0026-895X/98/010128-07$3.00/0

MOLECULAR PHARMACOLOGY, **53:**128–134 (1998).

The Role of the Seventh Transmembrane Region in High Affinity Binding of a β_2-Selective Agonist TA-2005

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Received May 14, 1997; Accepted October 1, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

To determine the structural basis for binding subtype selective agonists in the β-adrenergic receptor (βAR), we examined the interaction of the mutant β_2AR and chimeric β_1/β_2AR with a selective β_2AR agonist, TA-2005 (8-hydroxy-5-[(1*R*)-1-hydroxy-2-[*N*-[(1*R*)-2-(*p*-methoxyphenyl)-1-methylethyl]amino]ethyl] carbostyril hydrochloride). The β_2AR mutant with Ala substituted for Ser204 (S204A) significantly decreased the affinities for TA-2005, des-8-hydroxy-TA-2005 derivative (compound I), and isoproterenol. In contrast, a S207A mutation slightly decreased the affinities for TA-2005 and compound I, although the affinity for isoproterenol was decreased dramatically. The EC_{50} values of TA-2005 to activate adenylyl cyclase were not changed in either the S204A- or S207A-β_2AR. In contrast with TA-2005, the EC_{50} values of compound I were reduced in the S204A-β_2AR but not in the S207A-β_2AR. These results suggest that Ser204 is important for high affinity binding but not necessary to activate adenylyl cyclase. Although TA-2005 was highly selective at the β_2AR, the compounds lacking *p*-methoxyphenyl-ethyl (compound II) or *p*-methoxyphenyl-methylethyl groups (compound III) on the amine portion of TA-2005 lost β_2AR subtype selectivity. When the second and seventh transmembrane (TM) region but not the TM1 region of the β_2AR were replaced with the corresponding regions of the β_1AR, the affinities of the chimeras for TA-2005 decreased compared with those of the wild type β_2AR. Furthermore, substitution of the TM7 region of the β_1AR with the corresponding region of the β_2AR significantly increased the affinities for TA-2005. The affinities for isoproterenol and compounds II and III were not affected in the chimeras. These data suggest that the TM7 region of the β_2AR plays an important role in β_2-selective agonist binding. To determine the specific amino acid which confers this high affinity binding of TA-2005 to the β_2AR, an alanine-scanning mutagenesis approach was employed. All amino acids that were different from those of the β_1AR were individually changed to alanine. One mutant receptor (Y308A-β_2AR) out of 10 point-mutated β_2ARs showed a dramatically reduced affinity for TA-2005. These results indicate that Tyr308 is an essential amino acid for high affinity binding of the β_2-selective agonist TA-2005.

ARs are members of the G protein-coupled receptor superfamily and are classified into three groups (i.e., α_1, α_2, and β) (Bylund *et al.*, 1994; Hieble *et al.*, 1995). βARs consist of three subtypes, β_1, β_2, and β_3. Endogenous agonists, norepinephrine, epinephrine, and the synthetic full agonist isoproterenol bind to the β_2AR and induced conformational changes to activate the G protein. Mutagenesis experiments have revealed that the binding sites of isoproterenol on the β_2AR seem to be located in the TM regions (Dixon *et al.*, 1987; Dohlman *et al.*, 1988). It is assumed that aspartic acid at position 113 in the TM3 region of the β_2AR form an ionic bond with the amino group of isoproterenol (Strader *et al.*, 1987, 1988, 1989a). It is also assumed that the catechol hydroxyl groups of the agonist isoproterenol interact with the side chains of Ser204 and Ser207 in the TM5 region of the receptor (Strader *et al.*, 1989b). Although the TM regions are well conserved among the three βAR subtypes (70% for β_1 versus β_2, 70% for β_1 versus β_3, 63% for β_2 versus β_3), the β_1AR and the β_2AR show different affinities for the various synthetic agonists and antagonists including the endogenous agonist norepinephrine (Emorine *et al.*, 1989; Frielle *et al.*, 1987; Kobilka *et al.*, 1987; Stiles and Lefkowitz, 1984). Frielle *et al.* (1988) have constructed a series of chimeric β_1/β_2ARs to analyze the binding domains of β_1- and β_2-selective ligands. The gradual replacement of the TM regions of the β_2AR with those of the β_1AR result in receptors that show a gradual loss of β_2AR selectivity and a gain in β_1AR selectivity. The β_1ARs with homologous replacement show a gradual loss of β_1AR selectivity and a gain in β_2AR selectivity. Frielle *et al.* (1988) have concluded that the TM4 region is a major determinant of the β_1 and β_2AR selectivity of agonist norepinephrine and that the TM6 or TM7 regions play a major role in determining β_2AR selectivity for the β_2AR antagonist ICI 118551 or a β_1AR selectivity for β_1AR antagonist betaxolol. Another

ABBREVIATIONS: AR, adrenergic receptor; TM, transmembrane; WT, wild type; CYP, cyanopindolol; DMEM, Dulbecco's modified Eagle's medium; CH, chimera.

group has employed the approach to randomly exchange the TM regions of the βARs and determine the binding characteristics of subtype selective antagonists in these chimeric receptors (Marullo *et al.*, 1990). They have shown that the TM region's contribution to the subtype selective binding of the β_1 and β_2ARs differed between ligands. However, the random exchange of the TM regions may underestimate or overestimate the contribution of some TM regions to binding of a subtype selective ligand, because more than one TM region mutates at once and it is possible that some TM region are not involved in the subtype selective binding. Thus, determining which regions of the βAR confer the subtype selectivity, especially for agonists, has so far been only tentative.

TA-2005 is a non-catechol β_2AR agonist with a *p*-methoxyphenyl group on the amine side chain and a 8-hydroxyl group on the carbostyril aromatic ring (see Fig. 1). We have previously shown that, compared with other β_1 and β_2ARs, TA-2005 has a high selectivity as well as a high affinity for the β_2AR in pharmacological and radioligand-binding studies using isolated guinea pig tissues (Kikkawa *et al.*, 1991). Based on *in vivo* studies, we have reported that TA-2005 has long lasting bronchodilating effects (Kikkawa *et al.*, 1994). Voss *et al.* (1992) have also reported that TA-2005 shows a high potency for the β_2AR and a long duration of action after removal of the drug using both guinea pig tracheal muscle relaxation and bovine trapezium muscle binding experiments.

In the present study we putatively assigned the binding sites of the β_2AR for carbostyril moiety of TA-2005 and determined the specific amino acid to be responsible for β_2-selective binding. We made several site-directed mutant β_2ARs and eight chimeric β_1/β_2ARs, which were expressed in COS-7 cells, then analyzed the binding characteristics of TA-2005 and derivatives for these receptors, and compared these characteristics with those of isoproterenol.

Experimental Procedures

Materials

[^{125}I]Iodocyanopindolol (2200 Ci/mmol) and [^{3}H]adenine (24.0–27.0 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). [^{14}C]cAMP (20.0–50.1 mCi/mmol) was obtained from DuPont-New England Nuclear Research (Boston, MA), American Radiolabeled Chemicals (St. Louis, MO), or Moravek Biochemicals (Brea, CA). The plasmid constructs pBC-β_1 and -β_2 encoding for 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 of Dr. S. Nagata (Osaka University, Osaka, Japan). (−)Isoproterenol, (±)propranolol, and DEAE-dextran were purchased from Sigma Chemical (St. Louis, MO). DMEM and gentamicin were from GIBCO/BRL (Grand Island, NY). Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS). *Taq* or *Pfu* DNA polymerases were from Takara (Siga, Japan) or Stratagene (La Jolla, CA), respectively. GTP was from Seikagaku (Tokyo, Japan). TA-2005 (8-hydroxy-5-[(1*R*)-1-hydroxy-2-[*N*-[(1*R*)-2-(*p*-methoxy-phenyl)-1-methylethyl] amino]ethyl] carbostyril, hydrochloride), compound I (5-[(1*R*)-1-hydroxy-2-[*N*-[(1*R*)-2-(*p*-methoxy-phenyl)-1-methylethyl-]amino]ethyl]quinolin-2(1*H*)-one, hydrochloride), compound II (5-[(2-amino-1-hydroxy)ethyl]-8-hydroxycarbostyril, hydrochloride), and compound III (5-[(1-hydroxy-2-isopropylamino)ethyl]-8-hydroxycarbostyril, hydrochloride) were synthesized at the Lead Optimization Research Laboratory, Tanabe Seiyaku (Saitama, Japan). The structures of these compounds are shown in Fig. 1.

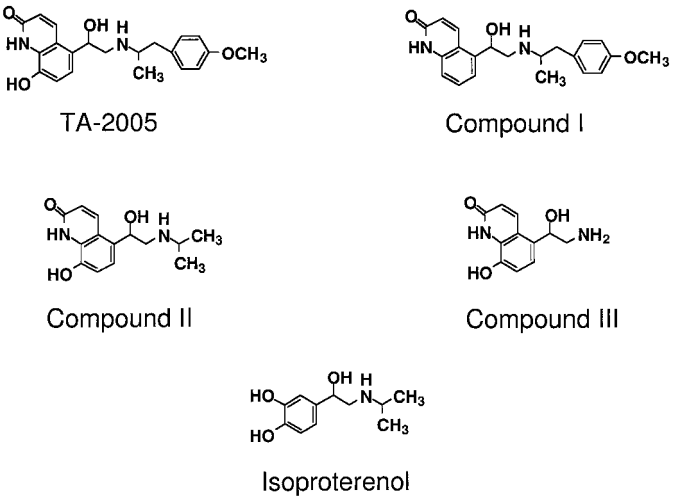

Fig. 1. The chemical structures of TA-2005, compounds I–III derivatives of TA-2005, and isoproterenol.

DNA constructions, cell transfection, and culture. The epitope sequence (YPYDVPDYA) recognized by monoclonal antibody 12CA5 (Wilson *et al.*, 1984) was inserted at the amino terminus of human β_1 and β_2ARs to evaluate the expression of the receptors (Barak *et al.*, 1994; Sato *et al.*, 1996; Von Zastrow and Kobilka, 1992). The epitope did not change the binding characteristics of β_1 and β_2ARs to the ligands (data not shown). Chimeric receptors and site-directed mutants of the β_1 and β_2ARs were constructed by the polymerase chain reaction method (Higuchi, 1989). The positions of the junctions for individual chimeric β_1 and β_2ARs are as follows (numbers refer to amino acid positions in the human β_1 and β_2AR sequences): CH1, β_1 1–84/β_2 60–413; CH2, β_2 1–71/β_1 97–131/β_2 107–413; CH3, β_2 1–295/β_1 347–381/β_2 331–413; CH4, β_2 1–71/β_1 97–131/β_2 107–295/β_1 347–381/β_2 331–413; CH5, β_2 1–59/β_1 85–477; CH6, β_1 1–96/β_2 72–106/β_1 132–477; CH7, β_1 1–346/β_2 296–330/β_1 382–477; CH8, β_1 1–96/β_2 72–106/β_1 132–346/β_2 296–330/β_1 382–477. The sequences of the amplified regions were confirmed by the dideoxy chain termination method (Sanger *et al.*, 1977). Chimeric and mutated cDNAs were inserted into the *Eco*RI and *Bam*HI or *Eco*RI and *Sal*I sites of the mammalian expression vector pCMV5. The alanine-scanning point mutants of the β_2AR were made by the Quick change method according to manufacture's instructions (Stratagene, La Jolla, CA). The two oligonucleotides (33–36 base pairs) and the *Bgl*II/*Eco*RV fragment of the β_2AR in pSL1190 (Pharmacia LKB, Uppsala, Sweden) were used as primers or as a template, respectively. After the sequences were confirmed by the dideoxy chain termination method, the rest of the coding regions were ligated to make point-mutated full-length β_2ARs. The resulting constructs were inserted into the *Xba*I site of pEF-BOS (Mizushima and Nagata, 1990). For the binding studies, these constructs were transfected into COS-7 cells by the DEAE-dextran method (Cullen, 1987). Before the day of transfection, the COS-7 cells were seeded at 1.5×10^6 cells per 100-mm dish. The concentration of the chimeric or mutated βAR cDNAs were 5 μg per 100-mm dish. All cells were maintained in DMEM containing 10% fetal bovine serum and gentamicin (10 μg/ml). Two to three days after the transfection, the cells were harvested for preparation of the crude membrane fraction. For the cAMP accumulation assay, the expression constructs of WT-, S204A-, and S207A-β_2ARs were transfected into the JEG-3 cells as described above, except that the concentration of DEAE-dextran was reduced to 250 μg/ml and the JEG-3 cells were seeded at 1.5–2.0 × 10^6 cells/100-mm dish. The JEG-3 cells were maintained in DMEM containing 10% fetal bovine serum and gentamicin (10 μg/ml).

Radioligand binding assay. The cells were rinsed twice with 10 ml of ice-cold phosphate-buffered saline and mechanically detached in 1 ml of an ice-cold buffer containing 5 mM Tris·HCl (pH 7.4) and 2

mM EDTA. The lysate was centrifuged at 45,000 × *g* for 10 min at 4°. The pellet containing membrane fraction was resuspended in 1 ml of buffer containing 75 mM Tris·HCl (pH 7.4), 12.5 mM $MgCl_2$ and 2 mM EDTA with Potter type homogenizer and stored at −80° until use. A competition binding assay was performed in duplicate using ~10 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 rapid filtration over Whatman GF/C filters and washing with an ice-cold 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 protein concentration was determined by the method of Lowry *et al.* (1951).

cAMP accumulation assay. Two days after the transfection, JEG-3 cells were incubated overnight with [^{3}H]adenine (2 μCi/ml). On day 4 the cAMP accumulation was measured in the absence of activator (basal activity) or in the presence of test compounds for 15 min at 37° with 1 mM 3-isobutyl-1-methylxanthine. The reaction was terminated by the addition of 1 ml of ice-cold stop solution containing 2.5% perchloric acid, 0.2 mM cAMP, and [^{14}C]cAMP (about 10,000 cpm). After being neutralized with 4.2 M KOH, the precipitate was removed by centrifugation at 5,000 rpm for 5 min at 4° in a microcentrifuge. The supernatant was sequentially processed by Dowex and by aluminum oxide columns for isolation of [^{3}H]cAMP.

Data analysis and statistics. All results are expressed as an arithmetic mean together with mean ± standard error of the mean for *n* determinations except the K_i and K_d values, which are expressed as geometric means with 95% confidence limits. Equilibrium dissociation constants were determined from the saturation isotherms. Radioligand binding data were analyzed by a nonlinear regression analysis to determine IC_{50} and K_i values using PRISM software (GraphPAD Software, San Diego, CA). Statistical significance was assessed with the analysis of variance for multiple comparisons; a probability value of $p < 0.05$ was considered as a significant difference.

Results

Interaction of TA-2005 with Ser204 and Ser207 of β_2AR. To investigate whether TA-2005 interacts with the same serine residues of the β_2AR as isoproterenol, we assessed the affinities of compound I, the des-8-hydroxy derivative of TA-2005, for the S204A- and S207A-β_2ARs. The K_d values of ^{125}I-CYP for the WT-, S204A-, and S207A-β_2ARs, respectively, were 56, 34, and 32 pM. TA-2005 had much higher affinity for the WT-β_2AR than for isoproterenol and the affinity of TA-2005 for the S204A-β_2AR was decreased 56-fold but only slightly for the S207A-β_2ARs (4-fold), as compared with the WT-β_2AR (Fig. 2) [see also Kikkawa *et al.* (1997)]. Isoproterenol bound to the S204A- and S207A-β_2ARs with 27- and 13-fold lower affinities, respectively, than to the WT-β_2AR. In the present study, although the affinity of compound I for the S204A-β_2AR was decreased 22-fold, the affinity for the S207A-β_2AR was essentially the same as that of the WT-β_2AR (Fig. 2).

The contribution of Ser204 and Ser207 to the binding of TA-2005 was also determined by a functional assay. We used JEG-3 cells for the cAMP accumulation assay with two reasons. First, the JEG-3 cells had low basal adenylyl cyclase activity. In addition there was no significant cAMP accumulation by isoproterenol stimulation, even with high concentration (100 μM) of isoproterenol. These two characteristics allowed us to detect small changes in the cAMP contents by agonist stimulation in transiently transfected cells. The EC_{50} values of isoproterenol in the S204A- and S207A-β_2ARs were increased by 12- and 4.3-fold, respectively (Fig. 3), consistent with the previous report (Strader *et al.*, 1989b). The EC_{50} values of TA-2005 and compound I were slightly increased in the S204A-β_2ARs (2.0- and 7.6-fold, respectively) but not in the S207A-β_2ARs. In the WT-, S204A-, and S207A-β_2ARs, both compound I and TA-2005 activated the adenylyl cyclase to the same extent as isoproterenol.

Selectivity for the WT-β_2AR. To determine which portion of the TA-2005 molecule is important for β_2AR selectivity, we synthesized two compounds that lack *p*-methoxyphenyl (compound II) or *p*-methoxyphenyl methylethyl groups (compound III), and examined the affinities of these compounds for the WT-β_1 and β_2ARs. TA-2005 showed a 53-fold higher selectivity for the β_2AR than for the β_1AR, whereas isoproterenol showed no selectivity for the β_1 and β_2ARs (Fig. 4, A and B). In contrast to TA-2005, compounds II and III completely lost their βAR subtype selectivity (Fig. 4, C and D).

Affinity for β_1/β_2AR chimeras. To determine the domain(s) of the β_2-receptor that interact with the *p*-methoxyphenyl group on the amine portion of TA-2005, eight β_1/β_2 chimeric receptors were constructed and expressed in COS-7 cells (see Fig. 5 for structures). The ligand-binding properties of the resultant chimeric receptors are summarized in Tables 1 and 2. The K_d values of the radioligand ^{125}I-CYP in the chimeric receptors were essentially the same as those of the WT-β_1 and β_2ARs except in CH4 with slightly low affinity of ^{125}I-CYP.

When the TM2 or TM7 regions of the β_2AR were replaced with the corresponding regions of the β_1AR (CH2 and CH3), the affinities of TA-2005 were significantly decreased by 7- and 8-fold, respectively (Table 1). In contrast, the affinities of isoproterenol and compound III did not change significantly in CH2 and CH3. The replacement of both the TM2 and TM7 regions of the β_2AR with those of the β_1AR (CH4) markedly decreased the affinity of TA-2005 (20-fold). The affinity of isoproterenol for CH4 increased, although the extent of increase was small (1.9-fold) (Table 1).

The affinities of TA-2005 were not increased by the replacement of the TM1 and TM2 regions of the β_1AR with

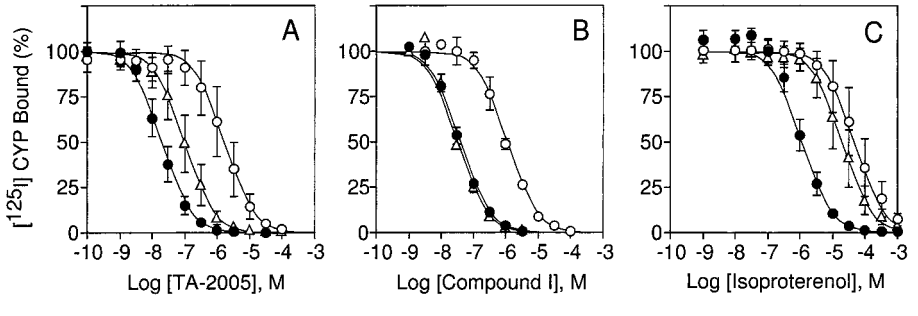

Fig. 2. Competition binding of TA-2005 (A), isoproterenol (B), and compound I (C) to the wild-type and mutant β_2ARs. Competition binding was performed with membranes prepared from COS-7 cells transfected with the WT-β_2AR (●), S204A-β_2AR (○), or S207A-β_2AR (△) expression constructs. The data are the mean ± standard error of three experiments done in duplicate.

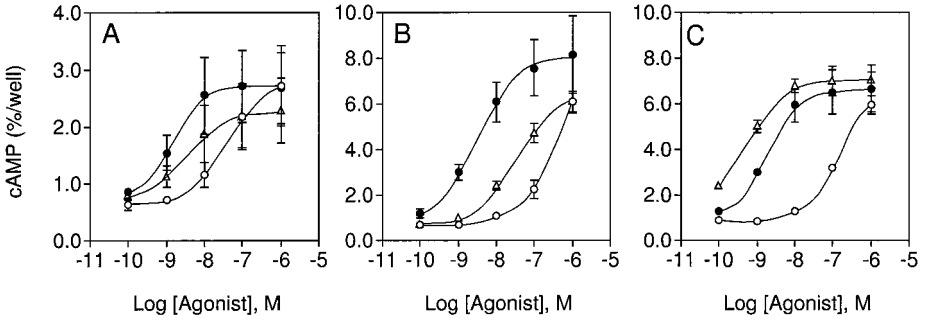

Fig. 3. Cyclic AMP production by TA-2005 (●), isoproterenol (○), and compound I (△) through activation of the WT-β_2 (A), S204A-β_2 (B), or S207A-β_2 (C) ARs. The cAMP accumulation assay was performed with JEG-3 cells transfected with one of the βAR constructs. The data are the mean ± standard error of three experiments done in duplicate.

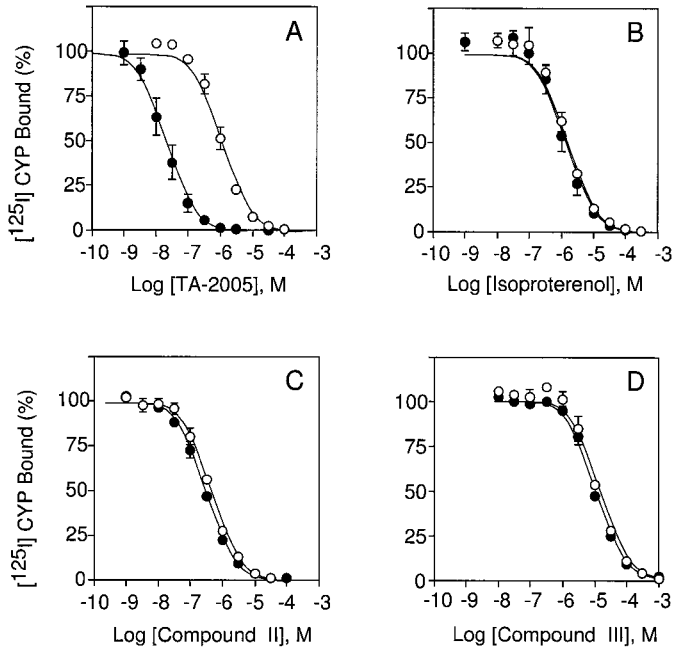

Fig. 4. Competition binding of TA-2005 (A), isoproterenol (B), compound II (C), and compound III (D) to the WT-β_1 (○) and WT-β_2 (●) ARs. Competition binding was performed with membranes prepared from COS-7 cells transfected with either of the βAR constructs. The data are the mean ± standard error of three experiments done in duplicate.

those of the β_2AR (CH5 and CH6), although these affinities would be expected to increase if these regions were involved in the β_2-selective binding (Table 2). When the TM7 region of the β_1AR was replaced with homologous region of the β_2AR (CH7), the affinity of TA-2005 increased 3-fold. Although the affinity of TA-2005 for CH8 was essentially the same as that for the WT-β_2AR, the affinities for all of the ligands were also increased in the CH8 receptor (Table 2). These nonspecific increases in the affinity for all of the ligands obscured the contribution of the TM2 region to the β_2 selectivity.

Alanine-scanning mutants of β_2ARs. There are 10 positions in the TM7 region of the β_2AR in which the amino acid residues are different from those of the β_1AR. To identify the amino acid which is important in β_2-selective agonist binding, each of the amino acids was changed to alanine. One mutant (Y308A-β_2AR) out of 10 different alanine-substituted mutants, in which Tyr308 was changed to alanine, showed a dramatically decreased affinity for TA-2005 (Table 3). Although the Y308A-β_2AR also showed the decreased affinity for isoproterenol, the extent of the decrease in affinity was smaller than that of TA-2005. Furthermore, the Y308F-β_2AR mutant, in which Tyr308 in the TM7 region of the β_2AR was replaced with the corresponding amino acid residue (Phe) of the β_1AR, showed decreased affinity for TA-2005 (Y308F-β_2AR: 61 nM versus WT-β_2AR: 12 nM, $p < 0.05$), although that of isoproterenol for Y308F-β_2AR was essentially the same as that of the WT-β_2AR (1800 and 900 nM, respectively). The replacement of Phe359 of the β_1AR with Tyr (F359Y-β_1AR), which is a complementary mutant of Y308F-β_2AR, increased the affinity for TA-2005 by 2.5-fold. However, we have also observed that F359Y-β_1AR decreased the affinity for ^{125}I-CYP by 19-fold, whereas it increased the affinity for isoproterenol by 7-fold (data not shown).

Discussion

Mutagenesis experiments have determined the binding sites of the nonselective agonists and selective antagonists for the β_2AR (Dixon *et al.*, 1989; Frielle *et al.*, 1988; Marullo *et al.*, 1990). However, the binding sites of the selective β_2AR agonists are little known. We have studied the binding sites of TA-2005, one of the most selective β_2AR agonists with a hydroxy-carbostyril structure. TA-2005 has one hydroxyl group and one amido group at positions corresponding to the *p*- and *m*-hydroxyl groups of isoproterenol, respectively (Fig. 1).

At first, we compared the binding sites of the hydroxy-carbostyril moiety of TA-2005 to those of the catechol moiety of isoproterenol. For this purpose, we synthesized compound I and constructed two site-directed mutants in which Ser204 or Ser207 in the TM5 region were changed to alanine. It was proposed that these serines should interact with the *m*- and *p*-hydroxyl groups of isoproterenol (Strader *et al.*, 1989b). Replacement of Ser204 or Ser207 with alanine decreased the affinities of isoproterenol and TA-2005, although the change in the affinity of TA-2005 for the S207A-β_2AR was smaller than that of isoproterenol (Kikkawa *et al.*, 1997). In contrast with binding experiments, the functional assay for TA-2005-stimulated cAMP accumulation showed no differences in the EC_{50} values of TA-2005 in the WT-β_2AR and the two mutants. These data suggest that Ser204 may be an important determinant for the high affinity binding of TA-2005 but not necessarily for the activation of adenylyl cyclase. It also suggests that one of the two serines in the TM5 region of the β_2AR may be enough to activate the adenylyl cyclase by TA-2005 with high affinity. Because the affinity of compound I for the S207A-β_2AR was similar to that for the WT-β_2AR, and the affinity of compound I decreased in the S204A-β_2AR, it seems that the 8-hydroxyl group of TA-2005 may interact with the Ser207 hydroxyl group of the β_2AR. However, it is necessary to analyze the interaction of another derivatives of TA-2005 with more mutants to determine the precise inter-

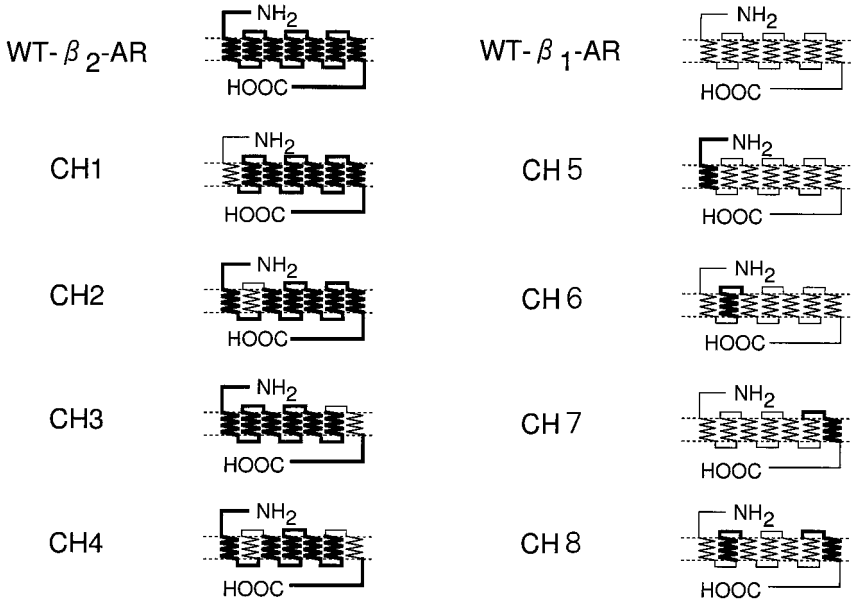

Fig. 5. Structures of chimeric β_1/β_2ARs. *Thin lines*, peptide sequences derived from the β_1AR; *thick lines*, peptide sequences derived from the β_2AR. The positions of the junctions are described in Experimental Procedures.

TABLE 1

Effects of substitution of the TM regions with corresponding portion of the β_1AR on the ligand-binding characteristics of the β_2AR

The binding of ligands to the WT- and CH-βARs was measured by competition with 50 pM ^{125}I-CYP. The data were analyzed using the nonlinear least-squares regression computer program as described in Experimental Procedures. The results are shown as the means and 95% confidence limits of three to four separate experiments.

Receptor	^{125}I-CYP (K_d)	Agonist (K_i)			
		TA-2005	Isoproterenol	Compound II	Compound III
	pM		*nM*		
WT-β_2AR	73 (43–120)	12[a] (1.8–82)	900[a] (200–4000)	170 (130–230)	6300 (4800–8400)
CH1	86 (44–170)	9.2 (7.2–12)	1400 (440–4600)	190 (98–370)	7900 (5300–12000)
CH2	100 (63–170)	78[b] (22–280)	1000 (530–1900)	130 (79–210)	3800 (1400–10000)
CH3	150 (100–210)	96[b] (15–610)	560 (190–1600)	320[c] (120–900)	8000 (5000–13000)
CH4	280[c] (280–420)	240[b] (84–690)	400[c] (230–710)	220 (79–590)	5200 (1700–16000)

[a] Data from Kikkawa *et al.* (1997) for the clarity.
[b] $p < 0.01$, [c] $p < 0.05$, significantly different from WT-β_2AR.

TABLE 2

Effects of substitution of the TM regions with corresponding portion of the β_2AR on the ligand-binding characteristics of the β_1AR

The binding of ligands to the WT- and CH-βARs was measured by competition with 50 pM ^{125}I-CYP. The data were analyzed using the nonlinear least-squares regression computer program as described under Experimental Procedures. The results are shown as the means and 95% confidence limits from three to four separate experiments.

Receptor	^{125}I-CYP (K_d)	Agonist (K_i)			
		TA-2005	Isoproterenol	Compound II	Compound III
	pM		*nM*		
WT-β_1AR	110 (62–180)	630[a] (310–1300)	950[a] (630–1400)	290 (200–430)	8400 (5300–13000)
CH5	410 (82–2000)	770 (290–2100)	450[b] (200–1000)	320 (140–720)	7600 (3000–19000)
CH6	110 (25–500)	540 (140–2100)	630 (450–870)	410 (210–810)	8700 (3700–21000)
CH7	280 (200–390)	220[b] (86–550)	1300 (360–4600)	390 (190–770)	8300 (4600–15000)
CH8	110 (20–630)	16[c] (1.9–140)	410[b] (250–650)	81[c] (57–120)	3800[b] (2600–5600)

[a] Data from Kikkawa *et al.* (1997) for the clarity.
[b] $p < 0.05$, [c] $p < 0.01$, significantly different from WT-β_1AR.

action sites for the hydroxy-carbostyril moiety and the TM5 region of the β_2AR.

To determine the structural basis for the β_2AR selectivity, we assessed the affinities of TA-2005 and its derivatives for a series of chimeric β_1/β_2ARs. Although TA-2005 had a 53 times higher affinity for the β_2AR than for the β_1AR, compound II and III which lacked the *p*-methoxyphenyl group of TA-2005 completely lost their β_2 selectivity. Kontoyianni *et*

TABLE 3

Effects of substitution of single amino acid in the TM7 region of the β_2AR with alanine on the ligand-binding characteristics

The binding of ligands to the WT- and mutant-βARs was measured by competition with 50 pM ^{125}I-CYP. The data were analyzed using the nonlinear least-squares regression computer program as described under Experimental Procedures. Results are shown as the means and 95% confidence limits from three separate experiments.

Receptor	^{125}I-CYP (K_d)	Agonist (K_i)	
		TA-2005	Isoproterenol
	pM	*nM*	
WT-β_2AR	31 (18–55)	4.3 (1.4–13)	240 (180–320)
E306A	33 (17–65)	4.7 (3.8–5.7)	200 (90–430)
V307A	35 (29–41)	6.3 (5.0–7.9)	330 (200–540)
Y308A	27 (23–31)	71[a] (45–110)	1300[a] (790–2000)
I309A	34 (30–39)	21[a] (13–32)	360 (190–670)
L310A	39 (24–66)	5.5 (3.3–9.2)	280 (71–1100)
L311A	28 (23–35)	12[a] (9.1–16)	360 (52–2400)
I314A	28 (24–31)	3.2 (2.1–4.8)	150 (93–240)
V317A	32 (24–43)	5.9 (4.1–8.6)	310 (240–400)
G320A	49[a] (48–50)	9.0[b] (4.9–17)	480[b] (320–720)
L324A	36 (33–39)	21[a] (15–29)	280 (180–440)

[a] $p < 0.01$, [b] $p < 0.05$, significantly different from WT-β_2AR.

al. (1996) have suggested that, based on a computer-modeling technique, the large 2-phenylethyl *N*-substituent of TA-2005 can lie in a pocket formed by the TM2 and TM7 regions. Their binding model supports our finding that the replacement of either the TM2 or the TM7 regions of the β_2AR, or both with homologous regions of the β_1AR significantly decreases the affinities of TA-2005 but not of compound II and III. There have been several reports that the TM2 region of the gonadotropin-releasing hormone receptor should be in close the proximity to the TM7 region and that both regions participate in hormone binding (Arora *et al.*, 1996; Awara *et al.*, 1996; Davidoson *et al.*, 1996; Zhou *et al.*, 1994).

To avoid the misleading conclusions that can arise from the use of loss-of-function mutants, in which chimeric receptor mutants lose their the β_2 selectivity, we made a series of gain-of-function mutants, in which the chimeric receptor mutants gained β_2 selectivity. The replacement of the TM7 but not the TM2 regions significantly increased the affinities of TA-2005. Although the β_1AR with both TM2 and TM7 regions of the β_2AR almost completely restored the β_2 selectivity to the level of the WT-β_2AR, the resultant chimeric receptor (CH8) also increased the affinity of isoproterenol. This suggests that the TM7 region of the β_2AR contributes to β_2-selective agonist binding, but the contribution of the TM2 region to this binding is not definitive. It has recently been reported that the long lipophilic side chain of salmeterol, a β_2-selective agonist, interacts with residues 149–158 within the TM4 region of the β_2AR (Green *et al.*, 1996). It has also been shown that the TM4 region is a domain necessary for the persistent binding of salmeterol to the β_2AR (i.e., exosite of the β_2AR). This region, however, does not seem to contribute to β_2-selective binding of salmeterol, because the β_2AR with the TM4 region of the β_1AR lost its ability to persistently bind salmeterol but still retained the β_2 selectivity. This suggests that the region that confers β_2 selectivity to the β_2AR is distinct from the exosite. These results also support our assumption that the TM7 region is important for the β_2-selective agonist TA-2005 in binding to the β_2AR with high affinity.

To identify the amino acid that is important for the high affinity binding of a β_2-selective agonist, each of the amino acids in TM7 region that are different from those of the β_1AR were changed to alanine. One mutant (Y308A-β_2AR) out of 10 alanine-substituted mutants significantly decreased its affinity for TA-2005. Furthermore, the affinity of TA-2005 for the Y308F-β_2AR was significantly decreased, although the affinity of isoproterenol was essentially the same as that of the WT-β_2AR. These results suggest that Tyr308 is a major determinant for the binding of the β_2-selective agonist TA-2005 and Tyr308 may interact with the side chains of N-substituted TA-2005. Although the affinity of TA-2005 increased in the F359Y-β_1AR, which is a complementary mutant of the Y308F-β_2AR, the affinities for ^{125}I-CYP and isoproterenol were also changed dramatically. This indicates that the replacement of Phe of the β_1AR with Tyr may cause an overall structural change of the β_1AR and the substituted Tyr may provide an additional binding site for the ligands. Photoaffinity labeling experiments have shown the direct interaction between the TM7 region and the aryloxy portion of the βAR antagonists such as pindolol, CGP-12177A, and CYP (Dohlman *et al.*, 1988; Hockerman *et al.*, 1996; Wong *et al.*, 1988). Each of the three photoaffinity labels, [^{125}I]iodocyanopindolol-diaserine, [^{125}I]iodoasidobenzylpindolol, and ^{125}I-asidophenyl CGP12177A was incorporated at the TM6 an TM7 regions of the purified βAR as well as other TM regions, depending on the photoaffinity label. These data support our assumption that *N*-substituent of the ligands can interact with Tyr308 in the TM7 region and contribute to subtype selective binding with high affinity.

From the results of chimeric and alanine-substituted mutants, we have concluded that Tyr308 in the TM7 region of the β_2AR is crucial for the high affinity binding of the β_2-selective agonist TA-2005. This is the first report to show that a specific residue in the TM7 region is involved in the binding of a β_2-selective agonist. It remains to be determined whether Tyr308 also plays an important role in the binding of other β_2-selective agonists such as salmeterol, formoterol and procaterol.

Acknowledgments

We are grateful to Drs. K. Naito and A. Saito (Tanabe Seiyaku) for their helpful suggestions, to Dr. P.W. Tsao for reviewing this manuscript, and to Dr. Inoue (Tanabe Seiyaku) who kindly synthesized the compounds for us. We also thank Dr. R. J. Lefkowitz for the pBC-β_1 and β_2 plasmids and Dr. S. Nagata for the pEF-BOS plasmid.

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