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Identification of key residues in transmembrane 4 responsible for the secondary, low affinity conformation of the human β1-adrenoceptor.

Jillian G Baker, Richard G W Proudman, Stephen J Hill

Cell Signalling, School of Life Sciences,

C Floor Medical School,

Queen's Medical Centre,

University of Nottingham,

Nottingham,

NG7 2UH, UK.

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Email: jillian.baker@nottingham.ac.uk

Running title: identification of secondary conformation of β1-AR

Corresponding author:

Jillian G Baker

Cell Signalling, School of Life Sciences

C Floor Medical School

University of Nottingham

Queen's Medical Centre

Nottingham

NG7 2UH

UK

Tel: 0115 8230085

Fax: 0115 8230081

Email: jillian.baker@nottingham.ac.uk

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Abbreviations

CHO: Chinese hamster ovary;

CGP12177: (-)-4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one;

CGP20712A: 2-hydroxy-5-(2-[{hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy)propyl}amino]ethoxy)benzamide;

CRE-SPAP: name of the reporter gene: cyclic AMP response element-upstream of a secreted placental alkaline phosphatase reporter gene

ICI118551: (-)-1-(2,3-[dihydro-7-methyl-1H-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol;

IBMX: 3-isobutyl-1-methylxanthine

sfm: serum free media

TM: transmembrane

WT: wildtype

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Abstract

The β1-adrenoceptor exists in two agonist conformations/states: (1) a high affinity state where responses to catecholamines (and other agonists e.g. cimaterol) are potently inhibited by β1-adrenoceptor antagonists and (2) a low affinity secondary conformation where agonist responses (particularly CGP12177) are relatively resistant to inhibition by β1-adrenoceptor antagonists. Although both states have been demonstrated in many species (including human), the precise nature of the secondary state is unknown and does not occur in the closely related β2-adrenoceptor. Here, using site directed mutagenesis and functional measurements CRE-SPAP production and ^{3}H-cAMP accumulation, we examined the pharmacological consequences of swapping transmembrane (TM) regions of the human β1 and β2-adrenoceptors, followed by single point mutations, in order to determine the key residues involved in the β1-adrenoceptor secondary conformation. We found that TM4 (particularly amino acids L195 and W199) had a major role in the generation of the secondary β1-adrenoceptor conformation. Thus unlike at the human β1-wildtype adrenoceptor, at β1-TM4 mutant receptors, cimaterol and CGP12177 responses were both potently inhibited by antagonists. CGP12177 acted as a simple partial agonist with similar K_B and EC_{50} values in the β1-TM4 but not β1-wildtype receptors. Furthermore pindolol switched from a biphasic concentration response at human β1-wildtype adrenoceptors to a monophasic concentration response in the β1-TM4 mutant receptors. Mutation of these amino acids to those found in the β2-adrenoceptor (L195Q and W199Y), or mutation of a single residue (W199D) in the human β1-adrenoceptor, thus abolished this secondary conformation and created a β1-adrenoceptor with only one high affinity agonist conformation.

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Introduction

The β1-adrenoceptor exists in two agonist conformations or states. Catecholamines activate the primary, high affinity, catecholamine conformation and these responses are readily inhibited by β-blockers (β-adrenoceptor antagonists). Much higher concentrations of β-blockers are required to inhibit secondary conformation agonist responses (e.g. response to CGP12177) and this conformation is therefore termed the secondary, low affinity, CGP12177 conformation (Pak and Fishman 1996; Granneman, 2001; Molenaar, 2003; Arch, 2004).

Conventional partial agonists need to saturate receptors to stimulate their maximum response and thus the concentrations required for half receptor occupancy (K_B) should normally be equal the concentration required to stimulate half maximum response (EC_{50}) value. However, early observations found that several β-blockers with partial agonist activity did not conform to this. The concentration of pindolol, for example, needed for the cardiostimulant effects in feline heart was 10-times greater than that needed to antagonise responses of the full agonist isoprenaline and thus was labelled as non-conventional partial agonist (Kaumann 1973; Kaumann and Blinks 1980; Kaumann and Molenaar 2008). CGP12177 (Staehelin et al., 1983) was also found to be a non-conventional partial agonist with even larger differences between K_B and EC_{50} (Kaumann 1983) and therefore has become an important tool in understanding β1-adrenoceptor pharmacology (Kaumann and Molenaar 2008). In 1996, Pak and Fishman, using transfected cells, showed that CGP12177 inhibited isoprenaline responses at low concentrations (i.e. with high affinity), but required 100-times higher concentrations to stimulate responses. Furthermore, CGP12177 agonist responses required higher concentrations of conventional β-blockers to antagonise them. They therefore proposed a high affinity and a low affinity state or conformation of the β1-adrenoceptor and this terminology has now been widely adopted (Granneman 2001; Kaumann et al., 2001).

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Studies using transfected cells have determined the site of action of many β-ligands (e.g. Konkar et al., 2000; Baker et al., 2003 Joseph et al., 2004a; Baker 2005a). Pindolol, alprenolol (and several other ligands) have biphasic concentration response relationships (Walter et al., 1984; Baker et al., 2003; Kaumann and Molenaar; 2008; Baker 2010a). The high affinity component (readily blocked by β-blockers) is thought to occur via the high affinity catecholamine conformation whilst the low affinity component (resistant to β-blockade) occurs via the secondary low affinity conformation (Walter et al., 1984; Baker et al., 2003).

These two β1-adrenoceptor conformations have also been demonstrated in rat, mouse, ferret and human heart (e.g. Kompa and Summers 1999; Kaumann et al., 2001; Lowe et al., 2002; Joseph et al., 2003; Sarsero et al., 2003; Molenaar et al., 2007), human blood vessels (e.g. Mallem et al., 2004; Kozlowska et al., 2003 and 2006) and in whole animals (e.g. Malinowska and Schlicker 1996; Zakrzeska et al., 2005). However the physiological or clinical relevance of this conformation remains unknown, even though plasma concentration of carvedilol (100ng/ml = 300nM) used in human cardiovascular diseases is sufficient to occupy this secondary conformation (Sawangkoon et al., 2000; Baker et al, 2003).

Limited mutagenesis studies have, in passing, noted secondary conformation effects and detected some overlap of the amino acids involved in the catecholamine conformation, although these studies are not all in agreement. D138 (transmembrane 3, TM3) and N363 (TM7) have been suggested by some (Baker et al., 2008), but not all (Joseph et al., 2004b) to be involved in the secondary conformation, as have mutations in TM5 (Kaumann and Molenaar 2008) and TM6 (Baker et al., 2008). However, the precise nature of this secondary conformation remains unknown from its location within the receptor sequence to the physiological relevance or potential clinical implications (Molenaar 2003; Molenaar et al., 2007).

Finally, although the β1-adrenoceptor of many species (rat, mouse, guinea pig, ferret, cat and human: Kaumann and Molenaar 2008), and the human β3-adrenoceptor (Baker 2005b) have been shown to exist

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in at least two agonist conformations, this is not true for all β-adrenoceptors. CGP12177 is a conventional partial agonist at the human β2 and turkey β-3C adrenoceptors, (i.e. with similar K_B values and EC_{50} values) suggesting interaction via a single conformation (Pak and Fishman 1996; Baker et al., 2002; Baker 2010b).

This study aimed to discover the primary region important for the secondary conformation of the β1-adrenoceptor. Using a chimeric receptor approach (Isogaya et al., 1999; Kaumann and Molenaar 2008), amino acids in the β1-adrenoceptor were mutated to those of the β2-adrenoceptor and the pharmacological consequences observed. Here we locate TM4, and specifically L195 and W199 within the human β1-adrenoceptor, as the major site responsible for the secondary conformation pharmacology.

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Materials and Methods

Materials

Molecular biology reagents were from Promega (Madison, WI, USA). Lipofectamine, OPTIMEM, pcDNA3.1 and Top 10F competent cells were from Life Technologies (Paisley, UK). The QuikChange mutagenesis kit was from Stratagene (La Jolla, CA) and foetal calf serum was from PAA Laboratories (Teddington, Middlesex, UK). ^{3}H-adenine and ^{14}C-cAMP were from Amersham International (Buckinghamshire, UK) and Ultima Gold XR scintillation fluid from PerkinElmer (Shelton, CT, USA). Cimaterol, CGP 20712A, bisoprolol and carvedilol were from Tocris Life Sciences (Avonmouth, UK). All other reagents were from Sigma Aldrich (Poole, Dorset, UK). Racemic ligands were used throughout.

Molecular biology

The cDNA sequence encoding the wildtype human β1-adrenoceptor (β1-WT) in pJG3.6 was a gift from Steve Rees (GlaxoSmithKline, Stevenage). This cDNA was subcloned as a HindIII/XbaI fragment into pcDNA3.1 and the sequence was confirmed by DNA sequencing. Mutations described in Table 1 were generated using QuikChange mutagenesis and BioLine PolyMate Additive for GC-rich templates (Baker et al., 2008). After subcloning in Top 10F' competent cells, each mutant β1-adrenoceptor cDNA was excised on Hind III/XbaI and subcloned into native pcDNA3.1 containing a neomycin selection marker. All mutations and sequences were confirmed by DNA sequencing using the School of Biomedical Sciences Sequencing Facility. In order to detect the most important areas of the human wildtype β1-adrenoceptor (β1-WT) involved in the secondary conformation, we made point mutations in the transmembrane (TM) regions of the receptor such that the each TM region resembled that of the wildtype human β2-adrenoceptor (β2-WT). Prediction of the transmembrane regions was performed using ExPASy topology prediction tools (www.expasy.org). For example, 6 point mutations were made in β1-WT (L63I, L64V, A66S, L71A, A74F, V81T) which effectively converted the TM1 region of this receptor to that of the β2-WT. This chimeric receptor was called β1-TM1 (i.e. β1-adrenoceptor but with TM1 of the β2-WT, Table 1). This was then replicated for each TM region such that we had eight β1 receptor

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constructs: the β1-WT and one with each of the seven TM regions in turn mutated to that of the β2-adrenoceptor (Table 1). A similar set of mutations were made starting with the β2-WT and creating seven chimeric receptors each with a TM region of the human β1-WT (Table 1). These constructs were expressed in CHO-CRE-SPAP cells (cells stably expressing a reporter construct that has six cyclic AMP response elements upstream of a secreted placental alkaline phosphatase reporter gene) and stable cell lines generated (see below).

Once important TM regions for the secondary site were identified, in order to determine which individual amino acids were involved, several single point mutations and chimeric receptors were made (Table 1) and these constructs expressed in CHO-CRE-SPAP cells either as stable mixed populations of cells (CRE-SPAP assays) or in transiently transfected cells (^{3}H-cAMP accumulation assays).

Cell culture: All CHO cells were grown in Dulbecco's modified Eagle's medium nutrient mix F12 (DMEM/F12) containing 10% foetal calf serum and 2mM L-glutamine in a 37°C humidified 5% CO_2 : 95% air atmosphere.

Generation of stable cell lines: CHO cells stably expressing a CRE-SPAP reporter gene were secondarily transfected with the wildtype human β1 or β2-adrenoceptor, or one of the full TM chimeric receptors (total 16 cell lines) using lipofectamine and OPTIMEM and selected for 3 weeks using resistance to geneticin (1mg/ml for the receptor) and hygromycin (200μg/ml for the CRE-SPAP reporter). Single clones were identified by dilution cloning and expanded to generate stable cell lines. These cell lines were used to identify the TM regions important for the secondary site. Receptor expression level in these stable cell lines was measured as previously described (Baker 2005b).

Generation of stable mixed populations of cells: Here, the same parent CHO-CRE-SPAP cells were transfected with the wildtype human β1 or β2-adrenoceptor, or a single point mutation or chimeric receptor and selected for 3 weeks using resistance to geneticin (1mg/ml for the receptor) and hygromycin

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(200µg/ml for the CRE-SPAP reporter). During this time the cells were passaged twice. The cells were then plated into 96-well plates for CRE-SPAP production experiments.

Generation of transient populations: For transiently transfected cells used in the ^{3}H-cAMP assays, the parent CHO-CRE-SPAP cells were transfected, as above, on day 1, the transfection reagents removed and replaced with media on day 2, the cells plated in to 24-well plates on day 3 and the ^{3}H-cAMP accumulation assay performed on day 4.

CRE-SPAP production

Cells were grown to confluence in, sterile, clear plastic, tissue culture treated 96-well plates. The cells were then serum starved by removal of the media and addition of 100µl serum-free media (sfm) per well for 24 hours before experimentation. At the start of each experiment, the sfm was removed from each well and 100µl sfm or 100µl sfm containing an antagonist at the final required concentration was added to each well and the cells incubated for 30 minutes at 37°C. Agonist in 10µl sfm was then added to each well and the plates incubated for 5 hours. After 5 hours, all drugs and media were removed from each well and 40µl sfm added to each well. The plates were then incubated for 1 hour at 37°C before being transferred to an oven preheated to 65°C and incubated for 30 minutes to destroy endogenous phosphatases. SPAP production was then measured by the addition of 100µl 5mM p-NPP per well (in diethanolamine buffer) and read on a Dynatech MRX plate reader at 405nM.

^{3}H-cAMP accumulation

Cells were grown to confluence in sterile, clear plastic, tissue culture treated 24-well plates. Cells were pre-labelled with ^{3}H-adenine by incubation for at least 2 hours with 2µCi/ml ^{3}H-adenine in media (0.5ml per well). The cells were washed, then 1ml sfm containing 1mM IBMX (3-isobutyl-1-methylxanthine) was added to each well and the cells incubated for 15 minutes at 37ºC. Agonists (in 10µl sfm) were added to each well and the plates incubated for 5 hours in order to maximise the responses (without altering the EC_{50} values or % maximum isoprenaline response observed; Baker, 2010a). The assay was

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terminated by adding 50µl concentrated HCl per well, the plates frozen, thawed and ^{3}H-cAMP separated from other ^{3}H-nucleotides by sequential Dowex and alumina column chromatography (using ^{14}C-cAMP to determine column efficiency). Isoprenaline (10µM) was used to define the maximal response in each plate of each experiment.

Data analysis

Agonist responses were best described by a one-site sigmoidal concentration response curve using the following equation:

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

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

The affinities of antagonists (K_B values) were calculated from the rightward shift of the agonist concentration response curve in the presence of a fixed concentration of antagonist using the following:

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

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].

When CGP12177 was used to antagonise the more efficacious agonists, clear partial agonism was seen (e.g. Figure 2). Here, the affinity was initially calculated by the method of Stephenson (1956):

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

where [P] is the concentration of CGP12177, $[A_1]$ is the concentration of the agonist at the point where CGP12177 alone causes the same agonist response, $[A_2]$ is the concentration of agonist causing a given

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response above that achieved by CGP12177 and $[A_3]$ the concentration of the agonist, in the presence CGP12177, causing the same stimulation as $[A_2]$.

The response to pindolol (and at times CGP12177) was best described by a two-site concentration response (e.g. Figure 6b)

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

where N is the percentage of site 1, [A] is the concentration of agonist and $EC1_{50}$ and $EC2_{50}$ are the respective EC_{50} values for the two agonist sites.

A two-site analysis was also used for the experiments e.g. Figure 6a:

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

where basal is the response in the absence of agonist, Ag is the response to a fixed concentration of agonist, [P] is the concentration of partial agonist (e.g. CGP12177), IC_{50} is the concentration of competing partial agonist that inhibits 50% of the response of the fixed agonist, PAg is the maximum stimulation by the competing partial agonist and EC_{50} is the concentration of competing agonist that stimulated a half maximal competing partial agonist response.

A 10μM (maximal) isoprenaline concentration was included in each plate for each separate experiment for CRE-SPAP production and ^{3}H-cAMP accumulation, to allow agonist responses to be expressed as a percentage of the isoprenaline maximum for each experiment. Data points in the Figures are presented as mean ± s.e.m. of triplicate determinations from a single experiment. Data in the text and Tables are mean ± s.e.m. of n separate experiments.

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Results

Demonstration of the two agonist conformations of the human β1-adrenoceptor – CRE-SPAP production in stable cell lines.

Cimaterol, a primary catecholamine conformation agonist (Baker 2005a), stimulated a response in cells stably expressing the human wildtype β1-adrenoceptor (β1-WT; log EC_{50} = -8.25 ± 0.14) that was 95.8 ± 2.9% that of the response to 10μM isoprenaline; top half Table 2a, Figure 1a). As expected, this β1 response was readily inhibited by the β1-selective antagonist CGP20712A (log K_B -9.18) and poorly by the β2-selective antagonist ICI118551 (log K_B -6.96). CGP12177, a partial agonist, also inhibited this cimaterol response with high affinity indicating high affinity for the catecholamine conformation of the receptor (log K_B -9.55; Table 2, Figure 2a). CGP12177 is however also known to activate the secondary conformation of the β1-adrenoceptor. At the β1-WT, CGP12177 stimulated a partial agonist response, log EC_{50} -8.18 ± 0.08, that was 73.8 ± 5.6% that of the maximum to isoprenaline (Figure 1b). This CGP12177 response was also inhibited by antagonists but in each case higher concentrations of antagonist were required yielding lower log K_B values (e.g. log K_B for CGP20712A was -7.16; bottom half Table 2a). The fact that 10-500 fold higher concentrations of antagonist were required to inhibit the CGP12177 rather than the cimaterol responses, and the discrepancy between the log K_B value and the log EC_{50} value for CGP12177 itself, demonstrate the high affinity and the lower affinity conformations of the human β1-adrenoceptor.

At the human β2-adrenoceptor, agonist responses to cimaterol and CGP12177 were also observed, however similar concentrations of antagonist were required to inhibit these responses (yielding similar log K_B values, Table 3a, Figure 1e and f), and the log K_B and log EC_{50} values for CGP12177 were similar suggesting that cimaterol and CGP12177 are acting through the same conformation of the β2-adrenoceptor.

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Identification of transmembrane regions important in the secondary conformation of the β1-adrenoceptor – CRE-SPAP production in stable cell lines.

To identify areas in the receptor important for the secondary conformation, β1/β2 chimeric receptors were examined. These receptors had mutations such that each TM was in turn swapped for that of the other receptor, e.g. β1-TM1 is the β1-adrenoceptor sequence containing mutations that make it equivalent to the TM1 region of the β2-adrenoceptor (Table 1). When the β1-TM chimeric receptors were examined (each stably expressed in a different cell line), cimaterol stimulated a full agonist response at all β1/2 receptors (β1-TM1 through to β1-TM7; Figure 1c, top half Table 2a) and this response was readily inhibited by CGP20712A and poorly by ICI118551 confirming that despite changing each transmembrane in turn to that of the β2-adrenoceptor, these chimeric receptors retained a β1-selective nature. There were some differences in the affinity of antagonists (e.g. affinity of ICI118551 at the β1-TM2, β1-TM3, β1-TM4, β1-TM6 and β1-TM7 receptors is substantially higher than at the β1-WT; top half Table 2a). This could be due to a general trend of these chimeras "losing" β1-subtype selectivity as judged by a range of ligands or due to a specific interaction between the ligand ICI118551 and specific amino acids in these chimeras. To determine which, several antagonist ligands (bisoprolol a less selective but still a β1-selective ligand; propranolol and carvedilol slightly β2-selective ligands) were examined in order to generate a pattern of affinity (top half Table 2a, Figure 1). The affinity of CGP20712A and bisoprolol remained high (similar to β1-WT, top half Table 2a and very different from values obtained at the β2-WT; top half Table 3a). Thus, the overall pattern for these chimeras is one of retaining β1-selective properties (and indeed ICI118551 was later found to interact with a specific amino acid in TM4 rather than a general pattern across several ligands – see below).

When the CGP12177 responses were examined, the data achieved for β1-TM4 was significantly different from that at the other chimeras ($p<0.001$, bottom half Table 2, Figure 1d). Firstly, the log EC_{50} for CGP12177 was very different from that at the other receptors (bottom half Table 2a). Secondly, the log K_B value and log EC_{50} values for CGP12177 in the β1-TM4 cells were similar (left column, Table 2b, and

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indeed similar to that seen in the β2-WT cells; Table 3a). Finally, the affinities of antagonists in the presence of CGP12177 at β1-TM4 were very similar to those obtained when cimaterol was used as the agonist in these cells (Table 2a). This is most easily seen in Table 2b where the ratios of the antagonist affinities are compared. Whereas the ratio of antagonist affinities for CGP20712A at β1-WT was log 2.02 (e.g. it has 100 fold different affinity for two conformations), it was log 0.01 at β1-TM4 suggesting that there is no difference in affinity and that the secondary conformation was not in existence. Thus β1-TM4 had a pharmacological profile consistent with CGP12177 acting as a partial agonist at a single high affinity conformation of the β1-adrenoceptor, i.e. in a similar manner to which CGP12177 acts at the single receptor conformation of the β2-WT adrenoceptor.

When the full β2-TM4 construct was expressed (11 amino acid changes), no function or binding was detectable despite multiple attempts. Therefore a construct containing 10 amino acid changes (all expect the last Y174W) was made which was then used as β2-TM4 (Table 1 and 3). The data obtained for the β2/β1 chimeras (Table 3a) suggest that the agonist responses and antagonist affinities obtained change very little in the β2-adrenoceptors with whole TM region changes.

Examination of the ratios of antagonist affinities at the two conformations is again helpful for detecting the most important changes – Tables 2b and 3b. Thus although there are some differences for the chimeric receptors (e.g.EC_{50}/K_B ratio for β1-TM7 and β2-TM7), by far the most overwhelming differences are in the β1-TM4 chimeric receptor and this is clearly shown when comparing the ratios of the log K_B and log EC_{50} values at the two conformations, as shown in Tables 2b and 3b. Also, here, although ICI118551 was found to have higher affinity at β1-TM4 (and indeed β1-TM7), the ratio of the affinities for the two conformations of the β1-adrenoceptor clearly shows that β1-TM4 is different from the other β1-chimeric receptors.

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Importantly, because these stable cell lines used here have different levels of receptor expression (Table 1), both of these methods of assessing the presence of the secondary conformation do not rely on the receptor expression levels being the same. Antagonist affinity measurements are independent of receptor expression level and, because CGP12177 is a partial agonist in all of these cell lines, its K_B value in a given cell line should be similar to its EC_{50} value in that cell line (i.e. ratio of zero) if the two are acting at a single conformation. A further piece of evidence can be obtained from comparing the log EC_{50} values for CGP12177 over the cell lines although this is more problematic, not least as it is likely to depend on receptor expression levels. Interestingly here, the log EC_{50} for CGP12177 at β1-TM4 is markedly more potent (left-shifted) even though its receptor expression level is the lowest of the β1 cell lines and thus, if anything, would be expected to have a less potent (i.e. right -shifted) log EC_{50} value. Even this softer evidence is therefore pointing to β1-TM4 as an important region for the secondary conformation.

Identification of the amino acids in TM4 involved in the secondary conformation

– CRE-SPAP production in stable mixed populations of cells.

In order to determine which of the amino acids in TM4 were responsible for this secondary conformation, intermediate stages of the production of the chimeric receptors were examined that had several, but not all, of the amino acids changes required to alter the TM domain from β1 to β2 (see Table 1 for the amino acid changes made). These constructs were expressed in parent CHO-SPAP cells and experiments performed in stable mixed populations of cells (4-24 separate populations of cells were generated for each chimeric receptor). Whereas the affinity for antagonists remained very similar when cimaterol was the agonist (primary catecholamine site, top half of Table 4a, with the exception of ICI118551), when the secondary site conformation was examined, (i.e. with CGP12177 as the agonist), the log EC_{50} value for CGP12177 became more potent (approaching that for the log K_B value) in β1-TM4 stage 5 (bottom half Table 4a). Secondly, the affinity of the other antagonists (log K_B values) also became higher with β1-TM4 stage 5 and full β1-TM4 chimeras (bottom half Table 4a). The affinity for ICI118551 was high at both conformations for β1-TM4 Stage 4, although the ratio (Table 4b) suggests the retention of both

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conformations of the receptor. This suggests that amino acids L195 and W199 are the most important for the secondary conformation.

To investigate the role of these specific amino acids further, each of these individual single point mutations were examined alone (in stable mixed populations) rather than on the background of the other TM4 mutations (Table 1 and 5, Figure 3 and 4). β1-L195Q and β1-W199Y had little effect on the cimaterol catecholamine conformation response (top half Table 5a; Figure 3). However, CGP12177 agonist responses were significantly more potent at β1-L195Q and β1-W199Y (bottom half Table 5a, Figure 4). Examining antagonist affinity values, whereas β1-V189T affected the affinity of ICI118551 at the catecholamine conformation, the affinity of CGP20712A, propranolol and CGP12177 were largely unaffected by any of the mutations (top half of Table 5a, Figure 3). However, the secondary conformation CGP12177 responses were significantly more sensitive to inhibition with the L195Q and W199Y mutations (bottom half Table 5a, Figure 4).

We also examined the impact of these specific mutations in combination with each other to determine whether the effects of each change were additive or whether one single change was sufficient to account for the secondary site. A combination of L195Q and W199Y largely achieved the same effect as the whole TM4 swap, with the exception of the affinity of ICI118551 (Table 5a). However, addition of the V189T mutation (thus making a triple β1V189T-L195Q-W199Y) effectively normalised the affinity of ICI118551 to that observed with the full TM4 swap (Figure 5a and b). This suggests that β1V189T-L195Q-W199Y has also lost the secondary conformation.

In addition to the simple substitution of β2-adrenoceptor residues for their equivalents in the β1-adrenoceptor, we also examined the effects of different substitutions at position 199. Thus at position 199, as well as mutating the tryptophan (W) to tyrosine (Y, as in the human β2-adrenoceptor) it was also mutated to another aromatic residue (phenylalanine, F), a hydrophobic residue (leucine, L), a basic

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residue (lysine, K), an amide (asparagine, N), an acid (aspartic acid, D) and the small amino acid alanine (A). Substitution of W for other residues had little effect on the nM EC_{50} for CGP12177 observed with the tyrosine (Y) residue from the β2-adrenoceptor and most appeared to have lost, or at least compromised, the secondary conformation (Figure 5c and d, Table 5a). Interestingly, the W199K and W199L mutations decreased the EC_{50} of cimaterol (top half Table 5a) whilst increasing the efficacy of CGP12177 which then appeared as a virtual full agonist in these two mutations (bottom half Table 5a).

Confirmation of loss of the secondary site with mutations V189T, L195Q and W199Y – ^{3}H-cAMP accumulation in transiently transfected cells.

So far, identification of the role of amino acids at positions 195, 199 and possibly 189 in the secondary conformation pharmacology of the β1-adrenoceptor has relied on observation of (a) different affinity values for antagonists in the presence of cimaterol and CGP12177 and (b) the comparison between the partial agonist log EC_{50} value and log K_B value for CGP12177. To confirm that these amino acids were indeed important, evidence was sought using a different assay to explore two further strands of evidence – the stimulation resulting from CGP12177 in the presence of a fixed concentration of cimaterol and the effect on the response to pindolol. To examine whether cimaterol and CGP12177 were competing at the same site of the receptor, CGP12177 concentration responses were examined in the presence of different fixed concentrations of cimaterol. Low concentrations of CGP12177 competed with cimaterol causing a reduction in the stimulatory response. However, higher CGP12177 concentrations stimulated responses, creating a "dip" in the curve, for the two conformation β1-WT adrenoceptor (Pak and Fishman, 1996; Figure 6a). This "dip" was absent in the single conformation β2-WT adrenoceptor and at β1-TM4 (Figure 6c and e; Table 6). The β1-V189T mutation alone had little effect on the two conformations (Figure 7a, Table 6), although the "dip" appeared reduced in both L195Q and W199Y mutations alone. However, the "dip" was absent from β1-V189T-L195Q-W199Y and β1-W199D (Figure 8a and c; Table 6) in keeping with a single conformation receptor. Interestingly, in the transiently transfected cells, the β1-WT ^{3}H-cAMP accumulation response to CGP12177 was best described by a two-component response (Figure 6a,

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Table 6). Hints that the CGP12177 response may, at times, be biphasic have been seen before (e.g. Figure 4 of Baker et al., 2013) and are also potentially present in the stable β1-WT cell lines and stable mixed β1-WT population data (Figure 1b and 4a respectively). Interestingly, mutations L195Q and W199Y (or W199D) removed the biphasic response of CGP12177 (Figure 7c and e, Figure 8c, Table 6). Finally, concentration responses to the biphasic ligand pindolol were examined (Figures 6b, d and f, Figure 7 b, d and f, Figure 8b and d, Table 6). Both the racemic and S-isomer of this ligand have previously been shown to stimulate agonist responses via both conformations of the β1-adrenoceptor (Walter et al., 1984; Baker 2010a). The data obtained with this agonist were consistent with the observations made with CGP12177 (apart from W199Y which continued to exhibit two-site pharmacology with pindolol; Table 6). However, this was not the case with W199D or combinations of W199Y with L195Q and/or V189T (Table 6).

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Discussion

The human β1-WT exists in at least two agonist conformations: a high affinity catecholamine conformation and a low affinity secondary (CGP12177) conformation (Pak and Fishman, 1996; Kaumann and Molenaar, 2008). This secondary conformation does not occur in the closely related human β2-WT (Pak and Fishman, 1996; Baker et al., 2002). This study attempted to identify the key area responsible for this secondary conformation using a β1/β2-chimeric receptor approach (Isogaya et al., 1999; Kaumann and Molenaar 2008).

At the β1-WT, the secondary conformation was clearly delineated from the primary catecholamine conformation by two pieces of evidence. Firstly, the cimaterol responses (catecholamine conformation agonist) were readily inhibited by antagonists whereas CGP12177 responses required much greater antagonist concentrations, yielding lower log K_B values (Table 2). Secondly, CGP12177 readily inhibited the cimaterol responses at low concentrations (log K_B -9.55), but required high concentrations to produce its agonist response (log EC_{50} -8.18) in keeping with previous studies (e.g. Pak and Fishman, 1996; Lowe et al., 2002; Baker et al., 2003; Joseph et al., 2004a; Baker, 2005). At the β2-WT, cimaterol and CGP12177 responses were inhibited by similar concentrations of antagonist and the EC_{50}/K_B discrepancy for CGP12177 was less marked suggesting competition of the two ligands at the same conformation.

When the TM-chimeras were examined, antagonism of cimaterol responses suggested that all receptors (β1-TM1 to β1-TM7 and β2-TM1 to β2-TM7, Tables 2 and 3) retained their respective pharmacological parent β1 or β2-subtype, even when one entire transmembrane region was altered (i.e. high affinity for CGP20712A at β1-adrenoceptors, high affinity for ICI118551 at β2-adrenoceptors). Significant changes were however seen in the secondary conformation in β1-TM4. Here, antagonists inhibited the cimaterol and CGP12177 responses to give similar log K_B values (Table 3), and the concentration of CGP12177 required to inhibit the cimaterol response (-9.63) approached that required to stimulate the agonist response (-9.27). Taken together this strongly suggests that the secondary conformation is absent in β1-

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TM4. Although direct comparison of partial agonist efficacy across cell lines is difficult, the log EC_{50} for CGP12177 (-9.27) is substantially more potent (left-shifted) at β1-TM4 than any other β1-TM-chimeric-receptor (-7.83 to -8.27; Table 2) even though the response is less efficacious (% isoprenaline maximum response achieved) and the receptor expression level is the lowest of the β1-cell lines. If CGP12177 were simply more efficacious at β1-TM4, the left-shifted EC_{50} would be accompanied by an increase in the maximum response obtained. The increase in potency, but decrease in efficacy, suggests that something different is happening at β1-TM4, and indeed these values are similar to those obtained for CGP12177 at the β2-WT and β2-chimeric-receptors (Tables 2 and 3). This differs from the comment in Kaumann and Molenaar (2008) regarding TM5.

Thus β1-TM4 appears to be a β1-adrenoceptor without the secondary conformation. When the β2-chimeras were examined (Table 3), the antagonist affinities (whether cimaterol or CGP12177 was the agonist), log EC_{50} and log K_B values for CGP12177 were similar for all β2-chimeric-receptors to that of β2-WT and β1-TM4. Thus although the secondary site was lost in β1-WT, it was not recreated in β2-WT by the TM4 swap, and therefore other areas of the receptor must contribute to this secondary conformation.

To locate the specific amino acids involved in the secondary conformation, intermediate stages in the generation of β1-TM4 were examined. The log EC_{50} for CGP12177 was more potent and the antagonist affinities were higher for β1-TM4 stage 5 and the full β1-TM4 chimeric receptor (Table 1 and 4). ICI118551 had a slightly different pattern with increased affinity at both conformations from β1-TM4 stage 4 onwards. This suggests that amino acids L195, W199 and maybe V189 are important for the secondary conformation. These mutations were therefore examined alone. For β1-L195Q and β1-W199Y, the antagonist affinities (log K_B) in the presence of CGP12177 were higher, and the log EC_{50} for CGP12177 more potent, than at β1-WT suggesting that both of these residues are important in the secondary conformation. ICI118551 had higher affinity for β1-V189T in the presence of both cimaterol

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and CGP12177, suggesting all-round (not conformation specific) higher affinity. This was seen in all receptors containing a V189T mutation (i.e. β1-TM4, β1-V189T, β1-V189T-L195Q, β1-V189T-W199Y and β1-V189T-L195Q-W199Y). ICI118551 also showed lower affinity at β2-TM4 (containing the reverse T164V mutation). V189T is therefore specifically involved in the affinity of ICI118551. CGP20712A and propranolol both had slightly higher affinity in the presence of CGP12177 at β1-V189T than β1WT, giving rise to lower ratios (Table 5b) and suggesting a potential minor role for V189T in the secondary conformation.

However, neither L195Q nor W199Y alone gave K_B or EC_{50} values that matched those of the β1-TM4 (Table 5). Double and triple mutations were therefore made. At β1-V189TL-195Q-W199Y, antagonist affinities and log EC_{50} values for CGP12177 were the same as for the β1-TM4 suggesting complete abolishment of the secondary conformation. β1-L195Q-W199Y also achieved this (with the exception of ICI118551 affinity because this lacks the V189T mutation). β1-V189T-L195Q and β1-V189T-W199Y both had the higher ICI118551 affinity, but the secondary conformation affinity for CGP20712A and propranolol did not reach that of β1-TM4. This suggests that whilst L195Q and W199Y are each important, their effects are additive and that both are needed to completely abolish the secondary conformation. The effect of V189T, whilst important for ICI118551 affinity, was minor for the secondary conformation.

The effect of charge at the W199 site was then assessed. The addition of a negatively charged group (W199D) greatly increased CGP20712A and propranolol affinity in the presence of CGP12177 to that of β1-TM4, β1-L195Q-W199Y and β1-V189T-L195Q-W199Y, suggesting that this single change could remove the secondary conformation (although ICI118551 affinity does not reach that of β1-TM4 or β1-V189T-L195Q-W199Y because V189T is missing). Thus the single amino acid change of W199D appeared to create a β1-adrenoceptor with β1 pharmacological characteristics (i.e. 100 fold higher affinity for CGP20712A than ICI118551) but that is completely devoid of the secondary conformation.

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Finally, to confirm L195Q, W199Y (and maybe V189T) involvement in the secondary conformation, ^{3}H-cAMP accumulation was investigated (Figures 6-8). At low concentrations, CGP12177 inhibited the stimulation of the catecholamine conformation by cimaterol, whilst at higher concentrations CGP12177 stimulated the secondary conformation resulting in a "dip" in the response to cimaterol in the presence of CGP12177 (Figure 6a Pak and Fishman 1996; Konkar et al., 2000; Baker et al., 2003; Baker 2005a). This "dip" was present for β1-V189T (Figure 7a) but reduced at β1-L195Q and β1-W199Y (Figure 7c and 7e). There was however no "dip" in β2-WT, β1-TM4, β1-L195Q-W199Y, β1-V189T-L195Q-W199Y or β1-W199D (Figures 6c, 6e, 8a and 8c). At all these receptors, CGP12177 competed with cimaterol to inhibit the cimaterol response at the same as (or just to the right of) that of the EC_{50} value, strongly suggesting competition at the same conformation.

Pindolol has a biphasic concentration response curve at the β1-adrenoceptor. The first (higher affinity) component is thought to occur via the catecholamine conformation whilst the second component via the secondary conformation (Walter et al., 1984; Baker et al., 2003). Therefore, the pindolol response should become a single component response at β1-TM4 and the other receptors lacking the secondary conformation. Indeed, pindolol was found to have a two-component concentration response curve at β1-WT and β1-V189T, but a single component response curve at β2-WT, β1-TM4, β1-L195Q-W199Y, β1-T189V-L195Q-W199Y, and β1-W199D confirming the crucial roles of L195 and W199 in the secondary conformation. Interestingly, for all β-adrenoceptors with a known sequence and two pharmacologically confirmed conformations, V, L and W, or similar, are found at equivalent positions for 189, 195 and 199 respectively, whilst those of one-conformation β-adrenoceptors have T, Q and Y (Table 7).

A recent turkey β1-adrenoceptor crystal structure suggests that TM4 is involved in a β1-dimer interface with TM5 (Huang et al., 2013). Figure 9a shows that the equivalent key turkey TM4 residues (V172, M178, and W182; Table 7) are largely outward facing, away from the binding pocket, but lie within the

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TM4/TM5 dimer interface (Figure 9b). However, the key binding residues in TM3 (D138) and TM7 (N363), that are essential for both the catecholamine and secondary conformation responses (Baker et al., 2008), are both inward-facing residues within the ligand binding pocket (Figure 9a). It is therefore interesting to speculate that both binding site activation and receptor dimerization are involved in the secondary conformation. However, more work is required to understand how and where molecules bind in order to create the distinct pharmacological patterns observed.

In conclusion, the β1-adrenoceptor exists in two agonist conformations and the key residues responsible for the secondary low affinity conformation are in TM4. The mutation β1-V189T has an effect on the affinity of ICI118551 and may have a minor role in secondary conformation pharmacology. However, the amino acids at positions 195 and 199 in TM4 both have a major role in the secondary conformation and together L195Q and W199Y (or indeed W199D alone), abolishes this secondary conformation and creates a β1-adrenoceptor with only one high affinity agonist conformation. From Huang et al., 2013 data, it is tempting to speculate that oligomerization involving TM4 may underlie the secondary β1-adrenoceptor conformation.

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Authorship contributions

Participated in research design: Baker

Conducted experiments: Baker, Proudman

Contributed new reagents or analytical tools: Baker, Proudman, Hill

Performed data analysis: Baker

Wrote or contributed to the writing of the manuscript: Baker, Hill

References

Arch JR (2004) Do low-affinity states of beta-adrenoceptors have roles in physiology and medicine? *Br J Pharmacol*. **143:** 517-518.

Baker JG, Hall IP, and Hill SJ. (2002) Pharmacological characterization of CGP 12177 at the human β2-adrenoceptor. *Br J Pharmacol*. **137:** 400-408.

Baker JG (2005a) Sites of action of β-ligands at the human β1-adrenoceptor. *J. Pharmacol Exp Ther*. **313:** 1163-1171.

Baker JG (2005b). Evidence for a secondary state of the human β3-adrenoceptor. *Mol. Pharmacol*. **68:** 1645-1655.

Baker JG (2010a) The selectivity of β-adrenoceptor agonists at the human β1, β2 and β3 adrenoceptors. *Br J Pharmacol* **160:** 148-161.

Baker JG (2010b) A full pharmacological analysis of the three turkey β-adrenoceptors and comparison with the human β-adrenoceptors. *PLoS ONE 5(11): e15487. doi:10.1371/journal.pone.0015487*

Baker JG, Hall IP, and Hill SJ (2003) Agonist actions of "β-blockers" provide evidence for two agonist activation sites or conformations of the human β1-adrenoceptor. *Mol Pharmacol* **63:** 1312-1321.

Baker JG, Proudman RGW, Hawley NC, Fischer PM, and Hill SJ (2008) Role of key transmembrane residues in agonist and antagonist actions at the two conformations of the human β1-adrenoceptor. *Mol Pharmacol*. **74:** 1246-1260.

Baker JG, Proudman RGW, and Hill SJ (2013) Impact of Polymorphic Variants on the Molecular Pharmacology of the Two-Agonist Conformations of the Human β1-Adrenoceptor. *PLoS ONE 8(11): e77582. doi:10.1371/journal.pone.0077582*

Granneman JG (2001) The putative beta4-adrenergic receptor is a novel state of the beta1-adrenergic receptor. *Am J Physiol Endocrinol Metab*. **280:** E199-202.

Huang J, Chen S, Zhang JJ, and Huang XY (2013) Crystal structure of oligomeric β1-adrenergic G protein-coupled receptor in ligand-free basal state. *Nature Struct Mol Biol.* **20:** 419-425.

Isogaya M1, Sugimoto Y, Tanimura R, Tanaka R, Kikkawa H, Nagao T, Kurose H (1999) Binding pockets of the beta(1)- and beta(2)-adrenergic receptors for subtype-selective agonists. *Mol Pharmacol.* **56:** 875-85.

Joseph SS, Lynham JA, Molenaar P, Grace AA, Colledge WH, and Kaumann AJ (2003) Intrinsic sympathomimetic activity of (-)-pindolol mediated through a (-)-propranolol-resistant site of the beta1-adrenoceptor in human atrium and recombinant receptors. *Naunyn Schmiedebergs Arch Pharmacol.* **368:** 496-503.

Joseph SS, Lynham JA, Colledge WH, and Kaumann AJ (2004a) Binding of (-)-[3H]-CGP12177 at two sites in recombinant human beta 1-adrenoceptors and interaction with beta-blockers. *Naunyn Schmiedebergs Arch Pharmacol.* **369:** 525-532.

Joseph SS, Colledge WH, and Kaumann AJ (2004b) Aspartate138 is required for the high-affinity ligand binding site but not for the low-affinity binding site of the beta1-adrenoceptor. *Naunyn Schmiedebergs Arch Pharmacol*. **370:** 223-226.

Kaumann AJ (1973) Adrenergic receptors of cardiac muscle. Two different mechanisms of β-blockers as partial agonists. *Acta Physiol Lat Amer* **23:** 619-620.

Kaumann AJ (1983) Cardiac β-adrenoceptors - experimental aspects. *Z Kardiol* **72:** 63-82.

Kaumann AJ, and Blinks JR. (1980) beta-Adrenoceptor blocking agents as partial agonists in isolated heart muscle: dissociation of stimulation and blockade. *Naunyn Schmiedebergs Arch Pharmacol*. **311:** 237-328.

Kaumann AJ, Engelhardt S, Hein L, Molenaar P, and Lohse M. (2001) Abolition of (-)-CGP 12177-evoked cardiostimulation in double beta1/beta2-adrenoceptor knockout mice. Obligatory role of beta1-adrenoceptors for putative beta4-adrenoceptor pharmacology. *Naunyn Schmiedebergs Arch. Pharmacol.* **363:** 87-93.

Kaumann AJ, and Molenaar P (2008) The low-affinity site of the beta1-adrenoceptor and its relevance to cardiovascular pharmacology. *Pharmacol Ther*. **118:** 303-336.

Kompa AR, and Summers RJ (1999) Desensitization and resensitization of beta 1- and putative beta 4-adrenoceptor mediated responses occur in parallel in a rat model of cardiac failure. *Br J Pharmacol*. **128:** 1399-1406.

Konkar AA, Zhengxian Z and Granneman JG (2000) Aryloxypropanolamine and catecholamine ligand interactions with the β1-adrenergic receptor: evidence for interaction with distinct conformations of β1-adrenergic receptors. *J Pharmacol Exp Ther*. **294:** 923-932.

Kozłowska H, Schlicker E, Kozłowski M, Baranowska M, and Malinowska B (2006) Potential involvement of a propranolol-insensitive atypical beta-adrenoceptor the vasodilator effect of cyanopindolol in the human pulmonary artery. *J Physiol Pharmacol*. **57:** 317-328.

Kozlowska H, Szymska U, Schlicker E, and Malinowska B (2003) Atypical beta-adrenoceptors, different from beta 3-adrenoceptors and probably from the low-affinity state of beta 1-adrenoceptors, relax the rat isolated mesenteric artery. *Br J Pharmacol*. **140:** 3-12.

Lowe MD, Lynham JA, Grace AA, and Kaumann AJ (2002) Comparison of the affinity of β-blockers for the two states of the β1-adrenoceptor in ferret ventricular myocardium. *Br J Pharmacol*. **135:** 451-461.

Malinowska B, and Schlicker E (1996) Mediation of the positive chronotropic effect of CGP 12177 and cyanopindolol in the pithed rat by atypical beta-adrenoceptors, different from beta 3-adrenoceptors. *Br J Pharmacol*. **117:** 943-949.

Mallem MY, Toumaniantz G, Serpillon S, Gautier F, Gogny M, Desfontis JC, and Gauthier C (2004) Impairment of the low-affinity state beta1-adrenoceptor-induced relaxation in spontaneously hypertensive rats. *Br J Pharmacol*. **143:** 599-605.

Molenaar P (2003) The 'state' of beta-adrenoceptors. *Br J Pharmacol*. **140:** 1-2.

Molenaar P, Chen L, Semmler AB, Parsonage WA, and Kaumann AJ (2007) Human heart beta-adrenoceptors: beta1-adrenoceptor diversification through 'affinity states' and polymorphism. *Clin Exp Pharmacol Physiol*. **34:** 1020-1028.

Pak MD, and Fishman PH (1996). Anomalous behaviour of CGP 12177A on beta 1-adrenergic receptors. *J Recept. Signal Transduction Res*. **16:** 1-23.

Sarsero D, Russell FD, Lynham JA, Rabnott G, Yang I, Fong KM, Li L, Kaumann AJ, and Molenaar P (2003) (-)-CGP 12177 increases contractile force and hastens relaxation of human myocardial preparations through a propranolol-resistant state of the beta 1-adrenoceptor. *Naunyn Schmiedebergs Arch Pharmacol.* **367:** 10-21.

Stephenson RP (1956) A modification of receptor theory. *Br J Pharmacol.* **11:** 5109-5116.

Staehelin M, Simons P, Jaeggi K, and Wigger N (1983) CGP-12177. A hydrophilic beta-adrenergic receptor radioligand reveals high affinity binding of agonists to intact cells. *J Biol Chem*. **258:** 3496-3502.

Swangkoon S, Miyamoto M, Nakayama T and Hamlin RL (2000) Acute cardiovascular effects and pharmacockinetics of carvedilol in healthy dogs. *Am J Vet Res* **61:** 57-60.

Walter M, Lemoine H, and Kaumann AJ (1984) Stimulant and blocking effects of optical isomers of pindolol on the sinoatrial node and trachea of guinea pig. Role of beta-adrenoceptor subtypes in the dissociation between blockade and stimulation. *Naunyn Schmiedebergs Arch Pharmacol*. **327:** 159-175.

Zakrzeska A, Schlicker E, Kwolek G, Kozłowska H, and Malinowska B (2005) Positive inotropic and lusitropic effects mediated via the low-affinity state of beta1-adrenoceptors in pithed rats. *Br J Pharmacol*. **146:** 760-768.

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Footnotes

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Figure legends

Figure 1

CRE-SPAP production in response to cimaterol (a, c and e) and CGP12177 (b, d and f) in the absence and presence of bisoprolol and propranolol in stable cell lines expressing β1-WT (a and b), β1-TM4 (c and d) and human β2-WT (e and f). Bars represent basal CRE-SPAP production, that in response to 10μM isoprenaline or that in response to bisoprolol or propranolol alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of a) 5, b) 4, c) 4, d) 7, e) 6 and f) 10 separate experiments.

This Figure shows that the greater concentrations of antagonist are required to inhibit the secondary conformation CGP12177 responses in the β1-WT, but that this is not the case for the β1-TM4 or β2-WT.

Figure 2

CRE-SPAP production in response to cimaterol in the absence and presence of CGP12177 in stable cell lines expressing a) β1-WT, b) β1-TM4 and c) β2-WT. Bars represent basal CRE-SPAP production, that in response to 10μM isoprenaline or that in response 1-30nM CGP12177 alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of a) 6, b) 8, and c) 9 separate experiments.

This Figure shows that CGP12177 inhibits cimaterol responses with similar high affinity at β1-WT, β1-TM4 and β2-WT.

Figure 3

CRE-SPAP production in response to cimaterol in the absence and presence of 10nM and 100nM CGP20712A and 1μM ICI118551 in stable mixed populations of cell lines expressing a) β1-WT, b) β1-V189T, c) β1-L195Q, d) β1-W199Y and e) β1-TM4. Bars represent basal CRE-SPAP production, that in response to 10μM isoprenaline or that in response to 10nM or 100nM CGP20712A or 1μM ICI118551

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alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of a) 24, b) 12, c) 12, d) 12 and e) 23 separate experiments.

This Figure shows that CGP20712A inhibits cimaterol responses in all of these receptors with similar ability, however ICI118551 is able to inhibit β1-V189T and β1-TM4 to a greater extent than the others.

Figure 4

CRE-SPAP production in response to CGP12177 in the absence and presence of 100nM and 1μM CGP20712A and 10μM ICI118551 in stable mixed populations of cell lines expressing a) β1-WT, b) β1-V189T, c) β1-L195Q, d) β1-W199Y and e) β1- TM4. Bars represent basal CRE-SPAP production, that in response to 10μM isoprenaline or that in response to 100nM or 1μM CGP20712A or 10μM ICI118551 alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of a) 21, b) 11, c) 11, d) 11 and e) 20 separate experiments.

This Figure shows that secondary conformation CGP12177 responses are more readily inhibited by CGP20712A and ICI118551 at β1-L195Q, β1-W99Y and β1-TM4 than at β1-WT suggesting that the secondary conformation is compromised in the β1-L195Q, β1-W99Y and β1-TM4 mutants. β1-V189T whilst remaining more resistant to CGP20712A inhibition (i.e. demonstrating preservation of the secondary conformation) is once again more sensitive to ICI118551 inhibition.

Figure 5

CRE-SPAP production in response to cimaterol (a and c) and CGP12177 (b and d) in the absence and presence of 10nM, 100nM or 1μM CGP20712A and 10μM ICI118551 in stable mixed populations of cell lines expressing β1-V189T-L195Q-W199Y (a and b) or β1-W199D (c and d). Bars represent basal CRE-SPAP production, that in response to 10μM isoprenaline or that in response to 10nM, 100nM or 1μM CGP20712A or 10μM ICI118551 alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of 8 separate experiments in each case.

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This Figure shows that CGP20712A and ICI118551 are able to inhibit cimaterol and CGP12177 responses to a similar degree at both β1-V189T-L195Q-W199Y and β1-W199D, unlike that seen at β1-WT (Figure 3a and Figure 4a). This suggests that whilst both are primarily β1-adrenoceptors (given that both are more readily inhibited by CGP20712A than ICI118551) the secondary conformation is compromised in both of these mutant receptors.

Figure 6

^{3}H-cAMP accumulation in response to CGP12177 (a, c and e) and pindolol (b, d and f) in transiently transfected cells expressing β1-WT (a and b), β1-TM4 (c and d) and β2-WT (e and f). The CGP12177 responses were examined in the absence and presence of 3, 10 and 30nM cimaterol (a and c) and 0.3, 1 and 3nM cimaterol (e). Bars represent basal ^{3}H-cAMP accumulation, that in response to 10μM isoprenaline or that in response to 0.3-30nM cimaterol alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of a) 6, b) 8, c) 4, d) 3, e) 4 and f) 4 separate experiments.

This Figure examines different evidence for the absence or presence of the secondary conformation. At β1-WT, low concentrations of CGP12177 (e.g. 3nM, -8.5) inhibit the response to cimaterol whereas higher concentrations of CGP12177 cause a stimulatory response (e.g. 100nM, -7 and above). This creates a “dip” in the curve (CGP12177+fixed concentration of cimaterol) and is strongly suggestive of interaction at two different conformations. This is absent at β1-TM4 and β2-WT suggesting single conformation interaction. Similarly the response to pindolol is biphasic at β1-WT but monophasic at β1-TM4 and β2-WT again suggesting two conformations at β1-WT but only one conformation at β1-TM4 and β2-WT.

Figure 7

^{3}H-cAMP accumulation in response to CGP12177 (a, c and e) and pindolol (b, d and f) in transiently transfected cells expressing β1-V189T (a and b), β1-L195Q (c and d) and β1-W199Y (e and f). The

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CGP12177 responses were examined in the absence and presence of 3, 10 and 30nM cimaterol. Bars represent basal ^{3}H-cAMP accumulation, that in response to 10μM isoprenaline or that in response to 3, 10 and 30nM cimaterol alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of a) 6, b) 5, c) 3, d) 3, e) 4 and f) 4 separate experiments.

This Figure shows that both the "dip" in the CGP12177+cimaterol curve and biphasic pindolol response are still present at the β1-V189T suggesting conservation of the secondary conformation. However both are reduced at β1-L195Q and β1-W199Y suggesting some loss of the secondary conformation at β1-L195Q and β1-W199Y (see Figure 6a for β1-WT comparison)

Figure 8

^{3}H-cAMP accumulation in response to CGP12177 (a, and c) and pindolol (b and d) in transiently transfected cells expressing β1-V189T-L195Q-W199Y (a and b) and β1-W199D (c and d). The CGP12177 responses were examined in the absence and presence of 3, 10 and 30nM cimaterol. Bars represent basal ^{3}H-cAMP accumulation, that in response to 10μM isoprenaline or that in response to 3, 10 and 30nM cimaterol alone. Data points are mean ± sem of triplicate determinations. These single experiments are representative of a) 3, b) 4, c) 6 and d) 4 separate experiments.

This Figure shows that the "dip" in the CGP12177+cimaterol curve and biphasic pindolol response, both present at β1-WT with two conformations (see Figure 6a), are absent in β1-V189T-L195Q-W199Y and β1-W199D suggesting that neither of these receptors have the secondary conformation.

Figure 9.

Location of equivalent key residues (V172, M178, W182) in the crystal structure of the turkey β1-adrenceptor described by Huang et al., (2013). This structure was obtained in the ligand-free state and in a lipid membrane-like environment and suggested that TM4 was involved in a dimer interface with TM5. Views of one protomer of the oligomeric structure (PDB; 4GPO) reported by Huang et al., (2013) were generated with Cn3D (NCBI).

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(a) Residues V172, M178, W182 (equivalent to the human V189, L195 and W199) and D121 and N329 (equivalent to D138 and N363, identified in a previous study as essential for both catecholamine and secondary conformation functional responses, Baker et al., 2008) are highlighted in yellow.
(b) View of V172, M178, W182 (highlighted in red) with the key residues for dimerization in TM5 (R205, A206, A210, I218, R229), TM4 (L171) and the proximal region of extracellular loop 2 (W181, R183) identified by Huang et al., (2013) highlighted in purple. Interestingly, V172 and W182 are on the same outward facing surface as the key dimerization residues with W182 sandwiched between the two residues (W181, R183) and V172 is next to another (L171) identified by Huang et al., (2013).

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Table 1
The chimeric β1/β2-adrenoceptor constructs – the amino acid changes made and where a stable cell line was made, the receptor expression level is given as fmol/mg protein

	Amino acid changes	Fmol/mg protein
β1-WT		556 ± 39
β1-TM1	L63I, L64V, A66S, L71A, A74F, V81T	372 ± 47
β1-TM2	M98T, S102C, L110A, T117A, I118H, V119I, V120L	567 ± 58
β1-TM3	L133F, V137I, L154V	1994 ± 214
β1-TM4	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G, V189T, L195Q, W199Y	233 ± 18
β1-TM5	R222Q, V230I, C238V, A241V, L245S	359 ± 30
β1-TM6	V332T, L342I, A343V, V345I, K347H, A348V, F349I	1207 ± 100
β1-TM7	R357E, L358V, F359Y, V360I, F361L, F362L, L365I, A368V, A371G, I375L	2078 ± 133
β1-A187G	A187G	
β1-V189T	V189T	
β1-L195Q	L195Q	
β1-W199Y	W199Y	
β1-TM4 stage 1	G177V, L178I, V179I	
β1-TM4 stage 2	G177V, L178I, V179I, C180L, T181M, A184I, I185V	
β1-TM4 stage 3	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G	
β1-TM4 stage 4	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G, V189T	
β1-TM4 stage 5	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G, V189T, L195Q	
β1-V189T-L195Q	V189T, L195Q	
β1-V189T-W199Y	V189T, W199Y	
β1-L195Q-W199Y	L195Q, W199Y	
β1-V189T-L195Q-W199Y	V189T, L195Q, W199Y	
β1-W199A	W199A	
β1-W199D	W199D	
β1-W199F	W199F	
β1-W199K	W199K	
β1-W199L	W199L	
β1-W199N	W199N	
β2-WT		268 ± 25
β2-TM1	I38L, V39L, S41A, A46L, F49A, T56V	138 ± 8
β2-TM2	T73M, C77S, A85L, A92T, H93I, I94V, L95V	405 ± 41
β2-TM3	F108L, I112V, V129L	1037 ± 113
*β2-TM4	V152G, I153L, I154V, L155C, M156T, I159A, V160I, G162A, T164V, Q170L	458 ± 64
β2-TM5	Q197R, I205V, V213C, V216A, S220L	849 ± 83
β2-TM6	T281V, I291L, V292A, I294V, H296K, V297A, I298F	728 ± 58
β2-TM7	E306R, V307L, Y308F, I309V, L310F, L311F, I314L, V317A, G320A, L324I	67 ± 5

*Making the full change here, i.e with Y174W, rendered the receptor non-functional (no binding and no functional responses). This chimera with 10 of the 11 amino acid substitutions was therefore used as the β2-TM4 mutant.

Table 2a

Log EC_{50} values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at the human β1-WT and chimeric β1/β2-adrenoceptors where mutations in the β1-WT mean that each TM region in turn is mutated to that of the β2-WT (see Table 1). Log K_B values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. This data was obtained from stable cell lines and n in the Table refers to the number of separate experiments.

This Table shows that the most consistent and highly significant difference between the β1-WT receptor and all of the TM-swap mutants is that obtained from β1-TM4 when CGP12177 is the agonist. As CGP12177 agonist responses occur at the secondary low affinity conformation of the β1-WT receptor, this suggests that the β1-TM4 receptor mutations alter this conformation in some way.

Cimaterol as agonist – β1 – stable cell lines																			
	Cimaterol Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B bisoprolol	n		Log K_B ICI118551	n		Log K_B propranolol	n		Log K_B carvedilol	n		Log K_B CGP12177	n
β1-WT	-8.25 ± 0.14	95.8 ± 2.9	8		-9.18 ± 0.07	4		-8.48 ± 0.17	5		-6.96 ± 0.04	5		-8.52 ± 0.08	12		-9.89 ± 0.13	5		-9.55 ± 0.12	8
β1-TM1	-7.91 ± 0.05*	96.5 ± 3.3	11		-9.35 ± 0.10	9		-8.65 ± 0.12	5		-6.96 ± 0.05	9		-8.66 ± 0.07	16		-9.88 ± 0.08	5		-9.77 ± 0.07	15
β1-TM2	-8.19 ± 0.03	98.9 ± 3.0	15		-8.78 ± 0.12	8		-8.15 ± 0.07	7		-7.47 ± 0.03*	14		-8.52 ± 0.03	24		-9.53 ± 0.18	8		-9.90 ± 0.09	17
β1-TM3	-8.82 ± 0.03*	102.4 ± 2.5	9		-9.34 ± 0.12	9		-8.43 ± 0.07	4		-7.31 ± 0.04*	10		-8.53 ± 0.06	15		-9.92 ± 0.16	5		-9.49 ± 0.06	13
β1-TM4	-8.15 ± 0.02	99.2 ± 1.8	10		-9.50 ± 0.13	11		-8.81 ± 0.04	5		-8.07 ± 0.06*	9		-8.99 ± 0.04*	15		-9.81 ± 0.12	6		-9.63 ± 0.08	17
β1-TM5	-8.48 ± 0.05	103.0 ± 1.7	9		-9.09 ± 0.04	11		-7.91 ± 0.03	5		-7.01 ± 0.06	11		-8.71 ± 0.04	17		-9.96 ± 0.09	6		-9.68 ± 0.09	17
β1 TM6	-8.59 ± 0.03*	103.2 ± 2.2	10		-8.52 ± 0.07	8		-8.10 ± 0.15	5		-7.92 ± 0.05*	12		-8.90 ± 0.05*	18		-10.13±0.09	6		-9.58 ± 0.06	14
β1-TM7	-8.54 ± 0.05	106.1 ± 2.5	10		-8.45 ± 0.08	7		-8.19 ± 0.06	5		-7.80 ± 0.05*	12		-8.95 ± 0.05*	18		-10.06±0.03	3		-9.39 ± 0.05	13
CGP12177 as agonist – β1 – stable cell lines																			
	CGP 12177 Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B bisoprolol	n		Log K_B ICI118551	n		Log K_B propranolol	n		Log K_B carvedilol	n			
β1-WT	-8.18 ± 0.08	73.8 ± 5.6	10		-7.16 ± 0.06	14		-5.83 ± 0.14	4		-5.90 ± 0.15	9		-6.18 ± 0.06	13		-7.25 ± 0.18	7			
β1-TM1	-7.83 ± 0.05	53.7 ± 3.9	10		-7.41 ± 0.13	11		-5.77 ± 0.18	4		-5.79 ± 0.09	3		-6.55 ± 0.12	13		-7.72 ± 0.13	7			
β1-TM2	-8.27 ± 0.09	53.5 ± 3.9	14		-7.13 ± 0.08	13		-6.72 ± 0.18	7		-5.87 ± 0.05	6		-6.87 ± 0.15*	18		-8.52 ± 0.15*#	7			
β1-TM3	-8.07 ± 0.03	70.5 ± 3.6	9		-7.45 ± 0.05	12		-5.83 ± 0.13	4		-5.83 ± 0.09	7		-6.31 ± 0.05	16		-7.45 ± 0.09	6			
β1-TM4	-9.27 ± 0.06*#	29.2 ± 1.4*#	10		-9.49 ± 0.08*#	9		-8.83 ± 0.21*#	7		-7.93 ± 0.11*#	11		-8.85 ± 0.09*#	15		-9.73 ± 0.21*#	7			
β1-TM5	-7.91 ± 0.03	60.1 ± 3.4	10		-6.68 ± 0.04*	11		-5.31 ± 0.13	5		-5.71 ± 0.15	8		-6.23 ± 0.09	15		-7.53 ± 0.16	7			
β1-TM6	-7.83 ± 0.02*	63.5 ± 2.8	11		-5.97 ± 0.08*#	12		-5.22 ± 0.14	3		-5.79 ± 0.04	11		-6.05 ± 0.06	19		-7.24 ± 0.18	7			
β1-TM7	-7.08 ± 0.02*#	68.5 ± 3.8	11		-6.86 ± 0.05	10		-5.67 ± 0.05	5		-6.67 ± 0.03*#	13		-6.42 ± 0.06	16		-7.35 ± 0.12	6			

*p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β1-WT. Thus the log EC_{50} for CGP12177 at β1-TM4 is different from that obtained from the β1-WT with p<0.001.

#p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing each value with all other values in this set. Thus the log EC_{50} value for CGP12177 at β1-TM4 is different from that obtained for β1-WT, β1-TM1, β1-TM2, β1-TM3, β1-TM5, β1-TM6 and β1-TM7 with p<0.001 in all cases.

Table 2b

The ratio of the log K_B value for CGP12177 (as an antagonist of cimaterol, thus at the high affinity conformation) and log EC_{50} value for CGP12177 (as an agonist of the secondary low affinity conformation) for responses seen at the β1-WT and β1-TM-chimeric receptors (data taken from Table 2a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log K_B for antagonism of cimaterol at high affinity site / log K_B for antagonism of CGP12177 at low affinity site).

This Table examines the ratio of the affinities of ligands at the two conformations of the β1-adrenoceptor and thus allows for differences in absolute affinity. For example, ICI118551 has a higher affinity at the β1-TM7 receptor (Table 2a) than the β1-WT receptor, however the ratio of the affinity in the presence of cimaterol and CGP 12177 is similar to that at the WT receptor suggesting that although the β1-TM7 mutation alters the affinity of ICI118551, it does not alter the secondary conformation. The most consistent change is seen with the β1-TM4 mutation where the ratios are all nearly 0 suggesting no difference in affinity i.e. no secondary conformation.

	Ratio of log K_B CGP12177 / log EC_{50} CGP12177		Ratio of log K_B (cimaterol as agonist) / log K_B (CGP12177 as agonist)				
			CGP20712A	bisoprolol	ICI118551	propranolol	carvedilol
β1-WT	1.37		2.02	2.65	1.06	2.34	2.64
β1-TM1	1.94		1.94	2.88	1.17	2.11	2.16
β1-TM2	1.63		1.65	1.43	1.60	1.65	1.01
β1-TM3	1.42		1.89	2.60	1.48	2.22	2.47
β1-TM4	0.36		0.01	-0.02	0.14	0.14	0.08
β1-TM5	1.77		2.41	2.60	1.30	2.47	2.43
β1-TM6	1.75		2.55	2.88	2.15	2.85	2.89
β1-TM7	2.31		1.59	2.52	1.13	2.53	2.71

Table 3a

Log EC_{50} values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at the human β2-WT and chimeric β2/β1-receptors where mutations in the β2-WT mean that each TM region in turn is mutated to that of the β1-WT (see Table 1). Log K_B values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. This data was obtained from stable cell lines and n in the Table refers to the number of separate experiments.

This Table shows that although there are some changes (e.g. β2-TM2 has higher affinity for CGP20712A than β2-WT), there is no highly significant consistent change in one mutant receptor compared to the others.

Cimaterol as agonist – β2 – stable cell lines																	
	Cimaterol Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B bisoprolol	n		Log K_B ICI118551	n		Log K_B propranolol	n		Log K_B CGP12177	n
β2-WT	-9.05 ± 0.08	101.5 ± 3.2	13		-6.03 ± 0.12	8		-6.85 ± 0.10	8		-9.56 ± 0.07	8		-9.65 ± 0.05	14		-10.00 ± 0.05	9
β2-TM1	-9.45 ± 0.06	105.3 ± 2.8	9		-6.46 ± 0.10	8		-6.82 ± 0.09	10		-9.74 ± 0.08	9		-9.56 ± 0.06	19		-9.74 ± 0.11	19
β2-TM2	-9.64 ± 0.08*	102.1 ± 1.8	12		-7.20 ± 0.11*	8		-7.25 ± 0.10	7		-9.30 ± 0.11	8		-9.87 ± 0.03	14		-10.01 ± 0.06	9
β2-TM3	-9.21 ± 0.13	98.3 ± 4.1	7		-6.57 ± 0.12	7		-7.05 ± 0.13	6		-9.71 ± 0.10	7		-9.81 ± 0.07	11		-10.00 ± 0.10	10
β2-TM4	-9.66 ± 0.06*	103.3 ± 2.4	13		-6.09 ± 0.09	10		-6.75 ± 0.03	9		-8.87 ± 0.04*	10		-9.34 ± 0.06	14		-9.83 ± 0.05	9
β2-TM5	-8.97 ± 0.09	100.6 ± 2.4	13		-6.63 ± 0.12	10		-7.04 ± 0.09	9		-9.50 ± 0.09	9		-9.46 ± 0.04	13		-9.82 ± 0.07	4
β2-TM6	-8.74 ± 0.05	101.0 ± 4.7	12		-7.05 ± 0.15*	7		-7.07 ± 0.10	9		-8.86 ± 0.12*	9		-9.52 ± 0.06	11		-9.59 ± 0.13	8
β2-TM7	-8.77 ± 0.06	107.4 ± 2.9	10		-6.54 ± 0.04	14		-7.06 ± 0.09	11		-9.18 ± 0.07	12		-9.30 ± 0.06*	17		-9.72 ± 0.10	13
CGP12177 as agonist – β2 – stable cell lines																		
	CGP 12177 Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B bisoprolol	n		Log K_B ICI118551	n		Log K_B propranolol	n			
β2-WT	-9.35 ± 0.09	23.5 ± 3.9	14		-6.12 ± 0.09	3		-6.60 ± 0.14	11		-9.31 ± 0.17	11		-9.02 ± 0.12	19			
β2-TM1	-9.48 ± 0.19	26.9 ± 1.7	9		-6.00 ± 0.16	9		-6.63 ± 0.12	12		-9.51 ± 0.17	12		-9.07 ± 0.08	20			
β2-TM2	-9.20 ± 0.09	12.6 ± 1.9	11		-6.79 ± 0.11	8		-7.00 ± 0.09	9		-9.03 ± 0.11	10		-9.32 ± 0.10	14			
β2-TM3	-9.19 ± 0.10	35.9 ± 3.3	9		-6.21 ± 0.29	4		-6.53 ± 0.12	8		-9.47 ± 0.16	8		-8.95 ± 0.09	21			
β2-TM4	-9.20 ± 0.07	38.5 ± 2.4*	13		-6.05 ± 0.12	8		-6.34 ± 0.09	7		-8.42 ± 0.08*	11		-8.34 ± 0.08*	18			
β2-TM5	-9.31 ± 0.07	28.8 ± 2.0	12		-6.47 ± 0.15	8		-6.98 ± 0.22	8		-9.31 ± 0.08	9		-8.75 ± 0.10	19			
β2-TM6	-8.81 ± 0.10	22.4 ± 1.6	8		-7.31 ± 0.34	5		-6.74 ± 0.11	5		-8.39 ± 0.27	4		-9.11± 0.11	4			
β2-TM7	-9.71 ± 0.12	41.3 ± 2.7*	9		-6.19 ± 0.15	10		-6.59 ± 0.16	10		-9.09 ± 0.14	9		-9.02 ± 0.08	22			

*p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β2-WT. Thus the log K_B value for CGP20712A at β2-TM2 obtained in the presence of cimaterol is different (p<0.001) from that obtained in the β2-WT receptor.
#p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing each value with all other values in this set (as for Table 2a). Unlike Table 2a for the β1-receptor. There are no values that fulfil this criterion for the β2-receptor.

Table 3b
The ratio of the log K_B value for CGP12177 (as an antagonist of cimaterol) and log EC_{50} value for CGP12177 for responses seen at the β2WT and β2TM-chimeric-receptors (data taken from Table 3a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log K_B for antagonism of cimaterol / log K_B for antagonism of CGP12177).

This Table examines the ratio of the affinities of ligands at the β2-adrenoceptor and thus allows for differences in absolute affinity. ICI118551 has lower affinity at the β2-TM4 receptor (Table 3a), but this occurred both in the presence of cimaterol and CGP12177 thus the ratio is similar to that at the other receptors. There is no large consistent change with any of the receptors.

	Ratio of log K_B CGP12177 / log EC_{50} CGP12177		Ratio of log K_B (cimaterol as agonist) / log K_B (CGP12177 as agonist)			
			CGP20712A	bisoprolol	ICI118551	propranolol
β2-WT	0.65		-0.09	0.25	0.25	0.63
β2-TM1	0.26		0.46	0.19	0.23	0.49
β2-TM2	0.81		0.41	0.25	0.27	0.55
β2-TM3	0.81		0.36	0.52	0.24	0.86
β2-TM4	0.63		0.04	0.41	0.45	1.00
β2-TM5	0.51		0.16	0.06	0.19	0.71
β2-TM6	0.78		-0.26	0.33	0.47	0.41
β2-TM7	0.01		0.35	0.47	0.09	0.28

Table 4a

Log EC_{50} values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at the β1-WT, the chimeric β1-TM4 receptor (containing the full TM4 mutations) and chimeric β1/β2-adrenoceptors with several amino acids in TM4 mutated to that of the β2-WT. Log K_B values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. Table 1 lists the detail of each mutation. This data was obtained from between 4 and 24 stable mixed populations of cells for each chimera, and n in the Table refers to the number of separate experiments.

This Table shows that within TM4, it is only the amino acid changes in Stage 5 and the full TM4 (i.e. L195Q and W199Y) that are very important for the alteration of the secondary conformation (i.e. responses to CGP12177 and antagonist affinities obtained in the presence of CGP12177). Stage 4 (addition of V189T to the other changes) affects the affinity of ICI18551. There is no change in the pharmacology of either conformation even when the majority of amino acids have been changed (up to and including Stage 3).

Cimaterol as agonist – stable mixed populations of cells															
	Cimaterol Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B ICI118551	n		Log K_B propranolol	n		Log K_B CGP12177	n
β1-WT	-8.29 ± 0.04	81.2 ± 2.1	24		-9.36 ± 0.07	35		-7.13 ± 0.06	31		-8.69 ± 0.08	30		-10.01 ± 0.06	33
β1-TM4 Stage 1	-8.26 ± 0.11	85.6 ± 3.9	4		-9.10 ± 0.09	4		-7.04 ± 0.09	6		-8.64 ± 0.14	4		-9.96 ± 0.33	3
β1-TM4 Stage 2	-8.20 ± 0.06	77.8 ± 1.5	12		-9.34 ± 0.10	18		-7.14 ± 0.06	14		-8.79 ± 0.11	12		-9.81 ± 0.09	14
β1-TM4 Stage 3	-8.13 ± 0.07	78.2 ± 3.9	11		-8.95 ± 0.09	13		-7.00 ± 0.07	10		-8.63 ± 0.07	11		-9.93 ± 0.06	15
β1-TM4 Stage 4	-8.08 ± 0.05	76.3 ± 1.5	12		-9.14 ± 0.08	18		-7.62 ± 0.05*	16		-8.59 ± 0.06	12		-9.77 ± 0.06	19
β1-TM4 Stage 5	-8.14 ± 0.06	78.2 ± 3.2	11		-8.97 ± 0.09	16		-7.71 ± 0.07*	15		-8.81 ± 0.04	11		-9.93 ± 0.08	19
β1-TM4	-8.32 ± 0.05	77.3 ± 1.9	23		-9.43 ± 0.09	35		-7.94 ± 0.06*	31		-9.08 ± 0.07	29		-9.98 ± 0.09	35
CGP12177 as agonist – stable mixed populations of cells															
	CGP12177 Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B ICI118551	n		Log K_B propranolol	n			
β1-WT	-8.12 ± 0.07	53.3 ± 1.5	21		-7.37 ± 0.08	37		-5.86 ± 0.08	22		-6.69 ± 0.08	27			
β1-TM4 Stage 1	-8.43 ± 0.06	50.4 ± 2.2	4		-7.32 ± 0.25	5		-6.21 ± 0.50	3		-6.17 ± 0.17	4			
β1-TM4 Stage 2	-8.46 ± 0.07	46.5 ± 1.9	11		-7.55 ± 0.12	17		-6.08 ± 0.11	10		-6.78 ± 0.09	20			
β1-TM4 Stage 3	-8.48 ± 0.08	45.9 ± 3.0	11		-7.13 ± 0.10	18		-5.90 ± 0.11	10		-6.88 ± 0.11	13			
β1-TM4 Stage 4	-7.93 ± 0.12	40.0 ± 1.6*	10		-7.26 ± 0.10	15		-6.57 ± 0.13*	10		-6.98 ± 0.13	18			
β1-TM4 Stage 5	-9.29 ± 0.06*	30.2 ± 2.3*	11		-8.74 ± 0.09*	19		-7.54 ± 0.09*	10		-8.18 ± 0.08*	25			
β1-TM4	-9.43 ± 0.06*	33.9 ± 1.5*	19		-8.98 ± 0.07*	39		-7.64 ± 0.08*	32		-8.39 ± 0.06*	40			

*p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β1-WT, thus the log EC_{50} for CGP12177 at β1-TM4 Stage 5 receptor is different from that obtained from the β1-WT with p<0.001

Table 4b

The ratio of the log K_B value for CGP12177 (as an antagonist of cimaterol, thus at the high affinity conformation) and log EC_{50} value for CGP12177 (as an agonist of the secondary low affinity conformation) for responses seen at the β1-WT and β1-TM4 chimeric receptors (data taken from Table 4a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log K_B for antagonism of cimaterol at high affinity site / log K_B for antagonism of CGP12177 at low affinity site).

This Table examines the ratio of the affinities of ligands and shows that the ratio of affinities between the two conformations is much less for β1-TM4 Stage 5 and beyond suggesting that only the amino acids in Stage 5 and beyond are important for the secondary conformation.

	Ratio of log K_B CGP12177 / log EC_{50} CGP12177		Ratio of log K_B (cimaterol as agonist) / log K_B (CGP12177 as agonist)		
			CGP20712A	ICI118551	propranolol
β1WT	1.89		1.99	1.27	2.00
β1-TM4 Stage 1	1.53		1.78	0.83	2.47
β1-TM4 Stage 2	1.35		1.79	1.06	2.01
β1-TM4 Stage 3	1.45		1.82	1.10	1.75
β1-TM4 Stage 4	1.84		1.88	1.05	1.61
β1-TM4 Stage 5	0.64		0.23	0.17	0.63
β1-TM4	0.55		0.45	0.30	0.69

Table 5a

Log EC_{50} values and % maximum response to isoprenaline for CRE-SPAP production for the agonists cimaterol and CGP12177 at β1-WT, the chimeric β1-TM4 receptor (containing the full TM4 mutations) and chimeric β1/β2-adrenoceptors with single point mutations and then combinations of these mutations. Log K_B values for several antagonists for inhibition of the cimaterol and CGP12177 responses are also given. This data was obtained from between 4 and 24 stable mixed populations of cells for each receptor and n in the Table refers to the number of separate experiments

This Table shows that the mutations L195Q and W199Y both alone and in combination have a very significant effect on the responses to CGP12177 and affinity of antagonist measured in the presence of CGP12177 (i.e. the secondary conformation of the β1-receptor). Every time the mutation V189T is present, the affinity of ICI118551 is increased, both at the catecholamine conformation (in the presence of cimaterol) and at the secondary conformation (presence of CGP12177).

Cimaterol as agonist – stable mixed populations of cells															
	Cimaterol Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B ICI118551	n		Log K_B propranolol	n		Log K_B CGP12177	n
β1-WT	-8.29 ± 0.04	81.2 ± 2.1	24		-9.36 ± 0.07	35		-7.13 ± 0.06	31		-8.69 ± 0.08	30		-10.01 ± 0.06	33
β1-A187G	-8.22 ± 0.08	78.9 ± 2.3	8		-9.23 ± 0.08	14		-7.02 ± 0.07	8		-8.72 ± 0.09	8		-9.99 ± 0.06	17
β1-V189T	-8.20 ± 0.04	80.6 ± 1.8	12		-9.26 ± 0.09	20		-7.73 ± 0.07*	15		-8.61 ± 0.05	14		-9.81 ± 0.08	27
β1-L195Q	-8.20 ± 0.05	80.6 ± 2.7	12		-8.97 ± 0.10	19		-7.12 ± 0.07	15		-8.86 ± 0.11	14		-9.85 ± 0.07	25
β1-W199Y	-8.46 ± 0.06	77.3 ± 2.4	12		-9.66 ± 0.08	19		-7.39 ± 0.07	16		-9.01 ± 0.12	15		-10.06 ± 0.05	26
β1-W199A	-8.63 ± 0.07*	85.4 ± 3.2	8		-9.51 ± 0.14	13		-7.26 ± 0.08	11		-9.12 ± 0.11	13		-10.07 ± 0.16	4
β1-W199D	-8.91 ± 0.07*	87.6 ± 3.5	8		-9.88 ± 0.08	12		-7.48 ± 0.11	10		-9.17 ± 0.10	12		-9.85 ± 0.12	8
β1-W199F	-8.71 ± 0.09*	80.0 ± 2.6	8		-10.14 ± 0.10*	14		-7.19 ± 0.12	12		-9.09 ± 0.13	14		-9.95 ± 0.16	11
β1-W199K	-7.64 ± 0.05*	86.4 ± 3.2	8		-9.46 ± 0.08	14		-6.41 ± 0.06*	9		-8.00 ± 0.12*	10		$	
β1-W199L	-7.60 ± 0.06*	75.2 ± 2.9	8		-10.06 ± 0.11*	12		-6.90 ± 0.11	11		-8.72 ± 0.06	12		$	
β1-W199N	-8.88 ± 0.06*	84.6 ± 5.4	8		-9.93 ± 0.09	11		-7.21 ± 0.08	12		-9.22 ± 0.10	13		-10.12 ± 0.12	12
β1-V189T-L195Q	-8.22 ± 0.04	87.0 ± 3.4	8		-9.49 ± 0.09	13		-7.67 ± 0.10*	11		-8.81 ± 0.11	14		-9.58 ± 0.09	12
β1-V189T-W199Y	-8.62 ± 0.07*	84.2 ± 2.1	8		-9.93 ± 0.08	12		-7.85 ± 0.08*	10		-8.80 ± 0.08	12		-10.10 ± 0.15	11
β1-L195Q-W199Y	-8.41 ± 0.06	79.6 ± 2.0	8		-9.68 ± 0.06	13		-7.24 ± 0.06	11		-9.04 ± 0.11	12		-9.82 ± 0.14	9
β1-V189T-L195Q-W199Y	-8.44 ± 0.04	82.7 ± 2.7	8		-9.85 ± 0.08	14		-7.92 ± 0.06*	11		-8.99 ± 0.09	14		-9.79 ± 0.11	12
β1-TM4	-8.32 ± 0.05	77.3 ± 1.9	23		-9.43 ± 0.09	35		-7.94 ± 0.06*	31		-9.08 ± 0.07	29		-9.98 ± 0.09	35

CGP12177 as agonist – stable mixed populations of cells															
	CGP12177 Log EC_{50}	% isop	n		Log K_B CGP20712A	n		Log K_B ICI118551	n		Log K_B propranolol	n			
β1-WT	-8.12 ± 0.07	53.3 ± 1.5	21		-7.37 ± 0.08	37		-5.86 ± 0.08	22		-6.69 ± 0.08	27			
β1-A187G	-8.21 ± 0.09	41.5 ± 3.1	7		-7.25 ± 0.11	12		-5.81 ± 0.08	7		-6.72 ± 0.12	10			
β1-V189T	-8.01 ± 0.07	44.1 ± 2.3	11		-7.64 ± 0.08	21		-6.36 ± 0.09*	16		-6.98 ± 0.08	24			
β1-L195Q	-9.00 ± 0.06*	32.9 ± 1.4*	11		-8.53 ± 0.09*	19		-6.64 ± 0.11*	13		-7.74 ± 0.12*	21			
β1-W199Y	-9.16 ± 0.05*	40.2 ± 2.3*	11		-8.46 ± 0.07*	23		-6.69 ± 0.09*	17		-7.90 ± 0.09*	29			
β1-W199A	-9.46 ± 0.11*	68.6 ± 4.0*	8		-8.79 ± 0.10*	12		-6.88 ± 0.11*	9		-7.96 ± 0.15*	9			
β1-W199D	-9.48 ± 0.08*	45.3 ± 3.3	8		-8.94 ± 0.09*	17		-6.98 ± 0.16*	13		-8.87 ± 0.12*	20			
β1-W199F	-8.95 ± 0.13*	58.6 ± 3.4	8		-8.10 ± 0.07*	13		-6.53 ± 0.10*	10		-7.61 ± 0.17*	12			
β1-W199K	-9.40 ± 0.05*	85.1 ± 2.5*	8		-8.13 ± 0.10*	18		-6.34 ± 0.09*	13		-7.47 ± 0.08*	20			
β1-W199L	-9.20 ± 0.06*	82.9 ± 3.2*	8		-8.12 ± 0.09*	20		-6.45 ± 0.08*	13		-7.56 ± 0.11*	20			
β1-W199N	-9.27 ± 0.12*	47.7 ± 3.2	8		-8.26 ± 0.11*	12		-6.67 ± 0.10*	10		-8.19 ± 0.09*	17			
β1-V189T-L195Q	-9.21 ± 0.06*	35.1 ± 2.6*	8		-8.64 ± 0.09*	15		-7.11 ± 0.08*	12		-8.17 ± 0.10*	14			
β1-V189T-W199Y	-9.07 ± 0.11*	43.2 ± 2.9	8		-8.70 ± 0.08*	18		-7.42 ± 0.14*	13		-7.82 ± 0.10*	16			
β1-L195Q-W199Y	-9.51 ± 0.06*	41.3 ± 2.3	8		-9.17 ± 0.12*	15		-6.94 ± 0.09*	12		-8.15 ± 0.11*	15			
β1-V189T-L195Q-W199Y	-9.40 ± 0.07*	39.2 ± 2.4*	8		-9.21 ± 0.12*	14		-7.49 ± 0.07*	12		-8.66 ± 0.11*	16			
β1-TM4	-9.43 ± 0.06*	33.9 ± 1.5*	19		-8.98 ± 0.07*	39		-7.64 ± 0.08*	32		-8.39 ± 0.06*	40			

[$]CGP12177 is very efficacious in these mutants, similar to that of cimaterol, and it is therefore not possible to measure a log K_B value for CGP12177 by a rightward shift of the cimaterol concentration response.

*p<0.001 One-way ANOVA with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β1-WT, thus the log K_B for ICI118551 at β1-V189T is different from that obtained from the β1-WT with p<0.001

Table 5b

The ratio of the log K_B value for CGP12177 (as an antagonist of cimaterol, thus at the high affinity conformation) and log EC_{50} value for CGP12177 (as an agonist of the secondary low affinity conformation) for responses seen at β1-WT, β1-TM4, and β1-receptors with point mutations in TM4 (data taken from Table 5a). Ratio of the affinity for antagonists at the two conformations is also given (i.e. log K_B for antagonism of cimaterol at high affinity site / log K_B for antagonism of CGP12177 at low affinity site).

This Table examines the ratios of the antagonist affinities and shows that the difference in affinity between the antagonists at the catecholamine conformation (in the presence of cimaterol) and secondary conformation (presence of CGP12177) is substantially less with the L195 and W199 mutations suggesting that these are important for the secondary conformation. Although V189T alters the affinity of ICI118551, the ratio of affinity does not change suggesting that V189T does not have a major role in the secondary conformation.

	Ratio of log K_B CGP12177 / log EC_{50} CGP12177		Ratio of log K_B (cimaterol as agonist) / log K_B (CGP12177 as agonist)		
			CGP20712A	ICI118551	propranolol
β1-WT	1.89		1.99	1.27	2.00
β1-A187G	1.78		1.98	1.21	2.00
β1-V189T	1.80		1.62	1.37	1.63
β1-L195Q	0.85		0.44	0.48	1.12
β1-W199Y	0.90		1.20	0.70	1.11
β1-W199A	0.61		0.72	0.38	1.16
β1-W199D	0.37		0.94	0.50	0.30
β1-W199F	1.00		2.04	0.66	1.48
β1-W199K	*		1.33	0.07	0.53
β1-W199L	*		1.94	0.45	1.16
β1-W199N	0.85		1.67	0.54	1.03
β1-V189T-L195Q	0.64		0.85	0.56	0.64
β1-V189T-W199Y	1.03		1.23	0.43	0.98
β1-L195Q-W199Y	0.31		0.51	0.30	0.89
β1-V189T-L195Q-W199Y	0.39		0.64	0.43	0.33
β1-TM4	0.55		0.45	0.30	0.69

Table 6

Log EC_{50} values and % maximum response to isoprenaline for ^{3}H-cAMP accumulation in response to CGP12177 and pindolol at β1-WT, β2-WT and β1-adrenoceptors with single point mutations or combinations of these mutations. This data was obtained from transiently transfected cells and n in the Table refers to the number of separate experiments, each being conducted in a separate transiently transfected population of cells.

This Table shows that the biphasic responses to CGP12177 and pindolol, and the "dip" in the curve seen when CGP12177 competed with a fixed concentration of cimaterol (see Figures 6-8) observed in the β1-WT are lost with several β1-TM4 mutant receptors, including the β1-L195Q-W199Y, β1-V189T-L195Q-W199Y and β1-W199D receptors.

	CGP12177 as agonist – transiently transfected cells							Pindolol as agonist – transiently transfected cells				
	Log EC_{50}1	Log EC_{50}2	% site 1	% max isop	Competition with cimaterol (dip)	n		Log EC_{50}1	Log EC_{50}2	% site 1	% max isop	n
β1WT	-9.28 ± 0.17	-7.36 ± 0.21	42.3 ± 7.2	53.0 ± 2.9	yes	6		-9.10 ± 0.04	-5.93 ± 0.18	57.1 ± 3.3	30.0 ± 2.0	8
β1V189T	-9.37 ± 0.12	-7.16 ± 0.06	45.1 ± 2.7	48.4 ± 4.6	yes	6		-8.87 ± 0.05	-5.78 ± 0.14	67.1 ± 3.3	22.3 ± 2.9	5
β1L195Q	-9.02 ± 0.09			37.4 ± 1.5	slight	3		-9.14 ± 0.08			27.9 ± 1.6	3
β1W199Y	-9.28 ± 0.11			41.0 ± 3.2	slight	4		-9.02 ± 0.21	-6.49 ± 0.18	71.7 ± 1.4	32.4 ± 3.0	4
β1W199D	-9.80 ± 0.06			49.9 ± 1.0	no	6		-8.96 ± 0.23			28.9 ± 1.8	4
β1L195Q-W199Y	-9.62 ± 0.05			39.9 ± 3.7	no	4		-9.33 ± 0.08			26.2 ± 3.0	4
β1V189T-L195Q-W199Y	-9.52 ± 0.05			35.3 ± 6.3	no	3		-9.11 ± 0.03			21.8 ± 1.4	4
β1TM4	-9.54 ± 0.06			30.9 ± 1.8	no	4		-9.25 ± 0.08			23.0 ± 3.1	3
β2WT	-9.81 ± 0.15			11.2 ± 1.6	no	4		-9.58 ± 0.06			11.6 ± 0.4	4

Table 7

Amino acids occurring at positions 189, 195 and 199 or equivalent positions in other β-adrenoceptors from several species.

	UniProtKB/TrEMBL accession number		Position 189		Position 195		Position 199
Two-conformation receptors							
Human β1	P08588		V 189		L 195		W 199
Rat β1	P18090		V 189		L 195		W 199
Mouse β1	P34971		V 189		L 195		W 199
Guinea pig β1	B0FL73		V 189		L 195		W 199
Cat β1	Q9TST6		V 189		L 195		W 199
Turkey β1	P07700		V 172		M 178		W 182
Turkey β4C	P43141		I 154		M 160		W 164
Human β3	P13945		V 168		M 174		W 178
One-conformation receptors							
Human β2	P07550		T 164		Q 170		Y 174
Turkey β3C	*		T 161		Q 167		Y 171

*Sequence from Figure 1, Baker 2010b

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Figure 1

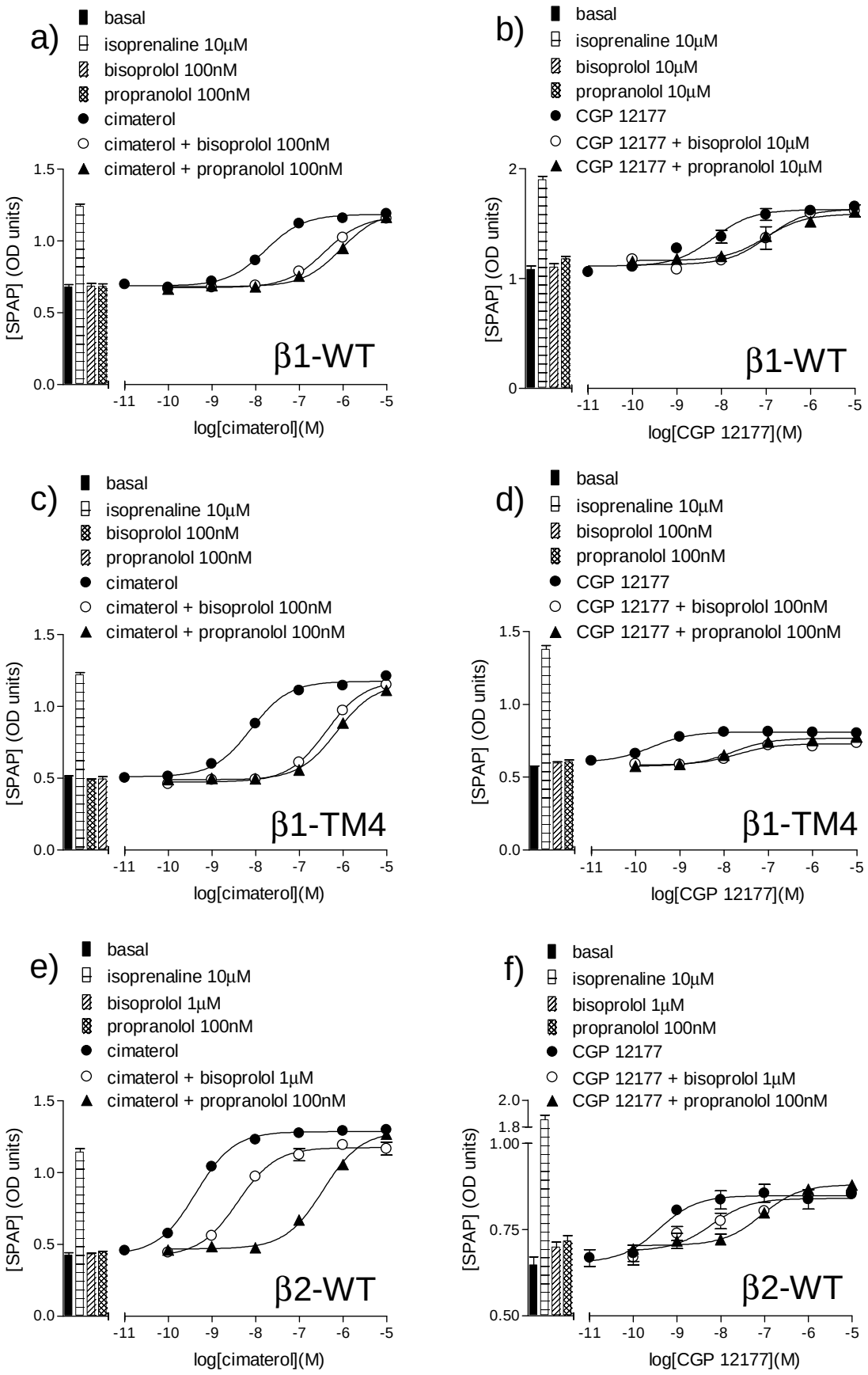

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Figure 2

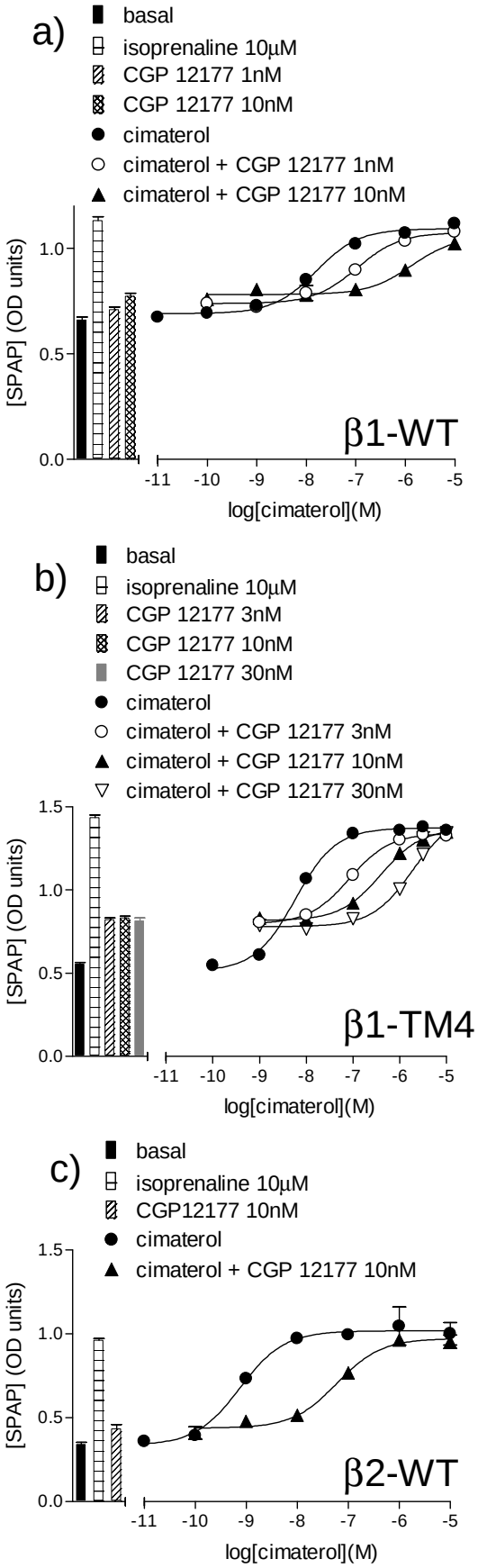

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Figure 3

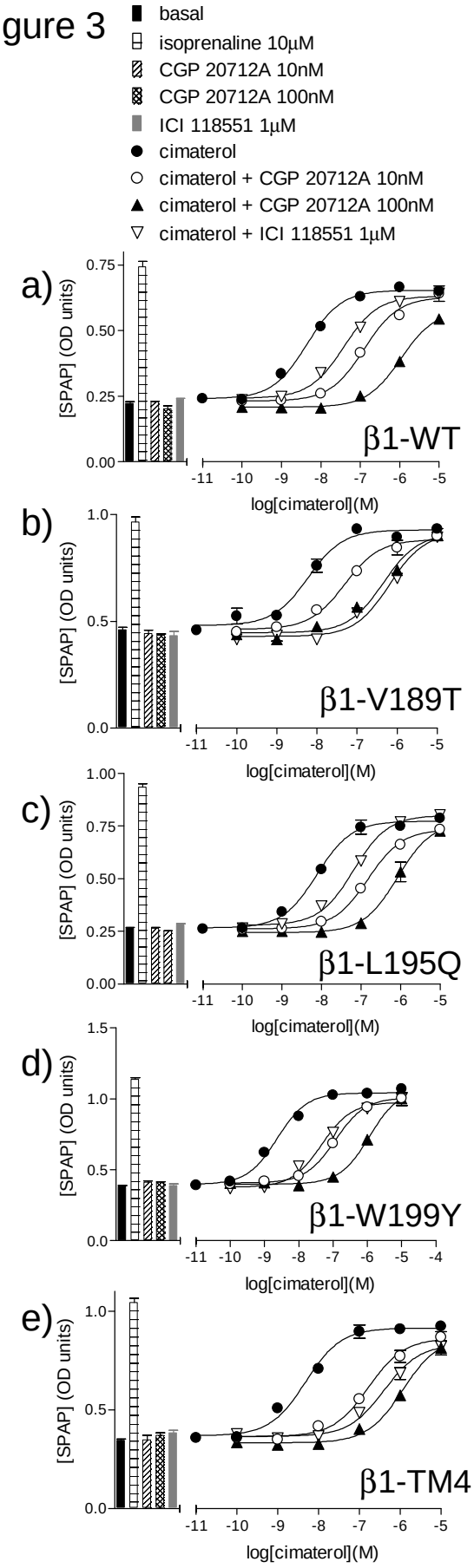

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Figure 4

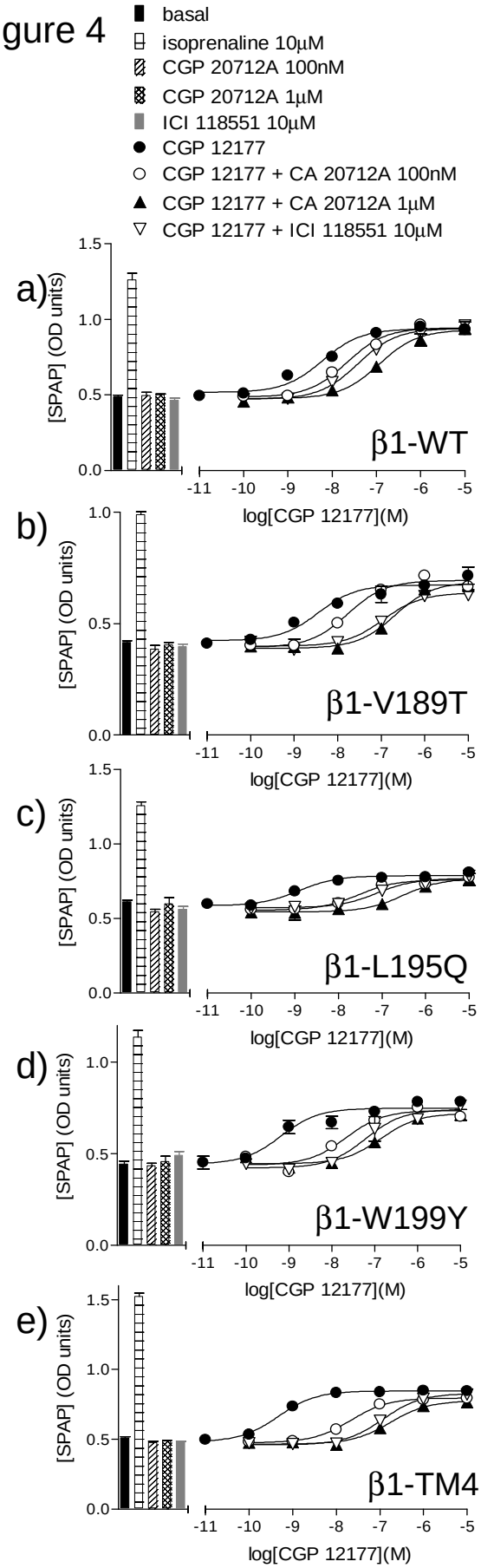

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Figure 5

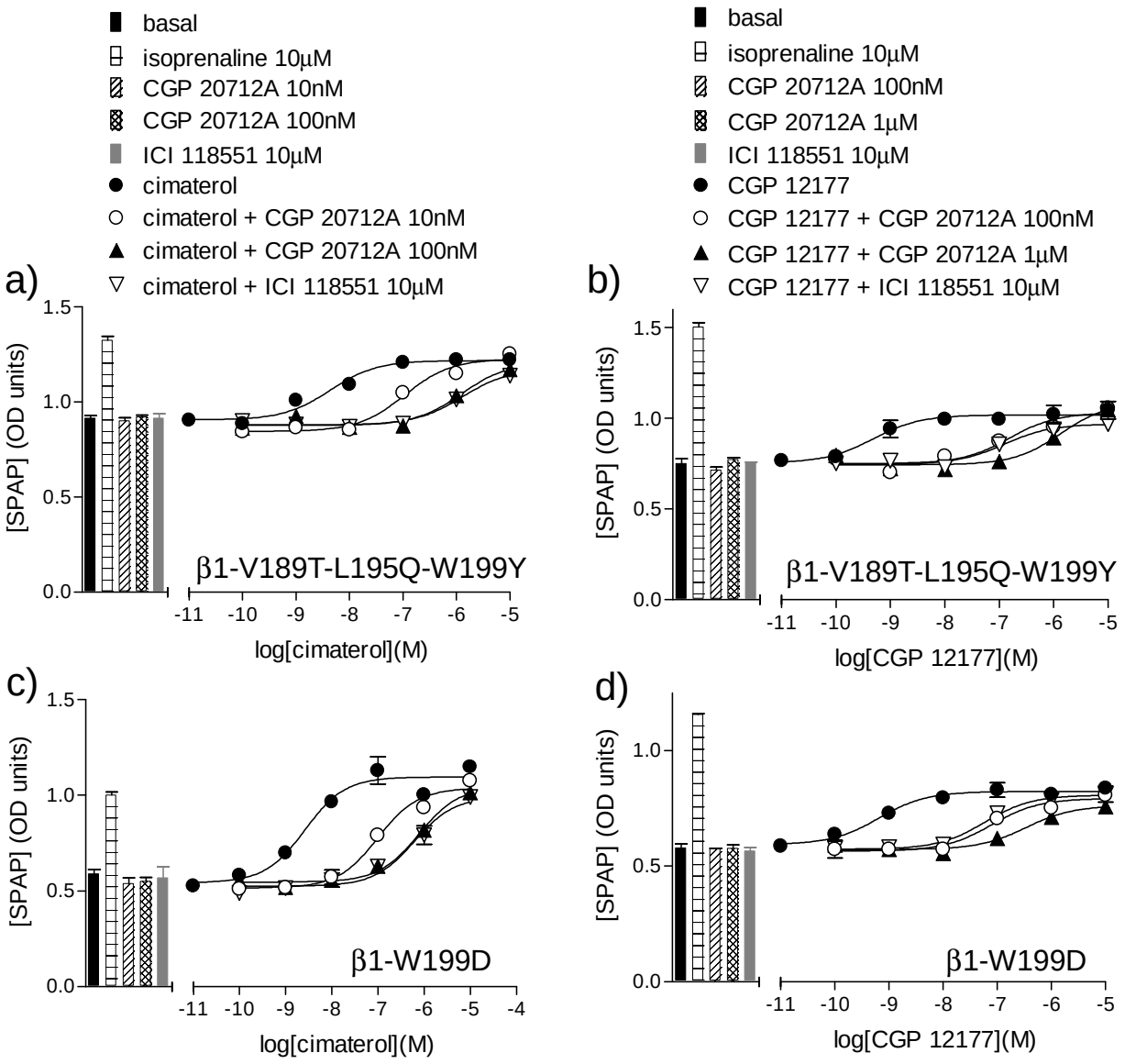

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Figure 6

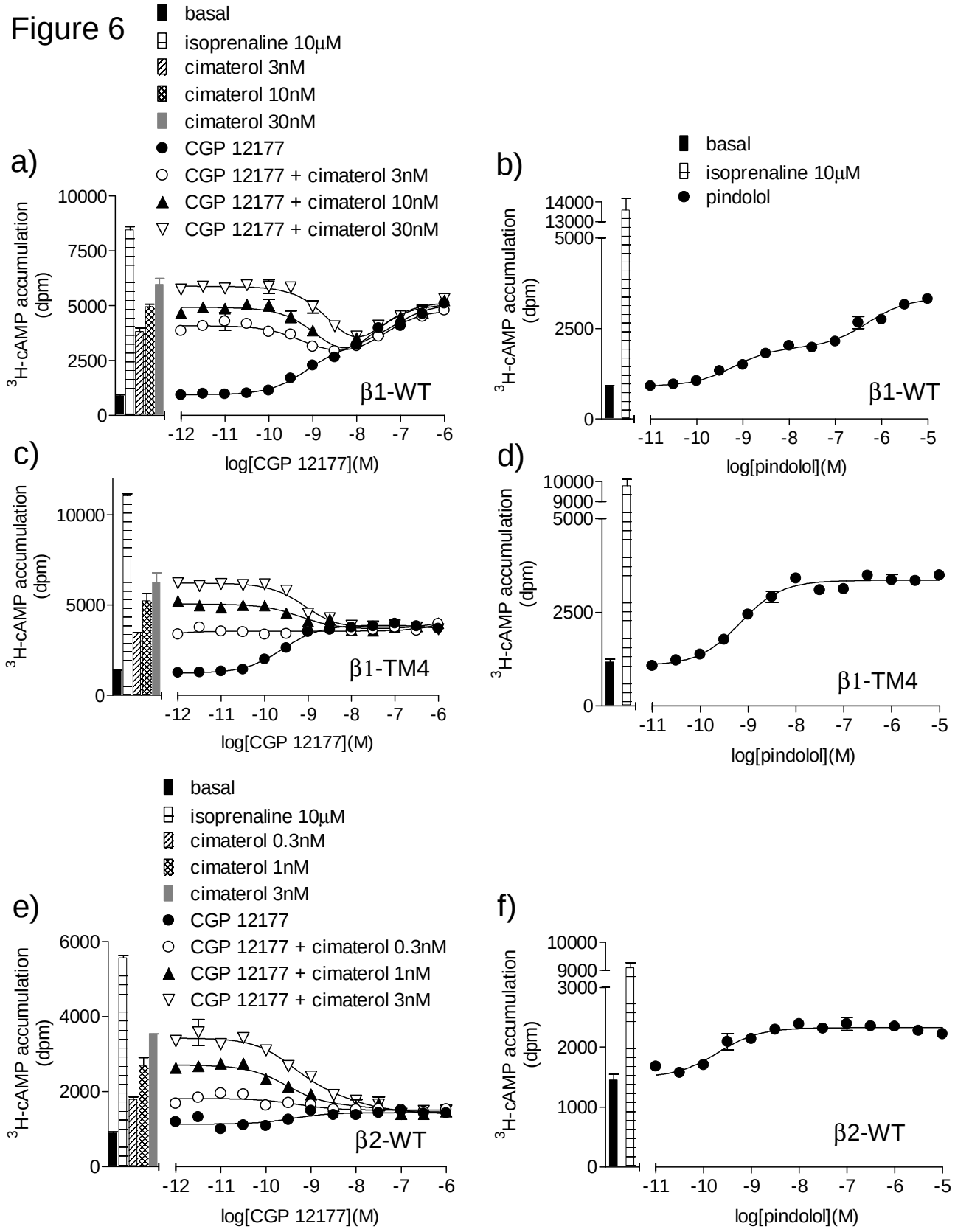

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Figure 7

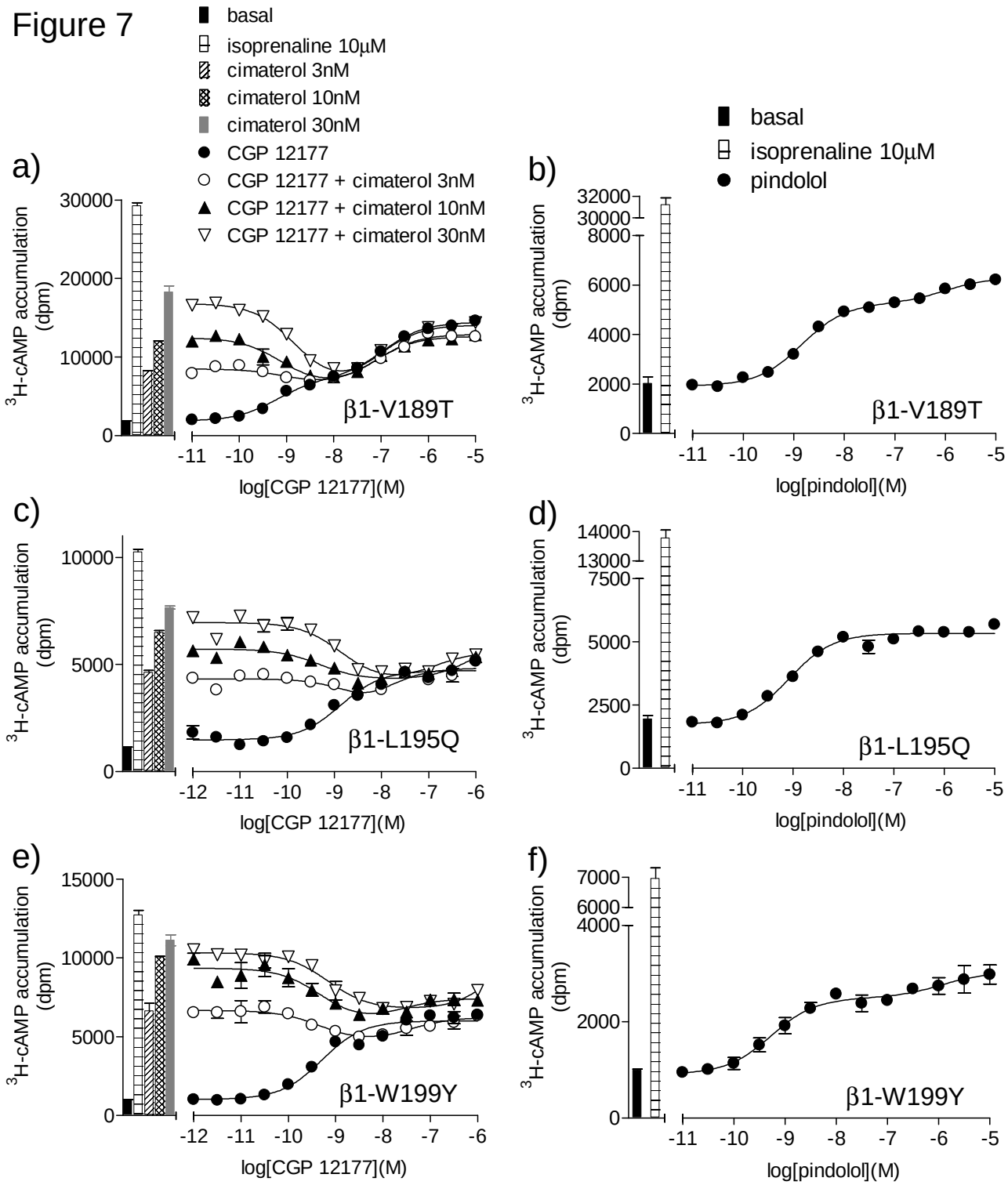

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Figure 8

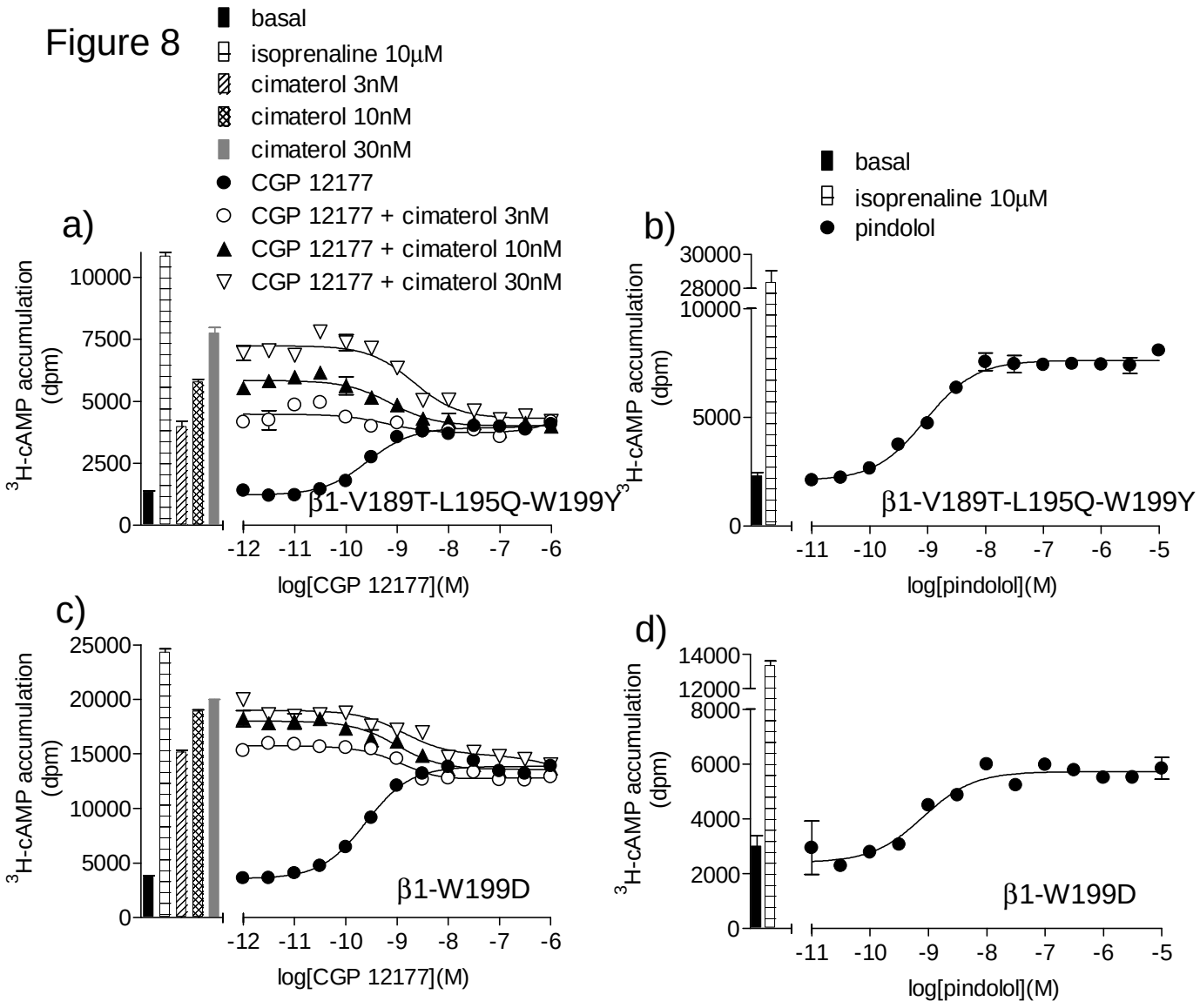

Figure 9.