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Identification of a Key Amino Acid of the β_2-Adrenergic Receptor for High Affinity Binding of Salmeterol

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ABSTRACT

Transmembrane domains (TMDs) I, II, and VII of the β_2-adrenergic receptor (β_2AR) were replaced, individually or in combination, with the corresponding regions of the β_1AR, and vice versa. The β_2-selective binding of salmeterol was not affected by the exchange of TMD I between the β_1- and β_2ARs. The affinity of salmeterol was slightly decreased (32-fold) by replacement of TMD II of the β_2AR with the homologous region of the β_1AR; the affinity was strongly decreased (1870-fold) for the β_2AR with TMD VII of the β_1AR. The affinity of salmeterol was partially restored by the introduction of TMD VII, but not TMD II, of the β_2AR into the β_1AR. By analyzing alanine-substituted mutants, we found that Tyr308 in TMD VII was mainly responsible for the high affinity binding of salmeterol. Two salmeterol derivatives with the ether oxygen at different positions in the side chain showed 33- and 64-fold decreased affinities for the wild-type β_2AR, and a derivative with no ether oxygen showed 147-fold decreased affinity for the wild-type β_2AR. These results indicate that Tyr308 in TMD VII is the major amino acid conferring the β_2-selective binding of salmeterol to the β_2AR and that the position of the ether oxygen in the side chain is also important for β_2-selective binding. A three-dimensional model of the salmeterol-β_2AR complex shows that the phenyl group of Tyr308 interacts with methylene groups near the protonated amine of salmeterol and the ether oxygen interacts with Tyr316.

The βARs belong to a family of G protein-coupled receptors and have been analyzed as prototypes of these receptors. The binding of agonists induces conformational changes, leading to functional coupling with G proteins. The ligand binding site of the βARs has been extensively characterized by a variety of techniques (Savarese and Fraser, 1992; Strader *et al*., 1994). Deletion mutagenesis experiments have shown that the hydrophilic intracellular and extracellular loops connecting the seven hydrophobic domains of the β_2AR are not required for ligand binding (Dixon *et al*., 1987). The findings indicated that the ligand binding domain of the β_2AR is located within the hydrophobic TMD of the β_2AR. Site-directed mutagenesis experiments have revealed the amino acids and regions of the β_2AR that are important for ligand binding and G protein coupling (Dohlman *et al*., 1988; Wong *et al*., 1988; Hockerman *et al*., 1996). Several key residues were identified by analyzing point-mutated β_2ARs; Asp113 in TMD III interacts with the protonated amine of the agonists, and two serine residues in TMD V (Ser204 and Ser207) form hydrogen bonds with the *meta*- and *para*-hydroxyl groups of the catechol ring (Strader *et al*., 1988, 1989).

Binding domains of subtype-selective ligands [i.e., β_1- and β_2-selective antagonists and a slightly selective agonist (norepinephrine)] have been analyzed by several groups. Frielle *et al*. (1988) reported that TMDs VI and VII of the βAR seem to be important for the high affinity binding of the β_1-selective antagonist betaxolol and the β_2-selective antagonist ICI118551. Those authors also showed that the β_1-selectivity of norepinephrine is largely determined by TMD IV of the β_1AR. Dixon *et al*. (1989) reported that TMD IV of the β_1AR is responsible for the β_1-selective binding of norepinephrine. Marullo *et al*. (1990) reported that single TMDs cannot be responsible for the high affinity binding of β_1- and β_2-selective antagonists, based on examination of β_1- and β_2AR CHs. However, those authors did not examine the binding domains of β_1- and β_2-selective agonists. Therefore, domains responsible for the high affinity binding of subtype-selective agonists have not been examined.

Salmeterol is a derivative of salbutamol with an aralkyloxyalkyl substitution at the amine group; it has a long duration of action and high selectivity for the β_2AR (Johnson,

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ABBREVIATIONS: βAR, β-adrenergic receptor; TMD, transmembrane domain; CYP, cyanopindolol; CH, chimera; WT, wild-type.

1995). Green *et al*. (1996) recently revealed that the anchoring region of salmeterol, referred to as the "exosite," is located in the inner part of TMD IV and accounts for the long duration of action. However, it has not been determined which region of the β_2AR is involved in the subtype-selective binding of salmeterol.

In this study, we examined the TMDs and the key amino acids that are responsible for the β_2AR selectivity of salmeterol and we investigated the role of the ether oxygen in the side chain of salmeterol. We found that Tyr308 in TMD VII was the major amino acid determining the high affinity binding of salmeterol and that the position of the ether oxygen in the side chain was also important for β_2-selective binding. We built a three-dimensional model of the salmeterol-β_2AR complex to account for this structural information.

Experimental Procedures

Materials. Salmeterol and salmeterol derivatives were kindly synthesized and provided by the Lead Optimization Research Laboratory, Tanabe Seiyaku (Saitama, Japan). *Thermus aquaticus* and *Pyrococcus furiosus* DNA polymerases were obtained from Takara (Shiga, Japan) and Stratagene (La Jolla, CA), respectively. ^{125}I-CYP was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). (±)-Propranolol and DEAE-dextran were obtained from Sigma Chemical (St. Louis, MO). The plasmids encoding the human β_1- and β_2ARs were kindly provided by Dr. R. J. Lefkowitz (Duke University, Durham, NC). The mammalian expression vector pEF-BOS was a gift from Dr. S. Nagata (Osaka University, Osaka, Japan).

Construction of chimeric β_1/β_2ARs and alanine-substituted β_2AR mutants. Chimeric β_1/β_2ARs were constructed by polymerase chain reaction, as described (Kikkawa *et al*., 1998). TMDs I, II, and VII of the β_2- or β_1AR were exchanged with homologous regions of the β_1- or β_2AR, respectively. The structures of these CHs are shown in Fig. 1. The positions and amino acids of the junctions for individual chimeric β_1- and β_2ARs are as follows: CH-1, β_1 Met1–Ala84/β_2 Lys60–Leu413; CH-2, β_2 Met1–Phe71/β_1 Ile97–Cys131/β_2 Glu107–Leu413; CH-3, β_2 Met1–Val295/β_1 Lys347–Pro381/β_2 Asp331–Leu413; CH-4, β_2 Met1–Phe71/β_1 Ile97–Cys131/β_2 Glu107–Val295/β_1 Lys347–Pro381/β_2 Asp331–Leu413; CH-5, β_2 Met1–Ala59/β_1 Lys85–Val477; CH-6, β_1 Met1–Phe96/β_2 Ile72–Cys106/β_1 Glu132–Val477; CH-7, β_1 Met1–Val346/β_2 His296–Pro330/β_1 Asp382–Val477; CH-8, β_1 Met1–Phe96/β_2 Ile72–Cys106/β_1 Glu132–Val346/β_2 His296–Pro330/β_1 Asp382–Val477. Alanine-substituted mutants of the β_2AR were constructed by polymerase chain reaction using the QuickChange site-directed mutagenesis kit (Stratagene). Briefly, 35–40-base primers encompassing the positions of mutation were used for mutagenesis. The plasmid containing the *Bgl*II-*Eco*RV fragment of the β_2AR in pSL1190 (Amersham Pharmacia Biotech) was used as a template. The sequences of the amplified regions were confirmed by the dideoxy chain termination method (Sanger *et al*., 1977). The fragments containing appropriate mutations were then ligated to construct a full-length β_2AR and were finally inserted into *Xba*I or *Eco*RI and *Bam*HI sites of mammalian expression vectors pEF-BOS (Mizushima and Nagata, 1990) or pCMV5, respectively. These constructs were transfected into COS-7 cells by the DEAE-dextran method (Cullen, 1987).

Membrane preparations and radioligand binding assays. The cells were rinsed twice with ice-cold phosphate-buffered saline and mechanically detached in ice-cold buffer containing 10 mM Tris·HCl, pH 7.4, 5 mM EDTA, 10 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor (type II-S), and 5 μg/ml leupeptin (lysis buffer). The lysate was centrifuged at 45,000 × *g* for 10 min at 4°. The pellet was rehomogenized in lysis buffer, with a Potter-type homogenizer, and stored at −80° until use. The competition binding assays were performed in buffer containing 75 mM Tris·HCl, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA, using 1–5 μg of membrane protein, 50 pM ^{125}I-CYP, and 0–100 μM unlabeled ligand in the presence of 100 μM GTP, for 60 min at 37°. The binding reaction was terminated by dilution and rapid filtration through Whatman GF/C filters; the filters were washed three times with solution containing 25 mM Tris·HCl, pH 7.4, and 1 mM $MgCl_2$. Nonspecific binding was determined in the presence of 5 μM (±)-propranolol. The radioactivity on the filters was counted with a γ-counter. The protein concentration was determined by the method of Lowry *et al*. (1951). Because the expression levels of endogenous βARs in COS-7 cells are <30 fmol/mg of protein (data not shown), the binding activities measured in this study are largely attributable to those of the exogenously expressed chimeric and mutated βARs.

Data analysis. All data are shown as mean ± standard error of the specified number of determinations. Equilibrium dissociation constants were determined from saturation isotherms. The competition curves were analyzed by nonlinear regression analysis, to determine EC_{50} and K_i values, using PRISM software (GraphPAD Software Inc., San Diego, CA). Statistical significance was assessed by one-way analysis of variance for multiple comparisons. Analysis of variance *post hoc* comparisons were evaluated with the Dunnett test.

Molecular modeling of the salmeterol-β_2AR complex. The positions of the α-carbon of the β_2AR were determined by overlaying

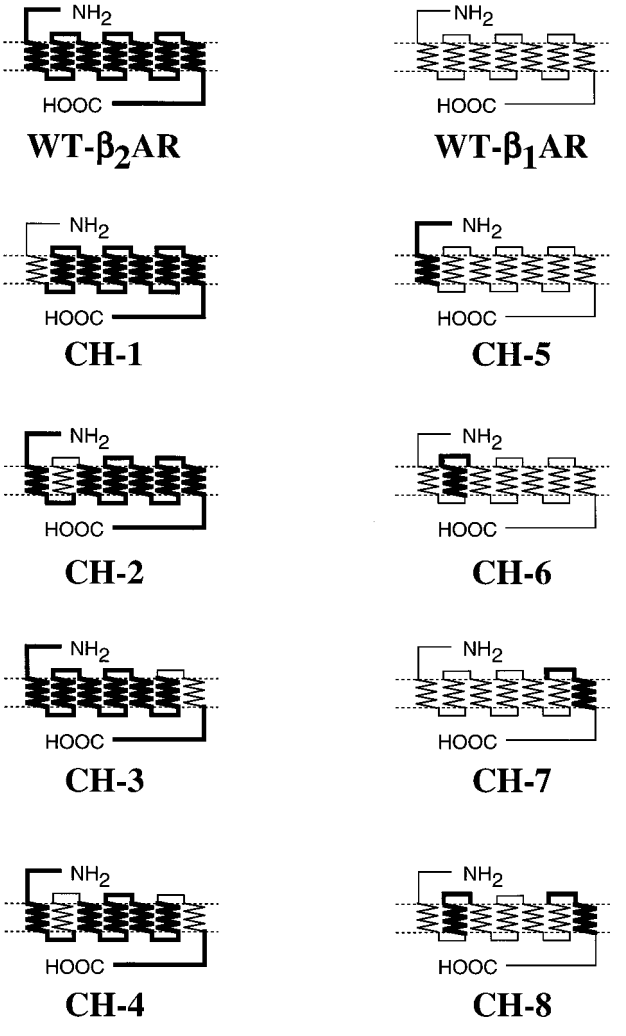

Fig. 1. Structures of β_1/β_2AR CHs. *Thin lines*, sequences of the β_1AR; *thick lines*, sequences of the β_2AR. The positions and amino acids of the junctions and the construction method are described in Experimental Procedures.

the amino acids in the TMDs of the β_2AR with those of bacteriorhodopsin, using Insight II molecular modeling software (MSI, San Diego, CA). The salmeterol-β_2AR complex was then manually obtained by specifying the following interactions between residues of the β_2AR and specific groups of salmeterol: Asp113 in TMD III with the amine group, two serines in TMD V with the saligenin moiety, and the inner half of TMD IV with the phenyl group of the side chain. Then constrains were released, and the reasonable model of the salmeterol-β_2AR complex was obtained by Monte Carlo simulation.

Results

Affinities of salmeterol for β_1/β_2AR CHs. Salmeterol showed very high selectivity for the WT β_2AR (β_1 K_i /β_2 K_i ratio of approximately 1500) (Table 1). To determine the domain(s) responsible for β_2-selectivity, we constructed several β_1/β_2AR CHs. Structures of various CHs are shown in Fig. 1. Although the affinity of salmeterol was slightly decreased by replacement of TMD I or II of the β_2AR (CH-1 or CH-2) with that of the β_1AR, the affinity of salmeterol was not affected by the introduction of TMD I or II of the β_2AR into the β_1AR (CH-5 or CH-6) (Table 1). This finding suggested that TMDs I and II of the β_2AR are not primarily involved in the β_2-selective binding of salmeterol. On the other hand, the CH (CH-3) that contained TMD VII of the β_1AR demonstrated greatly decreased affinity for salmeterol. The contribution of TMD VII of the β_2AR to the high affinity binding of salmeterol was confirmed by the finding that the affinity of salmeterol for the β_1AR was increased by introduction of TMD VII of the β_2AR into the β_1AR. The replacement of TMD VII plus TMD II further increased the affinity of salmeterol, suggesting that TMD II indirectly contributes to the β_2-selective binding of salmeterol by supporting a role of TMD VII. These results suggest that TMD VII of the β_2AR is a major region for the subtype-selective binding of salmeterol.

Analysis of alanine-substituted mutants. To identify the amino acid that is responsible for the high affinity binding of salmeterol, 10 amino acids in TMD VII of the β_2AR that are different from those of the β_1AR were individually changed to alanine. The positions of the mutated amino acids are indicated in Fig. 2. The K_d values of ^{125}I-CYP for the alanine-substituted β_2ARs were not significantly different from that for the WT β_2AR (Table 2). Eight mutants showed no significant differences in binding affinities for salmeterol. The affinities for salmeterol were decreased in two mutants. The mutation of Tyr308 to alanine (Y308A-β_2AR) significantly decreased the affinity for salmeterol (120-fold decrease). The change of Ile309 to alanine (I309A-β_2AR) resulted in a receptor that showed 16-fold decreased affinity for salmeterol, although the decrease in the affinity for salmeterol was not significant. The decreases in affinities for Y308A- and I309A-β_2ARs were smaller than that for the chimeric receptor (CH-3). The affinity for salmeterol was decreased further, but not additively, in the double mutant (Y308A/I309A-β_2AR) (Table 3). This finding indicates that two amino acids cooperatively contribute to the high affinity binding of salmeterol.

Effects of the position of the ether oxygen on β_2-selectivity. To examine the importance of the ether oxygen in the side chain of salmeterol for β_2-selectivity, we synthesized three salmeterol derivatives; two derivatives had ether oxygens in different positions and one derivative did not have an ether oxygen. Fig. 3 shows the structures of the three derivatives of salmeterol. It was reported that the duration of action of salmeterol is altered when the position of the ether oxygen is changed. Derivative 1 (in which the ether oxygen is positioned four carbons from the protonated amine) and derivative 2 (in which it is positioned eight carbons from the protonated amine) showed 33- and 64-fold decreased affinities for the WT β_2AR, respectively (Table 3). The affinity of derivative 3 (in which the ether oxygen was removed from the side chain) for the WT β_2AR was decreased 147-fold. The affinities of derivatives 1 and 3 for the WT β_1AR were essentially the same as that of salmeterol. The affinities of derivative 3 for the Y308A-, I309A-, and Y308A/I309A-β_2ARs were close to that for the WT β_1AR. The rank order of potency for the WT β_2AR was salmeterol > derivative 1 > derivative 2 > derivative 3; those for Y308A-, I309A-, and Y308A/I309A-

TABLE 1

Effects of replacement of TMDs with corresponding regions of the β_1AR on the binding characteristics of salmeterol for the β_2AR

The binding of salmeterol to the WT β_1- and β_2ARs and β_1/β_2 chimeric receptors was assayed by competition with 50 pM ^{125}I-CYP. The data were analyzed by a nonlinear least-squares regression computer program, as described in Experimental Procedures. The results are shown as the mean ± standard error of three or four separate experiments.

	^{125}I-CYP		Salmeterol, K_i
	K_d	B_{max}	
	pM	*pmol/mg of protein*	*nM*
WT β_2AR	23.2 ± 3.1	4.4 ± 0.4	1.5 ± 0.4
CH-1	56.1 ± 0.6	4.4 ± 0.1	18 ± 4
CH-2	15.8 ± 0.8	4.9 ± 0.01	49 ± 1
CH-3	47.3 ± 3.6	23.6 ± 1.4	2,800 ± 290[a]
CH-4	142 ± 14[a]	54.5 ± 5.9	1,300 ± 120[b]
CH-5	54.1 ± 3.2	28.3 ± 0.3	3,100 ± 410[a]
CH-6	66.7 ± 11.0[b]	6.6 ± 0.3	10,000 ± 690[a,c]
CH-7	129 ± 24[a,c]	2.2 ± 0.7	270 ± 68[c]
CH-8	40.6 ± 2.2	0.2 ± 0.1	25 ± 7[c]
WT β_1AR	36.9 ± 5.2	19.9 ± 5.6	2,200 ± 79

[a] $p < 0.001$, significantly different from the WT β_2AR.
[b] $p < 0.05$, significantly different from the WT β_2AR.
[c] $p < 0.001$, significantly different from the WT β_1AR.

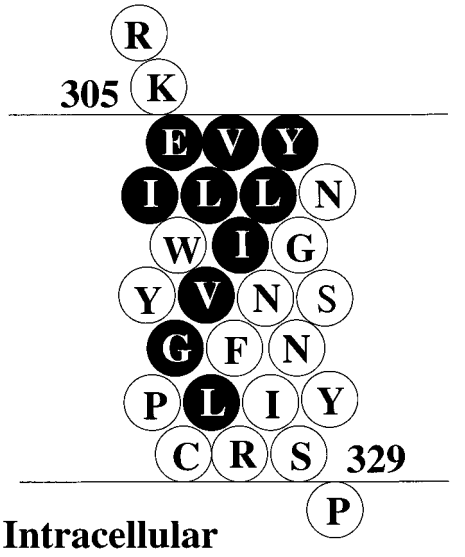

Fig. 2. Amino acid residues of TMD VII of the β_2AR. ●, amino acids different from those of the β_1AR.

β_2ARs were the same as that for the WT β_2AR. This indicates that the ether oxygen does not directly interact with Tyr308 and Ile309.

Molecular model of the salmeterol-β_2AR complex. To obtain structural information regarding the salmeterol-β_2AR complex, we built a molecular model using Insight II software (Fig. 4). It showed that the phenyl group of Tyr308, which is important for the β_2-selective, high affinity binding of salmeterol, seemed to interact with methylene groups in the side chain near the protonated amine of salmeterol, via hydrophobic interactions. Another feature of the model is a possible interaction of the ether oxygen with Tyr316 in TMD VII. The ether oxygen in the side chain could not directly interact with Tyr308 because of constraints on the structures of salmeterol and the β_2AR. The model can explain the decrease in the affinities of the β_2AR for salmeterol derivatives. Changes in the position of the ether oxygen would result in disruption of the interactions with Tyr316. This molecular model also suggested that Asp113 in TMD III, which interacts with the protonated amine of agonists, would become close to the ether oxygen and would compensate for the lost interaction of the ether oxygen with Tyr316.

TABLE 2

Affinity constants of salmeterol for WT and alanine-substituted β_2ARs

The K_d values for ^{125}I-CYP were measured in direct binding assays. The K_i values for salmeterol were determined in competition binding assays, as described in Experimental Procedures. The data were analyzed by the nonlinear least-squares regression computer program PRISM. The results are shown as the mean ± standard error of three separate experiments.

	^{125}I-CYP		Salmeterol, K_i
	K_d	B_{max}	
	pM	*pmol/mg of protein*	*nM*
WT β_2AR	23.2 ± 3.1[a]	4.4 ± 0.4[a]	1.5 ± 0.4[a]
E306A	22.3 ± 1.0	0.2 ± 0.02	3.0 ± 1.2
V307A	22.8 ± 2.2	2.6 ± 0.4	2.3 ± 0.5
Y308A	24.2 ± 4.7	4.1 ± 0.2	184 ± 60[b]
I309A	26.6 ± 2.7	3.3 ± 0.4	24 ± 3
L310A	25.7 ± 3.1	3.5 ± 0.5	2.0 ± 0.7
L311A	26.1 ± 4.7	3.9 ± 0.5	1.6 ± 0.3
I314A	21.5 ± 1.1	2.9 ± 0.6	2.0 ± 0.1
V317A	25.8 ± 2.6	3.6 ± 0.1	2.2 ± 0.7
G320A	44.7 ± 6.6[c]	6.2 ± 1.4	3.0 ± 1.4
L324A	32.9 ± 4.5	4.6 ± 1.3	4.7 ± 0.6
WT β_1AR	36.9 ± 5.2[a]	19.9 ± 5.6[a]	2,200 ± 79[a]

[a] These data are taken from Table 1, for comparison.
[b] $p < 0.001$, significantly different from the WT β_2AR.
[c] $p < 0.01$.

Discussion

We constructed a series of β_1/β_2 chimeric receptors and alanine-substituted β_2ARs, to examine the binding domain for the β_2-selective agonist salmeterol. We previously reported that the binding domains of the chimeric receptors for the agonist isoproterenol were largely preserved after TMDs of the β_1AR were exchanged with those of the β_2AR, or vice versa (Kikkawa *et al.*, 1998). The exchange of TMD I between the β_2AR and the β_1AR (CH-1 and CH-5) did not affect the binding characteristics of salmeterol. This suggests a small contribution of TMD I to the β_2-selective binding of salmeterol.

The affinity of salmeterol was slightly decreased in CH-2. This finding suggests that TMD II contributes to the high affinity binding of salmeterol, compared with the contribu-

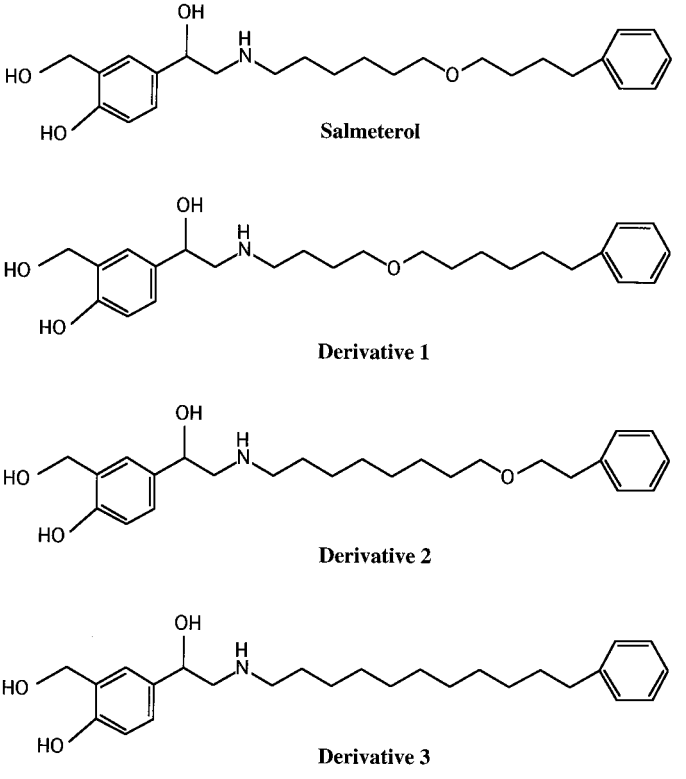

Fig. 3. Structures of salmeterol and its derivatives. The structures of salmeterol and three derivatives used in this study, in which the ether oxygen was moved or removed, are shown. The derivatives were synthesized and provided by the Lead Optimization Research Laboratory, Tanabe Seiyaku.

TABLE 3

Affinities of salmeterol and its derivatives for WT and alanine-substituted β_2ARs and the WT β_1AR

The binding of ^{125}I-CYP to the WT β_1- and β_2ARs and alanine-substituted β_2ARs was assayed in direct binding assays. The K_i values of salmeterol and its derivatives were determined by competition with 50 pM ^{125}I-CYP. The results are shown as the mean ± standard error of three or four separate experiments.

Parameter	WT β_2AR	Y308A-β_2AR	I309A-β_2AR	Y308A/I309A-β_2AR	WT β_1AR
K_d, ^{125}I-CYP (pM)	31.2 ± 2.9	29.8 ± 2.7	34.0 ± 2.4	17.4 ± 1.9	30.4 ± 3.8
B_{max}, ^{125}I-CYP (pmol/mg of protein)	4.4 ± 0.8	5.6 ± 0.4	5.1 ± 0.4	1.5 ± 0.1	19.4 ± 2.7
K_i (nM)					
Salmeterol	1.5 ± 0.4[a]	184 ± 60[a]	18.7 ± 1.3	530 ± 61	2,200 ± 79[a]
Derivative 1	50 ± 8	542 ± 79	133 ± 18	860 ± 99	2,800 ± 180
Derivative 2	96 ± 13	1,400 ± 240[b]	281 ± 21	1,300 ± 130[b]	5,300 ± 470[c]
Derivative 3	220 ± 48[c]	1,300 ± 480	3,370 ± 760[d]	1,700 ± 240[b]	2,300 ± 480

[a] These data are taken from Tables 1 and 2 for comparison.
[b] $p < 0.05$, significantly different from salmeterol.
[c] $p < 0.001$.
[d] $p < 0.01$.

tion of TMD I. However, the introduction of TMD II of the β_2AR did not increase the affinity for salmeterol, suggesting that TMD II does not contain the sites of direct contact with salmeterol. The affinity of salmeterol for the CH (CH-3) containing TMD VII of the β_1AR was essentially the same as that for the β_1AR. The introduction of TMD VII of the β_2AR into the β_1AR partially restored the high affinity binding of salmeterol, and the replacement of both TMD II and TMD VII of the β_1AR with the corresponding regions of the β_2AR further increased the affinity for salmeterol. It is important to produce not only loss-of-function mutants but also gain-of-function mutants, because the loss of binding activity may be the result of a nonspecific alteration of binding sites (Strader *et al.*, 1995). These data suggest that TMD VII plays a major role in the high affinity binding of salmeterol and TMD II plays a supportive role in the β_2-selective binding of salmeterol.

There have been several reports that specific amino acids

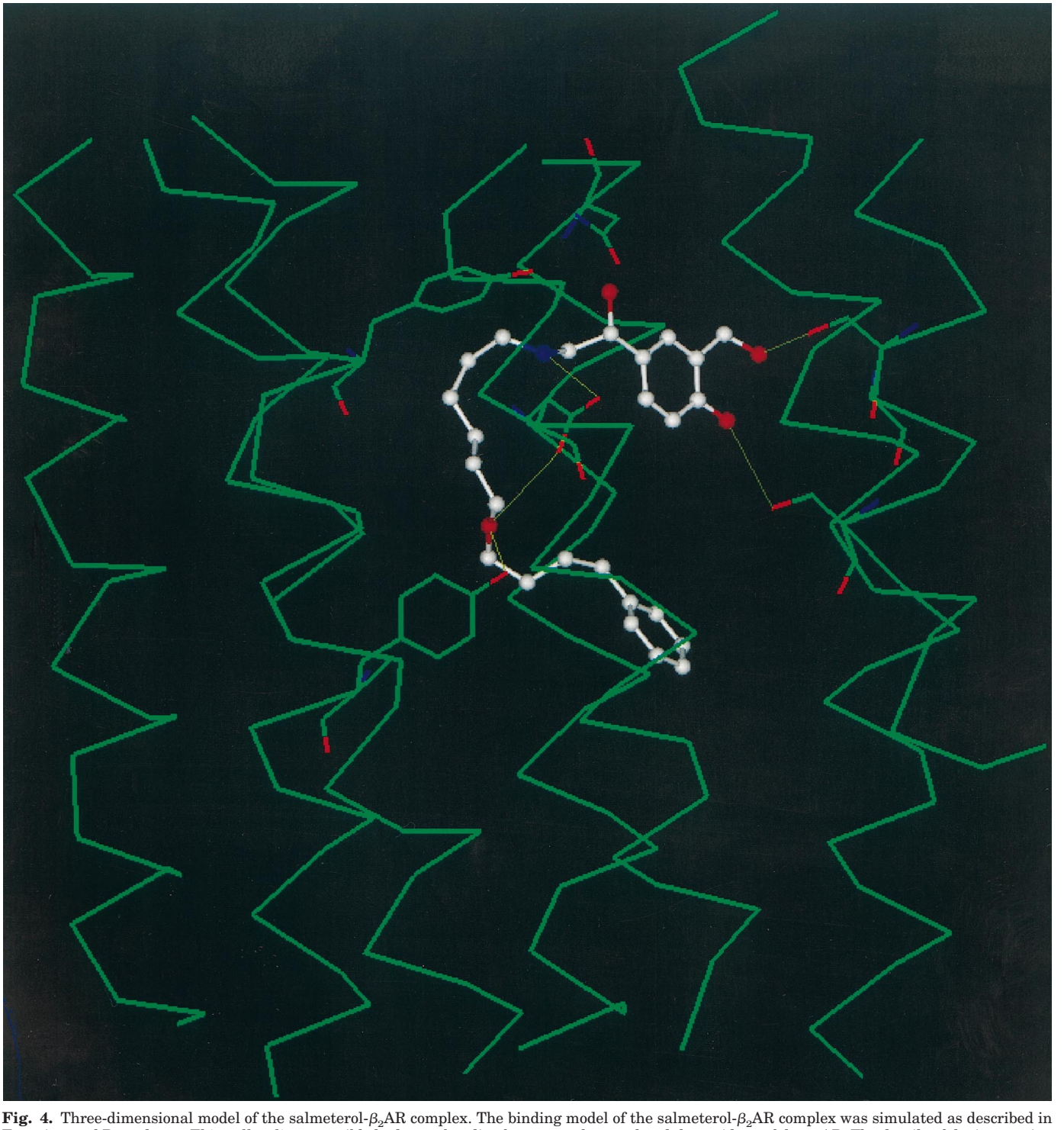

Fig. 4. Three-dimensional model of the salmeterol-β_2AR complex. The binding model of the salmeterol-β_2AR complex was simulated as described in Experimental Procedures. *Thin yellow lines*, possible hydrogen bonding between salmeterol and the residues of the β_2AR. The details of the interaction of salmeterol with the β_2AR are described in the text.

in TMDs II and VII are close in space and have functional interactions [i.e., the interactions of Asn87 in TMD II of the gonadotropin-releasing hormone receptor with Asp318 in TMD VII (Zhou *et al*., 1994), Asp120 in TMD II of the 5-hydroxytryptamine type 2A receptor with Asn376 in TMD VII (Sealfon *et al*., 1995), and Asp71 in TMD II of the thyrotropin-releasing hormone receptor with Asp316 in TMD VII (Perlman *et al*., 1997)]. It is possible that similar interactions between TMDs II and VII stabilize the structure of the β_2AR and form a binding pocket for salmeterol.

Salmeterol is a β_2-selective agonist with very long-lasting physiological actions (Johnson, 1995). The structural feature of salmeterol include a long side chain that has a phenyl group at the end, and the interaction of the phenyl group with the so-called exosite is assumed to govern the kinetics of salmeterol (Coleman *et al*., 1996). The exosite is an unique site that can participate in the persistent binding of salmeterol to the β_2AR. Green *et al*. (1996) reported recently that the exosite is located within the inner part of TMD IV of the β_2AR, because a chimeric β_1/β_2 receptor containing the inner part of TMD IV of the β_1AR lost the long duration of action. Interestingly, the binding characteristics of salmeterol for the CH were same as those for the WT β_2AR. Those authors indicated that the exosite in the inner part of TMD IV does not contribute to the subtype-selective binding, supporting our observation that the β_2-selective high affinity binding site is mainly located within TMD VII of the β_2AR.

The results with the chimeric receptors indicate that at least one of the amino acids in TMD VII is responsible for the high affinity binding of salmeterol. Analysis of the binding characteristics of alanine-substituted mutants revealed that Tyr308 contributes to the β_2-selective binding of salmeterol. We recently reported that the β_2-selective binding of TA-2005, a β_2-selective agonist, was mainly determined by Tyr308 in TMD VII of the β_2AR (Kikkawa *et al*., 1998). Considering the results with TA-2005, we concluded that Tyr308 is a critical amino acid conferring high affinity binding of β_2-selective agonists to the β_2AR. Because agonist binding domains are assumed to be located within TMDs, and the homologies of TMDs IV and VII (58–63%) between the β_1- and β_2ARs are lower than those of other TMDs (71–88%), TMD VII seems to be a good target for the design of β_2-selective agonists. According to a model proposed by Schwartz and Rosenkilde (1996), consisting of a deep pocket formed by TMDs III, IV, V, and VI and a shallow pocket formed by TMDs II, III, and VII, Ser204 and Ser207 in TMD V, Phe293 in TMD IV, and Asp113 in TMD III seem to face the deep pocket; Tyr308 in TMD VII, which is important for β_2-selectivity, faces the shallow pocket.

Rosenkilde *et al*. (1994) recently reported that point mutations in TMD II of the neurokinin-1 receptor induce a conformation of the receptor that impairs interconversion from an antagonist-bound form to an agonist-bound form. They showed that binding of agonist to the mutated receptor cannot shift the equilibrium of the receptor conformation toward the agonist-bound form when antagonists are already bound to the receptor. It is possible that mutation of Tyr308 of the β_2AR induced and fixed the conformation that showed high affinity for ^{125}I-CYP and low affinity for salmeterol. However, this possibility is unlikely, because the binding of isoproterenol was not affected by the mutation of Tyr308 to alanine (Kikkawa *et al*., 1998). This indicates that the decrease in the affinity of Y308A-β_2AR actually reflects a specific alteration of the binding site that is important for the high affinity binding of salmeterol.

Frielle *et al*. (1988) reported, using chimeric β_1/β_2ARs, that the majority of the βAR-selective binding of betaxolol and ICI118551 is determined by TMD IV, although multiple domains seem to be involved in the determination of the βAR-selective binding of these two antagonists. The contribution of TMD IV to subtype-selective antagonist binding was supported by the fact that TMD IV shows the greatest difference in primary amino acid sequences between the β_1- and β_2ARs (58% identity). It would be interesting to determine whether TMD IV of the β_2AR plays a role in the high affinity binding of salmeterol.

The affinities of the two derivatives of salmeterol with the ether oxygen at different positions were decreased 30–60-fold, and the affinity of the derivative of salmeterol with no ether oxygen was decreased approximately 150-fold. However, these derivatives still showed higher affinities for the β_2AR than for the β_1AR. Therefore, the position of the ether oxygen in the side chain is important for the β_2-selective high affinity binding of salmeterol, although it is not the sole determinant for β_2-selective binding. Johnson (1995) reported that 1) compounds in which ether oxygens are placed two or eight carbons from the protonated amine show reduced durations of action of $<$30 min, compared with $>$12 hr for salmeterol (in which the ether oxygen is located six carbons from the amine), 2) the saligenin head of salmeterol produces high β_2-selectivity, and 3) the kinetics at the receptor are governed by the side chain. It is apparent that the side chain containing the ether oxygen of salmeterol adopts a turn to interact with the amino acids in TMDs III, IV, and V. The results with alanine-substituted mutants suggested that candidate amino acids to interact with the ether oxygen were Tyr308 and Ile309 in TMD VII. However, the molecular modeling did not support this idea. Tyr308 could not directly interact with the ether oxygen of salmeterol because of the constraints on the flexible movement of the side chain, considering the well-established interaction sites. The present model predicted that the ether oxygen would interact with Tyr316 in TMD VII, instead of Tyr308. The model also suggested that Asp113 in TMD III interacted with the ether oxygen when more favorable interactions with Tyr316 were disrupted. It is necessary to perform more mutagenesis experiments to establish a model of the salmeterol-β_2AR complex, especially focusing on the long-lasting action of salmeterol. In conclusion, we demonstrated that Tyr308 in TMD VII is the key amino acid for the β_2-selective binding of salmeterol and that the position of the ether oxygen in the side chain plays an important role in the β_2-selectivity of salmeterol.

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