A Polymorphism of the Human \( \beta_2 \)-Adrenergic Receptor within the Fourth Transmembrane Domain Alters Ligand Binding and Functional Properties of the Receptor*

(Received for publication, April 13, 1993, and in revised form, June 15, 1993)

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The human \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)AR) has been extensively studied as a prototype of G-protein coupled receptors. Common features of these receptors are an extracellular N terminus, seven hydrophobic regions which are proposed to form transmembrane-spanning \( \alpha \)-helices, three intracellular and three extracellular loops, and an intracellular C terminus. Mutagenesis experiments with the adrenergic receptors have delineated specific domains which appear to be critical for various receptor functions. These include regions responsible for G-protein coupling (1-4), agonist-promoted phosphorylation and desensitization (5-7), sequestration (8-11), down-regulation (8, 10, 12), and ligand binding (2, 13-15).

With regard to the latter, several lines of evidence have shown that the individual transmembrane spanning domains contain key amino acids which are critical determinants of agonist and antagonist binding. Strader et al. (15, 16) have shown that Asp113 in the third transmembrane region, and Ser231 and Ser235 within the fifth transmembrane region of the \( \beta_2 \)AR, appear to interact with the amino and aromatic hydroxyl groups of catecholamine agonists, respectively. In addition to these direct interactions, there also appear to be contributions from other transmembrane domains which are necessary for formation of the hydrophobic binding pocket. Studies utilizing chimeric \( \alpha_2 \)/\( \beta_2 \)- and \( \beta_2 \)/\( \beta_2 \)-adrenergic receptors have suggested important roles for these other domains, presumably either as sites for direct interaction with ligands or as critical for maintenance of the overall receptor conformation necessary for ligand binding (2, 13). Computer modeling techniques have also predicted a number of interactions between the \( \beta_2 \)AR and agonists or antagonists (17, 18). Some of these interactions have been difficult to prove by mutagenesis. Of particular importance to this report is the prediction that Ser231 in the fourth transmembrane region forms a hydrogen bond with the \( \beta \)-hydroxyl group of agonists (18). Mutagenesis of Ser231 to Ala results in abnormal protein folding into the membrane, which has made the role of Ser231 in agonist binding testable (15).

Recently, we identified within a cohort of 107 subjects two uncommon polymorphisms of the \( \beta_2 \)AR gene which result in mutations of the receptor within transmembrane-spanning domains, one in the putative first transmembrane region (Val184 → Met); and one in the fourth transmembrane region (Thr146 → Ile) (19). Based upon studies such as those cited above, we postulated that the mutation of Thr146 to Ile might result in abnormal ligand binding. We report here the consequences of this natural polymorphism when stably expressed in a mammalian cell line.

**EXPERIMENTAL PROCEDURES**

*This work was supported by National Institutes of Health Grant R01-HL45967 (to S. B. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: \( \beta_2 \)AR, \( \beta_2 \)-adrenergic receptor; WT \( \beta_2 \)AR, wild-type \( \beta_2 \)AR; Ile146 \( \beta_2 \)AR, mutated \( \beta_2 \)AR containing Ile substituted for Thr at codon 184; Gs, stimulatory guanine nucleotide regulatory protein; GppNHp, 5'-guanylylimidodiphosphate; PBS, phosphate-buffered saline.

**Methods**

*Constructs and Transfections—We have previously identified polymorphisms of the human \( \beta_2 \)AR gene using a combination of temper-
polymorphism at nucleic acid 491, where a thymidine was found to be substituted for a cytidine, resulting in the substitution of Ile for Thr at amino acid residue 164. This mutation is denoted Ile'A. We mimicked this mutation in the wild-type βAR cDNA by site directed techniques similar to those described (3). The wild-type and mutant constructs were individually ligated into the expression vector pcDNA-Neo, and CHW-1102 cells were permanently transfected by a calcium phosphate precipitation technique (20,21). Clonal cell lines were isolated by their resistance to 300 µg/ml G418. The amount of DNA required for transfection, and the efficiency of transfection with the same construct, were the same as that for WT βAR. We found in transient transfections that 125I-CYP bound Ile'A with high affinity, and binding assays using this radioligand were used to screen the permanent clonal cell lines for the presence of the transfected receptor. Growth rates of the transfected cells and the stability of expression of βAR were equivalent for the two clonal lines chosen for these studies (1647 ± 193 versus 1335 ± 107 fmol/mg protein for WT βAR and Ile'A, respectively). In addition, the Kd for 125I-CYP as derived by Scatchard analysis was also equivalent between the receptors (25.12 ± 5.97 versus 27.68 ± 4.07 pM, respectively). In competition studies (performed in the presence of Gpp(NH)p), each receptor displayed agonist rank order affinities typical for the βAR subtype (i.e. isoproterenol > epinephrine > norepinephrine). However, Ile'A consistently displayed decreased affinities toward these agonists (Table I). The magnitude of the increases in the inhibition constant Ki (approximately 4-fold) was the same for all three catecholamines. Since the weight of evidence has suggested that the neighboring amino acid Ser166 plays a key role in binding of the β-hydroxyl group of catecholamines (17), we considered that the substitution of Ile at position 164 may perturb this interaction. We therefore studied two agonists, dopamine and dobutamine, which do not have β-hydroxyl groups. As shown in the table, in contrast to what was observed for catecholamines, neither dopamine nor dobutamine distinguished between WT βAR and Ile'A receptors.

We also examined the ability of the two receptors to form the high affinity ternary complex by performing agonist competition studies with isoproterenol in the absence of guanine nucleotide (Fig. 1). In the case of the WT βAR, shallow curves were obtained which were best fit to a two-site model with a high affinity component representing 17 ± 1.6% of receptors with an affinity of 0.90 ± 0.22 nM and a low affinity component representing 82.9 ± 1.7% of receptors with an affinity of 296 ± 58 nM (n = 6). In contrast, curves for Ile'A were steeper (mean Hill coefficient = 0.91) and displayed only a single low affinity component (Fig. 1). In the presence of Gpp(NH)p, WT βAR binding underwent a classic shift to steep curves with single low affinity binding components, whereas the single-site Ile'A curves displayed minor rightward shifts (Kd = 524 ± 44 nM to 772 ± 60 nM, p = not significant).

We next examined the binding characteristics for a group of βAR antagonists with diverse structures. Propranol, ICI118551, and CGP 20712 all clearly showed lower affinities for Ile'A as compared with WT βAR, and alprenolol displayed a similar trend (Table I, lower portion). On the other hand, pindolol and CGP12177 showed no differences in affinity for the two receptors. Given that all of these antagonists have β-hydroxyl groups, it is clear that other interactions have also been perturbed. One common feature of the antagonists (and also agonists) which demonstrate decreased affinity toward Ile'A is the absence of polar substituents on the aromatic rings.

To determine whether the differences in catecholamine binding found between WT βAR and Ile'A receptors trans-
\textbf{Table I}

Receptor binding affinities of agonists and antagonists for WT $\beta$AR and Ile164

Inhibition constants ($K_i$) for various adrenergic agonists and antagonists were determined in competition assays with the radioligand $^{125}$I-CYP and membranes expressing WT $\beta$AR or mutant (Ile164) receptors in the presence of 100 $\mu$M GppNHp. Structures of the ligands are shown on the left. Values given are means ± S.E. for three to six experiments, in nanomolar except as noted. *, $p < 0.05$ as compared with WT $\beta$AR.

| Agonist/Analog         | WT     | Ile164 |
|------------------------|--------|--------|
| isoproterenol          | isoproterenol | 68.2 ± 11.8 | 295 ± 90.4 |
| epinephrine            | epinephrine | 368 ± 39.6 | 1450 ± 79.0 | *
| norepinephrine         | norepinephrine | 10395 ± 1890 | 45000 ± 9910 | * |
| dopamine (uM)          | dopamine (uM) | 209 ± 61.8 | 238 ± 65.9 |
| dobutamine (uM)        | dobutamine (uM) | 2.31 ± 0.64 | 4.48 ± 1.48 |
| propranolol            | propranolol | 0.06 ± 0.01 | 0.20 ± 0.01 | * |
| alprenolol             | alprenolol | 0.11 ± 0.02 | 0.18 ± 0.04 |
| ICI 118551             | ICI 118551 | 0.43 ± 0.08 | 1.97 ± 0.35 | * |
| pindolol               | pindolol | 2.58 ± 0.25 | 3.48 ± 0.93 |
| CGP 12177              | CGP 12177 | 10.3 ± 2.44 | 9.74 ± 2.59 |
| CGP 20712              | CGP 20712 | 1110 ± 190 | 6580 ± 104 | * |

As shown in Fig. 2, there was an approximately 3-fold rightward shift in the epinephrine dose-response curve and a decrease in maximal stimulation of adenylyl cyclase for Ile164 versus WT $\beta$AR. For WT $\beta$AR, the mean EC$_{50}$ was 113.5 ± 24.8 nM; for Ile164, the corresponding value was 302 ± 95.8 nM (ratio = 2.88, $n = 6$, $p < 0.05$). Mean agonist-stimulated (i.e. maximal minus basal) activity for Ile164 was also reduced versus WT $\beta$AR (3.20 ± 0.46 versus 7.49 ± 1.02 pmol/min/mg protein, respectively, $n = 6$, $p < 0.05$). These differences in activity were not due to differences in receptor expression, which were equivalent (see above). It is of interest to note that the basal (i.e. agonist-independent) activity of Ile164 was also reduced as compared with WT $\beta$AR (1.88 ± 0.38 versus 5.06 ± 0.91 pmol/min/mg protein, respectively, $n = 6$, $p < 0.02$). As shown in Fig. 3, the lower basal adenylyl cyclase activities of Ile164 as compared with WT $\beta$AR was observed in multiple clonal cell lines having a broad range of receptor densities. With increasing expression of either receptor, basal levels of adenylyl cyclase increased, but the Ile164 mutant consistently displayed lower basal levels as compared with wild-type $\beta$AR expressed at a similar level. However, the basal activity of Ile164 is higher than that of...
nontransfected CHW cells (which was 0.71 ± 0.20 pmol/min/mg protein, p < 0.05). It should also be noted that forskolin-induced activation of Ile164 is not different from that of nontransfected CHW cells (15.15 ± 4.15 versus 14.54 ± 0.24 pmol/min/mg protein, p = not significant). Taken together, these data suggest that the mutant receptor displays perturbed nonagonist-dependent coupling to Gs in addition to that observed in the presence of agonist.

Although the mechanisms of receptor sequestration are not known with certainty, it is clear that the process is triggered by agonist binding (25). We therefore assessed the ability of Ile164 to undergo sequestration in response to saturating concentrations of epinephrine. Fig. 4 shows the results of epinephrine (10 μM)-induced sequestration experiments with WT β2-AR and Ile164. WT β2-AR expressed in CHW cells undergo ~15–20% sequestration under these conditions (5, 7, 8). Exposure of Ile164 to epinephrine resulted in ~65% less maximal sequestration as compared with WT β2-AR (5.8 ± 2.0 versus 16.4 ± 1.2% sequestered, respectively, n = 7, p < 0.001). It should be noted that these findings are not artifacts of differences in ligand binding between the two receptors since 125I-CYP and CGP12177 display equal affinities for WT β2-AR and Ile164, and saturating concentrations of propranolol were used in the sequestration assay.

As delineated in the above studies, there appears to be multiple potential aberrancies in the ligand binding interactions that have been perturbed in Ile164. One possible component of this distinction is the proposed interaction of Ser165 with the β-hydroxyl group of some ligands (17). To further evaluate this, the binding of the (+)-enantiomers of isoproterenol and propranolol were assessed. Both compounds

Fig. 1. Agonist competition with 125I-CYP for WT β2-AR and Ile164 receptors in the absence or presence of guanine nucleotide. Membranes prepared from cells expressing WT β2-AR or Ile164 were prepared and competition studies carried out as described under "Methods." WT β2-AR binding curves in the absence of GppNHP were shallow and displayed high and low affinity components, whereas Ile164 binding curves were steep and displayed a single low affinity binding site. In the presence of GppNHP, WT β2-AR binding curves displayed a shift to a single low affinity component, whereas Ile164 curves were unchanged. Shown is a single experiment representative of six performed. Mean data from six such experiments are presented under "Results."

Fig. 2. Depressed agonist-stimulated adenylyl cyclase activity of Ile164. Membranes from cells expressing WT β2-AR or Ile164 were exposed to various concentrations of epinephrine and adenylyl cyclase activities determined as described under "Methods." A single representative experiment is shown. For six such experiments, maximal adenylyl cyclase activities (pmol/min/mg) for WT β2-AR were 7.49 ± 1.02 and for Ile164 were 3.20 ± 0.46, p < 0.01.

Fig. 3. Basal levels of adenylyl cyclase in membranes from cells expressing Ile164 compared with WT β2-AR. Membranes were prepared from different clonal CHW cell lines expressing either Ile164 or WT β2-AR at various levels of expression and adenylyl cyclase activities determined in the absence of agonist as described under "Methods." Compared with WT β2-AR at similar levels of expression, basal adenylyl cyclase activity was always lower with the Ile164 receptor.

versus 16.4 ± 1.2% sequestered, respectively, n = 7, p < 0.001).
binding in G-protein coupled receptors in general, and adrenergic receptors in particular, reside in the transmembrane α-helices (reviewed in Refs. 26 and 27). Mutagenesis studies have identified transmembrane region 5 of the β2AR as being especially important regions of receptor/ligand interaction. For example, mutation of either Asp113 in transmembrane region 3 tested may suggest mechanisms for the changes in affinities of physiologic adrenergic agonists forms a chiral center. Accordingly, removal of the β-hydroxyl group, as seen with the ligands dopamine and dobutamine, or substitution with the (+)-enantiomer would be expected to eliminate the proposed hydrogen bond and thus result in equal affinities between the wild-type and mutant receptors. As noted under “Results,” competition studies with dopamine, dobutamine, and (+)-propranolol all failed to distinguish between WT β2AR and Ile164, consistent with this proposed interaction. Surprisingly, however, (+)-isoproterenol maintained a decreased affinity for the mutant as compared with the wild-type. Taken together, one interpretation of our results is that the Ile substitution at residue 164 has perturbed the role of Ser165, resulting in both its inability to interact with the β-hydroxyl group of some catecholamines and its inability to participate in the maintenance of proper receptor conformation. As discussed below, this latter effect may be manifested by altered binding of some agonists and by depressed agonist and nonagonist-dependent coupling of the receptor to Gs. This proposed mechanism of the effects of the Thr to Ile164 substitution (i.e. interference with the function of Ser165) is based on the majority of our results being consistent with the predicted role of Ser165 by computer modeling (17) and results of site-directed mutagenesis of Ser166 (15). We recognize, however, that Thr164 may have a direct interaction with some ligands which is perturbed by the substitution of Ile. Based on our findings, though, such a substitution would also affect nonagonist coupling to Gs, independent of Ser166. Although there is little supportive evidence for such, we recognize this possibility.

Examination of the divergent structures of the antagonists tested may suggest mechanisms for the changes in affinities observed. Antagonists with polar nitrogen substituents within the catecholamine aromatic regions (CGP12177, pindolol) fail to differentiate between WT β2AR and Ile164. At physiologic pH, these groups presumably have a net positive charge and thus potentially provide a stabilizing interaction with another, as of yet undetermined, side group within the receptor. This stabilizing interaction may be strong enough to effectively compensate for any distorting interaction at the proposed Ser166-β-hydroxyl hydrogen bond. Although CGP20712 does have nitrogen substituents on the aromatic catecholamine ring, it did manifest altered binding to the Ile164 mutant. However, this compound also has a bulky trifluoromethyl group which presumably negates any polar properties which may be contributed by the nitrogens (Table I).

In addition to the abnormalities in ligand binding noted above, substitution of Ile for Thr at position 164 also results in functional abnormalities of adenylyl cyclase activation. In agonist competition binding studies performed in the absence of guanine nucleotide, we were unable to detect a high affinity

**FIG. 4.** Agonist-induced receptor sequestration in CHW cells expressing WT β2AR or Ile164. Confuent cells were exposed to 10 μM epinephrine and 0.1 mM ascorbic acid in serum-free media for the indicated times. Cells were then extensively washed, detached, and whole cell [3H]-I-CYP binding studies were performed at 13 °C as described under “Methods.” Results from six independent experiments are shown. The maximal agonist induced sequestration for Ile164 was lower as compared with WT β2AR (5.8 ± 2.0% versus 16.4 ± 1.2%, p < 0.001).
binding site in the Ile164 mutant, suggesting an impairment in the ability of this receptor to form the high affinity agonist ternary complex. In adenylyl cyclase assays, such an impairment of physical coupling was manifested as a significantly depressed maximal agonist-stimulated activity as compared with WT \( \beta_2 \)AR, under conditions where receptor expressions were the same.

We suggest that in addition to impaired agonist binding, another abnormality is present in Ile164 which further contributes to its depressed function. We found that the basal (i.e. nonagonist-dependent) adenylyl cyclase activity of Ile164 was also depressed. We and others (Fig. 3 and Ref. 29) have noted that the basal adenylyl cyclase activity of nontransfected cells can be lower than that of transfected cells expressing high levels of \( \beta_2 \)AR. Thus although the tendency for the receptor to couple to Gs is markedly favored in the agonist-occupied form, no mutant exhibited impaired sequestration. Cheung et al. (30) have suggested that sequestration requires that the basal adenylyl cyclase activity of nontransfected cells, suggesting that the nonagonist-dependent adenylyl cyclase activity of Ile164 was in some way different than that of WT \( \beta_2 \)AR. Since it has been shown that mutating Ser166 causes the receptor to be in a markedly abnormal conformation (15), it is not unexpected that our mutating the adjacent Ile164 also results in a global, but clearly less dramatic, alteration in receptor function. Additionally, since the known regions of interaction with Gs are intracellular and are all somewhat remote from the mutation in transmembrane region 4, a global rather than local distortion of the receptor structure should be implicated.

We also observed depressed agonist-promoted sequestration of Ile164. Although the precise molecular determinants for this process have not yet been identified, several recent studies have suggested that sequestration requires that the \( \beta_2 \)AR undergo a conformational change after agonist binding. Neither physical or functional coupling to Gs appear to be necessary for sequestration. Campbell et al. (10) studied several mutations of the human \( \beta_2 \)AR in CHW cells; although impairment of adenylyl cyclase activity was associated with loss of ability to undergo down-regulation, no mutant exhibited impaired sequestration. Cheung et al. (11) noted loss of G-protein coupling and sequestration when the N-terminal portion of the third intracellular loop of the hamster \( \beta_2 \)AR was deleted; substitution of the analogous region of the M1 muscarinic receptor restored sequestration but not coupling. Taken together, these results suggest a conformational rather than functional dependence for the integrity of the sequestration response. The observed impairment of sequestration for the Ile164 mutant presented here is therefore most consistent with a perturbation in agonist-promoted triggering of an appropriate conformational change, rather than an indirect result of impaired coupling to Gs.

The current study is the first to delineate the functional characteristics of a naturally occurring mutation of any adrenergic receptor. The presence of a dysfunctional \( \beta_2 \)AR variant may explain, in part, the variability in responses to catecholamines which have been noted in physiologic studies in man (30). Based on the structures of the compounds studied, we cannot offer a unified mechanism for all of the mutated receptor's dysfunctions that we observed. It is clear, however, that this region (possibly dictated by Ser166) is important for ligand binding, agonist-mediated sequestration and coupling to Gs, and establishing overall receptor conformation.

Acknowledgments—We thank Catherine Strader for helpful discussion and Cheryl Theiss for excellent technical assistance.

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