Phosphorylation of GRK2 by PKA augments GRK2-mediated phosphorylation, internalization, and desensitization of VPAC2 receptors in smooth muscle

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Murthy KS, Mahavadi S, Huang J, Zhou H, Sriwai W. Phosphorylation of GRK2 by PKA augments GRK2-mediated phosphorylation, internalization, and desensitization of VPAC2 receptors in smooth muscle. Am J Physiol Cell Physiol 294: C477–C487, 2008. First published December 12, 2007; doi:10.1152/ajpcell.00229.2007.—The smooth muscle of the gut expresses mainly G protein-coupled vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide receptors (VPAC2 receptors), which belong to the secretin family of G protein-coupled receptors. The extent to which PKA and G protein-coupled receptor kinases (GRKs) participate in homologous desensitization varies greatly among the secretin family of receptors. The present study identified the novel role of PKA in homologous desensitization of VPAC2 receptors via the phosphorylation of GRK2 at Ser685. VIP induced phosphorylation of GRK2 in a concentration-dependent fashion, and the phosphorylation was abolished by blockade of PKA with cell-permeable myristoylated protein kinase inhibitor (PKI) or in cells expressing PKA phosphorylation-site deficient GRK2(S685A). Phosphorylation of GRK2 increased its activity and binding to Gβγ. VIP-induced phosphorylation of VPAC2 receptors was abolished in muscle cells expressing kinase-deficient GRK2(K220R) and attenuated in cells expressing GRK2(S685A) or by PKI. VPAC2 receptor internalization (determined from residual [32P]-labeled VIP binding and receptor biotinylation after a 30-min exposure to VIP) was blocked in cells expressing GRK2(K220R) and attenuated in cells expressing GRK2(S685A) or by PKI. Finally, VPAC2 receptor degradation (determined from residual [32P]-labeled VIP binding and receptor expression after a prolonged exposure to VIP) and functional VPAC2 receptor desensitization (determined from the decrease in adenylcyclase activity and cAMP formation after a 30-min exposure to VIP) were abolished in cells expressing GRK2(K220R) and attenuated in cells expressing GRK2(S685A). These results demonstrate that in gastric smooth muscle VPAC2 receptor phosphorylation is mediated by GRK2. Phosphorylation of GRK2 by PKA enhances GRK2 activity and its ability to induce VPAC2 receptor phosphorylation, internalization, desensitization, and degradation.

Homologous desensitization; vasoactive intestinal peptide; G protein-coupled receptor kinase; gastric muscle; G protein signaling; pituitary adenylate cyclase-activating peptide

Homologous desensitization of agonist-occupied receptors is initiated upon receptor phosphorylation by G protein-coupled receptor kinase (GRK) (19, 37, 38). Binding of phosphorylated receptors to β-arrestin uncouples the receptors from the G proteins and targets the receptors to clathrin-coated pits. β-Arrestin acts as a scaffold for other proteins including the terminal components of MAPKs and the cytosolic tyrosine kinase c-Src; the latter stimulates tyrosine phosphorylation of the large GTPase dynamin and promotes its ability to cleave clathrin-coated vesicles from the cell surface (20–23, 38). The vesicles fuse with endosomes, where the receptors are dephosphorylated and either slowly or rapidly recycled to the surface. Some receptors, however, are partly or wholly internalized via a clathrin-independent caveolar pathway involving the phosphorylation of dynamin and caveolin by c-Src (1, 2, 18, 40).

A role for second messenger-activated protein kinases, such as PKC or PKA, in receptor phosphorylation and/or internalization is highly variable and may depend on the cell type in which the receptor is expressed (9, 11, 39). β2-Adrenergic receptors (β2-ARs) are preferentially phosphorylated by PKA at low agonist concentrations and by both PKA and GRK2 at higher agonist concentrations; in addition, PKA phosphorylates GRK2 and enhances its activity (4, 7, 10, 45). Both kinases together with the receptor and other signaling molecules are bound to a kinase-anchoring protein 79, a PKA-anchoring protein that facilitates their interaction (8). β1-Adrenergic receptors (β1-ARs), however, are phosphorylated in equal measure by PKA and GRK2, with PKA-phosphorylated receptors targeted to caveolae and internalized via a β-arrestin-independent pathway (9, 39). Among class II receptors, G protein-coupled secretin receptors are preferentially phosphorylated by PKA and internalized via a GRK2/β-arrestin/clathrin-independent endocytic pathway (12, 42, 46). Their close homologs, vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide (PACAP) receptors (VPAC1 receptors), which possess equally high affinity for VIP and PACAP, are phosphorylated by GRK2 and internalized via a β-arrestin/clathrin-independent dependent pathway (12, 17, 43). This mechanism is obscured in cell lines overexpressing VPAC1 receptors, underlining the importance of studies on constitutively expressed receptors (43). PKA inhibitors have no effect on VPAC1 receptor phosphorylation, suggesting that PKA does not participate directly or indirectly in VPAC1 receptor phosphorylation (17). VPAC2 receptors also appear to be internalized via a clathrin pathway, but the roles of GRK2 and PKA in receptor phosphorylation and internalization have not been characterized (26, 27).

Here, we show that Gs protein-coupled VPAC2 receptors, predominantly expressed in smooth muscle cells of the gut (6, 44), are exclusively phosphorylated by GRK2 and that feedback phosphorylation of GRK2 at Ser685 by PKA leads to an

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increase in GRK2 activity and thus augments GRK2-mediated receptor phosphorylation, internalization, and desensitization of the functional response.

**MATERIALS AND METHODS**

Preparation of dispersed gastric smooth muscle cells. Gastric smooth muscle cells were isolated from the circular muscle layer of the rabbit stomach by sequential enzymatic digestion, filtration, and centrifugation as previously described (29, 32, 33). The Virginia Commonwealth University Institutional Animal Care and Use Committee approved the use of animals and protocol used in this study. Briefly, muscle strips were incubated at 31°C for 20 min in HEPES medium with type II collagenase (0.1%) and soybean trypsin inhibitor (0.1%). The partially digested strips were washed, muscle cells were allowed to disperse spontaneously for 30 min, and cells harvested by filtration through 500-μm Nitex and centrifuged twice at 350 g for 10 min. Dispersed smooth muscle cells were cultured in DMEM containing 10% FBS until they attained confluence and were then passaged once for use in various studies (13, 14).

Vector and GRK2 mutant constructs. Wild-type GRK2, dominant negative GRK2 [i.e., kinase-deficient GRK2(K220R)], PKA phosphorylation site-deficient GRK2(S685A), and the COOH-terminal fragment of GRK2 (GRK2(985–989)) were subcloned separately into the multiple cloning site (EcoRI) of the eukaryotic expression vector pEXV. A Myc tag was incorporated into the NH2 terminus of GRK2 constructs. Recombinant plasmid DNAs (2 μg each) were transiently transfected into smooth muscle cells in the first passage by lipofectamine Plus reagent for 48 h. Cells were cotransfected with 1 μg of pGreen Lantern-1 to monitor expression. Control cells were cotransfected with 2 μg of vector (pEXV) and 1 μg of pGreen Lantern-1 DNA. The transfecion efficiency (~75%) was monitored by the expression of green fluorescent protein using FITC filters (13, 14).

**Phosphorylation of VPAC2 receptors and GRK2.** Phosphorylation of VPAC2 receptors and GRK2 was determined from the amount of 32P incorporated into the protein after immunoprecipitation with specific GRK2, Myc, or VPAC2 receptor antibody. GRK2 was immunoprecipitated with GRK2 antibody in freshly dispersed muscle cells and with Myc antibody in cultured muscle cells overexpressing GRK2 constructs. Ten milliliters of smooth muscle cell suspension (3 × 10^6 cells/ml) were incubated with [32P]orthophosphate for 4 h at 31°C. Samples (1 ml) were then incubated with VIP (1 μM) for 5 min. Cell lysates were separated by centrifugation at 13,000 g for 10 min at 4°C, precleared with 40 μl of protein A-Sepharose, and incubated for 2 h at 4°C with antibody to VPAC2 receptors, GRK2, or Myc and with 40 μl of protein A-Sepharose for another 1 h. Immunoprecipitates were extracted with Laemmli sample buffer, boiled for 5 min, and separated by electrophoresis with SDS-PAGE. After a transfer to nitrocellulose membranes, the antibody to VPAC2 receptors, GRK2, or Myc antibody in cultured muscle cells was immunoprecipitated using GRK2 antibody in freshly dispersed cells or in the presence of the vector. Immunoprecipitates were washed four times in lysis buffer and boiled in Laemmli buffer. Samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed with the antibody to common GB. After an incubation with secondary antibody, proteins were visualized by ECL, and the intensity of the protein band on ECL film was determined by densitometry.

**Receptor internalization, recycling, and degradation.** Binding of 125I-labeled VIP (125I-VIP) to cultured muscle cells was performed as previously described (6, 13, 24, 29). Muscle cells were detached from culture dishes by an incubation with 0.53 mM EDTA in PBS at 37°C for 30 min. Cells were boiled in Laemmli sample buffer, centrifuged for 30 min, and supernatants were separated by SDS-PAGE. After a transfer to nitrocellulose membranes, the antibody to VIPAC2 receptors, GRK2, or Myc antibody in cultured muscle cells was immunoprecipitated using GRK2 antibody in freshly dispersed cells or in the presence of the vector. Immunoprecipitates were washed four times in lysis buffer and boiled in Laemmli buffer. Samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed with the antibody to common GB. After an incubation with secondary antibody, proteins were visualized by ECL, and the intensity of the protein band on ECL film was determined by densitometry.

**Biotin labeling of surface receptors.** Receptor internalization was also measured by biotinylation of cell surface receptors. Muscle cells were incubated with VIP at 37°C for different time periods; cells were then washed twice with PBS at 4°C to avoid further receptor internalization and recycling, and 125I-VIP binding to residual surface VAPC2 receptors was measured for 60 min at 4°C and compared with control 125I-VIP binding in the absence of treatment with VIP.

**Receptor recycling was determined by treating muscle cells with 10 μM VIP for 30 min at 37°C to promote internalization. Ligan-**

ds and receptors remaining on the cell surface were removed by two washes with 150 mM NaCl plus 5 mM acetic acid and three washes with PBS (all at 4°C), and cells were then allowed to recover for different time periods at 37°C. At the end of each recovery period, cells were resuspended in control medium at 4°C, and 125I-VIP binding to the recycled surface receptors was determined for 60 min.

**Receptor degradation was determined from residual 125I-VIP binding after a prolonged 5-h exposure to VIP. At intervals of 1 h, the 125I-VIP binding was determined in cells expressing wild-type or mutant GRK2. Amounts of VPAC2 receptors at each interval were determined by Western blot analysis.**

**Biotin labeling of surface receptors.** Receptor internalization was also measured by biotinylation of cell surface receptors. Muscle cells were incubated with VIP at 37°C for different time periods; cells were then washed twice with PBS at 4°C followed by treatment with 10 mM sodium periodate for 30 min in the dark. After being washed with PBS, cells were incubated for 30 min at 4°C with membrane-impermeable biotin LC-hydrazide (2 mM) to conjugate glycoproteins with biotin. Cells were then solubilized in lysis buffer, and the insoluble material was removed by centrifugation; soluble extracts were incubated with antibody to VAPC2 receptors. VAPC2 receptor immunoprecipitates were extracted with Laemmli sample buffer, boiled for 5 min, and separated by electrophoresis with SDS-PAGE. After a transfer to nitrocellulose membranes, the antibody to VIPAC2 receptors, GRK2, or Myc antibody in cultured muscle cells was immunoprecipitated using GRK2 antibody in freshly dispersed cells or in the presence of the vector. Immunoprecipitates were washed four times in lysis buffer and boiled in Laemmli buffer. Samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed with the antibody to common GB. After an incubation with secondary antibody, proteins were visualized by ECL.

**Assay for GRK2 activity.** GRK2 activity was measured in immunoprecipitates of GRK2 using rhodopsin as the substrate, as previously described (7). One-milliliter aliquots (3 × 10^6 cells/ml) of cultured muscle cells were incubated with VIP for 5 min, and GRK2 was immunoprecipitated using GRK2 antibody in freshly dispersed cells or in the presence of the vector. Immunoprecipitates were washed with phosphorylation buffer containing 10 mM MgCl2 and 40 mM HEPES (pH 7.4) and then incubated for 30 min with 1 μM rhodopsin. Kinase assays were initiated by the addition of kinase
buffer containing 30 mM Tris·HCl (pH 7.2), 8 mM MgCl₂, 1.4 mM EDTA, 1 mM EGTA, 10 μCi of [γ-32P]ATP (3,000 Ci/mmol), and 160 μM ATP, and the reaction was stopped with SDS sample buffer. Phosphorylated proteins were resolved by SDS-PAGE and quantitated.

**Materials.** 125I-VIP, [α-32P]ATP, 125I-labeled-cAMP, and 32P-orthophosphate were obtained from NEN Life Sciences Products (Boston, MA); polyclonal antibodies to GRK2, VPAC₂ receptors, and hemaglutinin (HA) were from Santa Cruz Biotechnology (Santa Cruz, CA); and monoclonal antibody to PKA phosphosubstrate was from Cell Signaling Technology (Beverly, CA). Western blot and chromatography material were from Bio-Rad Laboratories (Hercules, CA); collagenase and soybean trypsin inhibitor were from Worthington Biochemical (Freehold, NJ); and all other reagents were from Sigma.

**RESULTS**

Feedback phosphorylation of GRK2 by PKA. We have recently shown that the selective activation of PKA with a low concentration (1 μM) of isoproterenol induced GRK2 phos-

![Figure 1. Concentration-dependent phosphorylation of G protein-regulated kinase 2 (GRK2) induced by vasoactive intestinal peptide (VIP) in smooth muscle. Freshly dispersed gastric smooth muscle cells labeled with 32P were incubated with VIP (0.1 nM to 1 μM) for 5 min and then lysed, and GRK2 was immunoprecipitated with GRK2 antibody. GRK2 phosphorylation was identified in immunoprecipitates by autoradiography. Immunoblot analysis showed equal amounts of loaded protein. Radioactivity in the protein bands was expressed as the increase in counts per minute (cpm) above basal levels (502 ± 87 cpm). p-GRK2, phosphorylated GRK2. Values are expressed as means ± SE of 4 experiments. **P < 0.001, significant increase in GRK2 phosphorylation.**](./image.png)

![Figure 2. Blockade of VIP-induced GRK2 phosphorylation in cells expressing GRK2(S685A). Cultured smooth muscle cells expressing Myc-tagged wild-type (WT) GRK2 (control) or Myc-tagged phosphorylation-deficient GRK2(S685A) were treated with VIP (1 μM) for 5 min, and GRK2 was immunoprecipitated using the antibody to Myc. A: GRK2 phosphorylation was determined by autoradiography in GRK2 immunoprecipitates isolated from 32P-labeled cells and expressed as cpm. **p-GRK2 stabilization. Values are expressed as means ± SE of 4 experiments. **P < 0.001, significant increase in GRK2 phosphorylation.**](./image.png)

![Figure 3. Effect of VIP on GRK2(S685A) expression and phosphorylation. A: Western blot analysis showing equal levels of GRK2 expression in control and GRK2(S685A) cells. B: Autoradiography showing VIP-induced phosphorylation of GRK2(S685A). Values are expressed as means ± SE of 4 experiments. **P < 0.001, significant increase in GRK2 phosphorylation.**](./image.png)
phorylation at Ser\(^{685}\) in freshly dispersed and cultured gastric smooth muscle cells, whereas the selective activation of PKG with sodium nitroprusside had no effect (14). VIP interacts with both cognate Gs protein-coupled VPAC2 receptors and single-transmembrane Gi1/Gi2 protein-coupled NPR-C receptors in gastric smooth muscle cells to generate cAMP and cGMP, respectively, and activate both PKA and PKG (29, 48).

In the present study, treatment of 32P-labeled cultured gastric smooth muscle cells with VIP induced phosphorylation of GRK2 in a concentration-dependent fashion (Fig. 1). GRK2 phosphorylation, measured in immunoprecipitates using the antibody to Myc, was blocked in cells expressing PKA phosphorylation site-deficient GRK2(S685A) (Fig. 2A). Phosphorylation levels in cells overexpressing wild-type GRK2 appeared to be similar to that of freshly dispersed muscle cells, reflecting the low efficiency of the Myc antibody used to immunoprecipitate GRK2. However, when GRK2 was immunoprecipitated with the antibody to GRK2, phosphorylation and expression levels of GRK2 were greater, suggesting overexpression of GRK2 (Fig. 2A, inset). Selective phosphorylation of GRK2 by PKA was corroborated using a phosphosubstrate antibody specific for PKA phosphorylation sites: phosphorylation of GRK2 was blocked in cells expressing GRK2(S685A) (Fig. 2B).

cAMP generated in smooth muscle cells is degraded by two cAMP-specific phosphodiesterases (PDEs), PDE3A and PDE4D5 (33). VIP (10 nM) caused an increase (65 ± 5% increase above basal levels of 3.98 ± 0.45 pmol/mg protein) in cAMP levels, and the increase was significantly augmented (136 ± 15% increase above basal levels) by a combination of the PDE3 inhibitor milrinone (10 μM) and the PDE4 inhibitor rolipram (10 μM). Blockade of cAMP degradation by the combination of milrinone (10 μM) and rolipram (10 μM) significantly augmented GRK2 phosphorylation induced by a low concentration of VIP (10 nM), providing further support for the notion that phosphorylation was mediated via a feedback mechanism involving the activation of PKA (Fig. 3A).

Interdependence of GRK2 phosphorylation and GRK2:G\(\beta\gamma\) association. Since G protein \(\beta\gamma\)-subunits assist in the targeting of GRK2 to the membrane-bound receptor, we examined the effect of G\(\beta\gamma\):GRK2 association on GRK2 phosphorylation by PKA and the effect of GRK2 phosphorylation on G\(\beta\gamma\):GRK2 association. VIP-induced GRK2 phosphorylation was significantly inhibited in smooth muscle cells expressing G\(\beta\gamma\)-scavenging peptide (Fig. 3B).
enging peptide (COOH-terminal fragment of GRK2), suggesting that binding of Gβγ to GRK2 augments GRK2 phosphorylation by PKA (Fig. 3B).

Conversely, Gβγ:GRK2 association induced by VIP was significantly inhibited in smooth muscle cells expressing GRK2(S685A), suggesting that phosphorylation of GRK2 by PKA augments the GRK2 association with Gβγ (Fig. 4). Forskolin, which activates adenylyl cyclase directly, had no effect on GRK2:Gβγ association or GRK2 phosphorylation but augmented GRK2:Gβγ association and GRK2 phosphorylation induced by a low concentration of VIP (Fig. 5). These results imply that GRK2:Gβγ association and GRK2 phosphorylation were reciprocally regulated and were dependent on receptor activation: phosphorylation of GRK2 increased its association with Gβγ, which, in turn, enhanced GRK2 phosphorylation by PKA.

To determine whether the phosphorylation of GRK2 affects its activity, we measured the activity of GRK2 in response to VIP in both freshly dispersed muscle cells and cells expressing wild-type GRK2 and GRK2(S685). VIP caused an increase in GRK2 activity that was significantly attenuated by blockade of PKA with myristoylated protein kinase inhibitor (PKI), suggesting that the phosphorylation of GRK2 by PKA augmented GRK2 activity (Fig. 6). VIP also caused an increase in GRK2 activity in cultured muscle cells expressing wild-type GRK2 and GRK2(S684). However, the increase in GRK2 activity was significantly inhibited in cells expressing GRK2(S685A), providing further support for the notion that PKA-mediated phosphorylation augmented GRK2 activity (Fig. 6).
Phosphorylation of GRK2 by PKA augments VPAC2 receptor phosphorylation. VIP induced phosphorylation of VPAC2 receptors, and the phosphorylation was abolished in cells expressing GRK2(K220R) and partly inhibited in cells expressing GRK2(S685A), implying that the ability of GRK2 to phosphorylate VPAC2 receptors is partly dependent on its phosphorylation by PKA (Fig. 7). No phosphopeptide band was detected in VPAC2 receptor immunoprecipitates using phosphosubstrate antibody specific for PKA phosphorylation sites, confirming that VPAC2 receptors were phosphorylated by GRK2 but not by PKA. Furthermore, receptor-independent activation of PKA with forskolin had no effect on VPAC2 receptor phosphorylation, but it augmented VPAC2 receptor phosphorylation induced by a low concentration of VIP (10 nM; Fig. 8). Similar results were obtained with a cAMP analog, Sp-5,6-dichloro-1-β-d-ribofuransylbenzimidazole-3′,5′-monophosphorothioate, which selectively activates PKA (data not shown). These results show that VPAC2 receptors were phosphorylated exclusively by GRK2 and that GRK2 activity was augmented upon its phosphorylation by PKA.

Phosphorylation of GRK2 by PKA augments VPAC2 receptor internalization. VPAC2 receptor internalization was assessed by the decrease in [125I]-VIP binding to surface receptors after treatment with 1 μM unlabeled VIP. Pretreatment of cultured smooth muscle cells with VIP caused a decrease in [125I]-VIP binding that was completely reversed in cells expressing GRK2(K220R) and partly reversed in cells expressing GRK2(S685A), suggesting that receptor internalization was mediated by GRK2 and that the effect of GRK2 was augmented upon its phosphorylation by PKA (Fig. 9).

In cells expressing wild-type GRK2, receptor internalization (decrease in receptor binding to [125I]-VIP after a 30-min exposure to the ligand) was dependent on the concentration of VIP and was significantly attenuated in cells expressing GRK2(S685A) (Fig. 10A). VPAC2 receptor internalization was rapid [half-time (t_{0.5}): ~7 min] and maximal after a 20-min
exposure to VIP (21 ± 3% surface receptors and 76 ± 4% internalized receptors; Fig. 10B). In cell expressing GRK2(S685A), however, VPAC2 receptor internalization was slower (t0.5: ~20 min), and the maximal number of internalized receptors after a 30-min exposure to VIP was significantly lower (56 ± 6% surface receptors and 42 ± 5% internalized receptors; Fig. 10B).

A similar time course for VPAC2 receptor internalization was observed after biotin labeling of surface VPAC2 receptors.

Treatment of cells expressing wild-type GRK2 with VIP caused a rapid decrease in surface VPAC2 receptors (t0.5: ~10 min) and a maximal decrease after 30 min (~80% decrease in surface receptors). In cell expressing GRK2(S685A), however, VPAC2 receptor internalization was slower (t0.5: ~30 min), and the maximal decrease in surface receptors after a 30-min exposure to VIP was significantly lower (~50% decrease; Fig. 11).

Receptor recycling was determined after a 30-min exposure to VIP followed by a 60-min washout period; at 10-min intervals during this period, 125I-VIP binding to surface receptors was measured. In cells expressing wild-type GRK2, recycling was virtually complete within 60 min, whereas in cells expressing GRK2(S685A) recycling was complete within 30 min (Fig. 10B).

Receptor degradation determined from residual 125I-VIP binding after prolonged exposure to VIP in cells expressing wild-type GRK2 was minimal during the first hour and increased progressively to 80% after 5 h (Fig. 12). Degradation was greatly reduced (~50% after 5 h) in cells expressing GRK2(S685A) and was undetectable in cells expressing kinase-deficient GRK2(K220R). The pattern of receptor degradation was corroborated by immunoblot analysis of VPAC2 receptors.

Phosphorylation of GRK2 augments VPAC2 receptor desensitization. Desensitization of the response mediated by VPAC2 receptors was determined by direct measurement of adenylyl cyclase activity. VIP stimulated adenylyl cyclase activity of cultured smooth muscle cells in a concentration-dependent fashion (EC50: 3 nM; maximal effect at 1 μM VIP). After treatment of the cells with 1 μM VIP for 30 min, adenylyl cyclase activity decreased significantly at all concentrations; the response to 1 nM VIP was abolished, and the
response to 1 μM VIP was inhibited by 60% (Fig. 13). There was no desensitization of VIP-stimulated adenylyl cyclase activity in cells expressing GRK2(K220R) and only partial desensitization in cells expressing GRK2(S685A) (Fig. 13). Similarly, VIP-stimulated cAMP generation was significantly inhibited after treatment of the cells with 1 μM VIP for 30 min (data not shown). There was no desensitization of the cAMP response in cells expressing GRK2(K220R) and only partial desensitization in cells expressing GRK2(S685A) (data not shown). These results implied that VIP-induced desensitization resulted from GRK2-mediated phosphorylation and internalization of VPAC2 receptors, which were augmented via PKA-mediated GRK2 phosphorylation.

The notion that phosphorylation of GRK2 was mediated by PKA leading to increase in GRK2:Gβγ association and GRK2-mediated VPAC2 receptor phosphorylation and partially inhibited GRK2:Gβγ association and GRK2-mediated VPAC2 receptor phosphorylation (Fig. 14). PKI also partially inhibited the VIP-induced VPAC2 receptor internalization and desensitization (Fig. 14).

DISCUSSION

The extent to which second messenger-activated protein kinases such as PKA participate in receptor phosphorylation and internalization varies greatly among receptors. As noted above, agonist-occupied β2ARs are preferentially phosphorylated by PKA at low agonist concentrations and jointly by PKA and GRK2 at higher concentrations, whereas β1ARs are phosphorylated in equal measure by PKA and GRK2, with PKA-phosphorylated receptors targeted to caveolae for internalization (4, 7, 9, 10, 39, 45). Among class II receptors, secretin receptors can be phosphorylated by GRK2 in vitro, yet are preferentially phosphorylated by PKA in vivo and internalized via an endocytic pathway that is independent of GRK2, β-arrestin, or dynamin (12, 42, 46). In contrast, constitutively expressed VPAC1 receptors are phosphorylated by GRK2 and internalized via a β-arrestin/dynamin-dependent pathway; neither phosphorylation nor internalization of VPAC1 receptors is affected by PKA inhibitors (17, 25, 43).

We have previously shown that smooth muscle cells of the gut express predominantly VPAC2 receptors (6, 44). Here, we show that VPAC2 receptors differ from their homologs, secre-
tin and VPAC1 receptors, as well as from class I receptors. VPAC2 receptors, like VPAC1 receptors, are phosphorylated exclusively by GRK2 but, unlike secretin and β2AR phosphorylation that is mediated by GRK2 (7).

The inability of PKA to phosphorylate unoccupied or VIP-occupied VPAC2 receptors directly was evident in experiments where forskolin alone did not induce VPAC2 receptor phosphorylation. In the presence of VIP, however, forskolin augmented GRK2 phosphorylation, GRK2:Gβγ association, and receptor phosphorylation.

The essential role of GRK2 and the augmentatory role of PKA-mediated GRK2 phosphorylation were reflected in measurements of GRK2 activity and VPAC2 receptor internalization, recycling, and degradation as well as in the desensitization of the functional response to VIP. VIP-induced phosphorylation of GRK2 was blocked by PKI in freshly dispersed cells and by expression of GRK2(S685A) in cultured muscle cells. Internalization, determined from the decrease in 125I-VIP binding to VPAC2 receptors, was abolished in cells expressing kinase-deficient GRK2(K220R) and significantly inhibited in cells expressing PKA phosphorylation site-deficient GRK2(S685A). A similar inhibition was observed in experiments where VPAC2 receptors were labeled with biotin. VPAC2 receptor degradation, observed only upon prolonged exposure to VIP (1–5 h), reflected the pattern of receptor internalization and was significantly decreased in cells expressing GRK2(S685A) and undetectable in cells expressing kinase-deficient GRK2(K220R). Desensitization of the functional response (adenylyl cyclase activity and cAMP generation) also followed the pattern of internalization by decreasing in cells expressing GRK2(S685A) and was absent in cells expressing kinase-deficient GRK2(K220R). The effect of GRK2(S685) expression on VPAC2 receptor phosphorylation, internalization, and desensitization was mimicked by the selective blockade of PKA activity.

The binding of PKA to AKAP at the inner surface of the plasma membrane and the recruitment of GRK2 by Gβγ association, and VPAC2 phosphorylation, internalization, and desensitization were measured in the presence or absence of myristoylated PKI, as described in MATERIALS AND METHODS. GRK2 phosphorylation was measured in cells labeled with 32P and in nonlabeled cells. VPAC2 receptor internalization was assessed by the decrease in 125I-VIP binding to surface receptors after the treatment with VIP. cAMP was measured by radioimmunoassay, and the results are expressed as picomoles per milligram of protein. **P < 0.001, significant decrease in 125I-VIP binding and cAMP formation after VIP treatment; ###P < 0.001, significant attenuation of the decrease in 125I-VIP binding and cAMP formation by PKI.

![Fig. 14. Effect of PKA inhibitor (PKI) on the phosphorylation of GRK2 and its association with Gβγ as well as VPAC2 phosphorylation, internalization, and desensitization induced by VIP. VIP-induced GRK2 phosphorylation, GRK2:Gβγ association, and VPAC2 phosphorylation, internalization, and desensitization were measured in the presence or absence of myristoylated PKI, as described in MATERIALS AND METHODS. GRK2 phosphorylation was measured in cells labeled with 32P and in nonlabeled cells. VPAC2 receptor internalization was assessed by the decrease in 125I-VIP binding to surface receptors after the treatment with VIP. cAMP was measured by radioimmunoassay, and the results are expressed as picomoles per milligram of protein. **P < 0.001, significant decrease in 125I-VIP binding and cAMP formation after VIP treatment; ###P < 0.001, significant attenuation of the decrease in 125I-VIP binding and cAMP formation by PKI.](http://ajpcell.physiology.org/)
kinases. In this study, binding of Gβγ to GRK2 enhanced phosphorylation of GRK2 by PKA; in turn, phosphorylation of GRK2 enhanced its binding to Gβγ. This interdependence was evident by the decrease of GRK2 phosphorylation in cells expressing Gβγ-scavenging peptide and by the decrease in Gβγ:GRK2 association in cells expressing GRK2(S685A).

It is possible, as proposed by Cong et al. (7) for β2ARs, that AKAP79/150 acts as a scaffold that binds receptor and PKA (but not GRK2) and facilitates phosphorylation of receptor or GRK2 by PKA. While AKAP79/150 is an appropriate scaffold for receptor substrates of PKA such as β2ARs, a more plausible candidate where VPAC2 receptors are concerned is caveolin, which is known to act as a scaffold that binds various signaling proteins including receptors (e.g., β2AR), activated G protein subunits, AKAP79/150, adenylyl cyclase V/VI, PKC ε, L-type Ca2+ channel protein, and phosphatase 2B (3, 5, 15, 16, 28, 31, 34–36, 41, 47). In gastric smooth muscle homogenates, caveolin-3, adenylyl cyclase V/VI, PKA, GRK2, PKC ε, phosphatase 2B, and voltage-gated Ca2+ channel protein coimmunoprecipitated with AKAP79/150 (K. S. Murthy, unpublished observations).

In summary, G protein–coupled VPAC2 receptor phosphorylation, internalization, and desensitization exhibit features that distinguish VPAC2 receptors from other G protein–coupled class I and II receptors. VPAC2 receptor phosphorylation is mediated exclusively by GRK2 (Fig. 15). Feedback phosphorylation of GRK2 by PKA enhances GRK2 activity and its ability to mediate VPAC2 receptor phosphorylation, internalization, desensitization, and degradation.

GRANTS

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