The vasoactive intestinal polypeptide type-1 (VPAC\textsubscript{1}) receptor is a class II G protein-coupled receptor, distinct from the adrenergic receptor superfamily. The mechanisms involved in the regulation of the VPAC\textsubscript{1} receptor are largely unknown. We examined agonist-dependent VPAC\textsubscript{1} receptor signaling, phosphorylation, desensitization, and sequestration in human embryonic kidney 293 cells. Agonist stimulation of cells overexpressing this receptor led to a dose-dependent increase in cAMP that peaked within 5–10 min and was completely desensitized after 20 min. Cells cotransfected with the VPAC\textsubscript{1} receptor (VPAC\textsubscript{1}R) and G protein-coupled receptor kinases (GRKs) 2, 3, 5, and 6 exhibited enhanced desensitization that was not evident with GRK 4. Immunoprecipitation of the epitope-tagged VPAC\textsubscript{1} receptor revealed dose-dependent phosphorylation that was increased with cotransfection of any GRK. Agonist-stimulated internalization of the VPAC\textsubscript{1}R peaked in 10 min, and neither overexpressed β-arrestin nor its dominant-negative mutant altered internalization. However, a dynamin-dominant negative mutant did inhibit VPAC\textsubscript{1} receptor internalization. Interestingly, VPAC\textsubscript{1}R specificity in desensitization was not evident by study of the overexpressed receptor; however, we determined that human embryonic kidney 293 cells express an endogenous VPAC\textsubscript{1} receptor (VPAC\textsubscript{1}R) that did demonstrate dose-dependent GRK specificity. Therefore, VPAC\textsubscript{1} receptor regulation involves agonist-stimulated, GRK-mediated phosphorylation, β-arrestin translocation, and dynamin-dependent receptor internalization. Moreover, study of endogenously expressed receptors may provide information not evident in overexpressed systems.

The neuromodulator vasoactive intestinal polypeptide (VIP)\textsuperscript{1} is a potent vasodilator and has been shown to participate in regulating gastrointestinal motility, enzyme secretion, and blood flow (1–3). The type-1 VIP (VPAC\textsubscript{1}) receptor is a member of a family of G protein-coupled receptors (GPCRs), designated as class II. These receptors share a significant degree of sequence homology within the family (>50%), but are distinct from members of the larger rhodopsin/adrenergic receptor family (class I) (4). GPCRs are membrane proteins characterized by seven transmembrane-spanning domains and are named for their functional interaction with heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins). Agonist-activated GPCRs transduce extracellular signals into intracellular events through activation of G protein-regulated second messenger pathways or ion channels. Agonist activation of GPCRs also leads to the competing process whereby uncoupling of the receptor from its G protein results in attenuation, or desensitization, of signaling events (5). An important process in the desensitization of GPCRs is the phosphorylation of agonist-occupied receptors, followed by receptor internalization and, finally, eventual recycling to the plasma membrane as competent receptors (5).

G protein-coupled receptor kinases (GRKs) contribute to desensitization of GPCRs by phosphorylating agonist-activated receptors (6). Second messenger-dependent protein kinases, such as cAMP-dependent protein kinase and protein kinase C, can also phosphorylate GPCRs and dampen signaling; however, these processes are independent of receptor occupancy. GRK-mediated receptor phosphorylation promotes subsequent binding of arrestin proteins. Arrestins are cytosolic proteins that bind GRK-phosphorylated receptors to prevent G protein coupling, thereby quenching intracellular signaling, and that target GPCRs to clathrin-coated pits for internalization/sequestration, dephosphorylation and recycling (7). The mechanisms regulating these various processes are critical to the normal function of GPCRs.

We have previously demonstrated that the secretin receptor, also a class II GPCR, is regulated differently from many class I receptors (8, 9). Like the β\textsubscript{2}AR (class I), phosphorylation and desensitization of the secretin receptor was promoted by GRKs (9); however, unlike the β\textsubscript{2}AR, sequestration of secretin receptors was not increased by GRK overexpression nor was sequestration inhibited by a dominant negative (V53D) β-arrestin mutant (8). Instead, second messenger-dependent phosphorylation was important for sequestration of the secretin receptor, whereas GRKs and β-arrestin are critical to the internalization of the β\textsubscript{2}AR (class I) (8, 10).

In this paper we probe the roles of receptor phosphorylation by GRKs and β-arrestin recruitment in the regulation of VPAC\textsubscript{1} receptor signaling, desensitization, and sequestration. Moreover, we determined that human embryonic kidney (HEK 293) cells express an endogenous VPAC\textsubscript{1} receptor and used this receptor to demonstrate GRK specificity that was not evident.
in overexpressed systems. These findings suggest that the VPAC1 receptor is regulated by agonist-stimulated, GRK-mediated receptor phosphorylation, β-arrestin translocation, and dynamin-dependent receptor internalization likely via clathrin-coated pits.

EXPERIMENTAL PROCEDURES

Materials—General chemicals and reagents were from Sigma. Vasointestinal polypeptide was obtained from Peninsula Laboratories. HEK 293 cells were obtained from the American Type Culture Collection. Tissue culture supplies were obtained from Invitrogen. 

Plasmid Preparation—The full-length nucleotide sequence of the rat VPAC1 receptor (12) was amplified from rat heart cDNA by PCR using gene-specific oligonucleotides. An epitope-tagged rat VPAC1 receptor was provided for the study by Dr. H. Koh (12). The FLAG epitope was placed on the N-terminal region of the mature receptor following a modified influenza hemagglutinin signal sequence to produce a protein that could be recognized with commercially available anti-FLAG antibodies. The cDNAs were inserted into the pcDNA 1/Amp plasmid (Invitrogen) using HindIII and XhoI. Fidelity was demonstrated by dideoxy sequencing. GRK cDNAs were produced as previously described: GRKs 2 and 3 (13), GRK 4 (14), GRK 5 (15), and GRK 6 (16). β-Arrestin-1 and dynamin, as well as their dominant negative mutants, were used as previously described (8). β-Arrestin-green fluorescent protein (GFP) was produced as outlined by Barak et al. (17).

Cell Culture—HEK 293 cells were grown in modified Eagle’s medium (MEM), 10% fetal bovine serum (FBS), and 50 mg/liter gentamicin at 37 °C prior to experimentation. Labeling medium was prepared and identified by high performance liquid chromatography (9, 11). [3H]Adenine, [3H]-AMP, [3H]-[35S]deoxyadenosine 5′-O-(α-thio)triphosphate, and [32P]orthophosphate were obtained from PerkinElmer Life Sciences. Restriction enzymes were from Promega. Sequencing supplies were from United States Biochemical Corp./Amersham Biosciences. Polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR) materials were from PerkinElmer Life Sciences. Primers were obtained from Genosys.

Membrane Preparation/Binding—All steps were performed at 4 °C. Cells were aspirated, and cells were washed with 1 ml of PBS and preincubated in HEPES-buffered saline (0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na3PO4, pH 7.1) was added to the tube and mixed well. This mixture was added dropwise to the 100-mm dish of cells. The accumulation of cAMP in intact cells was quantified chromatographically by the method of Salomon (18).

Transfection—Transient transfections were performed with calcium phosphate co-precipitation. One to 10 μg of vector DNA was transferred into a 6-m1 Falcon tube with 450 μl of sterile water and 50 μl of 2.5 M CaCl2. One day after transfection, cells were split into appropriate plates following trypsin dissociation. Experiments were performed 24–48 h after transfection.

Membrane Preparation/Binding—All steps were performed at 4 °C. Plates were placed on ice, media aspirated, and cells washed with 10 ml of ice-cold PBS. Five to 10 ml of lysis buffer (10 ml Tris, 5 ml EDTA with protease inhibitors: 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.2 μg/ml benzamidine, and 0.25 mM sodium metabisulfite) 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 cps. 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) (Sorvall 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 (11). Briefly, using constant amount of HEK 293 membrane protein, competition displacement (using synthetic vasointestinal polypeptide, Peninsula Laboratories) of [35S]VIP binding was performed in triplicate tubes. Non-specific binding was defined in the presence of 1 μM unlabeled VIP. Data were analyzed using Graph Pad-Prism and LIGAND software as described (9, 11).

Adenyl Cyclase Assays—The accumulation of cAMP in intact cells was quantified chromatographically by the method of Salomon (18). Cells transiently transfected with the VPAC1 receptor or untransfected cells were washed with wash buffer (2–3 × 106 cells/1 ml) and labeled with [3H]adenine (1 μCi/ml) in MEM, 5% FBS, 50 mg/liter gentamicin (1 ml/well) for 12–16 h prior to experimentation. Labeling medium was aspirated, and cells were washed with 1 ml of PBS and preincubated in assay medium (1 ml/well, MEM, 0% FBS, 10 μl HEPES, 1 ml isobutylmethylamine) for 15–30 min. Cells were stimulated with appropriate agonist, and, at the end of the experimental duration, medium was aspirated and 1 ml of ice-cold stop solution (0.1 ml cAMP, 4 nCi/ml [3H]-AMP, 2.5% perchloric acid) was added to each well. Plates remained at 4 °C for 20–30 min, after which solution was transferred to 12 × 75-mm tubes containing 100 μl of 4.2 M KOH. Tubes were vortexed and stored at 4 °C for cAMP determination by column chromatography (19). Data were normalized to basal levels and using [3H]-cAMP for column efficiency as previously described (18).

Receptor Internalization by Immunofluorescence—HEK 293 cells transiently transfected with 5 μg of cDNA for FLAG-tagged VPAC1 receptor were plated onto 35-mm dishes containing a central glass well as described (17). Cells were maintained at 4 °C to prevent receptor internalization with agonist (100 nM VIP), primary antibody (IgG-M2-FLAG, Eastman Kodak Co.) and secondary antibody (Fab conjugated with fluorescein isothiocyanate, Organon Teknika). Sequential incubations were 30 min in duration and occurred in the sequence listed. Cells were washed three times with cold PBS after each antibody application. Immediately following the last PBS wash, cells were viewed using confocal microscopy (basal time point), whereas a second plate of identically treated cells was warmed at 37 °C for 1 h prior to imaging (60 min of treatment).

Immunofluorescent Colocalization of VIP Receptor with β-Arrestin-GFP—HEK 293 cells transiently transfected with 5 μg of cDNA for wild-type VIP receptor were plated onto glass coverslips contained in six-well plates. After an initial wash, cells were stimulated with 100 nM VIP for only 2 min. Cells were washed with 1 ml of ice-cold PBS, aspirated, and cells were washed with 1 ml of ice-cold stop solution (0.1 ml cAMP, 4 nCi/ml [3H]-AMP, 2.5% perchloric acid) and added to each well. Plates remained at 4 °C for 20–30 min, after which solution was transferred to 12 × 75-mm tubes containing 100 μl of 4.2 M KOH. Tubes were vortexed and stored at 4 °C for cAMP determination by column chromatography (19). Data were normalized to basal levels and using [3H]-cAMP for column efficiency as previously described (18).

Immunoprecipitation of the VIP Receptor with β-Arrestin—HEK 293 cells transiently transfected with 3 μg of cDNA for FLAG-tagged wild-type VIP receptor and 2 μg of cDNA for β-arrestin were treated with medium alone or stimulated with 100 nM VIP in a 37 °C incubator for 5, 10, or 20 min. One plate of HEK 293 cells was transfected only with 2 μg of cDNA β-arrestin and served as the negative control. After stimulation, cells were lysed with lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 20 μg/ml aprotinin, 0.1% Triton X-100) and protein samples from each sample were prepared as described (17). Cells were 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 VPAC1 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 (8). Cells were plated to a density of ~1.5 × 106 cells per well and exposed to agonist for the period of time indicated after washing cells with wash buffer. After washing, cells were exposed for 1 h to monoclonal IgG-M2-FLAG antibody (1:600 dilution, Kodak), washed three times with PBS, and detected with Fc specific, fluorescein-labeled goat anti-mouse antibody (1:200 dilution, Sigma). Cells were 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 transfected with the VPAC1 receptor, not exposed to agonist and another sample not exposed to primary antibody (IgG-M2-FLAG or 12CA5). Baseline fluorescence was subtracted from each sample.

Using RT-PCR to Identify the VIP Receptor Endogenously Expressed in HEK 293 Cells—RNA was prepared using RNAzol (Tel-Test, Inc., Friendswood, TX) and RT-PCR was performed using a PerkinElmer Life Sciences MMLV kit following the manufacturers’ protocols. Primers were designed to distinguish between the two subtypes of VIP receptors as follows: human VPAC1 receptor 463 TTTCTCCTGCCTGCTAATGTTGTC), and human VPAC2 receptor 414 TTCACTTCAGGCTTAA–––GCCACCCT––––AATTGTCC), and human VPAC2 receptor 414 TTCACTTCAGGCTTAA–––GCCACCCT––––AATTGTCC), and human VPAC2 receptor 414 TTCACTTCAGGCTTAA–––GCCACCCT––––AATTGTCC), and human VPAC2 receptor 414 TTCACTTCAGGCTTAA–––GCCACCCT––––AATTGTCC), and human VPAC2 receptor 414 TTCACTTCAGGCTTAA–––GCCACCCT––––AATTGTCC). Primer sequences for VPAC1 receptors were designed specifically to discriminate between the human VPAC1 and VPAC2 receptors. Using these primers and RT-PCR, we determined that HEK 293 cells endogenously express only the VPAC1 receptor. We then investigated the role of GRKs in the regulation of the endogenous VPAC1 receptor. To determine the specificity of GRK-dependent regulation of the endogenous VPAC1 receptor, we overexpressed GRKs 4, 5 and 6 in HEK 293 cells. As shown in Fig. 5A, overexpressing various amounts of GRK 5 or 6 caused attenuation of cAMP accumulation in response to agonist (Fig. 1A). The time-course experiments revealed a rapid increase in cAMP accumulation that declined with a t½ of 5 min, yielding complete cessation of further cAMP accumulation after ~10–15 min of agonist exposure, for both receptors when overexpressed in HEK 293 cells (Fig. 1B). By fluorescence-activated cell sorting analysis, the epitope-tagged VPAC1 receptor demonstrated a concomitantly rapid time course of internalization, with a t½ of 5 min and maximum loss of 50% by 15–20 min (Fig. 1C). Because the FLAG-tagged VIP type 1 receptor construct was similar to that of the wild-type receptor, we employed the FLAG tagged receptor to investigate VPAC1 regulation.

Vasoactive Intestinal Polypeptide (VIP) Receptor Phosphorylation—Receptor phosphorylation has been found to be important in the regulation of many GPCRs (6, 21). Using the epitope-tagged VPAC1 receptor, we observed a dose-dependent VPAC1 phosphorylation in response to agonist (Fig. 2A). The major phosphorylated protein in receptor-expressing cells migrated as a broad band of 60–80 kDa and is not present in immunoprecipitates from cells not expressing the epitope-tagged VPAC1 (Fig. 2B, far right lanes). Agonist-dependent receptor phosphorylation occurs with an EC50 of 1.1 nM (Fig. 2A). Interestingly, in cells not stimulated with agonist, there is a significant amount of basal receptor phosphorylation.

The Role of G Protein-coupled Receptor Kinases in VPAC1 Receptor Desensitization and Phosphorylation—We have previously shown that the secretin receptor, another class II GPCR, exhibits GRK specificity in its desensitization. In HEK 293 cells, GRKs 2, 3, and 5 attenuate secretin receptor signaling; however, GRKs 4 and 6 do not (9). VIP receptor signaling was attenuated in HEK 293 cells overexpressing FLAG-tagged VIP type 1 receptor by GRKs 2, 3, 5, and 6, as measured by cAMP accumulation after exposure to agonist for 10 min (Fig. 3, A and B). GRK 4 was relatively ineffective in diminishing VPAC1 signaling (Fig. 3B). Using immunoprecipitation of the receptor, we studied the effect of overexpressed GRKs on VPAC1 phosphorylation. In contrast to the secretin receptor, all the GRKs produced an increase in VPAC1 receptor phosphorylation (Fig. 4, A and B). PhosphorImager analysis of immunoprecipitated VPAC1 demonstrated that all the GRKs increased receptor phosphorylation 3–4-fold following stimulation with agonist for 10 min. The VPAC1 receptor undergoes a variable amount of GRK-dependent basal phosphorylation that hinders comparison of GRK specificity in cells overexpressing the rat VPAC1 receptor (Fig. 4).

The Role of G Protein-coupled Receptor Kinases 4, 5, and 6 in the Desensitization of the Endogenous VPAC1 Receptor in HEK 293 Cells—We designed primers specifically to discriminate between the human VPAC1 and VPAC2 receptors. Using these primers and RT-PCR, we determined that HEK 293 cells endogenously express only the VPAC1 receptor. We then investigated the role of GRKs in the regulation of the endogenous VPAC1 receptor. To determine the specificity of GRK-dependent regulation of the endogenous VPAC1, we overexpressed GRKs 4, 5, and 6 in HEK 293 cells. As shown in Fig. 5A, overexpressing various amounts of GRK 5 or 6 caused attenuation of cAMP accumulation in response to VIP. The effects of GRKs 5 and 6 were dose-dependent, and decreasing amounts of DNA transfected corresponded to decreased levels of expressed proteins (Fig. 5B). GRK 4 did not cause a significant change in cAMP accumulation, even at very high doses of transfected DNA and expressed protein.

The Role of β-Arrestin and Dynamin in VPAC1 Sequestration—β-Arrestin binds preferentially to phosphorylated receptor and promotes receptor internalization (22).
Endogenous VPAC₁R Regulation

β-arrestin on VPAC₁R signaling was initially studied on the endogenous VPAC₁R in HEK 293 cells overexpressing β-arrestin 1 and 2. As shown in Fig. 6, β-arrestins 1 and 2 decreased agonist-stimulated VPAC₁R cAMP accumulation by 27 and 39% of control levels, respectively. Neither arrestin construct caused a significant shift in EC₅₀.

To further examine the role of β-arrestin in VPAC₁ receptor internalization, we used transiently transfected HEK 293 cells overexpressing the FLAG-tagged VPAC₁ receptor and β-arrestin-1, dynamin I, or their respective dominant negative mutants, β-arrestin V53D or dynamin I K44A. Similar to results with the β₂-adrenergic receptor, overexpression of neither β-arrestin nor dynamin altered VPAC₁ receptor internalization when quantitated by fluorescence-activated cell sorting analysis as loss of cell surface receptor (Fig. 7). However, when the dominant negative constructs (β-arrestin V53D, dynamin I K44A) were overexpressed, both decreased internalization of the β₂-adrenergic receptor, but only dynamin K44A reduced VPAC₁ receptor endocytosis (Fig. 7). Although the VPAC₁R behaves differently from the β₂-adrenergic receptor, other receptors have shown this variance (8, 23, 24). This difference may be caused in part by different receptor affinities for β-arrestin. If the affinity of the VPAC₁ receptor for β-arrestin is significantly higher than that of the β₂-adrenergic receptor, then the overexpressed V53D protein may not be able to compete with endogenous β-arrestin and the endogenous β-arrestin may be able to target the receptor to clathrin-coated pits. In an attempt to resolve this, we performed additional experiments in COS 7 cells. These cells are known to have less β-arrestin than HEK 293 cells. Sequestration of the VPAC₁R in these cells revealed

Fig. 1. Signaling, desensitization, and sequestration of the wild-type rat VPAC₁R and N-terminal epitope-tagged rat VPAC₁R in transiently transfected HEK 293 cells. A, receptor signaling was determined by whole cell cAMP assays of transiently transfected HEK 293 cells as described under “Experimental Procedures.” Dose-response curves were performed on cells exposed to indicated concentrations of VIP for 10 min. Data are not normalized. EC₅₀ for the wild-type and FLAG-VPAC₁ receptor was 0.23 and 0.18 nM, respectively. B, desensitization of VIP-stimulated signal transduction. Time course of cAMP accumulation in transiently transfected HEK 293 cells expressing the wild-type and FLAG-tagged VIP receptors. Data are not normalized. C, sequestration of VIP receptor after agonist exposure. HEK 293 cells were transiently transfected with the FLAG-tagged VIP receptor. Data were normalized to surface receptor expression in cells not exposed to agonist. Each point represents the mean of three independent experiments, each with triplicate samples per time point. Data represent mean ± S.E. for each point.

Fig. 2. Agonist-stimulated FLAG-tagged vasoactive intestinal polypeptide receptor phosphorylation. Phosphorylation experiments were performed as noted under “Experimental Procedures.” Data were quantitated densitometrically using a Molecular Dynamics PhosphorImager. A, dose-response curve generated from four independent experiments. Agonist-dependent phosphorylation exhibited an EC₅₀ of 1.1 nM. Maximal phosphorylation was determined with 1 μM VIP for 15 min. Agonist stimulation increased phosphorylation 2–4-fold over basal. B, representative gel from one experiment. 0 represents no agonist; other numbers denote the log molar concentration of VIP. Numbers to the left are molecular mass in kilodaltons. The far right lanes (C, control) represent cells mock-transfected and exposed to agonist.
a similar pattern (data not shown). To investigate the role of β-arrestin in VPAC1 receptor internalization, we used immunofluorescence to study the fate of the VPAC1 receptor and β-arrestin after agonist stimulation.

β-Arrestin Translocation, Vasosative Intestinal Polypeptide Receptor Sequestration, and VPAC1R/β-Arrestin Colocalization—We stimulated HEK 293 cells overexpressing the VPAC1 receptor and observed rapid translocation of a β-arrestin-GFP fusion protein from the cytosol to the plasma membrane (Fig. 8A). β-Arrestin-GFP translocated from the cytosol to the plasma membrane robustly after stimulation with VIP, but only in cells overexpressing the VPAC1R. To determine more directly whether β-arrestin is targeted to the VPAC1 receptor, we transfected HEK 293 cells with the epitope-tagged VPAC1 receptor and β-arrestin-GFP. After 1 min of agonist exposure, the β-arrestin-GFP was found co-localized with the VPAC1 receptor at the cell membrane (Fig. 8B).

DISCUSSION

Although class II GPCRs are abundant and involved in the regulation of a variety of physiological processes, information on their regulation lags for the larger class I rhodopsin/adrenergic family of receptors. Study of GPCRs has relied on overexpressed heterologous cell systems that provide a controlled manner to investigate various aspects of receptor signal regulation. However, in some cases the overexpressed cell system may obscure molecular determinants involved in specific receptor regulation. For example, overexpression of a receptor not normally found in a specific cell type may not recapitulate receptor regulation in vivo. Similarly, receptor overexpression may alter endogenous regulatory proteins and either increase or decrease the phosphorylation of the receptor under study. Therefore, study of cell systems with endogenous receptor expression may provide the opportunity to ask specific questions not possible in the heterologous system.

Using the VPAC1R, we have investigated its regulation by overexpression in HEK 293 cells and by studying an endogenously expressed receptor. Placement of the FLAG epitope at the N terminus of VPAC1R did not significantly alter receptor binding or signaling properties, providing a useful tool in the study of VPAC1 receptor regulation. Prior investigators have noted diminished agonist binding to the VPAC1 with mutation of the N terminus (25, 26). We placed the epitope at the amino
notable that, although GRK 4 can phosphorylate VPAC1 receptor, GRKs 5 and 6, but not GRK 4, desensitize it preferentially. It is ever, when studied in an endogenously expressed fashion, phosphorylated and desensitized by GRKs 2, 3, and 5. How-

![Image](image_url)

**FIG. 5.** Endogenous VPAC1-stimulated cAMP accumulation in HEK 293 cells overexpressing GRK 4, 5, or 6. A, HEK 293 cells were transfected with GRK 4, 5, or 6 and stimulated with VIP (1 μM) for 10 min. No exogenous receptor was transfected in these experiments. Transient transfections of the various GRKs were performed using the amount of DNA noted in the figure (0.003–3 μg). cAMP accumulation was determined as noted under “Experimental Procedures” and is the mean ± S.E. of three independent experiments, each performed in triplicate. The 293 bar and the Empty bar represent the response of untransfected HEK 293 cells and HEK 293 cells transfected with empty vector to 1 μM VIP for 10 min, respectively. B, representative Western blot of G protein-coupled receptor kinase 5 overexpression in HEK 293 cells. HEK 293 cells were transfected with GRK 4, 5, or 6 and demonstrated dose-dependent expression. Transient transfections of the various GRKs were performed using the amount of DNA noted in the figure (0.003–3 μg) without any exogenous VPAC1 receptor. Proteins were resolved by SDS-polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose and immunoblotted with appropriate GRK antibodies (see “Experimental Procedures”).

terminus of the processed portion of the mature receptor to minimize interactions with sites important for agonist binding. The VPAC1R, like all class II receptors, is coupled to Gs and activates adenylyl cyclase, and appears to follow the paradigm of regulation established for class I GPCRs (27). In this paper we have demonstrated that each member of the GRK family can phosphorylate and desensitize the VPAC1R. The VPAC1R was not overexpressed in these experiments. In that study the authors demonstrated that the VPAC1R was desensitized and phosphorylated by a kinase sensitive to the cAMP-dependent protein kinase inhibitor bisindolylmaleimide (29). These authors postulated a role for GRKs in the phosphorylation and desensitization of the VPAC1R. The VPAC1R is desensitized and phosphorylated by a kinase sensitive to the cAMP-dependent protein kinase inhibitor bisindolylmaleimide (29). These authors postulated a role for GRKs in the phosphorylation and desensitization of the VPAC1R. The VPAC1R was not overexpressed in these experiments. How-

![Image](image_url)

**FIG. 6.** Effect of β-arrestin on endogenous VPAC1R-stimulated cAMP accumulation in HEK 293 cells not overexpressing the VPAC1R. HEK 293 cells overexpressing either β-arrestin 1 or β-arrestin 2 alone were exposed, in a dose-dependent manner, to VIP for 10 min to determine their ability to attenuate signaling of the endogenous VPAC1R. The VPAC1R was not overexpressed in these experiments. β-Arrestin 1 elicited a 27% reduction, whereas β-arrestin 2 reduced maximal cAMP levels by 39% of control. However, neither arrestin construct caused a significant shift in the EC50 for cAMP accumulation. Data are mean ± S.E. of three independent experiments, each done in triplicate.

![Image](image_url)

**FIG. 7.** Effect of β-arrestin and dynamin on VPAC1R internalization. HEK 293 cells were transiently transfected with wild-type VPAC1 and β-arrestin 1 mutant (V53D), dynamin, or the dominant-negative dynamin mutant (K44A) on the agonist promoted sequestration of the VPAC1 and the β2AR as assessed by flow cytometry. Flag-tagged VIP receptor or hemagglutinin-tagged β2AR was transiently transfected in HEK 293 cells with 5 μg of the following: empty vector (Empty), pCMV rat β-arrestin 1 (barr1), pcDNA1-AMP rat β-arrestin-1-V53D (V53D) (10), 8 μg of pCB1 rat dynamin 1 (Dynamin), or 8 μg of rat dynamin 1-K44A (K44A). Expression of mutant and wild-type β-arrestin-1 was monitored by immunoblot using an antibody for dynamin 1 (33). The data represent the mean ± S.E. of at least three independent experiments, with each point done in duplicate, for each group.

regulation of the related secretin receptor, where both GRK 4 and GRK 6 appear incapable of phosphorylating or desensitizing this receptor (9). Thus, VPAC1 receptor is among the relatively few receptors that have been shown to be regulated by GRK 6 (27).

Phosphorylation and desensitization of the human VIP type 2 (VPAC2) receptor has been demonstrated in response to agonist (29). In that study the authors demonstrated that the VPAC2 receptor is desensitized and phosphorylated by a kinase sensitive to the cAMP-dependent protein kinase inhibitor bisindolylmaleimide (29). These authors postulated a role for GRK-dependent receptor phosphorylation; however, the role of GRKs in the phosphorylation and desensitization of the VPAC2 receptor has not been reported. In this paper we have demonstrated that each member of the GRK family can phosphorylate
the VPAC₁ receptor. However, overexpression of the VPAC₁ receptor produced a significant amount of basal phosphorylation, and this may make studies on GRK-specific receptor phosphorylation less sensitive when tested in heterologous cell systems. HEK 293 cells possess an endogenous, and previously uncharacterized, VIP-type receptor. The presence of an endogenously expressing receptor provided a means to study the regulation of the VPAC₁ receptor at lower, and consistently reproducible, levels of expression. Using RT-PCR we identified this receptor to be the VPAC₁ receptor. Overexpression of GPCRs has been known to produce agonist-independent receptor phosphorylation, and this may be because of activation of mechanisms responsible for receptor regulation. Using the endogenous VPAC₁ receptor, we demonstrated GRK specificity by dose-dependent GRK expression and cAMP accumulation. The novelty of our study resides in the utility of the endogenous receptor to determine GRK specificity that would not have been evident in the classical approach using immunoprecipitation of overexpressed receptors from cells overexpressing individual GRKs. Additionally, in this case, the lack of GRK inhibitors precludes studying specificity by kinase inhibition. We had attempted to show secretin receptor GRK specificity by titrating GRK expression but were not successful. In those studies any amount of receptor overexpression resulted in an inability to produce graduated GRK expression. Furthermore, the lack of an endogenous receptor precluded the determination of receptor regulation similar to that obtained here for the VIP receptor.

Receptor phosphorylation causes β-arrestin translocation to many GPCRs (30), and β-arrestin was originally characterized as a desensitization protein (31). We investigated the role of β-arrestin in the regulation of the VPAC₁ by demonstrating profound and swift translocation of β-arrestin-GFP from the cytosol to the plasma membrane. Interestingly, when β-arrestin 1 or 2 were overexpressed in HEK 293 cells expressing only the endogenous receptor, either β-arrestin caused only a minor decrease in cAMP accumulation. This lack of effect on receptor signal termination is distinct from the effect of β-arrestin on GRK phosphorylated β₂-adrenergic receptors (22). This prompted us to investigate the role of β-arrestin in VIP recep-

colocalizes with β-arrestin in endocytic vesicles and by immunoprecipitation. A, HEK 293 cells overexpressing the VIP receptor and a β-arrestin-GFP conjugate were exposed to VIP (0.1 μM VIP) for the time indicated and followed with confocal microscopy. Within seconds of agonist exposure, the β-arrestin-GFP translocated from the cytosol (as shown at time 0) to the plasma membrane (as shown at 1 min). Translocation is rapid and persists from many minutes. B, the VIP receptor internalizes and is colocalized with β-arrestin in endocytic vesicles. Top panels, HEK 293 cells overexpressing the VIP receptor and β-arrestin-GFP prior to agonist exposure. In the left panel, the VIP receptor is localized to the plasma membrane (Texas Red, red). In the center panel, β-arrestin-GFP is distributed throughout the cytosol (green fluorescent protein, green). The right panel demonstrates no overlap (yellow) between VIP receptor staining and green fluorescent protein prior to agonist exposure. Middle panel, after exposure to VIP for 30 min, the VIP receptor is found within the cytosol located in vesicles (left panel). Similarly, β-arrestin-GFP has coalesced into vesicles within the cytoplasm (center panel) and as shown in the overlay, VIP receptor and β-arrestin are now co-localized in these endocytic vesicles (right panel). Lower panels, At higher magnification, the co-localization of the VIP receptor and β-arrestin-GFP is more evident in large doughnut-shaped vesicles. C, HEK 293 cells transiently transfected with 3 μg of cDNA for FLAG-tagged wild-type VIP receptor and 2 μg of cDNA for β-arrestin were stimulated with 100 nM VIP in a 37 °C incubator for 5, 10, or 20 min. One plate of HEK 293 cells was transfected only with 2 μg of cDNA β-arrestin and served as the control (lane 1). After stimulation, cells were lysed. Supernatant was immunoprecipitated with IgG-M2-FLAG antibody and separated on a 10% SDS-PAGE gel. After separation, proteins were transferred to nitrocellulose and immunoblotted with antibody to β-arrestin. An aliquot of the total cellular material was assayed for β-arrestin, and this is shown in the bottom panel.

Fig. 8. Agonist stimulation causes a rapid translocation of β-arrestin GFP to the plasma membrane and the VIP receptor

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tor trafficking, as it has recently been proposed that β-arrestin serves to target GPCRs to endocytic pathways (5).

We have demonstrated a role for β-arrestin in the regulation of VPAC₁ receptor regulation by co-localization of the receptor with arrestin using confocal microscopy and receptor-arrestin complex formation using immunoprecipitation. Clearly, β-arrestin forms a complex with the VPAC₁ receptor and this complex is transported into the cell during receptor endocytosis. This internalization of the receptor-arrestin complex may play a role in subsequent receptor signaling and appears to be maintained for some time during endocytic vesicle trafficking.

The molecular determinants for GPCR internalization include β-arrestin and dynamin, which act to promote GPCR targeting to clathrin-coated pit-dependent endocytosis (5). However, recent studies on the secretin receptor and the angiotensin II type 1A receptor suggest that all GPCRs may not manifest the same dependence on these components of receptor internalization (8, 23, 24). In HEK 293 cells overexpressing the VPAC₁ receptor, agonist stimulation causes receptor internalization in a prompt manner. Receptor internalization appears to occur via endocytic vesicles. Internalization of the VPAC₁ receptor is altered by overexpression of the dynamin GTPase-deficient mutant (K44A), but not by overexpression of the wild-type dynamin protein. This supports a dynamin-dependent clathrin-coated pit path for VPAC₁ receptor internalization and supports a role for clathrin-dependent receptor trafficking that was not apparent in studies on the secretin receptor.

Another significant difference between the VPAC₁R and the secretin receptor is the result obtained using the dominant-negative inhibitor of dynamin. Secretin receptor internalization was not affected by overexpression of K44A dynamin. Similarly, the muscarinic (M2) and angiotensin type 1A receptors internalize in the face of overexpression of this impaired dynamin protein. This finding has been used to support the hypothesis that certain GPCRs may use a dynamin-independent mechanism for receptor trafficking. However, our data on VPAC₁R internalization support a recent observation on the muscarinic and angiotensin type 1A receptors that, under appropriate conditions, the M2 and angiotensin type 1A receptors sequester in a dynamin-dependent manner and, once activated, these GPCRs are targeted to clathrin-coated pits that are pinched off at the plasma membrane by dynamin (32).

The VPAC₁ receptor, as a member of a distinct class of GPCRs, is phosphorylated in an agonist-dependent manner by specific GRKs and internalized via clathrin-coated pits. Unlike the secretin receptor and many other GPCRs, the VPAC₁ receptor is phosphorylated and desensitized by GRK 6, information that would not have been clear by limited study in the overexpressed cell system. The utility of an endogenously expressing receptor provides a novel mechanism to pursue information on GPCR regulation. Study of endogenously expressing proteins is likely to yield results that more closely recapitulate regulation in vivo. Therefore, it appears VPAC₁ receptor regulation follows a paradigm similar to that of the superfamily of class I GPCRs.

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