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# Site-directed mutagenesis of human β-adrenergic receptors: Substitution of aspartic acid-130 by asparagine produces a receptor with high-affinity agonist binding that is uncoupled from adenylate cyclase

(receptor conformational changes/protein secondary structure/gene expression/continuous expression/guanine nucleotide-binding regulatory proteins)

CLAIRE M. FRASER, FU-ZON CHUNG, CHENG-DIAN WANG, AND J. CRAIG VENTER

Section of Receptor Biochemistry and Molecular Biology, Laboratory of Molecular and Cellular Neurobiology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Park Building, Room 405, Bethesda, MD 20892

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ABSTRACT **By using oligonucleotide-directed mutagenesis, we have produced a point mutation (guanine to adenine) at nucleotide 388 of the gene for human β-adrenergic receptor (βAR) that results in a substitution of asparagine for the highly conserved aspartic acid at position 130 in the putative third transmembrane domain of the human βAR ([Asn$^{130}$]βAR). We have examined the functional significance of this mutation in B-82 cells continuously expressing the mutant [Asn$^{130}$]βAR. The mutant [Asn$^{130}$]βAR displayed normal antagonist binding but unusually high-affinity agonist binding (5- to 10-fold higher than wild-type βAR), consistent with a single class of high-affinity binding sites. The mutant βAR displayed guanine nucleotide-sensitive changes in agonist affinity (3- to 5-fold shift) implying an interaction between the βAR and the stimulatory guanine nucleotide-binding regulatory protein; however, the ability of guanine nucleotides to alter agonist affinity was attenuated. Addition of saturating concentrations of isoproterenol to cell cultures expressing mutant [Asn$^{130}$]-βARs had no effect on intracellular levels of cAMP, indicating that the mutant βAR is unable to affect stimulation of adenylate cyclase. These results indicate that substitution of the aspartic acid with asparagine at residue 130 of the human βAR dissociates the well-characterized guanine nucleotide effects on agonist affinity from those on activation of the stimulatory guanine nucleotide-binding regulatory protein and adenylate cyclase and suggests the existence of two distinct counterions for the amine portion of catecholamines that are associated with high- and low-affinity agonist binding states of βAR.**

The cloning and sequence analyses of the genes encoding adrenergic and muscarinic cholinergic receptors (1–10) have revealed marked similarities between these pharmacologically distinct proteins and have shown that they are members of a supergene family (10, 11). The sequence homology among these proteins is particularly striking in their putative transmembrane regions (10). By using oligonucleotide-directed mutagenesis to study the relationship between receptor structure and function, we have identified a highly conserved aspartic acid at position 79 in the putative second transmembrane domain of the human β-adrenergic receptor (βAR) that may act as a counterion for catecholamine binding and appears to be involved in agonist-induced conformational change(s) required for activation of the stimulatory guanine nucleotide-binding regulatory protein ($G_s$) (12) (Fig. 1). This mutant βAR displays low-affinity agonist binding that is unaffected by guanine nucleotides. However, addition of isoproterenol to cells expressing mutant [Asn$^{79}$]βARs produces a small receptor-specific increase in adenylate cyclase activity (12).

An aspartic acid at position 130 in the third transmembrane domain of the human βAR is also highly conserved among members of this gene family, being present in all β-adrenergic (1–4, 10), α-adrenergic (5), and muscarinic cholinergic receptors (6–10) sequenced to date (Fig. 1). In this report we describe a mutant human βAR in which an asparagine has been substituted for the conserved aspartic acid at position 130. This βAR displays unusually high-affinity binding of agonists that is attenuated by guanine nucleotides. However, the guanine nucleotide effects on agonist affinity observed with the mutant [Asn$^{130}$]βAR are markedly different than those on the wild-type βAR, suggesting that the coupling of the [Asn$^{130}$]βAR to $G_s$ is altered as a result of this mutation. In addition, [Asn$^{130}$]βAR are unable to mediate isoproterenol stimulation of adenylate cyclase catalytic activity. These data together with our previous site-directed mutagenesis study on the βAR (12) identify two loci present in transmembrane domains II and III that appear to be essential for normal catecholamine binding and are involved in agonist-induced βAR conformational changes associated with activation of $G_s$.

## MATERIALS AND METHODS

**Materials.** Materials were obtained from sources described in refs. 12 and 15.

**Receptor Mutagenesis and Expression.** For βAR expression, the entire coding region of the human $\beta_2$-adrenergic receptor ($\beta_2$AR) derived from the genomic clone LCV-517 (base pairs −6 to +1267) (1) was cloned into the *Mlu* I and *Eco*RV sites in the polylinker region of the expression vector pMSVneo (12). A single-base mutation was introduced at base 388 of the βAR coding region. This point mutation, which changed the codon from GAT to AAT and converted aspartic acid to asparagine, was introduced into the human $\beta_2$AR by oligonucleotide-directed mutagenesis (by using a 24-base oligonucleotide) as described by Kunkel (16). Authenticity of the mutation was confirmed by dideoxy sequencing. Cells were transfected and selected as described by Fraser *et al.* (15).

**Cell Culture.** B-82 cells, a murine L-cell line, and the transfected cell lines were grown in monolayer culture as described (15).

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Abbreviations: βAR and $\beta_2$AR, β- and $\beta_2$-adrenergic receptor, respectively; $G_s$, stimulatory guanine nucleotide-binding regulatory protein; [$^{125}$I]ICYP, [$^{125}$I]iodocyanopindolol; p[NH]ppG, guanosine 5′-[β, γ-imido]triphosphate; $PGE_1$, prostaglandin $E_1$.

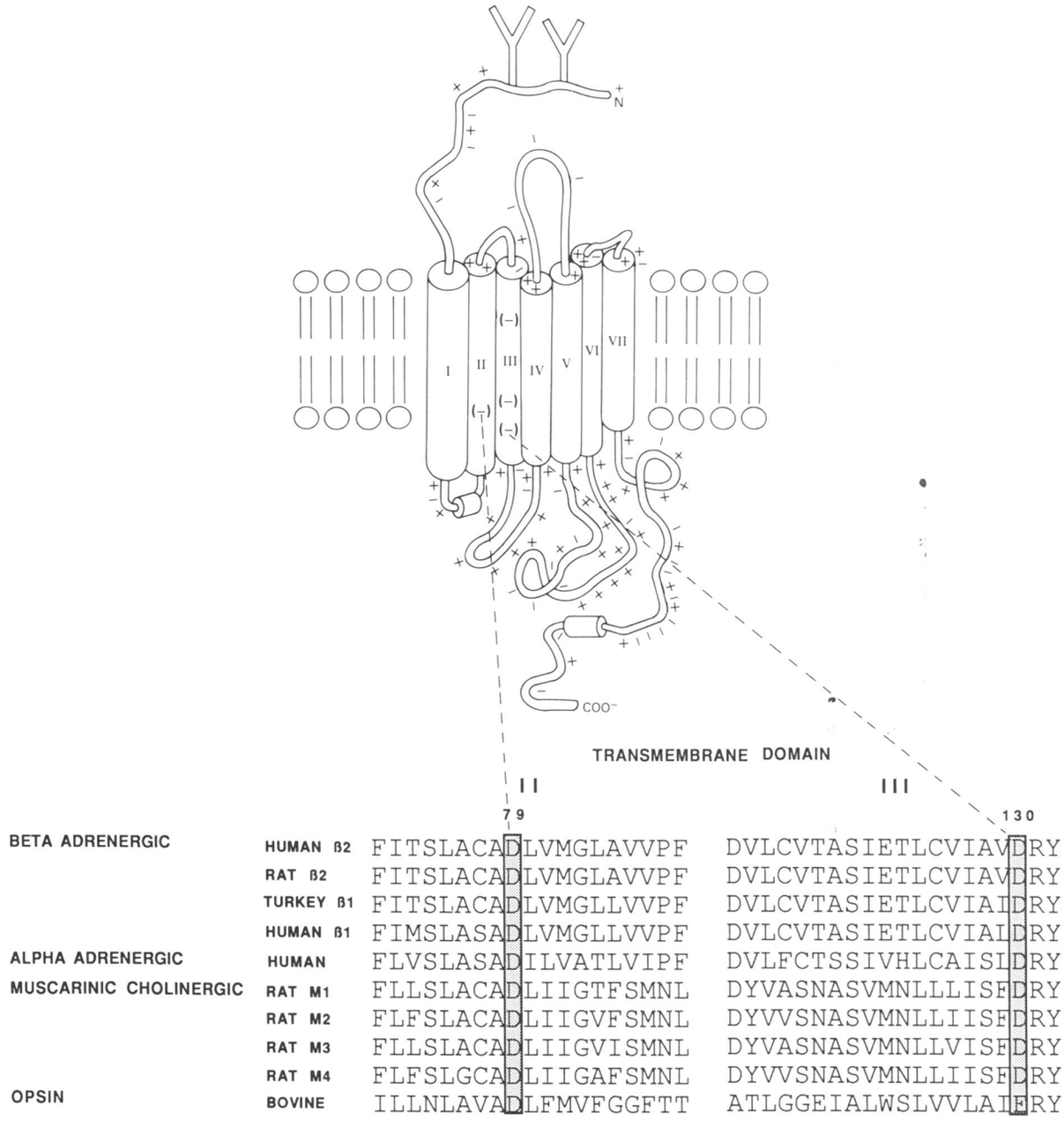


FIG. 1. Location of site-directed mutants and sequence homology of putative transmembrane regions II and III from a family of neurotransmitter receptors. (*Upper*) Schematic model for the predicted secondary structure of the human βAR. The seven putative transmembrane segments of the receptor are numbered and the approximate location of charged amino acid residues are indicated. The indicated charged residues are oriented so that they face into an aqueous core formed by the seven amphipathic transmembrane segments of the receptor (13). (*Lower*) Comparison of the deduced amino acid sequences of transmembrane segments II and III from the indicated receptors (1, 3–5, 10, 11, 14, 24). The shaded areas are the loci of site-directed mutagenesis for residues Asp-79 and Asp-130.

**Assays.** Cell membrane preparation and βAR ligand binding studies were performed as described by Fraser *et al.* (17). Protein was assayed by using the fluorescamine assay with bovine serum albumin as a standard (18). cAMP assays were performed as described (12, 15) with a radioimmunoassay kit for cAMP (New England Nuclear). Cells were dissolved in 0.2 M NaOH for determination of cellular protein.

## RESULTS

**Stable, Steroid-Inducible Receptor Expression.** By using the expression vector pMSVneo (12), we have obtained stable steroid-inducible expression of the mutant [$Asn^{130}$]βAR in B-82 cells, a murine cell line deficient in βAR but containing a prostaglandin $E_1$ ($PGE_1$)-stimulated adenylate cyclase (15, 19). The addition of 1 μM dexamethasone to transfected cell cultures resulted in a 15- to 20-fold increase in the density of βAR. As illustrated in Fig. 2, membranes prepared from cells grown in the absence of steroid displayed [$^{125}$I]iodocyanopindolol ([$^{125}$I]ICYP) binding equivalent to only 3 fmol/mg of protein as compared to membranes prepared from cells grown in the presence of 1 μM dexamethasone that displayed [$^{125}$I]ICYP binding equivalent to 42 fmol/mg of protein. The calculated $K_d$ for [$^{125}$I]ICYP binding to the mutant [$Asn^{130}$]-βAR was 33 pM, a value comparable to that reported for wild-type human βARs expressed in B-82 cells (ref. 12 and Table 1). The density of βARs in dexamethasone-stimulated cells expressing the wild-type βAR was 117 fmol/mg of protein, as reported (12).

**Agonist and Antagonist Binding to Wild-Type and Mutant βARs.** The binding of adrenergic agonists and antagonists to wild-type βAR and mutant [$Asn^{130}$]βAR was examined by

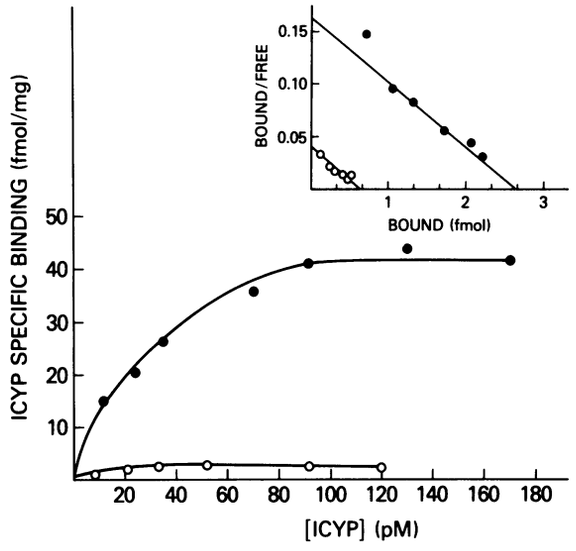


FIG. 2. Saturation isotherms of [$^{125}$I]ICYP binding to membranes expressing mutant [Asn$^{130}$]$\beta$ARs. Membranes from untreated cells (○) or from cells treated with 1 $\mu$M dexamethasone for 24 hr prior to harvesting (●) were incubated with the indicated concentrations of [$^{125}$I]ICYP in the presence and absence of 1 $\mu$M (−)-propranolol for 30 min at 30°C. Samples were filtered on Whatman GF/C filters by using a Brandel M-24R cell harvester and washed with 20 ml of isotonic phosphate-buffered saline. Data are representative of three experiments with each point in triplicate. (*Inset*) Scatchard analysis (20) of [$^{125}$I]ICYP binding to untreated (○) and dexamethasone-treated (●) cell membranes.

competition studies with the indicated ligands and [$^{125}$I]ICYP (Table 1). Wild-type $\beta$AR and mutant [Asn$^{130}$]$\beta$AR have essentially the same affinity for the (+)- and (−)-isomers of propranolol (Fig. 3*A*) and for the radioligand [$^{125}$I]ICYP (Table 1), indicating that this point mutation has no apparent effect on antagonist binding. As illustrated in Fig. 3*B*, the mutant [Asn$^{130}$]$\beta$AR displayed a significantly higher (5- to 10-fold) affinity for isoproterenol, epinephrine, and norepinephrine as compared to the wild-type $\beta$AR. The mutant [Asn$^{130}$]$\beta$AR also retained stereoselective binding of agonists. The (+)- and (−)-isomers of isoproterenol and epinephrine display essentially the same difference in potency for displacement of [$^{125}$I]ICYP as they did with the wild-type $\beta$AR (Table 1). Also, the same rank order of agonist potency was observed in both the wild-type $\beta$AR and mutant [Asn$^{130}$]$\beta$AR.

**Guanine Nucleotide Effects on Agonist Binding.** In membranes containing wild-type $\beta$ARs, isoproterenol binding was

Table 1. Human $\beta_2$ARs: Affinities for adrenergic agents

| Agent | $K_d$, nM | | | | | |
|---|---|---|---|---|---|---|
| | Wild-type $\beta$AR | | | [Asn$^{130}$]$\beta$AR | | |
| (−)-Isoproterenol | 17.6 | ± 4 | (3) | 2.5 | ± 0.2 | (3) |
| (−)-Isoproterenol/ p[NH]ppG | 120.0 | ± 18 | (2) | 9.3 | ± 3.2 | (2) |
| (+)-Isoproterenol | 200.0 | ± 33 | (2) | 32.5 | ± 15 | (2) |
| (−)-Epinephrine | 56.5 | ± 6 | (2) | 4.3 | ± 0.7 | (2) |
| (+)-Epinephrine | 500.0 | | (1) | 75.0 | | (1) |
| (−)-Norepinephrine | 219.0 | ± 27 | (2) | 55.0 | ± 4.8 | (2) |
| (−)-Propranolol | 0.22 | ± 0.1 | (2) | 0.36 | ± 0.15 | (2) |
| (+)-Propranolol | 36.8 | ± 3.0 | (2) | 65.0 | ± 15.0 | (2) |
| (±)-Iodocyanopindolol | 21.0 | ± 5.0 | (2) | 32.0 | ± 6.0 | (2) |

Affinity constant ($K_d$) for the radioligand [$^{125}$I]ICYP was determined directly in equilibrium binding studies. The $K_d$ for competing adrenergic agents was calculated from competition studies by using the method described by Cheng and Prusoff (21). Values represent the means ± SEM of the indicated number of experiments in parentheses with triplicate samples.

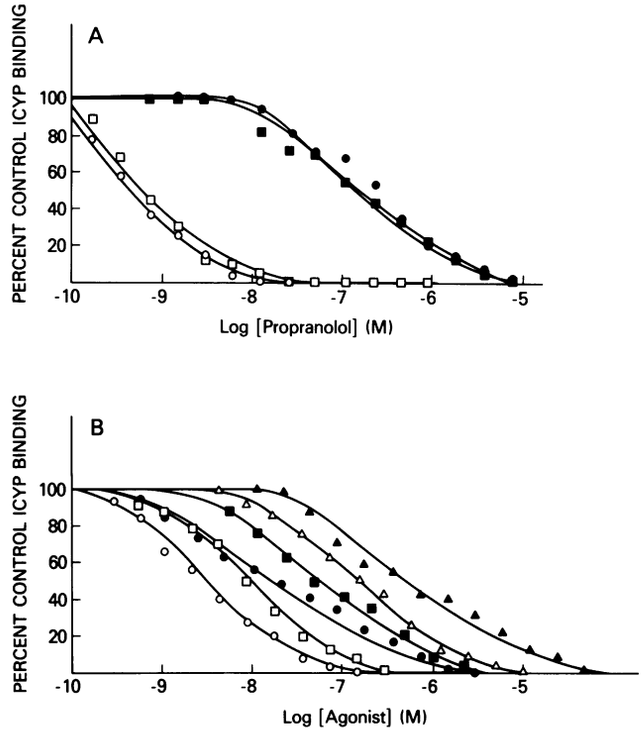


FIG. 3. Adrenergic agonist and antagonist competition for [$^{125}$I]ICYP binding to wild-type $\beta$ARs and mutant [Asn$^{130}$]$\beta$ARs. Membranes from dexamethasone-treated cells expressing wild-type $\beta$ARs or mutant [Asn$^{130}$]$\beta$ARs were incubated in the presence of 35 pM [$^{125}$I]ICYP and the indicated concentrations of adrenergic agents for 30 min at 30°C. Samples were filtered as described in Fig. 2. Total [$^{125}$I]ICYP binding in the absence of competing ligands was 5 fmol. Data are representative of two or three experiments with triplicate samples. (*A*) ● and ○, wild-type $\beta$ARs in the presence of various concentrations of (+)- and (−)-propranolol, respectively; ■ and □, [Asn$^{130}$]$\beta$ARs in the presence of (+)- and (−)-propranolol, respectively. (*B*) Competition binding between [$^{125}$I]ICYP and (−)-isoproterenol (○ and ●), (−)-epinephrine (□ and ■), and (−)-norepinephrine (△ and ▲), respectively, to wild-type $\beta$ARs and [Asn$^{130}$]$\beta$ARs is shown.

heterogeneous (Hill coefficient = 0.5–0.66) indicating the presence of low- and high-affinity receptor sites (Fig. 4). Competition binding studies with isoproterenol and [$^{125}$I]-

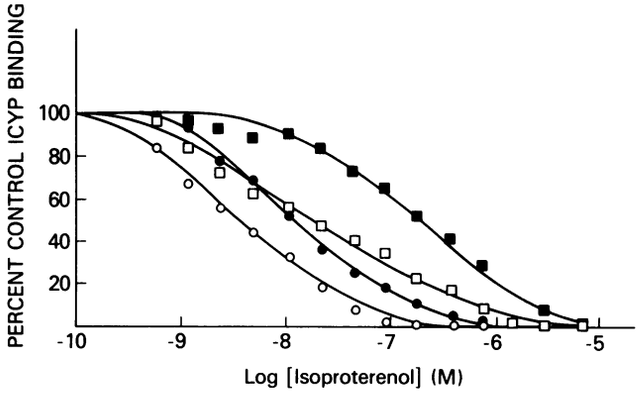


FIG. 4. Guanine nucleotide regulation of agonist affinity to wild-type $\beta$ARs and mutant [Asn$^{130}$]$\beta$ARs. [$^{125}$I]ICYP binding to wild-type $\beta$ARs and mutant [Asn$^{130}$]$\beta$ARs was determined at a concentration of 35 pM in the presence of various concentrations of (−)-isoproterenol in the presence or absence of 100 $\mu$M p[NH]ppG. ■ and ●, Binding of [$^{125}$I]ICYP to wild-type $\beta$ARs in the presence and absence of p[NH]ppG, respectively; □ and ○, binding of [$^{125}$I]ICYP to [Asn$^{130}$]$\beta$ARs in the presence and absence of p[NH]ppG, respectively. Samples were assayed as described in Fig. 2.

ICYP in the presence of 100 $\mu$M guanosine 5'-[$\beta,\gamma$-imido]triphosphate (p[NH]ppG) resulted in 5- to 10-fold shifts in the isoproterenol dose–response curve to the right and eliminated the majority of the high-affinity component of agonist binding (Hill coefficient = 0.86–1.0). The addition of exogenous guanine nucleotides to membranes containing the [Asn$^{130}$]$\beta$ARs caused a 3- to 5-fold rightward shift in the isoproterenol dose–response; however, in contrast to wild-type $\beta$AR, the Hill coefficient for isoproterenol binding to mutant [Asn$^{130}$]$\beta$AR was unchanged by the addition of p[NH]ppG (Fig. 4).

To further characterize the guanine nucleotide-induced shifts in agonist affinity, the dose–response effects of GTP and p[NH]ppG on agonist binding to wild-type $\beta$ARs and mutant [Asn$^{130}$]$\beta$ARs were examined. As illustrated in Fig. 5, GTP ($K_m = 2 \times 10^{-7}$M) was more potent than p[NH]ppG ($K_m = 10^{-5}$ M) in altering agonist affinity in membranes containing the wild-type $\beta$ARs. These data are consistent with those originally described by Lad *et al.* (22) on guanine nucleotide regulation of glucagon receptors. In contrast to the results observed with the wild-type $\beta$AR, the mutant [Asn$^{130}$]-$\beta$AR was less sensitive to the effects of guanine nucleotides on agonist affinity; GTP and p[NH]ppG exerted these effects only at concentrations $>10^{-5}$ M (Fig. 5).

**Activation of Adenylate Cyclase.** We have demonstrated (12, 15) that wild-type $\beta_2$ARs expressed in B-82 cells are able to mediate isoproterenol stimulation of adenylate cyclase in a dose-dependent manner. However, addition of isoproterenol to cultures expressing mutant [Asn$^{130}$]$\beta$ARs had no effect on intracellular concentrations of cAMP (Fig. 6), indicating that the mutant $\beta$AR was completely unable to affect activation of adenylate cyclase. Addition of $PGE_1$ to cultures expressing [Asn$^{130}$]$\beta$ARs produced a dose-dependent activation of adenylate cyclase identical to control B-82 cells (15) and that observed in cells expressing wild-type $\beta$ARs (12, 15), demonstrating the presence of functional $G_s$ and adenylate cyclase in these cells (Fig. 6).

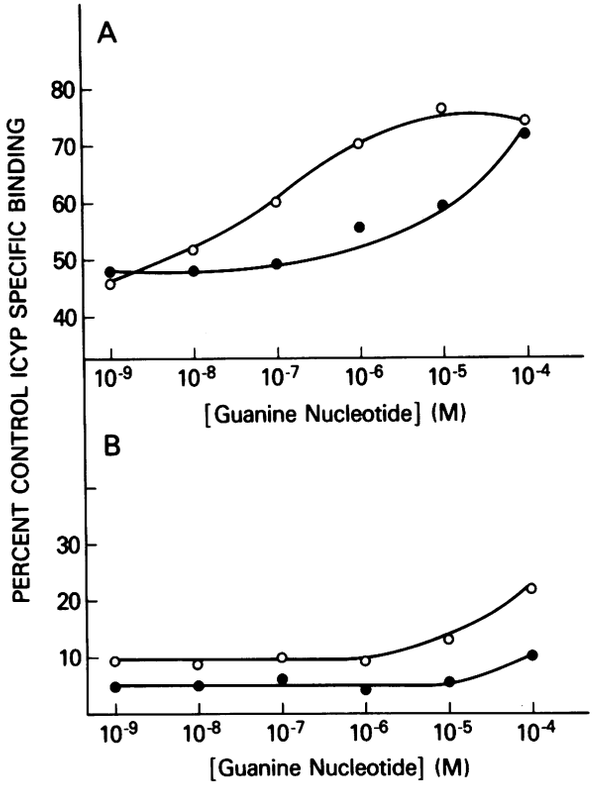


FIG. 5. Effect of various concentrations of guanine nucleotides on isoproterenol binding to wild-type $\beta$ARs and mutant [Asn$^{130}$]-$\beta$ARs. [$^{125}$I]ICYP binding to wild-type $\beta$ARs and mutant [Asn$^{130}$]-$\beta$ARs was determined at a concentration of 35 pM in the presence of $5 \times 10^{-8}$ M isoproterenol and the indicated concentrations of GTP (○) or p[NH]ppG (●). *A* and *B* represent data obtained with wild-type $\beta$ARs and mutant [Asn$^{130}$]$\beta$ARs, respectively.

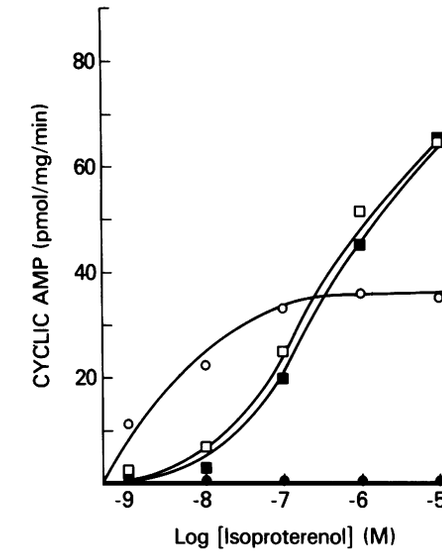


FIG. 6. Agonist-induced changes in cAMP concentrations in cells expressing wild-type $\beta$ARs and mutant [Asn$^{130}$]$\beta$ARs. Confluent cultures of cells expressing wild-type $\beta$ARs or mutant [Asn$^{130}$]$\beta$ARs were incubated with the indicated concentrations of isoproterenol (○, ●) or $PGE_1$ (□, ■). cAMP concentrations were determined with a RIA kit for cAMP (New England Nuclear). Data are expressed as pmol of cAMP per mg of cellular protein per min.

## DISCUSSION

We introduced a point mutation (guanine to adenine) at base 388 of the human $\beta_2$AR gene that resulted in a change of aspartic acid to asparagine at position 130 in the expressed $\beta$AR protein. We used the expression vector pMSVneo to obtain stable glucocorticoid-inducible expression of the mutant $\beta$AR in B-82 cells, a murine L cell line completely lacking $\beta$ARs. Agonist binding to the [Asn$^{130}$]$\beta$ARs was changed as a result of this point mutation, with the mutant $\beta$AR exhibiting unusually high-affinity binding ($K_d$ for isoproterenol = 2.5 nM). This high-affinity binding state of the $\beta$AR is consistent with a single class of binding sites (Hill coefficients = 0.85–1.00). These data contrast with those from wild-type $\beta$ARs expressed in B-82 cells where agonist binding is heterogenous (Hill coefficients = 0.5–0.66), and the affinity for isoproterenol is significantly lower ($K_d$ = 17.6 nM).

We have considered the possibility that the persistent high-affinity binding of agonists to the mutant [Asn$^{130}$]$\beta$AR represents an irreversibly coupled $\beta$AR–$G_s$ complex; however, addition of $PGE_1$ to transfected cells preincubated with isoproterenol produces the same dose-dependent change in intracellular levels of cAMP as in untreated cells (data not shown), indicating that $G_s$ is available for interaction with $PGE_1$ receptors. These data suggest that either $G_s$ is not limiting in these cells or it is not precoupled to the mutant $\beta$AR.

The [Asn$^{130}$]$\beta$AR displayed guanine nucleotide-sensitive agonist binding; however, the affinity of the receptor for isoproterenol in the presence of p[NH]ppG was still significantly higher than that of the wild-type $\beta$AR in the presence of p[NH]ppG (Fig. 4). In every experiment performed, the guanine nucleotide-induced shift in agonist dose–response curves with the mutant $\beta$AR was smaller than that seen with the wild-type $\beta$AR (3- to 5-fold vs. 5- to 10-fold) and was not

associated with changes in the Hill coefficient. The potency of GTP and p[NH]ppG for attenuation of agonist affinity with the mutant [$Asn^{130}$]βAR was also significantly reduced. These data demonstrate that substitution of aspartic acid with asparagine at position 130 of the human βAR has resulted in an altered conformation that displays high-affinity agonist binding and is unable to effectively interact with or activate $G_s$.

Our findings from the characterization of the [$Asn^{130}$]βAR contrast markedly with those from our study (12) on the properties of the mutant [$Asn^{79}$]βAR that displayed persistent low-affinity agonist binding but was able to activate adenylate cyclase. These data suggest that the βAR conformation displaying low-affinity agonist binding is able to activate adenylate cyclase, whereas the βAR conformation associated with high-affinity agonist binding may represent a desensitized or uncoupled state of βAR. This hypothesis is in apparent conflict with the proposed models for hormonal activation of adenylate cyclase that hypothesize that the state of high-affinity agonist binding is presumed to be required for activation of adenylate cyclase (23).

There are several intriguing possibilities that might account for the differences in agonist affinities observed among wild-type βARs and [$Asn^{79}$]- and [$Asn^{130}$]βARs. We proposed that Asp-79 in the βAR may serve as a counterion for the amine substituents of the catecholamines (12). However, our data suggest that there may be more than one counterion (Asp-79 and -130) for catecholamine binding. Depending upon which counterion interacts with the agonist, binding may have either a high (Asp-79) or a low (Asp-130) affinity. Substitution of Asp-79 with Asn changed the properties of the mutant βAR, so that only a single class of low-affinity agonist binding was observed (12). In contrast, substitution of Asp-130 with Asn produced a βAR that displayed only a single class of high-affinity agonist binding sites. Therefore, it is possible that conformational changes in βAR induced by agonist or $G_s$ can result in the differential exposure of one of these counterions for catecholamine binding.

In using site-directed mutagenesis to study the relationship between protein structure and function, it is important to be aware of the limitations of this powerful technique. Radical amino acid substitutions or deletion of long stretches of amino acids may alter protein folding or insertion into the membrane and thereby complicate data interpretation. The amino acid substitution chosen for this study (Asp → Asn) represents a change that replaces a negatively charged side chain with a neutral group of similar size. The finding that antagonist binding is unaltered and both adrenergic agonists and antagonists retain stereoselective binding with [$Asn^{130}$]-βAR argues that we have not markedly altered the structure of βAR required for ligand binding.

Conservation of Asp-79 and Asp-130 among members of the gene family of neurotransmitter receptors suggested to us that they play an important role(s) in maintenance of receptor structure, ligand binding, or activation mechanisms of these multicomponent systems. These data on Asp-130 and our study on Asp-79 (12) imply that these negatively charged amino acids play critical roles in agonist binding and in agonist-induced receptor conformational changes. The availability of established cell lines expressing these βAR mutants will allow a more detailed characterization of the precise role of Asp-79 and Asp-130 in βAR activation.

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