Protein Kinase C Phosphorylates RGS2 and Modulates Its Capacity for Negative Regulation of Go11 Signaling*

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RGS proteins (regulators of G protein signaling) attenuate heterotrimeric G protein signaling by functioning as both GTPase-activating proteins (GAPs) and inhibitors of G protein/effector interaction. RGS2 has been shown to regulate Goa-mediated inositol lipid signaling. Although purified RGS2 blocks PLC-β activity by the nonhydrolyzable GTP analog guanosine 5′-O-thiophosphate (GTPγS), its capacity to regulate inositol lipid signaling under conditions where GTPase-promoted hydrolysis of GTP is operative has not been fully explored. Utilizing the turkey erythrocyte membrane model of inositol lipid signaling, we investigated regulation by RGS2 of both GTP and GTPγS-stimulated Goa11 signaling. Different inhibitory potencies of RGS2 were observed under conditions assessing its activity as a GAP versus as an effector antagonist; i.e. RGS2 was a 10–20-fold more potent inhibitor of aluminum fluoride and GTP-stimulated PLC-β activity than of GTPγS-promoted PLC-β activity. We also examined whether RGS2 was regulated by downstream components of the inositol lipid signaling pathway. RGS2 was phosphorylated by PKC in vitro to a stoichiometry of approximately unity by both a mixture of PKC isozymes and individual calcium and phospholipid-dependent PKC isoforms. Moreover, RGS2 was phosphorylated in intact COS7 cells in response to PKC activation by 4β-phorbol 12β-myristate 13α-acetate and, to a lesser extent, by the P2Y2 receptor agonist UTP. In vitro phosphorylation of RGS2 by PKC decreased its capacity to attenuate both GTP and GTPγS-stimulated PLC-β activation, with the extent of attenuation correlating with the level of RGS2 phosphorylation. A phosphorylation-dependent inhibition of RGS2 GAP activity was also observed in proteoliposomes reconstituted with purified P2Y1 receptor and Goaβγ. These results identify for the first time a phosphorylation-induced change in the activity of an RGS protein and suggest a mechanism for potentiation of inositol lipid signaling by PKC.

A variety of hormone and neurotransmitter receptors transduce signals through heterotrimeric G proteins. In their inactive state, G proteins exist as heterotrimers consisting of α, β, and γ subunits with GDP bound to Go. Upon agonist occupation, the receptor promotes GDP/GTP exchange, and the active GTP-bound Go subunit and Gβγ dissociate to interact with target effector proteins. Signaling is terminated by the hydrolysis of GTP to GDP and the subsequent formation of the heterotrimer. Therefore, the magnitude and duration of signaling is determined by the length of time Go remains in the active GTP-bound conformation.

A recently identified family of proteins termed RGS (regulators of G protein signaling) proteins interact directly with Go subunits to decrease the lifetime of the active GTP-bound complex (1–4). RGS proteins attenuate heterotrimeric G protein signaling by functioning as both GTPase-activating proteins (GAPs) (5, 6) and inhibitors of G protein/effector interaction (6, 7). In vitro studies illustrate that RGS2 interacts with and functions as a GAP for Goa11 (8), and in vivo studies demonstrate that RGS2 is a more potent inhibitor of Goa11 signaling than is RGS4 in transfected cells (9). Members of the Goa family of G proteins transmit signals from numerous cell surface receptors, leading to activation of PLC-β isozymes and subsequent cleavage of membrane phosphatidylinositol 4,5-bisphosphate to the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (10). Inositol 1,4,5-trisphosphate initiates release of calcium from endoplasmic reticulum stores, and diacylglycerol, in conjunction with calcium and phospholipids, activates PKC (10). Purified RGS2 has been shown to attenuate GTPγS-stimulated inositol lipid signaling in reconstitution studies with both purified Goa11 and NG-108 cell membranes (7). However, the capacity of RGS2 to modify inositol lipid signaling under conditions where GTPase-promoted hydrolysis was operative was not established. Utilizing the well characterized turkey erythrocyte model of inositol lipid signaling (11–18), we have determined the effects of RGS2 on both GTP and GTPγS-stimulated Goa11 activation of PLC-β. Moreover, our results indicate that PKC promotes phosphorylation of RGS2, both in intact mammalian cells in response to PMA and in vitro with purified kinase. This modification in vitro inhibits the capacity of RGS2 to attenuate PLC-β activation and significantly reduces RGS2-promoted GAP activity.

EXPERIMENTAL PROCEDURES

Materials—Hexahistidine-tagged human RGS2 was purified after expression in Escherichia coli as described previously (19). PS and 1,2-dioleoyl-sn-glycerol were obtained from Avanti Polar Lipids

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‡ The abbreviations used are: G protein, guanine nucleotide-binding protein; PKC, protein kinase C; PLC, phospholipase C; GTPγS, guanosine 5′-O-thiophosphate; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; 2MeSATP, 2-methylthioadenosine diphosphate; 2MeSADP, 2-methylthioadenosine triphosphate; PMA, 4β-phorbol 12β-myristate 13α-acetate; GAP, GTPase-activating protein; NTA, nitrilotriacetic acid; MES, 4-morpholineethanesulfonic acid.

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membranes were washed with lysis buffer and 10 mM Hepes, pH 7.0, and assayed for inositol phosphate production as described previously. Washed erythrocytes were radiolabeled overnight in a 10 mM Hepes buffer (5 mM NaH$_2$PO$_4$, 5 mM MgCl$_2$, 1 mM EGTA, pH 7.4), and membranes were isolated by centrifugation at 13,500 g for 30 min and then added to 2× assay buffer (10 mM Hepes, pH 7.0, 424 μM CaCl$_2$, 910 μM MgSO$_4$, 2 mM EGTA, 115 mM KCl, 5 mM KH$_2$PO$_4$). The assay was initiated by the addition of 25 μl of buffer, GTPγS, GTPγS with agonist, or GTP with agonist and proceeded at 30 °C for 10 min. The reaction was stopped by the addition of 500 μl of ice-cold CICH$_2$/MeOH (1:2). 175 μl each of CHCl$_3$ and H$_2$O were added, and the samples were centrifuged at 1800 × g for 5 min. Inositol phosphates were isolated by anion exchange chromatography by transferring 400 μl of the aqueous upper phase to Bio-Rad AG1-X8 (200–400 mesh) columns containing 10 ml of H$_2$O. 10 ml of 200 mM ammonium formate, 100 mM formic acid were added, and the eluate was discarded. Inositol phosphates were eluted with 5 ml of 1.2 mM ammonium formate, 100 mM formic acid, and inositol phosphates were quantitated by liquid scintillation spectrometry.

**In Vitro Kinase Reactions**

For experiments with the mixture of calcium and phospholipid-dependent PKC isoforms or with the individual PKC isoforms (α, β, δ, and γ), histidine-tagged RGS2 (4–18 pmol) was incubated with PKC at 30 °C in a reaction containing 20 mM Tris, pH 7.5, 10 mM MgCl$_2$, 500 μM CaCl$_2$, 100 μg/ml PS, 20 μg/ml 1.2-dioleoyl-sn-glycerol, 200 mM calciun A, and 200 μM γ-[32P]ATP (1500 cpm/pmol) in a final volume of 20 μl. Concentrations of PKC and incubation times are as indicated in the figure legends. One unit of PKC activity is defined as the amount of enzyme required to transfer 1 μmol of phosphate from ATP to histone H1 per min at 30 °C. Reactions were terminated by the addition of 20 μl of 2× Laemmli sample buffer. Samples were separated by SDS-PAGE through 12.5% acrylamide according to the method of Laemmli (20), and the protein bands were visualized by silver or Coomassie stain. The gel was dried and exposed to autoradiography film to detect radioactive bands. For experiments to test the capacity of phosphorylated RGS2 to inhibit G$_{q1}$ signaling, RGS2 was phosphorylated by the PKC catalytic subunit, which does not require calcium and phospholipids for activation. Purified RGS2 (40 pmol) was incubated with the PKC catalytic subunit at 30 °C in a reaction containing 50 mM MES, pH 6.0, 12.5 mM MgCl$_2$, 1.25 mM EGTA, 200 mM calciun A, and 125 μM ATP in a final volume of 16 μl. Concentrations of Ca$^{2+}$ and incubation times are as indicated in the figure legends. Reaction mixtures were diluted in 10 μl Hepes, pH 7.0, 10 μM β-glycerophosphate, 200 mM calciun A, and bovine serum albumin (2 mg/ml) and mixed with turkey erythrocyte membranes at 4 °C for 30 min to obtain the indicated concentrations of RGS2. Membrane samples were assayed for inositol phosphate production as described above. β-Glycerophosphate and calciun A were included in PLC assays to phosphorylate RGS2 and inhibit phosphate wash. 32P-labeled proteoliposomes were isolated by centrifugation at 13,000 × g for 30 min. The resulting supernatant (1 ml) was incubated with 25–50 μl of Ni$^{2+}$-NTA resin with mixing for 1 h at 4 °C to isolate His$_6$-tagged RGS2. The Ni$^{2+}$-NTA resin was pelleted by centrifugation at 13,000 × g for 15 s, and the supernatant was aspirated. The pellet was washed three times with 25 mM imidazole, three times with 50 mM imidazole, and two times with 75 mM imidazole. RGS2 was eluted from the resin twice with 100 μl of 250 mM imidazole. Isolated proteins were resolved by SDS-PAGE (12.5% w/v) and subjected to protein staining or transferred electrophoretically to nitrocellulose. RGS2 was detected by Coomassie staining or by Western blot with an anti-penta-His monoclonal antibody. γ-[32P]ATP incorporation into RGS2 was assessed by autoradiography and PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

**GTPase Assays**

Purified recombinant human P2Y$_1$ receptor was reconstituted with Go$_{a}$ and Gβγ to proteoliposomes by a modification of the method described by Brandt et al. (21). Briefly, 15 pmol of P2Y1 receptor, 40 pmol of Go$_{a}$, and 150 pmol of Gβγ were combined with a mixture of phosphatidylethanolamine, phosphatidylserine, and cholesterol hemisuccinate in detergent solution. Proteoliposomes were formed by Sephadex G-50 gel filtration. RGS2 was phosphorylated by PKC essentially as described above and diluted ~20-fold to the indicated final concentrations in the assay. GTPase activity of the proteoliposomes was determined in the presence of either phosphorylated or mock-phosphorylated RGS2, with or without 100 μM 2MeSATP. Assays were incubated for 30 min at 30 °C and contained 20 mM Hepes, pH 8.0, 50 mM NaCl, 2 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 μM β-glycerophosphate, 10 μM microcystin, and 2 μM γ-[32P]GTP (~4500 cpm/pmol). The assays were terminated by the addition of 950 μl of a 4 °C solution of 5% activated charcoal in 20 mM H$_2$PO$_4$. Following centrifugation, liberated [γ-32P]GTP in the supernatant was quantified in a liquid scintillation counter.

**RESULTS**

Others reported previously that recombinant RGS2 blocks GTPγS-stimulated Go$_{a}$ activation of PLC-β in NG-108 cell membranes and in reconstitution assays with purified Go$_{a}$ and PLC-β (7). However, the effects of RGS2 on Go$_{a}$ signaling activity in the presence of GTP (i.e. under conditions where Go$_{a}$ GTPase activity is operative) have not been fully characterized. We have modified an established turkey erythrocyte membrane assay to investigate and compare the capacities of RGS2 to inhibit GTP and GTPγS-stimulated PLC-β activity. RGS2 was mixed with erythrocyte membranes at various concentrations, and incubations were carried out in the presence of aluminum fluoride, GTP plus 2MeSATP, GTPγS plus 2MeSATP, or GTPγS alone. RGS2 inhibited aluminum fluoride (IC$_{50}$ = 10 nM), GTP (IC$_{50}$ = 14 nM, with 2MeSATP) and GTPγS-stimulated (IC$_{50}$ = 192 and 223 nM, in the presence and absence of 2MeSATP, respectively) PLC-β activity in a concentration-dependent manner. However, the aluminum fluoride- and 2MeSATP plus GTPγS-promoted responses were inhibited by 10–20-fold lower concentrations of RGS2 than those necessary to inhibit GTPγS-stimulated PLC activity, both in the presence and absence of the P2Y$_1$ receptor agonist 2MeSATP (Fig. 1). Inositol lipid signaling in the turkey erythrocyte is also stimulated by isoproterenol through Go$_{a1}$ coupling to a β-adrenergic receptor (22–24). RGS2 attenuated isoproterenol plus GTPγS-stimulated inositol phosphate production half-maximally at an RGS concentration ~20-fold lower than that observed with 2MeSATP plus GTPγS stimulation (Fig. 2). Although the mechanism underlying this difference in potency...
and are representative of three experiments. Data are mean ± S.D. of triplicate determinations and are representative of three experiments. Phosphorylation of RGS2

PKC isozyme: - Mix α β1 β2 γ

FIG. 1. Inhibition of PLC-β activation in turkey erythrocyte membranes by RGS2. Turkey erythrocyte membranes were radiolabeled overnight in inositol-free DMEM with 0.5 mCi [3H]inositol, and membranes were prepared by hypotonic lysis as described under “Experimental Procedures.” Membranes were incubated with the indicated concentrations of RGS2 for 30 min at 4 °C, and PLC activity was assayed as described. Reactions were initiated by the addition of aluminum fluoride (A), 10 μM 2MeSATP plus 1 μM GTP (B), 10 μM 2MeSATP plus 10 μM GTP·S (C), or 100 μM GTP·S (D). The IC50 values for RGS2 obtained in A–D are summarized in E. Basal levels of inositol phosphate production with 10 mM Hepes, pH 7.0, were subtracted from the values presented. Data are mean ± S.D. of triplicate determinations and are representative of two or three experiments.

PKC concentration (Fig. 4A) and the time of incubation (Fig. 4B).

Due to lack of an antibody for immunoprecipitation of RGS2 from turkey erythrocytes, in vitro phosphorylation could not be assessed in these cells. However, a histidine-tagged RGS2 construct for expression in mammalian cells was engineered (see “Experimental Procedures”) that permitted isolation of RGS2 from cell lysates with Ni2⁺-NTA resin. COS7 cells were transiently transfected with His6-RGS2 and radiolabeled with [32P]P. Treatment of cells with PMA resulted in a concentration-dependent increase in phosphorylation of RGS2 (Fig. 5A), indicating PKC-dependent phosphorylation of RGS2 in intact cells. Pharmacological analysis indicates that COS7 cells possess an endogenous P2Y2 receptor that couples to Gq to initiate phosphoinositide hydrolysis and subsequently activate PKC.
Phosphorylation of RGS2

**Fig. 4.** Time and concentration dependence of PKC-promoted phosphorylation of RGS2. Purified RGS2 (4–9 pmol) was incubated at 30 °C with (A) the specified concentrations of PKC for 30 min or (B) 20 microunits of the calcium and phospholipid-dependent PKC for the indicated times as described under “Experimental Procedures.” Reactions were stopped by the addition of SDS-PAGE sample buffer, and the samples were processed as described in the legend to Fig. 2. Autoradiography results are presented and are representative of more than three experiments.

**Fig. 5.** In vivo phosphorylation of RGS2. COS7 cells transiently expressing His$_{10}$-RGS2 were radiolabeled with $[^{32}P]$P, and were treated as described under “Experimental Procedures” with vehicle (Me$_2$SO) or the indicated concentrations of PMA (A) or 100 μM UTP (B) for 20 min at 37 °C. The cells were lysed isotonically, and RGS2 was isolated with Ni$^{2+}$-NTA resin as described. Proteins were separated by SDS-PAGE, and RGS2 protein was detected by immunoblotting with an anti-His antibody (A). SDS-polyacrylamide gels were exposed to film or a PhosphorImager screen for $^{32}$P detection. $^{32}$P incorporation into RGS2 was quantitated as arbitrary PhosphorImager units (B). The autoradiograms are representative of three experiments, and the bar graphs present the mean ± S.E. of results quantitated and averaged from three separate experiments.

Incubation of $^{32}$P-labeled COS7 cells expressing RGS2 with the P2Y2 receptor agonist UTP also resulted in an ~80% increase in RGS2 phosphorylation (Fig. 5B), indicating that physiological activation of PKC through a G protein-coupled receptor promotes in vivo phosphorylation of RGS2.

To determine the effect of phosphorylation by PKC on the capacity of RGS2 to attenuate activation of PLC-β, RGS2 was phosphorylated to a stoichiometry near unity by PKC in vitro and reconstituted at the indicated concentrations with erythrocyte membranes as described above. Nonphosphorylated RGS2, which was incubated in a standard kinase reaction lacking PKC, inhibited GTP plus 2MeSATP-stimulated inositol phosphate production in a concentration-dependent manner (Fig. 6A), consistent with that observed with untreated RGS2 (Fig. 1B). Phosphorylation by PKC decreased the capacity of RGS2 to inhibit GTP-stimulated phosphoinositide hydrolysis (Fig. 6A). Concentration of PKC-dependent increases in phosphorylation of RGS2 (Fig. 4A) resulted in proportional decreases in RGS2-promoted attenuation of 2MeSATP plus GTP-stimulated inositol lipid signaling (Fig. 6B). Time-dependent increases in RGS2 phosphorylation (Fig. 4B) also produced corresponding decreases in RGS2-mediated inhibition of GTP-promoted inositol phosphate production (Fig. 6C).

The effect of phosphorylation by PKC on the activity of RGS2 was also determined under conditions where the RGS protein functions as an inhibitor of G protein/effector interaction but not as a GAP (i.e. in the presence of GTPγS). The significantly higher concentrations of RGS2 required to inhibit GTPγS alone and GTPγS plus 2MeSATP-stimulated PLC-β activity (Fig. 1C, D) limited our capacity to test the effect of phosphorylation on RGS2 activity with the nonhydrolyzable GTP analog under these conditions. However, the observation that RGS2 is a significantly more potent inhibitor of GTPγS-stimulated inositol phosphate production in the presence of isoproterenol than with 2MeSATP (Fig. 2) provided conditions for studying the capacity of RGS2 to inhibit PLC-β activity promoted by a nonhydrolyzable GTP analog. Nonphosphorylated RGS2 attenuated isoproterenol plus GTPγS-stimulated inositol phosphate production in a concentration-dependent manner, and phosphorylation by PKC increased the concentration of RGS2 required for half-maximal inhibition of PLC-β activation (Fig. 7A). The extent of the reversal of RGS2-mediated attenuation of inositol phosphate production correlated with the concentration of PKC (Fig. 7B) and time of incubation with PKC (Fig. 7C), consistent with a phosphorylation-dependent inhibition of RGS2 activity.

The mechanism of phosphorylation-dependent inhibition of RGS2 activity was further investigated via steady-state GTPase assays performed in proteoliposomes. Phosphorylated or mock-phosphorylated RGS2 was incubated with proteoliposomes containing purified P2Y$_1$ receptor and Go$_{q}$β$_1$γ$_{2}$-$\alpha$. The addition of unphosphorylated RGS2 and the P2Y$_1$ receptor agonist 2MeSADP stimulated GTP hydrolysis ~4-fold above the level observed with RGS2 alone (Fig. 8). Phosphorylation significantly reduced RGS2-promoted GTPase activity both in the presence and absence of agonist, demonstrating a phosphorylation-dependent inhibition of the GAP activity of RGS2.

**DISCUSSION**

RGS proteins inhibit heterotrimeric G protein signaling by functioning as both GAPs and effector antagonists (1–3). Previous studies have demonstrated that RGS2 acts as a GAP for Go$_{q}$ (8) and attenuates GTPγS-stimulated PLC-β activity (7). In the present study, we have examined the capacity of RGS2 to regulate inositol lipid signaling under conditions where GTPase-promoted hydrolysis was operative and have established that RGS2 is a much more potent inhibitor of P2Y receptor-stimulated PLC-β activity in the presence of GTP than in the presence of the hydrolysis-resistant GTP analog GTPγS. Similar concentrations of RGS2 were required for half-maximal inhibition of GTP-stimulated inositol lipid signaling and that occurring in the presence of aluminum fluoride, which mimics the structure of the α subunit at the transition state of the
GTP

trations 20-fold lower than those observed with 2MeSATP and inositol phosphate production half-maximally at RGS concentration in pancreatic acinar cells dialyzed with RGS4. G-selective inhibition by RGS4 of calcium release and PLC activation (27, 28).

the GDP-AlF4 observation that RGS proteins interact with higher affinity to GTPase reaction (26). Thus, RGS2 is a more potent inhibitor of inositol lipid signaling under conditions in which the G protein α subunit exists in the GTPase transition state, either transiently (GTP-promoted signaling) or stably (aluminum fluoride-stimulated signaling). Our results are consistent with the observation that RGS proteins interact with higher affinity to the GDP-AlF₄ complex than to the GTPγS-bound form of Go (5, 27, 28).

Zeng et al. (29) and Xu et al. (30) recently reported receptor-selective inhibition by RGS4 of calcium release and PLC activity in pancreatic acinar cells dialyzed with RGS4. Go₁₁-mediated inositol lipid signaling in the turkey erythrocyte membrane model is stimulated by both P2Y₂ and β-adrenergic receptors (22–24), and thereby provides conditions for more directly investigating potential receptor-selective activity of RGS2. RGS2 inhibited isoproterenol plus GTPγS-stimulated inositol phosphate production half-maximally at RGS concentrations 20-fold lower than those observed with 2MeSATP and GTPγS. While our results do not reveal the mechanism underlying these differences in RGS2 potency, they suggest the occurrence of receptor-selective activity of RGS2. The N-terminal domain of RGS4 was proposed to impart high affinity and receptor-selective inhibition of Gq signaling (29). Little similarity exists between the N-terminal domain of RGS4 and RGS2, and it will be important to establish the basis of the apparent receptor selectivity found in our study with RGS2.

PKC is activated as a downstream consequence of PLC activation and has been implicated in regulation of inositol lipid signaling (25). We previously demonstrated that membranes isolated from turkey erythrocytes pretreated with PMA exhibit a decreased capacity for Gα₁₁-mediated activation of purified, reconstituted PLC-β1 (18). Additionally, we illustrated that PLC-β is phosphorylated in intact erythrocytes in response to PMA treatment, and in vitro phosphorylation of PLC-β by PKC reduces its basal catalytic activity (17). We demonstrate here that RGS2 is phosphorylated stoichiometrically by PKC in vitro and in intact mammalian cells stimulated with PMA or the P2Y₂ receptor agonist UTP. Phosphorylation decreases the

GTPase reaction (26). Thus, RGS2 is a more potent inhibitor of inositol lipid signaling under conditions in which the G protein α subunit exists in the GTPase transition state, either transiently (GTP-promoted signaling) or stably (aluminum fluoride-stimulated signaling). Our results are consistent with the observation that RGS proteins interact with higher affinity to the GDP-AlF₄ complex than to the GTPγS-bound form of Go (5, 27, 28).

FIG. 6. Effect of phosphorylation by PKC on the inhibition of purinergic receptor-stimulated inositol phosphate production by RGS2. Purified RGS2 was incubated with 22 microunits of the catalytic subunit of PKC (●) or enzyme buffer (○) for 30 min at 30 °C (A), the indicated concentrations of the PKC catalytic subunit for the indicated times at 30 °C (B), or 22 microunits of PKC for the indicated times at 30 °C (C). Kinase reactions were diluted in 10 mM Hepes, 10 mM β-glycerophosphate, 200 mM calyculin A, and bovine serum albumin (2 mg/ml) and mixed with turkey erythrocyte membranes at an RGS2 concentration of 10 nM (B and C) or as indicated in the figure (A). Erythrocyte membrane/RGS2 samples were challenged with 10 μM 2MeSATP plus 1 mM GTP for 10 min at 30 °C. Values are presented as percentages of agonist-induced inositol phosphate production in the absence of RGS2 (3539 ± 93 cpm (A); 4793 ± 144 cpm (B); 2159 ± 278 cpm (C)). Basal levels of inositol phosphate production with 10 mM Hepes, pH 7.0, were subtracted from the values presented. Data are mean ± S.D. of triplicate determinations and are representative of 2–4 experiments.

FIG. 7. Effect of phosphorylation by PKC on the inhibition of β-adrenergic receptor-stimulated inositol phosphate production by RGS2. Purified RGS2 was incubated with 22 microunits of the catalytic subunit of PKC (●) or enzyme buffer (○) for 30 min at 30 °C (A), the indicated concentrations of the PKC catalytic subunit for the indicated times at 30 °C (B), or 22 microunits of PKC for the indicated times at 30 °C (C). Kinase reactions were diluted in 10 mM Hepes, 10 mM β-glycerophosphate, 200 mM calyculin A, and bovine serum albumin (2 mg/ml) and mixed with turkey erythrocyte membranes at an RGS2 concentration of 10 nM (B and C) or as indicated in the figure (A). Erythrocyte membrane/RGS2 samples were challenged with 10 μM 2MeSATP plus 1 mM GTPγS for 10 min at 30 °C. Values are presented as percentages of agonist-induced inositol phosphate production in the absence of RGS2 (7330 ± 432 cpm (A); 10,780 ± 1595 cpm (B); 13,498 ± 690 cpm (C)). Basal levels of inositol phosphate production with 10 mM Hepes, pH 7.0, were subtracted from the values presented. Data are mean ± S.D. of triplicate determinations and are representative of 2–4 experiments.
were centrifuged, and liberated \([32P]\)Pi in the supernatant was quantified. Incubation with 40 microunits of the PKC catalytic subunit or enzyme at a final concentration of 316 nM to proteoliposomes containing purified recombinant human P2Y1 receptor and G\(_{\alpha}\beta\gamma\)o, and GTPase activity was measured in the presence or absence of 100 \(\mu\)M 2MeSADP. Following a 30-min incubation at 30 °C, the assay was terminated by the addition of 5% activated charcoal in 20 mM H\(_2\)PO\(_4\). The samples were centrifuged, and liberated \([32P]\)Pi, in the supernatant was quantitated by liquid scintillation spectrometry. Data are mean ± S.D. of six measurements and are representative of two experiments.

Phosphorylation-dependent inhibition of RGS2 GAP activity in P2Y1 receptor/G\(_{\alpha}\beta\gamma\) vesicles. Purified RGS2 (7 µg) was incubated with 40 microunits of the PKC catalytic subunit or enzyme buffer for 30 min at 30 °C in a total reaction volume of 48 µl. Kinase reactions were diluted prior to use in the GTPase assay. Phosphorylated (solid bars) or mock-phosphorylated RGS2 (open bars) was added at a final concentration of 316 nM to proteoliposomes containing purified recombinant human P2Y1 receptor and G\(_{\alpha}\beta\gamma\)o, and GTPase activity was measured in the presence or absence of 100 \(\mu\)M 2MeSADP. Following a 30-min incubation at 30 °C, the assay was terminated by the addition of 5% activated charcoal in 20 mM H\(_2\)PO\(_4\). The samples were centrifuged, and liberated \([32P]\)Pi, in the supernatant was quantitated by liquid scintillation spectrometry. Data are mean ± S.D. of six measurements and are representative of two experiments.

Phosphorylation may provide a common method of regulation of RGS proteins, impacting on both their subcellular localization and their GAP activity. Pedram et al. (48) observed phosphorylation of RGS3 and RGS4 by cAMP-dependent protein kinase and implicated phosphorylation in translocation of these RGS proteins from a cytosolic to a membrane localization. Farquhar and colleagues reported that membrane-associated GAIP, but not soluble GAIP, exists as a phosphoprotein and that GAIP can be phosphorylated in vitro by purified casein kinase 2 and by isolated clathrin-coated vesicles (49). Benzing et al. (50) recently reported PKC-promoted phosphorylation of RGS7 and phosphorylation-dependent association of RGS7 and 14-3-3 proteins. Moreover, binding of 14-3-3 to phosphorylated RGS7 inhibited the capacity of RGS7 to promote GTP hydrolysis by G\(_{\alpha}\). Interestingly, several of the residues in the putative 14-3-3 binding motif of RGS7, including the serine residue phosphorylated by PKC, are conserved in RGS2 and other RGS proteins, suggesting another possible level of RGS2 regulation. Phosphorylation of RGS2 may be involved in cross-talk regulation between signaling pathways. More detailed studies will be needed to determine whether involvement of different PKC isoforms with different temporal patterns of activation and substrate selectivities also underlies our observations with PKC-promoted phosphorylation of RGS2.

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