RGS4 Inhibits Platelet-Activating Factor Receptor Phosphorylation and Cellular Responses†

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ABSTRACT: To define the role of regulators of G-protein signaling (RGS) in chemoattractant-mediated responses, RGS4 and the receptors for platelet-activating factor (PAFR), formylated peptides (FR), or interleukin-8 (CXCR1) were stably coexpressed in a rat basophilic leukemia (RBL-2H3) cell line. The data demonstrate that RGS4 inhibited responses by PAFR (i.e., phosphoinositide (PI) hydrolysis, Ca^{2+} mobilization) but not by FR or CXCR1. An N-terminal 33 amino acid deletion mutant of RGS4 (ΔRGS4), deficient in GAP (GTPase activating protein) activity and plasma membrane localization, had no effect on either PAFR, FR, or CXCR1. RGS4, but not ΔRGS4, also blocked phosphorylation of PAFR by platelet-activating factor (PAF) and, unexpectedly, by phorbol 12-myristate 13-acetate (PMA); it also blocked cross-phosphorylation by formylmethionylleucylphenylalanine (fMLP). A point mutant of RGS4 (N88S), deficient in GAP activity but not membrane localization, partially blocked PAFR phosphorylation but had no effect on PAFR-mediated PI hydrolysis and Ca^{2+} mobilization. Truncation of the cytoplasmic tail of PAFR (mPAFR) resulted in a loss of its susceptibility to inhibition by RGS4. Taken together, the data indicate that of the receptors studied, RGS4 selectively inhibited responses to PAFR, which preferentially couples to Gq. At the level of expression studied, RGS4 did not inhibit FR or CXCR1 which activates Gi to transduce cellular signals. Since the tail-deleted mutant of PAFR was not affected by RGS4, and RGS4 blocked homologous as well as heterologous phosphorylation of this receptor, it is possible that RGS4 interferes sterically with the cytoplasmic tail of PAFR. Thus, in addition to stimulating the GTPase activity of Gα, RGS4 prevents G protein activation by PAFR and the homologous and heterologous phosphorylation of this receptor.

Chemoattractant receptors couple to heterotrimeric guanine nucleotide binding proteins (G-protein) to mediate the chemotactic and cytotoxic function of leukocytes (*2*). Given the potent biological activities of these wandering cells, their activities are likely to be tightly regulated. Substantial evidence indicates that the duration of receptor signaling controls the ability of leukocytes to generate cytotoxic responses (*1, 3*). Receptor desensitization is one mechanism for the dampening of signal duration and subsequent cellular responses (*4*). Recent studies in neutrophils and transfected cells have demonstrated that inhibition of downstream effector activity also regulates leukocyte functions (*5*). A family of proteins termed regulators of G-protein signaling (RGS) have been shown to modulate the duration of G-protein-mediated signal transduction (*6*−*9*). Upon receptor activation, G-proteins exchange GDP for GTP at the Gα subunit and dissociate into Gα−GTP and G$\beta\gamma$ to activate effectors such as phospholipase C (PLC), phospholipase D (PLD), and ion channels (*1, 10*). RGS activates the GTPase activity (GAP) of the Gα subunit, facilitating its rapid reassociation with G$\beta\gamma$ (*6*−*9*). To date, over 20 members of the RGS family have been identified and characterized for their GAP activity. However, little is known about their mechanism of activation, regulation, or pattern of specificity.

In this work, we sought to determine the role of RGS4 in the regulation of the receptors for PAF (PAFR), fMLP[1] (FR), and interleukin-8 (CXCR1). For that purpose, wild-type RGS4, an N-terminal 33 amino acid deletion mutant, ΔRGS4, and a GAP-deficient mutant of RGS4, N88S, were expressed, along with FR and PAFR, in a rat basophilic leukemia cell line (RBL-2H3). The results presented here demonstrate that RGS4 inhibited responses to PAFR, including PI hydrolysis, Ca^{2+} mobilization, and receptor phosphorylation. RGS4 had no effect on the activation of FR or CXCR1. The data also indicate that the carboxy-terminal tail of PAFR is required for RGS4 modulation of PAFR function and that an additional role of RGS may be to protect receptors against phosphorylation.

MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphate (8500−9120 Ci/mmol) and *myo*-[2-^{3}H]inositol (24.4 Ci/mmol) were purchased from

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[1] Abbreviations: fMLP, formylmethionylleucylphenylalanine; FR, fMLP receptor; IL-8, interleukin-8; CXCR1, IL-8 receptor A; PAF, platelet-activating factor; PAFR, PAF receptor; RGS4, regulator of G-protein signaling 4; G protein, GTP-regulatory protein; Ptx, pertussis toxin; PMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate.

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DuPont NEN. Geneticin (G418) and all tissue culture reagents were purchased from Life Technologies, Inc. Polyclonal antibody against RGS4 was obtained from Santa Cruz Biotechnology. Monoclonal antibody against PAFR was obtained from Cayman Chemical. Monoclonal 12CA5 antibody, protein G-agarose, and protease inhibitors were purchased from Boehringer Manheim. Indo-1 acetoxymethyl ester and pluronic acid were purchased from Molecular Probes. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. All other reagents are from commercial sources.

cDNAs encoding wild-type RGS4, tagged with a green fluorescent protein (GFP) at the carboxy terminus (RGS4−GFP), and an N-terminus deletion (amino acid residues 1−33) mutant of RGS4, ΔRGS4−GFP, were generous gifts from Dr. Kendall J. Blumer. RGS4, a GAP-deficient mutant of RGS4, N88S, and RGS1 were kindly provided by Dr. John Kehrl.

Construction of the Epitope-Tagged (ET) Receptors and RGS. Hemagglutinin (HA) epitope-tagged FR, CXCR1, PAFR, and the cytoplasmic tail deletion mutant of PAFR (mPAFR) were constructed as described previously (*11*−*14*). RGS4−GFP and ΔRGS4−GFP were excised from the yeast expression vector pVT102U as *BamH1-Xho1* fragments and subcloned into the same site in the eukariotic expression vector, pcDNA3. To generate the N88S−GFP construct, N88S was amplified by PCR using the HA−N88S clone, obtained from Dr. John Kehrl as a template, excised with *BamH1-Xba1*, and cloned into the same site in the pVT102U−GFP vector. N88S−GFP was excised from pVT102U as a *BamH1-Xho1* fragment and subcloned into pcDNA3. The integrity of each molecule was verified by sequencing.

Cell Culture and Transfection. RBL-2H3 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/mL) (*11*). RBL-2H3 cells (1×10^7 cells) stably expressing FR (7300 ± 267 receptors/cell) and PAFR (6981 ± 412 receptors/cell) (PFR) or CXCR1 (8532 ± 152 receptors/cell) (*12, 14*) were electroporated in the presence of pcDNA3, containing either GFP, RGS4−GFP, N88S−GFP or ΔRGS4−GFP cDNAs (20 μg), or prCMV containing RGS1−GFP cDNA. Cells were cloned into single cell by flow cytometry (FACS) analysis. The intensity of the GFP expression was used to determine the levels of expression of RGS. GFP-positive and control PFR cells were also monitored by FACS for cell surface expression of the receptors, using specific antibodies against the amino terminus of FR, PAFR, or CXCR1.

Phosphoinositide Hydrolysis and Calcium Measurement. RBL-2H3 cells were subcultured overnight in 96-well culture plates (50 000 cells/well) in an inositol-free medium supplemented with 10% dialyzed fetal bovine serum and 1 μCi/mL [^{3}H]inositol. The generation of inositol phosphates was determined as reported (*11*−*14*). For calcium mobilization, cells (5×10^6) were removed, washed with HEPES-buffered saline, and loaded with 1 μM Indo I-AM in the presence of 1 μM pluronic acid for 30 min at room temperature. The cells were then washed and resuspended in 1.5 mL of buffer. Intracellular calcium increase in the presence and the absence of ligands was measured as described (*11*−*14*).

Phosphorylation of the Epitope-Tagged Receptors. Phosphorylation of ET-receptors was performed as described previously (*11*−*14*). Briefly, RBL-2H3 cells (2.5×10^6) expressing each combination of receptors were subcultured overnight in 60 mm tissue culture dishes. The following day, the cells were rinsed twice with 5 mL of phosphate free Dulbecco's modified Eagle's medium and incubated in the same medium, supplemented with [^{32}P]orthophosphate (150 μCi/dish) for 90 min in order to metabolically label the intracellular ATP pool. Labeled cells were then stimulated with or without agonists (IL-8 100 nM, PAF 100 nM, or fMLP 1 μM) for 5−7 min at 37 °C. The phosphorylated receptors were immunoprecipitated with the 12CA5 antibody, analyzed by SDS-gel electrophoresis (10%), and visualized by autoradiography (*11*−*14*).

RESULTS

Expression and Characterization of RGS in PAFR−FR−RBL−2H3 Cells (PFR−RBL). GFP-tagged RGS4, ΔRGS4, and N88S were expressed in RBL-2H3 cells stably coexpressing FR (7300 ± 267 receptors/cell) and PAFR (6981 ± 412 receptors/cell) (PFR cells) (*14*). GFP-positive cells were cloned into single cells by FACS. The intensity of GFP expression was used to determine and equalize the levels of expression of RGS. Single-cell clones expressing similar levels of RGS4, ΔRGS4, and N88S (GFP intensity $\sim 10^1$) were chosen for this study. RGS4 was not detectable in RBL or PFR expressing cells by Western blot (data not shown).

PAF and fMLP stimulated PI hydrolysis and intracellular Ca^{2+} mobilization in a dose-dependent manner in control PFR cells (Figure 1, panels A−D). The EC_{50} values were 19.14 ± 2 and 0.74 ± 0.3 nM for PI hydrolysis for fMLP and PAF, respectively, and 9.89 ± 1.3 and 0.57 ± 0.18 nM for Ca^{2+} mobilization for fMLP and PAF, respectively. PI hydrolysis and Ca^{2+} mobilization in response to PAF, but not fMLP, was inhibited by ∼50% in cells expressing RGS4 but not ΔRGS4 or N88S (Figure 1A−D). Neither PI hydrolysis to PAF nor that to fMLP was affected in PFR cells expressing GFP alone. In contrast, expression of the native RGS4 inhibited responses to PAF, but not to FMLP (data not shown). This suggests that the effect of RGS4−GFP on PAF responses is due to RGS4 not the GFP-tag.

Pretreatment of PFR, PFR−RGS4, PFR−N88S, and PFR−ΔRGS4 cells with pertussis toxin (Ptx) had no effect on PAF-mediated PI hydrolysis and Ca^{2+} mobilization. However, Ptx pretreatment completely inhibited the ability of fMLP to stimulate these responses (data not shown).

Expression of GFP−RGS1 in PFR cells had no effect on PAFR-mediated Ca^{2+} mobilization (Figure 1F) but inhibited the Ca^{2+} response to FR by ∼45% (Figure 1E). CXCR1 expressing RBL cells were also transfected with GFP-tagged RGS4 and ΔRGS4 (Figure 2). As was the case for FR, intracellular Ca^{2+} mobilization in response to interleukin-8 (IL-8) was not inhibited by RGS4 or ΔRGS4 (Figure 2). Overexpression of RGS4 or RGS1, but not ΔRGS4, in PFR cells (GFP intensity 10^3) inhibited PI hydrolysis (Figure 3) and Ca^{2+} mobilization (data not shown) in response to both PAF and fMLP. Partial inhibition was obtained with N88S (Figure 3).

Effect of RGS4 on FR and PAFR Phosphorylation. Cells expressing RGS4, ΔRGS4, or control cells were ^{32}P labeled

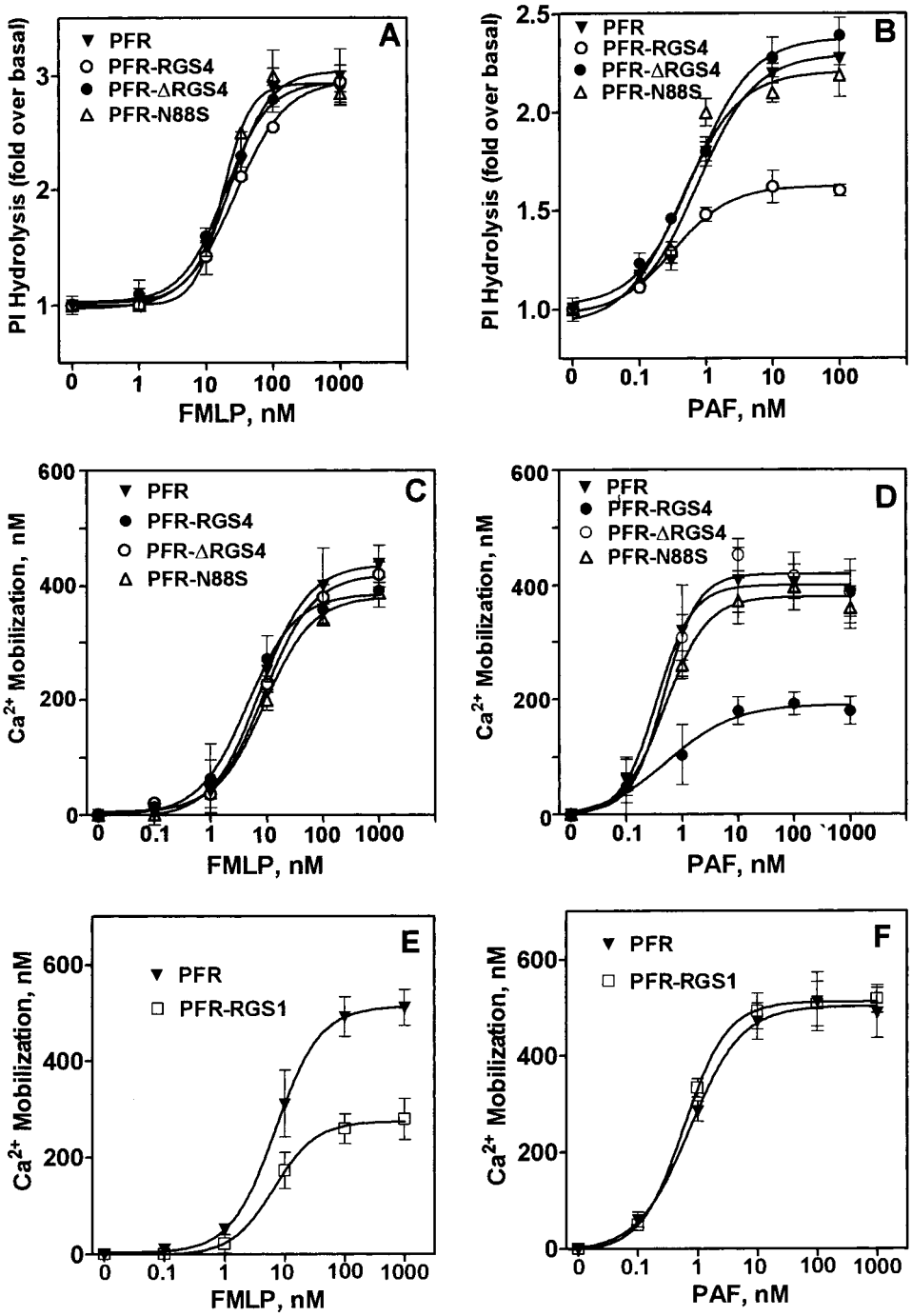

FIGURE 1: Expression and characterization of GFP-tagged RGS4, N88S, ΔRGS4, and RGS1 in RBL-2H3 cells stably expressing FR and PAFR. For phosphoinositide hydrolysis, RBL cells stably expressing FR and PAFR (PFR) and either RGS4−GFP, (PFR−RGS4), ΔRGS4−GFP (PFR−ΔRGS4), or N88S−GFP (PFR−N88S) were cultured overnight in the presence of [^{3}H]inositol (1 μCi/mL). Cells were preincubated (10 min, 37 °C) with a HEPES-buffered saline solution containing 10 mM LiCl in a total volume of 50 μL and stimulated with different concentrations of fMLP (A) or PAF (B) for 10 min. Supernatant was used to determine the release of [^{3}H]IPs. Data are represented as fold of stimulation over basal. The experiment was repeated four times with similar results. For intracellular Ca^{2+} mobilization cells (5×10^6) were loaded with indo-1 and stimulated with different concentrations of fMLP (C) or PAF (D) for 3 min. The experiment was repeated three times with similar results. Dose−response of FMLP (E)- and PAF (F)-mediated intracellular Ca^{2+} mobilization was determined in PFR and PFR cells expressing RGS1−GFP (PFR−RGS1) as described above. The results are from a representative experiment that was repeated three times.

and treated with different ligands (FMLP, 1 μM; PAF, 100 nM; or PMA, 100 nM) and immunoprecipitated with 12CA5 antibody. The identity of the phosphorylated bands for FR (~65 kDa) and PAFR (~45 kDa) was previously demonstrated by immunoprecipitation of iodinated and phosphorylated receptors in the presence and absence of the epitope-tag peptide (*11*, *13*, *14*). FR (~65 kDa) was phosphorylated by FMLP in the four cell lines (Figure 4A, lanes 2). PAFR (~45 kDa) was phosphorylated by PAF and PMA (lanes 3 and 4) and cross-phosphorylated by fMLP (lanes 2) in PFR and PFR−ΔRGS4 cells. Phosphorylation of PAFR by either PAF or PMA (lanes 3 and 4), or cross-phosphorylation by FR (lanes 2), was attenuated in PFR−RGS4 cells. N88S partially inhibited (~60%) PAFR phosphorylation by both PAF and PMA and cross-phosphorylation by FMLP, but had no effect on FR phosphorylation (Figure 4A and B).

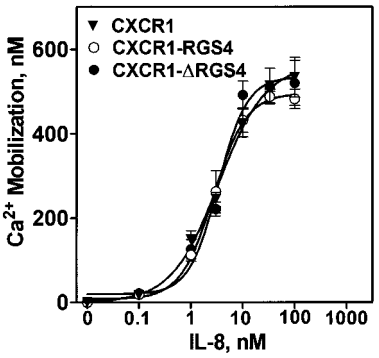

FIGURE 2: Effect of RGS4 and ΔRGS4 in CXCR1-mediated Ca^{2+} mobilization. RBL cells stably expressing CXCR1 and RGS4−GFP (CXCR1−RGS4) or ΔRGS4−GFP (CXCR1−ΔRGS4) were loaded with indo-I and dose−response of IL-8-mediated Ca^{2+} mobilization was measured. The experiment was repeated three times with similar results.

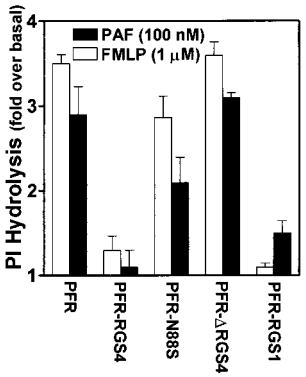

FIGURE 3: Effect of overexpression of RGS4, N88S, ΔRGS4, and RGS1 on FR- and PAFR-mediated phosphoinositide hydrolysis. PFR cells overexpressing RGS4, N88S, ΔRGS4, and RGS1 (100-fold higher than the cells used in Figure 1) were cultured overnight in the presence of [^{3}H]inositol (1 μCi/mL). fMLP- and PAF-mediated phosphoinositide hydrolysis were determined as in the legend of Figure 1. The results are from a representative experiment that was repeated three times.

Phosphorylation of both PAFR and FR was inhibited in PFR cells overexpressing RGS4 (GFP intensity 10^3) (data not shown).

Role of the Cytoplasmic Tail of PAFR on RGS4 Modulating Receptor Function. To assess the role of the C-tail of PAFR on RGS-mediated inhibition of the receptor, RGS4 and ΔRGS4 were expressed in RBL cells stably expressing a carboxyl terminal deletion mutant of PAFR, mPAFR (6211 ± 736 receptors/cell) (*14*). In contrast to PAFR, mPAFR was previously shown to induce greater GTPase activity and PI hydrolysis, sustained Ca^{2+} mobilization, and resistance to receptor phosphorylation and desensitization (*14*). Dose−response of PAF-mediated PI hydrolysis (Figure 5A) was not inhibited in mPAFR cells expressing RGS4 (mPAFR−RGS4, EC_{50} 1.57 ± 0.48 nM) as compared to control cells (mPAFR, EC_{50} 1.63 ± 0.61 nM), cells expressing ΔRGS4 (mPAFR−RGS4, EC_{50} 2.53 ±0.73 nM), or cells expressing N88S (mPAFR−N88S, EC_{50} 2.19 ± 0.41 nM). PAF-mediated Ca^{2+} mobilization to mPAFR was also unaffected in mPAFR−RGS4 cell relative to mPAFR, mPFR−ΔRGS4, and mPAFR−N88S cells (Figure 5B).

DISCUSSION

The data show that RGS4 stably expressed in RBL cells (RGS4−GFP intensity $\sim 10^1$) inhibited PAFR, but not FR- or CXCR1-mediated phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization (Figure 1). The specificity of RGS4 for PAFR was mirrored by the expression of RGS1 in PFR cells which inhibited Ca^{2+} mobilization to FR (~70%) but not PAFR (Figure 1E and F). PAFR expressed in RBL cells couples preferentially to Gq, whereas FR and CXCR1 interact with the Ptx-sensitive Gi to mediate signal transduction (*11*−*13*). This suggests that RGS4 specifically modulates responses to Gq but not Gi linked receptors in RBL cells whereas the reverse is true for RGS1 (Figures 1 and 2). Previous studies in vitro have shown that RGS4 promotes the GTPase activity of both Gαi and Gαq classes of G-proteins in a reconstituted system (*15*, *16*). Transient expression of RGS4 in several cell lines also blocked cellular responses, including MAP kinase activity, PLCβ activation, and chemotaxis to both Gq and Gi linked receptors (*17*−*20*). The lack of specificity of RGS4-mediated inhibition of cellular signals observed there relative to the data presented in this work may be due to the level of expression of RGS4. Supporting this contention is the fact that overexpression of RGS4 (GFP intensity $\sim 10^3$) in PFR cells blocked both PAF- and fMLP-mediated PI hydrolysis (Figure 3) and Ca^{2+} mobilization (data not shown). In addition, recent studies by Bowman et al. (*20*) using transfected L1/2 cells have shown that the ability of RGS to inhibit cell adhesion and migration to chemoattractants depends on its level of expression.

An interesting finding in the current studies is that RGS4, but not ΔRGS4 which lacks the first 33 amino acid residues of the N-terminus and plasma membrane localization (*21*), blocked PAFR phosphorylation (Figure 4). PAFR undergoes phosphorylation via three different processes: (1) homologous (or agonist-dependent), mediated by a G-protein coupled receptor kinase (GRK) and requiring the agonist-occupied form of the receptor; (2) heterologous (or agonist-independent), mediated by the second messenger activated kinases PKC or PKA; and (3) cross-phosphorylation or phosphorylation upon activation of another receptor, including FR and C5aR (*13*, *14*). The inhibition of homologous phosphorylation of the PAFR could be interpreted as inhibition of G$\beta\gamma$-mediated GRK translocation from the cytosol to the membrane (*22*, *23*). However, the inhibition of phosphorylation of PAFR by PMA-mediated PKC activation and by cross-phosphorylation through FR activation suggests that a more generalized process is involved. One possibility is that a direct interaction between the cytoplasmic tail of the PAF receptor and the N-terminus of RGS4 is responsible for the inhibition of PAFR phosphorylation, since removal of the cytoplasmic tail of the PAFR or the N-terminus of RGS4 prevented RGS4-mediated inhibition of PI hydrolysis and Ca^{2+} mobilization to PAF (Figures 1 and 5). Direct evidence for an association of RGS4 with PAFR was, however, not forthcoming since attempts to coimmunoprecipitate PAFR and RGS4 in PFR−RGS4 cells, and "pull down" experiments in Cos cells transiently expressing RGS4 and the GST-fusion caboxy-tail of the receptor, were unsuccessful (data not shown). A second possibility is that the complex RGS4−Gαq sterically hinders the cytoplasmic tail of the PAFR, thereby preventing the access of the kinases to the phosphorylation sites. Supporting that contention is the fact that the GAP-deficient mutant of RGS4, N88S, which had no effect on PAFR-mediated PI

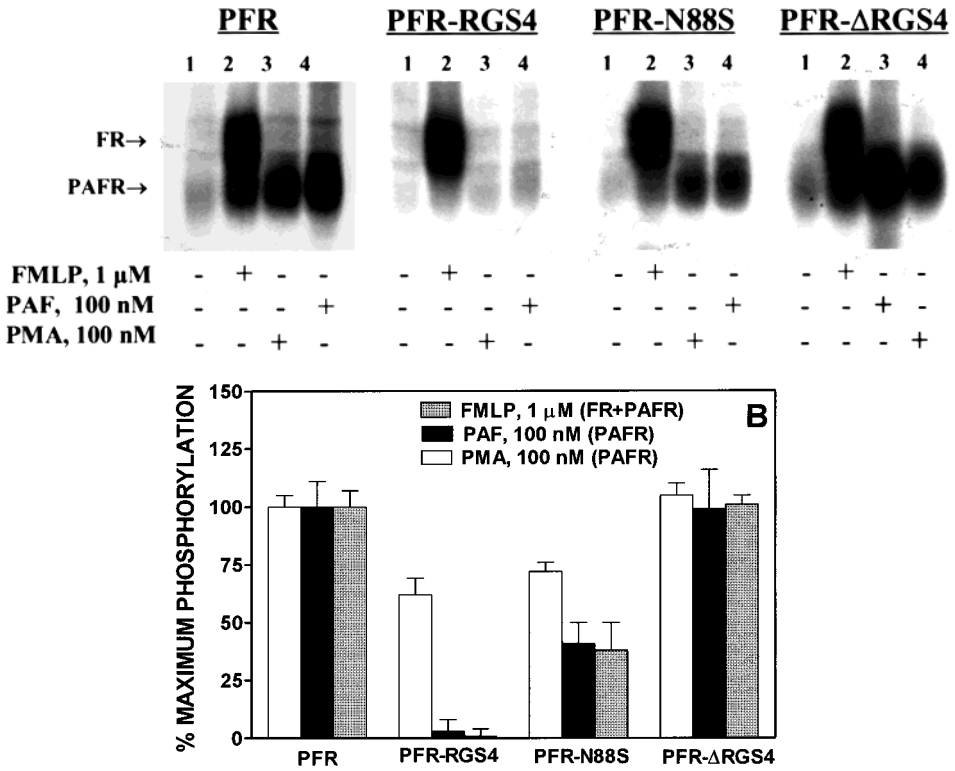

FIGURE 4: Effect of RGS4, N88S, and ΔRGS4 on FR and PAFR phosphorylation. (A) PFR, PFR−RGS4, PFR−N88S, and PFR−ΔRGS4 cells (5×10^6/60 mm plate) were ^{32}P labeled and incubated for 5 min with (lanes 2−4) or without (lane 1) stimulants. Cells were lysed, immunoprecipitated with 12CA5 antibody, electrophoresed into 10% SDS−polyacrylamide gel, and autoradiographed. The experiment was repeated four times with similar results. (B) Extent of phosphorylation as determined by Cerenkov counting of the excised phosphorylated bands. Data are mean ± S.E. of four separate experiments and are represented as percentage of maximum phosphorylation obtained with control PFR cells.

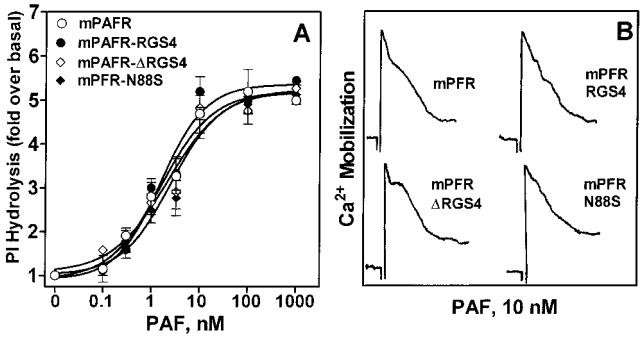

FIGURE 5: Effect of RGS4, N88S, and ΔRGS4 on a phosphorylation-deficient PAF receptor mutant (mPAFR). RBL cells expressing a phosphorylation-deficient PAF receptor mutant, mPAFR, were modified to express RGS4−GFP (mPAFR−RGS4), ΔRGS4−GFP (mPAFR−ΔRGS4), or N88S (mPAFR−N88S) as described in Figure 1. PAF-mediated PI hydrolysis (A) and intracellular Ca^{2+} mobilization (B) were measured as described in Figures 2 and 3. Data for PI hydrolysis are represented as fold stimulation over basal. The experiment was repeated four times with similar results. Ca^{2+} mobilization traces are representative of five experiments.

hydrolysis and Ca^{2+} mobilization, still blocked PAFR phosphorylation by ~60% (Figure 4). Xu et al. (*24*) reported that RGS4 blocked the initial activation of PLC and Ca^{2+} release, suggesting a possible interaction of RGS with the receptor−G-protein complex. Furthermore, a peptide from the amino terminus domain of RGS4 was shown to block carbachol-mediated intracellular Ca^{2+} mobilization in pancreatic acinar cells, suggesting that the N-terminus of RGS4 may interfere with the receptor−G-protein activation (*25*). It was recently shown that the N-terminal 33 amino acid of RGS4 forms an amphipathic α-helix which facilitates its membrane association (*26*). These data are consistent with the inhibitory effect of RGS4, observed in this work, versus ΔRGS4 and N88S.

In summary, the data herein suggest that expression of RGS4 in RBL cells can attenuate cellular responses to the Gq-linked chemoattractant receptor, PAFR, but not FR or CXCR1 which couple to Gi. Thus, various regulators of G-protein signaling may selectively affect the activity of different receptors as a consequence of the receptor's cytoplasmic tail and which G-protein the receptor is coupled to. The data also indicate that, in addition to the stimulation of the GTPase activity of Gα subunits of G-proteins, RGS4 also protects receptors from homologous and heterologous phosphorylation.

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