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Salmeterol's Extreme β2 Selectivity Is Due to Residues in Both Extracellular Loops and Transmembrane Domains

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

Cell Signalling, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, United Kingdom

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ABSTRACT

Salmeterol is a long-acting β2-agonist, widely used as an inhaled treatment of asthma and chronic obstructive pulmonary disease. It has very high β2-affinity (log K_D −8.95) and is very selective for the β2-adrenoceptor (1000-fold selectivity over the β1-adrenoceptor). This study used a mutagenesis approach to determine the exact amino acids in the human β2-adrenoceptor responsible for this very high selectivity. Wild-type β2- and β1-adrenoceptors, chimeric β2/β1-adrenoceptors, and receptors with single-point mutations were transfected into Chinese hamster ovary-K1 cells, and affinity and function were studied using [^{3}H]CGP 12177 [(−)-4-(3-*tert*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one] whole-cell binding and [^{3}H]cAMP accumulation. Extracellular loop 3 (and specifically amino acid K305) had the largest single effect by reducing salmeterol's affinity for the β2-adrenoceptor by 31-fold. H296 in transmembrane 6 also had a major effect (18-fold reduction in salmeterol affinity). Combining these, in the double mutant β2-H296K-K305D, reduced salmeterol's affinity by 275-fold, to within 4-fold of that of the β1-adrenoceptor, without affecting the affinity or selectivity of other β2-agonists (salbutamol, formoterol, fenoterol, clenbuterol, or adrenaline). Another important amino acid was Y308 in transmembrane 7, although this also affected the affinity and selectivity of other agonists. F194 in extracellular loop 2 and R304 in extracellular loop 3 also had minor effects. None of these mutations (including the double mutant β2-H296K-K305D) affected the efficacy or duration of action of salmeterol. This suggests that the high affinity and selectivity of salmeterol are due to specific amino acids within the receptor itself, but that the duration of action is at least in part due to other factors, for example lipophilicity.

Introduction

Salmeterol, a long-acting β2-agonist, is widely used as an inhaled treatment of asthma and chronic obstructive pulmonary disease because it improves symptoms and reduces hospital admissions (Johnson, 1995; Waldeck, 2002; Cazzola et al., 2012; Kew et al., 2014). β2-agonists (e.g., salbutamol) were first developed in the 1960s (Brittain et al., 1968; Hartley et al., 1968). By stimulating β2-adrenoceptors in the lungs, they mimic the action of adrenaline causing bronchodilation (Johnson, 1995). However, these drugs (which also include fenoterol and clenbuterol) have the major disadvantage that they are short acting and therefore require frequent dosing in patients and do not control nocturnal asthma (Bradshaw et al., 1987; Ball et al., 1991; Jack, 1991; Johnson, 1995; Waldeck, 2002). In the 1980s, long-acting β2-agonists were sought, and formoterol and salmeterol were subsequently developed. Salmeterol (which consists of salbutamol with a long side chain) (Fig. 1) was developed by Glaxo Group Research in a program specifically designed to identify long acting β2-compounds (Bradshaw et al., 1987; Ball et al., 1991, Jack, 1991; Johnson, 1995), and its long duration of action was confirmed in patients (Ullman and Svedmyr, 1988).

Ligand binding, affinity, efficacy, and selectivity have largely been ascribed to interactions occurring within the transmembrane (TM) domains of the β2-adrenoceptor. These conclusions followed mutagenesis studies (e.g., Dixon et al., 1987a; Strader et al., 1987; Dohlman et al., 1988; Marullo et al., 1990), photoaffinity label techniques (e.g., Wong et al., 1988), studies involving extra- and intracellular region deletions (Dixon et al., 1987b), and recognition of the similarity of the β-adrenoceptors to rhodopsin (Dixon et al., 1986). The β2-adrenoceptor receptor crystal structure also largely confirms these findings (Rasmussen et al., 2011; Katritch et al., 2013; Venkatakrishnan et al., 2013). Frielle et al. (1988) examined ligand affinities at six β2-β1 chimeric receptors, and found that mutants involving TM4 had lower affinity for noradrenaline, thus concluding that TM4 was the main region for β-agonist selectivity. Marullo et al. (1990) also identified TM4 as contributing to agonist binding and selectivity. However, neither of these studies is definitive because each chimera contained several TM, EL, and intracellular loop changes, making it difficult to pinpoint the precise region involved. Isogaya et al. (1998, 1999) therefore made different chimeras in which only one TM domain was changed and investigated TM1, TM2, and TM7 changes on ligand binding (Kikkawa et al., 1998). They found that TM2 and TM7 were important in agonist and antagonist affinity (and thus β2/β1 selectivity) and pinpointed Y308 in TM7 as a major contributor to β2 affinity, including that of salmeterol. However, these chimeras also

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ABBREVIATIONS: CGP 12177, (−)-4-(3-*tert*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one; CHO, Chinese hamster ovary; EL, extracellular loop; sfm, serum-free media; TM, transmembrane; WT, wild-type.

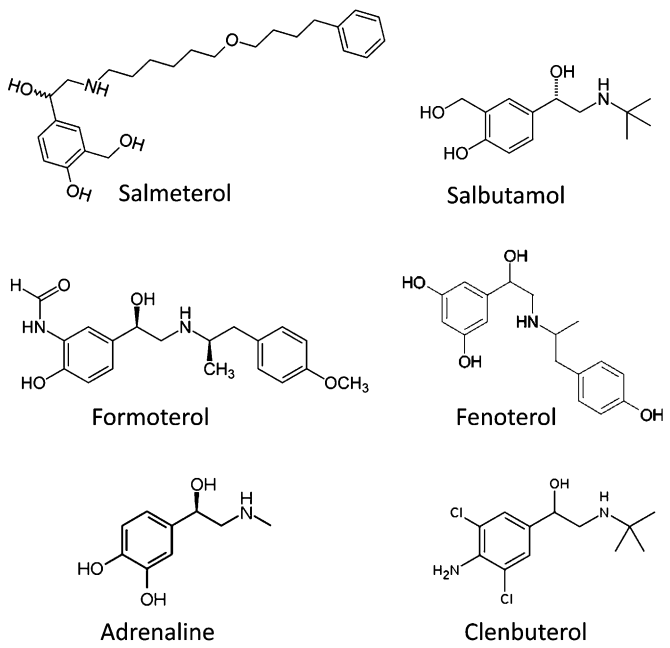

Fig. 1. Chemical structures of salmeterol and salbutamol, formoterol, fenoterol, adrenaline, and clenbuterol.

contained EL and intracellular loop changes in addition to the TM changes. Furthermore, the Y308 mutation (Isogaya et al., 1998) did not explain all of the high selectivity of salmeterol, and thus there must be selective binding occurring elsewhere in the β2-adrenoceptor.

Salmeterol is considered to have its long duration of action by binding to an exosite on the β2-adrenoceptor. The side chain is thought to anchor the molecule to the receptor in one region (exocite), whereas the head group binds to and activates the receptor via the normal orthosteric site (Jack, 1991; Johnson, 1995; Clark et al., 1996; Coleman et al., 1996). Studies have suggested that this exocite may be in TM4 (Green et al., 1996, 2001) or TM6/7 (Rong et al., 1999). Furthermore, Isogaya et al. (1998) suggested that it was the side chain, not the head group, that interacted with TM7. However, it is also recognized that salmeterol is very lipophilic and therefore partitions into the cell membrane, making washout of the drug problematic (Rhodes et al., 1992; Anderson et al., 1994; Johnson, 1995; Coleman et al., 1996; Sykes et al., 2014). This makes it difficult to discriminate between long duration due to true exocite binding or long duration due to membrane partition (Coleman et al., 1996), and therefore its long duration of action may indeed be partly due to both mechanisms (Rhodes et al., 1992; Rong et al., 1999).

This study therefore aimed to identify the regions of the human β2-adrenoceptor responsible for the high-affinity binding of salmeterol. As the selectivity of salmeterol for the human β2-adrenoceptor is 1000- to 3000-fold greater than that for the human β1-adrenoceptor (and this is due to selective affinity, not selective efficacy; Baker, 2010), a chimeric β2/β1 mutagenesis study was undertaken in which single TM or EL regions were examined in turn before progressing to single-point mutagenesis studies (Baker et al., 2014).

Materials and Methods

Molecular biology reagents were from Promega (Madison, WI). 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 fetal calf serum was from PAA Laboratories (Teddington, Middlesex, UK). [^{3}H]CGP 12177 [(−)-4-(3-*tert*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one], [^{3}H]adenine, and [^{14}C]cAMP were from Amersham International (Buckinghamshire, UK), and Microscint 20 and Ultima Gold XR scintillation fluid were from PerkinElmer (Shelton, CT). Formoterol and salmeterol were from Tocris Life Sciences (Avonmouth,

TABLE 1
The chimeric β1/β2-adrenoceptor constructs

	Amino Acid Changes
β2-WT	
β2-N	β2 but with full N terminus of the β1—see *Materials and Methods* for details
β2-EL1	M96W, K97G, M98R, T100E, F101Y, N103S, W105F
β2-EL2	T177E, H178S, Q179D, I182R, N183R, A186N, N187D, E188P, T189K, F194V
β2-EL3	Q299H, D300R, N301E, I303V, R304P, K305D
β2-TM1	I38L, V39L, S41A, A46L, F49A, T56V
β2-TM2	T73M, C77S, A85L, A92T, H93I, I94V, L95V
β2-TM3	F108L, I112V, V129L
β2-TM4[a]	V152G, I153L, I154V, L155C, M156T, I159A, V160I, G162A, T164V, Q170L
β2-TM5	Q197R, I205V, V213C, V216A, S220L
β2-TM6	T281V, I291L, V292A, I294V, H296K, V297A, I298F
β2-TM7	E306R, V307L, Y308F, I309V, L310F, L311F, I314L, V317A, G320A, L324I
β1-WT	
β1-N	β1 but with full N terminus of the β2—see *Materials and Methods* for details
β1-EL1	W121M, G122K, R123M, E125T, Y126F, S128N, F130W
β1-EL2	E202T, S203H, D204Q, R207I, R208N, N211A, D212N, P213E, K214T, V219F
β1-EL3	H350Q, R351D, E352N, V354I, P355R, D356K
β1-TM1	L63I, L64V, A66S, L71A, A74F, V81T
β1-TM2	M98T, S102C, L110A, T117A, I118H, V119I, V120L
β1-TM3	L133F, V137I, L154V
β1-TM4	G177V, L178I, V179I, C180L, T181M, A184I, I185V, A187G, V189T, L195Q, W199Y
β1-TM5	R222Q, V230I, C238V, A241V, L245S
β1-TM6	V332T, L342I, A343V, V345I, K347H, A348V, F349I
β1-TM7	R357E, L358V, F359Y, V360I, F361L, F362L, L365I, A368V, A371G, I375L

[a]Making the full change here, i.e., with Y174W, rendered the receptor nonfunctional (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 2

Affinity (log K_D values) of β-adrenoceptor ligands for the β2-WT and β1-WT and receptors containing whole TM or EL changes (see Table 1 for details) in stable cell lines

The K_D values of [^{3}H]CGP 12177 (measured from saturation binding) and the receptor expression levels are also given. The values are mean ± S.E.M. for n separate experiments.

	K_D [^{3}H]CGP 12177	n	Protein	Log K_D Salmeterol	n	Log K_D Salbutamol	n	Log K_D Formoterol	n	Log K_D Fenoterol	n
			fmol/mg								
β2-WT	0.21 ± 0.02	29	279 ± 20	−8.95 ± 0.05	30	−5.94 ± 0.03	33	−8.29 ± 0.03	32	−6.80 ± 0.02	30
β2-N	0.29 ± 0.04	9	389 ± 42	−9.14 ± 0.05	10	−5.90 ± 0.03	11	−8.25 ± 0.05	10	−6.86 ± 0.03	10
β2-EL1	0.36 ± 0.02	9	946 ± 97	−8.99 ± 0.04	10	−5.84 ± 0.03	11	−8.12 ± 0.06	10	−6.61 ± 0.02*	10
β2-EL2	0.30 ± 0.01	9	516 ± 35	−8.28 ± 0.06 *	10	−5.60 ± 0.04*	11	−7.74 ± 0.06*	10	−6.37 ± 0.03*	9
β2-EL3	0.28 ± 0.02	9	778 ± 63	−6.74 ± 0.03*,#	10	−5.68 ± 0.03*	11	−7.89 ± 0.05*	10	−6.51 ± 0.03*	10
β2-TM1	0.17 ± 0.06	15	138 ± 8	−9.11 ± 0.10	8	−5.83 ± 0.04	9	−8.18 ± 0.04	8	−6.82 ± 0.03	9
β2-TM2	0.22 ± 0.02	11	405 ± 41	−8.57 ± 0.08*	7	−5.76 ± 0.03	7	−8.27 ± 0.09	7	−7.16 ± 0.10*	7
β2-TM3	0.34 ± 0.03	10	1037 ± 113	−8.84 ± 0.05	8	−5.77 ± 0.07	8	−7.66 ± 0.05*	8	−6.15 ± 0.04*	7
β2-TM4	0.25 ± 0.03	11	458 ± 64	−8.96 ± 0.03	7	−5.97 ± 0.07	7	−8.30 ± 0.03	7	−6.87 ± 0.02	8
β2-TM5	0.32 ± 0.03	10	849 ± 83	−9.00 ± 0.05	7	−5.84 ± 0.03	8	−8.05 ± 0.07	8	−6.48 ± 0.03*	6
β2-TM6	0.75 ± 0.06	9	728 ± 58	−7.37 ± 0.03*	7	−5.89 ± 0.08	7	−8.06 ± 0.07	8	−6.65 ± 0.04	7
β2-TM7	0.14 ± 0.02	14	67 ± 5	−7.57 ± 0.06*	8	−5.83 ± 0.06	10	−7.57 ± 0.04*	9	−6.16 ± 0.03*	10
β1-WT	0.32 ± 0.02	28	534 ± 33	−5.80 ± 0.02	27	−4.80 ± 0.02	27	−6.02 ± 0.02	25	−5.09 ± 0.03	27
β1-N	0.50 ± 0.02	6	1309 ± 126	−5.60 ± 0.01*	7	−4.71 ± 0.02	7	−5.90 ± 0.02	6	−4.95 ± 0.05	7
β1-EL1	0.31 ± 0.01	9	497 ± 39	−5.69 ± 0.05	7	−4.74 ± 0.03	7	−6.10 ± 0.04	6	−5.14 ± 0.02	7
β1-EL2	0.58 ± 0.03	9	1100 ± 111	−5.72 ± 0.03	7	−4.74 ± 0.04	7	−6.08 ± 0.04	6	−5.13 ± 0.04	7
β1-EL3	0.54 ± 0.04	9	384 ± 30	−6.25 ± 0.06*	7	−5.01 ± 0.03*	7	−6.35 ± 0.06*	6	−5.43 ± 0.06*	7
β1-TM1	0.30 ± 0.02	11	372 ± 47	−5.75 ± 0.05	12	−4.74 ± 0.03	12	−6.03 ± 0.03	8	−5.05 ± 0.07	8
β1-TM2	0.26 ± 0.03	10	567 ± 58	−5.50 ± 0.03*	12	−5.09 ± 0.02*	12	−6.56 ± 0.05*	8	−5.54 ± 0.06*	8
β1-TM3	0.48 ± 0.03	10	1994 ± 214	−5.73 ± 0.02	12	−4.67 ± 0.03	12	−5.95 ± 0.03	8	−5.06 ± 0.07	8
β1-TM4	0.26 ± 0.02	12	233 ± 18	−5.91 ± 0.04	12	−4.84 ± 0.03	12	−6.31 ± 0.05*	8	−5.06 ± 0.08	8
β1-TM5	0.25 ± 0.02	12	359 ± 30	−5.82 ± 0.04	11	−4.86 ± 0.04	12	−6.17 ± 0.03	8	−5.20 ± 0.05	8
β1-TM6	0.29 ± 0.01	19	1207 ± 100	−5.89 ± 0.02	18	−5.01 ± 0.03*	18	−6.37 ± 0.03*	7	−5.45 ± 0.08*	7
β1-TM7	0.57 ± 0.04	9	2078 ± 133	−6.44 ± 0.02*	17	−5.42 ± 0.02*	18	−6.93 ± 0.05*	7	−5.86 ± 0.09*	7

*$P < 0.001$. One-way analysis of variance with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β2-WT or the β1-WT. Thus, the log K_D for salmeterol at the β2-EL2 is different from that obtained from the β2-WT with $P < 0.001$. Likewise, the log K_D for salmeterol at the β1-EL3 is different from that obtained from the β1-WT with $P < 0.001$; #$P < 0.001$. One-way analysis of variance with post hoc Newman-Keuls comparing each value with all other values in this set. Thus, the log K_D for salmeterol at β2-EL3 is different from that obtained for β2-WT, β2-N, β2-EL1, β2-EL2, β2-TM1, β2-TM2, β2-TM3, β2-TM4, β2-TM5, β2-TM6, and β2-TM7 with $P < 0.001$ in all cases.

UK). AG 50W-4X resin was from Bio-Rad (Hertfordshire, UK). All other reagents were from Sigma-Aldrich (Poole, Dorset, UK). Racemic ligands were used throughout.

Molecular Biology

The cDNA sequence encoding the wild-type (WT) human β1-adrenoceptor (β1-WT) in pJG3.6 was a gift of S. Rees (GlaxoSmithKline, Stevenage, UK). This cDNA was subcloned as a HindIII/XbaI fragment into pcDNA3.1, and the sequence was confirmed by DNA sequencing. The cDNA for the WT human β2-adrenoceptor (β2-WT) in pcDNA3.1 was obtained from the Missouri S&T cDNA Resource Centre (www.cdna.org). This cDNA was also subcloned as a HindIII/XbaI fragment into pcDNA3.1, and the sequence was confirmed by DNA sequencing. The mutations described in Table 1 were generated using QuikChange mutagenesis and BioLine PolyMate Additive for GC-rich templates (Baker et al., 2014). After subcloning in Top 10F competent cells, each mutant β2-adrenoceptor cDNA was excised on HindIII/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 Life Sciences Sequencing Facility.

To detect the most important areas of the β2-WT important for salmeterol interactions, we made point mutations in first the EL and second the TM regions of the receptor such that each EL or TM region resembled that of the β1-WT. Prediction of the EL and TM regions was performed using ExPASy topology prediction tools (www.expasy.org). For example, six-point mutations were made in β2-WT (I38L, V39L, S41A, A46L, F49A, T56V), which effectively converted the TM1 region of this receptor to that of the β1-WT. This chimeric receptor was called β2-TM1 (i.e., β2-adrenoceptor, but with TM1 of the β1-WT; Table 1). This was then replicated for each EL and TM region (Table 1). A similar set of mutations was made starting with the β1-WT and creating chimeric receptors each with an EL or TM region of the human β2-WT (Table 1). For the N-terminal swaps, a VspI site (ATTAAT) was introduced immediately downstream of the A66S (β1; TCATTA AT) or S41A (β2; GCATTAAT) mutations produced during phase 2 of the production of TM1 mutations (Table 1), which

TABLE 3

The ratios of affinity (given as fold change) compared with that obtained in the β2-WT or β1-WT receptors (data from Table 2)

Numbers in Roman text are a decrease in affinity compared with their respective WT, and numbers in italics are an increase in affinity compared with their respective WT. Thus, salmeterol has an affinity of 1.5-fold more at β2-N than β2-WT but 162-fold less at β2-EL3 than β2-WT. The β2/β1 selectivity for the ligands in these stable cell lines is also given.

	Salmeterol	Salbutamol	Formoterol	Fenoterol
β2-WT	1.0	1.0	1.0	1.0
β2-N	*1.5*	1.1	1.1	*1.1*
β2-EL1	*1.1*	1.3	1.5	1.5
β2-EL2	4.7	2.2	3.5	2.7
β2-EL3	162	1.8	2.5	1.9
β2-TM1	*1.4*	1.3	1.3	*1.0*
β2-TM2	2.4	1.5	1.0	*2.3*
β2-TM3	1.3	1.5	4.3	4.5
β2-TM4	*1.0*	*1.1*	*1.0*	*1.2*
β2-TM5	*1.1*	1.3	1.7	2.1
β2-TM6	38.0	1.1	1.7	1.4
β2-TM7	24.0	1.3	5.2	4.4
β1-WT	1.0	1.0	1.0	1.0
β1-N	1.6	1.2	1.3	1.4
β1-EL1	1.3	1.1	*1.2*	*1.1*
β1-EL2	1.2	1.1	*1.1*	*1.1*
β1-EL3	*2.8*	*1.6*	*2.1*	*2.2*
β1-TM1	1.1	1.1	*1.0*	1.1
β1-TM2	2.0	*1.9*	*3.5*	*2.8*
β1-TM3	1.2	1.3	1.2	1.1
β1-TM4	*1.3*	*1.1*	*1.9*	1.1
β1-TM5	*1.0*	*1.1*	*1.4*	*1.3*
β1-TM6	*1.2*	*1.6*	*2.2*	*2.3*
β1-TM7	*4.4*	*4.2*	*8.1*	*5.9*
β2/β1 selectivity	1413	14	186	51

generated [β1; L63I, L64V, A66S] or [β2; I38L, V39L, S41A] (see Table 1). Following mutagenesis, each receptor DNA was digested with VspI, the fragments were purified, and the alternative N terminal sequences were ligated to give a β1-adrenoceptor with the full β2-adrenoceptor N terminus (β1-N) or a β2-adrenoceptor with the full β1-adrenoceptor N terminus (β2-N). These constructs were expressed in Chinese hamster ovary (CHO)–K1 cells, and stable cell lines were generated (see below and Baker et al., 2014).

Once important EL or TM regions for the interaction of salmeterol were identified, to determine which individual amino acids were involved, several single-point mutations and chimeric receptors were made (Table 1), and these constructs were expressed transiently in CHO-K1 cells.

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

Generation of Stable Cell Lines. CHO-K1 cells were transfected with the WT human β1- or β2-adrenoceptor, or one of the full EL region or TM domain chimeric receptors (total 24 cell lines) using lipofectamine and OPTIMEM and selected for 3 weeks using resistance to geneticin (1 mg/ml for the receptor). Single clones were identified by

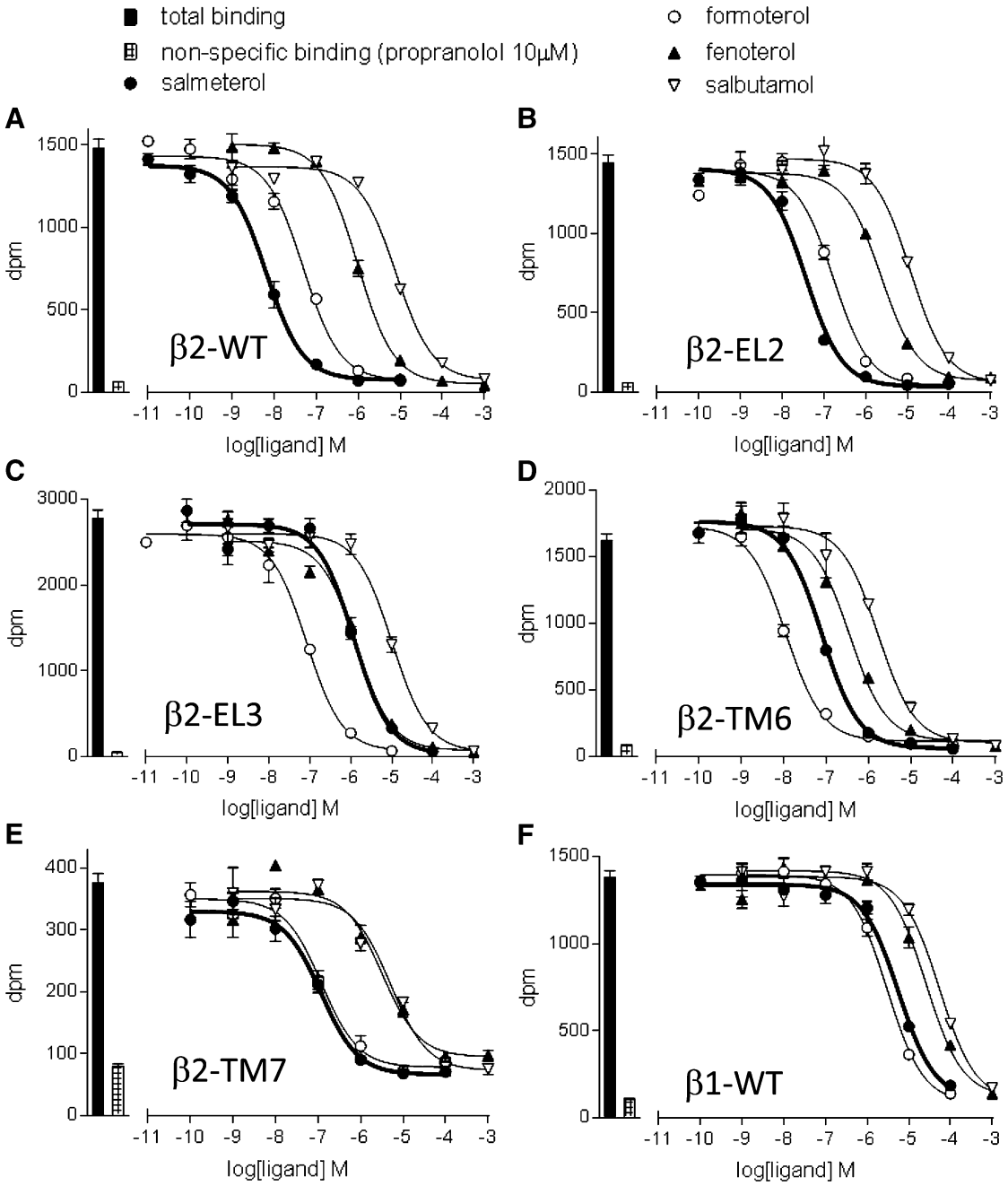

Fig. 2. Inhibition of [^{3}H]CGP 12177 whole-cell binding in response to salmeterol, formoterol, fenoterol, and salbutamol in stable cell lines expressing (A) β2-WT, (B) β2-EL2, (C) β2-EL3, (D) β2-TM6, (E) β2-TM7, and (F) β1-WT receptors. The salmeterol curve is in bold. Nonspecific binding was determined by 10 μM propranolol. The concentrations of [^{3}H]CGP 12177 present in these experiments were (A) 1.4 nM, (B) 1.3 nM, (C) 1.3 nM, (D) 0.7 nM, (E) 0.4 nM, and (F) 0.8 nM. Data points are mean ± S.E.M. of triplicate determinations. These single experiments are representative of (A) 30, (B) 9, (C) 10, (D) 7, (E) 8, and (F) 25 separate experiments.

TABLE 4

Affinity (log K_D values) of β-adrenoceptor ligands for the wild-type β-AR and receptors containing single and double point mutations in the extracellular loops in transiently transfected populations of cells

The K_D value of [^{3}H]CGP 12177 and the receptor expression level in these transient populations are also given. The values are mean ± S.E.M. for n separate experiments, and each separate n number has been obtained in a separate transiently transfected population of cells.

	K_D [^{3}H]CGP 121777 from Saturation	Protein		Log K_D Salmeterol	n	Log K_D Salbutamol	n	Log K_D Formoterol	n	Log K_D Fenoterol	n	Log K_D Adrenaline	n	Log K_D Clenbuterol	n
		fmol/mg													
β2-WT	0.18 ± 0.01	165 ± 13	30	−8.67 ± 0.03	67	−5.76 ± 0.03	38	−7.92 ± 0.03	52	−6.56 ± 0.02	46	−5.64 ± 0.05	17	−7.44 ± 0.04	20
Point mutations in β2 resulting in a change to β1 amino acids															
β2-EL2	0.17 ± 0.02	188 ± 33	6	−8.07 ± 0.07*	19	−5.59 ± 0.04	9	−7.51 ± 0.03	4	−6.19 ± 0.03	3	−5.20 ± 0.10	5	−7.26 ± 0.04	5
β2-F194V	0.24 ± 0.03	207 ± 47	7	−7.92 ± 0.05*	21	−5.50 ± 0.06	14	−7.57 ± 0.05*	12	−6.18 ± 0.03*	12	−5.37 ± 0.10	8	−7.13 ± 0.04	8
β2-EL3	0.22 ± 0.04	126 ± 21	6	−6.56 ± 0.04*,#	25	−5.54 ± 0.05	9	−7.61 ± 0.05*	9	−6.33 ± 0.03	8	−4.97 ± 0.08*	5	−7.10 ± 0.14	4
β2-Q299H	0.25 ± 0.03	216 ± 31	9	−8.30 ± 0.06*	10	−5.76 ± 0.03	7	−7.84 ± 0.05	13	−6.54 ± 0.04	12	ND		ND	
β2-D300R	0.27 ± 0.03	271 ± 47	9	−8.27 ± 0.10*	10	−5.74 ± 0.05	7	−7.99 ± 0.06	12	−6.59 ± 0.06	12	ND		ND	
β2-N301E	0.23 ± 0.03	176 ± 30	8	−8.44 ± 0.09	10	−5.69 ± 0.04	6	−7.83 ± 0.04	12	−6.46 ± 0.04	12	ND		ND	
β2-I303V	0.25 ± 0.04	224 ± 38	9	−8.34 ± 0.05*	10	−5.66 ± 0.05	7	−7.78 ± 0.05	13	−6.40 ± 0.04	13	ND		ND	
β2-R304P	0.22 ± 0.04	267 ± 59	9	−8.10 ± 0.04*	16	−5.65 ± 0.03	14	−7.71 ± 0.04	19	−6.29 ± 0.03*	18	−5.59 ± 0.07	11	−7.26 ± 0.03	10
β2-K305D	0.21 ± 0.03	159 ± 26	8	−7.18 ± 0.04*	15	−5.61 ± 0.03	12	−7.56 ± 0.04*	17	−6.43 ± 0.03	17	−5.30 ± 0.10	8	−7.33 ± 0.06	8
β2-F194V-K305D	0.19 ± 0.03	128 ± 21	9	−6.88 ± 0.03*	17	−5.54 ± 0.04	12	−7.33 ± 0.05*	20	−6.31 ± 0.04*	19	−5.16 ± 0.13	6	−7.25 ± 0.10	8
β2-R304P-K305D	0.22 ± 0.06	109 ± 12	7	−6.85 ± 0.06*	15	−5.50 ± 0.07	13	−7.41 ± 0.04*	13	−6.28 ± 0.04*	14	−5.19 ± 0.15	5	−7.22 ± 0.07	5
Point mutations in β2 resulting in a change to non-β1 amino acids															
β2-F194A	0.22 ± 0.03	170 ± 23	9	−7.36 ± 0.05*	10	−5.43 ± 0.04	11	−7.60 ± 0.06*	9	−6.17 ± 0.05*	9	−5.24 ± 0.10	8	−6.98 ± 0.03*	8
β2-K305A	0.16 ± 0.02	151 ± 45	8	−8.05 ± 0.09*	9	−5.52 ± 0.09	9	−7.74 ± 0.04	8	−6.44 ± 0.08	7	−5.54 ± 0.08	8	−7.30 ± 0.07	8
β2-K305E	0.22 ± 0.03	114 ± 16	9	−7.62 ± 0.07*	10	−5.28 ± 0.10*	11	−7.46 ± 0.04*	9	−6.34 ± 0.04	9	ND		ND	
β2-K305G	0.24 ± 0.04	77 ± 7	6	−7.51 ± 0.11*	9	−5.70 ± 0.06	9	−7.74 ± 0.09	5	−6.38 ± 0.12	5	−5.47 ± 0.05	5	−7.35 ± 0.07	5
β2-K305H	0.23 ± 0.03	157 ± 20	8	−7.57 ± 0.06*	9	−5.65 ± 0.11	10	−7.24 ± 0.07*	8	−6.49 ± 0.05	8	ND		ND	
β2-K305R	0.24 ± 0.03	135 ± 14	15	−8.37 ± 0.05*	12	−5.64 ± 0.05	16	−8.02 ± 0.06	14	−6.55 ± 0.05	11	−5.87 ± 0.07	6	−7.26 ± 0.11	4
β2-K305S	0.19 ± 0.04	83 ± 11	6	−7.57 ± 0.06*	9	−5.58 ± 0.07	8	−7.66 ± 0.11	5	−6.54 ± 0.09	3	−5.38 ± 0.07	5	−7.55 ± 0.10	5
β2-R304D	0.22 ± 0.02	94 ± 14	6	−8.30 ± 0.11*	8	−5.63 ± 0.10	6	−7.86 ± 0.07	6	−6.70 ± 0.11	4	−5.66 ± 0.08	6	−7.39 ± 0.06	8
β2-R304D-K305G	0.19 ± 0.02	58 ± 3	6	−7.56 ± 0.09*	6	−5.63 ± 0.15	6	−7.49 ± 0.18*	5	−6.42 ± 0.07	3	−5.38 ± 0.12	5	−7.50 ± 0.06	5
β1-WT	0.28 ± 0.02	731 ± 96	27	−5.67 ± 0.01	44	−4.74 ± 0.02	28	−5.86 ± 0.02	42	−4.90 ± 0.02	44	−4.74 ± 0.04	11	−6.58 ± 0.06	11
Point mutations in β1 resulting in a change to β2 amino acids															
β1-EL2	0.32 ± 0.01	1135 ± 179	6	−5.82 ± 0.05	9	−4.79 ± 0.08	5	−5.99 ± 0.10	4	−4.98 ± 0.06	3	−4.80 ± 0.05	5	−6.48 ± 0.02	5
β1-V219F	0.38 ± 0.04	1224 ± 261	9	−5.48 ± 0.02*	10	−4.76 ± 0.01	7	−5.80 ± 0.03	13	−5.06 ± 0.03	13	−4.89 ± 0.10	8	−6.51 ± 0.03	8
β1-EL3	0.33 ± 0.02	209 ± 11	6	−6.29 ± 0.07*	9	−4.90 ± 0.06	5	−6.14 ± 0.06*	5	−5.26 ± 0.03*	3	−5.24 ± 0.09*	4	−6.76 ± 0.05	5
β1-P355R	0.22 ± 0.02	283 ± 72	6	−5.77 ± 0.05	11	−4.82 ± 0.07	5	−5.98 ± 0.02	11	−4.98 ± 0.04	9	−4.76 ± 0.07	5	−6.56 ± 0.03	5
β1-D356K	0.69 ± 0.04	945 ± 137	9	−5.38 ± 0.02*	12	−4.48 ± 0.03*	7	−5.59 ± 0.03*	15	−4.64 ± 0.02*	15	−4.67 ± 0.06	8	−6.31 ± 0.04	8
β1-V219F-D356K	0.70 ± 0.06	631 ± 74	6	−5.52 ± 0.04*	9	−4.58 ± 0.02	5	−5.70 ± 0.03	9	−4.81 ± 0.07	7	−4.61 ± 0.02	5	−6.36 ± 0.09	5
β1-P355R-D356K	0.61 ± 0.03	191 ± 29	5	−5.53 ± 0.03*	9	−4.61 ± 0.07	5	−5.80 ± 0.03	9	−4.91 ± 0.07	7	−4.67 ± 0.10	5	−6.42 ± 0.06	5

ND, not determined.

*$P < 0.001$. One-way analysis of variance with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β2-WT or the β1-WT. Thus, the log K_D for salmeterol at the β2-EL2 is different from that obtained from the β2-WT with $P < 0.001$. Likewise, the log K_D for salmeterol at the β1-EL3 is different from that obtained from the β1-WT with $P < 0.001$; #$P < 0.001$. One-way analysis of variance with post hoc Newman-Keuls comparing each value with all other values in this set. Thus, the log K_D for salmeterol at β2-EL3 is different from that obtained at β2-WT and all other β2-mutant receptors with $P < 0.001$ in all cases.

dilution cloning and expanded to generate stable cell lines. These cell lines were used to identify the EL or TM regions important for salmeterol interaction.

Generation of Transient Populations. For transiently transfected cells, the parent CHO-K1 cells were transfected, as above, on day 1; the transfection reagents were removed and replaced with media on day 2; the cells were plated into 48- or 96-well plates on day 3; and the experiments were performed on day 4.

[^{3}H]CGP 12177 Whole-Cell Binding

Cells were grown to confluence in sterile white-sided, tissue culture–treated 96-well view plates. [^{3}H]CGP 12177 whole-cell competition binding was performed, as previously described (Baker, 2005), using [^{3}H]CGP 12177 in the range of 0.43–3.03 nM (total volume 200 μl per well). Cells were incubated with competing ligand in the presence of a fixed concentration of [^{3}H]CGP 12177 for 2 hours before being washed with 2 $\times$ 200 ml cold phosphate-buffered saline. Microscint 20 (100 ml) was then added to each well, and the plates were left for several hours before being counted on a Topcount for 2 minutes per well. For saturation experiments, concentrations of [^{3}H]CGP 12177 in the range of 0.005–42.8 nM were used. Propranolol (10 μM) was used to define nonspecific binding in all experiments. Receptor expression level was measured, and protein was determined by the method of Lowry et al. (1951).

For attempts at measuring duration of receptor binding, cells were incubated with either competing ligand in the presence of a fixed concentration of [^{3}H]CGP 12177 for 2 hours as above (control plate), or competing ligand alone for 2 hours (duration plate). After 2 hours, the control plate was washed as above, whereas the duration plate was washed with 2 $\times$ 200 ml warm media and [^{3}H]CGP 12177 alone was added to the wells (except nonspecific binding wells when propranolol was also added) and incubated for 2 hours. After this second 2-hour incubation, the plates were washed with cold phosphate-buffered saline and Microscint was added, as above. Total and nonspecific binding were determined in each plate, and, as the duration plate had more washes than the control plate (and thus more potential for cell loss), the data were normalized to the total and nonspecific binding values for each plate.

[^{3}H]cAMP Accumulation

Cells were grown to confluence in sterile, clear plastic, tissue culture–treated 48-well plates. Cells were prelabeled with [^{3}H]adenine by incubation for at least 2 hours with 2 μCi/ml [^{3}H]adenine in media (0.5 ml per well). The cells were washed with 1 ml serum-free media (sfm) per well, and then 0.5 ml sfm containing 1 mM 3-isobutyl-1-methylxanthine was added to each well. Agonists (in 5 μl sfm) were added to each well, and the plates were incubated for 5 hours at 37°C to maximize the responses (without altering the EC_{50} values or percentage of maximum isoprenaline response observed; Baker, 2010). The assay was terminated by adding 50 μl concentrated HCl per well, the plates were frozen and thawed, and [^{3}H]cAMP was separated from other [^{3}H]nucleotides by sequential AG 50W-4X resin and alumina column chromatography (using [^{14}C]cAMP to determine column efficiency; Baker, 2010). Isoprenaline (10 μM) was used to define the maximal response in each plate of each experiment.

Data Analysis

Whole-Cell Binding. The affinity of [^{3}H]CGP 12177 for each mutant was determined from saturation binding, using 10 μM propranolol to determine nonspecific binding, and all data points were performed in quadruplicate. Specific binding (SB; eq. 1) of [^{3}H]CGP 12177

TABLE 5

The ratios of affinity (as fold change) for ligands in Table 4 compared with that obtained in the β2-WT or β1-WT receptors

Numbers in Roman text are a decrease in affinity compared with WT, and numbers in italics are an increase in affinity compared with their respective WT. Thus, salmeterol has an affinity of 1.4-fold more at β1-EL2 than β1-WT but 129-fold less at β2-EL3 than β2-WT. The β2/β1 selectivity for the ligands in these transiently transfected cells is also given.

	Salmeterol	Salbutamol	Formoterol	Fenoterol	Adrenaline	Clenbuterol
β2-WT	1.0	1.0	1.0	1.0	1.0	1.0
β2-EL2	4.0	1.5	2.6	2.3	2.8	1.5
β2-F194V	5.6	1.8	2.2	2.4	1.9	2.0
β2-EL3	128.8	1.7	2.0	1.7	4.7	2.2
β2-Q299H	2.3	1.0	1.2	1.0		
β2-D300R	2.5	1.0	*1.2*	*1.1*		
β2-N301E	1.7	1.2	1.2	1.3		
β2-I303V	2.1	1.3	1.4	1.4		
β2-R304P	3.7	1.3	1.6	1.9	1.1	1.5
β2-K305D	30.9	1.4	2.3	1.3	2.2	1.3
β2-F194V-K305D	61.7	1.7	3.9	1.8	3.0	1.5
β2-R304P-K305D	66.1	1.8	3.2	1.9	2.8	1.7
β2-F194A	20.4	2.1	2.1	2.5	2.5	2.9
β2-K305A	4.2	1.7	1.5	1.3	1.3	1.4
β2-K305E	11.2	3.0	2.9	1.7		
β2-K305G	14.4	1.1	1.5	1.5	1.5	1.2
β2-K305H	12.6	1.3	4.8	1.2		
β2-K305R	2.0	1.3	*1.3*	1.0	*1.7*	1.5
β2-K305S	12.6	1.5	1.8	1.0	1.8	*1.3*
β2-R304D	2.3	1.3	1.1	*1.4*	*1.0*	1.1
β2-R304D-K305G	12.9	1.3	2.7	1.4	1.8	*1.1*
β1-WT	1.0	1.0	1.0	1.0	1.0	1.0
β1-EL2	*1.4*	*1.1*	*1.3*	*1.2*	*1.1*	1.3
β1-V219F	1.5	*1.0*	1.1	*1.4*	*1.4*	1.2
β1-EL3	*4.2*	*1.4*	*1.9*	*2.3*	*3.2*	*1.5*
β1-P355R	*1.3*	*1.2*	*1.3*	*1.2*	*1.0*	1.0
β1-D356K	1.9	1.8	1.9	1.8	1.2	1.9
β1-V219F-D356K	1.4	1.4	1.4	1.2	1.3	1.7
β1-P355R-D356K	1.4	1.3	1.1	*1.0*	1.2	1.4
β2/β1 selectivity	1000	10	115	46	7.9	7.2

at different concentrations of [^{3}H]CGP 12177 was fitted using the nonlinear regression program Prism 2.01 to the equation:

$$SB = \frac{(A \times B_{max})}{(A + K_D)} \quad (1)$$

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

The affinity of the other ligands was determined from competition binding. All data points were performed in triplicate, and each 96-well plate contained six determinations of total and nonspecific binding. A 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 using equation 2.

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

where A is the concentration of the competing ligand, IC_{50} is the concentration at which half of the specific binding of [^{3}H]CGP 12177 has been inhibited, and NS is the nonspecific binding.

From the IC_{50} value and the known concentration of [^{3}H]CGP 12177, a K_D value (concentration at which half the receptors are bound by the competing ligand) was calculated using equation 3:

$$K_D = \frac{IC_{50}}{1 + ([[^3H]CGP\ 12177]/K_D[^3H]CGP\ 12177)} \quad (3)$$

For the assessment of duration of binding, as the duration plate had more washes than the control plate and thus there was greater loss of cells from this plate, the sigmoidal concentration response curve for

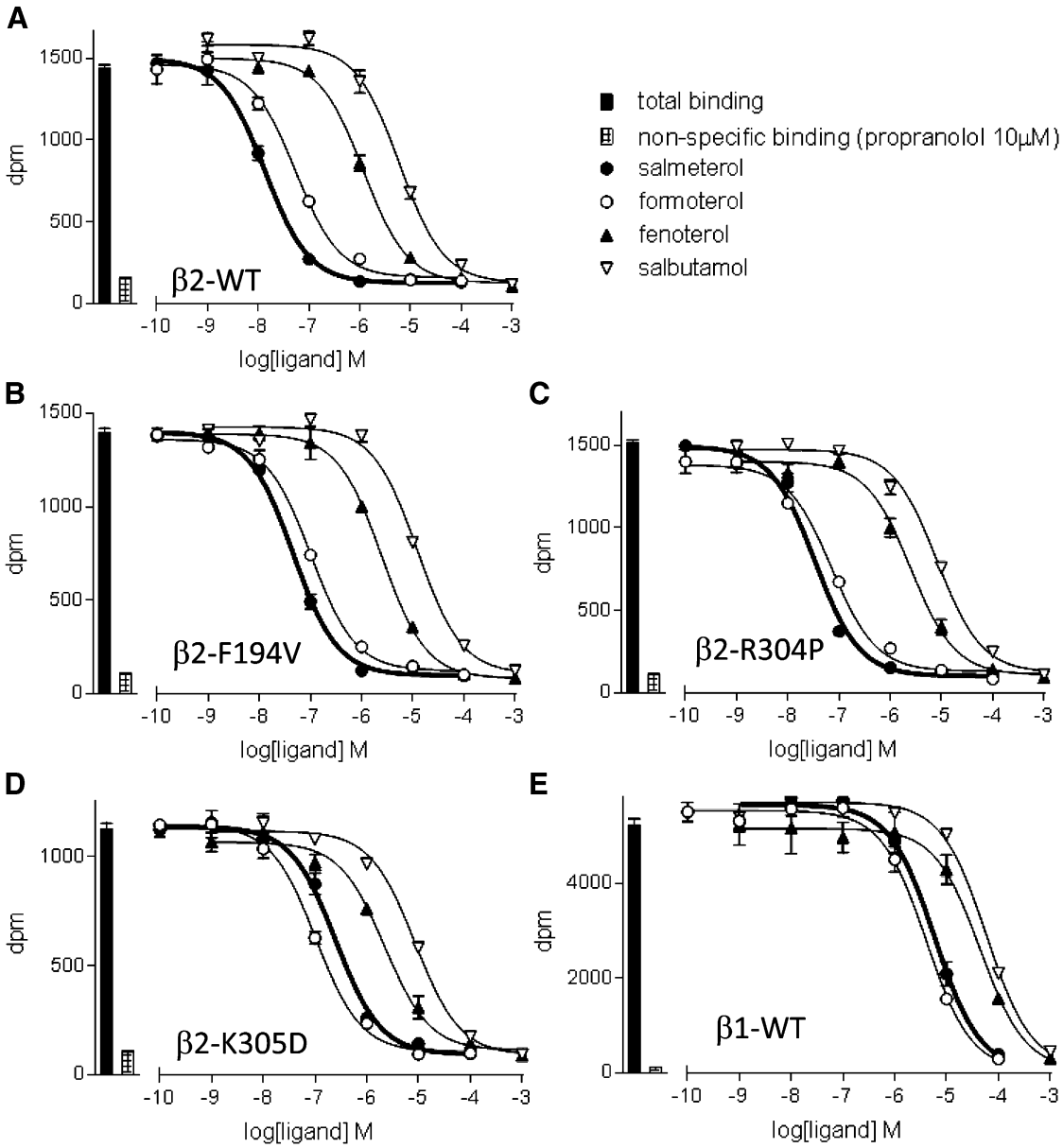

Fig. 3. Inhibition of [^{3}H]CGP 12177 whole-cell binding in response to salmeterol, formoterol, fenoterol, and salbutamol in transiently transfected cells expressing (A) β2-WT, the EL changes (B) β2-F194V, (C) β2-R304P, (D) β2K305D, and (E) β1-WT receptors. The salmeterol curve is in bold. Nonspecific binding was determined by 10 μM propranolol. Data points are mean ± S.E.M. of triplicate determinations, and the β2-WT and β1-WT experiments in this figure were run alongside these EL mutation experiments. The concentrations of [^{3}H]CGP 12177 present in these experiments were 0.7 nM throughout. These single experiments are representative of (A) 38, (B) 12, (C) 14, (D) 12, and (E) 28 separate experiments.

TABLE 6

Affinity (log K_D values) of β-adrenoceptor ligands for the wild-type β-AR and receptors containing single and double point mutations in TM6 and TM7 in transiently transfected populations of cells

The K_D values of [^{3}H]CGP 12177 and the receptor expression levels in these transient populations are also given. The values are mean ± S.E.M. for n separate experiments, and each separate n number has been obtained in a separate transiently transfected population of cells.

	K_D [^{3}H]CGP 121777 from Saturation	Protein		Log K_D Salmeterol	n	Log K_D Salbutamol	n	Log K_D Formoterol	n	Log K_D Fenoterol	n	Log K_D Adrenaline	n	Log K_D Clenbuterol	n
		fmol/mg													
β2-WT	0.18 ± 0.01	165 ± 13	30	−8.67 ± 0.03	67	−5.76 ± 0.03	38	−7.92 ± 0.03	52	−6.56 ± 0.02	46	−5.64 ± 0.05	17	−7.44 ± 0.04	20
Point mutations in β2 resulting in a change to β1 amino acids															
β2-TM6	0.47 ± 0.03	245 ± 48	9	−7.06 ± 0.04*	13	−5.70 ± 0.04	9	−7.96 ± 0.07	12	−6.42 ± 0.07	12	−5.36 ± 0.06	6	−7.47 ± 0.04	7
β2-T281V	0.16 ± 0.01	168 ± 38	8	−8.75 ± 0.11	8	−5.85 ± 0.04	8	−8.15 ± 0.09	6	−6.66 ± 0.05	5	−5.62 ± 0.06	5	−7.50 ± 0.03	7
β2-I291L	0.17 ± 0.02	121 ± 31	9	−8.63 ± 0.08	9	−5.91 ± 0.04	8	−8.17 ± 0.07	6	−6.41 ± 0.07	6	−5.52 ± 0.09	6	−7.48 ± 0.07	7
β2-V292A	0.21 ± 0.03	155 ± 38	8	−8.20 ± 0.07*	8	−5.61 ± 0.04	8	−7.90 ± 0.03	5	−6.17 ± 0.07*	5	−5.10 ± 0.03*	5	−7.33 ± 0.03	7
β2-I294V	0.19 ± 0.02	155 ± 44	9	−8.87 ± 0.07	8	−5.80 ± 0.04	9	−8.05 ± 0.07	7	−6.75 ± 0.03	6	−5.82 ± 0.01	5	−7.59 ± 0.03	8
β2-H296K	0.34 ± 0.02	148 ± 27	9	−7.41 ± 0.06*	13	−5.78 ± 0.04	14	−7.97 ± 0.06	12	−6.19 ± 0.03*	11	−5.53 ± 0.03	6	−7.39 ± 0.08	8
β2-V297A	0.13 ± 0.01	168 ± 26	9	−8.60 ± 0.09	8	−5.97 ± 0.03	9	−8.22 ± 0.06	7	−6.76 ± 0.05	6	−5.61 ± 0.11	5	−7.78 ± 0.06*	8
β2-I298F	0.20 ± 0.01	158 ± 29	9	−8.30 ± 0.08	8	−5.80 ± 0.05	9	−8.14 ± 0.07	7	−6.54 ± 0.05	6	−5.69 ± 0.05	5	−7.35 ± 0.06	8
β2-TM7	0.29 ± 0.06	96 ± 24	6	−7.21 ± 0.08*	15	−5.46 ± 0.05*	8	−7.08 ± 0.07*	17	−5.62 ± 0.08*	14	−5.52 ± 0.06	5	−7.17 ± 0.06	6
β2-E306R	0.28 ± 0.05	80 ± 15	6	−8.46 ± 0.13	7	−5.72 ± 0.05	7	−7.96 ± 0.16	7	−6.54 ± 0.16	5	−5.59 ± 0.08	6	−7.55 ± 0.08	5
β2-V307L	0.26 ± 0.04	107 ± 9	8	−8.49 ± 0.02	7	−5.58 ± 0.05	8	−7.84 ± 0.05	8	−6.40 ± 0.07	6	−5.64 ± 0.02	5	−7.34 ± 0.07	6
β2-Y308F	0.16 ± 0.03	160 ± 22	8	−7.61 ± 0.03*	12	−5.50 ± 0.03*	12	−7.16 ± 0.04*	12	−5.99 ± 0.05*	10	−5.37 ± 0.05	6	−7.14 ± 0.05	6
β2-I309V	0.23 ± 0.03	132 ± 21	7	−8.41 ± 0.06	8	−5.61 ± 0.06	8	−7.89 ± 0.05	8	−6.31 ± 0.03	6	−5.78 ± 0.06	6	−7.35 ± 0.03	5
β2-L310F	0.24 ± 0.03	165 ± 20	8	−8.55 ± 0.10	8	−5.65 ± 0.05	8	−7.73 ± 0.07	8	−6.47 ± 0.05	6	−5.53 ± 0.06	6	−7.38 ± 0.09	6
β2-I311F	0.24 ± 0.03	108 ± 14	8	−8.49 ± 0.07	8	−5.85 ± 0.05	8	−7.80 ± 0.06	8	−6.49 ± 0.09	6	−5.47 ± 0.03	6	−7.35 ± 0.09	6
β2-I314L	0.25 ± 0.03	137 ± 22	8	−8.49 ± 0.06	8	−5.63 ± 0.05	8	−7.92 ± 0.04	8	−6.37 ± 0.08	6	−5.52 ± 0.07	6	−7.24 ± 0.06	6
β2-V317A	0.21 ± 0.02	121 ± 4	7	−8.54 ± 0.08	8	−5.64 ± 0.04	8	−7.83 ± 0.06	8	−6.54 ± 0.04	6	−5.74 ± 0.07	6	−7.36 ± 0.07	6
β2-G320A	0.24 ± 0.04	159 ± 15	7	−8.57 ± 0.06	8	−5.61 ± 0.06	8	−7.74 ± 0.04	8	−6.45 ± 0.06	6	−5.40 ± 0.05	5	−7.43 ± 0.06	6
β2-L324I	0.23 ± 0.03	134 ± 12	8	−8.50 ± 0.05	7	−5.63 ± 0.05	7	−7.65 ± 0.03	7	−6.49 ± 0.06	5	−5.38 ± 0.06	6	−7.37 ± 0.07	6
β2-H296K-Y308F	0.23 ± 0.04	132 ± 43	6	−6.95 ± 0.06*	10	−5.68 ± 0.03	10	−7.54 ± 0.06*	10	−5.91 ± 0.06*	8	−5.56 ± 0.05	5	−7.03 ± 0.06*	5
β2-K305D-Y308F	0.14 ± 0.02	71 ± 8	8	−6.61 ± 0.08*	5	−5.41 ± 0.12*	5	−7.20 ± 0.15*	5	−5.84 ± 0.06*	5	−4.99 ± 0.11*	6	−7.27 ± 0.10	5
β2-H296K-K305D	0.21 ± 0.04	95 ± 31	5	−6.23 ± 0.10*	5	−5.83 ± 0.04	5	−7.76 ± 0.07	5	−6.32 ± 0.11	5	−5.17 ± 0.12*	5	−7.38 ± 0.08	5
Point mutations in β2 resulting in a change to non-β1 amino acids															
β2-H296A	0.16 ± 0.02	129 ± 25	10	−8.13 ± 0.06*	10	−5.84 ± 0.06	10	−7.71 ± 0.07	8	−6.43 ± 0.07	8	−5.67 ± 0.07	5	−7.45 ± 0.05	9
β1-WT	0.28 ± 0.02	731 ± 96	27	−5.67 ± 0.01	44	−4.74 ± 0.02	28	−5.86 ± 0.02	42	−4.90 ± 0.02	44	−4.74 ± 0.04	11	−6.58 ± 0.06	11
Point mutations in β1 resulting in a change to β2 amino acids															
β1-TM6	0.22 ± 0.03	604 ± 172	4	−5.83 ± 0.04*	11	−4.90 ± 0.06	4	−6.30 ± 0.04*	13	−5.16 ± 0.04*	11	−4.75 ± 0.04	5	−6.71 ± 0.03	5
β1-K347H	0.20 ± 0.03	611 ± 127	10	−6.00 ± 0.04*	9	−5.11 ± 0.03*	9	−6.16 ± 0.03*	5	−5.05 ± 0.04	7	−4.63 ± 0.04	5	−7.01 ± 0.06*	5
β1-TM7	0.44 ± 0.03	793 ± 82	6	−6.25 ± 0.02*	12	−5.26 ± 0.02*	6	−6.68 ± 0.03*	13	−5.59 ± 0.02*	13	−4.70 ± 0.05	6	−7.23 ± 0.03*	6
β1-F359Y	0.51 ± 0.04	1137 ± 96	6	−5.82 ± 0.02*	5	−4.95 ± 0.05*	5	−6.09 ± 0.04*	5	−4.84 ± 0.08	3	−4.46 ± 0.05	5	−6.66 ± 0.04	5
β1-K347H-F359Y	0.37 ± 0.05	497 ± 72	6	−6.01 ± 0.06*	5	−5.06 ± 0.04*	5	−6.34 ± 0.02*	5	−5.03 ± 0.13	3	−4.55 ± 0.10	5	−6.85 ± 0.04	5

*$P < 0.001$. One-way analysis of variance with post hoc Newman-Keuls comparing values from the mutant receptors with those obtained from the β2-WT or the β1-WT. Thus, the log K_D for salmeterol at the β2-TM6 is different from that obtained from the β2-WT with $P < 0.001$. Likewise, the log K_D for salmeterol at the β1-TM6 is different from that obtained from the β1-WT with $P < 0.001$.

both the control and duration plate was normalized to the total and nonspecific binding for each plate. Shorter-acting ligands that were removed and/or continue to dissociate from the receptor during the 2-hour [^{3}H]CGP 12177 incubation would result in more [^{3}H]CGP 12177 binding than the control, and thus a rightward shift of the concentration response curve. Longer-acting ligands that did not dissociate from the receptor during the wash or during the 2-hour [^{3}H]CGP 12177 incubation would result in similar [^{3}H]CGP 12177 binding as control, and thus the curve would be less, if at all, right-shifted. To give a relative measure of duration of action, the degree of rightward shift of the curve was noted.

[^{3}H]cAMP Accumulation Agonist Responses. Agonist responses were best described by a one-site sigmoidal concentration response curve using the following equation:

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

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.

A 10 μM (maximal) isoprenaline concentration was included in each plate for each separate experiment to allow agonist responses to be expressed as a percentage of the maximum response to the full agonist isoprenaline for each experiment. Data points in the figures are presented as mean ± S.E.M. of triplicate determinations from a single experiment.

Results

Identification of the Regions in the Human β2-Adrenoceptor Important for Salmeterol Affinity: [^{3}H]CGP 12177 Whole-Cell Binding in Stable Cell Lines. Salmeterol bound to the β2-WT receptor with high affinity (log K_D −8.95) and to the β1-WT with low affinity (log K_D −5.80), demonstrating its high β2 selectivity (1400-fold; Fig. 2A; Tables 2 and 3). To determine the key regions of the β2-AR responsible for this very selective high affinity, the affinity of salmeterol was examined in mutations in which the N terminus was swapped over, or each EL or each TM region in turn was mutated such that it contained the amino acid residues of the human β1-AR (Table 1). The β2-EL3 mutant receptor (i.e., the β2-AR but with EL3 of the β1-AR) had the largest effect with a reduction in salmeterol affinity of 162-fold (Fig. 2C; Tables 2 and 3). Table 2 gives the actual affinity (K_D) values obtained, but the ratios of the affinities observed for the mutant receptors compared with the WT receptors (Table 3) provide an easier way of picking out the major differences. Several other regions were also identified that decreased salmeterol affinity: these were TM6 (38-fold), TM7 (24-fold), and EL2 (4.7-fold; Fig. 2; Tables 2 and 3). The β2-TM2 mutant also has a very small effect with a reduction in salmeterol affinity of 2.4-fold.

The affinities of three other β2-selective agonists were also examined at these WT and chimeric receptors to determine whether the receptor regions identified were specific to salmeterol. Although the β2-EL2 and β2-EL3 receptors also reduced salbutamol, formoterol, and fenoterol affinity, these changes were less marked than those with salmeterol, with the largest changes being a 3.5-fold reduction in formoterol affinity at β2-EL2 and 2.5-fold at β2-EL3 (Tables 2 and 3). For the TM regions, the reduction in agonist affinity in β2-TM6 appeared to be specific to salmeterol, whereas formoterol and fenoterol also

TABLE 7

The ratios of affinity (as fold change) for ligands in Table 6 compared with that obtained in the β2-WT or β1-WT receptors

Numbers in Roman text are a decrease in affinity compared with WT, and numbers in italics are an increase in affinity compared with their respective WT. Thus, salmeterol has an affinity of 1.2-fold more at β2-T281V than β2-WT but 41-fold less at β2-TM6 than β2-WT. The β2/β1 selectivity for the ligands in these transiently transfected cells is also given.

	Salmeterol	Salbutamol	Formoterol	Fenoterol	Adrenaline	Clenbuterol
β2-WT	1.0	1.0	1.0	1.0	1.0	1.0
β2-TM6	40.7	1.1	*1.1*	1.4	1.9	*1.1*
β2-T281V	*1.2*	*1.2*	*1.7*	*1.3*	1.0	*1.1*
β2-I291L	1.1	*1.4*	*1.8*	1.4	1.3	*1.1*
β2-V292A	3.0	1.4	1.0	2.5	3.5	1.3
β2-I294V	*1.6*	*1.1*	*1.3*	*1.5*	*1.5*	*1.4*
β2-H296K	18.2	*1.0*	*1.1*	2.3	1.3	1.1
β2-V297A	1.2	*1.6*	*2.0*	*1.6*	1.1	*2.2*
β2-I298F	2.3	*1.1*	*1.7*	1.0	*1.1*	1.2
β2-TM7	28.8	2.0	6.9	8.7	1.3	1.9
β2-E306R	1.6	1.1	*1.1*	1.0	1.1	*1.3*
β2-V307L	1.5	1.5	1.2	1.4	1.0	1.3
β2-Y308F	11.5	1.8	5.8	3.7	1.9	2.0
β2-I309V	1.8	1.4	1.1	1.8	*1.4*	1.2
β2-L310F	1.3	1.3	1.5	1.2	1.3	1.1
β2-I311F	1.5	*1.2*	1.3	1.2	1.5	1.2
β2-I314L	1.5	1.3	1.0	1.5	1.3	1.6
β2-V317A	1.3	1.3	1.2	1.0	*1.3*	1.2
β2-G320A	1.3	1.4	1.5	1.3	1.7	1.0
β2-L324I	1.5	1.3	1.9	1.2	1.8	1.2
β2-H296K-Y308F	52.5	1.2	2.4	4.5	1.2	2.6
β2-K305D-Y308F	114.8	2.2	5.2	5.2	4.5	1.5
β2-H296K-K305D	275.4	*1.2*	1.4	1.7	3.0	1.1
β2-H296A	3.5	*1.2*	1.6	1.3	*1.1*	*1.0*
β1-WT	1.0	1.0	1.0	1.0	1.0	1.0
β1-TM6	*1.4*	*1.4*	*2.8*	*1.8*	*1.0*	*1.3*
β1-K347H	*2.1*	*2.3*	*2.0*	*1.4*	1.3	*2.7*
β1-TM7	*3.8*	*3.3*	*6.6*	*4.9*	1.1	*4.5*
β1-F359Y	*1.4*	*1.6*	*1.7*	1.1	1.9	*1.2*
β1-K347H-F359Y	*2.2*	*2.1*	*3.0*	*1.3*	1.5	*1.9*
β2/β1 selectivity	1000	10	115	46	8	7

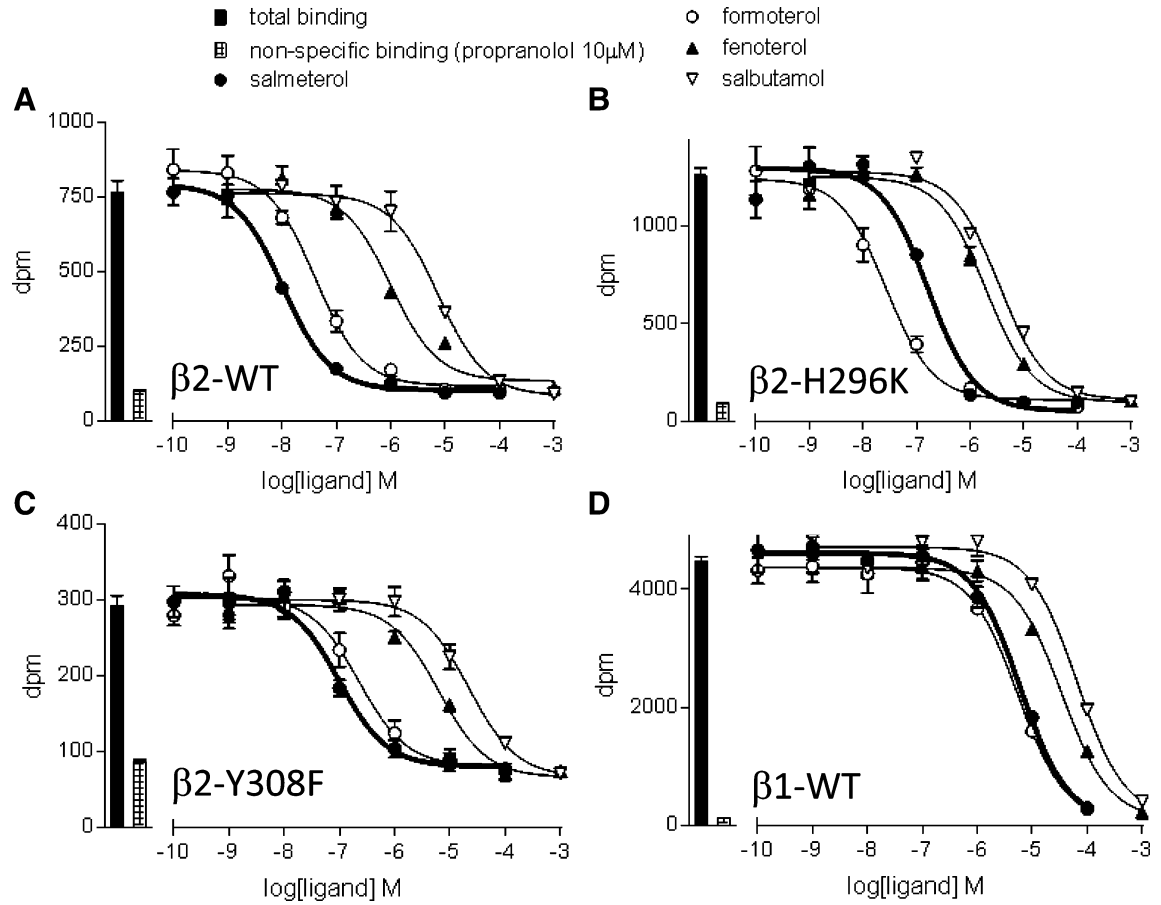

Fig. 4. Inhibition of [^{3}H]CGP 12177 whole-cell binding in response to salmeterol, formoterol, fenoterol, and salbutamol in transiently transfected cells expressing (A) β2-WT, the TM changes (B) β2-H296K, (C) β2-Y308F, and (D) β1-WT receptors. The salmeterol curve is in bold. Nonspecific binding was determined by 10 μM propranolol. Data points are mean ± S.E.M. of triplicate determinations, and the β2-WT and β1-WT experiments in this figure were run alongside these TM mutation experiments. The concentrations of [^{3}H]CGP 12177 present in these experiments were (A) 0.6 nM, (B) 0.7 nM, (C) 0.5 nM, and (D) 0.7 nM. These single experiments are representative of (A) 38, (B) 11, (C) 10, and (D) 28 separate experiments.

had reduced affinity in β2-TM7 (of 5.2- and 4.4-fold, respectively), although this once again was not as great as that seen for salmeterol.

Interestingly, when the reverse chimeric receptors were examined (i.e., β1-parent receptor with EL and TM regions in turn mutated to that of the β2-adrenoceptor), the changes observed were not as great. The single biggest changes observed were for the EL3 mutation, in which β1-EL3 had a 2.8 reciprocal increase in salmeterol affinity (larger than that for the other agonists) and β1-TM7 had a 4.4-fold increase in salmeterol affinity (accompanied by an increase of formoterol and fenoterol affinity of 8.1- and 5.9-fold, respectively; Tables 2 and 3).

It therefore appeared that β2-selective nature of salmeterol binding was due largely to amino acids in EL3 and TM6, with smaller and less salmeterol-specific contributions from the amino acids in EL2 and TM7. Given the very small nature of the TM2 effect (less than 3-fold), this region was not investigated further.

Identification of the Amino Acids Involved in the Extracellular Regions: [^{3}H]CGP 12177 Whole-Cell Binding in Transiently Transfected Cells. To determine exactly which amino acids were important in each region, the effect of individual single-point mutations was examined in each of the regions identified above—namely, EL2, EL3, TM6, and TM7. Thus, single amino acids of interest were mutated from that of the β2-WT to that of the β1-WT. Also, to check for salmeterol rather general β2-selective mutations, the ligands tested were increased to include two further β2-selective agonists, adrenaline and clenbuterol.

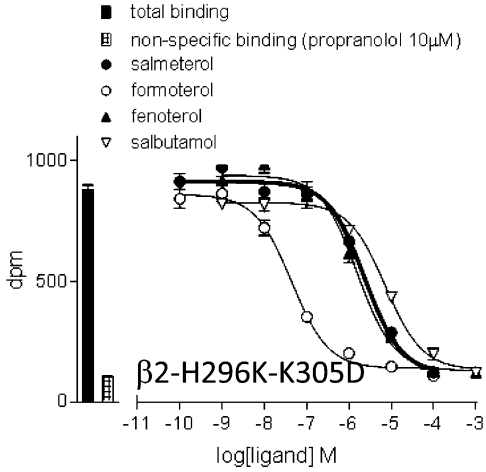

Fig. 5. Inhibition of [^{3}H]CGP 12177 whole-cell binding in response to salmeterol, formoterol, fenoterol, and salbutamol in transiently transfected cells expressing the double-mutant receptor β2-H296K-K305D. The salmeterol curve is in bold. Nonspecific binding was determined by 10 μM propranolol. Data points are mean ± S.E.M. of triplicate determinations, the concentration of [^{3}H]CGP 12177 was 0.7 nM, and this experiment is representative of five separate experiments.

For the β2-EL2, the first single amino acid change examined was F194V. The β2-F194V mutant receptor had a reduction in salmeterol affinity that was actually slightly greater than that of the whole EL swap (Fig. 3B; Tables 4 and 5). The other amino acid changes were assessed as two amino acid changes at a time, and no changes over that seen with F194V alone were observed; thus, the single-point mutations for the rest of EL2 were not examined individually (data not shown). Interestingly, β2-F194V caused a reduction in affinity for all six agonists examined. As seen in the stable cell lines, the reciprocal mutations (β1-EL2 and β1-F219V) did not restore salmeterol affinity. Thus, for the EL2, the single amino acid change of F194V in the β2-adrenoceptor caused a small reduction in affinity of all the agonists examined; however, the reduction in salmeterol affinity was the greatest.

Given the much larger effect of the EL3 changes, each amino acid mutation was examined individually. In this study, the mutation K305D caused the single biggest reduction in affinity of salmeterol of 31-fold (Fig. 3D; Table 4 and 5). Furthermore, this effect was far greater for salmeterol than any of the other ligands examined. Again, this is more easily picked out by looking at the affinity ratios shown in Table 5. The next largest change in salmeterol affinity was from the mutation of the adjacent amino acid R304P, which resulted in a 3.7-fold decrease in salmeterol affinity (Fig. 3C; Tables 4 and 5). A double-mutant receptor was therefore made, β2-R304P-K305D, to examine whether these effects were additive. The resultant affinity for salmeterol was indeed lower at this double mutation than either amino acid change alone, decreasing salmeterol affinity to within 2-fold of that achieved by the

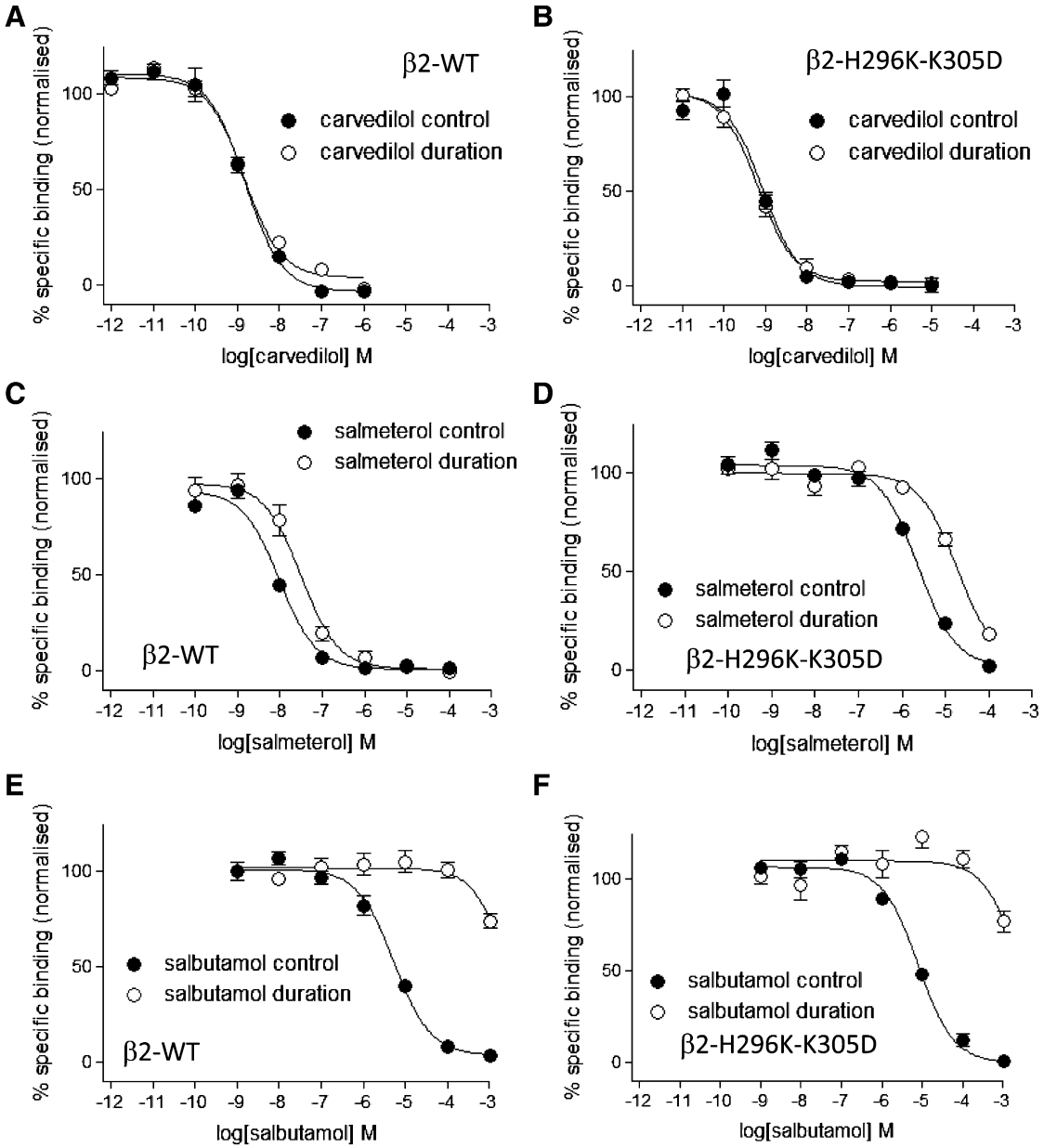

Fig. 6. Inhibition of specific [^{3}H]CGP 12177 whole-cell binding in response to (A) and (B) carvedilol, (C) and (D) salmeterol, (E) and (F) salbutamol in transiently transfected cells expressing β2-WT (A, C, and E) or the double-mutant receptor β2-H296K-K305D (B, D, and F). Nonspecific binding was determined by 10 μM propranolol. Data points are mean ± S.E.M. of triplicate determinations; the concentrations of [^{3}H]CGP 12177 were (A) 0.7 nM, (B) 0.7 nM, (C) 0.5 nM, (D) 0.7 nM, (E) 0.5 nM, and (F) 0.7 nM; and this experiment is representative of five separate experiments in each case.

whole β2-EL3 swap (Tables 4 and 5). Once again, the reciprocal β1 mutations either individually or with the double β1-P355R-D356K did not restore salmeterol affinity (Tables 4 and 5).

Finally, the effect of combining the two main extracellular loop (EL) region mutations (i.e., F194V from EL2 and K305D from EL3) was examined by making the double mutation β2-F194V-K305D. This again had a decrease in salmeterol affinity that was greater than either change alone, and once again the reciprocal β1 mutation (β1-V219F-D356K) did not restore salmeterol affinity (Tables 4 and 5).

Identification of the Amino Acids Involved in the Transmembrane Regions: [^{3}H]CGP 12177 Whole-Cell Binding in Transiently Transfected Cells. From the stable cell line experiments above, TM6 and TM7 were identified as having significant effects on the affinity of salmeterol. Single-point mutations were therefore made in each of these domains to examine the effect of each amino acid in turn. In TM6, whereas V292A and I298F both had small effects on the affinity of salmeterol, the mutation H296K caused the largest single effect, reducing the affinity of salmeterol to within 2-fold of that of the whole TM6 change (Fig. 4B; Tables 6 and 7). For TM7, the mutation Y308F caused the single biggest decrease in the affinity of salmeterol (Fig. 4C; Tables 6 and 7). The whole TM7 mutation (β2-TM7) actually caused a significant decrease in affinity of several agonists, and the Y308F was the single biggest contributor to this for each ligand.

The effect of combining the two major TM mutations was also examined (i.e., H296K from TM6 and Y308F from TM7 in the double mutant β2-H296K-Y308F) and resulted in a further decrease in salmeterol affinity (Tables 6 and 7). The addition of Y308F to the K305D mutation (β2-K305D-Y308F) also reduced the affinity of salmeterol more than K305D alone (Tables 6 and 7). Once again, the reciprocal β1 mutations (β1-K347H and β1-F359Y) either alone or in combination did not restore salmeterol affinity (Tables 6 and 7).

Effect of Combining the Major EL Mutation with the Major TM Mutation: [^{3}H]CGP 12177 Whole-Cell Binding in Transiently Transfected Cells. Finally, the effect of the double mutation β2-H296K-K305D, which combines the major EL, salmeterol-specific mutation with the major TM, and salmeterol-specific mutation, was examined, and salmeterol affinity was reduced by 275-fold, to within 4-fold of that at the β1-WT (Fig. 5; Tables 6 and 7).

Effects of Different Amino Acids: [^{3}H]CGP 12177 Whole-Cell Binding in Transient Transfected Cells. To date, all of the single-point mutations had involved the change in amino acid from that found in the β2 to that found in the β1-adrenoceptor. The effect of other amino acid changes in these same residues was also assessed. The effect of changing the amino acids to alanine in the major EL regions (F194A, K305A) and the salmeterol-specific TM region (H296A) was examined (Tables 4–7). Interestingly, the alanine in F194A reduced the affinity of salmeterol significantly more than the other compounds and thus produced a more salmeterol-specific change than that to the β1 amino acid V, whereas alanines at H296A and K305A had significantly less effect on the affinity of salmeterol than the K and D, respectively, from the β1-adrenoceptor. Also, the R304D mutation—i.e., moving the D one amino acid along to position 304—resulted in a salmeterol-specific reduction in affinity, but not as great as that seen for K305D. Furthermore, R304D was not able to produce any greater reduction in salmeterol affinity when K305 was also mutated to a glycine (β2-R304D-K305G).

Given that mutations at K305 had the single biggest and most selective effect on the affinity of salmeterol, the effect of changes to several different amino acids at this position was examined. This had differing effects, but the K305D mutation had the largest effect on the affinity of salmeterol (Tables 4 and 5).

Duration of Action of Salmeterol. To attempt to measure the impact of these mutations on the duration of action of salmeterol, the binding assay was adapted to include a washout period. Thus, as detailed in *Materials and Methods*, cells were either incubated with the competing ligand and [^{3}H]CGP 12177 for 2 hours (control), or the competing ligand alone for 2 hours before this was removed by washing prior to the addition of [^{3}H]CGP 12177 alone for an additional 2 hours (duration; Fig. 6). Under these conditions, short-acting ligands should be washed away and the radioligand bind unopposed (shifting the curve to the right), whereas long-acting ligands should remain bound to the receptor after the washout period such that, following the addition of the radioligand, inhibition of radioligand binding would still occur. Salbutamol and fenoterol

TABLE 8

[^{3}H]CGP 12177 whole-cell binding in transiently transfected cell populations

The measure of duration of binding is the log shift of the curve from the control curve to the duration curve run in parallel in each experiment. The log K_D values for carvedilol are also given. The values are mean ± S.E.M. for n separate experiments, and each separate n number has been obtained in a separate transiently transfected population of cells. Thus, salbutamol is short-acting in that the IC_{50} of the binding curve is shifted more than 2 log units (100-fold) to the right following washout at all receptors. Carvedilol, however, is long-acting as the duration curve remains in a similar place to the control curve (and the shift in IC_{50} is about 0).

	Salmeterol		Salbutamol		Formoterol		Fenoterol		Carvedilol		
	Shift (log)	n	Shift (log)	n	Shift (log)	n	Shift (log)	n	Log K_D	Shift (log)	n
β1-WT	0.86 ± 0.08	4	>2	4	>2	4	>2	4	−9.01 ± 0.12	0.26 ± 0.08	4
β2-WT	0.52 ± 0.14	5	>2	5	1.96 ± 0.12	5	2.65 ± 0.14	5	−9.62 ± 0.09	−0.03 ± 0.10	5
β2-EL3	0.84 ± 0.11	5	>2	5	1.75 ± 0.14	5	2.56 ± 0.05	5	−9.33 ± 0.01	−0.10 ± 0.06	5
β2-F194V	0.80 ± 0.08	5	>2	5	2.07 ± 0.08	5	2.68 ± 0.05	4	−9.61 ± 0.08	−0.12 ± 0.09	5
β2-H296K	0.57 ± 0.13	5	>2	5	1.70 ± 0.20	5	2.49 ± 0.08	5	−9.20 ± 0.11	−0.42 ± 0.02	5
β2-R304P	0.51 ± 0.10	4	>2	4	1.78 ± 0.18	4	2.53 ± 0.17	4	−9.48 ± 0.16	0.11 ± 0.08	4
β2-K305D	0.91 ± 0.07	5	>2	5	2.06 ± 0.08	5	2.48 ± 0.14	5	−9.42 ± 0.09	0.27 ± 0.10	5
β2-Y308F	0.78 ± 0.04	4	>2	4	2.28 ± 0.13	4	>2	4	−9.61 ± 0.07	0.13 ± 0.13	4
β2-F194V-K305D	1.07 ± 0.05	5	>2	5	2.30 ± 0.15	5	2.65 ± 0.07	5	−9.62 ± 0.12	0.17 ± 0.07	5
β2-R304P-K305D	0.91 ± 0.04	4	>2	4	2.10 ± 0.14	4	2.47 ± 0.10	4	−9.34 ± 0.07	0.08 ± 0.16	4
β2-H296K-Y308F	0.88 ± 0.13	5	>2	5	1.60 ± 0.19	5	>2	5	−9.47 ± 0.06	−0.31 ± 0.18	5
β2-K305D-Y308F	0.67 ± 0.09	5	>2	5	1.96 ± 0.21	4	>2	5	−9.62 ± 0.07	0.16 ± 0.11	4
β2-H296K-K305D	0.67 ± 0.09	5	>2	5	1.43 ± 0.16	5	2.07 ± 0.19	5	−9.39 ± 0.12	−0.43 ± 0.14	5

TABLE 9

Log EC_{50} and percentage of the maximum response to isoprenaline values obtained from [^{3}H]cAMP accumulation in transiently transfected populations of cells

The fold over basal for the maximal isoprenaline (Isop) response (10 μM) is also given for each receptor. The values are mean ± S.E.M. for n separate experiments, and each separate n number has been obtained in a separate transiently transfected population of cells.

	Isoprenaline Fold Over Basal	n	Salmeterol Log EC_{50}	% Isop	n	Salbutamol Log EC_{50}	% Isop	n	Formoterol Log EC_{50}	% Isop	n	Fenoterol Log EC_{50}	% Isop	n
β2-WT	4.40 ± 0.36	18	−9.69 ± 0.08	74.7 ± 1.8	18	−7.16 ± 0.06	82.2 ± 2.3	18	−9.40 ± 0.06	101.3 ± 2.4	17	−7.96 ± 0.08	94.2 ± 2.5	18
β1-WT	6.33 ± 0.64	14	−6.92 ± 0.03	69.2 ± 3.0	14	−6.32 ± 0.04	68.1 ± 2.4	14	−8.20 ± 0.06	96.1 ± 2.0	13	−7.43 ± 0.06	88.4 ± 2.1	13
Point mutations in β2 resulting in a change to β1 amino acids														
β2-EL2	4.14 ± 0.60	8	−9.13 ± 0.07	69.0 ± 1.9	8	−6.79 ± 0.05	64.9 ± 4.4	8	−9.28 ± 0.08	94.6 ± 7.9	8	−7.76 ± 0.09	78.7 ± 4.9	8
β2-F194V	5.12 ± 0.85	8	−8.82 ± 0.04	63.0 ± 2.8	8	−6.46 ± 0.10	61.0 ± 2.8	8	−8.97 ± 0.12	88.2 ± 5.2	8	−7.57 ± 0.10	82.7 ± 2.4	7
β2-EL3	4.37 ± 0.40	14	−7.88 ± 0.04	75.2 ± 2.0	14	−7.41 ± 0.06	83.9 ± 2.0	14	−9.72 ± 0.07	98.5 ± 1.8	13	−8.43 ± 0.06	90.1 ± 1.8	13
β2-R304P	7.15 ± 0.97	8	−9.60 ± 0.05	88.1 ± 2.6	8	−7.28 ± 0.07	83.2 ± 1.6	8	−9.32 ± 0.06	98.7 ± 2.6	8	−8.11 ± 0.11	91.3 ± 1.4	8
β2-K305D	5.25 ± 0.97	8	−8.00 ± 0.04	78.9 ± 2.6	8	−6.90 ± 0.09	77.4 ± 2.9	8	−9.09 ± 0.12	99.6 ± 2.2	8	−7.97 ± 0.11	88.9 ± 3.1	8
β2-TM6	5.26 ± 0.65	14	−8.46 ± 0.03	74.5 ± 2.3	14	−7.31 ± 0.07	81.6 ± 2.2	14	−9.44 ± 0.06	101.9 ± 2.8	13	−8.19 ± 0.11	92.1 ± 2.5	14
β2-T281V	2.60 ± 0.13	6	−9.92 ± 0.14	60.0 ± 3.5	6	−7.13 ± 0.08	73.3 ± 3.4	6	−9.47 ± 0.12	99.8 ± 3.4	5	−8.32 ± 0.17	88.5 ± 2.6	6
β2-I291L	2.70 ± 0.28	6	−9.96 ± 0.04	87.2 ± 2.4	5	−7.60 ± 0.10	83.2 ± 2.9	6	−9.62 ± 0.15	109.3 ± 1.8	5	−8.27 ± 0.09	90.4 ± 1.7	6
β2-V292A	3.47 ± 0.27	6	−9.41 ± 0.10	81.1 ± 3.0	6	−7.09 ± 0.07	80.9 ± 2.1	6	−9.47 ± 0.13	106.3 ± 2.8	5	−7.90 ± 0.10	92.0 ± 2.4	6
β2-I294V	2.31 ± 0.15	6	−10.06 ± 0.13	72.6 ± 4.6	5	−7.41 ± 0.08	75.5 ± 4.2	5	−9.76 ± 0.09	101.3 ± 3.7	4	−8.34 ± 0.16	93.9 ± 3.8	5
β2-H296K	2.92 ± 0.35	6	−8.69 ± 0.08	80.2 ± 6.1	6	−7.33 ± 0.05	85.0 ± 3.7	6	−9.11 ± 0.04	109.9 ± 3.2	5	−7.79 ± 0.05	99.5 ± 1.6	6
β2-V297A	2.44 ± 0.11	6	−9.78 ± 0.19	75.9 ± 3.9	6	−7.37 ± 0.14	77.9 ± 3.7	6	−9.69 ± 0.11	101.4 ± 4.9	5	−8.16 ± 0.08	85.7 ± 2.3	5
β2-I298F	2.64 ± 0.24	6	−9.66 ± 0.15	85.9 ± 3.7	5	−7.52 ± 0.10	84.5 ± 3.6	5	−9.87 ± 0.11	96.0 ± 4.7	5	−8.23 ± 0.16	89.1 ± 3.6	5
β2-TM7	2.04 ± 0.16	6	−8.88 ± 0.05	80.7 ± 4.6	6	−7.18 ± 0.05	75.7 ± 3.8	6	−8.96 ± 0.05	101.9 ± 3.3	6	−7.28 ± 0.15	97.5 ± 4.6	6
β2-V307L	2.69 ± 0.19	6	−9.88 ± 0.11	63.7 ± 1.3	6	−7.12 ± 0.07	79.1 ± 2.6	6	−9.45 ± 0.08	98.7 ± 3.9	6	−8.12 ± 0.13	94.4 ± 3.1	6
β2-Y308F	3.13 ± 0.26	6	−8.88 ± 0.04	76.7 ± 3.6	6	−6.74 ± 0.04	72.4 ± 5.0	6	−8.94 ± 0.15	99.3 ± 4.6	6	−7.61 ± 0.08	86.5 ± 4.3	6
β2-I309V	2.21 ± 0.15	6	−10.05 ± 0.08	81.4 ± 5.8	6	−7.55 ± 0.09	80.0 ± 4.6	6	−9.67 ± 0.14	99.6 ± 8.3	6	−8.10 ± 0.10	89.4 ± 8.3	6
β2-F194V-K305D	4.64 ± 0.49	8	−7.36 ± 0.05	66.2 ± 2.2	8	−6.28 ± 0.11	61.9 ± 2.3	8	−8.91 ± 0.10	96.1 ± 3.6	8	−7.62 ± 0.09	82.9 ± 2.9	8
β2-R304P-K305D	6.10 ± 0.61	8	−8.11 ± 0.07	76.7 ± 2.5	8	−7.35 ± 0.07	77.4 ± 2.0	8	−9.30 ± 0.07	93.4 ± 2.2	8	−8.26 ± 0.06	85.7 ± 2.5	8
β2-H296K-Y308F	6.07 ± 0.64	8	−8.22 ± 0.05	88.9 ± 1.9	8	−7.14 ± 0.04	87.8 ± 1.2	8	−9.00 ± 0.03	100.1 ± 3.5	8	−7.59 ± 0.06	94.4 ± 2.9	8
β2-K305D-Y308F	4.00 ± 0.35	6	−7.59 ± 0.08	74.7 ± 2.1	6	−6.71 ± 0.07	65.0 ± 2.8	6	−8.60 ± 0.12	106.2 ± 4.1	6	−7.69 ± 0.08	93.7 ± 3.7	6
β2-H296K-K305D	5.00 ± 0.43	8	−7.37 ± 0.06	78.1 ± 2.4	8	−7.41 ± 0.04	83.4 ± 2.5	8	−9.64 ± 0.09	92.8 ± 2.7	6	−7.96 ± 0.05	86.5 ± 3.5	6
β2-K305A	3.89 ± 0.69	8	−9.03 ± 0.06	66.6 ± 3.0	8	−6.90 ± 0.11	71.8 ± 3.2	8	−8.99 ± 0.13	97.5 ± 2.9	7	−7.71 ± 0.12	87.8 ± 4.4	8
β2-K305H	4.86 ± 0.51	8	−8.67 ± 0.04	82.1 ± 3.5	8	−7.04 ± 0.08	82.5 ± 2.2	8	−8.76 ± 0.08	103.3 ± 5.2	8	−8.11 ± 0.09	87.8 ± 2.2	8
β2-K305R	4.44 ± 0.64	8	−9.48 ± 0.04	80.7 ± 3.0	8	−6.92 ± 0.08	86.3 ± 2.5	8	−9.49 ± 0.10	102.8 ± 3.5	8	−7.97 ± 0.12	90.3 ± 2.9	7
β2-H296A	2.21 ± 0.11	6	−9.51 ± 0.10	83.4 ± 3.4	6	−7.13 ± 0.11	88.6 ± 1.6	6	−9.51 ± 0.09	99.2 ± 2.9	5	−7.90 ± 0.16	88.5 ± 2.8	6

were identified as short-acting ligands at all receptors, and carvedilol was shown to be a long-acting ligand at all receptors (Fig. 6; Table 8). The duration of salmeterol binding, however, remained relatively constant at all of the receptors (Fig. 6; Table 8). This suggests that the duration of binding is not closely aligned to the receptor-ligand affinity and that salmeterol's long duration of action may be related to another property, for example, its lipophilicity, and thus ability to partition into the cell membrane, forming a reservoir for future receptor binding after the free ligand had been washed away.

Functional Effects of the Mutations: [^{3}H]cAMP Accumulation in Transient Transfected Cells. Specific amino acids had been identified that reduced salmeterol-binding affinity. The effect of these mutations on the efficacy of salmeterol was examined by measuring [^{3}H]cAMP accumulation using the same preparation of transiently transfected cells as used in the [^{3}H]CGP 12177 binding assays above. The agonist responses were compared with the maximal response stimulated by the full agonist isoprenaline. Salmeterol stimulated a highly potent, partial agonist response at the β2-WT (log EC_{50} of -9.69, 74.7% maximal isoprenaline response; Fig. 7A; Table 9). The salmeterol response was, however, considerably less potent (i.e., right-shifted) at β2-F194V, β2-K305D, and β2-H296K, and even more so in the double mutant β2-H296K-K305D (Fig. 7; Table 9). Potency (EC_{50}) is, however, a complex measurement involving ligand affinity, receptor expression level, and tissue factors, as well as ligand efficacy (Johnson, 1995). The ratio of K_D/EC_{50} (Table 10), however, gives an indication of efficacy. The values obtained for the β2-WT ($-8.67/-9.69$) and β2-H296K-K305D ($-6.23/-7.37$) give K_D/EC_{50} ratios of 10 and 14, respectively, suggesting that salmeterol has similar efficacy at the two receptors.

Discussion

Salmeterol is widely used in the treatment of asthma and chronic obstructive pulmonary disease as a long-acting β2-agonist (Johnson, 1995; Waldeck, 2002; Cazzola et al., 2012; Kew et al., 2014). It is one of the most selective β-ligands discovered; however, the mechanism for this remains controversial. This study determined where this extreme selectivity occurred using a mutagenesis approach.

To determine the receptor regions important for salmeterol binding, experiments were performed in cells expressing either

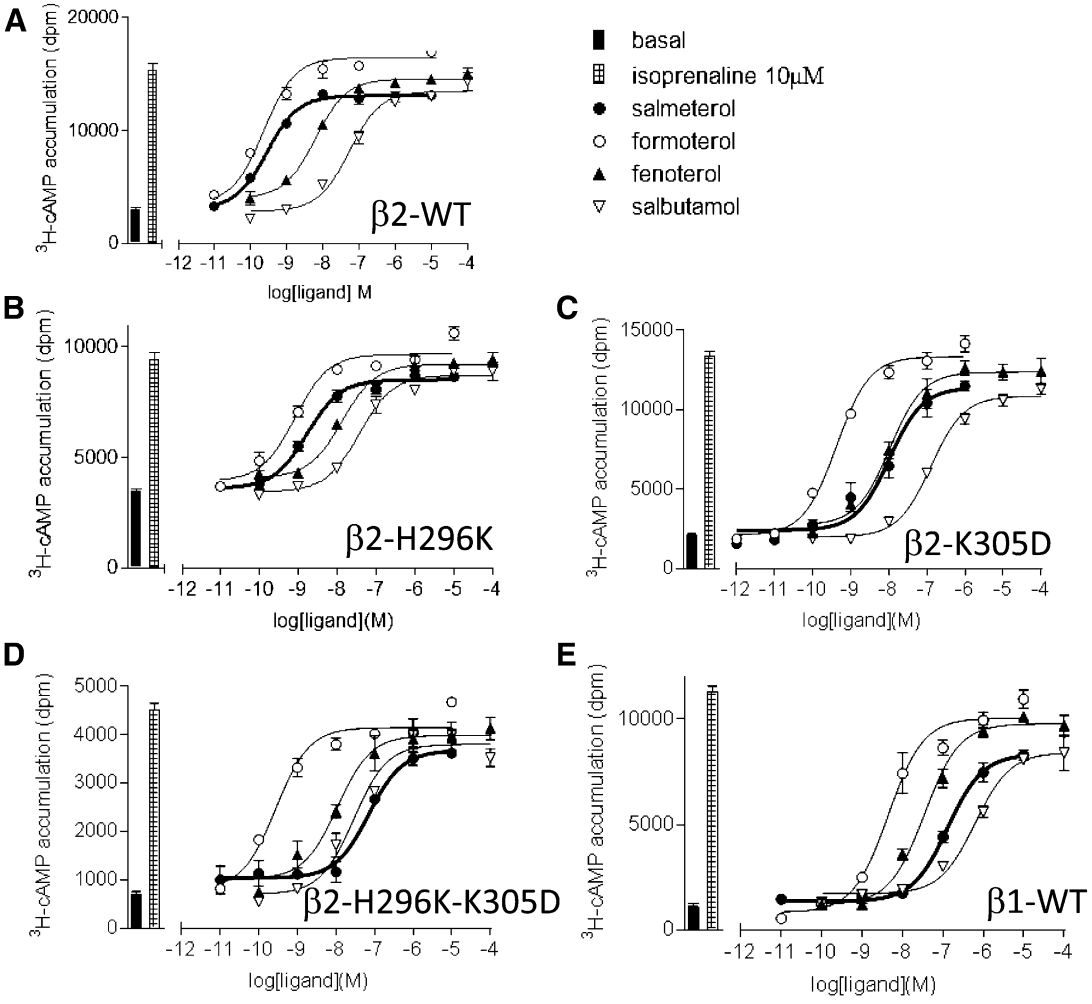

Fig. 7. [^{3}H]cAMP accumulation in response to salmeterol, formoterol, fenoterol, and salbutamol in transiently transfected cells expressing (A) β2-WT, (B) β2-H296K, (C) β2-K305D, (D) β2-H296K-K305D, and (E) β1-WT receptors. The salmeterol curve is in bold. Bars represent basal [^{3}H]cAMP accumulation, and that in response to 10 M isoprenaline alone. Data points are mean ± S.E.M. of triplicate determinations, and these single experiments are representative of (A) 17, (B) 5, (C) 8, (D) 6, and (E) 13 separate experiments.

the β1-WT, β2-WT, or a receptor in which either the N terminus, one EL, or TM domain had been mutated to that of the other receptor (Tables 1 and 2). The affinity and selectivity of salmeterol and three other agonists were studied—formoterol (long-acting, β2-selective agonist), salbutamol (short-acting, less β2-selective agonist from which salmeterol was developed), and fenoterol (short-acting agonist with some β2 selectivity; Jack, 1991; Johnson, 1995; Waldeck, 2002). The β2-WT and β1-WT affinities and selectivities were very similar to previous reports (e.g., Isogaya et al., 1998, 1999; Battram et al., 2006; Baker, 2010). By comparing the ligand affinities at each mutant with that of the WT parent (Table 3), EL3, TM6, TM7, and, to a lesser extent, EL2 were identified as important regions for salmeterol binding. TM7 and EL2 also affect other ligand affinities and were thus not salmeterol-specific. The equivalent β1 mutants did not recreate high-affinity salmeterol binding, although β1-TM7 had higher binding affinity for all ligands than β1-WT, again suggesting that TM7 has a more general role in β2/β1 affinity and selectivity. Others have previously demonstrated the importance of TM7 for ligand affinity (Isogaya et al., 1998, 1999; Kikkawa et al., 1998); however, all of their TM7 mutants also involve EL3 changes. They also identified TM2 as affecting ligand affinity (although the TM2 chimeras also had EL changes), and, although there was a small TM2 effect noted in this work (2.4-fold), we did not investigate this further, as other regions appeared to have greater effects.

To determine the precise amino acids involved, single-point mutations were made in the β2-WT. Importantly, similar reductions in affinity and selectivity were seen in the stable cell lines and transient transfections (compare Tables 5 and 7 with Table 3). Of these individual amino acid changes, β2-K305D from EL3 had the single biggest reduction in salmeterol affinity of 31-fold. Other major contributors to salmeterol affinity were H296K from TM6 (18-fold), Y308F from TM7 (11-fold), with more minor contributions from F194V from EL2 (5.6-fold), R304P from EL3 (3.7-fold), and possibly V292A from TM6 (3.0-fold).

Other ligands were also examined to determine whether these affinity changes were salmeterol-specific. Y308F reduced the formoterol affinity by 6-fold and fenoterol by 4-fold, and thus is important for several β2-ligands. This amino acid was identified by Isogaya et al. (1998, 1999), who also found reduced affinity of formoterol, procaterol, salmeterol, and TA-2005 (Kikkawa et al., 1998). H296K, however, appeared to have little effect on the affinity of the other ligands, and thus was more salmeterol-specific. F194V and R304P from EL2 and EL3, respectively, were more minor, and thus the salmeterol-specific nature was more difficult to judge.

Thus, two amino acids were identified that affected salmeterol affinity in a specific manner, H296K and K305D, one that affected salmeterol and other ligand affinities, Y308F, and two with minor effects—F194Vand R304P.

As K305D had the single biggest loss of salmeterol affinity, dual mutations were made with this and the other important mutations. The double EL mutations of β2-R304P-K305D and β2-F194V-K305D decreased salmeterol affinity by 66- and 61-fold, respectively, suggesting that the minor effects of F194V and R304P have a small additive effect to the K305D mutation (Table 5). The effect of these double-mutant receptors on the

TABLE 10

Ratios of K_D (obtained from transient binding; Tables 2–5)/EC_{50} (obtained from [^{3}H]cAMP accumulation; Table 9) in both a log and normal scale providing an indication of efficacy

Thus, from Table 9 salmeterol appears 10-fold less potent in β2-TM7 and β2-Y308F than at β2-WT, β2-V307L, and β2-I309V. However, when the effect of affinity is also taken into account (from Table 6), this change in potency is purely a measure of the change in affinity, and the apparent efficacy of salmeterol remains similar for all of these mutations.

	Salmeterol		Salbutamol		Formoterol		Fenoterol	
	Log Ratio	Ratio	Log Ratio	Ratio	Log Ratio	Ratio	Log Ratio	Ratio
β2-WT	1.02	10	1.40	25	1.48	30	1.40	25
β1-WT	1.25	18	1.58	38	2.34	219	2.53	389
β2-EL2	1.06	11	1.20	16	1.77	59	1.57	37
β2-F194V	0.90	8	0.96	9	1.40	25	1.39	25
β2-EL3	1.32	21	1.87	74	2.11	129	2.10	126
β2-R304P	1.50	32	1.63	43	1.61	41	1.82	66
β2-K305D	0.82	7	1.29	19	1.53	34	1.54	35
β2-TM6	0.40	3	1.61	41	1.48	30	1.77	59
β2-T281V	1.17	15	1.28	19	1.32	21	1.66	46
β2-I291L	1.33	21	1.69	49	1.45	28	1.86	72
β2-V292A	1.21	16	1.48	30	1.57	37	1.73	54
β2-I294V	1.19	15	1.61	41	1.71	51	1.59	39
β2-H296K	1.28	19	1.55	35	1.14	14	1.60	40
β2-V297A	1.18	15	1.40	25	1.47	30	1.40	25
β2-I298F	1.36	23	1.72	52	1.73	54	1.69	49
β2-TM7	1.67	47	1.72	52	1.88	76	1.66	46
β2-V307L	1.39	25	1.54	35	1.61	41	1.72	53
β2-Y308F	1.27	19	1.24	17	1.78	60	1.62	42
β2-I309V	1.64	44	1.94	87	1.78	60	1.79	62
β2-F194V-K305D	0.48	3	0.74	5	1.58	38	1.31	20
β2-R304P-K305D	1.26	18	1.85	70	1.89	78	1.98	95
β2-H296K-Y308F	1.27	19	1.46	29	1.46	29	1.68	48
β2-K305D-Y308F	0.98	10	1.30	20	1.40	25	1.85	71
β2-H296K-K305D	1.14	14	1.58	38	1.88	76	1.64	44
β2-K305A	0.98	10	1.38	24	1.25	18	1.27	19
β2-K305H	1.10	13	1.39	25	1.52	33	1.62	42
β2-K305R	1.11	13	1.28	19	1.47	30	1.42	26
β2-H296A	1.38	24	1.29	19	1.80	63	1.47	30

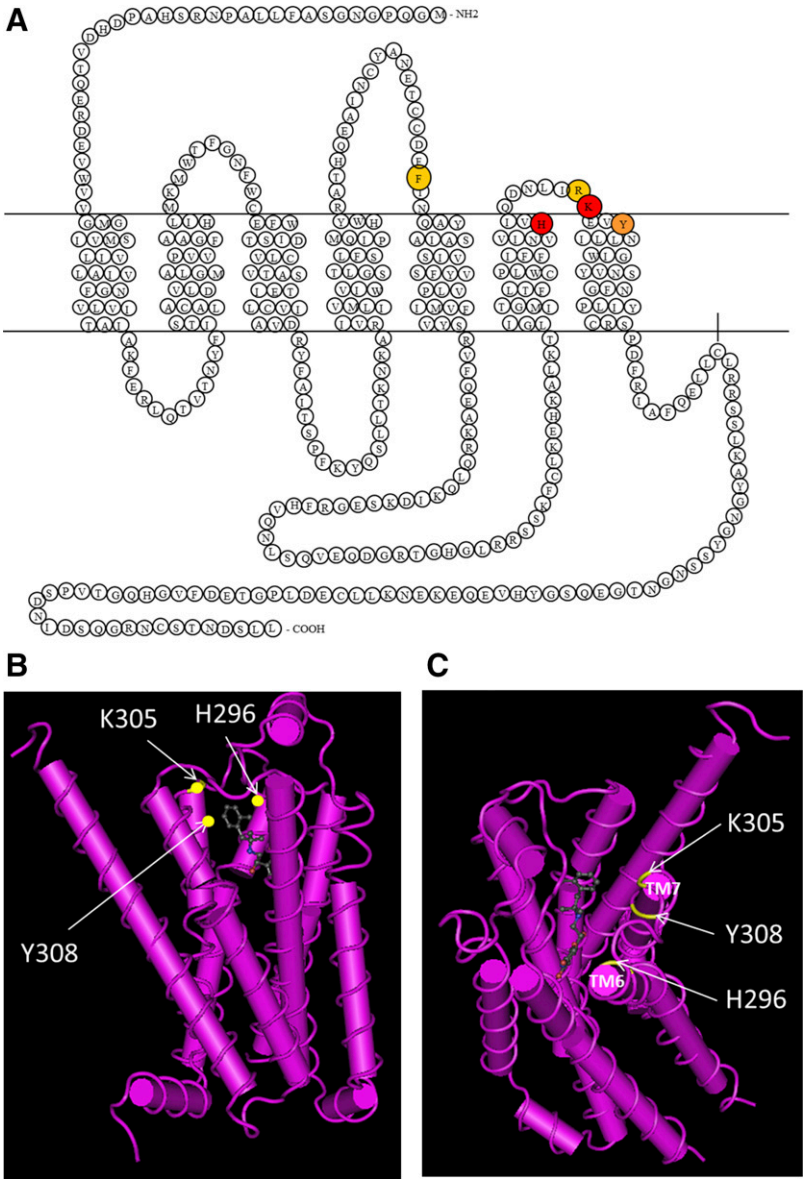

Fig. 8. (Afc) Snake diagram of the human β2-adrenoceptor with the location of the key residues involved in salmeterol selectivity highlighted. (B and C) Location of residues H296 (TM6), K305 (EL3), and Y308 (TM7) in the crystal structure of an agonist-bound, active state of a human β2-adrenoceptor–nanobody complex (Rasmussen et al., 2011). This crystal structure was obtained with the agonist BI-167107 bound to a variant of the β2-adrenoceptor (where the third intracellular loop was replaced by T4 lysozyme) in complex with nanobody 80 (Rasmussen et al., 2011). (B) Side and (C) top views of the β2-adrenoceptor (PDB ID 3POG; with the nanobody 80 sequence not included) reported by Rasmussen et al. (2011) were generated with Cn3D (National Centre for Biotechnology Information). The structure of BI-167107 is also shown. The position of residues H296, K305, and Y308 is shown in yellow.

other ligands was minimal with a potential borderline reduction of formoterol and adrenaline affinity (3- to 4-fold). The addition of Y308F to the salmeterol-specific mutations (β2-H296K-Y308F and β2-K305D-Y308F) decreased salmeterol affinity more than K305D and H296K alone (Table 7), but small changes in affinity for formoterol and fenoterol were also seen. However, when the two greatest and most salmeterol-specific changes were combined (β2-H296K-K305D), the reduction in salmeterol affinity was 275-fold and within only 4-fold of the affinity for β1-WT. This double mutant had a borderline 3-fold effect on adrenaline affinity and no effect on the affinity of the other ligands, making it salmeterol specific.

The effect of charge at position 305 was examined. The β2-amino acid lysine (K) is positively charged, whereas β1-aspartate (D) is negatively charged. Mutation to arginine (K305R), another positively charged amino acid with an exposed amine group, had little effect on salmeterol affinity (Table 4). Likewise, glutamate (K305E), another negatively charged amino acid, retained lower salmeterol affinity. Histidine (K305H), which at physiologic pH would not be charged, resulted in loss of salmeterol affinity, as did the other noncharged amino acids glutamate, glycine, and serine (Table 4). Thus, loss of the positive charge at position 305 affects the high affinity of salmeterol, potentially by disruption of a hydrogen bond to the oxygen in the side chain of salmeterol.

As affinity (K_D) is a measure of both how quickly a ligand associates with the receptor and how slowly it dissociates ($K_D = k_{off}/k_{on}$), dramatic affinity changes may cause significant changes in the duration of ligand action, and more specifically in the off rate. Attempts were therefore made to examine the duration of ligand action by incubating agonists and then removing the free ligand before the subsequent addition of [^{3}H]CGP 12177. Short-acting ligands, dissociating from the receptor during the wash, should no longer occupy the receptors enabling uninhibited [^{3}H]CGP 12177 binding. Moderate-duration ligands may partially dissociate from the receptor during the wash, but also further dissociate during the 2-hour [^{3}H]CGP 12177 incubation, creating a new equilibrium with

[^{3}H]CGP 12177, and a right-shifted binding curve. Long-acting ligands should not dissociate from the receptor, and the curves should superimpose. Whereas fenoterol and salbutamol appeared as short-acting compounds, carvedilol was shown to have a very long duration of action (Fig. 6; Table 8). The rank order of duration was carvedilol < salmeterol < formoterol < fenoterol and salbutamol, in keeping with previous studies (Bradshaw et al., 1987; Ball et al., 1991; Johnson, 1995).

However, a long duration of action may be a consequence of a long residence time on the receptor itself or, for the case of lipophilic ligands, partitioning into the lipophilic membrane (Rhodes et al., 1992; Anderson et al., 1994; Coleman et al., 1996; Sykes et al., 2014). Salmeterol had equal duration of action across all the receptors, despite large differences in affinity. Rebinding to the receptor of salmeterol that had previously partitioned into the membrane and therefore not removed by washing would prolong the apparent receptor residence time and mask changes in the duration of action at the receptor itself (Vauquelin, 2010; Vauquelin and Charlton, 2010; Sykes et al., 2014). This would also apply to previous studies that observed a long duration of action of salmeterol (e.g., Bradshaw et al., 1987; Ball et al., 1991; Coleman et al., 1996).

Finally, the impact of the mutations on ligand efficacy was examined by measuring [^{3}H]cAMP. Formoterol was consistently a full agonist (~100% of isoprenaline maximum; Table 9), whereas salbutamol and salmeterol were both partial agonists (as in previous studies; Johnson, 1995; Coleman et al., 1996; Baker, 2010; Cazzola et al., 2012). Salmeterol stimulated a partial agonist response at β2-H296K-K305D (78.1% of the isoprenaline maximum), which was very similar to that from β2-WT (74.7%) despite the large difference in log EC_{50} values (−7.37 versus −9.69, respectively; Table 9). There was no dramatic decrease in the degree of partial agonism for salmeterol, or change in K_D/EC_{50} ratio (Table 10), suggesting that the mutations did not affect salmeterol efficacy.

Advances have been made in the understanding of structure-activity relationships following the determination of the crystal structure of the human β2-adrenoceptor (Katritch et al., 2013; Venkatakrishnan et al., 2013). Following early work on inactive mutated receptors bound by inverse agonists, recent crystal structures include agonist-bound β2-adrenoceptors (Rosenbaum et al., 2011) or nanobody-stabilized β2-adrenoceptor active states (Rasmussen et al., 2011). Figure 8 shows the crystal structure of the human β2-adrenoceptor–T4 lysozyme construct in complex with nanobody 80 and the high affinity agonist BI167107 (Rasmussen et al., 2011) with residues K305, Y308, and H296 highlighted in yellow. All three residues are facing inward, toward the binding pocket at the top of TM6 and TM7 with K305 located in a key position between EL3 and TM7 (Fig. 8), and are thus located in an important position for determining receptor-ligand interactions.

In conclusion, salmeterol is a highly β2-selective ligand that specifically binds to H296K in TM6 and K305D in EL3. Combining these (β2-H296K-K305D) reduces salmeterol affinity by 275-fold, to within 4-fold of that of the β1-WT, without affecting the affinity of other agonists. Another important, although less salmeterol-specific, amino acid is Y308F in TM7. F194V in EL2 and R304P in EL3 have minor effects. These mutations (including β2-H296K-K305D) do not affect the efficacy of salmeterol.

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Authorship Contributions

Participated in research design: Baker, Hill.
Conducted experiments: Baker, Proudman.
Contributed new reagents or analytic tools: Baker, Proudman, Hill.
Performed data analysis: Baker.
Wrote or contributed to the writing of the manuscript: Baker, Hill.

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Address correspondence to: Dr. Jillian G. Baker, Cell Signalling, School of Life Sciences, C Floor Medical School, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, United Kingdom. E-mail: jillian.baker@nottingham.ac.uk