Arrangement of Transmembrane Domains in Adrenergic Receptors

SIMILARITY TO BACTERIORHODOPSIN*

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G protein-coupled receptors (GPCRs) have seven hydrophobic domains, which are thought to span the lipid bilayer as a helical transmembrane domains (TMDs). The tertiary structure of GPCRs has not been determined; however, molecular models of GPCRs have generally been based on bacteriorhodopsin, which is functionally unrelated to GPCRs but has a similar secondary structure. We sought to examine the validity of using bacteriorhodopsin as a scaffold for GPCR model building by experimentally determining the orientation of the TMDs of adrenergic receptors in the plasma membrane. In separate experiments, three sequential amino acid residues (Leu-310, Leu-311, Asn-312) in TMD VII of the β2 adrenoreceptor were mutated to the amino acids found in the homologous domain of the α2 adrenoreceptor (Phe, Phe, Phe). Exchange of Asn-312 and Leu-311 in the β2 adrenoreceptor resulted in nonfunctional proteins, most likely due to incompatibility of the introduced bulky phenylalanine side chain with adjacent structural domains in the β2 adrenoreceptor. This structural incompatibility was "repaired" by replacing the specific β2 TMD sequence with an α2 receptor sequence. TMD I and TMD II complemented the Asn-312 → Phe mutation, and TMD III and TMD VI complemented the Leu-311 → Phe mutation. These results indicate that TMDs I, II, III, and VI surround TMD VII in a counter-clockwise orientation analogous to the orientation of TMDs in bacteriorhodopsin.

Adrenergic receptors are one of the best characterized class of the family of G protein-coupled receptors for hormones and neurotransmitters. G protein-coupled receptors exhibit a common secondary structure consisting of seven hydrophobic segments that are thought to represent a helical membrane-spanning domains. In this paper the hydrophobic segments will be referred to as transmembrane domains (TMD). A high resolution three-dimensional structure of adrenergic and other G protein-coupled receptors is lacking because of the difficulties inherent in producing, purifying, and crystallizing integral membrane proteins. Without these data, investigators have used the structural information from an analogous protein, bacteriorhodopsin, as a template from which to generate molecular models of adrenoreceptors (1). The rationale for using bacteriorhodopsin, a prokaryotic proton pump, as a "structural scaffold" for human adrenoreceptors is based on the seven-transmembrane α helices in bacteriorhodopsin, which are thought to correspond topographically to the seven hydrophobic domains present in G protein-coupled receptors. But is bacteriorhodopsin structurally similar enough to employ as a model for G protein-coupled receptors? While bacteriorhodopsin does share with rhodopsin (a G protein-coupled receptor) the photoisomerization of the retinal chromophore, it has less than 10% sequence homology with G protein-coupled receptors and is not coupled to G proteins. When bacteriorhodopsin and rhodopsin are compared at a low resolution (9 Å) the projection footprints of the helical arrangements are different (2, 3).

Because of the lack of independent evidence that hormone and neurotransmitter G protein-coupled receptors are structurally analogous to bacteriorhodopsin, we sought to identify whether the membrane-spanning α helices of adrenoreceptors were arranged clockwise as depicted in several models (4, 5) or counterclockwise (6) (as for bacteriorhodopsin) when viewed from the outside of the cell. We have used a strategy involving αββββββ adrenoreceptor chimeric receptors to identify intramolecular interactions between specific amino acids on TMD VII and other TMDs. Our results provide evidence that adrenoreceptors exhibit the same helical orientation known to be present in bacteriorhodopsin.

EXPERIMENTAL PROCEDURES

Reagents—All reagents were from Sigma except bovine calf serum (Gemini Bio-Products, Calabasas, CA), gentamicin (Boehringer Mannheim), pGEM-3Z (Promega), pBVC 12 MI (a gift from Bryan Cullen), pCDNA-3 (Invitrogen), and goat anti-rabbit IgG (Jackson Immunoresearch Laboratories).

Construction of Mutant Receptors—Genes encoding human α2A (or C10) (7) and β2 adrenoreceptors (8) were cloned into the multiple cloning site of pGEM-3Z. The "single point" mutated receptors were constructed by the polymerase chain reaction using oligonucleotide primers with base mismatches and the corresponding receptor genes as the template. For production of the receptor chimeras, the two receptors were ligated between the putative TMDs using previously described polymerase chain reaction techniques (9). The DNA sequence of the mutant or chimeric receptors was confirmed by sequencing both strands.

Transfection of Mutant Receptors—COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and 25 mg/liter gentamicin. The constructs were cloned into pBVC 12 MI vectors or pCDNA-3 vectors, and the receptors were expressed transiently in COS-7 cells using DEAE-dextran-mediated transfection (10).

Immunocytochemistry—Forty-eight hours after transfection, culture medium was removed and the cells washed with PBS. 4% formaldehyde was added and removed after 45 min. After three washes with PBS, the wells were completely dried and the cells on the coverslip were permeabilized by adding 0.2% Nonidet P-40 in a 5% nonfat dried milk solution and left for 45 min. The primary antibody (rabbit antisemur directed to

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‡‡ The abbreviations used are: TMD, transmembrane domain; PBS, phosphate-buffered saline.
Adrenoreceptor Structure

The carboxyl-terminal 15 residues of the human β2 adrenergic receptor, at a 1:500 dilution, was applied to the coverslip in the dried milk solution for 45 min. The coverslip was washed 3 times with PBS, and then the secondary antibody (Texas Red conjugate of goat anti-rabbit IgG) at a 1:500 dilution, was applied to the coverslip in the dried milk solution for 2 min in the dark. The coverslip was washed three times with PBS and mounted on a microscope slide to identify the subcellular location of the fluorescent-stained protein by fluorescence microscopy.

Radiolabeled Ligand Binding Assay—Cells were harvested 3 days after transfection and rinsed twice with cold PBS, and then 30 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) was added. Cells were scraped off the flask with a disposable “policeman” and collected. The flask was rinsed with an additional 5 ml of lysis buffer, and the washings were pooled. Cells were homogenized by four 5-s bursts at full speed using a Polytron homogenizer. The nuclei were pelleted by centrifugation at 220 × g at 4°C, and the supernatant was removed and assayed for cAMP (Fig. 2). The supernatant was removed and assayed for cAMP by the scintillation proximity assay kit (Amersham Corp.). Epinephrine-stimulated cAMP accumulation was expressed as a percent of forskolin stimulation; these data were analyzed by a nonlinear least square curve-fitting technique, and EC50 values were determined using GraphPAD software (GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

The construction and functional analysis of chimeric receptors from α2 and β2 adrenoreceptors have provided useful information about adrenoreceptor structure (10–13). These two receptors are both activated by the catecholamine epinephrine but can be readily distinguished pharmacologically using subtype-selective agonists and antagonists. The α2 adrenoreceptor couples to Gα and thereby inhibits adenylyl cyclase while the β2 adrenoreceptor couples to Gα and activates adenylyl cyclase. The binding specificity of wild-type α2 adrenoreceptor can be converted to that of a β2 adrenoreceptor by mutating Phe-412 → Asn in the seventh transmembrane domain (11); however, the “mirror image” mutation (Asn-312 → Phe on the β2 adrenoreceptor) is nonfunctional requiring the additional substitution of TMDs I and II from the α2 adrenoreceptor (see CRS11 in Fig. 1) to restore function (12). CRS11 has β2 adrenoreceptor domains essential for coupling to Gα, and binding of epinephrine to CRS11 leads to activation of adenylyl cyclase (Fig. 1). Pharmacologically CRS11 behaves more like an α2 adrenoreceptor (12, 13). Thus the more bulky phenylalanine residue at position 312 in TMD VII forverts the additional substitu-

**FIG. 1. Functional properties of chimeric receptors.** Receptors were transfected into COS-7 cells as described under “Experimental Procedures.” The α2 adrenoreceptor sequence inserted into the β2 adrenoreceptor to make each chimera indicated (TM, transmembrane domain; AA, amino acid residue) and is shown as black-filled in the receptor diagrams. Transfection efficiency, quantified by immunocytochemistry, varied between 20 and 40%. Both the radiolabeled ligand binding assays (to determine the Kd values) and the adenylyl cyclase assays (to determine the EC50 values) were performed at least three times, each in triplicate. Maximal epinephrine-induced increase in adenylyl cyclase stimulation (maximal/basal) for functional receptors was as follows: 1.76 ± 0.20 for CRS11, 1.51 ± 0.08 for CRS121, and 2.40 ± 0.18 for CRS117. All of the receptor chimeras contain sequences previously shown to be required for Gα activation (10) and for binding to α2 antagonists such as atipamezole (12, 13). Epinephrine is an agonist for both α2 and β2 adrenoreceptors.

| α2 Sequence | Kd Atipamezole (nM± SE) | EC50 Epinephrine (μM) [95% confidence interval] |
|-------------|-------------------------|-----------------------------------------------|
| TM-I, II    | AA-312                  |                                              |
|             | 21 ±/−4                 | 0.37 [0.064 − 2.1]                            |
| TM-I, II    | AA-311, 312             |                                              |
|             | >100                    | >100                                          |
| TM-I, II, III| AA-311, 312             |                                              |
|             | >100                    | >100                                          |
| TM-I, II, VI| AA-311, 312             |                                              |
|             | >100                    | >100                                          |
| TM-I, II, III, VI| AA-311, 312 | 26 ±/−2                                       |
|             |                        | 0.27 [0.011 − 5.5]                            |
| TM-I, II    | AA-310, 312             |                                              |
|             | 31 ±/−4                 | 0.40 [0.059 − 2.8]                            |

The results are in agreement with more recent studies on the 5HT2A receptor demonstrating a specific interaction between TMD II and TMD VII (14). We originally depicted the interaction between TMD VII and TMDs I and II in a model where the TMDs were arranged in a clockwise orientation (12); however, orientation of the helices in either a counterclockwise (Fig. 2A) or clockwise (Fig. 2B) manner is consistent with these data.
The arrangement of the transmembrane domains (I-VII) as viewed from the outside of the cell. α2 domains are shown as black circles with white letters, and β2 domains are shown as open circles with black letters. The replacement of asparagine 312 in the β2 transmembrane domain VII by phenylalanine (F) requires exchange of transmembrane domains I and II with the α2 adrenoreceptor sequence to be functional (12). This suggests that TMDs I and II are adjacent to position 312 in TMD VII, and this can be accommodated by either a counterclockwise (A) or a clockwise (B) orientation. Substitution with the α2 residue phenylalanine (F) for leucine (L) at position 311 in the β2 transmembrane domain VII requires α2 transmembrane domains III and VI to be functional. This suggests that TMDs III and VI are adjacent to position 311 in TMD VII. The findings are most easily explained by a counterclockwise (C) rather than a clockwise (D) orientation of TMDs I, II, III, and VI around TMD VII.

To determine which of these two orientations pertained, we examined the effect of mutations at positions 311 and 310. The orientation of positions 311 and 310 shown in Fig. 2 is based on the assumption that TMD VII traverses the membrane as a right-handed α helix. Leu-311 was mutated to Phe (the amino acid in the homologous position of the α2 adrenoreceptor) to determine if complementary changes were required in the neighboring TMDs of this novel receptor (CRS112) to maintain receptor function. Immunocytochemical studies confirmed that CRS112 was expressed in the plasma membrane of the transfected COS-7 cells (data not shown), but membranes prepared from these receptors displayed neither α2 nor β2 antagonist binding, and adenylyl cyclase activity could not be stimulated with epinephrine (Fig. 1) even though this receptor retained agonist activation or binding function. This prediction was borne out since CRS117 (Fig. 1) has similar epinephrine-stimulated adenylyl cyclase activity and adrenergic ligand binding to that of CRS11.

A high resolution tertiary structure of G protein-coupled receptors has not yet been determined because of the difficulty in extracting large amounts of pure protein from the natural membranes for crystallographic studies. Cryoelectron microscopy of two-dimensional crystals has been used to study the structure of rhodopsin (2, 3); however, the resolution (9 Å) of the structure obtained from these studies is not yet sufficient to determine the arrangement of the TMDs. In the absence of high resolution biophysical data, structural models have been devised, based largely on the folding pattern of bacteriorhodopsin. The data presented here provide evidence for specific intramolecular interactions between position 311 in TMD VII of the β2 adrenoreceptor and TMDs III and VI and, together with our previous results (12), support the hypothesis that the human adrenoreceptor exhibits the same helical orientation that is known to be present in bacteriorhodopsin (15). This structural similarity increases the likelihood that bacteriorhodopsin is an appropriate scaffold on which to “drape” the adrenoreceptor sequence and provides the experimental underpinnings for rational drug design using a molecular model of adrenergic receptors based on bacteriorhodopsin.

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