THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 23, Issue of June 6, pp. 15011–15016, 1997

Printed in U.S.A.

Arrestin/Clathrin Interaction

LOCALIZATION OF THE CLATHRIN BINDING DOMAIN OF NONVISUAL ARRESTINS TO THE CARBOXYL TERMINUS*

(Received for publication, February 12, 1997)

Jason G. Krupnick, Oscar B. Goodman, Jr., James H. Keen, and Jeffrey L. Benovic‡

From the Departments of Biochemistry and Molecular Pharmacology and Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

We have recently demonstrated that the nonvisual arrestins, β-arrestin and arrestin3, interact with high affinity and stoichiometrically with clathrin, and we postulated that this interaction mediates internalization of G protein-coupled receptors (Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* 383, 447–450). In this study, we localized the clathrin binding domain of arrestin3 using a variety of approaches. Truncation mutagenesis demonstrated that the COOH-terminal half of arrestin3 is required for clathrin interaction. Assessment of the clathrin binding properties of various glutathione S-transferase-arrestin3 fusion proteins indicated that the predominant clathrin binding domain is contained within residues 367–385. Alanine scanning mutagenesis further localized this domain to residues 371–379, and site-directed mutagenesis demonstrated the critical importance of both hydrophobic (Leu-373, Ile-374, and Phe-376) and acidic (Glu-375 and Glu-377) residues in the arrestin3/clathrin interaction. These results are complementary to the observation that hydrophobic and basic residues in clathrin are critical for its interaction with nonvisual arrestins (Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) *J. Biol. Chem.* 272, 15017–15022). Lastly, an arrestin3 mutant in which Leu-373, Ile-374, and Phe-376 were mutated to Ala was significantly defective in its ability to promote β_2-adrenergic receptor internalization in COS-1 cells when compared with wild-type arrestin3. Taken together, these results implicate a discrete region of arrestin3 in high affinity binding to clathrin, an interaction critical for agonist-promoted internalization of the β_2-adrenergic receptor.

G protein-mediated signal transduction involves agonist activation of a seven transmembrane-spanning G protein-coupled receptor (GPR)[1] which, in turn, activates a heterotrimeric guanine nucleotide-binding protein (G protein). It is now well established that both the α and βγ subunits of G proteins modulate the activity of many effectors including adenylyl cyclases, phospholipases, ion channels, and cGMP phosphodiesterase (for review, see Refs. 1 and 2). Within seconds to minutes following agonist exposure, activated GPRs lose their ability to respond to agonist with their original sensitivity, a phenomenon commonly referred to as desensitization. Receptor desensitization is initiated by phosphorylation of the agonist-activated GPR by a family of enzymes known as G protein-coupled receptor kinases (GRKs) leading to high affinity binding of a second class of proteins known as arrestins (for review, see Refs. 3 and 4). It is thought that arrestin binding to the phosphorylated GPR sterically inhibits G protein binding (5). To date, four mammalian arrestins have been identified. These include two visual arrestins (arrestin and C- or X-arrestin) (6–8), which likely regulate photoreceptors based on their restricted localization, and two nonvisual arrestins (β-arrestin and arrestin3) (9–12), which are ubiquitous and likely regulate a wide variety of GPRs.

Another level of regulation of GPR signaling involves internalization of the activated receptor into a compartment distinct from the plasma membrane, a process known as sequestration (for review, see Ref. 13). Utilizing a variety of techniques, many studies have demonstrated that at least one mechanism by which GPRs internalize is via clathrin-coated pits (14–19). Interestingly, recent studies have demonstrated a role for sequestration in resensitization of the activated GPR, suggesting a model in which the desensitized receptor is dephosphorylated by an intracellular vesicle-derived phosphatase and then recycled back to the plasma membrane (20–23).

Recently, several studies have implicated GRKs and arrestins in the internalization of agonist-activated GPRs. Overexpression of GRK2 (β-adrenergic receptor kinase) enhanced sequestration of coexpressed m2 muscarinic acetylcholine receptor, whereas a dominant-negative β-adrenergic receptor kinase inhibited receptor sequestration (24). Similarly, overexpression of β-adrenergic receptor kinase rescued sequestration of a β_2-adrenergic receptor mutant (Y326A) defective in its ability to be sequestered, whereas sequestration of β_2-adrenergic receptor mutant Y326A lacking β-adrenergic receptor kinase phosphorylation sites was not rescued (25). Indeed, coexpression of either GRK3, GRK4, GRK5, or GRK6, but not the photoreceptor-specific GRK1, also enhanced sequestration of β_2-adrenergic mutant Y326A (26). It was further observed that overexpression of β-arrestin or arrestin3 (β-arrestin2) alone rescued the sequestration of β_2-adrenergic mutant Y326A, whereas putative dominant-negative mutants of these arrestins inhibited sequestration of the wild-type β_2-adrenergic

* This work was supported in part by National Institutes of Health Grants GM44944 and GM47417 (to J. L. B.), GM28526 (to J. H. K.), and National Institutes of Health Training Grant 5-T32-DK07705-03 (to J. G. K. and O. B. G., Jr.). 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.

‡ Established investigator of the American Heart Association. To whom correspondence should be addressed: Kimmel Cancer Institute, Thomas Jefferson University, 233 S. 10th St., Philadelphia, PA 19107. Tel.: 215-503-4607; Fax: 215-923-1098.

[1] The abbreviations used are: GPR, G protein-coupled receptor; G protein, guanine nucleotide-binding protein; GRK, G protein-coupled receptor kinase; PCR, polymerase chain reaction; bp, base pair(s); GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; ROS, rod outer segment.

receptor (27). Interestingly, overexpression of β-arrestin also enhanced internalization of the angiotensin type II receptor via clathrin-coated pits, a compartment that is not normally utilized by this receptor (28).

We recently proposed that arrestins could both desensitize agonist-activated GPRs and promote their sequestration by interacting not only with the GPR but also with clathrin, the major protein component of the clathrin-based endocytic machinery (29). We observed that β-arrestin and arrestin3, but not visual arrestin, interact specifically, stoichiometrically, and with high affinity and rapid kinetics with clathrin. Moreover, immunofluorescence analyses demonstrated that the activated β_2-adrenergic receptor, β-arrestin, and clathrin all colocalize in intact cells upon agonist addition, suggesting that the arrestin/clathrin interaction observed *in vitro* also occurs in cells in the presence of an activated receptor. Thus, β-arrestin and arrestin3 appear to act as a signal for internalization of agonist-activated GPRs by virtue of their ability to target the desensitized receptor to clathrin-coated pits. In this study, we localize the clathrin binding domain of arrestin3 to a small stretch of residues in the far COOH terminus of the molecule. Interestingly, this region is highly conserved between β-arrestin and arrestin3 but is largely absent in the visual arrestins. Characterization of the clathrin binding domain in arrestins should provide a powerful means of disrupting the arrestin/clathrin interaction in cells.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Leucine and [γ-^{32}P]ATP were purchased from DuPont NEN. pGEX4T-2 vector was obtained from Pharmacia Biotech. All restriction enzymes were purchased from Boehringer Mannheim. Sepharose 2B and all other chemicals were purchased from Sigma. SP6 polymerase and rabbit reticulocyte lysate were prepared as described previously (30, 31). 11-*cis*-Retinal was generously supplied by Dr. R. K. Crouch. Purified rhodopsin kinase was generously supplied by Drs. J. Pitcher and R. Lefkowitz.

Generation of Bovine Arrestin3 Mutants—Truncation mutants of arrestin3S (409 amino acids) (12) were generated by linearizing the arrestin3 cDNA in pGEM-2 with the restriction enzyme *Sfu*I or *Pvu*II to obtain mRNAs encoding residues 1–375 and 1–183, respectively. Alanine scanning mutants of arrestin3 were generated by a two-step PCR protocol using the arrestin3 cDNA as template. In the first reaction, an ~800-bp product was generated using a forward primer (42 or 43 bp beginning at base ~1120–1165 of arrestin3 cDNA) containing the specific base mutations and a 21-bp reverse primer beginning at base 400 of pGEM-2. This product was purified and used together with an *Acc*I-*Hin*dIII restriction fragment of arrestin3 cDNA as template for a second PCR. Here, an ~1,200-bp product was generated using a forward primer (22 bp beginning at base ~800 of arrestin3 cDNA) and the pGEM-2 reverse primer. This product was purified, digested with *Xho*I and *Hin*dIII, and an ~510-bp fragment purified on a 1.2% agarose gel. The ~510-bp fragment was then subcloned into the *Xho*I-*Hin*dIII-digested and phosphatase-treated arrestin3 cDNA. Site-directed mutants of arrestin3 were generated as described above except forward primers for the first PCR, which contained the specific base mutation(s), started at base ~1129–1152 and were 34–46 bp in length. All sequences were confirmed by DNA sequencing (Nucleic Acid Facility, Kimmel Cancer Center, Thomas Jefferson University).

In Vitro Transcription and Translation of Arrestin3—Linearization for generation of arrestin3 truncation mutants was as described above. Wild-type, alanine scanning, and site-directed mutant cDNAs of arrestin3 were linearized with *Hin*dIII and purified on a 1% agarose gel. *In vitro* transcription and translation of arrestin3 proteins were performed exactly as described previously (32). The specific activities and concentrations of *in vitro* synthesized proteins were calculated to be ~500–1,200 dpm/fmol and ~5–20 nM, respectively.

Generation of Glutathione S-Transferase (GST)-Arrestin3 Fusion Proteins—Specific sequences within the COOH-terminal half of bovine arrestin3 were amplified by PCR using a forward primer (~30 bp) containing an engineered *Sma*I restriction site and a reverse primer (~30 bp) containing both an engineered *Sma*I restriction site and a stop codon. PCRs were purified, digested with *Sma*I, repurified, and then subcloned into *Sma*I-cut and phosphatase-treated pGEX4T-2. All sequences were confirmed by DNA sequencing.

Production of GST-arrestin3 fusion proteins was carried out according to the manufacturer's instructions (Pharmacia). Briefly, *Escherichia coli* BL21 cells, transformed with specific GST-arrestin3 fusion protein constructs, were grown at 37 °C to an OD_{600} ~1 and then induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside for 2 h at room temperature. Cells (1 liter) were centrifuged for 30 min at 5,000 rpm, 4 °C and resuspended in 15 ml of ice-cold phosphate-buffered saline (PBS), 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 200 μg/ml benzamidine (buffer A). The cells were incubated 10 min on ice following the addition of lysozyme (1 mg/ml), and then Triton X-100 (1%) and dithiothreitol (5 mM) were added, and the cells were lysed by two freeze/thaw cycles. DNase was added to ~50 μg/ml, and the cells were incubated on ice for 30 min and then centrifuged for 30 min at 50,000 rpm at 4 °C to obtain a cleared lysate. The lysate was incubated with glutathione-Sepharose 4B (0.8 ml) for 60 min at 4 °C on a rotator. The sample was centrifuged for 1 min at 1,000 rpm, 4 °C, washed three times with buffer A containing 0.1% Triton X-100, two times with buffer A, and then either resuspended in 0.8 ml of buffer A (for experiments involving GST-arrestin3 beads) or eluted with 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by dye binding (Bio-Rad) using bovine serum albumin as standard.

Preparation of Clathrin Cages and Cage Binding Assay—The preparation of clathrin and clathrin cages (33) and the clathrin cage binding assay (34) have been described previously. Briefly, *in vitro* synthesized radiolabeled arrestins (~0.5 nM) or GST-arrestin3 fusion proteins (15 nM) were incubated with or without clathrin cages for 30 min at 22 °C in a total volume of 50 μl in 100 mM Na-MES, pH 6.8; 1 mM dithiothreitol; 1 μg/ml each of leupeptin, pepstatin, and antipain; and 0.1 mg/ml bovine serum albumin (in the case of GST-arrestin3 fusion proteins). Samples were then cooled on ice, loaded onto a 75-μl 0.2 M sucrose cushion in 100 mM Na-MES, pH 6.8, and centrifuged for 5 min at 100,000 rpm, 4 °C in a TLA100.1 rotor. Pellets were solubilized in 20 μl of SDS sample buffer, heated at 100 °C for 5 min, and run on 10% SDS-polyacrylamide gels. For *in vitro* synthesized radiolabeled proteins, gels were stained with Coomassie Blue, destained, enhanced with 20% 2,5-diphenyloxazole in glacial acetic acid, dried, and autoradiographed. Quantitation was carried out by PhosphorImager analysis (Molecular Dynamics). After subtracting out nonspecific sedimentation in the absence of clathrin cages (~9% of input), specific binding was normalized to the binding for wild-type arrestin. For GST-arrestin3 fusion proteins, proteins were transferred to a nitrocellulose membrane at 100 V for 1 h. Blots were blocked for 30 min with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (buffer B) and then incubated for 1 h with a 1:250 dilution of PF2 antibody (recognizes GST) in buffer B. Blots were washed three times for 5 min each with buffer B, incubated for 1 h with a 1:2,000 dilution of goat anti-rabbit secondary antibody in buffer B, washed four times for 5 min each with buffer B, incubated with ECL reagent (Amersham) for 1 min, and then exposed to film for 5–10 s. Films were scanned by densitometry (Molecular Dynamics) to quantitate binding. Nonspecific sedimentation was assessed in the absence of clathrin cages (~8% of input) and subtracted from all experimental values.

Preparation of Rod Outer Segment (ROS) Membranes—Bovine ROS membranes containing >95% rhodopsin were prepared as described previously (32). Phosphorylated rhodopsin was generated by incubating ROS membranes (~150 μg of rhodopsin) with purified recombinant rhodopsin kinase in a total volume of 500 μl in 20 mM Tris-HCl, pH 7.5, 2 mM EGTA, 7.5 mM $MgCl_2$, 100 μM ATP for 1 h at 30 °C under bright light illumination. Phosphorylated ROS membranes were centrifuged for 45 min at 50,000 rpm, 4 °C, washed with 1 ml of 20 mM Tris-HCl, pH 7.5, 2 mM EGTA, resuspended in 200 μl of 20 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM dithiothreitol, sonicated twice for 10 s on ice, and then regenerated with a 3-fold molar excess of 11-*cis*-retinal for 40 min at 37 °C in the dark followed by another 3-fold molar excess of 11-*cis*-retinal for 2 h at 37 °C in the dark. The stoichiometry of phosphorylation was assessed by incubating 5 μl of the initial phosphorylation reaction with 5–10 μCi of [γ-^{32}P]ATP under the exact conditions as described above. The incubation was stopped with SDS sample buffer, and the sample was electrophoresed on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue, destained, dried, and autoradiographed, and pmol of phosphate incorporated was determined by excising and counting the rhodopsin band. The stoichiometry of phosphorylation was calculated to be ~2.4 mol of phosphate/mol of rhodopsin.

Rhodopsin Binding Assay—The rhodopsin binding assay was carried out essentially as described (35). Briefly, *in vitro* synthesized radiolabeled arrestins (~0.5 nM) were incubated with ROS membranes (200 nM

phosphorylated rhodopsin) for 5 min at 37 °C in total volume of 50 μl in 50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 100 mM potassium acetate, 1.5 mM dithiothreitol. Samples were then cooled on ice and receptor-bound arrestins separated from unbound arrestins by Sepharose 2B column chromatography. Specific binding was calculated after subtracting out nonspecific counts in the absence of ROS membranes (~1.8 fmol).

Internalization Experiments in COS-1 Cells—The bovine arrestin3 expression construct in pBC12BI was described previously (29); the arrestin3-LIFA mutant (Leu-373, Ile-374, and Phe-376 mutated to Ala) was made by replacing an ~600-bp *Eco*RV-*Xho*I restriction fragment of pBC-arrestin3 with the corresponding fragment of arrestin3-LIFA. The human β_2-adrenergic receptor expression construct in pBC12BI was generously provided by Dr. B. Kobilka. COS-1 cells were grown in T75 flasks at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown to ~90% confluence and then transfected with the constructs using LipofectAMINE according to the manufacturer's instructions (Life Technologies, Inc.). Briefly, 12–13.5 μg of pBC-β_2-adrenergic receptor and 3 μg of pBC-arrestin3 or pBC-arrestin3-LIFA was incubated with 65 μl of LipofectAMINE in 5 ml of Dulbecco's modified Eagle's medium for 30 min at 22 °C. Five ml of Dulbecco's modified Eagle's medium was then combined with the DNA/LipofectAMINE and added to a T75 flask of Dulbecco's modified Eagle's medium-rinsed COS-1 cells. After 5 h at 37 °C the DNA-containing medium was replaced with complete medium.

Cells were harvested by trypsinization 48–72 h after transfection, washed twice with ice-cold PBS and resuspended in 1.2 ml of ice-cold PBS containing 0.1 mM ascorbic acid. The cells (0.5 ml) were incubated with or without 1 μM (−)isoproterenol at 37 °C for 15 min, washed twice with 50 ml of ice-cold PBS, and resuspended in 0.4–0.5 ml of cold PBS. Cell surface receptors were measured directly by incubation with 6–9 nM [^{3}H]CGP-12177 (Amersham) for 3 h at 14 °C with nonspecific binding assessed in the presence of 10 μM (−)alprenolol. Bound ligands were separated on glass fiber filters (Whatman, GF/C) by vacuum filtration on a Brandel cell harvester. In these studies, β_2-adrenergic receptor expression levels (mean ± S.E. in pmol/mg of protein) were 4.7 ± 0.7 for control, 4.9 ± 0.4 for arrestin3-, and 4.8 ± 0.6 for arrestin3-LIFA-coexpressing cells.

To assess arrestin3 expression, 0.1–0.2 ml of the trypsinized washed cells was pelleted by centrifugation, resuspended in 0.2 ml of cold lysis buffer (20 mM Hepes, pH 7.2, 10 mM EDTA, 0.2 M NaCl, 1% Triton X-100, 0.1 mg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride), and then lysed by freeze/thaw and vortexing. The sample was centrifuged to remove insoluble material, and 20 μg of the supernatant protein was electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. Immunoblotting was carried out exactly as described above for GST-arrestin3 fusion proteins except the primary monoclonal antibody was F4C1 (36) at a 1:2,000 dilution, and the secondary antibody was goat anti-mouse antibody. Protein concentrations were determined by Bio-Rad assay using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

We demonstrated previously that the nonvisual arrestins β-arrestin and arrestin3, but not visual arrestin, interact specifically with clathrin cages (29), an assembled form of clathrin resembling that found in coated pits. Thus, our initial approach to localize the clathrin binding domain in nonvisual arrestins entailed assessing the ability of β-arrestin/arrestin chimeras to interact with clathrin cages. A chimera consisting of the NH_2-terminal half of arrestin and the COOH-terminal half of β-arrestin bound to clathrin cages comparably to wild-type β-arrestin, whereas a chimera consisting of the NH_2-terminal half of β-arrestin and the COOH-terminal half of arrestin did not bind (data not shown). Moreover, a chimera consisting of the first 345 residues of arrestin with the last 78 amino acids of β-arrestin retained significant clathrin binding, whereas a chimera consisting of predominantly β-arrestin with the NH_2-terminal 47 and COOH-terminal 59 amino acids of arrestin bound weakly to clathrin cages (29). These results implicate the COOH-terminal half of β-arrestin, and more specifically the far COOH terminus, as being critical for its high affinity interaction with clathrin.

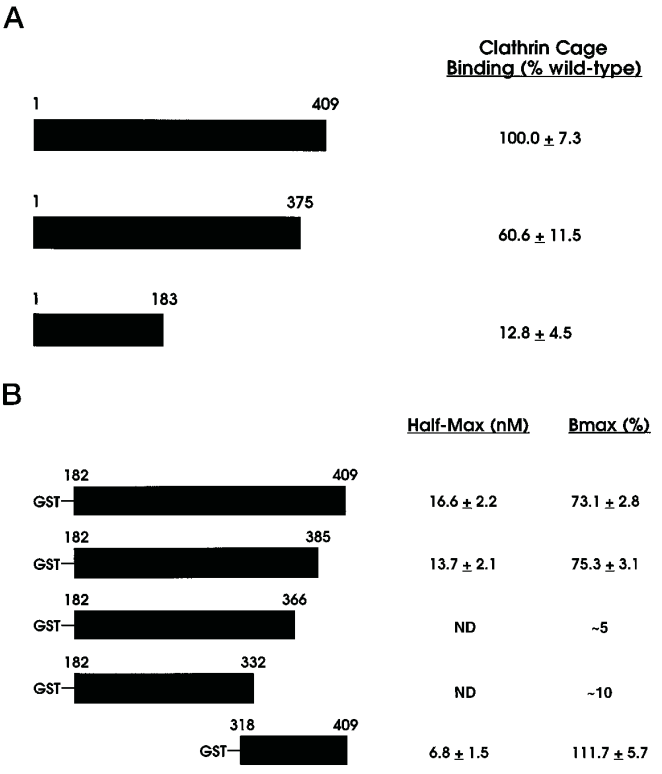

FIG. 1. **Initial localization of the clathrin binding domain of arrestin3 to the COOH terminus.** *Panel A*, wild-type arrestin3 (1–409) and the two truncation mutants (1–375 and 1–183) were *in vitro* transcribed and then translated with rabbit reticulocyte lysate in the presence of [^{3}H]leucine. *In vitro* translated proteins (~0.5 nM, ~500–1,200 dpm/fmol) were incubated with or without clathrin cages (200 nM clathrin) for 30 min at 22 °C. Samples were then processed as described under "Experimental Procedures," and gels were scanned by PhosphorImager analysis (Molecular Dynamics). Specific binding was normalized to the binding for wild-type arrestin3 (29.4 ± 6.8%). Data shown are the mean ± S.E. of four independent determinations. *Panel B*, GST-arrestin3 fusion proteins (15 nM) were incubated with or without clathrin cages (0–200 nM clathrin) for 30 min at 22 °C. Samples were processed and immunoblotted as described under "Experimental Procedures" using an antibody that recognizes GST. Films were scanned by densitometry (Molecular Dynamics), and the binding at each concentration of clathrin was determined by comparing the intensity of staining in experimental samples with the intensity of staining of GST-arrestin3 standards loaded on the same gel. Data points were then curve fitted to a hyperbolic function (Kaleidograph), and the half-maximal and B_{max} (percent of total input protein that binds) values determined. Data shown are the mean ± S.E. of three or four independent experiments. *ND*, not determined.

We chose to do a more detailed characterization of the clathrin binding domain with arrestin3 since it has an ~6-fold greater affinity for clathrin than β-arrestin (29). We initially sought to determine whether the clathrin binding domain in arrestin3 is also localized to the COOH-terminal domain by generating two truncation mutants, one lacking the last 34 amino acids and the other lacking the entire COOH-terminal half (Fig. 1*A*). Wild-type arrestin3 and the two truncation mutants were *in vitro* translated with rabbit reticulocyte lysate in the presence of [^{3}H]leucine and then assessed for interaction with clathrin cages. While truncation of the last 34 amino acids partially reduced clathrin binding (~40%), truncation of the entire COOH-terminal half of arrestin3 essentially eliminated clathrin binding (~90% reduced) (Fig. 1*A*). These results thus demonstrate that the clathrin binding domain in arrestin3, as described for β-arrestin, is contained within the COOH-terminal half of the protein and that at least a portion of this domain is localized within the last 34 amino acids of arrestin3.

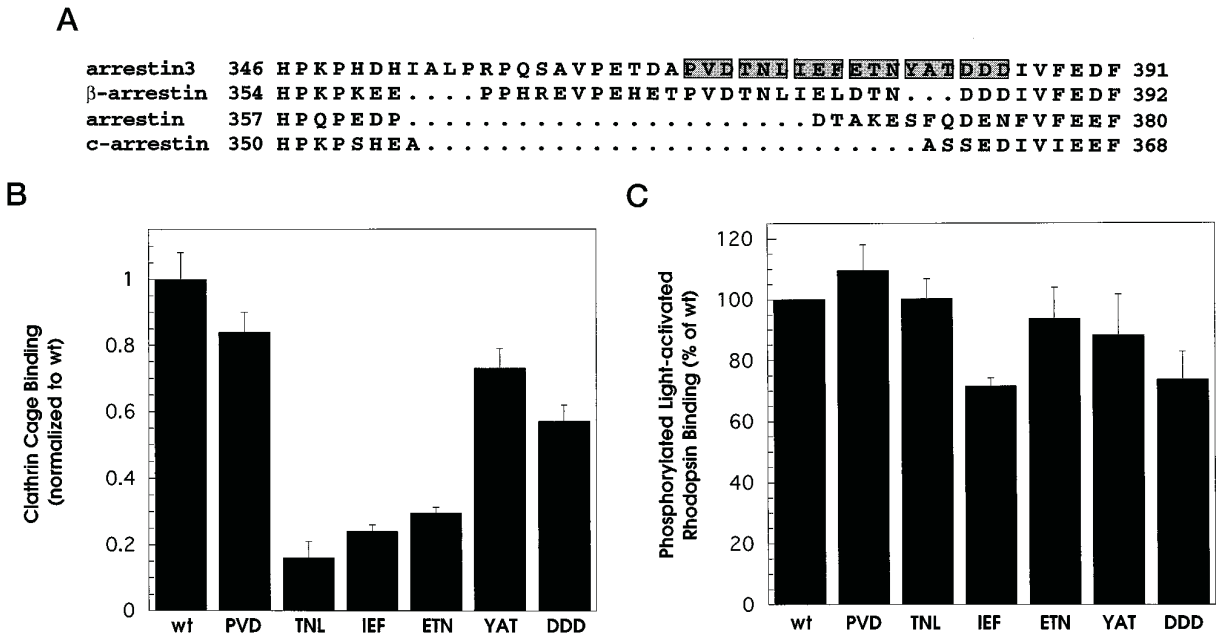

FIG. 2. **Analysis of clathrin cage and receptor binding properties of arrestin3 alanine scanning mutants.** *Panel A*, alignment of bovine arrestin3 (residues 346–391) with the corresponding region of bovine β-arrestin, bovine visual arrestin, and bovine cone arrestin. *Boxed* residues denote the six alanine scanning mutants that were generated. *Panel B*, wild-type (*wt*) arrestin3 and the arrestin3 alanine scanning mutants were *in vitro* transcribed and translated in the presence of [^{3}H]leucine (~800–1,000 dpm/fmol). The proteins (~0.5 nM) were then incubated with or without clathrin cages (200 nM clathrin) at 22 °C for 30 min and processed as described under "Experimental Procedures." Specific binding was normalized to the clathrin cage binding of wild-type arrestin3 (21.8 ± 3.0%). Data shown are the mean ± S.E. of four independent determinations. *Panel C*, wild-type and mutant arrestin3 (~0.5 nM) were incubated with or without phosphorylated ROS membranes (200 nM rhodopsin) at 37 °C for 5 min with constant illumination. Samples were cooled on ice and receptor-bound arrestins separated by Sepharose 2B column chromatography as described under "Experimental Procedures." Specific binding was normalized to the receptor binding for wild-type arrestin3 (4.75 ± 0.62 fmol). Data shown are the mean ± S.E. of three independent experiments.

We further localized the clathrin binding domain of arrestin3 by assessing the ability of GST-fusion proteins containing various portions of the arrestin3 COOH terminus (schematically illustrated in Fig. 1*B*) to interact with clathrin cages (Fig. 1*B*). A GST-arrestin3 fusion protein containing the entire COOH-terminal half of arrestin3 (residues 182–409) bound to clathrin cages with an affinity comparable to that for recombinant arrestin3 (29), further confirming that the clathrin binding domain of arrestin3 is localized to its COOH terminus. In contrast, GST alone or a GST-arrestin3 fusion protein containing the NH_2-terminal half of arrestin3 (residues 1–210) did not bind clathrin (data not shown). Although truncation of the 182–409 construct to residue 385 did not affect clathrin binding, truncation to residues 332 or 366 essentially eliminated clathrin binding. These results suggest that the predominant clathrin binding domain in arrestin3 is localized between residues 367 and 385. Indeed, GST-fusion proteins containing the far COOH terminus of arrestin3 (residues 318–409 or 340–409) also bound well to clathrin cages (Fig. 1*B* and data not shown). A similar pattern of clathrin binding was observed when GST-arrestin3 fusion proteins were analyzed for binding to free clathrin trimers, as the 182–409 and 318–409 constructs bound clathrin trimers, whereas the 182–332 and 182–366 constructs did not (data not shown). Moreover, the GST-arrestin3 fusion proteins that did bind clathrin cages demonstrated rapid kinetics ($t_{1/2} \leq 1$ min) (data not shown), similar to the kinetics of clathrin binding observed for purified recombinant arrestin3 (29).

A comparison of the far COOH-terminal domains of the four mammalian arrestins is depicted in Fig. 2*A*. The *shaded* portion represents the putative clathrin binding domain between residues 368 and 385 of arrestin3. Interestingly, β-arrestin and arrestin3 have greater than 80% homology in this region. In contrast, visual arrestin lacks a significant portion of this region and has less than 30% homology in the remaining residues, whereas cone arrestin lacks greater than 70% of this region. This likely explains the poor ability of visual arrestin to interact with clathrin (29). Furthermore, it is tempting to speculate that cone arrestin, similar to arrestin, will also not interact well with clathrin. The observation that β-arrestin and arrestin3 contain a putative clathrin binding domain but the visual arrestins do not suggests that during evolution either the nonvisual arrestins gained the ability to interact with clathrin, a function critical for their ability to promote internalization of GPRs (27–29), or that the visual arrestins lost this ability. The poor ability of arrestin to interact with clathrin is interesting in light of the apparent lack of internalization of rhodopsin as a mechanism of its regulation, a mechanism that is likely critical for the regulation of signaling of many other GPRs (17, 37–41).

Our initial approach toward identifying specific amino acid residues within region 368–385 which are critical for clathrin interaction involved alanine scanning mutagenesis in which three consecutive residues were mutated to alanine. The six alanine scanning mutants of arrestin3 generated are depicted in Fig. 2*A* (*shaded boxes*) and are named according to the three residues that were mutated. The mutants were *in vitro* translated with rabbit reticulocyte lysate in the presence of [^{3}H]leucine and then assessed for binding to clathrin cages (Fig. 2*B*). Although the alanine scanning mutants PVD, YAT, and DDD were modestly reduced in clathrin binding (15–40%), the mutants TNL, IEF, and ETN were ~70–85% reduced in clathrin binding. These results thus more precisely localize the clathrin binding site in arrestin3 to residues 371–379. To confirm a native tertiary structure of these alanine scanning mutants, their ability to bind to phosphorylated agonist-activated receptor was also analyzed. Since arrestin3 recognizes the phosphorylated and activated state of the photoreceptor rho-

dopsin with high affinity (42), we used phosphorylated light-activated rhodopsin to assess receptor binding. Previous studies have demonstrated that the primary sites in arrestins for interaction with the phosphorylated agonist-activated receptor are localized to the NH_2-terminal half of the molecule (42–44). Thus, we did not expect any of the alanine scanning mutants to be defective in receptor binding in the absence of alterations in overall protein conformation. Indeed, each of these mutants of arrestin3 bound to phosphorylated light-activated rhodopsin comparably to wild-type arrestin3, although the binding of IEF and DDD was reduced modestly (Fig. 2*C*).

To identify specific residues critical for arrestin3 interaction with clathrin, we performed site-directed mutagenesis within the three consecutive alanine scanning triplets TNL, IEF, and ETN. To assess the contribution of charged residues to the arrestin3/clathrin interaction, we mutated Glu-375 and Glu-377 to Lys, individually as well as in combination. To assess the contribution of hydrophobic amino acids to this interaction, we mutated Leu-373, Ile-374, and Phe-376 to Ala. Finally, to assess the potential contribution of hydrogen bonding, we generated mutants in which Thr-371, Thr-378, Asn-372, and Asn-379 individually were changed to alanine. These site-directed arrestin3 mutants were *in vitro* translated in the presence of [^{3}H]leucine and then assessed for clathrin cage binding (Fig. 3*A*). Whereas mutation of the individual Glu residues to Lys only modestly reduced clathrin binding (~20–30%), mutation of both Glu residues to Lys significantly reduced binding (~60% reduction). Similarly, mutation of individual Thr or Asn residues only modestly reduced clathrin binding (~10–30%). In contrast, an arrestin3 mutant containing Leu, Ile, and Phe mutated to Ala decreased clathrin binding by ~90%. As expected, none of the site-directed arrestin3 mutants was significantly affected in its ability to interact with phosphorylated light-activated rhodopsin, although the N379A mutant was reduced ~40% (Fig. 3*B*). These results thus implicate the hydrophobic residues Leu-373, Ile-374, and Phe-376 and the charged residues Glu-375 and Glu-377 as playing a primary role in high affinity interaction of arrestin3 with clathrin.

The results obtained in this study correlate well with results involving characterization of the arrestin binding domain in clathrin (46). In the latter study, a small region containing highly conserved residues in the clathrin heavy chain NH_2-terminal domain was found to be critical for clathrin interaction with nonvisual arrestins. Clathrin residues most important for arrestin binding included two highly conserved lysines and an invariant glutamine. Thus, it is likely that ionic and hydrophobic and/or hydrogen bonding interactions contribute to high affinity binding between nonvisual arrestins and clathrin.

Since previous studies have implicated arrestin/clathrin interaction in receptor internalization (29), we next sought to determine whether an arrestin3 mutant defective in clathrin binding would also be defective in its ability to promote internalization of the β_2-adrenergic receptor. The arrestin3 mutant containing Leu-373, Ile-374, and Phe-376 mutated to Ala (arrestin3-LIFA) was used for these studies since it was ~90% defective in clathrin binding (Fig. 3*A*) but unaffected in its ability to interact with receptor (Fig. 3*B*). COS-1 cells transfected with β_2-adrenergic receptor alone or together with wild-type arrestin3 or arrestin3-LIFA were treated with agonist for 15 min and then analyzed for cell surface receptors. When both arrestins were expressed at a comparable level (Fig. 4*A*), wild-type arrestin3 enhanced agonist-specific β_2-adrenergic receptor sequestration ~4-fold, whereas arrestin3-LIFA-promoted β_2-adrenergic receptor sequestration was ~65% reduced compared with the wild-type protein (Fig. 4*B*). These results thus confirm the critical requirement of clathrin binding for the ability of arrestin3 to promote internalization of agonist-activated GPRs.

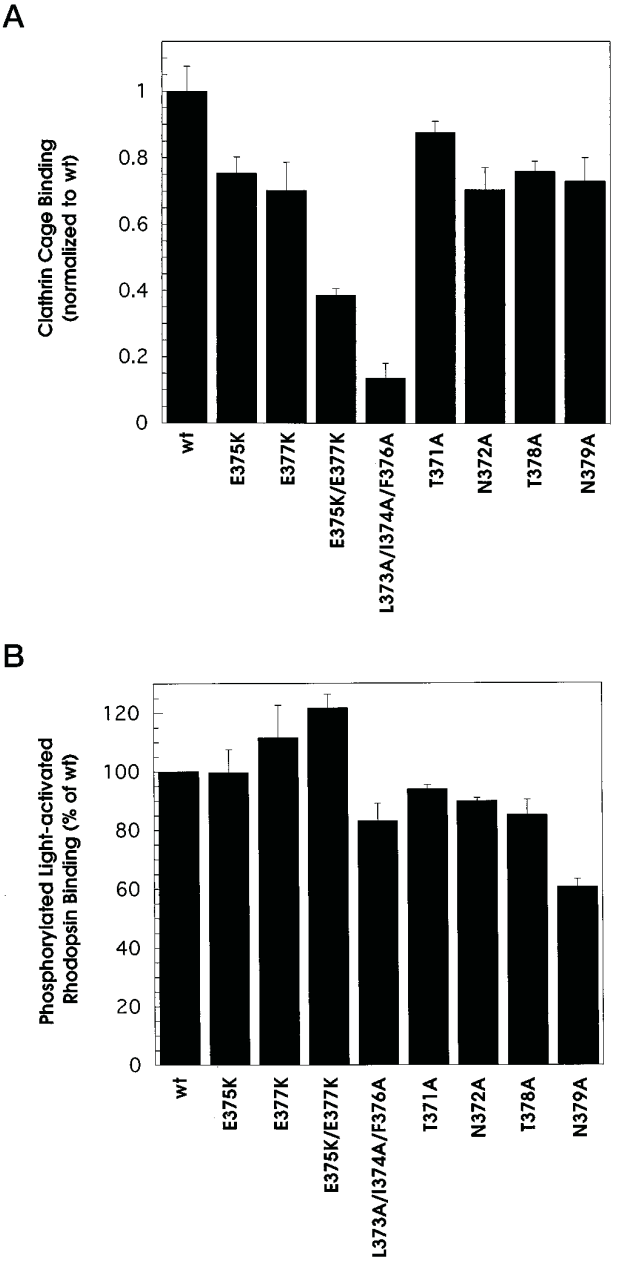

FIG. 3. **Analysis of clathrin cage and receptor binding properties of arrestin3 site-directed mutants.** *Panel A*, wild-type (*wt*) arrestin3 and arrestin3 site-directed mutants were *in vitro* transcribed and translated in the presence of [^{3}H]leucine (~1,000 dpm/fmol). The proteins (~0.5 nM) were incubated with or without clathrin cages (200 nM clathrin) at 22 °C for 30 min and then processed as described under "Experimental Procedures." Specific binding was normalized to the clathrin cage binding of wild-type arrestin3 (25.1 ± 1.3%). Data shown are the mean ± S.E. of four independent determinations. *Panel B*, wild-type and mutant arrestin3 (~0.5 nM) were incubated with or without phosphorylated ROS membranes (200 nM rhodopsin) at 37 °C for 5 min under constant illumination. Samples were cooled on ice and receptor-bound arrestins separated by Sepharose 2B column chromatography as described under "Experimental Procedures." Specific binding was normalized to the receptor binding for wild-type arrestin3 (3.38 ± 0.30 fmol). Data shown are the mean ± S.E. of three independent experiments.

In this study, we have localized the predominant clathrin binding domain of arrestin3 to a short stretch of residues in the far COOH terminus of the molecule (residues 373–377). These

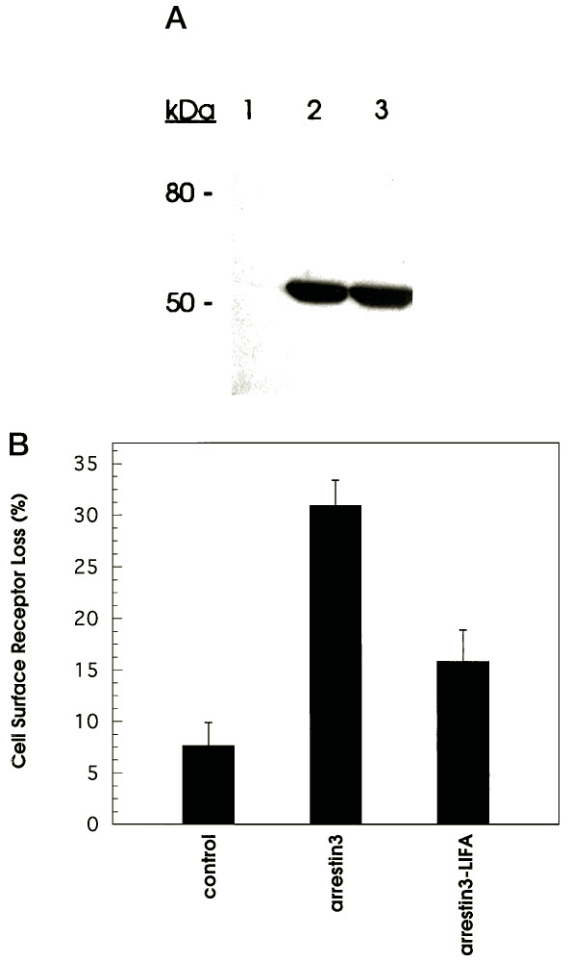

FIG. 4. **Arrestin3-promoted internalization of agonist-stimulated β_2-adrenergic receptor.** *Panel A*, COS-1 cells transiently transfected with 12 μg of pBC-β_2-adrenergic receptor without (*lane 1*) or with 3 μg of pBC-arrestin3 (*lane 2*) or pBC-arrestin3-LIFA (*lane 3*) were harvested 72 h after transfection. Cells were lysed, and 20 μg of protein was analyzed by immunoblotting using the arrestin monoclonal antibody F4C1 as described under "Experimental Procedures." *Panel B*, COS-1 cells transfected with pBC-β_2-adrenergic receptor alone or with pBC-arrestin3 or pBC-arrestin3-LIFA were harvested 48–72 h after transfection. The cells were incubated with 1 μM (−)isoproterenol at 37 °C for 15 min, washed extensively with ice-cold PBS, resuspended, and then analyzed for cell surface β_2-adrenergic receptors as described under "Experimental Procedures." The data represent the mean ± S.E. from five or six independent experiments.

findings should prove useful for designing dominant-negative arrestins to block specifically the arrestin/clathrin interaction in an effort to modulate the regulation of GPR signaling in cells. Since internalization of GPRs has been implicated in desensitization (37, 38, 45), and more recently, resensitization (20–23) of GPRs, the opportunity exists to prolong or attenuate receptor responsiveness to agonist using such arrestin mutants.

Acknowledgments—We thank Dr. V. Gurevich for providing the cDNAs for the β-arrestin/arrestin chimeras, Dr. R. Sterne-Marr for providing the GST-arrestin3 (318–409) construct, Drs. J. Pitcher and R. Lefkowitz for supplying purified recombinant rhodopsin kinase, L. Tran for purification of clathrin, and C. Carman and Drs. L. Kallal and A. Gagnon for helpful discussions.

REFERENCES

1. Birnbaumer, L. (1992) *Cell* **71,** 1069–1072
2. Muller, S., and Lohse, M. J. (1995) *Biochem. Soc. Trans.* **23,** 141–148
3. Hargrave, P. A., and McDowell, J. H. (1992) *FASEB J.* **6,** 2323–2331
4. Sterne-Marr, R., and Benovic, J. L. (1995) *Vitam. Horm.* **51,** 193–235
5. Kuhn, H., Hall, S. W., and Wilden, U. (1984) *FEBS Lett.* **176,** 473–478
6. Shinohara, T., Dietzschold, B., Craft, C. M., Wistow, G., Early, J. J., Donoso, L. A., Horwitz, J., and Tao, R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6975–6979
7. Murakami, A., Yajima, T., Sakuma, H., McLaren, M. J., and Inana, G. (1993) *FEBS Lett.* **334,** 203–209
8. Craft, C. M., Whitmore, D. H., and Wiechmann, A. F. (1994) *J. Biol. Chem.* **269,** 4613–4619
9. Lohse, M. J., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* **248,** 1547–1550
10. Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 17882–17890
11. Rapoport, B., Kaufman, K. D., and Chazenbalk, G. D. (1992) *Mol. Cell. Endocrinol.* **84,** R39–R43
12. Sterne-Marr, R., Gurevich, V. V., Goldsmith, P., Bodine, R. C., Sanders, C., Donoso, L. A., and Benovic, J. L. (1993) *J. Biol. Chem.* **268,** 15640–15648
13. Penn, R. B., and Benovic, J. L. (1997) in *Handbook of Physiology* (Conn, P. M., ed), Oxford University Press, New York, in press
14. von Zastrow, M., and Kobilka, B. K. (1992) *J. Biol. Chem.* **267,** 3530–3538
15. von Zastrow, M., and Kobilka, B. K. (1994) *J. Biol. Chem.* **269,** 18448–18452
16. Maloteaux, J. M., and Hermans, E. (1994) *Biochem. Pharmacol.* **47,** 77–88
17. Fonseca, M. I., Button, D. C., and Brown, R. D. (1995) *J. Biol. Chem.* **270,** 8902–8909
18. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) *J. Biol. Chem.* **271,** 19021–19024
19. Slowiejko, D. M., McEwen, E. L., Ernst, S. A., and Fisher, S. K. (1996) *J. Neurochem.* **66,** 186–196
20. Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993) *J. Biol. Chem.* **268,** 337–341
21. Pippig, S., Andexinger, S., and Lohse, M. J. (1995) *Mol. Pharmacol.* **47,** 666–676
22. Pitcher, J. A., Payne, E. S., Csortos, C., DePaoli-Roach, A. A., and Lefkowitz, R. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8343–8347
23. Krueger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 5–8
24. Tsuga, H., Kameyama, K., Haga, T., Kurose, H., and Nagao, T. (1994) *J. Biol. Chem.* **269,** 32522–32527
25. Ferguson, S. S. G., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A.-M., and Caron, M. G. (1995) *J. Biol. Chem.* **270,** 24782–24789
26. Menard, L., Ferguson, S. S. G., Barak, L. S., Bertrand, L., Premont, R. T., Colapietro, A.-M., Lefkowitz, R. J., and Caron, M. G. (1996) *Biochemistry* **35,** 4155–4160
27. Ferguson, S. S. G., Downey, W. E., III, Colapietro, A.-M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science* **271,** 363–365
28. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1996) *J. Biol. Chem.* **271,** 18302–18305
29. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383,** 447–450
30. Jackson, R. J., and Hunt, T. (1983) *Methods Enzymol.* **96,** 50–75
31. Zozulya, S. A., Gurevich, V. V., Zvyaga, T. A., Shirokova, E. P., Dumler, I. L., Garnovskaya, M. N., Natochin, M. Y., Shmukler, B. E., and Badalov, P. R. (1990) *Protein Eng.* **3,** 453–458
32. Gurevich, V. V., and Benovic, J. L. (1992) *J. Biol. Chem.* **267,** 21919–21923
33. Keen, J. H., Willingham, M. C., and Pastan, I. H. (1979) *Cell* **16,** 303–312
34. Goodman, O. B., Jr., and Keen, J. H. (1995) *J. Biol. Chem.* **270,** 23768–23773
35. Krupnick, J. G., Gurevich, V. V., Schepers, T., Hamm, H. E., and Benovic, J. L. (1994) *J. Biol. Chem.* **269,** 3226–3232
36. Dua, H. S., Lee, R. H., Lolley, R. N., Barrett, J. A., Abrams, M., Forrester, J. V., and Donoso, L. A. (1992) *Curr. Eye Res.* **11,** (suppl.) 107–111
37. Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2979–2983
38. Yang, J., Williams, J. A., Yule, D. I., and Logsdon, C. D. (1995) *Mol. Pharmacol.* **48,** 477–485
39. Berry, S. A., Shah, M. C., Khan, N., and Roth, B. L. (1996) *Mol. Pharmacol.* **50,** 306–313
40. Sternini, C., Spann, M., Anton, B., Keith, D. E., Jr., Bunnett, N. W., von Zastrow, M., Evans, C., and Brecha, N. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9241–9246
41. Tolbert, L. M., and Lameh, J. (1996) *J. Biol. Chem.* **271,** 17335–17342
42. Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R. Hosey, M. M., and Benovic, J. L. (1995) *J. Biol. Chem.* **270,** 720–731
43. Gurevich, V. V., and Benovic, J. L. (1993) *J. Biol. Chem.* **268,** 11628–11638
44. Gurevich, V. V., and Benovic, J. L. (1995) *J. Biol. Chem.* **270,** 6010–6016
45. Pak, Y., Kouvelas, A., Scheideler, M. A., Rasmussen, J., O'Dowd, B. F., and George, S. R. (1996) *Mol. Pharmacol.* **50,** 1214–1222
46. Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) *J. Biol. Chem.* **272,** 15017–15022