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Regulators of G-protein Signaling (RGS) 4, Insertion into Model Membranes and Inhibition of Activity by Phosphatidic Acid*

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Regulators of G-protein signaling (RGS) proteins are critical for attenuating G protein-coupled signaling pathways. The membrane association of RGS4 has been reported to be crucial for its regulatory activity in reconstituted vesicles and physiological roles *in vivo*. In this study, we report that RGS4 initially binds onto the surface of anionic phospholipid vesicles and subsequently inserts into, but not through, the membrane bilayer. Phosphatidic acid, one of anionic phospholipids, could dramatically inhibit the ability of RGS4 to accelerate GTPase activity *in vitro*. Phosphatidic acid is an effective and potent inhibitor of RGS4 in a $G\alpha_{i1}$-[γ-^{32}P]GTP single turnover assay with an $IC_{50} \sim 4$ μM and maximum inhibition of over 90%. Furthermore, phosphatidic acid was the only phospholipid tested that inhibited RGS4 activity in a receptor-mediated, steady-state GTP hydrolysis assay. When phosphatidic acid (10 mol %) was incorporated into m1 acetylcholine receptor-$G\alpha_q$ vesicles, RGS4 GAP activity was markedly inhibited by more than 70% and the EC_{50} of RGS4 was increased from 1.5 to 7 nM. Phosphatidic acid also induced a conformational change in the RGS domain of RGS4 measured by acrylamide-quenching experiments. Truncation of the N terminus of RGS4 (residues 1–57) resulted in the loss of both phosphatidic acid binding and lipid-mediated functional inhibition. A single point mutation in RGS4 (Lys^{20} to Glu) permitted its binding to phosphatidic acid-containing vesicles but prevented lipid-induced conformational changes in the RGS domain and abolished the inhibition of its GAP activity. We speculate that the activation of phospholipase D or diacylglycerol kinase via G protein-mediated signaling cascades will increase the local concentration of phosphatidic acid, which in turn block RGS4 GAP activity *in vivo*. Thus, RGS4 may represent a novel effector of phosphatidic acid, and this phospholipid may function as a feedback regulator in G protein-mediated signaling pathways.

The G-protein signaling pathway is one of the most important signaling cascades used to relay extracellular signals and sensory stimuli in eukaryotic cells. Classically, this signaling system is composed of three major components: G protein-coupled receptors, heterotrimeric G proteins, and second messenger-producing effectors (1). Recently, regulators of G-protein signaling (RGS)[1] have been identified as GTPase-activating proteins (GAP) for Gα subunits, thereby attenuating a number of G-protein-dependent signaling cascades (Ref. 2, and for review see Ref. 3). Members of this protein family share a conserved domain of ~120 amino acid residues, defined as the RGS domain. This domain retains GAP activity *in vitro* comparable with the full-length RGS protein (4). The structure of the RGS4·$G\alpha_{i1}$ complex in the presence of both GDP and AlF_4^- shed light on the mechanism whereby RGS proteins promote the intrinsic GTPase activity of Gα subunits (5). Flanking sequences outside the RGS domain exhibit considerable diversity and constitute additional domains that enable RGS proteins to interact with membranes or proteins beyond Gα subunits (6).

The membrane binding of RGS proteins is essential for their physiological functions. RGSZ1, a G_z-selective RGS protein in the brain, requires membrane association for its GAP activity toward G_z (7). The association of RGS4 with the plasma membrane occurs through an N-terminal α-helix that is also found in RGS16 and RGS2. Plasma membrane association is required for most RGS proteins to inhibit G-protein signaling *in vivo* and confers receptor selectivity (8–11). A recent study indicated that RGS4 binds to phospholipid surfaces in a slow, multistep process where the binding of RGS4 is initially reversible but becomes irreversible within minutes (12). However, whether RGS4 is simply bound to the membrane surface or can penetrate into the membrane bilayer remains unknown.

It has been previously reported that RGS4 binds to anionic phospholipid liposomes (13). However, relatively little is known about what modulates RGS4 activity on signaling pathways when bound to the membrane. RGS4 must bind to model membranes in a specific orientation for its optimal GAP activity *in vitro* (12). Popov and co-workers (14) reported that RGS4 binds to phosphatidylinositol 3,4,5-trisphosphate, another anionic phospholipid, at a site within the RGS domain distinct from and opposite to the RGS/Gα contact face. As a consequence, phosphatidylinositol 3,4,5-trisphosphate binding inhibits GAP activity of RGS4 (14, 15).

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[1] The abbreviations used are: RGS, regulator of G-protein signaling; GAP, GTPase-activating proteins; m1 AChR, muscarinic acetylcholine receptor; G-protein, guanine nucleotide-binding protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; CHS, cholesteryl hemisuccinate; DPC, 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine; SUVs, small unilamellar vesicles; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); mN, millinewton; PLD, phospholipase D; DGK, diacylglycerol kinase; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Pipes, 1,4-piperazinediethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay.

Using model membranes, we demonstrate here that RGS4 can insert into one leaflet of some anionic phospholipid membranes following its initial membrane association. Although both phosphatidylserine (PS) and phosphatidic acid (PA) are effective in promoting RGS4 insertion into the membrane bilayer, PA selectively inhibits its GAP activity in both single-turnover and reconstituted vesicle assays. PA-mediated inhibition of GAP activity of RGS4 requires its N-terminal domain. Also, a mutation of Lys^{20} in the N terminus of RGS4 blocks the PA-induced conformational change in the RGS domain and diminishes PA-conferred inhibition of its GAP activity. PA is a putative second messenger (21) and arises principally from two major signal transduction pathways via phospholipase D (PLD) (16) and diacylglycerol kinase (DGK) (17, 18), both of which are regulated by G protein-coupled receptors (19, 20). Based on our results, we speculate that RGS4 may represent a novel effector of phosphatidic acid.

EXPERIMENTAL PROCEDURES

Materials

Myristoylated $G\alpha_{i1}$, RGS4 wild type, and the N-terminal truncation mutant (Δ57RGS4) with an N-terminal His_6 tag were purified from *Escherichia coli* as described previously (12). Untagged RGS4 was a gift from Maurine Linder (Washington University School of Medicine). $G\alpha_q$ (22), $G\beta_1\gamma_2$ (23), and m1 AchR (24) were purified from Sf9 cells. PS (bovine brain), phosphatidylinositol (egg yolk), cardiolipin (bovine brain), cholesteryl hemisuccinate (CHS), dansyl-PE, imidazole, and Tween 20 were purchased from Sigma. Phosphatidylcholine (PC) (bovine brain), phosphatidic acid (egg yolk), phosphatidylglycerol (PG) (bovine brain), and 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine (DPC) were products of Avanti Polar Lipids Inc. GTPγS and GTP were purchased from Roche Molecular Biochemicals. [γ-^{32}P]GTP (30 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were obtained from PerkinElmer Life Sciences. Acrylamide (Ultrapure) was from Invitrogen. The antibody to the C terminus of RGS4 and secondary antibody were purchased from Santa Cruz Biotechnology. *p*-Nitrophenyl phosphate was from Ameresco. The 96-well microtiter plates were obtained from Nunc. Other reagents were of analytic grade available in China.

Site-directed Mutagenesis of RGS4

RGS4K20E was generated by the QuikChange mutagenesis kit (Stratagene) using the following primers (the mutated codons are in bold): primer 1, GCAAAGGATATG**GAA**CATCGGCTGGG, and primer 2, CCCAGCCGATG**TTC**CATATCCTTTGC. RGS4W59A was generated using Gene Editor (Promega). The mutagenic oligonucleotide (the mutated codon is in bold) was: GAAGTCAAGAAA**GCG**GCTGAATCGCTG. RGS4 coding regions of all constructs were sequenced to verify that only the desired mutations had been introduced.

Preparation of Liposomes

Lipids of desired compositions were mixed in chloroform/methanol (3:1) and dried under a stream of nitrogen. Residual solvents were removed under vacuum for 3–4 h. The phospholipid films were then resuspended in an appropriate buffer and vortexed thoroughly for hydration. Small unilamellar vesicles (SUVs) were prepared by sonicating lipid suspensions to optical clarity at ~2–3-min intervals. Large unilamellar vesicles were prepared according to Rietveld *et al.* (25). For centrifugation binding experiments, large unilamellar vesicles were made in 10 mM Pipes, pH 7.4, 50 mM NaCl, and 0.1 mM EDTA. For fluorescence measurements, SUVs were prepared in 10 mM Hepes, pH 7.4, and 1 mM dithiothreitol. Phospholipid concentration was determined after perchloric acid digestion (26).

Assays to Measure RGS4 Protein Binding to Phospholipid Vesicles

Centrifugation Assay—RGS4 (0.6–1.8 μg) was incubated for 45 min at 30 °C with 50 μl of large unilamellar vesicles with the desired components at varied concentrations. The mixture was subjected to centrifugation at 100,000 × *g* for 1 h at 4 °C. In a control experiment, RGS4 was centrifuged without phospholipid vesicle preincubation. The supernatants were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and Western blotted (27). The amount of unbound RGS4 was estimated by comparing the amount of RGS4 found in the supernatant with varying amounts of defined RGS4 standards. The bound RGS4 was determined by subtraction of the unbound protein from the total. The RGS4 in the supernatant from control experiments was used as the total to calculate the binding efficiency of RGS4 to vesicles.

Fluorescence Resonance Energy Transfer Method—Fluorescence resonance energy transfer between tryptophans in the RGS4 protein (excitation at 280 nm) and dansyl in the phospholipid (emission at 515 nm) was used to monitor the association of protein with phospholipid vesicles (28). Direct excitation of the dansyl group at 280 nm produced a reference emission intensity (F_0). Fluorescence energy transfer was expressed as $(F - F_0)/F_0$, where F and F_0 are the fluorescence emission intensities of phospholipid at 515 nm with and without addition of RGS4, respectively. Dansyl-PE was mixed at 5 mol % in SUVs with the desired phospholipid compositions. The measurements were performed at 30 °C in a Hitachi F4010 fluorescence spectrophotometer.

Binding of RGS4 or RGS4 Mutants to PA Assayed by ELISAs

All procedures were performed as described previously (29). In brief, PA solutions (10 μg/well) diluted in methanol were allowed to bind overnight at 4 °C. The following ELISA steps were performed at room temperature. The wells were blocked with 3% bovine serum albumin in phosphate-buffered saline, pH 7.5, for 2 h. RGS4 or RGS4 mutants were added to the wells and incubated for 1 h. Bound proteins were detected using anti-RGS4 C terminus goat polyclonal antibody (1:500) followed by donkey anti-goat IgG-alkaline phosphatase conjugate (1:8000) and developed using *p*-nitrophenyl phosphate. The resulting absorbance was read at 405 nm using an ELISA plate reader (Bio-Rad). The magnitude of the absorbance was directly related to the bound amount of RGS4 and/or its mutant proteins, and this measurement was used to determine the dissociation constant according to the one-site binding equation.

Insertion of RGS4 into Phospholipid Monolayer

The monolayer surface pressure (π), defined as the change in surface tension after spreading a monolayer on a water surface, was measured by the Wilhelmy plate method with a Han-2000 microbalance designed by Dr. X. H. Han in our laboratory (30). The temperature was controlled at 20.0 ± 1.0 °C. Phospholipid monolayers were formed from a chloroform/methanol (3:1, v/v) stock solution of PC, PS, or PA (250 μg/ml). The experiments were performed as follows. The circular trough used for the penetration assay was filled with 3 ml of Pipes buffer (10 mM Pipes, 50 mM NaCl, 0.1 mM EDTA, pH 7.4). Then, the phospholipid monolayers were prepared by carefully spreading aliquots of phospholipid solution on the buffer surface. After the film pressure stabilized at a constant value (defined as the initial surface pressure (π_i)), RGS4 was injected through a side sample hole into the subphase containing a magnetic stirring bar. The pressure changes were recorded until the increase of surface pressure ($\Delta\pi$) reached a maximal value.

To measure the ability of RGS4 to insert into the phospholipid monolayers, the values of $\Delta\pi$ at 270 nM RGS4 were plotted as a function of varied initial surface pressure (π_i). The plot of $\Delta\pi$ *versus* π_i yields a straight line with a negative slope that intersects the abscissa at a limiting surface pressure. This limiting surface pressure is named the exclusion pressure (π_{ex}) and is the point at which RGS4 no longer penetrates into the phospholipid monolayer.

Protein-induced Calcein Release Experiment

Calcein-enclosed vesicles were prepared and release experiments were performed as previously reported (31). Liposomes were made from PC, PA, PE, and CHS (3:3:1:1). Calcein-containing liposomes were separated from free calcein by AcA34 chromatography and used immediately. Fluorescence was monitored on a Hitachi F2000 fluorescence spectrophotometer. Excitation was at 490 nm and emission was monitored at 520 nm.

Fluorescence Spectroscopy

RGS4 contains two tryptophans at positions 59 and 92. Intrinsic tryptophan fluorescence of RGS4 was measured using a Hitachi F4500 fluorescence spectrophotometer with a 1-cm quartz fluorescence cuvette. RGS4 was incubated with the indicated liposomes at 30 °C for 30 min before measurements. For the quenching experiments, excitation was set at 295 nm instead of 280 nm to excite only Trp residues. After each addition of a quencher, the mixture was stirred and equilibrated for at least 5 min before the emission intensity at 340 nm was recorded. Background fluorescence in samples without protein was subtracted, and the data of emission fluorescence intensities were determined from the corrected spectra. Unless otherwise noted, the emission and exci-

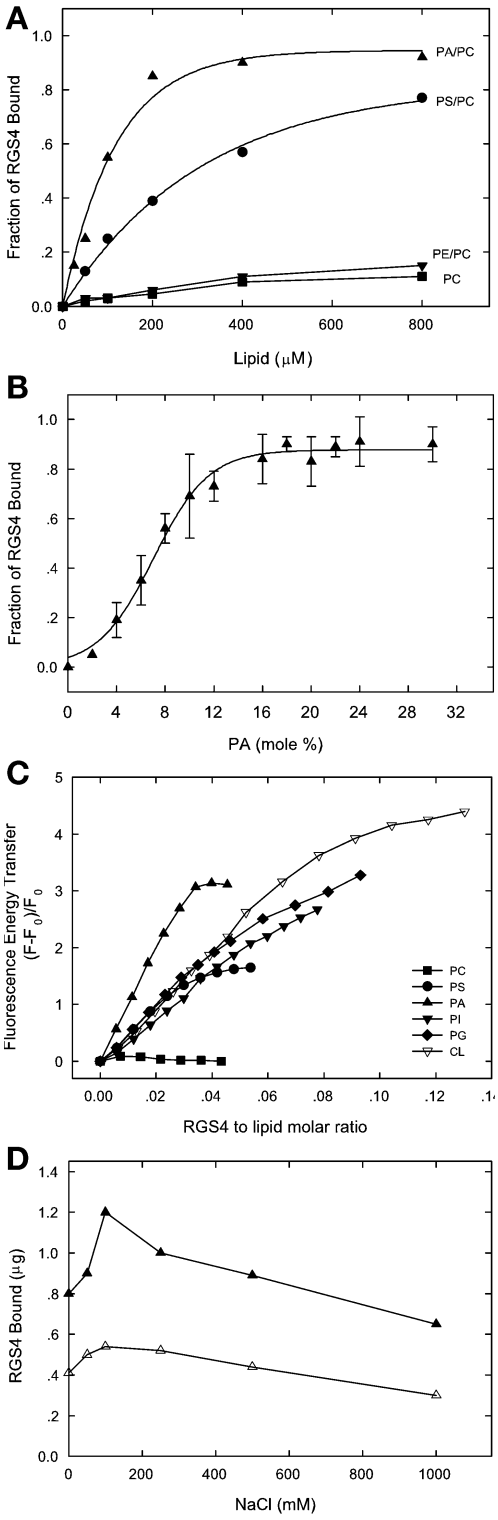

FIG. 1. **Association of RGS4 with vesicles containing different phospholipids.** *A*, binding of RGS4 to membranes was measured in vesicles isolated by centrifugation. Varying liposome concentrations (80 mol % of PC and 20 mol % of PA, PS, PE, or PC) were incubated with 0.5 μM RGS for 45 min at 30 °C. The extent of protein association with vesicles was determined relative to the amount of protein recovered in the absence of phospholipids (*y* axis = fraction of RGS4 bound). *B*, binding of RGS4 to liposomes as a function of PA molar percent. 0.5 μM RGS4 was incubated with vesicles containing PA/PE/CHS. The total lipid invariant was 400 μM, CHS was maintained at 10 mol %, and the mole percent of PA was varied from 0 to 30 mol %. Results shown are the mean of assays performed in triplicate. The data were fit to the modified Hill equation as described in the text. *C*, association of RGS4 with phospholipid vesicles measured by fluorescence resonance energy transfer. Dansyl-PE (5 mol %) was added to vesicles containing the indicated phospholipid. The measurements were performed at 30 °C. Excitation and emission wavelengths were set at 280 and 515 nm, respectively. F and F_0 are the fluorescence emission intensities of phospholipid vesicles at 515 nm with and without addition of RGS4. *D*, effect of ionic strength on RGS4-PA/PC liposomes interactions. Vesicles containing 400 μM PA/PC (PA, 20 mol %) were incubated with RGS4 in the presence of increasing concentrations of NaCl (0–1000 mM) and then assayed for binding efficiency. The experiments were carried out at RGS4 concentrations of 0.5 (*open triangles*) and 1.5 μM (*filled triangles*). Results are the mean of assays performed in duplicate.

tation slit widths were set at 5 and 10 nm, respectively. The temperature was maintained at 30 °C. The assay buffer was 10 mM Hepes, 1 mM dithiothreitol, pH 7.4.

For the depth quenching experiments, DPC was incorporated into the SUVs at a concentration of 10% (molar percent). Aliquots of SUVs with and without DPC were added to the cuvette containing RGS4 (3.2 μM). The F_0/F values were obtained from the corrected spectra described above by using the fluorescence intensities of samples without and with DPC labels.

Acrylamide, a hydrophilic quencher, was used to determine the conformational change in hydrophilic regions of RGS4. Acrylamide was added to the assay buffer containing 1.4 μM RGS4 or RGS4 mutants plus PA vesicles (100:1 molar ratio of vesicles to proteins). The acrylamide quenching data were corrected for dilution and background contributions and fitted by the Stern-Volmer equation (32): $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the concentration of quencher and K_{SV} is the Stern-Volmer quenching constant (M^{-1}).

RGS4 GAP Assays

Single Turnover [γ-^{32}P]GTPase Assays—Single turnover assays using ~100 nM $G\alpha_{i1}$-[γ-^{32}P]GTP were performed on ice as described previously (12). Briefly, purified $G\alpha_{i1}$ (2 μM) was first bound to [γ-^{32}P]GTP in 25 mM Hepes, pH 7.5, 10 mM EDTA, 0.05% Triton X-100, 50 mM NaCl, 1 mM dithiothreitol. The reaction mixture was chilled on ice and $G\alpha_{i1}$-[γ-^{32}P]GTP was immediately purified by centrifugal gel filtration. The rate of $G\alpha_{i1}$-[γ-^{32}P]GTP hydrolysis was measured in 25 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, and 1 mM free Mg^{2+} in the presence or absence of RGS4. 2 μM RGS4 was preincubated with the indicated lipids (25–750 μM) (Fig. 4) or 200 μM PA vesicles (Fig. 6) for 30 min at 4 °C before initiation of the reaction. In this assay, GAP activity is defined as the increase in the first-order hydrolysis rate constant (k_{hydrol}) or is approximated as an increase in the initial rate of hydrolysis (33).

Steady-state GTPase Assays—A presumably more physiological assay for GAP activity of RGS4 monitors the enhancement of agonist-stimulated, steady-state GTPase activity in proteoliposomes reconstituted with receptor and heterotrimeric G proteins. Reconstitution of m1 AchR with G_q was performed as described (22). We kept PE/CHS/PS at a constant molar ratio of 60:10:20 in our reconstituted vesicles and added PC, PS, PG, or PA at 10 mol % to evaluate the effect of different phospholipids on GAP activity of RGS4. RGS4 was added to the vesicles and incubated for 30 min at 30 °C prior to assay. Steady-state assays were carried out at 30 °C for 20 min. Data are given as increases in steady-state GTPase activity (34).

RESULTS AND DISCUSSION

Association of RGS4 with Anionic Phospholipid Membranes—RGS4 was incubated with vesicles composed of the indicated phospholipids, and then unbound RGS4 was separated from the membrane-bound population by sedimentation of vesicles using ultracentrifugation. The amount of RGS4 present in each supernatant was determined by Western blotting (not shown), and the amount of RGS4 bound under each condition was determined as described under "Experimental Procedures." To investigate whether the binding of RGS4 to membranes depends on the phospholipid composition of the vesicles, we first examined the ability of RGS4 to bind to vesicles containing PS/PC (PS, 20 mol %), PA/PC (PA, 20 mol %), PE/PC (PE, 20 mol %), or PC alone. Fig. 1*A* demonstrates that RGS4 did bind to vesicles containing anionic phospholipids in a concentration-dependent fashion. In contrast, no RGS4 was associated with vesicles composed exclusively of the neu-

tral phospholipids PC and PE, implying that electrostatic interaction plays a role in binding. These results are consistent with what has been observed previously (12, 13). The nature of the binding interaction between RGS4 and PA was further investigated using liposome association experiments. PE/CHS/PA liposomes (400 μM) containing constant CHS (10 mol %) and varying mole fractions of PA (0–30 mol %) were prepared and incubated with RGS4 (1 μM). The amount of RGS4 bound was quantitated as described above, and plotted as a function of the concentration of PA. The results of the liposome association experiment are shown in Fig. 1*B*. Notably, a significant increase in RGS4-PA association was observed at more than 10 mol % PA. The data was plotted and fit to the Hill equation for receptor-ligand binding (35). Using a sigmoidal fit, the half-maximal binding affinity (apparent K_d) of RGS4 for PA was estimated to be 8 $\pm$ 1 mol % PA. A Hill coefficient of about 2.5 was also obtained, suggesting positive cooperativity of binding of RGS4 to membranes. This observation may reflect cooperative sequestering of PA around RGS4.

To corroborate these results, we used fluorescence resonance energy transfer, a more sensitive assay, to monitor the association of RGS4 with vesicle membranes. Dansyl-PE, labeled at the head group of phospholipids, accepts tryptophan-donated energy from the protein and undergoes a change in its fluorescence intensity as the protein binds to phospholipid vesicles (28). As shown in Fig. 1*C*, there were significant increases in 515 nm fluorescence when anionic phospholipid vesicles were titrated with RGS4, whereas no fluorescence energy transfer was observed in the presence of the vesicles composed exclusively of PC. This result further confirms that RGS4 binding to phospholipid vesicles requires the presence of anionic phospholipids. Whereas we have not attempted to analyze the selectivity of RGS4 among phospholipids in great detail, PA was the most efficient anionic phospholipid tested in facilitating its binding to liposomes. This is surprising because other phospholipids, such as cardiolipin, have higher negative charge density than PA. We therefore studied the effect of increasing ionic strength on RGS4-PA interaction and the results are shown in Fig. 1*D*. At each concentration of RGS4 tested (0.5 and 1.5 μM), its binding to PA/PC (400 μM PA, 20 mol %) vesicles is weak in the absence of salt. RGS4/lipid vesicle binding progressively increases up to 100 mM NaCl, after which additional increases in salt concentration (up to 1 M) diminish the binding. Protein/lipid binding cannot be completely blocked even at 1 M NaCl, suggesting that other forces besides electrostatic interactions (*e.g.* hydrophobic interactions) may have a role in stabilizing the RGS4-PA interaction. It has been previously reported that PA can induce membrane regions to adopt a concave surface contour and to form a hexagonal phase (36). It is possible that such distinctive properties of PA may contribute to its strong enhancement of RGS4 binding to membranes. It should be mentioned that data shown in this paper used N-terminal His_6-tagged RGS4, but similar results were obtained in experiments using untagged RGS4 (data not shown).

Insertion of RGS4 into Phospholipid Monolayers—Whereas the binding of RGS4 to phospholipid vesicles appears to be dependent on anionic phospholipids, it is unclear whether RGS4 simply attaches on the surface of membranes or inserts into the phospholipid bilayer. To answer this question, we utilize the monolayer technique to evaluate the capability of RGS4 penetrating into membranes.

First we measured the penetration of RGS4 into an air/water interface without spread to the phospholipid monolayer to acquire the appropriate conditions of the following experiments. The maximal increase of surface pressure induced by RGS4 itself was found to be 23.5 mN/m and the minimal concentration of RGS4 to reach such a maximum was 270 nM. Therefore, we kept the initial surface pressures (π_i) of phospholipid monolayers spread onto the subphase surface at 23.5 mN/m or higher and the concentration of RGS4 injected into the subphase at 270 nM in the following experiments. Subsequently, we monitored the insertion of RGS4 into PA monolayers. Our results indicated that 270 nM RGS4 dramatically increased the surface pressure of the PA monolayer, whereas injecting only buffer or proteinase K-treated RGS4 did not alter the surface pressure (data not shown). It has been previously established that proteins that exclusively interact with the phospholipid surface headgroups do not increase the surface pressure of the monolayer (for review see Ref. 37). Therefore, a change in the surface pressure ($\Delta\pi$) following the addition of RGS4 indicates that this protein inserted into the phospholipid monolayer. We also measured $\Delta\pi$ for RGS4 under different initial surface pressures, π_i (Fig. 2). The resulting plot of $\Delta\pi$ *versus* π_i yielded a straight line with a negative slope that intersects the abscissa at the limiting surface pressure, π_{ex}. π_{ex} is the maximum surface pressure at which RGS4 can penetrate into the different phospholipid monolayers (37). Fig. 2 shows the plot of $\Delta\pi$ *versus* π_i for RGS4 insertion into anionic phospholipid monolayers. From these data, π_{ex} was calculated for RGS4 insertion into the following anionic phospholipids: 48 mN/m for PA, 50.5 mN/m for PS, and 33.6 mN/m for PC. The π_{ex} of a phospholipid monolayer with a surface pressure $\geq$35 mN/m is believed to be comparable with the packing density of biological membranes (37, 38). By comparing our values of π_{ex} measured for RGS4 with 35 mN/m, we extrapolate that RGS4 should have the potential to insert into biological membranes.

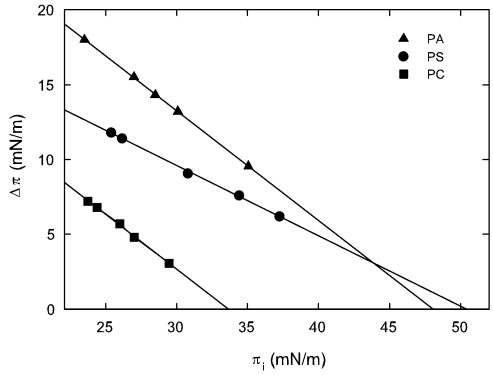

FIG. 2. **$\Delta\pi$-π_i plot of RGS4 insertion into different phospholipid monolayers.** 270 nM RGS4 was injected into the subphase and changes in the surface pressure ($\Delta\pi$) were recorded for the different phospholipid monolayers as indicated at different initial surface pressures (π_i). The plot of $\Delta\pi$ *versus* π_i yields a straight line with a negative slope that intersects the abscissa at a limiting surface pressure. This limiting surface pressure is named the exclusion pressure and is the point at which RGS4 no longer penetrates into the phospholipid monolayer.

Penetration of RGS4 into Anionic Phospholipid Vesicles—To further study RGS4 insertion into phospholipid bilayers, we measured RGS4 fluorescence quenching by DPC. DPC contains a doxylstearate labeled at carbon-5 of the fatty acid in PC, and this chemical side group quenches tryptophan (Trp) fluorescence. This quenching is primarily static rather than collisional. Only when the doxyl moiety (buried within the membrane bilayer) is no more than 5 Å from Trp residues can the Trp fluorescence be efficiently quenched (39). This method is only valid when at least one of the two RGS4 Trp residues insert into the membrane proximal to the quenching moiety embedded in the phospholipid bilayer.

Fig. 3*A* shows that Trp fluorescence of RGS4 is sharply decreased by DPC. This indicates that at least one Trp residue

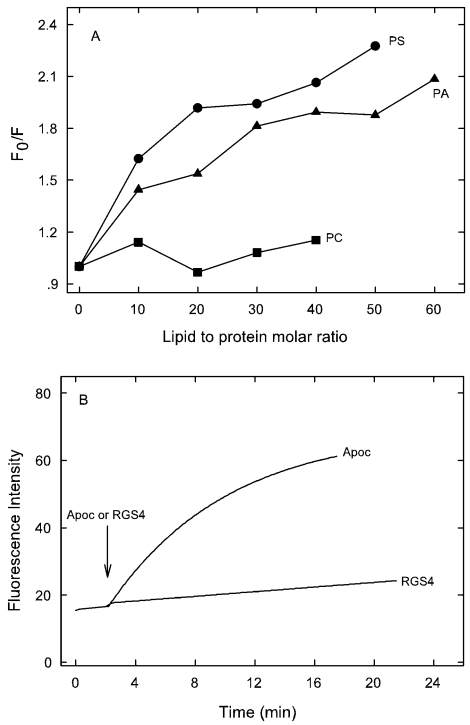

FIG. 3. **Demonstration of RGS4 inserting into anionic phospholipid vesicles.** *A*, quenching of Trp fluorescence of RGS4 by DPC. 3.2 μM RGS4 was added to phospholipid vesicles with and without DPC. F_0 and F represent the fluorescence intensities measured using RGS4/phospholipid membranes in the absence or presence of DPC, respectively. The excitation wavelength was 295 nm, and emission was monitored at 340 nm. *B*, RGS4 and apocytochrome *c* (*Apo c*)-induced calcein release from phospholipid bilayers. 50 μM Calcein-enclosed PC/PA/PE/CHS (3:3:1:1) vesicles were incubated until fluorescence intensity was stable. Then, RGS4 (1.5 μM) or apocytochrome *c* (0.4 μM) was added where indicated and the release of entrapped calcein was monitored following the increase of fluorescence at 520 nm.

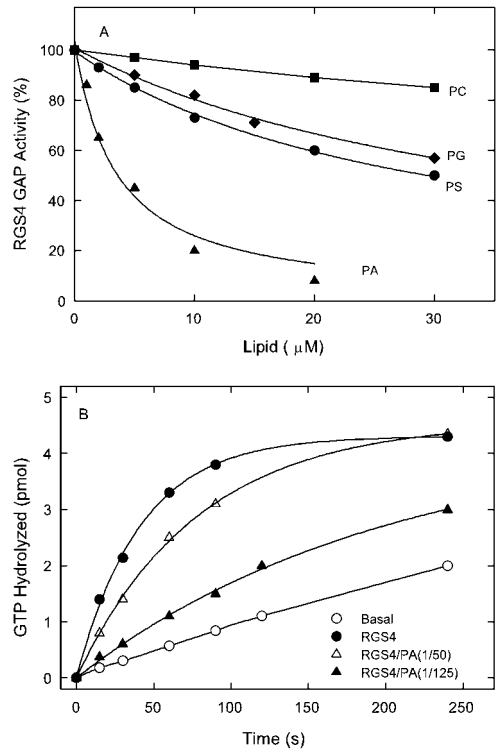

FIG. 4. **PA inhibits RGS4 GAP activity in single turnover assay**. *A*, RGS4 was incubated with different phospholipids for 30 min at 4 °C. The final RGS4 concentration in the assay was 0.08 μM and the phospholipid concentration varied from 2 to 30 μM. The first-order hydrolysis rate constant (k_{hydrol}) was determined in the presence (control) and absence of RGS4 (*basal*) and the difference was defined as GAP activity. The 100% GAP activity was equal to 1.2–1.5 min^{-1} in different experiments. These values are averages of three experiments with less than 10% standard error. *B*, RGS4 (2 μM) was incubated with 100–250 μM PA vesicles for 30 min at 4 °C. 2-μl aliquots were withdrawn and measured for GAP activity using $G\alpha_{i1}$-GTP as substrate. The final RGS4 and $G\alpha_{i1}$-GTP concentrations in the assay were 80 and 150 nM. The final concentration of PA shown was 4 (*open triangles*) or 10 μM (*filled triangles*). Control reactions contained neither RGS4 nor lipid (*open circles*) or no lipid (*filled circles*).

of RGS4 has inserted into the membrane bilayer of PA- or PS-containing phospholipid vesicles. In contrast, little to no changes in Trp fluorescence of RGS4 were observed using neutral phospholipid vesicles. This result shows that RGS4 has the intrinsic potential to penetrate into anionic phospholipid membranes. We also tested the quenching of RGS4 fluorescence by DPC with doxyl groups labeling the 12th carbon position of the fatty acid. Using this alternate label, only slight quenching was observed (data not shown). These data indicate that the N terminus of RGS4 inserted into the membrane bilayer, but not deeply such that Trp can be quenched by the 12-doxyl form of DPC.

To test whether RGS4 membrane insertion perturbs the phospholipid bilayer, the ability of RGS4 to release calcein encased in phospholipid vesicles was measured. Under initial conditions, 200 mM calcein was enclosed inside the phospholipid vesicles, which causes calcein to self-quench. The injection of membrane-penetrating proteins causes the dye to leak out of the vesicle, leading to a gradual increase of fluorescence intensity. We used apocytochrome *c* as a positive control to induce calcein leakage from the phospholipid vesicles (Fig. 3*B* and Ref. 40). In contrast (Fig. 3*B*), no calcein release was observed upon incubation with RGS4, indicating that RGS4 did not disrupt the integrity of the phospholipid bilayer. By combining the data obtained using 5- and 12-doxyl-DPC, and the failure of RGS4 to trigger leakage of entrapped calcein from vesicles, we propose that RGS4 can only insert into one leaflet of the phospholipid bilayer adjacent to the membrane surface. It has been reported that the inner leaflet of the plasma membrane of eukaryotic cells is composed of 30% anionic and 70% neutral phospholipids (41). Our results indicate that anionic phospholipids play essential roles in RGS4 insertion into the model membrane. Although RGS4 has a higher affinity for PA than to PS (Fig. 1), PS is the primary anionic phospholipid contained in the inner leaflet of the plasma membrane and may be one of the major binding sites tethering RGS4 to the plasma membrane *in vivo*.

PA Inhibits RGS4 GAP Activity—Growing evidence supports that the phospholipid environment of certain proteins can regulate their functions (42). Because RGS proteins are thought to act at the inner surface of biological membranes, it is reasonable to hypothesize that certain cell membrane phospholipids may influence the interaction between RGS proteins and their Gα targets. Therefore, we tested the effects of different anionic phospholipid vesicles on GAP activity of RGS4 (Fig. 4*A*). Using 80 nM RGS4, PA showed the greatest inhibitory activity with maximal inhibition exceeding 90% and an IC_{50} of ~4 μM (Fig. 4*A*). A 45% inhibition of GAP activity of RGS4 was observed in the presence of 4 μM PA (molar ratio of lipid:RGS4, 50:1), whereas 10 μM (molar ratio of 125:1) inhibited its GAP activity more than 90% (Fig. 4*B*). Other anionic phospholipids, PS and PG, could only weakly inhibit GAP activity of RGS4 (Fig. 4*A*) with maximal inhibition of less than 50% and a much lower potency ($IC_{50} > 30$ μM). Even at concentrations of up to 50 μM, neutral phospholipids (PC and PE) never inhibited GAP activity of RGS4 by more than 10%, thus they are considered essentially inactive. None of the phospholipids tested affected the

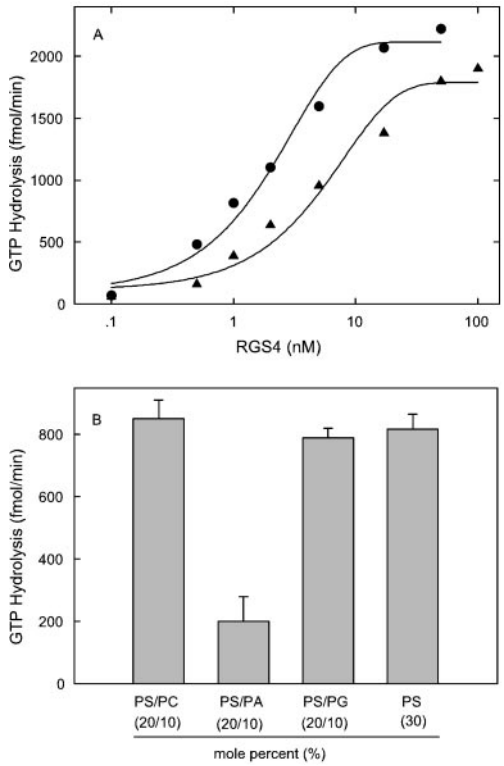

FIG. 5. **PA inhibits RGS4-stimulated GTP hydrolysis of m1 AChR-G_q vesicles.** *A*, m1 AChR-G_q vesicles (1.3 nM G_q and 0.35 nM m1 AchR reconstituted in PE/PS/CHS (60:30:10, *circles*), or 1.0 nM G_q and 0.3 nM m1 AchR reconstituted in PE/PS/PA/CHS (60:20:10:10, *triangles*); 6 μM final lipid concentration) were mixed with RGS4 (0.1–100 nM) and the mixture was preincubated at 30 °C for 30 min prior to assay. Carbachol-stimulated GTPase activity was assayed as described under "Experimental Procedures." For reference, GTPase activity in the absence of RGS4 was 60 fmol/min in PE/PS/CHS (60:30:10) vesicles, and 48 fmol/min in vesicles composed of PE/PS/PA/CHS (60:20:10:10). *B*, 1 nM RGS4 was incubated with 6 μM m1 AChR-G_q vesicles (PE/PS/CHS (60:30:10), PE/PS/PC/CHS (60:20:10:10), PE/PS/PG/CHS (60:20:10:10) or PE/PS/PA/CHS (60:20:10:10)) at 30 °C for 30 min prior to assay. Carbachol-stimulated GTPase activity was assayed as described under "Experimental Procedures." PE/PS/PG/CHS (60:20:10:10) vesicles contained 1.3 nM G_q and 0.3 nM m1 AchR. PE/PS/PC/CHS (60:20:10:10) vesicles contained 1.0 nM G_q and 0.28 nM m1AchR and the other vesicles were as described in *A*.

intrinsic GTPase activity of $G\alpha_{i1}$ (data not shown). The GAP assays shown in Fig. 4 used $G\alpha_{i1}$-GTP as a substrate, but similar results were obtained in experiments using $G\alpha_z$-GTP (data not shown). These data show that, although RGS4 can bind to anionic phospholipids and insert into membranes without great preference (Fig. 2), PA may play a distinctive and important role in regulating RGS4 function.

To further characterize the effect of PA on RGS4 activity, we performed receptor-mediated, steady-state GTP hydrolysis assays. These assays are presumably more physiological by monitoring RGS4-mediated enhancement of agonist-stimulated, steady-state GTPase activity in proteoliposomes reconstituted with receptor and heterotrimeric G proteins. PE/CHS/PS (60:10:20) were used for our reconstituted proteoliposomes because the binding of RGS4 through anionic phospholipids appears to be necessary for efficient GAP activity (12). To evaluate the effects of different phospholipids on RGS4 GAP activity, the remaining 10 mol % was PC, PS, PG, or PA. The agonist-stimulated GTPase activity of unilamellar phospholipid vesicles containing m1 AChR and heterotrimeric $G\alpha_q$ was measured in the presence of increasing concentrations of RGS4 (Fig. 5). When agonist-bound receptor drives GDP/GTP exchange in these vesicles, hydrolysis of G_q-bound GTP becomes rate-limiting and a GAP increases steady-state hydrolysis until the overall reaction again approaches the rate of receptor-catalyzed GDP/GTP exchange (43, 44). In m1 AChR-G_q vesicles (PE/CHS/PS, 60:10:30), 50 nM RGS4 increases agonist-stimulated GTPase activity about 40-fold with an EC_{50} ~1.5 nM (Fig. 5*A*). Partial replacement of PS by PA (PE/CHS/PS/PA, 60:10:20:10) inhibited RGS4 GAP activity by about 4-fold (EC_{50} ~ 7 nM). This data is consistent with RGS/phospholipid binding values shown in Fig. 1*B*. In contrast, 10 mol % of PG (PE/CHS/PS/PG, 60:10:20:10) or PC (PE/CHS/PS/PC, 60:10:20:10) exhibited no effect or slightly increased RGS4 GAP activity (Fig. 5*B*). Further decrease of PS (<15%) dramatically decreased both the efficiency of reconstitution and receptor-stimulated GTPγS binding of G_q, presumably because PS is essential for both the association of G proteins with membranes and the coupling between receptor and G_q (24, 44). It should be noted that RGS4 binding to all three proteoliposomes was virtually identical and almost 100% under the conditions used for GAP assay. The maximal GTPase activity was also similar for each vesicle composition, presumably because GDP/GTP exchange became rate-limiting (44). These results further support the idea that PA may play an important role in the regulation of RGS4-Gα interaction.

The N Terminus of RGS4 Is Required for PA Binding and Inhibition—The first 57 amino acids of RGS4 are essential for its localization to membranes (8, 11–13). To determine the importance of this region for interactions with PA, we generated both deletion (missing amino acids 1–57) and single point mutants of RGS4. All the RGS4 mutants used in our study reacted with anti-RGS4 C terminus goat polyclonal antibody with relatively equal affinity in an ELISA (data not shown). Therefore any differences that may be observed between proteins in the phospholipid-binding assay would be because of their differential binding to phospholipids and not because of a variation in their binding to the primary antibody. The RGS4 N-terminal truncation mutant Δ57RGS4 did not bind to PA (Fig. 6*A*). This agrees well with previous studies showing that the N terminus of RGS4 is required for membrane binding (12, 13). Furthermore, removing the first 57 amino acids resulted in the loss of the PA-conferred inhibitory effect on RGS4 GAP activity in a solution-based, single-turnover GTP hydrolysis assay (Fig. 6*B*). These results suggest that residues between 1 and 57 play an important role in both association of RGS4 with PA and PA-conferred inhibition of GAP activity of RGS4. Based on this result, we generated a series of mutations at the N terminus of RGS4. One RGS4 mutant, K20E (Lys^{20} → Glu) proved to be interesting. The protein-lipid binding assays indicated that this mutation did not have any significant effects on the initial binding of RGS4 to PA vesicles (Fig. 6*A*). Both wild type RGS4 and K20E mutant have similar dissociation constants for PA vesicles (K_d ~ 25 nM). K20E was also as effective as wild type RGS4 in the single-turnover GAP assay in the absence of PA vesicles (Fig. 6*B*). However, unlike wild type RGS4, PA did not inhibit GAP activity of RGS4K20E (Fig. 6*B,* molar ratio 100:1, lipid:RGS4). Although the RGS domain alone can function as a GAP, the N-terminal domain of RGS4 is required for efficient interaction with Gα (11, 12). Our data strengthens the argument for allosteric interactions between the N terminus and the RGS domain of RGS4 because Lys^{20} lies far from the RGS domain based on its primary structure.

PA Induces a Conformational Change in the Hydrophilic Domain of RGS4—The interaction of RGS4 with phospholipid vesicles changes its intrinsic fluorescence, most likely reflecting a conformational change of the protein (12). RGS4 has two Trp residues; one is at the 59th (Trp^{59}) position and the other is the 92nd (Trp^{92}) in the RGS domain. According to the crystal

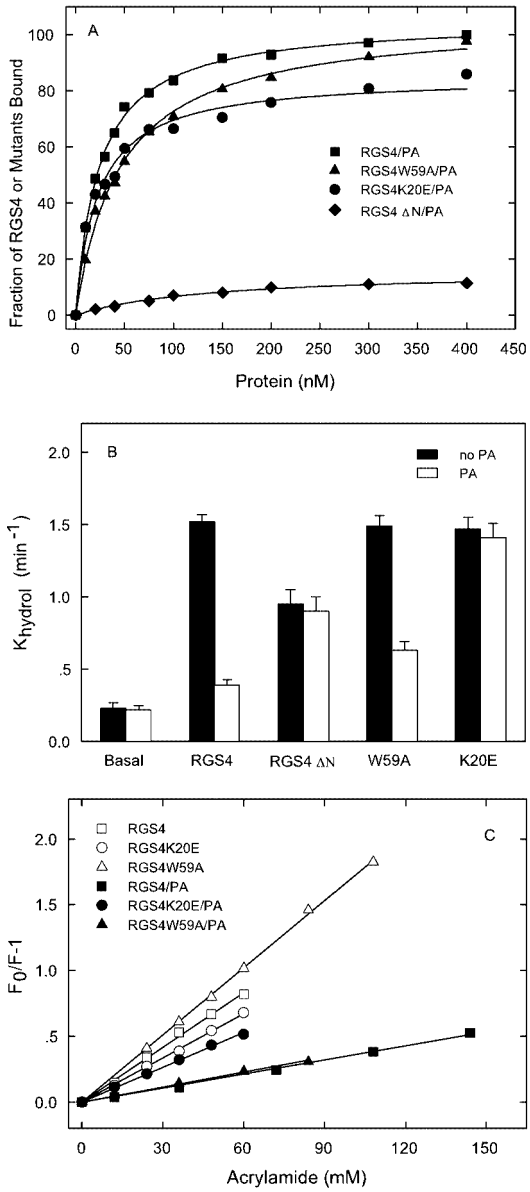

FIG. 6. **Mutation at Lys[20] of RGS4 blocked PA-induced conformational changes of the RGS domain and diminished PA-mediated inhibition of its GAP activity without affecting PA-RGS4 interaction.** *A*, binding of wild type RGS4 or its mutants to PA as determined by ELISA. Each well of an ELISA plate was coated with 10 μg of PA and blocked using bovine serum albumin. Then, the wells were incubated with increasing amounts of RGS4 or its mutants as indicated in the figure. The bound proteins were analyzed using an antibody to the C terminus of RGS4. *B*, the values of k_{hydrol} (min^{-1}) of RGS4 and its mutants in the presence and absence of PA. 2 μM RGS4 or its mutants were preincubated with 200 μM PA vesicles or buffer as a control for 30 min at 4 °C before initiation of the reaction, the molar ratios of PA vesicles to proteins were 100:1. GAP activity was assayed as in Fig. 4. Values shown are averages of three experiments ± S.D. *C*, acrylamide quenching of intrinsic fluorescence of RGS4 and mutants in the presence and absence of PA vesicles. The concentrations of RGS4 and its mutants were 1.4 μM, and the molar ratios of PA to proteins were all 100:1. Excitation wavelength, 295 nm; emission wavelength, 340 nm. F_0 and F represent the fluorescence intensities of RGS4 and its mutants without and with the quencher.

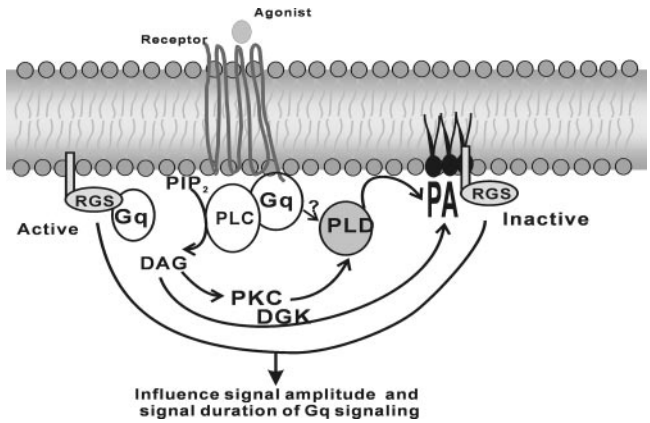

FIG. 7. **A model of RGS4 regulation by the anionic phospholipid PA.** Agonist-induced stimulation of G-protein-coupled receptors enhances GTP binding to Gα subunits. PLD may be activated by either $G\alpha_q$ or protein kinase C (*PKC*). PA is produced by both PLD and DGK as described in the text. We propose that RGS4 inserts into PA-containing membranes via its N terminus, leading to the potent inhibition of GAP activity. Thus, PA may act as a positive modulator for the output of G_q signaling.

structure, Trp^{59} is almost completely buried among helices 1, 2, 3, and 9, whereas Trp^{92} is relatively exposed to the hydrophilic environment opposite the site of Gα binding (5). Acrylamide is a neutral, efficient quencher of tryptophan fluorescence that is very sensitive to the surface exposure of Trp residues in proteins and has been used extensively to probe the conformational change in the hydrophilic region of proteins (32, 45). To further investigate the mechanism of PA-mediated inhibition of RGS4 GAP activity, acrylamide was used to determine whether there are conformational alterations around Trp residues upon RGS4 insertion into PA liposomes. As shown in Fig. 6*C*, the Stern-Volmer quenching constant (K_{SV}) for RGS4 in the absence of phospholipids is about 13.9 M^{-1}. Upon its insertion into PA vesicles, the value of K_{SV} was dramatically decreased to 3.5 M^{-1} indicating that Trp residues were much less accessible to acrylamide. In contrast, PC vesicles exhibited almost no effect on acrylamide fluorescence quenching (data not shown). When RGS4 was incubated with vesicles containing PA and PC (PA, 20 mol %), the value of K_{SV} was almost identical to those obtained in the presence of PA alone (data not shown). This implies that 20% PA content in the vesicles is sufficient to induce conformational changes in the hydrophilic regions of RGS4.

In a recent study, it is speculated that phospholipid-induced RGS4 conformational changes occur in the RGS domain, in proximity to Trp^{92} (12). We generated a RGS4W59A mutant ($Trp^{59} \rightarrow$ Ala) that contains only one Trp (Trp^{92}) in the RGS domain. This mutant protein behaved comparably with the wild type RGS4 in both binding to phospholipid membrane and as a GAP (Fig. 6). The results shown in Fig. 6*C* indicate that acrylamide-induced quenching in the RGS4W59A mutant ($Trp^{59} \rightarrow$ Ala) was comparable with wild type RGS4. This result supports the previous assumption (12) that the conformational change observed in wild type RGS4 was donated by the Trp^{92} residue in the RGS domain. After RGS4 and this mutant were incubated with PA vesicles, a sharp decrease in the fluorescence quenching by acrylamide was observed (Fig. 6*C*, K_{SV} decreased by 70–80%), and was accompanied by a remarkable inhibition of its GAP activity (Fig. 6*B*). In contrast, PA vesicles only slightly decreased the acrylamide-induced quenching of K20E fluorescence (Fig. 6*C*, K_{SV} decreased by approximately 10–15%) and did not affect RGS4 GAP activity at the same condition. These data indicate that the K20E mutant could not assume the more compact conformation around the Trp^{92} residue achieved by the wild type RGS4 and W59A mutant in the presence of PA vesicles.

From results presented here, it is clear that anionic phospholipid PA can inhibit GAP activity of RGS4. PA has been shown to modulate a large number of enzyme activities (*e.g.* phospholipase C, protein kinases, cyclic AMP-phosphodiester-

ase, Ras GTPase-activating protein, protein-tyrosine phosphatase (21, 29, 46, 47)), yet few reports have come up with a definitive physiological role for PA. Our results show that PA strongly inhibits GAP activity of RGS4 in both single-turnover and reconstitution vesicle assays. Replacement of PS by PA (10 mol %) in m1 AChR-G_q vesicles markedly inhibited RGS4 GAP activity by more than 70% with a 4-fold increase of EC_{50} during receptor-stimulated, steady-state GTP hydrolysis. In contrast, PS, PG, or PC had no such inhibitory effect. Based on these data and previous reports (14), we speculate that the RGS4/Gα interaction is influenced by the phospholipid microenvironment surrounding these proteins. We also propose that PA acts as a positive regulator of Gα-mediated signaling pathways by inhibiting RGS proteins. An example of PA being both generated by and acting on G_q-mediated signaling is shown in Fig. 7. In resting cells, PA only constitutes a minor portion of the total phospholipid pool, but there has been intense interest in the role of PA as a phospholipid second messenger. PA is generated principally from two mechanisms: the action of PLD on phosphatidylcholine (16) and directly converting diacylglycerol by DGK to PA (17, 18). In response to G protein receptor stimulation (as is shown for G_q-mediated signaling pathways in Fig. 7), both PLD and DGK are activated (48–51). PLD may be activated directly by $G\alpha_q$ or by the downstream activation of protein kinase C. PLD and/or DGK activation will increase the local concentration of PA, which can consequently block RGS4 GAP activity and therefore acts as a positive feedback regulator in G_q signaling. Thus, RGS4 may represent a novel PA effector and PA may be important in regulating the output of G_q signaling *in vivo*.

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