The Conserved Seven-Transmembrane Sequence $NP(X)_{2,3}Y$ of the G-Protein-Coupled Receptor Superfamily Regulates Multiple Properties of the β_2-Adrenergic Receptor†

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ABSTRACT: The β_2-adrenergic receptor (β_2AR) is a member of a large superfamily of seven transmembrane domain, G-protein-coupled receptors. Within the putative seventh transmembrane domain of the β_2AR is a sequence of amino acids, NPLIY, which is conserved with minor variations in all members of the superfamily. Previously it was demonstrated that mutation of tyrosine residue 326 to an alanine abolished agonist promoted sequestration of this mutant without affecting its ability to maximally stimulate adenylyl cyclase in membranes [Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., & Caron, M. J. (1994) *J. Biol. Chem.* **269**, 2790−2795]. In the present study we characterized the NPLIY amino acid sequence in an attempt to determine how it can affect the agonist-mediated sequestration of the β_2AR and to test whether it is a functional motif. We find that point mutations of the most conserved amino acids, N, P, and Y, in this sequence affect several other receptor properties in addition to sequestration. Mutation of asparagine 322 to an alanine resulted in complete uncoupling of the receptor, loss of high-affinity agonist binding, and abolition of receptor sequestration, down-regulation, and phosphorylation. In contrast, a conservative mutation of this residue to an aspartic acid (as found in the thrombin receptor) resulted in an improvement of G-protein coupling without adversely affecting other receptor properties. Substitution of proline residue 323 with an alanine residue resulted in a receptor with mild deficits in sequestration and coupling, a reduced agonist-mediated phosphorylation, and no change in down-regulation. A mutant receptor with tyrosine residue 326 changed to a phenylalanine sequestered at 25% the rate of wild type receptor and was also phosphorylated less well than wild type receptor in response to agonist. In contrast, the alanine mutant of tyrosine 326 does not sequester and is weakly phosphorylated in response to agonist, and it activates adenylyl cyclase less well than wild type receptor in whole-cell determinations. An insertion mutant, $^{325}IY \rightarrow {}^{325}IAY$, was inactive. These data suggest that the NPLIY sequence of the β_2AR functions as a motif that may represent a critical determinant for maintaining the normal conformation of the receptor but does not function as a specific sequestration recognition motif.

G-protein-coupled receptors have many common biophysical and biochemical properties (Birnbaumer et al., 1990; O'Dowd et al., 1991; Oliveira et al., 1994), which are probably consequences of conserved regions or motifs found in their primary amino acid structures (Probst et al., 1992). Several studies have identified regions among them that regulate the processes of ligand binding, G-protein coupling, and phosphorylation. The specific molecular determinants for some other processes that require agonist occupancy, such as sequestration and down-regulation have not yet been fully elucidated.

The β_2AR participates in a number of different processes which follow its exposure to agonist. These include high-affinity agonist binding, receptor−G-protein coupling, and coupling to adenylyl cyclase; receptor uncoupling (desensitization) due to receptor phosphorylation and β-arrestin binding; agonist-promoted sequestration (internalization); and down-regulation in response to continued exposure to agonist (Perkins et al., 1991; Pulvermuller et al., 1993; Lohse et al., 1990; Hausdorff et al., 1990a; Schwinn et al., 1992; Campbell et al., 1994; Mahan et al., 1985; Valiquette et al., 1990). The time frames over which these processes occur vary from milliseconds to hours, and this leads to the hypothesis that to some extent each process can be regulated independently of the others. Previous work with β_2AR mutants support this idea in that specific mutations can affect different parameters of receptor function. For example, removal of phosphorylation sites or mutations that uncouple the receptor do not necessarily prevent sequestration (Campbell et al., 1994; Cheung et al., 1988; Hausdorff et al., 1994). In addition, receptor down-regulation can occur independently of sequestration (Barak et al., 1994). However, there does appear to be some interdependence of these processes as β_2-AR mutants have been made that neither couple, sequester,

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[1] Abbreviations: β_2AR, β2-adrenergic receptor; Gpp(NHp), guanyl-5′-yl imidodiphosphate; CHO, Chinese hamster ovary; CHW, Chinese hamster fibroblast; PBS, phosphate-buffered saline; PKA, cAMP dependent protein kinase; βARK, β-adrenergic receptor kinase; IBMX, 1-methyl-3-isobutylxanthine.

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nor down-regulate (Mahan et al., 1985; Cheung et al., 1988; Hausdorff et al., 1994). This suggests that there are critical regions in the receptor that either direct or influence two or more receptor functions.

Among the most conserved regions of G-protein-coupled receptors is a sequence of amino acid residues, $NP(X)_{2,3}Y$, which is found toward the cytoplasmic face of the putative seventh transmembrane region. Almost without exception, the asparagine, proline, and tyrosine residues of this sequence are conserved (Probst et al., 1992). The inner two residues are generally hydrophobic in nature (leucine, isoleucine, valine), while the residues that flank the tyrosine and asparagine are variable. Previously we demonstrated that mutation of tyrosine residue 326 to an alanine resulted in a sequestration deficient β_2AR mutant ($Y^{326}A$-β_2AR), suggesting that this sequence may be involved in the regulation of receptor internalization. In this work we characterize the motif by making point mutations at all the conserved residues of the β_2AR NPLIY sequence. In doing so we have found that the NPLIY sequence, in addition to influencing agonist-promoted sequestration, affects other fundamental properties of the receptor such as high-affinity binding, receptor G-protein-coupling, and phosphorylation. This suggests that the $NP(X)_{2,3}Y$ sequence represents a critical determinant for maintaining the normal conformation of the β_2AR and mediating changes in that conformation in response to agonist. Thus, changes in the functional properties of receptor mutants, such as sequestration, could result from perturbations in the conformational state of the receptor.

EXPERIMENTAL PROCEDURES

Materials. [^{125}I]Pindolol and [^{3}H]adenine were purchased from DuPont NEN. Propranolol, isoproterenol, Gpp(NH)p, protein A, and fluorescein-labeled goat anti-mouse Fc specific antibody were obtained from Sigma Chemicals (St. Louis, MO). Cell culture media, antibiotics, and physiological buffers were purchased from Gibco-Life Technologies Inc. 12CA5 antibody was obtained from Babco. CGP12177 was purchased from RPI. Restriction enzymes were obtained from Promega and Hot Tub DNA polymerase from Amersham.

Plasmid Constructions. All mutant cDNA were created by site-directed mutagenesis using the polymerase chain reaction (Vallette et al., 1989) and a starting cassette of 12CA5 epitope-modified pBC-Nar cDNA as described previously (Barak et al., 1994; Hausdorff et al., 1990b). Mutations were confirmed by dideoxy sequencing analysis.

Cell Culture and Transfection. Human or mutant epitope-labeled β_2AR cDNA in the pBC plasmid were transfected with pSV-2neo (Hausdorff et al., 1990b) into CHO cells using coprecipitation with calcium phosphate. A 5−10 μg amount of β_2AR cDNA in the vector was resuspended in 450 μL of 18 MΩ water to which 50 μL of 2 M calcium chloride was added dropwise; 500 μL of 2× Hanks Balanced Salt Solution was added dropwise to this, and the solution added to 60%−80% confluent cells in 100 mm tissue culture dishes containing Ham's F-12 with 10 percent fetal bovine serum. Clones were selected in the presence of 0.5−1 mg of Geneticin/μL. A polyclonal mix of cells was evaluated for receptor expression by immunofluorescence using antibody against the 12CA5 epitope, and cells that fell into the 75−95 percentiles of expression using flow cytometric analysis were used for subcloning. Colonies originating from single cells were then subcloned and evaluated for homogeneity using immunofluorescence and [^{125}I]pindolol binding. Cells were maintained in Ham's F-12 with 10% fetal bovine serum as described in Barak et al. (1994).

Binding Studies and Adenylyl Cyclase Assay. Receptor binding assays were performed with [^{125}I]pindolol on membranes purified from CHO cell lines permanently expressing wild type or mutant β_2AR as previously described (Barak et al., 1994; Hausdorff et al., 1990b). Whole-cell adenylyl cyclase activity assays were performed as follows. CHO cells expressing wild type or mutant β_2AR and grown in six-well Falcon dishes at a density of 400 000 cells/well were treated overnight with 1−2 μCi of [^{3}H]adenine/mL/well in Ham's F-12 with 50 μg of penicillin/mL and 50 units of streptomycin/mL. The cells were then washed once with fresh media without serum and treated with varying concentrations of isoproterenol in media containing 10 mM HEPES, 1 mM IBMX, and 100 μM ascorbate at pH 7.4 for 30 min at 37 °C. The media was aspirated, and 1 mL of 4 °C stop solution containing 2.5% v/v perchloric acid, 0.1 mM cAMP, and 2 μCi [^{14}C]cAMP per 500 mL was added to each well for 20−30 min on ice. The cell lysate was added to tubes containing 100 μL of 4.2 M KOH and analyzed as previously described (Hausdorff et al., 1990b; Yu et al., 1993).

Sequestration and Down-Regulation. Radioligand binding was performed as in Barak et al. (1994). For immunofluorescence analysis of sequestration, cells were grown in 12-well Falcon dishes and used at a density of between 250 000−400 000 cells per well, with each well originally seeded with equal numbers of cells. Following aspiration and washing of each well with serum-free media, serum-free media with or without isoproterenol was added and the cells were incubated at 37 °C for the indicated times. The incubation was stopped by aspiration of the media and addition of ice-cold phosphate-buffered saline (PBS) to each well. The cells were washed 1 or 2 times with ice-cold PBS and then incubated with 28 μg of 12CA5 antibody/mL in Ham's F-12 at 4 °C for 40 min, washed twice with cold PBS, and then incubated with a 1:100 dilution of fluorescein-labeled goat anti-mouse antibody in Ham's F-12 for 40 min at 4 °C. The cells were then washed 3 or 4 times with PBS, gently scraped into 0.5−1 mL of PBS, and added to 0.25 mL of 8% formaldehyde in PBS. The cell suspension was gently resuspended and stored refrigerated in the dark until analyzed by flow cytometry (Barak et al., 1994; Murphy, 1990).

Whole-Cell Phosphorylation. CHO clones permanently expressing receptor and seeded in six-well dishes were labeled at 37 °C with a 0.75 mL solution of 66 μCi of [^{32}P]-orthophosphate/mL in phosphate-free DMEM for 60 min. The cells were then incubated for 15 min more with an additional 0.75 mL of phosphate-free DMEM containing 1 mM ascorbate ± 20 μM isoproterenol. The cells were washed with ice-cold PBS and scraped into 0.4 mL of RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of Pepstatin A/mL, pH 8.0). Cells were solubilized for 1 h at 4 °C and centrifuged twice at 436 000*g* for 15 min. The soluble fraction was precleared for 30−60 min with 100 μL of a 20% protein A suspension in RIPA

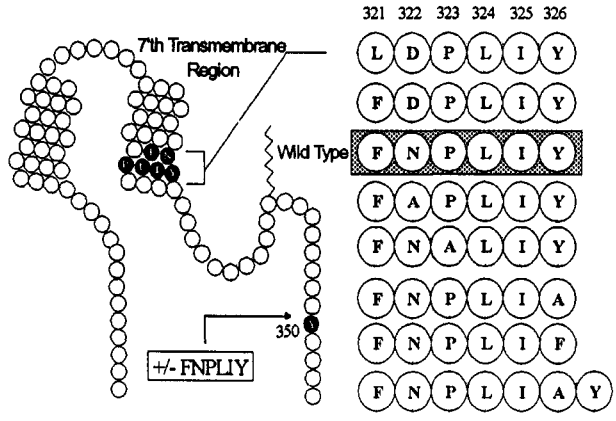

FIGURE 1: Seven-transmembrane region of the β_2AR showing the conserved NPLIY sequence along with mutations made in this area. An expanded view of the sixth and seventh transmembrane regions of the receptor is shown. The three most conserved residues of the NPLIY sequence are ^{322}N, ^{325}I, and ^{326}Y. The substitution N^{322}D corresponds to the sequence found in the thrombin receptor, whereas the insertion of ^{325}IA corresponds to a variant NPLIAY sequence of the endothelin receptor. The arrow located in the region of the cytoplasmic tail of the receptor shows the location where the FNPLIY site was inserted in an attempt to rescue the sequestration defective Y^{326}A mutant or to augment the sequestration of the wild type receptor.

buffer containing 2% (w/v) bovine serum albumin. Receptors in each sample were immunoprecipitated at 4 °C for 1−2 h with 8−12 μg of 12CA5 monoclonal antibody and 100 μL of a 20% suspension of protein A Sepharose. The immunoprecipitate was eluted from the Sepharose by heating the sample at 65 °C for 15 min in sample buffer containing 5% sodium dodecyl sulfate. The amount of sample buffer was adjusted to account for the amount of receptor and soluble protein in each sample. Protein amounts were determined using a Bio-Rad DC protein assay with bovine serum albumin as the standard. Proteins were resolved on 12.5% polyacrylamide gels. Phosphorylation levels were determined using a PhosphorImager (Molecular Dynamics). Phosphorylation levels in all experiments were normalized to the level obtained with the β_2AR.

RESULTS

Mutations. Figure 1 illustrates the different mutations that have been made to the ^{322}NPLIY sequence of the 12CA5 epitope-tagged β_2ARs used in the study (Barak et al., 1994; Kolodziej & Young, 1991; von Zastrow & Kobilka, 1992). In subsequent figures, the changes that occur in the signal transduction pathway of the β_2AR as a result of those mutations will be shown. This includes characterization of sequestration, binding, coupling, down-regulation, and phosphorylation.

Sequestration. The results from immunofluorescent studies are presented in Figure 2. The receptors could be classified into three groups on the basis of their behaviors. Group 1 includes those that sequestered normally. It contains in addition to the wild type receptor the N^{322}D-β_2AR as well as a mutant in which a second NPLIY sequence is inserted into its cytoplasmic tail at position 350. Group 2 includes those receptors that exhibited intermediate levels of sequestration, the P^{323}A and Y^{326}F mutants. Group 3 is formed by those receptors that did not sequester and includes the N^{322}A, I^{325}(IA), Y^{326}A, and its corresponding mutant in which an NPLIY sequence is inserted into the cytoplasmic tail at position 350. The placement of an NPLIY sequence in the cytoplasmic tail of the sequestration defective Y^{326}A-β_2AR mutant could not rescue its sequestration. A deletion mutant at position ^{325}I was also constructed but could not be characterized due to impaired expression.

Sequestration is a dynamic process and the sequestered fraction of receptors at equilibrium depends upon a ratio of internalization (k_f) and externalization (k_r) rate constants [$k_f/(k_r + k_f)$], and these constants can be derived from the sequestration kinetics. We determined the rates of sequestration of a subset of mutants representative of each group (Figure 2B). Analysis of the sequestration curves indicated that the reduced levels of sequestration observed at equilibrium for the group 2 mutants is the consequence of an impairment in their rate of internalization rather than in the rate at which they return to the cell surface. The wild type receptor and the N^{322}D (data not shown) have similar forward rate constants k_f and internalize with a $t_{1/2} = 1/k_f$ of 9−10 min. The group 2 P^{323}A internalized 60% slower with a $t_{1/2}$ of 16 min, while the $t_{1/2}$ of the Y^{326}F-β_2AR was 45 min. The externalization times ($1/k_r$) were 10, 10, and 16 min, respectively. In addition to impaired sequestration rates, group 2 mutants also had a decreased sequestration response to agonist (Figure 2C). The EC_{50} for the sequestration of the wild type β_2AR was 20 ± 10 nM, while the values for the P^{323}A and Y^{326}F were 170 ± 69 and 320 ± 140 nM, respectively.

Receptor−Ligand Binding. The decreased responsiveness of group 2 mutants to agonist stimulated sequestration suggested that they might be impaired in agonist binding or coupling to G-protein. Therefore, equilibrium binding experiments were performed to determine the dissociation constants for several of the mutant receptors (Figure 3B). The average K_D value for the binding of [^{125}I]pindolol to all the receptor clones was 32 ± 7 pM, and the K_D was not significantly different between any mutant receptor and that of the wild type. Agonist binding competition isotherms for the receptors are shown in Figure 3A, and the data are summarized in Table 1. The curves were fit to either a single- or two-site ligand binding model (Munson & Robard, 1980; DeLean et al., 1980). The isoproterenol competition curve for wild type β_2AR exhibits binding for both high- and low-affinity receptor populations. This binding could be shifted to a single low affinity state in the presence of the nonhydrolyzable GTP analogue Gpp(NH)p (Figure 3A,B), consistent with uncoupling from G-protein. Similar to the sequestration results, the mutant receptors fell into the same three groups as before when catagorized by their binding affinities. The first group of mutant receptors exhibited no change in their high- or low-affinity binding, except that the N^{322}D-β_2AR demonstrated a greater proportion of sites in the high-affinity state. Group 2 mutants had similar numbers of high-affinity sites compared to wild type receptor, but the affinity of these sites was reduced 5−6-fold (Table 1). The group 3 mutants N^{322}A and I^{325}(IA) exhibited no high-affinity binding. In the presence of Gpp(NH)p, Figure 3B, the agonist competition curves of all the mutants were consistent with a single low-affinity state, but the observed affinity constants of groups 2 and 3 mutants were mildly shifted 3−5-fold to the right from that observed for wild type receptor, suggesting that the motif is involved directly or indirectly in agonist binding.

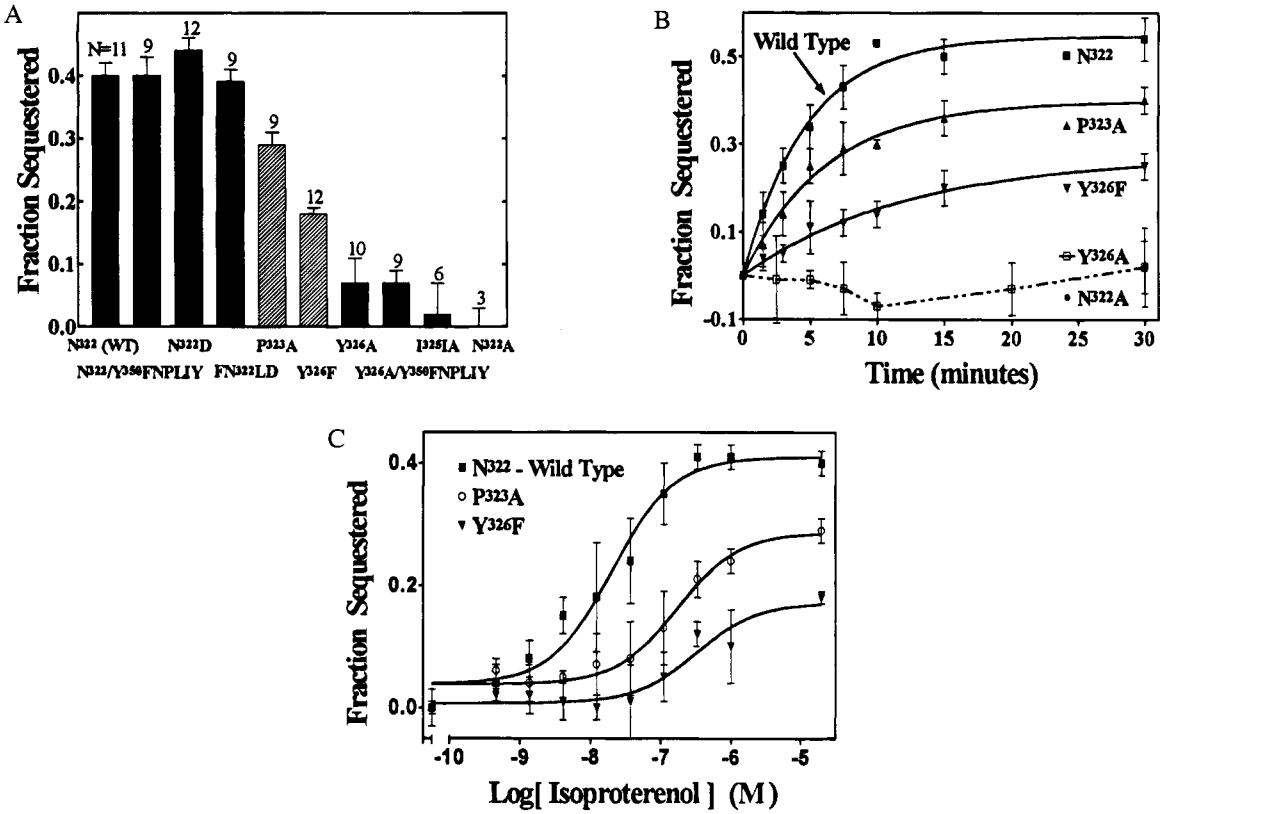

FIGURE 2: Sequestration properties of wild type and mutant β_2AR clones. A. CHO cells permanently transfected with the 12CA5, epitope-tagged β_2AR were exposed to either 0 or 20 μM isoproterenol for 30 min at 37 °C and then incubated with 12CA5 antibody followed by fluorescein-labeled secondary antibody. The number of receptors remaining in the plasma membrane of the cells was measured by flow cytometry. The fraction of sequestered receptors was calculated as 1 − (number of membrane receptors remaining after agonist treatment)/(number of membrane receptors present in matched untreated controls). B. Sequestration kinetics of mutant and wild type β_2AR as measured by flow cytometry. CHO cells containing β_2AR were exposed to 10 μM isoproterenol for the times indicated on the x-axis. The data were fitted to a single-exponential model defined by the differential equation $dR_f/dt = -k_f R_f + k_r R_i$ where R_T, R_f, and R_i represent total, surface, and sequestered receptor; $R_T = R_f + R_i$, and k_f and k_r represent the forward and reverse rate constants for internalization. The degree of sequestration is given by the fraction of sequestered receptor at 30 min, and the internalization rate is given by the slope of the curves at time $t = 0$. It is clear from the data that all the mutants sequester less rapidly (less steep slopes at $t = 0$) and to a smaller degree than the wild type receptor. The wild type receptor internalizes in approximately 10 min whereas the $P^{323}A$ receptor takes 16 min and the $Y^{326}F$ receptor takes 45 min. Neither the $Y^{326}A$ nor $N^{322}A$ receptors internalized. Curves are the averages ±SD of 3−5 separate experiments. C. The dependence of sequestration on agonist concentration was determined by measuring the steady state value of sequestered receptor in the cells following a 30 min exposure to isoproterenol. The concentration of agonist at which sequestration was half-maximal (EC_{50}) was shifted approximately 10-fold above the high-affinity binding constant for both wild type and mutant receptor. EC_{50} values were 20 ± 10 nM for wild type receptor, 170 ± 60 nM for the $P^{323}A$ mutant, and 320 ± 140 nM for the $Y^{326}F$ mutant. Results are the average ±SD of four experiments.

Table 1: Binding Data

clone	R_h	R_l	K_{dh}	K_{dl}	$K_{d+GppNHp}$	basal seq.
N^{322}-wild type	0.47 ± 0.06	0.53 ± 0.06	(2.5 ± 1.0) × 10^{-9}	(2.1 ± 0.5) × 10^{-7}	(1.4 ± 0.5) × 10^{-7}	0.10 ± 0.03
$N^{322}D$	0.68 ± 0.03	0.32 ± 0.03	(3.7 ± 0.9) × 10^{-9}	(2.9 ± 0.2) × 10^{-7}	(2.1 ± 0.2) × 10^{-7}	0.16 ± 0.05
$N^{322}A$	0	1.0		(7.2 ± 2.4) × 10^{-7}	(7.2 ± 2.4) × 10^{-7}	
$P^{323}A$	0.53 ± 0.03	0.47 ± 0.03	(12 ± 2) × 10^{-9}	(4.8 ± 0.4) × 10^{-7}	(7.2 ± 1.2) × 10^{-7}	0.06 ± 0.02
$Y^{326}F$	0.39 ± 0.04	0.61 ± 0.03	(15 ± 5) × 10^{-9}	(5.7 ± 0.6) × 10^{-7}	(6.6 ± 0.6) × 10^{-7}	0.06 ± 0.01
$I^{325}IA$	0	1.0		(3.7 ± 0.2) × 10^{-7}	(3.7 ± 0.2) × 10^{-7}	
N^{322}/Y^{350}FNPLIY	0.44 ± 0.05	0.56 ± 0.05	(2.0 ± 0.8) × 10^{-9}	(1.4 ± 0.2) × 10^{-7}		0.16 ± 0.05

Stimulation of Adenylyl Cyclase. Agonist binding studies (Figure 3, Table 1) demonstrated that the β_2AR could be uncoupled from G-protein depending upon the mutation made to the NPLIY sequence. Therefore, a subset of the mutants was investigated further by measuring their ability to stimulate whole-cell adenylyl cyclase, Figure 4. Basal cell cyclic AMP levels were unaffected by the mutations, and this included those clones which contained the $N^{322}D$. Isoproterenol was less potent at stimulating adenylyl cyclase in whole-cell preparations of the mutants $P^{323}A$, $Y^{326}A$, and $Y^{326}F$ than in wild type receptor. However the $N^{322}D$ and $P^{323}A$ could fully activate adenylyl cyclase, whereas the $Y^{326}A$ and $Y^{326}F$ only activated it to 60% of wild type levels. The $N^{322}A$ did not stimulate adenylyl cyclase activity at isoproterenol concentrations less than 10^{-6} M.

Down-Regulation. In response to long-term agonist exposure, wild type β_2AR is down-regulated (Lohse et al., 1990). We tested the mutant receptors in permanent clones of CHO cells (Figure 5) to see if they responded to agonist in a similar manner. Cells containing wild type or mutant receptor were exposed to 20 μM isoproterenol for 24 h, and the number of receptors remaining was assayed by ligand

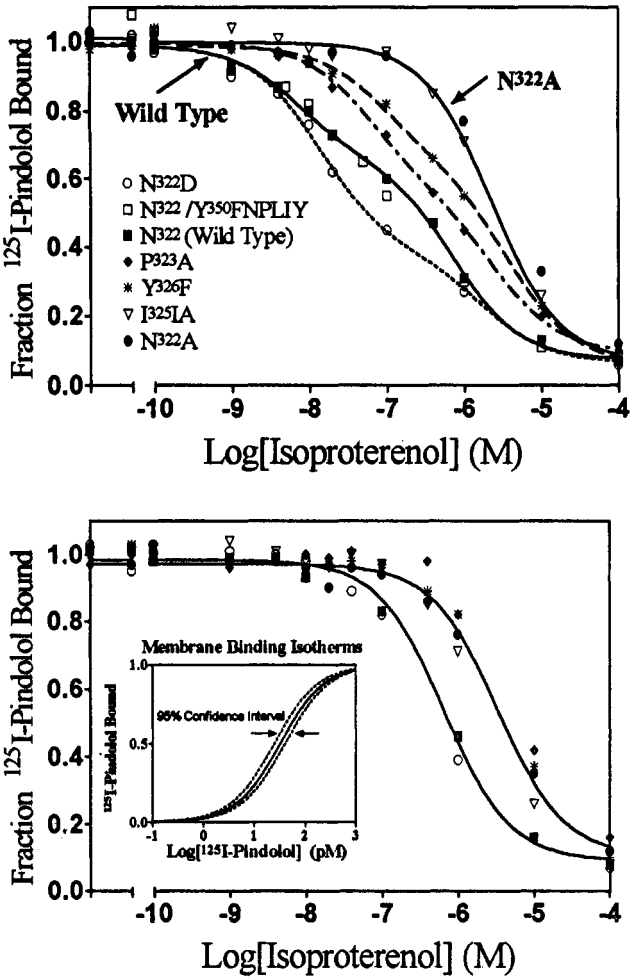

FIGURE 3: Comparison of the agonist binding to membranes containing mutated β_2AR receptors to membranes containing wild type β_2AR. A. Membranes containing receptor were exposed to increasing concentrations of the agonist isoproterenol (x-axis) in the presence of [^{125}I]pindolol, and the relative amount of [^{125}I]-pindolol remaining bound is plotted along the y-axis. A biphasic curve characteristic of a two-site binding model for the wild type receptor is plotted in the upper panel through the black squares (■). B. A decrease in receptor affinity for isoproterenol is also evident in the lower panel where only low-affinity binding occurs in the presence of Gpp(NH)p. Competitive binding data were fit with the computer program Ligand (Munson & Robard, 1980; DeLean et al., 1980), and each data set represents the average of multiple experiments ($N = 2-8$). Note the 95% confidence interval for antagonist binding isotherms of five NPLIY substitution mutants and wild type receptor (shown in the insert in the lower panel). The K_D for [^{125}I]pindolol binding for the clones were N^{322} (43 ± 18 pM), Y^{326}F (31 ± 12 pM), Y^{326}A (28 ± 18 pM), N^{322}A (22 ± 10 pM), P^{323}A (38 ± 22 pM), and N^{322}D (32 ± 11 pM). The means are not significantly different from that of the wild type with p values (two tailed student's t-test) of (0.28, 0.31, 0.07, 0.76, 0.31) respectively. Data were analyzed by the program Graphpad Prism and represent four separate experiments.

binding and compared to untreated controls. Approximately 50% of the total cellular complement of wild type receptors are down-regulated in response to agonist. A similar result was obtained for the N^{322}D , P^{323}A , Y^{326}A , and Y^{326}F mutants. The two mutant receptors, N^{322}A and I^{325}(IA), that exhibited no coupling or high-affinity binding were impaired in their ability to down-regulate.

Phosphorylation. Mutations made to residues within the NPLIY sequence affected agonist binding, coupling, and sequestration, events which occur both before and after receptor phosphorylation. Therefore, we tested if agonist

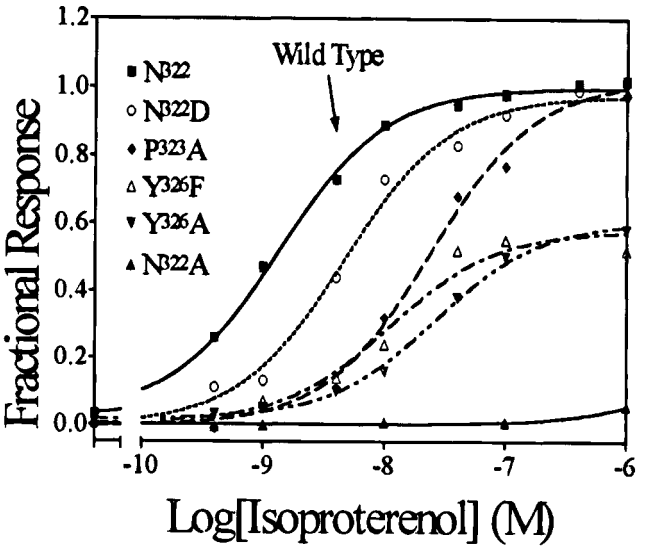

FIGURE 4: Whole-cell cyclase assay of permanently transfected CHO cells expressing mutant and wild type β_2AR. CHO cells were exposed to isoproterenol at concentrations between 0 and 1 μM for 30 min at 37 °C, and cAMP production was assayed as described. Results were normalized by the maximal response of the wild type expressing clone (■) at each concentration. Basal cyclase activity for the mutant receptor clones was the same as that of the wild type. The EC-50 and expression levels for the clones were as follows: N^{322} (1.4 ± 0.2 nM, 2.3 ± 0.2 pmol/mg), N^{322}D (6 ± 2 nM, 1.0 ± 0.4 pmol/mg), P323A (25 ± 5 nM, 1.4 ± 0.4 pmol/mg), Y^{326}F (11 ± 2 nM, 2 ● 0.5 pmol/mg), Y^{326}A (26 ± 4 nM, 0.9 ± 0.3 pmol/mg), N^{322}A (>4000 nM, 1.3 ± 0.2 pmol/mg). Curves represent the averages of two to four separate experiments.

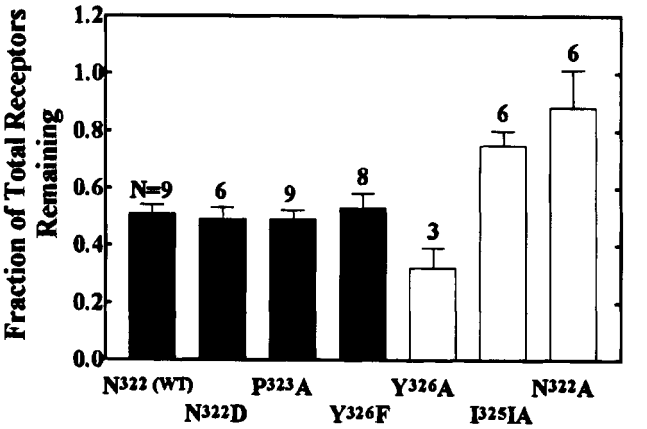

FIGURE 5: Down-regulation of wild type and mutant β_2AR in permanently transfected CHO cells. Clones containing wild type or mutant receptor were exposed to 0 or 20 μM isoproterenol for 24 h, and the amount of total cell β_2AR remaining after treatment was determined by [^{125}I]pindolol binding. Measurements were normalized by comparing them to untreated, matched controls. The extent of down-regulation is reflected by a decrease in the bar amplitude The N^{322}D, P^{323}A, Y^{326}F, and Y^{326}A clones (bars 2−5) down-regulate as least as well as the clone expressing wild type receptor (bar 1). The two apparently uncoupled receptors N^{322}A and I^{325}IA (bars 3, 7) also down-regulate to some extent. N equals the experimental population size used to determine each bar.

induced phosphorylation was also affected by mutations in this site. Figure 6 shows the extent of phosphorylation of a representative subset of mutants as compared to wild type receptor. Group 3, which comprised the nonsequestering mutants [Y^{326}A, I^{325}(IA), and N^{322}A], was phosphorylated in response to agonist to only 8%−21% of the level seen for the wild type receptor. However, the group 2 mutants P^{323}A and Y^{326}F were phosphorylated to 50% and 83%,

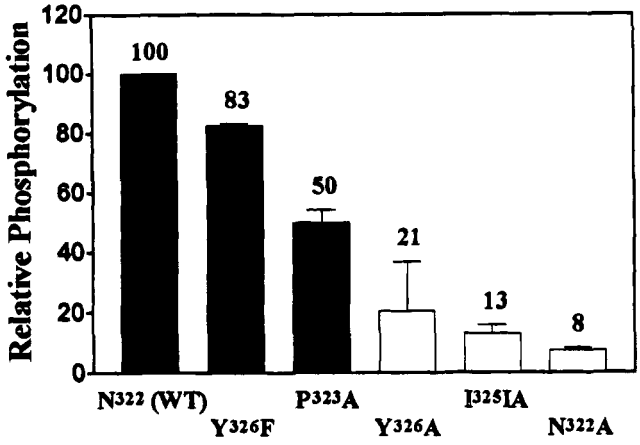

FIGURE 6: Whole-cell phosphorylation of wild type and mutant β_2AR in permanently transfected CHO fibroblasts. Cells were labeled with $^{32}PO_4$ and stimulated with or without 10 μM isoproterenol for 15 min at 37 °C. Receptors were immunoprecipitated and resolved on 12.5% PAGE. Agonist stimulated receptor phosphorylation was quantified for each receptor using a PhosphorImager. The data are the averages of three experiments and represent the net phosphorylation (agonist induced minus basal), and are presented as percentages compared to wild type receptor (100%).

respectively, of the level observed for the wild type receptors.

DISCUSSION

The amino acid sequence $NP(XX)_{2,3}Y$ recurs in the seventh transmembrane region throughout the superfamily of G-protein-coupled receptors. In this work we test the hypothesis, at least for the β_2AR, that the sequence behaves as a functional motif. We further characterize how the three conserved amino acids of this motif affect the regulation of processes associated with β_2AR function. We examined this by measuring the characteristics of mutant receptors in which these amino acids have been individually changed to alanine residues. These mutant receptors displayed changes in high- and low-affinity agonist binding, coupling to G-protein, their degree of phosphorylation, and their ability to sequester, to become down-regulated, and to activate adenylyl cyclase. These data suggest this sequence is a regulatory motif since the integrity of the sequence is crucial for normal β_2AR function. Moreover, the wide effects of the mutations in this motif suggest it plays a critical role in maintaining structural integrity of the β_2AR. We have also shown that insertion of copies of the motif in the β_2AR cytoplasmic tail does not enhance normal β_2AR sequestration nor does it permit rescue of the sequestration defect associated with the mutation in this sequence. This indicates that normal function of the motif depends upon its position in the receptor. Furthermore, the loss of function of an insertion mutant with an alanine placed proximal to tyrosine 326 indicates that the intramotif relationship among residues is a critical determinant governing receptor regulation, at least for the β_2AR.

We have previously shown that mutation of tyrosine 326 to alanine in the β_2AR blocks agonist mediated sequestration (Barak et al., 1994). One may then speculate that the NPLIY motif of the β_2AR may be a sequence recognized by the internalization machinery of the cell and that tyrosine 326 is critical for sequestration by analogy to the role that the tyrosine residues found in similar sequences play in growth factor receptor internalization (Trowbridge, 1991; Khan et al., 1989; Thies et al., 1989). It would appear from our data and studies on other G-protein-coupled receptors that the $NP-(X)_{2,3}Y$ motif is not specifically recognized as an internalization motif. This is a consequence of the following. As we mentioned above, the influence of the motif on the sequestration is position dependent. Placement of these residues in the cytoplasmic tail of the receptor does not affect sequestration. Second, mutations in this motif produce β_2-AR mutants that have unchanged affinity for antagonists, but are affected in their low-affinity interactions with agonists, suggesting significant changes in the conformational state of the receptor. Third, mutation of the motif in the β_2AR to the sequence found in the endothelin A receptor (NPLIAY) prevents normal sequestration rather than having no effect on it (Resnik et al., 1990; Marsault et al., 1993; Eguchi et al., 1993). Finally, studies of other G-protein-coupled receptors, including the gastrin-releasing peptide receptor (NPFALY motif), have shown that mutation of the tyrosine to an alanine does not affect the rate of agonist-mediated internalization (Slice et al., 1994).

Our data do indicate that whatever role tyrosine 326 plays in the internalization of the β_2AR, it is independent of tyrosine phosphorylation. Whereas mutation to alanine blocks receptor sequestration, the more conservative phenylalanine 326 mutation restores sequestered receptor levels to 50% of normal. Tyrosine differs from phenylalanine only in the aromatic hydroxyl group. This suggests that hydrogen bonding of this tyrosine may be important in maintaining a receptor conformation required for sequestration, or that it plays a role in the interaction of the receptor with another protein that directs sequestration. We did not study whether potential phosphorylation of this tyrosine may further facilitate β_2AR internalization. Tyrosine kinase phosphorylation has been shown to occur in the agonist-mediated internalization of non-G-protein-coupled receptors (Trowbridge, 1991) and may modulate the signal transduction pathway of G-protein-coupled receptors (Hausdorff et al., 1992). However, the extent to which tyrosine phosphorylation is involved in β_2AR regulation is unclear (Hadcock et al., 1991).

The processes in which these mutant receptors were defective, high- and low-affinity agonist binding, G-protein coupling, phosphorylation, ability to activate adenylyl cyclase, sequestration, and down-regulation, have previously been related to one another on a temporal basis in order to model receptor behavior. Some of these processes overlap in time so it may be more advantageous to relate the changes that occurred in the mutant receptors by categorizing the processes they undergo on a functional rather than temporal basis. Such a functional view of receptor behavior has recently been postulated in a limited manner by the modified ternary complex model (Samama et al., 1993), in which binding of receptor to agonist or G-protein only facilitates a certain subsequent behavior and absence of these interactions does not preclude it. In particular, in the absence of agonist the receptor (R) can form a state R* ($R \rightarrow R^*$) in which activation of adenylyl cyclase occurs. Thus the subsequent behavior (function) of the receptor is a consequence only of its actual state or conformation and does not depend on the manner in which that state was reached. Within the context of a more generalized functional model in which only the state of the receptor is important for determining further

behavior, the processes observed in these mutant receptors fall into two broad categories: (a) intrinsic processes (agonist binding, G-protein-coupling, and phosphorylation) in which the receptor directly participates in the interaction and the receptor remains accessible on the cell surface and (b) extrinsic processes (sequestration, down-regulation, and activation of adenylyl cyclase) in which the receptor is no longer accessible or does not directly participate in the interaction. In general, extrinsic processes are regulated by intrinsic ones.

From our experiments we cannot conclude or exclude that the NPLIY motif acts as a direct binding site for receptor associated proteins. Perhaps the motif influences the structural organization or accessibility of regions of the β_2AR that are important for G-protein coupling (the third intracellular loop) or ligand binding. The assumption that this motif is an important determinant of a normal receptor conformation by possibly interacting with other transmembrane or cytoplasmic regions offers a single explanation as to its effects on the intrinsic processes of agonist binding, G-protein coupling, and receptor phosphorylation.

It is clear from previous studies (Schwinn et al., 1992) and in agreement with our data that each of the above extrinsic processes should be affected in a negative manner as a result of decreased affinity of receptor for agonist or decreased coupling of receptor to G-protein. It is also known that phosphorylation, the third intrinsic process examined in this study, results in a diminished adenylyl cyclase response during homologous desensitization (Schwinn et al., 1992). Phosphorylation of the receptor apparently occurs after β-adrenergic receptor kinase (βARK) is targeted to the receptor by G-protein $\beta\gamma$ subunits (Inglese et al., 1992; Pitcher et al., 1992). If, as suggested, the mutant receptors interact abnormally with G-protein and consequently abnormally with βARK, could defective phosphorylation account for part of their inability to sequester? If so we should then observe that these mutant receptors are phorphorylated abnormally in response to agonist, which is just the case for group 2 and 3 mutants. It is also evident that the group 2 mutants with partially impaired sequestration are phosphorylated to a much greater extent than the group 3 mutants that do not sequester at all. Therefore phosphorylation of the β_2AR may be more intimately involved with sequestration than previously believed, and correction of the sequestration abnormalities of these mutants by enhancement of their phosphorylation would support this view (Tsuga et al., 1994; Ferguson et al., 1995).

It was thought that sequestration played a major role in receptor desensitization and uncoupling from effector (Hertel & Perkins, 1984). However, studies on receptors unable to internalize in response to agonist suggested that sequestration might play a more important role in receptor resensitization (Yu et al., 1993; Barak et al., 1994; Pippig et al., 1995). Our whole-cell adenylyl cyclase data support that idea, and indicate that under certain circumstances sequestration may significantly affect down stream events of receptor activities. Previously, we found that membrane preparations from the $Y^{326}A$ sequestration defective mutant maximally stimulated adenylyl cyclase as well as wild type receptor (Barak et al., 1994). However, this mutant receptor did not resensitize normally. In the present study we find that neither the $Y^{326}A$ nor the $Y^{326}F$-β_2AR stimulates adenylyl cyclase as well as wild type receptor in whole-cell determinations. This

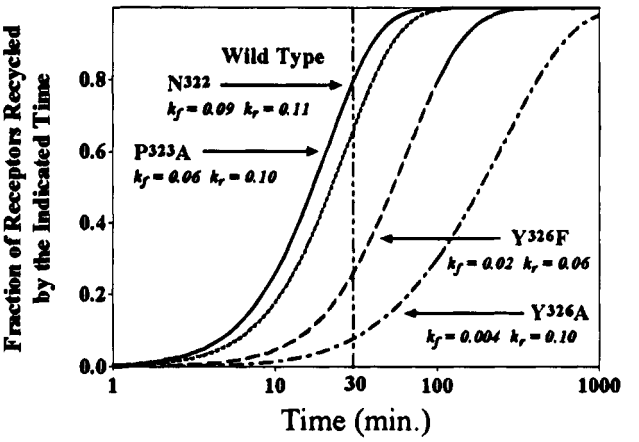

FIGURE 7: Relationship between the forward rate constant for sequestration and the probability that a receptor undergoes one round of internalization. The probability that a receptor will undergo at least one sequestration cycle by a given time (t) is plotted along the y-axis and is given by

$$P(t) = 1 - \frac{1}{(k_f - k_i)}[k_f e^{-k_i t} - k_i e^{-k_f t}]$$

where k_f is the forward rate constant for sequestration and k_i is the externalization rate constant This relationship can be derived in a straightforward manner by using a three-compartment model by considering the probability, $k\,\delta t$; that a receptor leaves an individual compartment during the infinitesimal time δt, and using this relationship to calculate its probability, $e^{-k\Delta t}$, of not arriving in the next compartment after a time interval Δt. If receptors are resensitized during each sequestration cycle, then the influence of the forward rate constants on resensitization of mutant receptor is shown by the rightward shift of the curves. Whereas the wild type clones would resensitize nearly 80% of receptors in 30 min (vertical line), the ^{326}F clones would only resensitize approximately 20% of the receptors. This suggests that sequestration-impaired clones should resensitize receptor less well than matched wild type clones, and this might result in decreased adenylyl cyclase activity.

difference is not adequately explained by differences in receptor expression, since the $Y^{326}F$-β_2AR and wild type receptor were expressed equally well. Instead, the differences in cyclase activity that were observed might be better explained by their impaired sequestration rates. Figure 7 illustrates a model that offers an explanation as to how an impairment in sequestration kinetics might influence whole-cell adenylyl cyclase activity. The model gives the probability that an agonist-exposed receptor, with a given internalization rate, will have traversed the sequestration pathway and returned to the cell surface by the indicated time. As an example, after 30 min have elapsed from the time of agonist stimulation more than 80% of wild-type receptors would be expected to have been resensitized and returned to the cell surface, whereas this number drops to less than 20% in the $Y^{326}F$ and less than 10% in the $Y^{326}A$ mutant population. In particular, the majority of the $Y^{326}A$ and $Y^{326}F$-β_2AR remain trapped and desensitized at or below the cell surface, whereas recycling of the wild type receptor results in a continuous supply of receptors that can couple to adenylyl cyclase. This scheme suggests that agonist promoted sequestration might play a more dynamic role in regulating G-protein-coupled receptor signaling than previously thought, especially in cells that may differ in the kinetic properties of the receptor recycling process.

In summary, the present work has examined the NPLIY amino acid sequence of the β_2AR. We find this sequence of amino acids functions as a motif that affects multiple

properties of the receptor, and suggest that a primary function of this motif may be in the maintenance of a receptor conformation that allows normal interaction with both agonist and G-protein. In addition we provide a model that describes one role receptor internalization and resensitization may play in regulating G-protein-coupled receptor signaling.

REFERENCES

Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., & Caron, M. G. (1994) *J. Biol. Chem. 269*, 2790.

Birnbaumer, L., Abramowitz, J., & Brown, A. M. (1990) *Biochim. Biophys. Acta 1031*, 163.

Campbell, P. T., Hnatowich, M., O'Dowd, B., Caron, M. G., Lefkowitz, R. J., & Hausdorff, W. P. (1994) *Mol. Pharmacol. 39*, 192.

Cheung, A. H., Sigal, I. S., Dixon, R. A. F., & Strader, C. D. (1988) *Mol. Pharmacol. 34*, 132.

DeLean, A., Munson, P. J., & Robard, D. (1980) *Am. J. Physiol. 235*, E97.

Eguchi, S., Hirata, Y., Imai, T., & Marumo, F. (1993) *Endocrinology 132*, 524.

Ferguson, S. S. G., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A. M., & Caron, M. (1995) *J. Biol. Chem.* (in press).

Hadcock, J. R., Port, J. D., & Malbon, C. C. (1991) *J. Biol. Chem. 266*, 11915.

Hausdorff, W. P., Caron, M. G., & Lefkowitz, R. J. (1990a) *FASEB 4*, 2881.

Hausdorff, W. P., Hnatowich, M., O'Dowd, B., Caron, M. G., & Lefkowitz, R. J. (1990b) *J. Biol. Chem. 265*, 1388.

Hausdorff, W. P., Pitcher, J. A., Luttrell, D. K., Linder, M. E., Kurose, H., Parsons, S. J., Caron, M., & Lefkowitz, R. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5720.

Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., & Lefkowitz, R. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2979.

Hertel, C., & Perkins, J. P. (1984) *Mol. Cell Endocrinol. 37*, 245.

Inglese, J., Koch, W. J., Caron, M. G., & Lefkowitz, R. J. (1992) *Nature 359*, 147.

Khan, M. N., Baquiran, G., Brule, C., Burgess, J., Foster, B., Bergeron, J. J. M., & Posner, B. I. (1989) *J. Biol. Chem. 264*, 12931.

Kolodziej, P. A., & Young, R. A. (1991) in *Methods in Enzymology* (Guthrie, C., & Fink, G. R., Eds.) Vol. 194, pp 508–519, Academic Press Inc., New York.

Lohse, M. J., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1990) *J. Biol. Chem. 265*, 3202.

Mahan, L. C., Koachman, A. M., & Insel, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 129.

Marsault, R., Feolde, E., & Frelin, C. (1993) *Am. J. Physiol. 264*, C687.

Munson, P. J., & Robard, D. (1980) *Anal. Biochem. 107*, 220.

Murphy, R. F. (1990) in *Flow Cytometry and Sorting* (Melamed, M. R., Lindmo, T., & Mendelsohn, M. L., Eds.) 2nd ed., pp 355–366, Wiley-Liss, Inc., New York.

O'Dowd, B., Hnatowich, M., & Lefkowitz, R. J. (1991) in *Encyclopedia of Human Biology*, pp 81–92, Academic Press Inc., New York.

Oliveira, L., Paiva, A. C. M., Sander, C., & Vriend, G. (1994) *Trends Pharmacol. Sci. 151*, 170.

Perkins, J. P., Hausdorff, W. P., & Lefkowitz, R. J. (1991) in *The β-Adrenergic Receptors* (Perkins, J. P., Ed.) pp 73–124, The Humana Press Inc., Totowa, NJ.

Pippig, S., Andexinger, S., & Lohse, M. J. (1995) *Mol. Pharmacol. 47*, 666.

Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., & Lefkowitz, R. J. (1992) *Science 257*, 1264.

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., & Sealfon, S. C. (1992) *DNA Cell Biol. 11*, 1.

Pulvermuller, A., Palczewski, K., & Hofmann, K. P. (1993) *Biochemistry 32*, 14082.

Resnik, T. J., Scott-Burden, T., Boulanger, C., Weber, E., & Buhler, F. R. (1990) *Mol. Pharmacol. 38*, 244.

Samama, P., Cotecchia, S., Costa, T., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 4625.

Schwinn, D. A., Caron, M. G., & Lefkowitz, R. J. (1991) in *The Heart and Cardiovascular System* (Fozzard, H. M., Haber, E., Jennings, R., Katz, A., & Morgan, H., Eds.) pp 1657–1684, Raven Press, Ltd., New York.

Slice, L. W., Wong, H. C., Sternini, C., Grady, E. F., Bunnett, N. W., & Walsh, J. H. (1994) *J. Biol. Chem. 269*, 21755.

Thies, R. S., Ulrich, A., & McClain, D. A. (1989) *J. Biol. Chem. 264*, 12820.

Trowbridge, I. S. (1991) *Curr. Opin. Cell Biol. 3*, 634.

Tsuga, H., Kameyama, K., Haga, T., Kurose, H., & Nagao, T. (1994) *J. Biol. Chem. 269*, 32522.

Valiquette, M., Bonin, H., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., & Bouvier, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5089.

Vallette, F., Mege, E., Reiss, A., & Adesnik, M. (1989) *Nucleic Acids Res. 17*, 723.

von Zastrow, M., & Kobilka, B. K. (1992) *J. Biol. Chem. 267*, 3530.

Yu, S. S., Lefkowitz, R. J., & Hausdorff, W. P. (1993) *J. Biol. Chem. 268*, 337.

BI951531G