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Evidence for a Secondary State of the Human β3-Adrenoceptor

Jillian G. Baker

Institute of Cell Signaling, Medical School, University of Nottingham, Queen's Medical Centre, Nottingham, United Kingdom

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ABSTRACT

There are three members of the β-adrenoceptor family, all of which are primarily coupled to G_s 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, whereas β2-adrenoceptors are able to induce signaling 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 Chinese hamster ovary cells. Several compounds described previously as β-antagonists have agonist properties at the β3-adrenoceptor. Antagonist affinity measurements varied at the β3-adrenoceptor in a manner similar 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, whereas other ligands [e.g., alprenolol and 1-(2-ethylphenoxy)-3-[[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2*S*)-2-propanol hydrochloride [SR 59230A]) stimulated responses that were more resistant to antagonism. Alprenolol inhibited fenoterol-induced β3-adrenoceptor responses while acting as an agonist at higher concentrations. This is highly suggestive of two active conformational states of the β3-adrenoceptor. (*S*)-4-[2-Hydroxy-3-phenoxypropylaminoethoxy]-*N*-(2-methoxyethyl)phenoxyacetamide (ZD 7114) stimulated a two-component response, of which the first component was more readily antagonized 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 seems similar to the human β1-adrenoceptor.

There are three members of the β-adrenoceptor family (β1-, β2-, and β3-adrenoceptors), which are all G-protein-coupled receptors primarily coupled to G_s 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 classic 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 classic 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 described previously as "β-blockers", differs between the two states: some ligands are antagonists at both conformations; others have agonist action at the secondary conformation while behaving as antagonists of the catecholamine state; and some seem to purely activate the catecholamine-state, while others clearly

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ABBREVIATIONS: CHO, Chinese hamster ovary; 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; CL 316243, 5-[(2*R*)-2-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid; CRE, cAMP response element; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's nutrient mix F-12; 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.

activate both conformations (Konkar et al., 2000; Baker et al., 2003a).

The β2-adrenoceptor has not been demonstrated to date 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 signaling cascades. Although 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-G_s-cAMP) while having agonist activity via a second separate pathway in the same cells (β2-p42/44 mitogen-activated protein kinase pathway) (Azzi et al., 2003; Baker et al., 2003b). In different tissues, the β2-adrenoceptor has also been shown to couple to G_i proteins under certain conditions (Brown and Harding 1992; Daaka et al., 1997; Hasseldine 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) seems 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 antiobesity drugs in humans (Arch, 2001). In addition, 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). In addition to agonists, several ligands generally considered 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 G_i 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 signaling 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 the pharmacology of a range of "β-blockers", examine antagonist affinity measurements, and look for evidence of differential signaling or the existence of different agonist conformations and thus determine whether the human β3-adrenoceptor is most similar to either the human β1 or β2-adrenoceptor or whether it has a completely different receptor phenotype altogether.

Materials and 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 GE Healthcare (Little Chalfont, Buckinghamshire, UK). The Luclite Plus Assay System was from PerkinElmer Life and Analytical Sciences (Groningen, the Netherlands). Betaxolol, bisoprolol, BRL 37344, cimaterol, CGP 12177, CL 316243, formoterol, ICI 118551, ICI 215001, ICI 89406, practolol, procaterol, pronethalol, salmeterol, SR 59230A, sotalol, and ZD 7114 were from Tocris Cookson (Avonmounth, Bristol, UK). Carvedilol was a gift from GlaxoSmithKline (Uxbridge, Middlesex, UK), and bupranolol was a gift from Professor Sian Harding (Imperial College, London, UK). Sigma Chemical (Poole, Dorset, UK) supplied all other reagents.

Cell Culture

CHO cells stably expressing both the human β3-adrenoceptor and a six cAMP response element-luciferase reporter gene (six CREs upstream of a luciferase response gene) were used throughout this study (a gift from Steve Rees, GlaxoSmithKline; CHO-β3 cells). The receptor coding sequence used for the original transfection was identical with that cited in the European Molecular Biology Laboratory entry number AY487247 and encodes for the full-length human β3-adrenoceptor protein (Swiss-Prot entry P13945). Cells were grown in Dulbecco's modified Eagle's medium/Ham's nutrient mix F-12 (DMEM/F12) containing 10% fetal calf serum and 2 mM 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 200 μl of DMEM/F12 containing 10% fetal calf serum and 2 mM L-glutamine in white-sided 96-well view plates. On the day of experimentation, this was removed and replaced with 200 μl of serum-free media (i.e., DMEM/F12 containing 2 mM L-glutamine only) containing the final required concentration of CGP 12177 and [^{3}H]CGP 12177 (11.97–27.65 nM), and the cells were incubated for 2 h at 37°C (in 5% CO_2). Total and nonspecific binding (defined by 100 μM ICI 118551) were measured in every experiment. The media and drugs were removed, and the cells were washed twice by the addition and removal of 2 × 200 μl of PBS/well. A white base was then added to the plate, followed by 100 μl of Microscint 20 per well. The plates were then counted on a Topcount (PerkinElmer Life and Analytical Sciences) for 2 min per well. The protein content was determined by the method described by Lowry et al. (1951).

CRE-Luciferase Production

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

neously to the wells, and the plates were incubated at 37°C for 5 h before luciferase production was detected.

[^{3}H]cAMP Accumulation

Cells were grown to confluence in 24-well plates in 1 ml of DMEM/F12 containing 10% fetal calf serum and 2 mM L-glutamine. The media were removed, and the cells were prelabeled with [^{3}H]adenine by incubation with 2 μCi/ml [^{3}H]adenine in serum-free media (0.5 ml/well) for 3 h at 37°C (5% CO_2). The [^{3}H]adenine was removed, and each well was washed by the addition and removal of 1 ml of serum-free media. One milliliter of serum-free media containing 1 mM 3-isobutyl-1-methylxanthine with or without the final required concentration of ICI 118551 was added to each well, and the cells were incubated for 30 min to 1 h at 37°C (5% CO_2). Agonist in 10 μl was added to each well, and the plates were incubated for 1 h at 37°C before the reaction was terminated by the addition of 50 μl of 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 described previously (Donaldson et al., 1988).

Data Analysis

Receptor Expression Level. Because the maximum concentration of [^{3}H]CGP 12177 obtainable is only 2-fold greater than 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 *percentage uninhibited binding* = $[(100 - NS)/([C]/IC_{50} + 1)] + NS$, where *NS* stands for nonspecific binding, [C] is the concentration of unlabeled 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 equation $K_D = IC_{50} - [[^3H]CGP\ 12177]$.

The B_{max} was then calculated from the K_D value for CGP 12177 in each experiment using the equation $B_{max} = \{(K_D + [C])/[C]\} \times$ specific binding and expressed as femtomoles per milligram of protein after protein was determined by the method described by Lowry et al. (1951).

One-Site Concentration-Response Curves. Sigmoidal agonist concentration-response curves were fitted through computer-assisted nonlinear regression using the program GraphPad Prism 2 (GraphPad Software Inc., San Diego, CA). The equation used was $Response = (E_{max} \times [A])/(EC_{50} + [A])$, where E_{max} 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 equation $DR = 1 + ([B]/K_D)$, 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 in which three to six different fixed concentrations of the same antagonist were used, Schild plots were constructed using the equation $\log(DR - 1) = \log[B] - \log(K_D)$. 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 (Figs. 7 and 8) were fitted to two sites with Prism 2 using the equation *Percentage maximal stimulation* = $\{[A] \times N/([A] + EC_{50}1)\} + \{[A] \times (100 - N)/([A] + EC_{50}2)\}$, where *N* is the percentage of site 1, [A] is the concentration of agonist, and $EC_{50}1$ and $EC_{50}2$ are the respective EC_{50} values for the two agonist sites.

A two-site analysis was also used for the experiments shown in Figs. 6 and 8 using the equation *response* = *basal* + $(Ag - Basal)\{1 - ([P]/([P] + IC_{50}))\} + PAg\{[P]/([P] + IC_{50})\}$, 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 10 μM (maximal) isoprenaline concentration was included in each plate for each separate experiment for CRE-luciferase and [^{3}H]cAMP accumulation (with the exception of Figs. 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 ± S.E.M. of triplicate determinations, and *n* in the text refers to the number of separate experiments.

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 was 677 ± 89 fmol/mg protein (n = 8).

Identification of Agonists, Partial Agonists, and Neutral Antagonists. 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 100 μM) were therefore

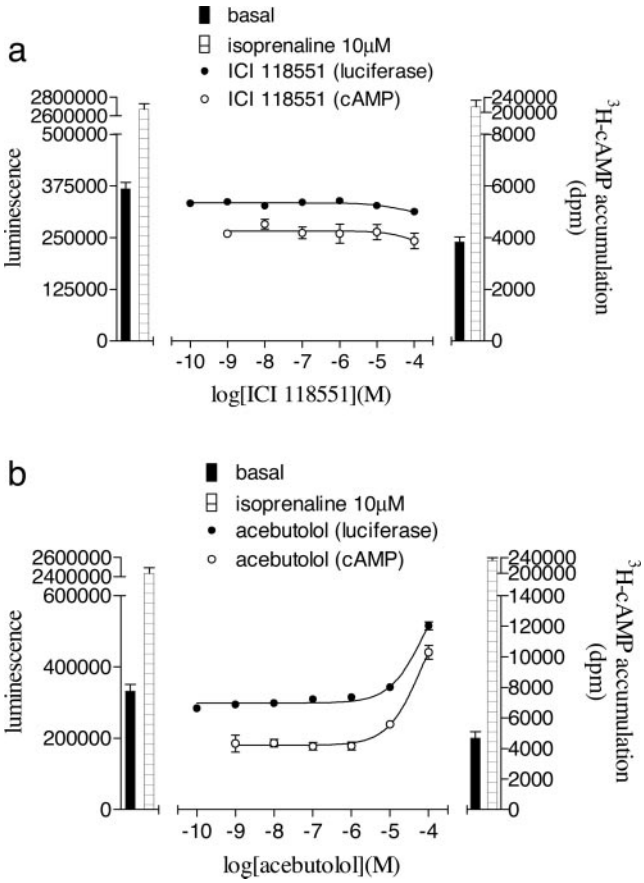

Fig. 1. CRE-luciferase production and [^{3}H]cAMP accumulation in response to ICI 118551 (a) and acebutolol (b) in CHO-β3 cells. Bars show basal responses and those in response to 10 μM isoprenaline. Data points are mean ± S.E.M. of triplicate values from a single experiment and are representative of three separate experiments in each case.

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; Fig. 1a and 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 100 μM ligand in each case (n = 3 for each ligand in each assay; Fig. 1b and Table 1). Several agonists were identified (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; Fig. 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) were more resistant to antagonism than the catecholamines (Table 2 and Figs. 2–4). 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.

To determine whether the agonist responses were competitively inhibited, fenoterol, BRL 37344, isoprenaline, and CGP 12177 responses were inhibited by six different concentrations (four 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 the following: 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 ± 0.004, n = 9: CGP 12177, 0.99 ± 0.02, 0.990 ± 0.003, n = 7, respectively; Fig. 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 and Fig. 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. To determine whether ligands can act as neutral antagonists of a putative β3-high-affinity state while 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, Fig. 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, Fig. 6a). A similar pattern was seen with SR 59230A (Fig. 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 two-component re-

TABLE 1

Log EC_{50} values and percentage of 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 given are mean ± S.E.M. of n determinations. Ligands with agonist activity at high concentrations (top of response not reached by 100 μM) include acebutolol, dopamine, ephedrine, ICI 89406, nadolol, and procaterol. Ligands with no intrinsic efficacy include atenolol, betaxolol, bisoprolol, bupranolol, CGP 20712A, ICI 118551, metoprolol, practolol, and sotalol.

Ligand	[^{3}H]cAMP Accumulation			CRE-Luciferase Production		
	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 ± 1.4	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	15
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

Fig. 2. CRE-luciferase production in CHO-β3 cells in response to fenoterol (a) BRL 37344 (b), isoprenaline (c), and CGP 12177 (d) in the absence and presence of 100 μM bisoprolol. Bars show basal luciferase activity, that in response to 10 μM isoprenaline, and that in response to 100 μM bisoprolol alone. Data points are mean ± S.E.M. from a single experiment in each case. These individual experiments are representative of four (a), four (b), five (c), and five (d) separate experiments.

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.

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***	8
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	−4.38 ± 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

***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ when comparing the log K_D value of the antagonist with that obtained in the presence of isoprenaline (one-way analysis of variance, Newman-Keuls post hoc).

sponse (log $EC_{50}1 = -7.30 \pm 0.03$, 68.5 ± 1.6% of the total response; log $EC_{50}2 = -4.78 \pm 0.06$; $n = 18$, Fig. 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 alongside 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 (Fig. 7b). A very similar response was seen at the level of [^{3}H]cAMP accumulation (log $EC_{50}1 = -7.49 \pm 0.09$, 61.6 ± 3.1% of the total response; log $EC_{50}2 = -5.41 \pm 0.25$; $n = 5$), in which ICI 118551 inhibited the first component to yield a log K_D value of −6.15 ± 0.09 (n = 4, Fig. 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) (Fig. 8, n = 3).

Lack of Responses in Cells without the Human β3-Adrenoceptor. As published previously (Baker et al., 2003a,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 had been detected previously using [^{3}H]CGP 12177 whole-cell binding in the parent CHO-K1 cells. In addition to those already published, full concentration-response curves (0.1 nM to 100 μM) to ZD 7114 and SR 59230A were examined in cells expressing a CRE reporter but not the β3-adrenoceptor, and no responses were seen at any ligand concentration (n = 3–4 for each ligand).

Discussion

Antagonists have been considered traditionally to bind to a receptor with a stable affinity that 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 (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 (Konkar et al., 2000; Lowe et al., 2002; Baker et al., 2003a,b,c).

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 to be β-antagonists had β3-adrenoceptor agonist activity (Table 1), again in agree-

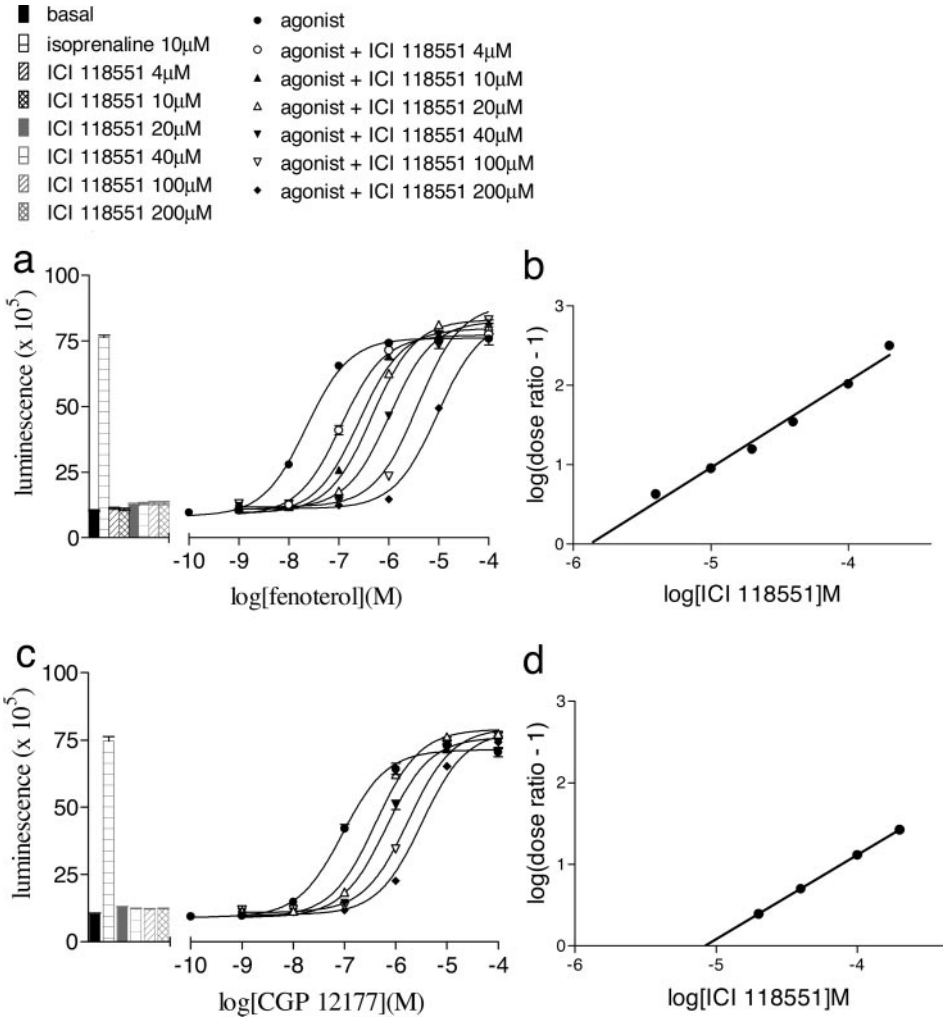

Fig. 3. CRE-luciferase production in CHO-β3 cells in response to fenoterol (a) and CGP 12177 (c) in the absence and presence 4, 10, 20, 40, 100, or 200 μM ICI 118551. Bars show basal luciferase activity, that in response to 10 μM isoprenaline alone, that in response to 4, 10, 20, 40, 100, and 200 μM 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 1.09 and 0.983 (a) and 0.95 and 0.985 (b), respectively. Data points are mean ± S.E.M. from a single experiment. Both a and b are representative of three separate experiments. In addition, another four separate experiments were also performed with each agonist and three different concentrations of ICI 118551. See text for summary of all data.

ment with previous studies (Blin et al., 1993; Gerhardt et al., 1999; Hutchinson et al., 2005). Also as seen in previous CRE-gene transcription studies (Baker et al., 2004), the efficacy of partial agonists seemed 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.

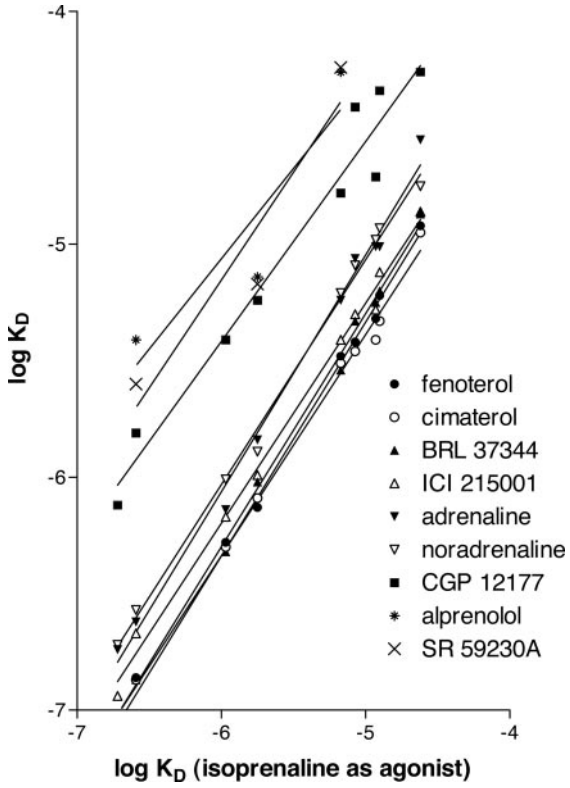

Fig. 4. Correlation between antagonist log K_D values with isoprenaline as agonist (*x*-axis) versus those obtained with agonists in Table 2 and alprenolol and SR 59230A from Table 3 (*y*-axis).

The endogenous catecholamines adrenaline and noradrenaline stimulated full-agonist responses similar to that of isoprenaline (Table 1). When these responses were antagonized 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. Because 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

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.

Agonist	cAMP Accumulation		CRE-Luciferase Production					
	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**	8	−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	−5.52 ± 0.09***	5
Carvedilol	−4.79 ± 0.19***	5	< −5	5	< −4.5	4	< −5	4

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, Newman-Keuls post hoc).

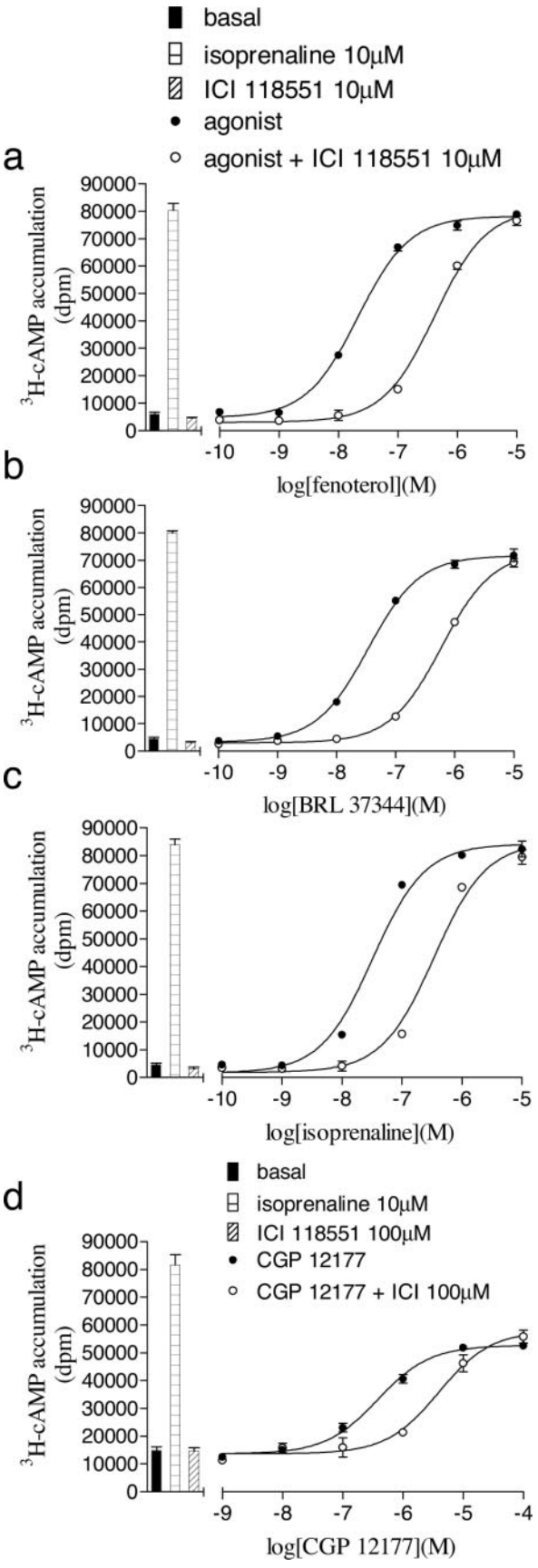

Fig. 5. [^{3}H]cAMP accumulation in CHO-β3 cells in response to fenoterol (a), BRL 37344 (b), isoprenaline (c), and CGP 12177 (d) in the absence and presence of 10 μM (a–c) and 100 μM ICI 118551 (d). Bars represent basal [^{3}H]cAMP accumulation and that in response to 10 μM isoprenaline, 10 μM ICI 118551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

change in the receptor that reduced antagonist affinity by 10-fold (Baker et al., 2003c). Because 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 (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 seem similar to β1-adrenoceptors.

Further examination of Table 3 suggests that β3-adrenoceptors indeed have pharmacological profiles similar to β1-adrenoceptors (Joseph et al., 2004; Baker, 2005a). First, all catecholamines induce full-agonist responses at both receptors that are inhibited to yield midrange antagonist affinity measurements at each receptor. Second, 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, whereas at the other end, there 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. Third, whereas 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 (Fig. 4; Baker, 2005a). The identical rank order for the high-affinity state ligands suggests that these ligands may be acting through a different conformation of the same receptor rather than a truly different third state, whereas 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. First, differences in antagonist affinity for the β1-adrenoceptor vary by 30- and 1000-fold, depending on the antagonist (Joseph et al., 2004; Baker, 2005a). In this β3-adrenoceptor study, although significant,

antagonist affinity measurements vary by only 4- to 50-fold (Tables 2 and 3). Second, 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 (Fig. 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.

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 and fenoterol were added simultaneously to CHO-β3 cells (Fig. 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 (Fig. 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 (Fig. 6). This suggests that (as with CGP 12177 and the β1-adrenoceptor) alprenolol is acting as a high-affinity state neutral antagonist while 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 two-component fit (Fig. 7). Because this response was seen at both [^{3}H]cAMP accumulation and CRE-gene transcription,

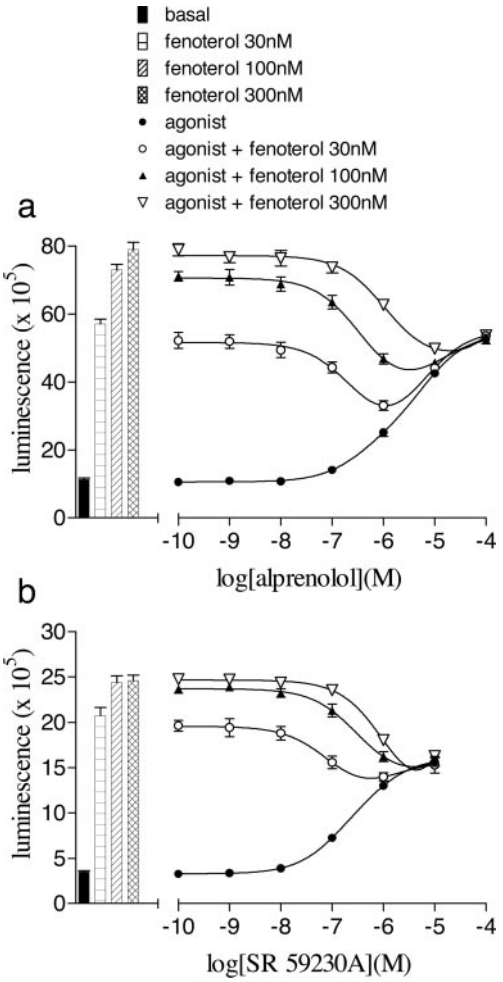

Fig. 6. CRE-luciferase production in CHO-β3 cells response to alprenolol (a) and SR 59230A (b) in the absence and presence of 30, 100, or 300 nM fenoterol. Bars represent basal luciferase production and that in response to 30, 100, or 300 nM fenoterol alone. Data points are triplicate determinations from a single experiment which are representative of four separate experiments in both a and b.

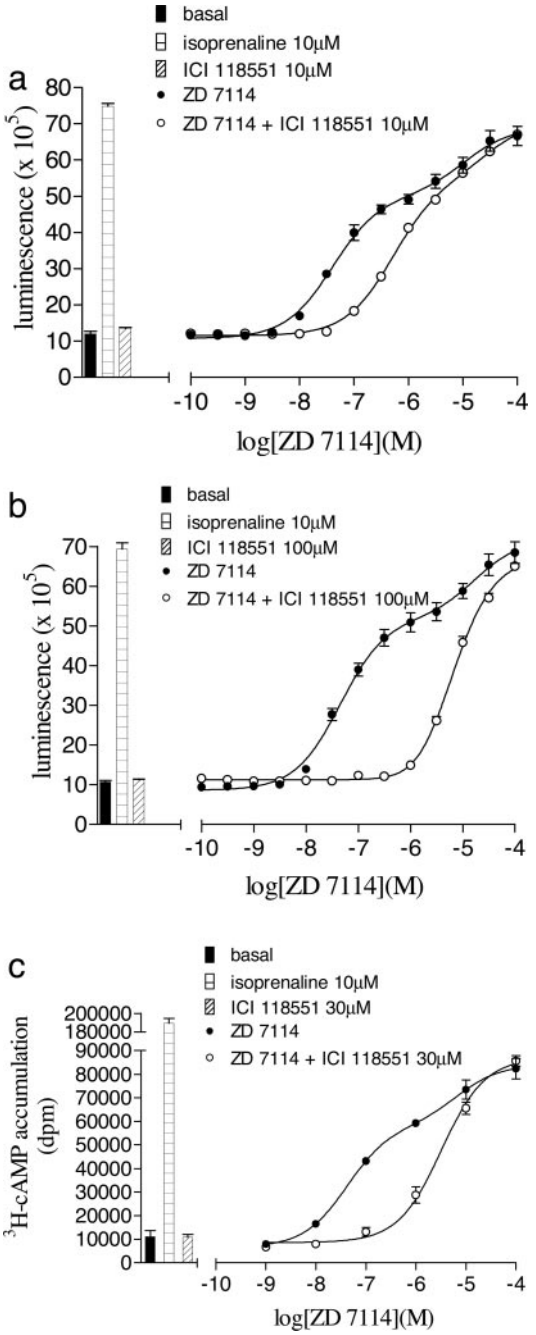

Fig. 7 CRE-luciferase production (a and b) and [^{3}H]cAMP accumulation (c) in CHO-β3 cells in response to ZD 7114 in the absence and presence of 10 μM ICI 118551 (a), 100 μM ICI 118551 (b), and 30 μM ICI 118551 (c). Bars show basal luciferase activity or [^{3}H]cAMP production, that in response to 10 μM isoprenaline, and that in response to 10 μM ICI 118551 (a), 100 μM ICI 118551 (b), or 30 μM ICI 118551 (c) alone. Data points are mean ± S.E.M. from a single experiment in each case. These individual experiments are representative of 18 (a), 3 (b), and 5 (c) separate experiments.

it suggests β3-G_s coupling only. The first component of the ZD 7114 response was more readily antagonized 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 toward 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 described previously as β-antagonists have agonist properties at the human β3-adrenoceptor (although with lower affinity than seen at human β1 and β2-adrenoceptors) (Hoffmann 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 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 in which both conformations coexist in living cells (rather than one predominantly in whole cells and another in membranes), and the ligands themselves seem to dictate the conformational state of the receptor.

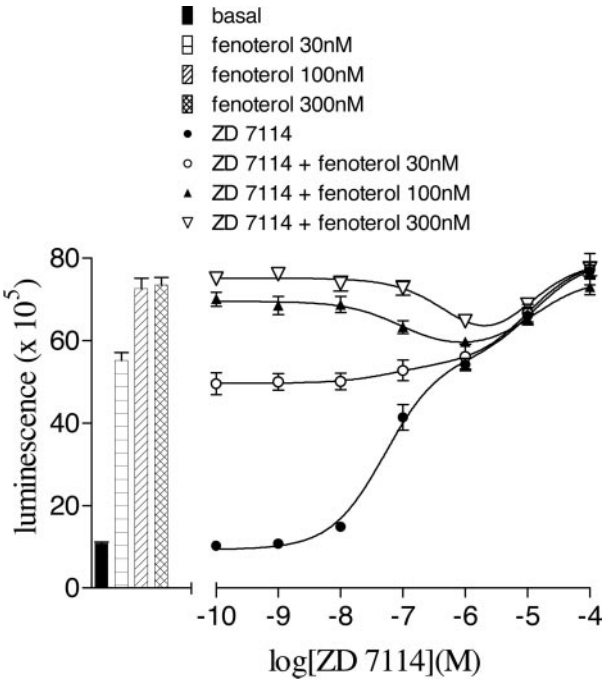

Fig. 8. CRE-luciferase production in CHO-β3 cells response to ZD 7114 in the absence and presence of 30, 100, or 300 nM fenoterol. Bars represent basal luciferase production and that in response to 30, 100, or 300 nM fenoterol alone. Data points are triplicate determinations from a single experiment which are representative of three separate experiments.

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Address correspondence to: Dr. Jillian G. Baker, Institute of Cell Signaling, Queen's Medical Centre, Nottingham, NG7 2UH, United Kingdom. E-mail: jillian.baker@nottingham.ac.uk