Cross-regulation of VPAC$_2$ receptor desensitization by M$_3$ receptors via PKC-mediated phosphorylation of RKIP and inhibition of GRK2

Jiean Huang, Sunila Mahavadi, Wimolpak Sriwai, John R. Grider, and Karnam S. Murthy

Departments of Physiology and Medicine, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia

Submitted 21 July 2006; accepted in final form 4 December 2006

Huang J, Mahavadi S, Sriwai W, Grider JR, Murthy KS. Cross-regulation of VPAC$_2$ receptor desensitization by M$_3$ receptors via PKC-mediated phosphorylation of RKIP and inhibition of GRK2. Am J Physiol Gastrointest Liver Physiol 292: G867–G874, 2007. First published December 14, 2006; doi:10.1152/ajpgi.00326.2006.—In gastrointestinal smooth muscle cells, VPAC$_2$ receptor desensitization is exclusively mediated by G protein-coupled receptor kinase 2 (GRK2). The present study examined the mechanisms by which acetylcholine (ACh) acting via M$_3$ receptors regulates GRK2-mediated VPAC$_2$ receptor desensitization in gastric smooth muscle cells. Vasoactive intestinal peptide induced VPAC$_2$ receptor phosphorylation, internalization, and desensitization in both freshly dispersed and cultured smooth muscle cells. Costimulation with ACh in the presence of M$_2$ receptor antagonist (i.e., activation of M$_3$ receptors) inhibited VPAC$_2$ receptor phosphorylation, internalization, and desensitization. Inhibition was blocked by the selective protein kinase C (PKC) inhibitor bisindolylmaleimide, suggesting that the inhibition was mediated by PKC, derived from M$_3$ receptor activation. Similar results were obtained by direct activation of PKC with phorbol myristate acetate. In the presence of the M$_2$ receptor antagonist, ACh induced phosphorylation of Raf kinase inhibitory protein (RKIP), increased RKIP-GRK2 association, decreased RKIP-Raf-1 association, and stimulated ERK1/2 activity, suggesting that, upon phosphorylation by PKC, RKIP dissociates from its known target Raf to associate with, and block the activity of, GRK2. In muscle cells expressing RKIP(S153A), which lacks the PKC phosphorylation site, RKIP phosphorylation was blocked and the inhibitory effect of ACh on VPAC$_2$ receptor phosphorylation, internalization, and desensitization and the stimulatory effect on ERK1/2 activation were abolished. This study identified a novel mechanism of cross-regulation of G$_c$-coupled receptor phosphorylation and internalization by G$_s$-coupled receptors. The mechanism involved phosphorylation of RKIP by PKC, switching RKIP from association with Raf-1 to association with, and inhibition of, GRK2.

IN THE GASTROINTESTINAL TRACT, the neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenyl cyclase-activating peptide (PACAP) are colocalized in a subset of myenteric neurons that innervate the smooth muscle, and their release is functionally linked to smooth muscle relaxation (1, 13). The biological actions of VIP and PACAP are mediated by a family of G protein-coupled receptors, which are designated as VPAC$_1$, VPAC$_2$, and PAC$_1$ receptors (9, 26). These receptors belong to class II of the G protein-coupled receptors known as the secretin receptor family that include receptors for secretin, gastric inhibitory peptide, glucagon, glucagon-like peptide 1, calcitonin, parathyroid hormone, growth hormone-releasing factor, and corticotrophin-releasing hormone (9, 23, 24, 26, 27). PAC$_1$ receptors exhibit high affinity for PACAP and low affinity for VIP, whereas VPAC$_1$ and VPAC$_2$ receptors exhibit equal affinity for VIP and PACAP (9).

Smooth muscle cells of the gut express VPAC$_2$ receptors, but not VPAC$_1$ and PAC$_1$ receptors (16, 25). We have recently shown that VPAC$_2$ receptors are desensitized and internalized by a mechanism distinct from those utilized by VPAC$_1$ or secretin receptors (15, 23, 24, 27, 31). VPAC$_2$ receptors are exclusively phosphorylated by G protein-coupled receptor kinase 2 (GRK2), resulting in internalization of receptors and desensitization of VIP-induced adenyl cyclase activity and cAMP formation. GRK2 consists of an NH$_2$-terminal domain, a central catalytic domain, and a membrane-targeting COOH-terminal domain, which contains a pleckstrin homology domain for interaction with the G$_{B_y}$ subunits (7). This interaction facilitates targeting of GRK2 to the membrane and enhances its catalytic activity toward the receptor. GRK2 activity and subcellular localization are regulated by different mechanisms including binding to G$_{B_y}$, activated receptor, phospholipids, Ca$^{2+}$-binding protein calmodulin, caseolin, and anchoring proteins (2, 5, 8, 12, 22). In addition, phosphorylation by various kinases such as extracellular signal-regulated kinases (ERK1/2), c-Src, and cAMP-dependent kinase (PKA) regulates GRK2 activity (5, 22). Phosphorylation by PKA or c-Src increased GRK2 activity, whereas phosphorylation by ERK1/2 decreased GRK2 activity.

In contrast to the direct phosphorylation by kinases, a recent study identified a novel mechanism for inhibition of GRK2 activity involving protein kinase C (PKC) and Raf kinase inhibitory protein (RKIP) (14). RKIP binds to Raf-1 and prevents the activation of the ERK1/2 cascade (4, 14, 28, 29). However, phosphorylation of RKIP by PKC dissociates RKIP from Raf-1 and switches its binding to GRK2, resulting in inhibition of GRK2 activity and stimulation of ERK1/2 activity (14). These studies suggest that when multiple receptors are simultaneously activated the net effect of kinases such as PKA, ERK1/2, and PKC could modulate GRK2 activity. In this study, we provide evidence that phosphorylation, internalization, and desensitization of G$_s$-coupled VPAC$_2$ receptors by GRK2 were inhibited by G$_s$-coupled M$_3$ receptors. The mechanism involves phosphorylation of RKIP by PKC and phosphorylation-dependent switching of RKIP from its association with Raf-1 to association with GRK2 and inhibition of GRK2 activity.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpgi.org 0193-1857/07 $8.00 Copyright © 2007 the American Physiological Society
MATERIALS AND METHODS

Dispersion and culture of gastric smooth muscle cells. Smooth muscle cells were isolated from the circular muscle layer of the rabbit stomach by sequential enzymatic digestion, filtration, and centrifugation as described previously (17, 18, 20). Briefly, smooth muscle strips were incubated at 31 °C for 20 min in HEPES medium [25 mM HEPES, 120 mM NaCl, 4 mM KCl, 2.6 mM KH2PO4, 0.6 mM MgCl2, 14 mM glucose, and 2.1% (vol/vol) Eagle’s essential amino acid mixture (pH 7.4)] containing type II collagenase (0.1%) and soybean trypsin inhibitor (0.1%). The partly digested strips were washed with 50 ml of enzyme-free medium, and muscle cells were allowed to disperse spontaneously for 30 min. Muscle cells were 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% fetal bovine serum until they attained confluence and were then passaged once for use in various studies (11, 20, 30).

RKIP mutant constructs. PKC phosphorylation site-deficient RKIP [RKIP(S153A)] was subcloned into the multiple cloning site (EcoRI) of the eukaryotic expression vector pEEXV. Smooth muscle cells were transiently transfected with recombinant plasmid DNAs (2 of the eukaryotic expression vector pEXV. Smooth muscle cells were in the first passage by use of Lipofectamine Plus reagent for 48 h. The 60 min at 4°C with 50 pM 125I-labeled VIP followed by separation of VIP (1 μM) plus M2 receptor antagonist methoctramine (0.1 μM) and then with PBS at 4°C followed by incubation with 0.53 mM EDTA in phosphate-buffered saline (PBS) for 30 min. Dispersed smooth muscle cells were cultured in DMEM containing 10% fetal bovine serum, and muscle cells were allowed to disperse spontaneously for 30 min. Muscle cells were 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% fetal bovine serum until they attained confluence and were then passaged once for use in various studies (11, 20, 30).

RKIP mutant constructs. PKC phosphorylation site-deficient RKIP [RKIP(S153A)] was subcloned into the multiple cloning site (EcoRI) of the eukaryotic expression vector pEEXV. Smooth muscle cells were transiently transfected with recombinant plasmid DNAs (2 μg each) in the first passage by use of Lipofectamine Plus reagent for 48 h. The cells were cotransfected with 1 μg of pGreen Lantern-1 to monitor the transfection efficiency (~75%) using FITC filters. Control cells were cotransfected with 2 μg of vector (pEXV) and 1 μg of pGreen Lantern-1 DNA (11, 30).

125I-labeled VIP binding to smooth muscle cells. Binding of 125I-labeled VIP to freshly dispersed and cultured muscle cells was done as described previously (17). Cultured muscle cells detached by incubation with 0.53 mM EDTA in phosphate-buffered saline (PBS) for 30 min were collected by centrifugation at 400 g and resuspended in DMEM containing BSA (0.1%), amastatin (10 μM), and phosphoramidon (1 μM). Cell aliquots (2-106 cells/ml) were incubated for 60 min at 4°C with 50 pM 125I-labeled VIP followed by separation of bound and free radioligands by rapid filtration through 5-μm polycarbonate Nucleopore filters. Nonspecific binding was calculated as the amount of radioactivity in the presence of 10 μM VIP. 125I-labeled VIP binding was measured in the control cells and in cells pretreated for 30 min with 1 μM VIP in the absence or presence of ACh (0.1 μM) plus M2 receptor antagonist methochromine (0.1 μM) to induce VPAC2 receptor internalization.

Biotin labeling of surface receptors. Muscle cells were treated with VIP (1 μM) for 30 min in the presence or absence of ACh or phorbol myristate acetate (PMA) and then with PBS at 4°C followed by treatment with 10 mM sodium periodate for 30 min in the dark. After the removal of periodate by a wash with PBS, cells were incubated for 30 min at 4°C with 2 μM biotin LC-hydrazide (membrane-impermeable derivative of biotin) to conjugate glycoproteins with biotin (6). Cells were then solubilized in lysis buffer and incubated with antibody to VPAC2 receptors. The VPAC2 receptor immunoprecipitates were separated by electrophoresis on SDS-PAGE and then transferred to a nitrocellulose membrane. Electron microscopy was performed on the immunoprecipitates as described previously (10). Muscle cells (2-106/ml) were treated with ACh (plus methochromine) in the presence or absence of the PKC inhibitor bisindolylmaleimide for 5 min, and cell lysates were prepared. The cell lysates were precleared by an incubation with 40 μl of protein A-Sepharose for another 2 h. The immunoprecipitates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibody to GRK2 or Raf-1. After incubation with secondary antibody, the proteins were visualized by enhanced chemiluminescence (11, 20).

Assay for adenyl cyclase activity. Adenyl cyclase activity was measured by using [α-32P]ATP as a substrate, as described previously (21). Muscle cell homogenates were incubated for 15 min at 37°C in 50 mM Tris-HCl (pH 7.4), 2 mM cAMP, 1 mM ATP, 1 mM isobutylmethylxanthine, 5 mM MgCl2, 100 mM KCl, 5 mM creatine phosphate, 50 U/ml creatine phosphokinase, and 0.5 mM [α-32P]ATP. The [32P]cAMP produced was collected by sequential chromatography on Dowex AG50W-4X and alumina columns. The results were expressed as picomoles of cAMP per milligram of protein per minute.

Radioimmunoassay for cAMP. cAMP levels were measured by radioimmunoassay as described previously (17, 21). Muscle cells were stimulated with 1 μM VIP for 60 s in the presence of 100 μM isobutylmethylxanthine, and the reaction was terminated with 10% trichloroacetic acid. The samples were centrifuged and supernatant was extracted with diethyl ether and then lyophilized. The lyophilized samples were suspended in 50 mM Na acetate (pH 6.2) and acetylated with triethylamine-acetic anhydride for 30 min. cAMP was measured in duplicate using 100-μl aliquots, and the results were expressed as picomoles per milligram of protein.

In vitro kinase assay for ERK1/2 activity. ERK1/2 activity was determined by immunokinetase assay as described previously (30). Freshly dispersed or cultured muscle cells were treated with ACh (plus methochromine) in the presence or absence of the PKC inhibitor bisindolylmaleimide for 5 min, and cell lysates were prepared. The cell lysates were precleared by centrifugation, and an aliquot was incubated with ERK1/2 antibody. The immunoprecipitates were washed twice with a phosphorylation buffer containing 10 mM MgCl2 and 40 mM HEPES (pH 7.4) and then incubated for 5 min with 5 μg of myelin basic protein. Kinase assay was initiated by the addition of 10 μCi of [γ-32P]ATP (3,000 Ci/mmol) and 20 μM ATP. 32P-labeled myelin basic protein was adsorbed onto phosphocellulose disks. The extent of phosphorylation was determined from the radioactivity on phosphocellulose disks by liquid scintillation.

Assay for PKC activity. PKC activity was measured in the particulate fraction as described previously (10). Muscle cells (2-106 cells/ml) were incubated with ACh in the presence of methochromine, and the reaction was terminated by rapid freezing. PKC activity was measured in membrane fractions by phosphorylation of myelin basic protein, and the results are expressed as cpm per milligram of protein.

Measurement of relaxation in dispersed smooth muscle cells. Inhibition of KCl-induced contraction (i.e., relaxation) by VIP was expressed as the decrease in maximal cell contraction induced by 20 mM KCl. The mean cell length of 50 muscle cells treated with KCl alone or KCl in the presence of VIP (1 μM) before and after desensitization VPAC2 receptors with VIP or VIP and ACh (plus methochromine) was measured by scanning micrometry and was compared with the length of untreated muscle cells (mean control cell length: 105 ± 4 μm) (17-19).

Materials. 125I-labeled VIP, [α-32P]ATP, 125I-labeled cAMP, and [32P]orthophosphate were obtained from NEN Life Sciences Products (Boston, MA); polyclonal antibodies to VPAC2 receptors, RKIP, GRK2, and Raf-1 were obtained from Santa Cruz Biotechnology.
Bands corresponding to VPAC2 receptor were identified by autoradiography. SB203580 (1 μM) or the p38 MAPK inhibitor PD98059 (10 μM) blocked the effect of PMA and ACh (Fig. 1), but not by the ERK1/2 inhibitor U0126 (10 μM) or the p38 MAPK inhibitor SB203580 (1 μM) (data not shown). Activation of PKC by PKC, however, did not induce GRK2 phosphorylation (data not shown). These results imply that GRK2-mediated VPAC2 receptor phosphorylation was inhibited by concomitant activation of PKC and that the inhibitory effect of PKC was not due to phosphorylation of GRK2. Treatment of cells with ACh in the absence of methoctramine had no significant effect on VPAC2 receptor phosphorylation (data not shown), suggesting that activation of M₃ receptors masks the effect of the M₃ receptors on VPAC2 receptor phosphorylation.

**Inhibition of GRK2-mediated VPAC2 receptor phosphorylation by PKC.** Treatment of freshly dispersed smooth muscle cells with VIP caused phosphorylation of VPAC2 receptor. Previous studies have shown that agonist-induced VPAC2 receptor phosphorylation was blocked in cells expressing dominant negative GRK2, but not in cells expressing dominant GRK3 or GRK5 (14, 31), suggesting that phosphorylation was mediated exclusively by GRK2. Stimulation of PKC by PMA or ACh in the presence of the M₂ receptor antagonist methoctramine (i.e., by activation of M₂ receptors) inhibited VPAC2 receptor phosphorylation (Fig. 1). The effect of PMA and ACh was blocked by the PKC inhibitor bisindolylmaleimide (1 μM) (Fig. 1), but not by the ERK1/2 inhibitor PD98059 (10 μM) or the p38 MAPK inhibitor SB203580 (1 μM) (data not shown). Ammonia activation of PKC by PKC, however, did not induce GRK2 phosphorylation (data not shown). These results imply that GRK2-mediated VPAC2 receptor phosphorylation was inhibited by concomitant activation of PKC and that the inhibitory effect of PKC was not due to phosphorylation of GRK2. Treatment of cells with ACh in the absence of methoctramine had no significant effect on VPAC2 receptor phosphorylation (data not shown), suggesting that activation of M₂ receptors

**Inhibition of VPAC2 receptor desensitization by PKC.** VIP caused an increase in adenyl cyclase activity in a concentration-dependent fashion (EC₅₀ 4 ± 1 nM), with a maximal stimulation at 1 μM VIP. After treatment of cells with VIP, there was a significant decrease in adenyl cyclase activity at all concentrations of VIP, suggesting that exposure of muscle cells to VIP induced desensitization of VPAC2 receptors (Fig. 3A). Treatment of muscle cell with VIP in the presence of ACh plus methoctramine attenuated VIP-induced desensitization of VPAC2 receptors (Fig. 3A). The effect of ACh was blocked by bisindolylmaleimide (data not shown), suggesting that attenuation was mediated by PKC. PMA or ACh, however, had no effect on adenyl cyclase activity stimulated directly by forskolin (forskolin: 35.1 ± 5.0 pmol·mg protein⁻¹·min⁻¹; forskolin plus ACh: 33.2 ± 5.2 pmol·mg protein⁻¹·min⁻¹; forskolin plus PMA: 34.4 ± 3.8 pmol·mg protein⁻¹·min⁻¹). The results imply that the attenuation of desensitization by PKC was not due to its direct effect on adenyl cyclase activity but rather to inhibition of agonist-induced phosphorylation and internalization of VPAC2 receptors.

Similarly, VIP-induced cAMP generation was greatly inhibited after pretreatment of cells with VIP; inhibition was attenuated by costimulation with ACh or PMA and the effect of ACh or PMA was blocked by bisindolylmaleimide (Fig. 3B). The basal levels of cAMP were similar before and after pretreatment of cells with VIP.

Further corroboration of M₃ receptor-induced attenuation of VPAC2 receptor desensitization was obtained in cells where only the M₃ receptor type was preserved by selective receptor protection (17). In cells where only M₃ receptors were preserved, VIP-induced cAMP generation was inhibited after pretreatment of cells with VIP and the extent inhibition (70 ± 4%) was similar to naive cells (72 ± 5%). Pretreatment of M₃ receptor-preserved cells with VIP in the presence of ACh attenuated VPAC2 receptor desensitization (36 ± 2% inhibition), suggesting that coactivation of M₃ receptors attenuates VPAC2 receptor desensitization.

Treatment of freshly dispersed smooth muscle cells with 20 mM KCl caused a peak contraction within 30 s. Contraction

---

**RESULTS**

**Inhibition of GRK2-mediated VPAC2 receptor phosphorylation by PKC.** Treatment of freshly dispersed smooth muscle cells with VIP caused phosphorylation of VPAC2 receptor. Previous studies have shown that agonist-induced VPAC2 receptor phosphorylation was blocked in cells expressing dominant negative GRK2, but not in cells expressing dominant GRK3 or GRK5 (14, 31), suggesting that phosphorylation was mediated exclusively by GRK2. Stimulation of PKC by PMA or ACh in the presence of the M₂ receptor antagonist methoctramine (i.e., by activation of M₂ receptors) inhibited VPAC2 receptor phosphorylation (Fig. 1). The effect of PMA and ACh was blocked by the PKC inhibitor bisindolylmaleimide (1 μM) (Fig. 1), but not by the ERK1/2 inhibitor PD98059 (10 μM) or the p38 MAPK inhibitor SB203580 (1 μM) (data not shown). Activation of PKC by PKC, however, did not induce GRK2 phosphorylation (data not shown). These results imply that GRK2-mediated VPAC2 receptor phosphorylation was inhibited by concomitant activation of PKC and that the inhibitory effect of PKC was not due to phosphorylation of GRK2. Treatment of cells with ACh in the absence of methoctramine had no significant effect on VPAC2 receptor phosphorylation (data not shown), suggesting that activation of M₂ receptors masks the effect of the M₃ receptors on VPAC2 receptor phosphorylation.

**Inhibition of VPAC2 receptor internalization by PKC.** VPAC2 receptor internalization was assessed in freshly dispersed muscle cells by the decrease in 125I-labeled VIP binding to the surface receptors after treatment with VIP. Pretreatment with VIP caused a decrease in 125I-labeled VIP binding, suggesting agonist-induced internalization of VPAC2 receptors. Stimulation of PKC by PMA or ACh in the presence of methoctramine inhibited VPAC2 receptor internalization (Fig. 2A). The effect of PMA and ACh on VPAC2 receptor internalization was blocked by bisindolylmaleimide (Fig. 2A). In addition, the selective M₃ receptor antagonist 4-diphenylacetoxy-N-methylpiperideine also blocked the effect of ACh on VPAC2 receptor internalization (Fig. 2A). The results imply that VPAC2 receptor internalization was inhibited by concomitant activation of M₃ receptors via PKC.

A similar pattern of VPAC2 receptor internalization was obtained by measurements of biotin labeling of surface VPAC2 receptors. Treatment of muscle cells with VIP for 30 min induced a significant decrease in surface VPAC2 receptors. Stimulation of PKC with PMA or ACh in the presence of methoctramine significantly attenuated the decrease in surface VPAC2 receptors (Fig. 2B).

**Inhibition of VPAC2 receptor desensitization by PKC.** VIP caused an increase in adenyl cyclase activity in a concentration-dependent fashion (EC₅₀ 4 ± 1 nM), with a maximal stimulation at 1 μM VIP. After treatment of cells with VIP, there was a significant decrease in adenyl cyclase activity at all concentrations of VIP, suggesting that exposure of muscle cells to VIP induced desensitization of VPAC2 receptors (Fig. 3A). Treatment of muscle cell with VIP in the presence of ACh plus methoctramine attenuated VIP-induced desensitization of VPAC2 receptors (Fig. 3A). The effect of ACh was blocked by bisindolylmaleimide (data not shown), suggesting that attenuation was mediated by PKC. PMA or ACh, however, had no effect on adenyl cyclase activity stimulated directly by forskolin (forskolin: 35.1 ± 5.0 pmol·mg protein⁻¹·min⁻¹; forskolin plus ACh: 33.2 ± 5.2 pmol·mg protein⁻¹·min⁻¹; forskolin plus PMA: 34.4 ± 3.8 pmol·mg protein⁻¹·min⁻¹). The results imply that the attenuation of desensitization by PKC was not due to its direct effect on adenyl cyclase activity but rather to inhibition of agonist-induced phosphorylation and internalization of VPAC2 receptors.

Similarly, VIP-induced cAMP generation was greatly inhibited after pretreatment of cells with VIP; inhibition was attenuated by costimulation with ACh or PMA and the effect of ACh or PMA was blocked by bisindolylmaleimide (Fig. 3B). The basal levels of cAMP were similar before and after pretreatment of cells with VIP.

Further corroboration of M₃ receptor-induced attenuation of VPAC2 receptor desensitization was obtained in cells where only the M₃ receptor type was preserved by selective receptor protection (17). In cells where only M₃ receptors were preserved, VIP-induced cAMP generation was inhibited after pretreatment of cells with VIP and the extent inhibition (70 ± 4%) was similar to naive cells (72 ± 5%). Pretreatment of M₃ receptor-preserved cells with VIP in the presence of ACh attenuated VPAC2 receptor desensitization (36 ± 2% inhibition), suggesting that coactivation of M₃ receptors attenuates VPAC2 receptor desensitization.

Treatment of freshly dispersed smooth muscle cells with 20 mM KCl caused a peak contraction within 30 s. Contraction
was measured as the mean decrease in cell length (control cell length: 105 ± 4 μm; mean decrease in cell length: 36 ± 2 μm). Treatment of muscle cells for 60 s with VIP (1 μM) inhibited KCl-induced contraction (i.e., relaxation) by 72 ± 5%. Pretreatment of cells with VIP for 30 min to desensitize VPAC2 receptors significantly decreased relaxation (28 ± 5%) in response to subsequent addition of VIP, suggesting desensitization of functional response. Pretreatment of cells with VIP and ACh (in the presence of methoctramine) attenuated desensitization and partly restored relaxation (56 ± 3%).

Phosphorylation of RKIP by PKC Switches RKIP from Raf-1 to GRK2

In the absence of GRK2 phosphorylation by PKC, we examined the mechanism for the PKC-mediated attenuation of
agonist-induced phosphorylation, internalization, and desensitization of VPAC2 receptors. Previous studies have shown that RKIP is a physiological inhibitor of Raf kinase (14, 28, 29). Phosphorylation of RKIP at Ser153 by PKC results in the dissociation of RKIP from its known target Raf-1 to associate with GRK2 and inhibition of GRK2 activity (14). ACh induced phosphorylation of RKIP, and the phosphorylation was blocked by bisindolylmaleimide (Fig. 4). In the basal state, RKIP was associated with Raf-1 but not with GRK2. Treatment with ACh induced RKIP-GRK2 association and decreased RKIP-Raf-1 association; the effect of ACh was blocked by bisindolylmaleimide, suggesting that PKC-induced phosphorylation switches RKIP from Raf-1 to GRK2 (Fig. 4). ACh-induced phosphorylation of RKIP and its switching from RKIP to GRK2 were blocked in cells expressing RKIP(S153A) (Fig. 4).

**Phosphorylation of RKIP by PKC Inhibits GRK2-Mediated VPAC2 Receptor Phosphorylation, Internalization, and Desensitization**

The effect of phosphorylated RKIP on GRK2-mediated VPAC2 receptor phosphorylation, internalization, and desensitization was examined in cells expressing RKIP(S153A).

As in freshly dispersed smooth muscle cells, VIP induced VPAC2 receptor phosphorylation in cultured smooth muscle cells. Activation of M3 receptor (ACh in the presence of M2 receptor antagonist) inhibited VIP-induced VPAC2 receptor phosphorylation (Fig. 5). ACh-induced decrease in VPAC2 receptor phosphorylation was blocked in cells expressing RKIP(S153A) (Fig. 5).

Fig. 4. ACh-induced dissociation of Raf kinase inhibitory protein (RKIP) from Raf-1 and association with G protein-coupled receptor kinase 2 (GRK2) is mediated by PKC-dependent phosphorylation of RKIP. Cultured smooth muscle cells expressing wild-type RKIP or PKC phosphorylation-deficient RKIP [RKIP(S153A)] were treated with ACh (in the presence of methoctra- mine). In some experiments, bisindolylmaleimide (1 μM) was included with ACh. Cells were lysed and immunoprecipitated with antibody to RKIP and association of RKIP with GRK2 or Raf-1. Phosphorylation of RKIP was identified in immunoprecipitates by autoradiography as described in MATERIALS AND METHODS. The experiments were repeated more than 3 times with similar results. Representative blots are shown.

Pretreatment of cultured muscle cells with VIP caused a decrease in 125I-VIP binding and VIP-induced cAMP formation, suggesting agonist-induced internalization and desensitization of VPAC2 receptors (Figs. 6 and 7). Activation of PKC via M3 receptors inhibited VPAC2 receptor internalization and PKC phosphorylation (2,098 ± 76 cpm/mg protein above basal levels of 560 ± 89 cpm/mg protein). The effect of ACh on PKC activity was blocked by bisindolylmaleimide (438 ± 87 cpm/mg protein above basal levels).

Phosphorylation of RKIP by PKC Augments ACh-Induced ERK1/2 Activation

We examined the possibility that ACh-induced switching of RKIP from Raf-1 could result in the augmentation of ACh-induced ERK1/2 activity. In freshly dispersed muscle cells, ACh induced activation of ERK1/2 (2.098 ± 257 cpm/mg protein above basal levels of 560 ± 89 cpm/mg protein). The effect of ACh on ERK1/2 activity was blocked by bisindolylmaleimide (438 ± 87 cpm/mg protein above basal levels). Similar PKC-sensitive activation of ERK1/2 by ACh was...
obtained in cultured muscle cells (Fig. 8). In cells expressing RKIP(S153A), however, activation of ERK1/2 by ACh was greatly inhibited (74% inhibition) and bisindolylmaleimide had no significant effect on the residual ERK1/2 activity. Activation of PKC by ACh was similar in both the wild-type and RKIP(S153A), expressing cells suggesting that RKIP had no effect on PKC activity (Fig. 8). These results provide evidence that activation of ERK1/2 by ACh was mediated by PKC via phosphorylation of RKIP and subsequent switching of RKIP from Raf-1, thus unmasking activation of Raf-1-dependent pathways.

**DISCUSSION**

This study shows that activation of PKC, whether by contractile agonists (e.g., ACh) or PMA, inhibited VPAC2 receptor phosphorylation, internalization, and desensitization in gastric muscle cells. The inhibition was mediated by phosphorylation of RKIP via PKC and subsequent dissociation of RKIP from Raf-1 and association with GRK2. In the basal state, RKIP is associated with Raf-1. After phosphorylation by PKC, RKIP dissociates from its target Raf-1 to associate with GRK2 (14). Association of RKIP with GRK2 inhibits the ability of GRK2 to phosphorylate VPAC2 receptors leading to inhibition of VPAC2 receptor internalization and desensitization.

The essential role of RKIP and the inhibitory phosphorylation by PKC were reflected in measurements of VPAC2 receptor phosphorylation, internalization, and desensitization. ACh-induced inhibition of VPAC2 receptor phosphorylation, internalization (determined from the decrease in 125I-labeled VIP binding to VPAC2 receptors after VIP treatment), and desensitization (determined from the decrease in adenyl cyclase activity and cAMP formation) was reversed by pretreatment of cells with the PKC inhibitor bisindolylmaleimide as well as in cells expressing RKIP(S153A).

In addition, ACh-induced dissociation of RKIP from Raf-1 and association with GRK2 were inhibited by bisindolylmaleimide and in cells expressing RKIP(S153A). After activation of PKC, phosphorylation-deficient RKIP remained bound to Raf-1 and did not switch to GRK2. These studies also provide evidence for the mechanism by which PKC derived from activation of Gq-coupled receptors activates ERK1/2 and an explanation of how PKC can physiologically regulate ERK1/2 function in smooth muscle cells. The mechanism involves phosphorylation of RKIP by PKC on Ser153 and loss of inhibition on Raf-1 by switching phosphorylated RKIP from Raf-1 to GRK2. Previous studies have shown that although Raf-1 was directly phosphorylated by PKC, mutation of the phosphorylation sites had no effect on PKC-induced activation Raf-1, suggesting that phosphorylation of Raf-1 is not necessary for activation (4, 14).

RKIP is a member of the phosphatidylethanolamine-binding protein family and a physiological inhibitor of Raf-1 and GRK2 (14). In the basal state, RKIP binds specifically Raf-1, but not Ras, and thus inhibits the activation of ERK1/2 pathway (4, 14, 28, 29). RKIP is not a kinase or a substrate for Raf-1, but it specifically inhibits Raf-1 interaction with its downstream targets.
downstream target MEK and thus suppresses ERK1/2 activity. Translocation and activation of PKC results in phosphorylation of membrane-bound (phospholipid-bound) RKIP on Ser153 and removal RKIP’s inhibition of Raf-1 (14). In vitro studies have shown that RKIP is a substrate for both classical and atypical PKCs, including PKC-α, -βI, -βII, -γ, -ζ, but not novel PKCs, including PKC-δ, -ɛ, and -η, suggesting that RKIP phosphorylation by PKC is isoform specific (4). Phosphorylation by PKC also switches RKIP’s association from Raf-1 to GRK2, leading to inhibition of GRK2 activity (14). It was estimated that the cellular stoichiometries are sufficient for Raf-1 to bind all RKIP molecules under basal conditions and for RKIP to bind all GRK2 molecules upon stimulation. In vitro studies have shown that GRK2, but not GRK5, PKA, or PKC, was specifically inhibited by RKIP (14). Studies using purified RKIP have also shown that RKIP inhibited GRK2-mediated phosphorylation of the rhodopsin receptor (14). A physiological role for RKIP as a GRK2 inhibitor was demonstrated in cardiomyocytes where downregulation of endogenous RKIP expression by a RKIP antisense or short interfering RNA restrained β-adrenergic signaling and contractile activity (14).

GRK2 plays an important role in agonist-induced phosphorylation of the receptor resulting in internalization and desensitization of G protein-coupled receptors (2, 7, 8). GRK2 requires Gβγ for its recruitment to the membrane and activation. Recent studies (31) have shown that, in gastric smooth muscle cells, Ga-coupled VPAC2 receptor phosphorylation, internalization, and desensitization were exclusively mediated by activation of GRK2 and that the effect of GRK2 is augmented by feedback phosphorylation of GRK2 on Ser685 by PKA. The mechanism by which PKA-induced phosphorylation augments GRK2 activity appears to be by increasing its binding to Gβγ and recruitment to the membrane (3, 31). GRK2, however, is not directly phosphorylated by PKC (data not shown). In contrast to PKA, PKC inhibits GRK2 activity and the mechanism of inhibition does not appear to involve inhibition of its interaction with Gβγ. Since the binding domain for RKIP is in the NH2-terminus domain of GRK2, which is important for catalytic activity and for interaction with the receptor, association of RKIP with GRK2 could inhibit the catalytic activity and/or interaction with the receptor for phosphorylation (14).

In summary, the present studies provide evidence that in gastrointestinal smooth muscle concomitant activation of Ga-coupled M3 receptors cross-regulates signaling by Ga-coupled VPAC2 receptors. Activation of PKC by Ga-coupled receptors inhibits VPAC2 receptor phosphorylation, internalization, and desensitization and the mechanism involves PKC-dependent switching of RKIP from Raf-1 to GRK2 and inhibition of GRK2 activity (Fig. 9). The functional significance of this mechanism may reside where there is overlap in the release of Ga-coupled excitatory transmitters (e.g., Ach) with the Ga-coupled inhibitory transmitters (e.g., VIP) during the peristalsis of the gut. These findings are also important in the interpreta-

Fig. 9. Cross-regulation of VPAC2 receptor desensitization by muscarinic M3 receptors (M3 R) via RKIP. Phosphorylation, internalization, and desensitization of VPAC2 receptors are exclusively mediated by GRK2. Concomitant activation of muscarinic M3 receptors leads to stimulation of PKC and phosphorylation of RKIP. Phosphorylation-dependent switching of RKIP from Raf-1 to GRK2 unmasks the stimulation of the ERK1/2 pathway by Raf-1 and inhibits phosphorylation, internalization, and desensitization of VPAC2 receptors by GRK2.
tion of the results in experiments designed to characterize the inhibitory function of VIP in muscle preparations pretreated with contractile agonists.

GRANTS

This work was supported by Grant DK-28300 from the National Institute of Diabetes and Digestive and Kidney Diseases.

REFERENCES


