An aromatic residue, tyrosine 326 in the prototypical human β2-adrenergic receptor, exists in a highly conserved sequence motif in virtually all members of the G protein-coupled receptor family. The potential role of this conserved aromatic amino acid residue in the cellular processes of sequestration (a rapid internalization of the surface receptor) and down-regulation (a slower loss of total cellular receptors) associated with agonist-mediated desensitization of the β2-adrenergic receptor was assessed by replacing tyrosine residue 326 with an alanine residue (β2AR-Y→A326A). This mutation completely abolishes agonist-mediated receptor sequestration without affecting the ability of the receptor to activate maximally adenylyl cyclase, to undergo rapid desensitization, and to down-regulate in response to agonist. The only other major change associated with the mutated receptor is a complete loss of the ability to resestinate following rapid desensitization. These results imply that this tyrosine residue, which is part of a highly conserved sequence motif in G protein-coupled receptors, may be responsible for their agonist-mediated sequestration and that sequestration and down-regulation of the receptor are dissociable phenomena. The lack of resestination in the sequestration-defective β2-adrenergic receptor mutant strongly suggests that the sequestration pathway is an important mechanism by which cells re-establish the normal responsiveness of G protein-coupled receptors following the removal of agonist.

Closely related members of the large superfamily of G protein-coupled receptors exhibit high degrees of amino acid conservation, especially in regions implicated in ligand binding and interactions with G proteins. Other residues whose roles have remained obscure, are also conserved across virtually all members of this superfamily (1, 2). One such residue, tyrosine 326 of the prototypical human β2-adrenergic receptor (β2AR),1 is present at the proposed junction of the seventh transmembrane domain and proximal part of the carboxyl terminus of virtually all G protein-coupled receptors. Aromatic residues in various sequence motifs, NPXY or YXXΦ, have been implicated in the cellular recycling of receptors for the proteins low density lipoprotein, epidermal growth factor, and insulin (3, 4). It has been assumed that these motifs direct the plasma membrane receptors to clathrin-coated pits. Recently, it has been demonstrated that the epidermal growth factor receptor interacts with the amino-terminal domain of α-adaptin, a component of the endocytic machinery of clathrin-coated pits, in response to the epidermal growth factor activation (5).

G protein-coupled receptors are known to undergo cellular processing, especially in association with the phenomenon of desensitization in response to prolonged agonist occupancy of the receptor. Several temporally distinguishable cellular events have been characterized for β2AR desensitization (6). Sequestration is observable within minutes of agonist occupancy. It is manifested by a loss of cellular binding sites for hydrophilic ligands (plasma membrane receptors) but not hydrophobic ligands (total cellular receptors). Sequestration quickly follows the more rapid (seconds to minutes) uncoupling of the receptor from its ability to activate the G protein. Whereas the rapid uncoupling process is due to phosphorylation of the β2AR by cAMP-dependent protein kinase and specific receptor kinases (β-adrenergic receptor kinase), receptor phosphorylation is not required for sequestration; conversely, sequestration is not required for the onset of rapid desensitization (7, 8). Prolonged (>1 h) exposure of cells to agonist results in the progressive loss of total cellular receptors in a process termed down-regulation. It has been proposed that β2AR sequestration may be an obligatory process that precedes down-regulation (9, 10). In the present study we sought to evaluate the potential role of the tyrosine 326 residue in these processes. This tyrosine residue, which exists in the sequence NPLY in the β2AR or a closely related sequence in other receptors, is conserved in the majority of the G protein-coupled receptors so far characterized (Fig. 1).

**EXPERIMENTAL PROCEDURES**

*125I*-Cyanopindolol and *125I*-pindolol were purchased from DuPont NEN. Protein G, propranolol, and isoproterenol were obtained from Sigma. Gpp(NH)p and GTPγS were from Boehringer Mannheim. Microcystin-LR was purchased from Calbiochem. 12CA5 ascites were purchased from Babco.

**Plasmid Constructions**

Mutant and epitope-labeled cDNA were modified by site-directed mutagenesis using the polymerase chain reaction (11). A BgII/EcoRV cassette of pBC-NAR (12) was used to create the Tyr326→Ala mutation and was re-ligated into the 12CA5 epitope-tagged construct (13, 14).
This construct was then blunt end-ligated at the SacI and SalI sites into the HindIII polyclinker site of the neomycin resistance expression plasmid pRC-CMV (Invitrogen). Constructs were verified by dyeoxy sequencing analysis.

**Cell Culture and Transfection**

Chinese hamster ovary (CHO) cells were obtained from ATCC. Human epitope-labeled βA-R in pBC or mutant βA-R cDNA in pRC CMV plasmids were transfected, respectively, with or without pSIV-2neo (12) into CHO cells using electroporation with calcium phosphate. Clones were selected in the presence of 1 mg/ml G418 (Life Technologies, Inc.). Colonies originating from single cells were subcloned and evaluated for receptor expression and homogeneity using 125I-pindolol binding and immunofluorescence. Cells were maintained following clonal selection in Ham’s F-12 with 10% fetal bovine serum, 50 μg/ml penicillin/streptomycin (50 units), and 500 μg/ml Geneticin® (Life Technologies, Inc.). Experiments reported in this paper were performed on at least two different clonal cell lines for each of the mutant and wild type receptors. Expression levels varied between 0.5-0.7 pmol of receptor/mg of membrane protein for the cells expressing the mutant receptor and 0.6-1.0 pmol of receptor/mg of membrane protein for the wild type receptors. This level of receptor expression corresponds to ~2,500-5,000 receptors/cell.

**Binding Studies**

Binding assays using 125I-cyanopindolol were performed as previously described (12). Levels of functionally expressed receptors were assessed in two ways. First, total cellular receptor expression was assayed by ligand binding with 125I-pindolol which, because of its hydrophobicity, can measure both surface receptors and intracellular receptors (12). Second, relative fluorescence quantitation using flow cytometry with the 12CA5 antibody was performed on the same aliquots of cells on which 125I-pindolol binding was performed. In these experiments a constant and equal ratio of the 125I-pindolol binding/relative surface fluorescence (0.94 ± 0.12; n = 3) was obtained for both mutant and wild type receptor-expressing cells. This suggests that both cell types express proportionally equivalent amounts of functional surface receptor and similar amounts of total receptors. In addition, total cellular wild type and mutant receptors were immunoprecipitated using the monoclonal antibody 12CA5 (Baeco) and revealed by Western blotting experiments using an antibody raised against the carboxyl tail of human βA-R. The results show roughly equivalent amounts of the same molecular weight species of wild type and mutant receptors (data not shown).

**Sequestration and Down-regulation**

**Radioligand Binding**—Whole cell radioligand binding was performed with 125I-pindolol for 3 hours at 12–13 °C in the presence or absence of either 100–600 μg CGP 12177 or 200 μM propranolol essentially as described (8). Cells were treated with isoproterenol either in suspension or while still attached to flasks. If first detached using phosphate-buffered saline (PBS) with 5 μM EDTA, the resulting antibody fractions were labeled, following treatment with isoproterenol, at 4 °C. Antibody labeling was terminated by 1 M Tris buffer, pH 8.5. The resulting antibody fractions were dialyzed against PBS and stored for use at 1.57 mg/ml. Live cell suspensions were labeled, following treatment with isoproterenol, at 4 °C for 40–60 min with 1:100 dilutions of antibody in Ham’s F-12 medium, washed with cold Dulbecco’s PBS by centrifugation and subsequently labeled with a 1:100 dilution of affinity-purified, Fc-specific, fluorescein-labeled goat anti-mouse antibody (Sigma). Cells were washed and fixed in 3.6% formaldehyde and analyzed within 1 h on a Becton-Dickinson flow cytometer. Baseline cell fluorescence intensity was determined with washed unlabeled cells, and cells incubated only with the fluorescein-labeled goat anti-mouse antibodies.

**Photography**—Live cells on glass coverslips were pretreated in the absence or presence of 10 μM isoproterenol for 30 min in Ham’s F-12 medium at 37 °C, chilled to 4 °C, exposed sequentially for 40 min (with an intervening wash) to 1:100 dilutions of 12CA5 mouse monoclonal antibody (1.75 mg/ml) and affinity-purified goat anti-mouse Fc-specific antibody (4 mg/ml), washed, and fixed in 4% formaldehyde in phosphate-buffered saline for 10 min at 4 °C. Photographic exposures of coverslips were done under identical conditions, and fluorescence intensities converted to pseudocolor images over a dynamic range of 1–256 following background subtraction. In order to facilitate the detection of the expressed receptors by means other than ligand binding, both wild type βA-R and mutant βA-R were produced by site-directed mutagenesis in CHO cells expressing either wild type or mutant βA-R were grown to confluence in T-75-cm flasks. Prior to experiments, cells were put in serum-free Ham’s F-12 medium for 10–24 h. Cells expressing wild type or mutant βA-R were incubated with 0.1 mM ascorbic acid and 10 μM isoproterenol for 20 min to induce desensitization. At the end of the incubation, the medium was removed and cells were washed extensively with cold PBS. Cells that were to be assessed for desensitization were washed twice with warm PBS (~30 ml) to remove the agonist and allowed to continue incubation in fresh serum-free medium for 20 min as previously described (7). The resensitization period was terminated by washing with cold PBS. Cells were scrapped into a lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 1 mM microcin-LR) and centrifuged at 40,000 x g for 25 min at 4 °C. The pellets were resuspended in cold 75 mM Tris (pH 7.4), 2 mM EDTA, and 12.5 mM MgCl2 using a Teflon pestle. Membrane preparations were assayed for agonist-stimulated adenyl cyclase as previously described (12, 15). Data treatment was done as previously reported (15).

**RESULTS AND DISCUSSION**

A mutant βA-R AR were produced by site-directed mutagenesis by replacing tyrosine 326 in the sequence NPLIY with an alanine residue to yield the construct βA-R-Y326A. In order to facilitate the detection of the expressed receptors by means other than ligand binding, both wild type βA-R and mutant βA-R were epitope-tagged on the amino terminus using the 12CA5 epitope as previously described (Fig. 1) (13, 14, 16). As shown in Fig. 2, in CHO cells expressing comparable numbers of βA-R at the cell surface, a dramatic difference was observed in the ability of these two receptors to sequester in response to agonist. Exposure...
FIG. 2. Time course of agonist-mediated sequestration of wild type and mutant β2-AR. CHO cells expressing wild type and mutant β2-AR were incubated with 10 μM isoproterenol (Iso) for the indicated times. A, sequestration of wild type and mutant β2-AR on CHO cells exposed to isoproterenol as visualized by immunofluorescence imaging. Red corresponds to regions of highest receptor density decreasing progressively to yellow, green, blue, and black (no receptors). Relative fluorescence intensities were as follows: wild type with no isoproterenol pretreatment = 1, wild type with isoproterenol pretreatment = 0.16, mutant without and with isoproterenol pretreatment = 1 and 0.97, respectively. Photographs were taken at the same magnification. Apparent differences in cell size relate to the fields chosen. B, sequestration of wild type and mutant β2-AR in CHO cells exposed to isoproterenol as assessed by receptor binding. Total receptor number and cell surface receptor number were assessed with 125I-pindolol and CGP 12177 in the upper panel (7) and surface receptors were measured on a Becton Dickinson flow cytometer using the monoclonal antibody 12CA5 in the lower panel. Results are expressed as the mean ± S.E. of 5–7 (top panel) and 3–4 (bottom panel) experiments. C, time course of agonist-mediated down-regulation of wild type and mutant β2-AR. CHO cells expressing wild type and mutant β2-AR were incubated with 10 μM isoproterenol for the indicated times. Total receptor population was assessed in whole cells with 125I-pindolol (top panel). Surface receptors were assessed by flow cytometry (bottom panel) using 12CA5 antibody directed against the engineered epitope tag at the NH2 terminus of the receptor. In these experiments, the first time point examined was at three h following agonist exposure. Results are expressed as mean ± S.E. of 3–9 (top panel) and 3–5 (bottom panel) experiments.

**A** No Isoproterenol Isoproterenol

**Wild Type**

**Y326 - A Mutant**

**B** Radioligand Binding

**C** Radioligand Binding

**Fluorescence**

**Fluorescence**

**Time (Minutes)**

**Time (hours)**

sure of cells to 10 μM isoproterenol for up to 90 min produced a rapid internalization or sequestration of 30–50% of the cell surface wild type β2-AR, while sequestration was totally absent in cells expressing the mutant receptor. Essentially the same lack of sequestration was observable whether surface receptors were assessed by ligand binding using the hydrophilic ligand CGP 12177 or by flow cytometry using the monoclonal antibody 12CA5 (Fig. 2B). As shown in Fig. 2A, the lack of agonist-mediated sequestration with the mutant receptor is observable directly by fluorescence microscopy. Cells expressing the mutant receptor do not lose cell surface fluorescence following agonist treatment. In contrast to the effect of the mutation on sequestration, both wild type and mutant β2-AR were similarly and efficiently down-regulated upon prolonged exposure (up to 24 h) to the agonist isoproterenol (Fig. 2C). These results strongly suggest a role for this region of the receptor in the process of sequestration and demonstrate that sequestration and down-regulation are dissociable phenomena.

In order to assess whether abolition of receptor sequestration was due to the specific mutation of the aromatic residue in this sequence motif or indirectly due to a major impairment of other essential receptor functions, we determined whether any of the signal transduction properties of the mutated receptor were significantly altered. As shown in Fig. 3, the β2-AR-Y326A mutant can interact with a G protein. In agonist competition curves for labeled antagonist (125I-cyanopindolol) binding in membranes, both wild type and mutant receptors display biphasic competition curves that could be shifted to monophasic curves in the presence of the guanine nucleotide Gpp(NH)p. This is the hallmark of receptor/G protein interaction. However, whereas the KD values derived for the agonist high (KDH) and low affinity states (KDL) were not statistically different between the wild type and mutant receptors (see legend to Fig. 3), the proportions of each receptor exhibiting high affinity for the
agonist isoproterenol were different with 37 and 15%, respectively, for the wild type and mutant receptors. These results indicate that the mutant receptor may have a reduced ability to couple to the G protein.

The mutant β2AR-Y326A was as effective as the wild type β2AR in mediating maximal agonist stimulation of adenylyl cyclase. A 5–7-fold stimulation of the enzyme activity was observed at maximal isoproterenol concentrations (wild type: basal = 28 ± 8 pmol/mg min; maximal activation = 129 ± 24 pmol/mg min; β2AR-Y326A mutant: basal = 18 ± 3 pmol/mg min; maximal activation = 124 ± 17 pmol/mg min; mean ± S.E., n = 8). However, as might be expected from the lower proportion (fraction) of receptors existing in a high affinity state (Fig. 3), the agonist isoproterenol was less potent in stimulating adenylyl cyclase via the mutant versus the wild type receptor (EC50 = 27 ± 3 nM for wild type β2AR; EC50 = 267 ± 33 nM for β2AR-Y326A; mean ± S.E., n = 8) (Fig. 4). Although functionally coupled, the mutant receptor appears to be less well coupled than the wild type receptor. However, it is unlikely that this impairment in coupling is responsible for the complete absence of sequestration of the mutant receptor. Indeed, previous studies from our laboratories (17, 19) have established that β2AR that are partially or completely uncoupled from G proteins and adenylyl cyclase stimulation as a result of other mutations are nonetheless totally normal in their agonist-mediated sequestration patterns.
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cyclase and a decrease in the maximal response. Moreover, following a 20-min exposure to 10 μM isoproterenol, we have demonstrated agonist-promoted phosphorylation of both wild type and Y236A mutant receptors (data not shown).

Whereas the onset of desensitization does not appear to involve sequestration, other evidence has previously suggested that sequestration might be more important to the process of resensitization of the adenyl cyclase response (15, 20). To test this possibility, we examined the ability of both wild type and mutant receptor-expressing β2AR cells to resensitize following removal of the desensitizing agonist. Fig. 4 reveals a dramatic difference in the behavior of the two receptors. Whereas the wild type β2AR was resensitized 20 min following removal of the agonist, over the same period of time no detectable recovery of responsiveness was observed with the β2AR-Y236A mutant. These data strongly suggest an involvement of sequestration in the process of resensitization of the β-adrenergic receptor-coupled adenyl cyclase response. Our results are also in line with recent observations of Yu et al. (21), who showed that, by selectively inhibiting sequestration of the β2AR in CHO cells or creating a sequestration-defective β2AR mutant by site-directed mutagenesis of serine and threonine residues in the C terminal tail of the receptor, they could essentially block resensitization of responsiveness of adenyl cyclase.

In this study we show that the presence of the tyrosine residue in the highly conserved sequence motif NPXXY in the β2AR appears to be required for agonist-mediated sequestration of the receptor. Alteration of this sequence by mutagenesis appears to interfere specifically with the process of sequestration since the mutated receptor was able to interact effectively with G proteins and stimulate adenyl cyclase. Although slight differences in these activation parameters were observed with the mutated receptor, the magnitude of these changes could not explain the total lack of sequestration of the β2AR.

The nature of the cellular compartment in which G protein-coupled receptors may sequester has not been extensively characterized. Recently, however, von Zastrow and Kobilka (13) have shown that β2AR internalized in response to agonist can be identified in the same endosomes as constitutively recycling transferrin receptors. Constitutively recycling receptors such as the transferrin and low density lipoprotein receptors contain tyrosine residues in motifs (YXRFX NPXY) that have been proposed to form tight turn conformations recognized by the endocytotic machinery (21, 22). By contrast, the secondary structural environment of the conserved tyrosine residue in G protein-coupled receptors (mutated in this study) most likely differs from this paradigm, since this tyrosine residue appears consistently in a motif in which it is 2 or 3 residues removed from conserved Asn and Pro residues (NPX-Y). Moreover, whereas in constitutively recycling receptors the tyrosine-containing motifs are usually 15–20 residues removed from the transmembrane domains (23), the conserved tyrosine residue in G protein-coupled receptors is in close proximity to the seventh transmembrane domain of these receptors. The structural differences between the internalization motifs of constitutively recycling receptors and the tyrosine-containing sequences of G protein-coupled receptors might suggest distinct internalization mechanisms for these receptors; it is interesting, therefore, to speculate that such differences might underlie the constitutive or agonist-mediated character of the internalization phenomena.

The highly conserved nature of the sequence surrounding the conserved tyrosine residue in a large number of the G protein-coupled receptors suggests that it may serve as a "sequestration signal" in all these receptors. The prevalence of this conserved sequence also raises the possibility that sequestration may be a common mechanism of G protein-coupled receptor trafficking in response to agonist stimulation. However, mutations in other regions of the receptor, specifically Ser, Thr, and Gin residues in the proximal half of the carboxyl terminus of the β2AR, also lead to a loss of agonist-mediated sequestration (15). In addition, related receptors, all of which possess the tyrosine residue in the conserved sequence motif, have been found to sequester to various degrees as exemplified by the various α2AR subtypes (24, 25) and the EP1a and EP2a prostaglandin E receptor subtypes (26). Altogether, these results suggest that while the motif is apparently necessary for sequestration, it may not be sufficient. Illustratively, the human β2AR, which contains the tyrosine residue in the conserved motif, fails to sequester. However, when a chimeric construct of the β2 receptor with the C terminal tail of the β2AR is expressed in cells, the resulting chimeric receptor undergoes agonist-mediated sequestration (14).

The results observed with the sequestration-defective β2AR-Y236A mutant point to a specific role of sequestration in the overall process of desensitization. Originally, removal of surface receptors through the process of sequestration was thought to play a major role in desensitization. As shown in this work (Fig. 4) and in other work using selective inhibitors of sequestration (12, 19), cells can desensitize normally without receptor sequestration. The lack of desensitization in the sequestration-defective β2AR-Y236A mutant strongly suggests that the sequestration pathway is an important mechanism by which cells reestablish the normal responsiveness of G protein-coupled receptors following removal of the stimulus. Since short term desensitization to agonist is mediated by phosphorylation of the receptor, it is interesting to speculate that the compartment where receptors are sequestered may be associated with dephosphorylation of receptor (15, 20). Our data are consistent with the proposal of Sibley et al. (20), who have shown that sequestered receptors in isolated vesicles are less phosphorylated than desensitized receptors still in the plasma membrane. Moreover, these isolated vesicles containing sequestered receptors show higher receptor phosphatase activity than do plasma membranes (20). Thus, rather than representing an important element in the onset of desensitization, sequestration may promote resensitization by transporting uncoupled receptors from the plasma membrane to a compartment in which they can be dephosphorylated and from which they can then recycle back to the cell surface as signaling-competent receptors.

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