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Identification of residues required for ligand binding to the β-adrenergic receptor

(agonist/mutations/expression/guanine nucleotide binding protein/conformation)

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ABSTRACT **The functional significance of conserved polar amino acids within the putative transmembrane region of the β-adrenergic receptor (βAR) was examined by oligonucleotide-directed mutagenesis of the hamster gene encoding βAR and expression of the mutant genes in COS-7 cells. Although a substitution of aspartate at position 113 with an asparagine residue did not affect expression or processing of the protein, the resulting mutant βAR did not show detectable binding toward the antagonist iodocyanopindolol. Replacement of the aspartate and asparagine residues at positions 79 and 318, respectively, had no effect on the affinity of the receptor toward antagonists but reduced the affinity of the receptor toward agonists by 1 order of magnitude. Furthermore, we observed that substitution of the proline at position 323 with a serine residue resulted in improper or incomplete processing of the βAR, presumably reflecting a role for this residue in the folding of the receptor. Together with our previous results from deletion mutagenesis studies, these observations indicate that the ligand binding site involves the transmembrane region of the βAR.**

The β-adrenergic receptor (βAR) is a member of a large class of hormone receptors that are located at the plasma membrane and that exert their intracellular effects through an interaction with guanine nucleotide binding proteins (G proteins; see ref. 1 for review). Different G-protein-linked receptors recognize different ligands as agonists and stimulate distinct classes of G proteins. In the case of βAR, upon binding catecholamine agonists, the receptor catalyzes the formation of the GTP complex of G_s, which in turn stimulates adenylate cyclase activity. βAR antagonists appear to act by competing with agonists for binding to the receptor and failing to stimulate adenylate cyclase. Two subtypes of βAR occur in mammalian tissue. The β_2 subtype, found to predominate in lung tissue, binds epinephrine preferentially to norepinephrine, whereas the β_1 subtype, which predominates in heart tissue, binds both agonists with similar affinities.

The recent cloning of the genes encoding the hamster (2) and human β_2AR (3), the avian βAR (4), and the porcine M1 muscarinic cholinergic receptor (MAR) (5) and the deduction of the primary sequences of these proteins suggest a structural basis for the mechanistic similarities among the G-protein-linked receptors. These hormone receptors share sequence homology with each other and with the visual opsins, which transduce their signals through the activation of the G-protein transducin (6). Most of the conservation in sequence among these proteins is found within seven hydrophobic domains of approximately 20–25 residues in length. Based on the model proposed for the opsins (7), these hydrophobic regions of the receptors, which are linked by more divergent hydrophilic regions of various lengths, would alternately traverse the membrane with the N terminus of the βAR exposed externally and the C terminus exposed intracellularly (2).

The cloning and expression of the gene for the βAR allows a genetic approach to the determination of the structural basis for ligand binding. Previous work from this laboratory has shown that most of the hydrophilic regions of the hamster βAR, up to 30% of the residues, can be deleted without affecting the binding of ligands (8). In this study we have mutated several of the conserved polar residues located within the hydrophobic regions. We show that aspartate-113 plays a critical role for antagonist binding to the βAR. In addition, we report that substitution of aspartate-79 or asparagine-318, while not affecting antagonist binding, results in a decrease in the affinity of the receptor for agonists.

MATERIALS AND METHODS

Mutagenesis and Expression Vectors. All general recombinant DNA procedures were performed as described (9). The nucleotide sequence for the hamster βAR gene has been published (2). The expression vector for the hamster βAR, pSVβAR (referred to as pSVHAM in ref. 8) was derived by inserting the hamster βAR gene into the simian virus 40 (SV40)-derived expression vector pSVL and has been described (8). Oligonucleotide-directed mutagenesis by previously described procedures was utilized to introduce nucleotide substitutions (8, 10). The nucleotide sequences of the mutant plasmids were confirmed by dideoxysequencing (11).

Mammalian Cell Expression. COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (DMEM/BS) in 5% CO_2/95% air at 37°C. Twenty-four hours prior to transfection, the cells were trypsinized, and 10^6 cells were plated into 75-cm^2 flasks. Plasmid DNA was transfected by the calcium phosphate method (12). Plasmid DNA (15 μg) was mixed with 15 μg of calf thymus DNA (Sigma) in Hepes-buffered saline and was precipitated with 125 mM $CaCl_2$ at 22°C for 30 min. The DNA was added to the monolayers of COS-7 cells and incubated for 4 hr at 37°C. The medium was aspirated, and 15% glycerol in DMEM/BS was added for 3 min. The monolayers were washed five times with DMEM/BS and overlaid with 15 ml of fresh DMEM/BS. The cells were incubated at 37°C for 72 hr.

Membrane Preparation. COS-7 cells growing in monolayer culture were washed three times with phosphate-buffered saline, scraped from the flask with a rubber policeman, and centrifuged at 2000 × *g* for 5 min. The cell pellet was

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Abbreviations: βAR, β-adrenergic receptor; ICYP, iodocyanopindolol; G protein, guanine nucleotide binding protein; MAR, M1 muscarinic cholinergic receptor.

resuspended in 15 mM Tris/2 mM $MgCl_2$/0.3 mM EDTA, pH 7.5, at a concentration of 10^8 cells per ml by vigorous mixing, frozen in liquid nitrogen, and thawed at 25°C, with continuous vigorous mixing during the freezing process. The resulting membrane preparation was washed once in 75 mM Tris/12.5 mM $MgCl_2$/1.5 mM EDTA, pH 7.5 (TME buffer) and resuspended at a protein concentration of 2 mg/ml. All binding assays were performed on freshly prepared membranes. Protein concentrations were determined by the method of Lowry (13).

Protein Immunoblotting. COS-7 membranes were prepared as described above. Nuclei were removed from the membrane preparation by centrifugation over a cushion of 60% sucrose at 2500 × *g* for 15 min, with full recovery of the βAR in the supernatant as assessed by binding with iodocyanopindolol (ICYP) in which the iodine is ^{125}I [(^{125}I)ICYP] (see below). The membranes in the sucrose supernatant were pelleted by centrifugation at 15,000 × *g* for 30 min, resuspended at a concentration of 10–20 mg/ml in 65 mM Tris·HCl, pH 6.5/3% $NaDodSO_4$/10% glycerol/5% 2-mercaptoethanol, and allowed to denature for 1 hr at 4°C before proteins were separated on 10% polyacrylamide gels by the method of Laemmli (14). After electrophoresis, proteins were transferred to nitrocellulose, and the immunoreactive receptor was detected by protein immunoblotting (15). The antibody used in the immunoblotting was raised to a peptide corresponding to the C terminus of the hamster βAR, with the sequence Cys-Leu-Asp-Ser-Gln-Gly-Arg-Asn-Nle-Ser-Thr-Asn-Asp-Ser-Pro-Leu (8), in which Nle is norleucine.

Ligand Binding. Saturation binding of (^{125}I)ICYP to COS-7 cell membranes was measured by generating binding isotherms using (^{125}I)ICYP (New England Nuclear) under conditions described in the legend to Fig. 3 (ref. 16). Data were analyzed by computer-fitting using an iterative nonlinear regression program. The binding of nonradioactive ligands to the membranes was measured by competition with (^{125}I)-ICYP under the conditions described in the legend to Fig. 4. Competition binding data were analyzed by using the iterative program LIGAND (17). Binding of [^3H]dihydroalprenolol (New England Nuclear) and (−)-4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^3H]benzimidazol-2-one, designated [^3H]CGP-12177 (Amersham) were measured as described for (^{125}I)ICYP at 20 nM and 50 nM, respectively.

RESULTS

Mutagenesis. Within the hydrophobic regions of the βAR and MAR are a few conserved aspartate and glutamate residues that potentially could interact with the positively charged amino group present on the ligands for these receptors. The aspartate residue at position 79 within the second hydrophobic region is conserved in all of the known βAR sequences, the MAR, and the opsins (Fig. 1). Within the third hydrophobic region, glutamate-107 is conserved among the βAR sequences, and an aspartate residue occurs at the analogous position of the MAR. Finally, aspartate-113 is conserved among the βAR and MAR sequences but not with the opsins. Analogy with rhodopsin suggests that residues within the seventh hydrophobic region may also be involved in ligand binding. Whereas the lysine residue that is involved in Schiff base formation with retinal in rhodopsin is not conserved with the receptor sequences, a residue at a similar position in the βAR, asparagine-318, is conserved among the sequences of the hormone receptors (see Fig. 1). This same region contains a number of amino acid homologies (Fig. 1), including the proline at position 323, which has been suggested to be important for maintaining the conformation of the opsins. By oligonucleotide-directed mutagenesis, alterations were made in the hamster βAR gene, creating genes encoding the mutants [Ala^{79}]βAR, [Ala^{107}]βAR, [Asn^{113}]-βAR, [Lys^{318}]βAR, and [Ser^{323}]βAR. For expression, the gene fragments encoding these mutant proteins were inserted into the simian virus 40-derived expression plasmid pSVL, which had been used by our laboratory previously to express βAR in mammalian cells (8).

Expression. We have described previously the characterization of deletion mutants of the hamster βAR by transient expression in COS-7 cells. In a similar manner, COS-7 cells were transfected with pSVβAR and the mutant plasmids described above. After 72 hr the transfected cells were harvested, and membrane preparations were examined by immunoblot analysis for the expression of βAR protein. As previously observed, transfection with pSVβAR resulted in

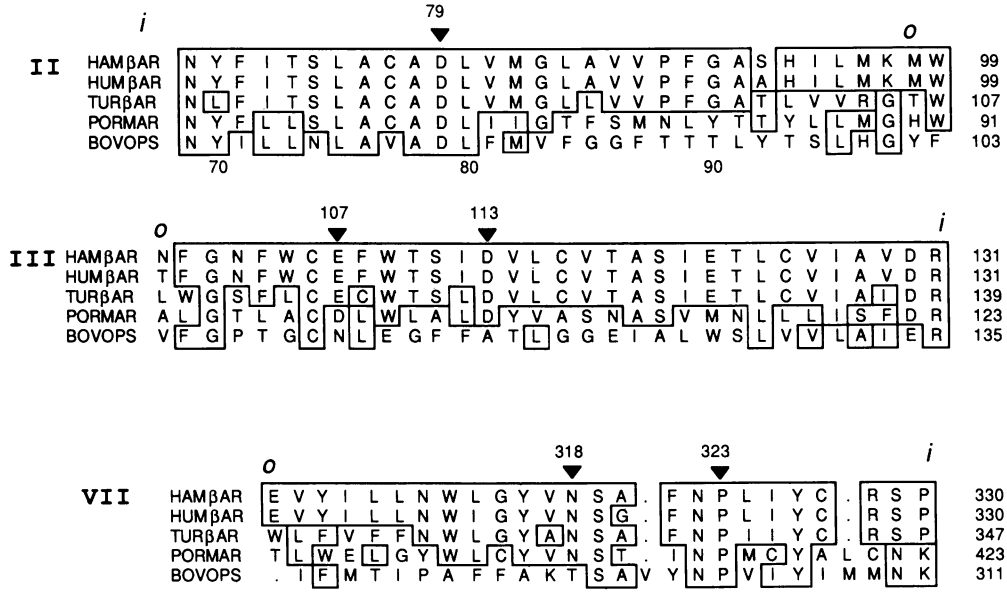

FIG. 1. Amino acid sequences of the second, third, and seventh hydrophobic domains of the hamster βAR (HAMβAR), human βAR (HUMβAR), turkey βAR (TURβAR), porcine muscarinic cholinergic receptor (PORMAR), and bovine opsin (BOVOPS). The sequences are aligned for maximum homology: the alignment of the entire sequences has been presented elsewhere (8). Boxes are drawn to indicate homology; the positions of the substituted amino acids are indicated.

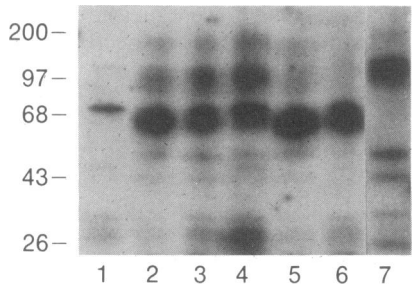

FIG. 2. Protein immunoblotting of the mutant and wild-type βAR. After electrophoresis, proteins were transferred to nitrocellulose, and the immunoreactive receptor was detected by protein immunoblotting with an antibody to a synthetic peptide corresponding to the C terminus of the βAR, with the serum used at a final dilution of 1:1000, followed by ^{125}I-labeled protein A (New England Nuclear) at a final concentration of 10^6 cpm/25 ml. Immunoreactive bands were detected by autoradiography. The immunoreactivity was completely blocked by incubation with a peptide corresponding to residues 404–418 of the βAR (the antigenic peptide) but not by a peptide corresponding to residues 1–10 of the βAR (data not shown). Membranes applied to each lane were from COS-7 cells transfected with pSVL (lane 1) and pSVL containing the following inserts: βAR (lane 2); [Ala^{79}]βAR (lane 3); [Ala^{107}]βAR (lane 4); [Asn^{113}]βAR (lane 5); [Lys^{318}]βAR (lane 6); and [Ser^{323}]βAR (lane 7). Protein amounts (mg) and (^{125}I)ICYP binding sites (fmol) per lane were respectively 0.6 and 42 (lane 1), 0.5 and 400 (lane 2), 0.8 and 400 (lane 3), 1.6 and 400 (lane 4), 2.8 and 80 (lane 5), 0.8 and 400 (lane 6), and 1.6 and 50 (lane 7). The positions of molecular mass standards are designated.

expression of an immunoreactive polypeptide with an apparent molecular mass of 67 kDa, which was barely visible in the control cells transfected with pSVL (see Fig. 2). The expressed polypeptide appeared to be identical in size with the fully glycosylated βAR purified from hamster lung (not shown). Transfection with plasmids containing genes encoding the mutant [Ala^{79}]βAR, [Ala^{107}]βAR, [Asp^{113}]βAR, and [Lys^{318}]βAR resulted in similar increases in expression of the same 67-kDa immunoreactive polypeptide (Fig. 2). With the [Ser^{323}]βAR mutant, the 67-kDa band was not observed, and two novel immunoreactive polypeptides with apparent molecular masses of 44 kDa and 50 kDa appeared in addition to a band at 150 kDa. The two lower molecular mass polypeptides probably represent nonglycosylated and partially glycosylated forms of the mutant βAR, and the 150-kDa band may be due to aggregation.

Analysis of Ligand Binding. The ability of the expressed βAR proteins to bind the labeled antagonist (^{125}I)ICYP was examined by using membrane preparations from the transfected COS-7 cells described above. Shown in Fig. 3 are (^{125}I)ICYP saturation curves for the membranes prepared from these cells. In this experiment membranes from cells transfected with the gene for the wild-type βAR had binding sites equivalent to 650 fmol/mg of protein compared to 30 fmol/mg for the control cells transfected with pSVL. Similar increases in (^{125}I)ICYP binding sites were observed for expression of [Ala^{79}]βAR, [Ala^{107}]βAR, and [Lys^{318}]βAR (Fig. 3) and were consistent with levels of protein expression revealed by the immunoblot analysis. The dissociation constants for ICYP binding by these mutant proteins were identical to that of the wild-type βAR ($K_d = 6 \times 10^{-11}$ M). In contrast to these results, no increase in the number of binding sites for (^{125}I)ICYP was observed for the mutant [Asn^{113}]-βAR, which was expressed at levels comparable to those of the wild-type receptor. For this mutant, binding of the antagonists [^{3}H]dihydroalprenolol and [^{3}H]CGP-12177 was also not observed (data not shown). In addition, [Ser^{323}]βAR, which, as noted above, appeared to be incompletely processed, also did not bind (^{125}I)ICYP and was not characterized further.

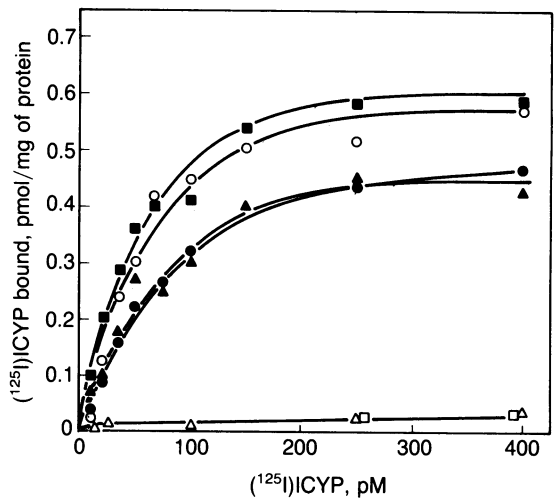

FIG. 3. Saturation binding of (^{125}I)ICYP to COS-7 membranes. COS-7 cell membranes were prepared by freeze/thaw lysis and resuspended in TME buffer as described. (^{125}I)ICYP binding was measured for 90 min at 23°C by filtration on GF/C glass fiber filters as described. Saturation isotherms were generated by using (^{125}I)-ICYP at 10–400 pM in 250 μl of TME buffer containing 10–20 μg of membrane protein, representing 2–4 fmol of (^{125}I)ICYP binding activity at saturation, except in the case of the pSVL control and of [Asn^{113}]βAR, where the protein concentration had to be increased 5–10 fold to provide detectable (0.3–0.5 fmol) levels of (^{125}I)ICYP binding. Nonspecific binding, defined in the presence of 10 μM (−)-alprenolol, was typically 2–5% of the total for cells expressing levels of βAR comparable to that of the wild-type βAR and 15–25% for pSVL and [Asn^{113}]βAR. The following receptors were used: pSVL (△), βAR (●), [Ala^{79}]βAR (▲), [Ala^{107}]βAR (○), [Asn^{113}]βAR (□), and [Lys^{318}]βAR (■).

The interaction of the mutant receptors, which retained their (^{125}I)ICYP binding activity, with other adrenergic ligands was examined by competition binding of these ligands with (^{125}I)ICYP (Fig. 4). Consistent with the results for (^{125}I)ICYP binding, all of the mutant receptors had affinities toward the antagonists propranolol and alprenolol similar to those of the wild-type receptor (Fig. 4 *A* and *B*). Whereas the mutant receptor [Ala^{107}]βAR had the same affinity toward the agonists isoproterenol and epinephrine as that of the wild-type βAR, the affinities of the mutants [Ala^{79}]βAR and [Lys^{318}]βAR for these agonists were reduced (Fig. 4 *C* and *D*). The EC_{50} values for the displacement of (^{125}I)ICYP binding from these latter mutants were shifted 1 order of magnitude to lower affinity than that of the wild-type βAR. The affinity of these mutant receptors for the agonist norepinephrine was similarly 1 order of magnitude lower than that of the wild-type βAR (data not shown), demonstrating that all of the mutant receptors maintained their β_2 subtype specificity.

DISCUSSION

In this report we substituted conserved negatively charged residues that are located within the hydrophobic regions of the βAR with uncharged amino acids. These aspartates and glutamates are located within the hydrophobic regions II and III. Since the Schiff base in rhodopsin involves the lysine residue from the VII region (see ref. 7 for a review), we also probed the functional significance of several of the conserved residues within the analogous region of the hamster βAR.

The expression of a properly glycosylated receptor requires its correct insertion into the membrane of the Golgi apparatus and can be a sensitive measure of the folding properties of a mutant protein. Of the mutations we studied here, only the substitution of proline residue 323 with a serine

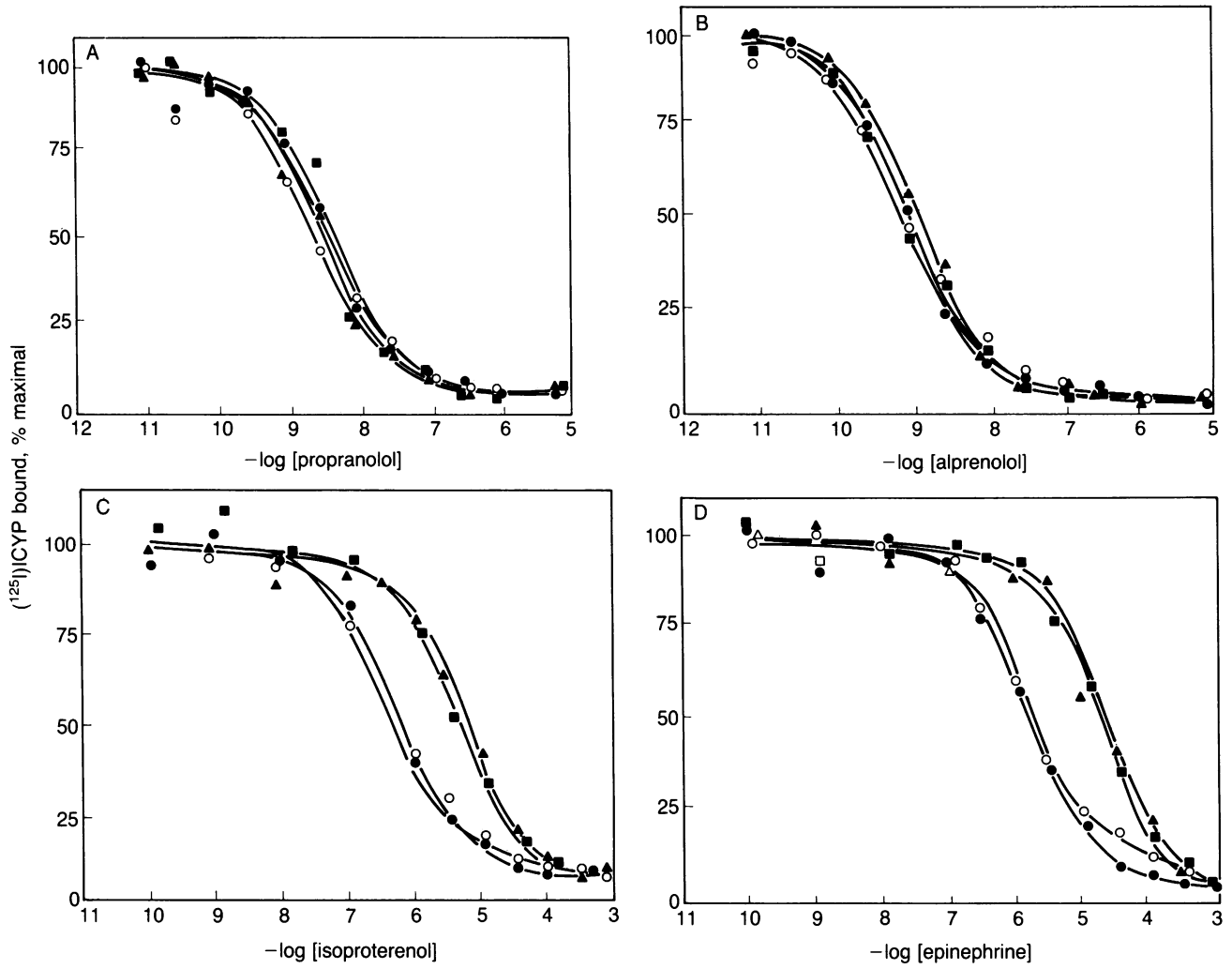

FIG. 4. Competition binding of antagonists propranolol (*A*) and alprenolol (*B*) and agonists isoproterenol (*C*) and epinephrine (*D*) to wild-type and mutant βARs. Binding was performed as described for 90 min at 23°C in a final volume of TME buffer of 250 μl, containing 40 pM (^{125}I)ICYP, 10–20 μg of membrane protein, and competing ligands at the concentrations indicated along the *x* axes above. Bound ligand was determined after filtration on GF/C glass-fiber filters. Data were fit by using the iterative program LIGAND (17). The following receptors were used: hamster βAR (●), [Ala^{79}]βAR (▲), [Ala^{107}]βAR (○), and [Lys^{318}]βAR (■). Data shown are representative of two to four similar experiments, with a variation among experiments of ±10%.

residue appeared to affect receptor processing. For this mutant, an immunoreactive polypeptide of the expected molecular mass of 67 kDa was not observed. The two immunoreactive polypeptides detected with molecular masses 44 and 50 kDa comigrate with nonglycosylated and partially glycosylated βAR (ref. 18; unpublished results). Further studies using radiolabeled sugars will help to define the exact nature of these processing defects. The prevalence of prolines within the hydrophobic regions of integral membrane proteins and within the signal peptides of secreted proteins has been noted previously (19). Our data suggest that the proline at position 323 is necessary for proper insertion of the polypeptide into the membrane.

Whereas the substitution of the aspartate-113 residue with asparagine did not have any apparent effect on the processing of the βAR, the resultant protein showed no detectable binding of antagonists. On the basis of these results, we suggest that binding of adrenergic ligands to the βAR involves a hydrogen bond between the carboxylate group of aspartate-113 and the protonated amino group of the ligand. A similar role is proposed for aspartate-121 of the MAR in binding the quaternary amino group of the muscarinic ligands. No aspartate residue is present at an analogous position in rhodopsin, where the amino group of the "ligand" is contributed by the lysine-296 on opsin itself. Because the agonist binding determinations performed in this work require competition with a radiolabeled antagonist, the agonist-binding properties of mutant [Asn^{113}]βAR remain to be determined.

Replacements of aspartate-79 and asparagine-318 resulted in proteins that had wild-type affinities for antagonists but reduced the affinity for the agonists examined by 1 order of magnitude. In the COS-7 cell expression system, at most only 10% of the cells were expressing the βAR from the transfected gene. In these cells the receptor was apparently in large excess over G_s. The wild-type βAR expressed in this manner showed only the low-affinity state for agonists indicative of a lack of coupling with G_s (8). Consequently, the decreases in agonist affinity observed for [Ala^{79}]βAR and [Lys^{318}]βAR probably reflect intrinsic alterations in the affinity of the receptor for the agonist, rather than an uncoupling of the receptor from G_s. The change in agonist affinities observed could reflect a change either in a direct interaction between the substituted residue and the ligand or in the stability of the agonist-induced conformation of the receptor. The observation that the decrease in affinity was similar for the three agonists examined argues against a change in any interaction involving the divergent amino

substituents of the ligands. The low frequency of expression and the high background β_2-adrenergic-responsive adenylate cyclase activity in the COS-7 cells preclude studying the coupling of the mutant receptors to adenylate cyclase in this system (8). An analysis of the abilities of the mutants described here to stimulate adenylate cyclase awaits expression of the mutant proteins in stable cell lines that have low-background βAR activity (8).

In summary, the results presented here support a critical role for the residues in the βAR hydrophobic region in the binding of ligands. The observation that aspartate-113 cannot be substituted by an asparagine residue without loss of ligand binding activity suggests that this residue is part of the βAR ligand binding site. The peptide sequence N-terminal to aspartate-113 is not conserved between the mammalian β_2AR, and the turkey βAR, which has ligand binding characteristics similar to those of the mammalian β_1AR. Amino acid substitutions within this region of sequence divergence could help to define the structural basis for the βAR subtypes.

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