Ca\textsuperscript{2+}/Calmodulin Reverses Phosphatidylinositol 3,4,5-Trisphosphate-dependent Inhibition of Regulators of G Protein-signaling GTPase-activating Protein Activity*

Regulators of G protein signaling (RGS proteins) are GTPase-activating proteins (GAPs) for \( G_i \) and/or \( G_q \) class G protein \( \alpha \) subunits. RGS GAP activity is inhibited by phosphatidylinositol 3,4,5-trisphosphate (PIP\textsubscript{3}) but not by other lipid phosphoinositides or diacylglycerol. Both the negatively charged head group and long chain fatty acids (C16) are required for binding and inhibition of GAP activity. Amino acid substitutions in helix 5 within the RGS domain of RGS4 reduce binding affinity and inhibition by PIP\textsubscript{3} but do not affect inhibition of GAP activity by palmitoylation. Conversely, the GAP activity of a palmitoylation-resistant mutant RGS4 is inhibited by PIP\textsubscript{3}. Calmodulin binds all RGS proteins we tested in a Ca\textsuperscript{2+}-dependent manner but does not directly affect GAP activity. Indeed, Ca\textsuperscript{2+}/calmodulin binds a complex of RGS4 and a transition state analog of \( G_i \)-GDP-AlF\textsubscript{4}\. Ca\textsuperscript{2+}/calmodulin reverses PIP\textsubscript{3}-mediated but not palmitoylation-mediated inhibition of GAP activity. Ca\textsuperscript{2+}/calmodulin competition with PIP\textsubscript{3} may provide an intracellular mechanism for feedback regulation of Ca\textsuperscript{2+} signaling evoked by G protein-coupled agonists.

Heterotrimeric G proteins link 7-transmembrane domain receptors to intracellular effector proteins in all mammalian cell types. These pathways are required for rapid responses to hormones and neurotransmitters. Signaling is initiated by agonist binding to receptors, which catalyze the exchange of GTP for GDP on the \( \alpha \) subunit. Activated \( G_{i}\)-GTP and \( G_{q}\) \( \beta \gamma \) subunits then regulate effector proteins that generate second messengers and subsequent downstream responses (reviewed in Ref. 1). The intensity, duration, and specificity of \( G \) protein-mediated signaling depend on a newly identified class of proteins, termed regulators of G protein signaling (RGS\textsuperscript{1} proteins), which are GTPase-activating proteins (GAPs) for \( G \) subunits (Refs. 2–4 and reviewed in Ref. 5).

A diverse family of more than 20 RGS proteins are expressed in mammals (6). The RGS proteins share a common sequence feature of about 130 amino acids, referred to as the RGS domain (RGS box), which is responsible for GAP activity (7–9). Many RGS proteins, including RGS4, can accelerate GTP hydrolysis on both \( G_i \) and \( G_q \) class \( \alpha \) subunits, whereas other RGS proteins are more selective catalysts (reviewed in Refs. 5, 10, and 11). Both full-length RGS4 and its RGS domain can inhibit Ca\textsuperscript{2+} signaling evoked by either \( G_i \)- or \( G_q \)-coupled agonists (12). The GAP activity of RGS proteins provided a molecular explanation of their role as inhibitors of G protein signaling, but mechanisms for how RGS GAP activity is regulated in cells remain obscure.

In several genetic systems, RGS proteins appear to act as feedback regulators of G protein signaling (7, 13, 14). Because RGS4 inhibits Ca\textsuperscript{2+} signaling in mammalian cells (11–17), we tested whether calmodulin, which regulates many Ca\textsuperscript{2+}-responsive proteins stimulated by \( G_q \)-coupled agonists (18), could bind RGS4 and regulate its activity. We report that calmodulin binds in a Ca\textsuperscript{2+}-dependent manner to all RGS proteins we tested, including RGS1, RGS2, RGS4, RGS10, RGS16, and GAIP. Surprisingly, Ca\textsuperscript{2+}/calmodulin binding did not influence the GAP activity of RGS proteins.

Calmodulin regulates membrane association of many intracellular proteins (19–21), and it has been observed that RGS proteins associate with the inner surface of the plasma membrane (22–25). A cluster of positively charged residues on the surface of helices 4 and 5 in the RGS domain was noted in the x-ray crystal structure of RGS4 (26) that could bind acidic lipids in the plasma membrane. Here we demonstrate that dipalmitoylphosphatidylinositol \( 3',4,5\)-trisphosphate (diC\textsubscript{16}-PIP\textsubscript{3} or Pip\textsubscript{3}) binds RGS4 and inhibits its GAP activity in a concentration-dependent manner, in contrast to other phosphatidylinositol phosphates. Pip\textsubscript{3} inhibited GAP activity of other RGS proteins, including RGS1, RGS2, RGS10 and GAIP, but not RGS16. Amino acid substitutions in helix 5 of RGS4 reduced Pip\textsubscript{3} binding (10-fold) and Pip\textsubscript{3}-dependent inhibition of GAP activity. A potential mechanism for feedback regulation of G protein-mediated Ca\textsuperscript{2+} signaling was suggested by the observation that Pip\textsubscript{3}-dependent inhibition of GAP activity was reversed by Ca\textsuperscript{2+}/calmodulin. The concerted action of

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‡ The abbreviations used are: RGS, regulators of G protein signaling; Pip\textsubscript{3} or diC\textsubscript{16}-Pip\textsubscript{3}, dihexadecanoylphosphatidylinositol 3,4,5-trisphosphate; Pip\textsubscript{3}, 1-\alpha-phosphatidylinositol 4,5-bisphosphate; Pip\textsubscript{3}, 1-\alpha-phosphatidylinositol 4-phosphate; Pl, 1-\alpha-phosphatidylinositol, DAG, 1-stearyl-2-arachidonoyl-sn-glycerol; GTPγS, guanosine 5′-triphosphosulphate 3,4,5-trisphosphate; diC\textsubscript{16}-Pip\textsubscript{3}, dioctanoylphosphatidylinositol 3,4,5-trisphosphate; diC\textsubscript{8}–3,4-Pip\textsubscript{3}, dioctanoylphosphatidylinositol 3,4-bisphosphate; diC\textsubscript{8}–3,5-Pip\textsubscript{3}, dioctanoylphosphatidylinositol 3,5-bisphosphate; diC\textsubscript{8}–4,5-Pip\textsubscript{3}, diocanoylphosphatidylinositol 4,5-bisphosphate; PC, 1-\alpha-phosphatidylcholine; PS, 1-\alpha-phosphatidyl-L-serine; PG, 1-\alpha-phosphatidyl-sn-glycerol; CaM, calmodulin; GAP, GTPase-activating protein; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; dansyl-CaM, dansylated calmodulin; PAGE, polyacrylamide gel electrophoresis; SUVs, small unilamellar lipid vesicles; PLCβ, phospholipase Cβ; RU, response units.
Ca\textsuperscript{2+}/calmodulin and PIP\textsubscript{2} may regulate RGS GAP activity to initiate [Ca\textsuperscript{2+}i] oscillations evoked by G protein–coupled agonists.

MATERIALS AND METHODS

Calmodulin, Peptides, and Inositol Lipids—Bovine brain calmodulin and calmodulin covalently attached to agarose beads (CaM-agarose) were from Sigma. Peptide P\textsubscript{i}, MCKGLAGLPASCLRSAKDMKHRL-GLFLQKDSDC, was described (12), and a scrambled sequence of P\textsubscript{i}, MLDQFLK3SACAGKLHRGCGSFLRKLCSD, \(\Delta P\) was synthesized by C. Slaugter (UT Southwestern). L-\(\alpha\)-Phosphatidylinositol (PC), L-\(\alpha\)-phosphatidylethanolamine (PE), L-\(\alpha\)-phosphatidylserine (PS), L-\(\alpha\)-phosphatidylcholine (PC), and clusters of positively charged amino acids similar to calmodulin-binding sites in other proteins (Table I; see Ref. 18). We used synthetic peptides containing the above mutation as a primer and cloned into the pQE60 expression vector (Qiagen) as described for RGS4 (9). RGS16 expression and purification were similar to RGS4 (9). Recombinant RGS1 and RGS2 were kindly provided by Drs. K. J. Blumer, S. Heximer (Washington University), and D. Fosdyke (Queens University), respectively (16). The fragment coding for mutant RGS4 K112E/K113E was generated by polymerase chain reaction using the oligonucleotide containing the above mutation as a primer and cloned into the pQE60 expression vector (Qiagen) as described for RGS4 (9). RGS4 C95V protein was kindly provided by Dr. E. Ross (UT Southwestern).

Ca\textsuperscript{2+}/Calmodulin Binding—Concentration of calmodulin was measured fluorimetrically using the extinction coefficient 3060 M\textsuperscript{-1} cm\textsuperscript{-1} at 278 nm in presence of 1 mM EGTA (30). Concentration of RGS4 was measured using Bradford reagent from Bio-Rad. Band shift gel analysis of calmodulin binding to RGS4 and RGS2 in 4 \(\times\) urea, in the presence of 0.1 mM Ca\textsuperscript{2+} or 2 mM EDTA, was carried out as described (30). RGS binding to calmodulin-agarose was detected by SDS-PAGE of supernatants as follows: 15 \(\mu\)l of wet calmodulin-agarose beads (26 \(\mu\)g of CaM) washed with buffer (10 mM HEPES, pH 7.4, 0.1 mM CaCl\textsubscript{2}, 1 mM DTT) were added to the beads. The supernatant was removed, and the beads were washed with RGS4 (1.5 nmol) equal in amount to coupled calmodulin-binding sites in other proteins (Table I; see Ref. 18). We used synthetic peptides containing the above mutation as a primer and cloned into the pQE60 expression vector (Qiagen) as described for RGS4 (9). RGS4 C95V protein was kindly provided by Dr. E. Ross (UT Southwestern). The fluorescent detection of RGS4–calmodulin interaction was performed using Perkin-Elmer LS 50B and Hitachi F-2000 fluorescent spectrophotometers at 25 ± 0.1 °C. Both excitation and emission slit widths were 10 nm. For internal RGS4 tryptophan fluorescence measurements, the excitation and the emission wavelengths were 283 and 336 nm, respectively. Typically, aliquots of CaM solution were gradually added to the solution of RGS4 in the assay cuvette with constant agitation, and after each addition the mixture was equilibrated inside the instrument with the excitation beam shutter closed. The fluorescence measurements were made after 5–15 min. The excitation beam shutter was opened only long enough to get accurate readings. Measurements were repeated until reproducible readings were obtained. Data in Fig. 2 were corrected for dilution (which did not exceed 7%) and for fluorescence of calmodulin alone.

Calmodulin was dansylated as described (31) and extensively dialyzed against 10 mM HEPES, pH 7.4. Fluorescence measurements of dansylated calmodulin (dansyl-CaM) were at 355 (excitation) and 500 nm (emission). All measurements were corrected for background fluorescence observed in control experiments. The Scatchard plot analysis of the fluorescence affinity measurement data indicated a single binding site interaction (or 2 sites with similar affinities) and fit well to linear approximations within the experimental error.

RESULTS AND DISCUSSION

| Table I | Putative calmodulin binding regions in RGS domain proteins |
|---------|-----------------------------------------------------------|
| Protein | Sequence |
| rRGS4   | (97) EYKTKRSPKSLPSRKKST (114) |
| hGAIP   | (125) EFKKIRSATKLASRAHHI (116) |
| rRGS1   | (107) EYKKKIKSPSKLSPKAKKI (114) |
| rRGS2   | (118) DFKKTSQPSKLSRKKST (135) |
| hRGS3   | (429) DFKKVRSSQKLSARASKK (446) |
| hRGS10  | (688) DFKKVRSSQKLSARASKK (85) |
| mRGS16  | (99) EFKKTSQPSKLSRKKST (116) |

The active amino acid residues used were: -, negative; +, positive; h, hydrophobic; X, any; -, gap in alignment.
found that RGS4 bound calmodulin in a Ca\(^{2+}\)-dependent manner (the complex was dissociated by 2 mM EGTA) in a standard band shift assay for calmodulin binding (Fig. 1; Ref. 30). RGS4 binding to Ca\(^{2+}\)/calmodulin was corroborated using calmodulin coupled to agarose beads. RGS4 binding to calmodulin-agarose beads required Ca\(^{2+}\) and was stable to buffer washes, but bound RGS4 could be eluted from beads either with EGTA or SDS (Fig. 1C). We found that a previously characterized amphi-aphatic calmodulin-binding peptide from CaM kinase II (34) competed with RGS4 binding to calmodulin-agarose beads (Fig. 1E, 4th and 5th lanes). An RGS4 N-terminal 33 amino acid peptide (P\(_{1-33}\)), which conveys high affinity and receptor-selective regulation of G\(_{q}\) signaling (12), bound Ca\(^{2+}\)/calmodulin (Fig. 1D) and competed with RGS4 for binding to calmodulin-agarose beads (Fig. 1E, 6th and 7th lanes). By contrast, a scrambled sequence composed of the same amino acids (\(\phi P_{1-33}\)) did not bind to calmodulin beads (data not shown) and did not compete with RGS4 binding (Fig. 1E, 8th and 9th lanes). Interestingly, RGS4 apparently formed a heterotrimeric complex with G\(_{q}\)-GDP-AIF\(_{4}\) and Ca\(^{2+}\)/calmodulin (Fig. 1F), consistent with their predicted distinct binding sites on RGS4.

We used fluorescence spectroscopy to quantitate binding interactions between RGS4 and Ca\(^{2+}\)/calmodulin. The fluorescence of two tryptophan residues within the RGS domain of RGS4 (4Box) was quenched by titration with Ca\(^{2+}\)/calmodulin (which lacks tryptophan), indicative of RGS4-Ca\(^{2+}\)/calmodulin binding in solution (Fig. 2). A sharp inflection in the titration curve indicates formation of a stable, equimolar complex between Ca\(^{2+}\)/calmodulin and 4Box (20 mM NaCl, 10 mM HEPES, pH 7.4). Upon further addition of Ca\(^{2+}\)/calmodulin to 4Box, the slope of the fluorescence titration curve paralleled that of unbound RGS4 and Ca\(^{2+}\)/calmodulin (calculated as the sum of their fluorescence signals measured separately). This behavior is consistent with the prediction of a single Ca\(^{2+}\)/calmodulin-binding site in the RGS domain. By contrast, in the absence of salt, the fluorescence intensity of RGS4 (Fig. 2) or 4Box (data not shown) did not change at higher molar ratios of Ca\(^{2+}\)/calmodulin, suggesting additional, low affinity calmodulin-binding sites on RGS4. These low affinity interactions are probably electrostatic because they were not detected at higher ionic strength (Fig. 2, 4Box, and data not shown).

In addition to the RGS domain, Ca\(^{2+}\)/calmodulin apparently binds to the N-terminal 33 amino acids of RGS4 because the peptide P\(_{1-33}\) binds in a Ca\(^{2+}\)-dependent manner both to calmodulin beads (Fig. 1) and in solution, as detected by two-dimensional heteronuclear single quantum coherence NMR using [\(^{15}\)N]calmodulin.\(^2\) Although P\(_{1-33}\) has no residues with fluorescent properties convenient for affinity measurements, we used a dansylated derivative of calmodulin (dansyl-CaM, see Ref. 31) which allowed us to compare the binding properties of P\(_{1-33}\) and RGS4 in similar conditions. The apparent K\(_d\) values (Table II) were calculated from Scatchard linear transformations of titration curves (Fig. 3). In the presence of 100 mM NaCl, RGS4 and P\(_{1-33}\) bind to Ca\(^{2+}\)/dansyl-CaM with similar affinities (K\(_d\) ~5 \(\mu\)M). The affinity of P\(_{1-33}\) binding within the accuracy of measurements did not change with ionic strength, whereas RGS4 bound Ca\(^{2+}\)/dansyl-CaM almost 5 times stronger than P\(_{1-33}\) in 20 mM NaCl, consistent with our observations that a decrease in salt concentration strengthened the interaction of RGS4 with Ca\(^{2+}\)/calmodulin.

Calmodulin binds to many RGS proteins in a Ca\(^{2+}\)-dependent manner but with different salt dependences. Binding assays indicated that RGS4, RGS16, and GAIP had similar affin-
Complexes were stable to extensive washing with buffer, but data not shown. Because the N terminus of RGS4 is not required for Gα binding or for Ca2+/calmodulin binding (35). Surprisingly, we found that dioctanoylphosphatidylinositol 3,4,5-trisphosphate (IP3) mimicked IP3 binding to Rac and RhoA, which only barely detected the inhibitory activity of 4-monophosphorylated phosphatidylinositol 4,5-bisphosphate (IP4, the other reaction product of PLC). Inhibition of RGS GAP activity by Ca2+/calmodulin was relatively weak (even without salt). Only RGS1 and RGS2 bound to calmodulin-agarose beads in high salt (150 mM KCl). Stable interaction was lost by dialysis of recombinant RGS4 into patch clamped pancreatic acinar cells potently inhibited Ca2+ signaling evoked by Gq- and Go-coupled receptor agonists (12, 16). This suggested the possibility that endogenous RGS proteins might be relatively inactive prior to agonist stimulation of Ca2+ signaling and that recombinant RGS proteins escaped this inhibition. To identify inhibitors of RGS4 GAP activity on Goq in the single turnover assay, we tested various compounds related to and including either the substrate or products of PLCβ. We found that RGS4 GAP activity was inhibited by preincubation with an analog of PIP3, diC16-PIP3 (30 μM PIP3, at 0 °C for several minutes, Fig. 5A), or phosphocholine vesicles containing 20% PIP3 (Fig. 5B). Inhibition of RGS4 GAP activity was dependent on the concentration of PIP3 (Fig. 5C). The kinetic curve of GTP hydrolysis in the presence of PIP3 closely approximated the basal activity of Go without RGS4. This behavior indicated negligible dissociation of PIP3 from RGS4 during the assay (5 min) because GTP hydrolysis was initiated by a rapid 20-fold dilution of the RGS-PIP3 incubation mixture into a solution containing Goq1-GTP. In control experiments, the intrinsic GTPase activity of Goq1 was unaffected by PIP3 (Fig. 5D). The GAP activity of each RGS protein that was tested in the single turnover assay, except RGS16, was inhibited by PIP3 (Fig. 5D and data not shown).

By contrast to PIP3 inhibition of RGS GAP activity, no effect was observed following coincubation of RGS4 with 400 μM PIP2 from bovine brain (Fig. 5B), and only 2–3-fold inhibition was observed following coincubation with PIP2 micelles (9 mM). We barely detected the inhibitory activity of 4-monophosphorylated phosphatidylinositol 4,5-bisphosphate (IP4, the other reaction product of PLC). As summarized in Table III, several synthetic PIP2 lipids were also without effect (assayed at 400 μM in phosphocholine vesicles), including dioctanoylphosphatidylinositol 4,5-bisphosphate (diC8–3,4,5-PIP2), dioctanoylphosphatidylinositol 3,5-bisphosphate (diC8–3,5-PIP2), and dioctanoylphosphatidylinositol 4,5-bisphosphate (diC8–4,5-PIP2). RGS4 GAP activity was also not affected by a lipid head group derivative, 1-stearoyl-2-arachidonoyl-sn-glycerol (diacylglycerol, DAG), which is one of the reaction products of PLCβ. No inhibitory activity was detected using PI or highly charged group derivatives of PIP2 lacking the fatty acyl moieties, including ionositol 1,3,4,5-tetraakisphosphate (IP1), or glycerophosphoinositol 3,4,5-trisphosphate (IP6), the other reaction product of PLCβ (data not shown). PIP2 binding to Rac1 and RhoA was shown to have similar requirements for both electrostatic and hydrophobic interactions (35). Surprisingly, we found that dioctanoylphosphatidylinositol 3,4,5-trisphosphate (diC8-PIP3), which only differed from PIP2 (diC16-PIP2) in the length of the fatty acid chains, did not inhibit RGS4 GAP activity; nor did diC16-3,5-

### Table II

| Interaction | [NaCl] |
|-------------|--------|
|             | 20 mM | 50 mM | 100 mM |
| P1–33 + dansyl-CaM | 4.9 | 4.9 | 4.9 |
| RGS4 + dansyl-CaM   | 1.1 | 2.3 | 4.7 |

![Fig. 3: Dansylated Ca2+/calmodulin binds RGS4 N terminus](Image)

Fluorescent titration of 0.5 μM dansylated CaM (dansyl-CaM) with P1–33 peptide (A) or RGS4 (B) in 10 mM HEPES, pH 8.0, 1 mM DTT, 0.1 mM CaCl2, at different concentrations of NaCl. Dansylated CaM (dansyl-CaM) in 20 mM NaCl was calculated based on fractional decrease in fluorescence intensity.

![Fig. 4: RGS4 GAP activity is not inhibited by Ca2+/calmodulin](Image)

RGS4 GAP activity was not inhibited by Ca2+/calmodulin. RGS4 (0.8 μM in A) or R4αN (N-terminal 56-amino acid truncation; 20 μM in B) was preincubated with Ca2+/CaM (1.6 μM in A or 75 μM in B) before initiating a single turnover GAP assay. Open squares, RGS4 with CaM; closed squares, RGS4 without CaM; closed circles, basal Gαi1-GTP hydrolysis. Final RGS4 concentrations were 22 nM (A) and 220 nM (B).
**Fig. 5. DiC16-PIP₃ inhibits RGS4 GAP activity.** Single turnover assay with Go₁₂-GTP. RGS4 (2 μM) was preincubated with 30 μM diC16-PIP₃ micelles (open squares) or 70 μM diC16-PIP₃ (open circles) on ice in 6-μl volume for 20 min. Control reactions contained no PIP₃ (squares) or no RGS4 (filled circles) (A). Single turnover assay with RGS4 and Go₁₂-GTP in the presence of PC lipid vesicles contained 20% diC16-PIP₃ (open circles) or 20% PIP₂ (filled squares). RGS4 (0.72 μM) was preincubated with 10-μl vesicles on ice in 11-μl volume for 20 min. Control reactions contained no lipid (open squares) or neither RGS nor lipid (filled circles) (B). DiC16-PIP₃ inhibition of RGS4 GAP activity is concentration-dependent in GAP assays with PIP₃ (0.9, 2.7, and 9.1%) incorporated into PC/PG (9:1) vesicles (C). GAP activity of RGS proteins in the presence of diC16-PIP₃, RGS1 (2 μM), RGS10 (0.5 μM), GAIP (0.24 μM), and RGS16 (2 μM) were preincubated with or without 200 μM PIP₃ on ice for 20 min. GAP activity was assayed as in A.

**TABLE III**

| Lipid tested         | RGS4 binding | RGS4 GAP activity |
|----------------------|--------------|-------------------|
| diC16-3,4,5-PIP₃     | +            | −                 |
| 4,5-PIP₃ (brain)     | −            | +/−               |
| 4-PIP (brain)        | NT           | +                 |
| P1 (brain)           | NT           | +                 |
| diC8-3,4,5-PIP₃      | −            | +                 |
| diC8-3,4-PIP₃        | −            | +                 |
| diC8-3,5-PIP₂        | −            | +                 |
| diC8-4,5-PIP₂        | −            | +                 |
| Gro-3,4,5-IP₃        | NT           | +                 |
| 1,3,4,5-IP₄          | NT           | +                 |
| DAG                  | NT           | +                 |

*Inhibition detectable above 1 mM 4,5-PIP₃.  
**Marginal effect at 7 mM P1. ±, binding detected or RGS4 GAP activity is normal; −, binding or GAP not detected; NT, not tested.

**Pip₃.** Thus, diC16-PIP₃ is the only phospholipid which inhibited RGS4 GAP activity, and its activity appears to require both the long chain fatty acyl moiety and the highly charged head group.

**RGS4 Is a Pip₃-binding Protein**—Protein-lipid binding affinities were estimated by surface plasmon resonance measurements on the BIAcore (Biacore, Inc.). RGS4 coupled to the carboxymethylated dextran surface of the BIAcore chip bound diC16-PIP₃ (Fig. 6A) but not diC8-PIP₃, PIP₂, or other phosphoinositides, consistent with the observation that RGS GAP activity was most sensitive to inhibition by diC16-PIP₃. The association phase of diC16-PIP₃ binding was typically complete within several minutes, whereas dissociation was slow (Fig. 6A). No diC16-PIP₃ binding was detected on a blank chip. The $K_d$ value of diC16-PIP₃ binding to RGS4 (44 ± 19 nM; Table IV) was calculated from the on and off rates extracted from the binding curves (Fig. 6A, and data not shown).

**Ca²⁺/CaM Antagonizes the Pip₃ Inhibition of RGS4 GAP Activity**—The positively charged patch on the surface of helixes 4 and 5 in the RGS domain of RGS4 (residues 99–113; Ref. 26) appeared to be a good candidate for binding not only to calmodulin but also to Pip₃. We found that Pip₃ inhibition of RGS4 GAP activity was reversed by coincubation of RGS4 and Pip₃ (micelles or phosphocholine vesicles) with Ca²⁺/calmodulin (Fig. 7). Ca²⁺/calmodulin and Pip₃ apparently compete for binding to helixes 4 and 5 in RGS4. To test this model further, we introduced amino acid substitutions of glutamate for lysine residues at positions 112 and 113 in helix 5 of RGS4. This mutant protein (RGS4 K112E/K113E) retained GAP activity and calmodulin binding, but it bound Pip₃ with almost 10-fold lower affinity than did wild type RGS4 (Table IV). Similarly, low binding affinity was observed with RGS16. The comparatively weak binding of these proteins to Pip₃ correlated with their relative insensitivity to Pip₃ inhibition of GAP activity (Figs. 5D and 6B). Helixes 4 and 5 are conserved in many RGS domains (Table I) and may provide an important regulatory feature of RGS proteins because Ca²⁺/calmodulin has been shown to serve as a molecular switch that regulates lipid-protein interactions (19–21).

**Pip₃ Inhibits GAP Activity of Palmitoylation-resistant RGS Proteins**—We propose that Pip₃-mediated inhibition of GAP activity acts by a concerted mechanism in which the highly charged head group interacts with the RGS domain residues in helixes 4 and/or 5 to position the palmitoyl moiety of Pip₃ near the binding site. Palmitoylation of a nearby cysteine residue inhibited RGS4 and RGS10 GAP activity (36). This cysteine residue in helix 4 (Cys²⁶ in RGS4) is conserved in the RGS domain of all mammalian RGS proteins except RGS6 and RGS7 (6). Substitution of cysteine for valine (C95V) prevents covalent modification by palmitate at this position in RGS4. Covalent modification by palmitate at this position in RGS4.
Before initiating the assay, RGS4 (K112E/K113E) are less sensitive than wild type RGS4 (RGS4 K112E/K113E (B, open bars) and RGS16 after 90 s (see Fig. 5D) to inhibition by PC/PIP3 vesicles (8:2). Single turnover GAP assay as in Fig. 5A. Binding curves for RGS16 and RGS4 K112E/K113E were normalized to 2500 RU of coupled RGS4 (A). The GAP activities of mutant RGS4 K112E/K113E (B, open bars) and RGS16 after 90 s (see Fig. 5D) are less sensitive than wild type RGS4 (B, solid bars) to inhibition by PC/PIP3 vesicles (8:2). Single turnover GAP assay as in Fig. 5A.

TABLE IV

| Protein       | $k_a$ (10$^3$ M$^{-1}$ s$^{-1}$) | $k_d$ (s$^{-1}$) | $K_d$ (nM) |
|---------------|-------------------------------|-----------------|------------|
| RGS4          | 12.1 ± 7.5                    | 4.8 ± 1.6       | 44 ± 19    |
| RGS4 K112E/K113E | 3.2 ± 1.4                    | 12.6 ± 1.1      | 430 ± 140  |
| RGS16         | 5.2 ± 2.5                     | 7.9 ± 2.7       | 163 ± 38   |

FIG. 6. RGS4 binds PIP$_3$. BIAcore sensorgrams of diC16-PIP$_3$ (4 μM) binding to RGS proteins immobilized on the chip (background binding subtracted). Binding curves for RGS16 and RGS4 K112E/K113E were normalized to 2500 RU of coupled RGS4 (A). The GAP activities of mutant RGS4 K112E/K113E (B, open bars) and RGS16 after 90 s (see Fig. 5D) are less sensitive than wild type RGS4 (B, solid bars) to inhibition by PC/PIP3 vesicles (8:2). Single turnover GAP assay as in Fig. 5A.

FIG. 7. Ca$^{2+}$/CaM reverses inhibition of RGS4 GAP activity by diC16-PIP$_3$. Single turnover GAP assay with PIP$_3$ incorporated into lipid vesicles, as in Fig. 5C. Before initiating the assay, RGS4 was incubated for 20 min on ice with PC/PIP$_3$ vesicles (8:2) alone or with of 100 μM Ca$^{2+}$/CaM (A). Single turnover GAP assay with PIP$_3$ micelles. Before initiating the assay, RGS4 (5 μM) was incubated with 50 μM Ca$^{2+}$/CaM (5 μM) for 2 min on ice with 40 μM PIP$_3$ (B and C, open circles), 40 μM PIP$_3$, and 100 μM Ca$^{2+}$/CaM (B, open squares), or 40 μM PIP$_3$, 100 μM CaM, and 2 μM EGTA (C, open squares). Control incubations contained no lipid (B and C, filled squares) or no RGS4 (B and C, filled circles). 2 μl of each incubation mix were used for GAP assay.

FIG. 8. DiC16-PIP$_3$ inhibits GAP activity of a palmitoylation-resistant RGS4 mutant. RGS4 C95V (1 μM) was preincubated with 80 μM PIP$_3$ (open circles) on ice in 5-μl volume for 20 min. Control reaction contained no PIP$_3$ (squares) or no RGS (filled circles) (A). RGS4 K112E/K113E GAP activity is inhibited by palmitoylation. RGS4 K112E/K113E (3 μM) was incubated with 50 μM palmitoyl-CoA (Pal-CoA) for 1 h at 30 °C (open circles). 2 μl of the reaction mix were used in GAP assay. Control reactions contained no RGS4 K112E/K113E (filled circles) or no palmitoyl-CoA (filled squares) (B). RGS16 is susceptible to inhibition by palmitoylation. RGS16 (6 μM) was incubated with 50 μM palmitoyl-CoA for 1 h at 30 °C (open circles). 2 μl of the reaction mix were used in GAP assay. Control reactions contained no RGS4 (filled circles) or no palmitoyl-CoA (Pal-CoA) (filled squares) (C). Ca$^{2+}$/CaM does not reverse inhibition of RGS by palmitoylation. RGS4 (2 μM) was preincubated with 25 μM palmitoyl-CoA for 1 h at 30 °C (open circles) or in the presence of 100 μM Ca$^{2+}$/CaM (triangles). 3 μl of each reaction mix were used in GAP assay. Control reactions contained no RGS4 (filled circles) or no palmitoyl-CoA (Pal-CoA) (filled squares) (D). Single turnover assay as in Fig. 5A.

Ca$^{2+}$/CaM-mediated inhibition as is wild type protein (Fig. 8A). The GAP activities of RGS16 and the mutant RGS4 K112E/K113E, which were relatively insensitive to inhibition by PIP$_3$, responded like wild type protein to inhibition by palmitoylation (Fig. 8, B and C). In contrast to RGS4 interaction with PIP$_3$, inhibition of GAP activity by palmitoylation was not prevented by addition of Ca$^{2+}$/calmodulin (Fig. 8D). This is presumably because Ca$^{2+}$/calmodulin competes with PIP$_3$ binding to RGS4 but cannot displace the covalent modification of RGS4 by palmitate. The feedback mechanisms that regulate the palmitoylation of RGS proteins in vivo are unknown, but we propose that PIP$_3$-mediated inhibition of RGS GAP activity may be reversed in a Ca$^{2+}$-dependent manner through binding of Ca$^{2+}$/calmodulin.
mechanism to initiate oscillations over a wide range of frequencies.

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FIG. 9. A model of RGS regulation by Ca2+/calmodulin and PIP3. Agonist-bound receptor promotes GTG binding to the Go subunit and subsequent dissociation of Go and Gβγ. The effector protein PLCβ may be activated by either Go or Gβγ from G class proteins. PLCβ catalyzes the hydrolysis of PIP2 to produce DAG and IP3, which binds IP3R to release Ca2+ from intracellular stores. We propose that endogenous RGS proteins may be inactive prior to agonist-evoked Ca2+ signaling, but as the local concentration of intracellular Ca2+ elevates, it binds calmodulin, which can displace PIP3, from helices 4 and 5 of RGS proteins, and thereby restores RGS GAP activity. Calmodulin binding may also reposition RGS within the receptor complex to enhance activity. RGS-mediated inhibition of G protein signaling would decrease [Ca2+], allowing dissociation of calmodulin and re-binding of PIP2 to inhibit RGS GAP activity.

Feedback Regulation of RGS GAP Activity by Ca2+/Calmodulin and PIP3—Negative regulation of RGS GAP activity by PIP2 may be part of a reset mechanism that allows a new wave of G protein signaling in response to agonist. G protein-coupled receptors, such as formyl-methionyl-leucyl-phenylalanyl receptors in neutrophils, can elicit both Ca2+ release and a rapid and large accumulation of PIP3 by activating the effector proteins PLCβ and PI-3 kinase, respectively (reviewed in Ref. 37). Ca2+ release from internal stores is one of the initial responses to PLCβ activation either by Go or Gβγ from G class proteins. As the local concentration of Ca2+ elevates in response to PLCβ activity, we postulate that Ca2+/calmodulin binding to RGS4 and other RGS proteins displaces PIP2 to restore GAP activity (modeled in Fig. 9). Ca2+/calmodulin binding may also enhance GAP activity by relocating RGS proteins within the receptor signaling complex to be in proximity to their Go-GTP substrates. Feedback inhibition of G protein-mediated PLCβ activation by RGS proteins would allow [Ca2+]i to decrease and promote the dissociation of calmodulin from RGS proteins. We hypothesize that PIP2 released after receptor stimulation could then bind RGS proteins to inhibit their GAP activity. If agonist stimulation persists, this may reactivate G protein signaling and allow another burst of Ca2+ release from internal stores. If agonist is no longer present, PIP2-mediated inhibition of RGS GAP activity may reset the signaling pathway to allow a robust cellular response to subsequent agonist stimulation. Feedback regulation of RGS GAP activity may provide an intracellular...
