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Involvement of Asn-293 in stereospecific agonist recognition and in activation of the β_2-adrenergic receptor

(stereospecificity/intrinsic activity/isoproterenol/propranolol/ligand binding)

KERSTIN WIELAND*, HELENE M. ZUURMOND†, CORNELIUS KRASEL*, AD P. IJZERMAN†, AND MARTIN J. LOHSE*‡

*Department of Pharmacology, University of Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany; and †Leiden/Amsterdam Center for Drug Research, Division of Medicinal Chemistry, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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ABSTRACT **To investigate the molecular mechanism for stereospecific binding of agonists to β_2-adrenergic receptors we used receptor models to identify potential binding sites for the β-OH-group of the ligand, which defines the chiral center. Ser-165, located in transmembrane helix IV, and Asn-293, situated in the upper half of transmembrane helix VI, were identified as potential binding sites. Mutation of Ser-165 to Ala did not change the binding of either isoproterenol isomer as revealed after transient expression in human embryonic kidney (HEK)-293 cells. In contrast, a receptor mutant in which Asn-293 was replaced by Leu showed substantial loss of stereospecific isoproterenol binding. Adenylyl cyclase stimulation by this mutant after stable expression in CHO cells confirmed the substantial loss of stereospecificity for isoproterenol. In a series of agonists the loss of affinity in the Leu-293 mutant receptor was strongly correlated with the intrinsic activity of the compounds. Full agonists showed a 10–30-fold affinity loss, whereas partial agonists had almost the same affinity for both receptors. Stereospecific recognition of antagonists was unaltered in the Leu-293 mutant receptor. These data indicate a relationship between stereospecificity and intrinsic activity of agonists and suggest that Asn-293 is important for both properties of the agonist–receptor interaction.**

β_2-Adrenergic receptors (β_2-AR) are often studied as a model system for the large superfamily of G-protein-coupled receptors. These receptors most likely contain seven transmembrane α-helices, and their topography has been verified using biochemical and immunological techniques to identify extra- and intracellular domains (1, 2). The binding of agonists to these receptors has been studied in much detail to understand the molecular mechanisms of ligand docking and receptor activation (3). A series of mutagenesis experiments plus the analysis of large numbers of ligands has allowed the identification of several of the amino acids involved in agonist binding to β_2-AR (reviewed in refs. 4 and 5). The current concept of agonist binding proposes that the positively charged nitrogen interacts with Asp-113 in transmembrane domain III (6, 7), and that the two catechol-OH-groups form hydrogen bonds with Ser-204 and Ser-207 in transmembrane domain V (8).

Despite these advances, one of the key properties of agonists at these receptors has not been clarified: their stereospecific binding. Stereospecificity of β-adrenergic agonists is defined by their β-OH-group, which is located at the chiral center. β_2-AR bind their agonists such as isoproterenol in a stereospecific manner, with the (−)isomer being about 40-times more potent than the (+)isomer, both in the high-affinity state (equivalent to the receptor/G-protein complex) and in the low-affinity state (9). In addition to its potential role in stereospecificity, the β-OH-group might also be involved in the agonistic properties of these compounds (3). The amino acids that interact with this β-OH-group and may therefore be responsible for stereospecificity have not been identified.

Apart from relatively low-resolution electron diffraction results obtained with rhodopsin (10), there are no biophysical data on G-protein-coupled receptors. Several authors have therefore developed computer models of G-protein-coupled receptors to predict their structure and modes of interaction with their ligands. The first group of such models uses the known structure of bacteriorhodopsin, which also contains seven transmembrane α-helices (11), to develop a backbone of the transmembrane helices of G-protein-coupled receptors (12, 13). The second group of models avoids the use of bacteriorhodopsin as a template because it is a developmentally probably unrelated archaebacterial proton pump and not a receptor (14). The latter models are based essentially on sequence alignments of many G-protein-coupled receptors and their hydrophobicity profiles or their polarity conserved positions (15, 16). Finally, both sets of information have often been used in attempts to obtain models of G-protein-coupled receptors (reviewed in refs. 17 and 18).

Obviously, all of these models contain a great deal of speculation. However, assuming that the helices are arranged in a circular and sequential (anti-clockwise when viewed from the top) manner [which is the prevailing hypothesis even though a different order has also been proposed by Pardo *et al.* (19)], then anchoring agonists at helices III and V, as described above, leaves essentially helices IV and VI as potential anchoring points for the β-OH-group.

Trumpp-Kallmeyer *et al.* (13) have proposed that Ser-165 in helix IV binds the β-OH-group, but this hypothesis has not yet been investigated experimentally. In the present study, we have used computer modelling of the β_2-AR to identify Asn-293 as another potential interaction site for this β-OH-group, and have investigated the roles of these amino acids by site-directed mutagenesis.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. CHO-10001 cells were kindly provided by M. Gottesman (National Institutes of Health). ^{125}Iodo-cyanopindolol (^{125}I-CYP) and [α-^{32}P]ATP were obtained from New England Nuclear, and the latter was purified as described by Walseth and Johnson (20). Stereoisomers of isoproterenol (>99% purity) and dobutamine were purchased from Research Biochemicals (Natick, MA), and stereoisomers of propranolol (>98.5% purity) were from Sigma. The following compounds were kindly provided by: (−)- and (+)epi-

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Abbreviations: β_2-AR, (human) β_2-adrenergic receptor; ^{125}I-CYP, ^{125}Iodo-cyanopindolol.

‡To whom reprint requests should be addressed.

nephrine (O. E. Brodde, Halle, Germany) and (−)- and (+)norepinephrine (A. Kaumann, Cambridge, U.K.)

Modelling of the Human β_2-AR. Since no structural information on G-protein-coupled receptors was available at the outset of these studies, a putative model of the β_2-AR was generated according to the method of Lewell (21). It was based on the atomic coordinates of bacteriorhodopsin as obtained from the Brookhaven Protein Data Bank (reference code 1BRD). Because there is no significant sequence homology between bacteriorhodopsin and the β_2-AR, the sequence alignment proposed by Lewell with rhodopsin as an intermediate was used, and the side chains of bacteriorhodopsin were substituted by those of the human β_2-AR. Unfavorable steric interactions were removed and subsequently energy minimizations were performed both in the presence and in the absence of the reference ligand (−)isoproterenol according to the procedures described for the modelling of the A_1 adenosine receptor (12). No rotations or translational motions of the helical backbones were allowed.

To obtain docking of (−)isoproterenol into the β_2-AR cavity (see Fig. 1), three distance constraints were applied in line with mutagenesis data (7, 8). Thus, the distance between the positively charged nitrogen atom of (−)isoproterenol and the negatively charged Asp-113 (both terminal oxygen atoms) was 3.5 Å (ionic interaction), and the distance between Ser-204 (oxygen atom) and the meta-OH (hydrogen atom) of the catechol group of (−)isoproterenol was 2.1 Å (hydrogen bond), as was the distance between the catechol para-OH-group and Ser-207. Further energy minimization after removal of the distance constraints preserved these interactions.

All manipulations were carried out using the software package QUANTA/CHARMM, version 3.3.1 (Molecular Simulations, Waltham, MA) on a Silicon Graphics XZ4000 workstation.

Mutagenesis of β_2-AR cDNA. The cDNA for the human β_2-AR (22) was cloned into the expression vector pBC-CMV-SK (23) to generate the vector pBC-CMV-β_2AR. Mutation of the codon for amino acid 165 was carried out according to Kunkel (24). Site-directed mutagenesis of the codon for amino acid 293 took advantage of the fact that a unique *Hpa*I restriction site is located directly adjacent to this codon: The vector was linearized at this site with *Hpa*I, and the gap was bridged with a 38-mer mutant oligonucleotide containing in its center the codon CTC (Leu) for amino acid 293. A piece of mutant receptor cDNA corresponding to the entire coding region was then generated by polymerase chain reaction (PCR) under standard conditions (30 cycles) using the oligonucleotide-annealed linear vector (100 ng) as a template and primers corresponding to nucleotides 1–18 (forward) and 1242–1225 (reverse) of the receptor cDNA. A 318-bp *Bgl*II–*Eco*RV fragment containing the mutated region was excised from the PCR products and inserted into the corresponding sites of pBC-CMV-β_2AR. The sequences of all resultant cDNAs were verified by automated sequencing.

Transfection of Human Embryonic Kidney (HEK)-293 Cells and Generation of Stable CHO Cell Lines. For initial studies, wild-type and mutant β_2-AR were transiently expressed in HEK-293 cells. Transfection of these cells by the calcium phosphate/*N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid method (25) resulted 2 days later in the expression of receptors at a density of several pmol/mg membrane protein. More detailed studies were done with CHO cell lines stably expressing these receptors. These cell lines were obtained by transfecting CHO-10001 cells with the respective expression vectors plus pSV2*neo* using DOTAP (Boehringer Mannheim) as transfection reagent and G418 (GIBCO) to select positive clones as described earlier (23, 26). Clones with comparable densities (about 0.2 pmol/mg membrane protein) were selected for the experiments. Additional clones were also tested to check for potential artifacts in individual clones.

Radioligand Binding Studies. Ligand binding to β_2-AR was analyzed using ^{125}I-CYP and crude cell membranes prepared as described earlier (27) using an incubation time of 1 hr at 30°C. Saturation studies were done with radioligand concentrations from 2 pM to 200 pM, using 1 μM (−)propranolol to define nonspecific binding. Competition studies were done with a radioligand concentration of 30 pM. Unless stated otherwise, all radioligand binding assays contained 100 μM GTP to uncouple β_2-AR from G_s and thereby generate monophasic competition curves for agonists, as well as antagonists.

Adenylyl Cyclase Assays. The function of β_2-AR was assessed by determining their capacity to stimulate the adenylyl cyclase activity in membranes prepared from the CHO cell lines stably expressing the receptor variants. Membranes were prepared as above, and adenylyl cyclase activity was determined by measuring the generation of [^{32}P]cAMP from [α-^{32}P]ATP as described (28). The incubation was done for 30 min at 30°C.

Data Analysis. Radioligand binding data were analyzed by nonlinear curve fitting using the program SCTFIT, which allows analysis for models containing multiple-binding sites (29, 30). Concentration-response curves for adenylyl cyclase stimulation were analyzed by nonlinear curve fitting to the Hill equation as described (31).

RESULTS

To identify amino acids in the human β_2-AR that might bind the β-OH-group of β-adrenergic receptor agonists, computerized visualization of the receptors and their ligand binding pocket was performed as described in *Materials and Methods*. At the outset of the present study, no structural information on any G-protein-coupled receptor was available. Therefore, the coordinates of bacteriorhodopsin were used to generate the backbone for the β_2-AR. Fig. 1 shows the helical backbone with the ligand (−)isoproterenol fitted to its putative binding site formed by Asp-113, Ser-204, and Ser-207. Anchoring the ligand to the respective side chains still leaves rotational freedom of the ligand due to the flexibility of the ethanolamine side chain. This allowed visualization of amino acids that could interact with the β-OH-group. Ser-165 represents one possible interacting amino acid as suggested by Trumpp-Kallmeyer *et al.* (13). However, by changing the torsion angles in the ligand, the β-OH-group could be brought into even closer vicinity to Asn-293, situated in transmembrane helix VI. Energy minimization yielded a putative hydrogen bond between the hydrogen atom of the β-OH-group and the oxygen atom of the carboxamide function in Asn-293, suggesting that this residue might be important for stereospecificity in agonist binding. No other amino acids were identified in this model that could form a bond with the β-OH-group.

To test the role of these amino acids, mutants of the human β_2-AR were generated in which Ser-165 was replaced by Ala, or Asn-293 was replaced by Leu. The mutations resulted in side chains that were similar in size to those in the wild-type receptor, but incapable of forming a hydrogen bond. Transient expression of these receptors in HEK-293 cells revealed that the mutations did not significantly affect the affinity for ^{125}I-CYP (Table 1). Competition for ^{125}I-CYP binding by the stereoisomers of isoproterenol was used to investigate the ability of the receptor variants to bind agonists in a stereospecific manner (Table 1). (−)Isoproterenol was almost 40-fold more potent than (+)isoproterenol at wild-type receptors, and very similar data were obtained for the 165-Ala mutant. In contrast, in the 293-Leu mutant the stereospecificity of isoproterenol was reduced to about 6-fold. This reduction was essentially due to a loss in affinity of the (−)stereoisomer, whereas the affinity of the (+)stereoisomer was only slightly altered. These data are compatible with a stereospecific in-

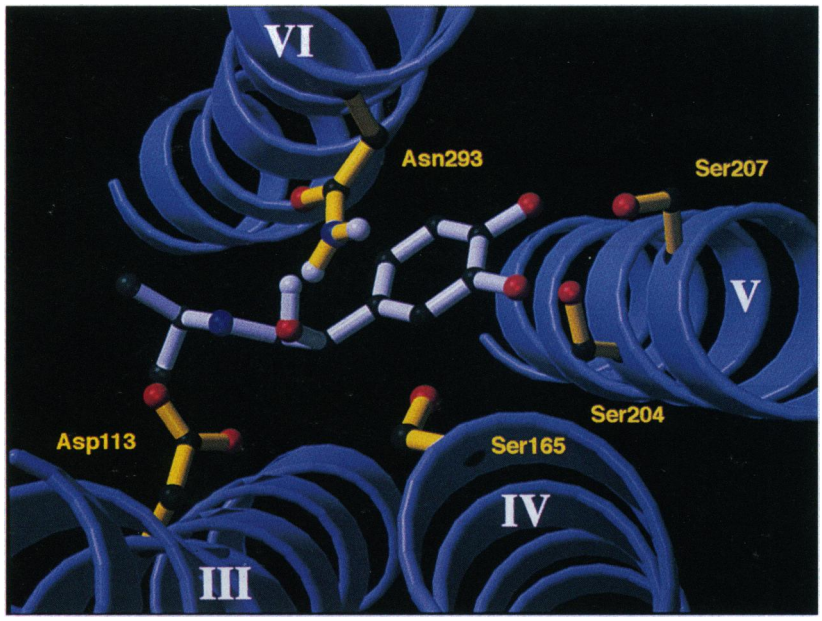

FIG. 1. Model visualizing the binding of (−)isoproterenol to the human β_2-AR. The receptor α-helical backbone (helices III–VI) is viewed from the extracellular side, tilted by ≈20°. (−)Isoproterenol and the amino acids thought to be involved in ligand binding are indicated: Asp-113 (helix III) and Ser-204 and Ser-207 (helix V). Also indicated are Ser-165 (helix IV) and Asn-293 (helix VI), the residues that might be in close vicinity to the β-OH-group of (−)isoproterenol. The atoms are represented as follows: white, hydrogen atoms connected to either oxygen or nitrogen (most hydrogens are not shown to improve clarity); black, carbon atoms; blue, nitrogen atoms; red, oxygen atoms. The figure was generated using the programs MOLSCRIPT and RASTER3D.

teraction of the side chain of Asn-293, but not of Ser-165, with the β-OH-group in isoproterenol.

To test whether a contribution of Ser-165 to agonist binding might be visible in 293-Leu mutant receptors, we also generated a double mutant (165-Ala, 293-Leu). However, this double mutant was not much different from the 293-Leu mutant. The affinities of both isomers of isoproterenol were 1.7-fold lower than in the 293-Leu mutant, and the stereospecificity was the same, indicating that in the 293-Leu receptor mutant Ser-165 also does not participate in ligand binding.

Wild-type and 293-Leu mutant receptors were then stably expressed in CHO cells and clones with comparable receptor densities (182 ± 12 and 170 ± 20 fmol/mg membrane protein) were used for functional analysis of the two receptor variants. The maximal stimulation of adenylyl cyclase activity by (−)isoproterenol and by forskolin was similar in CHO cell membranes containing either wild-type or Leu-293 mutant receptors, indicating that the mutant receptor was fully capable of activating G_s (Fig. 2, legend). Concentration-response curves of the isomers of isoproterenol revealed a very marked stereospecificity of the wild-type receptor (Fig. 2). This appears to be due to the fact that (+)isoproterenol is a partial agonist in these assays and to a significant receptor reserve in these cells. Using wild-type receptors we found a 134-fold higher potency of (−)isoproterenol as compared with (+)isoproterenol, again mainly due to a loss in the potency of (−)isoproterenol. This stereoselectivity was reduced to 13-fold for the 293-Leu mutant. As in the binding experiments, this reduction was mainly due to a loss in the potency of (−)isoproterenol, whereas the potency of (+)isoproterenol was only marginally reduced.

The differential effects of the 293-Leu mutations on the stereoisomers of isoproterenol might be due either to their chiral character or to their different intrinsic activities. Therefore, we tested a series of compounds in radioligand-binding assays and in adenylyl cyclase assays on wild-type and 293-Leu mutant receptors. These experiments showed a reduced stereospecificity also for epinephrine and norepinephrine (Table 2). They further revealed that full agonists had the greatest loss of affinity in the mutant compared with the wild-type receptor, whereas partial agonists were only moderately affected by the mutation (Fig. 3).

The affinities of dobutamine (a partial β-adrenergic receptor agonist lacking the β-OH-group) and dopamine (the parent compound of norepinephrine lacking the β-OH-group, which is a very weak β_2-AR agonist) were not altered in the 293-Leu mutant receptor (Table 2). Overall, there was a good correlation between the intrinsic activity of a series of agonists and the mutation-induced loss of affinity (Fig. 3). Because in all cases the (+)isomers of these compounds have a lower intrinsic activity than the (−)isomers, this observation is equivalent to the loss of agonist stereospecificity reported above.

Table 1. Binding parameters for ^{125}I-CYP and the stereoisomers of isoproterenol for wild-type and mutant β_2-AR

Receptor	^{125}I-CYP K_D, pM	(−)Isoproterenol K_i, μM	(+)Isoproterenol K_i, μM	Stereospecificity, $K_{i(+)}/K_{i(-)}$
Wild type	7.1 ± 1.8	0.28 ± 0.07	10.6 ± 2.4	38.1 ± 9.2
165-Ala	12.0 ± 2.7	0.19 ± 0.03	8.0 ± 3.8	39.8 ± 13.8
293-Leu	7.3 ± 3.1	2.5 ± 0.36	14.4 ± 1.3	5.8 ± 1.0
165-Ala/293-Leu	11.1 ± 3.7	4.2 ± 0.71	24.9 ± 3.3	5.9 ± 1.5

Saturation studies with 2–200 pM ^{125}I-CYP and competition studies using 30 pM ^{125}I-CYP were done on cell membranes prepared from HEK-293 cells transiently expressing the receptor variants. The data were analyzed by nonlinear curve fitting to obtain estimates for the affinity (K_D for ^{125}I-CYP, K_i for isoproterenol). Stereospecificity was calculated as the ratio of the K_i-values for the (+)- and (−)stereoisomer. Data are means ± SEM, n = 3.

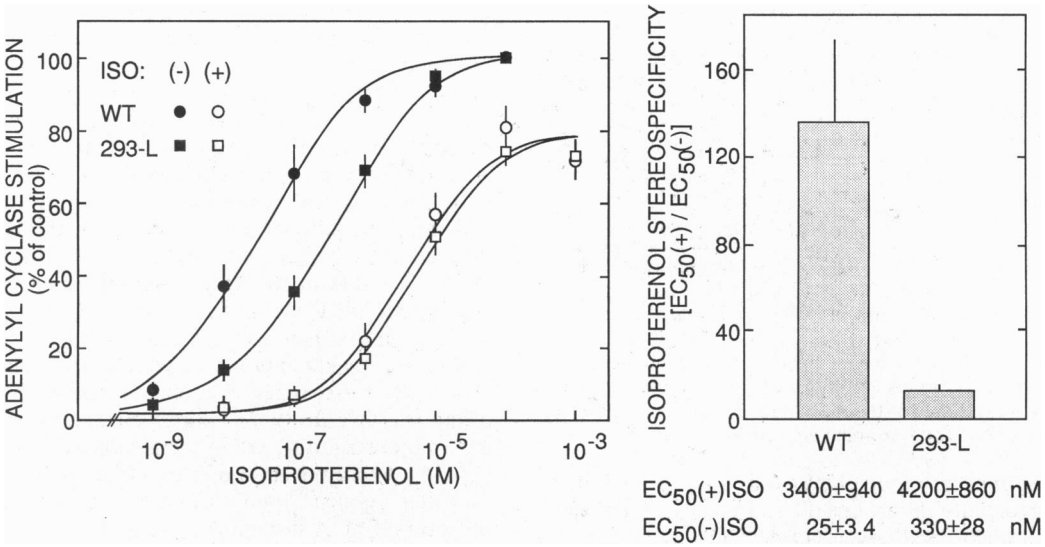

FIG. 2. Stimulation of adenylyl cyclase activity by isoproterenol via wild-type and 293-Leu mutant β_2-AR. (*A*) Stimulation of adenylyl cyclase activity in membranes prepared from CHO cells expressing wild-type (circles) or 293-Leu mutant (squares) β_2-AR by (−)isoproterenol (solid symbols) or (+)isoproterenol (open symbols). Maximal activation by (−)isoproterenol (set to 100%) was 114 ± 5 and 127 ± 9 pmol cAMP/mg of protein/min in the wild-type and mutant receptors, respectively. Stimulation by 10 μM forskolin in these membranes was 202 ± 7 and 229 ± 15 pmol cAMP/mg of protein/min. Data are means ± SEM of four independent experiments with duplicate samples. (*B*) Stereospecificity of the adenylyl cyclase stimulation by isoproterenol via wild-type or 293-Leu mutant β_2-AR. The data shown in *A* were analyzed by nonlinear curve fitting and the stereospecificity for isoproterenol was calculated as the ratio of the EC_{50} values for the (+) and the (−)stereoisomer. These EC_{50} values are indicated below the panel. Data are means ± SEM, $n = 3$.

No such relationship was found for a group of antagonists. We tested the stereoisomers of two neutral antagonists, alprenolol and metoprolol, and of propranolol that reduced basal adenylyl cyclase activity in membranes prepared from cells expressing wild-type or 293-Leu mutant receptors (data not shown). The 293-Leu mutation caused modest alterations in the affinities of these compounds, but their stereospecific recognition was not altered when compared with wild-type receptors (Table 3). This indicates that the changes in stereospecificity induced by this mutation were restricted to agonists.

DISCUSSION

Binding of agonists, as well as antagonists, to β_2-AR receptors is generally thought to occur to the transmembrane α-helices. This was initially concluded from studies showing that mutant receptors obtained by deletion of extracellular segments were still capable of binding ligands (32). Later studies involving site-directed mutagenesis identified specific amino acid residues that appear to interact with specific determinants of β-adrenergic agonists. These include the anchoring points

Table 2. Affinities of agonists for wild-type and 293-Leu mutant β_2-AR

Agonists	Affinity K_i, μM	
	Wild type	293-Leu
With a β-OH-group		
(−)Epinephrine	0.17 ± 0.01	6.2 ± 1.1
(+)Epinephrine	2.1 ± 0.24	31 ± 5.0
stereospecificity	13 ± 1.3	5.0 ± 0.4
(−)Norepinephrine	3.9 ± 0.80	43 ± 2.8
(+)Norepinephrine	174 ± 6.3	800 ± 110
stereospecificity	49 ± 9.3	18 ± 1.3
(±)Clenbuterol	0.024 ± 0.008	0.025 ± 0.007
(+)Clenbuterol	0.56 ± 0.26	0.30 ± 0.12
(±)Terbutaline	5.4 ± 2.4	8.3 ± 2.6
Without a β-OH-group		
(±)Dobutamine	66 ± 21	63 ± 11
dopamine	400 ± 150	550 ± 210

The affinities (and stereospecificities when two pure isomers were available) were determined in competition experiments with ^{125}I-CYP and calculated as in Table 1.

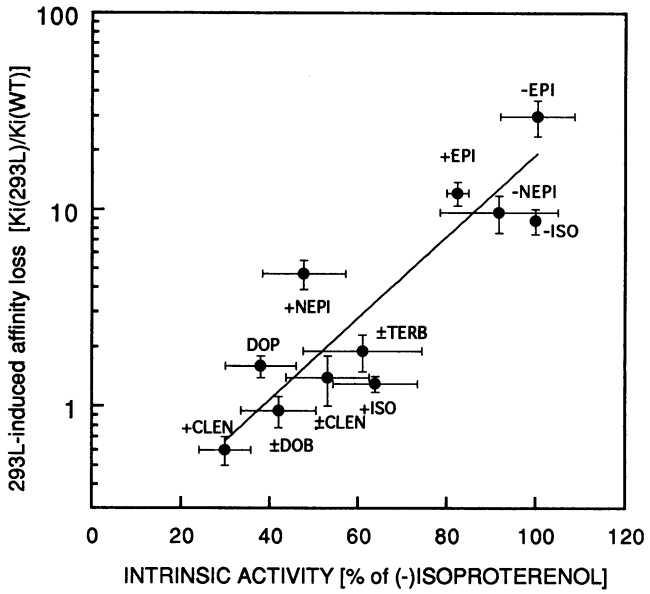

FIG. 3. Comparison of the intrinsic activities of agonists and the loss in affinity induced by the 293-Leu mutation in the human β_2-AR. Intrinsic activities were determined as the maximal stimulation of adenylyl cyclase activity in membranes prepared from CHO cells stably expressing the respective receptors (see Fig. 2). They were normalized to the maximal activity of (−)isoproterenol tested in the same experiments. The mutation-induced loss in affinity was determined from inhibition of ^{125}I-CYP binding to wild-type and 293-Leu mutant receptors and is expressed as K_i(293-Leu mutant)/K_i(wild-type). Abbreviated compounds are as follows: CLEN, clenbuterol; DOB, dobutamine; DOP, dopamine; EPI, epinephrine; ISO, isoproterenol; NEPI, norepinephrine; TERB, terbutaline. Data are means ± SEM of three to six experiments.

Table 3. Stereoselective binding of antagonists to wild-type and 293-Leu mutant β_2-AR

Compound receptor	Stereospecificity, K_i(+)isomer/K_i(−)isomer	
	Wild type	293-Leu
Propranolol	104 ± 27	112 ± 41
Alprenolol	22 ± 1	19 ± 1
Metoprolol	21 ± 4	25 ± 3

Membranes were prepared from CHO cells stably expressing wild-type or 293-Leu mutant β_2-AR (182 ± 12 and 170 ± 20 fmol/mg membrane protein, respectively), and ^{125}I-CYP binding was measured in the presence of 10^{-12} to 10^{-4} M of the (−) and (+)isomers of the indicated antagonists. The data were analyzed by nonlinear curve fitting to obtain estimates for the affinities of the compounds (K_i). Stereospecificity was calculated as the ratio of the K_i values for the (+) and the (−)stereoisomer. Data are means ± SEM, n = 3.

Asp-113 for the amine group of the ligands and Ser-204 and Ser-207 for the catechol-OH-groups (7, 8). In contrast to these well-accepted interactions, there is no certainty regarding a potential interaction site for the β-OH-group of agonists that might explain the well-known and pharmacologically very relevant stereospecificity of agonist binding.

Computer modelling has led Trumpp-Kallmeyer *et al.* (13) to suggest Ser-165 in transmembrane helix IV as a potential anchoring point for this β-OH-group. However, it has been difficult to verify this hypothesis, because mutation of Ser-165 to Ala resulted in a β_2-AR mutant that failed to be expressed at significant levels (8). Green *et al.* (33) have investigated a naturally occurring Thr-164-Ile mutant human β_2-AR, which showed a 4-fold decreased affinity for isoproterenol, but only modest decreases for dobutamine and dopamine. The authors postulate that an alteration at position 164 might affect binding at Ser-165, and that their data indicate a role for Ser-165 in stereospecific-agonist binding. However, the stereospecificity of isoproterenol was entirely maintained in the Ile-164 receptor variant, and, furthermore, the G-protein-coupling of this mutant was significantly impaired, indicating a more generalized alteration of receptor structure. In contrast to these hypotheses, we now find that the 165-Ala β_2-AR was indistinguishable from the wild-type receptor in its affinities for ^{125}I-CYP and the stereoisomers of isoproterenol, suggesting that Ser-165 does not participate in ligand binding.

A potential role of transmembrane helix VI in binding the β-OH-group was suggested by the model shown in Fig. 1 implying that Asn-293 might be capable of forming the postulated hydrogen bond. It should be stressed that such receptor models are at best crude approximations, which can be used as means of visualization of emerging experimental data, such as structure–activity relationships of β-adrenergic ligands and data from mutagenesis studies. Interestingly, in the model developed by Baldwin (15) on the basis of sequence comparisons of G-protein-coupled receptors plus emerging structural information on rhodopsin, Asn-293 faces the central portion of the receptor lumen at the same horizontal level as the three other anchoring points (i.e. Asp-113, Ser-204, and Ser-207). Its position is entirely compatible with an orientation of the side chain toward the ligand as proposed here.

Replacement of Asn-293 with Leu resulted in a receptor that displayed reduced stereospecificity for agonists. This effect is apparently accompanied by very few alterations in other properties of the mutant receptor. In particular, the 293-Leu mutant receptor retained unaltered binding of agonists that lack the β-OH-group (dobutamine and dopamine) as well as of the antagonist radioligand ^{125}I-CYP, showed the same stereoselectivity for several antagonists, and was fully functional in mediating activation of adenylyl cyclase. All of these properties are compatible with the view that Asn-293 plays a role in forming a hydrogen bond with the β-OH-group of agonists. Although our data suggest that Asn-293 is important for stereospecific agonist binding, replacement with Leu did not completely abolish this stereoselectivity. This suggests that other interaction sites contribute to such stereoselectivity. Such interactions could occur with the polypeptide backbone of transmembrane helix VI or with additional amino acid side chains. However, the most likely side chain candidate, Ser-165, does not apparently contribute to such interactions.

Among the adrenergic receptors, only the three β-adrenergic receptor subtypes, but not the α1- or α2-receptors, contain an Asn residue in the corresponding position in transmembrane helix VI. This Asn occurs in the upper part of this helix, in a region that is more divergent than the rest of this highly conserved transmembrane helix. The α-adrenergic receptors also show stereospecific agonist binding, even though the differences in affinity for the stereoisomers of epinephrine, the most potent agonist, are in most subtypes only about 10-fold (34–36). These observations are compatible with the hypothesis that agonists bind differently to α- and β-adrenergic receptors (37). A homologous Asn also occurs in some other G-protein-coupled receptors. For example, it is present in all cloned adenosine receptors where it has been implicated in ligand binding (12).

In all compounds examined, the (−)isomer of agonists proved to have not only higher affinity but also higher intrinsic activity than the (+)isomer. Thus, the loss of affinity in the 293-Leu mutant receptors was always greater for agonists of higher intrinsic activity. Interestingly, partial agonists that lack the β-OH-group, such as dobutamine and dopamine, also fitted well in the correlation given in Fig. 3. Therefore, the mutant receptors reveal a relationship between the intrinsic activity of agonists and the presence and correct orientation of the β-OH-group, which is "active" in the (−)isomers of the agonists studied here. We speculate that it is the interaction between this β-OH-group and Asn-293 that contributes to the formation of an active receptor. The end of the third cytosolic loop and the beginning of transmembrane helix VI have been shown in many studies to be the most critical region for G-protein activation (38–40). Asn-293 is situated in the middle of helix VI, and it is easy to imagine that an interaction of this amino acid with agonists might cause an altered conformation of this critical G-protein activator region.

In summary, our studies have identified Asn-293 of the human β_2-AR as a major determinant for agonist stereospecificity as well as intrinsic activity. No stereospecificity determinants have been reported in other G-protein-coupled receptors, and future studies will have to show whether there are general principles that govern stereoselective ligand binding of such receptors. The identification of an interaction point for the β-OH-group does not only contribute to our understanding of ligand binding to the β_2-AR but also reveals clues as to how this binding might result in the formation of the active state of the receptor.

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