Heterologous desensitization of response mediated by selective PKC-dependent phosphorylation of $G_{i-1}$ and $G_{i-2}$

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Murthy, K. S., J. R. Grider, and G. M. Makhlouf. Heterologous desensitization of response mediated by selective PKC-dependent phosphorylation of $G_{i-1}$ and $G_{i-2}$. Am J Physiol Cell Physiol 279: C925–C934, 2000.—This study examined the ability of protein kinase C (PKC) to induce heterologous desensitization by targeting specific G proteins and limiting their ability to transduce signals in smooth muscle. Activation of PKC by pretreatment of intestinal smooth muscle cells with phorbol 12-myristate 13-acetate, cholecystokinin octapeptide, or the phosphatase 2A inhibitor, calyculin A, selectively phosphorylated $G_{i-1}$ and $G_{i-2}$ but not $G_{o}$ or $G_{z}$, and blocked inhibition of adenylyl cyclase mediated by somatostatin receptors coupled to $G_{z}$, and opioid receptors coupled to $G_{i-2}$, but not by muscarinic $M_2$ and adenosine $A_1$ receptors coupled to $G_{i-2}$. Phosphorylation of $G_{o}$ and $G_{z}$ and blockage of cyclase inhibition were reversed by calphostin C and bisindolylmaleimide, and additionally by selective inhibitors of PKCa and PKC. Blockade of inhibition was prevented by downregulation of PKC. Phosphorylation of Go-subunits by PKC also affected responses mediated by $\beta$-subunits. Pretreatment of muscle cells with cAMP (4–23), a selective agonist of the natriuretic peptide clearance receptor, NPR-C, which activates phospholipase C (PLC)-$\beta$3 via the $\beta_3$-subunits of $G_{i-1}$ and $G_{i-2}$, inhibited the PLC-$\beta$ response to somatostatin and [d-Pen2,5]enkephalin. The inhibition was partly reversed by calphostin C. Short-term activation of PKC had no effect on receptor binding or effector enzyme (adenylyl cyclase or PLC-$\beta$) activity. We conclude that selective phosphorylation of $G_{o}$ and $G_{z}$ by PKC partly accounts for heterologous desensitization of responses mediated by the $\alpha$- and $\beta$-subunits of both G proteins. The desensitization reflects a decrease in reassociation and thus availability of heterotrimeric G proteins.

G proteins; smooth muscle; signal transduction
decreasing the availability of the heterotrimeric G protein in the plasma membrane. The present study examined whether \( G_{i1}, G_{i2}, G_{i3}, \) and \( G_o \) were targets of phosphorylation by PKC in intestinal smooth muscle, and whether phosphorylation of one or more of these G proteins resulted in heterologous desensitization. The results indicate that \( G_{i1} \) and \( G_{i2} \), but not \( G_{i3} \) or \( G_o \), are phosphorylated by PKC, resulting in attenuation of subsequent responses mediated by both G proteins.

**MATERIALS AND METHODS**

*Preparation of dispersed smooth muscle cells.* Smooth muscle cells were isolated from the circular muscle layer of rabbit intestine by sequential enzymatic digestion, filtration, and centrifugation as described previously (23–26). Aliquots (0.5 ml) containing 10⁶ cells/ml were incubated with various agents, and the reaction was terminated after 60 s with 60% cold trichloroacetic acid (vol/vol). The partly digested strips were washed with 100 ml of enzyme-free medium, and the muscle cells were released by disperse spontaneously for 30–60 min. The cells were harvested by filtration through a 500-μm Nitex mesh followed by two 10-min centrifugations at 350 g.

*Measurement of cAMP in dispersed smooth muscle cells.* cAMP was measured in dispersed cells by radioimmunoassay as described previously (23, 24, 26). Aliquots (0.5 ml) containing 10⁶ cells/ml were incubated with various agents, and the reaction was terminated after 60 s with 60% cold trichloroacetic acid (vol/vol). The supernatant was extracted three times with 2 ml of diethyl ether and lyophilized. The samples were reconstituted for radioimmunoassay in 500 μl of 50 mM sodium acetate (pH 6.2) and acetylated with triethylamine/acetic acid (vol/vol). The mixture was centrifuged at 2,000 g for 15 min at 4°C. The supernatant was extracted three times with 2 ml of diethyl ether and lyophilized. The samples were reconstituted for radioimmunoassay in 500 μl of 50 mM sodium acetate (pH 6.2) and acetylated with triethylamine/ acetic anhydride (2:1 vol/vol) for 30 min. cAMP was measured in duplicate with the use of 100-μl aliquots and expressed as pmol/10⁶ cells.

*Measurement of adenyl cyclase activity in muscle membranes.* Smooth muscle cells were incubated with phorbol 12-myristate 13-acetate (PMA, 1 μM) for 10 min, and a crude homogenate was prepared in 50 mM Tris·HCl (pH 7.4), 1 mM ATP, 2 mM cAMP, 100 μM isobutyl methyl xanthine, 5 mM MgCl₂, 100 mM NaCl, 5 mM creatine phosphate, and 50 μM creatine kinase. Adenyl cyclase activity was measured in the presence of [³²P]ATP and 1 mM GTP by an adaptation of the method of Salomon et al. (38). The reaction was terminated after 15 min by addition of stop solution containing 2% SDS, 45 mM ATP, and 1.5 mM cAMP. [³²P]cAMP was separated by sequential chromatography on Dowex AG50W-4X and alumina columns. The samples were measured by scintillation counting, and the results were expressed as picomoles of cAMP per milligram of protein per minute.

*Measurement of inositol phosphates.* Total inositol phosphate formation in smooth muscle cells was measured by ion-exchange chromatography as described previously (31). Ten milliliters of cell suspension (2×10⁶ cells/ml) were labeled with myo-[³H]inositol (15 μCi/ml) for 3 h at 31°C. After centrifugation at 350 g for 10 min, the cells were resuspended in 10 ml of fresh HEPES medium. After treatment with a cAMP (4–23) for 10 min, the cells were centrifuged at 350 g for 5 min. [β-²⁵¹]enkephalin (DPDPE), somatostatin, or cyclopentyladenosine (CPA) was then added to 0.5 ml of cell suspension and the samples incubated for 30 s. The reaction was terminated with 940 μl of chloroform:methanol:HCl (50: 100:1, vol/vol/vol). After addition of chloroform (310 μl) and water (310 μl), the samples were vortexed, and the phases were separated by centrifugation at 1,000 g for 15 min. The upper aqueous phase was applied to a column that contained 1 ml of 1:1 slurry of Dowex AG1-X8 resin (100–200 mesh in formate form) and distilled water. The column was washed with 10 ml of water followed by 10 ml of 5 mM sodium tetraborate-60 mM ammonium formate. Inositol phosphates were eluted with 5 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 (cpm)/10⁶ cells or percentage increase above basal levels.

**Immunoblotting of [³²P]labeled G subunits.** Ten milliliters of smooth muscle cell suspension (2–3×10⁶ cells/ml) were incubated with [³²P]orthophosphate for 4 h at 31°C. One-milliliter samples were then incubated with PMA for 10 min and microcentrifuged for 2 min. The samples were washed three times with phosphate-buffered saline and incubated in lysis buffer that contained 150 mM NaCl, 50 mM sodium phosphate (pH 7.2), 2 mM EDTA, 1 mM dithiothreitol, 10 mg/ml aprotinin, 0.2 mg/ml leupeptin, 0.5% SDS, 1% sodium deoxycholate, 1% Triton X-100, 5 mM NaF, 2 mM Na₂PO₄, and 2 mM Na₂S/O₃. The protein lysate was boiled for 5 min and incubated on ice for 1 h. The supernatant was preclariﬁed by incubation with 40 μl of protein A-Sepharose for 6 h at 4°C and incubated overnight with Gα₁, Gα₂, Gα₃, or Gα₃ antibody at a final concentration of 4 μg/ml, and then with 40 μl of Protein A-Sepharose for another 1 h. The immunoprecipitates were collected, washed ﬁve times with 1 ml of wash buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM Tris·HCl, pH 7.4), extracted with Laemml buffer, boiled for 5 min, and separated by 12% SDS-PAGE. After transfer to nitrocellulose membranes, [³²P]-labeled G proteins were visualized by autoradiography, and radioactivity was measured and expressed as cpm.

**Agonist-stimulated G protein activity.** Agonist-stimulated G protein activity was measured as previously described (23, 26, 34). Membranes were obtained from control muscle cells, and cells were treated for 10 min with PMA (1 μM). The membranes were incubated at 37°C with 60 nM [³⁵S]GTP·γ·S alone or with somatostatin (1 μM) for 15 min at 37°C. The cell lysate was boiled for 5 min and incubated on ice for 1 h. The supernatant was preclariﬁed by incubation with 40 μl of protein A-Sepharose for 6 h at 4°C and incubated overnight with Gα₁, Gα₂, Gα₃, or Gα₃ antibody at a final concentration of 4 μg/ml, and then with 40 μl of Protein A-Sepharose for another 1 h. The immunoprecipitates were collected, washed ﬁve times with 1 ml of wash buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM Tris·HCl, pH 7.4), extracted with Laemml buffer, boiled for 5 min, and separated by 12% SDS-PAGE. After transfer to nitrocellulose membranes, [³²P]-labeled G proteins were visualized by autoradiography, and radioactivity was measured and expressed as cpm.

**Radioligand binding.** Radioligand binding to dispersed smooth muscle cells was measured as described previously (23). Muscle cells were suspended in HEPES medium containing 1% bovine serum albumin (BSA). For competitive binding, triplicate aliquots (0.5 ml) of cell suspension (10⁶/ml) were incubated for 15 min with 50 pM [¹²⁵I]somatostatin-14 alone or in the presence of unlabeled somatostatin-14 (10 μM). Saturable [¹²⁵I]somatostatin binding was examined with the use of radioligand concentrations in the range of 10–400 pM in the presence or absence of unlabeled somatostatin (10 μM). Bound and free radioligand were separated by rapid filtration under reduced pressure through 5-μm polycarbonate Nucleopore filters followed by repeated washing (4 times) with 3 ml of ice-cold HEPES medium that contained 0.2% BSA. Nonspecific binding was measured as the amount of radioactivity associated with the muscle cells in the presence of 10 μM of unlabeled ligand. Specific binding was calculated as the difference between total and nonspecific binding (24±6%).

**Materials.** [¹²⁵I]somatostatin, [³²P]ATP, [³²P]cAMP, [³²P]H₂PO₄, [³⁵S]GTP·γ·S, and [²⁻³¹]H]inositol were obtained from NEN Life Sciences Products (Boston, MA); Dowex AG50W-4X (Bio-Rad Laboratories, Hercules, CA); and Sigma Chemical Co. (St. Louis, MO). Other reagents were obtained as described previously (23, 31).
AG50W-4X and Dowex AG1-X8 resin were from Bio-Rad (Hercules, CA); and G protein and phosphotyrosine antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Myristoylated peptide inhibitors of PKCα, PKCβγ, PKCe, and PKCδ were gifts from Drs. D. A. Dartt and D. Zoukhri, Harvard Medical School.

RESULTS

Selective phosphorylation of Gαi-1 and Gαi-2 by PKC. The ability of PMA to induce phosphorylation of inhibitory G proteins (Gαi-1, Gαi-2, Gαi-3, and Gαo) was examined in dispersed intestinal smooth muscle cells labeled with 32P. Treatment of the cells with PMA (1 μM) for 10 min caused a significant increase in phosphorylation of Gαi-1 (178 ± 10% above basal; P < 0.001) and Gαi-2 (181 ± 11%; P < 0.001), but not Gαi-3 or Gαo (Fig. 1). The increase in phosphorylation of Gαi-1 and Gαi-2 was time and concentration dependent and was abolished by calphostin C (Figs. 1 and 2). Experiments in which muscle cells were treated with PMA followed by immunoprecipitation with phosphotyrosine antibodies and Western blot with Gαi-1 and Gαi-2 antibodies (n = 3), or immunoprecipitation with Gαo, did not disclose any evidence of tyrosine phosphorylation, implying that PKC phosphorylation of Gαi-1 and Gαi-2 was direct and did not involve tyrosine phosphorylation.

A similar pattern of selective phosphorylation of Gαi-1 and Gαi-2, but not Gαi-3 or Gαo, was observed after a 10-min treatment of smooth muscle cells with cholecystokinin octapeptide (CCK-8; 1 nM), which ac-
tivates Gq/11 and stimulates phosphoinositide hydrolysis and PKC activity (25, 31) (Fig. 1). CCK-induced phosphorylation of Goα1-i and Goα2-i was inhibited by calphostin C and by myristoylated pseudosubstrate peptide inhibitors of various PKC isozymes, including Ca2+-dependent PKCa and Ca2+-independent PKCe; a selective inhibitor of PKCδ had no effect on phosphorylation of either G protein (Fig. 3). The effects of PKCa and PKCe inhibitors were additive.

Treatment of the muscle cells with the phosphatases 1/2A inhibitor, calyculin A (10 μM), also caused a

time-dependent increase in phosphorylation of Goα1-i (211 ± 26%) and Goα2-i (216 ± 14%) that was maximal within 5 min (Fig. 4). Calyculin-induced phosphorylation was abolished by bisindolylmaleimide (1 μM), a competitive inhibitor of the ATP-binding site of PKC.

**Blockade by PMA of agonist-stimulated activation of Go1-i and Go2-i:** The effect of pretreatment with PMA on activation of Go1-i by somatostatin and Go2-i by DPDPE was determined in solubilized smooth muscle membranes. As previously shown (23, 26), somatostatin and DPDPE increased the binding of [35S]GTPγS to
Go$_{i,1}$ and Go$_{i,2}$, respectively. Pretreatment of the cells for 10 min with PMA (1 μM) before membrane isolation inhibited the increase in $[^35$S]GTP$_S$ binding to Go$_{i,1}$ and Go$_{i,2}$ induced by the corresponding agonists (Fig. 5).

**Blockade by PMA of Go$_{i,1}$- and Go$_{i,2}$-mediated inhibition of adenylyl cyclase.** To determine whether phosphorylation was associated with a decrease in signaling by Go$_{i,1}$ and Go$_{i,2}$, smooth muscle cells were treated with PMA for 10 min and then with forskolin (10 μM) for 1 min, either alone or in combination with various agonists. Four agonists were used: somatostatin to activate Go$_{i,1}$ and Gs (23), DPDPE to activate Go$_{i,2}$ and Gs (26), acetylcholine to activate Go$_{i,3}$ (via M2 receptors), and CPA to activate Go$_{i,3}$ (via A1 receptors) (23, 24, 26, 27).

Forskolin-stimulated cAMP (22.2 ± 1.8 pmol/10^6 cells above a basal level of 4.5 ± 0.2) was inhibited 80 ± 5% by somatostatin, 82 ± 2% by DPDPE, 75 ± 4% by CPA, and 76 ± 2% by acetylcholine (Fig. 6). Pretreatment of the cells with PMA (1 μM) had no significant effect on basal (4.4 ± 0.6 pmol/10^6 cells) or forskolin-stimulated (21.3 ± 1.7 pmol/10^6 cells) cAMP levels, but it partially reversed the inhibition induced by DPDPE to 33 ± 5% ($P < 0.01$ from control inhibition) and somatostatin to 43 ± 7% ($P < 0.01$) without affecting inhibition induced by either CPA or acetylcholine (Fig. 6). The effect of PMA was suppressed by calphostin C (1 μM) (Fig. 6). Pretreatment with the inactive phorbol ester, 4α-phorbol, had no significant effect on inhibition of forskolin-stimulated cAMP induced by all four ligands (76 ± 2% to 82 ± 2% inhibition). The reversal of cAMP inhibition mediated by Go$_{i,1}$ and Go$_{i,2}$ paralleled the selective phosphorylation of these two G proteins by PKC. It is worth noting that reversal of cAMP inhibition was partial, reflecting the fact that Go$_i$, which partly mediates the effects of somatostatin and opioid peptides, was not subject to phosphorylation by PKC.

The measurements were repeated in dispersed smooth muscle cells derived from muscle strips incubated for 24 h with 0.1 μM PMA to downregulate PKC. The prolonged treatment with PMA did not affect basal (5.0 ± 0.4 pmol/10^6 cells) or forskolin-stimulated (23.2 ± 1.5 pmol/10^6 cells) cAMP levels, or the inhibition of cAMP induced by DPDPE or somatostatin. Treatment of these cells with 1 μM PMA for 10 min did not reverse the inhibition of cAMP induced by DPDPE (81 ± 4%) or somatostatin (76 ± 3%), providing further support for the notion that blockade of cAMP inhibition was mediated by PKC (Fig. 7).

Control studies were done to determine whether other protein targets (e.g., receptor or adenylyl cyclase) in the signaling pathway besides Go$_{i,1}$ and Go$_{i,2}$ were affected by PKC. Treatment with PMA had no effect on forskolin-stimulated adenylyl cyclase activity in smooth muscle membranes (Fig. 8), or as noted above, on basal and forskolin-stimulated cAMP levels in dispersed smooth muscle cells, consistent with the notion...
that adenylyl cyclase types V/VI expressed in gastrointestinal smooth muscle and directly activated by forskolin is not inhibited by PKC (27). However, adenylyl cyclase activity stimulated by GTP (1 mM) in membranes, which reflected net activation via Gs and inhibition via Gi/Go, was significantly augmented (72 ± 4%; \( P < 0.001 \)) by pretreatment of the cells with PMA, consistent with selective inactivation of one or more inhibitory G proteins by PKC (Fig. 8). Similar results were obtained on treatment of muscle cells with pertussis toxin (200 ng/ml for 60 min; GTP alone: 7.5 ± 0.5 pmol cAMP·mg protein\(^{-1}\)·min\(^{-1}\); GTP after pertussis toxin treatment: 14.0 ± 1.4 pmol cAMP·mg protein\(^{-1}\)·min\(^{-1}\)). Finally, 10-min treatment with PMA (1 \( \mu \)M) had no direct effect on saturation or competitive somatostatin binding to somatostatin receptors on dispersed smooth muscle cells (Fig. 9). A fit of the data to a two-site model yielded dissociation constant (\( K_d \)) values of 0.21 ± 0.03 nM and 12.6 ± 5.8 nM for high and low affinity sites, and \( B_{\text{max}} \) values of 270 ± 65 and 3,225 ± 628 fmol/mg protein. The values were closely similar after treatment with PMA: \( K_d \) 0.23 ± 0.04 and 13.7 ± 6.2 nM; density of binding sites, \( B_{\text{max}} \) 288 ± 54 and 3,408 ± 607 fmol/mg protein.

**Blockade by agonists and phosphatase inhibitors of \( G_{i,1} \)- and \( G_{i,2} \)-mediated inhibition of adenylyl cyclase.** Activation of PKC with CCK-8 elicited similar results to those with PMA. Pretreatment for 10 min with a maximal concentration of CCK-8 (1 nM) partially reversed the inhibition of forskolin-stimulated cAMP induced by DPDPE to 30 ± 8% \( (P < 0.01) \) from control.
inhibition) and somatostatin to 37 ± 8% \((P < 0.01)\) without affecting inhibition induced by either CPA or acetylcholine (Fig. 6). The effect of CCK was suppressed by calphostin C and by myristoylated pseudosubstrate peptide inhibitors of PKC\(\alpha\) and PKC\(\epsilon\), but not PKC\(\delta\) (Figs. 6 and 10) (47). The effectiveness of these inhibitors paralleled their ability to block CCK-induced phosphorylation of G\(_{\alpha_1}\) and G\(_{\alpha_2}\) (Fig. 3).

The role of PKC in reversing the inhibition of cAMP mediated by G\(_{\alpha_1}\) and G\(_{\alpha_2}\) was corroborated by studies with calycin A. Pretreatment of muscle cells with 10 \(\mu\)M calycin A for 10 min partially reversed the inhibition of forskolin-stimulated cAMP induced by DPDPE to 34 ± 3% \((P < 0.01\) from control inhibition) and somatostatin to 45 ± 5%, but not that induced by CPA or acetylcholine (Fig. 11). The effect of calycin A was suppressed by bisindolylmaleimide, which blocks the catalytic site of PKC, but not by calphostin C, which blocks the regulatory diacylglycerol-binding site (Fig. 11).

**Inhibition of G\(_{\alpha_1}\) and G\(_{\alpha_2}\)-mediated phospholipase C-\(\beta\) activity by PKC.** The studies described above disclosed the role of PKC in attenuating inhibition of adenylyl cyclase activity mediated by the \(\alpha\)-subunits of G\(_{\alpha_1}\) and G\(_{\alpha_2}\). We next examined whether G protein phosphorylation by PKC attenuated the ability of the \(\beta\gamma\)-subunits of both G proteins to activate phospholipase C-\(\beta3\) (PLC-\(\beta3\)). Our previous studies had shown that the \(\beta\gamma\)-subunits of G\(_{\alpha_1}\), G\(_{\alpha_2}\), and G\(_{\alpha_3}\) selectively activated PLC-\(\beta3\) in smooth muscle (23–26).

DPDPE caused a significant increase in PLC-\(\beta\) activity (746 ± 29 cpm/10^6 cells [\(^3\)H]inositol phosphates). The PLC-\(\beta\) response was inhibited by 39 ± 7% after activation of PKC by pretreatment of the cells for 10 min with acetylcholine; the inhibition was completely reversed by calphostin C. Similar inhibition of the PLC-\(\beta\) response to DPDPE was observed after stimulation of PKC activity with CCK-8 (46 ± 8%), substance P (35 ± 7%), and CPA (33 ± 8%).

cANP-(4–23), a selective agonist of the natriuretic peptide clearance receptor, NPR-C, was recently shown to activate PLC-\(\beta3\) via the \(\beta\gamma\)-subunits of G\(_{\alpha_1}\) and G\(_{\alpha_2}\) (32, 33). Pretreatment of the cells for 10 min with cANP-(4–23) (1 \(\mu\)M) inhibited the PLC-\(\beta\) response to both DPDPE and somatostatin by 61 ± 6% and 64 ± 7%, respectively; the inhibition was partially reversed by calphostin C to 33 ± 4% and 29 ± 3%, respectively \((P < 0.01\) from control inhibition), implying that it was only partly mediated by PKC (Fig. 12). In contrast, the PLC-\(\beta\) response to CPA was not affected by pretreatment of the cells with cANP-(4–23). The lack of effect of PKC on a response mediated by G\(_{\alpha_3}\) is consistent with the lack of PKC-dependent phosphorylation of this G protein.

**DISCUSSION**

This study demonstrates that PKC-dependent phosphorylation of the \(\alpha\)-subunits of G\(_{\alpha_1}\) and G\(_{\alpha_2}\) in smooth muscle limits the ability of the G proteins to transduce signals. PKC-dependent phosphorylation was confined to G\(_{\alpha_1}\) and G\(_{\alpha_2}\) and was not observed with G\(_{\alpha_3}\) or G\(_{\gamma}\).
Selective phosphorylation of Go_{i-1} and Go_{i-2} resulted in heterologous desensitization of responses mediated by these two G proteins and could contribute to homologous desensitization.

Previous studies had shown that PKC was capable of phosphorylating Go_{i-2} in some cells only; phosphorylation of Go or Go_{11} was not detected, and phosphorylation of Go_{i-3} and Go was not examined (4–6, 22, 40, 45). The present study showed that G_{i-1} and G_{i-2}, but not Go_{i-3} or Go_{o}, were directly phosphorylated by PKC in smooth muscle and not via tyrosine kinase(s).

The role of PKC-dependent phosphorylation in heterologous desensitization of response was demonstrated in this study by activation of PKC with a phorbol ester (PMA) or an agonist (CCK-8), followed by treatment of the cells with agonists (somatostatin, DPDPE, CPA, and acetylcholine) known to activate specific G proteins. Pretreatment of muscle cells with PMA or CCK-8 caused rapid phosphorylation of Go_{i-1} and Go_{i-2}, but not Go_{o} or Go_{i-3}, and reversed the inhibition of adenyl cyclase mediated by somatostatin receptors that couple to G_{i-1} and G_{i-2} (23) and opioid δ-receptors that couple to G_{i-2} and G_{o} (26), but not muscarinic M_{3} and adenosine A_{2} receptors that couple to Go_{i-3} (24, 27). Reversal of the inhibition induced by somatostatin and DPDPE was not complete, reflecting phosphorylation of Go_{i-1} and Go_{i-2}, but not Go_{o}. Suppression of dephosphorylation with calycin A increased phosphorylation of Go_{i-1} and Go_{i-2} but not Go_{i-3}; the increase in phosphorylation of Go_{i-1} and Go_{i-2} was inhibited by bisindolylmaleimide, which blocks the ATP-binding site of PKC, implying that phosphorylation was mediated by receptor-independent, endogenous PKC activity normally contained by endogenous phosphatase activity.

In vitro studies on isolated α-subunits of various G proteins have shown that Go_{12}, a ubiquitous G protein, and Go_{2}, which is predominantly expressed in platelets and neurons, are readily phosphorylated by PKC (11, 16, 19, 20). The functional consequences of phosphorylation were alluded to but were not examined in situ. The primary sites of Go_{2} and Go_{12} phosphorylation (Ser^{27} and Ser^{38}) are located in the NH2-terminal domain of Go that determines the binding of α- and βγ-subunits; phosphorylation of these residues impedes the reassociation of α- and βγ-subunits (11, 16). Ser^{16} in Go_{i-1} and Go_{i-2} is analogous to Ser^{16} in Go_{2} and Ser^{38} in Go_{12}; its phosphorylation might, therefore, be expected to impede reassociation of α- and βγ-subunits (11, 16). Furthermore, the rate of GTP/GDP exchange in G_{i-1} and G_{i-2} is more rapid than in G_{12} and G_{2}. This makes it possible for PKC, whether activated by a phorbol ester or an agonist, to phosphorylate dissociated α-subunits, impede the reassociation of α- and βγ-subunits, and reduce the availability of the trimeric species for subsequent activation by other receptors, thereby leading to heterologous desensitization of responses mediated by a specific G protein. As shown in Fig. 5, treatment with PMA decreased the ability of somatostatin and DPDPE to activate Go_{i-1} and Go_{i-2}, respectively, reflecting a decrease in the availability of the trimeric species.

The decrease in the availability of G proteins that results from phosphorylation of Go_{i-1} and Go_{i-2} also led to a decrease in responses mediated by βγ-subunits. The notion was tested with the NPR-C agonist, cANP(4–23), which selectively activates G_{i-1} and G_{i-2} and stimulates PLC-β activity via the βγ-subunits of both G proteins (32, 33). Treatment of smooth muscle with cANP-(4–23) inhibited the PLC-β response to subsequent activation of Go_{i-1} by somatostatin or activation of Go_{i-2} by DPDPE. The PLC-β response was only partly restored when PKC activity was blocked with calphostin C. We postulate that residual inhibition reflects sequestration of Go_{i-1} and Go_{i-2} by binding to caveolin-3 (8, 17, 29). In contrast, inhibition of the PLC-β response to DPDPE after treatment of muscle cells with acetylcholine (that activates Go_{i-1} and Go_{i-2} via M_{3} and M_{2} receptors, respectively) could be fully restored by calphostin C, because under these conditions, phosphorylation but not caveolin sequestration of Go_{i-1} and Go_{i-2} would be induced.

No evidence was obtained to suggest that other protein targets in the signaling pathways initiated by somatostatin or DPDPE (e.g., receptors or effector enzymes) were affected by PKC. Adenyl cyclase type V/VI expressed in gastrointestinal smooth muscle is not a PKC substrate (27, 41), and its activity was not affected by pretreatment with PMA or agonists. However, GTP-stimulated adenyl cyclase activity, which reflected the balance of activation via Go and inhibition via G_{o}, was significantly augmented by PKC consistent with selective inactivation or reduction in the availability of Go_{i-1} and Go_{i-2}. Although PKC-dependent phosphorylation of somatostatin and opioid receptors has been reported in some cells (14, 43), no effect was detected on the affinity or density of somatostatin receptors after treatment of muscle cells with PMA.
Where the phosphoinositide pathway was concerned, prior activation of PKC inhibited PLC-β3 activity stimulated by somatostatin-3 and opioid δ-receptors coupled to Gβγ1,1 and Gβγ1,2 (23, 26, and this study), but had no effect on PLC-β3 activity stimulated by A1 and M2 receptors coupled to Gβγ1,3 (24, 27, 29). The pattern implied that inhibition of PLC-β activity by PKC in smooth muscle was G protein specific and was not exerted directly on PLC-β isozymes. This is in contrast to other cell types, where PKC was shown to inhibit directly mammalian or avian PLC-β isozymes (12, 39). The results leave open the question of whether phosphorylation by PKC could additionally target RGS proteins and enhance or attenuate their ability to accelerate GTP hydrolysis. Inhibition of response that results from acceleration of GTP hydrolysis by a cognate, phosphorylated RGS may not be readily distinguishable from inhibition that results from phosphorylation of the corresponding G protein.

PKC-dependent, G protein-specific heterologous desensitization could occur physiologically when neurotransmitters are delivered sequentially to target smooth muscle cells. During peristalsis, neurotransmitters (e.g., acetylcholine and tachykinins) that activate PKC could influence the response to other neurotransmitters, such as opioid peptide, which activates G1,2, and vasoactive intestinal peptide, which activates Gs via VPAC2 receptors (42), and Gγ1,1 and Gγ1,2 via NPR-C (33).

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