A Role for Receptor Kinases in the Regulation of Class II G Protein-coupled Receptors

PHOSPHORYLATION AND DESENSITIZATION OF THE SECRETIN RECEPTOR*

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The secretin receptor is a member of a structurally distinct class of G protein-coupled receptors designated as Class II. The molecular mechanisms of secretin receptor signal termination are unknown. Using transiently transfected HEK 293 cells expressing the secretin receptor, we investigated its mechanisms of desensitization. Binding of [125I]-secretin to plasma membranes of receptor-expressing cells was specific, with a Kd of 2 nM. Secretin evoked an increase in cellular cAMP with an EC50 of 0.4 nM. The response was maximal by 20 min and desensitized rapidly and completely. Immunoprecipitation of a functional, N-terminal epitope-tagged secretin receptor was used to demonstrate agonist-dependent receptor phosphorylation, with an EC50 of 14 nM. Pretreatment with protein kinase A or C inhibitors failed to alter secretin-stimulated cAMP accumulation. G protein-coupled receptor kinases (GRKs) are known to be involved in the desensitization of Class I G protein-coupled receptors; therefore, the effect of cotransfection of GRKs on secretin-stimulated cAMP signaling and phosphorylation was evaluated. GRKs 2 and 5 were the most potent at augmenting desensitization, causing a 40% reduction in the maximal cAMP response to secretin. GRK 5 also caused a shift in the EC50 to the right (p < 0.05). GRK 4 and GRK 6 did not alter dose-dependent signaling, and GRK 3 was intermediate in effect. Receptor phosphorylation correlated with desensitization for each GRK studied, whereas second messenger-dependent kinase phosphorylation appeared to be less important in secretin receptor signal termination.

We demonstrate agonist-dependent secretin receptor phosphorylation coincident with profound receptor desensitization of the signaling function in HEK 293 cells, suggesting a role for receptor phosphorylation in this paradigm. Although GRK activity appears important in secretin receptor desensitization in HEK 293 cells, protein kinases A and C appear to play only a minor role. These results demonstrate that the GRK-arrestin system regulates Class II G protein-coupled receptors.

The gastrointestinal hormone secretin stimulates pancreatic water and bicarbonate secretion, leading to neutralization of acidic chyme in the intestine. Secretin also plays a role in gastric acid release and intestinal motility. Secretin exerts these effects by binding to specific heptahelical membrane receptors and activating the heterotrimeric G protein, Gs, leading to elevation of cellular cAMP levels. Receptor sequence analysis has divided G protein-coupled receptors (GPCRs) into sub-families: I) rhodopsin/β-adrenergic, II) secretin/glucagon, and III) the metabotropic glutamate receptor families (1, 2). The secretin/glucagon receptor subfamily comprises a structurally distinct class of receptors, designated Class II. This group consists of receptors for secretin, glucagon, calcitonin, parathyroid hormone, pituitary adenylyl cyclase-activating peptide, vasoactive intestinal polypeptide, and others. These receptors lack many of the structural signature sequences present in the prototypic rhodopsin/β-adrenergic receptor family of GPCR (Class I) (1).

GPCR signaling is dynamically regulated. The rapid process by which GPCR-mediated signals are attenuated is termed desensitization. Although the mechanisms of desensitization have been well characterized for the β-adrenergic receptor family, the mechanisms regulating Class II GPCR signal transduction are largely unknown. Typically, signal termination occurs via two distinct pathways (2). Mechanisms that modulate only stimulated GPCRs are termed homologous desensitization. Receptor phosphorylation by G protein-coupled receptor kinases (GRKs) is believed to be a major component of this rapid diminished responsiveness for Class I receptors (3, 4). Other modes of signal attenuation that involve second messenger-dependent protein kinases acting on both active and unstimulated receptors are termed heterologous desensitization (2, 5). Both protein kinase A and protein kinase C have been shown to play a role in this mode of desensitization (2). With the cloning of the secretin receptor (6), it is now possible to investigate the molecular basis of its desensitization.

Previous work on the secretin receptor has been hampered by lack of appropriate biochemical tools to demonstrate specific receptor protein phosphorylation. Recently, Ozcelebi et al. (7) demonstrated a secretin-stimulated phosphorylated protein that migrated at 57,000–62,000 on SDS-polyacrylamide gel electrophoresis, consistent with the predicted molecular weight of the secretin receptor. This band was not present when a C-terminal-truncated mutant secretin receptor was stimulated with agonist. In contrast, Holtmann et al. (8) studied a mutant secretin receptor with the C-terminal putative phosphorylation

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1 The abbreviations used are: GPCR, G protein-coupled receptors; GRK, GPCR kinase; HEK, human embryonic kidney; dATPγS, deoxyadenosine 5’-[α-thio]triphosphate; PCR, polymerase chain reaction; PKA and PKC, protein kinase A and C, respectively.
sites removed and noted that desensitization was still present. This persistent desensitization suggested that phosphorylation at these sites might not be important in the signal termination of the secretin receptor.

Many questions remain concerning the regulation of the secretin receptor. Is this receptor a substrate for GRK phosphorylation? Are specific GRK-dependent processes involved? Does receptor phosphorylation correlate with desensitization? What is the role of second messenger-dependent phosphorylation? In this paper we demonstrate agonist-dependent secretin receptor phosphorylation by immunoprecipitation of an N-terminal FLAG-tagged secretin receptor, coincident with profound functional receptor desensitization, suggesting a role for receptor phosphorylation in desensitization. Signaling, as determined by cAMP accumulation in human embryonic kidney cells (HEK 293 cells), appears to be unaffected by second messenger-dependent kinases, whereas agonist-activated G protein-coupled receptor kinases play a significant role. GRK-specific phosphorylation of the secretin receptor is shown to correlate with signal attenuation. Understanding the molecular basis for secretin receptor regulation may provide information relevant to an entire class of structurally distinct receptors and should aid in our understanding of the processes they regulate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Basic chemicals and reagents were from Sigma. Peptides (secretin, glucagon, vasoactive intestinal polypeptide) were obtained from Peninsula Labs. HEK 293 cells were obtained from the American Tissue Culture Collection. Tissue culture supplies were obtained from Life Technologies, Inc. Labeled secretin (125I) is prepared and purified by high performance liquid chromatography (9). [3H]adenine, [3H]cAMP, [3H]insulin, [3H]glucose, and [3H]leucine were obtained from Amersham Life Science, Inc. Polymerase chain reaction (PCR) materials were from Perkin-Elmer (Roche Molecular Systems).

**Plasmid Preparation**—Using the known cDNA sequence for the rat secretin receptor (6), oligonucleotides were synthesized, and the full-length cDNA sequence was amplified from rat heart cDNA by PCR. An epitope-tagged rat secretin receptor was prepared as described (10) by placing the FLAG epitope on the N-terminal region of the mature receptor following a modified influenza hemaglutinin signal sequence to produce a protein that could be recognized with commercially available anti-FLAG antibodies. Fidelity was demonstrated with deoxy sequencing. The cDNAs were inserted into the pcDNA 1Amp plasmid (Invitrogen) using HindIII and BamHI. GRK cDNAs were produced as described previously: GRK 2 and GRK 3 (11), GRK 4 (12), GRK 5 (13), and GRK 6 (14). Plasmid purification was performed with Qiagen reagents.

**Cell Culture**—HEK 293 cells were grown in 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 (10% fetal bovine serum, 50 μg/ml gentamicin) at 37 °C in 95% air, 5%

**Adenylyl Cyclase Assays**—The accumulation of cAMP in intact cells was measured chromatographically by the method Salomon (15). Cells were labeled with [3H]adenine (1 μCi/ml) in modified Eagle’s medium, 5% fetal bovine serum, 50 μg/ml gentamicin (1 ml/well) 12–16 h before experimentation. To assay the accumulation of cAMP, labeling media was aspirated, and cells were washed with 1 ml of phosphate-buffered saline and preincubated in 1 ml of media/well (modified Eagle’s medium, 0% fetal bovine serum, 10 mM HEPES, 1 mM isobutylmethylxanthine; 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 nCi/ml [3H]cAMP, 2.5% perchloric acid) was placed in each well. Plates remained on ice at 4 °C for 20–30 min, after which solution was transferred to 12 × 75 tubes containing 100 μl of 4.2 ml KOH. Tubes were vortexed and stored at 4 °C for cAMP determination by column chromatography (15). For experiments requiring pretreatment with protein kinase inhibitors, transfected cells were incubated in 30 μM H89 or staurosporine for 20 min and then stimulated in the presence of H89 or staurosporine at doses noted for 10 min. Control experiments were performed in parallel to ensure activity of the inhibitors. Data is normalized for total cellular uptake using [3H]cAMP as described previously (15).

**Western Blotting**—Cellular proteins were resolved by SDS-polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose and then subjected to immunoblotting with appropriate GRK antisera (12, 16, 17).

**Cell Phosphorylation**—Cells were labeled with [32P]orthophosphate (66 μCi/ml) for 1 h in phosphate-free modified Eagle’s medium, 20 mM HEPES, pH 7.4, at 37 °C. Agonist was applied as indicated in figure legends. Treatment was stopped by placing the cells at 4 °C and washing with ice-cold phosphate-buffered saline (3 ml/well) twice and then adding 400 μl/well of radiolabeled precipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 μg/ml EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM disodium pyrophosphate, protease cocktail A, 10 μg/ml benzamidine) resuspended in 2 ml/well of 800 μl of 1.5 ml tubes on ice and rotated on an inversion wheel for 1 h. Solubilized material was transferred to Beckman TLA 100.2 tubes for centrifugation at 200,000 g for 15 min at 4 °C. The supernatant was transferred to 1.5-ml tubes on ice with 100 μl of protein A-Sepharose beads (Pharmacia Biotech Inc.) in 300 μl/well of 3 ml/well of 200 μl of centrifugation supernatant. The supernatant was transferred to 1.5-ml tubes on ice with 100 μl of protein A-Sepharose beads and 16 μg of monoclonal IgM-2F2-FLAG (Eastman Kodak Co.). Samples were placed on an inversion wheel at 4 °C. After 2 h, beads were pelleted, and supernatant was discarded. Beads were washed three times with ice-cold radioimmunoprecipitation buffer. SDS-polyacrylamide gel electrophoresis sample buffer was added to each sample to provide the same membrane protein/volume of sample for gel loading. Immune complexes were dissociated by heating to 65 °C for 10 min and resolved on a 1-mm thick, 10% SDS-polyacrylamide gel electrophoresis gel. Dried gels were analyzed quantitatively with a Molecular Dynamics PhosphorImager.

**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 phosphate-buffered saline. 5–10 ml of lysis buffer (10 mM Tris, 5 mM EDTA with protease inhibitors: 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 10 μg/ml benzamidine, 0.2 mM 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 at a 14,000,16,000 rpm. Material was centrifuged at 3000–4000 x g for 10 min to remove unlysed cells and nuclei. Supernatant was transferred to 15 × 100-mm tubes on ice and centrifuged at 15,000 rpm (40,000 x g) (Serval 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 (9). Briefly, using a constant amount of HEK 293 membrane protein, cold displacement (porcine secretin; Peninsula Labs) of 125I-porcine secretin binding was performed in duplicate 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 (9).
**RESULTS**

**Signaling and Desensitization of the Rat Secretin Receptor**—Binding studies with cell membranes prepared from HEK 293 cells transiently transfected with the native rat secretin receptor and the N-terminal FLAG secretin receptor cDNA demonstrated an identical K_d for secretin binding (2.3 nM) with a V_{max} of 0.5–1.0 pmol/mg of protein (data not shown). Ligand binding was specific and not significantly competed for by either excess glucagon or vasoactive intestinal polypeptide (data not shown). Secretin elicited dose-dependent whole cell cAMP accumulation, with an EC_{50} of 0.4 nM and 0.07 nM for the native and N-terminal FLAG-tagged rat secretin receptor, respectively, with identical V_{max} values (Fig. 1A). The rate of cAMP accumulation decreased with a half-time of 7 min, and no further cAMP accumulation occurred after 20 min (Fig. 1B). Cross-desensitization experiments using either vasoactive intestinal polypeptide or glucagon as the stimulus in cells coexpressing these receptors indicate that this desensitization is homologous (data not shown). Given the rapid nature of secretin receptor desensitization, receptor phosphorylation was examined as a likely mechanism for signal attenuation.

**Secretin Receptor Phosphorylation**—Studies of desensitization of the rhodopsin and adrenergic receptors have demonstrated the importance of receptor phosphorylation (2–4). The addition of the FLAG epitope to the N terminus of the rat secretin receptor provides the opportunity to demonstrate unequivocal secretin receptor protein phosphorylation by immunoprecipitation (Fig. 2, A and B). The major phosphorylated protein in receptor-expressing cells runs as a broad band of 55–65 kDa and is not present in immunoprecipitates from cells not expressing FLAG-secretin receptor. Agonist-dependent phosphorylation occurs with an EC_{50} of 14 nM (Fig. 2B). There is a small component of basal (agonist-independent) phosphorylation; however, agonist stimulation increased this signal 4–10-fold (Fig. 2A). The functional N-terminal FLAG-tagged receptor represents a suitable tool for studying specific secretin receptor protein phosphorylation.

**The Role of Second Messenger-dependent Protein Kinases in Secretin Receptor Desensitization/Phosphorylation in HEK 293 Cells**—Phosphorylation of Class I GPCRs can occur by second messenger-dependent mechanisms or by G protein-coupled receptor kinase activity (2, 4). In HEK 293 cells transiently transfected with the secretin receptor and pretreated with 30 μM H89, a PKA inhibitor, no significant enhancement in cAMP generation was observed. Similarly, 1 μM staurosporine, a PKC inhibitor, had no significant effect on cAMP accumulation (Fig. 3). Dose-response curves were generated to investigate the potential effects of these kinases over the full range of agonist concentrations. The EC_{50} for cAMP accumulation was 0.3 nM for secretin alone, or in the presence of H89 or staurosporine.
FIG. 3. cAMP accumulation in HEK 293 cells preincubated with PKA and PKC inhibitors. cAMP accumulation was performed in whole cell assays as described under “Experimental Procedures.” Secretin receptor-transfected cells were preincubated in serum-free modified Eagle’s medium, 10 mM HEPES, 1 mM isobutylmethylxanthine (assay medium) and then stimulated with secretin at the doses indicated. +H89- and + staurosporine-treated cells were preincubated with 30 μM H89 or 1 μM staurosporine in assay medium for 20 min and then stimulated with secretin in the continued presence of H89 or staurosporine at the secretin doses noted for 10 min. Data are means of three independent experiments, with each point performed in triplicate and normalized as a fraction of maximal. Maximal cAMP accumulation was determined in cells exposed to 1 μM secretin with no inhibitor preincubation for 10 min in assay medium. Experimental values for mean basal cAMP accumulation ± S.D. for three independent experiments (determined with 0.1 μM secretin) in secretin, +H89, and +staurosporine were 0.09 ± 0.05, 0.15 ± 0.11, and 0.12 ± 0.06 and for maximal secretin stimulation (determined at 1 μM secretin), 8.94 ± 0.47, 9.95 ± 0.46, and 9.92 ± 1.15, respectively.

and only a minor increase in the maximal cAMP, accumulation was evident at high agonist concentrations (Fig. 3). Under these conditions, H89 and staurosporine can effectively inhibit protein kinase A and C. Despite the lack of effect of PKA and PKC inhibition on secretin receptor signaling, the effect of the kinase inhibitors on receptor phosphorylation was examined. Interestingly, preincubation with 30 μM H89 and 1 μM staurosporine produced a 50% decrease in secretin receptor phosphorylation (Fig. 4, A and B). This effect was present whether cells were stimulated with high (0.1 μM) or low (1 nM) secretin concentrations for 2 min. These results suggest that in HEK cells, phosphorylation of the secretin receptor by PKA or PKC occurs but does not appreciably modulate its signaling efficiency. However, in COS 7 cells similarly transfected with the rat secretin receptor, preincubation with these protein kinase inhibitors significantly augmented secretin-stimulated cAMP accumulation, indicating that the second messenger-regulated protein kinases may be involved in receptor regulation in other cell types (data not shown). The ability of PKA and PKC to regulate secretin receptor signaling in COS 7, but not in HEK 293 cells, is likely due to the relatively low content of GRKs in COS 7 cells as compared with HEK 293 cells (18).

Role of G Protein-coupled Receptor Kinases in Secretin Receptor Desensitization and Phosphorylation in HEK 293 Cells—The lack of effect of PKA and PKC inhibitors on cAMP signaling in HEK 293 cells transfected with the secretin receptor suggests that GRKs might play the predominant role in receptor phosphorylation in these cells. This is supported by the short time course of desensitization and the fact that receptor phosphorylation occurred within 1 min of agonist stimulation (data not shown) (19). Therefore, we examined the potential involvement of G protein-coupled receptor kinases on both phosphorylation and signaling by co-transfecting HEK 293 cells with each of five different GRKs (GRK 2–6). Since GRK-mediated phosphorylation has been shown to occur within seconds to minutes, phosphorylation was examined at 2 min after agonist exposure. As shown in Fig. 5, secretin receptor phosphorylation varied by GRK subtype. GRKs 2, 3, and 5 significantly enhanced agonist-stimulated receptor protein phosphorylation. Expression of GRKs 3 and 5 increased phosphorylation up to -15 fold compared with the level apparent with endogenous cellular GRKs. However, overexpression of GRKs 4 and 6 did not significantly increase basal or agonist-stimulated secretin receptor phosphorylation (Fig. 5, A and B). GRK 5 was the only GRK to have an effect on basal phosphorylation (Fig. 5B). In the absence of agonist, overexpressed GRK 5 doubled basal secretin receptor phosphorylation. Coexpression of members of the GRK 2 subfamily (GRK 2 and 3) with the secretin receptor caused significant signal attenuation, reducing maximal cAMP accumulation by 39 and 26%, respectively (Fig. 6 and Table I). These GRKs also caused a shift in the secretin EC50 to the right (Fig. 6). The EC50 shifted from 0.48 nM for the native receptor to 0.69 nM with cotransfection of GRK 2 and 3, respectively (Fig. 6A, Table I). This is in contrast to GRKs 4 and 6, which had no significant effect on signal generation. The VMAX was 89 and 92% with EC50 0.78 and 0.85 nM for GRK 4 and 6, respectively (Table I). GRK 5 was the most potent of the GRKs evaluated, in reducing both VMAX (40% reduction) and shifting the EC50 (to 1.37 nM) (Fig. 6B, Table I).

FIG. 4. Secretin receptor phosphorylation by immunoprecipitation. HEK 293 cells overexpressing secretin receptor with preincubation of 30 μM H89 or 1 μM staurosporine (Stauro) or media (vehicle) receptor phosphorylation was determined as under “Experimental Procedures.” Preincubation was for 18 min, and stimulation with secretin (0.1 μM) was performed for 2 min. A, representative gel of receptor protein demonstrating basal (–) and agonist-stimulated (+) receptor phosphorylation. One band of precipitable radioreactivity is apparent in each treatment group corresponding to the size of the phosphorylated secretin receptor (50–60 kDa, Bio-Rad low range markers). B, phosphorImager analysis of two independent experiments. Data are mean ± S.E. and normalized to basal receptor phosphorylation in the absence of agonist and preincubation with vehicle (vehicle). Similar data were generated with 1 nM agonist stimulation (not shown).

DISCUSSION

In this paper we show that homologous desensitization of the secretin receptor, a Class II G protein-coupled receptor, is mediated by phosphorylation via G protein-coupled receptor kinases in HEK 293 cells. In these cells, PKA and PKC inhibitors did not significantly alter secretin receptor signaling. Direct assessment of phosphorylation of the secretin receptor was performed with a N-terminal FLAG-tagged rat secretin receptor, which retains the properties of the native receptor.
Receptor phosphorylation correlates with desensitization of cAMP signaling for all GRKs investigated. Agonist-dependent receptor phosphorylation occurs in the presence of PKA and PKC inhibition; moreover, these inhibitors do not appear to alter signal attenuation. These data suggest that receptors of the Class II family of G protein-coupled receptors can be regulated by mechanisms similar to those of the rhodopsin/β adrenergic (Class I) receptor family.

The native secretin receptor and the N-terminal FLAG-tagged secretin receptor bound ligand identically, consistent with the $K_d$ determined for the secretin receptor by others (6, 7). The N-terminal portion of the native secretin receptor has been shown to be important for ligand binding and signaling (20). It is therefore interesting that addition of the FLAG epitope does not alter ligand binding but does enhance receptor signaling. The additional FLAG residues caused a shift in the EC$_{50}$ to the left, indicating an increased ability to stimulate cAMP accumulation. The mechanism for this enhanced signaling is unclear. The presence of this epitope may alter the conformation of the native receptor or the agonist-bound receptor to promote signaling and adenylyl cyclase activation. Alterations in receptor sequence have been shown to result in both increased and decreased signaling (8, 20). Despite these subtle differences, the N-terminal FLAG-tagged receptor is an excellent tool for the study of secretin receptor phosphorylation, desensitization, and cellular trafficking.

Termination of G protein-coupled receptor-mediated signaling is facilitated by different mechanisms (3, 4, 19, 21). Evidence that phosphorylation plays a role in GPCR desensitization has been obtained from several members of the large Class I receptor family (11, 13, 19, 22). Phosphorylation of GPCRs has been shown to involve two types of serine/threonine protein kinases: second messenger-activated protein kinases (PKA and PKC) and the GRKs that phosphorylate agonist-occupied GPCRs (2–4). A family of these GRKs exist that have been shown to phosphorylate GPCRs but differ significantly in their mechanisms of membrane association and/or activation (3, 4).

![Figure 5](image1.png)

**FIG. 5.** Secretin receptor phosphorylation by immunoprecipitation. HEK 293 cells overexpressing either no exogenous GRK or secretin receptor (Control) or cells overexpressing the secretin receptor plus one of GRK 2, 3, 4, 5, or 6. Receptor phosphorylation was determined as under "Experimental Procedures." Whole cell stimulation with secretin was performed for 2 min. A, representative gel of receptor protein demonstrating basal (−) and agonist-stimulated (+) receptor phosphorylation. One band of precipitable radioactivity is apparent in each treatment group corresponding to the size of the phosphorylated secretin receptor (50–60 kDa, Bio-Rad-Low range markers). B, PhosphorImager analysis of three independent experiments. Data are mean ± S.E. and normalized to basal receptor phosphorylation in the absence of agonist and no overexpressed GRK (Empty).

![Figure 6](image2.png)

**FIG. 6.** cAMP accumulation in HEK 293 cells cotransfected with empty vector or with GRKs. cAMP accumulation was measured as under "Experimental Procedures." Whole cell stimulation with secretin was performed at doses indicated for 2 min. All curves represent three independent experiments, with each experimental point performed in triplicate. Data are mean ± S.E. and normalized to maximal stimulation, determined with secretin receptor cotransfected with empty vector and stimulated with 1 μM secretin. A, secretin receptor-only transfected cells compared with cells cotransfected with 5 μg of GRK 2 or GRK 3. B, secretin receptor-only transfected cells compared with cells cotransfected with 5 μg of GRK 4, GRK 5, or GRK 6. Experimental values for mean cAMP accumulation for basal and maximal stimulation for the three independent experiments (determined with 0.1 μM and 1 μM secretin, respectively) for secretin, GRK 2, GRK 3, GRK 4, GRK 5, and GRK 6 were 0.05, 2.52; 0.03, 1.86; 0.04, 2.19; 0.07, 2.63; 0.04, 1.70; 0.05, 2.97.

**TABLE I**

| Transfection | EC$_{50}$ (95% CI) | V$_{max}$ (±S.E.) |
|--------------|------------------|------------------|
| + Empty vector | 0.48 (0.31–0.73) | 0.92 (±0.03) |
| + GRK 2 | 0.70 (0.42–1.13) | 0.61 (±0.02) |
| + GRK 3 | 1.28 (0.57–1.88) | 0.74 (±0.02) |
| + GRK 4 | 0.78 (0.42–1.47) | 0.92 (±0.04) |
| + GRK 5 | 1.37 (0.95–1.97) | 0.60 (±0.02) |
| + GRK 6 | 0.85 (0.49–1.47) | 0.89 (±0.04) |
Here we show that in HEK 293 cells transfected with the secretin receptor, desensitization of the secretin-mediated signal is rapid and accompanied by significant phosphorylation of the receptor protein. The time course for the attenuation of the signal is consistent with that which has been shown for the \( \beta_2 \)-adrenergic receptor in such cells as the BEAS-2B cells (human airway epithelial cells) but is slower than that found in Chinese hamster ovary cells permanently expressing the secretin receptor (8, 23). We have also noted that kinetic differences exist between HEK 293 cells and COS 7 cells transiently expressing the rat secretin receptor. These slower rates in Cos 7 cells are consistent with recent findings from our laboratory that demonstrated that the complement of GRKs in COS 7 cells is significantly lower than that of many other cell lines (18).

That secretin receptor signaling in HEK 293 cells is modulated by GRK-mediated phosphorylation is supported by two lines of evidence. First, inhibitors of PKA and PKC had essentially no effect on the secretin-mediated accumulation of cAMP, suggesting these kinases do not play a dominant role in regulating the receptor signaling function in HEK 293 cells. PKA has been shown to be effective in the desensitization of the D1\( _A \) dopamine and the \( \beta \)-adrenergic receptor (22). The effectiveness of PKA and PKC in 293 cells is evident in the reduction in receptor phosphorylation by inhibitors (Fig. 4). The role of second messenger-regulated phosphorylation of the receptor in 293 cells is unknown. Second, overexpression of specific GRKs enhanced the blunting of the receptor response as well as increased receptor phosphorylation. Among the GRKs tested, GRK 2, 3, and 5 produced the largest enhancement of desensitization and the greatest enhancement of phosphorylation. On the other hand, GRKs 4 and 6 were without effect on signaling and did not cause any significant increase in receptor phosphorylation over endogenous kinases. These data are reminiscent of data obtained with adrenergic, dopaminergic, and angiotensin receptors (13, 22, 24) in which preferential action of the various GRKs has been demonstrated. GRK mediated inhibition of the secretin-dependent attenuation of the receptor response and phosphorylation in HEK 293 cells is consistent with the rapid time course of receptor desensitization. The half-time for the GRK-mediated desensitization of the \( \beta_2 \)-adrenergic receptor is 15 s, whereas that for the PKA-mediated response is several-fold slower (19, 22, 25). The role of receptor sequestration in desensitization has been reviewed recently (21). Sequestration is enhanced by phosphorylation in the adrenergic receptor family (21). Previous work on the \( \beta_2 \)-adrenergic and the secretin receptors demonstrate only 10–15% surface receptor loss by 2–3 min of agonist exposure (23). Also, using a fluorescent secretin agonist, Holtmann et al. (8) demonstrated that label remained at the cell surface after 1 min of agonist stimulation. Our desensitization and phosphorylation experiments were performed at 2 min, and sequestration is likely not a predominant feature of this signal attenuation. These data support a role for GRKs in the desensitization process. The use of specific GRK phosphorylation inhibitors was attempted (K220R and a C-terminal GRK 2 construct); however, the effect of agonist-induced phosphorylation is so dramatic (up to 15-fold stimulation over basal), we were unable to block the effect of endogenous kinase activity. Mutation of the phosphorylation sites may provide further information; however, the secretin receptor contains no less than 22 putative phosphorylation residues in its intracellular domains. Perturbation of all these sites would require characterization of receptor binding, signaling, and desensitization properties of all these mutants. Additionally, site-directed mutagenesis is known to alter the conformation of receptor proteins, and this may alter phosphorylation and/or desensitization.

The direct demonstration of secretin receptor phosphorylation by specific immunoprecipitation of an N-terminal FLAG-tagged receptor correlates well with previous data by Ozcelebi et al. (7) on agonist-dependent phosphorylation of a protein, with the size expected for the transfected secretin receptor in COS 7 cells and Chinese hamster ovary cells. The effect of overexpressed GRK 2, 3 and 5 on the agonist-mediated response and consequent phosphorylation, implies a role for phosphorylation by these kinases in signal termination of the secretin receptor. Holtmann et al. (8) have proposed internalization of the secretin receptor as the main mode of desensitization rather than receptor phosphorylation. This contention was based on the observation that in a stimulation/restimulation protocol, a C-terminal-truncated mutant secretin receptor was capable of desensitizing and internalizing a fluorescently labeled agonist similar to the wild-type receptor. However, interpretation of these results may have been confounded by the incomplete removal of bound agonist before restimulation (8). Whereas sequestration/internalization is an important phenomenon in the desensitization process, evidence suggests that it plays a more important role in the GPCR resensitization process (21). Indeed, phosphorylation of GPCRs facilitates the interaction of the phosphorylated receptors with arrestin proteins, which uncouple the receptor/G-protein interaction. At the same time though, phosphorylation and arrestin protein binding provide a trigger for phosphorylated receptor internalization and resensitization (21). Thus, phosphorylation and arrestin binding play a dual role in that not only do they uncouple the response but also act as a trigger or adaptor to mediate the resensitization process (21).

G protein-coupled receptors are ubiquitous. Presently GPCRs are known to be activated by peptides and protein hormones, monoamine and amino neurotransmitters, calcium ions, light photons, tastants, and odorants. To ensure the stability of the cellular environment, cells must be able to terminate signals in a timely manner to prevent overstimulation and to be prepared for the acquisition of new information. The secretin receptor has an important role in many physiologic processes of the gastrointestinal tract, pancreas, and biliary epithelium (26, 27). Understanding the role of GRKs in Class II receptor signal transduction should provide insight into the regulation of pancreatic fluid secretion, bile flow, and other gastrointestinal neuroendocrine responses.

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A Role for Receptor Kinases in the Regulation of Class II G Protein-coupled Receptors: PHOSPHORYLATION AND DESENSITIZATION OF THE SECRETIN RECEPTOR

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