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ORIGINAL PAPER

Regulation of $G\beta\gamma_i$-Dependent PLC-β3 Activity in Smooth Muscle: Inhibitory Phosphorylation of PLC-β3 by PKA and PKG and Stimulatory Phosphorylation of $G\alpha_i$-GTPase-Activating Protein RGS2 by PKG

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Abstract In gastrointestinal smooth muscle, agonists that bind to G_i-coupled receptors activate preferentially PLC-β3 via Gβγ 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-β3-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 Gβγ-scavenging peptide, or $G\alpha_i$ minigene but not $G\alpha_q$ minigene. Isoproterenol and S-nitrosoglutathione (GSNO) induced phosphorylation of PLC-β3 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-β3 activity by direct phosphorylation of PLC-β3. 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 $G\alpha_i$-GTPase activity, enhance $G\alpha\beta\gamma_i$ trimer formation, and inhibit $G\beta\gamma_i$-dependent PLC-β3 activity.

Keywords Phospholipase-C · Muscle relaxation · Nitric oxide · Muscle contraction · G protein

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Introduction

Contraction of smooth muscle is mediated by Ca^{2+}/calmodulin-dependent activation of myosin light chain (MLC) kinase and phosphorylation of MLC_{20}, a prerequisite in acto–myosin interaction [1–4]. Mobilization of intracellular Ca^{2+} by main contractile agonists such as acetylcholine, which activate G_q-coupled m3 receptors, is mediated via $G\alpha_q$-dependent activation of PLC-β1 isoform, whereas agonists such as adenosine, which activate G_i-coupled A_1 receptors, is mediated via $G\beta\gamma_i$-dependent activation of PLC-β3 isoform [5–9].

The strength and duration of G protein signaling are regulated by 2 major mechanisms that accelerate intrinsic GTPase activity of Gα subunits: (i) through PLC-β1, which possesses GAP (GTPase-activating protein activity) in its C-terminal region and which stimulates $G\alpha_q$-GTPase activity and functions as both effector and terminator of $G\alpha_q$ signaling [10, 11], and (ii) through family of GTPase-activating proteins (GAP) known as regulators of G protein signaling (RGS), which bind via their RGS domain to Gα subunits and, like PLC-β1, stimulate the intrinsic GTPase activity of Gα subunits [12, 13]. Accelerated hydrolysis of GTP to GDP by RGS

proteins leads to re-association of Gα and Gβγ and rapid cessation of Gα and Gβγ 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 are 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-β1) as well as in cross-regulation between signaling pathways (e.g., ERK1/2-mediated regulation of PLC-β1, and PKA- and PKG-mediated regulation of PLC-β3) [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 are 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-β1 activity was inhibited indirectly by both PKA and PKG via phosphorylation of RGS4 at Ser52 and acceleration of $Gα_q$ inactivation [27]. The regulation of $Gβγ_i$-mediated PLC-β3 activity is not clear. In the present study, we demonstrate that in gastric muscle cells, PLC-β3, unlike PLC-β1, is directly phosphorylated by both PKA and PKG. PLC-β3 is indirectly regulated via phosphorylation of RGS2 at Ser46/Ser64 by PKG only. Phosphorylation of PLC-β3 and RGS2 leads to inhibition of $Gβγ_i$-mediated PI hydrolysis, Ca^{2+} release, and initial muscle contraction.

Materials and Methods

Materials

[^{125}I]cAMP, [^{32}P]Pi, and [^{3}H]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-1X8 resin (100–200 mesh in formate form), chromatography material, and protein assay kit were from Bio-Rad Laboratories, Hercules, CA; antibodies to PLC-β3 and RGS2, and $Gα_{i3}$ 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 μm 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 U/ml), streptomycin (200 μg/ml), gentamycin (100 μg/ml), amphotericin B (2.5 μg/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 Gα-GTPase-resistant $Gα_{i3}$(Q204L) were subcloned into the multiple cloning site (EcoR1) of the eukaryotic expression vector pcDNA3. Recombinant plasmid DNAs were transiently transfected into the muscle cells in primary culture using Effectene Transfection Reagent (QIAGEN) for 48 h [27, 38]. Cells were

co-transfected with 2 μg of pcDNA3 vector and 1 μg 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 residues of $G\alpha_i$ in random order was synthesized and ligated into pcDNA3.1(+) as a control minigene. All Gα 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 lipofectamine^{TM}2000 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 milliliter of cell suspension (2×10^6 -cells/ml) was labeled with myo-[^{3}H] inositol (15 μCi/ml) for 90 min at 31 °C, and then, cells were centrifuged at $350\times g$ for 10 min to remove excess [^{3}H]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 labeled with [^{3}H]myo-inositols (1.5 μCi/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-1X8 resin (100–200 mesh in formate form) and distilled water. Total inositol phosphates were eluted with 6 ml of 0.8 M ammonium formate–0.1 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 milliliter (3×10^6 cells/ml) of cell suspension was treated with agonist in the presence of 100 μM isobutylmethylxanthine, either alone or in combination with CPA (1 μM). The reaction was terminated with 6 % cold trichloroacetic acid (v/v) and the supernatants were extracted three times with water-saturated diethyl ether to remove the tricholoroacetic acid and the samples were then lyophilized and frozen at −20 °C. The samples were reconstituted for radioimmunoassay in 50 μl 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-μl 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-β3 and RGS2

Protein phosphorylation was determined from the amount of ^{32}P incorporated into each protein after immunoprecipitation with specific antibody to PLC-β3 or RGS2 as previously described [27]. Freshly dispersed cells were incubated with [^{32}P]Pi for 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 μl/ml). Cell lysates were separated by centrifugation at $13{,}000\times g$ for 10 min at 4 °C, precleared with 40 μl of protein A-Sepharose, and incubated with PLC-β3 or RGS2 antibody for 2 h at 4 °C and with 40 μl of protein A-Sepharose for another 1 h. 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-β3 or RGS2 was visualized by autoradiography.

$G\alpha_{i3}$: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 °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 $15{,}000\times g$ 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 $G\alpha_{i3}$. 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 $^{45}Ca^{2+}$ (10 µCi/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) was added 10 min before CPA. The decrease in $^{45}Ca^{2+}$ 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 ± SE 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.

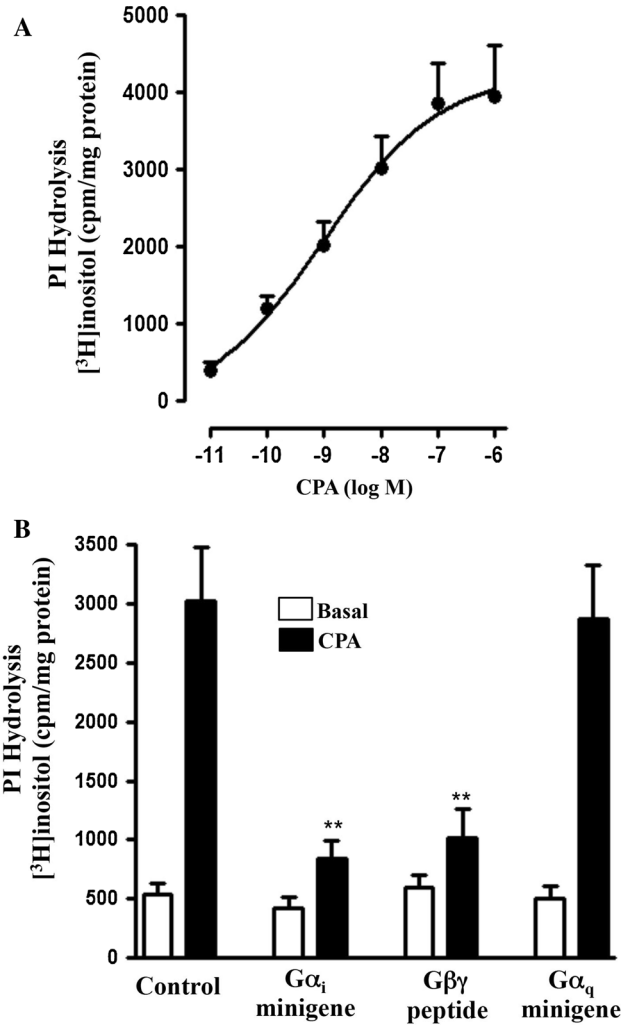

Fig. 1 Stimulation of PI hydrolysis by CPA via $G\beta\gamma_i$. **a** Freshly dispersed cells labeled with myo[^{3}H]inositol were treated with different concentrations of CPA for 1 min. PI hydrolysis was measured as increase in the water-soluble inositol phosphates by ion-exchange chromatography. Results are expressed as cpm/mg protein above basal levels of 623 ± 84 cpm/mg protein. **b** Cultured muscle expressing control vector, $G\alpha_i$ minigene, $G\alpha_q$ minigene, or carboxyl-terminal sequence of GRK2(495–689), a $G\beta\gamma$-scavenging peptide ($G\beta\gamma$ peptide), were labeled with myo[^{3}H]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 mean ± SE of 4 experiments. $^{**}p < 0.01$ significant inhibition compared to response to CPA in control cells

Results

Stimulation of PI hydrolysis by Cyclopentyl Adenosine (CPA) Via $G\beta\gamma_i$

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 ± 1 nM) (Fig. 1a). A maximal stimulation of 3956 ± 654

above basal levels of 623 ± 84 cpm/mg protein was obtained at 1 μM 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-β1 via $G\alpha_q$, whereas activation of A_1 receptors stimulates PLC-β3 isoform via Gβγ [4, 5, 7]. The involvement of Gβγ 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 Gβγ-scavenging peptide. CPA (1 μM) caused stimulation of PI hydrolysis in cultured muscle cells which was similar to that in dispersed muscle cells. CPA-induced stimulation of PI hydrolysis was significantly inhibited in cells expressing $G\alpha_i$ minigene (76 ± 5 % inhibition, $p < 0.01$) or Gβγ peptide (81 ± 6 % inhibition, $p < 0.01$), but not in cells expressing $G\alpha_q$ minigene (3 ± 5 % inhibition, NS) (Fig. 1b). Control studies showed that stimulation of PI hydrolysis in response to $G\alpha_q$-coupled m3 receptor activation by acetylcholine was significantly inhibited (78 ± 7 %, $p < 0.01$) in cells expressing $G\alpha_q$ minigene (485 ± 32 % increase in PI hydrolysis in control cells and 75 ± 6 % increase in cells expressing $G\alpha_q$ minigene). Previous studies have shown that adenosine-stimulated PI hydrolysis in muscle membranes was selectively blocked by $G\alpha_{i3}$- and Gβγ-specific antibodies, as well as by a PLC-β3-specific antibody, but not by antibodies to other PLC-β isoforms or G protein subunits, suggesting activation of PLC-β3 via $G\beta\gamma_i$ [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 μM)-induced PI hydrolysis in a concentration-dependent manner (Fig. 2a). Maximal inhibition induced by GSNO (87 ± 6 % inhibition) was significantly higher than isoproterenol (53 ± 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 Gs/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 μM)-induced PI hydrolysis in a concentration-dependent manner (Fig. 2b). Maximal inhibition induced by 8-pCPT-cGMP (80 ± 4 % inhibition) was significantly higher than cBIMPS (56 ± 5 % inhibition) confirming the results with isoproterenol and GSNO.

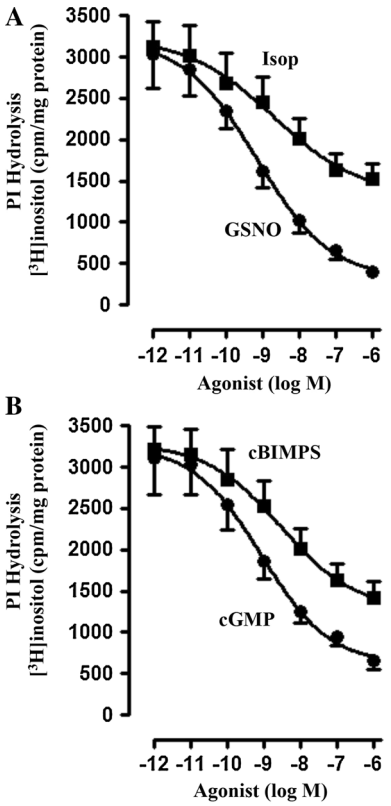

Fig. 2 Inhibition of PI hydrolysis by cAMP-(PKA) and cGMP-dependent (PKG) protein kinases. Freshly dispersed cells labeled with myo[^{3}H]inositol were treated with different concentrations of PKA activators (isoproterenol (Isop) or cBIMPS) or PKG activators (GSNO or 8-pCPT-cGMP (cGMP)) for 10 min and then treated with CPA (1 μM) 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 (3,381 ± 502–3,425 ± 506 cpm/mg protein) above basal levels (589 ± 84–681 ± 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 mean ± SE of 4–5 experiments

GSNO (10 μM)-induced inhibition of PI hydrolysis was blocked by a selective inhibitor of PKG, Rp-cGMPS (1 μM), but was not affected by the PKA inhibitor, myristoylated PKI 14–22 amide (1 μM) (Fig. 3a). In contrast, isoproterenol (10 μM)-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

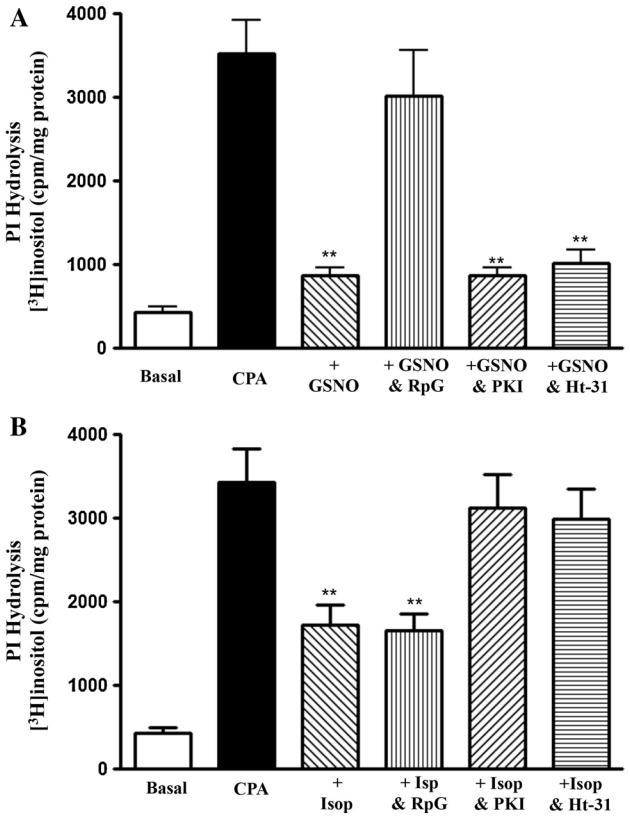

Fig. 3 Inhibition of CPA-induced PI hydrolysis by cAMP-(PKA) and cGMP-dependent (PKG) protein kinases. Freshly dispersed cells labeled with myo[^{3}H]inositol were incubated with GSNO (10 μM) or isoproterenol (10 μM) for 10 min in the presence of PKA inhibitor, PKI 14–26 amide (PKI, 1 μM), PKG inhibitor, 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 mean ± SE of 4–5 experiments. $^{**}P < 0.001$ significant inhibition of CPA-induced PI hydrolysis

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

Phosphorylation of PLC-β3 and RGS2 by PKA and PKG

Both GSNO (10 μM) and isoproterenol (10 μM) phosphorylated PLC-β3 in dispersed muscle cells (Fig. 4).

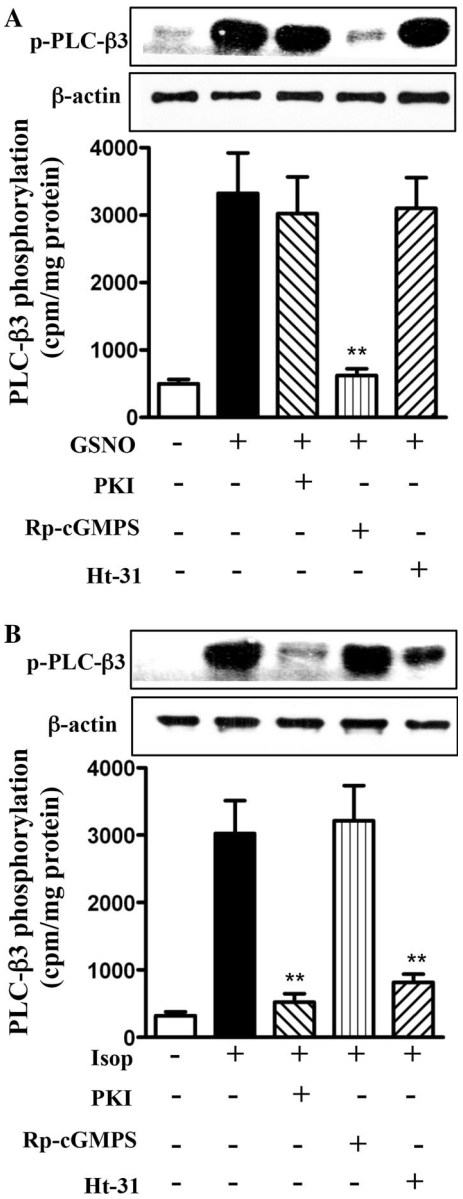

Fig. 4 Phosphorylation of PLC-β3 by PKA and PKG. Muscle cells labeled with ^{32}P were incubated for 10 min with isoproterenol (Isop, 10 μM) or GSNO (10 μM) in the presence or absence of selective inhibitors of PKA (myristoylated PKI, 1 μM), PKG (Rp-cGMPS, 1 μM), or AKAP–PKA interaction (Ht-31, 10 μM). 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 ± SE of 4 experiments. $^{**}P < 0.01$ significant inhibition of GSNO- or isoproterenol-induced PLC-β3 phosphorylation

Phosphorylation of PLC-β3 by GSNO was blocked by Rp-cGMPS but was not affected by myristoylated PKI or Ht-31 (Fig. 4a). In contrast, phosphorylation of PLC-β3 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

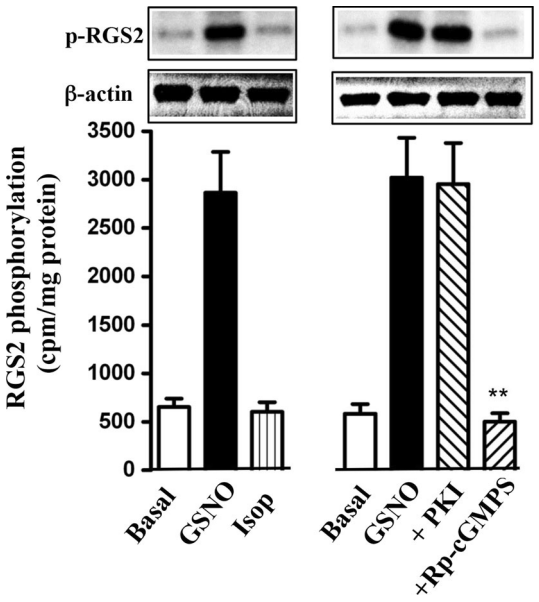

Fig. 5 Selective phosphorylation of RGS2 by PKG. Muscle cells labeled with ^{32}P were incubated for 10 min with isoproterenol (Isop, 10 μM) or GSNO (10 μM) in the presence or absence of selective inhibitors of PKA (myristoylated PKI, 1 μM), or PKG (Rp-cGMPS, 1 μM). RGS2 immunoprecipitates were separated on SDS-PAGE, ^{32}P-labeled RGS2 (p-RGS2) was identified by autoradiography, and radioactivity was expressed as cpm/mg protein. Values are mean ± SE of 4 experiments. $^{**}p < 0.01$ significant inhibition of GSNO-induced RGS2 phosphorylation

Ht-31 and GSNO-induced inhibition of PI hydrolysis by Rp-cGMPS.

GSNO, but not isoproterenol, also phosphorylated RGS2, an activator of Gα-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 $G\alpha_{i3}$.GTP leading to rapid hydrolysis and inactivation of $G\alpha_{i3}$ 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 ± 45 % increase) the PI hydrolysis in response to CPA compared to cells expressing control vector (728 ± 15 % 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 Gβγ-mediated PI hydrolysis (Fig. 6a). GSNO-induced inhibition of PI hydrolysis was significantly attenuated by RGS2 siRNA (48 ± 3 % inhibition vs. 85 ± 6 % inhibition in control cells), suggesting that

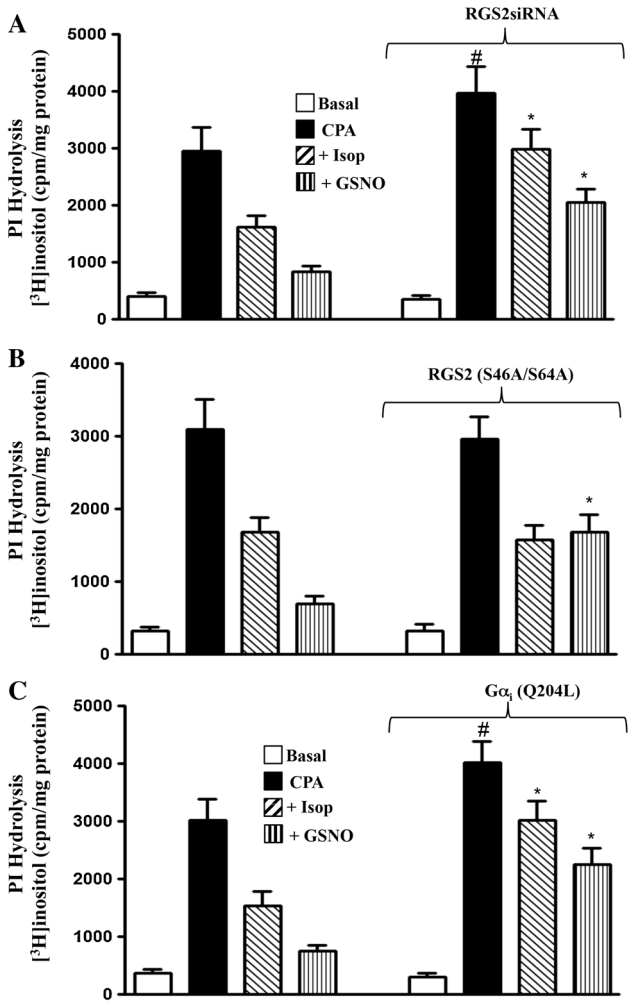

Fig. 6 Inhibition of PI Hydrolysis by PKG via RGS2 Phosphorylation and increase in $G\alpha_{i3}$.GTPase activation. Cultured muscle cells expressing RGS2 siRNA (**a**), phosphorylation-deficient RGS2(S46A/S64A) (**b**), and $G\alpha_i$-GTPase-resistant $G\alpha_i$(Q240L) (**c**) were labeled with myo[^{3}H]inositol. 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 mean ± 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

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 ± 3 % inhibition vs. 56 ± 4 % 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 ± 58 % increase vs. 853 ± 38 % increase in control cells) (Fig. 6b). These results suggest that stimulation of

Gαi-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 ± 5 % inhibition) compared to control cells (84 ± 8 % 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 ± 3 % inhibition vs. 53 ± 6 % 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 $G\alpha_i$ mutant (Q204L) significantly augmented (1,016 ± 24 % increase) the PI hydrolysis in response to CPA compared to cells expressing control vector (634 ± 28 % increase) (Fig. 6c). The increase reflects augmentation of Gβγ-dependent PI hydrolysis due to inhibition of GTP hydrolysis and resultant Gαβγ trimer formation. Inhibition of PI hydrolysis by GSNO was significantly attenuated in cells expressing $G\alpha_{i3}$(Q204L) (50 ± 4 % inhibition) compared to control cells (75 ± 5 % 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 ± 3 % inhibition) compared to control cells (53 ± 5 % 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 ± 35 % increase above basal levels of 2.62 ± .031 pmol/mg protein) was significantly inhibited (43 ± 3 %) in the presence of CPA (452 ± 21 % increase) (Fig. 7). Expression of $G\alpha_i$(Q204L) had no effect on isoproterenol-stimulated cAMP formation (824 ± 28 % increase), but the inhibition by CPA was augmented (74 ± 5 % 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 ± 42 % increase), but the inhibition by CPA was augmented (78 ± 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 ± 42 % increase) or inhibition (45 ± 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 of PI hydrolysis by isoproterenol.

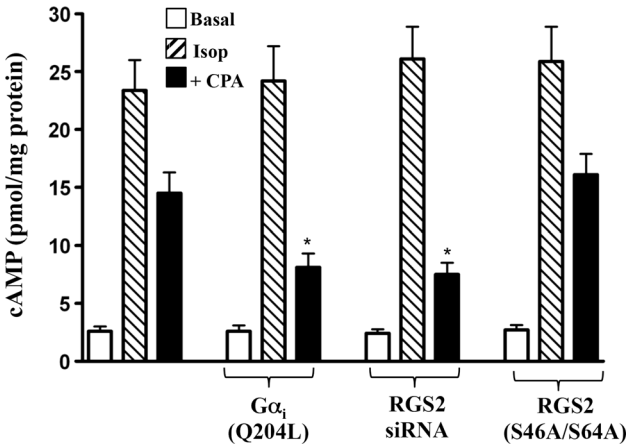

Fig. 7 Activation of $G\alpha_{i3}$ by CPA is regulated by $G\alpha_i$-GTPase-activating protein RGS2. Cultured muscle cells expressing $G\alpha_i$-GTPase-resistant $G\alpha_i$(Q240L), RGS2 siRNA, or phosphorylation-deficient RGS2(S46A/S64A) were treated with isoproterenol (Isop, 10 μM) in the presence 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 mean ± SE of 4–5 experiments. *$p < 0.05$ significant augmentation of CPA-induced inhibition of cAMP formation compared to response in control cells

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.

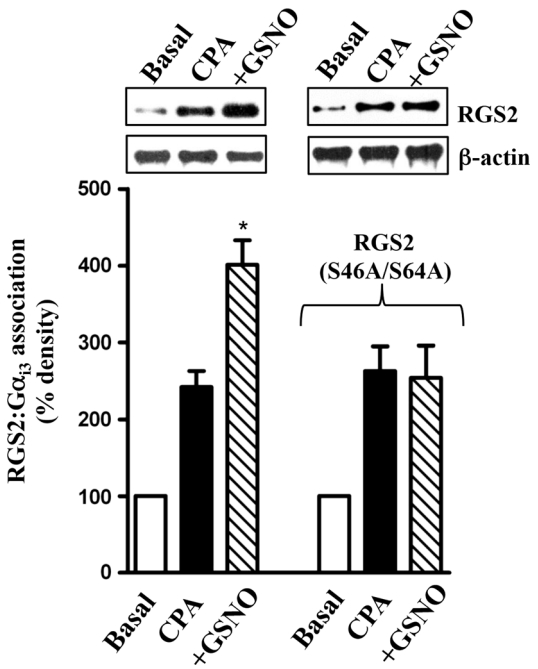

Fig. 8 Phosphorylation of RGS2 by PKG increases its association with $G\alpha_{i3}$-GTP. Smooth muscle cells expressing wild-type RGS2 or phosphorylation-deficient RGS2(S46A/S64A) were treated with CPA (1 μM) in the presence or absence of GSNO (10 μM). Immunoprecipitates derived from 500 μg of proteins using $G\alpha_{i3}$ 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-$G\alpha_{i3}$ by GSNO

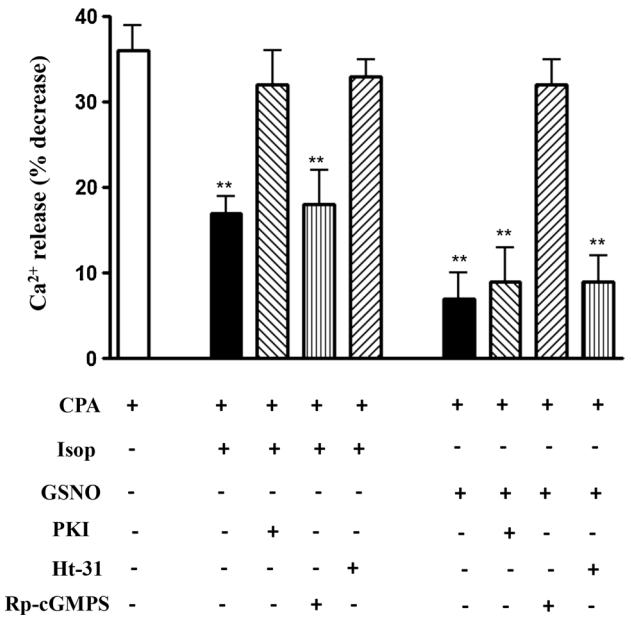

Fig. 9 Inhibition of CPA-induced Ca^{2+} release by PKA and PKG. Dispersed muscle cells were incubated with $^{45}Ca^{2+}$ (10 μCi/ml) for 90 min to obtain steady-state Ca^{2+} levels, and then, CPA (1 μM) was added and the reaction was terminated after 30 s. In some experiments, cells were pretreated with GSNO (10 μM) or isoproterenol (Isop, 10 μM) for 10 min in the presence or absence of inhibitors of PKA (PKI, 1 μM), PKG (Rp-cGMPS, 1 μM), or AKAP–PKA interaction (Ht-31, 10 μM). The decrease in $^{45}Ca^{2+}$ content, representing net Ca^{2+} efflux, was expressed as decrease in steady-state Ca^{2+} levels (2.23 ± 0.31 nmoles/10^6cells). $**p < 0.001$ significant inhibition of CPA-induced Ca^{2+}release

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

CPA-induced activation of PLC-β3 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 ± 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 ± 4 % inhibition) and isoproterenol (53 ± 4 % inhibition) (Fig. 9). Inhibition of CPA-induced Ca^{2+} release by GSNO and isoproterenol could reflect inhibition of PLC-β3 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 ± 4 % inhibition) or Ht-31 (14 ± 2 % inhibition), but was not affected by Rp-cGMPS (48 ± 2 % inhibition) (Fig. 9). In contrast, GSNO-induced inhibition of Ca^{2+} release was blocked by Rp-cGMPS (7 ± 4 % inhibition), but was not affected by myristoylated PKI (74 ± 3 % inhibition) or Ht-31 (71 ± 6 % inhibition) (Fig. 9).

Increase in cytosolic Ca^{2+} in smooth muscle causes stimulation of Ca^{2+}/calmodulin-dependent MLC kinase activity and increases MLC_{20} 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 ± 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 %

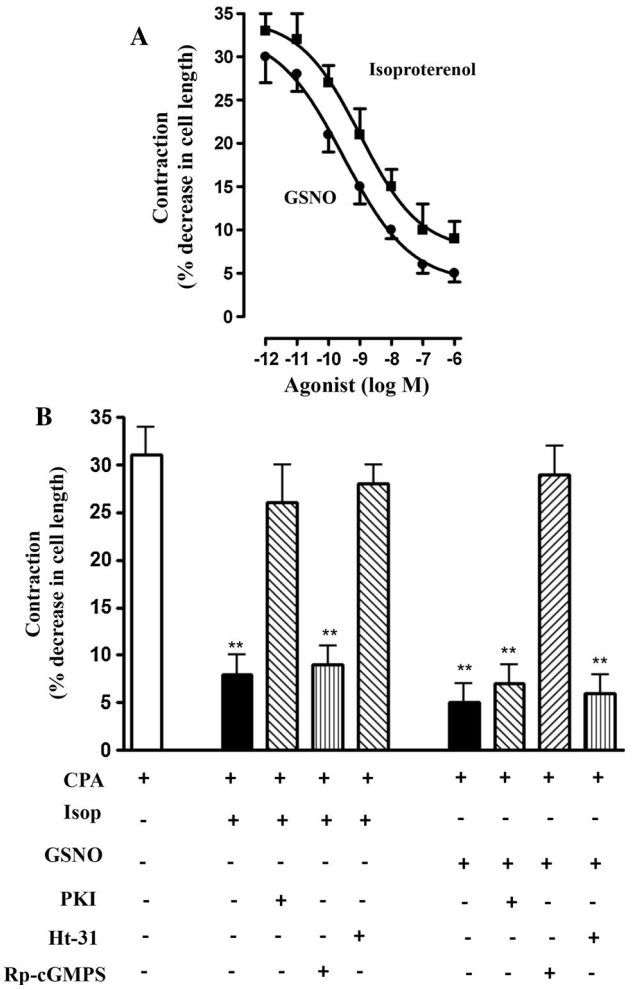

Fig. 10 Inhibition of CPA-induced contraction by PKA and PKG. **a** Dispersed muscle cells were incubated with different concentrations of GSNO or isoproterenol (Isop) for 10 min and then treated with CPA for 30 s. Contraction was measured by scanning micrometry as decrease in cell length and expressed as percent decrease from the basal length. CPA caused a significant decrease in cell length (32 ± 3 % decrease) from the basal length of 105 ± 6 μm. GSNO and isoproterenol inhibited CPA-induced contraction in a concentration-dependent manner. **b** Muscle cells were treated with isoproterenol (Isop, 10 μM) or GSNO (10 μM) in the presence or absence of selective inhibitors of PKA (PKI, 1 μM), PKG (Rp-cGMPS, 1 μM), or AKAP–PKA interaction (Ht-31, 10 μM) 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

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 $G\alpha_q$ or PLC-β3 via $G\beta\gamma_i$ in response to contractile agonists results in the generation of

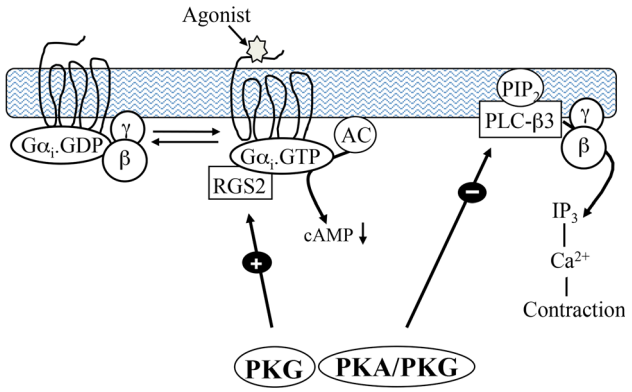

Fig. 11 Mechanism for inhibition of Gβγ-mediated PLC-β3 by PKA and PKG. Both PKA and PKG phosphorylated PLC-β3 leading to inhibition of PI hydrolysis (PLC-β3 activity), IP_3 generation, IP_3-dependent Ca^{2+} release, and muscle contraction. In addition, PKG, but not PKA, phosphorylated RGS2 leading to stimulation of intrinsic $G\alpha_i$-GTPase activity and termination of α-mediated inhibition of adenylyl cyclase (AC) activity and βγ-mediated stimulation of PI hydrolysis

inositol 1,4,5-trisphosphate (IP_3), IP_3-dependent release of Ca^{2+}, activation of Ca^{2+}/calmodulin-dependent activation of MLC kinase, and phosphorylation of MLC_{20}, 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 Ca^{2+} mobilization leading to inhibition of MLC kinase activity and MLC_{20} 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 Ca^{2+}. We have identified 3 such targets in the Ca^{2+}-dependent contraction: (i) RGS4, which accelerates inactivation of $G\alpha_q$-GTP and termination of $G\alpha_q$ signaling [27]; (ii) IP_3 receptor 1 (IP_3 R-I), which regulates Ca^{2+} release from sarcoplasmic stores in response to IP_3 generation [49, 50]; and (iii) phospholamban, which regulates sarco-endoplasmic reticulum Ca^{2+} pump (SERCA) activity [51]. Neither $G\alpha_q$ nor PLC-β1 is phosphorylated by PKA or PKG [27, 52, 53]. Phosphorylation of RGS4 by both PKA and PKG leads to increase in $G\alpha_q$-GTPase activity and decrease in PLC-β1 activity and IP_3 generation. Phosphorylation of IP_3RI by PKG, but not PKA, leads to decrease in the IP_3-mediated Ca^{2+} release, whereas phosphorylation of phospholamban by PKG leads to increase in SERCA activity and Ca^{2+} sequestration. PKA and PKG are also shown to regulate plasmalemmal Ca^{2+} and K^+ channels [54, 55]. Phosphorylation of one or more of these targets should lead to a decrease in cytosolic Ca^{2+} and Ca^{2+}-dependent muscle contraction (Fig. 11).

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 A_1, opioid μ, δ, κ, and somatostatin sst_3) coupled to G_i and mobilize Ca^{2+} via activation of PLC-β3 by $G\beta\gamma_i$ [6–9]. In the present study, we have shown that, unlike PLC-β1, PLC-β3 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 $G\alpha_{i3}$-GTP, accelerates inactivation of $G\alpha_{i3}$, and trimer formation and thus, terminates both $G\alpha_i$-dependent inhibition of adenylyl cyclase activity and $G\beta\gamma_i$-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 of PKG activators was selectively blocked by PKG inhibitor, Rp-cGMPS; and (iii) PKG, but not PKA, also phosphorylated $G\alpha_i$-GTPase-activating protein RGS2 and enhanced its association with $G\alpha_{i3}$-GTP leading to inactivation of $G\beta\gamma_i$-dependent PI hydrolysis. The notion that PKG-induced phosphorylation of RGS2 results in the inhibition of $G\beta\gamma_i$-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. Inhibition of CPA-induced PI hydrolysis by GSNO was partly reversed in cells expressing $G\alpha_{i3}$(Q204L), RGS2 (S46A/S64A), or RGS2 siRNA providing evidence that PKG-mediated phosphorylation of RGS2 accelerates $G\alpha_i$-GTPase activity and inhibits $G\beta\gamma_i$-dependent PLC-β3 activity. Increase in $G\alpha_i$-GTP hydrolysis causes termination of Gβγ signaling, because the regions of Gβγ that interact with effectors such as PLC-β3 and Gα overlap and Gβγ interacts with either Gα or PLC-β3, but not with both simultaneously [18].

The ability of PKA to phosphorylate PLC-β3 and inhibit its activity was reported in previous studies [20–22]. Phosphorylation of PLC-β3 at Ser1105 was shown to inhibit PLC-β3 activation by $G\alpha_q$ without affecting activation by $G\beta\gamma_i$ suggesting that PKA inhibits $G\beta\gamma_i$-dependent activity via a distinct mechanism that does not involve phosphorylation at Ser1105. Gβγ interacts with more than one region of PLC-β3 and differs from the $G\alpha_q$, which interacts with C-terminus of PLC-β3 [18]. Possible involvement of phosphorylation sites in the N-terminal region of PLC-β3 was also examined and found to be that one of the potential sites Ser26 was without any effect on stimulation of PLC-β3 activity by Gβγ [21, 22]. Thus, the identification of sites of phosphorylation and mechanism of inhibition of Gβγ-stimulated PLC-β3 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-β3 phosphorylation and inhibition of its activity by Ht-31 suggest that PKA-induced phosphorylation of PLC-β3 and inhibition of CPA-induced PLC-β3 activity require the association of PLC-β3 with AKAP [56].

Both $G\alpha_q$- and Gβγ-stimulated PLC-β activity is terminated by intrinsic GTPase activity of Gα 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 Gα 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 Gβγ-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-Iα, 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 $G\alpha_i$ [57, 58]. 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 $G\alpha_i$ [57]. RGS2-mediated suppression of $G\alpha_{i/o}$ was also reported in previous studies: it regulates presynaptic Ca^{2+} channels via Gβγ subunits derived from $G\alpha_{i/o}$ in hippocampal neurons [59], carbachol-stimulated ERK and Akt activity via $G\alpha_{i/o}$ in COS cells [60], and parathyroid hormone-stimulated cAMP function by RGS2 via $G\alpha_s$ in osteoblasts [61]. RGS2 protein, in addition, can bind effector enzymes such as adenylyl cyclase to regulate

their activity despite lacking GAP activity toward $G\alpha_s$ [57]. 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 Gβγ-dependent PI hydrolysis to mediate muscle relaxation. Both PKA and PKG phosphorylate PLC-β3. 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 $G\alpha\beta\gamma$. 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 vivo PKG, but not PKA, phosphorylates IP_3R-I and inhibits Ca^{2+} release [50]. Although phosphorylation of IP_3R-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-β3 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-β3 phosphorylation and RGS2 phosphorylation to inhibit IP_3 formation underscores this proximal step in mediating relaxation.

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