Sequential activation of heterotrimeric and monomeric G proteins mediates PLD activity in smooth muscle

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Murthy, K. S., H. Zhou, J. R. Grider, and G. M. Makhlouf. Sequential activation of heterotrimeric and monomeric G proteins mediates PLD activity in smooth muscle. Am J Physiol Gastrointest Liver Physiol 280: G381–G388, 2001.—The identity of G proteins mediating CCK-stimulated phospholipase D (PLD) activity was determined in intestinal smooth muscle cells, CCK-8 activated Gq11, G13, and G12, and the monomeric G proteins Ras-homology protein (RhoA) and ADP ribosylation factor (ARF). Activation of RhoA, but not ARF, was mediated by G13 and inhibited by G12 antibody, CCK-stimulated PLD activity was partly mediated by RhoA and could be inhibited to the same extent (47 ± 2% to 53 ± 6%) by 1) a dominant negative RhoA mutant, 2) RhoA antibody or Gα12 antibody, and 3) Clostridium botulinum C3 exoenzyme. PLD activity was also inhibited by ARF antibody, and the effect was additive to that of RhoA antibody or C3 exoenzyme. PLD activity was inhibited by calphostin C, bisindolylmaleimide I, and a selective protein kinase C (PKC)-α inhibitor; the inhibition was additive to that of ARF and RhoA antibodies and C3 exoenzyme. In contrast, activated G12 was not coupled to RhoA or ARF, and Gα12 antibody augmented PLD activity. Thus agonist-stimulated PLD activity is mediated additively by G13-dependent RhoA and by ARF and PKC-α and is modulated by an inhibitory G12-dependent pathway.

G12: protein kinase C; PLD: phospholipase D; intestinal smooth muscle
The existence of pathways involving sequential coupling of heterotrimERIC (G_{13} and G_{12}) and monomeric (RhoA and ARF) G proteins to activation of PLD in smooth muscle has not been determined. In a recent study of cultured vascular smooth muscle, PLD activity induced by angiotensin II was shown to be partly inhibited by RhoA and G_{12}a2 antibodies, as well as by G\beta and pp60^src antibodies, suggesting involvement of G\betaG-Src and G_{12}RhoA pathways (34). In the present study, we examined the roles of G_{13} and G_{12} in activation of RhoA and ARF and of both monomeric G proteins in activation of PLD. CCK-8 was shown to stimulate PLD activity additively via RhoA, ARF, and PKC-\alpha. CCK activated both G_{13} and G_{12}, but only G_{13} was coupled to activation of RhoA, Rho kinase (ROK), and PLD. ARF activation was not mediated by either G_{13} or G_{12}, and activation of G_{12} resulted in inhibition of PLD.

**MATERIALS AND METHODS**

**Dispersion of intestinal smooth muscle cells.** Smooth muscle cells were isolated from the circular muscle layer of rabbit jejunum and reincubated for 40–60 min to allow spontaneous dispersion of muscle cells. The cells were harvested by filtration through 500-\mu m Nitex mesh, centrifuged twice for 10 min at 350 \times 1, and resuspended in HEPES medium containing 1.5 mM ATP and 1.5 mM phosphoenolpyruvate. Muscle cells were homogenized in a solution containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, 2 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 20 \mu g/ml leupeptin, and 20 \mu g/ml aprotinin. The suspension was centrifuged at 100,000 \times g for 30 min at 4°C, and the supernatant was collected as the cytosolic fraction. Pellets were resuspended, and proteins were extracted by incubation for 30 min in the homogenization buffer containing 1% Triton X-100 and 1% sodium cholate. The extract was centrifuged at 1,000 \times g for 10 min, and the supernatant was collected as the particulate fraction. Proteins (80–100 \mu g) were resolved by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After incubation in 5% nonfat dry milk to block nonspecific antibody binding, the blots were incubated first with antibodies to RhoA or ARF and then with secondary antibodies conjugated with horseradish peroxidase. The bands were identified by enhanced chemiluminescence.

**RhoA and ARF activities were measured in muscle cells incubated for 3 h in low-phosphate (0.12 mM Na\textsubscript{2}HPO\textsubscript{4}) buffer containing 10 mM HEPES, 2.5 mM glucose, 1% BSA, and 10 mCi of [\textsuperscript{35}S]PO\textsubscript{4}.** Aliquots (2 \times 10^6) cells were treated with CCK-8 (1 nM) for 10 min, and the reaction was stopped with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 250 mM Na\textsubscript{2}EDTA, 150 mM NaCl, 2 mM EGTA, 10 mM Mg\textsubscript{2}+O\textsubscript{4}, 1 mM Na\textsubscript{2}PO\textsubscript{4}, 1 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1% Triton X-100, 0.5% Nonidet, 1 mM phenylmethylsulfonyl fluoride, 10 \mu g/ml leupeptin, and 20 \mu g/ml aprotinin. RhoA and ARF were immunoprecipitated separately, using specific antibodies, washed three times with lysis buffer, and boiled for 20 min at 68°C in buffer containing 5 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP. GTP and GDP were separated on polyethylene-cellulose plates developed with 1 mM KH\textsubscript{2}PO\textsubscript{4} (pH 3.4) and measured by autoradiography.

**Assay for PLD activity.** PLD activity was determined by the formation of phosphatidylethanolamine (EtP), a specific product of PLD activity in the presence of ethanol. Muscle cells (2 \times 10^6 cells/ml) were incubated with \textsuperscript{[3]H}myristic acid (2 \mu Ci/ml) for 3 h and then with 150 mM ethanol for 15 min at 31°C in HEPES medium. The cells were then centrifuged at 350 \times g for 10 min to remove excess \textsuperscript{[3]H}myristic acid and resuspended in fresh medium. CCK-8 (1 nM) was added for 10 min, and the reaction was terminated by the addition of 1.8 ml of chloroform-methanol-HCl (100:200:2, vol/vol/vol) and extracted by the method of Bligh and Dyer (2) as described previously (26). The organic phase was dried under N$_2$ and analyzed for \[3\H]EtP by TLC on silica gel plates (dipped in 1% potassium oxalate), with ethyl acetate-2,2,4-trimethylpentane-acetic acid-water (13:2:10) as a running solvent. \[3\H]EtP was identified using unlabeled standards, which were sprayed with 0.1% 1,2-dichlorofluorescein in isopropyl alcohol and visualized under ultraviolet light at 357 nm. The spots corresponding to EtP were scraped and counted by liquid scintillation.

**Transfection of dominant negative RhoA cDNA into cultured smooth muscle.** Dominant negative RhoA cDNA was subcloned into the multiple cloning site (Eco I) of the eukaryotic expression vector pEXV. A myc tag was incorporated into the NH$_2$ terminus. Recombinant plasmid DNA was transiently transfected into the muscle cells in primary culture using Lipofectamine Plus reagent. Cells were cotransfected with 2 \mu g of pEXV-myc tag RhoA dominant negative and 1 \mu g of pGreen Lantern-1 for 48 h. Control cells were cotransfected with 2 \mu g pEXV vector and 1 \mu g of pGreen Lantern-1 DNA. Transfection efficiency was monitored by the expression of the green fluorescent protein using FITC filters. In the RhoA dominant negative mutant, asparagine was substituted for serine at position 19 (N19RhoA).

**Materials.** \textsuperscript{3}H]myristic acid (22.4 Ci/mmol) and carrier-free \textsuperscript{32}P[Pi] were obtained from NEN Life Science Products (Boston, MA). Collagenase type II and soybean trypsin inhibi-
Expression and receptor-mediated activation of $G_{13}$ and $G_{12}$ in intestinal smooth muscle. Previous studies (24) have shown that several G proteins ($G_{q/11}$, $G_{s}$, $G_{i-1}$, $G_{1-2}$, and $G_{1-3}$) are expressed in intestinal smooth muscle, where they are coupled to various receptors. Western blot analysis in the present study showed that $G_{13}$, $G_{12}$, and $G_{q/11}$ are also expressed in intestinal smooth muscle.

Receptor-mediated translocation and activation of RhoA and ARF. Western blot analysis showed that the monomeric G proteins RhoA and ARF were present mainly in the cytosolic fraction in the resting state, but increased significantly in the membrane fraction after stimulation of the muscle cells with CCK-8 (Figs. 2 and 3).

CCK-induced translocation of RhoA and ARF to the membrane was accompanied by a significant increase in the activities of both G proteins as indicated by the increase in the incorporation of $[^{32}P]GTP$ (Figs. 2 and 3). RhoA activity was inhibited 74 ± 9% ($P < 0.01$) by preincubation of permeabilized muscle cells for 1 h with $G_{13}$ antibody (5 µg/ml), whereas ARF activity was not affected (Figs. 2 and 3). Preincubation with $G_{12}$ antibody had no effect on either RhoA or ARF activity (Figs. 2 and 3).

$G_{13}$-dependent activation and $G_{12}$-dependent inhibition of PLD. CCK caused a sustained fourfold increase in PLD activity as determined by the formation of $[^{3}H]PEt$ [basal: 455 ± 71 counts/min (cpm)/10^6 cells; CCK-8: 2,415 ± 158 cpm/10^6 cells]. CCK-stimulated PLD activity was inhibited in a concentration-dependent fashion by the PLD inhibitor CCG-16 with an EC_{50} of 0.1 µM. PLD activity was also inhibited in a
concentration-dependent fashion by preincubation of permeabilized muscle cells for 1 h with G<sub>a</sub>13 antibody (0.1–10 µg/ml); a maximal inhibition of 47 ± 2% (P < 0.001) was elicited with 5 µg/ml of antibody (Fig. 4). In contrast, preincubation with G<sub>a</sub>12 antibody (10 µg/ml) increased PLD activity by 33 ± 2% (P < 0.001). Preincubation with antibodies to G<sub>a</sub>q/11, G<sub>i</sub>α1–2, G<sub>i</sub>α3, and G<sub>b</sub> had no effect on PLD activity.

RhoA- and ARF-dependent activation of PLD. The ability of RhoA to activate PLD was examined 1) in cultured smooth muscle cells transfected with a dominant negative mutant of RhoA (N19RhoA), 2) in permeabilized, freshly dispersed smooth muscle cells incubated for 1 h with RhoA antibody, and 3) in intact smooth muscle cells incubated for 3 h with the Clostridium botulinum C3 exoenzyme.

Transfection of cultured muscle cells with a dominant negative RhoA mutant inhibited CCK-stimulated PLD activity by 49 ± 8% (P < 0.01; n = 5) (Fig. 5). Preincubation of freshly dispersed permeabilized smooth muscle cells with RhoA antibody (0.1–10 µg/ml) inhibited CCK-stimulated PLD activity in a concentration-dependent fashion with a maximal inhibition of 53 ± 6% (P < 0.01) at 5 µg/ml (Fig. 4).
stimulated by GTP and [3H]PEt in the presence of ethanol. Results are expressed as cpm/10^6 cells.

The inhibition by 29% (1 nM) was determined by the formation of [3H]PEt in the presence of ethanol. Results are expressed as cpm/10^6 cells. Preincubation of muscle cells for 3 h with C3 exoenzyme caused additive inhibition. A combination of C3 exoenzyme and RhoA antibody was not additive (30% and 31%, respectively). The pattern implied that PKC-dependent activation of PLD was mediated by PKC-α.

The ability of PKC to activate PLD was additive to that of either RhoA or ARF. A combination of calphostin C with RhoA antibody or C3 exoenzyme inhibited CCK-stimulated PLD activity by 75% and 73%, respectively, and a combination with ARF antibody inhibited PLD activity by 58% (Fig. 9). Combining calphostin C with both RhoA and ARF antibodies inhibited PLD activity by 83% (Fig. 9), whereas a combination of calphostin C with both C3 exoenzyme and ARF antibody inhibited PLD activity by 86%.

**DISCUSSION**

This study shows that agonist-induced, sustained activation of PLD involves a distinct set of heterotrimERIC and monomeric G proteins. The pathways involved are depicted schematically in Fig. 10. CCK-8 activated the heterotrimeric G proteins G_{13} and G_{12}.
and the monomeric G proteins RhoA and ARF. The α-subunit of G13, but not G12, was coupled to sequential activation of RhoA and PLD. RhoA was the dominant activator of PLD, accounting for 50% of the response, and its effect appeared to be mediated by ROK. ARF also activated PLD, but its effect was not mediated by either G13 or G12. The effects of RhoA and ARF were additive to those of PKC-α, the specific isoform that mediates activation of PLD by PKC. Unexpectedly, the activation of G12 by CCK-8 resulted in inhibition of PLD. The evidence is summarized as follows.

Receptor-mediated activation of G13 and G12 and its relation to PLD activity. CCK-8 activated three heterotrimeric G proteins (Gq/11, G13, and G12), only one of which, G13, was coupled to sustained activation of PLD. Gα13 antibody inhibited CCK-stimulated PLD activity, whereas Gα12 antibody increased CCK-stimulated PLD activity, suggesting that G12 mediated an inhibitory pathway. Antibodies to the α-subunits of Gq/11, G11, G13, and G5, and a common antibody to Gβ had no effect.

Coupling of G13 to RhoA but not ARF. CCK-8 induced translocation of RhoA and ARF to the membrane and activated both monomeric G proteins as indicated by the increase in GTP binding to RhoA and ARF. Gα13 antibody inhibited activation of RhoA; neither Gα13 nor Gα12 antibody had any effect.
on ARF. Thus in intestinal smooth muscle, only G\textsubscript{13} and RhoA were sequentially coupled.

**Activation of PLD via RhoA and ARF.** Agonist-stimulated PLD activity was mediated additively by RhoA and ARF. RhoA antibody, the *Clostridium botulinum* C3 exoenzyme, and a dominant negative RhoA mutant transfected into cultured muscle cells inhibited agonist-stimulated PLD activity to the same extent (49% to 53%). The extent of inhibition was similar to that obtained with G\textsubscript{13} antibody (47%), consistent with sequential activation of G\textsubscript{13}, RhoA, and PLD. ARF antibody inhibited PLD activity to a lesser extent (29%), and its effect was additive to that of RhoA antibody or C3 exoenzyme (75% to 78%), suggesting that RhoA and ARF activate PLD via distinct mechanisms. The effect of RhoA appeared to be mediated by ROK and was inhibited by HA-1077, a preferential inhibitor of ROK (30, 33).

Neither the mechanism by which CCK induced activation of ARF and its translocation to membranes nor the mechanism by which ARF activated PLD was identified. As noted above, G\textsubscript{12} and G\textsubscript{13} antibodies did not block receptor-mediated activation of ARF. It seemed unlikely that G\beta\gamma subunits that can bind to ARF were involved in its activation, because a common G\beta antibody had no effect on CCK-stimulated PLD activity.

**Activation of PLD by PKC.** Activation of PLD by PKC has been demonstrated in various cell types and represents a feedback mechanism, because DAG, the main activator of PKC, is largely generated by dephosphorylation of PA, the primary product of PLD activity (1, 9, 32). Previous studies (26) on intestinal circular and longitudinal smooth muscle have shown that agonist-stimulated PLD activity was partly inhibited by calphostin C. The present study confirmed that PLD activity was partly inhibited by calphostin C, as well as by selective inhibitors of PKC-\alpha. The involvement of PKC-\alpha, and to a lesser extent PKC-\betaI and -\betaII, has been demonstrated in other cell types (1, 9, 32). In the present study, the inhibition of PLD activity by calphostin C was additive to that of RhoA or ARF antibodies, applied separately or in combination.

The mechanism by which PKC activates PLD has not been fully established. In vitro studies suggest that the stimulatory activity of PKC resides in its regulatory domain, because PKC fragments devoid of catalytic domain retain their stimulatory activity in vitro, and inhibitors of catalytic activity appear to be ineffective (8). In vivo, however, as in the present study, blockers of the regulatory and catalytic domains of PKC inhibited PLD activity (1, 8, 9). Because no evidence exists for direct phosphorylation of PLD by PKC, the effectiveness of both types of inhibitors suggests that PKC may act indirectly on PLD via an intermediate susceptible to stimulatory phosphorylation.

A recent study by Usio-Fukai et al. (34) showed that angiotensin II-stimulated PLD activity in cultured vascular smooth muscle was inhibited by antibodies to G\textsubscript{12}, G\beta, RhoA, and c-src, suggesting involvement of G\textsubscript{12}-RhoA and G\beta\gamma-Src pathways. However, the roles of G\textsubscript{13}, ARF, or PKC and their interplay with the RhoA/ROK pathway were not examined. In intestinal smooth muscle, G\textsubscript{12}, unlike G\textsubscript{13}, mediated an inhibitory PLD response, whereas in cultured vascular smooth muscle, G\textsubscript{12} mediated a stimulatory response. Differential involvement of G\textsubscript{12} and G\textsubscript{13} in activation of monomeric G proteins has been reported (4, 10, 17, 18, 28) in other cell types, and as noted earlier, appears to be both cell and agonist specific.

The functional significance of agonist-stimulated, sustained activation of PLD resides in the ability of its primary product, PA, to generate DAG and thus activate PKC. We and others (15, 16, 25, 35) have provided evidence that specific isoforms of PKC are involved in sustained contraction of vascular and visceral smooth muscle. Sustained contraction of intestinal smooth muscle induced by G protein-coupled agonists is mediated by the Ca\textsuperscript{2+}-independent isoform, PKC-\epsilon, whereas sustained contraction induced by phorbol esters and growth factors (e.g., epidermal growth factor) is mediated by PKC-\alpha, and possibly other Ca\textsuperscript{2+}-dependent isoforms (25). Preliminary evidence suggests that agonist-stimulated PKC-\epsilon activity and sustained contraction of intestinal smooth muscle are mediated by a pathway involving sequential activation of G\textsubscript{13}, RhoA, and PLD and could be inhibited by GDP\betaS, G\textsubscript{13} and RhoA antibodies, and by PLD and PKC inhibitors (27).

Recent studies (11, 14, 16, 33) have provided further evidence of a functional linkage between RhoA, PKC, and sustained muscle contraction; activation of ROK inhibits myosin light chain phosphatase via the PKC target protein CPI-17, resulting in phosphorylation of myosin light chain and sustained contraction.

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**REFERENCES**


