Vasoactive intestinal polypeptide type–1 receptor (VPAC1R) regulation: desensitization, phosphorylation and sequestration

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Summary

The VPAC₁ receptor is a Class II G protein-coupled receptor, distinct from the adrenergic receptor superfamily. The mechanisms involved in the regulation of the VPAC₁ receptor are largely unknown. We examined agonist-dependent VPAC₁ receptor signaling, phosphorylation, desensitization, and sequestration in HEK 293 cells. Agonist stimulation of cells overexpressing this receptor led to a dose-dependent increase in cAMP that peaked within 5-10 minutes and was completely desensitized after 20 minutes. Cells cotransfected with the VPAC₁R and G protein receptor kinases 2,3,5 and 6 exhibited enhanced desensitization that was not evident with GRK 4. Immunoprecipitation of the epitope-tagged VPAC₁ receptor revealed dose-dependent phosphorylation that was increased with cotransfection of any GRK. Agonist-stimulated internalization of the VPAC₁R peaked in 10 minutes and neither overexpressed β-arrestin nor its dominant-negative mutant altered internalization. However, a dynamin-dominant negative mutant did inhibit VPAC₁ receptor internalization. Interestingly, VPAC₁R specificity in desensitization was not evident by study of the overexpressed receptor, however, we determined that HEK 293 cells express an endogenous VPAC₁R that did demonstrate dose-dependent GRK specificity. Therefore, VPAC₁ 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.
INTRODUCTION

The neuromodulator vasoactive intestinal polypeptide (VIP) 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 (VPAC1) 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 protein kinase A (PKA) and protein kinase C (PKC), 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 β2AR (Class I), phosphorylation and desensitization of the secretin receptor was promoted by GRKs (9); however, unlike the β2AR, 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 β2AR (Class I) (8,10).

In this paper we probe the roles of receptor phosphorylation by GRKs and β-arrestin recruitment in the regulation of VPAC1 receptor signaling, desensitization and sequestration. Moreover, we determined that HEK 293 cells express an endogenous VPAC1 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. Vasoactive intestinal polypeptide was obtained from Peninsula Labs. Human embryonic kidney cells (HEK 293 cells) were obtained from the American Tissue Culture Collection. Tissue culture supplies were obtained from Life Technologies. Labeled vasoactive intestinal polypeptide (125-Iodine) was prepared and purified by HPLC (9,11). [2,8 3H]adenine, [3H]cAMP, [8-14C]cAMP, [α-35S]deoxyadenosine 5’-(α-thiol)triphosphate, and [32P]orthophosphate were obtained from DuPont NEN. Restriction enzymes were from Promega. Sequencing supplies were from US Biochemicals/Amersham. Polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR) materials were from Perkin-Elmer (Roche Molecular Systems). Primers were obtained from Genosys.

Plasmid Preparation - The full-length nucleotide sequence of the rat VIP (VPAC1) receptor (12) was amplified from rat heart cDNA by PCR using gene specific oligonucleotides. An epitope-tagged rat VPAC1 was prepared as described for the rat secretin receptor (9). The FLAG epitope was placed 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. The cDNAs were inserted into the pcDNA 1/Amp plasmid (Invitrogen) using Hind III and Xba I. 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, dynamin, as well as their dominant negative mutants were used as previously described (8). β-arrestin-GFP was produced as outlined in Barak et al (17). Plasmid purification was performed with Qiagen reagents.
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 in 95% air, 5% CO₂. One day after transfection cells were split into appropriate plates following trypsin dissociation. Experiments were performed 24-48 hours 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 sterile water and 50 µl 2.5 M CaCl₂. Then 500 µl of 2X 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 drop wise to the 100 mm dish of cells.

Membrane Preparation/Binding - All steps were performed at 4°C. Plates were placed on ice, media aspirated, and cells washed with 10 ml 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, 0.7 µg/ml pepstatin A, 10 µg/ml benzamidine, 0.2 mM PMSF) 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 secs at 14-16,000 cps. Material was centrifuged at 300-400 x g for 10 min to remove unlysed cells and nuclei. Supernatant was transferred to 13x100 mm tubes on ice and centrifuged at 18,000 rpm (40,000 x 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 vasoactive intestinal polypeptide, Peninsula Labs) of ¹²⁵I-VIP binding was performed in triplicate tubes. Non-specific
binding was defined in the presence of 1 µM unlabeled VIP. Data were analyzed using GraphPad-Prism and LIGAND software as described (9,11).

**Adenylyl 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 were plated to a density of approximately 2-3x10⁵ cells per well and labeled with [³H]-adenine (1 µCi/ml) in MEM, 5% FBS, 50 mg/liter gentamicin (1 ml/well) for 12 to 16 hours prior to experimentation. Labeling media was aspirated, cells washed with 1 ml PBS and preincubated in assay media (1 ml/well, MEM, 0% FBS, 10 mM HEPES, 1 mM IBMX) for 15-30 minutes. 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 [¹⁴C]cAMP, 2.5% perchloric acid) was added to each well. Plates remained at 4°C for 20-30 minutes, after which solution was transferred to 12x75 mm tubes containing 100 µl of 4.2 M KOH. Tubes were vortexed and stored at 4°C for cAMP determination by column chromatography (18). Data is normalized for total cellular uptake of [³H]adenine and using [¹⁴C]cAMP for column efficiency as previously described (18).

**VIP receptor internalization by immunofluorescence** – HEK 293 cells transiently transfected with 5 µg cDNA for FLAG-tagged VPAC₁ were plated onto 35 mm dishes containing a central glass well as described (17). Cells were maintained at 4°C to prevent receptor internalization while incubating with agonist (100 nM VIP), primary antibody (IgG-M2-FLAG, Kodak, Inc) and secondary antibody (Fab conjugated with FITC, Organo Teknica). Sequential 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 hour prior to imaging (60 min treatment).

**Immunofluorescent colocalization of VIP receptor with β-arrestin-GFP** – HEK 293 cells transiently transfected with 5 µg cDNA for wild-type VIP receptor were plated onto glass coverslips contained in 6 well plates. After an initial wash, cells were stimulated with 100 nM VIP in a 37°C incubator. After 30 and 60 min, cells were fixed in 4% paraformaldehyde for 25 min. Cells were successively incubated for 1 hour at room temperature with primary antibody (IgG-M2-FLAG) and secondary antibody (Texas red conjugated goat anti-mouse, Molecular Probes Inc.) dissolved in solubilization buffer consisting of 0.2% Triton X-100 and 1% bovine serum albumin in phosphate-buffered saline. Cells were washed for 20 min in solubilization buffer after each antibody incubation. Immediately following the last wash, glass coverslips containing the cells were mounted onto glass slides for viewing using a Zeiss laser scanning confocal microscope. β-arrestin-GFP (1 µg cDNA) was coexpressed with wild-type VPAC₁ (5 µg cDNA) and an identical protocol was followed except that cells were stimulated with VIP alone for only 2 min.

**Immunoprecipitation of the VIP receptor with β-arrestin** – HEK 293 cells transiently transfected with 3 µg cDNA for FLAG tagged wild-type VIP receptor and 2 µg cDNA β-arrestin were treated with media alone or stimulated with 100 nM VIP in a 37°C incubator for 5, 10, 20 minutes. One plate of HEK 293 cells was transfected only with 2 µg cDNA β-arrestin and served as the control. After stimulation, cells were lysed with lysis buffer (50 mM TRIS, pH 8.0, 5 mM EDTA, 0.05% SDS, 200 mM NaCl, 10 mM NaF, 10 mM Na₂ pyrophosphate and 1% Triton-X-100). Supernatant was immuno-precipitated 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 (31).

**Western Blotting** - Cellular proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred to nitrocellulose and then subjected to immunoblotting with appropriate GRK antiserum (14,19,20). Blots were revealed by chemiluminescence.

**Receptor expression** - In plasmid co-transfection experiments, receptor expression was determined by flow cytometry analysis of a sample from each transfection group (9). The fluorescence was determined by incubation for 1 hour at 4°C with monoclonal IgG-M2-FLAG (1:600 dilution, Kodak), washed three times with PBS, and detected with Fc specific, fluorescein-labeled goat anti-mouse (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 untransfected and/or a sample of HEK 293 cells transfected with the VPAC₁ 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 approximately 1-1.5x10⁶ cells per well and exposed to agonist for the appropriate time. After washing with iced PBS, cells on ice were exposed for 1 hour to monoclonal IgG-M2-FLAG antibody (1:600 dilution, Kodak) or 12CA5 (1:500 dilution, Boehringer Mannheim), 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 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 Perkin Elmer (Boston, MA) MMLV kit following manufacturers protocols. Primers were designed to distinguish between the two subtypes of VIP receptors as follows: human VPAC₁ receptor 463-486 5'GCCACCCCTTCTGCGCCACAGCT, and 1115-1092 5'TTCACTTCAGGCTTTAAATTGTCC; human VPAC₂ receptor 414-438 5'ATGTCTTCTTGCAACAGGAAGCATA, and 1067-1044 5'TGGTATTTGGAGGAGATGCTGATG. PCR was initiated by adding 0.5ul of Taq polymerase (Perkin Elmer, Inc.). PCR was run at 96°C for 2 minutes, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C. Program was terminated with 7 minutes at 72°C. Samples were resolved with a 1% agarose gel and ethidium bromide and examined under UV light.

**β-arrestin-GFP Translocation** – Transfected HEK 293 cells were plated onto 35 mm dishes containing a central glass well as described (17). Cells in Dulbecco’s modified medium, pH 7.4, buffered with 20 mM HEPES were stimulated with 1 µM agonist while being viewed on a Zeiss laser scanning confocal microscope.

**Receptor Phosphorylation** – Cells were plated to a density of approximately 1-1.5x10⁶ cells per well and labeled with [³²P] orthophosphate (66 µCi/well) for one hour in phosphate-free
MEM, 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 radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM disodium pyrophosphate, 5 µg/ml leupeptin, 0.7 µg/ml pepstatin A, 10 µg/ml benzamidine). Lysed cells from two wells (800 µl) were transferred to 1.5 ml tubes on ice and rotated on an inversion wheel for one hour. Solubilized material was transferred to Beckman TLA 100.2 tubes for centrifugation at 200,000 x g for 15 minutes at 4°C. The supernatant was transferred to 1.5 ml tubes on ice with 100 µl protein A-Sepharose beads (Pharmacia Biotech Inc.) in 3% bovine serum albumin and radioimmune precipitation buffer. An aliquot of supernatant was removed for protein determination (Bio-rad DC protein assay kit). After a one hour pre-clearing, beads were pelleted, and the supernatant was transferred to 1.5 ml tubes with 100 µl of protein A-Sepharose beads and 16 µg of monoclonal IgG-M2-FLAG (Kodak). Samples were placed on an inversion wheel at 4°C. After two hours, beads were pelleted, and supernatant was discarded. Beads were washed three times with ice-cold radioimmune precipitation 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 minutes and resolved on a 1-mm thick, 10% SDS-polyacrylamide gel. Dried gels were analyzed quantitatively with a Molecular Dynamics PhosphoImager.

In plasmid co-transfection experiments, receptor expression was determined by flow cytometry analysis of a sample from each transfection group. The fluorescence was determined by incubation for one hour at 37 °C with monoclonal IgG-M2-FLAG (1:500 dilution, Kodak),
three washes with phosphate-buffered saline, and detection with Fc-specific, fluorescein-labeled goat anti-mouse antibody (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 within one hour on a Becton-Dickson flow cytometer. Base-line fluorescence was determined from a sample of HEK 293 cells untransfected and/or a sample of HEK 293 cells transfected with the rat VIP receptor but not exposed to primary antibody. Baseline fluorescence was subtracted from each sample. Receptor fluorescence was normalized to total cellular protein determined from an aliquot of each transfection sample before immunoprecipitation. Gel lanes were loaded with the same amount of receptor protein.
RESULTS

**Characterization of VPAC₁ receptor constructs: receptor binding, signaling and sequestration.**

Binding studies with cell membranes prepared from HEK 293 cells transiently transfected with wild-type rat VPAC₁ and the N-terminal FLAG VPAC₁ receptor cDNA resulted in an identical $K_D$ for VIP binding (4 nM, data not shown). The wild-type and N-terminal FLAG VIP type 1 receptor produced an EC$_{50}$ for cAMP accumulation of 0.2 nM in response to vasoactive intestinal polypeptide (Figure 1A). Time course experiments revealed a rapid increase in cAMP accumulation that declined with a $t_{1/2}$ of 5 minutes, yielding complete cessation of further cAMP accumulation after approximately 10-15 minutes of agonist exposure, for both receptors when overexpressed in HEK 293 cells (Figure 1B). By FACS analysis, the epitope-tagged VPAC₁ receptor demonstrated a concomitantly rapid time course of internalization, with a $t_{1/2}$ of 5 minutes and maximum loss of 50% by 15 to 20 minutes (Figure 1C). Since 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 (VPAC₁) 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 (Figure 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 (Figure 2B, last lane). Agonist-dependent receptor phosphorylation occurs with an EC$_{50}$ of 1.1 nM (Figure 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 minutes (Figure 3A and 3B). GRK 4 was relatively ineffective in diminishing VPAC₁ signaling (Figure 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 (Figure 4A and 4B). PhosphoImager analysis of immunoprecipitated VPAC₁ demonstrated that all the GRKs increased receptor phosphorylation 3- to 4-fold following stimulation with agonist for 10 minutes. 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 (Figure 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. In order 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 Figure 5A, overexpressing various amounts of GRK 5 or GRK 6 caused attenuation of cAMP accumulation in response to VIP. The effects of GRK 5 and 6 were dose-dependent, and decreasing amounts of DNA
transfected corresponded to decreased levels of expressed proteins (Figure 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). The effect of β-arrestin on VPAC₁ signaling was initially studied on the endogenous VPAC₁ in HEK 293 cells overexpressing β-arrestin 1 and 2. As shown in Figure 6, β-arrestin 1 and 2 decreased agonist-stimulated VPAC₁ cAMP accumulation by 27% and 39% of control levels, respectively. Neither arrestin construct caused a significant shift in EC₅₀.

In order 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-1 V53D or dynamin I K44A. Similar to results with the β₂-adrenergic receptor, overexpression of neither β-arrestin nor dynamin altered VPAC₁R internalization when quantitated by FACS analysis as loss of cell surface receptor (Figure 7). However, when the dominant negative constructs (β-arrestin V53D, dynamin 1 K44A) were overexpressed, both decreased internalization of the β₂-adrenergic receptor, but only dynamin K44A reduced VPAC₁ receptor endocytosis (Figure 7). Although the VPAC₁ behaves differently from the β₂-adrenergic receptor, other receptors have shown this variance (8,23,24). This difference may be due in part to 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 overexpresssed 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 resole 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 a similar pattern (data not shown). In order 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, vasoactive intestinal polypeptide receptor sequestration and VPAC₁/β-arrestin colocalization by immunofluorescence microscopy and immuno-precipitation – We stimulated HEK 293 cells overexpressing the VPAC₁ receptor and observed rapid translocation of a β-arrestin-green fluorescent (GFP) fusion protein from the cytosol to the plasma membrane (Figure 8A). β-arrestin-GFP translocates from the cytosol to the plasma membrane robustly after stimulation with VIP, but only in cells overexpressing the VIP₁R. In order to determine more directly if β-arrestin is targeted to the VPAC₁ receptor, we transfected HEK 293 cells with the epitope-tagged VPAC₁ receptor and β-arrestin-GFP. After one-minute of agonist exposure, the β-arrestin-GFP is found co-localized with the VPAC₁ receptor at the cell membrane (Figure 8B). Similarly, after 30 minutes of agonist exposure, β-arrestin is clearly co-localized with the VPAC₁ receptor in endocytic vesicles inside the cell (Figure 8B).

In order to support an interaction of the VPAC₁ receptor with β-arrestin, we immunoprecipitated the VIP receptor and tested for co-immunoprecipitation of over expressed β-arrestin. HEK 293 cells transiently transfected with both FLAG tagged wild-type VIP receptor and β-arrestin were stimulated with 100 nM VIP in a 37°C incubator for 5, 10, 20 minutes. One plate of HEK 293 cells was transfected with β-arrestin only and served as the control. As
demonstrated in Figure 8C, immunoprecipitation of the VIP receptor brings down β-arrestin, suggesting the presence of a receptor-arrestin complex.

**DISCUSSION**

Although Class II GPCRs are abundant and involved in the regulation of a variety of physiological processes, information on their regulation lags that known for the larger Class I rhodopsin/adrenergic family of receptors. Study of GPCRs has relied on over-expressed heterologous cell systems that provide a controlled manner to investigate various aspects of receptor signal regulation. However, in some cases the over-expressed 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 over-expression 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 VPAC_1 R, 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 VPAC_1 R did not significantly alter receptor binding or signaling properties, providing a useful tool in the study of VPAC_1 receptor regulation. Prior investigators have noted diminished agonist binding to the VPAC_1 with mutation of the N-terminus (25,26). We placed the epitope at the amino terminus of the processed portion of the mature receptor in order to minimize interactions with sites important for agonist binding. The VPAC_1 R, 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 demonstrate that agonist –
dependent receptor phosphorylation, arrestin translocation and consequent receptor internalization, regulates the VPAC1 receptor. By immunoprecipitation, we demonstrate agonist dependent VPAC1 phosphorylation with an EC$_{50}$ of 1.1 nM. Our receptor immunoprecipitation revealed a broad band of approximately 70 kDa, similar to Fabre et al (28).

Agonist-induced receptor phosphorylation occurs by either G protein-coupled receptor kinases (GRKs) or second-messenger dependent kinases. The VPAC1 receptor, when overexpressed, is rather promiscuous with respect to kinase phosphorylation. Like many GPCRs, the VPAC1 is phosphorylated and desensitized by GRKs 2, 3 and 5. However, when studied in an endogenously expressed fashion, GRKs 5 and 6, but not GRK 4, desensitize it preferentially. It is notable that although GRK 4 can phosphorylate VPAC1 receptor, when both proteins are over-expressed, GRK 4 appears incapable of functionally desensitizing the endogenously expressed VPAC1 receptor. This is in marked contrast to the 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 demonstrate that the VPAC2 receptor is desensitized and phosphorylated by a kinase sensitive to the PKA 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 due to 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 over-expressed receptors from cells over-expressing individual GRKs. Also, 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 are 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 receptor 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 effected 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 supports 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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Abbreviations:
VIP, vasoactive intestinal polypeptide; GPCRs, G protein-coupled receptors; β₂AR, β₂-adrenergic receptor; HEK, human embryonic kidney; dATPαS, deoxyadenosine 5’-(α-thiol) triphosphate; PCR, polymerase chain reaction; GRK, G protein-coupled receptor kinase; PKA, cAMP-dependent protein kinase; CCP, clathrin-coated pit; GFP, green fluorescent protein; PBS, phosphate-buffered saline, HA, hemagglutin
REFERENCES


Figure Legends

Figure 1. Signaling, desensitization and sequestration of the wild-type rat VPAC$_1$ and N-terminal epitope-tagged rat VPAC$_1$ 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 minutes.  Data are not normalized.  EC$_{50}$ for the wild-type and FLAG-VIP receptor was 0.23 nM 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 normalized to surface receptor expression in cells not exposed to agonist.  Each point represents the mean of 3 independent experiments, each with triplicate samples per time point.  Data represent mean +/- S.E. for each point.

Figure 2. Agonist-stimulated FLAG-tagged vasoactive intestinal polypeptide receptor phosphorylation.  Phosphorylation experiments were performed as noted in the “Experimental Procedures.”  Data were quantitated densitometrically using a Molecular Dynamics PhosphoImager.  A, Dose-response curve generated from four independent experiments.  Agonist-dependent phosphorylation exhibited an EC$_{50}$ of 1.1 nM.  Maximal phosphorylation was determined with 1 uM VIP for 15 minutes.  Agonist stimulation increased phosphorylation 2- to 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 kilo Daltons.  The last lanes (C, control) represent cells mock transfected and exposed to agonist.
Figure 3. Effect of GRKs on cAMP accumulation in HEK 293 cells cotransfected with rat VPAC₁ and GRK. cAMP accumulation was measured as under “Experimental Procedures.” Whole cell stimulation with VIP was performed at doses indicated for 10 minutes. All curves represent mean +/- S.E. for three independent experiments, with each experimental point performed in duplicate. **A**, HEK 293 cells transfected with VPAC₁ alone or cotransfected with 5 µg of GRK 2 or GRK 3. **B**, HEK 293 cells transfected with VPAC₁ alone or cotransfected with 5 µg of GRK 4, GRK 5 or GRK 6. The EC₅₀ for cAMP accumulation for receptor alone was, 0.11 nM. The EC₅₀ for cAMP accumulation for each GRK was; GRK 2, 0.13 nM; GRK 3, 0.28 nM; GRK 4, 0.08 nM; GRK 5, 0.32 nM; and GRK 6, 0.07 nM. The Vₓ for cAMP accumulation (mean +/- S.E.) for receptor-alone was, 0.91 +/- 0.08. The Vₓ for cAMP accumulation for each receptor/GRK cotransfection was; GRK 2, 0.47 +/- 0.04; GRK 3, 0.26 +/- 0.03; GRK 4, 0.70 +/- 0.06; GRK 5, 0.16 +/- 0.02; and GRK 6, 0.20 +/- 0.02.

Figure 4. Effect of GRK on VPAC₁ phosphorylation receptor by immunoprecipitation. HEK 293 cells overexpressing the epitope tagged VPAC₁ with or without (Control) exogenous GRK were incubated with media alone (Basal) or 1 µM VIP for 10 minutes (VIP) and subject to immunoprecipitation as noted in the “Experimental Procedures.” **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 VPAC₁ (60-80 kDa). **B**, PhosphoImager analysis of four independent experiments. Data are mean +/- S.E. and normalized to basal receptor phosphorylation in the absence of agonist and no overexpressed GRK (Control, basal).

Figure 5. Endogenous VPAC₁-stimulated cAMP accumulation in HEK 293 cells overexpressing GRKs 4, 5 or 6. **A**, HEK 293 cells were transfected with GRK 4, 5 or 6 and
stimulated with VIP (1 µM) for 10 minutes. 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 to 3 µg). cAMP accumulation was determined as noted in the “Experimental Procedures” and is the mean +/- S.E. of 3 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 minutes, respectively. B, Representative Western blot of G protein-coupled receptor kinase 5 overexpression in HEK 293 cells. HEK 293 cells were transfected with either 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 to 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 antisera (see Experimental Procedures).

Figure 6. Effect of β-arrestin on endogenous VPAC₁-stimulated cAMP accumulation in HEK 293 cells not overexpressing the VIP. HEK 293 cells overexpressing either β-arrestin 1 or β-arrestin 2 alone were exposed, in a dose-dependent manner, to VIP for 10 minutes to determine their ability to attenuate signaling of the endogenous VPAC₁. The VPAC₁ was not overexpressed in these experiments. β-arrestin 1 elicited a 27 % reduction in Vmax, while 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 3 independent experiments, each done in triplicate.

Figure 7. Effect of β-arrestin and Dynamin on VPAC₁ internalization. HEK 293 cells were transiently transfected with wild-type VPAC₁ and either β-arrestin 1, β-arrestin 1 mutant
(V53D), dynamin or the dominant-negative dynamin mutant (K44A) on the agonist promoted sequestration of the VPAC₁ and the β₂-adrenergic receptor (β₂-AR) as assessed by flow cytometry. FLAG-tagged VIP receptor or HA-tagged β₂-AR was transiently transfected in HEK 293 cells with 5 µg of the following: empty vector (control/empty), pCMV rat β-arrestin 1 (βarr), 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 β-arrestin-1 (31). Expression of mutant and wild-type dynamin was monitored by immunoblot using an antibody for dynamin 1 (33). The data represent the mean +/- S.E. of at least 3 independent experiments, with each point done in duplicate, for each group.

Figure 8. Agonist stimulation causes a rapid translocation of β-arrestin Green Fluorescent Protein (GFP) to the plasma membrane and the VIP receptor colocalizes with β-arrestin in endocytic vesicles and by immuno-precipitation. 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 zero) to the plasma membrane (as shown at time one minute). Translocation is rapid and persists from many minutes. B, The VIP receptor internalizes and is colocalized with β-arrestin in endocytic vesicles. Top figures, 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). In the center panel, β-arrestin-GFP is distributed throughout the cytosol (Green Fluorescence). The right panel demonstrates no overlap (yellow) between VIP receptor staining and Green Fluorescent Protein prior to agonist exposure. Middle figures, After exposure to VIP for 30 minutes, the VIP receptor localizes to the plasma membrane (Texas Red). In the center panel, β-arrestin-GFP is distributed throughout the cytosol (Green Fluorescence). The right panel demonstrates no overlap (yellow) between VIP receptor staining and Green Fluorescent Protein prior to agonist exposure.
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 and β-arrestin are now co-localized in these endocytic vesicles (right panel). Lower figures, 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 cDNA for FLAG tagged wild-type VIP receptor and 2 μg cDNA β-arrestin were stimulated with 100 nM VIP in a 37°C incubator for 5, 10, 20 minutes. One plate of HEK 293 cells was transfected only with 2 μg 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.
Figure 1A

CAMP Accumulation (mean CA/Tu x 100) vs Log M [VIP]

- Wild-type
- FLAG-tagged
Figure 1C

The figure shows a graph with the y-axis labeled as VPAC1 receptor sequestration (% loss of surface receptors) and the x-axis labeled as Time (min). The graph depicts a curve that increases with time, reaching a plateau after approximately 30 minutes. Error bars are present, indicating variability in the data.
Figure 4

A

B

Phosphorylation (Fold over Basal)

<table>
<thead>
<tr>
<th>Control</th>
<th>GRK 2</th>
<th>GRK 3</th>
<th>GRK 4</th>
<th>GRK 5</th>
<th>GRK 6</th>
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<td>+</td>
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</table>

- Basal
- VIP
Figure 5A

CAMP Accumulation (mean CA/TU x 100)

Amount of GRK transfected (μg)

GRK 4  GRK 5  GRK 6

Figure 5B

GRK expression
Figure 6

CAMP Accumulation (mean CA/TU x 100)

- Control
- β-arrestin 1
- β-arrestin 2

Log M [VIP]
Figure 7

Sequestration (% loss receptors)

VPAC₁  β₂AR

Empty  βarr1  V53D  Dynamin  K44A
Figure 8B

VPAC1 β-arrestin Overlay

No Agonist

VIP (1 μM)

VIP (1 μM)
M2 Flag IP, blot βArr Ab

Lysate, blot βArr Ab
Vasoactive intestinal polypeptide type–1 receptor (VPAC1R) regulation: Desensitization, phosphorylation and sequestration
Michael A. Shetzline, Julia K. L. Walker, Kenneth J. Valenzano and Richard T. Premont
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