Properties of Secretin Receptor Internalization Differ from Those of the β2-Adrenergic Receptor*

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Julia K. L. Walker‡, Richard T. Premont, Larry S. Barak, Marc G. Caron§, and Michael A. Sheteline

From the Howard Hughes Medical Institute, Departments of Cell Biology and Medicine, Divisions of Gastroenterology and Cardiology, Duke University Medical Center, Durham, North Carolina 27710

The endocytic pathway of the secretin receptor, a class II GPCR, is unknown. Some class I G protein-coupled receptors (GPCRs), such as the β2-adrenergic receptor (β2-AR), internalize in clathrin-coated vesicles and this process is mediated by G protein-coupled receptor kinases (GRKs), β-arrestin, and dynamin. However, other class I GPCRs, for example, the angiotensin II type 1A receptor (AT1A), exhibit different internalization properties than the β2-AR. The secretin receptor, a class II GPCR, is a GRK substrate, suggesting that like the β2-AR, it may internalize via a β-arrestin and dynamin directed process. In this paper we characterize the internalization of a wild-type and carboxyl-terminal (COOH-terminal) truncated secretin receptor using flow cytometry and fluorescence imaging, and compare the properties of secretin receptor internalization to that of the β2-AR. In HEK 293 cells, sequestration of both the wild-type and COOH-terminal truncated secretin receptors was unaffected by GRK phosphorylation, whereas inhibition of cAMP-dependent protein kinase mediated phosphorylation markedly decreased sequestration. Addition of secretin to cells resulted in a rapid translocation of β-arrestin to plasma membrane localized receptors; however, secretin receptor internalization was not reduced by expression of dominant negative β-arrestin. Thus, like the AT1A, secretin receptor internalization is not inhibited by reagents that interfere with clathrin-coated vesicle-mediated internalization and in accordance with these results, we show that secretin and AT1A receptors colocalize in endocytic vesicles. This study demonstrates that the ability of secretin receptor to undergo GRK phosphorylation and β-arrestin binding is not sufficient to facilitate or mediate its internalization. These results suggest that other receptors may undergo endocytosis by mechanisms used by the secretin and AT1A receptors and that kinases other than GRKs may play a greater role in GPCR endocytosis than previously appreciated.

The importance of phosphorylation as a regulatory mechanism for desensitizing receptor signaling has been well established for class I G protein-coupled receptors (GPCRs),1 such as the β2-adrenergic receptor (β2-AR) (1, 2), as well as for class II GPCRs such as the secretin receptor (3). The role of phosphorylation in restoring GPCR signaling ability is less clear. GPCR phosphorylation is predominantly directed by two different classes of kinases, the G protein-coupled receptor kinases (GRKs) and the second messenger-dependent protein kinases. GPCR phosphorylation by cAMP-dependent protein kinase (PKA) appears to directly diminish the ability of the receptor to signal. In contrast, GRK phosphorylation increases receptor affinity for arrestin proteins, and it is this interaction of the receptor with arrestin that uncouples the receptor from the G protein (4). Additionally, arrestin directs some class I GPCRs, like the β2-AR, to a clathrin-mediated endocytic pathway in which receptors are internalized, dephosphorylated, and subsequently recycled to the plasma membrane as competent receptors (5, 6). However, whether arrestins are required for endocytosis or desensitization of class II GPCRs has not been examined.

β-Arrestin 1 and 2 are members of the arrestin protein family that regulate both the signaling and trafficking of GPCRs. β-Arrestin translocates from the cytosol to agonist-activated GPCRs where it subsequently targets the GPCR-β-arrestin complex to clathrin-coated pits (CCPs), presumably by its ability to bind the clathrin heavy chain and the β-adaptin subunit of the AP2 clathrin adaptor protein complex (7, 8). The roles of β-arrestin and CCPs in GPCR endocytosis have been established by fluorescence colocalization studies and through functional cellular assays that employ mutants of β-arrestin and dynamin (9–11). These studies demonstrate that endocytosis through CCPs is dependent upon the ability of dynamin, a GTPase, to catalyze the pinching off of the CCP (12). This process is blocked by a dominant negative mutant, dynamin K44A, that is defective in GTP binding. Similarly, β-arrestin mutants that have lost the ability to support GPCR trafficking to CCPs also function as dominant negative inhibitors of sequestration. In a series of experiments, Zhang et al. (9) demonstrated that expression of either dynamin K44A or β-arrestin V53D prevented endocytosis of the β2-AR but not the angiotensin II type 1A receptor (AT1A), despite the ability of the AT1A (also a class I GPCR) to interact with β-arrestin. Thus, class I GPCRs that desensitize by β-arrestin binding can internalize with distinct properties.

Secretin receptors internalize following agonist stimulation (13, 14), but the endocytic pathway utilized remains uncharac-

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‡ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Duke University Medical Center, Box 3287, Durham, NC 27710. Tel.: 919-684-5433; Fax: 919-681-8641.

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1 The abbreviations used are: GPCR, G protein-coupled receptor; β2-AR, β2-adrenergic receptor; GRK, G protein-coupled receptor kinase; PKA, cAMP-dependent protein kinase; CCP, clathrin-coated pit; AT1A, angiotensin II type 1A receptor; HEK, human embryonic kidney; GFP, green fluorescent protein; PBS, phosphate-buffered saline; HA, hemagglutinin.
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terized. The ability of secretin receptors to robustly desensitize in the presence of GRKs suggests that they may internalize through a mechanism similar to that of the β-Ar. In this study we investigate the role of receptor kinases and β-arrinets in the regulation of secretin receptor endocytosis. The internalization of wild-type and COOH-terminal truncated secretin receptors was measured in the presence of exogenously over-expressed GRKs, dominant negative mutants of dynamin and β-arrestin, or in the presence of PKA inhibitors. Our results demonstrate that, unlike the β-Ar, secretin receptor internalization is not inhibited by β-arrestin-V53D or dynamin-K44A. Moreover, they suggest a dependence of secretin receptor internalization on PKA activity. These results imply that β-arrestin-mediated desensitization is separable from its GPCR trafficking function and that kinases other than GRKs may regulate GPCR endocytosis.

EXPERIMENTAL PROCEDURES

Materials—General chemicals and reagents were from Sigma. Secretin was obtained from Peninsula Labs. Human embryonic kidney cells (HEK 293 cells) were obtained from the American Type Culture Collection. Tissue culture supplies were obtained from Life Technologies. Labeled secretin ([35S]ormethon) was prepared and purified by high performance liquid chromatography (3, 15). [2,8-3H]Adenosine, [3H]cAMP, [β,γ-32P]Orthophosphate were obtained from NEN Life Science Products. [3H]adenosine 5′-triphosphate was obtained from New England Nuclear. Polyethylene glycol (PEG) was obtained from U. S. Biochemical Corp./Amersham Pharmacia Biotech. Polymerase chain reaction materials were from Perkin-Elmer (Roche Molecular Systems).

Membrane Preparation—The FLAG epitope-tagged rat secretin receptor, the HA epitope-tagged β-Ar, and β-arrestin-green fluorescent protein (β-arrestin-GFP) have been described previously (3, 16, 17). The COOH-terminal truncated secretin receptor was prepared from the NH2-terminal, FLAG-tagged secretin receptor by polymerase chain reaction to include amino acids 1–398. The cDNAs were inserted into the pcDNA I/Amp plasmid (Invitrogen) using HindIII and BamHI. Fidelity was demonstrated by dideoxy sequencing. GRK cDNAs were produced as described previously (GRK 2 (18) and GRK 5 (19)). Plasmid purification was performed with Qiagen reagents.

Secretin receptor-green fluorescent protein (secretinR-GFP) was prepared by inserting the rat secretin receptor into pEGFP-N3 vector (CLONTECH) via BamHI and XhoI restriction sites by the method previously described (17). Fidelity was demonstrated by dideoxy sequencing and function was confirmed by cAMP accumulation in response to secretin agonist by whole cell linkage (see methods below). AT1 R/GFP was prepared in a manner similar to that described by Barak et al. (17).

Cell Culture—HEK 293 cells were grown in modified Eagle’s medium (modified Eagle’s medium: 10% fetal bovine serum, 50 μg/ml gentamicin) at 37 °C in 95% air, 5% CO2. One day after transfection cells were split into appropriate plates following trypsin dissociation. Experiments were performed 24–48 h after transfection.

Transfection—Transient transfections were performed with calcium phosphate co-precipitation. One to 10 μg of vector DNA was transferred into a 6-ml Falcon tube with 450 μl of sterile water and 50 μl of 2.5 M CaCl2. Then 500 μl of 2 × HEPES-buffered saline (0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na2PO4, pH 7.1) was added to the tube and mixed well. This mixture was added dropwise to the 100-mm dish of cells. Cells were plated to a density of approximately 2–3 × 105 cells to each well for cAMP accumulation experiments and 1–1.5 × 106 cells per well for phosphorylation and sequestration experiments.

Membrane Preparation/Binding—All steps were performed at 4 °C. Plates were placed on ice, media was aspirated, and the cells were washed with 10 ml of ice-cold PBS. Five to 10 ml of lysis buffer (10 mM Tris, 5 mM EDTA with protease inhibitors: 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml benzamidine, 10 μg/ml phenylmethylsulfonyl fluoride) were added to each plate. With a cell lifter, cells were scraped off the plate and placed in 15-ml conical tubes on ice. Cell fragments were homogenized with a Polytron PT 3000 for 20–30 s at 14,000–16,000 g. Material was centrifuged at 300–400 × g for 10 min to remove unlysed cells and nuclei. Supernatant was transferred to 13 × 100-mm tubes on ice and centrifuged at 18,000 rpm (40,000 × g) (Sorval SM24 rotor) for 30 min at 4 °C. Supernatant was discarded and the membrane pellet was resuspended in binding buffer, for immediate assay, or lysis buffer and stored at −80 °C.

Membrane binding was performed as published (3). Briefly, using a constant amount of HEK 293 cell membrane protein, competition displacement (porcine secretin, Peninsula Labs) of [3H]-porcine secretin binding was performed in triplicate tubes. Non-specific binding was defined in the presence of 1 μM unlabeled porcine secretin. Data was analyzed using Graph Pad-Prism and LIGAND software as described (3, 15).

Adenyl Cyclase Assay—The accumulation of cAMP in intact cells was quantitated chromatographically by the method of Solomon (20).

Membrane bound cAMP (1 μc/ml) in modified Eagle’s medium, 5% fetal bovine serum, 50 μg/ml gentamicin (1 ml/well) 12 to 16 h prior to experimentation. To assay the accumulation of cAMP, labeling media was aspirated, cells were washed with 1 ml of PBS, and preincubated in 1 ml of media per well (modified Eagle’s medium, 0% fetal bovine serum, 10 mM HEPES, 1 mM IBMX, assay medium) for 15–30 min. Cells were stimulated with appropriate agonist, and at the end of the experimental duration, media was aspirated and 1 ml of ice-cold stop solution (0.1 mM cAMP, 4 mM/ml [3H]cAMP, 2.5% perchloric acid) was placed in each well. Plates remained on ice for 20–30 min, after which solution was transferred to 12 × 75 tubes containing 100 μl of 4.2 M KOH. Tubes were vortexed and stored overnight, at 4 °C, prior to cAMP determination by column chromatography (20). Data is normalized for total cellular uptake using [3H]cAMP as described previously (20).

Western Blotting—Cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose and then subjected to immunoblotting with appropriate GRK (21), dynamin (22), and β-arrestin antisera (23).

Receptor Expression—In plasmid co-transfection experiments, receptor expression was determined by flow cytometry analysis of a sample from each transfection group (3). The fluorescence was determined by incubation for 1 h at 4 °C with monoclonal IgG-M2-FLAG (1:600 dilution, Kodak), three washes with PBS, and detection with Fc-specific, fluorescein-labeled goat anti-mouse (1:200 dilution, Sigma). Cells were then washed, removed from the plate with 10 mM Tris, pH 7.4, 5 mM EDTA and fixed with 3.6% formaldehyde. Samples were analyzed on a Becton-Dickson flow cytometer. Baseline fluorescence was determined from a sample of HEK 293 cells untransfected and/or a sample of HEK 293 cells transfected with the secretin receptor not exposed to primary antibody (IgG-M2-FLAG). Baseline fluorescence was subtracted from each sample.

Receptor Internalization/Sequestration—Sequestration is defined as the number of receptors removed from the cell surface after agonist exposure, as determined by flow cytometry. Cells are incubated with agonist for the appropriate time. After washing with ice-cold PBS, cells on ice are exposed for 1 h at 4 °C to monoclonal IgM-M2-FLAG (1:600 dilution, Kodak) or 12CA5 (1:500 dilution, Roche Molecular Biochemicals), three washes with PBS, and detection with Fc-specific, fluorescein-labeled goat anti-mouse (1:200 dilution, Sigma). Cells were then removed from the plate with 10 mM Tris, pH 7.4, 5 mM EDTA and fixed with 3.6% formaldehyde. Samples were analyzed on a Becton-Dickson flow cytometer. Baseline fluorescence was determined from a sample of HEK 293 cells transfected with the secretin receptor not exposed to agonist and another sample not exposed to primary antibody (IgG-M2-FLAG). Baseline fluorescence was subtracted from each sample.

Secretin Receptor Internalization by Immunofluorescence—HEK 293 cells transiently transfected with 2.5 μg of cDNA for secretin wild-type receptor or 3.5 μg of cDNA for COOH-terminal truncated secretin receptor were plated onto 35-mm dishes containing a central glass well as described (16). Cells were maintained at 4 °C to prevent receptor internalization while incubating with agonist (100 μM secretin); primary antibody (IgG-M2-FLAG, Kodak, Inc.), and secondary antibody (Fabs conjugated with fluorescein isothiocyanate, Organo Technica). Incubations were 30 min in duration and occurred in the sequence listed. Cells were washed 3 times with cold PBS after each antibody application. Immediately following the last PBS wash, cells were viewed using confocal microscopy (basal time point), while a second plate of identically treated cells was warmed at 37 °C for 1 h prior to imaging (60 min treatment).

Immunofluorescent Colocalization of Secretin Receptor with Angiotensin Receptors, β-Arrestin-GFP, or β-Ar-AFs—HEK 293 cells transiently transfected with 3.0 μg of cDNA for secretin wild-type receptor and 4.8 μg of cDNA for angiotensin II type 1A-GFP receptor (AT1 R/GFP) were plated on glass coverslips contained in 6-well plates. After an initial wash, cells were stimulated with 100 nM secretin and 100 nM angiotensin II.
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was defined as the cAMP accumulation in response to 0.1 nM secretin to HEK 293 cell membranes transiently transfected with native, FLAG-tagged wild-type, or COOH-terminal truncated FLAG-tagged secretin receptor cDNA demonstrated similar K_d values. The half-time for complete desensitization of wild-type or truncated receptors was 0.1 and 0.05 nM, respectively.

EC_{50} for cAMP accumulation was approximately 0.1 nM for both the wild-type and the truncated receptors. Our previous data for the native receptor (non-epitope tagged) revealed an EC_{50} of 0.4 nM. Truncation of the COOH terminus did not produce any difference in the kinetics of cAMP accumulation compared with the wild-type (Fig. 1C). Having demonstrated that the addition of an NH2-terminal FLAG or the truncation of the COOH-terminal tail did not appreciably alter the binding or the signaling of the secretin receptor, we employed these FLAG epitope-tagged receptor tools to investigate the mechanism of secretin receptor internalization.

Secretin Receptor Sequestration—Fluorescence and corresponding interference and fluorescence overlay micrographs (Fig. 2, A and B) demonstrate that the FLAG epitope-tag on the secretin receptor does not hinder endocytosis of the wild-type

RESULTS

Characterization of Secretin Receptor Constructs—In order to demonstrate receptor movement from the plasma membrane to intracellular compartments, epitope-tagged wild-type and COOH-terminal truncated secretin receptor cDNAs were prepared. Quantifying cell surface epitopes has been used with other receptors and represents a non-radioactive method of monitoring receptor sequestration (9, 16, 18). The truncated receptor contains amino acids 1–398, and is devoid of 10 serines and threonines in the COOH-terminal tail, which eliminates over 50% of the total intracellular sites for potential serine/threonine kinase-mediated phosphorylation. Binding studies were performed on cell membranes prepared from HEK 293 cells overexpressing the NH2-terminal FLAG epitope-tagged wild-type and COOH-terminal truncated secretin receptors. When compared with the membranes prepared from cells transfected with native (non-epitope tagged) secretin receptor, competition binding resulted in similar K_d values. K_d values ranged from 2.5 nM for the FLAG-truncated secretin receptor to 5.3 nM for the native receptor (Fig. 1A).

In order to investigate the signaling of these receptor constructs, dose-response and time course of cAMP production were studied. Signaling experiments did not demonstrate any significant difference between the wild-type and truncated secretin receptors (Fig. 1B). The EC_{50} for cAMP accumulation ranged from 2.5 nM for the FLAG-truncated secretin receptor to 5.3 nM for the native receptor (Fig. 1A).

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Secretin Receptor Sequestration—Fluorescence and corresponding interference and fluorescence overlay micrographs (Fig. 2, A and B) demonstrate that the FLAG epitope-tag on the secretin receptor does not hinder endocytosis of the wild-type
receptor, or the COOH-terminal truncated receptor, into vesicles. As measured by flow cytometry, the wild-type secretin receptor sequestered to a maximum of 58 ± 4% after 20 to 30 min. The half-time of sequestration was 8 min (Fig. 3A). In order to determine the role of the COOH-terminal portion of the secretin receptor on sequestration, we studied the sequestration of the truncated secretin receptor during a 1-h period. Truncation did not appear to alter the kinetics of receptor sequestration, however, total surface receptor internalization at all time points beyond 30 min (Fig. 3A) was decreased 10–15% compared with wild-type (p < 0.02, nine experiments, each done in duplicate). The COOH-terminal region of the secretin receptor contains multiple potential phosphorylation sites. Others have shown an association between receptor phosphorylation and receptor internalization (18). We next investigated the effect of truncation (loss of COOH-terminal phosphorylation sites) on secretin receptor phosphorylation and internalization.

**Effect of GRK-mediated Phosphorylation on Secretin Receptor Sequestration**—Secretin receptor phosphorylation is increased by specific GRKs (3). In order to investigate the effect of increased receptor phosphorylation on receptor sequestration, we overexpressed GRK 2 and GRK 5 in HEK 293 cells. As shown in Fig. 3, B (for wild-type receptor) and C (for C-terminal truncated receptor), kinases known to augment phosphorylation of the secretin receptor did not increase receptor internalization for either the wild-type or truncated receptor. Although overexpression of GRKs enhances secretin receptor desensitization (3), it does not alter receptor sequestration. At all time points studied (up to 60 min), no significant effect of overexpressed GRKs on sequestration was observed. Expression of GRKs was documented by Western blotting using GRK specific antisera (not shown) as described under “Experimental Procedures.” Thus, enhanced GRK-mediated phosphorylation of the secretin receptor, unlike the β2-AR (18), does not appear to augment receptor sequestration.

**Effect of the PKA Inhibitors, H89 and Staurosporin, on Secretin Receptor Sequestration**—G protein-coupled receptor desensitization can be mediated by PKA and/or PKC. We have shown that PKA inhibitors reduce phosphorylation of the secretin receptor (3). However, these inhibitors had no effect on secretin receptor signaling in HEK 293 cells (3). Therefore, we tested the effect of PKA inhibition on secretin receptor sequestration. The PKA inhibitors, H89 and staurosporin, significantly decreased sequestration of both the wild-type and truncated secretin receptors (Fig. 4). Interestingly, sequestration of the COOH-terminal truncated secretin receptor was more profoundly affected by H89 than the wild-type receptor. Staurosporin (1 μM) blocks the activity of both PKA and PKC (24). However, compared with the inhibition of sequestration by H89, additional PKC inhibition did not promote further inhibition of sequestration. These data suggest that there may be an association between cAMP-dependent protein kinase phosphorylation and sequestration of the secretin receptor.

**Effect of β-Arrestin and GRK-dependent Phosphorylation on Sequestration of the COOH-terminal Truncated Receptor in the Presence of PKA Inhibition**—Given that inhibition of PKA activity inhibits secretin receptor internalization, we attempted to reverse this by overexpressing β-arrestin, GRK 2, or β-arrestin and GRK 2 combined. Overexpression of either β-arrestin or GRK 2 increased receptor internalization (Fig. 5) in the presence of H89 but the effects were not additive. This result demonstrates that β-arrestin and GRK 2 can enhance secretin receptor sequestration; however, their involvement only becomes apparent when PKA dependent phosphorylation is prevented.

**Effect of Hypertonic Sucrose on Secretin Receptor Sequestration**—Hypertonic medium reduces the clustering of surface receptors into endosomes (25). Hypertonic sucrose similarly inhibited sequestration of the β2-AR, the secretin wild-type, and the COOH-terminal truncated receptors (Fig. 4) suggesting that, like other GPCRs, the secretin receptor does internalize by clustering surface receptors into endosomes.

**The Role of β-Arrestin and Dynamin in Secretin Receptor Sequestration**—β-Arrestin translocates to agonist activated β2-AR (26). Using a recently developed β-arrestin translocation assay (16), we found that agonist activation of wild-type or truncated secretin receptors initiated rapid movement of β-arrestin-GFP from the cytosol to the plasma membrane and that this translocation did not require overexpression of GRKs (Fig. 6). Addition of agonist to cells not overexpressing the secretin...
receptor caused no translocation of β-arrestin-GFP (micrograph not shown). H89-mediated inhibition of PKA activity did not alter the translocation of β-arrestin-GFP to the agonist-activated wild-type or COOH-terminal truncated secretin receptor (micrograph not shown). Fluorescence micrographs show co-localization of β-arrestin-GFP and secretin wild-type receptors at the plasma membrane (Fig. 7). Thus, β-arrestin interacts with the agonist-activated secretin receptor. How-

**FIG. 3.** Influence of GRKs on the agonist-promoted sequestration kinetics of secretin wild-type and COOH-terminal truncated receptors as assessed by flow cytometry. A, secretin wild-type or truncated receptors were transiently transfected in HEK 293 cells. Cells were exposed to 0.1 μM porcine secretin for the times indicated on the x axis. The half-time for complete sequestration of wild-type and truncated receptors was 7.6 min (range 5.6 to 12.2) and 8.2 min (range 5.6 to 15.3), respectively. The data was analyzed by Graph-Pad Prism, one phase exponential association, $R^2 = 0.74$ for wild-type and $R^2 = 0.64$ for truncated receptors. The data represent the mean ± S.E. of four independent experiments done in duplicate.

**FIG. 4.** Effect of protein kinase A inhibition (H89 and staurosporin) and sucrose on the agonist-promoted sequestration of secretin and β2-ARs. Secretin wild-type or truncated receptors or β2-ARs were transiently transfected in HEK 293 cells and then preincubated with either 30 μM H89 or 1 μM staurosporin for 15 min, or 0.45M sucrose for 30 min. Cells expressing wild-type or truncated secretin, or β2-ARs, were then stimulated for 30 min with 0.1 μM porcine secretin or 10 μM isoproterenol, respectively. Sequestration was assessed by flow cytometry using the anti-FLAG (M2) and anti-12CA5 monoclonal antibodies. The data represent the mean ± S.E. of three independent experiments each performed in duplicate.

**FIG. 5.** Effect of overexpressed β-arrestin, GRK 2, or both β-arrestin and GRK 2 on the agonist-promoted sequestration of the COOH-terminal truncated secretin receptor pretreated with the PKA inhibitor H89. HEK 293 cells transiently expressing truncated secretin receptor alone, or in combination with β-arrestin, GRK 2, or β-arrestin and GRK2 were preincubated with 30 μM H89 for 15 min. Cells were then stimulated for 30 min with 0.1 μM porcine secretin. Sequestration was assessed by flow cytometry using the anti-FLAG (M2) monoclonal antibody. The data represent the mean ± S.E. of three independent experiments each performed in duplicate.
In this paper we provide direct evidence demonstrating internalization of agonist-activated secretin receptors. Although agonist-activated secretin and β2-ARs are each phosphorylated by GRKs and subsequently recruit β-arrestin, secretin receptors internalize with different properties, implying that the aforementioned characteristics are not sufficient to predict the sequestration pathway of a GPCR. Furthermore, the majority of secretin receptors internalize into endosomes which do not contain β2-ARs, and vice versa. In addition, we demonstrate co-localization of secretin and AT1A Rs in endocytic vesicles, suggesting that the pathway or mechanism used by these two
receptors may also be common to other GPCRs. Finally, we present the unique finding that PKA activity regulates sequestration of a GPCR.

The role of β-arrestin in GPCR regulation has been extensively studied, primarily using the prototypical β2-AR (6). Agonist activation and GRK phosphorylation of the β2-AR trigger the translocation of β-arrestin from the cytosol to the plasma membrane where it binds and uncouples the receptor from its heterotrimeric G protein (1, 2, 16). The receptor is thus desensitized and must be internalized, processed through endocytic vesicles, and dephosphorylated before it is returned to the plasma membrane as a functional receptor (5, 27).

β-Arrestin plays a role in regulating this internalization/resensitization process by directing the β2-AR to CCPs (7, 10). β-Arrestin interacts directly with clathrin; however, the importance of this reaction for receptor internalization is not clear, as β-arrestin constructs lacking the inherent clathrin binding motif can still support β2-AR internalization (7, 8). The β2-ARβ-arrestin complex also recruits and binds the β2-adaptin subunit of AP-2 (8). AP-2 is a protein complex which serves as an adaptor between receptors and the clathrin lattice during clathrin-coated pit formation (28). Through its interaction with AP-2 and clathrin, β-arrestin may serve as a docking protein which links the β2-AR to the clathrin lattice.

The clathrin lattice of coated pits contains a uniform distri-
Given the present results and our previous finding that PKA-mediated phosphorylation of the secretin receptor does not affect signaling (3), we suggest that the primary role of PKA-mediated phosphorylation of the secretin receptor in HEK 293 cells is to promote receptor sequestration. Further studies delineating PKA phosphorylation sites on the secretin receptor, followed by mutagenesis studies, should determine if the effect of PKA activity on receptor sequestration is direct. Putative PKA phosphorylation sites are located at two sites within the intracellular regions of the secretin receptor. One potential site of PKA-mediated phosphorylation is removed by truncation of the secretin receptor. Presently, though, we cannot rule out the possibility that PKA may promote sequestration of the secretin receptor through phosphorylation of some adaptor-like protein that targets the receptor to endocytic vesicles. Regardless of the mechanism, the fact that secretin receptor sequestration is associated with PKA activity raises the possibility that secretin receptor internalization may be subject to heterologous regulation.

In conclusion, regulation of internalization of the secretin receptor, a class II GPCR, differs substantially from that of the prototypical class I β2-AR. Like the AT1R, the dominant negative mutants β-arrestin-V53D and dynamin-K44A do not inhibit the sequestration of the secretin receptor. These results imply that an endocytic pathway or mechanism alternative to that of the β2-AR is common to other GPCRs and may have unique implications for receptor recycling and/or down-regulation. The secretin receptor should represent an important tool for delineating this mechanism. This study also provides the novel finding that internalization of secretin receptors is dependent upon PKA activity.
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REFERENCES

1. Lohse, M. J., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1990) J. Biol. Chem. 265, 3202–3211
2. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) FASEB J. 4, 2881–2889
3. Shetzline, M. A., Premont, R. T., Walker, J. K. L., Vigna, S. R., and Caron, M. G. (1998) J. Biol. Chem. 273, 6756–6762
4. Premont, R. T., Inglese, J., and Lefkowitz, R. J. (1995) FASEB J. 9, 2881–2889
5. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. G. (1997) J. Biol. Chem. 272, 27005–27014
6. Ferguson, S. S. G., Barak, L. S., Zhang, J., and Caron, M. G. (1996) Can. J. Physiol. Pharmacol. 74, 1095–1110
7. 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
8. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S. G., Caron, M. G., and Barak, L. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3712–3717
9. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1996) J. Biol. Chem. 271, 18302–18305
10. Ferguson, S. S. G., Downey, W. E., III, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) Science 271, 363–366
11. von Zastrow, M., and Kohilkas, R. K. (1992) J. Biol. Chem. 267, 3530–3538
12. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
13. Holtmann, M. H., Roettger, B. F., Pinon, D. I., and Miller, L. J. (1996) J. Biol. Chem. 271, 23566–23571
14. Mundell, S. J., and Kelly, E. (1998) Br. J. Pharmacol. 125, 1594–1600
15. Farouk, M., Vigna, S. R., McVey, D. C., and Meyers, W. C. (1992) Gastroenterology 102, 963–968
16. Barak, L. S., Ferguson, S. S. G., Zhang, J., and Caron, M. G. (1997) J. Biol. Chem. 272, 27497–27506
17. Barak, L. S., Ferguson, S. S. G., Zhang, J., Martenson, C., Meyer, T., and Caron, M. G. (1997) Mol. Pharmacol. 51, 177–184
18. 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
19. Tiberi, M., Nash, S. R., Bertrand, L., Lefkowitz, R. J., and Caron, M. G. (1996) J. Biol. Chem. 271, 3771–3778
20. Salomon, Y. (1991) Methods Enzymol. 195, 22–28
21. Premont, R. T., Koch, W. J., Inglese, J., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6832–6841
22. Sontag, J. M., Fykse, E. M., Ushkaryov, Y., Liu, J. P., Robinson, P. J., and Sudhof, T. C. (1994) J. Biol. Chem. 269, 4547–4554
23. Attwood, H., Arriza, J. L., Aski, 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
24. Hudaka, H., and Kobayashi, R. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 377–397
25. Daukas, G., and Zigmond, S. H. (1985) J. Cell Biol. 101, 1673–1679
26. Zhang, J., Barak, L. S., Anborgh, P. H., Laporte, S. A., Caron, M. G., and Ferguson, S. S. G. (1999) J. Biol. Chem. 274, 10999–11006
27. Krueger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 5–8
28. Schmid, S. L. (1997) Annu. Rev. Biochem. 66, 511–548
29. Hinshaw, J. E., and Schmid, S. L. (1995) Nature 374, 190–192
30. Clark, R. B., Kunkel, M. W., Friedman, J., Goka, T. J., and Johnson, J. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1442–1446
31. Goretzki, L., and Mueller, B. M. (1997) J. Cell Sci. 110, 1395–1402