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Evidence for a secondary state of the human β3-adrenoceptor.

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Abbreviations:

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

CL 316243, 5-[(2*R*)-2-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid:

CRE, cyclic AMP response element;

DMEM/F12, Dulbecco's modified Eagles medium/nutrient mix F12;

GPCR, G-protein coupled receptor;

ICI 118551, (-)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol;

ICI 215001, (*S*)-4-[2-hydroxy-3-phenoxypropylaminoethoxy]phenoxyacetic acid;

ICI 89406, *N*-[2-[3-(2-cyanophenoxy)-2-hydroxypropylamino]ethyl-N'-phenylurea

SR 59230A, 1-(2-ethylphenoxy)-3-[[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2*S*)-2-propanol hydrochloride

ZD 7114, (*S*)-4-[2-hydroxy-3-phenoxypropylaminoethoxy]-N-(2-methoxyethyl)phenoxyacetamide

Abstract

There are three members of the β-adrenoceptor family all of which are primarily coupled to Gs-proteins. Recent studies using the huge range of β-ligands now available have given remarkable new insights into their pharmacology. β1-adrenoceptors exist in at least two active conformations while β2-adrenoceptors are able to induce signalling via different agonist-induced receptor conformational states and their affinity for antagonists can be altered by highly efficacious agonists. This study therefore examined the pharmacology of the human β3-adrenoceptor stably expressed in CHO cells. Several compounds previously described as β-antagonists have agonist properties at the β3-adrenoceptor. Antagonist affinity measurements varied at the β3-adrenoceptor in a similar manner to those observed at human β1-adrenoceptors and unlike those seen at β2-adrenoceptors. Some ligands e.g. fenoterol and cimaterol were more readily inhibited by all antagonists whilst other ligands (e.g. alprenolol and SR 59230A) stimulated responses that were more resistant to antagonism. Alprenolol inhibited fenoterol induced β3-adrenoceptor responses whilst acting as an agonist at higher concentrations. This is highly suggestive of two active conformational states of the β3-adrenoceptor. ZD 7114 stimulated a two-component response of which the first component was more readily antagonised than the second. Taken together, these experiments suggest that the human β3-adrenoceptor exists in at least two different agonist conformations with a similar high and low-affinity pharmacology analogous to, if not as pronounced as, the β1-adrenoceptor. Both conformations are present in living cells and can be distinguished by their pharmacological characteristics. In this respect, the human β3-adrenoceptor appears similar to the human β1-adrenoceptor.

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Introduction

There are three members of the β-adrenoceptor family (β1, β2 and β3-adrenoceptors) which are all G-protein coupled receptors primarily coupled to Gs-proteins and have been studied extensively. However, recent studies using the huge range of β-ligands now available have given remarkable new insights into their pharmacology.

The β1-adrenoceptor is now considered to exist in at least two active conformations and although the structural nature of these two conformations or states remains unknown, they have different pharmacological properties (Granneman, 2001; Molenaar, 2003; Arch, 2004). All reports so far have described ligands as having higher affinity for the classical catecholamine-conformation than the secondary (low-affinity, formally the putative β4-adrenoceptor) conformation. Furthermore, measurements of antagonist affinity revealed that agonist responses occurring via the catecholamine-conformation are readily inhibited by classical antagonists whereas those occurring via the secondary low-affinity state are relatively resistant to antagonism (Konkar et al., 2000; Lowe et al, 2002; Baker et al., 2003a; Baker, 2005a). Finally, the efficacy of ligands, including many drugs previously described as "β-blockers" differs between the two states: some ligands are antagonists at both conformations; others have agonist action at the secondary-conformation whilst behaving as antagonists of the catecholamine-state; some appear to purely activate the catecholamine-state whilst others clearly activate both conformations (Konkar et al., 2000; Baker et al., 2003a).

The β2-adrenoceptor has not to date been demonstrated to have two active conformations with distinct ligand affinity and efficacy differences akin to the β1-adrenoceptor. However, recent studies have demonstrated that ligands are able to induce different conformational states of the receptor that activate different intracellular signalling cascades. Whilst many agonists and inverse agonists have been described that either increase or decrease intracellular cAMP respectively (Jasper et al., 1990; Chidiac et al., 1994; Bond et al., 1995; Baker et al., 2003b), other ligands have been described that simultaneously act as inverse agonists of one pathway (β2-Gs-cAMP) whilst having agonist activity via a second separate pathway in the same cells (β2-p42/44 MAP Kinase pathway; Azzi et al., 2003; Baker et al.,

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2003b). In different tissues, the β2-adrenoceptor has also been shown to couple to Gi-proteins under certain conditions (e.g. Daaka et al., 1997; Brown and Harding 1992; Hassledine et al., 2003). Furthermore studies of antagonist affinity have revealed that the affinity of an antagonist for the receptor changes in a time-dependent, phosphorylation-dependent manner, a property not shared by the β1-adrenoceptor (Baker et al., 2003c; Baker 2005a).

The expression pattern of the β3-adrenoceptor is somewhat contentious but reports include expression in gut, gall bladder, brain, urogenital tissue, skeletal muscle and the heart, however its primary function (at least in rodents) appears to be in thermogenesis as a consequence of its expression in adipose tissue (Strosberg 1997; Gauthier et al., 2000; Arch 2001). Highly selective β3-agonists may therefore have potential as anti-obesity drugs in man (Arch 2001). Also, β3-adrenoceptors may be important in cardiovascular pharmacology especially if β3-adrenoceptors are increased in human heart failure (Gauthier et al., 2000; Moniotte et al., 2001; Moniotte and Balligand, 2002). As well as agonists, several ligands generally considered as β-adrenoceptor antagonists have been demonstrated to have agonist properties at the β3-adrenoceptor (Blin et al., 1993; Gerhardt et al., 1999; Hutchinson et al., 2005). There are however important differences between rodent and human β3-adrenoceptors in their physiology and pharmacological function in both adipose tissue and the heart (Strosberg 1997; Gauthier et al., 2000; Arch 2001). The β3-adrenoceptor has also be shown to couple to Gi-proteins in certain tissues/conditions (rodent, Chaudhry et al., 1994; Hutchinson et al., 2002; human, Gauthier et al., 1996) and a recent study of mouse β3-adrenoceptor function has revealed the existence of different downstream signalling cascades (similar to that of the human β2-adrenoceptor) that are targeted by different ligands (Hutchinson et al., 2005). Finally, studies of adenylyl cyclase and membrane binding have revealed different potencies for ligands leading to the suggestion of a two-state model of human β3-adrenoceptor (not dissimilar to that of the human β1-adrenoceptor), but with one state that exists in membranes and a second state that predominates in whole cells (Arch 2002).

The aim of this study was to systematically examine the pharmacology of the human β3-adrenoceptor expressed in CHO cells and determine 1) the pharmacology of a range of "β-blockers" 2) examine antagonist affinity measurements and 3) look for

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evidence of differential signalling or the existence of different agonist conformations and thus determine whether the β3-adrenoceptor is most similar to either the β1 or β2-adrenoceptor or whether it has a completely different receptor phenotype altogether.

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Methods

Materials

Fetal calf serum was from PAA laboratories (Teddington, Middlesex, UK). ^{3}H-adenine, ^{3}H-CGP 12177 and ^{14}C-cAMP were obtained from Amersham International (Buckinghamshire, UK). The Luclite Plus Assay System was from PerkinElmer (Groningen, Netherlands). Betaxolol, bisoprolol, BRL 37344; cimaterol, CGP 12177, CL 316243, formoterol, ICI 118551, ICI 215001, ICI 89406, practolol, procaterol, pronethalol, salmeterol, SR59230A, sotalol and ZD 7114 were from Tocris Cookson (Avonmounth, Bristol, UK). Carvedilol was a gift from GlaxoSmithKline and bupranolol a gift from Prof Sian Harding (Imperial College, London). Sigma Chemicals (Poole, Dorset, UK) supplied all other reagents.

Cell Culture

CHO cells stably expressing both the human β3-adrenoceptor and a six CRE-luciferase reporter gene (six cyclic AMP response elements (CRE) upstream of a luciferase response gene) were used throughout this study (gift from Steve Rees, GlaxoSmithKline = CHO-β3 cells). The receptor coding sequence used for the original transfection was identical to that cited in EMBL AY487247 and encodes for the full length human β3-adrenoceptor protein (Swiss-Prot entry P13945). Cells were grown in Dulbecco's modified Eagles medium/Nutrient mix F12 (DMEM/F12) containing 10% fetal calf serum and 2mM L-glutamine in a humidified 5% CO_2 : 95% air atmosphere at 37°C. A control CHO cell line expressing a CRE-reporter gene but not the human β3-adrenoceptor was also used.

^{3}H-CGP 12177 whole cell binding.

CHO-β3 cells were grown to confluence in 200µl DMEM/F12 containing 10% fetal calf serum and 2mM L-glutamine in white-sided 96-well view plates. On the day of experimentation, this was removed and replaced with 200µl serum-free media (i.e. DMEM/F12 containing 2mM L-glutamine only) containing the final required concentration of CGP 12177 and ^{3}H-CGP 12177 (11.97 – 27.65nM) and the cells incubated for 2 hours at 37°C (5% CO_2). Total and non-specific binding (defined by 100µM ICI 118551) were measured in every experiment. The media and drugs were removed and the cells washed twice by the addition and removal of 2 x 200µl

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PBS/well. A white base was then added to the plate, followed by 100µl Microscint 20 per well, The plates were then counted on a Topcount (Packard) 2 minutes per well. The protein content was determined by the method of Lowry et al., (1951).

CRE-luciferase production

CHO-β3 cells were grown to confluence in white-sided 96-well view plates in 200µl DMEM/F12 containing 10% fetal calf serum and 2mM L-glutamine. On the day of experimentation, this was removed and replaced with 200µl serum-free media or 200µl serum-free media containing an antagonist at the final required concentration and the cells incubated for 1 hour at 37°C (5% CO_2). Agonist in 20µl (diluted in serum free media) was then added to each well and the plate incubated at 37°C (5% CO_2) for a further 5 hours. The media and drugs were removed, a white base added to the plate and luciferase activity detected using a PerkinElmer luciferase Luclite kit, counted on a Topcount (2 seconds per well). For the experiments seen in Figures 6 and 8, all drugs were added simultaneously to the wells and the plates incubated at 37°C for 5 hours before luciferase production was detected.

^{3}H-cAMP accumulation

Cells were grown to confluence in 24-well plates in 1ml DMEM/F12 containing 10% fetal calf serum and 2mM L-glutamine. The media was removed and the cells pre-labelled with ^{3}H-adenine by incubation with 2µCi/ml ^{3}H-adenine in serum-free media (0.5ml per well) for 3 hours at 37°C (5% CO_2). The ^{3}H-adenine was removed, each well washed by the addition and removal of 1ml serum-free media. 1ml serum-free media containing 1mM IBMX with or without the final required concentration of ICI 118551, was added to each well and the cells incubated for 30 minutes - 1 hour 37°C (5% CO_2). Agonist in 10µl was added to each well and the plates incubated for 1 hour at 37°C before the reaction was terminated by the addition of 50µl concentrated HCl per well. The plates were then frozen, thawed and ^{3}H-cAMP separated from other ^{3}H-nucleotides by Dowex and alumina column chromatography, with each column being corrected for efficiency by comparison with ^{14}C-cAMP recovery as previously described (Donaldson et al., 1988).

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Data Analysis

Receptor expression level

As the maximum concentration of ^{3}H-CGP 12177 obtainable is only two-fold over its K_D value at the human β3-adrenoceptor (Baker 2005b), the expression level of the transfected human β3-adrenoceptor was determined from CGP 12177 displacement of ^{3}H-CGP 12177. Displacement curves were fitted using the equation:

$$\% \text{ uninhibited binding} = \frac{(100 - NS)}{([C] / IC_{50} + 1)} + NS$$

where NS = non-specific binding, [C] is the concentration of cold CGP 12177 and IC_{50} the concentration at which half of the specific binding has been inhibited.

From the IC_{50} value and known concentration of ^{3}H-CGP 12177, the K_D value for CGP 12177 was calculated using the following equation:

$$K_D = IC_{50} - [^3\text{H-CGP 12177}].$$

The B_{MAX} was then calculated from the K_D value for CGP 12177 in each experiment using the following equation:

$$B_{MAX} = \frac{K_D + [C]}{[C]} \times \text{specific binding}$$

and expressed as fmol/mg protein after protein was determined by the method of Lowry et al., (1951).

One-site concentration responses curves

Sigmoidal agonist concentration-response curves were fitted using the following equation through computer-assisted non-linear regression using the programme Graphpad Prism 2:

$$\text{Response} = \frac{Emax \times [A]}{EC_{50} + [A]}$$

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

Antagonist K_D values were then calculated from the shift of the agonist concentration responses in the presence of a fixed concentration of antagonist using the following equation:

$$DR = 1 + \frac{[B]}{K_D}$$

where DR (dose ratio) is the ratio of the agonist concentration required to stimulate an identical response in the presence and absence of a fixed concentration of antagonist [B].

In experiments where 3 to 6 different fixed concentrations of the same antagonist were used, Schild plots were constructed using the following equation:

$$\text{Log } (DR-1) = \log [B] - \log (K_D)$$

These points were then fitted to a straight line. A slope of 1 then indicates competitive antagonism (Arunlakshana and Schild, 1959).

Two site agonist curves

Concentration-response curves for ZD 7114 (Figure 7) were fitted to two sites with Prism 2 using the equation:

$$\% \text{ maximal stimulation} = \frac{[A].N}{([A] + EC1_{50})} + \frac{[A].(100-N)}{([A] + EC2_{50})}$$

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.

A two-site analysis was also used for the experiments shown in Figures 6 and 8 using the following equation:

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$$\text{Response} = \text{Basal} + (\text{Ag} - \text{Basal})\left[1 - \frac{[P]}{([P] + IC_{50})}\right] + \text{PAg}\left[\frac{[P]}{([P] + EC_{50})}\right]$$

where basal is the response in the absence of agonist, Ag is the response to a fixed concentration of agonist, [P] is the concentration of partial agonist (e.g. alprenolol), IC_{50} is the concentration of competing partial agonist that inhibits 50% of the response of the fixed agonist, PAg is the maximum stimulation by the competing partial agonist and EC_{50} is the concentration of competing agonist that stimulated a half maximal competing partial agonist response.

A 10μM (maximal) isoprenaline concentration was included in each plate for each separate experiment for CRE-luciferase and ^{3}H-cAMP accumulation (with the exception of Figures 6 and 8), to allow agonist responses to be expressed as a percentage of the isoprenaline maximum for each experiment. All data are presented as mean $\pm$ s.e.m. of triplicate determinations and n in the text refers to the number of separate experiments.

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Results

Expression level of human β3-adrenoceptors in CHO-β3 cells

In CHO-β3 cells, the log K_D value for CGP 12177 was -6.65 ± 0.07 (n=8) and the receptor expression level 677 ± 89 fmol/mg protein (n=8).

Identification of agonists, partial agonists and neutral antagonists

In order to investigate changes in antagonist affinity measurements, full and partial agonists and neutral antagonists of the human β3-adrenoceptor were required. Using CHO-β3-luciferase cells, six or seven point concentration responses (to a maximum of 100μM) were therefore constructed with a range of ligands and both CRE-luciferase production and ^{3}H-cAMP accumulation were measured. Several ligands did not stimulate any change in either ^{3}H-cAMP accumulation or CRE-luciferase production (n=3 for each ligand in each assay, Figure 1a, Table 1). Other ligands stimulated an increase in both ^{3}H-cAMP accumulation and CRE-luciferase production at high concentrations however the top of the concentration response was not reached with 100μM ligand in each case (n=3 for each ligand in each assay, Figure 1b, Table 1). Several agonists were identified (see Table 1). Of those ligands with no intrinsic activity, ICI 118551 had one of the highest affinities (Baker 2005b) and was therefore used as the main neutral antagonist in further studies.

Antagonist affinity measurements – CRE-luciferase production

Isoprenaline stimulated an increase in CRE-luciferase production that was 8.5 ± 0.5 fold over basal (log EC_{50} = -7.48 ± 0.05, n=15, Figure 2). These responses were inhibited by several antagonists to give log K_D values as shown in Table 2. However, when the ability of antagonists to inhibit other agonists were examined, the responses to some agonists (e.g. fenoterol) were more readily inhibited, and others (e.g. CGP 12177) more resistant to antagonism than the catecholamines (Table 2, Figures 2-4). In order to investigate this further, the ability of three of the antagonists to inhibit a larger range of agonists was studied (Table 3). A range of antagonist affinities can clearly be seen with some agonists more resistant to antagonism than CGP 12177.

In order to determine whether the agonist responses were competitively inhibited, fenoterol, BRL 37344, isoprenaline and CGP 12177 responses were inhibited by 6

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different concentrations (4 in the case of CGP 12177) of ICI 118551 and Schild plots constructed. In all cases the Schild slopes confirmed competitive inhibition (Schild slope and r^2 values are: fenoterol 1.12 ± 0.02, 0.989 ± 0.003, n=7; BRL 37344 1.04 ± 0.02, 0.988 ± 0.003, n=7: isoprenaline 0.99 ± 0.03, 0.975 ± 0.04, n=9: CGP 12177 0.99 ± 0.02, 0.990 ± 0.003, n=7 respectively; Figure 3).

Antagonist affinity measurements – ^{3}H-cAMP accumulation.

The antagonist affinity of ICI 118551 was examined in a second shorter-term assay, ^{3}H-cAMP accumulation. Here a similar, almost identical, range of log K_D values were obtained for ICI 118551 in the presence of the different agonists as seen in the CRE-gene transcription experiments (Table 3, Figure 5).

Evidence for two agonist-conformations of the β3-adrenoceptor

The range of antagonist affinity values is similar to that seen at the β1-adrenoceptor which is now regarded to have two-agonist states. In order to determine whether ligands can act as neutral antagonists of a putative β3-high-affinity state whilst being agonists of a low-affinity conformation of the β3-adrenoceptor (as seen at the β1-adrenoceptor), alprenolol (partial agonist, bottom of Table 3) was added to cells simultaneously with a fixed concentration of fenoterol (top of Table 3, Figure 6a) in a manner analogous to that in β1-adrenoceptor experiments (Pak and Fishman 1996; Konkar et al., 2000; Baker et al., 2003a; Baker, 2005a). Alprenolol was found to inhibit the maximum response stimulated by fenoterol at concentrations 10-fold lower than that required to stimulate its agonist response (i.e. inhibitory component to the left of the EC_{50} for alprenolol, n=4, Figure 6a). A similar pattern was seen with SR 59230A (Figure 6b, n=4).

Two-site agonist responses.

When ZD 7114 was examined as an agonist (CRE-luciferase production), the concentration response observed best fits to a 2-component response (log $EC_{50}1$ = -7.30 ± 0.03, 68.5 ± 1.6% of the total response; log $EC_{50}2$ = -4.78 ± 0.06; n=18, Figure 7a). ICI 118551, betaxolol and timolol inhibited the first component of this response to yield log K_D values of -6.03 ± 0.04 (n=11), -5.33 ± 0.04 (n=3) and -6.70 ± 0.10 (n=4) respectively which would place the first component of ZD 7114 along side

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BRL 37344 in Table 3. Significantly higher concentrations of ICI 118551 were required to achieve only small shifts of the second component of the ZD 7114 response (Figure 7b). A very similar response was seen at the level of ^{3}H-cAMP accumulation (log $EC_{50}1$ = -7.49 ± 0.09, 61.6 ± 3.1% of the total response; log $EC_{50}2$ = -5.41 ± 0.25; n=5) where ICI 118551 inhibited the first component to yield a log K_D value of -6.15 ± 0.09 (n=4, Figure 7c). When ZD 7114 was added with a fixed concentration of fenoterol, the inhibition of fenoterol by ZD 7114 was to the right of the first component i.e. as expected if the first component of ZD 7114 and fenoterol were competing at the same site (Figure 8, n=3).

Lack of responses in cells without the human β3-adrenoceptor.

As previously published (e.g. Baker et al., 2003a and b), no responses were seen in response to any of the ligands examined at either the level of cAMP or CRE-gene transcription in parent cell lines (both the parent CHO-K1 cell line and cells transfected only with a CRE-reporter). Furthermore, no specific binding has previously been detected using ^{3}H-CGP 12177 whole cell binding in the parent CHO-K1 cells. In addition to those already published, full concentration response curves (0.1nM – 100μM) to ZD 7114 and SR 59230A were examined in cells expressing a CRE-reporter but not the β3-adrenoceptor line and no responses were seen at any ligand concentration (n=3-4 for each ligand).

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Discussion

Antagonists have traditionally been considered to bind to a receptor with a stable affinity which represents the ligand-receptor chemical interaction and is therefore fixed regardless of competing ligands (Kenakin et al., 1995). Thus antagonist affinity measurements (K_D values) have been used to determine the receptors/receptor subtypes present in tissues and changes in this value have been used to demonstrate the presence of different receptors/receptor subtypes (e.g. Black et al., 1972). However, recent studies of β1 and β2-adrenoceptors have shown that antagonist affinity measurements do change within a single tissue or cell line (e.g. Konkar et al., 2000; Lowe et al., 2002; Baker et al., 2003a, b and c).

In order to investigate the human β3-adrenoceptor, full and partial agonists and neutral antagonists were identified. Many of those identified were in agreement with previous studies (Blin et al., 1993). As seen with β1 and β2-adrenoceptors, several ligands usually considered as β-antagonists had β3-adrenoceptor agonist activity (Table 1) again in agreement with previous studies (Blin et al., 1993; Gerhardt et al., 1999; Hutchinson et al., 2005). Also as seen in previous CRE-gene transcription studies (e.g. Baker et al., 2004), the efficacy of partial agonists appeared greater in downstream CRE-gene transcription than at the second messenger level (^{3}H-cAMP; Table 1). No ligands were found to have inverse agonist activity in either assay in this CHO-human-β3 cell line.

The endogenous catecholamines adrenaline and noradrenaline stimulated full agonist responses similar to that of isoprenaline (see Table 1). When these responses were antagonised by ligands with no or minimal intrinsic efficacy, the antagonist affinities obtained were very similar, suggesting that the three catecholamines were binding to and activating the receptor in a similar manner (Table 2). However, when this was extended to several other full-agonists, the antagonist affinity measurements differed. Some agonist responses e.g. fenoterol and cimaterol were more readily inhibited than the catecholamines by all antagonists. CGP 12177 responses were more resistant to inhibition (Table 2). This is very similar to findings at human β1-adrenoceptors expressed in CHO-cells (Baker 2005a).

To investigate this further, a wider range of agonists, including partial agonists, were inhibited by three antagonists, ICI 118551 (no intrinsic efficacy, higher affinity), betaxolol (no intrinsic efficacy, lower affinity) and timolol (minimal agonist efficacy, higher affinity). For all three antagonists, the affinity measurements obtained depended on the agonist present. As the only difference between each experiment was the agonist, this suggests that the agonists themselves were responsible for inducing different receptor conformations to which the antagonists could either bind with high-affinity (e.g. in the presence of fenoterol, top of Table 3) or low-affinity (e.g. in the presence of alprenolol, bottom of Table 3).

Changing antagonist affinity occurs at both β1 and β2-adrenoceptors. With β2-adrenoceptors, antagonist affinity measurements were the same in short-term cAMP assays however in longer-term assays (cAMP and CRE-gene transcription), efficacious agonists caused an efficacy-driven change in the receptor that reduced antagonist affinity by ten-fold (Baker et al., 2003c). As antagonist affinity measurements differed at the β3-adrenoceptor in this CRE-gene transcription study, shorter-term β3-adrenoceptor cAMP responses were therefore examined. The log K_D values for ICI 118551 in the cAMP assays were very similar to those obtained in long-term gene transcription assay (Table 3). There is therefore no time-dependent change in antagonist affinity. Also the efficacy of the competing agonists was unrelated to the antagonist affinity value obtained (see Tables 1 and 3). The reason for antagonist affinity variation is therefore not the same as that seen at β2-adrenoceptors.

A range of antagonist affinity measurements also occurs at β1-adrenoceptors although, these values are not affected by agonist efficacy (Joseph et al., 2004; Baker 2005a). The most likely explanation for β1-antagonist affinity differences is that ligands activate the two β1-adrenoceptor states (the antagonist-sensitive catecholamine-conformation and the antagonist-resistant low-affinity-conformation) to differing degrees. Thus, unlike β2-adrenoceptors, there is a range of antagonist affinities at both β1 and β3-adrenoceptors that are not related to agonist efficacy, time of incubation or response measured. In this regard, β3-adrenoceptors therefore appear similar to β1-adrenoceptors.

Further examination of Table 3 suggests that β3-adrenoceptors indeed have similar pharmacological profiles to β1-adrenoceptors (Joseph et al., 2004; Baker 2005a). Firstly, all catecholamines induce full agonist responses at both receptors that are inhibited to yield mid-range antagonist affinity measurements at each receptor. Secondly, at both receptors, at one end of the spectrum (top of Table 3) are agonists that are more readily inhibited than catecholamine responses by all antagonists examined whilst at the other end are agonists whose responses are all more resistant to inhibition (thereby giving rise to lower antagonist affinity measurements). If β3-adrenoceptors are truly like β1-adrenoceptors (Konkar et al., 2000; Granneman, 2001; Baker et al., 2003a; Molenaar 2003; Arch 2004; Baker 2005a), then agonists at the top of Table 3 are acting through a "high-affinity" state and those at the bottom of Table 3 via a "low-affinity" state of the β3-adrenoceptor. Agonists in the middle of Table 3 may therefore be acting through both putative states of the β3-adrenoceptor. Thirdly, whilst the rank order of antagonist affinities for high-affinity-state agonists is the same as for the catecholamines in both β1 and β3-adrenoceptors, it is different for low-affinity state ligands (Figure 4; Baker 2005a). The identical rank order for the high-affinity-state ligands suggest that these ligands may be acting through a different conformation of the same receptor rather than a truly different third state, whilst the low-affinity ligands are indeed acting via a truly different state of the two receptors.

There are however some differences from previous β1-adrenoceptor findings. Firstly, differences in antagonist affinity for the β1-adrenoceptor vary by 30 and 1000-fold depending on the antagonist (Joseph et al., 2004; Baker et al., 2005a). In this β3-adrenoceptor study, although significant, antagonist affinity measurements vary by only 4-50 fold (Tables 2 and 3). Secondly, the correlation between antagonist affinity at the β1-high-affinity-state and β1-low-affinity-state is very poor (Baker 2005a) suggesting two very different receptor states or conformations. In this β3-adrenoceptor study, the correlation of antagonist affinities at the high and low affinity-states (Figure 4) is again not as marked as that seen at β1-adrenoceptors. However, given the similarities to β1-adrenoceptors, evidence of a potential secondary β3-adrenoceptor state was sought.

In order to determine whether fenoterol (top of Table 3) was stimulating a response through the same β3-adrenoceptor state as alprenolol (bottom of Table 3), alprenolol

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and fenoterol were added simultaneously to CHO-β3 cells (Figure 6). If they were competing through the same state, alprenolol would inhibit fenoterol responses at concentrations just above those required to stimulate an alprenolol response (i.e. IC_{50} to the right of EC_{50}). However, alprenolol inhibited the stimulation of fenoterol at lower concentrations (i.e. to the left) of those expected (Figure 6). This strongly suggests that the agonist effects of alprenolol and fenoterol are not competing at the same conformation. Similar results are seen with SR 59230A (Figure 6). This suggests that (as with CGP 12177 and the β1-adrenoceptor), alprenolol is acting as a high-affinity-state neutral antagonist whilst stimulating agonist responses at higher concentrations via a low-affinity-state of the β3-adrenoceptor.

ZD 7114 stimulated a response that was best described by a 2-component fit (Figure 7). As this response was seen at both ^{3}H-cAMP accumulation and CRE-gene transcription, it suggests β3-Gs-coupling only. The first component of the ZD 7114 response was more readily antagonised than the second, which is very reminiscent of alprenolol and pindolol responses at β1-adrenoceptors (Baker et al., 2003a). Furthermore, if an estimate of antagonist affinity is made from the parallel shift of the first component, the resulting log antagonist K_D values place this first component of ZD 7114 towards the top of Table 3. Therefore ZD 7114 may be an agonist of two different states of the β3-adrenoceptor, one with higher affinity for both ZD 7114 and antagonists than the other. Finally, when ZD 7114 and fenoterol were added simultaneously, the initial fenoterol inhibition by ZD 7114 was to the right of the initial ZD 7114 stimulation (as would be expected for the same state competition) but to the left for the second component (suggesting that the second component of the ZD 7114 response and fenoterol were acting at different states).

Thus, these results show that several compounds previously described as β-antagonists have agonist properties at the human β3-adrenoceptor (although with lower affinity than seen at human β1 and β2-adrenoceptors, Hoffman et al., 2004; Baker 2005b). Antagonist affinities measurements vary at β3-adrenoceptors but unlike β2-adrenoceptors these do not depend on time of incubation or agonist efficacy. The two-component ZD 7114 response and fenoterol/alprenolol competition experiments suggest that the human β3-adrenoceptor pharmacology is best explained by considering it to have at least two different agonist states with a similar high and

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low-affinity pharmacology analogous to, if not as pronounced as, the β1-adrenoceptor. This is similar to the previous suggestion of two β3-adrenoceptor binding states (Arch, 2002) but where both conformations co-exist in living cells (rather than one predominantly in whole cells and another in membranes) and the ligands themselves appear to dictate the conformational state of the receptor.

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Footnotes

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Legends for Figures

Figure 1

CRE-luciferase production and ^{3}H-cAMP accumulation in response to a) ICI 118551 and b) acebutolol in CHO-β3 cells. Bars show basal responses and those in response to 10μM isoprenaline. Data points are mean ± s.e.m. of triplicate values from a single experiment and are representative of 3 separate experiments in each case.

Figure 2

CRE-luciferase production in CHO-β3 cells in response to a) fenoterol b) BRL 37344, c) isoprenaline and d) CGP 12177 in the absence and presence of 100μM bisoprolol. Bars show basal luciferase activity, that in response to 10μM isoprenaline, and that in response to 100μM bisoprolol alone. Data points are mean ± s.e.m. from a single experiment in each case. These individual experiments are representative of a) 4, b) 7, c) 5 and d) 5 separate experiments.

Figure 3

CRE-luciferase production in CHO-β3 cells in response to a) fenoterol and c) CGP 12177 in the absence and presence 4μM, 10μM, 20μM, 40μM, 100μM or 200μM ICI 118551. Bars show basal luciferase activity, that in response to 10μM isoprenaline alone, that in response to 4μM, 10μM, 20μM, 40μM, 100μM and 200μM ICI 118551 alone. b) and d) show the Schild plot of a) and c) respectively. The Schild slopes and r^2 values for these experiments are a) 1.09 and 0.983 and b) 0.95 and 0.985 respectively. Data points are mean ± s.e.m. from a single experiment. Both a) and b) are representative of 3 separate experiments. In addition, a further 4 separate experiments were also performed with each agonist and 3 different concentrations of ICI 118551, see text for summary of all data.

Figure 4

Correlation between antagonist log K_D values with isoprenaline as agonist (x-axis) versus those obtained with agonists in Table 2 and alprenolol and SR 59230A from Table 3 (y-axis).

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Figure 5

^{3}H-cAMP accumulation in CHO-β3 cells in response to a) fenoterol, b) BRL 37344, c) isoprenaline and d) CGP 12177 in the absence and presence of 10μM (a-c) and 100μM (d) ICI 118551. Bars represent basal ^{3}H-cAMP accumulation and that in response to 10μM isoprenaline, (a-c) 10μM ICI 118551 or (d) ICI 118551 100μM alone. Data points are mean ± s.e.m. of triplicate determinations. Each of these single experiments is representative of a) 3, b) 3, c) 4 and d) 5 separate experiments.

Figure 6

CRE-luciferase production in CHO-β3 cells response to a) alprenolol and b) SR 59230A in the absence and presence of 30nM, 100nM or 300nM fenoterol. Bars represent basal luciferase production and that in response to 30nM, 100nM or 300nM fenoterol alone. Data points are triplicate determinations from a single experiment which are representative of a) 4 and b) 4 separate experiments.

Figure 7

a) and b) CRE-luciferase production and c) ^{3}H-cAMP accumulation in CHO-β3 cells in response to ZD 7114 in the absence and presence of a) 10μM ICI 118551, b) 100μM ICI 118551 and c) 30μM ICI 118551. Bars show basal luciferase activity or ^{3}H-cAMP production, that in response to 10μM isoprenaline, and that in response to a) 10μM ICI 118551, b) 100μM ICI 118551 or c) 30μM ICI 118551 alone. Data points are mean ± s.e.m. from a single experiment in each case. These individual experiments are representative of a) 18 b) 3 and c) 5 separate experiments.

Figure 8

CRE-luciferase production in CHO-β3 cells response to ZD 7114 in the absence and presence of 30nM, 100nM or 300nM fenoterol. Bars represent basal luciferase production and that in response to 30nM, 100nM or 300nM fenoterol alone. Data points are triplicate determinations from a single experiment which are representative of 3 separate experiments.

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	^{3}H-cAMP accumulation				CRE-luciferase production		
ligand	Log EC_{50}	%isoprenaline	n		Log EC_{50}	%isoprenaline	n
fenoterol	-7.56±0.05	98.5±1.0	4		-7.56±0.03	102.6±1.6	14
terbutaline	-5.53±0.06	97.5±0.4	6		-5.68±0.13	110.6±3.0	4
salbutamol	-5.76±0.08	88.8±3.0	4		-5.76±0.12	108.2±2.0	4
tulobuterol	-5.40±0.05	76.6±4.6	5		-5.40±0.12	107.7±3.5	4
cimaterol	-6.73±0.08	86.2±2.5	6		-6.71±0.04	106.3±1.8	7
BRL 37344	-7.38±0.05	79.2±3.4	4		-7.52±0.03	98.4±1.7	13
pronethalol	-5.34±0.07	7.8±3.7	9		-4.82±0.16	24.2±11.0	3
ICI 215001	-7.06±0.06	60.3±2.9	5		-7.27±0.03	89.2±2.8	6
clenbuterol	-5.90±0.07	77.7±2.8	4		-5.91±0.10	109.3±2.2	4
dobutamine	-6.27±0.01	87.3±1.6	5		-6.29±0.08	106.9±3.4	4
CL 316243	-6.03±0.04	75.8±4.5	6		-6.17±0.12	99.8±3.8	4
salmeterol	-6.23±0.07	84.2±2.5	5		-6.15±0.07	139.2±1.9	4
formoterol	-7.72±0.08	99.6±3.5	4		-7.72±0.10	113.0±2.8	4
noradrenaline	-7.14±0.07	96.9±2.2	6		-7.29±0.04	106.9±2.1	8
adrenaline	-6.55±0.06	96.5±1.8	5		-6.67±0.04	109.5±2.1	7
isoprenaline	-7.35±0.05	100	5		-7.48±0.05	100	12
labetolol	-5.32±0.06	9.8±0.5	10		-4.97±0.09	37.4±2.1	5
pindolol	-5.67±0.06	39.3±1.0	8		-5.69±0.05	90.1±4.0	8
CGP 12177	-6.57±0.09	61.4±1.5	6		-6.93±0.03	101.7±1.4	15
SR 59230A	-7.21±0.04	12.3±0.8	9		-6.49±0.06	37.5±5.7	10
alprenolol	-5.69±0.07	17.5±1.3	6		-5.56±0.09	54.1±5.5	10
carvedilol	-7.19±0.22	8.0±0.5	9		-6.10±0.12	44.9±6.5	6
propanolol	-5.49±0.10	7.5±0.5	12		-5.10±0.10	23.1±7.1	3
timolol	-5.55±0.14	5.7±0.5	6		-4.77±0.07	20.4±11.5	3
Ligands with agonist activity at high concentrations (top of response not reached by 100μM)							
Acebutolol, dopamine, ephedrine, ICI 89406, nadolol, procaterol							
Ligands with no intrinsic efficacy							
Atenolol, betaxolol, bisoprolol, bupranolol, CGP 20712A, ICI 118551, metoprolol, practolol, sotalol							

Table 1
Log EC_{50} values and % isoprenaline maximum values for a range of β-ligands when measured at both ^{3}H-cAMP accumulation and CRE-luciferase production in CHO-β3 cells. Values are mean ± s.e.m. of n determinations

antagonist	fenoterol	n	cimaterol	n	BRL 37344	n	ICI 215001	n	isoprenaline	n	adrenaline	n	noradrenaline	n	CGP 12177	n
bupranolol	-7.12±0.05 **	4	-7.05±0.09 *	4	-7.01±0.04	4	-6.94±0.03	3	-6.72±0.07	5	-6.74±0.09	4	-6.72±0.09	4	-6.12±0.08 ***	5
timolol	-6.86±0.02	5	-6.87±0.04	6	-6.87±0.08	6	-6.67±0.04	5	-6.59±0.06	5	-6.62±0.08	7	-6.57±0.08	8	-5.81±0.07 ***	8
nadolol	-6.28±0.07 *	4	-6.30±0.07 *	4	-6.32±0.08 *	5	-6.17±0.06	3	-5.97±0.06	5	-6.14±0.08	4	-6.01±0.09	4	-5.41±0.08 ***	5
ICI 118551	-6.04±0.02 ***	36	-6.09±0.06 ***	5	-5.95±0.02 ***	35	-5.99±0.05 *	4	-5.75±0.03	39	-5.84±0.08	6	-5.89±0.04	7	-5.17±0.04 ***	33
betaxolol	-5.48±0.08 *	5	-5.51±0.09 *	6	-5.54±0.08 *	5	-5.41±0.08	5	-5.17±0.06	6	-5.24±0.05	6	-5.21±0.05	7	-4.78±0.08 ***	5
bisoprolol	-5.42±0.04 ***	4	-5.46±0.05 ***	4	-5.33±0.07 **	4	-5.30±0.03 *	4	-5.07±0.04	5	-5.06±0.04	4	-5.09±0.03	4	-4.41±0.08 ***	5
CGP 20712A	-5.32±0.03 **	4	-5.41±0.05 ***	4	-5.25±0.05 **	4	-5.28±0.04 **	4	-4.93±0.03	4	-5.01±0.05	4	-4.98±0.11	4	-4.71±0.06 *	5
sotalol	-5.22±0.03 *	4	-5.33±0.03 **	4	-5.20±0.03 *	4	-5.12±0.03	4	-4.90±0.08	5	-5.01±0.08	5	-4.93±0.10	5	-4.34±0.07 ***	5
metoprolol	-4.92±0.03 *	4	-4.95±0.05 *	4	-4.87±0.03	4	-4.86±0.06	4	-4.62±0.04	6	-4.55±0.10	4	-4.75±0.13	4	-4.26±0.07 ***	6

Table 2

Log K_D values for antagonists with no or minimal intrinsic efficacy as determined in CRE-luciferase production experiments in the presence of a range of agonists. Values are mean ± s.e.m. of n determinations.

*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$ when comparing the log K_D value of the antagonist with that obtained in the presence of isoprenaline (one-way analysis of variance (ANOVA), Neuman-Keul's post hoc).

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	cAMP accumulation				CRE-luciferase production							
agonist	Log K_D ICI 118551	n			Log K_D ICI 118551	n		Log K_D betaxolol	n		Log K_D timolol	n
fenoterol	-6.24±0.03	3			-6.04±0.02 ***	36		-5.48±0.08	5		-6.86±0.02	5
terbutaline	-6.24±0.09	5			-6.13±0.05 ***	4		-5.29±0.04	3		-6.93±0.09	4
salbutamol	-6.30±0.03	3			-6.13±0.06 ***	4		-5.28±0.10	3		-6.88±0.05	4
tulobuterol	-6.28±0.06	4			-6.13±0.04 ***	4		-5.25±0.00	3		-6.83±0.05	4
cimaterol	-6.33±0.07 *	5			-6.09±0.06 ***	5		-5.51±0.09	6		-6.87±0.04	6
BRL 37344	-6.13±0.04	3			-5.95±0.02 ***	35		-5.54±0.08	5		-6.87±0.08	6
pronethalol	-6.05±0.06	6			N.D			N.D.			N.D.	
ICI 215001	-6.04±0.08	4			-5.99±0.05	4		-5.41±0.08	5		-6.67±0.04	5
clenbuterol	-6.23±0.06	3			-5.99±0.04	4		-5.17±0.04	3		-6.70±0.04	4
dobutamine	-6.12±0.06	4			-5.97±0.07	4		-5.13±0.00	3		-6.60±0.04	4
CL 316243	-6.08±0.08	5			-5.96±0.11	4		-5.17±0.04	3		-6.72±0.05	4
salmeterol	-6.13±0.06	4			-5.94±0.05	4		-5.33±0.04	3		-6.59±0.10	4
formoterol	-6.21±0.04	3			-5.92±0.08	4		-5.25±0.00	3		-6.77±0.05	4
noradrenaline	-5.83±0.08	5			-5.89±0.04	7		-5.21±0.05	7		-6.57±0.08	8
adrenaline	-5.99±.0.03	4			-5.84±0.08	6		-5.24±0.05	6		-6.62±0.08	7
isoprenaline	-5.87±0.05	4			-5.75±0.03	39		-5.17±0.06	6		-6.59±0.06	5
labetolol	-5.53±0.08 *	6			-5.47±0.04 ***	5		-4.82±0.08 **	5		N.D.	
pindolol	-5.29±0.05 ***	7			-5.24±0.06 ***	8		-4.57±0.09 ***	7		-5.80±0.07 ***	8
CGP 12177	-4.87±0.07 ***	5			-5.17±0.04 ***	33		-4.78±0.08 **	5		-5.81±0.07 ***	8
SR 59230A	-5.13±0.14 ***	6			-5.17±0.04 ***	6		-4.24±0.12 ***	5		-5.60±0.05 ***	4
alprenolol	-5.23±0.06 ***	5			-5.14±0.03 ***	6		-4.26±0.09 ***	5		-5.14±0.03 ***	5
carvedilol	-4.79±0.19 ***	5			< -5	5		< -4.5	4		< -5	4

Table 3
Log K_D values for ICI 118551 (determined from ^{3}H-cAMP accumulation and CRE-luciferase production experiments) and timolol and betaxolol (determined in CRE-luciferase experiments) in the presence of a range of agonists. Values are mean ± s.e.m. of n determinations. N.D. = not determined.
*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$ when comparing the log K_D value of the antagonist with that obtained in the presence of isoprenaline (one-way analysis of variance (ANOVA), Neuman-Keul's post hoc).

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Fig 1

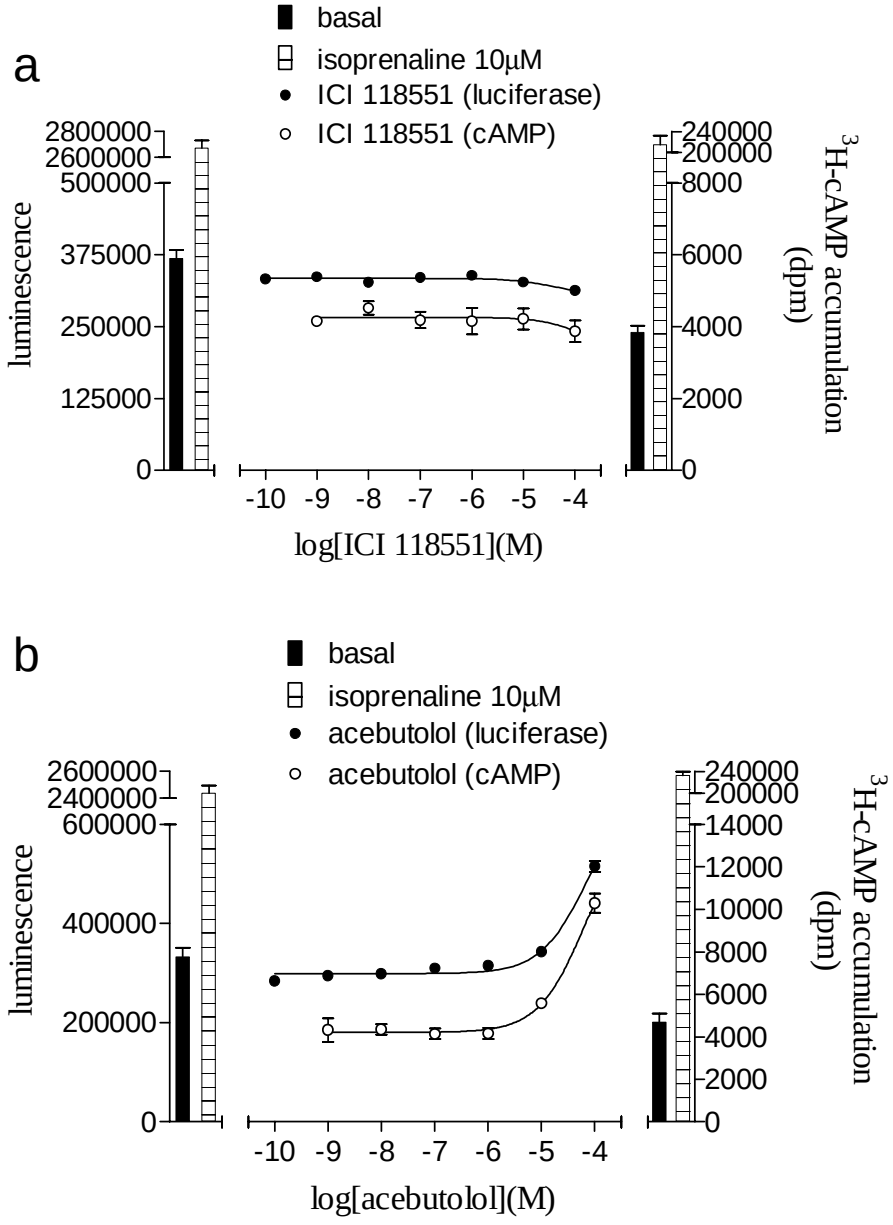

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Fig 2

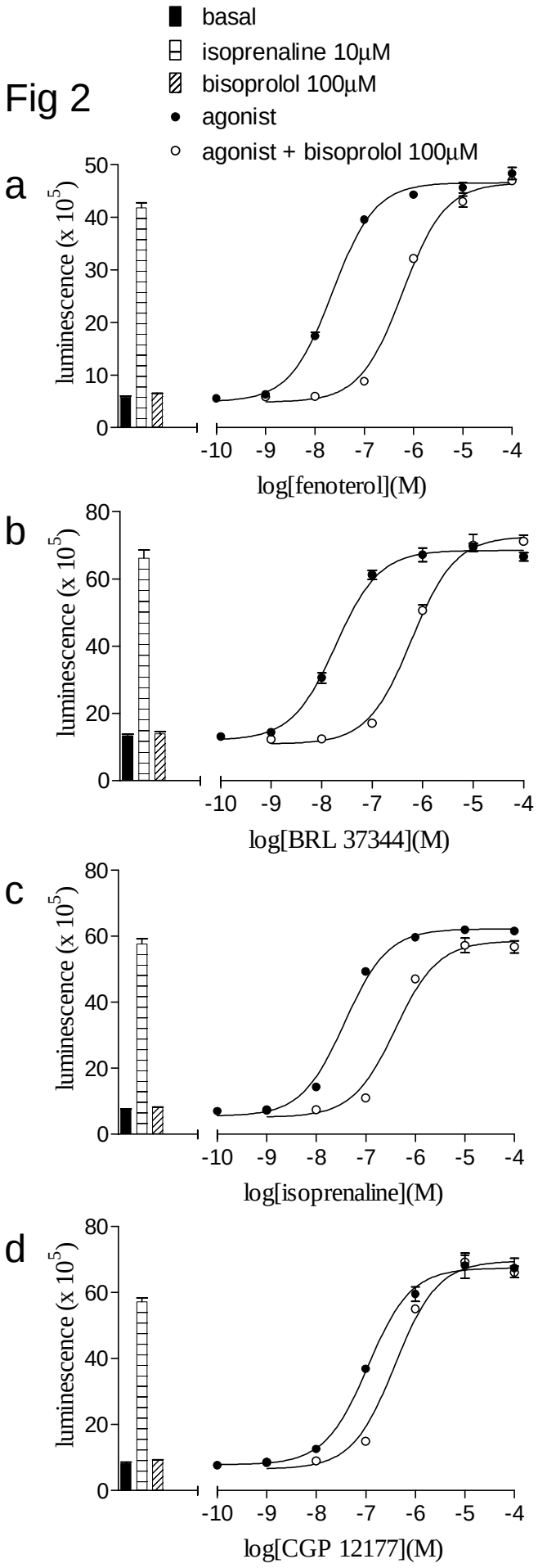

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Fig 3

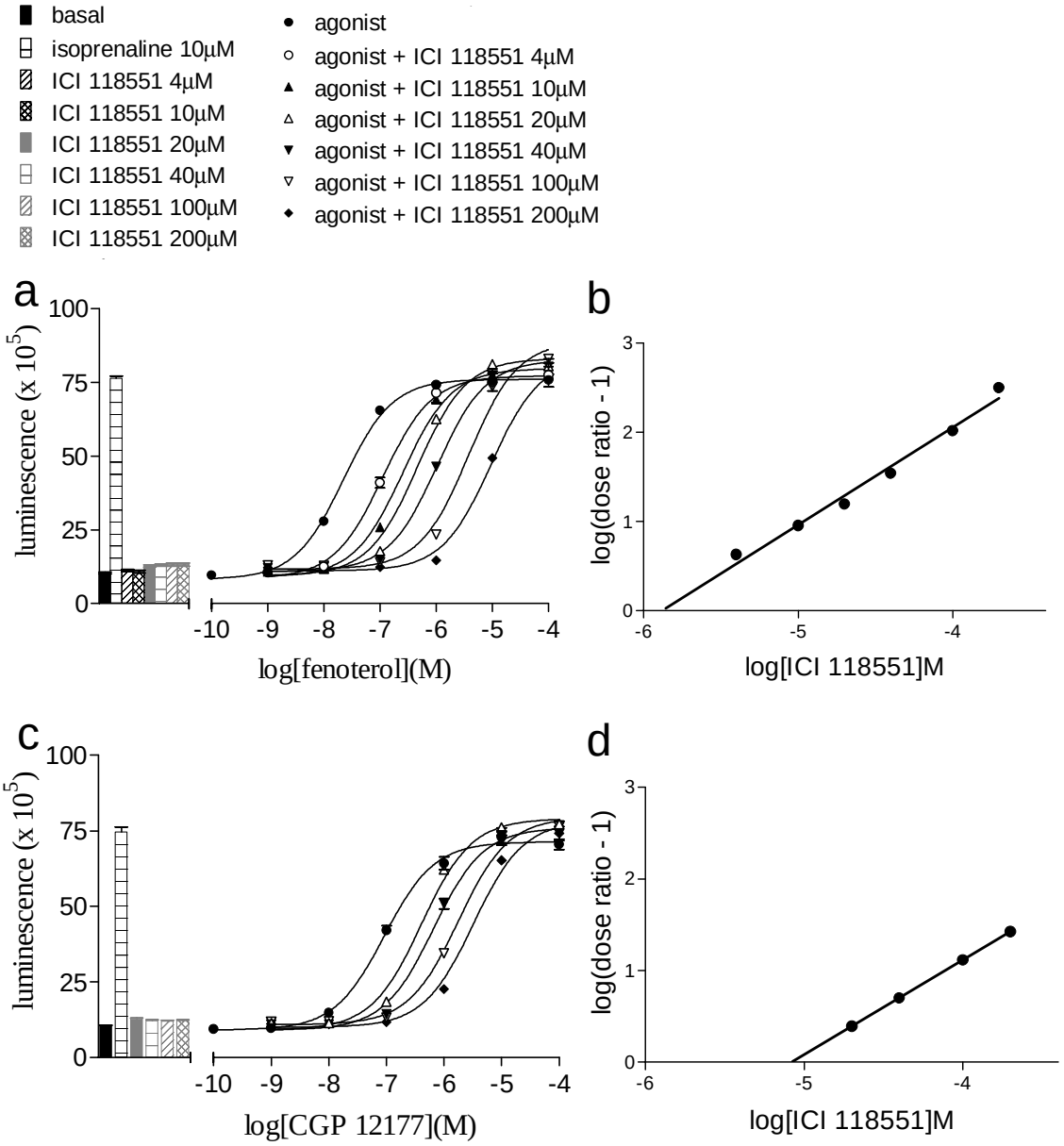

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Fig 4

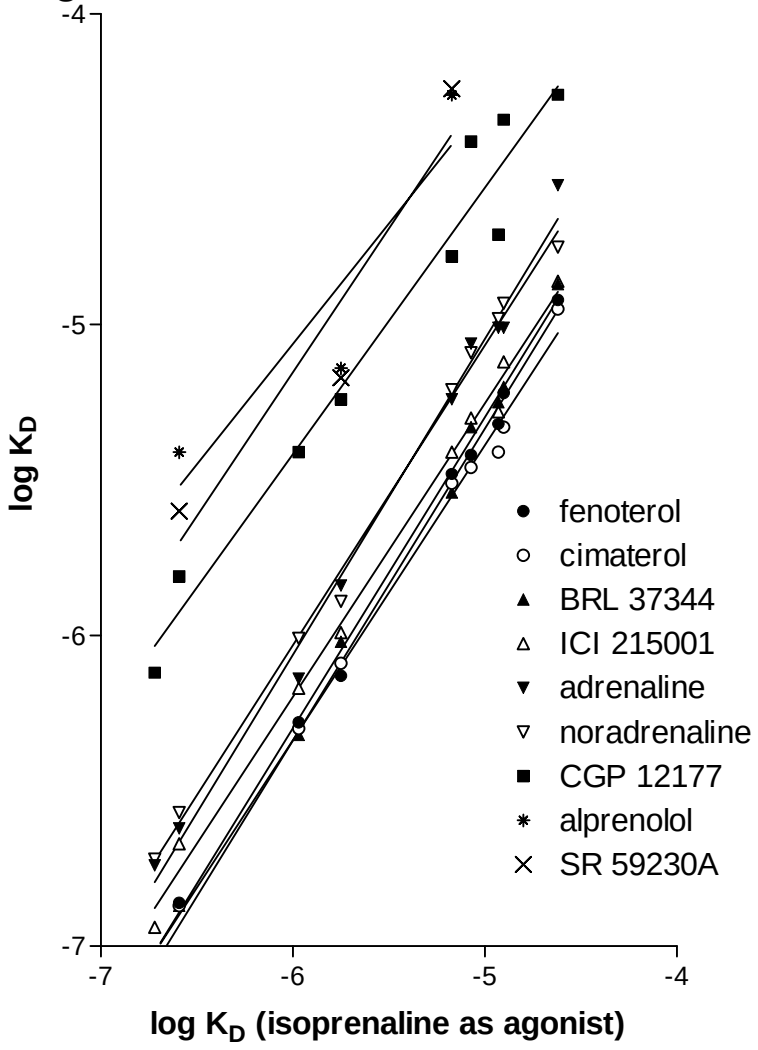

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Fig 5

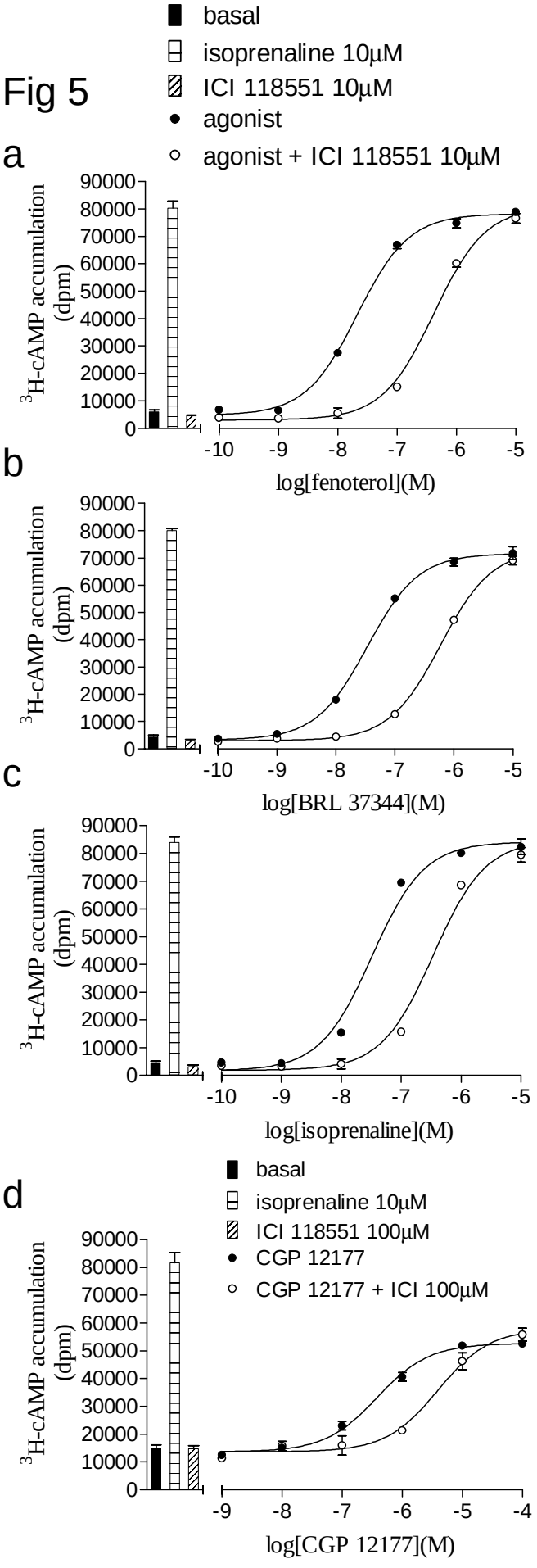

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Fig 6

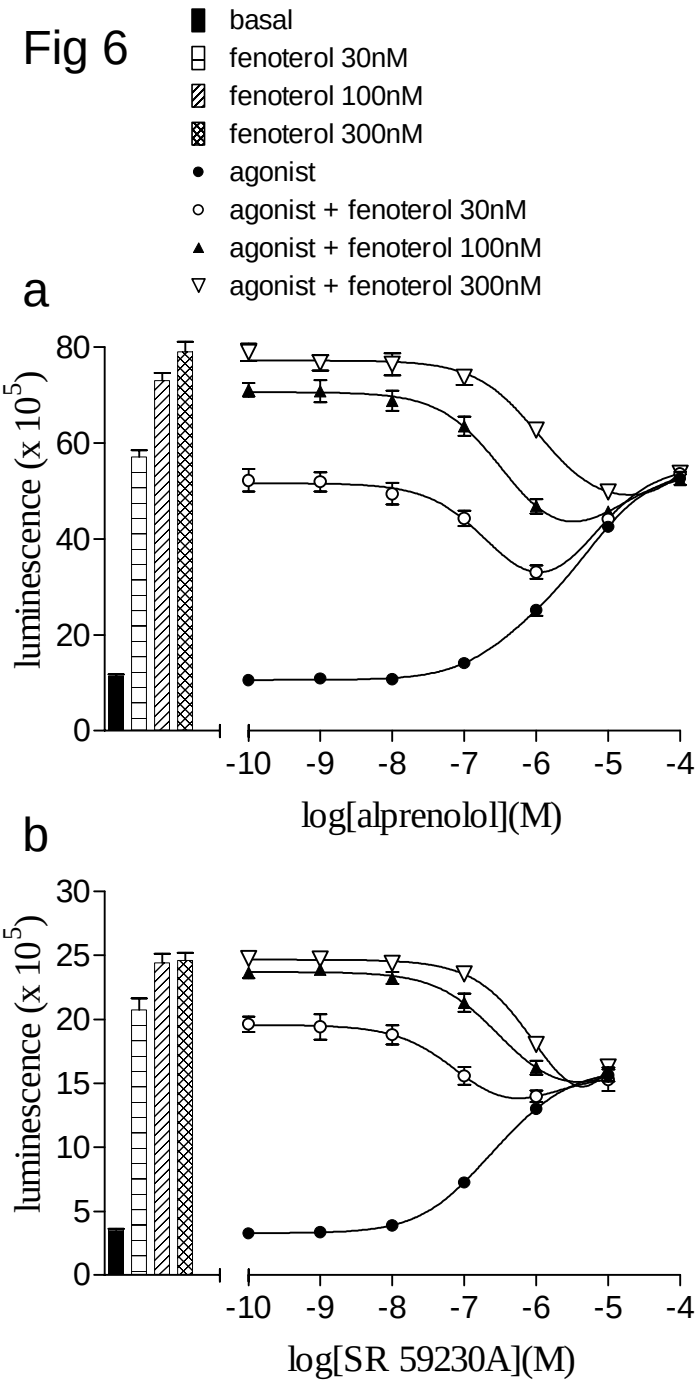

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Fig 7

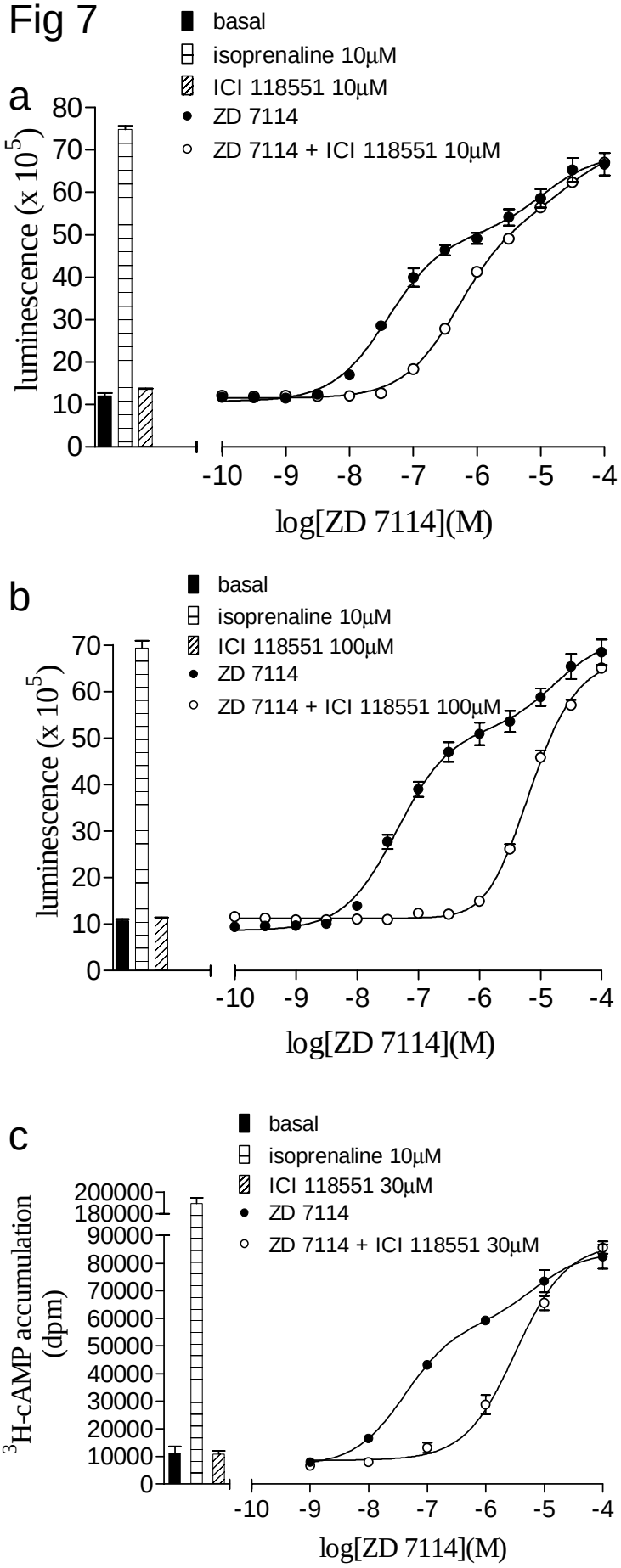

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Fig 8