Molecular Pharmacology Fast Forward. Published on August 7, 2008 as DOI: 10.1124/mol.108.048371
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Role of key transmembrane residues in agonist and antagonist actions at the two conformations of the human β1-adrenoceptor.

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Running title: β1-adrenoceptor point mutations

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Abbreviations:

BSA, bovine serum albumen
cAMP, adenosine-3',5'-cyclic monophosphate
CHO, Chinese hamster ovary
CRE, cyclic AMP response element;
DMEM/F12, Dulbecco's modified Eagles medium/nutrient mix F12;
L158,870, 1-(3'4'-dihydroxylphenyl)-3-methyl-1-butanone;
GPCR, G-protein coupled receptor
PBS, Phosphate buffered saline
pNPP 4-nitrophenyl phosphate
SPAP, secreted placental alkaline phosphatase
CGP 12177, 4-[3-[(1,1-Dimethylethyl)amino]2-hydroxypropoxy]-1,3-di hydro-2H-benzimidazol-2-one hydrochloride
CGP 20712A, 1-[2-((3-Carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-prop anol dihydrochloride
TM, transmembrane spanning region

Abstract 250 words

Introduction 737 words

Discussion 1497 words

Figures 9

Tables 5

References 40

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Abstract

Studies with CGP 12177 at the human β1-adrenoceptor have provided evidence for two binding modes or conformations that have markedly different pharmacological properties. Here, key transmembrane residues (D104, D138, S228, S229, S232, F341, N344 and N363) have been mutated to provide structural insights into the nature of these conformations. ^{3}H-CGP 12177 binding and CRE-mediated reporter gene studies confirmed that CGP 12177 was a neutral antagonist (log K_D = -9.18) at the "catecholamine site" and an agonist at the "CGP 12177 site" (log EC_{50} = -8.12). Agonist responses to isoprenaline and CGP 12177 had different sensitivities to β1-antagonists (e.g. CGP 20712A; log K_D = -8.65 and -7.26 respectively). Site-directed mutagenesis showed that N363 and D138 were key residues for binding of agonists and antagonists, and were also essential for the agonist actions of CGP 12177. S228A and S229A in TM5 reduced the binding of CGP 12177 and had an identical effect on its agonist and antagonist actions. Both N344A and F341A in TM6 abolished the ability of CGP 20712A to discriminate between responses elicited by isoprenaline and CGP 12177. The fact that both D138 and N363 are absolutely required for CGP 12117 binding in both agonist and antagonist modes leads to the conclusion that the secondary agonist binding site for CGP 12117 must overlap with the catecholamine binding site. Modelling studies provide a basis for these overlapping sites with either the *tert*-butylamino group or the hydroxyethyloxy and imidazolone portions of CGP 12177 capable of forming polar interactions with D138 and N363.

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Introduction.

The β1 and β2-adrenoceptors are classical examples of G protein-coupled receptors (GPCRs) that couple to $G\alpha_S$ G-proteins and stimulate adenylyl cyclase activity (Kobilka, 2007). Recent studies of the human and rodent β1-adrenoceptors, however, have shown that this receptor exists in at least two conformations that have markedly different pharmacological properties (Granneman, 2001). The initial evidence for multiple binding conformations on the β1-adrenoceptor came from detailed studies with the aryloxypropanolamine CGP 12177 (Pak and Fishman, 1996). This compound is a high affinity neutral antagonist of the classical "catecholamine" binding site or conformation of the β1-adrenoceptor, however, at higher concentrations it activates a secondary site or conformation and produces an agonist response that is relatively resistant to antagonism by other classical β-antagonists such as CGP 20712A and propranolol (Pak and Fishman, 1996; Konkar et al., 2000; Baker et al., 2003).

Many of our thoughts concerning the specific sites of interaction of ligands with the β-adrenoceptors has come from site-directed mutagenesis studies of the β2-adrenoceptor. The main binding conformations for catecholamines at the β2-adrenoceptor have been identified as Asp 113 in transmembrane 3 (TM3), Ser 204, 207 (and probably 203) in TM5 and Asn 293 in TM6 (Savarese and Fraser, 1992; Sato et al., 1999). In the human β2-adrenoceptor, Asp 113 in TM3 has been shown to be the anchor point for the protonated amine function of catecholamines such as isoprenaline and adrenaline (Savarese amd Fraser, 1992; Sato et al., 1999). Mutation of Asp 113 to Asn or Glu in the β2-adrenoceptor produced a marked decrease in the potencies of agonists and the affinity of antagonists (Strader et al., 1988, 1989). Similar observations have been made with Ser and His substitutions at this position, but these mutants can also be fully activated by catechol-containing esters and ketones (e.g. L-159,084 and L-158,870) that do not activate the wild-type receptor (Strader et al., 1991; Elling et al., 1999).

The anchor points for the catechol hydroxyl groups of catecholamines have been classically associated with Ser 204 and 207 (TM5) respectively (Savarese and Fraser, 1992; Sato et al., 1999). However, evidence has also been obtained that Ser 203 may also (in addition to Ser 204) interact with the *meta*-OH of catecholamines (Sato et al., 1999; Liapakis et al., 2000). Removal of the OH at position 203 also selectively reduced the binding affinity of antagonists with a nitrogen in their heterocyclic ring structure (e.g. pindolol and CGP 12177; Liapakis et al., 2000).

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Asn 293 in TM6 of the human β2-adrenoceptor has been proposed to be important for the stereoselectivity of catecholamines and binding of the β-OH group (Wieland et al., 1996).

In addition to these essential catecholamine anchor points, other amino-acids have also been found to be important for ligands interactions at the β2-adrenoceptor. Asp 79 in TM2 of the β2-adrenoceptor has been shown to produce different effects on agonist and antagonists properties (Strader et al., 1988; Chung et al., 1988). Phe 290 in TM6 is important for interaction with the aromatic ring of catecholamines (Shi et al., 2002; Swaminath et al., 2005; Kobilka, 2007). This residue may also be an important part of the rotamer "toggle switch" which allows receptor activation as a consequence of relative movements in the positions of TM3 and TM6 during agonist stimulation (Shi et al., 2002). Finally, Asn 312 in TM7 of the human β2-adrenoceptor has been shown to be important for the high affinity binding of the aryloxypropanolamine family of β-adrenoceptor antagonists (Suryanarayana and Kobilka, 1993; Elling et al., 1999). Interestingly, the human 5-HT1A receptor has an asparagine residue in an homologous position in TM7 which is responsible for high affinity binding of aryloxypropanolamine β-antagonists to the 5-HT1A receptor (Kuipers et al., 1997; Guan et al., 1992). A point mutation from phenylalanine in the wild-type receptor to Asn in the same position of the α2-adrenoceptor also increases its affinity for this family of β-adrenoceptor antagonists (Suryanarayana et al., 1991).

The human β1-adrenoceptor has analogous residues to those described above for the human β2-adrenoceptor in TMs 2,3,5,6 and 7 (Figure 1). Here a series of point mutations was made in the human β1-adrenoceptor and experiments undertaken to investigate whether: (1) the equivalent amino acid residues are essential for catecholamine binding and activation of the human β1-adrenoceptor; (2) these residues are required for secondary site agonist responses i.e. those to CGP 12177 and (3) the binding of the antagonist ligands propranolol and CGP 20712A are affected by these mutations.

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Methods.

Materials

Fetal calf serum was from PAA laboratories (Teddington, Middlesex, UK). ^{3}H-CGP 12177 was obtained from GE Healthcare (Buckinghamshire, UK). Cimaterol, CGP 20712A and isoprenaline were from Tocris Cookson (Avonmouth, Bristol, UK). Lipofectamine and hygromycin were from Invitrogen (Paisley, UK). OPTIMEM and neomycin were from Gibco (Paisley UK). Propranolol, CGP 12177 and all other cell culture reagents were from Sigma Chemicals (Poole, Dorset, UK). L158,870 (1-(3'4'-dihydroxylphenyl)-3-methyl-1-butanone) was synthesized by Cedric Lion, School of Pharmacy, University of Nottingham, UK.

Generation of β1-adrenoceptor mutations

The cDNA sequence encoding the human human β1-adrenoceptor in pJG3.6 was a gift from Steve Rees (GSK, Stevenage, UK). This cDNA was subcloned as a Hind III/XbaI fragment into pcDNA3.1 (Invitrogen) and the sequence confirmed by DNA sequencing. The wild type β1-adrenoceptor sequence was confirmed to contain the S49 and G389 polymorphisms. Mutations (as described in Figure 1a) were generated using the Stratagene QuikChange mutagenesis kit and Bioline PolyMate Additive for GC rich templates. Mutants are named as (wild type residue) (residue number) (mutant residue) where the residues are given in the single letter amino acid code. Sequence of primers (5' to 3') designed to synthesize two complimentary oligonucleotides containing the relevant mutations are provided in Supplementary Information. Following subcloning in Top F' competent cells (Invitrogen), the mutant β1-AR cDNA was excised on Hind III/XbaI and subcloned into native pcDNA3.1 containing either a neomycin (all mutations) or zeocin (additional N363A construct) selection marker. An N-terminal myc-tagged WT human β1-adrenoceptor was generated by PCR amplification from β1-adrenoceptor WT pcDNA3.1 vector template using a 5' forward primer (see Supplementary Information) comprising a Hind III site, a Kozac sequence, a nucleotide sequence encoding the myc tag peptide sequence (MEQLISEEDL) and the first 30 bases of the β1-adrenoceptor sequence. The PCR product was digested with Hind III/XbaI and ligated into pCDNA3.1. To construct N-myc tagged versions of the D138A, D138S and N363A mutant constructs, OliI/XbaI fragments of each mutant construct was subcloned into the 5'myc-β1-adrenoceptor pcDNA3.1 vector at OliI/XbaI. All mutations and sequences were confirmed by DNA sequencing.

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Cell Culture

The parent cell line used for all experiments was a clonal CHO-K1 cell line stably expressing a CRE-SPAP reporter gene (on a hygromycin selection marker, Baker et al., 2002). 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 T75 of these cells was transfected for 24 hours with 10 ml OPTIMEM, 100µl lipofectamine and 10µg of DNA for either the wild-type human β1-adrenoceptor or with a mutant of the receptor containing a single point mutation (each on a neomycin selection marker). Transfected cells were selected for 2-3 weeks using resistance to neomycin (1mg/ml; for β1 receptor or mutant) and hygromycin (200µg/ml; for CRE-SPAP reporter gene) during which time they were passaged twice. Thus stable mixed populations of cells were generated and the number of each detailed in Table 1. Cells were grown for at least 24 hours in the absence of selection antibiotics before any experiments were conducted.

Four separate stable mixed population cell lines were created expressing both D138A and N363A. In these mixed transfections, 5µg DNA for D138A on a neomycin marker and 5µg DNA for N363A on a zeocin selection marker were co-transfected. Cells were selected for 3 weeks in neomycin (1mg/ml; for the D138A mutant), zeocin (500µg/ml; for the N363A mutant) and hygromycin (200µg/ml; for CRE-SPAP reporter gene) during which time they were passaged twice.

For binding experiments using dopamine with the N344A mutant, cells were transfected as above with either the WT β1-adrenoceptor DNA or β1N344A DNA for 24 hours. The transfection media was then removed and the cells allowed to recover in media for 24 hours. The cells were then plated into white-sided 96well view plates for binding experiments on the following day. Thus, these experiments were performed on transiently transfected cells.

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

Cells were grown to confluence in white-sided 96-well view plates as described previously (Baker 2005a). Immediately prior to experimentation the media was removed from each well. For saturation binding studies, 100µl serum free media (i.e. DMEM/F12 containing 2mM L-glutamine only) or serum free media containing 2-20µM CGP 20712A was added to each well,

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immediately followed by 100µl serum free media containing ^{3}H-CGP 12177. This gave final well concentrations of 1-10µM CGP 20712A (to define non-specific binding) and ^{3}H-CGP 12177 in the range 0.018 – 76.63nM. In competition studies, 100µl serum-free media containing the competing ligand was added and immediately followed by the addition of 100µl serum-free media containing ^{3}H-CGP 12177 (to give a final concentration of ^{3}H-CGP 12177 of 0.90 – 5.85nM). Total and non-specific binding (as defined by 1-10µM CGP 20712A) were measured in every experiment. All cells, for both the saturation and competition studies, were then incubated for 2 hours at 37°C in a humidified 5% CO_2 : 95% air atmosphere. After 2 hours, the media and drugs were removed and the cells washed twice by the addition and removal of 2 x 200µl 4°C PBS/well. A white base was then added to the plate, followed by 100µl Microscint 20 (Perkin Elmer) per well and a sealant film placed over the wells. The plates were left overnight at room temperature it in dark then counted the following day on a Topcount (Packard) 2 minutes per well at 21°C. The protein content was determined by the method of Lowry et al (1951).

CRE-SPAP gene transcription

Cells were grown to confluence in 96-well plates. Once confluent, the media was removed and replaced with 100µl serum free media (i.e. DMEM/F12 containing 2mM L-glutamine) and the cells incubated for a further 24 hours. On the day of experimentation, the serum-free media was removed and replaced with 100µl serum-free media or 100µl serum-free media containing antagonists at the final required concentration and the cells incubated for 30 minutes at 37°C (5% CO_2). Agonists in 10µl (diluted in serum free media) were then added to each well and the plate incubated at 37°C for 5 hours (5% CO_2). After 5 hours, the media and drugs were removed, 40µl serum-free media was added to each well and the cells incubated for a further 1 hour at 37°C. The plates were then incubated at 65°C for 30 minutes to destroy any endogenous phophatases. The plates were then cooled to 37°C. 100µl 5mM pNPP in diethanolamine buffer was added to each well and the plates incubated at 37°C until the yellow colour developed. The plates were then read on a Dynatech MRX plate reader at 405nm.

Confocal immunocytochemistry localisation/detection of myc-tagged receptors.

CHO-CRE-SPAP cells were seeded into 6-well plates containing a glass coverslip. The following day, the cells were transfected with either the N-terminal myc-tagged WT β1-receptor or an N-terminal myc-tagged D138A, D138S or N363A mutant of the receptor for 24 hours (with 2ml OPTIMEM, 20µl lipofectamine and 2µg DNA per well). A double transfect of N-terminal myc-D138A with untagged N363A was also performed. The control mock transfection contained

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OPTIMEM and lipofectamine but no DNA. After 24 hours, the cells were washed and left for 24 hours to recover in media.

The following day the cells were washed with 1ml PBS per well, then fixed with 1ml 4% formaldehyde per well for 20 minutes at room temperature. The cells were washed 3 times with BPS then blocked with 1ml PBS containing 3%BSA and 1% glycine per well for 20 minutes at room temperature. The cells were washed 3 times with BPS then 1ml 10% goat serum (in PBS) added to each well for 20 minutes at room temperature. Primary antibody (mouse anti-c-myc from an in house mouse hybridoma cell line used at 1:100 in 10% goat serum) was then added to each well and the cells left at 4°C overnight. The next day, the cells were washed 3 times with PBS, then 1ml secondary antibody (goat anti-mouse rhodamine red-X at 1:500 in 10% goat serum) was added to each well and left in the dark at room temperature for 1hour. The cells were then washed 3 times with PBS, then the coverslips removed from the wells and mounted on glass slides using 10µl 50%glycerl/50%PBS mix. The coverslips were then examined on a Zeiss LSM510 laser scanning microscope with a Zeiss 40 x 1.3NA oil immersion lens, using a HeNe 543nm laser on 100% power, a pin hole of 1 Airey unit and capturing all fluorescence emission passing through a 560nm long pass filter.

Data Analysis

^{3}H-CGP 12177 whole cell binding

To determine the K_D of CGP 12177 in each mutation, saturation curves for specific ^{3}H-CGP 12177 binding were fitted to the following equation using GraphPad Prism 2:

$$\text{Specific binding} = \frac{B_{max} \times [^{3}\text{H-CGP}]}{([^{3}\text{H-CGP}] + K_D)}$$

B_{max} is the maximum specific binding, K_D is the dissociation constant of ^{3}H-CGP 12177 and [^{3}H-CGP] is the concentration of ^{3}H-CGP 12177.

The binding affinity of other ligands was assessed by examining the inhibition of specific binding of ^{3}H-CGP 12177 fitted to the following equation:

$$\text{\% of specific binding} = 100 - \frac{100 \times [A]}{[A] + IC_{50}}$$

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where [A] is the concentration of competing ligand and IC_{50} is the concentration which inhibits specific binding by 50%. Ligand dissociation constants (K_D) were then determined from the following expression:

$$K_D = \frac{IC_{50} \times K_{D\text{-}CGP}}{(K_{D\text{-}CGP} + [^3H\text{-}CGP])}$$

where $[^3H\text{-}CGP]$ and $K_{D\text{-}CGP}$ are the concentration and dissociation constant of ^{3}H-CGP 12177 respectively.

CRE-gene transcription

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

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

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 affinity (K_D values) were 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 cases where clear partial agonism was seen, the antagonist (partial agonist) affinity was initially calculated by the method of Stephenson (1956) using the following equation:

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$$K_D \text{ partial agonist} = \frac{Y \times [P]}{1 - Y} \qquad \text{where } Y = \frac{[A_2] - [A_1]}{[A_3]}$$

where [P] in the concentration of the partial agonist, $[A_1]$ in the concentration of the agonist at the point where the fixed partial agonist causes the same response, $[A_2]$ is the concentration of agonist causing a given response above that achieved by the partial agonist and $[A_3]$ the concentration of the agonist, in the presence of the partial agonist, causing the same stimulation as $[A_2]$.

This analysis assumes that both the full agonist and partial agonist are interacting with the same site on the receptor. However, in the case where partial agonist activity is mediated at a separate site to the "catecholamine" site this is not appropriate. As a consequence, the dissociation constant of the partial agonist for the catecholamine site was calculated by analysis of the parallel shift of the upper part of the concentration response curve to the full agonist (i.e. the responses greater than that obtained by the partial agonist alone). Some mutations in the receptor may alter the sites involved in the partial agonist response, both sets of values are presented in the manuscript.

A 3μM (maximal) forskolin concentration was included in each CRE-gene transcription plate of each separate 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.

Molecular modelling

A homology model of the β1-adrenoceptor was constructed based on a recently reported β2-adrenoceptor X-ray crystal structure (Cherezov et al., 2007; PDB accession code 2RH) as a template, using the facilities of the SWISS-MODEL server (Schwede et al., 2003). The ligand-binding site in this model was defined by the position of the adrenoceptor ligand carazolol present in the 2RH1 complex. Docking studies were carried out using the β1-adrenoceptor ligand-binding site with the programmes FRED Receptor v. 2.2.3, OMEGA v. 2.2.1 (Kirchmair et al, 2006), FRED v. 2.2.3 (McGann et al., 2003) , and VIDA v. 3.0.0, from OpenEye Scientific Software, Santa Fe, NM, USA. Models of inactive and active conformations of the β2-adrenoceptor (Fig. 9) were kindly provided by Professor C.A. Reynolds, Department of

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Biological Sciences, University of Essex, Colchester, UK (Gouldson et al., 2004). 3D-Structure illustrations (Figures 8 and 9) were prepared using the PyMOL programme [W.L. DeLano, The PyMOL Molecular Graphics System (2002) on World Wide Web http://www.pymol.org].

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Results.

All stable mixed populations were investigated by whole cell binding (Table 1, 2) and in a CRE-reporter functional assay (Table 3-5). The effects of three agonists were investigated: (1) isoprenaline, the classical catecholamine (site 1) agonist; (2) cimaterol, a more site 1 selective agonist (Baker 2005b); and (3) CGP 12177, the classical site 2 agonist (Konkar et al., 2000; Baker et al., 2003). CGP 12177 is also a high affinity neutral antagonist of site 1 (Pak and Fishman 1996; Konkar et al., 2000; Baker et al., 2003; Baker 2005b). In ligand binding studies low concentrations of ^{3}H-CGP 12177 were therefore used to only occupy site 1 and give an indication of catecholamine (site 1) affinity.

Wild-type human β_1-adrenoceptor.

In saturation binding studies, ^{3}H-CGP 12177 was found to have high affinity for the wild-type human β1-adrenoceptor (K_D of 0.31±0.04nM, n=11, Figure 2a, Table 2). This is similar to previous studies in stable clonal cell lines in CHO cells expressing the wild type receptor. The affinity of the other ligands, as measured in whole cell binding assay, was also similar to previously reported values (Figure 2c, Table 1, Baker, 2005b). When the function of all three agonists was examined, CGP 12177 was able to stimulate a lower maximum response when compared to isoprenaline and cimaterol and thus appeared as a partial agonist, again in keeping with previous studies. All agonist responses were inhibited by propranolol and CGP 20712A, however, as expected, the concentrations required to inhibit the CGP 12177 responses were considerably higher than those required to inhibit the isoprenaline and cimaterol responses (Tables 3-5). The resulting antagonist affinity measurements (log K_D values) were therefore markedly different at the two sites or conformations, as would be expected from previous studies (Konkar et al., 2000; Baker et al., 2003, Baker 2005b). Furthermore, cimaterol was more readily inhibited than isoprenaline (Tables 3 and 4), again in keeping with previous studies (Baker 2005b). CGP 12177 was also able to inhibit the isoprenaline and cimaterol responses to give log K_D values similar to the affinity of ^{3}H-CGP 12177 obtained in the binding studies indicating high affinity for site 1 (Tables 2-4). This was true regardless of whether the affinity measurements in functional studies were determined by the partial agonist method of Stephenson (which assumes that both ligands are competing at the same site) or when the shift of the upper part of the concentration response curve to the isoprenaline or cimaterol alone was considered (therefore allowing for the fact that the agonist response to CGP 12177 was occurring at a different site).

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Also, as previously found, the K_D and EC_{50} values obtained for CGP 12177 were not the same suggesting that this agonist was binding at more than one site. The difference in the affinity of propranolol and CGP 20712A to inhibit isoprenaline, cimaterol and CGP 12177 agonist responses, and the discrepancy between the log K_D and EC_{50} values for CGP 12177 defines the two sites of the β1-adrenoceptor. All values obtained in this study using mixed populations of cells were similar to those obtained in previous studies using stable clonal cell lines (Baker et al., 2003; Baker 2005b).

Transmembrane 2 mutations: Aspartate 104

The aspartate at position 79 of the human β2-adrenoceptor is important for agonist binding (Strader et al., 1988; Chung et al., 1988). The equivalent in the human β1-adrenoceptor is the aspartate at position 104. This was therefore mutated to alanine (D104A) and asparagine (D104N). Apart from a minor reduction in the affinity for CGP 20712A, the binding affinities for all ligands was very similar to that seen for the wild-type receptor (Table 2). For the functional responses, the maximum responses stimulated by each ligand was less then for the wild-type receptor WT>D104A>D104N (Tables 3-5). The log EC_{50} values also moved progressively rightward with reducing maxima. This is consistent with a reduction in coupling of the receptor to its downstream effector, the Gs-protein. Interestingly, the B_{max} values from ^{3}H-CGP 12177 binding to site 1 were in the opposite order (Table 1) to the change in efficacy (D104N>D104A>WT) indicating that this was not a consequence of a reduced receptor reserve. Thus mutations at position D104 of the β1-adrenoceptor do not significantly alter the binding of ligands (including agonist binding) to the receptor but the coupling to signalling cascades is reduced.

Transmembrane 3 mutations : Aspartate 138

In the human β2-adrenoceptor, the anchor point for the protonated amine function of catecholamines such as isoprenaline has been identified as Aspartate 113 in transmembrane region 3 (Savarese and Fraser, 1992; Sato et al., 1999). The equivalent residue in the human β1-adrenoceptor is Aspartate 138. This was therefore mutated to alanine, glutamate, histidine, asparagine or serine. This had a catastrophic effect on the β1-adrenoceptor function. It abolished all specific binding in all mutations of the receptor and no functional responses were seen to isoprenaline (to concentrations of 100μM), cimaterol (to concentrations of 10μM) or CGP 12177 (to concentrations of 100μM). Strader et al., (1991) showed that a serine mutation in the

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equivalent position of the human β2-adrenoceptor can respond to a catechol ester that was capable of forming hydrogen bonds with the serine residue. We therefore used this molecule, L158,870 (1-(3’4’-dihydroxylphenyl)-3-methyl-1-butanone), to activate the serine mutant (D138S) of the β1-adrenoceptor. High concentrations of L158,870 stimulated an increase in CRE-gene transcription with the D138S mutation that was not seen with either the wild-type receptor or any of the other D138 mutations (Figure 3). Furthermore, CGP 20712A was not able to antagonise this response confirming the lack of binding of classical β-antagonists (Figure 3). This therefore suggests that the D138S mutation reaches the cell surface and is able to couple to intracellular signalling pathways mediating CRE-gene transcription. In order to confirm that the D138 mutations were indeed reaching the cell surface, N-terminally myc-tagged receptors were transiently expressed in the parent CHO-CRE-SPAP cells and detected by immunohistochemistry. Both D138A and D138S mutations reached the cell surface in a similar manner to the WT receptor (Figure 4). Thus mutations at position D138 of the β1-adrenoceptor abolish all binding and functional responses to both catecholamine site and secondary CGP 12177 site agonists and antagonists. It is always possible that interpretation of the involvement a a particular residue in ligand binding or activation by mutagenesis may be confounded by the mutation inducing global or local conformational changes, however, the fact that a variety of D138 substitutions (alanine, glutamate, histidine, asparagine or serine) produce the same effect suggests that this is an effect at the level of this particular amino acid.

Transmembrane 5 mutations: Serines 228, 229 and 232.

The anchor points for the catechol hydroxyl groups of catecholamines in the β2-adrenoceptor are Ser 203, 204 and 207 (Savarese and Fraser, 1992; Sato et al., 1999). The equivalent amino acids in the β1-adrenoceptor are the serines at positions 228, 229, 232 and were therefore similarly mutated to alanines. Loss of the serine at position 228 or 229 resulted in a reduction in the affinity of CGP 12177 for site 1 of the β1-adrenoceptor (as measured by both the saturation binding of ^{3}H-CGP 12177, Table 2, Figure 2b, and CGP 12177 inhibition of isoprenaline and cimaterol agonist responses, Tables 3-4; Figure 5d). This is consistent with the observation in the β2-adrenoceptor of reduced antagonist affinity of those antagonists with a nitrogen in their heterocyclic ring structure in the Ser 203 mutations (e.g. pindolol and CGP 12177; Liapakis et al., 2000). This was not however universal for all ligands but rather appears ligand-dependent. For example, the affinity for isoprenaline at both of these two mutations was reduced when measured in the binding assay in keeping with the loss of catechol anchor points (Table 2). This is also in

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keeping with the increase in EC_{50} value for isoprenaline observed in the functional assay (Figure 5a). Cimaterol, however, (which has no catechol hydroxyl groups) actually had high affinity for the S228A mutation (in keeping with the observed leftward shift in the EC_{50} value; Table 2, Figure 5c). The maintenance of the EC_{50} values in the cimaterol response suggests that the S228A mutation retained its coupling efficiency. Interestingly, propranolol however had a markedly increase in affinity for the S228A mutation (as measured in both the binding and functional assays; Figure 2d, Tables 2-5).

CGP 12177 stimulated a response in CRE-gene transcription in both S228A and S229A mutations comparable in magnitude to that at the wild-type receptor (Table 5). The CGP 12177 responses still required higher concentrations of antagonist to inhibit the responses suggesting the second site was still present. The EC_{50} of this response was however right-shifted suggesting a possible reduction in affinity of CGP 12177 for the second site of the β1-adrenoceptor. Interestingly, propranolol also had higher affinity for the site 2 in the S228A mutation, but it remained 20 fold lower affinity than for site 1.

Loss of the serine in the S232A mutant did not result in a loss of CGP 12177 binding affinity (in either binding or functional assays, Tables 2-5). There was however a reduction in isoprenaline affinity, again in keeping with a loss of a catechol hydroxyl anchor. CGP 20712A had higher affinity for this mutation in both binding and functional assays. CGP 12177 stimulated an agonist response and although CGP 20712A antagonised this response with higher affinity at this mutation than the wild-type β1-adrenoceptor, the 20-fold discrepancy remained suggesting the second site was still present.

Thus, serines 228, 229 and 232 are important for catecholamine binding to the β1-adrenoceptor. Individual mutations affect ligand affinities (e.g. S228A has higher affinity for propranolol and Ser 232 has higher affinity for CGP 20712A than the wild-type receptor). Ser 228 and Ser 229, but not Ser 232, appear to be important for CGP 12177 binding to site 1 of the β1-adrenoceptor. The agonist response to CGP 12177 remains however, as does the discrepancy in antagonist affinities suggesting that site 2 remains in all mutants. The decrease in EC_{50} potency for CGP 12177 in S228A and S229A (receptor coupling otherwise appearing to be maintained) may also suggest a reduction in the affinity of CGP 12177 for site 2 of the β1-adrenoceptor.

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Transmembrane 6 mutations: phenylalanine 341 and asparagine 344.

In the human β2-adrenoceptor, phenylalanine at position 290 is important for interactions involving the aromatic ring of catecholamines and has been proposed to be involved in the rotamer "toggle switch" which allows receptor activation (Shi et al., 2002; Swaminath et al., 2005; Kobilka, 2007). When the equivalent phenylalanine in the β1-receptor (at position 341) was mutated to an alanine (F341A), the affinity for the agonists isoprenaline and cimaterol and that for propranolol (measured in both the binding and functional assays) was unchanged. The affinity for CGP 12177 at site 1 was reduced (as determined from both binding and as an antagonist of isoprenaline and cimaterol agonist responses; Tables 2-4). CGP 12177 still stimulated an agonist response so the second site appeared to be present (Figure 6d). However the affinity for CGP 20712A was reduced at site 1 and increased at site 2 such that the affinities were similar whether measured in the presence of cimaterol or CGP 12177 (Figure 6c and d). This suggests that CGP 20712A could no longer differentiate between the two sites of the β1-adrenoceptor in the F341A mutant.

Asparagine 293 of the human β2-adrenoceptor has been proposed to be important for the stereoselectivity of catecholamines and binding of the β-OH group (Wieland et al., 1996). The equivalent residue in the human β2-adrenoceptor is at position 344. When this is mutated to alanine (N344A), the affinity of CGP 12177 and isoprenaline was reduced (Table 2). The affinity of propranolol however was increased (Table 2). Isoprenaline retained its agonist properties, although at a lower potency, in keeping with the lower affinity. The isoprenaline and cimaterol responses were inhibited by CGP 20712A, propranolol and CGP 12177 to give lower, higher, and lower antagonist affinity measurements respectively (compared with wild-type) in the functional assay as would be expected from the binding studies (Tables 3,4). CGP 12177 retained its agonist activity although the concentration of CGP 20712A required to antagonise this response was similar to that required to block the site 1 agonist responses (Tables 4 and 5). As with the F341A mutation, N344A also removes the ability of CGP 20712A to discriminate between sites 1 and 2. Thus in TM6, both Asn 344 and Phe 341 are important for the high affinity of CGP 12177 and CGP 20712A (but not propranolol) for the "catecholamine" site of the β1-adrenoceptor. Neither mutation, however, has any effect on the agonist actions of CGP 12177 at the secondary "CGP 12177" site of the β1-adrenoceptor. The N344A mutation also had no effect on the binding affinity of dopamine (which lacks a chiral β-hydroxyl group) determined by inhibition of ^{3}H-CGP 12177 binding in transiently transfected cells (log K_D values: WT -3.64 ± 0.03, n=8;

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N344A -3.28 ± 0.02, n=8). Parallel studies with CGP 20712A confirmed that similar log K_D values were determined (WT -8.97 ± 0.07; N344A -8.81 ± 0.06, n=9) to those obtained in stable mixed populations used in the rest of the study (Table 2).

Transmembrane 7 mutations: Asparagine 363.

Asparagine 312 in TM7 of the human β_2-adrenoceptor has been shown to be important for the high affinity binding of the aryloxypropanolamine family of ligands, such as propranolol and CGP 12177 (Suryanarayana and Kobilka, 1993). The equivalent amino-acid in the human β1-adrenoceptor is at position 363. When this was mutated to either alanine, cysteine, tyrosine or phenylalanine, there was a complete loss of all specific binding of ^{3}H-CGP 12177. To confirm that the N363 mutations are capable of reaching the cell surface, an N-terminal myc tagged N363A mutant was transfected into the parent CHO-CRE-SPAP cells. Clear membrane expression was observed with this mutation (Figure 4).

Isoprenaline and cimaterol retained some agonist potency but this was greatly reduced when compared with the wild-type receptor (Figure 7). Propranolol and CGP 12177 were not able to antagonise these responses in the manner they did to the wild-type response. Finally, CGP 12177 was no longer able to stimulate an agonist response. Thus Asn 363 is an important residue for the interactions of both agonists and antagonists with the human β1-adrenoceptor. Furthermore, it is vital for the interaction of drugs with both the catecholamine and the CGP 12177 agonist sites of this receptor.

Potential role for β1-adrenoceptor dimerisation in the agonist actions of CGP 12177.

Molecular modelling studies have indicated that aryloxypropanolamine β-blockers such as pindolol and CGP 12177 can adopt either an extended or a folded conformation (Hockerman et al., 1996). It is therefore possible that the antagonist and agonist actions of CGP 12177 could represent different interactions with dimeric and monomeric forms of the β1-adrenoceptor. Studies with other GPCRs have shown that domain swapped dimers can be established in which two ligand binding domains are created upon the mutual exchange of transmembrane domains from both receptors (Maggio et al., 1993; Gouldson et al., 2000). The β1-adrenoceptor has been shown to form dimers (He et al., 2002) and the availability of two amino acids (D138 and N363) in different transmembrane domains that are essential for the binding of aryloxypropanolamine β-blockers provided an opportunity to test this hypothesis. Co-transfection of D138A and N363A β1-adrenoceptor mutants, however, did not reinstate any binding of ^{3}H-CGP 12177 (Table 1) or

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agonist activity of CGP 12177 (Table 5). Co-expression of these two separate mutant β1-adrenoceptors produced essentially the same weak agonist response to cimaterol and isoprenaline as that observed with the N363A mutant alone (Table 3 and 4). These data confirm that the 363A mutant is still reaching the cell surface. However, in order to test whether the D138A mutant is also reaching the cell surface under these conditions, a N-myc D138A mutant was contransfected with an untagged N363A and cell surface expression monitored using immunohistochemistry (Figure 4). These data confirm that the D138A mutant can still reach the surface in the presence of N363A mutants. These data suggest that β1-adrenoceptor monomers and dimers are not responsible for the differential effects of CGP 12177 at the "catecholamine site" and "CGP 12177 agonist site" of the β1-adrenoceptor.

Molecular modelling

In order to rationalise the results from our mutational studies we conducted molecular modelling experiments using a homology model of the β1-adrenoceptor. This model was obtained using a recently determined experimental structure of the homologous β2-adrenoceptor in complex with carazolol (Cherezov et al., 2007) as the template. Docking back individual members of a multi-conformer database of carazolol (Figure 1b) to the β1-adrenoceptor model produced highly-scoring ligand poses (stereochemical configurations) very similar to that observed in the experimental β2-adrenoceptor complex structure (Figures 8a-b). While this is not surprising based on the high homology between the β1- and β2-adrenoceptors in the ligand-binding region, it validates both the β1 model and the docking strategy employed.

Carazolol has been reported to be a partial inverse β1- and β2-adrenergic agonist (Rosenbaum et al., 2007) and one would therefore expect carazolol-bound adrenoceptor structures to represent conformations that are different from the fully inactive (Figure 9a) and active (Figure 9b) receptor forms. Comparison of the experimental carazolol–β2-adrenoceptor complex structure with models of inactive and active β2-adrenoceptor forms confirms this (not shown). Activation of class A GPCRs is believed to involve a contraction of the TM helices at the extracellular receptor side, resulting from rotational and translational movements of helices TM4, TM5, TM6, and TM7 (Gouldson et al 2004). Docking of the agonist isoprenaline into the ligand-binding site of our β1-adrenoceptor model reliably produced the expected ligand poses at the catecholamine site when docking constraints inferred from the effects of mutations at D138, S228, S229, S232, and N363 on isoprenaline agonism were imposed (Figure 8c). However, the putative hydrogen bonds between the ligand catechol ring hydroxyl moieties and the TM5 serine side chains on the

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one hand, and the polar interactions between the ligand propanolamine groups and the D138 and N363 side chains on the other, were somewhat too long [O–O and N–O distances > 3.5 Å; for actual hydrogen bonds these are typically 2.7–3.3 Å; Williams and Ladbury, 2003], suggesting closer proximity between TM3, TM5, and TM7 in active forms of the β1-adrenoceptor. Similar conclusions can be drawn from the modelled binding mode of the other β-adrenergic agonist, cimaterol (Figure 8d).

Similar docking experiments with the antagonist propranolol, but including only constraints that demanded ligand NH and OH groups within 1.5-Å of where the corresponding propanolamine groups of carazolol in the complex structure are observed, resulted in binding poses for propranolol that were similar to those for carazolol (Figure 8e). Again this result was not surprising considering the structural similarities between these compounds. The naphthyl system of propranolol faces the serine residues of TM5 but is incapable of making polar contacts. This and the close proximity of one of the naphthyl benzene rings to S228 explains the enhanced affinity of propranolol for the S228A mutant β1-adrenoceptor.

Top-ranking CGP 12177 poses (Figure 8f) suggested that the benzimidazolone system of this compound is capable of facing the TM5 Ser residues in alternative orientations. Unlike the catechol system of isoprenaline, which can form multiple hydrogen bonds with these Ser side chains, the imidazolone system in the CGP 12177 poses would be likely to hydrogen-bond with a single serine hydroxyl. Complete removal of all constraints upon CGP 12177 docking, while still predominantly producing carazolol-like binding poses, also resulted in geometrically and energetically plausible binding poses that positioned the ligand in such a way that the hydroxyethyloxy and imidazolone portions formed polar contacts with D138 and N363, respectively, whereas the *tert*-butylamino group was bound in the region between the TM5 Ser residues and the TM6 N344 and F341 residues, without however forming any polar contacts in that region (Figure 8g). This binding pose is potentially relevant to the CGP 12177 agonist secondary site, which is comparatively insensitive to TM5 Ser mutations but requires both D138 and N363.

The antagonist CGP 20712A is structurally more different (larger) than either carazolol, isoprenaline, or CGP 12177 (Figure 1b), and also contains more freely rotatable bonds (increased flexibility). It can thus be expected that the carazolol-based receptor conformation is less relevant to CGP 20712A and docking studies using this conformation are less meaningful (Bissantz et al.,

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2003). Nevertheless, assuming propanolamine interactions of CGP 20712A with the D138/N363 clamp as for carazolol, docking to the β1-adrenoceptor model resulted in binding poses that position the ligand 1-methyl-2-phenyl-4-(trifluoromethyl)-1*H*-imidazole system in the vicinity of the TM6 residues F341 and N344 (Figure 8h), whose mutations uniquely affect the ability of CGP 20712A to discriminate between isoprenaline- and CGP 12177-mediated agonism.

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Discussion.

This study examined the effect of single point mutations of amino acids within the transmembrane regions of the human β1-adrenoceptor on the actions of agonists and antagonists at the two conformations (catecholamine and CGP 12177 sites) of this receptor. These studies show that the binding of isoprenaline and cimaterol to the catecholamine site or conformation of human β1-adrenoceptor shares many of the ligand binding characteristics of the human β2-adrenoceptor (Savarese and Fraser, 1992; Sato et al., 1999). The anchor point for the protonated amine of isoprenaline and cimaterol is likely to be Asp138 in TM3 of the β1-adrenoceptor whilst the catechol hydroxyl groups of isoprenaline appear to associate with three serine residues in TM5 in an analogous fashion to the β2 adrenoceptor (Savarese and Fraser, 1992; Sato et al., 1999).

In the β2-adrenoceptor, Asn293 in TM6 has been proposed to be important for binding of the β-OH group of isoprenaline (Wieland et al., 1996). However, mutation of the equivalent residue in the β1-adrenoceptor (N344A) to alanine had little effect on the EC_{50} or binding affinity for isoprenaline or cimaterol. More strikingly, mutation of asparagine 363 in TM7 (thought to be important for antagonist binding, see below) increased the EC_{50} for isoprenaline (i.e. decreased the potency by two orders of magnitude) indicating an important role for this residue at the β1-adrenoceptor. It seems likely, therefore, that Asn363 is important for the binding of the β-OH group of isoprenaline and cimaterol at the β1-adrenoceptor. Interestingly, the crystal structure of an engineered β2-adrenoceptor bound to carazolol has confirmed that Asn312 (equivalent to Asn 363 in the β1-adrenoceptor) forms a close contact with the β-OH group of carazolol (Rosenbaum et al., 2007). Furthermore, molecular modelling with isoprenaline placed into the β2-adrenoceptor binding site indicated that Asn293 (equivalent to Asn 344 in the β1-adrenoceptor) was too distant to form productive contacts with the modelled β2-adrenoceptor (Rosenbaum et al., 2007). Docking of isoprenaline into the ligand-binding site of our β1-adrenoceptor model also reliably produced the expected ligand poses at the catecholamine site when docking constraints inferred from the effects of mutations at D138, S228, S229, S232, and N363 on isoprenaline agonism were imposed (Figure 8c).

Mutation of Phe341 to alanine in TM6 had little effect on agonist responses to isoprenaline and cimaterol at the β1-adrenoceptor. The β2-adrenoceptor equivalent residue (Phe290 in TM6) is

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important for interaction with the aromatic ring of catecholamines and has been proposed to be involved in the rotamer "toggle switch" which allows receptor activation (Shi et al., 2002; Swaminath et al., 2005; Kobilka, 2007). Asp79 in TM2 of the β2-adrenoceptor is also important for agonist action (Strader et al., 1988; Chung et al., 1998). In the β1-adrenoceptor, mutation of the equivalent residue (D104A and D104N) increased the EC_{50} for isoprenaline 10-fold consistent with a decrease in efficacy. For cimaterol, the maximum responses for D104A and D104N were reduced to a much larger extent and the EC_{50} values were again increased by an order of magnitude. Therefore, mutations of D104 appear to decrease agonist efficacy at the catecholamine site.

Three β-antagonists (propranolol, CGP 1177 and CGP 20712A) were evaluated as antagonists of isoprenaline and cimaterol responses at the catecholamine site of the β1-adrenoceptor. All D138 mutations totally abolished the binding of ^{3}H-CGP 12177 to the β1-adrenoceptor. Functional responses to L158,870 in the D138S mutation were also not antagonised by CGP 20712A. Mutation of Ser228 increased propranolol affinity by an order of magnitude whilst that for CGP 12177 decreased by the same amount. The lower affinity of CGP 12177 in the S228A mutant is consistent the model shown in Figure 8f and with previous data from the β2-adrenoceptor where the affinity of antagonists with a nitrogen in their heterocyclic ring decreased (Liapakis et al., 2000).

Asparagine 363 in TM7 was important for the binding of antagonists. No specific binding of ^{3}H-CGP 12177 was detected in the N363 mutants. Both CGP 12177 and propranolol were unable to antagonise the isoprenaline and cimaterol responses in the N363A or N363C mutants. Both N344A and F341A in TM6 had different effects on the affinity of these three antagonists. N344A had no effect on CGP 20712A binding to the catecholamine site but increased propranolol affinity and decreased CGP 12177 affinity. In contrast, in the F341 mutation, the binding affinity of CGP 20712A and CGP 12177 were reduced by nearly an order of magnitude, whilst that of propranolol was little affected. Thus, these mutations decreased the binding affinity of CGP 12177 and CGP 20712A to the catecholamine site, but this effect was not shared by propranolol. These mutations, however, do not influence the agonist actions of CGP 12177 at the secondary CGP 12177 site.

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As both D138 and N363 are both absolutely required for CGP 12177 binding in both agonist and antagonist modes, the secondary agonist binding site for CGP12177 must overlap with the catecholamine binding site. The modelled binding pose in Figure 8g shows that polar interactions between CGP12177 and D138/N363 may occur in a different way from the canonical interactions of propanolamines with these residues. However, the binding pose shown with CGP 12177 in Figure 8f suggests that this molecule can also interact with D138/N363 in an analogous way to CGP 20712A and propranolol. These different poses provide a potential framework for the differential pharmacology of CGP 12177 at the catecholamine site (Figure 8f) and the secondary CGP 12177 site (Figure 8g). In keeping with these different poses, mutations of the serine residues in TM5 (228, 229 and 232) had little effect on the agonist efficacy of CGP 12177, however the S228A mutant decreased ^{3}H-CGP 12177 affinity for the catecholamine site (Figure 8f). Mutations N344A and F341A had little effect on the CGP 12177 agonist properties, however both D104A and D104N markedly reduced the efficacy of CGP 12177 at the secondary site. This suggests that D104 is important in coupling both site 1 (via isoprenaline and cimaterol) and site 2 (via CGP 12177) to functional responses.

Extensive biochemical and modelling studies have shown that GPCRs are highly dynamic structures and that structural plasticity is relevant to receptor activation (Kobilka and Deupi, 2007). The so-called 7TM toggle switch, involving $W^{6.48}$ (W337 and W286 in the β1 and β2-adrenoceptors, respectively) and certain other residues adjacent on the same face of TM6 (Figure 9), is central to receptor activation (Schwartz et al., 2006). In inactive receptors, including *e.g.* the new β2-adrenoceptor–carazolol complex structure (Cherezov et al., 2007), this Trp side chain lies between TM3 and TM6. Upon activation, the side chain rotamer state of $W^{6.48}$ (and nearby TM6 residues) changes, permitting an inward movement (tilting) at the extracellular side of TM5, and subsequently of TM4 and TM7, and the $W^{6.48}$ side chain is believed to be positioned at the interface between TM5 and TM6 in active receptor conformations. In the structurally best-characterised GPCR, rhodopsin, the ligand retinal interacts directly with the toggle switch residues on TM6, but it remains unclear to what extent this applies to adrenoceptor ligands. In the case of carazolol, the distal benzene ring of the carbazole system interacts with $F^{6.52}$ (F341 in the β1-adrenoceptor), whose rotamer state is coupled to that of $W^{6.48}$. Our modelling studies (Figure 8) suggest that the other antagonists modelled act similarly. Presumably agonists at the catecholamine site stabilise the active receptor conformation, in which $W^{6.48}$ lies between TM5 and TM6, by tethering TM5 to TM7, rather than by direct interaction with the toggle residues. This interpretation is consistent with our observation that isoprenaline and cimaterol agonism

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does not require the F341 or N344 side chains, which are not in close proximity to isoprenaline and cimaterol in the predicted binding poses (Figure 8c and d).

Thus, the major conclusion from this study is that the residues involved in the interaction of CGP 12177 with the secondary agonist binding site must overlap with the residues defining the catecholamine binding site via which isoprenaline and cimaterol produce their agonist effects. The modelled binding pose in Figure 8g shows that polar interactions between CGP12177 and D138/N363 may occur in a way that is different from the interactions used by other propanolamine-based antagonists in antagonising the catecholamine site. Nevertheless, such binding poses do not offer a clear explanation of how receptor agonism is achieved by CGP 12177 (i.e. tethering of TM5 and TM6 to TM3 and TM7) in the pose shown in Figure 8g since there are no pronounced interactions between the *tert*-butylamine portion of CGP 12177 with either the serine residues of TM5 nor the toggle switch residues of TM6. Nevertheless, it is possible that this group directly induces a rotamer change in N344 and/or F341, that can be transmitted to W337 thus leading to receptor activation. A remaining issue is why the binding of CGP 12177 to the high affinity catecholamine site does not block its binding to the lower affinity agonist site. This will require further work but may involve the structural changes in the catecholamine site caused by CGP 12177 binding at higher concentrations to the non-overlapping residues.

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Acknowledgements

We thank Cedric Lion for the synthesis of L158,870 (1-(3’4’-dihydroxylphenyl)-3-methyl-1-butanone) and Marleen Groenen for assistance with cell culture.

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Footnotes:

We thank the BBSRC (BB/C5077853/1) for financial support.

JGB is a Wellcome Trust Clinician Scientist Fellow.

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Figure legends

Figure 1

(a) Schematic representation on the human β1-adrenoceptor showing sites where mutations were made.

(b) Chemical structures of adrenoceptor ligands discussed in this study.

Figure 2

Whole cell ^{3}H-CGP 12177 binding to cells expressing the wild-type human β1-adrenoceptor (a,c) or the S228A mutation (b,d). (a,b) Whole cell ^{3}H-CGP 12177 saturation binding. Graphs show total binding, non-specific binding (as determined by the presence of 1μM CGP 20712A (a) or 10μM CGP 20712A (b) and the resultant specific binding. Data points are mean ± s.e.m. of quadruplicate determinations from a single experiment which is representative of 11 (a) and 15 (b) separate experiments. The reduced affinity for ^{3}H-CGP 12177 in the S228A mutation can clearly be seen. (c,d) Whole cell ^{3}H-CGP 12177 competition binding. Bars represent total binding and non-specific binding as determined in the presence of 1μM CGP 20712A (c) and 10μM CGP 20712A (d). Data points are mean ± s.e.m. of triplicate determinations from a single experiment which is representative of 4 (c) and 16 (d) separate experiments. The concentrations of ^{3}H-CGP 12177 used were (c) 1.75nM and (d) 5.25nM. It can be seen that whilst CGP 20712A is more potent in the wild-type human β1-adrenoceptor, the single S228A mutation results in propranolol becoming the more potent.

Figure 3

CRE-SPAP production in cells expressing the wild-type human β1-adrenoceptor (a) or the D138S mutated receptor (b). Bars show the basal response, that in response to 3μM forskolin and that in response to 1μM CGP 20712A alone. L158,870 did not stimulate any response in the wild-type receptor and the response seen in the D138S mutation that was not inhibited by 1μM CGP 20712A. Bars represent mean ± s.e.m. of triplicate values from a single experiment which is representative of 3 separate experiments in each case. In all four experiments there was a significant stimulation of the D138S mutant at 100 μM L158870 ($p < 0.05$, paired t test, $n = 4$ experiments).

Figure 4

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Confocal images showing localisation by immunocytochemistry of N-terminal myc-tagged receptors - wild type (WTβ1), D138A, D138S, N363A, and co-expression of myc-tagged D138A with non-tagged N363A. Control mock transfected cells are also shown. These images are representative of 4 separate experiments. Receptors can be seen to reach the cell surface in all but the mock transfected cells.

Figure 5

CRE-SPAP production in cells expressing the wild-type human β1-adrenoceptor (a,c) or the S228A mutated receptor (b,d) in response to isoprenaline (a,c) or cimaterol (b,d).
Bars represent CRE-SPAP production under basal conditions, that in response to 3μM forskolin and that in response to either CGP 20712A or CGP 12177 alone. Data points are means ± s.e.m. of triplicate determinations from a single experiment and are representative of a) 10, b) 16, c) 12 and d) 16 separate experiments. It shows that although the isoprenaline response is 10fold less potent in the S228A mutation than the wild-type receptor, the affinity of CGP 20712A is not greatly affected. The cimaterol response is however unaffected by the S228A mutation, but the affinity of CGP 12177, as measured by a shift in the cimaterol response is greatly reduced in the S228A mutation (NB concentration of CGP 12177 is 10nM in c) but 100nM in d)).

Figure 6

CRE-SPAP production in cells expressing the wild-type human β1-adrenoceptor (a,b) or the F341A mutated receptor (c,d) in response to cimaterol (a,c) and CGP 12177 (b,d). Bars represent CRE-SPAP production under basal conditions, that in response to 3μM forskolin and that in response to CGP 20712A alone. Data points are means ± s.e.m. of triplicate determinations from a single experiment and are representative of 12 (a), 25 (b), 14 (c) and 14 (d) separate experiments. It shows the typical wild-type pharmacology of the two-sites of the human β1-adrenoceptor: the cimaterol response (site 1) is readily antagonised by CGP 20712A, the CGP 12177 response (site 2) in comparison required higher concentrations of CGP 20712A to antagonise the response. For the F341A mutant, the cimaterol response was also relatively resistant to antagonism by CGP 20712A whilst that to CGP 12177 remained as for the wild-type receptor.

Figure 7

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CRE-SPAP gene transcription in response to isoprenaline (a) or cimaterol (b) in cell expressing the wild-type receptor, the N344A mutant or the N363A mutant alone. Data points are mean ± s.e.m. of triplicate determinations from a single experiment and are expressed as % response in relation to 3μM forskolin for each cell line. These separate experiments are representative of 8 (a) and 5 (b) separate experiments. This shows that the isoprenaline and cimaterol responses have reduced potency at the N344A mutant (which may partly be explained by the reduced affinity – table 1) however the effect of the N363A mutation is more pronounced, particularly for cimaterol.

Figure 8.
Docking studies with the β1-adrenoceptor homology model. The ligand-binding site in the extracellular receptor portion is shown as a cartoon model, including the side chains of residues that were the subject of mutation (plus the toggle residue $W337^{6.48}$) as grey CPK sticks. The carazolol binding pose in (a) and (b, gray CPK-coloured molecular surface) corresponds closely to that observed experimentally in the β2-adrenoceptor (Cherezov et al., 2007). Predicted binding poses for isoprenaline (c), cimaterol (d), propranolol (e), CGP 12177 (f and g), and CGP 20712A (h) are shown as green CPK stick models; an alternative antagonist pose for CGP 12177 in (f) is shown in cyan CGP. The pose for CGP 12177 in (h) may be relevant to agonist activity of this ligand.

Figure 9.
Models of inactive (a) and active (b) β2-adrenoceptor conformations are depicted looking into the ligand binding site from the extracellular side (Gouldson et al., 2004). In (a) the side chains of key residues implicated in ligand binding are shown as green CPK sticks and are labelled using the Ballesteros-Weinstein convention (Ballesteros and Weinstein, 1995). In (b) a modelled isoprenaline (gray CPK sticks) binding pose is shown, including polar contacts (broken lines) between the agonist and the propanolamine clamp $D^{3.32}$, $N^{7.39}$ (D138 and N363 in the β1-adrenoceptor, respectively) and the catecholamine recognition site on TM5, *i.e.* $S^{5.42}$, $S^{5.43}$, and $S^{5.46}$ (S228, S229, and S232 in the β1-adrenoceptor, respectively).

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	Bmax (fmoles/mg protein)	n
WT	510 ± 59	9
D104A	818 ± 102	8
D104N	1296 ± 140	7
D138A	No ^{3}H-CGP 12177 binding	3
D138E	No ^{3}H-CGP 12177 binding	3
D138H	No ^{3}H-CGP 12177 binding	3
D138N	No ^{3}H-CGP 12177 binding	3
D138S	No ^{3}H-CGP 12177 binding	3
S228A	818 ± 78	8
S229A	443 ± 66	6
S232A	582 ± 55	6
F341A	699 ± 37	8
N344A	436 ± 63	8
N363A	No ^{3}H-CGP 12177 binding	7
N363C	No ^{3}H-CGP 12177 binding	3
N363F	No ^{3}H-CGP 12177 binding	3
N363T	No ^{3}H-CGP 12177 binding	3
D138A + N363A	No ^{3}H-CGP 12177 binding	4

Table 1
Bmax values (mean ± s.e.m. in fmol/mg protein) obtained from saturation experiments on the various stable mixed populations used throughout this study. n = the number of stable mixed populations made for each given receptor mutant and the wild type (WT) β1-adrenoceptor.

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mutation	K_D ^{3}H-CGP 12177	n		Log K_D CGP 20712A	n		Log K_D propranolol	n		Log K_D isoprenaline	n		Log K_D cimaterol	n
Wildtype β1	0.31 ± 0.04nM	11		-8.97 ± 0.04	7		-7.94 ± 0.10	4		-6.09 ± 0.06	7		-6.31 ± 0.07	4
D104A	0.37 ± 0.03nM	10		-8.36 ± 0.07	8		-7.99 ± 0.07	4		-5.75 ± 0.03	7		-6.09 ± 0.06	4
D104N	0.51 ± 0.03nM	10		-8.54 ± 0.05	8		-8.06 ± 0.06	4		-5.86 ± 0.07	7		-6.20 ± 0.06	4
S228A	7.92 ± 0.33nM	15		-9.17 ± 0.07	17		-9.42 ± 0.04	16		-5.00 ± 0.04	17		-6.95 ± 0.05	16
S229A	2.20 ± 0.28nM	15		-9.36 ± 0.09	4		-7.89 ± 0.05	5		-5.16 ± 0.10	6		-5.97 ± 0.08	6
S232A	0.30 ± 0.03nM	15		-9.44 ± 0.11	7		-8.16 ± 0.06	6		-5.11 ± 0.06	6		-6.01 ± 0.08	6
F341A	1.66 ± 0.09nM	15		-7.89 ± 0.10	15		-7.97 ± 0.03	16		-6.05 ± 0.05	16		-6.14 ± 0.02	16
N344A	1.59 ± 0.17nM	14		-8.87 ± 0.09	6		-8.94 ± 0.09	6		-5.32 ± 0.09	6		-5.92 ± 0.07	6

Table 2

K_D and log K_D values from ^{3}H-CGP 12177 whole cell binding experiments in the stable mixed population of each construct.
The K_D values for ^{3}H-CGP 12177 were calculated from the saturation experiments. The log K_D values for the other ligands were calculated from the IC_{50} values obtained from competition experiments and converted to K_D values as outlined in the methods. There was no specific binding to any of the D138 or N363 mutants. There was also no specific binding seen when D138A and N363A were co-transfected. Values are mean ± s.e.m. from separate experiments. The total number of stable populations of the mutant are shown in Table 1.

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mutation	Log EC_{50} isoprenaline	% forskolin	n		Log K_D CGP20712A	n		Log K_D propranolol	n		*Log K_D CGP 12177	**Log K_D CGP 12177	n
Wildtype	-7.80 ± 0.16	66.2 ± 3.3	12		-8.65 ± 0.19	10		-7.80 ± 0.21	6		-9.18 ± 0.14	-9.13 ± 0.14	9
D104A	-6.59 ± 0.20	65.7 ± 5.4	11		-7.94 ± 0.15	11		-7.57 ± 0.10	5		-8.93 ± 0.12	-8.86 ± 0.11	9
D104N	-6.63 ± 0.13	55.4 ± 4.2	12		-8.65 ± 0.09	11		-7.97 ± 0.15	6		-9.14 ± 0.15	-9.19 ± 0.08	10
S228A	-6.65 ± 0.11	66.7 ± 2.0	16		-8.64 ± 0.10	29		-8.84 ± 0.06	28		-8.08 ± 0.10	-7.92 ± 0.10	21
S229A	-6.97 ± 0.09	91.6 ± 8.8	9		-8.93 ± 0.18	9		-7.28 ± 0.14	8		-8.37 ± 0.05	-8.24 ± 0.07	7
S232A	-6.90 ± 0.15	86.6 ± 13.0	9		-9.35 ± 0.17	9		-7.96 ± 0.10	9		-8.87 ± 0.20	-8.93 ± 0.22	5
F341A	-7.41 ± 0.06	65.0 ± 1.7	15		-7.56 ± 0.10	14		-7.65 ± 0.10	18		-8.38 ± 0.04	-8.26 ± 0.05	29
N344A	-7.05 ± 0.14	95.0 ± 12.1	9		-8.19 ± 0.17	9		-8.59 ± 0.26	9		-8.56 ± 0.08	-8.58 ± 0.13	6
N363A	-5.44 ± 0.06	56.5 ± 8.2	8		-6.36 ± 0.13	4							
N363C	-4.96 ± 0.04	43.5 ± 1.4	4		-6.52 ± 0.04	3							
N363F	No response		3										
N363T	-4.65 ± 0.07	32.3 ± 5.9	4										
D138A+N363A	-5.57 ± 0.20	68.4 ± 12.4	5		No inhibition			No inhibition			No inhibition		

Table 3

Log EC_{50} values and % maximum of 10µM forskolin response for isoprenaline-stimulated responses in stable mixed populations expressing each mutant. Log K_D values for CGP 20712A, propranolol and CGP 12177 obtained from parallel shifts of the isoprenaline response are also shown. No agonist response to isoprenaline was seen in any of the D138 mutants. Values are mean ± s.e.m. from separate experiments. The total number of stable populations of the mutants are shown in Table 1.

* calculated by the partial agonist method Stephenson (1956) which assumes the two ligands are competing at the same site

** calculated from a parallel shift of the top part of the curve thus allowing for the CGP 12177 agonist response to be occurring via a different site to the agonism of isoprenaline

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mutation	Log EC_{50} cimaterol	% forskolin	n		Log K_D CGP20712A	n		Log K_D propranolol	n		*Log K_D CGP 12177	**Log K_D CGP 12177	n
WT	-8.23 ± 0.06	62.2 ± 4.1	12		-9.22 ± 0.05	15		-8.53 ± 0.08	8		-9.97 ± 0.12	-9.73 ± 0.09	5
D104A	-7.12 ± 0.05	40.5 ± 3.2	12		-8.59 ± 0.15	11		-8.62 ± 0.09	8		-9.70 ± 0.21	-9.66 ± 0.21	4
D104N	-6.95 ± 0.11	17.2 ± 2.2	11										
S228A	-8.67 ± 0.06	62.2 ± 1.8	16		-8.95 ± 0.09	30		-9.48 ± 0.04	29		-8.38 ± 0.05	-8.28 ± 0.05	27
S229A	-7.48 ± 0.07	68.3 ± 6.4	12		-9.87 ± 0.06	7		-8.24 ± 0.19	6		-8.76 ± 0.14	-8.54 ± 0.15	7
S232A	-7.96 ± 0.08	69.3 ± 6.3	15		-10.05 ± 0.09	10		-8.66 ± 0.11	11		-10.06 ± 0.14	-9.94 ± 0.17	7
F341A	-7.59 ± 0.03	57.7 ± 2.0	14		-7.76 ± 0.05	16		-8.38 ± 0.09	12		-8.78 ± 0.05	-8.64 ± 0.05	25
N344A	-7.45 ± 0.13	73.8 ± 5.4	14		-8.96 ± 0.07	11		-9.14 ± 0.10	13		-8.69 ± 0.13	-8.44 ± 0.11	9
N363A	-6.02 ± 0.11	26.0 ± 9.2	5										
D138A+N363A	-6.40 ± 0.17	30.7 ± 3.3	5										

Table 4

Log EC_{50} values and % maximum of 10µM forskolin response for cimaterol-stimulated responses in stable mixed populations expressing each mutant. Log K_D values for CGP 20712A, propranolol and CGP 12177 obtained from parallel shifts of the cimaterol response are also shown. No agonist response to cimaterol was seen in any of the D138 mutants, or to N363C, N363F or N363T mutant. Values are mean ± s.e.m. from separate experiments. The total number of stable populations of the mutant are shown in Table 1.

* calculated by the partial agonist method Stephenson (1956) which assumes the two ligands are competing at the same site

** calculated from a parallel shift of the top part of the curve thus allowing for the CGP 12177 agonist response to be occurring via a different site to the agonism of cimaterol

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	Log EC_{50}CGP 12177	% forskolin	n		Log K_D CGP 20712A	n		Log K_D propranolol	n
WT	-8.12 ± 0.04	32.9 ± 1.9	33		-7.26 ± 0.10	25		-6.12 ± 0.07	29
D104A	-7.57 ± 0.16	8.01 ± 1.1	20		-6.75 ± 0.15	5			
D104N	-6.89 ± 0.19	6.58 ± 0.09	10						
S228A	-6.93 ± 0.05	30.5 ± 0.8	16		-7.45 ± 0.08	29		-7.40 ± 0.06	30
S229A	-7.29 ± 0.12	46.1 ± 6.4	13		-7.70 ± 0.11	14		-6.48 ± 0.11	7
S232A	-8.66 ± 0.09	46.1 ± 4.3	15		-8.03 ± 0.08	16		-6.86 ± 0.14	4
F341A	-8.11 ± 0.04	26.7 ± 1.8	14		-7.58 ± 0.10	27		-7.07 ± 0.06	27
N344A	-7.95 ± 0.07	30.0 ± 2.6	29		-8.21 ± 0.10	29			
D138A+N363A	No response		10						

Table 5
Log EC_{50} values and % maximum of 10µM forskolin response for CGP 12177-stimulated responses in stable mixed populations expressing each mutant. Log K_D values for CGP 20712A and propranolol were obtained from parallel shifts of the CGP 12177 response are also shown. No agonist response to CGP 12177 was seen in any of the D138 or N363 mutants. Values are mean ± s.e.m. from separate experiments. The total number of stable populations of the mutants are shown in Table 1.

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Figure 1a

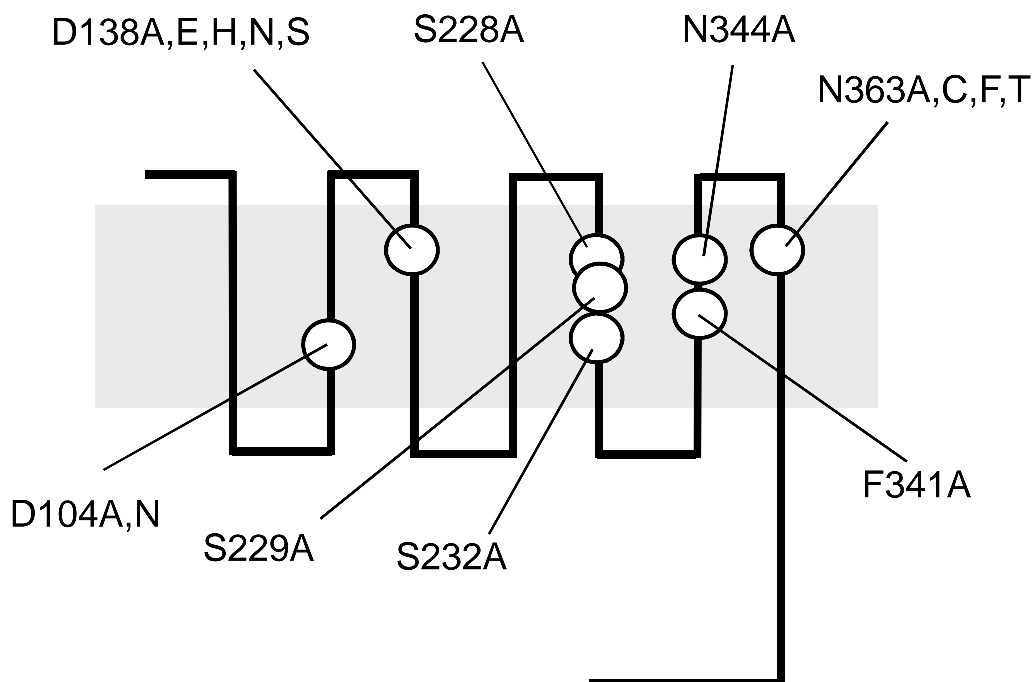

Figure 1b

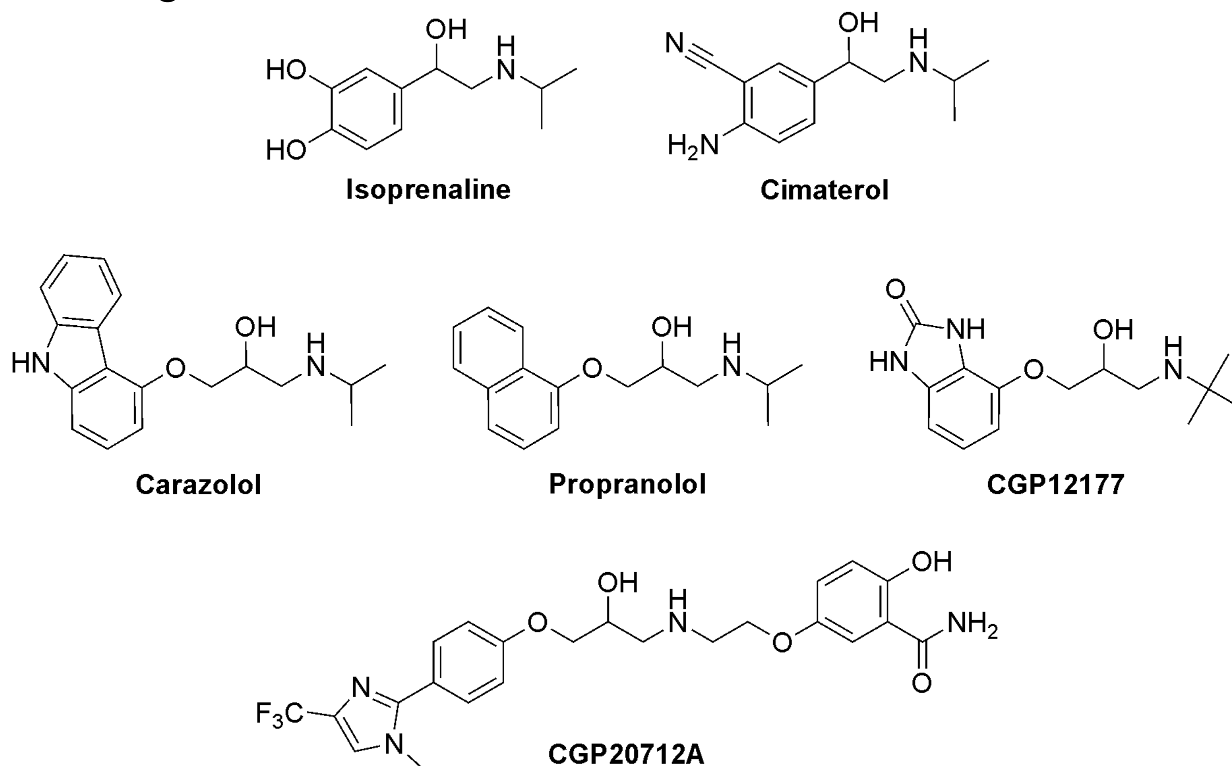

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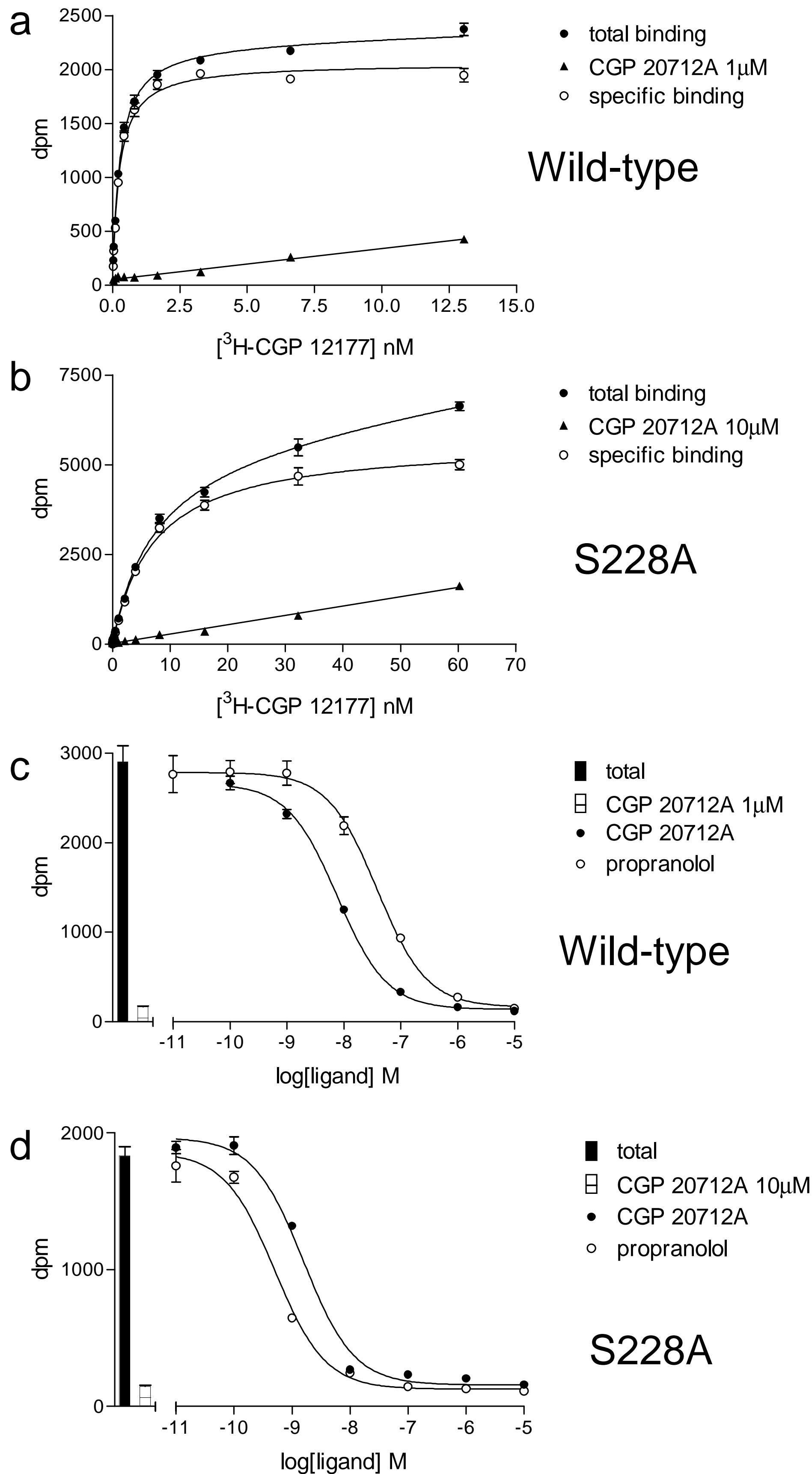
Figure 2



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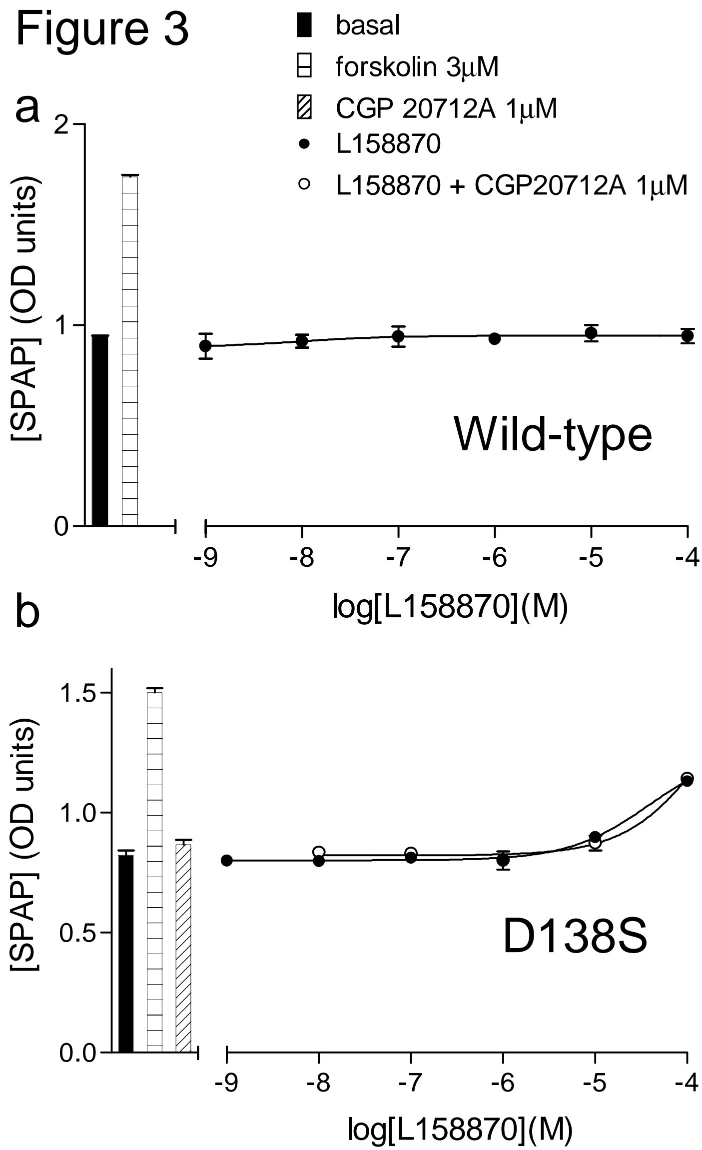

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Figure 4

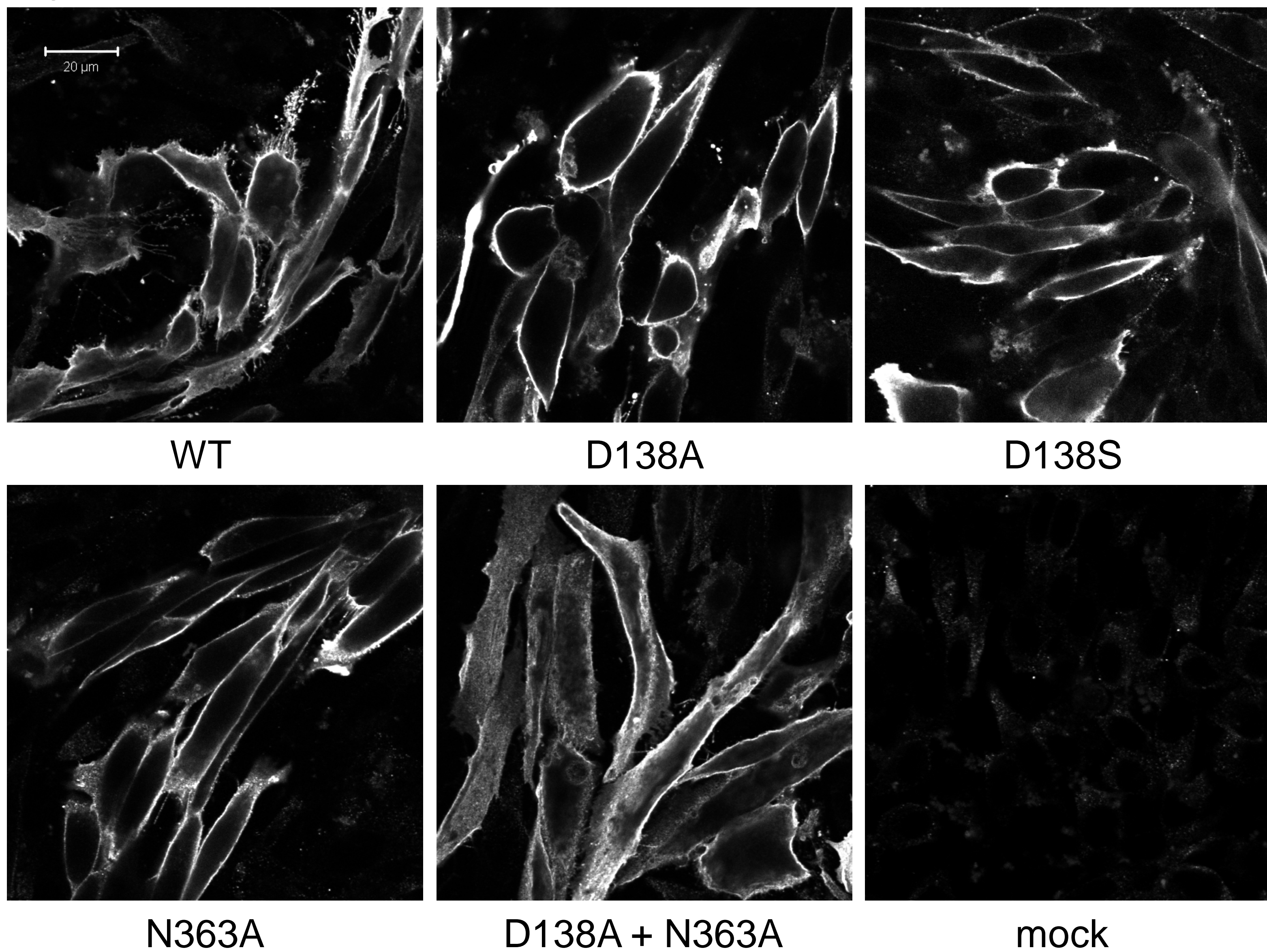

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Figure 5

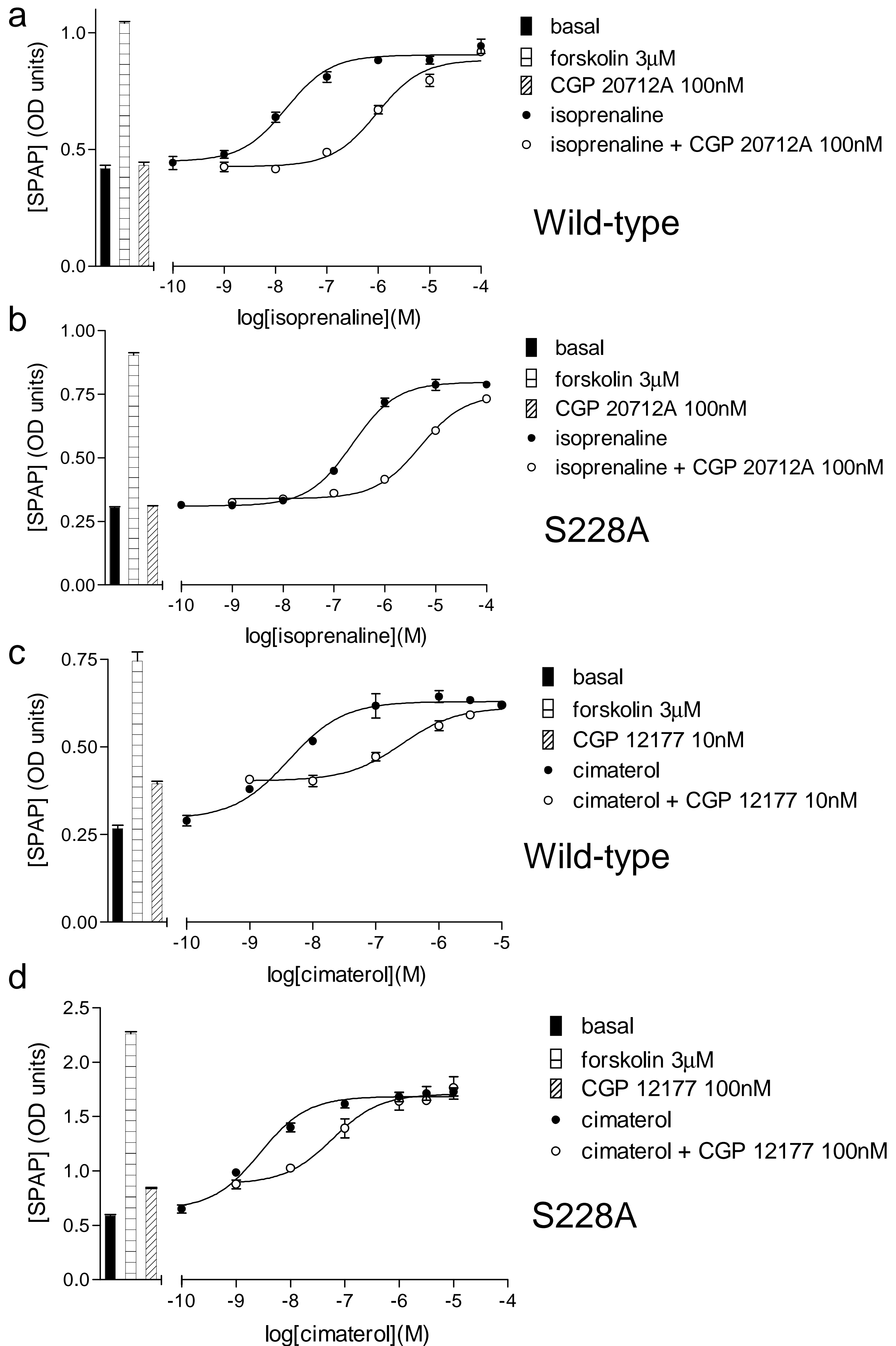

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Figure 6

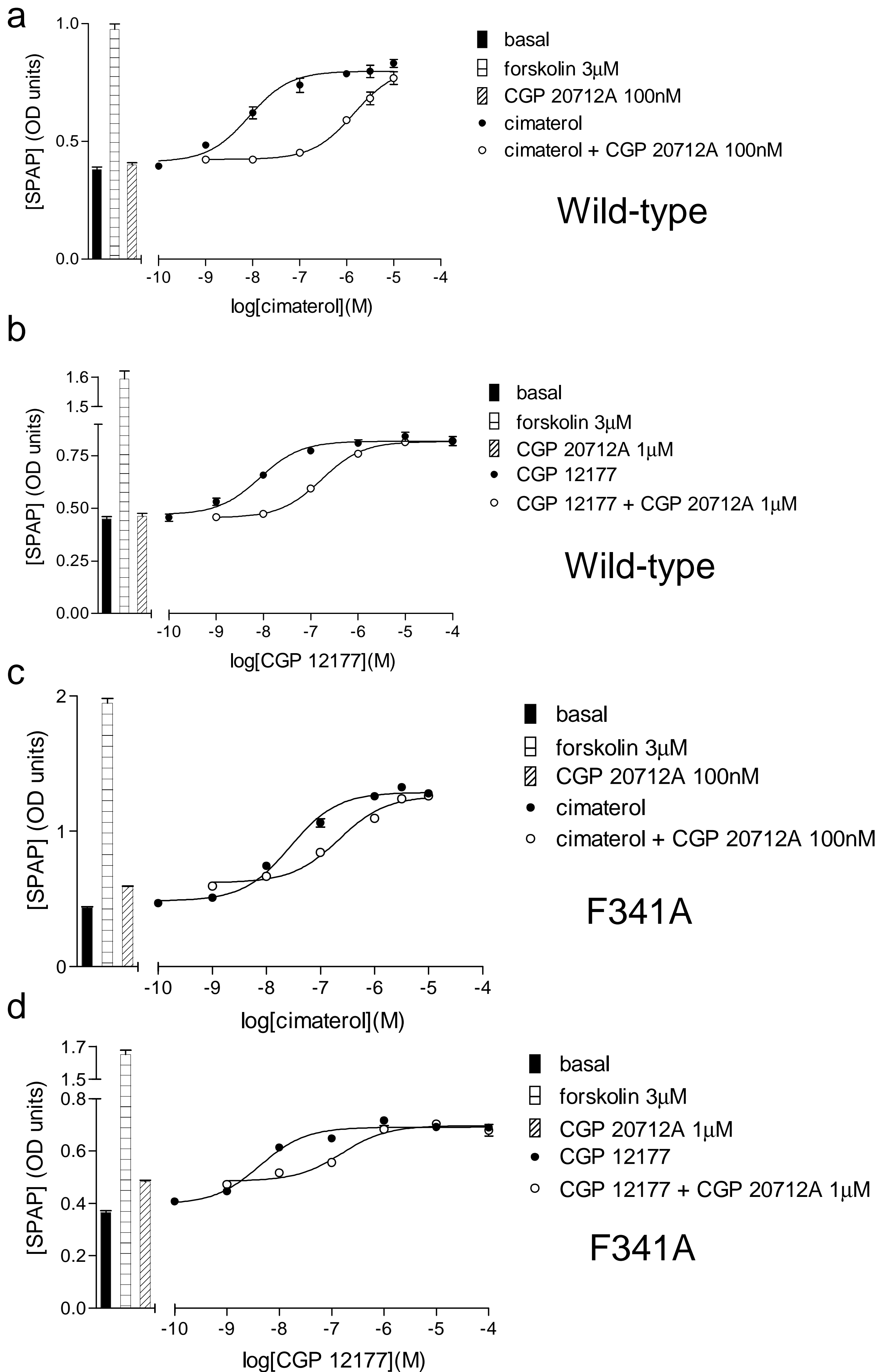

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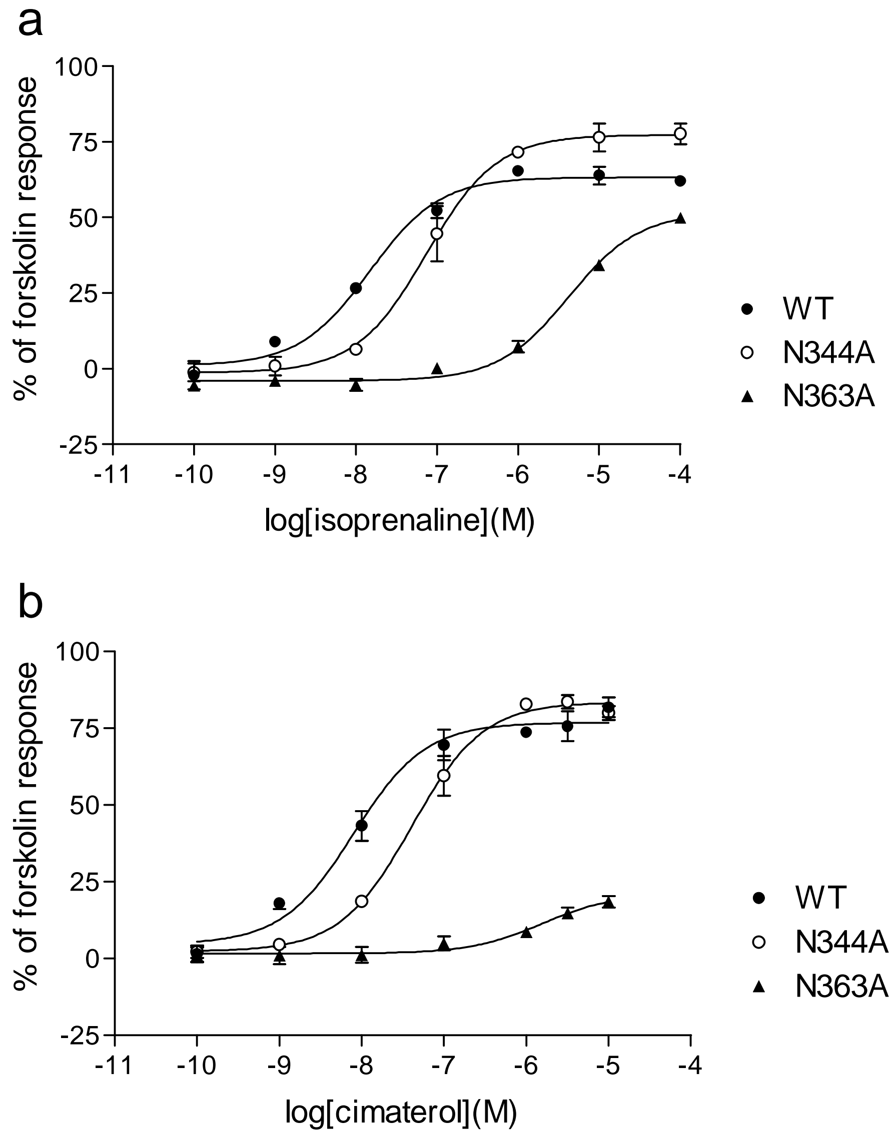
Figure 7



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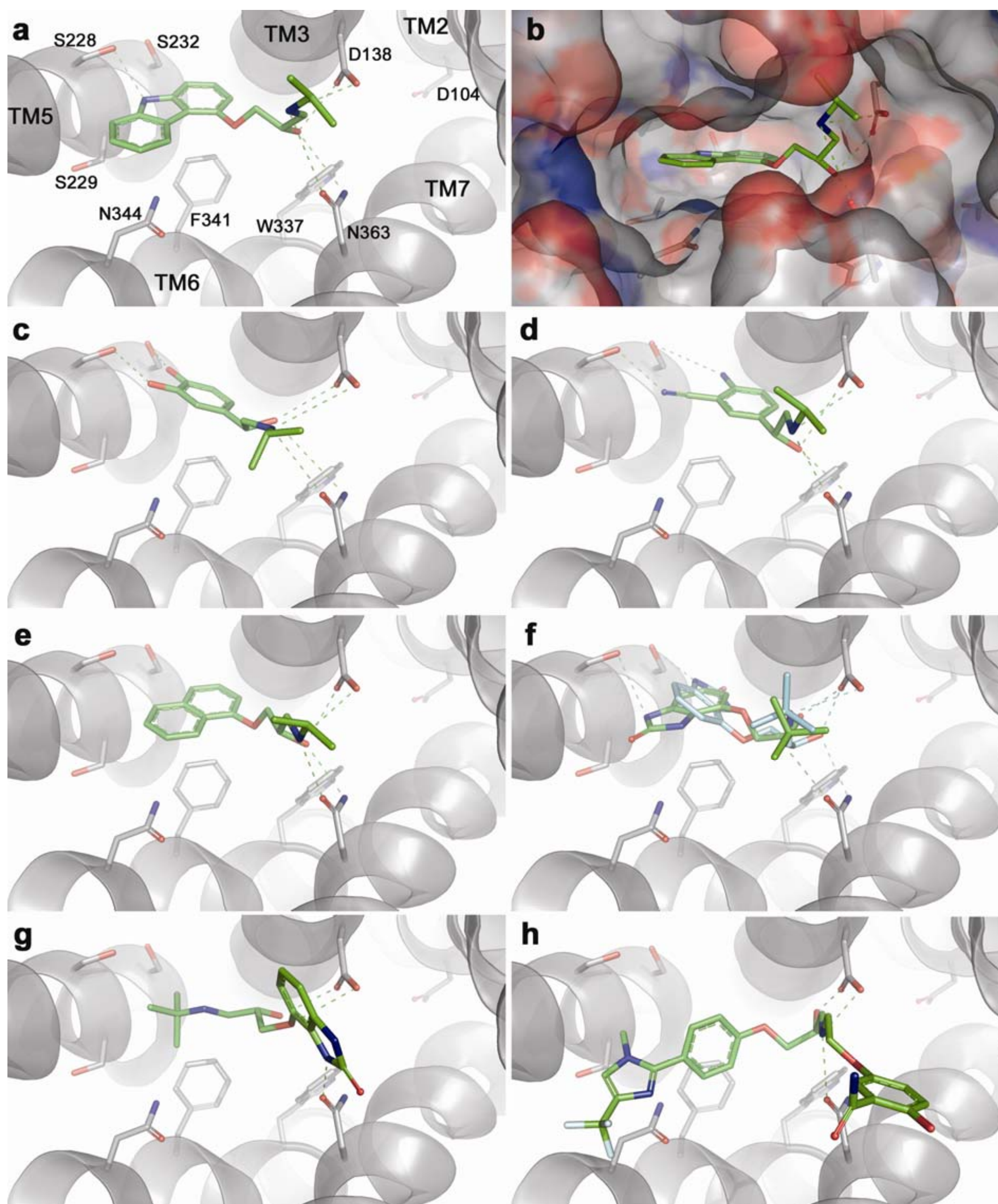

Figure 8

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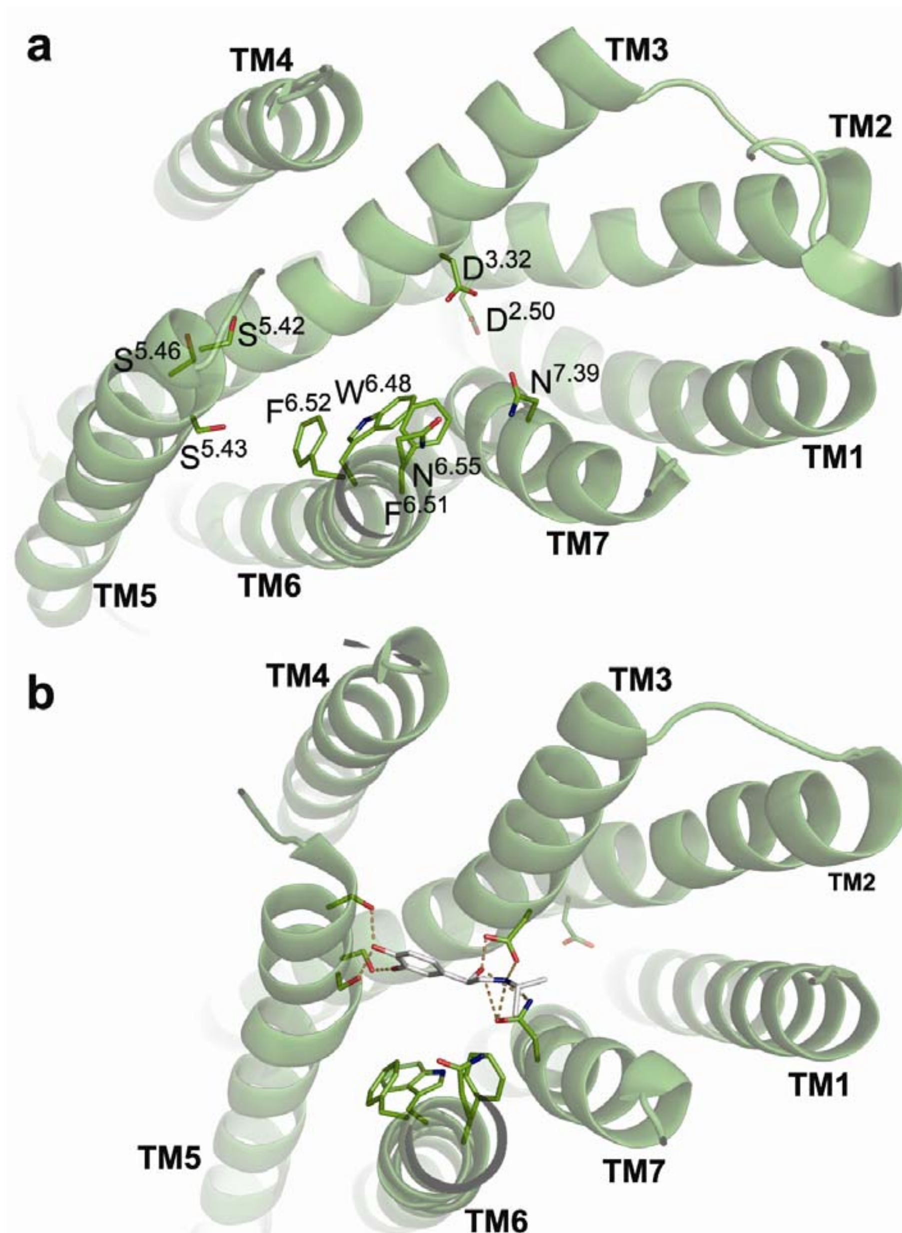

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Figure 9