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Sites of action of β-ligands at the human β1-adrenoceptor.

Jillian G Baker

Institute of Cell Signalling

Medical School

University of Nottingham

Queen's Medical Centre

Nottingham

NG7 2UH

UK

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Correspondence to:

Dr Jillian G Baker

Institute of Cell Signalling,

Queen's Medical Centre,

Nottingham,

NG7 2UH,

UK

Tel: +44-115-9709799

Fax: +44-115-9194493

E-Mail: jillian.baker@nottingham.ac.uk

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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;

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;

PKA, protein kinase A;

SPAP, secreted placental alkaline phosphate;

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

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Abstract

Antagonist affinity measurements have traditionally been considered important in defining the receptor or receptor-subtypes present within cells or tissues. Any change in this value has normally been taken as evidence for the presence of a second receptor. However, highly efficacious ligands induce a time and phosphorylation-dependent change in the β2-adrenoceptor resulting in 10-fold lower affinity for antagonists. Also the β1-adrenoceptor is now considered to exist in two different active conformations which are distinguished by their pharmacological properties. In this study, the site of action of a range of β-agonists and β-antagonists was determined using the human β1-adrenoceptor stably expressed in CHO cells with CRE-reporter genes. Adrenaline and noradrenaline were confirmed as having agonist actions via the catecholamine site whilst all antagonists had higher affinity for the catecholamine rather than secondary site. However, the rank order of affinity for the two sites was different suggesting that they are indeed separate entities. The measurements of antagonist affinity at the catecholamine site however were found to depend upon the agonist present. For example, xamoterol, cimaterol, terbutaline and formoterol agonist responses were more readily antagonised by CGP 20712A than the catecholamine responses themselves. This however was not related to agonist efficacy as has previously been reported for the human β2-adrenoceptor. It may be therefore that some agonists (e.g. cimaterol) purely activate the catecholamine site, others purely activate the secondary site (e.g. CGP 12177) whilst the others (e.g. catecholamines) activate both sites to differing degrees.

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Introduction

Antagonist affinity measurements have traditionally been considered important in defining the receptor or receptor-subtypes present within cells or tissues. Antagonist affinity measurements (K_D values) were therefore considered to be constant for a given receptor-antagonist interaction regardless of which agonists were present or which downstream responses were measured (Kenakin et al., 1995). As a consequence, any change in this value has been taken as evidence for the presence of a second receptor population (Arunlakshana and Schild, 1959; Black et al., 1965, 1972). However, previous studies with the β2-adrenoceptor have suggested that this is not always the case (Baker et al., 2003a). Here, short-term cAMP accumulation studies gave similar values for antagonist affinity regardless of which competing agonist was present. However, in longer-term gene transcription assays, isoprenaline and adrenaline required 10-fold higher antagonist concentrations to inhibit the responses that when salbutamol or terbutaline were used. It transpired that this depended upon the efficacy of the competing agonist. Highly efficacious ligands (isoprenaline and adrenaline) induced a time-dependent, phosphorylation-dependent change in the β2-adrenoceptor resulting in 10-fold lower affinity for antagonists (Baker et al., 2003a).

This affinity assumption also does not hold for the β1-adrenoceptor as it is now considered to exist in two different active conformations (Granneman, 2001; Molenaar, 2003; Arch, 2004). Although the molecular nature of these conformations is unknown, they are distinguishable by their pharmacological properties. Agonist responses occurring via the classical "catecholamine" site of the β1-adrenoceptor are readily inhibited by classical β-antagonists whilst agonist responses occurring via the "secondary" (low-affinity) site are relatively resistant to antagonism (Granneman, 2001). This was first demonstrated with CGP 12177 (originally considered as a β-antagonist; Staehelin et al., 1983). CGP 12177 inhibits isoprenaline-induced β1-adrenoceptor responses as a high affinity neutral antagonist, however at higher concentrations (i.e. with lower affinity) it produces an agonist response which is relatively resistant to antagonism by classical β-antagonists (Pak and Fishman, 1996; Konkar et al., 2000a, b; Lowe et al., 2002; Baker et al., 2003b). Further studies from knockout animals and transfected cell systems receptors showed that both

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pharmacological profiles were dependent upon the presence of only the β1-adrenoceptor and not a further subtype of β-receptor, hence the current two-state receptor model (Pak and Fishman, 1996; Kaumann and Molenaar, 1997; Kaumann et al., 1998; Cohen et al., 2000; Granneman, 2001; Kaumann, 2001; Konkar et al., 2000a, b; Baker et al., 2003b). This is very different from the human β2-adrenoceptor, where CGP 12177 is a typical partial agonist (i.e. similar K_D values from binding (0.14nM), partial antagonism (0.09-0.17nM) and agonism (EC_{50} 0.22nM; Baker et al., 2002).

The secondary low-affinity site of the β1-adrenoceptor may be important in its own right as certain physiological events appear specifically linked to β1-low-affinity state, rather than catecholamine site, actions (e.g. relaxation in blood vessels, Kozlowska et al., 2003; Mallem et al., 2004). Furthermore, the plasma concentration of carvedilol (100ng/ml = 300nM) used in human cardiovascular diseases is sufficient to activate this secondary site (Sawangkoon et al., 2000; Baker et al, 2003b). However, whereas the β1-low-affinity state appears to signal via Gs-proteins in recombinant cell systems (Pak and Fishman, 1996, Konkar et al., 2000a; Baker et al., 2003b) this conformation may also couple to Gi-proteins in rat heart and blood vessels (Kompa and Summers, 1999; Mallem et al., 2004).

Since the discovery of the two β1-adrenoceptor sites, several studies have examined the site of action of different ligands. Most classical antagonists and adrenaline, noradrenaline, isoprenaline and dobutamine were found to preferentially act via the catecholamine site whilst LY 362884 agonist actions occurred via the secondary site (Konkar et al., 2000a; Lowe et al., 2002; Joseph et al., 2004). Several "β-antagonists" also have intrinsic agonist actions of their own (Lowe, et al., 2002; Baker et al., 2003b). Some of these agonist responses were potently antagonised by neutral β1-antagonists (suggesting catecholamine site agonism e.g. acebutolol), others were relatively resistant to antagonism (suggesting secondary site activation e.g. carvedilol) whilst other compounds had agonist activity at both sites (alprenolol, pindolol; Baker et al., 2003b). Thus now, when evaluating the overall pharmacology of any particular β-adrenoceptor ligand at the β1-adrenoceptor, both the site of action and efficacy at each site must be determined.

The aim of this study was therefore to determine the site of action of several β-agonists and β-antagonists at the human β1-adrenoceptor using stably transfected recombinant cell systems, then explore whether the antagonist affinity measurements for these antagonists varied at the two sites of the β1-receptor in an efficacy-dependent manner analogous to that seen at the human β2-adrenoceptor.

Methods

Materials

Cell culture reagents were from Sigma Chemicals (Poole, Dorset, UK) except fetal calf serum which was from PAA laboratories (Teddington, Middlesex, UK). ^{3}H-adenine and ^{14}C-cAMP were obtained from Amersham International (Buckinghamshire, UK). The Luclite Plus Assay System was from PerkinElmer (Groningen, Netherlands). CGP 12177, betaxolol, pronethalol, bisoprolol, ICI 118551, sotalol, salbutamol, timolol and xamoterol 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 supplied all other reagents.

Cell Culture

CHO cells stably expressing both the human β1-adrenoceptor (at 79fmol/mg protein) and a six CRE-luciferase reporter gene (six cyclic AMP response elements (CRE) upstream of a luciferase response gene) were used for the luciferase experiments (CHO-β1-luciferase cells; Baker et al., 2003b). CHO cells stably expressing the human β1-adrenoceptor (at 1147fmol/mg protein) and a six CRE-SPAP reporter gene (six CRE upstream of a secreted placental alkaline phosphate (SPAP) reporter gene) were used for the SPAP and cAMP experiments (CHO β1-SPAP cells; Baker et al., 2003b). Both cells lines 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.

CRE-luciferase production

CHO-β1-luciferase 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. This was removed and replaced with 200μl serum-free media (i.e. DMEM/F12 containing 2mM L-glutamine only) 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). 20μl of agonist (diluted in serum free media) was then added to each well and the plate incubated at 37°C (5% CO_2) for 5 hours. The media and drugs were removed, a white base added to the plate and luciferase activity detected as described previously

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(Baker et al., 2003b). For the experiments seen in Figure 3, serum free media or a fixed concentration of agonist in serum free media was added to the wells and immediately followed by the addition of 20µl CGP 12177 and the plates incubated at 37°C for 5 hours.

CRE-SPAP production

CHO-β1-SPAP 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 from each well, replaced with 1ml serum free media and incubated for 24 hours (37°C, 5% CO_2). On the morning of experimentation, the serum free media was removed from each well and replaced with either a further 1ml of serum free media or 1ml serum free media containing CGP 20712A at the final required concentration and the cells incubated for 1 hour at 37°C (5% CO_2). Agonist in 10µl was then added to each well and the plates incubated for 5 hours (37°C, 5% CO_2). SPAP secretion was then determined as described previously (McDonnell et al., 1998).

^{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 for 2 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 CGP 20712A, was added to each well and the cells incubated for 1 hour 37°C (5% CO_2). Agonist in 10µl was added to each well and the plates incubated for 10 minutes 37°C before the reaction was terminated by the addition of 50µl concentrated HCl per well.
The plates were then frozen 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

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

Equation 1: $\text{Response} = \frac{\text{Emax} \times [A]}{EC_{50} + [A]}$

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:

Equation 2: $DR = 1 + \frac{[B]}{K_D}$

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 different fixed concentrations of antagonist were used, Schild plots were constructed using the following equation:

Equation 3: $\text{Log}(DR-1) = \log[B] - \log(K_D)$

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

A two-site analysis was used for the experiments shown in Figure 3 using the following equation:

Equation 4:

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

where basal is the response in the absence of agonist or antagonist, Ag is the response to a fixed concentration of agonist, [C] is the concentration of CGP 12177, IC_{50} is the concentration of CGP 12177 that inhibits 50% of the response of the fixed agonist,

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CGP_{stim} is the maximum stimulation by CGP 12177 and EC_{50} is the concentration of CGP 12177 that stimulated a half maximal CGP 12177 response.

A 10μM (maximal) isoprenaline concentration was included in each plate for each separate experiment for CRE-luciferase, CRE-SPAP and ^{3}H-cAMP accumulation (with the exception of Figure 3), 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.

Results

CRE-luciferase production

Isoprenaline stimulated an increase in luciferase activity in CHO-β1-luciferase cells that was 6.2 ± 0.3 fold over basal (log EC_{50} -8.02 ± 0.06, n=19). Adrenaline (log EC_{50} -6.85 ± 0.05, 102.8 ± 1.1% isoprenaline maximum, n=21), noradrenaline (log EC_{50} -7.30 ± 0.05, 103.2 ± 1.2% isoprenaline maximum, n=20) and cimaterol (log EC_{50} -7.65 ± 0.02, 93.0 ± 1.4% isoprenaline maximum, n=20) stimulated similar responses. CGP 12177 (log EC_{50} -7.43 ± 0.02, n=17) stimulated a response 37.8 ± 1.6% that of isoprenaline and thus was a partial agonist in this cell system.

The β1-selective antagonist CGP 20712A inhibited the isoprenaline, adrenaline and noradrenaline responses to yield very similar log K_D values of -9.19 ± 0.12, n=9, -9.12 ± 0.09, n=14 and -9.15 ± 0.09, n=12 respectively. These values are markedly different from that obtained from antagonism of the CGP 12177 response (log K_D -7.09 ± 0.09, n=7, $p < 0.0001$ ANOVA Neuman-Keul's post hoc). Similar results were obtained with the other β-antagonists studied (see Table 1, Figure 1 and Figure 2) suggesting that adrenaline and noradrenaline were stimulating responses via the same site as isoprenaline i.e. the catecholamine site of the β1-adrenoceptor. When cimaterol was the agonist, all of the antagonist K_D values obtained were significantly different from those obtained with isoprenaline as agonist (see Table 1). However, all of the receptor-ligand interactions appeared to be competitive as the Schild slopes achieved were not significantly different from unity (e.g. Figure 2). This is similar to previous studies (Konkar et al., 2000a, b; Lowe et al., 2002)

CGP 12177 was then added to fixed concentrations of the agonists (adrenaline, noradrenaline and cimaterol). At low concentrations, CGP 12177 inhibited the agonist stimulation. However, at higher concentrations of CGP 12177, its stimulatory effects were clearly seen (Figure 3). In each case, the concentration of CGP 12177 required to inhibit the agonist was about two orders of magnitude less than that required to cause the stimulation.

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CRE-SPAP production

Isoprenaline stimulated an increase in CRE-SPAP production (log EC_{50} -8.46 ± 0.12, 2.4 ± 0.1 fold over basal, n=13) that was inhibited by CGP 20712A to yield a log K_D value of -9.24 ± 0.08 (n=13). Adrenaline and noradrenaline responses were similarly inhibited by CGP 20712A (see Table 2). A range of other β-agonists were then examined and the responses to each antagonised by CGP 20712A. The results obtained demonstrate a range of K_D values for CGP 20712A with xamoterol and cimaterol being the most potently antagonised and SR 59230A and CGP 12177 being the least (see Figure 4 and Table 2).

^{3}H-cAMP accumulation.

Isoprenaline stimulated an increase in ^{3}H-cAMP accumulation (10 minutes agonist incubation) that was 10.1 ± 1.9 fold over basal (log EC_{50} -8.27 ± 0.16, n=4). This response was antagonised by increasing concentrations of CGP 20712A of yield a log K_D value for CGP 20712A of -9.25 ± 0.07, n=12. This was a competitive inhibition as the Schild slope obtained was 0.95 ± 0.05, n=4 (Figure 5). Cimaterol stimulated a response (log EC_{50} -8.19 ± 0.07, 97.9 ± 1.7% isoprenaline maximum, n=4) that was antagonised by CGP 20712A to yield a log K_D value of -9.48 ± 0.06 (n=10; Schild slope 0.98 ± 0.07, n=3, Figure 5). Thus although this too appeared competitive, the log K_D value for CGP 20712A was significantly different (p = 0.02, unpaired t-test) from that obtained when isoprenaline was the agonist. The response to noradrenaline was antagonised by CGP 20712A to yield a log K_D value similar to that obtained when isoprenaline was the agonist. However, the increasing concentrations of CGP 20712A caused a reduction in the maximum response to noradrenaline unmasking the lower efficacy of this ligand compared with isoprenaline and cimaterol (Figure 5, Table 3). A log K_D value was not obtained for CGP 20712A when tulobuterol was the agonist as even in the presence of the lowest concentration of CGP 20712A the maximum tulobuterol response was significantly reduced showing a low efficacy for this ligand at the human β1-adrenoceptor (Figure 5, Table 3).

Discussion

It is now accepted that the β1-adrenoceptor has two separate sites or conformations to which ligands can bind (Pak and Fishman, 1996; Konkar et al., 2000a, b; Granneman, 2001; Joseph et al., 2004). Agonists stimulating responses through the catecholamine site are readily inhibited by antagonists in a manner similar to that of isoprenaline. Agonist responses occurring via the secondary site are relatively resistant to inhibition with antagonists in a similar manner to those of CGP 12177. Thus, in a system with only the β1-adrenoceptor present, the site of action of a new agonist can be determined by comparing inhibition of its response with inhibition of isoprenaline and CGP 12177 responses (Konkar et al., 2000a,b; Lowe et al., 2002; Baker et al., 2003b; Joseph et al., 2004). In this study, the agonist actions of adrenaline and noradrenaline were inhibited in a very similar manner to that of isoprenaline and are therefore acting through the catecholamine site of the human β1-adrenoceptor. This is in agreement with previous findings in the ferret (Lowe et al., 2002).

All of the 12 antagonists used in this study have higher affinity (K_D value) for the catecholamine site than the secondary site (Table 1). This is also true for CGP 12177 itself and carvedilol (which are neutral antagonists of the catecholamine site and agonists of the secondary site) and alprenolol and pindolol (which are agonists of both sites; Pak and Fishman, 1996; Konkar et al., 2000a; Lowe et al., 2002; Baker et al., 2003b). Thus regardless of the efficacy of the ligand, no ligand has yet been described that is able to bind to the secondary site with greater affinity. However, the rank order of antagonist affinities at the catecholamine site (which is identical with isoprenaline, adrenaline and noradrenaline), is markedly different from the rank order of affinities at the secondary site (Table 1 and Figure 6 where the poor correlation is clearly demonstrated). ICI 118551, bupranolol and nadolol all have higher than expected affinity, and atenolol a lower than expected affinity for the secondary site than if it was just a low-affinity mirror of the catecholamine site. This is similar to findings recently reported by Joseph et al., (2004) also at the human β1-adrenoceptor but there appear to be some differences between species (c.f. Lowe et al., 2002 in the ferret heart, this report and Joseph et al., (2004) using the human β1-adrenoceptor). Thus, as well as the differences in ligand efficacy at the two sites, the different ligand

affinities also suggest that the secondary site is indeed a separate pharmacological entity.

Cimaterol stimulated responses that were inhibited by all antagonists to yield much higher affinity values than those achieved with CGP 12177, suggesting that cimaterol is not acting via the secondary site. However, all of the K_D values obtained showed a consistently higher affinity than when the catecholamines were present. This raises the possibility that cimaterol is acting through a third, higher-affinity conformation of the β1-adrenoceptor and that many ligand-dependent states of the β1-adrenoceptor may exist (Molenaar, 2003; Arch 2004; Figure 6). The rank order of antagonist affinity (Table 1; Figure 6) however is the same for cimaterol as for the catecholamines, suggesting that cimaterol is likely to stimulate the β1-adrenoceptor via the catecholamine site. Cimaterol also behaved exactly the same as the catecholamines in the presence of CGP 12177 (Figure 3). Here, CGP 12177 inhibited all of the responses (as it is a high affinity neutral antagonist of the catecholamine site) at concentrations substantially below that required for CGP 12177 stimulation via the secondary site (Pak and Fishman, 1996; Konkar et al., 2000a: Baker et al., 2003b). Thus again, cimaterol appears to be acting via the catecholamine site.

Interestingly, in all 3 cases presented in Figure 3 and that previously reported in Baker et al., (2003b), the maximum stimulation achieved by CGP 12177 was always higher in the presence of the agonist than in its absence, even though the higher CGP 12177 concentrations should be completely inhibiting the agonist-catecholamine site stimulation. This however was not seen when examining the pharmacology of the rat β1-adrenoceptor expressed in CHO cells in membrane cAMP assays (Konkar et al., 2000a). The reason for this is unknown, but may yet be explained when better understanding of the molecular structure of the secondary site and its interaction with signalling complexes is known.

In order to explore the discrepancy in antagonist affinity in the presence of cimaterol (rather than isoprenaline, adrenaline or noradrenaline) further, experiments were undertaken with CHO-β1-SPAP cells. This not only validated this finding in a completely separate cell line, at a different receptor expression level and with a different reporter gene, but the higher receptor expression allowed easier examination

of other agonist responses. By comparing the affinity of CGP 20712A as an antagonist of the agonist responses with the affinity of it as an antagonist of the isoprenaline responses (to denote the catecholamine site) and CGP 12177 responses (to denote the secondary site), it can be seen that most of the β-agonists were stimulating responses through the catecholamine site. There was however a full range of antagonist affinity values obtained (see Table 2). This is at odds with the accepted dogma that antagonist affinity is an innate property of that receptor-ligand interaction and is immune to influences from the competing agonist (Kenakin et al., 1992).

A change in antagonist affinity (K_D) measurement within the same assay, that depended purely upon which competing agonist was reported at the human β2-adrenoceptor (Baker et al., 2003a). At the β2-adrenoceptor highly efficacious ligands induced a time-dependent, phosphorylation-dependent change in the β2-adrenoceptor that resulted in a 10-fold lower affinity for antagonists (Baker et al., 2003a). In order to determine whether the apparent changes in antagonist affinity at the catecholamine site of the β1-adrenoceptor in this study (Table 2) were also dependent upon the efficacy of the competing ligand, short-term cAMP accumulation assays were performed on 4 agonists; cimaterol from the top of Table 2, tulobuterol from the middle, and isoprenaline and noradrenaline

Firstly, study of the EC_{50} values of the agonists suggests that receptor desensitisation was not the cause. In the β2-adrenoceptor study (Baker et al., 2003a), the concentration response curves of less efficacious agonists became left shifted over time (i.e. EC_{50} in long term assays was to the left of the EC_{50} obtained in same cells in short term assays). This is likely to be due to the magnification in the signalling cascade from cAMP to CRE-gene transcription that occurs at the level of PKA (Yuan et al., 1994; January et al., 1998). At the β2-adrenoceptor, concentration response curves of the more efficacious agonists however became right shifted over time suggesting receptor desensitisation. In this β1-adrenoceptor study, the concentration responses of all 4 agonists became left-shifted over time. Secondly, the log K_D values for CGP 20712A at the β1-adrenoceptor were different even in the short-term cAMP assay suggesting that the time of agonist incubation was therefore not a factor (Table 3). Thirdly, there was no correlation between agonist efficacy and antagonist affinity. Isoprenaline and cimaterol appeared the most efficacious of the ligands (parallel shifts

were seen with increasing concentrations of CGP 20712A) despite being at opposite extremes of Table 2. Noradrenaline appeared less efficacious than isoprenaline and tulobuterol (from the middle of the table) a partial agonist whose low efficacy and lack of receptor reserve was made clearly evident by the collapsing concentration response curves with increasing CGP 20712A concentrations (similar to that seen in Hopkinson et al., 2000 and Baker et al., 2003a). The agonist efficacy (isop = cimat > noradren > tulo) and time of incubation therefore was not responsible for the change in antagonist affinity ranking (cimat > tulo > isop = noradren).

In conclusion, there is increasing evidence that the secondary site of the human β1-adrenoceptor is indeed a separate entity from the catecholamine site. There is a different rank order in which antagonists bind to the two sites (Table 1), there are different efficacies of ligands at the two sites (Baker et al., 2003b) and CGP 12177 in the presence of a catecholamine-site agonist seems to give a greater response than alone. However, no ligand, regardless of efficacy has yet been described to bind to the secondary site with higher affinity than the catecholamine site. Furthermore, the measurement of antagonist affinity at the catecholamine site depends upon the agonist present (Table 2). The reason for this is not known but clearly has a different explanation from that seen at the human β2-adrenoceptor (c.f. Table 3 and Baker et al., 2003a). However, it appears that all of these agonist responses are occurring primarily via the catecholamine site rather than a separate third conformation of the human β1-adrenoceptor. One explanation could be that some agonists (e.g. cimaterol) purely activate the catecholamine site, others purely activate the secondary site (e.g. CGP 12177) whilst the others activate both sites to differing degrees. Clearly more investigation into the nature of the two sites of the β1-adrenoceptor is needed.

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Footnotes

JGB is a Wellcome Trust Clinical Scientist Fellow

Legends for Figures

Figure 1

CRE-luciferase activity in CHO-β1-luciferase cells response to a) isoprenaline (isop), b) adrenaline (adren), c) noradrenaline (norad), d) cimaterol (cimat) and e) CGP 12177 in the absence and presence of 100nM (a-d) or 10μM (e) betaxolol (betax). Bars show basal luciferase activity, that in response to 10μM isoprenaline, and that in response to 100nM (a-d) or 10μM (e) betaxolol 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, d) 6 and e) 5 separate experiments.

Figure 2

CRE-luciferase activity in CHO-β1-luciferase cells in response to a) isoprenaline (isop), b) adrenaline (adren), c) noradrenaline (norad), d) cimaterol (cimat) and e) CGP 12177 in the absence and presence of various concentrations of carvedilol (carv). Bars show basal luciferase activity, that in response to 10μM isoprenaline alone. The response to each carvedilol concentration alone was assessed in each experiment (0.3nM to 10μM) and was never significantly different from basal and for simplicity is not shown. The Schild slopes are a) 0.91 ± 0.08 (n=4), b) 1.01 ± 0.03 (n=4), c) 0.97 ± 0.06 (n=3), d) 1.03 ± 0.08 (n=3) and e) 1.01 ± 0.08 (n=4). Data points are mean ± s.e.m. from a single experiment in each case. These individual experiments are representative of a) 4, b) 4, c) 3, d) 3 and e) 4 separate experiments.

Figure 3

CRE-luciferase production in CHO-β1-luciferase cells response to CGP 12177 in the absence and presence of fixed concentrations of a) 1μM adrenaline (adren), b) 300nM and 1μM noradrenaline (norad) and c) 10nM, 30nM and 100nM cimaterol (cimat). Bars represent basal CRE-luciferase production and that in response to each of the agonists as detailed in the individual legends. Data points are triplicate determinations from a single experiment which are representative of a) 3, b) 3 and c) 4 separate experiments.

Figure 4

CRE-SPAP production in CHO-β1-SPAP cells response to a) xamoterol, b) formoterol, c) salbutamol, d) tulobuterol, e) BRL 37344, f) fenoterol and g) dobutamine in the presence and absence of 10nM CGP 20712A. Bars represent basal CRE-SPAP production and that in response to 10μM isoprenaline and 10nM CGP 20712A. Data points are mean ± s.e.m. of triplicate determinations from single experiments, each representative of a) 11, b) 12, c) 8, d) 11, e) 11, f) 12 and g) 12 separate experiments.

Figure 5

^{3}H-cAMP accumulation in CHO-β1-SPAP cells response to a) cimaterol, b) tulobuterol, c) isoprenaline and d) noradrenaline in the absence and presence of 3nM, 30nM or 300nM CGP 20712A. Bars represent basal ^{3}H-cAMP accumulation and that in response to 10μM isoprenaline, 3nM CGP 20712A, 30nM CGP 20712A or 300nM CGP 20712A alone. The Schild slope are a) 0.98 ± 0.07, n=3 and c) 0.95 ± 0.05, n=4. Calculation of Schild slopes is not possible for b) and d) due to non-competitive antagonism. Data points are mean ± s.e.m. of triplicate determinations. Each of these single experiments is representative of a) 3, b) 4, c) 4 and d) 4 separate experiments.

Figure 6

Correlation between antagonist log K_D values with isoprenaline as agonist (x-axis) versus those obtained with adrenaline, noradrenaline, cimaterol or CGP 12177 as agonist (y-axis). The log K_D values are those described in Table 1. The correlation coefficients and slopes are as follows: 0.98 and 1.06 for isoprenaline vs adrenaline; 0.99 and 1.04 for isoprenaline vs noradrenaline; 0.99 and 1.04 for isoprenaline vs cimaterol: and 0.76 and 0.98 for isoprenaline vs CGP 12177 respectively.

antagonist	agonist														
	cimaterol	n		isoprenaline	n		adrenaline	n		noradrenaline	n		CGP 12177	n
carvedilol	-9.92 ± 0.05 **	14		-9.41 ± 0.05	18		-9.61 ± 0.04 #	16		-9.48 ± 0.04	13		-7.29 ± 0.05 **	18
CGP 20712A	-9.61 ± 0.06 *	13		-9.19 ± 0.12	9		-9.12 ± 0.09	14		-9.15 ± 0.09	12		-7.09 ± 0.09 **	7
timolol	-9.03 ± 0.08 *	9		-8.62 ± 0.14	9		-8.54 ± 0.05	9		-8.59 ± 0.08	9		-6.28 ± 0.04 **	6
bupranolol	-9.22 ± 0.08 **	7		-8.54 ± 0.13	8		-8.64 ± 0.08	8		-8.72 ± 0.12	8		-7.29 ± 0.06 **	6
betaxolol	-8.87 ± 0.06 **	4		-8.30 ± 0.10	7		-8.42 ± 0.08	5		-8.29 ± 0.09	6		-5.73 ± 0.03 **	5
propranolol	-8.65 ± 0.03 **	14		-8.15 ± 0.07	17		-8.42 ± 0.04 *	13		-8.33 ± 0.04 #	13		-6.44 ± 0.06 **	23
bisoprolol	-8.51 ± 0.02 *	5		-8.04 ± 0.10	7		-8.22 ± 0.07	6		-8.03 ± 0.11	7		-5.53 ± 0.07 **	7
metoprolol	-7.98 ± 0.03 **	4		-7.54 ± 0.07	7		-7.40 ± 0.08	6		-7.42 ± 0.09	6		-5.26 ± 0.05 **	7
nadolol	-7.90 ± 0.05 *	6		-7.50 ± 0.14	8		-7.34 ± 0.08	8		-7.44 ± 0.11	8		-5.93 ± 0.06 **	7
atenolol	-7.06 ± 0.08 **	5		-6.82 ± 0.15	7		-6.89 ± 0.07	6		-6.80 ± 0.10	6		-3.82 ± 0.04 **	6
ICI 118551	-7.17 ± 0.04 **	9		-6.77 ± 0.12	9		-6.60 ± 0.05	13		-6.62 ± 0.06	11		-5.80 ± 0.08 **	6
sotalol	-6.26 ± 0.05 *	4		-5.76 ± 0.07	5		-5.71 ± 0.11	4		-5.75 ± 0.13	4		-3.66 ± 0.09 **	5

Table 1

Log K_D values of 12 antagonists in the presence of 5 different agonists from CRE-luciferase activity experiments using CHO-β1-luciferase cells. Values are mean ± s.e.m. from n separate determinations.

** = $p < 0.001$, * = $p < 0.01$, # = $p < 0.05$ when comparing the K_D value of the antagonist with that obtained with isoprenaline as agonist (one-way analysis of variance, Neuman-Keul's post hoc)

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agonist	Log EC_{50}	% isoprenaline	Log K_D CGP 20712A		n
xamoterol	-8.53 ± 0.07	83.3 ± 5.0	-9.91 ± 0.09	****	11
cimaterol	-8.79 ± 0.9	98.8 ± 3.9	-9.82 ± 0.09	****	12
terbutaline	-6.24 ± 0.10	82.3 ± 5.0	-9.76 ± 0.05	****	9
formoterol	-8.80 ± 0.08	95.2 ± 2.3	-9.72 ± 0.08	***	12
clenbuterol	-7.83 ± 0.10	83.5 ± 5.7	-9.70 ± 0.08	***	11
salbutamol	-6.50 ± 0.04	86.0 ± 4.0	-9.67 ± 0.08	**	8
pronethalol	-7.13 ± 0.10	56.6 ± 3.0	-9.60 ± 0.11	*	9
tulobuterol	-6.70 ± 0.11	86.5 ± 3.7	-9.59 ± 0.06	**	11
BRL 37344	-6.82 ± 0.09	83.9 ± 5.1	-9.52 ± 0.08	*	11
procaterol	-6.14 ± 0.05	76.8 ± 4.3	-9.52 ± 0.12		10
fenoterol	-7.85 ± 0.10	89.1 ± 2.5	-9.49 ± 0.08	*	12
ZD 7114	-8.42 ± 0.07	73.0 ± 5.4	-9.39 ± 0.14		14
isoprenaline	-8.46 ± 0.12	100	-9.24 ± 0.08		13
adrenaline	-7.52 ± 0.07	99.5 ± 4.2	-9.19 ± 0.08		10
noradrenaline	-7.79 ± 0.11	98.5 ± 4.7	-9.09 ± 0.09		12
dobutamine	-7.18 ± 0.12	98.7 ± 2.2	-9.09 ± 0.08		12
SR 59230A	-8.17 ± 0.11	66.3 ± 4.8	-8.16 ± 0.12	****	12
CGP 12177	-8.63 ± 0.11	79.1 ± 3.5	-7.66 ± 0.07	****	11

Table 2

Log EC_{50} values and % isoprenaline maximum response of CRE-SPAP production from CHO-β1-SPAP cells to a range of β-agonists. Log K_D values obtained from CGP 20712A antagonism of these agonists is also shown. Values are mean ± s.e.m. of n determinations.

**** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ when comparing the K_D value of CGP 20712A with that obtained with isoprenaline as agonist (un-paired t-test)

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agonist	Log EC_{50}	% isoprenaline	Log K_D CGP 20712A	n
cimaterol	-8.19 ± 0.07	97.9 ± 1.7	-9.48 ± 0.06 *	10
isoprenaline	-8.27 ± 0.16	100	-9.25 ± 0.07	12
noradrenaline	-7.59 ± 0.18	101.9 ± 1.0	-9.19 ± 0.14	7
tulobuterol	-6.58 ± 0.06	56.4 ± 4.8		4

Table 3

Log EC_{50} values, % isoprenaline maximum response and log K_D values for CGP 20712A at the level of ^{3}H-cAMP accumulation from CHO-β1-SPAP cells. Values are mean ± s.e.m. of n determinations.

* $p = 0.02$ when comparing the K_D value of CGP 20712A from antagonism of cimaterol with that obtained with isoprenaline as agonist (un-paired t-test)

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Fig 1

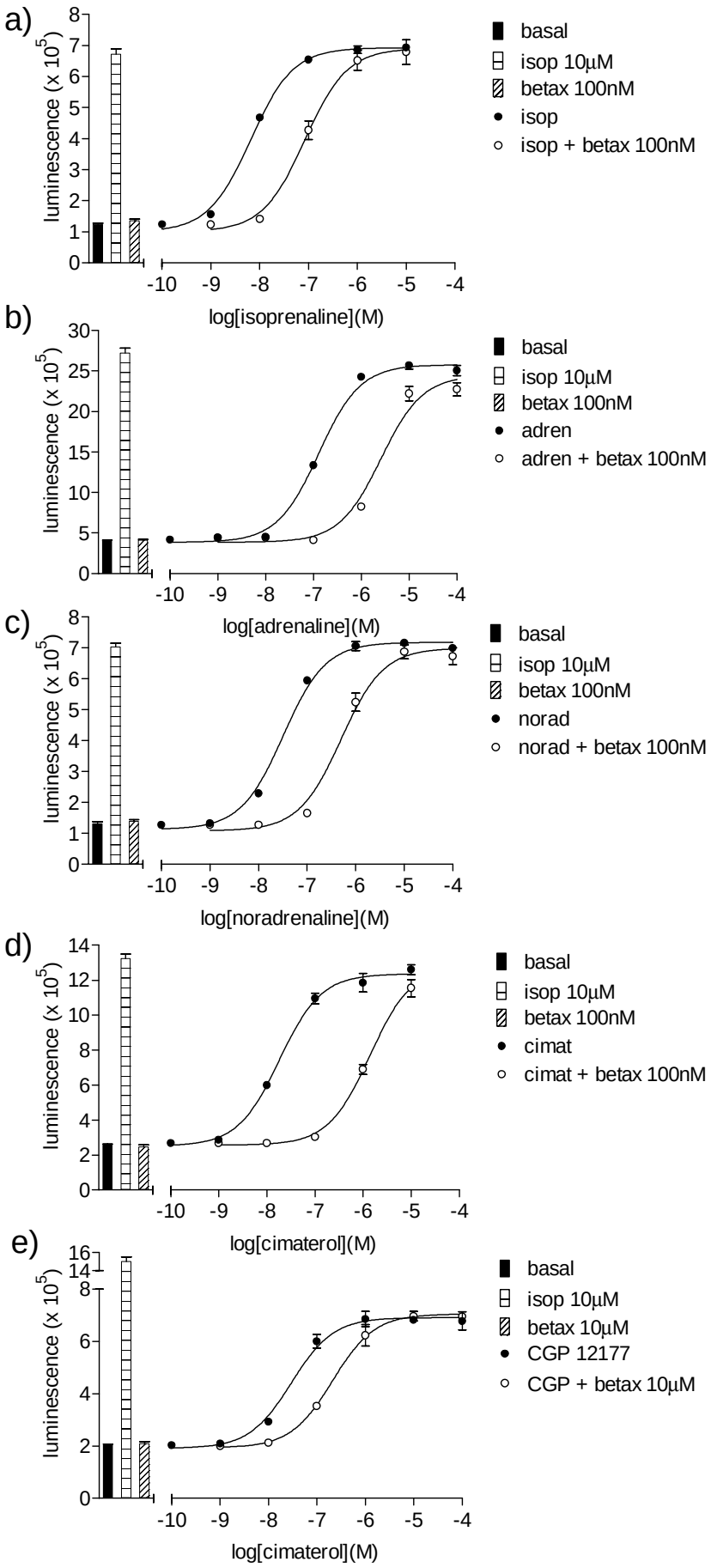

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Fig 2

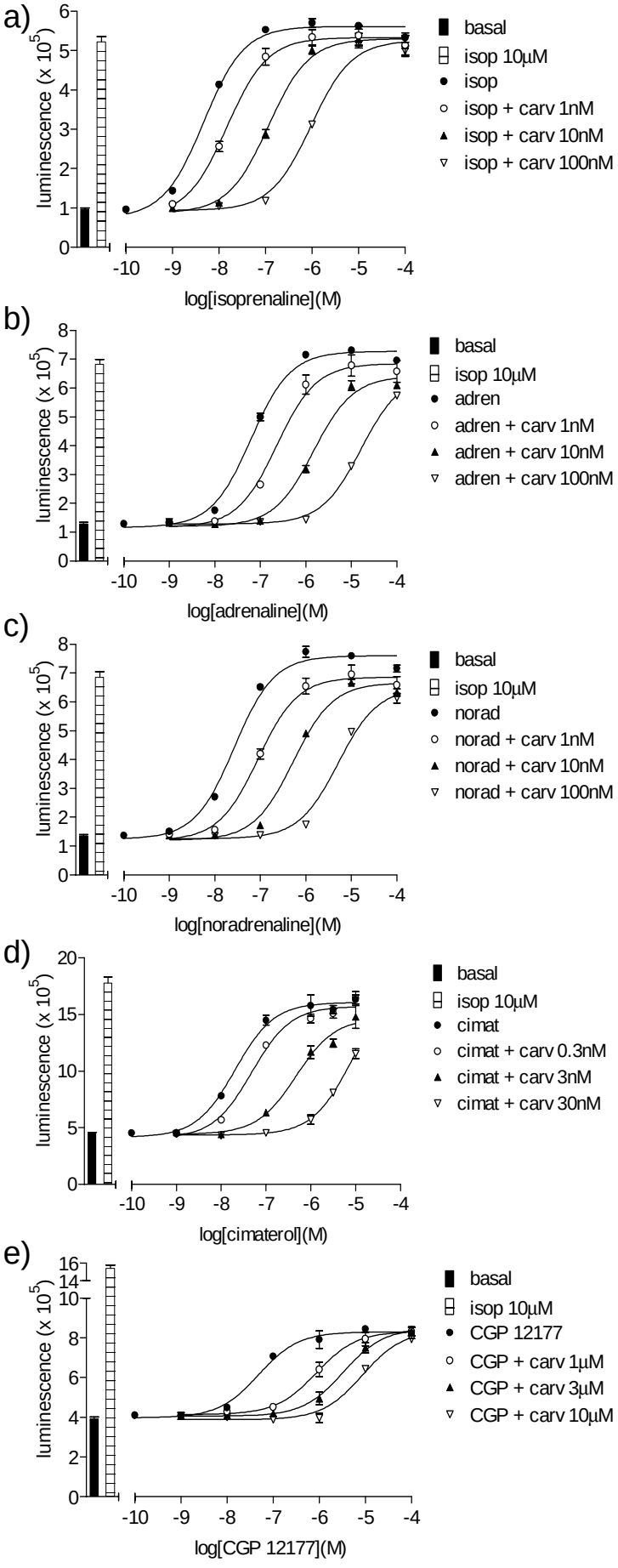

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Fig 3

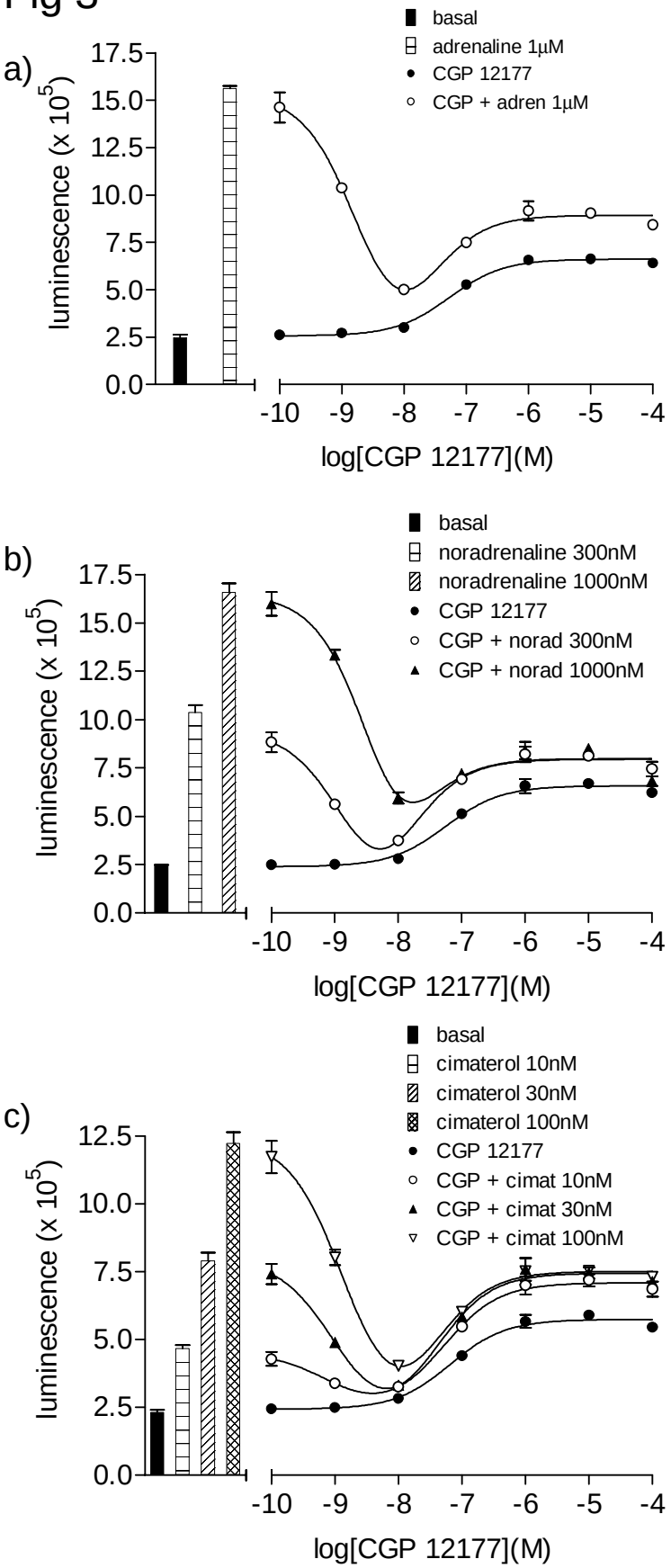

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Fig 4

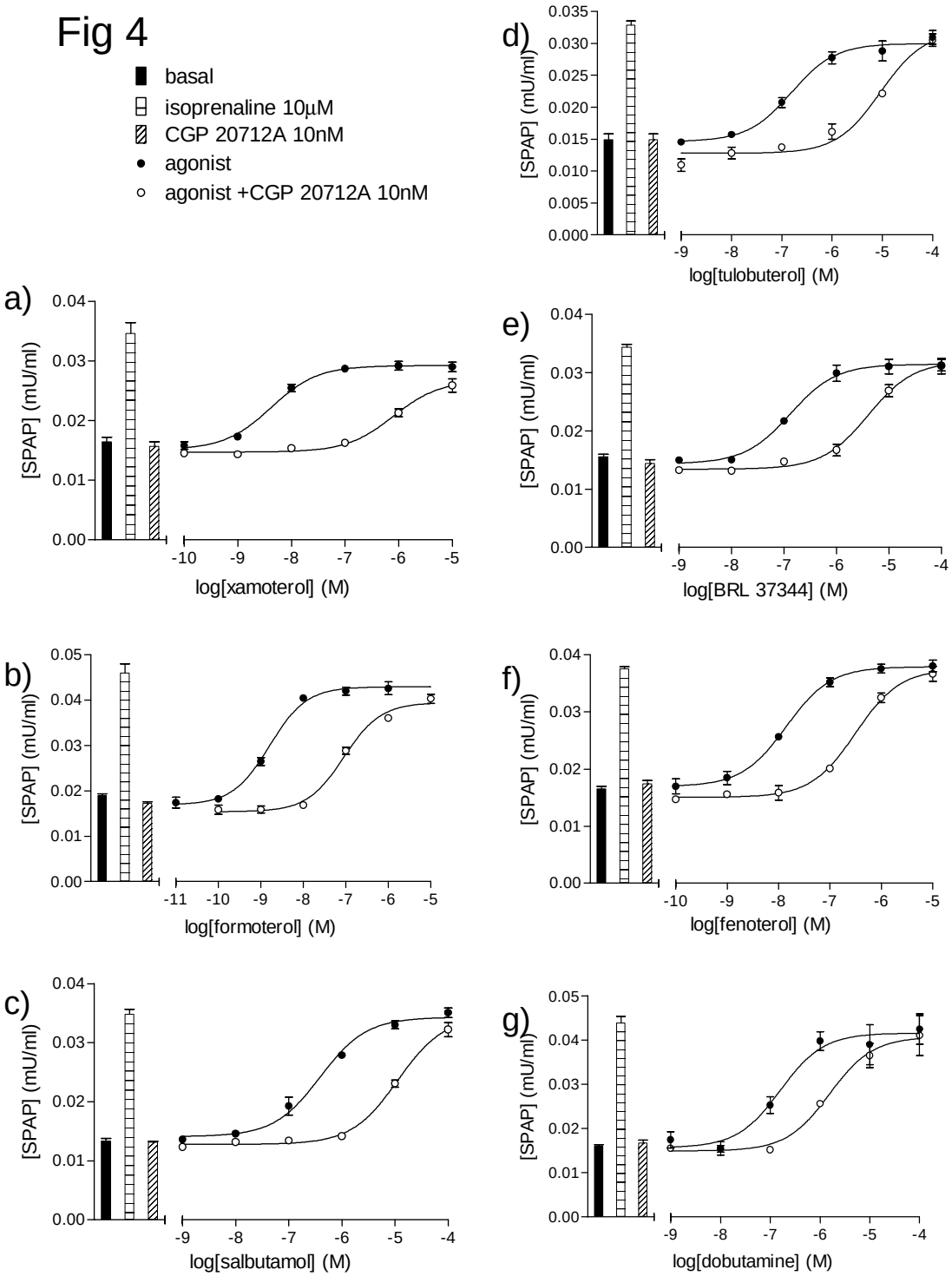

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Fig 5

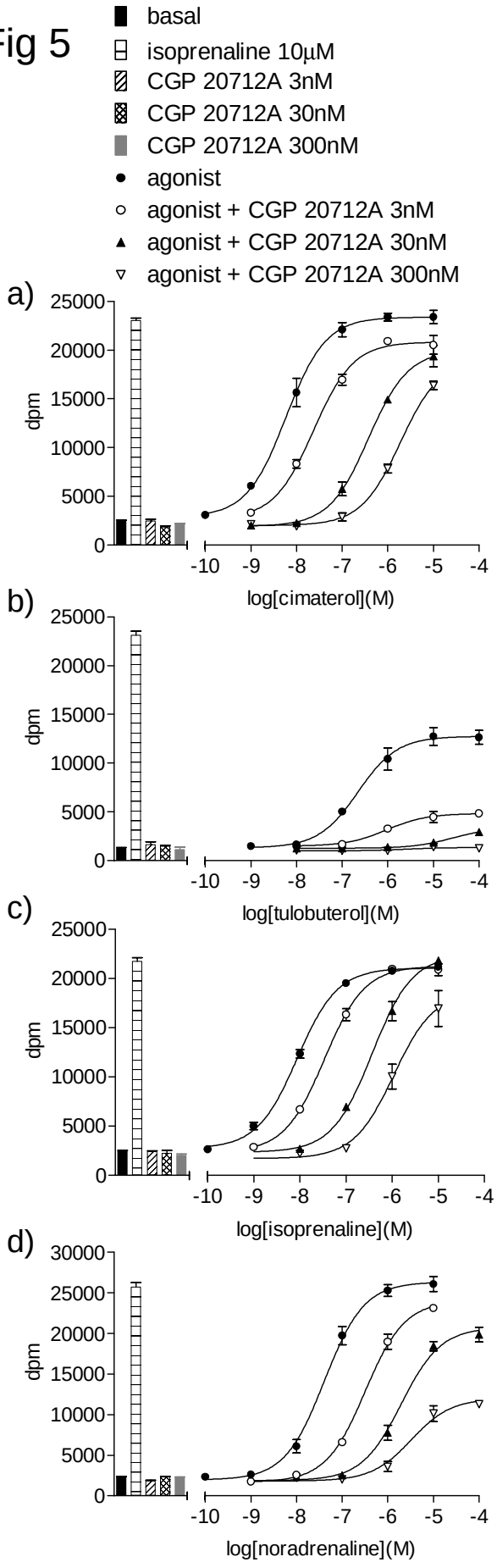

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Fig 6