Characterization of the Residues in Helix 8 of the Human \( \beta_1 \)-Adrenergic Receptor That Are Involved in Coupling the Receptor to G Proteins*

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Several key amino acids within amphipathic helix 8 of the human \( \beta_1 \)-adrenergic receptor (\( \beta_1 \)-AR) were mutagenized to characterize their role in signaling by G protein-coupled receptors. Mutagenesis of phenylalanine at position 383 in the hydrophobic interface to histidine (F383H) prevented the biosynthesis of the receptor, indicating that the orientation of helix 8 is important for receptor biosynthesis. Mutagenesis of aspartic acid at position 382 in the hydrophilic interface to leucine (D382L) reduced the binding and uncoupled the receptor from G protein activation. Mutagenesis of the basic arginine residue at position 384 to glutamine (R384Q) or to glutamic acid (R384E) increased basal and agonist-stimulated adenylyl cyclase activities. R384Q and R384E displayed features associated with constitutively active receptors because inverse agonists markedly reduced their elevated basal adenylyl cyclase activities. R384Q and R384E displayed features associated with constitutively active receptors because inverse agonists markedly reduced their elevated basal adenylyl cyclase activities.

Isoproterenol increased the phosphorylation and promoted the desensitization of the Gly389 or Arg389 allelic variants of the wild type \( \beta_1 \)-AR but failed to produce these effects in R384Q and R384E, because these receptors were maximally phosphorylated and desensitized under basal conditions. In contrast to the membranous distribution of the wild type \( \beta_1 \)-AR, R384Q and R384E were localized mostly within intracellular punctate structures. Inverse agonists restored the membranous distribution of R384Q and R384E, indicating that they recycled normally when their constitutive internalization was blocked by inverse agonists. These data combined with computer modeling of the putative three-dimensional organization of helix 8 indicated that the amphipathic character of helix 8 and side chain projections of Asp382 and Arg384 within the hydrophilic interface might serve as a tethering site for the G protein.

GPCR for mediating the functions of catecholamines. Activation of the \( \beta_1 \)-AR increases heart rate and myocardial contractility and increases the secretion of renin, all of which contribute to its important role in regulating cardiac function and blood pressure (3). The \( \beta_1 \)-AR is a prominent member of class A GPCR, which are structurally related to the visual receptor rhodopsin that propagates its signal through the activation of the G protein transducin (1). The three-dimensional crystal structure of dark-adapted rhodopsin offers a structural template for other GPCRs, including the assignment of secondary structural elements and the location of highly conserved amino acids (4). This model can accommodate most of the essential elements of functional importance in G protein activation. Therefore, an evaluation of the \( \beta_1 \)-AR can be modeled after rhodopsin.

The cytoplasmic surface of class A GPCR includes three loops and a C-terminal tail. The first, second, and third loops connect adjacent transmembrane helices. A crucial finding in the crystal structure of rhodopsin was the confirmation that residues in helix 7 and the C-terminal domain form a cytoplasmic helical structure termed helix 8 (4). Helix 8 is bound by helix 7 at its N terminus and by two palmitoyl groups inserted in the membrane bilayer at its C terminus (5). In the human \( \beta_1 \)-AR it is predicted that helix 8 is preceded by a short linker sequence of 3–4 amino acids that begin after the tyrosine residue at position 377 of the NPXYX motif. The linker sequence is followed by a stretch of 10 amino acids that are predicted to form an \( \alpha \)-helical structure that constitutes a novel hallmark of class A of the GPCR superfamily (4). In rhodopsin, helix 8 was involved in coupling rhodopsin to transducin because synthetic peptides corresponding to helix 8 competed with metabotropic helix II in binding transducin (6). In addition, site-directed mutagenesis within helix 8 of rhodopsin indicates that helix 8 is involved in rhodopsin-mediated activation of transducin (7–9). Analysis of the role of helix 8 in other GPCR revealed a number of diverse functions attributed to this domain. Helix 8 was involved in regulating surface expression of the melanin concentrating hormone receptor-1 (10) and in the internalization of the bradykinin receptor (11, 12). Residues within the hydrophilic interface of putative helix 8 of angiotensin II, bradykinin, and oxytocin receptors were involved in coupling these receptors to Gs (12–14).

The studies described earlier have focused on the role of helix 8 in GCs-coupled GPCR. Here we characterized site-directed mutants of helix 8 in coupling the human \( \beta_1 \)-AR to the stimulatory regulatory G protein (Gs). Our detailed analyses show that proper orientation of helix 8 regulates the biosynthesis of the \( \beta_1 \)-AR, and the charges of key residues in the hydrophilic interface of helix 8 are involved in proper expression and coupling of the \( \beta_1 \)-AR to Gs.

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2 The abbreviations used are: GPCR, G protein-coupled receptors; \( \beta_1 \)-AR, \( \beta_1 \)-adrenergic receptor; WT \( \beta_1 \)-AR, wild type \( \beta_1 \)-AR; helix \( \beta_8 \), eighth cytoplasmic \( \alpha \)-helical loop; DMEM, Dulbecco’s modified Eagles medium; ICYP, [\(^{125}\)I]iodocyanopindolol, KD, receptor binding affinity, \( K_m \), maximal density of receptors, \( K_s \), high affinity binding site for agonists, \( K_L \), low affinity binding site for agonists, EC\(_{50}\), concentration of agonist that elicits half-maximal responses; G\(_S\), stimulatory G protein; BLT-1, leukotriene B\(_4\) receptor; FITC, fluorescein isothiocyanate; GTP\(_{\gamma}\)S, guanosine 5’-3-O-(thio)triphosphate.
**Mapping Helix 8 in the Human β1-AR**

Cyclic AMP Accumulation and Adenylyl Cyclase Assays—Transiently transfected cells in 6-well plates were switched to DMEM + 25 mM HEPES for 2 h. Appropriate drugs in DMEM/HEPES, supplemented with 300 μM of the phosphodiesterase inhibitor ibosulfamethoxynithane, were added to the cells for 10 min at 37 °C. The reaction was stopped, and 1 ml of 0.1 N cold HCl was added followed by freezing of the entire plate in liquid nitrogen. Frozen plates were quickly thawed at 65 °C to break the cells, and the cell extract was lyophilized. The dry pellet was resuspended in assay buffer, and cyclic AMP was quantified by radioimmunoassay (RIKENEN Assay System, DuPont).

For the determination of adenylyl cyclase activity, membranes were prepared from cells without phenylmethylsulfonyl fluoride. Fifty mg of membrane proteins were incubated in a final volume of 0.1 ml in buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 10 mM phosphocreatine, 1 mM cyclic AMP, 2 mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.4 mM EGTA, 2 mg/ml creatine kinase, 0.2 mM ATP containing 2 μCi of [α-32P]ATP, 1 mM GTP, and increasing concentrations of isoproterenol from 0.1 nM to 1 mCi. The assay was initiated by the addition of membranes and terminated after 10 min (16). Cyclic AMP that was formed was separated from ATP by column chromatography, and its specific activity was determined as cyclic AMP formed in pmol/min/mg protein.

**Intact Cell Phosphorylation and β1-AR Immunoprecipitation**—Cell cultures were switched to phosphate-free DMEM supplemented with 12.5 mM HEPES for 60 min. Then they were incubated with 100 μCi of [32P]Pi/ml for 2 h at 37 °C in equilibrate the [32P]ATP pools. The cells were exposed to 1 mM ascorbic acid (control) or 10 μM isoproterenol for 10 min at 37 °C, followed by rapid aspiration of this medium. The cells were lysed with 1 ml/plate of ice-cold RIPA, and SDS buffer (20 mM Tris-HCl, 3.1 were transiently transfected into HEK-293 cells using the Cytofect method (Clontech). The sequence of the mutated β1-AR cDNA—cDNA—Cytofectene at room temperature for 20 min. Then

**Preparation of Membranes and Radioligand Binding Assays**—Transiently transfected or stable cell lines on 10-cm culture plates were cultured in DMEM supplemented with 10% fetal bovine serum until they reached 90% confluence. The WT β1-AR or its point mutants in pcDNA 3.1 were transiently transfected into HEK-293 cells using the Transforn system (Clontech). The sequence of the mutated β1-AR cDNA—cDNA was verified by dideoxy sequencing, followed by cloning of the mutated full-length β1-AR cDNAs into the mammalian expression vector pcDNA-3.1 (Invitrogen).

**Cell Culture and Transient Transfections—**HEK-293 cells were cultured in DMEM supplemented with 10% fetal bovine serum until they were ~90% confluent. The WT β1-AR or its point mutants in pcDNA 3.1 were transiently transfected into HEK-293 cells using the Cytodex gene reagent (Bio-Rad) as follows. Plasmid DNA (5 μg) was diluted into 200 μl of DMEM and then mixed with an equal volume of DMEM containing 12 μl of Cytodex at room temperature for 20 min. Then 4 ml of DMEM was added, and the DNA-lipid complex was layered over the cells for 5 h at 37 °C, followed by the addition of an equal volume of DMEM + 10% fetal bovine serum. Stable cell lines for Gly389, G389R, D382L, R384E, and R384Q were used where indicated.

**Preparation of Membranes and Radioligand Binding Assays**—Transiently transfected or stable cell lines on 10-cm culture plates were washed twice with 10 ml of ice-cold PBS, then scraped from the plates, and pelleted by centrifugation at 4,000 × g for 10 min. The cell pellets were suspended in 10 ml of hypotonic buffer composed of 20 mM HEPES, pH 7.4, 2 mM MgCl2, 1 mM EDTA, and 1 mM 2-mercaptoethanol supplemented with 10 μg/ml leupeptin and 10 μg/ml aprotinin with or without 1 mM phenylmethylsulfonyl fluoride for 10 min on ice. The cells were lysed by 30 up-and-down strokes in a glass-glass homogenizer and then centrifuged at 2,500 × g for 5 min. The supernatant was recentrifuged at 15,000 × g for 20 min to pellet the membranes.

Binding of [3H]iodocyanopindolol (ICYP) to 0.5 μg of membranes was measured in 50 mM Tris-HCl, pH 7.4, plus 10 mM MgCl2 binding buffer containing 0.1 mM ascorbic acid for 2 h at 25 °C. For saturation binding experiments, ICYP concentrations ranging between 5 and 300 nM were used to calculate the Kd and the Bmax values for ICYP binding by parametric fitting of the data using the Prism 4 software (Graph-Pad Corp.).

For competition binding experiments, ICYP 70 nM was competed with 24 increasing concentrations of isoproterenol ranging from 0.01 nM to 10 μM. The IC50 (high) and IC50 (low) values for isoproterenol were derived from two-compartment competition to the GTP data. The IC50 values were converted to the corresponding K_i (high) and K_i (low) values using Equation 1.

\[
K_i = \frac{IC_{50} \ (nm) \ (1 + \text{concentration of ICYP})}{IC_{50} \ (nm)} \quad (\text{Eq. 1})
\]

Each saturation and competition experiment was in triplicate, and each was replicated between three and five times to determine the means ± S.E.
**Mapping Helix 8 in the Human β1-AR**

**TABLE 1**

| GPCR         | Helix 8          |
|--------------|------------------|
| hβ1-AR       | YCRS- [P] D F R  |
| hβ3-AR       | YCRS- [P] D F R  |
| hα1a-AR      | YPCSS [R] F K R |
| hα1b-AR      | YPCSS [K] R F Q |
| hα1c-AR      | YPCSS [Q] R F Q |
| hα2a-AR      | YTFN [H] D F R |
| hα2b-AR      | YTFN [Q] D F R |
| hα2c-AR      | YTFN [F] D R F |
| hD1-AR       | YAFN [A] D F R  |
| hD2-AR       | YTFN [I] D F R  |
| hD3-AR       | YTFN [E] F R Q |
| hD4-AR       | YTVF [A] E F R  |
| rMCH-1R      | YIVLC [T] F R   |

**GPCR** | **Helix 8**
-------------------
| boRHO | YIMMN [K] P |
| hM1-R | YALCN [K] R |
| hM2-R | YALCN [A] T |
| hM3-R | YALCN [T] K |
| hM4-R | YALCN [A] F |
| hA1-R | YAFRI [Q] F |
| hA2-R | YAYRI [R] F |
| hSHT1a-R | YAYFN [K] D |
| hSHT1b-R | YTMSN [E] D |
| hSP-R | YCCLN [D] F |
| hBR1-R | YVVG [K] F |
| hOX-R | YTFLG [H] P |
| hANG1-R | YGFLG [K] F |
| hBLT1-R | RSRAG [V] F |

To ascertain that the recycled receptor trafficked back to the membrane, coverslips were incubated with alprenolol for 45 min and then permeabilized in Tris-buffered saline containing 1% Triton X-100, 2% normal goat serum, and 1% bovine serum albumin (20). To estimate the effect of the inverse agonists on the distribution of receptors, a circular boundary was drawn around the inner circumference of control and inverse agonist-treated cells to define a 300-nm-wide membranous delimited area (21). Fluorescence intensity measurements were calculated in the areas outside and inside the boundary to estimate the effect of the inverse agonist on this parameter. The data represent the mean of triplicate determinations ± S.E. from at least four independent experiments.

Analysis of Immunocytochemical Data—All analyses were performed blind to the stimulation history of the culture. Microscope fields had 1–3 cells displaying generally healthy morphology. Six to 10 cells were imaged per culture, and n = 10 cultures were processed per condition. Confocal microscopy was performed on all the slides using a Zeiss Axiosvert LSM 510 (100 × 1.4 DIC oil immersion objective). FITC was excited with the 488-nm argon laser and imaged through the 520-nm long-pass emission filter. Thresholds were set by visual inspection and kept constant for each condition. Z-stacks of images were exported as TIFF files, and individual sections were analyzed with Zeiss LSM 510 and NIH Image 1.6 software as described in Gardner et al. (19).

**Molecular Modeling of Wild Type and Mutant β1-AR—Homology modeling was performed with the program SegMod (22). The program was used to optimally align the sequences of the human β1-AR and bovine rhodopsin, and then the sequence of the β1-AR was threaded onto the rhodopsin structure based upon the 1F88 (4) and the 1HZX (23) models of rhodopsin oligomers deposited in the Protein Data Bank. The resulting homology model was then refined according to SegMod protocols. The figures were produced using MOLSCRIPT (24) and rendered with RASTER3D (25) that was also used to estimate the distances in angstroms between the various amino acids.

Data Analysis—Quantitative data were summarized and presented as means ± S.E. from at least four determinations each from triplicate
expressions. Least squares linear regression and nonlinear curves were calculated by the Prism 4 program (Graphpad version 4.05) to estimate the $K_{\text{diss}}$ of each $\beta_1$-AR in stimulating the activity of adenylyl cyclase. Statistical comparisons were analyzed by two-way analysis of variance with post hoc tests using Graphpad Prism program. A value of $p < 0.05$ was considered statistically significant.

**RESULTS**

The crystal structure of bovine rhodopsin indicates that a partially exposed $\alpha$-helical termed helix 8 is located between TM7 and the palmitoylated cysteines in the C terminus. The corresponding region in the human $\beta_1$-AR is the sequence between Pro$^{381}$ and Leu$^{390}$ (Table 1). The distribution of side chains in helix 8 of rhodopsin exhibits an amphiphilic pattern, where charged polar groups cluster on one side and hydrophobic ones cluster on the other side of the helix (4, 23). Based upon the rhodopsin model, it is expected that the corresponding hydrophobic amino acids in the human $\beta_1$-AR, Phe$^{383}$ and Phe$^{387}$, will be buried in the hydrophobic core of the helix facing the inner leaf of the membrane, whereas the charged polar groups Arg$^{384}$, Lys$^{385}$, and Gln$^{386}$ would cluster on the other side of the $\alpha$-helix that is exposed to the cytoplasmic environment. As shown in Table 1, the identity and location of the two hydrophobic Phe residues are conserved in class A of GPCR. On the hydrophilic side of the helix, the residue corresponding to Arg$^{384}$ is always a basic amino acid such as Arg or Lys, whereas the residues corresponding to Lys$^{385}$ and Gln$^{386}$ are not conserved.

To test the validity of this model in predicting the structure-activity relationship of these residues, 11 point mutations were introduced into putative helix 8 of the N-terminally FLAG-tagged human $\beta_1$-AR (Table 2). We used the Biotecnix 3D (Gentech Corp.) computer program to verify that none of the point mutations disrupted the predicted $\alpha$-helical secondary structure of helix 8.

**Expression and Ligand Binding Properties of Helix 8 Mutants---Immunoblot analyses of detergent extracts prepared from HEK-293 cells that were transiently transfected with the various helix 8 mutants revealed that monomers and dimers of the 73-kDa $\beta_1$-AR were expressed (Fig. 1). The expression patterns of the various helix 8 mutants were comparable with that of the WT $\beta_1$-AR with the exception of the F383H mutant. The highly conserved hydrophobic Phe$^{383}$ when mutated to a charged basic His residue (F383H) was not synthesized as a mature protein and did not result in detectable radioligand binding activity (Table 2).

The other point mutants were tested for the pharmacological character of their radioligand binding profiles (Table 2). Helix 8 mutants bound the radiolabeled $\beta$-AR antagonist $[\text{125I}]$ICYP with $K_I$ values between 15 and 25 nM, which were comparable with the $K_I$ value of the WT $\beta_1$-AR. In addition, the $B_{\text{max}}$ values of the various helix 8 mutants were comparable with the $B_{\text{max}}$ values of the WT $\beta_1$-AR.

The high and low affinity states for isoproterenol binding to the $\beta_1$-AR were modeled by two-site analysis of the competition isotherms of isoproterenol (Fig. 2). The percentage of receptors in the high affinity state for agonists was comparable among the different helix 8 mutants (Table 2). The affinities of some point mutants to isoproterenol, however, were not comparable (Fig. 2). Specifically, mutagenesis of the negatively charged amino acid Asp$^{382}$ to a nonpolar hydrophobic Leu (D382L) produced a mutant $\beta_1$-AR with ~200-fold lower affinity to isoproterenol than the WT $\beta_1$-AR (Fig. 2 and Table 2).

**Table 2**

| Expression vector | $IC_{50}$ high | $IC_{50}$ low | $K_D$ | $B_{\text{max}}$ | $K_I$ high | $K_I$ low |
|-------------------|---------------|---------------|-------|-----------------|------------|-----------|
| WT $\beta_1$-AR   | 1.4 ± 0.4     | 50            | 0.4 ± 0.1 | 50              | 151 ± 4    | 1.3 ± 0.4 |
| D382L             | 290 ± 37      | ND            | 83.2 ± 2 | ND              | 17.2 ± 5   | 1.5 ± 0.5 |
| R384E             | 0.08 ± 0.003  | 47            | 0.01 ± 0.005 | 53     | 13.8 ± 5   | 1 ± 0.2  |
| R384Q             | 0.015 ± 0.003 | 60            | 0.04 ± 0.003 | 40     | 27 ± 2    | 1.2 ± 0.25 |
| R384K             | 21 ± 4        | 55            | 1.5 ± 0.7 | 45               | 23 ± 4     | 0.58 ± 0.2 |
| R384F             | 7 ± 0.04      | 54            | 1 ± 0.3  | 46               | 19 ± 5     | 0.47 ± 0.2 |
| R384H             | 6 ± 0.4       | 36            | 0.7 ± 0.1 | 64               | 20 ± 4     | 1.2 ± 0.3 |
| K385R             | 0.8 ± 0.04    | 32            | 0.4 ± 0.06 | 68     | 19 ± 3     | 1.1 ± 0.3 |
| K385E             | 0.9 ± 0.05    | 32            | 1.1 ± 0.2 | 68               | 17 ± 3     | 0.6 ± 0.25 |
| K385M             | 31 ± 5        | 52            | 4.2 ± 0.3 | 48               | 16 ± 4     | 0.8 ± 0.2 |
| G389R             | 0.5 ± 0.06    | 70            | 0.2 ± 0.03 | 30     | 13 ± 2     | 1.1 ± 0.3 |

The dissociation constants ($K_I$) of isoproterenol for the high and low affinity binding sites were calculated from the corresponding $IC_{50}$ values as described under “Experimental Procedures.” $K_I$ high and $K_I$ low indicate the percentage of high and low affinity binding sites, respectively. The results are mean ± S.E. of eight triplicate independent determinations for WT $\beta_1$-AR, R384E, R384Q, and G389R or three determinations for the other mutants. The values for the high affinity binding sites are in nM, while the values for the low affinity binding sites are in pmol/mg.
neutralization of its charge with an amino acid of comparable mass such as Phe (R384F) reduced the binding affinity of isoproterenol to these mutants by 5–20-fold, and these differences in the $K_I$ were statistically significant ($p < 0.05$). Therefore, it is possible to alter the binding affinity of the $\beta_1$-AR to agonists through selective mutations in Arg384.

In the next series of experiments, we determined the effect of mutating the surface-exposed Lys385 on the binding affinities of isoproterenol. Mutagenesis of Lys385 to another basic amino acid such as Arg (K385R) or to an acidic amino acid such as Glu (K385E) did not alter the binding affinities of these mutants to isoproterenol (Table 2). However, mutagenesis of Lys385 to the hydrophobic Met residue (K385M) markedly reduced the binding affinity of isoproterenol (Fig. 2). We also investigated the effect of Gly389 or Arg389 on the binding affinity of isoproterenol because these residues are expressed in this position at an allelic frequency of 0.26 and 0.76, respectively (26). The affinity of isoproterenol for the Arg389 allele was ~3-fold higher than its affinity for the Gly389 allele, in agreement with other more detailed studies on this allelic variation (26).

**Coupling of Point Mutants in Helix 8 to Gs/Adenylyl Cyclase**—Activation of the $\beta_1$-AR promotes the conversion of GDP-Gs into GTP-Gs leading to the activation of adenylyl cyclase that catalyzes the conversion of ATP into cyclic AMP. To investigate the role of helix 8 in coupling the $\beta_1$-AR to Gs, basal and isoproterenol-stimulated cyclic AMP accumulation were measured in cells expressing comparable amounts (between 0.8 and 1.3 pmol of $\beta_1$-AR/mg protein) of WT $\beta_1$-AR or its helix 8 mutants (Fig. 3A). Isoproterenol produced a graded increase in cyclic AMP accumulation in cells expressing the WT $\beta_1$-AR that was maximal at 50 nM. Expression of either F383H or D382L did not increase cyclic AMP accumulation in response to isoproterenol because the F383H mutation prevented the biosynthesis of the $\beta_1$-AR, and the D382L mutation apparently uncoupled the receptor from Gs. Based on their binding parameters in Table 2, the Arg384 point mutants were divided into mutations that either increased or decreased the binding affinity of isoproterenol for the $\beta_1$-AR. These differences were manifested in their respective potencies in promoting cyclic AMP accumulation. Arg384 mutations that reduced the affinity of isoproterenol in binding to the $\beta_1$-AR, such as R384F and R384K, were associated with markedly diminished receptor-Gs coupling efficacy and with 70% reduction in maximal levels of cyclic AMP accumulation. Arg384 muta-

**FIGURE 2.** Competition binding isotherms of isoproterenol to the wild type $\beta_1$-AR or its helix 8 mutants. [3H]ICYP binding was determined as described under “Experimental Procedures” on membranes derived from HEK-293 cells expressing the WT $\beta_1$-AR or mutants in helix 8 of the $\beta_1$-AR. The results were representative of three to eight independent experiments whose mean values are reported in Table 2.

**FIGURE 3.** Dose-response curves of isoproterenol on cyclic AMP accumulation. HEK-293 cells grown on 24-well plates and expressing the WT $\beta_1$-AR or its helix 8 point mutants at comparable receptor densities were stimulated with increasing concentrations of isoproterenol, and their cyclic AMP levels were measured as described under “Experimental Procedures.” The results represent the means ± S.E. of four to six separate determinations each performed on triplicate samples.
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Table 3

| Construct | Membrane adenylyl cyclase activity | Change from basal |
|-----------|-----------------------------------|------------------|
|           | Basal | Metoprolol | pmol/min/mg | %                  |
| WT β1-AR  |       |            | 16 ± 3      | 12 ± 2*             | -25 ± 3 |
| R384E     | 85 ± 13 | 32 ± 4* | 52 ± 4         |
| R384Q     | 72 ± 15 | 32 ± 4* | -55 ± 6        |

* p < 0.05 versus basal.
** p < 0.008 versus basal.
*** p < 0.004 versus basal.

Inverse agonism of metoprolol on R384E and R384Q mutants of the β1-AR

Membranes were prepared from cells expressing either the WT β1-AR or R384E and R384Q. The membranes were incubated at 37 °C for 15 min in the absence or presence of 100 μM metoprolol, washed three times in ice-cold PBS + 10 mM HEPES, pH 7.4, followed by determining the activity of adenylyl cyclase in response to 10 μM isoproterenol as described under “Experimental Procedures.” Values are mean ± S.E. of four determinations, each in triplicate.

The basal activities of adenylyl cyclase for R384E or R384Q were 62 ± 11 pmol/mg/min. Maximal activation of adenylyl cyclase by R384Q was 218 ± 38 pmol/mg/min (n = 5), which was attained at 10 nM or less of isoproterenol. Similarly, maximal activation of adenylyl cyclase by R384E occurred at 12 nM isoproterenol (EC50 = 0.4 ± 0.06 nM), but the absolute values for its maximal activation were comparable with those achieved by the WT β1-AR (Fig. 4B). The profile of the other Arg384 and Lys385 point mutants in activating adenylyl cyclase was comparable (EC50 = 79 ± 17 nM), but their maximal activation of the cyclase was 45 ± 11% of that attained by the WT β1-AR.

The allosteric ternary complex model for GPCR activation predicts that constitutively active GPCR display increased affinity for agonists and decreased affinity for inverse agonists as compared with wild type GPCR (27). Metoprolol is a selective β1-AR blocker that is widely used for the management of essential hypertension, which acted as a weak inverse agonist in cells expressing constitutively active β1-AR mutants (28). Basal adenylyl cyclase activity in the presence of 100 μM metoprolol was reduced by 25 ± 3% in WT β1-AR membranes, but the magnitude of metoprolol-mediated inhibition of adenylyl cyclase in R384E or R384Q membranes was significantly higher at 52 ± 4 and 55 ± 6%, respectively (Table 3). Therefore, mutagenesis of R384E or R384Q produced a gain of function profile that is more commonly associated with constitutively active receptors.

Desensitization of Helix 8 Point Mutants—Resistance to agonist-mediated desensitization could account for the functional differences between the various helix 8 mutants because desensitization controls the duration of the cellular signal generated by the GPCR (29). Desensitization was studied by comparing adenylyl cyclase activities in membranes prepared from cells that were exposed to buffer or 10 μM isoproterenol for 20 min (Fig. 5). In the case of the Gly389 variant of the WT β1-AR, desensitization was manifested by a 48% reduction of maximal adenylyl cyclase activity and by a right-ward shift of the dose-response curve that increased the EC50 from 15 ± 3 to 32 ± 6 nM (Fig. 5A). The EC50 value of G389R in activating adenylyl cyclase also increased from 5 ± 2 to 23 ± 6 nM in membranes prepared from cells exposed previously to isoproterenol (Fig. 5B). The dose-response curves of R384E or R384Q were neither shifted to the right nor appreciably desensitized, because the EC50 values in both cases were between 2 and 5 nM (Fig. 5, C and D). The dose-response curves for the other Arg384 point mutants such as R384F or Lys385 point mutants such as K385E in activating
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**FIGURE 5.** Short term desensitization of adenylyl cyclase activity in membranes expressing the wild type $\beta_1$-AR and Arg$^{384}$ or Lys$^{385}$ mutants. Intact cells were prestimulated (closed symbols) or not (open symbols) with 10 $\mu$m isoproterenol for 20 min at 37°C. Then membranes were prepared on ice, and adenylyl cyclase activities were determined at increasing concentrations of isoproterenol as described under “Experimental Procedures.” Dose-response curves of isoproterenol for the Gly/Arg$^{389}$ variants of the wild type $\beta_1$-AR and Arg$^{384}$, R384Q, R384F, and K385E are expressed as percent maximal adenylyl cyclase value after subtraction of basal activity. Basal and maximal adenylyl cyclase activities, respectively, were as follows: Gly$^{389}$, 16 ± 4 and 145 ± 23 pmol/mg/min; G389R, 21 ± 6 and 172 ± 30; R384E, 65 ± 13 and 180 ± 25; R384Q, 62 ± 12 and 210 ± 38; R384F, 9 ± 3 and 24 ± 5; and K385E, 15 ± 4 and 152 ± 21. These values represent the adenylyl cyclase activities in untreated membranes. The figure shows the mean ± S.E. values derived from three separate determinations, each in triplicate.

Adenylyl cyclase were shifted to the right causing a 3–4-fold reduction in their EC$_{50}$ values and about 50% reduction in their maximal adenylyl cyclase activities (Fig. 5, E and F). Therefore, neutralization or reversal of the charge of Arg$^{384}$ resulted in constitutively active $\beta_1$-ARs that were resistant to agonist-induced desensitization.

**Phosphorylation of Helix 8 Point Mutants**—Another characteristic of constitutively active and desensitized GPCR is that they are constitutively phosphorylated (30). To determine whether the different extent of desensitization among the various helix 8 mutants could be explained by differences in their phosphorylation, basal and isoproterenol-mediated phosphorylation of these receptors was determined in cells expressing equivalent amounts of receptor densities (Fig. 6). As shown in Fig. 6, exposure of cells expressing Gly$^{389}$ to isoproterenol resulted in receptor phosphorylation that was rapid (maximal response occurred in -3 min) and resulted in a 530 ± 80% increase in $^{32}$P incorporation above basal levels ($n = 6$). Basal phosphorylation of G389R was higher than Gly$^{389}$, which is expected because basal cyclic AMP levels of G389R were higher (Fig. 3). The phosphorylation of G389R in response to isoproterenol was increased by 200 ± 43% above basal ($n = 3$), whereas in the F383H mutant we did not observe appreciable $^{32}$P incorporation either in the presence or absence of isoproterenol. The magnitudes of the increase in the Lys$^{385}$ mutants were comparable with that observed in the WT $\beta_1$-AR (data not shown). Basal phosphorylation levels of the constitutively active R384E and R384Q were significantly higher than the WT $\beta_1$-AR (410 ± 50%, $n = 3$). The incorporation of $^{32}$P into R384E and R384Q did not appreciably increase in response to isoproterenol, indicating that these receptors were already maximally phosphorylated. Therefore, mutagenesis of R384E and R384Q resulted in constitutively phosphorylated and desensitized $\beta_1$-AR mutants.

**Distribution and Recycling of Helix 8 $\beta_1$-AR Mutants**—The distribution of the WT $\beta_1$-AR and the other helix 8 mutants in HEK-293 cells was determined by confocal microscopy (Fig. 7). The cells were transfected for 2 days with the various FLAG-tagged $\beta_1$-AR constructs or with empty pCMV-Tag 2 that expresses a 37-amino acid FLAG-tagged peptide. The pCMV-Tag2 vector was used to compare the distribution of the FLAG tag alone with that of the FLAG-tagged $\beta_1$-AR. To visualize the various FLAG-tagged constructs, the cells were labeled for 1 h at 37°C with FITC-conjugated anti-FLAG IgG and then fixed and imaged with the Zeiss LSM-510 (Fig. 7A). The images revealed that the various FLAG epitopes were expressed in the cell membrane (Fig. 7A, panels a, j, s, b', and k'). To confirm these data, the cells were exposed to mild acidic conditions to remove the FITC-labeled anti-FLAG antibody from the extracellular lining of the cell membrane (Fig. 7A, panels b, k, t, c', and f'). FITC anti-FLAG IgG labeling was stripped from cells expressing the various FLAG-tagged $\beta_1$-AR (Fig. 7A, panels k, t, c', and f'). However, the localization of the FLAG peptide in cells expressing pCMV-Tag-2 was consistent through out the entire experiment (Fig. 7A, panels a–i).

Exposing the various cell lines to isoproterenol for 30 min promoted the internalization of Gly$^{389}$, G389R, and the other helix 8 mutants into distinct intracellular punctate vesicular structures that are associated with the internalized GPCR phenotype (Fig. 7A, panels l, u, d', and m').
Mapping Helix 8 in the Human $\beta_{1}$-AR

FIGURE 6. Isoproterenol-mediated phosphorylation of the wild type and its helix 8 mutants in HEK-293 cells. HEK-293 cells expressing the various FLAG-tagged $\beta_{1}$-AR were metabolically labeled with $^{32}$P$\textsuperscript{32}$PO$_4$ in phosphate-free DMEM and exposed to either buffer (−) or 10 $\mu$M isoproterenol (ISO) (+) for 10 min. Cells were lysed in radioliumme precipitation buffer, and equal amounts of protein were incubated overnight with anti-FLAG M2 IgG conjugated to agarose. Anti-FLAG immunoprecipitates were subjected to SDS-PAGE, and the gels were dried and subjected to autoradiography. The amount of $^{32}$P incorporated into each $\beta_{1}$-AR monomer band from $n = 3$ separate experiments was plotted. Statistical analyses were based on analysis of variance with post hoc tests.

FIGURE 7. Localization and recycling of the wild type $\beta_{1}$-AR and its helix 8 variants in HEK-293 cells by confocal microscopy. A, HEK-293 cells cultured on poly-l-lysine-coated coverslips were transfected with the FLAG-tagged $\beta_{1}$-AR or the empty pCMV Tag2 FLAG vector, which expresses a fusion protein composed of the FLAG peptide (DYKDDDDK) fused to a 23-amino acid peptide. The slides were incubated with FITC-anti FLAG M2 antibody for 1 h at 37 °C and then exposed to 10 $\mu$M isoproterenol (ISO) for 30 min. The cells were acid-washed (0.5 M NaCl, 0.2 M acetic acid for 4 min at 4 °C) to strip off the antibody bound to the extracellular lining of the cell membrane. $\beta_{1}$-AR recycling was initiated by exposing the cells to 100 $\mu$M of the $\beta$-adrenergic antagonist alprenolol at 37 °C. After each period, the cells were fixed and then visualized by confocal microscopy. At the end of the recycling period, the cells in panels i, r, a, f, and t were subjected to a second acid wash to strip the antibody from the recycled receptors. Each image is a representative image of at least 10 separate experiments involving five or more cells. $t_{\%}$ quantification of $\beta_{1}$-AR recycling kinetics by confocal recycling assays. The LSM-510 software was used to determine the pixel densities after each recycling period. The time constants ($\tau$) for $\beta_{1}$-AR recycling were determined by fitting the data to a single exponential decay function of the form $\gamma = y_0 + Ae^{-t/\tau}$, where $y_0$ and $A$ are constants. Each scale bar represents 5 $\mu$m.

As expected, the internalized $\beta_{1}$-AR constructs were resistant to acid treatment (Fig. 7A, panels m, v′, e′, and i′).

To determine the recycling kinetics of the various $\beta_{1}$-AR constructs, the cells were exposed to the $\beta$-antagonist alprenolol to segregate receptor recycling from internalization. Gly$^{389}$ and G389R recycled rapidly (Fig. 7A, panels n–q and w–z) and completely as indexed by acid stripping (Fig. 7A, panels r and a′). The recycling of R384F and K385E appeared to be similar to that of the WT $\beta_{1}$-AR (Fig. 7A, panels f′–l′ and o′–r′). The recycling kinetics of Gly389, G389R, R384F, and K385E were comparable at $t_{\%}$ of 18 ± 5 min (Fig. 7B).

In separate sets of experiments, we determined the cellular localization of the R384E, R384Q, and D382L mutants in permeabilized cells to observe cell surface and internalized $\beta_{1}$-AR populations (Fig. 8A). Unlike the WT $\beta_{1}$-AR, which was localized to the cell surface (Fig. 8A, panel a), these mutant receptors were mainly located in intracellular cytoplasmic vesicles (Fig. 8A, panels e, i, and m). Because the inverse agonist metoprolol markedly reduced basal adenylyl cyclase activities of R384E and R384Q (Table 3), we investigated whether metoprolol would also affect cellular localization of these receptors. Cells expressing the $\beta_{1}$-AR, as described in Fig. 8A, were treated with 100 $\mu$M of metoprolol for 2 h, fixed, permeabilized, and then labeled with FITC to detect intracellular and membranous populations of the $\beta_{1}$-AR. The images reveal that continuous exposure to 100 $\mu$M metoprolol for 2 h did not affect the distribution of cells.
the WT β1-AR (Fig. 8A, panel b) but caused the redistribution of intracellular R384E and R384Q to the cell membrane (Fig. 8A, panels f and j). A circular boundary was drawn around the inner circumference of cell expressing R384E and R384Q to determine the distribution of these receptors between membranous and intracellular compartments. The density of the pixels residing inside the boundary versus those residing outside the boundary was used as an index for internalized and membranous β1-AR, respectively. To estimate the effect of the inverse agonist on intracellular β1-AR distribution, internal pixel densities in control cells were arbitrarily set as 100, and the data for each inverse agonist was calculated as percent of this value. In slides (panels d, h, l, and p), total cellular pixel densities were divided by the internal pixel densities in control cells to calculate their percentile values. The data represent the mean ± S.E. from five slides each involving between 6 and 10 cells. Each scale bar represents 5 μm.

The phenomenon of inverse agonist-mediated redistribution of the internalized constitutively active GPCR is referred to as “externalization” (31). If externalized R384E and R384Q are inserted properly into the cell membrane, then FITC-conjugated anti-FLAG IgG bound to the N-terminal FLAG epitope will be oriented extracellularly. In this case, a second acid wash would strip FITC-IgG from the externalized receptor population. In agreement with these assumptions, we observed reduced cell fluorescence in metoprolol-treated R384E and R384Q cells after a brief acid treatment (Fig. 8A, panels h and l). Metoprolol-mediated translocation of R384Q to the plasma membrane resulted in a 24% increase in the density of cell surface-binding sites on R384Q expressing cells as measured by [3H]CGP-12177 binding (data not shown). We screened another inverse agonist CGP-20712A (28) and a pure antagonist (propranolol) for their ability to “externalize” R384E and R384Q. CGP-20712A was equally efficacious to metoprolol in “externalizing” R384E and R384Q (Fig. 8A, panels g and k), whereas propranolol did not cause significant externalization (data not shown). The effect of the inverse agonists metoprolol or CGP-20712A on the distribution of D382L was also investigated (Fig. 8A, panels n and o). The intracellular distribution of D382L was not altered by the inverse agonist metoprolol as determined by the pixel distribution method (Fig. 8A, panel n) or by the acid-strip method (Fig. 8A, panel p, and Fig. 8B). CGP-20712A reduced by 30% the density of intracellular pixels of D382L (Fig. 8B).

The predicted three-dimensional structure of the 8th α-helix in Fig. 9 reveals that the highly conserved hydrophobic residues Phe383 and Phe387 were oriented toward the hydrophobic milieu of the inner cell membrane or lipid raft. In addition, helix 8 is bordered on one side by the linker sequences of Cys379, Ser380, and Pro381 that project from TM7 and at its C terminus by a pair of palmitoylated cysteines that are buried into the cytoplasmic face of the membrane. These structures at both ends of helix 8 tether the predicted helix to the membrane, and along with the hydrophobic phenyl alanines orient helix 8 into a partially buried α-helix on one side and an exposed α-helix on the opposite side. Our predictions indicate that the residues in the exposed top portion of helix 8 are Lys385 and Arg/Glu389. The other residues such as Ala386 and Leu390 are aligned on one side of the 8th α-helix (Fig. 9A), whereas Asp382, Arg384, and Gln388 lie on the other side of putative helix 8 (Fig. 9B).
Mapping Helix 8 in the Human $\beta_1$-AR

FIGURE 9. Structural model of the 8th $\alpha$-helix relative to the (Asp/Glu)-Arg-Tyr motif in helix 3 of the human $\beta_1$-AR. The amino acids in the transmembrane helices of the human $\beta_1$-AR and rhodopsin were aligned according to the three-dimensional data of Palczewski et al. (4) by the SegMod computer program. Using the aligned sequence of the $\beta_1$-AR, we threaded the sequence into that of the rhodopsin structure and refined it to build a structurally valid model. The figure was produced using MOLSCRIPT and rendered with RASTER3D computer programs. $A$ shows a side-view distribution of the amino acids in helix 8 relative to the (Asp/Glu)-Arg-Tyr motif in the transmembrane helix III and to the other transmembrane helices of the human $\beta_1$-AR. $B$ represents the side view for the distribution of helix 8 amino acids after a 180° rotation around the vertical axis. The distances in Å for the amino acids in helix 8 and Arg156 of the (Asp/Glu)-Arg-Tyr motif were as follows: Pro381, 16.83; Asp382, 17.4; Phe383, 19.10; Arg384, 21.88; Lys385, 23.02; Ala386, 23.83; Phe387, 25.1; Gly/Arg389, 28.4 and Leu300, 30.1.

DISCUSSION

Several reports indicate that the putative 8th $\alpha$-helix formed between the NPXXY motif in TM7 and by membrane insertion of conserved palmitoylated cysteines may be involved in the interaction of GPCR with their cognate G proteins. Synthetic peptides from the C terminus of the $\alpha$-subunit of transducin interacted with rhodopsin and kept it in the active state (9). This interaction, however, was abolished in mutants of rhodopsin in which the Asn310, Lys311, and Gln312 sequences in helix 8 were replaced with the corresponding sequences (Ser, Pro, and Asp) in the $\beta_1$-AR or the $\beta_2$-AR. In other experiments, the substitution of the Asn-Lys-Gln sequence at the N terminus of helix 8 in rhodopsin with the Ser-Pro-Asp sequence inhibited the activation of transducin by rhodopsin (8). These data are intended to supplement other reports showing that G protein subunits interact directly or indirectly with helix 8 (6). Discrete point mutations within the Asn310-Lys311-Gln312 sequence in helix 8 of rhodopsin engineered in Lys311 revealed that Lys311 mutants were not defective in transducin activation (8). In another study, it was shown that both Asn310 and Lys311 were required for correct folding of rhodopsin (32). Therefore, the Asn-Lys-Gln sequence is involved in proper folding of rhodopsin, but the specific residue(s) in this sequence that are involved in promoting rhodopsin-transducin interactions are still obscure.

The sequence that corresponds to the Asn-Lys-Gln sequence of rhodopsin in the $\beta_1$-AR and in the $\beta_2$-AR is Ser$^{380}$-Pro$^{381}$-Asp$^{382}$ (Table 1). We analyzed the role of Asp$^{382}$ in $\beta_1$-AR-mediated activation of $G_o$ because this residue is outside the Ser-Pro sequence that was involved in proper folding of rhodopsin. D382L was deficient in its activation of $G_o$, indicating that Asp$^{382}$ was a potential player in coupling helix 8 to $G_o$. Analysis of the role of the N terminus of predicted helix 8 in the human leukotriene B$_4$ (BLT-1) receptor indicates that this domain is involved in sensing the status of the BLT-1 receptor coupling to the G protein because deletion of the N-terminal sequence of helix 8 prevented GTP$\gamma$S-mediated inhibition of leukotriene B$_4$ binding (33, 34). These mutant BLT-1 receptors also exhibited high $B_{max}$ values accompanied with more prolonged intracellular signaling than in the wild type BLT-1 receptor (33).

The role of the highly conserved Phe residue that projects into the intracellular leaflet of the plasma membrane has generated modest attention because mutagenesis of Phe$^{312}$ in rhodopsin and Phe$^{318}$ in the melanin-concentrating hormone receptor or Phe$^{313}$ in the angiotensin II$_A$ receptor to Ala produced properly folded and biologically active receptor molecules (10, 13, 35). We opted instead to test for the effect of a charge substitution at this position, and we found out that it prevented the biosynthesis of the $\beta_1$-AR perhaps by interfering with the proper folding of the receptor. Therefore, a hydrophobic uncharged residue at this position is required for proper expression of the GPCR, indicating that the formation of an amphiphilic 8th $\alpha$-helix is important for folding of the full-length receptor.

To determine the role of the highly conserved Arg$^{384}$ residue, we engineered a variety of mutations to test the effect of different chemical structures on the coupling of the $\beta_1$-AR to $G_s$. Mutagenesis of the basic Arg$^{384}$ to the two other basic amino acids, Lys and His, or to uncharged Phe indicated that side chain projections play a major role in coupling the $\beta_1$-AR to $G_s$. Our results strongly indicate that Arg at this position provides normal basal levels and displays the highest affinity for isoproterenol in binding to the receptor among those Arg$^{384}$ mutants with similar basal activities (Table 2). Furthermore, Arg$^{384}$ promoted the highest efficacy in activating adenylyl cyclase among this group of Arg$^{384}$ mutants (Fig. 3). The mutation involving a charge reversal to glutamic acid produced a receptor with an exceedingly high affinity in binding to isoproterenol and high basal levels of adenylyl cyclase activity. R384E and R384Q displayed characteristics associated with the constitutively active $\beta_1$-AR phenotype. The pharmacological character of constitutively active $\beta_1$- and $\beta_2$-AR was gleaned from mutagenesis of Leu$^{322}$ in helix 6 of the $\beta_1$-AR into Lys and from compound mutations in the cytoplasmic neck of helix 6 of the $\beta_1$-AR (36). The involvement of residues in the cytoplasmic neck of helix 6 in coupling the $\beta$-AR to $G_s$ and in constitutive activation is well documented (37), but our finding that a single point mutation in helix 8 can constitutively activate the $\beta_1$-AR has not been described before. R384E and R384Q displayed the pharmacological profile predicted by the allosteric ternary complex model for constitutively active receptors (36). This model predicts that constitutively active receptors manifest increased affinity for agonists and increased intrinsic activity for inverse agonists as compared with wild type GPCR (27). In agreement with the predictions of the allosteric ternary complex model, the affinity of isoproterenol in binding to R384E and R384Q was markedly increased by 50–75-fold, whereas the distribution of the low versus high affinity sites was maintained. Another prediction of the ternary complex model stipulates that $\beta_1$-adrenergic inverse agonists would cause stronger inhibition of basal adenylyl cyclase activity in the R384E and R384Q mutants than in the WT $\beta_1$-AR (27, 28). The inverse agonist metoprolol preferentially reduced the basal activity of adenylyl cyclase in the R384E and R384Q mutants, indicating enhanced activity of inverse agonists toward the constitutively active $\beta_1$-AR phenotype (Table 3).

Another characteristic of the R384E and R384Q mutants was their resistance to desensitization, which we attribute to constitutive desensitization of these $\beta_1$-AR constructs (30). This finding is surprising con-
considering that the Gly48 variant of the β1-AR displayed the pharmacological character of a constitutively active receptor but was desensitized to a greater extent than the Ser48 variant of the WT β1-AR (38); but this finding is in agreement with the ternary complex model that stipulates that constitutively active receptors are also constitutively desensitized (30).

There were major qualitative differences, however, in the desensitization of adenylyl cyclase in WT β1-AR versus R384E and R384Q. The fold increase in adenylyl cyclase activity in control WT β1-AR in response to isoproterenol was 2-7-fold over basal, whereas in the desensitized state the fold increase was reduced to 5-7-fold (Fig. 5). The fold increase in isoproterenol-mediated activation of adenylyl cyclase in control or desensitized R384E and R384Q mutants was 3-fold. We attribute the resistance of the R384E and R384Q mutants to desensitization to two complementary processes. The first is that these receptors were constitutively phosphorylated. The counts/min of [32P] incorporated into R384E and R384Q in the absence of isoproterenol were equivalent to the counts/min incorporated into the WT β1-AR after isoproterenol. Furthermore, the phosphorylation of R384E and R384Q did not increase after isoproterenol. Because desensitization is a consequence of β-adrenergic receptor kinase-mediated phosphorylation (39), basal phosphorylation of R384E and R384Q was already maximal, which blunted further desensitization. The second process is that desensitization involves agonist-mediated sequestration and internalization of the GPCR (40). This phenomenon was exhibited by the WT β1-AR, which was rapidly internalized with a t1/2 of 5-7 min and recycled in a cAMP-dependent protein kinase-dependent manner with a t1/2 of 18 min (19) (Fig. 7A). The R384E and R384Q mutants, however, were already internalized, and their internal receptor pixels did not appreciably increase after isoproterenol. What accounts for constitutive internalization of R384E and R384Q? To address this question we exposed cells expressing R384E and R384Q to the inverse agonist metoprolol. If R384E and R384Q mimic the behavior of the agonist-activated WT β1-AR, then they would be constitutively activated, internalized, and recycled. Thus treating R384E and R384Q with an inverse agonist would be expected to restore their membranous distribution because inverse agonists generate a strong inhibitory effect on constitutively active GPCR (41). What we observed was the redistribution of R384E and R384Q to the cell surface, indicating that when inverse agonists blocked constitutive internalization, R384E and R384Q recycled back and were re-inserted into the cell membrane. Therefore, the R384E and R384Q mutations produced a β1-AR phenotype that was constitutively internalized and recycled. These receptors were not degraded because the densities of R384E and R384Q in broken cells were not increased, even though metoprolol increased the densities of R384E and R384Q in membrane fractions by ~25%. The behavior of the constitutively active β1-AR in this regard was different from the behavior of the constitutively active β2-AR. The constitutively active β2-AR was associated with constitutive down-regulation and desensitization that resulted in low expression levels (30). This phenotype of the β2-AR was reversed by overnight treatment with an inverse agonist that resulted in strong up-regulation of receptor levels and in the stimulation of agonist-promoted activation of adenylyl cyclase (30). Therefore, in this regard, the β2-AR resembled the behavior of the constitutively active angiotensin II1A receptor, which was constitutively internalized and recycled, and its density was not appreciably increased by inverse agonists (31). What is the role of the amino acid corresponding to Arg384 in coupling other GPCR to their cognate G protein? In the angiotensin II1A receptor, Lys310 corresponds to Arg384 in the human β1-AR (Table 1). K310Q and K311Q mutants displayed normal binding parameters and displayed normal activity in activating phospholipase C in response to angiotensin II (13). The corresponding mutations in the melanin-concentrating hormone receptor1, R319Q, or R319E reduced the Bmax by 60%, increased the EC50 for calcium efflux by 7-fold, and altered the distribution of these receptors from the plasma membrane into the perinuclear zone and cytoplasm (10). Thus, Arg384 functions in a receptor or G protein-specific manner.

Inverse agonists or antagonists did not alter the distribution of D382L (Fig. 8A, panels m–p). D382L displayed low binding affinities to agonists and did not increase cyclic AMP or activate cAMP-dependant protein kinase in response to isoproterenol. Because cAMP-dependant protein kinase is required for efficient recycling of β1-AR through the endosomal pathway (19), we speculate that the D382L was not efficiently delivered to membranes by the biosynthetic and/or by the recycling routes. Whatever is the structural basis for the role of helix 8 in coupling the GPCR to G proteins is currently obscure. A fundamental observation gleaned from docking the three-dimensional structures of rhodopsin and transducin is that transducin binds to rhodopsin dimers in such a way that one molecule of rhodopsin binds transducin and the other serves as a docking platform (42). In this complex, the activation of one signaling rhodopsin in the oligomer is needed for productive coupling with transducin. The contact sites between transducin and rhodopsin involve the tips of helices 3, 6, and 8 (42). There were additional interactions between Glu395 and Lys366 of the γ-subunit of transducin with Gln311 and Thr319, respectively, in helix 8 of rhodopsin (42). In addition, mutational and cross-linking studies indicate that the Asn310–Gln312 region in helix 8 of rhodopsin interacts with residues 340–350 in the C-terminal sequence of the α-subunit of transducin (8, 9). These and other pieces of data indicate that helix 8 participates in binding to transducin and in rhodopsin-mediated activation of transducin. The analogue situation in other GPCR is not known because rhodopsin is the only GPCR for which the crystal structure has been determined. A similar situation might occur in the human β1-AR because our data in Fig. 1 reveal that dimers and monomers of the human β1-AR were expressed in HEK-293 cells. Furthermore, modeling of helix 8 of the human β1-AR on the three-dimensional structure of rhodopsin revealed that many amino acids were conserved. Our modeling data indicate that the most functionally active amino acids in predicted helix 8 of the β1-AR were Asp382, Phe383, and Arg384. Asp382 and Arg384 were assigned to the same side of the 8th α-helix and may form a groove that is involved in coupling the β1-AR to Gs.

Currently there is no definite rule that defines the coupling between the GPCR and a given G protein. In rhodopsin, a stretch of three consecutive amino acids (Asn310–Lys311–Gln312) in the N terminus of helix 8 was shown to interact directly with the C terminus of the G protein transducin (8, 9). The amino acids in putative helix 8 of selected GPCR that have been implicated in coupling the GPCR to G proteins are shaded in Table 1. In the angiotensin II1A receptor, a string of three amino acids (Tyr312–Phe313–Leu314) in the C terminus of helix 8 was essential for the coupling and activation of Gi (13). In the melanin-concentrating hormone receptor1, a succession of three amino acids (Arg319–Lys320–Arg321) participated equally in cell expression, whereas Arg319 and Lys320 were partially involved in coupling the receptor to Gi and Gs (10). In the oxytocin receptor, the charged amino acids His335, Glu339, and Arg343 that are separated from another by one another of the putative α-helix were involved in oxytocin-mediated activation of phospholipase C by Gi (14). In the bradykinin1 receptor, Arg311 in helix 8 (11) and Tyr320 in the C-terminal tail (43) were involved in coupling the receptor to phosphatidylinositol hydrolysis via Gi

The GPCR with the most intriguing correlations between the structure of its hypothetical helix 8 and mutant receptor function is the BLT-1 receptor (34). The putative helix 8 of the BLT-1 receptor is linked to the NPXY motif of helix 7 by a long linker and lacks the cysteine residue, which is palmitoylated in many GPCR (44). Substitution of Leu304 and Leu305 in helix 8 by alanine caused constitutive BLT-1 recep-
In the human β1-AR, three consecutive amino acids (Asp\(^{382}\)-Phe\(^{383}\)-Arg\(^{384}\)) were implicated in regulating the expression, localization, and coupling of this receptor to G\(_s\). Thus several structural determinants within helix 8 appear to influence the proper recognition of the G protein by the β1-AR. The involvement of different motifs within predicted helix 8 in binding to G proteins is dictated by differences in amino acid composition and sequences of helix 8 among the various GPCR and by the diversity of the G proteins that interact with these receptors. One would expect that the G\(_q\)-coupling motif in helix 8 would be different from the G\(_s\)-coupling motif because these G proteins have different α-, β-, and γ-subunits with varying contact points among them to the GPCR.

The role of putative helix 8 in coupling the GPCR to G proteins illustrates the importance of amino acids outside the classical GPCR binding pocket in regulating receptor-mediated signaling effects. These observations along with the realization that GPCR and G proteins do not exist as 1:1 complexes but rather as heterodimers, involving more than one molecule of each, provides additional mechanisms for GPCR-mediated activation of the G protein. In this regard, helix 8 may serve as a novel site for allosteric modulation of the GPCR (47). Allosteric modulation relies on the organization of multipoprotein subunits into discrete conformations capable of undergoing reversible transitions in the absence of ligand (48). The activity of these complexes can be regulated by ligand binding to domains that are topographically distinct from the binding pocket, "the orthosteric site," within the hydrophobic core of the class A GPCR. The amino acids of helix 8 lie outside the catecholamine binding pocket (49) and are involved in heterooligomerization of the GPCR with the G protein. Furthermore, distinct motifs in helix 8 are used by each GPCR in coupling to their specific G proteins. These observations suggest that allosteric ligands designed to interact with helix 8 might be specific for each GPCR because of the diverse nature of helix 8 sequences among the various GPCR subclasses (50) and the distinctiveness of their G protein binding and interacting pockets. These novel allosteric agents, could either "tune up" or "tune down" the signaling of the agonist-occupied GPCR with more selectivity than traditionally "orthosteric" agents (50).