ORIGINAL ARTICLE

The pharmacological effects of the thermostabilising (m23) mutations and intra and extracellular (β36) deletions essential for crystallisation of the turkey β-adrenoceptor

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Abstract The X-ray crystal structure of the turkey β-adrenoceptor has recently been determined. However, mutations were introduced into the native receptor that was essential for structure determination. These may cause alterations to the receptor pharmacology. It is therefore essential to understand the effects of these mutations on the pharmacological characteristics of the receptor. This study examined the pharmacological effects of both the m23 mutations and the β36 deletions, both alone and then in combination in the β36–m23 mutant used in the crystallisation and structure determination of the turkey β-adrenoceptor. Stable CHO-K1 cell lines were made of each of the receptor mutants and the affinity and efficacy of ligands assessed by ^{3}H-CGP 12177 whole cell ligand binding, ^{3}H-cAMP accumulation, and CRE-SPAP gene transcription assays. The m23 mutations reduced affinity for agonists, partial agonists and neutral antagonists by about tenfold whilst the β36 deletions alone had no effect on ligand affinity. Both sets of changes appeared to reduce the agonist activation of the receptor. Both the m23 and the β36 receptors retained two active agonist-induced receptor conformations similar to that of the original tβtrunc receptor. The combined β36–m23 receptor bound ligands with similar affinity to the m23 receptor; however, agonist activation was only observed with a few agonists including the catecholamines. Although the

combination of mutations severely reduced the activation ability, the final crystallised receptor (β36–m23) was still a fully functional receptor capable of binding agonist and antagonist ligands and activating intracellular agonist responses.

Keywords β-adrenoceptor · Conformational thermostabilisation · Crystal structure · Secondary site · m23 mutations · β36 deletions

Abbreviations

BRL37344	(R*,R*)-(±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid
BRL35135A	(R*,R*)-[4-2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]-acetic acid methyl ester
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
CL316243	disodium (R,R)-5-(2-[{2-(3-chlorophenyl)-2-hydroxyethyl}-amino]propyl)-1,3-benzodioxole-2,2,dicarboxylate
CYP	cyanopindolol
ICI118551	(−)-1-(2,3-[dihydro-7-methyl-1 H-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol
ICI215001	(S)-4-[2-hydroxy-3-phenoxypropylaminoethoxy]phenoxyacetic acid hydrochloride
L755507	4-[[(Hexylamino)carbonyl]amino]-N-[4-[2-[[(2S)-2-hydroxy-3-(4-hydroxyphenoxy)propyl]amino]ethyl]phenyl]-benzenesulfonamide

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SDZ21009	4-[3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-1 H-indole-2-carboxylic acid 1-methylethyl ester
SPAP	secreted placental alkaline phosphates
SR59230A	1-(2-Ethylphenoxy)-3-[[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2S)-2-propanol
tβtrunc	erythrocyte turkey β-adrenoceptor with a C-terminal truncation (Warne et al. 2008)
ZD2079	4-[2-[[(2R)-2-Hydroxy-2-phenylethyl]amino]ethoxy]-benzeneacetic acid hydrochloride
ZD7114	(S)-4-[2-Hydroxy-3-phenoxypropylaminoethoxy]-N-(2-methoxyethyl)phenoxyacetamide hydrochloride

Introduction

There are three turkey β-adrenoceptors (Baker 2010a). The first one described, commonly simply referred to as the turkey β-adrenoceptor (Bilezikian and Aurbach 1973a, b; Schramm et al. 1972), is a Gs-coupled G protein-coupled receptor (GPCR) that has recently been crystallised and its structure determined to 2.7 Å resolution (Warne et al. 2008). Crystallisation required milligramme quantities of pure, homogenous receptor, in a single conformation, that was stable in detergent (Warne et al. 2009). The native turkey β-adrenoceptor, which is significantly more stable than the human β_1 adrenoceptor (Serrano-Vega and Tate 2009), was still too unstable in short chain detergents for crystallisation and it also contained flexible regions that would prevent the formation of well-diffracting crystals. Therefore, the receptor was modified in a series of stages to increase both its stability and to also reduce the amount of unstructured regions.

Initially, part of the very large C-terminus was deleted and a C116L mutation made to make the starting construct: tβtrunc construct (Baker 2010a; Warne et al. 2008). Deletion of the N-terminus, made the β6 construct, which significantly increased heterologous expression in the baculovirus expression systems (Warne et al. 2003). This β6 construct was then modified, firstly by introducing 6-point mutations to increase the thermostability (the m23 mutations; Serrano-Vega et al. 2008, Fig. 1b) and secondly residues in intracellular loop three, C-terminus and the palmitoylation site were removed (β36 deletions; Warne et al. 2009, Fig. 1c). Combination of the receptor truncations (β36 deletions) and the thermostabilising mutations (m23 mutations) to form the construct β36–m23 (Fig. 1d) allowed crystallisation and structure determination (Warne et al. 2008, 2009).

The turkey β-adrenoceptor has several characteristics similar to that of the human β_1-adrenoceptor. If the amino acid sequences are compared, there is 82% identity between the transmembrane regions of both receptors with all the residues essential for catecholamine binding being absolutely conserved (Warne et al. 2008). Certain pharmacological characteristics are also very similar between the two receptors. For example, CGP12177 is an agonist at both receptors and its responses are similarly resistant to inhibition by classical antagonists (Baker 2005a; 2010a; Konkar et al. 2000; Lowe et al. 2002). In addition, both receptors show a significant difference between their affinity (K_D value) for CGP12177 and its partial agonist EC_{50} value in functional assays (Baker et al. 2003, Baker 2010a; Konkar et al. 2000). Furthermore, several ligands appear to stimulate a two-component concentration response curve in both receptors, suggesting that they appear to exist in at least two agonist-induced conformations (Arch 2004; Baker et al. 2003, 2010a, b; Molenaar 2003; Walter et al. 1984). There are, however, a few notable differences between the receptors, for example the affinity of CGP20712A (a human β_1-selective adrenoceptor antagonist) and ICI118551 (a human β_2-selective antagonist) are similar at the turkey β-adrenoceptor (tβtrunc).

Structural information on GPCRs is very helpful in the field of drug discovery and GPCR structures will potentially assist in computer-aided drug design (Congreve and Marshall 2010). However, the mutations introduced into the native receptor that were essential for structure determination may cause alterations to the receptor pharmacology. Therefore, for the maximum benefit to be realised from the structure, it is essential to understand the effects of the mutations on the pharmacological characteristics of the receptor. Here, we examine in detail the pharmacological effects of both the m23 mutations and the β36 deletions, both alone and in combination in the β36–m23 mutant used in the crystallisation and structure determination of the turkey β-adrenoceptor (Warne et al. 2008). This study looks at the binding characteristics of both agonists and antagonists to assess affinity changes, functional assays to examine receptor ligand efficacy and an analysis of the two agonist conformations observed for some ligands.

Methods

Materials

Foetal calf serum which was from PAA Laboratories (Teddington, Middlesex, UK). Microscint 20 scintillation fluid from PerkinElmer (Shelton, CT, USA). [3H]-CGP12177, [3H]-adenine and [14C]-cAMP were from Amersham International (Buckinghamshire, UK). Betaxolol, bisoprolol, bucindolol, BRL37344, carvedilol, cimaterol, formoterol, ICI 215001, isoprenaline, L755507, salmeterol, SDZ21009,
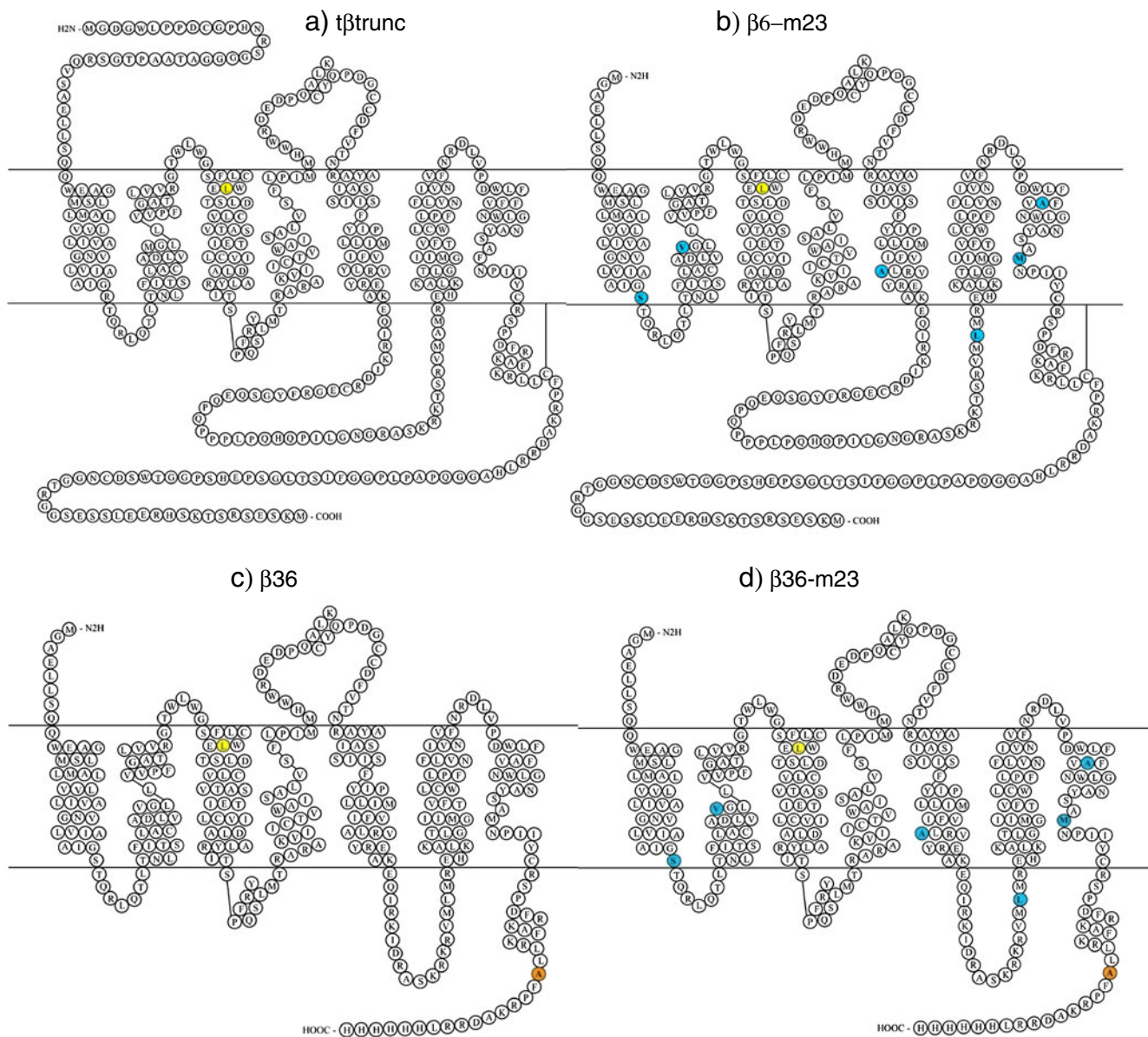
Fig. 1 The amino acid sequence of the turkey β-adrenoceptor after deletion of the C-terminal 50 amino acid residues (tβtrunc) and the three other mutants, β6–m23, β36 and β36–m23 used in this study.

Thermostabilising point mutations are coloured *blue*, the original C116L mutation in *yellow* and the C358A mutation to remove the palmitoylation sites in *orange*

SR59230A, ZD2079 and ZD7114 were from Tocris Life Sciences (Avonmouth, UK). Carazolol was from US Biological (Swampscott, MA, USA) nebivolol and butoxamine were a gift from Stefano Evangelista (Menarini Ricerche Spa, Florence, Italy); zinterol was a gift from Dr. Torsten Christ (Department of Pharmacology and Toxicology, Dresden University of Technology, Germany.). All other reagents were from Sigma Chemicals (Poole, Dorset, UK).

Constructs, cell lines and cell culture

As the full-length turkey β-adrenoceptor did not express (Baker 2010a), the starting point for this study

(tβtrunc) was a C-terminally truncated version of the turkey β-adrenoceptor that has 59 amino acids deleted from the C-terminus (residues 425–483) and one-point mutation C116L (Fig. 1a; Warne et al. 2008). This improved functional expression of the turkey β-adrenoceptor by threefold in the baculovirus expression system. In order to improve expression of the receptor for structural studies, 30 amino acid were deleted from the N-terminus (residues 2–32; construct β6; Warne et al. 2003). The thermostability of the receptor was increased by the inclusion of 6 point mutations introduced into the receptor (R67S, M90V, Y227A, A282L, F327A, F338M), which are referred to as the m23 mutations (construct=β6–m23; Serrano-Vega et al. 2008). This in-

Table 1 Log K_D values obtained from ^{3}H-CGP12177 whole cell binding studies in CHO cells stably expressing either the tβtrunc, β36, β6–m23 or β36–m23 adrenoceptors

	tβtrunc[a]	β6–m23	n	β36	n	β36–m23	n
Acebutolol	−5.85	−4.91±0.05	5	−5.81±0.06	6	−4.72±0.07	7
Adrenaline	−6.01	−3.81±0.07	11	−5.88±0.04	7	−3.88±0.08	6
Alprenolol	−7.96	−7.40±0.05	5	−8.09±0.02	5	−7.35±0.07	7
Atenolol	−5.40	−4.57±0.05	5	−5.23±0.03	6	−4.29±0.11	6
Betaxalol	−6.90	−6.38±0.01	5	−6.53±0.04	6	−5.85±0.07	6
BRL35135A	−6.32	−5.37±0.10	5	−6.86±0.11	5	−6.00±0.10	5
BRL37344	−5.19	−4.03±0.07	5	−5.25±0.07	6	−4.27±0.04	6
Bisoprolol	−6.70	−5.66±0.05	5	−6.37±0.05	6	−5.38±0.04	6
Bupranolol	−8.43	−7.78±0.04	5	−8.42±0.05	6	−7.61±0.08	6
Bucindolol	−9.43	−8.71±0.02	4	−9.39±0.07	6	−8.56±0.08	6
Butoxamine	−5.24	−4.93±0.07	5	−5.22±0.06	6	−4.78±0.10	5
Carazolol	−10.20	−9.09±0.04	13	−10.08±0.05	6	−9.23±0.10	6
Carvedilol	−9.43	−8.69±0.07	5	−9.58±0.06	5	−8.72±0.09	5
CGP20712A	−7.53	−6.59±0.05	11	−7.47±0.07	8	−6.38±0.09	8
Cimaterol	−7.17	−5.44±0.04	8	−7.33±0.06	9	−5.56±0.06	9
CL316243	≥4	≥4	4	≥4	6	≥4	6
Clenbuterol	−6.74	−5.56±0.02	5	−6.89±0.06	6	−5.66±0.05	6
S-Cyanopindolol	−10.89	−9.85±0.07	12	−10.81±0.07	6	−10.01±0.11	6
Denopamine	−6.29	−5.13±0.04	7	−6.29±0.05	7	−4.99±0.06	6
Dobutamine	−5.69	−5.40±0.01	5	−5.74±0.06	6	−5.49±0.03	6
Fenoterol	−6.36	−4.33±0.04	6	−6.33±0.06	6	−4.55±0.10	8
Formoterol	−7.33	−5.32±0.07	6	−7.42±0.07	6	−5.58±0.07	6
ICI118551	−7.20	−6.69±0.03	5	−7.16±0.04	6	−6.35±0.03	6
ICI215001	−5.79	−5.40±0.06	6	−5.68±0.05	6	−4.96±0.07	7
Isoprenaline	−6.86	−5.13±0.05	7	−6.67±0.13	7	−5.16±0.05	7
Labetolol	−7.51	−6.48±0.03	5	−7.49±0.04	6	−6.48±0.07	6
L755507	−7.17	−7.00±0.06	6	−7.40±0.06	7	−6.96±0.07	6
Metaproterenol	−5.64	−3.57±0.03	6	−5.71±0.08	6	−3.49±0.08	5
Metoprolol	−6.42	−5.68±0.03	6	−6.10±0.05	7	−5.27±0.07	7
Nadolol	−7.99	−7.33±0.02	5	−7.98±0.03	6	−7.08±0.09	6
Noradrenaline	−6.60	−4.40±0.03	6	−6.29±0.05	7	−4.53±0.11	7
Nebivolol	−8.25	−8.01±0.01	4	−8.35±0.05	6	−7.73±0.09	6
Pindolol	−8.55	−7.92±0.06	5	−8.56±0.06	6	−8.01±0.05	6
Pronethalol	−6.85	−6.11±0.03	5	−6.93±0.04	6	−6.12±0.08	6
Propranolol	−8.51	−7.92±0.05	6	−8.59±0.05	7	−7.78±0.07	7
Ractopamine	−6.75	−5.82±0.06	6	−6.85±0.03	6	−5.71±0.10	6
Salbutamol	−4.99	−4.16±0.06	6	−5.35±0.06	6	−4.20±0.07	6
Salmeterol	−5.92	−5.31±0.05	7	−6.36±0.08	8	−5.51±0.06	6
SDZ21009	−9.27	−8.57±0.06	5	−9.41±0.04	6	−8.62±0.08	6
SR59230A	−7.39	−7.10±0.05	6	−7.81±0.06	7	−7.50±0.10	7
Sotalol	−6.14	−5.47±0.03	5	−6.06±0.05	6	−5.21±0.05	6
Terbutaline	−4.61	≥3	3	−5.07±0.08	6	≥3	5
Timolol	−8.76	−7.95±0.03	5	−8.90±0.04	5	−7.82±0.05	6
Tulobuterol	−5.60	−4.82±0.03	5	−5.69±0.08	7	−5.00±0.10	6
Xamoterol	−6.58	−5.42±0.03	6	−6.60±0.05	6	−5.27±0.09	6
ZD2079	−3.77	≥4	4	−4.15±0.07	3	≥4	6
ZD7114	−7.30	−6.55±0.04	5	−7.07±0.04	6	−6.28±0.08	6
Zinterol	−6.40	−5.42±0.05	6	−6.52±0.06	6	−5.40±0.06	6

Values represent mean ± SEM for n separate experiments

[a] The tβtrunc data is taken from Baker (2010a)

creased the thermostability of the receptor and its tolerance to short-chain detergents by increasing kinetic barriers to the unfolded state (Balaraman et al. 2010; Serrano-Vega et al. 2008). The second round of changes involved removing potentially disordered flexible regions from the intracellular portion of the receptor. As well as the 30 amino acid deletion from the N-terminus (β6 construct), 30 amino acids were also deleted from the third intracellular loop (residues 244–271 and 277–278), a further 56 amino acids deleted from the C-terminus (residues 369–424) and a point mutation C358A introduced to remove the palmitoylation site (Warne et al. 2009). These are known as the β36 deletions (construct=β36; Warne et al. 2009). Both of these sets of changes were then combined into the final product (β36–m23) from which the X-ray crystal structure was determined (Fig. 1; Warne et al. 2008, 2009). Each of these constructs were then transfected into a Chinese hamster ovary K1 cells (CHO-K1) cell line stably expressing a six cAMP response element – secreted placental alkaline phosphatise reporter (CRE-SPAP) reporter gene using Lipofectamine and OPTIMEM as per manufacturer's instructions and a stable cell line of each mutation made by dilution cloning.

In order to check that there were no significant changes in the pharmacology of the receptor during the generation of the stable cell lines, CHO-K1 cells were also transiently transfected with each receptor construct and the binding of ligands examined under transient conditions (initial binding experiments only, see "Results" section). Here, the parent CHO-SPAP cells were transfected on day 1, the transfection reagents removed and replaced with media on day 2, the cells plated in to white-sided 96-well plates on day 3 and a binding assay performed on day 4.

All cell lines were grown in Dulbecco's modified Eagle's medium nutrient mix F12 containing 10% foetal calf serum and 2 mM L-glutamine in a 37°C humidified 5% CO_2: 95% air atmosphere.
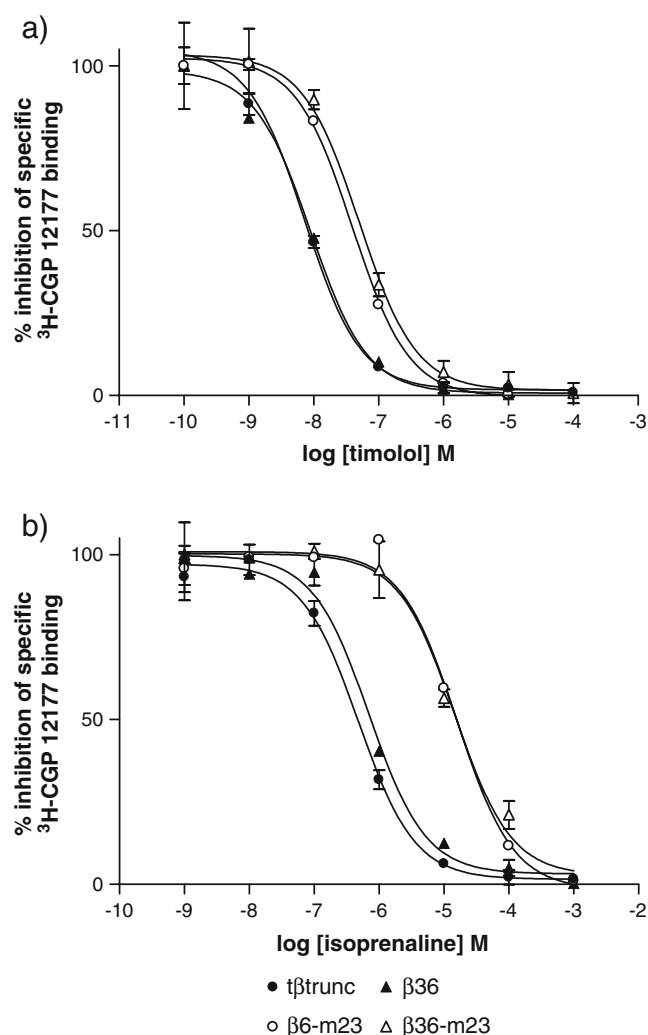
^{3}H-CGP12177 whole cell binding

Cells were grown to confluence in white-sided tissue culture treated 96-well view plates. ^{3}H-CGP12177 whole cell competition binding was performed as previously described (Baker 2005b) using ^{3}H-CGP12177 in the range of 0.932–3.030 nM (total volume 200 μl per well). For saturation experiments, concentrations of ^{3}H-CGP12177 in the range of 0.004–50.41 nM were used. The receptor expression level of the β6–m23 line was significantly higher than the other three cell lines, thus potentially posing a problem of ligand depletion. The total well volume to surface area of cells was therefore increased by carrying out an identical method, across all four cell lines, in 24-well plates (total volume of 2 ml per well) and 48-well plates

(total volume per well of 1.6 ml). For each individual cell line, the different methods yielded similar K_D values ^{3}H-CGP 12177, suggesting that ligand depletion was not a major factor. The results presented are therefore the mean ± SEM of the results from whole cell binding carried out in 24-, 48- and 96-well plates.

CRE-SPAP production

Cells were grown to confluence in clear plastic tissue culture treated 96-well plates and CRE-SPAP secretion into the media measured between 5 and 6 h after the addition of agonist as previously described (Baker 2010a).

Fig. 2 Inhibition of ^{3}H-CGP12177 specific binding to whole cells by **a** the antagonist timolol and **b** the agonist isoprenaline at each of the turkey β-adrenoceptors mutants. Nonspecific binding was determined by 10 μM propranolol. The concentration of 3H-CGP 12177 present in these experiments was 1.6 nM. Data points are mean ± SE mean of triplicate determinations. These single experiments are representative of **a** five and **b** seven separate experiments

Table 2 Log EC$_{50}$ values and % isoprenaline maximal responses obtained from ^{3}H-cAMP accumulation assays in CHO tβtrunc cells, β6–m23 cells, β36 cells and β36–m23 cells

	tβtrunc binding Log EC$_{50}$ cAMP	% isop max	n	β6–m23 Log EC$_{50}$ cAMP	% isop max	n	β36 Log EC$_{50}$ cAMP	% isop max	n	β36–m23 Log EC$_{50}$ cAMP	% isop max	n
Fenoterol	−7.95±0.08	114±5	6	−5.80±0.09	81±7	7	−6.91±0.02	117±2	6	100 μM	7.4±1.4	3
Cimaterol	−8.67±0.10	114±4	6	−7.01±0.06	82±8	6	−7.77±0.06	108±2	6	−6.23±0.18	11±2	7
Ractopamine	−7.87±0.08	114±5	3	−6.66±0.08	42±5	4	−7.22±0.05	57±4	3	100 μM	No response	3
Dobutamine	−6.73±0.09	112±7	5	−6.02±0.02	30±4	5	−6.13±0.04	88±3	4	100 μM	No response	3
Adrenaline	−7.16±0.11	110±2	5	−5.63±0.08	105±4	5	−6.29±0.07	116±4	5	−4.44±0.02	47±3	5
Formoterol	−8.85±0.06	108±6	5	−7.50±0.12	97±2	5	−7.92±0.03	106±4	5	10 μM	34±2	3
Noradrenaline	−7.89±0.05	107±5	5	−6.16±0.06	96±2	5	−6.92±0.09	119±6	6	−4.99±0.02	50±4	5
Zinterol	−7.64±0.05	101±1	3	−6.40±0.04	66±2	3	−6.88±0.03	62±3	3	10 μM	No response	3
Isoprenaline	−8.40±0.08	100	13	−7.23±0.07	100	13	−7.53±0.10	100	14	−5.85±0.05	100	14
BRL37344	−6.32±0.11	97±3	3	−5.10±0.12	60±8	4	−5.48±0.05	53±5	3	100 μM	No response	3
Salbutamol	−6.12±0.06	97±2	4	−4.79±0.02	35±6	4	−5.41±0.02	38±3	4	100 μM	No response	3
Salmeterol	−7.05±0.02	91±2	3	−5.59±0.07	25±9	3	−6.34±0.06	38±5	3	10 μM	No response	3
Clenbuterol	−7.63±0.09	86±4	5	−6.47±0.10	41±7	5	−7.10±0.05	24±2	5	100 μM	No response	4
Tulobuterol	−6.40±0.08	78±6	5	−5.40±0.13	13±2	4	−5.79±0.07	18±2	4	100 μM	No response	3
ZD7114	−7.80±0.09	76±1	3	−7.03±0.04	6.1±0.7	3	−7.42±0.10	34±5	7	100 μM	No response	3
Xamoterol	−7.22±0.08	61±7	4	−5.76±0.17	8.3±1.5	4	−6.94±0.09	13±1	4	100 μM	No response	3
Denopamine	−6.81±0.02	45±3	4	10 μM	11±2	6	−6.64±0.09	6.5±0.3	4	10 μM	No response	3
CGP20712A	−8.21±0.19	6.3±0.7	5	−7.31±0.16	2.7±0.7	4	100 μM	No response	3	100 μM	No response	3
Propranolol	−8.53±0.14	6.1±1.4	5	100 μM	3.3±1.0	6	−8.11±0.44	3.3±1.0	5	100 μM	No response	3
Bisoprolol	No response		3	−6.63±0.39	0.4±0.1	4	100 μM	No response	3	100 μM	No response	3
ICI118551	No response		3	100 μM	No response	4	100 μM	No response	3	100 μM	No response	3

Values are mean ± SEM of n separate determinations

[3H]-cAMP accumulation

Cells were grown to confluence in clear plastic tissue culture treated 24-well plates. Following [3H]-adenine pre-labelling, [3H]-cAMP accumulation in the wells was measured as previously described (Baker 2010a). [3H]-cAMP was separated from other [3H]-nucleotides by sequential Dowex and alumina column chromatography as previously described (Donaldson et al. 1988).

Data analysis

Whole cell binding–saturation binding

Saturation binding curves of the total and nonspecific binding (as determined by the presence of 10 μM propranolol) were performed in order to determine the specific binding K_D value for [3H]-CGP12177 and the receptor

expression level. All data points on each binding curve were performed in quadruplicate. Specific binding (SB, Eq. 1) of [3H]-CGP12177 at different concentrations of [3H]-CGP 12177 was fitted using the nonlinear regression programme Prism 2.01 to the equation:

$$SB = \frac{(A \times B_{MAX})}{(A + K_D)} \tag{1}$$

where, A is the concentration of [3H]-CGP12177, B_{MAX} is the maximal specific binding and K_D is the dissociation constant of [3H]-CGP12177. Protein was determined by using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) as per manufacturers' instructions.
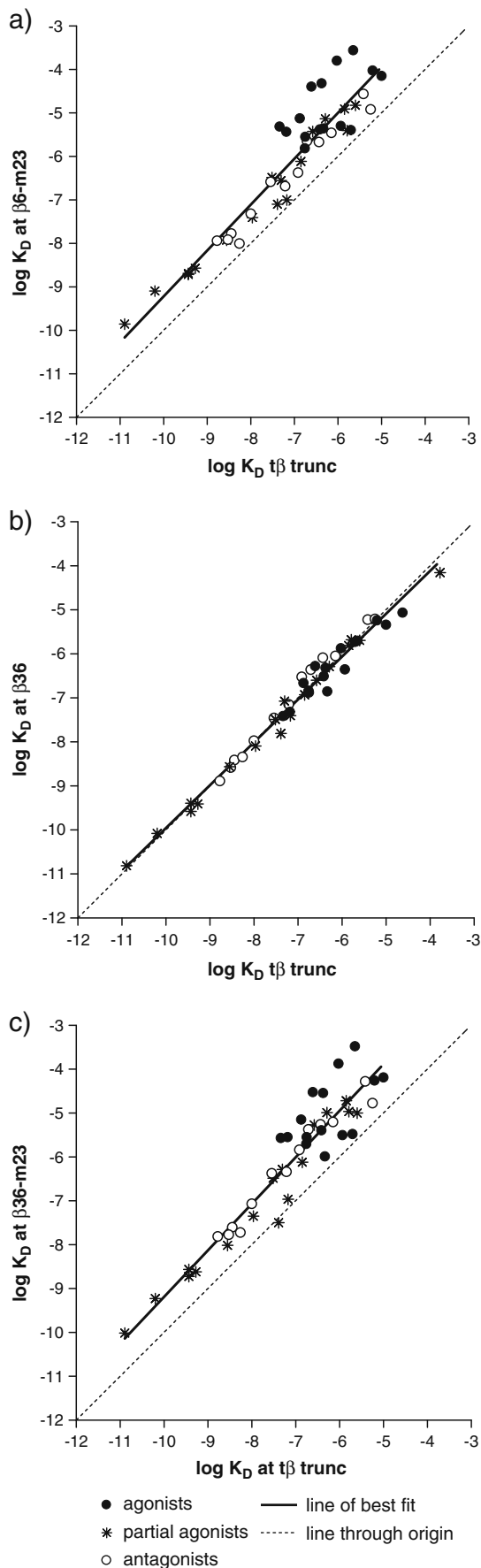
Whole cell binding–competition binding

All data points on each binding curve were performed in triplicate and each 96-well plate also contained six

Table 3 % isoprenaline maximal responses obtained from [3H]-cAMP accumulation assays in CHO tβtrunc cells, β6–m23 cells, β36 cells and β36–m23 cells, in response to a maximum concentration of ligand

	Concentration used	tβtrunc % isop max	n	β6–m23 % isop max	n	β36 % isop max	n	β36–m23 % isop max	n
Metaproterenol	100 μM	124±6	4	92±3	4	141±6	4	6.1±0.3	4
Terbutaline	1 mM	120±3	4	75±3	4	100±4	4	2.3±0.3	4
BRL35135A	10 μM	99±3	4	81±4	4	80±6	4	12±1	4
ICI215001	10 μM	75±3	4	10±1	4	33±3	4	No response	4
ZD2079	100 μM	73±9	4	25±2	4	38±2	4	0.4±0.1	4
L755507	10 μM	59±8	4	5±0.3	4	10±1	4	No response	4
Bucindolol	10 μM	49±1	4	17±1	4	12±1	4	No response	4
SR59230A	1 μM	38±2	4	1.4±0.1	4	4.9±0.7	4	No response	4
Pindolol	10 μM	31±1	4	1.5±0.1	4	3.4±0.8	4	No response	4
Alprenolol	10 μM	31±2	4	1.3±0.3	4	4.9±0.8	4	No response	4
Acebutolol	100 μM	30±1	4	1.2±0.2	4	6.7±0.4	4	No response	4
Labetolol	100 μM	29±1	4	6.1±0.6	4	3.5±0.6	4	No response	4
Pronethalol	100 μM	29±1	4	4.8±0.3	4	2.4±0.7	4	No response	4
SDZ21009	10 μM	24±1	4	4.4±0.2	4	3.7±0.3	4	No response	4
Carvedilol	10 μM	12±0.4	4	0.6±0.1	4	No response	4	No response	4
Butoxamine	100 μM	7.1±0.1	4	No response		No response	4	No response	4
Bupranolol	10 μM	4.4±0.2	4	No response	4	No response	4	No response	4
Timolol	10 μM	5.0±0.3	4	0.4±0.08	3	No response	4	No response	4
Nadolol	10 μM	2.6±0.3	4	No response	4	No response	4	No response	4
Atenolol	100 μM	2.1±1	4	No response	4	No response	4	No response	4
Betaxolol	100 μM	1.7±0.4	4	0.5±0.1	4	No response	4	No response	4
Nebivolol	10 μM	1.2±0.3	4	No response	4	No response	4	No response	4
Metoprolol	100 μM	No response	4	No response	4	No response	4	No response	4
Sotalol	100 μM	No response	4	No response	4	No response	4	No response	4

Values are mean ± SEM of n separate determinations

◀ **Fig. 3** Correlation plots of the log K_D values from ^{3}H-CGP12177 whole cell binding for the turkey β-adrenoceptor mutants compared with each other. Ligands are shown as either full agonists (*filled circles*), partial agonists (*asterisks*) or antagonists (*open circles*). Categorisation into these classes was performed from data in Tables 2 and 3, where ligands are defined as agonists if they stimulated more than 85% of the response at the human β1-adrenoceptor, as partial agonists if they stimulated 10–85% of the full response and as antagonists if they stimulated less than 10% of a full agonist response at the tβtrunc receptor

determinations of total and nonspecific binding. In all cases where a K_D value is stated, the competing ligand completely inhibited the specific binding of ^{3}H-CGP12177.

A one-site sigmoidal response curve was then fitted to the data using Graphpad Prism 2.01 and the IC_{50} was then determined as the concentration required to inhibit 50% of the specific binding.

$$\% \text{ of uninhibited binding} = 100 - \frac{(100 \times A)}{A + IC_{50}} + NS \quad (2)$$

where, A in the concentration of the competing ligand, IC_{50} is the concentration at which half of the specific binding of ^{3}H-CGP12177 has been inhibited and NS is the nonspecific binding.

From the IC_{50} value and the known concentration of ^{3}H-CGP12177, a K_D value (concentration at which half the receptors are bound by the competing ligand) was calculated using the equation:

$$K_D = \frac{IC_{50}}{1 + \left(\left[^3H - CGP12177\right]/K_D{}^3H - CGP12177\right)} \quad (3)$$

Functional assays—^{3}H-cAMP accumulation and CRE-SPAP production

Most agonist responses were best described by a one-site sigmoidal concentration response curve (Eq. 4)

$$\text{Response} = \frac{E_{MAX} \times [A]}{EC_{50} + [A]} \quad (4)$$

Where E_{MAX} 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 affinity of antagonists was calculated (K_D values) from the shift of the agonist concentration responses in the presence of a fixed concentration of antagonist using Eq. 5:

$$DR = 1 + \frac{[B]}{K_D} \quad (5)$$

where, dose ratio (DR) is the ratio of the agonist concentration required to stimulate an identical response
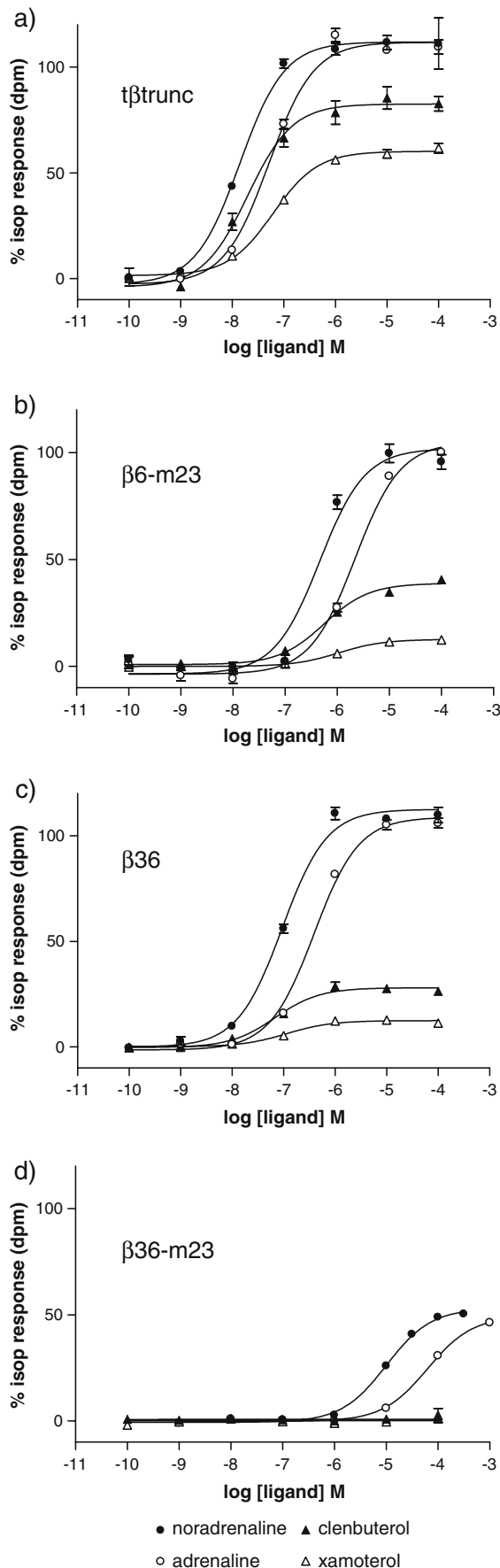
◀ **Fig. 4** ^{3}H-cAMP accumulation in response to noradrenaline, adrenaline, clenbuterol and xamoterol in **a** tβtrunc cells, **b** β6–m23 cells, **c** β36 cells and **d** β36–m23 cells expressed as a percentage of the maximal response to isoprenaline in each experiment. Data points are mean ± SE mean of triplicate determinations. Each experiment is representative of **a** four, **b** four, **c** four and **d** three separate experiments

in the presence and absence of a fixed concentration of antagonist [B].

In experiments where three different fixed concentrations of the same antagonist were used, Schild plots were constructed using the following Eq. 6:

$$\log(\text{DR} - 1) = \log[B] - \log(K_D) \tag{6}$$

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

When CGP 12177 was used to antagonise the more efficacious agonists where clear partial agonism was seen, the affinity was calculated by the method of Stephenson (1956) using Eq. 7:

$$K_D \text{ partial agonist} = \frac{Y \times [P]}{1 - Y} \text{ where } Y = \frac{[A_2] - [A_1]}{A_3} \tag{7}$$

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

Several of the responses were however best fitted to a two-site concentration response—Eq. 8 (e.g. Fig. 9).

$$\% \text{ maximal stimulation} = \frac{[A] \times N}{([A] + \text{EC1}_{50})}$$
$$+ \frac{[A] \times (100 - N)}{([A] + \text{EC2}_{50})} \tag{8}$$

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 experiments, e.g. Fig. 10 using Eq. 9:

$$\text{Response} = \text{Basal} + (\text{Ag} - \text{Basal})$$
$$\times \left(1 - \frac{[P]}{([P] + \text{IC}_{50})}\right)$$
$$+ \text{PAg}\left(\frac{[P]}{([P] + \text{EC}_{50})}\right) \tag{9}$$

Table 4 Log EC$_{50}$ values and % isoprenaline maximal responses obtained from CRE-SPAP production in CHO tβtrunc cells

tβtrunc[a]	Log EC$_{50}$	% isop max	n	Log K_D propranolol	n	Log K_D CGP20712A	n	Log K_D ICI118551	n	Log K_D CGP12177	n
Fenoterol	−8.17±0.02	120±5	3	−8.84±0.05	9	−8.20±0.00	4	−7.43±0.05	5	−9.66±0.09	9
Formoterol	−8.96±0.04	115±5	5	−8.77±0.03	10	−8.20±0.10	5	−7.44±0.09	6	−9.65±0.07	8
BRL37344	−6.45±0.05	108±4	8	−8.72±0.05	6	−8.04±0.07	75	−7.25±0.08	8	−9.44±0.11	11
Cimaterol	−8.63±0.05	122±5	7	−8.70±0.05	9	−8.24±0.09	5	−7.49±0.04	6	−9.54±0.07	14
Clenbuterol	−7.66±0.05	102±4	12	−8.52±0.05	16	−7.82±0.09	13	−7.32±0.10	12	−9.40±0.07	15
Salmeterol	−6.86±0.05	112±3	13	−8.42±0.08	10	−7.71±0.08	11	−7.15±0.10	9	−9.32±0.10	9
Salbutamol	−6.33±0.07	105±4	10	−8.41±0.10	10	−7.93±0.09	6	−7.19±0.08	6	−9.34±0.11	10
Adrenaline	−7.13±0.08	117±3	8	−8.38±0.12	13	−7.35±0.12	9	−6.82±0.05	7	−9.19±0.06	8
Noradrenaline	−7.74±0.04	113±4	12	−8.35±0.07	16	−7.51±0.08	11	−6.98±0.07	13	−9.05±0.10	11
Isoprenaline	−8.11±0.12	100	10	−8.21±0.10	18	−7.25±0.13	8	−6.72±0.12	10	−9.19±0.11	12
Dobutamine	−6.53±0.06	124±5	3	−8.19±0.11	9	−7.53±0.09	5	−6.80±0.09	4	−8.94±0.08	7
CGP12177	−8.38±0.08	53±4	20	−7.23±0.12	18	−6.91±0.15	12	−6.36±0.17	11		

Log K_D values for propranolol, CGP20712, ICI118551 and CGP12177 as antagonists of the agonist responses are also given. Values are mean ± SEM of n separate determinations

[a] Data table from Baker 2010a

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 CGP12177, IC$_{50}$ is the concentration of CGP12177 that inhibits 50% of the response of the fixed agonist, PAg is the maximum stimulation by the CGP12177 and EC$_{50}$ is the concentration of competing agonist that stimulated a half maximal CGP12177 response.

A 10 μM (maximal) isoprenaline concentration was included in two to three wells in each plate for each separate experiment for ^{3}H-cAMP accumulation and six wells of each plate for CRE-SPAP production and, to allow agonist responses to be expressed as a percentage of the isoprenaline maximum for each experiment. All data are presented as mean ± SEM of triplicate determinations (except saturation binding experiments where determinations were from quadruplicate wells) and n in the text refers to the number of separate experiments.

Results

Ligand affinity—^{3}H-CGP12177 whole cell binding

Binding studies were performed on both transiently transfected cells and the stable cell lines to ensure that the stable cell lines were indeed a true reflection of the pharmacology of the transfected receptors and that no alteration of the

Table 5 Log EC$_{50}$ values and % isoprenaline maximal responses obtained from CRE-SPAP production in CHO β6–m23 cells

β6–m23	Log EC$_{50}$	% isop max	n	Log K_D propranolol	n	Log K_D CGP20712A	n	Log K_D ICI118551	n	Log K_D CGP12177	n
Fenoterol	−6.10±0.06	92±2	12	−8.33±0.06	9	−7.25±0.06	9	−7.03±0.04	3	−9.32±0.07	4
Formoterol	−7.69±0.08	107±2	18	−8.24±0.02	13	−7.19±0.07	10	−6.97±0.04	4	−9.03±0.11	8
BRL37344	−5.37±0.07	84±2	12	−8.43±0.06	7	−7.30±0.05	6	−7.14±0.07	3	−9.18±0.07	3
Cimaterol	−7.25±0.15	96±2	21	−8.29±0.04	13	−7.34±0.07	7	−6.99±0.03	4	−9.13±0.05	11
Clenbuterol	−6.69±0.04	77±3	19	−8.14±0.08	11	−7.28±0.14	8	−7.10±0.04	3	−8.88±0.07	7
Adrenaline	−5.81±0.08	97±2	14	−8.03±0.04	14	−7.11±0.08	3	−6.76±0.08	5	−8.71±0.09	7
Noradrenaline	−6.27±0.10	98±3	15	−7.72±0.05	14	−6.87±0.08	5	−6.54±0.09	5	−8.61±0.13	7
Isoprenaline	−7.39±0.11	100	21	−7.63±0.10	13	−6.74±0.13	7	−6.55±0.14	4	−8.84±0.22	5
Dobutamine	−5.90±0.06	55±6	10	−7.94±0.06	4	−6.62±0.11	1	−6.71±0.04	4		
CGP12177	−6.81±0.06	20±2	15	−6.03±0.11	6	−6.42±0.20	4	−5.39±0.16	6		

Log K_D values for propranolol, CGP20712, ICI118551 and CGP12177 as antagonists of the agonist responses are also given. Values are mean ± SEM of n separate determinations

Table 6 Log EC$_{50}$ values and % isoprenaline maximal responses obtained from CRE-SPAP production in CHO β36 cells

β36	Log EC$_{50}$	% isop max	n	Log K_D propranolol	n	Log K_D CGP20712A	Log K_D ICI118551	n	Log K_D CGP12177	n
Fenoterol	−7.88±0.03	91±5	3	−8.69±0.04	9	−7.72±0.16	4	−7.45±0.07	5	−9.27±0.07	9
Formoterol	−8.66±0.06	90±3	4	−8.54±0.05	10	−8.10±0.06	5	−7.38±0.12	6	−9.20±0.04	9
BRL37344	−6.21±0.02	90±5	3	−8.74±0.04	6	−8.03±0.09	3	−7.41±0.05	4	−9.35±0.06	8
Cimaterol	−8.51±0.02	87±3	6	−8.71±0.03	10	−7.90±0.09	5	−7.29±0.04	6	−9.24±0.05	13
Clenbuterol	−7.27±0.03	72±3	6	−8.48±0.03	16	−7.72±0.07	8	−7.22±0.07	7	−8.96±0.08	10
Adrenaline	−6.72±0.08	95±3	6	−7.99±0.10	13	−6.89±0.17	7	−6.77±0.09	8	−8.97±0.09	10
Noradrenaline	−7.43±0.05	102±4	7	−8.00±0.05	17	−7.25±0.09	7	−6.89±0.09	9	−8.83±0.07	10
Isoprenaline	−7.80±0.11	100	6	−8.04±0.11	14	−6.94±0.24	7	−6.87±0.12	8	−8.73±0.15	9
Dobutamine	−6.35±0.02	102±3	3	−8.21±0.06	9	−7.33±0.05	5	−6.88±0.06	4	−8.69±0.07	7
CGP12177	−7.74±0.11	15±1	10	−6.95±0.12	12	−6.23±0.12	8	−6.32±0.17	8		

Log K_D values for propranolol, CGP20712, ICI118551 and CGP12177 as antagonists of the agonist responses are also given. Values are mean ± SEM of n separate determinations

receiver pharmacology had taken place during the process of making the cell line. The results from eight separate transiently transfected cell populations (both K_D of ^{3}H-CGP12177 from saturation studies and K_D values of betaxolol, bisoprolol, clenbuterol, CGP20712A, formoterol, ICI118551, metoprolol and xamoterol from competition studies) showed a similar pattern of log K_D values to those obtained from the stable cell line (Baker et al. 2008), so only the stable cell line data are presented here.

^{3}H-CGP12177 bound specifically to all four turkey β-adrenoceptors mutants to give K_D values (from all 96-, 48- and 24-well plate methods combined) of 0.32±0.02 nM ($n=$ 28) for the tβtrunc (receptor expression level of 148 fmol/mg protein; Baker 2010a), 0.85±0.09 nM ($n=27$) for the β6–

m23 (2,785 fmol/mg protein), 0.34±0.02 nM ($n=26$) for the β36 (134 fmol/mg protein) and 0.88±0.08 nM ($n=28$) for the β36–m23 (254 fmol/mg protein) in the stable cell lines generated. The value presented in Baker (2010a) for the tβtrunc receptor of 0.42 nM is from the 96-well plate method alone. The affinity of a range of other ligands (including agonists, partial agonists and neutral agonists, see below) was then assessed (Table 1, Fig. 2).

Agonist efficacy—^{3}H-cAMP accumulation and CRE-SPAP production

The agonist response to 24 ligands (seven-point concentration–response curves) was examined for each of the

Table 7 Log EC$_{50}$ values and % isoprenaline maximal responses obtained from CRE-SPAP production in CHO β36–m23 cells

β36–m23	Log EC$_{50}$	% isop max	n	Log K_D propranolol	n	Log K_D CGP20712A	n	Log K_D ICI118551	n	Log K_D CGP12177	n
Fenoterol	100 μM	18±2	5								
Formoterol	−5.97±0.05	90±4	4	−7.95±0.00	3	−6.95±0.06	4	−6.74±0.16	4	−8.94±0.03	4
BRL37344	No response		3								
Cimaterol	10 μM	32±3	8								
Clenbuterol	No response		3								
Adrenaline	−4.67±0.03	76±3	6	−7.94±0.10	10	−6.87±0.21	3	−6.69±0.12	4		
Noradrenaline	−5.20±0.04	75±2	9			−6.85±0.08	9	−6.78±0.08	9	−9.10±0.11	6
Isoprenaline	−6.20±0.02	100	10	−8.02±0.04	18	−6.66±0.04	16	−6.64±0.03	17	−9.05±0.03	20
Dobutamine	No response		3								
CGP12177	No response		11								

Log K_D values for propranolol, CGP20712, ICI118551 and CGP12177 as antagonists of the agonist responses are also given. Values are mean ± SEM of n separate determinations. For fenoterol and cimaterol, the response at the maximum concentration of 100 and 10 μM respectively is given as the top of the concentration response curve at these concentrations had not been reached

Table 8 Log EC$_{50}$ values and % isoprenaline maximal responses obtained from CRE-SPAP production in CHO tβtrunc, β6–m23, β36 and β36–m23 ce0lls

	tβtrunc Log EC$_{50}$	% isop max	n	β6–m23 Log EC$_{50}$	% isop max	n	β36 Log EC$_{50}$	% isop max	n	β36–m23 Log EC$_{50}$	% isop max	n
Salmeterol	−6.86±0.05	112±3	13	−5.75±0.05	61±4	13	−6.57±0.02	74±3	8	No response	0	5
Terbutaline	−6.09±0.08	106±4	3	−4.61±0.08	61±8	6	−5.89±0.03	78±0.3	3	No response	0	3
Salbutamol	−6.33±0.07	105±4	10	−4.99±0.04	50±2	12	−5.89±0.04	64±6	3	No response	0	3
Tulobuterol	−6.48±0.08	77±10	3	−5.13±0.04	29±5	7	−6.08±0.03	58±3	3	No response	0	3
ZD7114	−7.82±0.12	63±3	3	−7.15±0.04	15±3	3	−7.40±0.08	56±5	3	No response	0	3
Xamoterol	−7.26±0.07	68±1	3	−5.77±0.12	21±9	3	−6.96±0.13	51±5	3	No response	0	3
Denopamine	−6.70±0.07	58±3	5	10 μM	10±3	4	−6.29±0.14	40±2	4	No response	0	3
Labetolol	−7.58±0.19	35±5	3	−6.56±0.04	13±2	4	−7.48±0.03	9.6±0.5	3	No response	0	3
S-Cyanopindolol	−10.22±0.16	34±5	7	−7.96±0.31	16±3	3	−9.62±0.09	6.5±0.6	3	No response	0	3
Pronethalol	−7.23±0.16	27±2	3	No response	0	3	No response			No response	0	3
Carazolol	−8.34±0.34	25±3	7	No response	0	3	No response			No response	0	3

For denopamine in β6–m23 cells, the response at the maximum concentration of 10mM is given as the top of the concentration response curve had not been reached by this maximum concentration. Values are mean ± SEM of n separate determinations

turkey β-adrenoceptor mutants (Table 2). A further 24 ligands were examined at maximum concentrations only (Table 3). This enabled ligands to be classified into full agonists (those ligands that stimulated a greater than 85% maximum response to isoprenaline in the cAMP assays at the tβtrunc receptor), partial agonists (those that stimulated between 10–85% maximum responses at the tβtrunc receptor) and antagonists (those ligands that stimulated less than 10% maximum response at the tβtrunc receptor). This allowed the study of ligand affinity to be further examined to assess whether a particular subset of ligands (e.g. agonists) were disproportionately affected by the mutations (Fig. 3).

Initially, the ligands were incubated for 30 min with each cell line; however, as many responses were found to be very small, the response window was increased by extending the incubation time to 5 h. This does not affect the position (EC$_{50}$ value) or maximum response of the concentration response curve in relation to isoprenaline or forskolin for the ligands, it merely increases the size of the response window enabling small responses to be observed more accurately (Baker 2010b). For example, the response to isoprenaline at 30 min and 5 h in each lines was, respectively: tβtrunc, −8.30±0.10 ($n=8$) and −8.54±0.13 ($n=5$); β6–m23, −7.23±0.09 ($n=8$) and −7.22±0.11 ($n=5$); β36, −7.48±0.14 ($n=8$) and −7.59±0.16 ($n=6$); and β36–m23, −5.84±0.02 ($n=8$) and −5.88±0.11 ($n=6$). The numbers given in Table 2 are therefore a mean of all the 30-min and 5-h data.

Direct comparison of data between cell lines is not possible (even in these CHO cells) because levels of receptor expression vary and the coupling of receptors to effectors may also differ between the different cell lines.

Nevertheless, the rank order for the efficacy of ligands can be established by listing the ligands by the percentage of the maximum isoprenaline response observed. This was felt to be more appropriate than using Furchgott's efficacy ratio method (Furchgott 1966) as many of the ligands in this study stimulated partial agonist responses. Tables 2 and 3 are thus the response to agonist ligands presented in rank order of maximum response to isoprenaline as seen in the tβtrunc receptor cell line. Although there appear to be a few exceptions to the rank order using this method, they are generally in the ligands stimulating full agonist responses (e.g. dobutamine and ractopamine which have been shown to exhibit similar efficacy to BRL37344 and salbutamol at other β-adrenoceptors; Baker 2010b) and probably reflects the order in which the full agonists are presented. Generally, however the rank order of ligands, particularly once partial agonism is considered, is similar between tβtrunc and the mutant receptors, with the m23 mutations and β36 deletions causing a decrease in the amount of agonism seen and the β36–m23 receptor being the most severely affected (Tables 2 and 3, Fig. 4).

As the cell lines were all constructed from the same parent cell line, assessing the response to forskolin (which directly stimulates adenylyl cyclase thereby being

Fig. 5 CRE-SPAP production in response to clenbuterol in the absence and presence of propranolol in **a** tβtrunc cells, **b** β6–m23 cells, **c** β36 cells and **d** β36–m23 cells. *Bars* represent basal CRE-SPAP production, that in response to 10 μM isoprenaline and that in response 10, 100, 1,000 or 30, 300, 3,000 nM propranolol alone. Data points are mean ± SEM of triplicate determinations. The Schild slopes for each experiment are **a** 0.95, **b** 0.98 and **c** 1.00. Each experiment is representative of **a** five, **b** five, **c** five, and **d** three separate experiments
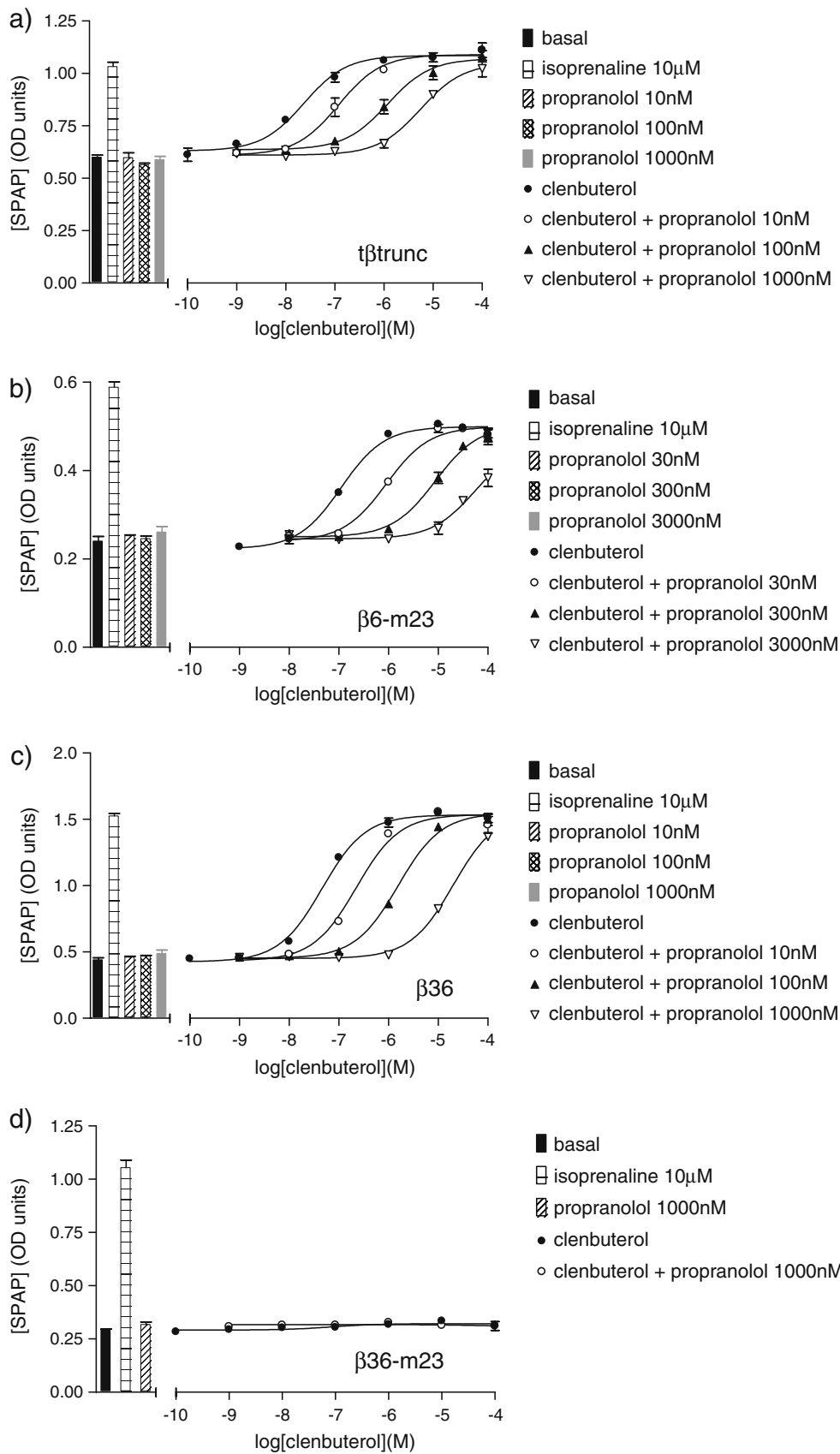
independent of the level of receptor expression and receptor–effector coupling and should therefore be constant in all lines) and comparing this to isoprenaline in each line might give information about receptor–effector coupling. For example, for equal receptor–effector coupling, a line with very high receptor expression would be expected to have a larger response (compared with forskolin) and left-shifted EC_{50} value for the agonist.

The response to 10 μM forskolin was similar across all four cell lines (40.1±3.9-fold over basal for tβtrunc; 37.8±2.0-fold over basal for β36; 37.6±2.2-fold over basal for β6–m23 and 40.7±3.9-fold over basal for β36–m23 cell line). Interestingly, the β6–m23 cell line has a significantly higher receptor expression level than the other three cell lines and generated responses to isoprenaline that were far greater than the maximum response to forskolin (isoprenaline maximum was 197.8±10.5% of the forskolin response, $n=8$). The other cell lines generated lower total amounts of ^{3}H-cAMP in response to isoprenaline relative to the forskolin response in each line, in keeping with their lower receptor expression levels (tβtrunc isoprenaline response was 35.9±4.5% forskolin, $n=5$; β36 cell line

isoprenaline response was 85.3±4.3 % of forskolin, $n=6$; and the β36–m23 cell line isoprenaline response was 107.4±14.6% of the forskolin response, $n=6$). Thus, despite having a lot more receptor available and being able to stimulate a maximum response far in excess of that in the tβtrunc line, the concentration of isoprenaline required to stimulate a response was significantly greater in the β6–m23 cells than in the tβtrunc cells (log EC_{50} −8.40 in tβtrunc cells vs. −7.23 in β6–m23 cells). This suggests that the m23 mutations reduce the receptor's ability to generate a response either by reduced affinity, reduced ability to become activated or reduced receptor–effector coupling.

Isoprenaline stimulated CRE-SPAP gene transcription responses were 2.3- (Baker 2010a), 2.6±0.01 ($n=21$)-, 4.2±0.2 ($n=6$)- and 2.7±0.10 ($n=10$)-fold over basal for tβtrunc, β6–m23, β36 and β36–m23 receptors respectively (Tables 4, 5, 6 and 7). Once again (better seen in Table 8 where partial agonists were examined), the m23 mutations and β36 deletions generated lower agonist responses than the tβtrunc cell line and the β36–m23 mutant was the most severely affected; however, the rank order of agonist efficacy remained largely undisturbed.
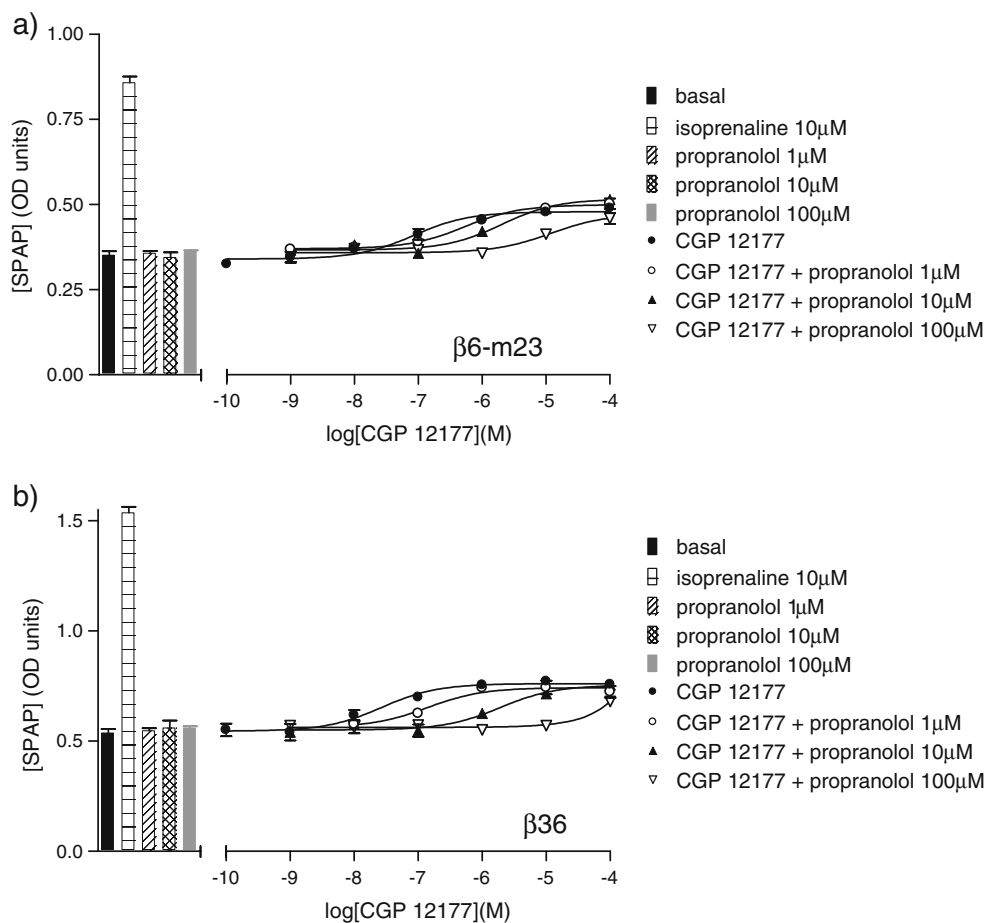

Fig. 6 CRE-SPAP production in response to cimaterol in **a** β6–m23 and **b** β36 cells in the absence and presence of CGP12177. Bars represent basal CRE-SPAP production that in response to 10 μM iso-prenaline and that in response 1, 10, 30 or 100 nM CGP12177 alone. Data points are mean ± SEM of triplicate determinations. Each experiment is representative of **a** five and **b** five separate experiments
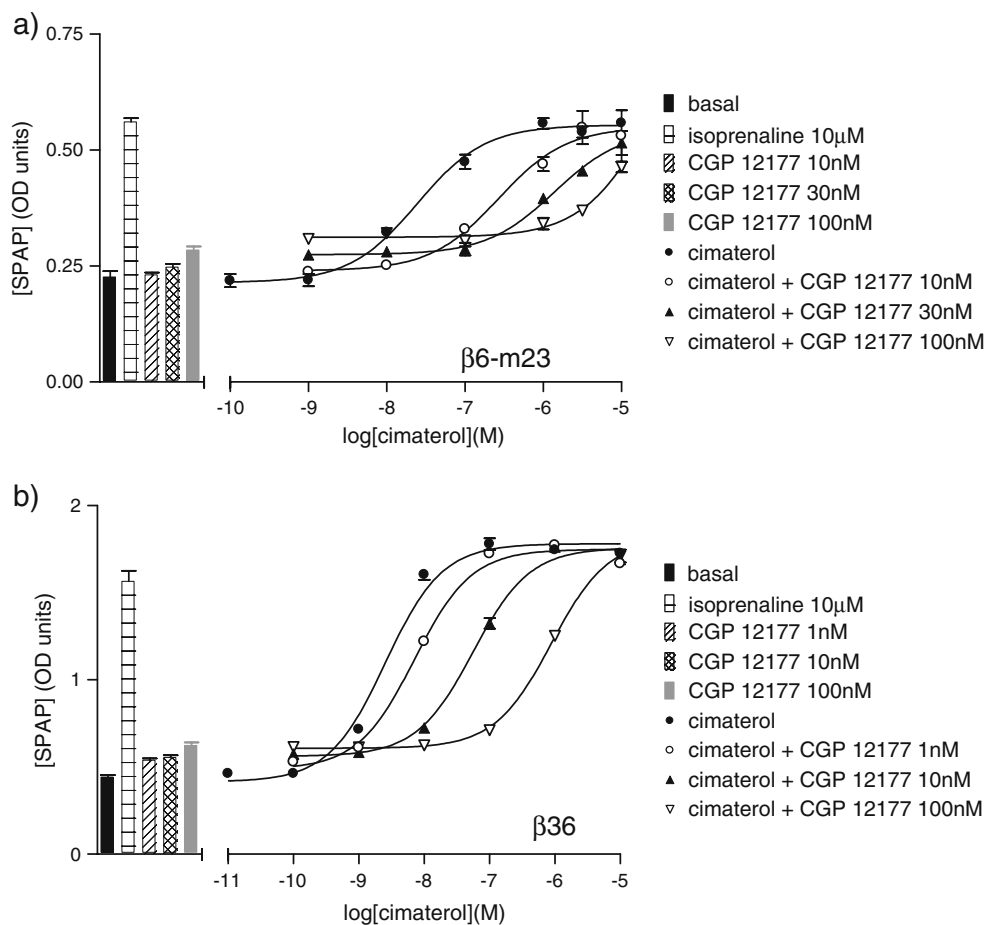
Evidence for two agonist-induced conformations—[3]H-cAMP accumulation and CRE-SPAP production

Next, the β36 deletions and the m23 mutations were each examined separately to determine the effects of these mutations on the two agonist-induced conformations of the tβtrunc receptor. Several agonists stimulated CRE-SPAP responses that were inhibited by propranolol, CGP20712A and ICI118551 (Fig. 5, Tables 4, 5, 6 and 7) in a competitive manner. CGP12177 also acted as an antagonist, inhibiting both of these mutant receptor responses with high affinity (Fig. 6, Tables 4, 5, 6 and 7). Also, CGP12177 itself stimulated a partial agonist response at both of these mutants and in each case the log EC_{50} value was significantly higher than the K_D value for CGP12177 at the mutant receptor (Fig. 7, Tables 4, 5, 6 and 7). In addition, these CGP12177 agonist responses required significantly higher concentrations of propranolol, CGP20712A and ICI118551 to be inhibited than the other agonists (Fig. 7, Tables 4, 5, 6 and 7). This is in keeping with two agonist-induced conformations (a catecholamine site conformation and a secondary CGP12177 site conformation) on each of the β36 and β6–m23 receptor mutants.

When these mutations were combined in the β36–m23 mutant receptor, many agonists did not stimulate any response (Fig. 5d, Tables 7 and 8). In addition, CGP12177 itself did not stimulate any agonist response at the β36–m23 receptor making it difficult to determine whether this mutant does indeed have two agonist-induced conformations. However, responses were seen to a few agonists and these responses were inhibited by classical antagonists in a competitive manner (propranolol, ICI118551 and CGP20712A, Fig. 8a, Table 7). In addition, CGP12177 also inhibited these agonist responses with high affinity in a similar manner to the original tβtrunc receptor (Fig. 8b, Table 7).

Further evidence for two sites or conformations was also sought by using the ^{3}H-cAMP accumulation assay, which has the largest response window, thus making the detailed examination of agonist responses possible. S-Cyanopindolol was the ligand bound to the β36–m23 receptor from which the X-ray structure was determined (Warne et al. 2008) and it stimulated an ^{3}H-cAMP accumulation response that was best described by a two-component response curve (Fig. 9a). CGP12177 and carazolol similarly stimulated two-component response curves (Table 9). Two component response curves were also seen for β6–m23 and β36 receptors (Fig. 9b and c,

Fig. 7 CRE-SPAP production in response to CGP12177 in **a** β6–m23 and **b** β36 cells in the absence and presence of propranolol. *Bars* represent basal CRE-SPAP production, that in response to 10 μM isoprenaline and that in response 1, 10 or 100 μM propranolol alone. Data points are mean ± SEM of triplicate determinations. Each experiment is representative of **a** five and **b** five separate experiments

Fig. 8 CRE-SPAP production in response to isoprenaline in β36–m23 cells in the absence and presence of **a** propranolol and **b** CGP12177. *Bars* represent basal CRE-SPAP production, that in response to 10 μM isoprenaline and that in response 10, 100 1,000 nM propranolol or 1, 10 or 100 nM CGP12177 alone. Data points are mean ± SEM of triplicate determinations. Each experiment is representative of **a** seven and **b** seven separate experiments. The Schild slopes for each experiment are **a** 1.00 and **b** 1.05
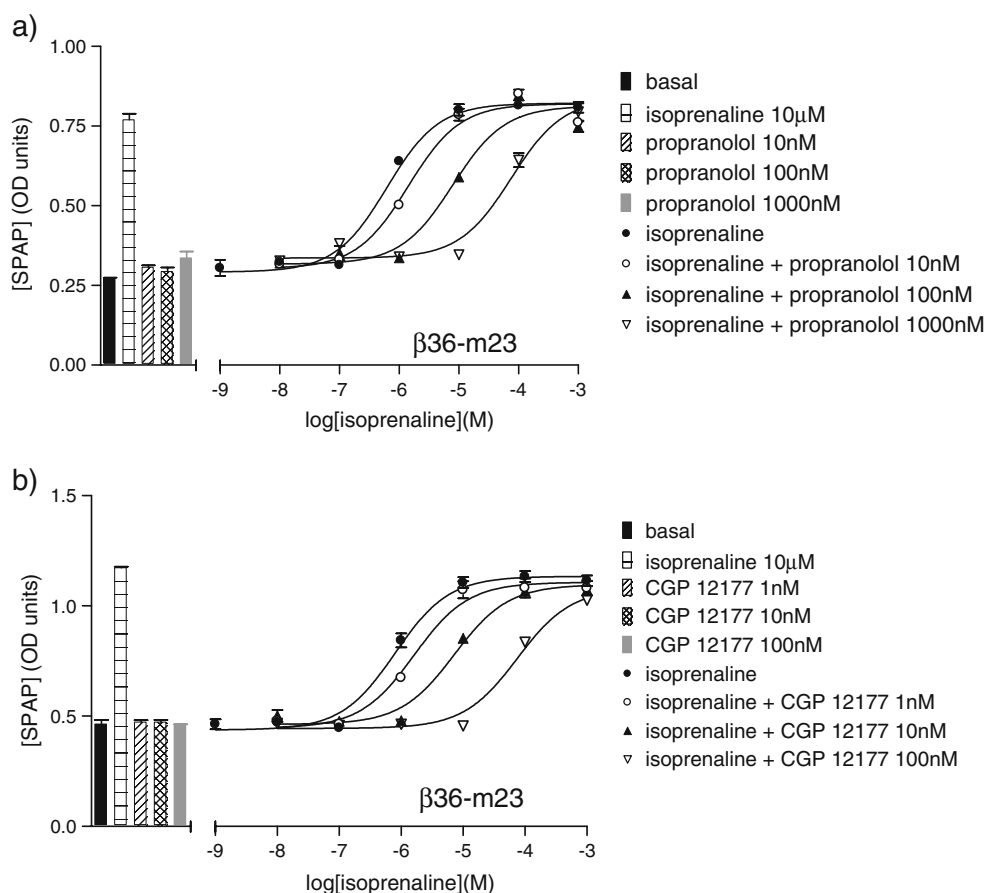
Table 9). No responses to cyanopindolol, carazolol or CGP12177 were seen for the β36–m23 receptor (Fig. 9d).

Finally, at the original tβtrunc receptor, the stimulatory responses to cimaterol were inhibited by low concentrations of CGP12177, with higher concentrations of CGP12177 causing the CGP12177 agonist response (Fig. 10a). This pattern, which can only be explained by two agonist sites or conformations (Baker 2010a), was also seen at the β6–m23 and β36 mutant receptors (Fig. 10b, c). Similar patterns were also seen with isoprenaline, clenbuterol and formoterol as agonists. Only the inhibition of the agonist responses to isoprenaline, cimaterol and formoterol were seen at the β36–m23 receptor (Fig. 10d).

Parent CHO cells

There was no specific binding of ³H-CGP12177 to the parent CHO cells (i.e. CHO cells without a transfected receptor). In addition, in the ³H-cAMP accumulation cAMP assay and the CRE-SPAP gene transcription assay, the parent CHO cells responded to forskolin but no responses were seen to any of the other ligands used in this study (Baker 2010a).

Discussion

Effect of the β36 deletions and m23 mutations on ligand affinity

The X-ray crystal structure of the turkey β-adrenoceptor was recently determined but several mutations were required to stabilise the protein before the crystallisation process could occur (Warne et al. 2008, 2009). To realise the full potential of this structure, it is important to understand the extent to which these mutations affects the pharmacology. Firstly, ligand affinity was examined. The β36 deletions made no difference to the ability of ligands to bind (compared to the tβtrunc construct). This was true for agonists, partial agonists and neutral antagonists. In the correlation plot (Fig. 3b), the ligands are clearly seen to have the same affinity for both the tβtrunc and β36

Fig. 9 ³H-cAMP accumulation in response to cyanopindolol in **a** ▶ tβtrunc cells, **b** β6–m23 cells, **c** β36 cells and **d** β36–m23 cells. Bars represent basal ³H-cAMP accumulation and that in response to 10 μM isoprenaline. Data points are mean ± SE mean of triplicate determinations. Each experiment is representative of **a** four, **b** 12, **c** six and **d** three separate experiments
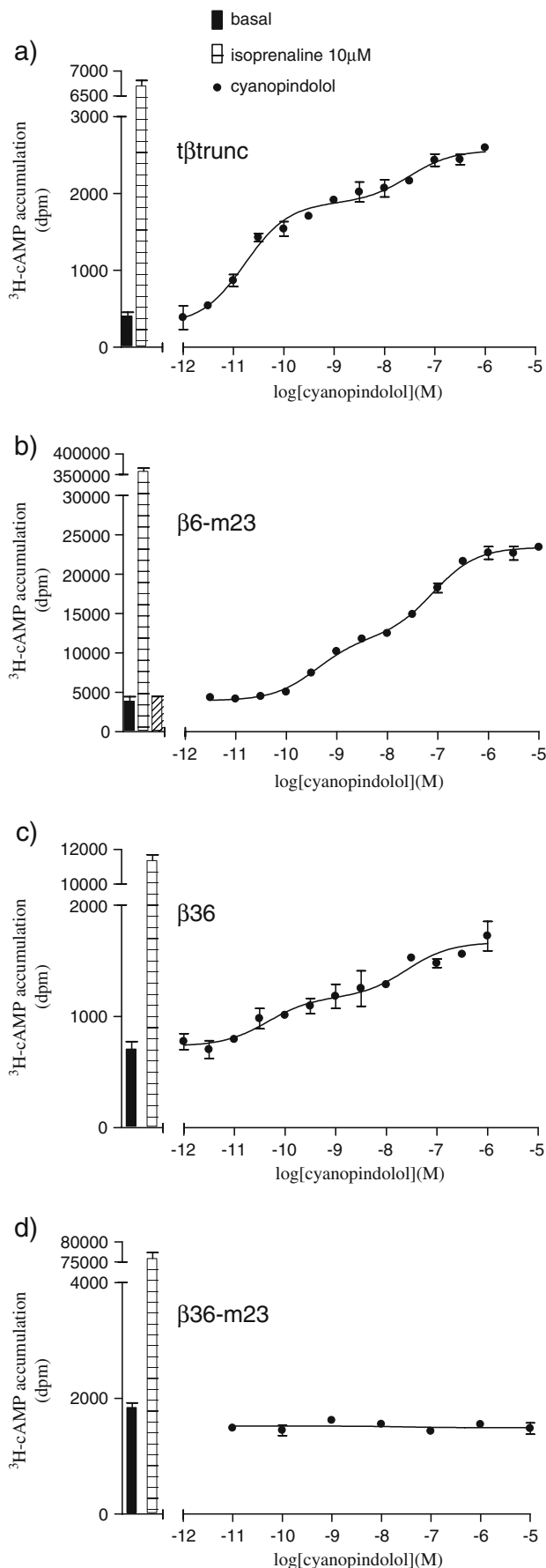
receptor and the line of best-fit passes through the origin. This is consistent with the view that the N-terminus, C-terminus and most of cytoplasmic loop 3 do not interact directly or indirectly with the ligand-binding pocket.

The m23-thermostabilising mutations resulted in a lower affinity for all ligands–agonists, partial agonists or neutral antagonists. The line of best fit (Fig. 3a, c) does not pass through the origin for either β6–m23 or β36–m23 receptors because all the ligands have about tenfold lower affinity. Interestingly, several of the most efficacious agonists (e.g. fenoterol, cimaterol, adrenaline, formoterol, noradrenaline and isoprenaline) have a greater than tenfold reduction in affinity with the m23 mutations, but this is not uniform across all agonists.

Previous ligand binding data for β6–m23 (Serrano-Vega et al. 2008) showed that cyanopindolol and dihydroalprenolol bound to the thermostabilised receptor with similar affinities to the unmutated receptor, whereas the binding of the agonists isoprenaline and noradrenaline was weaker by 650- and 2,470-fold, respectively. The significant difference between these results and the results presented here is most likely related to the environment in which these assays were performed. The binding experiments of Serrano-Vega et al. (2008) were performed on *Escherichia coli*-expressed receptors after solubilisation in the detergent dodecylmaltoside, whereas the binding experiments performed here were in living mammalian whole cells.

Effect of the β36 deletions and m23 mutations on ligand efficacy and receptor activation

Next, the effect on ligand efficacy (or ability for receptor activation) was investigated. As agonist efficacy depends on many factors, including the level of receptor expression (Clarke and Bond 1998; Kenakin 1999, 2002), it is difficult to directly compare across cell lines. However, in both functional assays, the catecholamines isoprenaline, adrenaline and noradrenaline were able to stimulate agonist response via all four receptors, including β36–m23 although much higher concentrations of agonist were required to achieve this.

As ligand affinity is reduced in the β6–m23 and β36–m23 mutants, all other things being equal, higher concentrations of agonist would be expected to be required to stimulate a response compared to the tβtrunc or β36 mutant. This was indeed observed, even in the β6–m23 line with its higher receptor expression level. Looking further at the other agonist ligands, with a range of efficacies from full to weak partial agonists (e.g. Tables 3 and 8), progressively fewer ligands were able to stimulate agonist responses via the β36 and β6–m23 mutants alone. For the β6–m23 receptor, the reduced ability of the receptor to activate a response, whilst partly being

Table 9 Log EC_{50} values and % isoprenaline maximal responses obtained in response to S-cyanopindolol, carazolol and CGP 12177 from ^{3}H-cAMP accumulation responses in CHO tβtrunc, β6–m23, β36 and β36–m23 cells

Ligand	receptor	Log EC_{50} 1	Log EC_{50} 2	% site 1	% isoprenaline max	n
S-Cyanopindolol	tβtrunc	-10.72 ± 0.06	-7.84 ± 0.13	67 ± 1	39 ± 1	4
	β6–m23	-9.24 ± 0.06	-6.88 ± 0.11	44 ± 2	9.2 ± 1.0	12
	β36	-10.42 ± 0.36	-7.46 ± 0.36	66 ± 5	7.4 ± 4.9	6
	β36–m23	No response				3
Carazolol	tβtrunc	-9.96 ± 0.06	-6.70 ± 0.08	43 ± 2	21 ± 1	8
	β6–m23	-8.69 ± 0.24	-6.14 ± 0.18	25 ± 3	2.5 ± 0.2	8
	β36	-9.92 ± 0.52	-6.52 ± 0.21	43 ± 10	3.4 ± 0.5	5
	β36–m23	No response				3
CGP 12177	tβtrunc	-9.18 ± 0.03	-6.73 ± 0.17	56 ± 3	51 ± 3	4
	β6–m23	-7.96 ± 0.11	-5.94 ± 0.15	35 ± 2	11 ± 2	11
	β36	-9.26 ± 0.15	-6.78 ± 0.15	54 ± 5	11 ± 5	6
	β36–m23	No response				9

Where the response is best described by a two-component response, the log EC_{50} 1 and log EC_{50} 2 are given along with the % of the response occurring at site 1. Values are mean ± SEM of n separate determinations

due to the reduction in affinity, is also consistent with the theory that the m23 mutations increase the stability of the receptor and thus increase the energy barrier required for receptor activation. However, as the β36 deletions did not affect ligand affinity, the reduced ability of the β36 mutant to activate responses may be a direct effect of the mutations reducing the receptor–G-protein interaction, consistent with extensive data implicating cytoplasmic loop 3 and the C-terminus of GPCRs in the receptor–G-protein activation process.

When the two were combined, the effect was even more striking with very few agonists being able to elicit a response from the β36–m23 mutant.

The effect of the β36 deletions and m23 mutations on the two agonist-induced conformations

The human β1-adrenoceptor and the tβtrunc exist in two pharmacologically active agonist-induced conformations, consisting of a high-affinity site where catecholamines stimulate responses and classical β-adrenoceptor antagonists bind with high affinity and a secondary site where CGP12177 stimulates an agonist response and classical antagonists have much higher affinity (Arch 2004; Baker 2005a, 2010a; Granneman 2001; Kaumann and Molenaar 2008; Konkar et al. 2000; Lowe et al. 2002; Molenaar 2003). Thus, CGP12177 binds to the tβtrunc catecholamine site with high affinity (K_D 0.32 nM from ^{3}H-CGP12177 binding and 0.22–1.5 nM from antagonism of agonist responses; Baker 2010a), but requires higher concentrations to stimulate agonist responses (EC_{50} 4.17 nM), suggesting that this CGP12177 agonist response is occurring via a secondary site (Baker 2010a). Furthermore, the concentrations of antagonists required to inhibit the CGP12177 response are significantly higher than those required to inhibit the other agonist responses (Baker 2010a).

In the β6–m23 and β36 mutants, CGP12177 bound with high affinity in both the binding assay and as an antagonist in the functional CRE-SPAP assay suggesting that CGP12177 binds with high affinity to the catecholamine site on both mutants. CGP12177 also stimulated agonist responses via both of these mutations but required a higher concentration (higher EC_{50} value) than expected from its K_D value at each receptor (β6–m23 K_D 0.85 nM on binding, K_D 0.48–1.45 nM on CRE-SPAP but EC_{50} of 155 nM on CRE-SPAP; β36 K_D 0.34 nM on binding, K_D 0.54–2.04 nM on CRE-SPAP but EC_{50} of 18.2 nM on CRE-SPAP). Also, the concentration of antagonists required to inhibit the CGP12177 agonist responses were significantly higher than those required to inhibit the other agonists. This suggests that the β6–m23 receptor and the β36 receptor both exist in at least two agonist-induced conformations. When both mutations are combined (β36–m23), CGP12177 was able to bind with high affinity and antagonise catecholamine responses with high affinity, suggesting that CGP12177 binds with high affinity catecholamine site on the receptor. However, CGP12177 itself did not simulate an agonist response via the β36–m23 receptor. This could be because either the ability of the β36–m23 receptor to stimulate responses is too poor to allow detection of partial agonist responses or because the combination of the β36 deletions and the m23 mutations has disrupted the secondary site on the turkey receptor.

Further evidence for two receptor conformations comes from ligands that stimulate two-component concentration response curves (Baker et al. 2003; Walter et al. 1984)—examples at the tβtrunc receptor being CGP12177 itself, S-cyanopindolol and carazolol (Baker 2010a). CGP12177, carazolol and S-cyanopindolol all stimulated two-component agonist response curves via both the β6–m23 and β36 receptors. This again suggests that neither mutation cause a major disruption to the secondary site on the receptor. Once again, no response was seen in the

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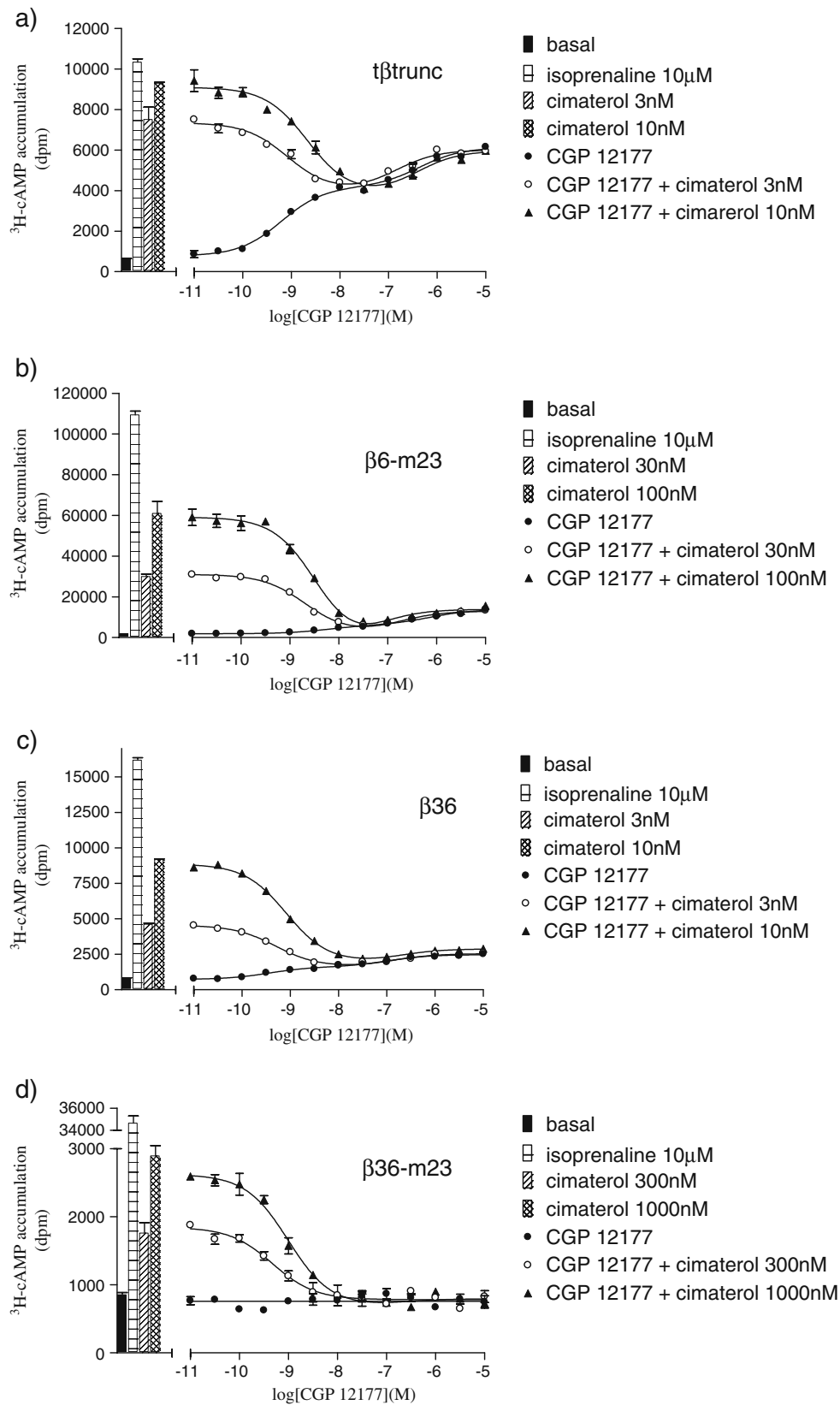
Fig. 10 ^{3}H-cAMP accumulation in response to CGP12177 in **a** tβtrunc cells, **b** β6–m23 cells, **c** β36 cells and **d** β36–m23 cells in the absence and presence of cimaterol. *Bars* represent basal ^{3}H-cAMP accumulation, that in response to 10 μM isoprenaline and that in response to 3, 10, 30, 100, 300 or 1,000 nM cimaterol. Data points are mean ± SE mean of triplicate determinations. Each experiment is representative of **a** four, **b** six, **c** six and **d** four separate experiments

combined β36–m23 mutant. Again this could be because the combination of mutations disrupted the secondary site or because the ability of this heavily mutated receptor to stimulate agonist responses is too reduced to be able to examine these partial agonists.

The final evidence for two receptor conformations comes from the inhibition of a catecholamine site agonist response by CGP12177 at lower concentrations than those required to stimulate the CGP12177 agonist response (Baker 2010a; Konkar et al. 2000). When the β36 and β6–m23 mutants were examined alone, this was also the case. Thus CGP12177 at low concentrations inhibited, e.g. the cimaterol response (occurring via the catecholamine site), but higher concentrations of CGP12177 were required to stimulate a CGP12177 agonist response, occurring via the secondary site. This again suggests that both of these mutant receptors exist in two agonist-induced conformations. For the combined β36–m23 mutant, CGP12177 inhibited the cimaterol response with high affinity, consistent with it binding to the catecholamine site, but the lack of the stimulation in response to CGP12177 once again precluded the demonstration of the secondary site in the β36–m23 receptor.

Conclusion

This detailed pharmacological analysis of the effects of the β36 deletions and the m23 thermostabilising mutations alone, and in combination, shows that the final crystallised receptor (β36–m23), whilst having severely reduced ability to activate responses compared to the original tβtrunc receptor, is still a fully functional receptor, capable of binding agonist and antagonist ligands and activating intracellular agonist responses. The m23 mutations reduced affinity for agonists, partial agonists and neutral antagonists whilst the β36 deletions alone had no effect on ligand affinity. Both sets of changes appeared to reduce the agonist activation of the receptor—the β36 deletions most likely by reducing receptor–G-protein activation and the m23 mutations by both reducing ligand affinity and receptor activation (most likely by the increased receptor stability and thus an increased activation energy requirement). Finally, both the β6–m23 and the β36 receptors retained two active agonist-induced receptor conformations similar to that of the original tβtrunc receptor, although due to the severely reduced activation ability of the β36–m23 mutant receptor, this feature is difficult to assess in the final combined mutant.

Thus, the original contention that the β36–m23 receptor is preferentially in an antagonist-binding conformation appears to be more complex than previously anticipated, not least because only a proportion of the agonists tested showed a marked reduction in affinity compared to the antagonists. The pharmacological characterisation also suggests that the structure of β36–m23 with cyanopindolol bound may represent the receptor bound with a weak partial agonist. Finally, this work provides a solid foundation for the analysis of further structures of the mutated turkey β-adrenoceptor with different bound ligands.

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