Phosphatidylinositol 3,4,5-trisphosphate and Ca^{2+} /calmodulin competitively bind to the regulators of G-protein-signalling (RGS) domain of RGS4 and reciprocally regulate its action

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RGS (regulators of G-protein signalling) are a diverse group of proteins, which accelerate intrinsic GTP hydrolysis on heterotrimeric G-protein α subunits. They are involved in the control of a physiological behaviour known as ‘relaxation’ of G-protein-gated K^+ channels in cardiac myocytes. The GTPase-accelerating activity of cardiac RGS proteins, such as RGS4, is inhibited by $\text{PtdIns}(3,4,5)\text{P}_3$ (phosphatidylinositol 3,4,5-trisphosphate) and this inhibition is cancelled by Ca^{2+} /calmodulin (CaM) formed during membrane depolarization. G-protein-gated K^+ channel activity decreases on depolarization owing to the facilitation of GTPase-activating protein activity by RGS proteins and vice versa on hyperpolarization. The molecular mechanism responsible for this reciprocal control of RGS action by $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM, however, has not been fully elucidated. Using lipid–protein

co-sedimentation assay and surface plasmon resonance measurements, we show in the present study that the control of the GTPase-accelerating activity of the RGS4 protein is achieved through the competitive binding of $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM within its RGS domain. Competitive binding occurs exclusively within the RGS domain and involves a cluster of positively charged residues located on the surface opposite to the $\text{G}\alpha$ interaction site. In the RGS proteins conserving these residues, the reciprocal regulation by $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM may be important for their physiological regulation of G-protein signalling.

Key words: calmodulin, G-protein, phosphoinositide, reciprocal regulation, regulators of G-protein signalling (RGS).

INTRODUCTION

Heterotrimeric G-protein signalling is a major mechanism in cellular signal transduction [1]. Many elaborate studies have been conducted to examine the main elements of this system, including G-protein-coupled receptors, the G-proteins themselves and effector molecules. However, physiological modulation and tuning of the trimeric G-protein cycle had not been considered until a family of proteins termed RGS (regulators of G-protein signalling) were discovered [2–4]. RGS proteins accelerate intrinsic GTPase activity on the heterotrimeric G-protein α subunit (GAP, GTPase-activating protein) and, thus, negatively regulate G-protein-mediated cell signalling. To date, more than 20 subtypes of mammalian RGS proteins have been identified. The different subtypes vary in their molecular structure, tissue distribution and intracellular localization and, thus, may play divergent functional roles in different tissues although they share the conserved ‘RGS domain’ of approx. 120 amino acids, which is responsible for their GAP activity. The problem is that if RGS proteins were unrestrictedly active, they would completely suppress G-protein-mediated cell signalling [2]. Therefore it is important to understand how the actions of RGS proteins are controlled under various physiological conditions.

G-protein-gated K^+ (K_G) channels are directly activated by the $\beta\gamma$ subunits released from pertussis toxin-sensitive G-proteins [5–7]. They contribute to neurotransmitter-induced deceleration of heart beat, formation of slow inhibitory post-synaptic potentials in neurons and inhibition of hormone release in endocrine cells. In cardiac myocytes, hyperpolarization under voltage clamp results

in a fast as well as a slow increase in the K_G channel current. The instantaneous increase in current is due to the release of channel blockade by Mg^{2+} and polyamines, which are common to many inwardly rectifying K^+ channels. The second, slower increase in current is known as ‘relaxation’ and is characteristic of native K_G channels. We have shown that RGS proteins are responsible for this relaxation behaviour of K_G channels [8–11]. In the resting state, the phospholipid, $\text{PtdIns}(3,4,5)\text{P}_3$ (phosphatidylinositol 3,4,5-trisphosphate), binds to RGS proteins and inhibits their action. On depolarization, Ca^{2+} flows into the cell across the plasma membrane and complexes with CaM (calmodulin). The Ca^{2+} /CaM complex recovers the function of RGS proteins by removing $\text{PtdIns}(3,4,5)\text{P}_3$ -mediated inhibition [10–14]. This is the mechanism underlying the characteristic temporal ‘relaxation’ of native K_G channel current. The mechanism of the reciprocal control of RGS activity by $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM is however still unknown.

The objective of the present study was to perform a detailed analysis of the interactions between an RGS protein (RGS4) and phospholipids. Lipid–protein co-sedimentation revealed specific binding of $\text{PtdIns}(3,4,5)\text{P}_3$, but not other phosphoinositides, to RGS4. This binding was weakened by Ca^{2+} /CaM. Quantitative analysis suggested that $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM competitively bind to the RGS domain. A pair of positively charged residues within the RGS domain ($\text{Lys}^{99}/\text{Lys}^{100}$ in RGS4) may be involved in this competitive binding. Since these residues are conserved in certain types of RGS proteins, their reciprocal control by $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM may be important in the physiological regulation of the G-protein cycle.

Abbreviations used: ACh, acetylcholine; CBB, Coomassie Brilliant Blue; CaM, calmodulin; diC16, dihexadecanoyl; GAP, GTPase-activating protein; GST, glutathione S-transferase; K_G , G-protein-gated K^+ ; Kir, inward rectifier K^+ ; PC, $\text{L}-\alpha$ -phosphatidylcholine; PE, $\text{L}-\alpha$ -phosphatidylethanolamine; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; $\text{PtdIns}(3,4,5)\text{P}_3$, phosphatidylinositol 3,4,5-trisphosphate; $\text{PtdIns}(5)\text{P}$, phosphatidylinositol 5-phosphate; RGS, regulators of G-protein signalling; RU, resonance unit; SPR, surface plasmon resonance for brevity, the single-letter system for amino acids has been used, e.g. K^{99} means Lys^{99} .

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MATERIALS AND METHODS

Reagents

Bovine brain CaM and CaM covalently attached to agarose beads (CaM-agarose), neomycin sulphate, PC (L- α -phosphatidylcholine) and PE (L- α -phosphatidylethanolamine) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). diC16 (dihexadecanoyl)-PtdIns(3,4,5) P_3 , diC16-PtdIns(4,5) P_2 (phosphatidylinositol 4,5-bisphosphate), diC16-PtdIns(3,4) P_2 (phosphatidylinositol 3,4-bisphosphate), diC16-PtdIns(5) P (phosphatidylinositol 5-phosphate), diC16-PtdIns(3) P (phosphatidylinositol 3-phosphate) and diC16-PtdIns were purchased from Echelon Research Laboratories (Salt Lake City, UT, U.S.A.).

Generation of liposomes

Phospholipids were dissolved in a mixture of chloroform/methanol (2:1, v/v). Soluble lipids were evaporated under a stream of nitrogen gas, and resuspended by vortexing in a sonication buffer containing (in mM) 150 NaCl, 10 Hepes/NaOH (pH 7.4) and EGTA or CaCl₂. The free Ca²⁺ concentration of the buffer was adjusted to approx. 10 μ M or less than 0.5 nM with CaCl₂ and EGTA respectively. Small unilamellar liposomes were generated by sonication of the lipid suspension in a water bath at room temperature (25 °C) for 20 min.

Expression and purification of RGS4 proteins

GST (glutathione S-transferase) fusion constructs of RGS4 were prepared by PCR tagging of RGS4 cDNA (kindly provided by Dr C. Doupnik, University of South Florida, FL, U.S.A.) with *Bam*HI and *Eco*RI sites at the 5'- and 3'-end respectively. These were subcloned into the pGEX-2T vector (Amersham Biosciences, Uppsala, Sweden). GST-RGS4 (wild-type and mutants) and GST were expressed in *Escherichia coli* and purified from the cell lysates through a glutathione-Sepharose column (Amersham Biosciences). Truncated mutants of RGS4 were produced by PCR to incorporate start and stop codons. The point mutations for K99/K100 and K112/K113 (where K is the single-letter code for lysine) in RGS4 cDNA were introduced by mutagenic oligonucleotide primers using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.). The sequences were verified by ABI Dye terminator cycle sequencing with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Lipid-protein co-sedimentation assay and immunoblot analysis

Lipid-protein co-sedimentation assays were performed as described previously [15]. Briefly, liposomes (PE/PC = 4:1; total 100 μ g) containing the indicated ratio (wt %) of phosphoinositides were mixed with 5 μ g of GST-RGS4 (in 100 μ l; final concentration ~1 μ M) and incubated for 30 min at 4 °C. In some experiments, CaM was added to the reaction mixture. Then the mixture was ultracentrifuged at 100 000 g for 1 h. Proteins in the supernatant (s) and the precipitate (p) were separated by SDS/PAGE and transferred on to PVDF membranes. The PVDF membranes were incubated with anti-GST polyclonal antibodies (Amersham Biosciences) diluted to 1:2000 in 'buffer A' [5% (w/v) skimmed milk and 0.2% (w/v) Lubrol PX in 50 mM Tris/HCl (pH 8.0) and 80 mM NaCl]. After washing three times with buffer A for 10 min each, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Wako Pure Chemicals, Osaka, Japan) diluted to 1:2000 (v/v) in buffer A for 2 h at room temperature, followed by three washes with 2% Lubrol PX and 0.2% (w/v) SDS in 50 mM Tris/HCl

(pH 8.0) and 150 mM NaCl. Immunoreactive bands were developed with the SuperSignal chemiluminescence kit (Pierce, Rockford, IL, U.S.A.), and densitometrically analysed using NIH Image version 1.62 software (NIH, Bethesda, MD, U.S.A.).

Ca²⁺/CaM binding assay

Binding assays of GST-RGS4 and CaM-agarose were performed as described previously [10]. In brief, purified GST-RGS4 proteins (wild-type and mutants) were incubated with CaM-agarose beads (20 μ g of CaM) at 4 °C for 1 h in a binding buffer (in mM: 150 NaCl, 1 dithiothreitol, 10 Hepes/NaOH, pH 7.4, EGTA and CaCl₂). EGTA and CaCl₂ were added to the buffer to adjust its free Ca²⁺ concentration to approx. 10 μ M or less than 0.5 nM. The beads were extensively washed and incubated with SDS/PAGE loading buffer for 5 min at 95 °C. The bound protein was separated by SDS/PAGE and visualized by CBB (Coomassie Brilliant Blue) R-250 staining.

G α subunit-binding assay

G α_2 was purified from bovine brain as described previously [16]. G α_2 (5 μ g) was incubated with GST-RGS4 (wild-type and mutants) proteins immobilized on glutathione-Sepharose beads (Amersham Biosciences) for 3 h at 4 °C, in a binding buffer (in mM: 100 NaCl, 1 MgCl₂, 1 dithiothreitol, 50 Tris/HCl, pH 8.0). The binding buffer contained either 10 μ M GDP (sodium salt; Sigma-Aldrich), 10 μ M guanosine 5'-O-(3-thiophosphate) (GTP γ S, lithium salt; Boehringer Mannheim, Mannheim, Germany), or 10 μ M GDP with AlF₄⁻. When AlF₄⁻ was applied, a cocktail of 100 μ M AlCl₃ (Sigma-Aldrich) and 10 mM NaF (Sigma-Aldrich) was added to the solution. The beads were extensively washed and incubated with SDS/PAGE loading buffer for 5 min at 95 °C. The bound protein was separated by SDS/PAGE, and visualized by CBB staining or transferred on to PVDF membranes. The membranes were immunoblotted with monoclonal antibody specific for G α_2 (Clone AS/7; NEN Life Science Products, Boston, MA, U.S.A.) or with anti-GST polyclonal antibodies.

CD measurements

CD spectroscopy measurements were performed as reported previously [17]. Far-UV CD spectra of the GST-RGS4 mutants were measured with a Jasco J-720WI spectropolarimeter at 20 °C, controlled with a Peltier thermostat (Jasco PTC-348WI). The buffer used was 20 mM Hepes/NaOH (pH 7.3) containing 150 mM NaCl. Far UV spectra were obtained at a protein concentration of approx. 0.15 mg/ml using a 2 mm quartz cell. The CD spectra shown are averages of four measurements. The molar absorption coefficients of the mutants were calculated from amino acid sequences by the method of Gill and von Hippel [18].

SPR (surface plasmon resonance)

Surface plasmon resonance measurements were performed using BIACORE X instrument (Biacore, Uppsala, Sweden) at 25 °C. Purified bovine CaM (Sigma-Aldrich) was immobilized on the surface of the carboxymethylated dextran chip (CM5) using standard carbodi-imide chemistry according to the manufacturer's instructions. The mean amount of immobilized CaM protein was 1000 RU (resonance units). GST-RGS4 (2.5 μ M) dissolved in a buffer (in mM: 150 NaCl, 0.01 CaCl₂, 10 Hepes/NaOH, pH 7.4), was passed over the chip surface at a flow rate of 20 μ l/min. The results were fitted and analysed by BIA evaluation 3.0 software (Biacore). The chip was regenerated after each binding experiment by the injection of 100 mM EGTA.

Figure 1 RGS4 specifically binds PtdIns(3,4,5)P₃

(A) Lipid–protein co-sedimentation assay. Purified GST–RGS4 protein (5 μg /final 1 μM) was incubated with PC/PE liposomes containing the indicated percentage (wt %) of PtdIns(3,4,5)P₃. On ultracentrifugation, supernatant (s) and precipitant (p) fractions were separated by SDS/PAGE, transferred on to a PVDF membrane, and immunoblotted with anti-GST antibodies. Increasing amounts of PtdIns(3,4,5)P₃ in PC/PE lipid vesicles recruited RGS4 into the precipitate (p) fraction from the supernatant (s) fraction, which suggests that RGS4 binds to PtdIns(3,4,5)P₃ (Aa). The binding is inhibited by the presence of neomycin (10 mM) (Ab). (B) GST–RGS4 shows little binding of PC/PE vesicles containing other phosphoinositides, such as PtdIns(4,5)P₂ (Ba), PtdIns(3,4)P₂ (Bb) or PtdIns(5)P (Bc). Arrowheads (A, B) show the position of GST–RGS4. (C) Relative amount of bound GST–RGS4 protein (= precipitant fraction/total fraction, designated as [B]) was determined by densitometry in lipid vesicles containing 0.5 % of each phosphoinositide. Results are shown as mean values obtained from five independent experiments, and error bars (C) represent $\pm$ S.E.M.

Electrophysiology in *Xenopus* oocytes

Electrophysiological assays using *Xenopus* oocytes were performed as described previously [8,9]. All animal work was in accordance with the guidelines for the use of laboratory animals of the Osaka University Graduate School of Medicine. The frogs were deeply anaesthetized by immersion in water containing 0.35 % tricaine (Sigma–Aldrich) and oocytes were surgically removed under clean conditions. After the operation, the frogs were returned to fresh water to allow them to recover from anaesthesia. Adequate time for healing was allowed between each procedure. The frogs were humanely killed after the final collection of oocytes. The constructs of Kir3.1 (inward rectifier K⁺ channel 3.1), Kir3.4, m₂-muscarinic receptor and RGS4 (wild-type and mutants) were transcribed *in vitro* with T7 RNA polymerase and an mRNA capping kit (Stratagene, La Jolla, CA, U.S.A.). cDNAs of rat Kir3.4 and porcine m₂-receptor were kindly provided by Dr D. Clapham (Harvard University, Boston, MA, U.S.A.) and Dr T. Kubo (Showa Pharmaceutical University, Tokyo, Japan) respectively. A mixture of 160 ng of RGS4, 80 ng of m₂-receptor and 8 ng each of Kir3.1 and Kir3.4 cRNAs was injected into the oocytes which had been defolliculated in 1 mg/ml collagenase solution (Wako Pure Chemicals). After injection, the oocytes were maintained in modified Barth's solution for 72–96 h at 18 °C and assayed with the two-electrode voltage clamp method using a commercially available amplifier (CEZ-1250; Nihon Kodon, Tokyo, Japan). Pipettes were filled with 3 M KCl. The bath solution contained (in mM): 90 KCl, 1 CaCl₂, 3 MgCl₂, 0.15 niflumic acid, 5 Hepes/KOH, pH 7.4). ACh (acetylcholine)-induced K_G channel currents were obtained by subtracting the control currents recorded during voltage steps from those in the presence of ACh.

Statistics

All results are means obtained from *n* experiments and error bars represent S.E.M. Statistical differences were evaluated by unpaired Student's *t*-tests. Statistical probability of *P* < 0.05 was considered a significant difference.

RESULTS

Specific interaction between RGS4 and PtdIns(3,4,5)P₃

To examine the interaction between RGS4 and PtdIns(3,4,5)P₃ quantitatively, we used a protein–lipid co-sedimentation assay (Figure 1Aa). In this assay, proteins were incubated with lipid vesicles (PC/PE = 1/4) containing a given quantity of PtdIns(3,4,5)P₃. After ultracentrifugation, lipid vesicles and bound protein were co-precipitated. As the percentage of PtdIns(3,4,5)P₃ in lipid vesicles was increased, the amount of co-sedimented GST–RGS4 protein increased (Figures 1Aa and 1C). This suggests that GST–RGS4 binds to PtdIns(3,4,5)P₃ but not to PC or PE. The carrier protein GST did not bind to PtdIns(3,4,5)P₃ even at the highest PtdIns(3,4,5)P₃ concentration (results not shown).

Neomycin, an antibiotic that firmly binds to the polar head of phosphoinositides [19,20], decreased the amount of RGS4 protein co-sedimented with the lipid vesicle (Figures 1Ab and 1C) significantly. This suggests that neomycin inhibits the interaction between RGS4 and PtdIns(3,4,5)P₃. PtdIns(4,5)P₂, PtdIns(3,4)P₂ and PtdIns(5)P, which possess fewer positive charges than PtdIns(3,4,5)P₃, exhibited little binding capacity for RGS4 (Figures 1B and 1C). These results suggest that the negative charge in the acidic structure of PtdIns(3,4,5)P₃ is necessary for electrostatic interaction with the RGS4 protein.

Taken together, our findings support specific binding of RGS4 protein to PtdIns(3,4,5)P₃, but not to other phosphoinositides. Since the proteins were incubated with lipid vesicles containing given concentrations [0.1–0.5 % (w/w)] of PtdIns(3,4,5)P₃ during the co-sedimentation assay, the observed binding was more specific and more physiological than previous studies using protein–lipid overlay assay, where all phosphoinositides had been shown to bind to RGS4 [11].

Competitive binding of PtdIns(3,4,5)P₃ and Ca^{2+} /CaM to RGS4

Ca^{2+} /CaM has been shown to bind to RGS4 and to reverse its inhibition by PtdIns(3,4,5)P₃ [10,11]. We tested the effect of Ca^{2+} /CaM on the interaction between RGS4 and PtdIns(3,4,5)P₃. In

Figure 2 RGS4–PtdIns(3,4,5)P₃ interaction is competitively inhibited by Ca²⁺/CaM

(A) GST–RGS4 (5 μg/final 1 μM) was co-incubated with PC/PE liposomes containing indicated percentage (wt %) of PtdIns(3,4,5)P₃, and CaM (20 μg/final 10 μM) in the presence (b, d) or absence (a, c) of 10 μM free Ca²⁺. These data demonstrate concentration-dependent specific binding between GST–RGS4 and PtdIns(3,4,5)P₃ in the absence of Ca²⁺ (Aa), which is abrogated in the presence of Ca²⁺ and CaM (Ab). CaM is present in supernatant fractions, irrespective of the presence (Ad) or absence (Ac) of Ca²⁺, suggesting that CaM does not complex with PtdIns(3,4,5)P₃ or RGS4–PtdIns(3,4,5)P₃. Arrowheads mark the position of GST–RGS4 (a, b) and CaM (c, d). (B) Concentration-dependent inhibitory effects of CaM. Precipitants of co-sedimentation assays in the presence of indicated concentrations of PtdIns(3,4,5)P₃ (wt %) were immunoblotted with anti-GST antibodies (Ba). CaM was increased from 1 up to 10 μM. [B] versus [PtdIns(3,4,5)P₃] plot (Bb) and Eadie–Hofstee plot ([B] versus [B]/[PtdIns(3,4,5)P₃] plot) (Bc) are shown in the presence of 0 μM (●), 1 μM (○), 3 μM (■) and 10 μM (□) of CaM. Results are shown as mean values obtained from five ([PtdIns(3,4,5)P₃] = 0 μM and 0.2 μM), three ([PtdIns(3,4,5)P₃] = 0.5 μM) and two ([PtdIns(3,4,5)P₃] = 1.0 μM) independent experiments. Error bars represent S.E.M. The [B] versus [PtdIns(3,4,5)P₃] plot was fitted by the following Michaelis–Menten equation with the least-squares method:

$$[B] = \frac{V_{\max} \times [PtdIns(3,4,5)P_3]}{K_m + [PtdIns(3,4,5)P_3]}$$

where $V_{\max}$ and K_m are 1.26 and 0.14 ([CaM] = 0 μM), 1.27 and 0.23 ([CaM] = 1 μM), 1.21 and 0.33 ([CaM] = 3 μM), and 0.88 and 0.44 ([CaM] = 10 μM), respectively. The regression lines of the Eadie–Hofstee plot (with 0–3 μM of CaM) intersect at almost the same point on the y-axis, indicating a competitive block pattern (Bc). Dixon plot, i.e. $[B]^{-1}$ versus [CaM], in the presence of PC/PE vesicles containing 0.1% (▽), 0.2% (▼) and 0.5% (★) of [PtdIns(3,4,5)P₃] (Bd). Using the intercepts of these plots, the K_i value for CaM inhibition was estimated as approx. 2.8×10^{-6} M. Results are shown as mean values obtained from five ([PtdIns(3,4,5)P₃] = 0 and 0.2 μM) or three ([PtdIns(3,4,5)P₃] = 0.5 μM) independent experiments. Error bar in each symbol represents ± S.E.M.

a co-sedimentation assay, GST–RGS4, PtdIns(3,4,5)P₃ and CaM were co-incubated in the presence or absence of Ca²⁺ (Figure 2A). In the absence of Ca²⁺, GST–RGS4 bound to PtdIns(3,4,5)P₃ (Figure 2Aa). The binding was significantly less in the presence of Ca²⁺ (Figure 2Ab). Since all CaM remained in the supernatant (Figures 2Ac and 2Ad), neither CaM–PtdIns(3,4,5)P₃ nor CaM–

RGS4–PtdIns(3,4,5)P₃ complexes could have formed. On the other hand, CaM binds to GST–RGS4 in the presence of Ca²⁺ ([10,12] and the present study, Figure 2Ac) and the binding of Ca²⁺/CaM to RGS4 in the supernatant may have prevented the binding of RGS4 to PtdIns(3,4,5)P₃ in the lipid precipitant (Figure 2Ab).

Figure 3 SPR measurement of the interaction between CaM and RGS4

SPR measurements of binding of GST-RGS4 proteins ($2.5 \mu\text{M}$) to CaM in the presence (broken line) or absence (continuous line) of $\text{PtdIns}(3,4,5)\text{P}_3$ ($5 \mu\text{M}$). A 1000 RU increase in the sensorgram is approx. equivalent to 1.2 ng of protein binding to the sensor chip. CaM was immobilized with the sensor chip (~ 1000 RU) and GST-RGS4 ($2.5 \mu\text{M}$) dissolved in a buffer (in mM: 150 NaCl, 0.01 CaCl_2 , 10 HEPES/NaOH, pH 7.4) was passed over the chip surface at a flow rate of $20 \mu\text{l}/\text{min}$, followed by washing with the same buffer. This result is representative of five independent experiments.

We also analysed the effect of Ca^{2+} /CaM on the binding between RGS4 and $\text{PtdIns}(3,4,5)\text{P}_3$ in a quantitative manner (Figure 2B). GST-RGS4 and lipid vesicles were co-incubated with the indicated doses of CaM in the presence of Ca^{2+} . The percentage of $\text{PtdIns}(3,4,5)\text{P}_3$ in PC/PE lipid vesicles was denoted as $[\text{PtdIns}(3,4,5)\text{P}_3]$. As the concentration of CaM was increased, the $\text{PtdIns}(3,4,5)\text{P}_3$ -bound fraction of GST-RGS4 protein ($[B]$) decreased (Figures 2Ba and 2Bb). An Eadie-Hofstee plot according to the Michaelis-Menten enzyme reaction is shown in Figure 2(Bc), where $[B]$ is plotted versus $[B]/[\text{PtdIns}(3,4,5)\text{P}_3]$. Linear regression showed that the relationship for 0, 1 and $3 \mu\text{M}$ CaM intersected at the same point on the y-axis, strongly suggesting that CaM is a competitive inhibitor of $\text{PtdIns}(3,4,5)\text{P}_3$ binding to RGS4 at these concentrations (Figure 2Bc). The estimated K_i value of CaM from a Dixon plot [21] was approx. 2.8×10^{-6} M (Figure 2Bd). At higher concentrations of CaM, however, the inhibitory mechanism appeared to be a mixture of competitive and non-competitive interactions.

That there was a competitive interaction between $\text{PtdIns}(3,4,5)\text{P}_3$ and CaM for RGS4 was confirmed with SPR analysis (Figure 3). In this experiment, we examined the inhibitory effects of $\text{PtdIns}(3,4,5)\text{P}_3$ on the binding between RGS4 and Ca^{2+} /CaM. CaM was first immobilized on to the sensor chip. The amount of immobilized CaM in each experiment was kept at approx. 1000 RU and GST-RGS4 diluted in a binding buffer (containing Ca^{2+}) was applied in the presence or absence of $\text{PtdIns}(3,4,5)\text{P}_3$ ($5 \mu\text{M}$), and then the sensor chip was washed with the binding buffer. If proteins bound to the sensor chip, the resonance unit would increase. Under controlled conditions, GST-RGS4 slowly bound to CaM, and could barely be washed off with the binding buffer (Figure 3, continuous line). On the other hand, in the presence of $\text{PtdIns}(3,4,5)\text{P}_3$, the binding of GST-RGS4 to CaM was weakened and easily washed off (broken line). These results clearly suggest that $\text{PtdIns}(3,4,5)\text{P}_3$ weakens the binding between RGS4 and CaM.

The results shown in Figures 2 and 3 suggest that CaM and $\text{PtdIns}(3,4,5)\text{P}_3$ bind to RGS4 in a competitive manner. Since both CaM and $\text{PtdIns}(3,4,5)\text{P}_3$ are reported to target positively charged residues [22–24], CaM and $\text{PtdIns}(3,4,5)\text{P}_3$ might bind to the same portion of the RGS4 protein.

Crucial residues of RGS4 involved in the competitive binding of $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM

Our next step was to identify the RGS4 binding site for $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM. We first examined whether the RGS domain of RGS4 (residues 51–177) would bind to $\text{PtdIns}(3,4,5)\text{P}_3$ using co-sedimentation. The RGS domain of RGS4 bound to $\text{PtdIns}(3,4,5)\text{P}_3$ with almost the same capacity as full-length RGS4 and binding was weakened by Ca^{2+} /CaM. These results suggest that the binding of $\text{PtdIns}(3,4,5)\text{P}_3$ to RGS4 and its competitive blocking by Ca^{2+} /CaM occur within the RGS domain (results not shown).

Next, we tried to identify residues within the RGS domain responsible for the interaction with $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM. Since $\text{PtdIns}(3,4,5)\text{P}_3$ binding of RGS4 was weakened with neomycin (Figure 1) we tested whether $\text{PtdIns}(3,4,5)\text{P}_3$ uses electrostatic interaction with positively charged residues [19,20]. Two pairs of positive charges are found within the RGS domain of RGS4, K99/K100 and K112/K113 (Figure 4A). According to the crystal structure of the protein [25], K99 and K100 lie in the C-terminal portion of helix 4, whereas K112 and K113 are located in helix 5 (Figure 4B). We examined the effect of charge displacement by replacing lysine (K) with glutamate (E).

Using CD spectroscopy measurements, we first examined whether these mutant proteins were properly folded (Figure 5). The far-UV CD spectra of GST and GST-RGS4 were characteristic of an α -helix protein, consistent with the predominant α -helical structure shown by X-ray crystallographic analysis (Figure 4B) [25,26]. Far-UV CD spectra of each mutant GST-RGS4 were similar to those of wild-type GST-RGS4, suggesting that the two mutations did not significantly alter the folding structure of RGS4 protein.

Whereas the K112E (Lys¹¹² $\rightarrow$ Glu)/K113E mutation partially decreased the binding of $\text{PtdIns}(3,4,5)\text{P}_3$ to the RGS domain (Figure 6Ab) as reported previously [12], the K99E/K100E mutations completely abolished the interaction between the RGS domain and $\text{PtdIns}(3,4,5)\text{P}_3$ (Figure 6Aa). The K99E/K100E mutation also abolished the binding of Ca^{2+} /CaM to the RGS domain, whereas K112E/K113E had only a marginal effect (Figure 6B). Furthermore, we found that the K99E/K100E mutation greatly impaired its binding of $G_{i\alpha_2}$, whereas the K112E/K113E mutant could bind $G_{i\alpha_2}$ as potentially as the wild-type RGS4 domain (Figure 6C). The charge neutralizing mutation, K99Q/K100Q, of RGS4 also impaired its binding to both $\text{PtdIns}(3,4,5)\text{P}_3$ and the G_{α} subunit (results not shown). Since the binding of the RGS domain to G_{α} subunit is required for the GAP action of the RGS proteins [2–4], the K99E/K100E mutant possesses little GAP activity. We, therefore, could not determine the effect of $\text{PtdIns}(3,4,5)\text{P}_3$ - and Ca^{2+} /CaM-binding to the K99E/K100E mutant on the regulation of GAP activity. Nevertheless, considering that there are only two positive charge clusters within the RGS domain, i.e. K99/K100 and K112/K113, and that the K112E/K113E mutation has only a limited effect on the binding of $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM, it is conceivable that K99/K100 is crucial for $\text{PtdIns}(3,4,5)\text{P}_3$ and Ca^{2+} /CaM interaction.

Co-expression of RGS protein is a prerequisite for reconstituting the agonist concentration-dependent relaxation behaviour of the K_G current in *Xenopus* oocytes [8,9] (Figure 7a). It appears as if this specific property is a reflection of the reciprocal control of RGS action by inhibitory $\text{PtdIns}(3,4,5)\text{P}_3$ and stimulatory Ca^{2+} /CaM (see the Introduction section). In the presence of wild-type RGS4, on sudden hyperpolarization (from +40 to -100 mV), K_G channel current increases first instantaneously to one level (I_{ins}) and then slowly reaches a steady state (I_{max}) (Figure 7a). The slow increase, referred to as 'relaxation', is a unique

Figure 4 Candidates for the interaction site between $\text{PtdIns}(3,4,5)P_3$ and CaM within the RGS domain

Positive charge clusters within the RGS domain. (A) Alignment of RGS family protein sequences. RGS proteins can be grouped into five subfamilies, i.e. RZ, R4, R7, R12 and RA [2]. The more N-terminal cluster of positive charge (boxed in blue) is more conserved than the more C-terminal cluster (boxed in red). (B) Crystal structure of the RGS4 RGS domain (PDB no. 1AGR, [23]). The blue ribbon represents the core structure. Carbon, nitrogen and oxygen atoms are denoted by green, blue and red balls respectively. Positively charged residues are highlighted by a Corey–Pouling–Keltun space-filling model.

characteristic of the K_G current and reflects a time-dependent recovery from the decrease in available K_G channel numbers associated with depolarization. The relaxation behaviour depends on the agonist concentration; it is more prominent when the K_G current is activated by low concentrations of agonist (10^{-8} M ACh) compared with high concentrations (10^{-6} M) (Figures 7a and 7e). This behaviour could be observed in oocytes expressing K112E/K113E mutant RGS4 (Figures 7c and 7e) but not in oocytes expressing the K99E/K100E mutant (Figures 7b and 7e). The expression levels of wild-type and two mutant RGS4

Figure 5 Far-UV CD spectra of wild-type and mutant GST-RGS4

CD measurements of GST and GST-RGS4 (wild-type) proteins (A), and wild-type and mutant (K99E/K100E and K112E/K113E) GST-RGS4 proteins (B). The absorption coefficients of the mutants were calculated from amino acid sequences by the Gill and von Hippel method [16].

proteins have been confirmed to be comparable (results not shown). These results confirm those in Figure 6(C), suggesting that K99E/K100E can associate neither with $\text{PtdIns}(3,4,5)P_3$ nor with the $G\alpha$ subunit.

DISCUSSION

In the present study, we have revealed that $\text{PtdIns}(3,4,5)P_3$ and the Ca^{2+} /CaM complex competitively bind to the RGS4 protein in a manner which depends on the RGS domain. In a previous study [11], we showed that purified RGS4 protein, when added to inside-out patches of cardiac myocyte membrane, inhibited K_G channel activity due to its GAP action. This inhibitory effect of RGS protein was abolished by the addition of $\text{PtdIns}(3,4,5)P_3$, and it was completely restored when Ca^{2+} /CaM was subsequently applied. We now show that prevention of $\text{PtdIns}(3,4,5)P_3$ -mediated inhibition of a RGS by Ca^{2+} /CaM is attributable to the displacement of $\text{PtdIns}(3,4,5)P_3$ from the RGS domain by the binding of Ca^{2+} /CaM. The inhibitory effect of RGS on K_G channel activity in the presence of both $\text{PtdIns}(3,4,5)P_3$ and Ca^{2+} /CaM is identical with that observed in the presence of RGS alone (Figure 2 in [11]). The binding of $\text{PtdIns}(3,4,5)P_3$ may, therefore, cause a conformational change of the RGS domain to inhibit its GAP activity. The exchange of $\text{PtdIns}(3,4,5)P_3$ for Ca^{2+} /CaM may restore the conformation of the RGS domain to its $\text{PtdIns}(3,4,5)P_3$ -free state and thus recover its GAP activity.

Figure 6 Analyses of K99E/K100E and K112E/K113E mutants of the RGS4-RGS domain

(A) Mutant (K99E/K100E and K112E/K113E) GST-RGS4 proteins were incubated with PC/PE liposomes containing indicated amounts (wt %) of PtdIns(3,4,5) P_3 . Following ultracentrifugation, supernatant (s) and precipitated (p) fractions were separated by SDS/PAGE, transferred on to a PVDF membrane and immunoblotted with anti-GST antibodies. The K99E/K100E mutant lost its ability to bind PtdIns(3,4,5) P_3 (Aa), whereas K112E/K113E retained its interaction with PtdIns(3,4,5) P_3 (Ab). (B) Wild-type and mutant (K99E/K100E and K112E/K113E) GST-RGS4 proteins were incubated with beads to which CaM was covalently attached (CaM-agarose) in the presence (10 μM) or absence of Ca^{2+} (less than 0.5 nM). Wild-type and K112E/K113E mutant proteins bound CaM-agarose in a Ca^{2+} -dependent manner, but K99E/K100E showed markedly weakened binding capacity. (C) Wild-type (WT) and mutant (K99E/K100E and K112E/K113E) GST-RGS4 proteins (~45 kDa) immobilized by glutathione-Sepharose were incubated with purified bovine $G_{i\alpha_2}$ subunit (~40 kDa) in the presence of 10 μM GDP, 10 μM GTP γ S or 10 μM GDP-AlF $_4^-$. CBB staining of the SDS gel (Ca), immunoblot with anti-GST-RGS4 (Cb) and with anti- $G_{i\alpha_2}$ (Cc) are shown. Wild-type and K112E/K113E mutant bound purified bovine $G_{i\alpha_2}$ subunit in the presence of GDP-AlF $_4^-$, a prerequisite for the GAP activity of RGS proteins. The K99E/K100E mutation showed no $G_{i\alpha}$ binding.

Two clusters of positive charges in RGS4, i.e. K99/K100 and K112/K113, are candidates for the binding sites of PtdIns(3,4,5) P_3 and CaM within the RGS domain. Replacement of the positive charges with negative ones at the latter site (K112E/K113E) had no effect on binding or RGS4-induced K_G current relaxation. The same changes in the first lysine cluster (K99E/K100E) impaired RGS domain binding of PtdIns(3,4,5) P_3 /CaM and also of $G_{i\alpha}$ subunits. Therefore it seems reasonable to assume that K99/K100 is involved in the competitive binding of PtdIns(3,4,5) P_3 and CaM. The functional importance of the K99/K100 site in the RGS domain is further supported by the fact that this position is also responsible for the interaction between Axin-RGS and the third Ser-Ala-Met-Pro repeat of adenomatous polyposis coli protein [27]. Since the K99 and K100 of RGS4 are well conserved in different RGS proteins, the reciprocal regulation of RGS by PtdIns(3,4,5) P_3 and CaM shown in the present study might be important for the physiological control of different RGS subtypes. Recent reports show other regulatory mechanisms of RGS, such as the interaction with 14-3-3 [28,29] and subcellular translocation

[30,31]. Thus, different RGS proteins may possess distinct regulatory mechanisms. Further studies are necessary to clarify which types or families of RGS proteins are physiologically regulated by the reciprocal interaction between PtdIns(3,4,5) P_3 and Ca^{2+} /CaM on the conserved dilysine.

Various kinds of phospholipid binding proteins have been identified and almost all of them possess particular binding modules for phospholipids, and these include the pleckstrin homology domain [32], the FYVE (Fab1p, YOTB, Vac1p and EEA1) domain [33] and the PX domain [34]. These modules determine the specific subcellular localization of the host proteins by binding their respective target phospholipids, which are enriched in certain membrane microdomains. Other proteins without these modules also bind to phospholipids. These phospholipid-binding proteins contain a brief non-specific motif, which includes a cluster of positive charges. This type of phospholipid-protein interaction has been shown to be important in the functional modulation of various target proteins [35]. Examples include PtdIns(4,5) P_2 regulation of ion transporters, such as the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [36]

Figure 7 Effects of the K99E/K100E and K112E/K113E mutations on the ACh concentration-dependent relaxation behaviour of K₆ channel currents reconstituted in *Xenopus* oocytes

K₆ currents were reconstituted by expressing Kir3.1/Kir3.4 and muscarinic (m₂) receptor, in the presence of wild-type (a), K99E/K100E (b) or K112E/K113E (c) RGS4. K₆ current was elicited by 10⁻⁸ M (left) or 10⁻⁶ M (right) ACh, and measured with the voltage clamp methods shown in (d). On stepping the voltage to -100 mV, inward current from +40 mV first changed instantaneously (I_{ins}) and then slowly increased to a steady-state (I_{max}). This slow increase corresponded to 'relaxation'. In each current trace, open arrowheads indicate the zero-current level. Scale bars, 1 s and 1 µA. (e) I_{ins}/I_{max} ratios for currents elicited by either 10⁻⁸ M (black bars) or 10⁻⁶ M (white bars) ACh. *n* = 5 for each condition. Bars indicate means ± S.E.M.; *, *P* < 0.05; n.s., not significant.

and inward rectifier K⁺ (Kir) channels [37–39]. Although some Kir channels such as the ATP-sensitive Kir6.x are regulated by PtdIns(3,4,5)P₃ in addition to PtdIns(4,5)P₂ [40], its physiological significance is still unclear. Therefore the PtdIns(3,4,5)P₃–RGS domain interaction described here may be one of the first examples of physiological regulation by PtdIns(3,4,5)P₃ in a protein lacking specific binding modules.

CaM often activates its target proteins by relieving inhibition, which results from intramolecular domains or exogenous elements. An example of the former is CaM activation of CaM-dependent protein kinase II, where CaM binds to the autoinhibitory domain of CaM-dependent protein kinase II and relocates it [41,42]. An example of the latter is CaM activation of endothelial nitric oxide synthase, where CaM removes calveolin-1, an extrinsic inhibitory molecule, from the target protein [43,44]. However, in this example, the mode of action of CaM has not been fully examined. The reciprocal control of RGS4 by PtdIns(3,4,5)P₃ and CaM shown here indicates for the first time that CaM and inhibitory molecules share the same binding site on their target protein and interact competitively to control its function.

The candidate-binding site on RGS4 (K99/K100) is located in the C-terminal portion of helix 4 of the RGS domain. This is in line with the recently proposed notion that the opposite (back or 'B site') surface of the RGS protein is as equally important as the RGS domain Gα-interacting 'A site' [45]. Structural studies are needed to clarify how the binding of either PtdIns(3,4,5)P₃ or Ca²⁺/CaM at the B site controls the conformation of the RGS domain and thus regulates the function of the A site.

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