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Role of Key Transmembrane Residues in Agonist and Antagonist Actions at the Two Conformations of the Human β1-Adrenoceptor[S]

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ABSTRACT

Studies with 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride (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 (Asp104, Asp138, Ser228, Ser229, Ser232, Phe341, Asn344 and Asn363) have been mutated to provide structural insights into the nature of these conformations. [^{3}H]CGP 12177 binding and cAMP response element-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 Asn363 and Asp138 were key residues for binding of agonists and antagonists, and they were also essential for the agonist actions of CGP 12177. S228A and S229A in transmembrane-spanning region (TM) 5 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 Asp138 and Asn363 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. Modeling 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 Asp138 and Asn363.

The β1 and β2-adrenoceptors are classic 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 classic "catecholamine" binding site or conformation of the β1-adrenoceptor; at higher concentrations, however, it activates a secondary site or conformation and produces an agonist response that is relatively resistant to antagonism by other classic 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 have come from site-directed mutagenesis studies of the β2-adrenoceptor. The main binding conformations for catecholamines at the β2-adrenoceptor have been identified as Asp113 in trans-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CGP 12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride; CGP 20712A, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; TM, transmembrane-spanning region; L-158,870, 1-(3′4′-dihydroxylphenyl)-3-methyl-1-butanone; WT, wild type; CHO, Chinese hamster ovary; CRE, cAMP response element; SPAP, secreted placental alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; F12, nutrient mix F12; PBS, phosphate-buffered saline.

membrane-spanning region (TM) 3, Ser204, Ser207 (and probably Ser203) in TM5, and Asn293 in TM6 (Savarese and Fraser, 1992; Sato et al., 1999). In the human β2-adrenoceptor, Asp113 in TM3 has been shown to be the anchor point for the protonated amine function of such catecholamines as isoprenaline and adrenaline (Savarese amd Fraser, 1992; Sato et al., 1999). Mutation of Asp113 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-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 traditionally been associated with Ser204 and Ser207 (TM5), respectively (Savarese and Fraser, 1992; Sato et al., 1999). However, there is evidence that Ser203 may also (in addition to Ser204) 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 the heterocyclic ring structure (e.g., pindolol and CGP 12177; Liapakis et al., 2000). 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 ligand interactions at the β2-adrenoceptor. Asp79 in TM2 of the β2-adrenoceptor has been shown to produce different effects on agonist and antagonists properties (Chung et al., 1988; Strader et al., 1988). Phe290 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" that allows receptor activation as a consequence of relative movements in the positions of TM3 and TM6 during agonist stimulation (Shi et al., 2002). Finally, Asn312 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). It is noteworthy that the human 5-HT1A receptor has an asparagine residue in an homologous position in TM7 that is responsible for high-affinity binding of aryloxypropanolamine β-antagonists to the 5-HT1A receptor (Guan et al., 1992; Kuipers et al., 1997). 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 residues analogous to those described above for the human β2-adrenoceptor in TMs 2, 3, 5, 6, and 7 (Fig. 1). Here, a series of point mutations was made in the human β1-adrenoceptor, and experiments were 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 were affected by these mutations.

Materials and Methods

Materials

Fetal calf serum was from PAA Laboratories (Teddington, Middlesex, UK). [^{3}H]CGP 12177 was obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Cimaterol, CGP 20712A, and isoprenaline were from Tocris Cookson (Avonmounth, Bristol, UK). Lipofectamine, hygromycin, Opti-MEM, and neomycin were from Invitrogen (Paisley, UK). Propranolol, CGP 12177, and all other cell culture reagents were from Sigma Chemicals (Poole, Dorset, UK). L158,870 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 (GlaxoSmithKline, Stevenage, UK). This cDNA was subcloned as a HindIII/XbaI fragment into pcDNA3.1 (Invitrogen), and the sequence was confirmed by DNA sequencing. The wild-type β1-adrenoceptor sequence was confirmed to contain the Ser49 and Gly389 polymorphisms. Mutations (as described in Fig. 1a) were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and Bioline PolyMate Additive for GC-rich templates. Mutants are named as (wild-type residue) (residue number) (mutant residue). Sequence of primers (5′ to 3′) designed to synthesize two complimentary oligonucleotides containing the relevant mutations are provided in Supplemental Information. After subcloning in Top F′ competent cells (Invitrogen), the mutant β1-adrenoceptor cDNA was excised on HindIII/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 Supplemental Informa-

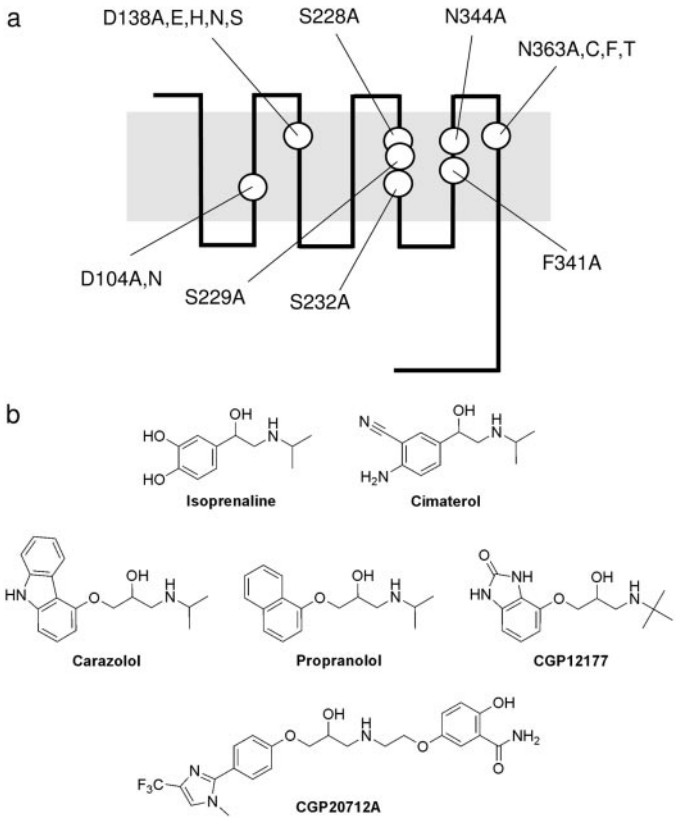

Fig. 1. a, schematic representation on the human β1-adrenoceptor showing sites where mutations were made. b, chemical structures of adrenoceptor ligands discussed in this study.

tion) comprising a HindIII 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 HindIII/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 were subcloned into the 5′myc-β1-adrenoceptor pcDNA3.1 vector at OliI/XbaI. All mutations and sequences were confirmed by DNA sequencing.

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 Eagle's medium (DMEM)/nutrient mix F12 (F12) containing 10% fetal calf serum and 2 mM L-glutamine in a humidified 5% CO_2/95% air atmosphere at 37°C. A tissue culture flask T75 of these cells was transfected for 24 h with 10 ml of Opti-MEM, 100 μl of lipofectamine, and 10 μg 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 to 3 weeks using resistance to neomycin (1 mg/ml; for β1 receptor or mutant) and hygromycin (200 μg/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 h 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, 5 μg of DNA for D138A on a neomycin marker and 5 μg of DNA for N363A on a Zeocin selection marker were cotransfected. Cells were selected for 3 weeks in neomycin (1 mg/ml; for the D138A mutant), Zeocin (500 μg/ml; for the N363A mutant), and hygromycin (200 μg/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 h. The transfection media were then removed, and the cells were allowed to recover in media for 24 h. The cells were then plated into white-sided 96-well view plates for binding experiments on the following day. Thus, these experiments were performed on transiently transfected cells.

TABLE 1

B_{max} values (mean ± S.E.M.) obtained from saturation experiments on the various stable mixed populations used throughout this study

	B_{max}	n
	fmol/mg protein	
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

n = the number of stable mixed populations made for each given receptor mutant and the wild-type (WT) β1-adrenoceptor.

[^{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 before experimentation, the media were removed from each well. For saturation binding studies, 100 μl of serum-free media (i.e., DMEM/F12 containing 2 mM L-glutamine only) or serum-free media containing 2 to 20 μM CGP 20712A were added to each well, immediately followed by 100 μl of serum-free media containing [^{3}H]CGP 12177. This gave final well concentrations of 1 to 10 μM CGP 20712A (to define nonspecific binding) and [^{3}H]CGP 12177 in the range of 0.018 to 76.63 nM. In competition studies, 100 μl of serum-free media containing the competing ligand were added and immediately followed by the addition of 100 μl of serum-free media containing [^{3}H]CGP 12177 (to give a final [^{3}H]CGP 12177 concentration of 0.90–5.85 nM). Total and nonspecific binding (as defined by 1–10 μM CGP 20712A) were measured in every experiment. All cells for both the saturation and competition studies were then incubated for 2 h at 37°C in a humidified 5% CO_2/95% air atmosphere. After 2 h, the media and drugs were removed and the cells washed twice by the addition and removal of 200 μl of 4°C PBS/well. A white base was then added to the plate, followed by 100 μl of Microscint 20 (PerkinElmer Life and Analytical Sciences, Waltham, MA) per well and a sealant film placed over the wells. The plates were left overnight at room temperature in the dark, then counted the following day on a TopCount liquid scintillation counter (PerkinElmer Life and Analytical Sciences) for 2 min 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 were removed and replaced with 100 μl of serum-free media (i.e., DMEM/F12 containing 2 mM L-glutamine), and the cells were incubated for a further 24 h. On the day of experimentation, the serum-free media was removed and replaced with 100 μl of serum-free media or 100 μl of serum-free media containing antagonists at the final required concentration, and the cells were incubated for 30 min at 37°C (5% CO_2). Agonists in 10 μl (diluted in serum free media) were then added to each well, and the plate was incubated at 37°C for 5 h (5% CO_2). After 5 h, the media and drugs were removed, 40 μl of serum-free media were added to each well, and the cells were incubated for a further 1 h at 37°C. The plates were then incubated at 65°C for 30 min to destroy any endogenous phosphatases. The plates were then cooled to 37°C. One hundred microliters of 5 mM 4-nitrophenyl phosphate in diethanolamine buffer was added to each well, and the plates were incubated at 37°C until the yellow color developed. The plates were then read on a Dynatech MRX plate reader at 405 nm.

Confocal Immunocytochemistry Localization/Detection of Myc-Tagged Receptors

CHO-CRE-SPAP cells were seeded into six-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 h (with 2 ml of Opti-MEM, 20 μl of lipofectamine, and 2 μg of DNA per well). A double transfect of N-terminal myc-D138A with untagged N363A was also performed. The control mock transfection contained Opti-MEM and lipofectamine but no DNA. After 24 h, the cells were washed and left for 24 h to recover in media.

The following day, the cells were washed with 1 ml of PBS per well, then fixed with 1 ml of 4% formaldehyde per well for 20 min at room temperature. The cells were washed three times with PBS then blocked with 1 ml of PBS containing 3% bovine serum albumin and 1% glycine per well for 20 min at room temperature. The cells were washed three times with PBS then with 1 ml of 10% goat serum (in PBS) added to each well for 20 min 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 were left at 4°C overnight. The next day, the cells were washed three times with PBS; then, 1 ml of secondary antibody (goat anti-mouse rhodamine red-X at 1:500 in 10% goat serum) was added to each well, and the wells were left in the dark at room temperature for 1 h. The cells were then washed three times with PBS, then the coverslips removed from the wells and mounted on glass slides using 10 μl of a 50/50 glycerl/PBS mix. The coverslips were then examined on a Zeiss LSM510 laser scanning microscope with a Zeiss 40 $\times$ 1.3 numerical aperture oil immersion lens, using a HeNe laser emitting at 543 nm on 100% power, a pinhole of 1 Airey unit, and capturing all fluorescence emission passing through a long-pass filter at 560 nm.

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 Prism 2 (GraphPad Software, San Diego, CA): Specific binding = $B_{max} \times \{[[^3H]CGP]/([[^3H]CGP] + K_D)\}$. 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: % of specific binding = $100 - \{(100 \times [A])/([A] + IC_{50})\}$, where [A] is the concentration of competing ligand and IC_{50} is the concentration that inhibits specific binding by 50%. Ligand dissociation constants (K_D) were then determined from the following expression: $K_D = \{(IC_{50} \times K_{D\text{-}CGP})/(K_{D\text{-}CGP} + [[^3H]CGP])\}$, where [[^{3}H]CGP] and $K_{D\text{-}CGP}$ are the concentration and dissociation constants 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 nonlinear regression using Prism 2: Response = $\{(E_{max} \times [A])/EC_{50} + [A])\}$, where E_{max} is the maximal response, [A] is the agonist concentration, and EC_{50} is the concentration of agonist that produces 50% of the maximal response.

Antagonist 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 equation $DR = 1 + ([B]/K_D)$, where DR (dose ratio) is the ratio of the agonist concentration required to stimulate an identical response in the presence and absence of a fixed concentration of antagonist [B].

In cases in which clear partial agonism was seen, the antagonist (partial agonist) affinity was initially calculated by the method of Stephenson (1956) using the equation K_D partial agonist = $(Y \times [P])/1 - Y$, where $Y = \{([A_2] - [A_1])/[A_3]\}$, [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 $[A_3]$ is 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 in which 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 3 μM (maximal) forskolin concentration was included in each CRE-gene transcription plate of each separate 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.

Molecular Modeling. 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 programs 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). Models of inactive and active conformations of the β2-adrenoceptor (Fig. 9) were kindly provided by Professor C.A. Reynolds, Department of Biological Sciences, University of Essex, Colchester, UK (Gouldson et al., 2004). Three-dimensional structure illustrations (Figs. 8 and 9) were prepared using the PyMOL program (http://www.pymol.org).

Results

All stable mixed populations were investigated by whole cell binding (Tables 1 and 2) and in a CRE-reporter functional assay (Table 3–5). The effects of three agonists were investigated: 1) isoprenaline, the classic catecholamine (site 1) agonist; 2) cimaterol, a more site 1-selective agonist (Baker 2005b); and 3) CGP 12177, the classic 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 $\pm$ 0.04 nM, n = 11; Fig. 2a, Table 2). This result is similar to that in 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 (Fig. 2c, Table 2; Baker, 2005b). When the function of all three agonists was examined, CGP 12177 was able to stimulate a lower maximum response compared with 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 was expected based on 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

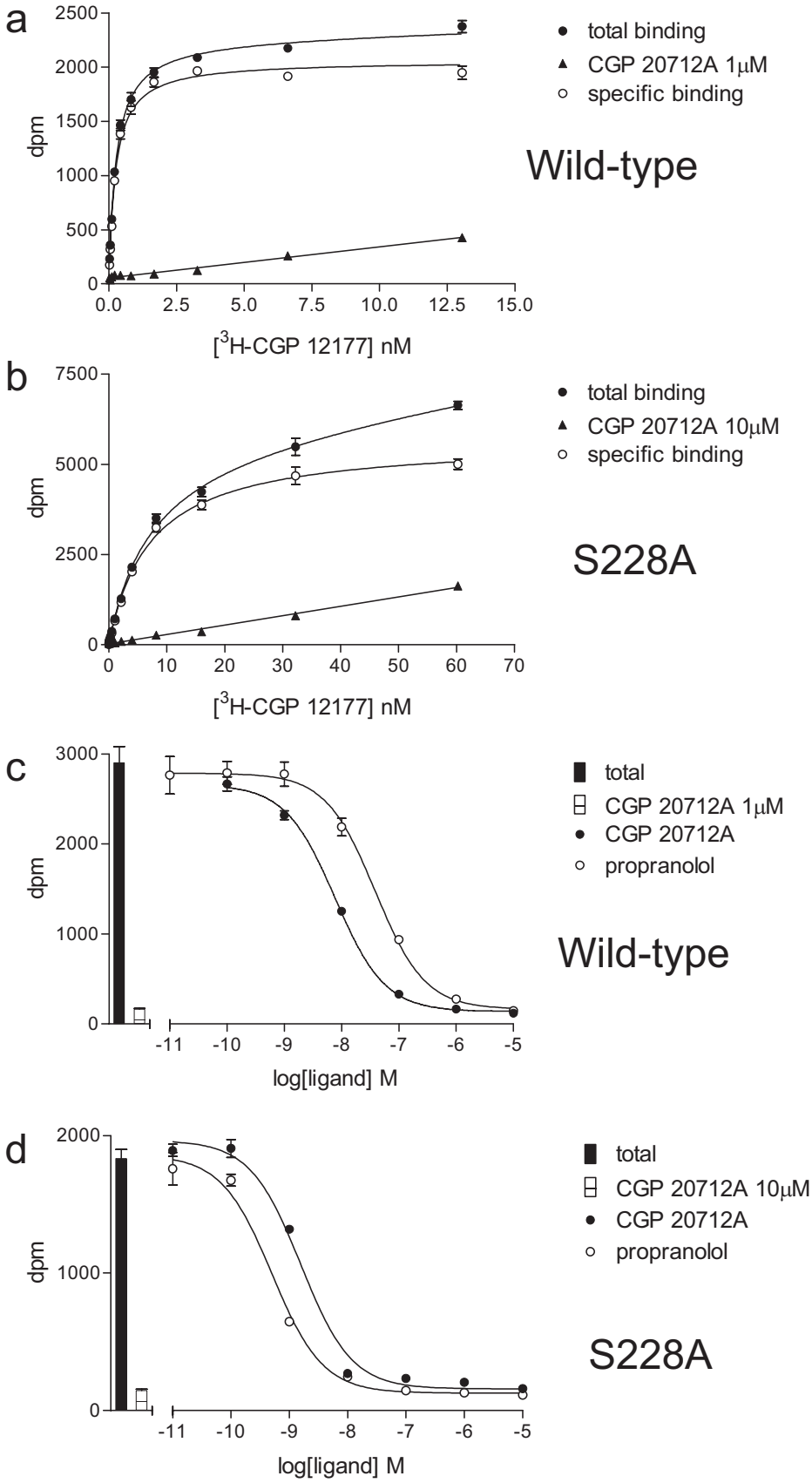

Fig. 2. Whole-cell [^{3}H]CGP 12177 binding to cells expressing the wild-type human β1-adrenoceptor (a and c) or the S228A mutation (b and d). a and b, whole-cell [^{3}H]CGP 12177 saturation binding. Graphs show total binding, nonspecific binding (as determined by the presence of 1 μM CGP 20712A (a) or 10 μM CGP 20712A (b) and the resultant specific binding. Data points are mean ± S.E.M. of quadruplicate determinations from a single experiment that 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 and d, whole-cell [^{3}H]CGP 12177 competition binding. Bars represent total binding and nonspecific binding as determined in the presence of 1 μM CGP 20712A (c) and 10 μM CGP 20712A (d). Data points are mean ± S.E.M. of triplicate determinations from a single experiment that is representative of 4 (c) and 16 (d) separate experiments. The concentrations of [^{3}H]CGP 12177 used were 1.75 nM (c) and 5.25 nM (d). It can be seen that although CGP 20712A is more potent in the wild-type human β1-adrenoceptor, the single S228A mutation results in propranolol becoming the more potent.

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). In addition, 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 define 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).

TABLE 2

K_D and log K_D values from [^{3}H]CGP 12177 whole cell binding experiments in the stable mixed populations 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 Asp138 or Asn363 mutants. There was also no specific binding seen when D138A and N363A were cotransfected. Values are mean ± S.E.M. from separate experiments. The total number of stable populations of the mutant is shown in Table 1.

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 ± 0.04 nM	11	−8.97 ± 0.04	7	−7.94 ± 0.10	4	−6.09 ± 0.06	7	−6.31 ± 0.07	4
D104A	0.37 ± 0.03 nM	10	−8.36 ± 0.07	8	−7.99 ± 0.07	4	−5.75 ± 0.03	7	−6.09 ± 0.06	4
D104N	0.51 ± 0.03 nM	10	−8.54 ± 0.05	8	−8.06 ± 0.06	4	−5.86 ± 0.07	7	−6.20 ± 0.06	4
S228A	7.92 ± 0.33 nM	15	−9.17 ± 0.07	17	−9.42 ± 0.04	16	−5.00 ± 0.04	17	−6.95 ± 0.05	16
S229A	2.20 ± 0.28 nM	15	−9.36 ± 0.09	4	−7.89 ± 0.05	5	−5.16 ± 0.10	6	−5.97 ± 0.08	6
S232A	0.30 ± 0.03 nM	15	−9.44 ± 0.11	7	−8.16 ± 0.06	6	−5.11 ± 0.06	6	−6.01 ± 0.08	6
F341A	1.66 ± 0.09 nM	15	−7.89 ± 0.10	15	−7.97 ± 0.03	16	−6.05 ± 0.05	16	−6.14 ± 0.02	16
N344A	1.59 ± 0.17 nM	14	−8.87 ± 0.09	6	−8.94 ± 0.09	6	−5.32 ± 0.09	6	−5.92 ± 0.07	6

TABLE 3

Log EC_{50} values and percentage maximum of 10 μM 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 Asp138 mutants. Values are mean ± S.E.M. from separate experiments. The total number of stable populations of the mutants are shown in TABLE 1.

Mutation	Log EC_{50} isoprenaline	Forskolin	*n*	Log K_D CGP20712A	*n*	Log K_D propranolol	*n*	Log K_D CGP 12177[a]	Log K_D CGP 12177[b]	*n*
		%								
Wild type	−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	N.I.		N.I.		N.I.		

N.I., no inhibition.

[a] Calculated by the partial agonist method of Stephenson (1956), which assumes that the two ligands are competing at the same site.

[b] 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 from the agonism of isoprenaline.

TABLE 4

Log EC_{50} values and percentage maximum of 10 μM 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 Asp138 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 is shown in Table 1.

Mutation	Log EC_{50} cimaterol	Forskolin	*n*	Log K_D CGP20712A	*n*	Log K_D propranolol	*n*	Log K_D CGP 12177[a]	Log K_D CGP 12177[b]	*n*
		%								
Wild type	−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							

[a] Calculated by the partial agonist method of Stephenson (1956), which assumes that the two ligands are competing at the same site.

[b] 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 from the agonism of cimaterol.

Transmembrane 2 Mutations

Aspartate 104. The aspartate at position 79 of the human β2-adrenoceptor is important for agonist binding (Chung et al., 1988; Strader 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 were 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 G_s protein. It is noteworthy that the B_{max} values from [^{3}H]CGP 12177 binding to site 1 were in the order (Table 1) opposite the change in efficacy (D104N>D104A>WT), indicating that this was not a consequence of a reduced receptor reserve. Thus mutations at position Asp104 of the β1-adrenoceptor do not significantly alter the binding of ligands (including agonist binding) to the receptor, but the coupling to signaling cascades is reduced.

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 100 μM), cimaterol (to concentrations of 10 μM), or CGP 12177 (to concentrations of 100 μM). Strader et al. (1991) showed that a serine mutation in the 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, 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 Asp138 mutations (Fig. 3). Furthermore, CGP 20712A was not able to antagonize this response, confirming the lack of binding of classic β-antagonists (Fig. 3). Therefore, this suggests that the D138S mutation reaches the cell surface and is able to couple to intracellular signaling pathways mediating CRE gene transcription. To confirm that the Asp138 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 manner similar to the that of the WT receptor (Fig. 4). Thus, mutations at position Asp138 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 of a particular residue in ligand binding or activation by mutagenesis may be confounded by the mutation inducing global or local conformational changes; however, a variety of Asp138 substitutions (alanine, glutamate, histidine, asparagine or serine) produces the same effect, which 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 Ser203, Ser204, and Ser207 (Savarese and Fraser, 1992; Sato et al., 1999). The equivalent amino acids in the β1-adrenoceptor are the serines at positions 228, 229, and 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, Fig. 2b) and CGP 12177 inhibition of isoprenaline and cimaterol agonist responses (Tables 3–4; Fig. 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 Ser203 mutations (e.g., pindolol and CGP 12177; Liapakis et al., 2000). This was not, however, universal for all ligands but appears to be ligand-dependent. For example, the affinity for isoprenaline at each 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 keeping with the increase in EC_{50} value for isoprenaline observed in the functional assay (Fig. 5a). Cimaterol, however (which has no catechol hydroxyl groups), actually had high affinity for the S228A mu-

TABLE 5

Log EC_{50} values and percentage maximum of 10 μM 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 Asp138 or Asn363 mutants. Values are mean ± S.E.M. from separate experiments. The total number of stable populations of the mutants is shown in Table 1.

	Log EC_{50}CGP 12177	Forskolin	*n*	Log K_D CGP 20712A	*n*	Log K_D propranolol	*n*
		%					
Wild type	−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	N.R.		10				

N.R., no response.

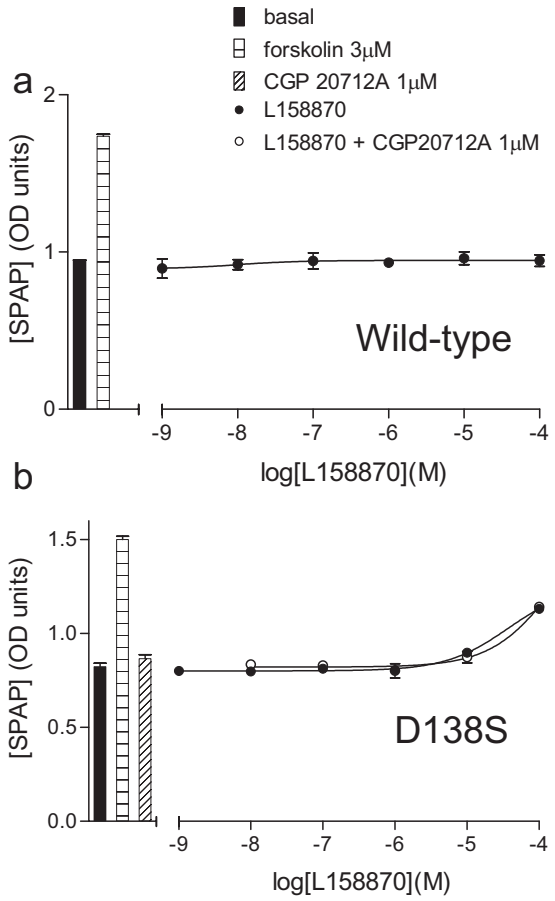

Fig. 3. CRE-SPAP production in cells expressing the wild-type human β1-adrenoceptor (a) or the D138S mutated receptor (b). Bars show the basal response, that in response to 3 μM forskolin, and that in response to 1 μM CGP 20712A alone. L158,870 did not stimulate any response in the wild-type receptor and the response seen in the D138S mutation was not inhibited by 1 μM CGP 20712A. Bars represent mean ± S.E.M. of triplicate values from a single experiment that is representative of three 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).

tation (in keeping with the observed leftward shift in the EC_{50} value; Table 2, Fig. 5c). The maintenance of the EC_{50} values in the cimaterol response suggests that the S228A mutation retained its coupling efficiency. Propranolol, however, had a marked increase in affinity for the S228A mutation (as measured in both the binding and functional assays; Fig. 2d, Tables 2–5).

CGP 12177 stimulated a response in CRE-gene transcription in both S228A and S229A mutations comparable in magnitude with that at the wild-type receptor (Table 5). The CGP 12177 responses still required higher concentrations of antagonist to inhibit the responses, suggesting that the second site was still present. The EC_{50} of this response, however, was right-shifted, suggesting a possible reduction in affinity of CGP 12177 for the second site of the β1-adrenoceptor. It is noteworthy that propranolol also had higher affinity for site 2 in the S228A mutation, but it remained 20-fold lower than that 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 antagonized this response with higher affinity at this mutation than the wild-type β1-adrenoceptor, the 20-fold discrepancy remained, suggesting that 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 Ser232 has higher affinity for CGP 20712A than the wild-type receptor). Ser228 and Ser229, but not Ser232, seem 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

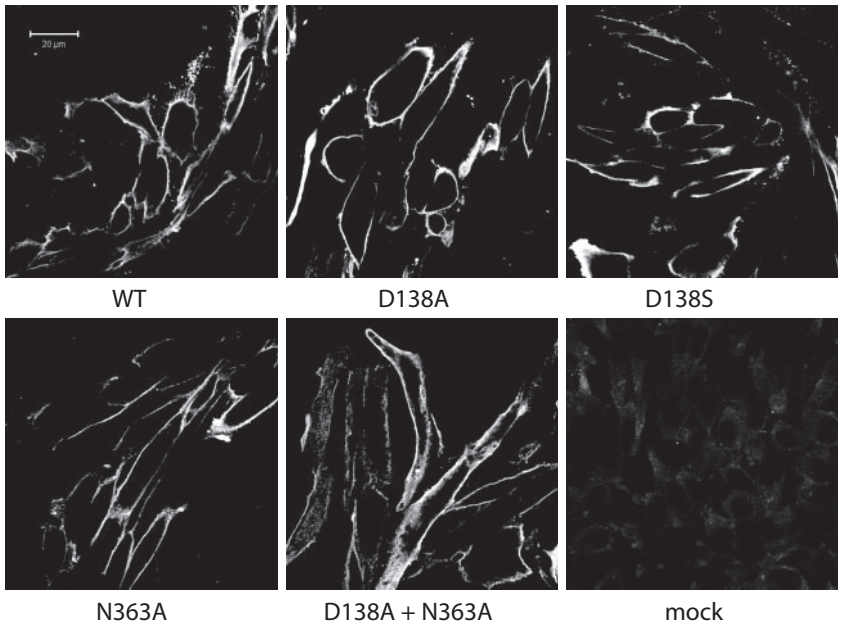

Fig. 4. Confocal images showing localization by immunocytochemistry of N-terminal myc-tagged receptors—wild type (WTβ1), D138A, D138S, N363A, and coexpression of myc-tagged D138A with nontagged N363A. Control mock-transfected cells are also shown. These images are representative of four separate experiments.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

(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.

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" that 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 (Fig. 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 (Fig. 6, c 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 cat-

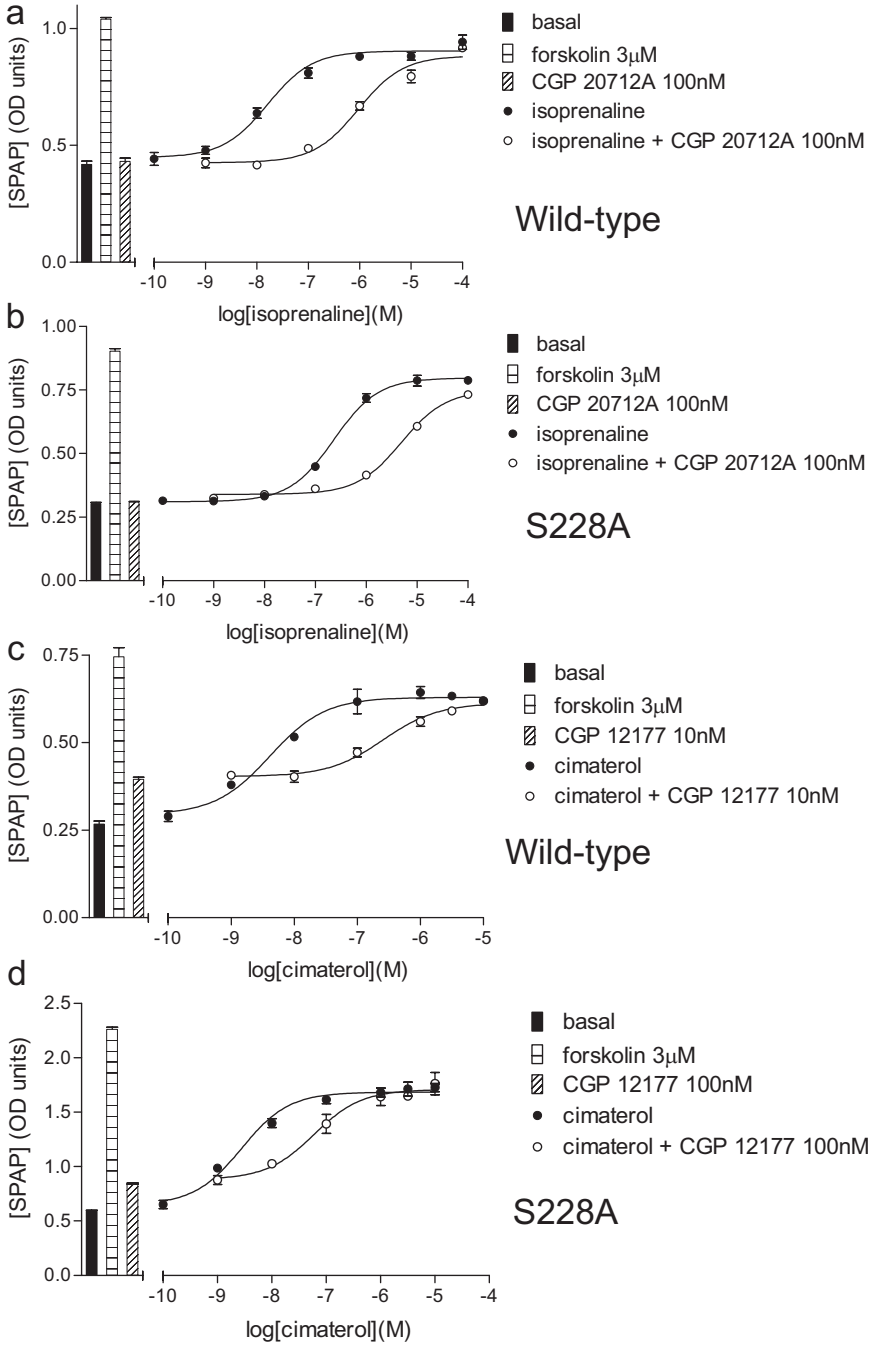

Fig. 5. CRE-SPAP production in cells expressing the wild-type human β1-adrenoceptor (a and c) or the S228A mutated receptor (b and d) in response to isoprenaline (a and b) or cimaterol (c and d). Bars represent CRE-SPAP production under basal conditions, that in response to 3 μM forskolin, and that in 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 10 (a), 16 (b), 12 (c) and 16 (d) separate experiments. It shows that although the isoprenaline response is 10-fold less potent in the S228A mutation than the wild-type receptor, the affinity of CGP 20712A is not greatly affected. The cimaterol response, however, is little affected 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 10 nM in c but 100 nM in d).

echolamines 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 was mutated to alanine (N344A), the affinity of [^{3}H]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

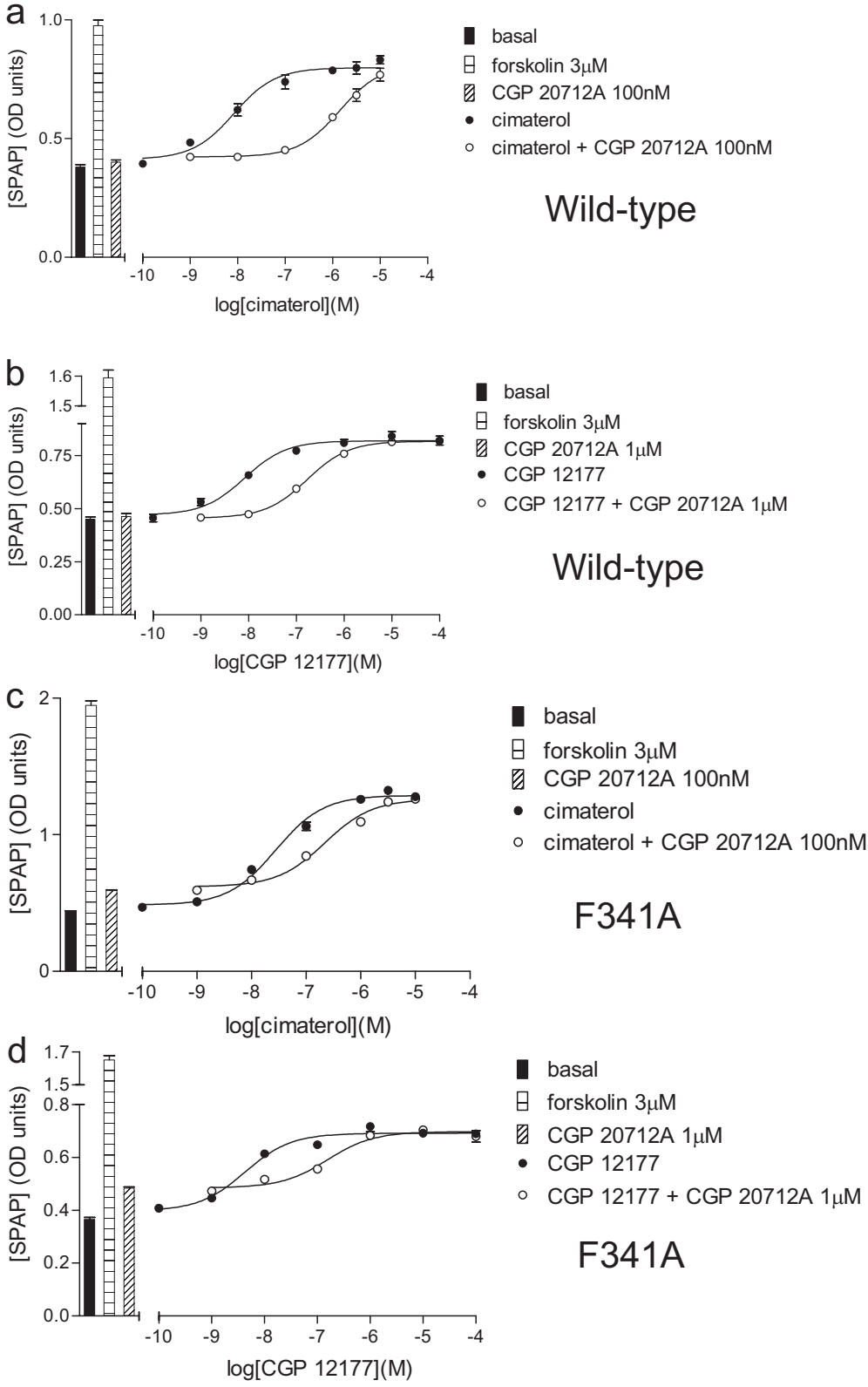

Fig. 6. CRE-SPAP production in cells expressing the wild-type human β1-adrenoceptor (a and b) or the F341A mutated receptor (c and d) in response to cimaterol (a and c) and CGP 12177 (b and d). Bars represent CRE-SPAP production under basal conditions, that in response to 3 μM forskolin and that in response 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 antagonized by CGP 20712A; the CGP 12177 response (site 2) by comparison required higher concentrations of CGP 20712A to antagonize the response. For the F341A mutant, the cimaterol response was also relatively resistant to antagonism by CGP 20712A, whereas that to CGP 12177 remained as for the wild-type receptor.

and 4). CGP 12177 retained its agonist activity, although the concentration of CGP 20712A required to antagonize this response was similar to that required to block the site 1 agonist responses (Tables 3–5). As with the F341A mutation, N344A also removes the ability of CGP 20712A to discriminate between sites 1 and 2. Thus in TM6, both Asn344 and Phe341 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, $n = 8$; N344A, -3.28 ± 0.02, $n = 8$). Parallel studies with CGP 20712A confirmed that log K_D values (WT, -8.97 ± 0.07; N344A, -8.81 ± 0.06, $n = 9$) similar to those obtained in stable mixed populations used in the rest of the study were determined (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 Asn363 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 (Fig. 4).

Isoprenaline and cimaterol retained some agonist potency, but this was greatly reduced compared with the wild-type receptor (Fig. 7). Propranolol and CGP 12177 were not able to antagonize 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 Dimerization in the Agonist Actions of CGP 12177

Molecular modeling 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 (Asp138 and Asn363) in different transmembrane domains that are essential for the binding of aryloxypropanolamine β-blockers provided an opportunity to test this hypothesis. Cotransfection of D138A and N363A β1-adrenoceptor mutants, however, did not reinstate any binding of [^{3}H]CGP 12177 (Table 1) or agonist activity of CGP 12177 (Table 5). Coexpression 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 N363A mutant is still reaching the cell surface. However, to test whether the D138A mutant is also reaching the cell surface under these conditions, an *N*-myc D138A mutant was contransfected with an untagged N363A, and cell surface expression was monitored using immunohistochemistry (Fig. 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 Modeling

To rationalize the results from our mutational studies, we conducted molecular modeling experiments using a homology

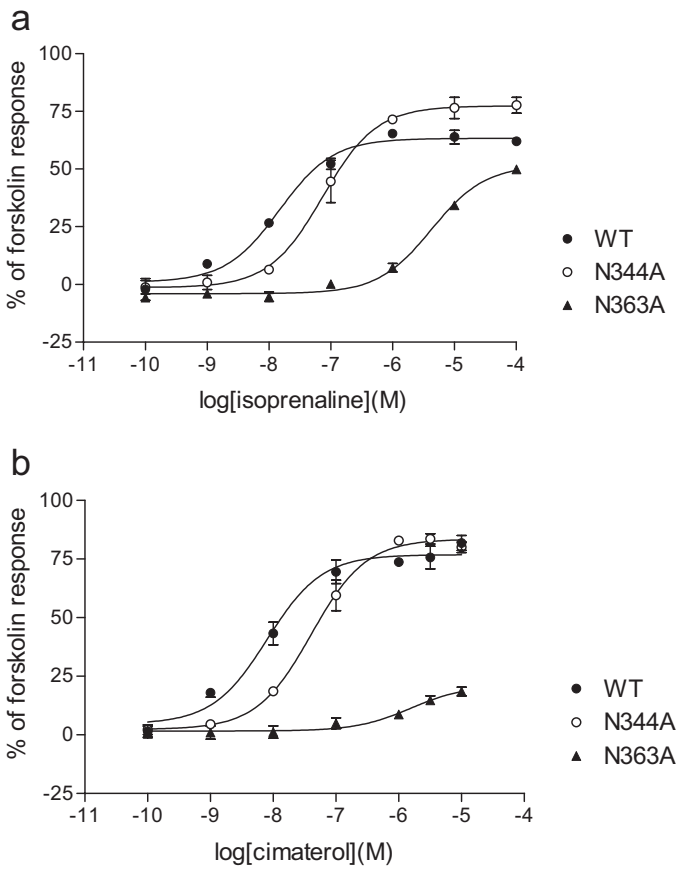

Fig. 7. CRE-SPAP gene transcription in response to isoprenaline (a) or cimaterol (b) in cells expressing the wild-type receptor, the N344A mutant, or the N363A mutant alone. Data points are mean ± S.E.M. of triplicate determinations from a single experiment and are expressed as percentage response in relation to 3 μM 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.

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 multiconformer database of carazolol (Fig. 1b) to the β1-adrenoceptor model produced highly scoring ligand poses (stereochemical configurations) very similar to those observed in the experimental β2-adrenoceptor complex structure (Figs. 8, a and b). Although 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 (Fig. 9a) and active (Fig. 9b) receptor forms. Comparison of the experimental carazolol–β2-adrenoceptor complex structure with models of inactive and active β2-adrenoceptor forms confirms this (data 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 Asp138, Ser228, Ser229, Ser232, and Asn363 on isoprenaline agonism were imposed (Fig. 8c). However, the putative hydrogen bonds between the ligand catechol ring hydroxyl moieties and the TM5 serine side chains on the one hand and the polar interactions between the ligand propanolamine groups and the Asp138 and Asn363 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 modeled binding mode of the other β-adrenergic agonist, cimaterol (Fig. 8d).

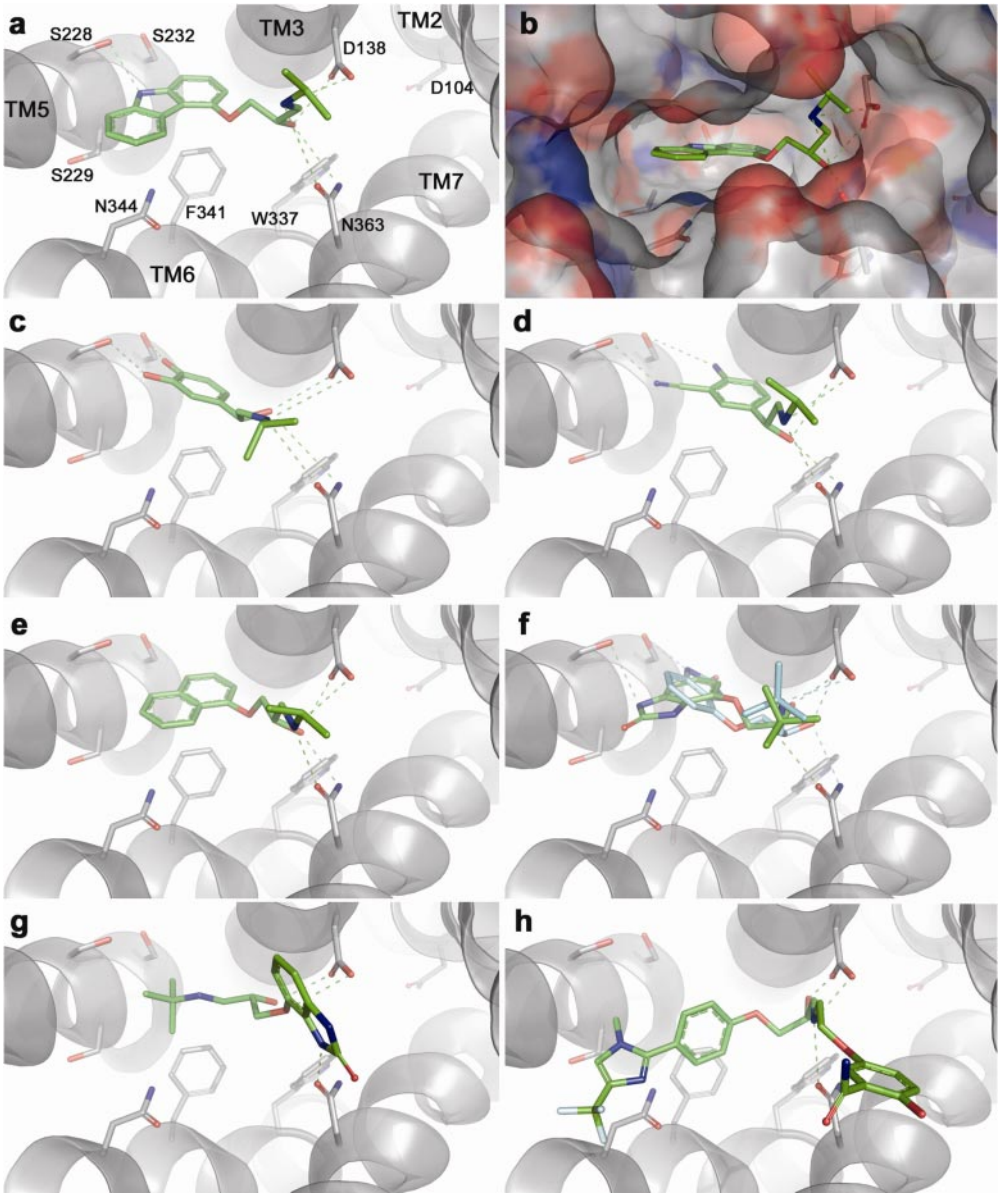

Fig. 8. Docking studies with the β1-adrenoceptor homology model. The ligand-binding site in the extracellular receptor portion is shown as a cartoon model, including the side chains of residues that were the subject of mutation (plus the toggle residue Trp337$^{6.48}$) as gray CPK sticks. The carazolol binding pose in a and b (gray CPK-colored 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.

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 (Fig. 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 Ser228 explain the enhanced affinity of propranolol for the S228A mutant β1-adrenoceptor.

Top-ranking CGP 12177 poses (Fig. 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, although 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 Asp138 and Asn363, respectively, whereas the *tert*-butylamino group was bound in the region between the TM5 Ser residues and the TM6 Asn344 and Phe341 residues, without, however, forming any polar contacts in that region (Fig. 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 Asp138 and Asn363.

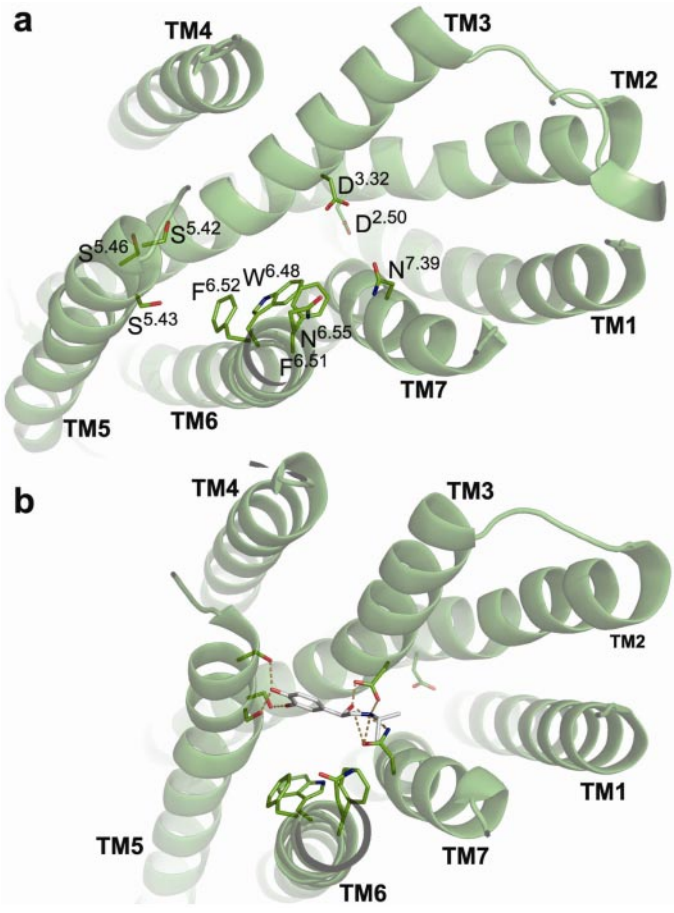

Fig. 9. Models of inactive (a) and active (b) β2-adrenoceptor conformations are depicted looking into the ligand binding site from the extracellular side (Gouldson et al., 2004). In a, the side chains of key residues implicated in ligand binding are shown as green CPK sticks and are labeled using the Ballesteros and Weinstein (1995) convention. In b, a modeled isoprenaline (gray CPK sticks) binding pose is shown, including polar contacts (broken lines) between the agonist and the propanolamine clamp $Asp^{3.32}$, $Asn^{7.39}$ (Asp138 and Asn363 in the β1-adrenoceptor, respectively) and the catecholamine recognition site on TM5 [i.e., $Ser^{5.42}$, $Ser^{5.43}$, and $Ser^{5.46}$ (Ser228, Ser229, and Ser232 in the β1-adrenoceptor, respectively)].

The antagonist CGP 20712A is structurally more different (larger) than either carazolol, isoprenaline, or CGP 12177 (Fig. 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 that docking studies using this conformation are less meaningful (Bissantz et al., 2003). Nevertheless, assuming propanolamine interactions of CGP 20712A with the Asp138/Asn363 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 Phe341 and Asn344 (Fig. 8h), whose mutations uniquely affect the ability of CGP 20712A to discriminate between isoprenaline- and CGP 12177-mediated agonism.

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, whereas the catechol hydroxyl groups of isoprenaline seem 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 2 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. It is noteworthy that 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 modeling

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 modeled 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 Asp138, Ser228, Ser229, Ser232, and Asn363 on isoprenaline agonism were imposed (Fig. 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 important for interaction with the aromatic ring of catecholamines and has been proposed to be involved in the rotamer "toggle switch" that 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 (Chung et al., 1988; Strader et al., 1988). 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 Asp104 seem to decrease agonist efficacy at the catecholamine site.

Three β-antagonists (propranolol, CGP 12177, and CGP 20712A) were evaluated as antagonists of isoprenaline and cimaterol responses at the catecholamine site of the β1-adrenoceptor. All Asp138 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 antagonized by CGP 20712A. Mutation of Ser228 increased propranolol affinity by an order of magnitude whereas that for CGP 12177 decreased by the same amount. The lower affinity of CGP 12177 in the S228A mutant is consistent with the model shown in Fig. 8f and with previous data from the β2-adrenoceptor in which 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 Asn363 mutants. Neither CGP 12177 nor propranolol was able to antagonize the isoprenaline and cimaterol responses in the N363A or N363C mutants. 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 Phe341 mutation, the binding affinities of CGP 20712A and CGP 12177 were reduced by nearly an order of magnitude, whereas 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.

Because both Asp138 and Asn363 were 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 modeled binding pose in Fig. 8g shows that polar interactions between CGP12177 and Asp138/Asn363 may occur in a different way from the canonical interactions of propanolamines with these residues. However, the binding pose shown with CGP 12177 in Fig. 8f suggests that this molecule can also interact with Asp138/Asn363 in a way analogous to that of CGP 20712A and propranolol. These different poses provide a potential framework for the differential pharmacology of CGP 12177 at the catecholamine site (Fig. 8f) and the secondary CGP 12177 site (Fig. 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 (Fig. 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 Asp104 is important in coupling both site 1 (via isoprenaline and cimaterol) and site 2 (via CGP 12177) to functional responses.

Extensive biochemical and modeling 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 $Trp^{6.48}$ (Trp337 and Trp286 in the β1 and β2-adrenoceptors, respectively) and certain other residues adjacent on the same face of TM6 (Fig. 9), is central to receptor activation (Schwartz et al., 2006). In inactive receptors, including for example 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 $Trp^{6.48}$ (and nearby TM6 residues) changes, permitting an inward movement (tilting) at the extracellular side of TM5, and subsequently of TM4 and TM7, and the $Trp^{6.48}$ side chain is believed to be positioned at the interface between TM5 and TM6 in active receptor conformations. In the structurally best-characterized GPCR, rhodopsin, the ligand retinal interacts directly with the toggle switch residues on TM6, but the extent to which this applies to adrenoceptor ligands remains unclear. In the case of carazolol, the distal benzene ring of the carbazole system interacts with $Phe^{6.52}$ (Phe341 in the β1-adrenoceptor), the rotamer state of which is coupled to that of $Trp^{6.48}$. Our modeling studies (Fig. 8) suggest that the other antagonists modeled act similarly. Presumably agonists at the catecholamine site stabilize the active receptor conformation, in which $Trp^{6.48}$ 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 does not require the Phe341 or Asn344 side chains, which are not near isoprenaline and cimaterol in the predicted binding poses (Fig. 8, c 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 modeled binding pose in Fig. 8g shows that polar interactions between CGP12177 and Asp138/Asn363 may occur in a way that is different from the interactions used by other propanolamine-based antagonists in antagonizing 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 Fig. 8g because there are no pronounced interactions between the *tert*-butylamine portion of CGP 12177 with either the serine residues of TM5 or the toggle switch residues of TM6. Nevertheless, it is possible that this group directly induces a rotamer change in Asn344 and/or Phe341 that can be transmitted to Trp337, 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 nonoverlapping residues.

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