of onset of action (Cote *et al.*, 2009). The reason for the increase in potency for all three catecholamines between 10 and 30 min is not known; however, given this change for both the long acting ligands and the catecholamines, 30 min was chosen as the incubation time for investigation of majority of the other ligands (Tables 3–5).

The percentage maximum response obtained was the same for the ligands at each receptor at each time-point. However, the counts collected after 5 h agonist incubation were greater than those after 30 min incubation, thus allowing a bigger window to examine the small responses. Weak partial agonists and those with a two-component agonist response curves were therefore incubated for 5 h to allow more accurate measurement (with no change in either log EC_{50} values or percentage maximum response obtained).

Many of the agonists stimulated full (as compared to isoprenaline) concentration–response curves. However, many ligands often considered as 'antagonists' stimulated partial agonist responses (e.g. nebivolol, cyanopindolol, carazolol, oxprenolol and bucindolol) (Tables 3–5, arranged in order of 'efficacy ratio' for each receptor). For the human β_1- and β_3-adrenoceptors, several of these partial agonist responses were found to best fit a two-component concentration–response curve (e.g. Figures 5 and 6). This is similar to previous findings which suggest that the first high-affinity

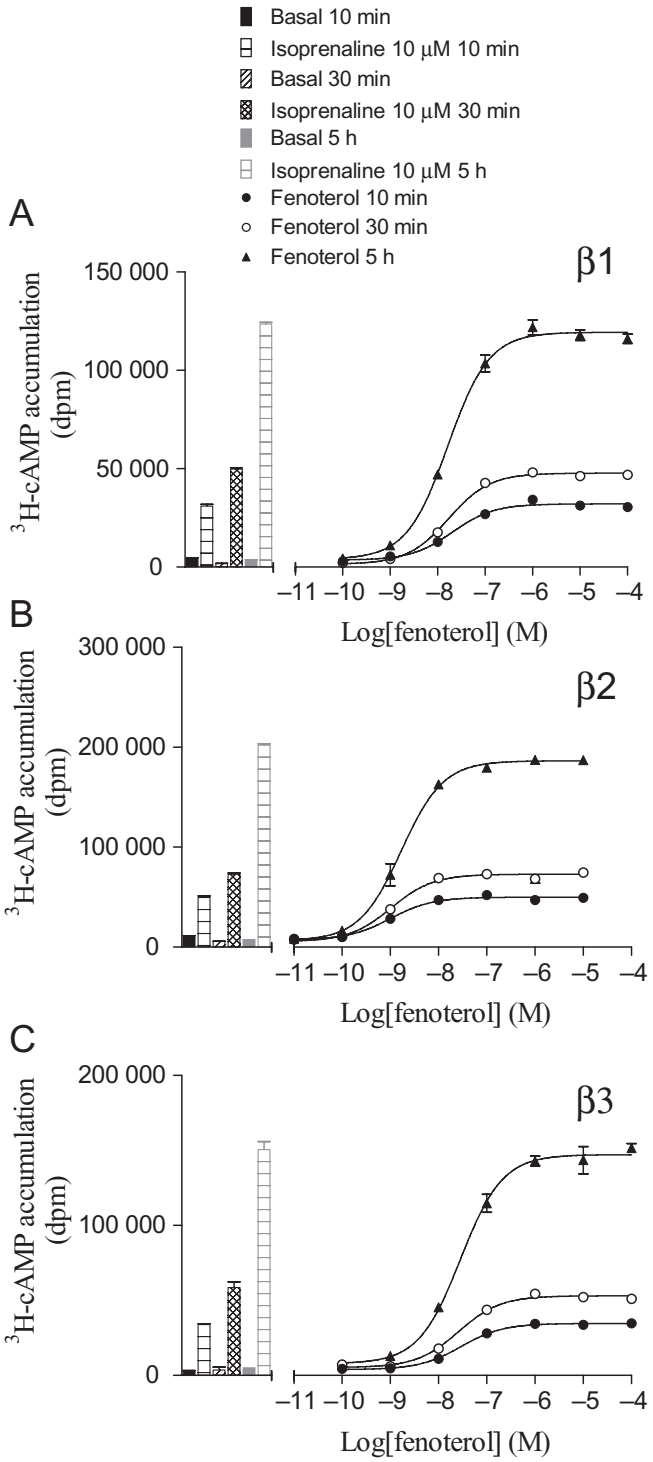

Figure 3 [^{3}H]-cAMP accumulation in response to fenoterol after 10 and 30 min, and 5 h incubation in (A) CHO β_1 cells, (B) CHO β_2 cells and (C) CHO β_3 cells. The columns represent basal [^{3}H]-cAMP accumulation and that in response to 10 μM isoprenaline at each time-point. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of (A) four, (B) four and (C) three separate experiments.

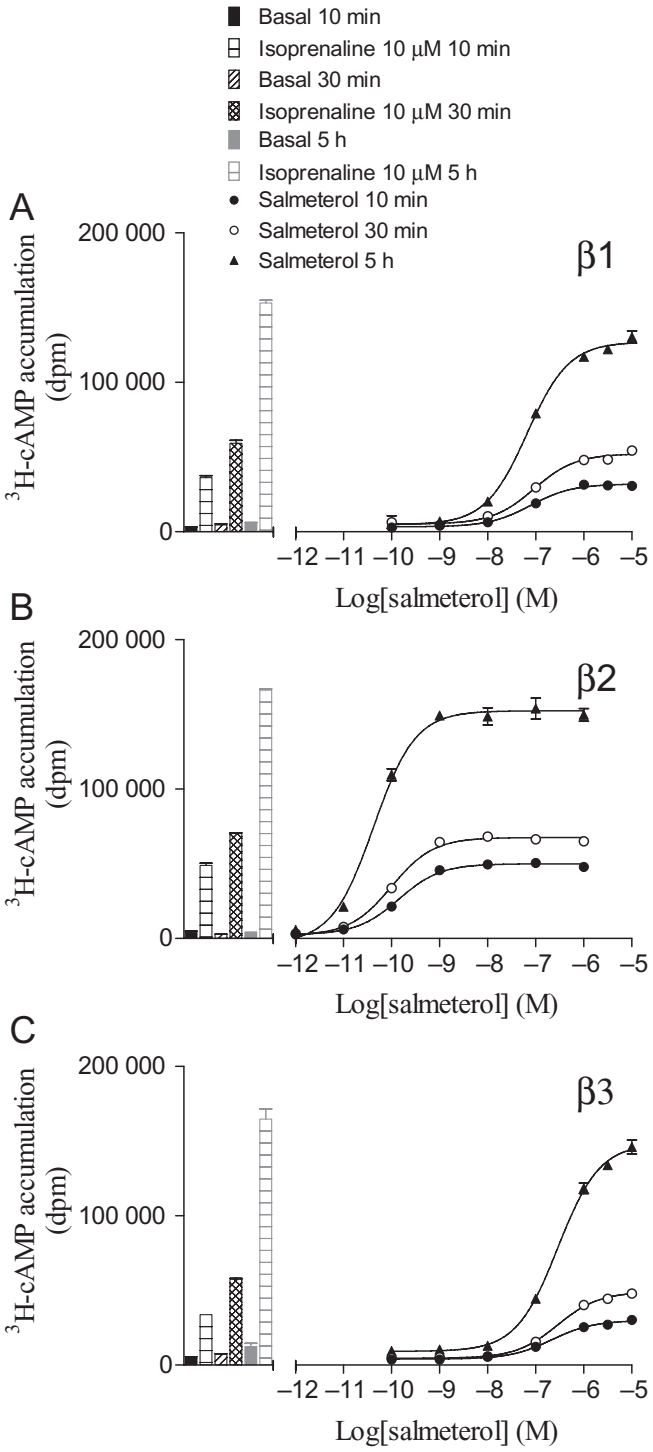

Figure 4 [^{3}H]-cAMP accumulation in response to salmeterol after 10 and 30 min, and 5 h incubation in (A) CHO β_1 cells, (B) CHO β_2 cells and (C) CHO β_3 cells. The columns represent basal [^{3}H]-cAMP accumulation and that in response to 10 μM isoprenaline at each time-point. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of (A) four, (B) four and (C) three separate experiments.

component is acting via the catecholamine conformation of the receptor, and the second low-affinity component via a secondary agonist conformation (Baker *et al.*, 2003a; Baker, 2005b).

S-pindolol. Racemic ligands were used in the majority of cases, but the different enantiomers of pindolol have previously been shown to have different properties in certain studies (Walter *et al.*, 1984; Joseph *et al.*, 2003). S-pindolol was

Table 3 Log K_D values from [^{3}H]-CGP 12177 whole-cell binding (from Table 1), log EC_{50} values and % isoprenaline maximal responses obtained from [^{3}H]-cAMP accumulation and intrinsic efficacy ratios (K_D/EC_{50}) for cells expressing the human β_1-adrenoceptor

β_1	*Log K_D from binding (β_1)*	*Log EC_{50} from cAMP (β_1)*	*% Isoprenaline maximal response*	n	*Efficacy ratio (log)*
Isoprenaline	–6.06	–8.59 ± 0.10	100	11	2.53
Metaproterenol	–4.71	–7.21 ± 0.07	113.0 ± 4.9	4	2.50
Fenoterol	–5.04	–7.53 ± 0.07	106.0 ± 1.7	4	2.49
Adrenaline	–5.15	–7.61 ± 0.10	101.7 ± 2.1	8	2.46
Noradrenaline	–5.74	–7.94 ± 0.13	102.4 ± 3.6	10	2.20
Formoterol	–6.11	–8.29 ± 0.08	111.4 ± 3.3	4	2.18
Terbutaline	–3.90	–5.76 ± 0.09	110.0 ± 2.2	4	1.86
Cimaterol	–6.57	–8.42 ± 0.08	109.7 ± 3.6	4	1.85
Octopamine	–3.91	–5.73 ± 0.03	107.2 ± 2.3	4	1.82
Ractopamine	–6.97	–8.74 ± 0.09	103.2 ± 1.5	5	1.77
Ritodrine	–4.48	–6.20 ± 0.06	103.9 ± 2.0	5	1.72
BRL 35135A	–6.08	–7.79 ± 0.10	104.0 ± 5.0	6	1.71
Isoxsuprine	–4.87	–6.54 ± 0.10	99.4 ± 4.5	5	1.67
Denopamine	–6.12	–7.73 ± 0.06	121.2 ± 4.1	5	1.61
Dobutamine	–5.23	–6.81 ± 0.08	97.4 ± 3.6	4	1.58
Salbutamol	–4.68	–6.21 ± 0.05	103.7 ± 3.8	4	1.53
Dopamine	–3.57	–5.02 ± 0.03	112.5 ± 4.9	5	1.45
TAK 677	–7.44	–8.86 ± 0.08	108.4 ± 1.6	8	1.42
L 755507	–6.23	–7.63 ± 0.05	101.6 ± 3.0	5	1.40
BRL 37344	–5.19	–6.54 ± 0.02	100.7 ± 8.6	4	1.35
Bamethane	–4.30	–5.63 ± 0.04	108.5 ± 5.3	4	1.33
Salmeterol	–5.73	–7.03 ± 0.09	98.1 ± 1.7	5	1.30
Zinterol	–5.96	–7.23 ± 0.10	113.5 ± 3.8	5	1.27
Procaterol	–4.81	–5.80 ± 0.09	106.7 ± 4.6	5	0.99
Tulobuterol	–5.62	–6.59 ± 0.09	97.8 ± 2.7	5	0.97
L 748337	–5.44	–6.23 ± 0.03	43.4 ± 1.7	5	0.79
Butoxamine	–4.85	–5.56 ± 0.12	11.3 ± 0.7	10	0.71
Clenbuterol	–6.62	–7.29 ± 0.04	99.9 ± 5.1	4	0.67
Methoxyphenamine	–3.94	–4.59 ± 0.04	9.6 ± 0.8	7	0.65
ZD 2079	–4.21	–4.79 ± 0.03	81.7 ± 4.9	4	0.58
AZ 40140d	–6.69	–7.23 ± 0.08	98.3 ± 5.6	6	0.54
Oxprenolol	–7.96	Site 1 –8.46 ± 0.03 Site 2 –6.03 ± 0.12 Site 1 65.4 ± 0.8%	37.1 ± 3.4	6	0.50
ZD 7114	–7.58	–8.05 ± 0.08	72.0 ± 2.9	4	0.47
SR 59230A	–7.54	–7.96 ± 0.06	52.8 ± 1.9	4	0.42
Pindolol	–8.57	Site 1 –8.84 ± 0.08 Site 2 –6.12 ± 0.11 Site 1 38.4 ± 1.4%	51.2 ± 4.1	6	0.27
Nebivolol	–9.06	–8.97 ± 0.09	2.8 ± 0.3	5	–0.09
BAAM	–7.65	–7.51 ± 0.07	34.4 ± 3.1	5	–0.14
Bucindolol	–9.31	Site 1 –9.11 ± 0.07 Site 2 –7.46 ± 0.07 Site 1 65.3 ± 4.9%	73.9 ± 1.3	6	–0.20
SDZ 21009	–8.94	Site 1 –8.50 ± 0.11 Site 2 –6.79 ± 0.10 Site 1 48.9 ± 3.8%	67.8 ± 2.5	12	–0.44
Carazolol	–9.69	Site 1 –9.15 ± 0.09 Site 2 –7.39 ± 0.06 Site 1 21.9 ± 2.4%	38.1 ± 4.9	8	–0.54
Cyanopindolol	–10.39	Site 1 –9.63 ± 0.11 Site 2 –8.43 ± 0.13 Site 1 42.4 ± 7.5%	62.7 ± 6.3	7	–0.76

Values represent mean ± SEM of *n* separate experiments. For several ligands, the concentration–response curve was best described by a two-component curve. In these cases, the log EC_{50} values are given for each component, the percentage of the response represented by the first component and the percentage of the total response in relation to the isoprenaline stimulation.
The ligands are arranged in order of intrinsic efficacy.

therefore examined. This enantiomer was found to have [^{3}H]-CGP 12177 binding affinities of –9.16 ± 0.09 (n = 5) and –9.55 ± 0.04 (n = 5) at the β_1- and β_2-adrenoceptors respectively. When the [^{3}H]-cAMP accumulation response was examined, S-pindolol stimulated a biphasic concentration–response curve (log $EC_{50}1$ = –9.09 ± 0.09, log $EC_{50}2$ = –6.43 ± 0.09, 43.3% site 1, 53.5 ± 1.6% isoprenaline maximum) at the β_1-adrenoceptor very similar to that of racemic pindolol (Table 3). Thus, as expected, S-pindolol appears to have slightly higher affinity than racemic pindolol, and the isomer S-pindolol appears to be able to activate both conformations of the β_1-adrenoceptor.

Parent CHO cells

There was no specific binding of [^{3}H]-CGP 12177 to the parent CHO cells (i.e. CHO cells without a transfected receptor). In

Table 4 Log K_D values from [^{3}H]-CGP 12177 whole-cell binding (from Table 1), log EC_{50} values and % isoprenaline maximal responses obtained from [^{3}H]-cAMP accumulation and intrinsic efficacy ratios (K_D/EC_{50}) for cells expressing the human β_2-adrenoceptor

β_2	*Log K_D from binding (β_2)*	*Log EC_{50} from cAMP (β_2)*	*% Isoprenaline maximal response*	n	*Efficacy ratio (log)*
Fenoterol	–7.03	–8.90 ± 0.07	100.9 ± 2.7	4	1.87
Adrenaline	–6.13	–7.93 ± 0.07	101.9 ± 1.8	8	1.80
Terbutaline	–5.51	–7.29 ± 0.02	102.7 ± 3.7	4	1.78
Metaproterenol	–5.30	–7.07 ± 0.06	109.2 ± 3.7	5	1.77
Salbutamol	–6.01	–7.72 ± 0.07	95.8 ± 2.4	4	1.71
Cimaterol	–7.26	–8.94 ± 0.07	96.6 ± 3.5	4	1.68
Isoprenaline	–6.64	–8.22 ± 0.11	100	12	1.58
Formoterol	–8.63	–10.08 ± 0.02	104.3 ± 2.8	4	1.45
Zinterol	–8.04	–9.48 ± 0.07	105.3 ± 4.6	5	1.44
Procaterol	–7.11	–8.43 ± 0.06	107.9 ± 2.0	6	1.32
BRL 35135A	–7.38	–8.69 ± 0.05	86.3 ± 3.4	5	1.31
Clenbuterol	–7.90	–9.18 ± 0.04	95.3 ± 3.1	4	1.28
AZ 40140d	–7.14	–8.40 ± 0.09	95.6 ± 2.0	7	1.26
Ritodrine	–5.81	–6.82 ± 0.06	91.4 ± 2.9	6	1.01
Noradrenaline	–5.41	–6.36 ± 0.04	103.4 ± 2.1	11	0.95
Tulobuterol	–6.83	–7.60 ± 0.08	89.3 ± 1.8	7	0.77
Isoxsuprine	–5.93	–6.65 ± 0.06	68.1 ± 2.4	5	0.72
Dopamine	–3.93	–4.64 ± 0.03	89.8 ± 3.5	6	0.71
TAK 677	–7.32	–8.03 ± 0.06	89.6 ± 1.9	8	0.71
Ractopamine	–6.93	–7.63 ± 0.05	83.3 ± 2.6	5	0.70
Salmeterol	–9.26	–9.89 ± 0.08	94.1 ± 2.3	6	0.63
Bamethane	–4.83	–5.38 ± 0.08	71.5 ± 2.3	5	0.55
Dobutamine	–5.84	–6.32 ± 0.05	64.8 ± 2.7	4	0.48
Octopamine	–4.03	–4.45 ± 0.08	32.0 ± 2.9	5	0.42
BRL 37344	–6.51	–6.88 ± 0.03	80.1 ± 4.1	5	0.37
ZD 7114	–7.31	–7.65 ± 0.12	2.2 ± 0.4	7	0.34
Methoxyphenamine	–4.32	–4.62 ± 0.07	10.0 ± 0.8	10	0.30
L 755507	–6.83	–7.05 ± 0.07	3.0 ± 0.2	6	0.22
Butoxamine	–6.23	–6.41 ± 0.17	1.4 ± 0.4	5	0.18
Oxprenolol	–8.97	–9.10 ± 0.03	5.9 ± 0.3	4	0.13
L 748337	–6.47	–6.44 ± 0.07	9.5 ± 2.2	6	–0.03
Pindolol	–9.23	–9.12 ± 0.10	9.9 ± 0.9	9	–0.11
Denopamine	–5.83	–5.65 ± 0.08	11.2 ± 0.7	5	–0.18
BAAM	–8.57	–8.24 ± 0.08	6.9 ± 0.1	5	–0.33
SR 59230A	–8.45	–8.10 ± 0.10	3.1 ± 0.2	4	–0.35
Bucindolol	–9.99	–9.40 ± 0.06	16.2 ± 1.2	5	–0.59
Carazolol	–10.49	–9.75 ± 0.07	1.9 ± 0.3	4	–0.74
SDZ 21009	–10.28	–9.39 ± 0.09	3.7 ± 0.4	13	–0.89
Cyanopindolol	–11.09	–10.03 ± 0.03	9.9 ± 1.7	4	–1.06
ZD 2079	>–4	Stimulation at 100 μM	16.1 ± 1.1	4	
Nebivolol	–7.92	No response		7	

Values represent mean ± SEM of *n* separate experiments.
The ligands are arranged in order of intrinsic efficacy.

the [^{3}H]-cAMP accumulation assay, the parent CHO cells responded to forskolin (stimulation 21.5 ± 2.2-fold over basal, $n = 10$), but there was no response to any of the other ligands.

Discussion

Several recent studies have examined the selectivity (affinity) of β-antagonists for the β-adrenoceptors in different recombinant cell systems, and concluded that many clinically available β-blockers are not very selective (Smith and Teitler, 1999; Schnabel *et al.*, 2000; Hoffmann *et al.*, 2004; Baker, 2005a). This study set out to examine the binding and function of β-adrenoceptor agonists, measured under similar conditions, to determine if agonists are subtype selective and whether it was affinity or intrinsic efficacy that made them so. Whole-cell techniques were used, as this is how agonists would behave in the clinical situation (i.e. with GTP present in living cells). This probably means that measured K_D values obtained from binding represent the binding affinity for the basal *R* state of the receptor (rather than the *R** state; Leff, 1995; Leff *et al.*, 1997).

The affinity measurements can be compared across the three cell lines, as affinity from ligand binding is a direct measure. From these studies, it can be seen that several very selective β_2- and β_3-agonists exist on the basis of affinity; however, there are few β_1-selective agonists. Competition binding curves were all best described by a one-site sigmoidal response curve for the human β_1- and β_2-adrenoceptors; however, AZ 40140d, L 755507, L 748337 and TAK 677 were found to have a two-component displacement curve (Table 1; Figure 2). The human β_3-receptor has previously been suggested to exist in at least two agonist conformations (Baker, 2005b), and it may be that this represents binding to two different agonist conformations of this receptor. The β_1-adrenoceptor also exists in at least two conformations (Pak

Table 5 Log K_D values from [^{3}H]-CGP 12177 whole-cell binding (from Table 1), log EC_{50} values and % isoprenaline maximal responses obtained from [^{3}H]-cAMP accumulation and intrinsic efficacy ratios (K_D/EC_{50}) for cells expressing the human β_3-adrenoceptor

β_3	*Log K_D from binding (β_3)*	*Log EC_{50} from cAMP (β_3)*	*% Isoprenaline maximal response*	n	*Efficacy ratio (log)*
Fenoterol	–5.39	–7.63 ± 0.07	100.5 ± 4.0	4	2.24
Terbutaline	–3.68	–5.85 ± 0.08	101.4 ± 2.7	4	2.17
Salbutamol	–3.98	–6.01 ± 0.06	97.2 ± 3.4	4	2.03
Metaproterenol	–4.06	–6.08 ± 0.07	95.8 ± 6.2	6	2.02
Formoterol	–5.82	–7.82 ± 0.05	99.0 ± 3.2	4	2.00
Isoprenaline	–5.52	–7.41 ± 0.08	100	10	1.89
Zinterol	–6.27	–8.09 ± 0.10	101.2 ± 3.4	5	1.82
Ritodrine	–4.73	–6.54 ± 0.08	99.1 ± 3.1	6	1.81
Adrenaline	–4.70	–6.48 ± 0.07	102.1 ± 2.0	8	1.78
Cimaterol	–5.28	–6.96 ± 0.06	98.2 ± 3.4	4	1.68
Dopamine	–3.10	–4.76 ± 0.07	90.5 ± 2.5	6	1.66
Noradrenaline	–5.53	–7.17 ± 0.09	104.1 ± 1.8	6	1.64
L 755507	Site 1 –8.60 Site 2 –6.20 57.7% site 1	–10.10 ± 0.05	101.1 ± 3.4	8	1.50
ZD 2079	–4.59	–6.00 ± 0.04	97.9 ± 1.4	4	1.41
Dobutamine	–5.09	–6.37 ± 0.06	87.3 ± 3.7	4	1.28
Procaterol	–3.99	–5.22 ± 0.04	91.6 ± 5.3	4	1.23
Bamethane	–4.42	–5.61 ± 0.08	98.3 ± 5.0	5	1.19
Ractopamine	–5.82	–7.00 ± 0.07	88.8 ± 2.1	5	1.18
AZ 40140d	Site 1 –8.94 Site 2 –6.11 47.4% Site 1	–9.99 ± 0.09 Site 2 max not reached	104.4 ± 2.4	9	1.05
Oxprenolol	–6.25	Site 1 –7.30 ± 0.04 Site 2 –5.62 ± 0.13 Site 1 56.6 ± 1.9%	47.7 ± 8.3	6	1.05
BRL 37344	–6.45	–7.47 ± 0.09	79.7 ± 3.6	6	1.02
Denopamine	–5.30	–6.27 ± 0.08	92.4 ± 3.4	5	0.97
TAK 677	Site 1 –8.59 Site 2 –5.65 57.4% Site 1	–9.54 ± 0.06	96.0 ± 2.2	7	0.95
Tulobuterol	–4.72	–5.65 ± 0.06	83.0 ± 3.6	6	0.93
BRL 35135A	–6.73	–7.61 ± 0.03	77.5 ± 2.5	6	0.88
Clenbuterol	–5.35	–6.19 ± 0.04	86.1 ± 2.6	4	0.84
Isoxsuprine	–5.29	–6.08 ± 0.06	71.4 ± 1.9	5	0.79
ZD 7114	–6.78	Site 1 –7.55 ± 0.06 Site 2 –5.64 ± 0.20 Site 1 74.8 ± 3.8%	58.2 ± 2.0	10	0.77
Carazolol	–8.35	Site 1 –8.84 ± 0.07 Site 2 –6.96 ± 0.08 Site 1 30.6 ± 3.3%	75.7 ± 1.1	8	0.49
Cyanopindolol	–8.36	Site 1 –8.84 ± 0.12 Site 2 –7.01 ± 0.05 Site 1 27.6 ± 2.7%	57.0 ± 2.1	8	0.48
L 748337	Site 1 –8.04 Site 2 –5.46 54.2% Site 1	Site 1 –8.43 ± 0.09 Site 2 –5.54 ± 0.12 Site 1 60.9 ± 4.4%	42.6 ± 0.9	8	0.39
Pindolol	–7.08	Site 1 –7.43 ± 0.12 Site 2 –5.63 ± 0.11 Site 1 44.5 ± 2.9%	67.2 ± 3.6	6	0.35
Salmeterol	–6.33	–6.60 ± 0.05	85.5 ± 1.4	5	0.27
SDZ 21009	–7.10	Site 1 –7.37 ± 0.04 Site 2 –5.75 ± 0.11 Site 1 52.4 ± 4.2%	87.3 ± 3.7	12	0.27
SR 59230A	–7.37	–7.59 ± 0.07	35.5 ± 1.9	4	0.22
BAAM	–6.40	–6.58 ± 0.07	33.5 ± 2.0	4	0.18
Bucindolol	–8.04	Site 1 –8.19 ± 0.08 Site 2 –6.43 ± 0.17 Site 1 61.4 ± 2.4%	80.4 ± 3.7	6	0.15
Octopamine	IC_{50} = –3	–5.38 ± 0.07	91.7 ± 2.0	4	
Butoxamine	>–4	No response		11	
Methoxyphenamine	–3.96	No response		8	
Nebivolol	–7.04	No response		8	

Values represent mean ± SEM of *n* separate experiments. For several ligands, the concentration–response curve was best described by a two-component curve. In these cases, the log EC_{50} values are given for each component, the percentage of the response represented by the first component and the percentage of the total response in relation to the isoprenaline stimulation.
The ligands are arranged in order of intrinsic efficacy.

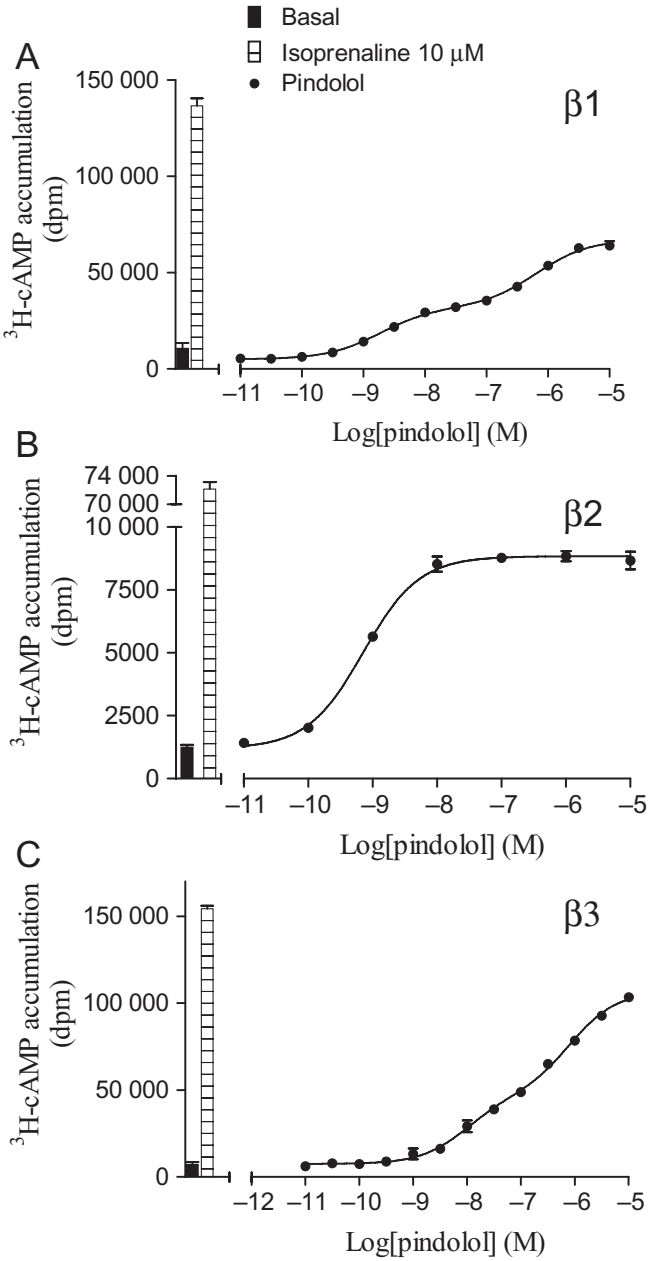

Figure 5 [^{3}H]-cAMP accumulation in response to pindolol after 5 h incubation in (A) CHO β_1 cells, (B) CHO β_2 cells and (C) CHO β_3 cells. The columns represent basal [^{3}H]-cAMP accumulation and that in response to 10 μM isoprenaline. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of (A) six, (B) nine and (C) six separate experiments.

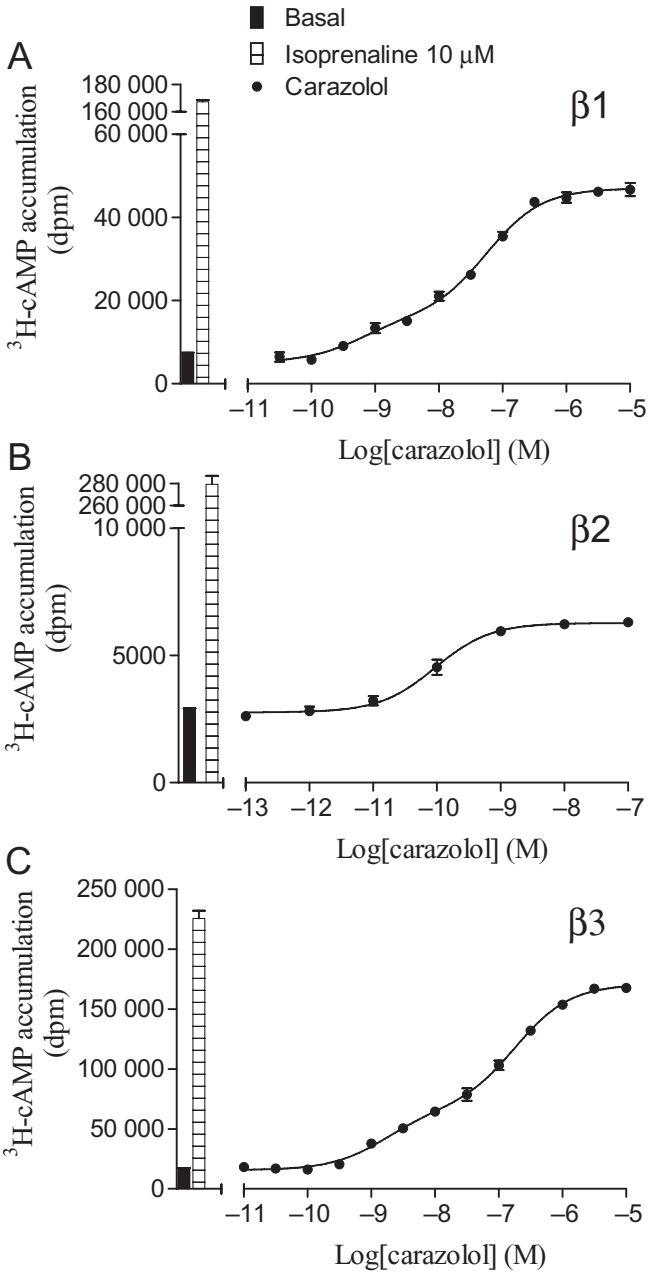

Figure 6 [^{3}H]-cAMP accumulation in response to carazolol after 5 h incubation in (A) CHO β_1 cells, (B) CHO β_2 cells and (C) CHO β_3 cells. The columns represent basal [^{3}H]-cAMP accumulation and that in response to 10 μM isoprenaline. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of (A) eight, (B) four and (C) eight separate experiments.

and Fishman, 1996; Granneman, 2001; Baker *et al.*, 2003a; Molenaar, 2003; Arch, 2004; Baker, 2005c; Kaumann and Molenaar, 2008). However, the affinity between these conformations is greater for the β_1- than the β_3-adrenoceptor, and this probably explains why [^{3}H]-CGP 12177 at the concentrations used only demonstrated binding to one conformation at the β_1-adrenoceptor. As several GPCRs have allosteric functions, it is also possible that there is a degree of allosteric interference from the radioligand or the competing agonist affecting the binding of the other.

Recent evidence has also shown that the functional response measured from a given receptor–ligand interaction can vary depending on which cellular response is being measured. For example, propranolol acts as an inverse agonist at the human β_2-adrenoceptor by decreasing cAMP production below basal, but at the same time stimulates the ERK pathway (e.g. Azzi *et al.*, 2003; Baker *et al.*, 2003b). Thus, the binding of a ligand (e.g. adrenaline) may generate a plethora of active 'states' of the receptor, some of which may be coupled to different G-proteins or intracellular effector mechanisms (e.g.

β-arrestin), and these in turn may have different allosteric influences on the receptor itself. As the human β-adrenoceptors are all G_s-coupled receptors, the robust, G_s-coupled, rapid upstream cAMP response was used as a measure of functional efficacy in this study. As the whole-cell binding assay and the cAMP accumulation assay were both performed in the same confluent intact living cells, the 'states' of the receptor induced by the competing ligand (e.g. adrenaline) in the binding assay would be the same 'states' as those induced by the same ligand measured in the cAMP assay. However, the binding assay will only detect the state that [^{3}H]-CGP 12177 is able to bind to. Thus, the [^{3}H]-CGP 12177 binding assay might not detect all of the active states generated by the binding of the agonist (e.g. adrenaline). The cAMP assay measured the response generated from the plethora of states generated from, for example, adrenaline. So, although adrenaline will generate the same plethora of states in both assays, potentially, the group of states detected in the binding assay might not overlap completely with the states that induce the production of cAMP. There is however likely to be substantial overlap as [^{3}H]-CGP 12177 is completely displaceable by virtually all the ligands (Table 1), and CGP 12177 itself stimulates a cAMP response (Supporting Information Tables S2–S4).

When examining the cAMP responses, several things must be considered before comparisons are made. Three different cell lines have been used (one for each receptor), and each has a different receptor expression level and, because they are different cell lines, will have different effector coupling efficiencies. Direct comparisons of functional responses across the three cell lines are therefore not possible. However, a rank order of intrinsic efficacy can be determined from a ratio between affinity (K_D from binding) and the EC_{50} from the functional response (Furchgott, 1966; Kenakin, 1982; 1999b; Strange, 2008). Thus, at the β_1-adrenoceptor, fenoterol (log K_D –5.04, log EC_{50} –7.53) has an 'intrinsic efficacy ratio' of 309 (log 2.49), and salbutamol has an intrinsic efficacy ratio of 33.9 (log 1.53). Therefore, fenoterol has *c*. 10-fold more intrinsic efficacy at the human β_1-adrenoceptor than salbutamol. Thus, although intrinsic efficacy has not been measured directly, a meaningful measure has been obtained to allow comparison between ligands.

Tables 3–5 are arranged in order of intrinsic efficacy for each receptor. At the top of the table are full agonists with the highest intrinsic efficacy. As the intrinsic efficacy of the ligands decreases, there comes a stage when they are no longer efficacious enough to stimulate a maximum response. The maximum responses then decrease until there is no response at all (as for nebivolol at β_2- and β_3-receptors). Here, nebivolol is a neutral antagonist at these receptors in this assay. This pattern is as would be predicted (Kenakin, 1999b; Strange, 2008), but there are some notable exceptions (butoxamine and methoxyphenamine at β_1, bucindolol at β_2 and oxprenolol and bucindolol at β_3).

In addition, the agonist concentration–response curves are not always best described by a one-site sigmoidal response curve. Several agonist concentration–response curves at both the human β_1- and β_3-adrenoceptors are best described by a two-component response. This is similar to previous findings (Walter *et al*., 1984; Baker *et al*., 2003a; Baker, 2005b) where both of these receptors (but as yet not the human β_2-adrenoceptor) have been described as having at least two active agonist conformations (Pak and Fishman, 1996; Granneman, 2001; Arch, 2004; Kaumann and Molenaar, 2008). Interestingly, some compounds appear to bind to or induce two conformations of the receptor, while others stimulate a two-component response, and L 748337 is best described by a two-component response in both binding and [^{3}H]-cAMP accumulation. Ligands are known to have different affinities and different efficacies for the two sites (agonist or antagonist; Baker *et al*., 2003a; Baker, 2005b,c), so the differences probably represent ligands binding to the two components with similar affinity, but one conformation being more functionally active (efficacious) than the other and vice versa.

Finally, comparisons of the rank order of ligands for the three different receptors provide information about relative intrinsic efficacies. Fenoterol is a full and efficacious agonist at the β_1-adrenoceptor, ranking third out of the agonists studied. It was also a full agonist at the β_2- and β_3-adrenoceptors with the highest intrinsic efficacy (i.e. top of Tables 4 and 5, rank 1). Thus, fenoterol is a full agonist with high intrinsic efficacy across all three β-adrenoceptors. Likewise, BAAM is a partial agonist at all three receptors, with rankings of 37, 34 and 36 at β_1, β_2 and β_3, respectively, and can therefore be said to have low intrinsic efficacy at all three receptors.

Salmeterol appears very selective for the human β_2-adrenoceptor in the cAMP assay (log EC_{50} values of –7.03, –9.89 and –6.60 for β_1, β_2 and β_3 respectively). This could be due to either selective affinity or selective intrinsic efficacy for the β_2-adrenoceptor over the other subtypes, or a mixture of both. The affinity measurements (log K_D values of –5.73, –9.26 and –6.33 for β_1, β_2 and β_3, respectively), show that salmeterol has high affinity for the β_2-adrenoceptor. The intrinsic efficacy of the compound is relatively poor in all cases (rank order 22 at β_1, 21 at β_2 and 33 at β_3). Thus, overall, salmeterol is a highly selective β_2-adrenoceptor agonist because of its higher β_2-affinity and not because of higher β_2-intrinsic efficacy. A similar reasoning can be applied to formoterol, although this agonist has higher intrinsic efficacy at all three receptors (rank 6, 8 and 5 at β_1, β_2 and β_3). This is similar to the finding of Kenakin and Beek (1980) where prenaterol was found to have equal intrinsic efficacy at β_1- and β_2-adrenoceptors.

Of the agonists studied here, there was a general trend that those with highest intrinsic efficacy were so across all three receptor subtypes (i.e. at the top of Tables 3–5, e.g. fenoterol, terbutaline, metaproterenol and adrenaline), and those with lower intrinsic efficacy likewise tended to have it across all three receptors (bottom of the tables). Thus, the approximate rank order of ligands is similar across all three receptors. However, in order to examine this further, the efficacy ratios (log K_D/log EC_{50}) were plotted for the receptors. Figure 7A (affinity values) shows that salmeterol has high selectivity for β_2-adrenoceptors based on affinity, whereas denopamine, AZ 40140d and isoprenaline have little affinity-based selectivity. Figure 7B (efficacy ratios) shows that isoprenaline and salmeterol are equally efficacious at the human β_1- and β_2-adrenoceptors. Denopamine is the most selective ligand for β_1-receptors, with regard to intrinsic activity and efficacy, and clenbuterol, procaterol, zinterol, AZ 40140d and salbutamol

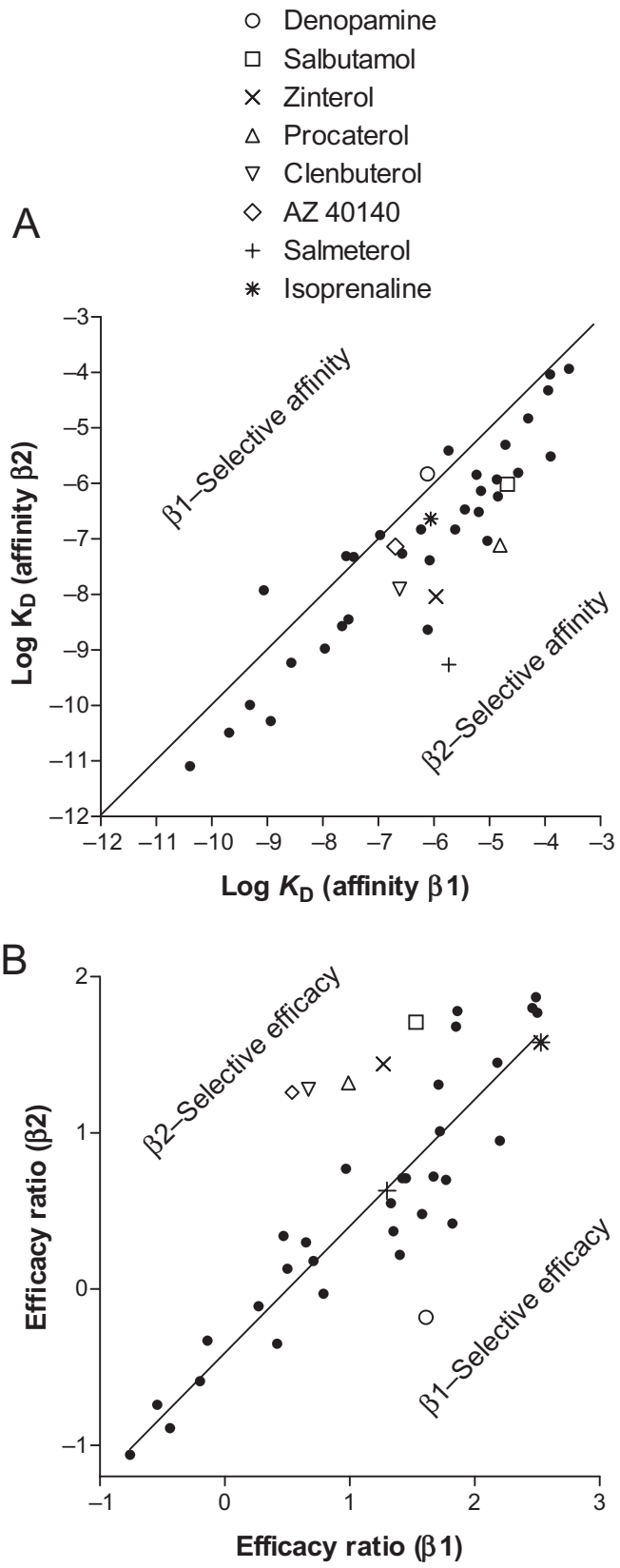

Figure 7 (A) Plot of log K_D for compounds from Table 1 for human β_1- versus human β_2-adrenoceptors. The line is through the origin and represents equal affinity, so compounds occurring to the right and below are β_2-selective. Thus, salmeterol can be seen to be highly β_2-selective based on affinity, whereas isoprenaline, denopamine and AZ 40140d have little selectivity based on affinity. (B) Plot of efficacy values (log K_D/log EC_{50}) for the same compounds (taken from Tables 3 and 4). The line is that of best fit – the slope is not 1 nor does it go though the origin as this represents a function of efficacy (i.e. differences in cell line which include receptor number, receptor–effector coupling, etc.). Denopamine can be seen to be β_1-selective based on efficacy, whereas AZ 40140d is β_2-selective, and isoprenaline and salmeterol were non-selective based on efficacy.

are more selective for the β_2-adrenoceptor than the β_1-adrenoceptor based on intrinsic activity and efficacy. Although difficult to directly compare, the selectivity based on affinity appears to be greater than the selectivity based on efficacy.

There are two current theories on the matter of efficacy: conformational selection and conformational induction (Clarke and Bond, 1998; Hunyady *et al*., 2003).

Conformational selection suggests that receptors spontaneously exist in basal (*R*) and pre-determined active forms [*R** in two-state (Leff, 1995) and *R*** in three-state models (Leff *et al*., 1997)] in the cell membranes that are in equilibrium with each other. Agonists bind with greater affinity to *R** (or *R***) over *R*, and therefore reset the equilibrium with more *R** present, and hence a response is generated. Here, intrinsic efficacy is explained on the basis of selective affinity where 'intrinsic efficacy' is actually the ratio of affinity for *R** over *R*. Conformational (or agonist) induction suggests that receptors are in a basal state in the cell membrane, and the binding of the ligand creates the agonist *R** state of the receptor. Here, efficacy is the ability of the ligand to change the receptor conformation. This model allows for an infinite number of active receptor states, but suggests that there is something distinct about the chemical interaction that gives some molecules the receptor-activating property of intrinsic efficacy. However, both models suggest that it should be possible to develop agonist ligands that are selective because of selective intrinsic efficacy.

In conclusion, therefore, agonist ligands can, in theory, appear selective for different receptor subtypes for different reasons: selective binding affinity or selective intrinsic efficacy. This study shows that some β-adrenoceptor agonists (e.g. salmeterol) are highly selective purely because of selective binding affinity. Others have very similar affinity but appear subtype selective because of a degree of selective intrinsic efficacy. Thus, it is possible to develop selective ligands based on either affinity or intrinsic efficacy, and there is therefore scope for the development of better, more selective agonists with both of these properties.

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Conflicts of interest

The author has no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Agonist, source, catalogue number, CAS RN number and isomeric status for each of the agonists used.

Table S2 Log EC_{50} values and % maximal isoprenaline responses measured from [^{3}H]-cAMP accumulation in CHO β_1 cells at 10 min, 30 min and 5 h.

Table S3 Log EC_{50} values and % maximal isoprenaline responses measured from [^{3}H]-cAMP accumulation in CHO β_2 cells at 10 min, 30 min and 5 h.

Table S4 Log EC_{50} values and % maximal isoprenaline responses measured from [^{3}H]-cAMP accumulation in CHO β_3 cells at 10 min, 30 min and 5 h.

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