Published in final edited form as:

Cell Biochem Biophys. 2014 November ; 70(2): 867–880. doi:10.1007/s12013-014-9992-6.

Regulation of $G\beta\gamma_i$ -dependent PLC- $\beta 3$ Activity in Smooth Muscle: Inhibitory Phosphorylation of PLC- $\beta 3$ by PKA and PKG and Stimulatory Phosphorylation of $G\alpha_i$ -GTPase Activating Protein RGS2 by PKG

Ancy D. Nalli, Divya P. Kumar, Othman Al-Shboul, Sunila Mahavadi, John F. Kuemmerle, John R. Grider, and Karnam S. Murthy

Department of Physiology, VCU Program in Enteric Neuromuscular Sciences, Virginia Commonwealth University, Richmond, Virginia

Abstract

In gastrointestinal smooth muscle, agonists that bind to G_i -coupled receptors activate preferentially PLC- $\beta 3$ via $G\beta\gamma$ to stimulate phosphoinositide (PI) hydrolysis and generate inositol 1,4,5-trisphosphate (IP_3) leading to IP_3 -dependent Ca^{2+} release and muscle contraction. In the present study, we identified the mechanism of inhibition of PLC- $\beta 3$ -dependent PI hydrolysis by cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG). Cyclopentyl adenosine (CPA), an adenosine A_1 receptor agonist caused an increase in PI hydrolysis in a concentration-dependent fashion; stimulation was blocked by expression of the carboxyl terminal sequence of GRK2 (495–689), a $G\beta\gamma$ -scavenging peptide, or $G\alpha_i$ minigene but not $G\alpha_q$ minigene. Isoproterenol and S-nitrosoglutathione (GSNO) induced phosphorylation of PLC- $\beta 3$, and inhibited CPA-induced PI hydrolysis, Ca^{2+} release and muscle contraction. The effect of isoproterenol on all three responses was inhibited by PKA inhibitor, myristoylated PKI, or AKAP inhibitor, Ht-31, whereas the effect of GSNO was selectively inhibited by PKG inhibitor, Rp-cGMPS. GSNO, but not isoproterenol also phosphorylated $G\alpha_i$ -GTPase activating protein, RGS2 and enhanced association of $G\alpha_{i3}$ -GTP and RGS2. The effect of GSNO on PI hydrolysis was partly reversed in cells (i) expressing constitutively active GTPase-resistant $G\alpha_i$ mutant (Q204L), (ii) phosphorylation-site deficient RGS2 mutant (S46A/S64A), or (iii) siRNA for RGS2. We conclude that PKA and PKG inhibit $G\beta\gamma_i$ -dependent PLC- $\beta 3$ activity by direct phosphorylation of PLC- $\beta 3$. PKG, but not PKA also inhibits PI hydrolysis indirectly by a mechanism involving phosphorylation of RGS2 and its association with $G\alpha_i$ -GTP. This allows RGS2 to accelerate G_i -GTPase activity, enhance $G\beta\gamma_i$ trimer formation and inhibit $G\beta\gamma_i$ -dependent PLC- $\beta 3$ activity.

Introduction

Contraction of smooth muscle is mediated by Ca^{2+} /calmodulin-dependent activation of myosin light chain (MLC) kinase and phosphorylation of MLC₂₀, a prerequisite in actomyosin interaction [1–4]. Mobilization of intracellular Ca^{2+} release by main contractile

agonists such as acetylcholine, which activate G_q -coupled m_3 receptors is mediated via G_{α_q} -dependent activation of PLC- β_1 isoform, whereas agonists such as adenosine, which activate G_i -coupled A_1 receptors is mediated via G_{β_i} -dependent activation of PLC- β_3 isoform [5–9].

The strength and duration of G protein signaling is regulated by 2 major mechanisms that accelerate intrinsic GTPase activity of G_{α} subunits: i) through PLC- β_1 , which possesses GAP (GTPase activating protein activity) in its C-terminal region and which stimulates G_{α_q} -GTPase activity, and functions as both effector and terminator of G_{α_q} signaling [10, 11], and ii) through family GTPase activating proteins (GAP) known as regulators of G protein signaling (RGS), which bind via their RGS domain to G_{α} subunits and, like PLC- β_1 , stimulate the intrinsic GTPase activity of G_{α} subunits [12, 13]. Accelerated hydrolysis of GTP to GDP by RGS proteins leads to re-association of G_{α} and G_{β} and rapid cessation of G_{α} and G_{β} signals. RGS proteins also bind to receptors via their N-terminal domain and thus, function as scaffolding proteins to modulate G protein signaling in a receptor-specific manner [13, 14]. In pancreatic acini, for example, RGS4 was found to be more effective in terminating G protein signals coupled to cholinergic receptors than G proteins coupled to bombesin and cholecystokinin [15].

The strength and duration of PLC- β activity is also regulated by a number of kinases, which function both in negative feedback regulation of PLC- β isoforms (e.g., protein kinase C mediated regulation of PLC- β_1) as well as in cross-regulation between signaling pathways (e.g., ERK1/2-mediated regulation of PLC- β_1 , and PKA and PKG mediated regulation of PLC- β_3) [16–24]. Regulation of PLC- β activity by protein kinases appears to be PLC- β isoform specific [21, 22]. The strength and duration of RGS activity is also regulated by intracellular mediators. RGS4 proteins bind Ca^{2+} /calmodulin complex and PIP3: the latter inhibits RGS GAP activity and the Ca^{2+} /calmodulin complex antagonizes binding of PIP3 to RGS without affecting the GAP activity [25]. Regulation of RGS proteins by phosphorylation has also been reported in several studies. The results suggest that the effect of phosphorylation on RGS function is kinase, site- and cell-specific. For example: i) phosphorylation of RGS4 increased GAP activity in cardiac myocytes, and smooth muscle [26, 27]; ii) phosphorylation of RGS16 at S53 and S194 inhibited GAP activity, whereas phosphorylation at Y168 and Y177 by src kinase increased GAP activity [28–30], and iii) phosphorylation of RGS2 at Ser46 and Ser64 by PKG increased GAP activity, whereas phosphorylation by PKC inhibited GAP activity [31–34]. RGS2 is ubiquitously expressed and a role for RGS2 in pathogenesis of hypertension has been implicated in humans. Studies using *Rgs2*^{-/-} mice and RGS2 knockdown in both human and mouse cells have suggested that it regulates GAP activity and increased vascular tone due to increased response to angiotensin II receptor [32–37].

We have reported that in smooth muscle PLC- 1 activity was inhibited indirectly by both PKA and PKG via phosphorylation of RGS4 at Ser52 and acceleration of G_q inactivation [27]. The regulation of G_{β_i} -mediated PLC- β_3 activity is not clear. In the present study we demonstrate that in gastric muscle cells PLC- 3, unlike PLC- 1, is directly phosphorylated by both PKA and PKG. PLC- β_3 is indirectly regulated via phosphorylation of RGS2 at

Ser46/Ser64 by PKG only. Phosphorylation of PLC- β 3 and RGS2 leads to inhibition of G β γ -mediated PI hydrolysis, Ca²⁺ release, and initial muscle contraction

Materials and Methods

Materials

[¹²⁵I]cAMP, [³²P]Pi, and [³H]myo-inositol were obtained from PerkinElmer Life Sciences, Boston, MA; Collagenase CLS type II and soybean trypsin inhibitor were obtained from Worthington, Freehold, NJ; Western blotting, Dowex AG-1 X 8 resin (100–200 mesh in formate form), chromatography material and protein assay kit from Bio-Rad Laboratories, Hercules, CA; antibodies to PLC- β 3 and RGS2, and G α_{i3} were from Santa Cruz biotechnology, Santa Cruz, CA; myristoylated PKI 14–22 amide, Rp-cGMPS and Ht-31 were from Calbiochem, La Jolla, CA; RNAqueous™ kit was from Ambion, Austin, TX; Effectene Transfection Reagent, QIAEX®II Gel extraction Kit and QIAprep®Spin Miniprep Kit were from QIAGEN Sciences, MD; PCR reagents were from Applied Biosystems, Roche; SuperScript™ II Reverse Transcriptase and TOPO TA Cloning® Kit Dual Promoter were from Invitrogen, CA; EcoR1 was from New England Bio Labs; All other chemicals were from Sigma, St. Louis, MO.

All animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Preparation of Dispersed Gastric Smooth Muscle Cells

Smooth muscle cells from the circular muscle layer of the rabbit antrum were isolated by sequential collagenase digestion, filtration, and centrifugation as described previously [5–9]. The partly digested tissues were washed twice with 50 ml of collagenase-free smooth muscle buffer and the muscle cells were allowed to disperse spontaneously for 30 min in collagenase-free medium. Cells were harvested by filtration through 500 μ m Nitex and centrifuged twice at 350 g for 10 min to eliminate broken cells and organelles. Dispersed muscle cells were cultured in DMEM containing penicillin (200 μ U/ml), streptomycin (200 μ g/ml), gentamycin (100 μ g/ml), amphotericin B (2.5 μ g/ml) and 10% fetal bovine serum (DMEM-10). All experiments were done on cells in the first passage.

Transfection of RGS Mutant, Minigene Constructs and RGS2 siRNA into Cultured Smooth Muscle Cells

Wild type RGS2, phosphorylation-deficient RGS2(S46A/S64A) and G α -GTPase resistant G α_{i3} (Q204L) were subcloned into the multiple cloning site (EcoR1) of the eukaryotic expression vector pcDNA3. Recombinant plasmid DNAs were transiently transfected into the muscle in primary culture using Effectene Transfection Reagent (QIAGEN) for 48 h [27, 38]. Cells were co-transfected with 2 μ g of pcDNA3 vector and 1 μ g of pGreen Lantern-1 DNA. Transfection efficiency was monitored by the expression of the green fluorescent protein using FITC filters. Control cells were transfected with vector alone. Analysis by fluorescence microscopy showed that approximately 80% of the cells were transfected.

The cDNA sequences encoding the last COOH-terminal 11 amino acids of $G\alpha_q$, and $G\alpha_i$ were amplified by PCR and verified by DNA sequencing as previously described [38–41]. The oligonucleotide sequence corresponding to the COOH-terminal 11 amino acid residuals of G_i in random order was synthesized and ligated into pcDNA3.1(+) as a control minigene. All $G\alpha$ minigene constructs used for transfection experiments were purified with an endotoxin-free maxiprep kit (Qiagen) following the manufacturer's protocol.

The RNAi-Ready pSIREN-DNR-DsRed-Express Vector (BD Biosciences, Clontech) encoding RGS2 small-interfering RNA was inserted between BamH1 and EcoR1 restriction sites and transfected into cultured gastric smooth muscle cells with lipofectamineTM2000 reagent (Invitrogen) according to the manufacturer's recommendation. To check the specificity of the siRNA, empty vector without the siRNA sequence was used as control. Successful knockdown of RGS2 protein was verified by western blot.

Assay for Phosphoinositide (PI) Hydrolysis (PLC- β activity)

Total inositol phosphates were measured in rabbit gastric circular muscle cells by anion exchange chromatography using the method of Berridge et al [42] as described previously [5–9]. Ten ml of cell suspension (2×10^6 cells/ml) were labelled with myo- $[^3H]$ inositol (15 Ci/ml) for 90 min at 31 C and then cells were centrifuged at 350 g for 10 min to remove excess $[^3H]$ inositol and resuspended in 10 ml of fresh medium. CPA was added at different concentration to 0.5 ml of cell suspension for 1 min. Cultured smooth muscle cells were labelled with $[^3H]$ myo-inositols (1.5 μ Ci/ml) for 24 h in inositol-free DMEM medium, and then treated with CPA for 1 min. In some experiments cells were preincubated with isoproterenol or GSNO for 10 min and then with CPA for 1 min. The reaction was terminated by the addition of chloroform:methanol:HCl (50:100:1 v/v/v). The upper aqueous phase was applied to a column containing 1 ml of 1:1 slurry of Dowex AG-1 X8 resin (100–200 mesh in formate form) and distilled water. Total inositol phosphates were eluted with 6 ml of 0.8 μ M ammonium formate-0.1 μ M formic acid. The eluates were collected into scintillation vials and counted in gel phase after addition of 10 ml of scintillant. The results were expressed as counts per minute per mg protein.

Assay for Adenylyl Cyclase Activity

Adenylyl cyclase activity was measured by the formation of cAMP in response to agonists by radioimmunoassay using $[^{125}I]$ cAMP as described previously [6–9]. One ml (3×10^6 cells/ml) of cell suspension was treated with agonist in the presence of 100 μ M isobutylmethyl xanthine, either alone or in combination with CPA (1 μ M). The reaction was terminated with cold 6% trichloroacetic acid (v/v) and the supernatants were extracted three times with water-saturated diethyl ether to remove the trichloroacetic acid and the samples were then lyophilized and frozen at -20°C . The samples were reconstituted for radioimmunoassay in 50 μ l of 50 mM sodium acetate (pH 6.2) and acetylated with triethylamine/acetic anhydride (2:1 v/v) for 30 min. Cyclic AMP was measured in duplicates using 100 μ l aliquots and the results were computed from a standard curve using Prizm[®]. The results are expressed as pmol of cAMP/mg protein.

Phosphorylation of PLC- β 3 and RGS

Protein phosphorylation was determined from the amount of ^{32}P incorporated into each protein after immunoprecipitation with specific antibody to PLC- β 3 or RGS2 as previously described [27]. Freshly dispersed cells were incubated with [^{32}P]Pi 4 h. One milliliter of samples was incubated with isoproterenol (10 μM) or GSNO (10 μM) for 10 min and the reaction was terminated by rapid centrifugation. The pellet was homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 % SDS, 0.5% sodium deoxycholate, 1% NP-40, 10 mM sodium pyrophosphate, and protease inhibitor cocktail (2 $\mu\text{l/ml}$). Cell lysates were separated by centrifugation at 13,000 g for 10 min at 4 $^{\circ}\text{C}$, precleared with 40 μl of protein A-Sepharose, and incubated with PLC- β 3 or RGS2 antibody for 2 h at 4 $^{\circ}\text{C}$ and with 40 μl of protein A-Sepharose for another one hour. The immunoprecipitates were extracted with Laemmli sample buffer, boiled for 5 min, and separated by electrophoresis on SDS-PAGE. After transfer to nitrocellulose membranes [^{32}P]PLC- β 3 was visualized by autoradiography.

G α_{i3} :RGS2 Association

Smooth muscle cells (3×10^6 cells/ml) treated with CPA in the presence or absence of GSNO (10 μM) or isoproterenol (10 μM) were lysed after an incubation for 30 min at 4 $^{\circ}\text{C}$ in 10 mM Tris (pH 7.5), 50 mM NaCl, 1% Triton X-100, and 60 mM octyl glucoside, and lysates were centrifuged at 15,000 g for 30 min. The supernatant was precleared by incubation with 40 μl of protein A-Sepharose for 4 h and then incubated overnight with the antibody to G α_{i3} . Protein A-Sepharose was then added, and the mixture was incubated for 2 h and then centrifuged at 13,000 g for 5 min. Immunoprecipitates were washed four times in lysis buffer and boiled in Laemmli buffer. Samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed with the antibody to RGS2. After incubation with secondary antibody, proteins were visualized by ECL, and the intensity of the protein band on ECL film was determined by densitometry [27].

Ca $^{2+}$ Release in Dispersed Muscle Cells

Ca $^{2+}$ release was measured in intact muscle cells by an adaptation of the method of Poggioli and Putney [43] as described previously [44]. The cells were incubated with $^{45}\text{Ca}^{2+}$ (10 $\mu\text{Ci/ml}$), and Ca $^{2+}$ uptake was measured at intervals for 90 min when a steady state was attained. After 90 min, CPA (1 μM) was added and the reaction was terminated after 30 s. Isoproterenol (10 μM) or GSNO (10 μM) were added 10 min before CPA. The decrease in $^{45}\text{Ca}^{2+}$ content, representing net Ca $^{2+}$ efflux, was expressed as nanomoles per 10^6 cells.

Measurement of Contraction in Dispersed Smooth Muscle Cells

Contraction in freshly dispersed gastric circular smooth muscle cells was determined by scanning micrometry as previously described [5–9]. An aliquot (0.4 ml) of cells containing approximately 10^4 cells/ml was treated with 100 μl of medium containing various concentrations of CPA for 30 s and the reaction was terminated with 1% acrolein at a final concentration of 0.1%. In some experiments cells were incubated with isoproterenol (10 μM) or GSNO (10 μM) for 10 min and then treated with CPA for 30 s. The mean lengths of 50 muscle cells treated with agonists were measured by scanning micrometry and compared

with the mean lengths of untreated cells. The contractile response was expressed as the percent decrease in mean cell length from control cell length.

Statistical Analysis

The results were expressed as means S.E. of n experiments and analyzed for statistical significance using Student's t -test for paired and unpaired values. Each experiment was done on cells obtained from different animals. A probability of $p < 0.05$ was considered significant.

Results

Stimulation of PI hydrolysis by Cyclopentyl Adenosine (CPA) via $G\beta_i$

Treatment of dispersed gastric muscle cells with adenosine A_1 selective receptor agonist CPA caused an increase in PI hydrolysis in a concentration-dependent manner ($EC_{50} 3 \pm 1$ nM) (Fig. 1A). A maximal stimulation of 3956 ± 654 above basal levels of 623 ± 84 cpm/mg protein was obtained at $1 \mu M$ CPA. Previous studies have shown that A_1 receptors are coupled to pertussis toxin-sensitive G_{i3} and muscarinic m3 receptors are coupled to G_q in gastrointestinal smooth muscle [7]. Activation of m3 receptors stimulates PLC- $\beta 1$ via G_{α_q} , whereas activation of A_1 receptors stimulates PLC- $\beta 3$ isoform via $G\beta$ [4, 5, 7]. The involvement of $G\beta$ derived from G_{i3} in the activation of PI hydrolysis by A_1 receptor agonist CPA was examined in cultured muscle cells expressing G_i minigene or carboxyl terminal sequence of GRK2 (495–689), a $G\beta\gamma$ -scavenging peptide. CPA ($1 \mu M$) caused stimulation of PI hydrolysis in cultured muscle cells was similar to that in dispersed muscle cells. CPA-induced stimulation of PI hydrolysis was significantly inhibited in cells expressing G_{α_i} minigene ($76 \pm 5\%$ inhibition, $p < 0.01$) or $G\beta$ peptide ($81 \pm 6\%$ inhibition, $p < 0.01$), but not in cells expressing G_{α_q} minigene ($3 \pm 5\%$ inhibition, NS) (Fig. 1B). Control studies showed that stimulation of PI hydrolysis in response to G_{α_q} -coupled m3 receptor activation by acetylcholine was significantly inhibited ($78 \pm 7\%$, $p < 0.01$) in cells expressing G_{α_q} minigene ($485 \pm 32\%$ increase in PI hydrolysis in control cells and $75 \pm 6\%$ increase in cells expressing G_{α_q} minigene). Previous studies have shown that adenosine-stimulated PI hydrolysis in muscle membranes was selectively blocked by $G_{\alpha_{i3}}$ - and $G\beta$ -specific antibodies, as well as by a PLC- $\beta 3$ -specific antibody, but not by antibodies to other PLC- β isoforms or G protein subunits, suggesting activation of PLC- $\beta 3$ via $G\beta_i$ [7].

Inhibition of CPA-stimulated PI Hydrolysis by PKA and PKG

Selective activators of cAMP/PKA (isoproterenol) or cGMP/PKG (GSNO) pathway were used to examine the effect of PKA and PKG on CPA-stimulated PI hydrolysis [27, 45]. Pretreatment of cells with isoproterenol or GSNO for 10 min inhibited CPA ($1 \mu M$)-induced PI hydrolysis in a concentration-dependent manner (Fig. 2A). Maximal inhibition induced by GSNO ($87 \pm 6\%$ inhibition) was significantly higher than isoproterenol ($53 \pm 5\%$ inhibition), suggesting that PKG is more effective in the inhibition of CPA-induced PI hydrolysis. An alternative explanation is that the effect of isoproterenol via G_s /cAMP/PKA pathway was attenuated by simultaneous activation of $G_{\alpha_{i3}}$ by CPA and inhibition of cAMP formation [7].

To further examine if PKG is more effective than PKA to mediate inhibition of PI hydrolysis in response to CPA, cell permeable analogs of cAMP and cGMP that selectively activate PKA (cBIMPS) or PKG (8-pCPT-cGMP) were used. Both cBIMPS and 8-pCPT-cGMP caused inhibition of CPA (1 μ M)-induced PI hydrolysis in a concentration-dependent manner (Fig. 2B). Maximal inhibition induced by 8-pCPT-cGMP ($80\pm4\%$ inhibition) was significantly higher than cBIMPS ($56\pm5\%$ inhibition) confirming the results with isoproterenol and GSNO.

GSNO (10 μ M)-induced inhibition of PI hydrolysis was blocked by a selective inhibitor of PKG, Rp-cGMPS (1 μ M) but was not affected by the PKA inhibitor, myristoylated PKI 14–22 amide (1 μ M) (Fig. 3A). In contrast, isoproterenol (1 μ M)-induced inhibition of PI hydrolysis was blocked by myristoylated PKI, but was not affected by Rp-cGMPS (Fig. 3B), suggesting selective activation of PKG by GSNO and PKA by isoproterenol. Previous studies showed that A kinase anchoring protein (AKAP) plays an important role in targeting PKA to different subcellular compartments and mediating the PKA effects on target proteins [46, 47]. A short peptide (Ht-31) derived from PKA-binding amphipathic helix of AKAP was used to disrupt PKA docking to AKAP to examine the involvement of AKAP in PKA induced inhibition of PI hydrolysis [46, 47]. Pretreatment of cell with Ht-31 reversed the effect of isoproterenol, but not GSNO, on CPA-induced PI hydrolysis, suggesting the involvement of AKAP in PKA mediated inhibitory effect (Fig. 3B).

Treatment of cells with Rp-cGMPS (1 μ M), myristoylated PKI (1 μ M), or Ht-31 (10 μ M) in the absence of isoproterenol or GSNO had no effect on either basal (456 ± 58 to 612 ± 89 cpm/mg protein) or CPA-stimulated (3325 ± 425 to 3502 ± 564 cpm/mg protein) PI hydrolysis.

Phosphorylation of PLC- β 3 and RGS2 by PKA and PKG

Both GSNO (10 μ M) and isoproterenol (10 μ M) phosphorylated PLC- β 3 in dispersed muscle cells (Fig. 4). Phosphorylation of PLC- β 3 by GSNO was blocked by Rp-cGMPS but was not affected by myristoylated PKI or Ht-31 (Fig. 4A). In contrast, phosphorylation of PLC- β 3 by isoproterenol was blocked by myristoylated PKI and Ht-31, but was not affected by Rp-cGMPS (Fig. 4B). This pattern of inhibition is consistent with reversal of isoproterenol-induced inhibition of PI hydrolysis by myristoylated PKI or Ht-31, and GSNO-induced inhibition of PI hydrolysis by Rp-cGMPS.

GSNO, but not isoproterenol, also phosphorylated RGS2, an activator of G α -GTPase (Fig. 5). Phosphorylation of RGS2 by GSNO was blocked by Rp-cGMPS, but was not affected by myristoylated PKI (Fig. 5).

Inhibition of CPA-induced PI Hydrolysis by PKG Is Mediated via RGS2 Phosphorylation at Ser46/Ser64

We examined the hypothesis that phosphorylation of RGS2 increased its association with G α _{i3}.GTP leading to rapid hydrolysis and inactivation of G α _{i3} using several approaches. In the first approach, cells were transfected with RGS2 siRNA to suppress the endogenous RGS2 levels. Expression of RGS2 siRNA significantly augmented ($1211\pm45\%$ increase) the PI hydrolysis in response to CPA compared to cells expressing control vector ($728\pm15\%$

increase), suggesting that RGS2 is involved in the augmentation of $G\alpha_{i3}$ -GTPase activity and that suppression of $G\alpha_i$ -GTPase activity by RGS2 siRNA augments $G\beta$ -mediated PI hydrolysis (Fig. 6A). GSNO-induced inhibition of PI hydrolysis was significantly attenuated by RGS2 siRNA ($48\pm3\%$ inhibition vs. $85\pm6\%$ inhibition in control cells), suggesting that inhibition of PI hydrolysis by PKG was partly mediated via activation of RGS2. Isoproterenol-induced inhibition of PI hydrolysis was also attenuated by RGS2 siRNA ($26\pm3\%$ inhibition vs. $56\pm4\%$ inhibition in control cells) (Fig. 6A).

In the second approach cells were transfected with phosphorylation-deficient RGS2(S46A/S64A) to preclude the effect of PKG. Expression of RGS2(S46A/S64A) had no effect on CPA-induced increase in PI hydrolysis ($811\pm58\%$ increase vs. $853\pm38\%$ increase in control cells) (Fig. 6B). These results suggest that stimulation of $G\alpha_i$ -GTPase activity was not affected by RGS2(S46A/S64A). GSNO-induced inhibition of PI hydrolysis, however, was significantly attenuated in cells expressing RGS2(S46A/S64A) ($46\pm5\%$ inhibition) compared to control cells ($84\pm8\%$ inhibition), suggesting that phosphorylation increased the function of RGS2 and that inhibition of PI hydrolysis by GSNO was partly mediated via phosphorylation of RGS2 at Ser46/Ser64 (Fig. 6B). Isoproterenol-induced inhibition of PI hydrolysis was not affected by RGS2 (S46A/S64A) ($51\pm3\%$ inhibition vs. $53\pm6\%$ inhibition in control cells) (Fig. 6B).

In the third approach constitutively active $G\alpha_{i3}$ mutant (Q204L) that is resistant to GTPase activity was used [48]. Expression of constitutively active G_i mutant (Q204L) significantly augmented ($1016\pm24\%$ increase) the PI hydrolysis in response to CPA compared to cells expressing control vector ($634\pm28\%$ increase) (Fig. 6C). The increase reflects augmentation of $G\beta$ -dependent PI hydrolysis due to inhibition of GTP hydrolysis and resultant $G\alpha\beta$ trimer formation. Inhibition of PI hydrolysis by GSNO was significantly attenuated in cells expressing $G\alpha_{i3}$ (Q204L) ($50\pm4\%$ inhibition) compared to control cells ($75\pm5\%$ inhibition), suggesting that inhibition of PI hydrolysis by GSNO was partly mediated via activation of $G\alpha_i$. GTPase activity, possibly via RGS2 phosphorylation. Isoproterenol-induced inhibition of PI hydrolysis was also attenuated by $G\alpha_{i3}$ (Q204L) ($26\pm3\%$ inhibition) compared to control cells ($53\pm5\%$ inhibition).

Although there was no effect of isoproterenol on RGS2 phosphorylation, its inhibitory effect on CPA-induced PI hydrolysis was attenuated in cells expressing RGS2 siRNA or $G\alpha_i$ mutant (Q204L). This could be due to augmentation of $G\alpha_i$ function by suppression of RGS2 or expression of $G\alpha_i$ -GTPase resistant $G\alpha_{i3}$ (Q204L) leading to greater inhibition of cAMP. This notion was confirmed by measurements of $G\alpha_{i3}$ function in control cells and cells expressing $G\alpha_{i3}$ (Q204L) or RGS2 siRNA. The function of $G\alpha_{i3}$ was measured as inhibition of isoproterenol-stimulated cAMP formation by CPA.

In control cells, isoproterenol-stimulated cAMP formation ($795\pm35\%$ increase above basal levels of $2.62\pm.031$ pmol/mg protein) was significantly inhibited ($43\pm3\%$) in the presence of CPA ($452\pm21\%$ increase) (Fig. 7). Expression of $G\alpha_i$ (Q204L) had no effect on isoproterenol-stimulated cAMP formation ($824\pm28\%$ increase), but the inhibition by CPA was augmented ($74\pm5\%$ inhibition) resulting in less cAMP than the control cells (Fig. 7). Similarly, expression of RGS2 siRNA had no effect on isoproterenol-stimulated cAMP

formation ($978 \pm 42\%$ increase), but the inhibition by CPA was augmented ($78 \pm 7\%$ inhibition) resulting in less cAMP than the control cells (Fig. 7). This attenuation of isoproterenol-stimulated cAMP levels and resultant decrease in PKA activity could explain the reduction in the inhibition of PI hydrolysis by isoproterenol in cells expressing $G\alpha_{i3}(Q204L)$ or RGS2 siRNA compared to control cells. In contrast, expression of RGS2(S46A/S64A) had no effect on either isoproterenol-stimulated cAMP levels ($851 \pm 42\%$ increase) or inhibition ($45 \pm 6\%$) of isoproterenol-induced cAMP formation by CPA compared to control cells (Fig. 7). This is consistent with the lack of effect of RGS2(S46A/S64A) expression on inhibition PI hydrolysis by isoproterenol.

Augmentation of CPA-induced $G\alpha_{i3}$ -RGS2 Association by PKG

Activation of A_1 receptors with CPA increased $G\alpha_{i3}$ -RGS2 association. In the presence of GSNO, CPA-induced association of $G\alpha_{i3}$ -RGS2 was augmented (Fig. 8). Expression of RGS2(S46A/S64A) did not affect the increase in the association of RGS2 with $G\alpha_{i3}$ in response to CPA, but blocked the stimulatory effect of GSNO on RGS2: $G\alpha_{i3}$ association (Fig. 8). The pattern reflected that PKG-mediated phosphorylation of RGS2 caused greater association with activated $G\alpha_{i3}$ -GTP, and suggested that inhibition of PI hydrolysis by PKG was partly due to increased hydrolysis of $G\alpha_{i3}$ -GTP by RGS2. Isoproterenol had no effect on CPA-induced increase in the association of $G\alpha_{i3}$ -GTP consistent with the lack of effect of isoproterenol on phosphorylation of RGS2.

Inhibition of CPA-induced Ca^{2+} Release and Contraction by PKA and PKG

CPA-induced activation of PLC- β_3 in smooth muscle cells leads to generation of IP_3 , and IP_3 -induced Ca^{2+} release from sarcoplasmic stores. Treatment of cells loaded with $^{45}Ca^{2+}$ for 30 s with CPA induced Ca^{2+} release ($36 \pm 3\%$ release) from the resting steady-state Ca^{2+} levels (2.23 ± 0.31 nmoles/ 10^6 cells). CPA-induced Ca^{2+} release was significantly inhibited by pretreatment of cells with GSNO ($78 \pm 4\%$ inhibition) and isoproterenol ($53 \pm 4\%$ inhibition) (Fig. 9). Inhibition of CPA-induced Ca^{2+} release by GSNO and isoproterenol could reflect inhibition of PLC- β_3 activity and/or inhibition of IP_3 -induced Ca^{2+} release. Inhibition of Ca^{2+} in response to GSNO was significantly higher than the inhibition in response to isoproterenol. The results are consistent with the greater inhibition of CPA-induced PI hydrolysis by GSNO and preferential phosphorylation of IP_3 receptor I (IP_3RI) by PKG leading to inhibition of IP_3 -mediated Ca^{2+} release [49,50]. Isoproterenol-induced inhibition of Ca^{2+} release was blocked by myristoylated PKI ($11 \pm 4\%$ inhibition) or Ht-31 ($14 \pm 2\%$ inhibition)), but was not affected by Rp-cGMPS ($48 \pm 2\%$ inhibition) (Fig. 9). In contrast, GSNO-induced inhibition of Ca^{2+} release was blocked by Rp-cGMPS ($7 \pm 4\%$ inhibition), but was not affected by myristoylated PKI ($74 \pm 3\%$ inhibition) or Ht-31 ($71 \pm 6\%$ inhibition) (Fig. 9).

Increase in cytosolic Ca^{2+} in smooth muscle causes stimulation of Ca^{2+} /calmodulin-dependent MLC kinase activity, increase MLC₂₀ phosphorylation and muscle contraction. Treatment of cells with CPA caused a 33 ± 4 μm decrease in muscle cell from the control cell length of 105 ± 6 μm . CPA-induced muscle contraction was inhibited (i.e., relaxation) by isoproterenol and GSNO in a concentration-dependent manner (Fig. 10A). Maximal inhibition of contraction was similar with isoproterenol ($72 \pm 6\%$ relaxation) and GSNO

(83±5% relaxation). This is in contrast to the greater inhibition of PI hydrolysis by GSNO compared to isoproterenol and this could be due to the fact that PKA could act on more than one locus downstream of PLC-β3 activation to mediate relaxation. Isoproterenol-induced inhibition of contraction was blocked by myristoylated PKI (16±4% inhibition) or Ht-31 (9±5% inhibition), but was not affected by Rp-cGMPS (71±6% inhibition) (Fig. 10B). In contrast, GSNO-induced inhibition of contraction was blocked by Rp-cGMPS (7±2% inhibition), but was not affected by myristoylated PKI 14–22 amide (77±4% inhibition) or Ht-31 (80±3% inhibition) (Fig. 10B).

Discussion

Activation of PLC-β1 via Gα_q or PLC-β3 via Gβ_i in response to contractile agonists results in the generation of inositol 1,4,5-trisphosphate (IP₃), IP₃-dependent release of Ca²⁺, activation of Ca²⁺/calmodulin-dependent activation of MLC kinase and phosphorylation of MLC₂₀, an essential step in smooth muscle contraction [1–4]. Activation of cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG) in response to relaxant agonists results in the inhibition of Ca²⁺ mobilization leading to inhibition of MLC kinase activity and MLC₂₀ dephosphorylation, an essential step in smooth muscle relaxation [4, 45]. Inhibition by PKG and/or PKA reflects their action on various targets in the signaling pathways that increase cytosolic Ca²⁺. We have identified 3 such targets in the Ca²⁺-dependent contraction: i) RGS4, which accelerates inactivation of G_q-GTP and termination of Gα_q signaling [27]; ii) IP₃ receptor 1 (IP₃ R-I), which regulates Ca²⁺ release from sarcoplasmic stores in response to IP₃ generation [49, 50]; and iii) phospholamban, which regulates sarco-endoplasmic reticulum Ca²⁺ pump (SERCA) activity [51]. Neither Gα_q nor PLC-β1 is phosphorylated by PKA or PKG [27, 52, 53]. Phosphorylation of RGS4 by both PKA and PKG leads to increase in Gα_q-GTPase activity and decrease in PLC-1 activity and IP₃ generation. Phosphorylation of IP₃RI by PKG, but not PKA leads to decrease in the IP₃-mediated Ca²⁺ release, whereas phosphorylation of phospholamban by PKG leads to increase in SERCA activity and Ca²⁺ sequestration. PKA and PKG are also shown to regulate plasmalemmal Ca²⁺ and K⁺ channels [54, 55]. Phosphorylation of one or more of these targets should lead to a decrease in cytosolic Ca²⁺ and Ca²⁺-dependent muscle contraction.

Muscle contraction in the gastrointestinal tract can be modulated by various endogenous agonists, such as adenosine, opioid peptides, and somatostatin. These agonists activate specific receptors (adenosine A₁, opioid, , , and somatostatin sst₃) coupled to G_i, and mobilize Ca²⁺ via activation of PLC-3 by G_i [6–9]. In the present study, we have shown that, unlike PLC-1, PLC-3 is phosphorylated by PKA and PKG and the phosphorylation leads to inhibition of PLC-β3 activity. PLC-β3 activity is also regulated via phosphorylation of RGS2 at Ser^{46/64} by PKG only. Phosphorylation of RGS2 increases its association with Gα_{i3}-GTP, accelerates inactivation of Gα_{i3}, and trimer formation and thus, terminates both Gα_i-dependent inhibition of adenylyl cyclase activity and Gβ_i-dependent PLC-β3 activity. The evidence can be summarized as follows: i) activators of PKA and PKG induced phosphorylation of PLC-β3 and inhibited PI hydrolysis; ii) the effect of PKA activators was selectively blocked by the PKA inhibitor, myristoylated PKI, whereas the effect PKG activators was selectively blocked by PKG inhibitor, Rp-cGMPS; iii) PKG, but not PKA

also phosphorylated $G\alpha_i$ -GTPase activating protein RGS2 and enhanced its association with G_{i3} -GTP leading to inactivation of $G\beta_i$ -dependent PI hydrolysis. The notion that PKG-induced phosphorylation of RGS2 results in the inhibition of $G\beta_i$ -dependent PI hydrolysis was corroborated using 3 different complimentary approaches. In the first approach, we used GTPase-resistant $G\alpha_i$ mutant $G\alpha_{i3}(Q240L)$ to block $G\alpha_i$ -GTP hydrolysis by RGS2, in the second approach phosphorylation-deficient RGS2(S46A/S64A) was used to block PKG-mediated phosphorylation and augmentation of $G\alpha_i$ -GTP association and in the third approach RGS2 expression was suppressed to inhibit $G\alpha_i$ -GTP hydrolysis. CPA-induced PI hydrolysis was partly reversed in cells expressing $G\alpha_{i3}(Q240L)$, RGS2 (S46A/S64A) or RGS2 siRNA providing evidence that PKG-mediated phosphorylation of RGS2 accelerates $G\alpha_i$ -GTPase activity and inhibits $G\beta_i$ -dependent PLC- β_3 activity. Increase in $G\alpha_i$ -GTP hydrolysis causes termination of $G\beta$ signaling because the regions of $G\beta$ that interact with effectors such as PLC- β_3 and $G\alpha$ overlap and $G\beta$ interacts with either $G\alpha$ or PLC- β_3 , but not with both simultaneously [56].

The ability of PKA to phosphorylate PLC- β_3 and inhibit its activity was reported in previous studies [20–22]. Phosphorylation of PLC- β_3 at Ser1105 was shown to inhibit PLC- β_3 activation by $G\alpha_q$ without affecting activation by $G\beta_i$ suggesting that PKA inhibits $G\beta_i$ -dependent activity via a distinct mechanism that does not involve phosphorylation at Ser1105. $G\beta$ interacts with more than one region of PLC- β_3 and differ from the $G\alpha_q$, which interacts with C-terminus of PLC- β_3 [56]. Possible involvement of phosphorylation sites in the N-terminal region of PLC- β_3 was also examined and found to be that one of the potential sites Ser26 was without any effect on stimulation of PLC- β_3 activity by $G\beta$ [21, 22]. Thus, the identification of sites of phosphorylation and mechanism of inhibition of $G\beta$ -stimulated PLC- β_3 activity awaits further work. A-kinase anchoring proteins (AKAP) are signaling scaffolds that regulate cAMP signaling by targeting PKA to specific cellular substrates [46,47]. Blockade of PKA-, but not PKG-induced PLC- β_3 phosphorylation and inhibition of its activity by Ht-31 suggests that PKA-induced phosphorylation of PLC- β_3 and inhibition of CPA-induced PLC- β_3 activity requires the association of PLC- β_3 with AKAP [57].

Both $G\alpha_q$ - and $G\beta$ -stimulated PLC- β activity is terminated by intrinsic GTPase activity of $G\alpha$ subunits, and the rate of inactivation is significantly augmented by the GTPase activating property of PLC- β isoforms as well as by RGS proteins [10–14]. RGS proteins bind to switch regions in the activated $G\alpha$ subunits via their conserved RGS domain and accelerate the intrinsic GTPase activity [12, 13]. Our studies show that phosphorylation of RGS2 by PKG augments the association of $G\alpha_{i3}$.RGS2 leading to acceleration of GTP hydrolysis to GDP and termination of both $G\alpha_i$ - and $G\beta$ -dependent effector activity.

Accumulating evidence shows that termination of G protein signaling by RGS2 plays an important role in the regulation of vascular smooth muscle tone [32–37]. An inverse correlation between RGS2 expression and blood pressure has been suggested: RGS2 knockout mice exhibit hypertension phenotype and the effect of PKG to inhibit Ca^{2+} release and muscle contraction was greatly suppressed in these mice [35–37]. Our studies show that, unlike PKA, PKG-mediated inhibition of PI hydrolysis was also mediated by phosphorylation of RGS2. Suppression of RGS2 expression and expression of phosphorylation-deficient RGS2 (S46A/S64A) shed further light on the role of PKG-

mediated phosphorylation of RGS2 in the inhibition of PI hydrolysis by GSNO, but not isoproterenol. The N-terminal domain of RGS2 binds directly to the N-terminal leucine-zipper domain of PKG-I, the predominant PKG isoform in smooth muscle [32]. RGS2 possesses poor GAP activity for $G\alpha_i$ *in vitro* and this is attributed to the structural hindrance of RGS to bind to switch regions of $G\alpha_i$ [58, 59]. The N-terminus 78 amino acid of RGS2 is important for membrane targeting and function. Phosphorylation of RGS2 at the N-terminus by PKG may increase its membrane targeting, facilitate binding of RGS2 and/or increase its GTPase activating potency for $G\alpha_i$ [58]. RGS2-mediated suppression of $G\alpha_{i/o}$ was also reported in previous studies: it regulates presynaptic Ca^{2+} channels via $G\beta$ subunits derived from $G\alpha_{i/o}$ in hippocampal neurons [60], carbachol-stimulated ERK and Akt activity via $G\alpha_{i/o}$ in COS cells [61], and parathyroid hormone stimulated cAMP function by RGS2 via $G\alpha_s$ in osteoblasts [62]. RGS2 protein, in addition, can bind effector enzymes such as adenylyl cyclase to regulate their activity despite lacking GAP activity toward $G\alpha_s$ [58]. Recent studies, using endothelium-specific RGS2 knockout mice, have shown that RGS2 deficiency impairs vascular relaxation by augmentation of $G\alpha_i$ signaling in endothelium [31].

In summary, we have identified a mechanism by which PKA and PKG inhibit $G\beta$ -dependent PI hydrolysis to mediate muscle relaxation. Both PKA and PKG phosphorylate PLC- β_3 . PKG, but not PKA also phosphorylates RGS2, stimulating its binding to $G\alpha_i$ -GTP to enhance intrinsic GTPase activity of $G\alpha_{i3}$, and thus promoting reconstitution of heterotrimer $G\alpha\beta$. Both PKA and PKG also inhibited CPA-induced Ca^{2+} release, and muscle contraction in freshly dispersed gastric muscle cells. Our previous studies have shown that in *in vivo* PKG, but not PKA phosphorylates IP₃R-I and inhibits Ca^{2+} release [50]. Although phosphorylation of IP₃R-I results in the decrease in Ca^{2+} and contraction, and RGS2 is one of the several molecules that mediate the actions of PKG, the importance of PLC- β_3 inhibition by direct phosphorylation and indirect regulation via RGS2 resides in their location at the start of the signaling cascade leading to suppression of multiple distal signaling molecules concomitantly. The convergence of PLC- β_3 phosphorylation and RGS2 phosphorylation to inhibit IP₃ formation underscores this proximal step in mediating relaxation.

Acknowledgments

This study was supported by grants from the National Institutes of Diabetes, and Digestive and Kidney Diseases DK28300 and DK15564 to Karnam S. Murthy.

References

1. Somlyo AV, Khromov AS, Webb MR, Ferenzi MA, Trentham DR, He ZH, Sheng S, Shao Z, Somlyo AP. Smooth muscle myosin: regulation and properties. *Philos Trans R Soc Lond B Biol Sci.* 2004; 29:1921–1930. [PubMed: 15647168]
2. Kamm KE, Stull JT. Dedicated myosin light kinases with diverse cellular functions. *J Biol Chem.* 2001; 276:4527–4530. [PubMed: 11096123]
3. de, Godoy MA.; Rattan, S. Role of rho kinase in the functional and dysfunctional tonic smooth muscle. *Trends Pharmacol Sci.* 2011; 32:384–393. [PubMed: 21497405]
4. Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. *Annu Rev Physiol.* 2006; 68:345–374. [PubMed: 16460276]

5. Murthy KS, Makhoul GM. Functional characterization of phosphoinositide-specific phospholipase C- β 1 and β 3 in intestinal smooth muscle. *Am J Physiol.* 1995; 269:C969–978. [PubMed: 7485467]
6. Murthy KS, Makhoul GM. Coexpression of ligand-gated P2X and G protein-coupled P2Y receptors in smooth muscle. Preferential activation of P2Y receptors coupled to phospholipase C (PLC)- β 1 via $G\alpha/11$ and to PLC- β 3 via $G\beta\gamma/3$. *J Biol Chem.* 1998; 273:4695–4704. [PubMed: 9468531]
7. Murthy KS, Makhoul GM. Adenosine A1 receptor-mediated activation of phospholipase C- β 3 in intestinal muscle: dual requirement for α and β gamma subunits of G_i . *Mol Pharmacol.* 1995; 47:1172–1179. [PubMed: 7603457]
8. Murthy KS, Coy DH, Makhoul GM. Somatostatin receptor-mediated signaling in smooth muscle. Activation of phospholipase C- β 3 by $G_{\beta\gamma}$ and inhibition of adenylyl cyclase by $G_{\alpha}11$ and $G_{\alpha}10$. *J Biol Chem.* 1996; 271:23458–23463. [PubMed: 8798553]
9. Murthy KS, Makhoul GM. Opioid μ , δ and κ receptor-induced activation of phospholipase C- β 3 and inhibition of adenylyl cyclase is mediated by G_i 2 and G_o in smooth muscle. *Mol Pharmacol.* 1996; 50:870–877. [PubMed: 8863832]
10. Bernstein G, Blank JL, Jhon DY, Exton JH, Rhee SG, Ross EM. Phospholipase C- β 1 is a GTPase-activating protein for $G_q/11$, its physiologic regulator. *Cell.* 1992; 70:411–418. [PubMed: 1322796]
11. Chidiac P, Ross EM. Phospholipase C- β 1 directly accelerates GTP hydrolysis by $G_{\alpha}11$ and acceleration is inhibited by $G_{\beta\gamma}$ subunits. *J Biol Chem.* 1999; 274:19639–19643. [PubMed: 10391901]
12. Berman B, Gilman AG. Mammalian RGS proteins: barbarians at the gate. *J Biol Chem.* 1998; 273:1269–1272. [PubMed: 9430654]
13. Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol Rev.* 2002; 54:527–559. [PubMed: 12223533]
14. Kach J, Sethakorn N, Dulin NO. A finer tuning of G-protein signaling through regulated control of RGS proteins. *Am J Physiol Heart Circ Physiol.* 2012; 303:H19–35. [PubMed: 22542620]
15. Xu X, Zeng W, Popov S, Berman DM, Davignon I, Yu K, Yowe D, Offermanns S, Muallem S, Wilkie TM. RGS proteins determine signaling specificity of G_q -coupled receptors. *J Biol Chem.* 1999; 274:3549–3556. [PubMed: 9920901]
16. Litosch I. Novel mechanisms for feedback regulation of phospholipase C- β activity. *IUBMB Life.* 2002; 54:253–260. [PubMed: 12587975]
17. Rebecchi MJ, Pentyala SN. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev.* 2000; 80:1291–1335. [PubMed: 11015615]
18. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem.* 2001; 70:281–312. [PubMed: 11395409]
19. Liu M, Simon MI. Regulation by cAMP-dependent protein kinase of a G protein-mediated phospholipase C. *Nature.* 1996; 382:83–87. [PubMed: 8657310]
20. Ali H, Fisher I, Haribabu B, Richardson RM, Snyderman R. Role of phospholipase C β 3 phosphorylation in the desensitization of cellular responses to platelet-activating factor. *J Biol Chem.* 1997; 272:11706–11709. [PubMed: 9115222]
21. Yue C, Dodge KL, Weber G, Sanborn BM. Phosphorylation of serine 1105 by protein kinase A inhibits phospholipase C β 3 stimulation by $G_{\alpha}11$. *J Biol Chem.* 1998; 273:18023–18027. [PubMed: 9660757]
22. Yue C, Ku CY, Liu M, Simon MI, Sanborn BM. Molecular mechanism of the inhibition of phospholipase C β 3 by protein kinase C. *J Biol Chem.* 2000; 275:30220–30225. [PubMed: 10893237]
23. Ryu SH, Kim UH, Wahl MI, Brown AB, Carpenter G, Huang KP, Rhee SG. Feedback regulation of phospholipase C- β by protein kinase C. *J Biol Chem.* 1990; 265:17941–17945. [PubMed: 2211670]
24. Xu A, Suh PG, Marmy-Conus N, Pearson RB, Seok OY, Cocco L, Gilmour RS. Phosphorylation of nuclear phospholipase C β 1 by extracellular signal-regulated kinase mediates the mitogenic action of insulin-like growth factor I. *Mol Cell Biol.* 2001; 21:2981–2990. [PubMed: 11287604]

25. Ishii M, Fujita S, Yamada M, Hosaka Y, Kurachi Y. Phosphatidylinositol 3,4,5-trisphosphate and Ca²⁺/calmodulin competitively bind to the regulators of G-protein-signalling (RGS) domain of RGS4 and reciprocally regulate its action. *Biochem J*. 2005; 385:65–73. [PubMed: 15324308]
26. Tokudome T, Kishimoto I, Horio T, Arai Y, Schwenke DO, Hino J, Okano I, Kawano Y, Kohno M, Miyazato M, et al. Regulator of G-protein signaling subtype 4 mediates antihypertrophic effect of locally secreted natriuretic peptides in the heart. *Circulation*. 2008; 117:2329–2339. [PubMed: 18443239]
27. Huang J, Zhou H, Mahavadi S, Sriwai W, Murthy KS. Inhibition of Gα₁₂-dependent PLC-β activity by PKG and PKA is mediated by phosphorylation of RGS4 and GRK2. *Am J Physiol Cell Physiol*. 2007; 292:C200–208. [PubMed: 16885398]
28. Chen C, Wang H, Fong CW, Lin SC. Multiple phosphorylation sites in RGS16 differentially modulate its GAP activity. *FEBS Lett*. 2001; 504:16–22. [PubMed: 11522288]
29. Derrien A, Druey KM. RGS16 function is regulated by epidermal growth factor receptor-mediated tyrosine phosphorylation. *J Biol Chem*. 2001; 276:48532–48538. [PubMed: 11602604]
30. Derrien A, Zheng B, Osterhout JL, Ma YC, Milligan G, Farquhar MG, Druey KM. Src-mediated RGS16 tyrosine phosphorylation promotes RGS16 stability. *J Biol Chem*. 2003; 278:16107–16116. [PubMed: 12588871]
31. Osei-Owusu P, Sun X, Drenan RM, Steinberg TH, Blumer KJ. Regulation of RGS2 and second messenger signaling in vascular smooth muscle cells by cGMP-dependent protein kinase. *J Biol Chem*. 2007; 282:31656–31665. [PubMed: 17681944]
32. Tang KM, Wang GR, Lu P, Karas RH, Aronovitz M, Heximer SP, Kaltenbronn KM, Blumer KJ, Siderovski DP, Zhu Y, Mendelsohn ME. Regulator of G protein signaling-2 mediate vascular smooth muscle relaxation and blood pressure. *Nat Med*. 2003; 9:1506–1512. [PubMed: 14608379]
33. Sun X, Kaltenbronn KM, Steinberg TH, Blumer KJ. RGS2 is a mediator of nitric oxide action on blood pressure and vasoconstrictor signaling. *Mol Pharmacol*. 2005; 67:631–639. [PubMed: 15563583]
34. Cunningham ML, Waldo GL, Hollinger S, Hepler JR, Harden TK. Protein kinase C phosphorylates RGS2 and modulates its capacity for negative regulation of Gα₁₁ signaling. *J Biol Chem*. 2001; 276:5438–5444. [PubMed: 11063746]
35. Obst M, Tank J, Plehm R, Blumer KJ, Diedrich A, Jordan J, Luft FC, Gross V. NO-dependent blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol*. 2006; 290:R1012–1019. [PubMed: 16269576]
36. Gross V, Tank J, Obst M, Plehm R, Blumer KJ, Diedrich A, Jordan J, Luft FC. Autonomic nervous system and blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol*. 2005; 288:R1134–1142. [PubMed: 15661972]
37. Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, Oliveira-dos-Santos A, Penninger JM, Muslin AJ, Steinberg TH, et al. Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J Clin Invest*. 2003; 111:1259. [PubMed: 12697745]
38. Zhou H, Murthy KS. Distinctive G protein-dependent signaling in smooth muscle by sphingosine 1-phosphate receptors S1P1 and S1P2. *Am J Physiol Cell Physiol*. 2004; 286:C1130–1138. [PubMed: 15075212]
39. Sriwai W, Mahavadi S, Al-Shboul O, Grider JR, Murthy KS. Distinctive G protein-dependent signaling by protease-activated receptor 2 (PAR2) in smooth muscle: feedback inhibition of RhoA by cAMP-independent PKA. *PLOS ONE*. 2013; 8:e66743. [PubMed: 23825105]
40. Gilchrist A, Bunemann M, Li A, Hosey MM, Hamm HE. A dominant-negative strategy for studying roles of G proteins in vivo. *J Biol Chem*. 1999; 274:6610–6616. [PubMed: 10037756]
41. Gilchrist A, Vanhauwe J, Li A, Thoma TT, Voyno-Yasenetskaya T, Hosey MM, Hamm HE. Gα_{minigenes} expressing C-terminal peptides serve as specific inhibitors of thrombin-mediated endothelial activation. *J Biol Chem*. 2001; 276:25672–25679. [PubMed: 11274183]
42. Berridge MJ, Downes CP, Hanley MR. Lithium amplifies agonist dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J*. 1982; 206:587–595. [PubMed: 7150264]
43. Poggioli J, Putney JW Jr. Net calcium fluxes in rat parotid acinar cells: evidence for a hormone-sensitive calcium pool in or near the plasma membrane. *Pflugers Arch*. 1982; 392:239–243. [PubMed: 6803225]

44. Murthy KS, Severi C, Grider JR, Makhoul GM. Inhibition of IP₃ and IP₃-dependent Ca²⁺ mobilization by cyclic nucleotides in isolated gastric muscle cells. *Am J Physiol.* 1993; 264:G967–974. [PubMed: 8388644]
45. Murthy KS, Makhoul GM. Interaction of cA-kinase and cG-kinase in mediating relaxation of dispersed smooth muscle cells. *Am J Physiol.* 1995; 268:C171–180. [PubMed: 7840145]
46. Welch EJ, Jones BW, Scott JD. Networking with AKAPs: context-dependent regulation of anchored enzymes. *Mol Interv.* 10:86–97. [PubMed: 20368369]
47. Hausken ZE, Dell'Acqua ML, Coghlan VM, Scott JD. Mutational analysis of the A-kinase anchoring protein (AKAP-binding site on RII: classification of side chain determinants for anchoring and isoform selective association with AKAPs. *J Biol Chem.* 1996; 271:29016–29022. [PubMed: 8910553]
48. Ogier-Denis E, Petiot A, Bauvy C, Codogno P. Control of the expression and activity of the G α -interacting protein (GAIP) in human intestinal cells. *J Biol Chem.* 1997; 272:24599–24603. [PubMed: 9305927]
49. Komalavilas P, Lincoln TM. Phosphorylation of the inositol 1, 4, 5-trisphosphate receptor. cGMP-dependent protein kinase mediates cAMP and cGMP dependent phosphorylation in intact rat aorta. *J Biol Chem.* 1996; 271:21933–21938. [PubMed: 8702997]
50. Zhou H, Murthy KS. Selective phosphorylation of IP₃ receptor type I (IP₃RI) *in vivo* by cGMP-dependent protein kinase in gastric smooth muscle. *Am J Physiol Gastrointest Liver Physiol.* 2003; 284:G221–230.
51. Coyer J. Phosphorylation states of phospholamban. *Ann N Y Acad Sci.* 1998; 853:79–91.
52. Lounsbury KM, Schlegel B, Poncz M, Brass LF, Manning DR. Analysis of G α by site-directed mutagenesis. Sites and specificity of protein kinase C-dependent phosphorylation. *J Biol Chem.* 1993; 268:3494–3498. [PubMed: 8429024]
53. Jhon DY, Lee HH, Park D, Lee CW, Lee KH, Yoo OJ, Rhee SG. Cloning, sequencing, purification and Gq-dependent activation of phospholipase C- β 3. *J Biol Chem.* 1993; 268:6654–6661. [PubMed: 8454637]
54. Xiong Z, Sperelakis N. Regulation of L-type calcium channels of vascular smooth muscle cells. *J Mol Cell Cardiol.* 1995; 27:75–91. [PubMed: 7760390]
55. Fukao M, Mason HS, Britton FC, Kenyon JL, Horowitz B, Keef KD. Cyclic GMP-dependent protein kinase activates cloned BKCa channels expressed in mammalian cells by direct phosphorylation at serine 1072. *J Biol Chem.* 1999; 274:10927–10935. [PubMed: 10196172]
56. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem.* 2001; 70:281–312. [PubMed: 11395409]
57. Ku CY, Sanborn BM. Progesterone prevents the pregnancy-related decline in protein kinase A association with rat myometrial plasma membrane and A-kinase anchoring protein. *Biol Reprod.* 2002; 67:605–609. [PubMed: 12135903]
58. Kehrl JH, Sinnarajah S. RGS2: a multifunctional regulator of G-protein signaling. *Int J Biochem Cell Biol.* 2002; 34:432–438. [PubMed: 11906816]
59. Heximer SP, Watson N, Linder ME, Blumer KJ, Hepler JR. RGS2/G0S8 is a selective inhibitor of G α q function. *Proc Natl Acad Sci U S A.* 1997; 94:14389–14393. [PubMed: 9405622]
60. Han J, Mark MD, Li X, Xie M, Waka S, Rettig J, Herlitze S. RGS2 determines short-term synaptic plasticity in hippocampal neurons by regulating Gi/o-mediated inhibition of presynaptic Ca²⁺ channels. *Neuron.* 2006; 51:575–586. [PubMed: 16950156]
61. Anger T, Klintworth N, Stumpf C, Daniel WG, Mende U, Garlisch CD. RGS protein specificity towards Gq- and Gi/o-mediated ERK 1/2 and Akt activation, *in vitro*. *J Biochem Mol Biol.* 2007; 40:899–910. [PubMed: 18047785]
62. Roy AA, Nunn C, Ming H, Zou MX, Penninger J, Kirshenbaum LA, Dixon SJ, Chidiac P. Up-regulation of endogenous RGS2 mediates cross-desensitization between Gs and Gq signaling in osteoblasts. *J Biol Chem.* 2006; 281:32684–32693. [PubMed: 16950788]

Figure 1. Stimulation of PI Hydrolysis by CPA via Gβ_i

(A) Freshly dispersed cells labelled with myo[³H]inositol were treated with different concentrations of CPA for 1 min. PI hydrolysis was measured as increase in the water soluble inositol phosphates by ion-exchange chromatography. Results are expressed as cpm/mg protein above basal levels of 623±84 cpm/mg protein. (B) Cultured muscle expressing control vector, Gα_i minigene, Gα_q minigene or carboxyl terminal sequence of GRK2 (495–689), a Gβγ-scavenging peptide (Gβγ peptide) were labelled with myo[³H]inositol and treated with CPA (1 μM) for 1 min. PI hydrolysis was measured as described above. Results are expressed as cpm/mg protein. Values are means ± SE of 4 experiments. **p<0.01 significant inhibition compared to response to CPA in control cells.

Figure 2. Inhibition of PI Hydrolysis by cAMP- (PKA) and cGMP-dependent (PKG) Protein Kinases

Freshly dispersed cells labelled with myo[³H]inositol were treated with different concentrations of PKA activators (isoproterenol (Isop) or cBIMPS) or PKG activators (GSNO or 8-pCPT-cGMP (cGMP)) for 10 min and then treated with CPA (1 μ M) for 1 min. PI hydrolysis was measured as increase in the water soluble inositol phosphates by ion-exchange chromatography. CPA caused a significant increase in PI hydrolysis (3381 ± 502 to 3425 ± 506 cpm/mg protein) above basal levels (589 ± 84 to 681 ± 88 cpm/mg protein). Activators of both PKA and PKG inhibited PI hydrolysis in a concentration-dependent manner. Results are expressed as cpm/mg protein. Values are means $\pm$ SE of 4–5 experiments.

Figure 3. Inhibition of CPA-induced PI Hydrolysis by cAMP- (PKA) and cGMP-dependent (PKG) Protein Kinases

Freshly dispersed cells labelled with myo[^3H]inositol were incubated with GSNO (10 μM) or isoproterenol (10 μM) for 10 min in the presence of PKA inhibitor, PKI 14–26 amide (PKI, 1 μM), PKG inhibitor, Rp-cGMPS (RpG, 1 μM) or AKAP-PKA inhibitor, Ht-31 (10 μM) and then treated with CPA for 1 min. PI hydrolysis was measured as increase in the water soluble inositol phosphates by ion-exchange chromatography. Results are expressed as cpm/mg protein. Values are means $\pm$ SE of 4–5 experiments. ** $P < 0.001$ significant inhibition of CPA-induced PI hydrolysis.

Figure 4. Phosphorylation of PLC-β3 by PKA and PKG

Muscle cells labelled with ^{32}P were incubated for 10 min with isoproterenol (Isop, 10 μM) or GSNO (10 μM) in the presence or absence of selective inhibitors of PKA (myristoylated PKI, 1 μM), PKG (Rp-cGMPS, 1 μM) or AKAP-PKA interaction (Ht-31, 10 μM). PLC-β3 immunoprecipitates were separated on SDS-PAGE, ^{32}P -labeled PLC-β3 (p-PLC-β3) was identified by autoradiography, and radioactivity was expressed as cpm/mg protein. Values are mean $\pm$ SE if 4 experiments. ** $P < 0.01$ significant inhibition of GSNO- or isoproterenol-induced PLC-β3 phosphorylation.

Figure 5. Selective phosphorylation of RGS2 by PKG

Muscle cells labelled with ^{32}P were incubated for 10 min with isoproterenol (Isop, 10 μM) or GSNO (10 μM) in the presence or absence of selective inhibitors of PKA (myristoylated PKI, 1 μM), or PKG (Rp-cGMPS, 1 μM). RGS2 immunoprecipitates were separated on SDS-PAGE, ^{32}P -labeled RGS2 (p-RGS2) was identified by autoradiography, and radioactivity was expressed as cpm/mg protein. Values are mean $\pm$ SE of 4 experiments.

** $P < 0.01$ significant inhibition of GSNO-induced RGS2 phosphorylation.

Figure 6. Inhibition of PI Hydrolysis by PKG via RGS2 Phosphorylation and increase in Gα_{i3}.GTPase activation

Cultured muscle cells expressing RGS2 siRNA (A), phosphorylation-deficient RGS2(S46A/S64A) (B), Gα_i-GTPase resistant Gα_i(Q204L) (C) were labeled with myo[³H]inositol. Cells were incubated with isoproterenol (Isop, 10 μM) or GSNO (10 μM) and then treated with CPA (1 μM) for 1 min. PI hydrolysis was measured as increase in the water soluble inositol phosphates by ion-exchange chromatography. Results are expressed as cpm/mg protein. Values are means ± SE of 4–5 experiments. # p<0.05 significant augmentation of CPA-

induced PI hydrolysis compared to response in control cells. * $p < 0.05$ significant attenuation of isoproterenol- or GSNO-induced inhibition of PI hydrolysis compared to control cells

Figure 7. Activation of Gα_{i3} by CPA is regulated by Gα_i-GTPase activating protein RGS2
 Cultured muscle cells expressing Gα_i-GTPase resistant Gα_i(Q240L), RGS2 siRNA, or phosphorylation-deficient RGS2(S46A/S64A) were treated with isoproterenol (Isop, 10 μM) in the presence or absence of CPA (1 μM) for 1 min. cAMP was measured by radioimmunoassay as described in the Methods. Results are expressed as pmol/mg protein. Values are means ± SE of 4–5 experiments. * p<0.05 significant augmentation of CPA-induced inhibition of cAMP formation compared to response in control cells.

Figure 8. Phosphorylation of RGS2 by PKG increases its association with Gα_{i3}-GTP
 Smooth muscle cells expressing wild type RGS2 or phosphorylation-deficient RGS2(S46A/S64A) were treated with CPA (1 μM) in the presence or absence of GSNO (10 μM). Immunoprecipitates derived from 500 μg of proteins using Gα_{i3} antibody were separated by SDS-PAGE and immunoblotted using RGS2 antibody. Values are mean ± SE of 4 experiments. *p<0.05 significant increase of CPA-induced association of RGS2-Gα_{i3} by GSNO.

Figure 9. Inhibition of CPA-induced Ca²⁺ Release by PKA and PKG

Dispersed muscle cells were incubated with ⁴⁵Ca²⁺ (10 μ Ci/ml) for 90 min to obtain steady-state Ca²⁺ levels and then CPA (1 μ M) was added and the reaction was terminated after 30 s. In some experiments cells were pretreated with GSNO (10 μ M) or isoproterenol (Isop, 10 μ M) for 10 min in the presence or absence of inhibitors of PKA (PKI, 1 μ M), PKG (Rp-cGMPS, 1 μ M) or AKAP-PKA interaction (Ht-31, 10 μ M). The decrease in ⁴⁵Ca²⁺ content, representing net Ca²⁺ efflux was expressed as decrease in steady-state Ca²⁺ levels (2.23 $\pm$ 0.31 nmoles/10⁶cells). **P<0.001 significant inhibition of CPA-induced Ca²⁺ release.

Figure 10. Inhibition of CPA-induced contraction by PKA and PKG

(A) Dispersed muscle cells were incubated with different concentrations of GSNO or isoproterenol (Isop) for 10 min and then treated with CPA for 30 s. Contraction was measured by scanning micrometry as decrease in cell length and expressed as percent decrease from the basal length. CPA caused a significant decrease in cell length ($32 \pm 3\%$ decrease) from the basal length of $105 \pm 6 \mu\text{m}$. GSNO and isoproterenol inhibited CPA-induced contraction in a concentration-dependent manner. (B) Muscle cells were treated with isoproterenol (Isop, $10 \mu\text{M}$) or GSNO ($10 \mu\text{M}$) in the presence or absence of selective inhibitors of PKA (PKI, $1 \mu\text{M}$), PKG (Rp-cGMPS, $1 \mu\text{M}$) or AKAP-PKA interaction (Ht-31, $10 \mu\text{M}$) for 10 min followed by CPA for 30 s. Muscle cell length was measured by scanning micrometry. Results are expressed as percent decrease in cell length (basal cell length 105 ± 6

μm). Values are mean ±SE of 6 experiments. **P<0.001 significant inhibition of CPA-induced contraction.

Figure 11. Mechanism for inhibition of $G\beta\gamma$ -mediated PLC- $\beta 3$ by PKA and PKG

Both PKA and PKG phosphorylated PLC- $\beta 3$ leading to inhibition of PI hydrolysis (PLC-3 activity), IP_3 generation, IP_3 -dependent Ca^{2+} release and muscle contraction. and stimulate the intrinsic GTPase activity of G_q . In addition, PKG, but not PKA, phosphorylated RGS2 leading to stimulation of intrinsic G_i -GTPase activity and termination of α -mediated inhibition of adenylyl cyclase (Ac) activity and $\beta\gamma$ -mediated stimulation of PI hydrolysis.