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Mutagenesis and Modelling of the α_{1b}-Adrenergic Receptor Highlight the Role of the Helix 3/Helix 6 Interface in Receptor Activation

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ABSTRACT

Computer simulations on a new model of the α1b-adrenergic receptor based on the crystal structure of rhodopsin have been combined with experimental mutagenesis to investigate the role of residues in the cytosolic half of helix 6 in receptor activation. Our results support the hypothesis that a salt bridge between the highly conserved arginine (R143$^{3.50}$) of the E/DRY motif of helix 3 and a conserved glutamate (E289$^{6.30}$) on helix 6 constrains the α1b-AR in the inactive state. In fact, mutations of E289$^{6.30}$ that weakened the R143$^{3.50}$-E289$^{6.30}$ interaction constitutively activated the receptor. The functional effect of mutating other amino acids on helix 6 (F286$^{6.27}$, A292$^{6.33}$, L296$^{6.37}$, V299$^{6.40}$, V300$^{6.41}$, and F303$^{6.44}$) correlates with the extent of their interaction with helix 3 and in particular with R143$^{3.50}$ of the E/DRY sequence.

The α1b-adrenergic receptor (AR) belongs to the rhodopsin family of G protein-coupled receptors (GPCRs). GPCRs are structurally characterized by seven transmembrane helices connected by alternating extracellular and intracellular loops. Whereas ligand binding involves the extracellular portion of the receptor, the intracellular regions mediate the interaction of the receptor with G proteins as well as other signaling and regulatory proteins.

A GPCR-mediated biological response involves a series of events (i.e., receptor activation, receptor-G protein interaction, and receptor-induced G protein activation) for which a detailed mechanism still remains elusive at the molecular level. Whereas residues located in the helical bundle and at the boundary between the membrane and the cytosol may play a role in the "conformational switch" underlying receptor activation [i.e., the transition from the inactive (R) to active (R*) state], amino acids in the intracellular loops are believed to be more directly involved in receptor-G protein interaction and/or receptor induced G protein activation. The combination of these two latter events, which cannot be unequivocally separated experimentally, is generally indicated as receptor-G protein coupling.

Biochemical and biophysical experiments on rhodopsin (Farrens et al., 1996; Sheikh et al., 1996) and the β_2-AR (Jensen et al., 2001) suggest that their activation, i.e., the transition from the inactive (R) to active (R*) state, involves a rearrangement of helices 3 and 6 (reviewed by Gether, 2000). Such a transition would result from the release of constraining interactions between these two helices.

The recently published structure of rhodopsin in its inactive state (Palczewski et al., 2000) suggests that a salt bridge between the highly conserved arginine of the E/DRY motif of helix 3 and a glutamate on helix 6 represents an important interaction constraining the positions of helices 3 and 6. The interaction pattern involving this conserved arginine in the inactive state of rhodopsin is not consistent with that found in the wild-type model of the α_{1b}-AR previously achieved by following an ab initio approach (Scheer et al., 1996, 1997, 2000; Fanelli et al., 1998). In fact, according to this model, the ground state of the α_{1b}-AR would be stabilized by the interactions between R143$^{3.50}$ and some amino acids forming a highly conserved polar pocket (i.e., D91$^{2.50}$ and Y348$^{7.53}$).

To advance our understanding of the molecular mechanisms underlying the α_{1b}-AR function, we recently built a homology model of the receptor based on the crystal structure of rhodopsin and have interpreted a number of experimental results in the context of this new model (Greasley et al., 2001). In this study, we have challenged the predictions of the α_{1b}-AR homology model concerning the potential role in receptor activation of residues in the cytosolic half of helix 6

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The receptor coordinates are available upon request from fanelli@unimo.it.

ABBREVIATIONS: AR, adrenergic receptor(s); GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle's medium; [^{125}I]HEAT, [^{125}I]iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone; MD, molecular dynamics; IP, inositol phosphates.

and in particular of those that are predicted to be located at the helix 3/helix 6 interface. Our results provide significant insight into the packing of helices 3 and 6 as well as into some of the structural constraints stabilizing the inactive state of the α_{1b}-AR.

Experimental Procedures

Materials. COS-7 cells were from the American Type Culture Collection (Manassas, VA); DMEM, gentamicin, fetal bovine serum and restriction enzymes were from Invitrogen (Carlsbad, CA). *Pwo* polymerase was from Roche Applied Science (Mannheim, Germany); [^{125}I]HEAT and [^{3}H]inositol from PerkinElmer Life Sciences (Boston, MA); epinephrine was from Sigma (St. Louis, MO); and prazosin was from RBI/Sigma (Natick, MA).

Mutagenesis of the α_{1b}-AR. The cDNA of the hamster α_{1b}. AR (Cotecchia et al., 1992) was mutated using polymerase chain reaction-mediated mutagenesis and *Pwo* DNA polymerase. The constructs were subcloned in the pRK5 expression vector and mutations confirmed by automated DNA sequencing of the entire portion amplified with polymerase chain reaction (Microsynth GmbH, Balgach, Switzerland).

Cell Culture and Transfection. COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum and gentamicin (100 μg/ml) and transfected using the DEAE-dextran method. For inositol phosphate determination, COS-7 cells (0.15×10^6) were seeded in 12-well plates. The quantity of transfected receptor encoding DNA was 0.6 to 3 μg/10^6 cells. In each experiment, the wild-type α_{1b}-AR was expressed using varying quantities of DNA, thereby allowing us to directly compare the properties of the mutated receptors with those of the wild-type receptor expressed at comparable levels within the same experiment.

Ligand Binding. Membrane preparations derived from cells expressing the α_{1b}-AR or its mutants and ligand binding assays using [^{125}I]HEAT were performed as described previously (Cotecchia et al., 1992). Prazosin (10^{-6}M) was used to determine nonspecific binding. [^{125}I]HEAT at a concentration of 250 pM was used for measuring receptor expression at a single concentration and 80 pM for competition binding analysis. Saturation analysis and competition curves were analyzed using Prism 3.02 (GraphPad Software, San Diego, CA).

Inositol Phosphate Measurements. Transfected cells were labeled for 12 h with [*myo*-^{3}H]inositol at 4 μCi/ml in inositol-free DMEM supplemented with 1% fetal bovine serum. Cells were preincubated for 10 min in PBS containing 20 mM LiCl and then stimulated for 45 min with different concentrations of epinephrine ranging from 10^{-10} to 10^{-4}M. Total inositol phosphates were extracted and separated as described previously (Cotecchia et al., 1992).

Homology Modeling of the α_{1b}-AR and Its Mutants. The homology model of the α_{1b}-AR was built using the program MODELLER (Sali and Blundell, 1993) and the structure of rhodopsin (Palczewski et al., 2000) as a template, as described recently (Greasley et al., 2001).

Eight different chimeric rhodopsin/α_{1b}-AR templates were probed in which the e2 loop, the i3 loop, and, in some cases, the i2 loop were extracted from the input structure of the ab initio model described previously (Fanelli et al., 1998). Furthermore, helix 5 was elongated by 10 amino acids in the chimeras using the α_{1b}-AR sequence after deleting the 226-to-235 segment of rhodopsin. For each of the eight different templates, MODELLER generated 25 models. Among the 200 models finally obtained, 20 models were selected showing low restraint violations and low numbers of main- and side-chain bad conformations or close contacts. These models were completed by the addition of the polar hydrogens and subjected to automatic and manual rotation of the side chain torsion angles when in bad conformations, as well as to energy minimization and molecular dynamics (MD) simulations according to the computational protocol employed for simulating the ab initio α_{1b}-AR model (Fanelli et al., 1998). About 450 MD trial runs were performed to select the proper input structure for the wild-type α_{1b}-AR. This structure was employed to build the input structure of the receptor mutants by substituting in turn each of the target amino acids. For each receptor mutant, different starting conformations of the mutated side chain were probed by MD simulations. These conformations were assigned by using different rotamer libraries and checking for the absence of bad contacts between the mutated side chain and its neighboring amino acid residues. The structure of the wild-type receptor and its mutants averaged over the last 100 ps of the 150-ps MD trajectory were finally minimized and considered for the comparative analysis.

In this study, the amino acids are labeled according to a double numbering system. In addition to numbering their position in the receptor sequence, the amino acids in the helical bundle are labeled with superscript numbers indicating the relative position of each amino acid in the helix (Ballesteros and Weinstein, 1995). According to this numbering system, every amino acid identifier starts with the helix number, followed by the position relative to a reference residue among the most conserved amino acid in that helix. That reference residue is arbitrarily assigned the number 50.

Results

Expression of Receptor Mutants. The wild-type and mutated α_{1b}-ARs were expressed in COS-7 cells and tested for their ability to bind the radioligand [^{125}I]HEAT, epinephrine, and prazosin. Saturation binding experiments indicated that the K_D of [^{125}I]HEAT was approximately 80 pM for all the receptors studied (results not shown), whereas the IC_{50} values for epinephrine varied as indicated in Table 1. The affinity of prazosin for the different receptor mutants was similar to that for the wild-type α_{1b}-AR (results not shown). Receptor coupling to the G_q/PLC pathway was assessed as the ability of the receptor mutants to mediate epinephrine stimulated inositol phosphates (IP) accumulation (Table 1).

Transfections using 3 μg of DNA per 1×10^6 cells resulted in the expression of all receptor mutants at levels ranging from 60 to over 300 fmol/well. In each experiment, the wild-type α_{1b}-AR was expressed using two quantities of DNA (0.6 and 3 μg of DNA/1x10^6 cells), resulting in low (between 60 and 120 fmol/well) and high (between 200 and 350 fmol/well) levels of expression. This allowed us to directly compare the properties of the mutated receptors with those of the wild-type α_{1b}-AR expressed at comparable levels within the same experiment.

Mutagenesis of Helix 6 Amino Acids Directed toward Helix 3. Fig. 1 shows the average minimized structure of the α_{1b}-AR built on the recent crystal structure of rhodopsin. A striking feature of the model is the interaction of R143$^{3.50}$ of the E/DRY motif with both the adjacent D142$^{3.49}$ and E289$^{6.30}$. The R143$^{3.50}$-E289$^{6.30}$ interaction would be expected to constrain the relative positions of helix 3 and helix 6. E289$^{6.30}$ can make additional intrahelix interactions with K285$^{6.26}$ and/or K290$^{6.31}$.

Helix 6 shows a significant bend at the highly conserved P309$^{6.50}$ (Fig. 1). Because of this bend, the cytosolic half of this helix (from K285$^{6.26}$ to W307$^{6.48}$) is more tightly packed toward helix 3 than its extracellular half.

In this study, we have investigated the role of amino acids in the cytosolic half of helix 6 (i.e., residues 285$^{6.26}$ to 304$^{6.45}$) and, in particular, those residues directed toward helix 3. There are only four amino acids belonging to this interface,

F286$^{6.27}$, E289$^{6.30}$, L296$^{6.37}$, and F303$^{6.44}$. There is a lack of periodicity in the sequential order of these residues because the main axis of helix 3 is tilted with respect to the main axis of the cytosolic half of helix 6 (Fig. 1).

The first and most solvent accessible residue is F286$^{6.27}$. In the structure of the wild-type receptor, F286$^{6.27}$ makes van der Waals' contacts with V147$^{3.54}$ and R148, the first amino acid in the i2 loop. To test its structure-functional role, F286$^{6.27}$ was mutated to alanine or to glutamate that would introduce an ionic interaction. Mutating F286$^{6.27}$ to Ala increased the maximal epinephrine-induced IP response by 50% and, to a lesser extent, the constitutive activity. In contrast, the E mutation impaired the maximal epinephrine-induced IP response by 30% (Table 1).

For both mutants of F286$^{6.27}$, the affinity of epinephrine was dramatically increased by more than 100-fold. However, whereas the potency of epinephrine at the F286A mutant was also markedly increased by 30-fold, it was only increased by 4-fold at the F286E. Overall, these results suggest that receptor activation is favored by the mutation of F286$^{6.27}$ to Ala but impaired by its mutation to Glu.

The results of computer simulation of the F286A and F286E mutants could help to interpret, at least in part, the effects of the mutations on receptor activation. The F286A mutant displayed the breakage of the D142$^{3.49}$-R143$^{3.50}$ interaction, whereas the R143$^{3.50}$-E289$^{6.30}$ interaction was conserved. In contrast, the mutation of F286$^{6.27}$ to Glu favored the formation of a salt bridge between the replacing glutamate and R148 in the second intracellular loop. This interaction might reinforce the link between the cytosolic halves of helices 3 and 6, therefore impairing receptor activation.

The results of the computational analysis cannot provide an explanation for the marked increase in the affinity for epinephrine displayed by the F286A and F286E mutants. Both mutants shared some perturbations in the interaction pattern involving R143$^{3.50}$ compared with that of the wild-type receptor. However, the potential relationship between these perturbations and the increased affinity of the agonist remains speculative.

The homology model suggests that the inactive state of the α_{1b}-AR is constrained by the charge reinforced H-bonding interaction between E289$^{6.30}$ and R143$^{3.50}$ of the E/DRY sequence. However, computer simulations suggest that the formation of the salt bridge is strongly dependent on the starting conformation of R143$^{3.50}$. In other words, the arginine should have a folded conformation in the input structure as found in the rhodopsin structure so as to maintain the salt bridge interaction during MD simulations. However, this conformation (dictated by the tight packing of the cytosolic extensions of helices 3 and 6 in the inactive state of rhodopsin) does not correspond to any of the arginine conformations most frequently found in protein structures (Dunbrack and Karplus, 1993). Whether the conformation and the interaction pattern of the arginine belonging to the E/DRY motif are just peculiar to the available rhodopsin structure will await the structural resolution of other closely related GPCRs.

To challenge the predictions of the model, E289$^{6.30}$ was mutated into several amino acids. The receptor mutants in which E289$^{6.30}$ was mutated into Ala, Phe, Lys, and Arg displayed the typical features described for the active state (R*) of a GPCR (Samama et al., 1993), including a marked increase in the constitutive and agonist dependent activity of

TABLE 1

Functional properties of the α_{1b}-AR (wild-type) and its mutants

The receptors were expressed in COS-7 cells. Receptor expression was measured using 250 pM [^{125}I]HEAT on membrane preparations derived from transfected cells from one well of a six-well dish (approximately 150 μg of protein). IP accumulation was measured after incubation in the absence (basal) or presence of 10^{-4} M epinephrine (epinephrine-induced IP) for 45 min. The IP accumulation is expressed as the percentage increase in IP levels above those of mock-transfected cells. For determining the EC_{50} values. the concentrations of epinephrine ranged from 10^{-12} to 10^{-4} M. Results for receptor expression and IP accumulation are the mean ± S.E. of at least three independent experiments. The IC_{50} values (mean ± S.E) of epinephrine are from 15 and 3 independent experiments for the wild-type and mutated receptors, respectively. The EC_{50} values (mean ± S.E) are from nine and three independent experiments for the wild-type and mutated receptors, respectively.

Receptor	Expression	IP		Epinephrine	
		Basal	Epinephrine-Induced	IC_{50}	EC_{50}
	fmol/well		%	*μM*	*nM*
Wild-type (low)	128 ± 9	14 ± 2	313 ± 69	4.7 ± 0.6	29 ± 6
Wild-type (high)	347 ± 58	17 ± 2	432 ± 44		
F286A	155 ± 27	59 ± 21*	643 ± 89*	0.03 ± 0.001*	0.9 ± 0.01*
F286E	447 ± 23	19 ± 5	291 ± 35*	0.04 ± 0.002*	6.6 ± 0.1*
E289A	162 ± 16	426 ± 72*	1633 ± 194*	0.19 ± 0.04*	0.7 ± 0.1*
E289Q	242 ± 43	25 ± 3	669 ± 62*	1.9 ± 0.1	32 ± 6
E289F	69 ± 18	252 ± 11*	976 ± 43*	0.07 ± 0.002*	0.9 ± 0.2*
E289D	275 ± 44	167 ± 72*	726 ± 130*	0.13 ± 0.01*	0.7 ± 0.02*
E289R	234 ± 27	301 ± 90*	1054 ± 297*	0.09 ± 0.02*	0.9 ± 0.3*
E289K	121 ± 37	1386 ± 292*	2385 ± 79*	0.06 ± 0.01*	0.2 ± 0.01*
E289R/ R143E	420 ± 34	28 ± 15	30 ± 16*	0.07 ± 0.02*	N.D.
A292E	352 ± 75	44 ± 16	89 ± 27*	3.2 ± 1	N.D
L296A	512 ± 96	13 ± 6	106 ± 22*	4 ± 0.5	N.D.
L296F	478 ± 70	14 ± 4	120 ± 31*	3.2 ± 0.5	N.D.
V299A	380 ± 69	52 ± 24	441 ± 43	1.2 ± 0.1	9 ± 0.4
V299F	411 ± 92	39 ± 7	449 ± 47	0.04 ± 0.01*	5.5 ± 0.5*
V300A	317 ± 24	27 ± 8	415 ± 101	6 ± 0.8	13 ± 2
V300F	246 ± 10	36 ± 19	309 ± 35	1.2 ± 0.2	23 ± 0.5
F303G	355 ± 185	7 ± 4	8 ± 2*	0.09 ± 0.01*	N.D
F303A	379 ± 92	8 ± 4	48 ± 18*	0.12 ± 0.01*	n.d.
F303L	443 ± 88	225 ± 73*	652 ± 165*	0.16 ± 0.03*	1.5 ± 0.2*
F303Y	409 ± 56	9 ± 7	227 ± 91*	10 ± 0.2	520 ± 5*

* $P < 0.05$ paired Student's t test compared with the wild-type receptor.
N.D., not determined.

the receptor as well as increased affinity and potency of the agonist (Table 1 and Fig. 2a). In agreement with the experimental findings, the model predicts that mutating E289$^{6.30}$ to the neutral amino acids Ala and Phe or to the positively charged residues Lys and Arg breaks the salt bridge interactions of R143$^{3.50}$ and should therefore constitutively activate the α_{1b}-AR.

In the E289D mutant, the charge-reinforced H-bonding interaction between positions 143$^{3.50}$ and 289$^{6.30}$ occurs less frequently than in the wild-type receptor, probably because of the shortening of the targeted amino acid side chain. In addition, the breakage of the R143$^{3.50}$-D142$^{3.49}$ interaction is very frequently observed in the simulations of this mutant. These effects correlate, at least in part, with the experimental findings showing that the E289D displays a moderate level of constitutive activation as well as increased affinity and potency of epinephrine (Table 1 and Fig. 2a).

The mutation of E289$^{6.30}$ to Gln did not significantly change the constitutive activity of the receptor or its affinity for epinephrine. However, the E289Q displayed a significant 50% increase in the maximal agonist-induced response, which is one of the features of the active state (R*) of the receptor (Samama et al., 1993). The results of computer simulations did not provide an unequivocal explanation of the effects resulting from this mutation. In fact, it was frequently observed that either the reversible or irreversible neutralization of E289$^{6.30}$ resulting from its protonation or mutation to Gln, respectively, can break both the salt bridge interactions involving R143$^{3.50}$ and the residues at positions 142$^{3.49}$ and 289$^{6.30}$. However, in some simulations, H-bonding interactions were observed between the replacing glutamine at position 289$^{6.30}$ and R143$^{3.50}$. This suggests that, despite the neutralization of the charge, the mutation of E289$^{6.30}$ to glutamine does not induce a destabilization between the cytosolic ends of helices 3 and 6 sufficient to trigger constitutive activity.

These findings are overall in good agreement with those of a recent study reporting that mutations of the equivalent glutamate (E268$^{6.30}$) of the β2-AR into Ala significantly increased the constitutive activity of the receptor (Ballesteros et al., 2001). In contrast, the mutation of the glutamate to Gln resulted only in a modest increase of constitutive activity compared with the Ala mutation without any significant change in the affinity of the agonist. It would be interesting to investigate whether other mutations of E268$^{6.30}$ in the

Fig. 1. Homology model of the α_{1b}-AR. In the lower left view, the receptor is seen from a direction parallel to the membrane surface. Each of the upper views shows the helical bundle from the intracellular side in a direction parallel to the membrane surface. Spheres represent the β-carbon atoms of the mutated amino acids. The lower right view displays the amino acids of helices 3 and 6 considered in this study. Van der Waals' spheres whose radius has been reduced by 40% depict each side chain. The effect of mutations at each residue is depicted by their color: white, no effect; green, constitutively active; red, impaired receptor mediated signaling; and violet, either impairing or constitutively activating depending upon the substituent amino acid.

β2-AR would result in greater levels of constitutive activation.

We then investigated whether swapping the positively and negatively charged residues reconstitutes their interaction, resulting in a functional mutated receptor. However, the E289R/R143E mutant was totally impaired in its ability to mediate an IP response (Table 1 and Fig. 2a). These findings agree with the predictions of the model demonstrating that positions 143 and 289 are not interchangeable. In fact, all MD simulations of the E289R/R143E mutant failed to reconstitute the salt bridge between positions $143^{3.50}$ and $289^{6.30}$, whereas new links between helices 3 and 6 were formed. In addition, the impairing effect of the double mutation is probably caused by the requirement for a positive charge at position $143^{3.50}$ of the α_{1b}-AR for agonist-induced receptor-G protein coupling, as demonstrated previously (Scheer et al., 2000).

The next amino acid in helix 6 that faces helix 3 is $L296^{6.37}$. To test its functional role, $L296^{6.37}$ was mutated to Ala or Phe, which reduces or increases its size, respectively. Both mutations of $L296^{6.37}$ to Ala and Phe resulted in a decrease in IP response of greater than 70% (Table 1). At the same time, the epinephrine affinity of these mutants was unchanged. Computer simulations indicated that the L296A mutant displayed increased proximity of the helix 3 and 6 backbones while conserving both the interactions involving $R143^{3.50}$. The mutation of $L296^{6.37}$ to Phe introduces additional interactions between the mutated amino acid and residues on helices 2 and 7 but leaves the interactions of $R143^{3.50}$ unaltered. Thus, $L296^{6.37}$ seems to have an important structural role in controlling the helix 3-helix 6 packing that is perturbed by mutations of this residue to either Ala or Phe.

The final residue in the cytosolic half of helix 6 to directly face helix 3 is $F303^{6.44}$. Previous studies have shown that mutation of this residue into Asn or Gly totally impairs the IP response but markedly increases the affinity of epinephrine (Chen et al., 1999, 2000). To further delineate the role of $F303^{6.44}$, we mutated it to Ala, Gly, Leu, and Tyr (Table 1 and Fig. 2b). Consistent with the previously published findings, the F303G receptor displayed a 50-fold increase in affinity for epinephrine and an almost complete loss of receptor mediated signaling. The F303A mutant was functionally similar

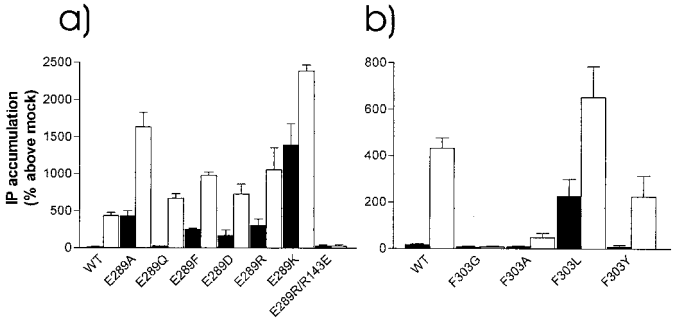

Fig. 2. Inositol phosphate response of the wild-type and mutated α_{1b}-AR. Cos-7 cells were transiently transfected with DNA encoding the wild-type α_{1b}-AR (WT) and its mutants. IP accumulation was measured in the absence (■) or presence (□) of 10^{-4}M epinephrine for 45 min. The IP levels are expressed as percentage over those of cells transfected with the plasmid pRK5 (mock). The scale (%) is different in a and b. Receptor expression measured in membrane preparations was as indicated in Table 1. The results are the mean ± S.E. of three independent experiments.

to the F303G. The mutation of $F303^{6.44}$ to Tyr impaired the receptor mediated maximal response to epinephrine by about 50%. Whereas the affinity of epinephrine was not significantly changed at the F303Y mutant, its potency was decreased by almost 20-fold (Table 1). These findings strongly suggest that the mutation of $F303^{6.44}$ to Tyr, which maintains the aromaticity of the side chain but introduces a polar functional group, impairs receptor activation. In contrast, the mutation of $F303^{6.44}$ to Leu, which maintains the hydrophobicity but abolishes the aromaticity, increased the constitutive as well as agonist-induced activity of the receptor. This mutation resulted also in a 30-fold increase in affinity and a 20-fold increase in potency of epinephrine (Table 1).

In the average minimized structure of the wild-type α_{1b}-AR, $F303^{6.44}$ together with $W307^{6.48}$ and $F310^{6.51}$ forms a cluster of aromatic amino acids on helix 6, making a chain of intrahelical interactions. The most extracellular residue in this chain, $F310^{6.51}$, may be directly involved in the agonist binding as suggested by both the computational and experimental results (Chen et al., 1999). In addition, $F303^{6.44}$ interacts with $S132^{3.39}$ and $I133^{3.40}$ as well as with $N344^{7.49}$ of the NPXXY motif. The results of computer simulations suggest that the F303A and F303G mutations enhance the packing of helices 3 and 6 while retaining both the $R143^{3.50}$-$D142^{3.49}$ and $R143^{3.50}$-$E289^{6.30}$ interactions found in the wild-type receptor. Substituting $F303^{6.44}$ with Tyr does not disrupt the interaction pattern of $R143^{3.50}$. This is consistent with experimental findings showing that the F303Y is not constitutively active. The decreased ability of epinephrine to activate this mutant might be caused, at least in part, by the fact that the phenolic hydroxy group of $Y303^{6.44}$ might perform H-bonds with $N344^{7.49}$ of the NPXXY motif, which is involved in the activation of the receptor (Scheer et al., 1996). This interaction might induce changes in the side chain fluctuations at either or both of these functionally important positions, compared with the wild-type.

Interestingly, the mutation of $F303^{6.44}$ to Leu perturbs the helix 3/helix 6 and the helix 6/helix 7 packing interactions in the vicinity of the mutated residue. This change is propagated along the helix 3/helix 6 interface, destabilizing both the $R143^{3.50}$-$D142^{3.49}$ and $R143^{3.50}$-$E289^{6.30}$ interactions. Therefore, computer simulations suggest that $F303^{6.44}$ lies in a position important for the transfer of information from the binding site of the agonist to the cytosolic portions of the helix 3-helix 6 interface.

Mutagenesis of Amino Acids on Helix 6 That Face Helices 2, 5, or 7. In this study, we also considered other amino acids in the cytosolic half of helix 6 that do not directly face helix 3 but rather are directed toward helix 2 ($A292^{6.33}$), helix 5 ($V300^{6.41}$), or helix 7 ($V299^{6.40}$).

$A292^{6.33}$ lies in the proximity to $R143^{3.50}$ of the E/DRY motif. Consequently, the mutation of $A292^{6.33}$ to Glu may be expected to reinforce the link between helices 3 and 6, thereby stabilizing the inactive state of the receptor. In agreement with this prediction, the A292E mutant displays a profoundly impaired IP response without any significant change in the affinity of epinephrine (Table 1).

As for $V299^{6.40}$ and $V300^{6.41}$, their positions in the wild-type receptor suggests that $V299^{6.40}$ rather than $V300^{6.41}$ plays a potential structural role. In fact, $V299^{6.40}$ lies between $I347^{7.52}$ and $Y348^{7.53}$ of the NPXXY highly conserved motif in helix 7. In contrast, $V300^{6.41}$ lies between $I219^{5.54}$

and L220$^{5.55}$ in helix 5. To test the functional effect of decreasing and increasing the size at these interhelical positions, V299$^{6.40}$ and V300$^{6.41}$ were mutated to Ala and Phe, respectively. Computer simulations of V299F, V300A, and V300F produce average arrangements retaining the wild-type interaction patterns of R143$^{3.50}$. In contrast, the V299A mutant retains only the R143$^{3.50}$-E289$^{6.30}$ interaction. Thus, simulations suggest that mutations at position 300$^{6.41}$ have higher propensity to behave like the wild-type than mutations at position 299$^{6.40}$.

In agreement with this, experimental mutagenesis indicated that the mutations of V299$^{6.40}$ and V300$^{6.41}$ into either Ala or Phe did not impair receptor activation (Table 1). However, the mutation of V299$^{6.40}$ into Phe increased the affinity for epinephrine by 100-fold. The potency of epinephrine was also significantly increased at the V299F mutant, but only by 5-fold (Table 1). These findings suggest that neither V299 nor V300 plays a crucial role in receptor activation. However, the structural change induced by the mutation of V299$^{6.40}$ to Phe has a marked effect on agonist binding resulting in a large increase in its affinity.

Discussion

Using predictions from a model of the α_{1b}-AR based on the crystal structure of rhodopsin and site-directed mutagenesis, we have investigated the role in receptor activation of residues on the cytosolic half of helix 6 either facing helix 3 (F286$^{6.27}$, E289$^{6.30}$, L296$^{6.37}$ and F303$^{6.44}$) or other helices (A292$^{6.33}$, V299$^{6.40}$, and V300$^{6.41}$). Our findings support the hypothesis that receptor activation involves the weakening or breaking of interhelical interactions between the cytosolic halves of helices 3 and 6. On the opposite, mutations in helix 6 that strengthen these interactions stabilize the inactive state of the receptor resulting in impaired receptor activation.

The majority of the mutations reported in this study resulted in important changes in the basal and/or agonist-induced IP response of the receptor as well as in the affinity and/or potency of epinephrine. Because the mutated residues are not predicted to be part of the α_{1b}-AR-Gq interface (Fanelli et al., 1999), a direct effect of the mutations on receptor-G protein coupling could be excluded. The computational and pharmacological analysis of the receptor mutants support the hypothesis that the mutations herein reported can either have a direct effect on the process of receptor activation [i.e., the transition from the inactive (R) to active (R*) state] or induce other structural changes having an impact on receptor function.

The Role of E289$^{6.30}$ on Helix 6 in Receptor Activation. A novel finding of our study is that mutations of E289$^{6.30}$ on the cytosolic end of helix 6 of the α_{1b}-AR can markedly increase the constitutive activity of the receptor (Table 1). All the E289$^{6.30}$ mutants (with the exception of E289Q) displayed all the properties previously considered the hallmark of the active state of the receptor (R*): increased constitutive activity, increased efficacy of the agonist, increased affinity and potency of the agonist (Samama et al., 1993). Altogether, these findings identify E289$^{6.30}$ as an important player in the activation process of the α_{1b}-AR.

The results of this study add new information to the mechanism of activation of the α_{1b}-AR, that is different, at least in part, from that proposed in our previous studies (Scheer et al., 1996, 1997). The differences mainly concern the structural features of the inactive states of the α_{1b}-AR. In fact, in the ab initio model of the α_{1b}-AR described previously, the arginine of the E/DRY motif was directed toward helix 2 and we predicted that its interaction with a conserved aspartate (D91$^{2.50}$) on this helix was an important constraint in maintaining the receptor in its inactive state.

However, the crystal structure of rhodopsin in its inactive state (Palczewski et al., 2000) suggests that the arginine of E/DRY sequence is too far apart for interacting with the conserved aspartate on helix 2, given that it is involved in interactions with both the adjacent E136$^{3.49}$ and a glutamate in helix 6, E247$^{6.30}$. Thus, the interaction between R135$^{3.50}$ and E247$^{6.30}$ is suggested to be one of the critical constraints keeping rhodopsin in the inactive state.

Similar to the rhodopsin structure, in the homology model of the wild-type α_{1b}-AR, D91$^{2.50}$ is too far to interact with R143$^{3.50}$, because the distance between the γ-carbon atom of D91$^{2.50}$ and the ζ-carbon atom of R143$^{3.50}$ is 22.8 Å (Fig. 1). Instead, in the inactive state of the receptor R143$^{3.50}$ makes a salt bridge with both the adjacent D142$^{3.49}$ and E289$^{6.30}$ on helix 6. The experimental findings described herein seem to favor the predictions of the homology model, because mutations of both D142$^{3.49}$ on helix 3 (Scheer et al., 1996) and of E289$^{6.30}$ on helix 6 markedly increase the constitutive activity of the receptor. Therefore, whereas the D142$^{3.49}$-R143$^{3.50}$ and R143$^{3.50}$-E289$^{6.30}$ interactions constrain the receptor in the inactive states, their breakage would contribute to receptor activation.

The functional role of the glutamate homologous to E289$^{6.30}$ in rhodopsin remains to be elucidated by site-directed mutagenesis. However, very recently it has been reported that mutations of the equivalent glutamate (E268$^{6.30}$) of the β2-AR into Gln and A significantly increased the constitutive activity of the receptor (Ballesteros et al., 2001). Moreover, spontaneous mutations of D564$^{6.30}$ in the human lutropin/choriogonadotropin receptor result in constitutive activation of the receptor and are associated with precocious puberty in males (Yano et al., 1995; Shenker et al., 1993). Altogether these findings strongly suggest that in the rhodopsin family of GPCRs where the arginine is almost ubiquitous and the glutamate/aspartate is highly conserved, these two residues are likely to play an important role in receptor activation.

The Role of Other Amino Acids at the Helix3/Helix 6 Interface. Our results suggest that the integrity of F286$^{6.27}$, that is the most solvent accessible residue on helix 6, is important in receptor activation contributing to maintain the inactive state of the receptor. In fact, our results suggest that the mutation of F286$^{6.27}$ to Ala or to Glu could favor or impair receptor activation weakening or reinforcing the link between the cytosolic halves of helices 3 and 6, respectively.

We have found that structural variability at position 296$^{6.37}$ of the α_{1b}-AR on the cytosolic half of helix 6 is poorly tolerated. In fact, the substitution of L296$^{6.37}$ with Ala or Phe profoundly impaired the receptor-mediated IP response. Leucine, isoleucine, or valine is generally found at the equivalent position in GPCRs belonging to the rhodopsin family. A previous random mutagenesis study on the C5a receptor demonstrated that a conservation of hydrophobicity at this position is necessary to maintain productive receptor-G pro-

tein coupling (Baranski et al., 1999). Another study has suggested that a leucine at this position contributes to the selectivity of the M1, M3, and M5 muscarinic cholinergic receptors for G_q (Blin et al., 1995). Computer simulations performed in this study suggested that the integrity of L296$^{6.37}$ plays a fundamental role in maintaining the helix 3/helix 6 packing around R143$^{3.50}$.

Our experimental findings also suggest that structural variability at position 303$^{6.44}$ of the α_{1b}-AR is not well tolerated, in accordance with the high degree of conservation of this residue in the rhodopsin family of GPCRs. Interestingly, mutations of F303$^{6.44}$ had different effects on the activation and agonist binding properties of the α_{1b}-AR. Because F303$^{6.44}$ belongs to a chain of aromatic amino acids near the binding site of epinephrine, it is not surprising that mutations of F303$^{6.44}$ can have an effect on the affinity of the agonist. In fact, mutations of F303$^{6.44}$ to Gly, Ala, and Leu markedly increased the affinity of epinephrine (Table 1). However, this increase in agonist affinity was correlated with increased constitutive activity only for the F303L mutant, in which the interaction pattern of R143$^{3.50}$ was perturbed by the mutation. In contrast, the nonconstitutively active F303A and F303G mutants, in which the packing of helices 3 and 6 was enhanced and the interaction pattern of R143$^{3.50}$ was conserved, were profoundly impaired in their activation properties. Altogether, our findings suggest that F303$^{6.44}$ is involved in the transfer of the agonist-induced conformational change along the helix 3/helix 6 interface. The analysis of the structural features of the constitutively active F303L mutant might provide insight into the conformational changes triggered by the agonist that probably can modify the helix3/helix6 and helix 6/helix7 packing.

The Role of Amino Acids on Helix 6 Facing Helices 2, 5, and 7. The mutational analysis of other residues in the cytosolic half of helix 6 suggests that in contrast to those residues facing helix 3, the majority of amino acids facing helix 2 (A292$^{6.33}$), helix 5 (K290$^{6.31}$, A293$^{6.34}$, and V300$^{6.41}$) or helix 7 (R288$^{6.29}$ and V299$^{6.40}$) do not play a prominent structure-functional role. In fact, we have reported recently that mutations of R288$^{6.29}$ and K290$^{6.31}$ to Ala or Glu did not significantly change the functional properties of the α_{1b}-AR (Greasley et al., 2001). In this study, we have found that mutating V299$^{6.40}$ and V300$^{6.41}$ to Ala or Phe did not markedly change the receptor-mediated IP response (Table 1). The only exceptions are residues that lie at almost the same level as R143$^{3.50}$ with respect to the membrane (i.e., A292$^{6.33}$ and A293$^{6.34}$). In fact, it has been shown previously that all mutations of A293$^{6.34}$ increased the constitutive activity of the α_{1b}-AR (Kjelsberg et al., 1992) Mutation of A293 to Glu induces the formation of an intrahelix salt bridge between the replacing glutamate and K290$^{6.31}$. The latter residue loses its interaction with E289$^{6.30}$, which consequently loses its charge reinforced H-bonding interaction with R143$^{3.50}$, gaining new intrahelix interactions with both K285$^{6.26}$ and the adjacent R288$^{6.29}$. Thus, the constitutive activity of the A293E mutant seems to be correlated with a rearrangement of charged amino acid side chains that results in the loss of the helix 3-helix 6 interactions. In contrast, the impairing effect of mutating A292$^{6.33}$ to Glu might be correlated with the possible formation of a salt bridge between the replacing glutamate and R143$^{3.50}$, thereby reinforcing the link between helices 3 and 6.

Conclusions

Taken together, the results of this study suggest that the role in activation of the amino acids in the cytosolic half of helix 6 of the α_{1b}-AR is strongly correlated to the extent of their structural/dynamic connection with helix 3 and the arginine of the E/DRY sequence. Consistent with the hypothesis previously inferred from the ab initio model, the homology model of the α_{1b}-AR suggests that the active receptor states would be characterized by the release or the weakening of the interactions that, in the inactive state, constrain the motion of the fully conserved arginine of the E/DRY motif. In general, we have observed that the weakening or breakage of the R143$^{3.50}$-E289$^{6.30}$ interaction is associated with the breakage of the R143$^{3.50}$-D142$^{3.49}$ interaction in those mutants displaying a high degree of constitutive activity (e.g., A293E, F303L, E289A, E289R, E289K). In contrast, in some weakly constitutively active mutants (e.g., F286A), only the breakage of the R143$^{3.50}$-D142$^{3.49}$ interaction was found, suggesting that this is one of the early events in the transition of the α_{1b}-AR from its inactive to active states.

It is worth noting that a dramatic detachment between the cytosolic halves of helices 3 and 6, as observed in the light-induced active state of rhodopsin (Farrens et al., 1996), has never been observed in our simulations. This agrees with the finding that in rhodopsin, the constitutively activating mutation of E134 to glutamine induces helix motions different from those caused by photoactivation (Kim et al., 1997). Indeed, the E134Q active mutant did not display the dramatic increase in distance between helices C and Phe as observed upon photoactivation of the wild-type rhodopsin (Farrens et al., 1996).

According to both the ab initio and homology models of the α_{1b}-AR, in the most active mutants, the perturbations in the interactions of R143$^{3.50}$ are associated with the increase in solvent accessibility of amino acids in the cytosolic extension of helix 6 and, for the ab initio model only, in the N-terminal portion of the second intracellular loop (Fanelli et al., 1999). However, this increase in solvent accessibility very rarely involves R143$^{3.50}$, suggesting that the main role of this amino acid is to mediate the allosteric transition between the inactive ad active receptor states rather than directly binding the G-protein. The hypothesis suggested by the theoretical models is that the change in the interaction pattern of R143 would be instrumental to allow amino acids in the third intracellular loop, including R254 and K258 to assume the proper configuration for binding and/or activating the G protein (Fanelli et al., 1999; Greasley et al., 2001). However, the role of the highly conserved arginine of the E/DRY in the activation process of GPCRs awaits full elucidation.

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