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Protein Kinase C Phosphorylates RGS2 and Modulates Its Capacity for Negative Regulation of Gα_{11} 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 Gα_q-mediated inositol lipid signaling. Although purified RGS2 blocks PLC-β activation 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 Gα_{11} 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-βt activity than of GTPγS-promoted PLC-βt 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 P2Y$_2$ receptor agonist UTP. *In vitro* phosphorylation of RGS2 by PKC decreased its capacity to attenuate both GTP and GTPγS-stimulated PLC-βt 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 P2Y$_1$ receptor and G$\alpha_q\beta\gamma$. 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 Gα. Upon agonist occupation, the receptor promotes GDP/GTP exchange, and the active GTP-bound Gα subunit and G$\beta\gamma$ 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 Gα remains in the active GTP-bound conformation.

A recently identified family of proteins termed RGS (regulators of G protein signaling) proteins interact directly with Gα subunits to decrease the lifetime of the active GTP-bound complex (1–4). RGS proteins attenuate heterotrimeric G protein[1] 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 Gα_q (8), and *in vivo* studies demonstrate that RGS2 is a more potent inhibitor of Gα_q signaling than is RGS4 in transfected cells (9). Members of the G$_q$ 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 Gα_q 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 Gα_{11} activation of PLC-βt. 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-βt 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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[1] 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; 2MeSADP, 2-methylthioadenosine diphosphate; 2MeSATP, 2-methylthioadenosine triphosphate; PMA, 4β-phorbol 12β-myristate 13α-acetate; GAP, GTPase-activating protein; NTA, nitrilotriacetic acid; MES, 4-morpholineethanesulfonic acid.

(Alabaster, AL). 2MeSATP and isoproterenol were purchased from RBI (Natick, MA). GTP, GTPγS, FuGENE transfection reagent, and PKC (calcium- and phospholipid-dependent enzyme) purified from bovine brain were purchased from Roche Molecular Biochemicals. Calyculin A, PKC (catalytic subunit) purified from rat brain, and PKC isoforms (α, β1, β2, and γ) were obtained from Calbiochem. *myo*-[^{3}H]inositol was purchased from American Radiolabeled Chemicals (St. Louis, MO). Histidine-tagged (His$_{10}$) RGS2 plasmid DNA (pcDNA3.1(−); Invitrogen) was constructed from a bacterial RGS2 expression construct (provided by Dr. Scott Heximer) via standard cloning techniques. Ni^{2+}-NTA resin and the penta-His monoclonal antibody were purchased from Qiagen (Valencia, CA).

[^{3}H]Inositol Labeling of Turkey Erythrocytes and Membrane Preparation—Turkey erythrocytes were collected and washed as described previously (12). Washed erythrocytes were radiolabeled overnight in inositol-free DMEM supplemented with *myo*-[^{3}H]inositol (500 μCi/ml packed cells) at 37 °C in a 95% O_2, 5% CO_2 atmosphere with rapid stirring. Erythrocytes were lysed hypotonically in 40 ml of ice-cold lysis buffer (5 mM NaH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4), and membranes were isolated by centrifugation at 13,500 × *g* for 10 min. The membranes were washed with lysis buffer and 10 mM Hepes, pH 7.0, and then resuspended (2 ml/ml packed erythrocytes) in 10 mM Hepes, pH 7.0, for use in the PLC assay.

Assay of Phospholipase C-β Activity in Turkey Erythrocyte Membranes—Turkey erythrocyte membranes (12.5 μl/assay) were mixed with an equal volume of RGS2 diluted to the indicated concentration in 10 mM Hepes, pH 7.0. Membrane/RGS samples were incubated at 4 °C 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_2PO_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 $CHCl_3$/MeOH (1:2). 175 μl each of $CHCl_3$ and H_2O 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_2O. 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 M ammonium formate, 100 mM formic acid, and [^{3}H]inositol phosphates were quantitated by liquid scintillation spectrometry.

In Vitro Kinase Reactions—For experiments with the mixture of calcium and phospholipid-dependent PKC isozymes or with the individual PKC isoforms (α, β1, β2, 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 nM calyculin A, and 200 μM [γ-^{32}P]ATP (~1500 cpm/pmol) in a final volume of 20 μl. Concentrations of PKC and incubation times are as listed 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 Gα_{11} 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 nM calyculin A, and 125 μM ATP in a final volume of 16 μl. Concentrations of PKC and incubation times are as indicated in the figure legends. Reaction mixtures were diluted in 10 mM Hepes, pH 7.0, 10 mM β-glycerophosphate, 200 nM calyculin 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 calyculin A were included in PLC assays with phosphorylated RGS2 to inhibit phosphatase activity.

[^{32}P]P$_i$ Labeling and Isolation of RGS2 from Mammalian Cells—COS7 cells were transiently transfected using FuGENE transfection reagent and His$_{10}$-RGS2 plasmid DNA (4 μg/100-mm plate), essentially as directed by the manufacturer. Forty-eight h post-transfection, phosphate-free DMEM (4 ml/100-mm plate) was applied for 1 h at 37 °C, and the medium was supplemented with 500 μCi of [^{32}P]P$_i$ for an additional 3 h. Drugs were added directly to the medium as indicated, and incubation at 37 °C continued for 20 min. Following drug treatment, the medium was aspirated, and the cells were lysed isotonically in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 5 mM β-mercaptoethanol, 5 mM NaF, 10 mM β-glycerophosphate, 10 nM microcystin, 200 μM phenylmethylsulfonyl fluoride, 10 μM tosylphenyl chloromethyl ketone, 1 μM pepstatin A, 2 μM leupeptin), and the lysates were centrifuged at 35,000 × *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$_{10}$-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) gel) 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. ^{32}P incorporation into RGS2 was assessed by autoradiography and PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

GTPase Assays—Purified recombinant human P2Y$_1$ receptor[2] was reconstituted with Gα_q and G$\beta_1\gamma_2$ into proteoliposomes by a modification of the method described by Brandt *et al.* (21). Briefly, 15 pmol of P2Y1 receptor, 40 pmol of Gα_q, and 150 pmol of G$\beta_1\gamma_2$ 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 2MeSADP. 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 mM β-glycerophosphate, 10 nM microcystin, and 2 μM [γ-^{32}P]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_3PO_4. Following centrifugation, liberated [^{32}P]P$_i$ in the supernatant was quantified in a liquid scintillation counter.

RESULTS

Others reported previously that recombinant RGS2 blocks GTPγS-stimulated Gα_q activation of PLC-β in NG-108 cell membranes and in reconstitution assays with purified Gα_q and PLC-β1 (7). However, the effects of RGS2 on Gα_q signaling activity in the presence of GTP (*i.e.* under conditions where Gα_q 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[3] to inhibit GTP and GTPγS-stimulated PLC-βt activation. 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-βt activity in a concentration-dependent manner. However, the aluminum fluoride- and 2MeSATP plus GTP-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 Gα_{11} 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

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[2] G. L. Waldo and T. K. Harden, manuscript in preparation.

[3] Human RGS2 was used in this study; however, we have cloned turkey RGS2, which is ~69% identical and 80% homologous to the human homologue (G. L. Waldo and T. K. Harden, unpublished observations).

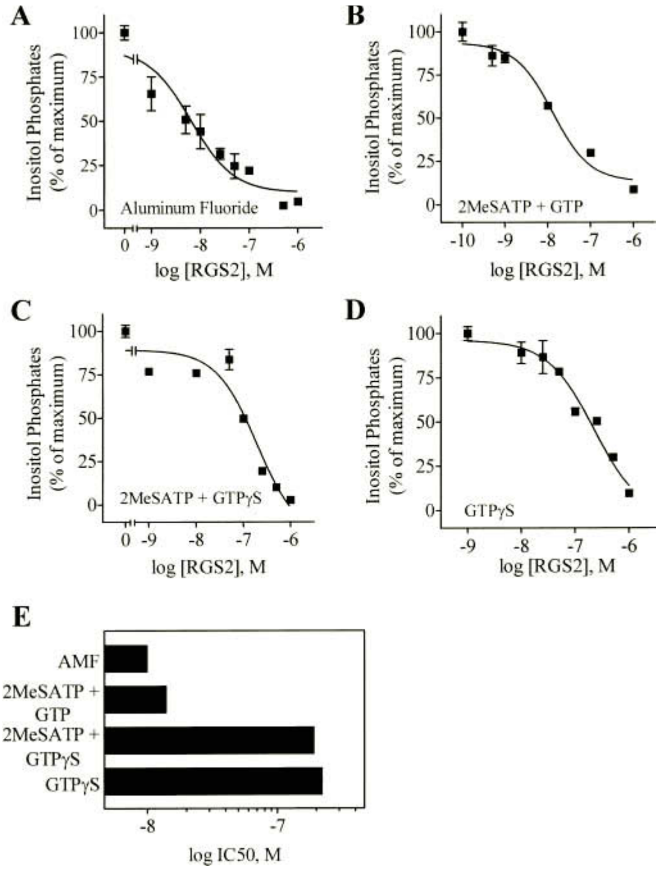

FIG. 1. **Inhibition of PLC-β activation in turkey erythrocyte membranes by RGS2.** Turkey erythrocyte membranes were radiolabeled overnight in inositol-free DMEM with 0.5 mCi [^{3}H]inositol, and membranes were prepared by hypotonic lysis as described under "Experimental Procedures." Membranes were 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 mM GTP (*B*), 10 μM 2MeSATP plus 10 μM GTPγS (*C*), or 100 μM GTPγS (*D*). The IC$_{50}$ 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 three experiments.

has not been fully investigated, this result suggests a receptor-selective component to inhibition by RGS2.

Activation of PKC either indirectly through Ca^{2+}-mobilizing receptors or directly by phorbol esters results in desensitization of receptor and G protein-promoted phosphoinositide hydrolysis (25). Utilizing a turkey erythrocyte membrane reconstitution assay, we previously reported that activation of PKC in intact cells inhibits the capacity of Gα_{11} to activate purified PLC-β1. While the effects of PKC were localized to the membrane, we were unable to identify the membrane target for PKC (18). Therefore, since the erythrocyte membrane assay provides a reliable assay of Gα_{11}-interacting RGS proteins, we determined if RGS2 was a substrate for PKC *in vitro* and utilized the erythrocyte assay to identify phosphorylation-dependent changes in RGS-promoted attenuation of inositol phosphate production. RGS2 was phosphorylated to a stoichiometry near unity (0.77 ± 0.25 mol phosphate/mol RGS2) by a bovine brain preparation consisting of a mixture of PKC isoforms (Fig. 3). Individual calcium and phospholipid-dependent PKC isoforms (α, β1, β2, and γ) were also utilized and phosphorylated RGS2 to approximately the same level (Fig. 3). Minimal phosphorylation was detected in the absence of PKC, indicating that little or no endogenous kinase activity was present in the preparation of RGS2. The extent of phosphorylation of RGS2 was dependent on PKC concentration (Fig. 4*A*) and the time of incubation (Fig. 4*B*).

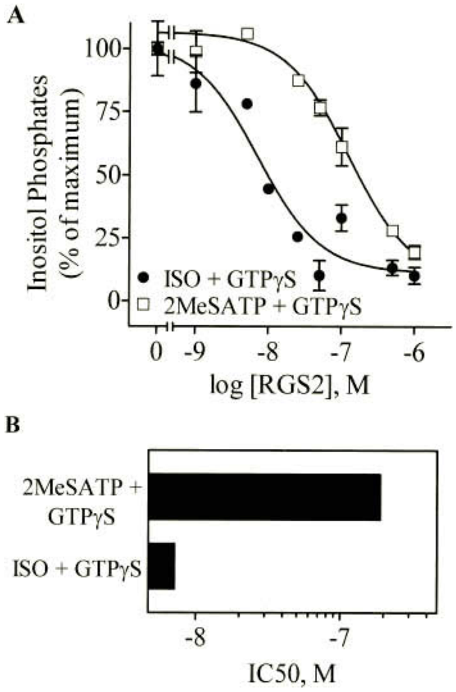

FIG. 2. **Inhibition of β-adrenergic receptor-mediated PLC-β activation in turkey erythrocyte membranes by RGS2.** *A*, turkey erythrocyte membranes were radiolabeled overnight in inositol-free DMEM with 0.5 mCi of [^{3}H]inositol, and membranes were prepared by hypotonic lysis as described under "Experimental 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 100 μM isoproterenol (*ISO*) plus 10 μM GTPγS (●) or 10 μM 2MeSATP plus 10 μM GTPγS (□). *B*, comparison of the IC$_{50}$ values of RGS2 for inhibition of purinergic and β-adrenergic receptor-mediated activation of PLC-βt. 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 three experiments.

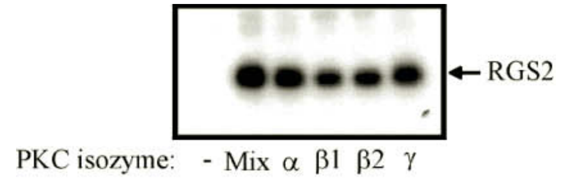

FIG. 3. **Phosphorylation of RGS2 by PKC.** RGS2 (17 pmol) was incubated with 20 microunits of a mixture of calcium and phospholipid-dependent PKC isoforms (*Mix*) or with individual purified PKC isoforms (α, β1, β2, and γ) for 30 min at 30 °C as described under "Experimental Procedures." The reaction products were separated by SDS-PAGE, stained with Coomassie Blue, and exposed to x-ray film. Autoradiography results are presented and are representative of two or three experiments.

Due to lack of an antibody for immunoprecipitation of RGS2 from turkey erythrocytes, *in vivo* 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 Ni^{2+}-NTA resin. COS7 cells were transiently transfected with His$_{10}$-RGS2 and radiolabeled with [^{32}P]P$_i$. Treatment of cells with PMA resulted in a concentration-dependent increase in phosphorylation of RGS2 (Fig. 5*A*), indicating PKC-dependent phosphorylation of RGS2 in intact cells. Pharmacological analysis indicates that COS7 cells possess an endogenous P2Y2 receptor that couples to G$_q$ to initiate phosphoinositide hydrolysis and subsequently activate PKC.

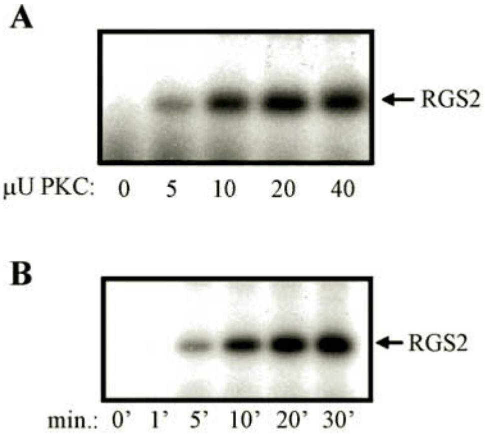

FIG. 4. **Time and concentration dependence of PKC-promoted phosphorylation of RGS2.** Purified RGS2 (4–9 pmol) was incubated at 30 °C with (*A*) the specified concentrations of PKC for 30 min or (*B*) 20 microunits of the calcium and phospholipid-dependent PKC for the indicated times as described under "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.

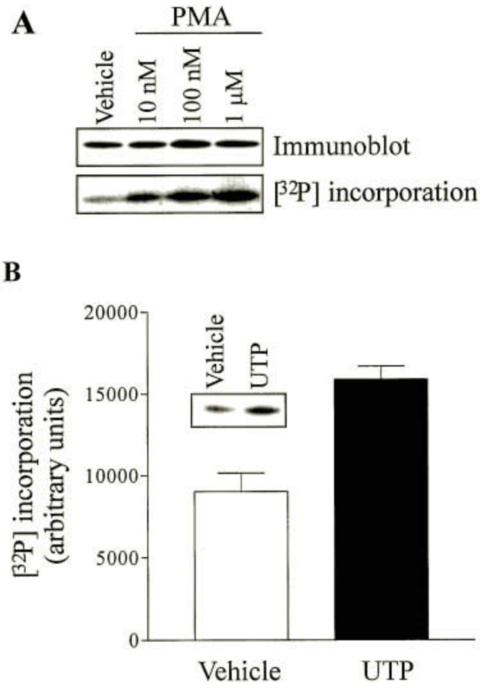

FIG. 5. ***In vivo* phosphorylation of RGS2.** COS7 cells transiently expressing His$_{10}$-RGS2 were radiolabeled with [^{32}P]P$_i$ and were treated as described under "Experimental Procedures" with vehicle (Me$_2$SO) or the indicated concentrations of PMA (*A*) or 100 μM UTP (*B*) for 20 min at 37 °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. 5*B*), 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-βt, 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. 6*A*), consistent with that observed with untreated RGS2 (Fig. 1*B*). Phosphorylation by PKC decreased the capacity of RGS2 to inhibit GTP-stimulated phosphoinositide hydrolysis (Fig. 6*A*). Concentration of PKC-dependent increases in phosphorylation of RGS2 (Fig. 4*A*) resulted in proportional decreases in RGS2-promoted attenuation of 2MeSATP plus GTP-stimulated inositol lipid signaling (Fig. 6*B*). Time-dependent increases in RGS2 phosphorylation (Fig. 4*B*) also produced corresponding decreases in RGS2-mediated inhibition of GTP-promoted inositol phosphate production (Fig. 6*C*).

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-βt activation (Fig. 1, *C*–*E*) 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-βt activation 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-βt activation (Fig. 7*A*). The extent of the reversal of RGS2-mediated attenuation of inositol phosphate production correlated with the concentration of PKC (Fig. 7*B*) and time of incubation with PKC (Fig. 7*C*), 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 G$\alpha_q\beta_1\gamma_2$. 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 Gα_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

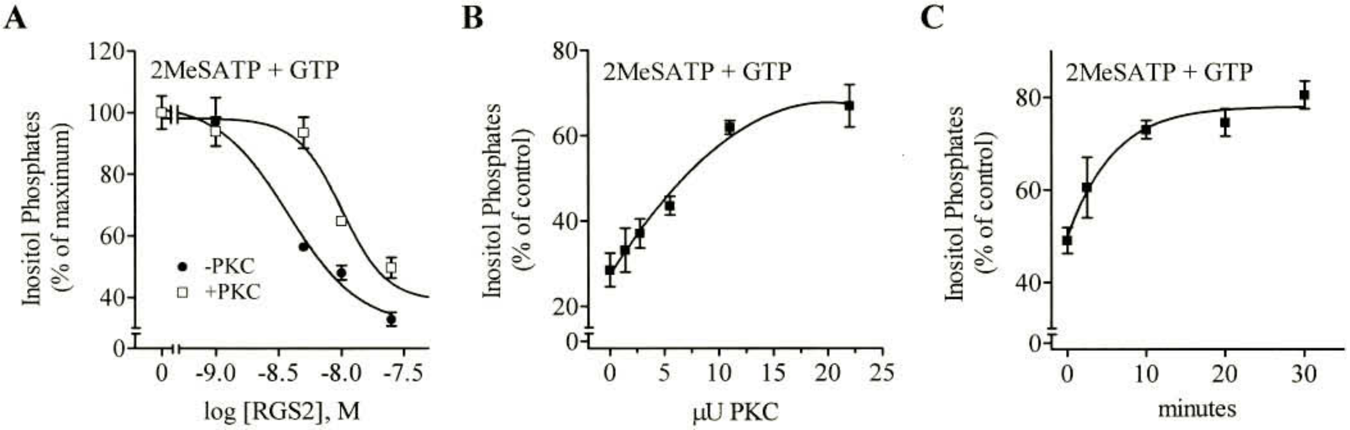

FIG. 6. **Effect of phosphorylation by PKC on the inhibition of purinergic receptor-stimulated inositol phosphate production by RGS2.** Purified RGS2 was incubated with 22 microunits of the catalytic subunit of PKC (□) or enzyme buffer (●) for 30 min at 30 °C (*A*), the indicated concentrations of the PKC catalytic subunit for 30 min 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 nM 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.

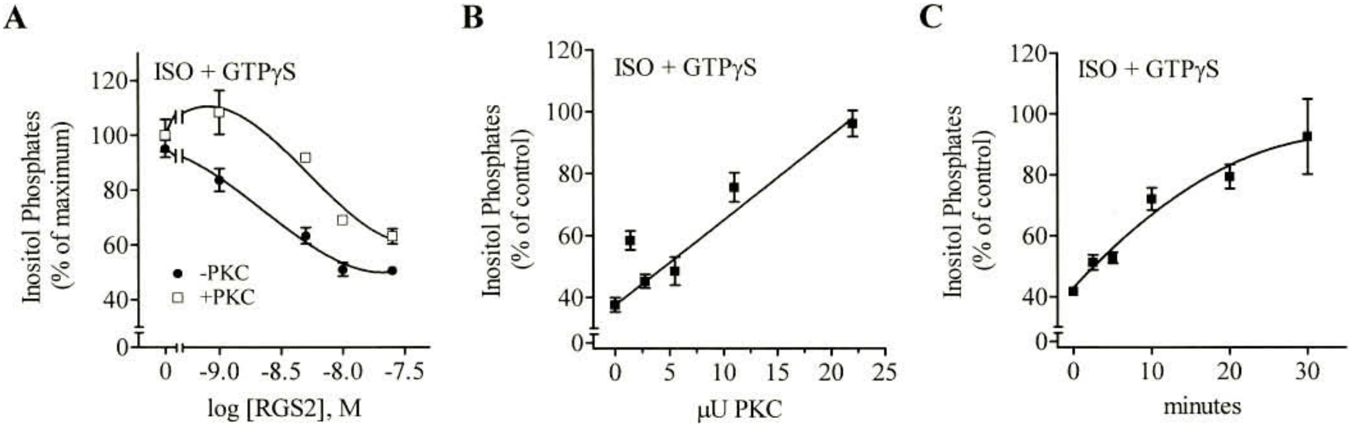

FIG. 7. **Effect of phosphorylation by PKC on the inhibition of β-adrenergic receptor-stimulated inositol phosphate production by RGS2.** Purified RGS2 was incubated with 22 microunits of the catalytic subunit of PKC (□) or enzyme buffer (●) for 30 min at 30 °C (*A*), the indicated concentrations of the PKC catalytic subunit for 30 min 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 nM 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 100 μM isoproterenol (*ISO*) plus 10 μM 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*); 13498 ± 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.

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-AlF_4^- complex than to the GTPγS-bound form of Gα (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. Gα_{11}-mediated inositol lipid signaling in the turkey erythrocyte membrane model is stimulated by both $P2Y_1$ 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 G_q 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 Gα_{11}-mediated activation of purified, reconstituted PLC-β1 (18). Additionally, we illustrated that PLC-βt is phosphorylated in intact erythrocytes in response to PMA treatment, and *in vitro* phosphorylation of PLC-βt 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 $P2Y_2$ receptor agonist UTP. Phosphorylation decreases the

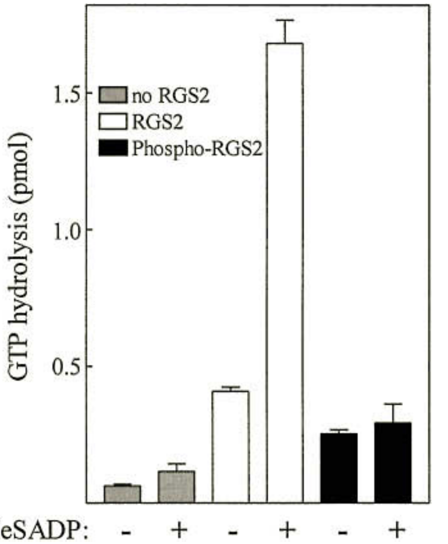

FIG. 8. **Phosphorylation-dependent inhibition of RGS2 GAP activity in P2Y1 receptor/G$\alpha_q\beta\gamma$ vesicles.** Purified RGS2 (7 μg) was incubated with 40 microunits of the PKC catalytic subunit or enzyme buffer for 30 min at 30 °C in a total reaction volume of 48 μl. Kinase reactions were diluted 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$\alpha_q\beta_1\gamma_2$, and GTPase activity was measured in the presence or absence of 100 μM 2MeSADP. Following a 30-min incubation at 30 °C, the assay was terminated by the addition of 5% activated charcoal in 20 mM H_3PO_4. The samples were centrifuged, and liberated $[^{32}P]P_i$ in the supernatant was quantitated by liquid scintillation spectrometry. Data are mean ± S.D. of six measurements and are representative of two experiments.

capacity of reconstituted RGS2 to attenuate PLC-βt activity in turkey erythrocyte membranes and significantly reduces RGS2 GAP activity in P2Y$_1$ receptor/G$\alpha_q\beta\gamma$ vesicles, supporting a role for phosphorylation in regulation of RGS protein activity. This result may be surprising in light of the general conception that RGS proteins have a "desensitizing" activity on G protein-mediated signaling and the observations in our laboratory and numerous others illustrating that PKC activation generally promotes desensitization of inositol lipid signaling (31–34). Phosphorylation of RGS2 by PKC would potentiate receptor-stimulated inositol lipid hydrolysis, and therefore, our results suggest that inositol lipid signaling *in vivo* is regulated temporally by a balance of PKC-promoted inhibitory and stimulatory signals. Such a view is not inconsistent with reports suggesting that receptor-promoted phospholipid signaling is not simply a result of straightforward negative feedback regulation by PKC. Rapid desensitization of phosphoinositide hydrolysis is often only partial and accumulation of inositol 1,4,5-trisphosphate is biphasic (35–37). Moreover, levels of intracellular calcium have been shown to oscillate in the presence of hormone (38–41), and PKC has been strongly implicated in these calcium oscillations (42–45). Thus, studies of both receptor-promoted inositol 1,4,5-trisphosphate production and intracellular calcium levels are consistent with the concept that the role of PKC in regulating phosphoinositide hydrolysis may be more complex than mere signal inactivation.

PKC is activated as a consequence of signaling events other than G$_q$-promoted phosphoinositide hydrolysis, and phospholipid signaling does not occur in isolation in the intact cell. Thus, *in vivo* 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.

Precedent exists for sensitivity of RGS protein-G protein interactions to protein phosphorylation state. Gα_z is phosphorylated by PKC *in vitro*, thereby reducing the capacity of RGSZ1 to accelerate Gα_z GTPase activity (46, 47). The effect of Gα_z phosphorylation on its interaction with RGSZ1 also suggests that stimulation of PKC potentiates Gα_z signaling by lengthening the time that the G protein remains in the active GTP-bound conformation. Therefore, while the target of PKC-promoted phosphorylation in our study is the RGS protein rather than the G protein, the observation that PKC-mediated phosphorylation of RGS2 decreases its capacity to attenuate Gα_{11} signaling is consistent with the sensitivity of RGS-G protein interactions to phosphorylation demonstrated with Gα_z and RGSZ1. Phosphorylation of RGS2 decreased its ability to inhibit GTP-promoted PLC-βt activation and its capacity to promote GTP hydrolysis in vesicles containing the P2Y$_1$ receptor and G$\alpha_q\beta\gamma$. In addition to inhibition of RGS2 activities under conditions where GTPase activity was operative, phosphorylation also attenuated RGS2 inhibition of GTPγS-stimulated signaling. While the details of the association are still unclear, these observations suggest that PKC-promoted phosphorylation of RGS2 affects interactions with G$\alpha_{q/11}$ in such a way that RGS2 cannot promote GTPase activity or block G$\alpha_{q/11}$ interactions with its effector PLC-β as effectively as in the nonphosphorylated state.

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 cGMP-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 Gα_i. 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 by PKC in the intact cell. Although the precise mechanisms have not been fully elucidated, phosphorylation by PKC probably plays an important role in regulating cellular RGS2 activity.

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