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$G\alpha_{12/13}$- and Rho-Dependent Activation of Phospholipase C-ε by Lysophosphatidic Acid and Thrombin Receptors

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ABSTRACT

Because phospholipase C ε (PLC-ε) is activated by $G\alpha_{12/13}$ and Rho family GTPases, we investigated whether these G proteins contribute to the increased inositol lipid hydrolysis observed in COS-7 cells after activation of certain G protein-coupled receptors. Stimulation of inositol lipid hydrolysis by endogenous lysophosphatidic acid (LPA) or thrombin receptors was markedly enhanced by the expression of PLC-ε. Expression of the LPA_1 or PAR1 receptor increased inositol phosphate production in response to LPA or SFLLRN, respectively, and these agonist-stimulated responses were markedly enhanced by coexpression of PLC-ε. Both LPA_1 and PAR1 receptor-mediated activation of PLC-ε was inhibited by coexpression of the regulator of G protein signaling (RGS) domain of p115RhoGEF, a GTPase-activating protein for $G\alpha_{12/13}$ but not by expression of the RGS domain of GRK2, which inhibits $G\alpha_q$ signaling. In contrast, activation of the G_q-coupled M1 muscarinic or $P2Y_2$ purinergic receptor was neither enhanced by coexpression with PLC-ε nor inhibited by the RGS domain of p115RhoGEF but was blocked by expression of the RGS domain of GRK2. Expression of the Rho inhibitor C3 botulinum toxin did not affect LPA- or SFLLRN-stimulated inositol lipid hydrolysis in the absence of PLC-ε but completely prevented the PLC-ε-dependent increase in inositol phosphate accumulation. Likewise, C3 toxin blocked the PLC-ε-dependent stimulatory effects of the LPA_1, LPA_2, LPA_3, or PAR1 receptor but had no effect on the agonist-promoted inositol phosphate response of the M1 or $P2Y_2$ receptor. Moreover, PLC-ε-dependent stimulation of inositol phosphate accumulation by activation of the epidermal growth factor receptor, which involves Ras- but not Rho-mediated activation of the phospholipase, was unaffected by C3 toxin. These studies illustrate that specific LPA and thrombin receptors promote inositol lipid signaling via activation of $G\alpha_{12/13}$ and Rho.

Many extracellular hormones, neurotransmitters, and growth factors exert their physiological effects by mechanisms that in part involve phospholipase C-catalyzed breakdown of phosphatidylinositol $(4,5)P_2$ into the Ca^{2+}-mobilizing second-messenger inositol $(1,4,5)P_3$ and the protein kinase C-activating second-messenger diacylglycerol (Irvine et al., 1987; Rhee, 2001). For example, extracellular stimuli that activate members of the large family of seven transmembrane-spanning heterotrimeric G protein-coupled receptors (GPCRs) activate PLC-β isozymes by the release of α-subunits of the G_q family of G proteins (Smrcka et al., 1991; Taylor et al., 1991; Waldo et al., 1991) or by the release of $G\beta\gamma$ dimers from activated G_i (Blank et al., 1992; Boyer et al., 1992; Camps et al., 1992). In contrast, PLC-γ enzymes are activated by tyrosine phosphorylation after activation of receptor and nonreceptor tyrosine kinases (Meisenhelder et al., 1989; Wahl et al., 1989).

PLC-ε, which possesses Ras-associating (RA) domains at its carboxyl terminus, was initially identified in *Caenorhabditis elegans* as a Ras-binding protein (Shibatohge et al., 1998). Mammalian PLC-ε is activated by coexpression with Ras (Kelley et al., 2001; Song et al., 2001) and by activators of GEFs that in turn promote the formation of active Rap or Ras (Schmidt et al., 2001; Evellin et al., 2002; Keiper et al., 2004). For example, G_s-coupled GPCRs promote PLC-ε-dependent inositol lipid signaling through activation of the

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ABBREVIATIONS: GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle's medium; LPA, lysophosphatidic acid; EGF, epidermal growth factor; PLC, phospholipase C; RGS, regulator of G protein signaling; GEF, guanine nucleotide exchange factor; C3 toxin, C3 botulinum toxin; p115-RGS, the RGS domain of p115 Rho guanine nucleotide exchange factor; GRK2-RGS, the RGS domain of G protein receptor kinase-2; RA, Ras-associating; PDZ, postsynaptic density-95/disc-large/zona occludens.

cAMP-activated GEF, EPAC, which in turn activates Rap1A (Schmidt et al., 2001). Initial studies of mammalian PLC-ε revealed activation by $G\alpha_{12}$ and $G\alpha_{13}$ but not by $G\alpha_q$ (Lopez et al., 2001; Wing et al., 2001), and $G\beta\gamma$ also has been shown to activate this PLC isozyme (Wing et al., 2001).

Coexpression of Rho family GTPases with PLC-ε results in marked stimulation of inositol lipid hydrolysis (Wing et al., 2003). PLC-ε mutants that lack functional RA domains retain activation by Rho, indicating that Rho family GTPases regulate this PLC isozyme by a mechanism distinct from that used by Ras and Rap. Observation of GTP-dependent activation of purified PLC-ε by purified RhoA illustrates that the stimulatory action of Rho in inositol lipid signaling is direct (Seifert et al., 2004). GEFs for Rho are downstream effectors of $G\alpha_{12/13}$ (Hart et al., 1998; Fukuhara et al., 1999; Booden et al., 2002; Suzuki et al., 2003; Dutt et al., 2004). Thus, the observation of Rho-dependent activation of PLC-ε suggests that GPCRs that activate $G\alpha_{12/13}$ promote inositol lipid signaling through the activation of Rho.

With the goal of establishing whether receptor-mediated regulation of inositol lipid signaling occurs through a mechanism involving $G\alpha_{12/13}$, Rho, and PLC-ε, we studied the regulation of PLC-ε-promoted inositol lipid hydrolysis by endogenous and recombinant GPCRs expressed in COS-7 cells. The results of these studies are consistent with the idea that $G\alpha_{12/13}$- and Rho-dependent activation of PLC-ε occurs downstream of both LPA- and thrombin-activated receptors and that the regulation of PLC-ε by $G\alpha_{12/13}$ occurs at least in part through activation of Rho.

Materials and Methods

Materials. Expression vectors (in pcDNA3.1) for the human M1 muscarinic cholinergic, LPA_1, LPA_2, and LPA_3 receptors were purchased from the University of Missouri–Rolla cDNA Resource Center (Rolla, MO). An expression vector encoding the human $P2Y_2$ receptor was described previously (Nicholas et al., 1996). The plasmid encoding wild-type epidermal growth factor (EGF) receptor is described by Carter and Sorkin (1998). A pCMV-Script vector encoding FLAG-tagged rat PLC-ε was generously provided by Grant Kelley, State University of New York (Syracuse, NY). An expression vector for C3 botulinum toxin was obtained from Channing Der, University of North Carolina (Chapel Hill, NC). cDNA encoding the first 240 amino acids of human p115RhoGEF was subcloned in-frame with an N-terminal tandem hemagglutinin-epitope tag into a modified pcDNA3.1 vector (Hains et al., 2004). cDNA encoding amino acids 45 to 178 of bovine GRK2 (designated GRK2-RGS) in frame with an N-terminal hemagglutinin-epitope tag in pcDNA3 was kindly provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). 1-Oleoyl-L-α-lysophosphatidic acid sodium salt (LPA) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in water containing 1.0% fatty acid-free bovine serum albumin. The PAR1 receptor agonist peptide SFLLRN was synthesized as the carboxyl amide and purified by reverse-phase high-pressure liquid chromatography (University of North Carolina Peptide Facility, Chapel Hill, NC). UTP, carbachol, and EGF were purchased from Sigma-Aldrich. All other reagents were from sources noted previously (Wing et al., 2001, 2003; Seifert et al., 2004).

Cell Culture and Transfection of COS-7 Cells. COS-7 cells were plated in 12- or 96-well culture dishes and maintained in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 10% CO_2/90% air atmosphere. The indicated DNA expression vectors were transfected into COS-7 cells using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) at a ratio of 3:1 (FuGENE/DNA) following the manufacturer's protocol. Empty-vector DNA was used as necessary to maintain a constant total amount of DNA per well.

Measurement of [^{3}H]Inositol Phosphates. Approximately 24 h after transfection, the medium was replaced with inositol- and serum-free DMEM containing 1 μCi/well [*myo*-^{3}H]inositol (American Radiolabeled Chemicals, St. Louis, MO). Phospholipase C activity was quantified 12 h after labeling by incubation in inositol-free DMEM containing 10 mM LiCl either in the absence of a receptor agonist or in the presence of 10 μM LPA, 50 μM SFFLRN, 100 μM carbachol, 100 μM UTP, or 100 ng/ml EGF. The reaction was stopped after 30 to 60 min by aspiration of the medium and addition of ice-cold 50 mM formic acid. After neutralization with 150 mM ammonium hydroxide, the accumulation of [^{3}H]inositol phosphates was quantified by Dowex chromatography as described previously (Wing et al., 2001).

Western Blotting. COS-7 cells were seeded at 60,000 cells/well in a 12-well plate and transfected 24 h later with pcDNA3, myc-PLC-ε, or myc-PLC-ε with 0.3, 3, or 30 ng of C3 toxin using FuGENE 6 transfection reagent as described previously. Forty-eight hours after transfection, the cells were lysed on ice in 200 μl of lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 1% Triton X-100, containing protease inhibitors). COS-7 cell lysates were sonicated in an ice-water bath for 5 min and then centrifuged at 13,000*g* for 20 min at 4°C. The supernatant was removed, mixed 1:1 with 5× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose. Western blotting was performed using anti-myc clone 9E10 (Roche Applied Science) and anti-α-tubulin clone B-5-1-2 (Sigma-Aldrich) primary antibodies, a secondary anti-mouse IgG antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and enhanced chemiluminescence (Denville Scientific Inc., Metuchen, NJ).

Results

PLC-ε-Dependent Promotion of Inositol Lipid Signaling by Endogenous LPA and Thrombin Receptors. We reported recently that Rho GTPases directly activate PLC-ε (Wing et al., 2003; Seifert et al., 2004). To begin to address potential GPCR-mediated regulation of this PLC isozyme through a Rho-dependent signaling pathway, we screened COS-7 cells for the functional presence of GPCRs that exhibit PLC-ε-dependent activation of inositol lipid signaling. Incubation of cells with histamine, prostaglandin E_2, carbachol, adenosine, norepinephrine, somatostatin, or a combination of P2Y receptor agonists (UTP, 2MeSADP, ATP, UDP, and UDP-glucose) all failed to elevate inositol phosphates in a PLC-ε-dependent manner (data not shown). In contrast, as was reported recently by Kelley et al. (2004), incubation of PLC-ε-expressing cells with LPA (10 μM) or with the agonist peptide SFLLRN (50 μM) resulted in an increase in inositol phosphate levels compared with the very low responses observed with LPA or SFLLRN in control cells (Fig. 1).

PLC-ε-Dependent Stimulation of Inositol Phosphate Accumulation by Molecularly Defined LPA and Thrombin Receptors. Because LPA is the cognate agonist for at least three different GPCRs, we individually expressed the LPA_1, LPA_2, or LPA_3 receptors in COS-7 cells with the goal of determining the extent to which these signaling proteins exhibit PLC-ε-dependence in their action. LPA-stimulated inositol phosphate accumulation was reproducibly increased in LPA_1 receptor-expressing cells compared with empty vector-transfected cells and was enhanced by ~10-fold in LPA_2 or LPA_3 receptor-expressing cells (Fig. 2). Coexpression of the LPA_1 receptor with PLC-ε markedly enhanced the

inositol phosphate response to LPA. Coexpression with PLC-ε also increased the LPA-promoted inositol lipid signaling response in LPA_2 or LPA_3 receptor-expressing cells, although the enhancement observed with PLC-ε was both variable and of much smaller magnitude (i.e., from no effect to approximately 2-fold increases in response) than that observed with the LPA_1 receptor (Fig. 2). To determine whether the lack of effect of the LPA_2 and LPA_3 receptors on PLC-ε activation was due to a depletion of phosphatidylinositol (4,5)P_2, we examined the ability of other $G\alpha_q$-coupled receptors to promote inositol lipid signaling. $G\alpha_q$-coupled receptors such as $P2Y_4$ and $P2Y_{11}$ produced much larger responses than the LPA_2 and LPA_3 receptors (data not shown), suggesting that the system is not saturated. In addition, selective inhibition of the large $G\alpha_q$-stimulated response of the LPA_2 and LPA_3 receptors by the RGS domain of GRK2 (Carman et al., 1999; Hains et al., 2004) did not uncover a PLC-ε-dependent response by these receptors (data not shown). Therefore, the LPA_1 receptor promotes signaling responses in COS-7 cells that are markedly dependent on the presence of PLC-ε, whereas the inositol lipid signaling response in LPA_2 or LPA_3 receptor-expressing cells is less affected by the expression of PLC-ε.

Given the effects of PLC-ε on agonist-promoted responses of the molecularly defined LPA receptors, we also tested the activities of the PAR1 receptor, another GPCR known to couple to $G\alpha_{12/13}$- and Rho-regulated pathways, and two receptors, the M1 muscarinic cholinergic receptor and the nucleotide-activated $P2Y_2$ receptor, known to primarily activate G_q-regulated signaling pathways. Expression of the PAR1 receptor in the presence of PLC-ε resulted in a marked increase in SFLLRN-promoted inositol phosphate accumulation (Fig. 3A). In contrast, expression of the M1 muscarinic receptor (Fig. 3B) or $P2Y_2$ receptor (Fig. 3C) resulted in a marked increase in agonist-promoted inositol lipid response that was not further augmented by coexpression with PLC-ε. The large increase in basal [^{3}H]inositol phosphate accumulation illustrated in Fig. 3C is due to basal release of ATP and UTP from COS-7 cells, which in turn activates the expressed $P2Y_2$ receptor (Lazarowski et al., 1995, 2000; Alvarado-Castillo et al., 2005).

RGS Protein-Selective Inhibition of PLC-ε-Dependent Signaling by the LPA_1 and PAR1 Receptors. Activation of both LPA (Anliker and Chun, 2004; Riobo and Manning, 2005) and thrombin (Trejo, 2003; Riobo and Manning, 2005) receptors has been reported previously to result in activation of $G\alpha_{12/13}$- and Rho-promoted signaling pathways. To address the potential roles of $G\alpha_{12/13}$ and $G\alpha_q$ in the responses to these GPCRs, we applied the RGS domain of p115RhoGEF (p115-RGS), which acts as a GTPase-activating protein for $G\alpha_{12}$ and $G\alpha_{13}$ (Kozasa et al., 1998; Hains et al., 2004), and GRK2-RGS, which is known to bind selectively to $G\alpha_q$ and inhibit $G\alpha_q$ signaling (Carman et al., 1999; Hains et al., 2004). Coexpression of p115-RGS with the LPA_1 receptor or PAR1 receptor had no effect on agonist-stimulated inositol phosphate accumulation in the absence of PLC-ε (Fig. 4, A and B). However, the elevated agonist-promoted inositol phosphate response observed in cells coexpressing these receptors with PLC-ε was essentially completely inhibited by coexpression with p115-RGS. In contrast, p115-RGS had no effect on agonist-stimulated inositol phosphate responses promoted by the M1 muscarinic receptor (Fig. 4C) or $P2Y_2$ receptor (data not shown) in the absence or presence of PLC-ε. Conversely, expression of $G\alpha_q$-binding GRK2-RGS significantly inhibited M1 (Fig. 4C) and $P2Y_2$ (data not shown) receptor-promoted signaling but had no effect on the inositol lipid signaling response to the LPA_1 (Fig. 4A) or PAR1 (Fig. 4B) receptor, either in the absence or presence of the expression of PLC-ε.

C3 Toxin Inhibits $G\alpha_{12/13}$-, LPA-, and Thrombin-Promoted Activation of PLC-ε. The data presented thus far implicate $G\alpha_{12/13}$ in the mechanism of activation of PLC-ε by LPA and thrombin receptors. In contrast, the M1 muscarinic and $P2Y_2$ receptors apparently regulate inositol lipid signaling by mechanisms that involve neither $G\alpha_{12/13}$ nor PLC-ε. To address the potential role of Rho in GPCR-promoted activation of PLC-ε, we used C3 toxin to inactivate Rho. Expression of GTPase-deficient mutants of $G\alpha_{12}$ ($G\alpha_{12}$Q229L) or $G\alpha_{13}$ ($G\alpha_{13}$Q226L) had no effect on the accumulation of inositol phosphates in control COS-7 cells (Fig. 5A). However, coexpression of these $G\alpha_{12}$ or $G\alpha_{13}$ mutants with PLC-ε resulted in a marked increase in [^{3}H]inositol phosphate accumulation compared with [^{3}H]inositol phosphate levels in cells transfected with PLC-ε alone. The capacity of $G\alpha_{12}$ and $G\alpha_{13}$ to activate PLC-ε was lost with the transfection of increasing amounts of an expression vector for C3 botulinum toxin (Fig. 5B). Loss of responsiveness was not due to a

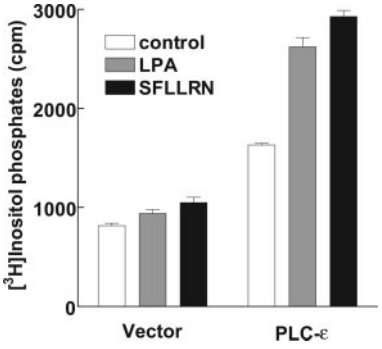

Fig. 1. PLC-ε-dependent stimulation of inositol phosphate accumulation by endogenous LPA and thrombin receptors in COS-7 cells. [^{3}H]Inositol phosphate accumulation was measured as described under *Materials and Methods* in COS-7 cells transfected with empty vector or with an expression vector for PLC-ε. Incubations were in the presence of vehicle, 10 μM LPA, or 50 μM SFLLRN.

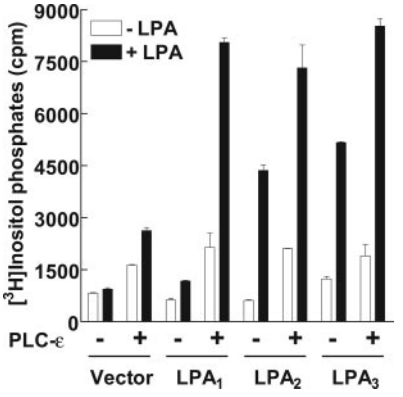

Fig. 2. PLC-ε-dependence of LPA-stimulated [^{3}H]inositol phosphate accumulation in cells expressing empty vector, LPA_1, LPA_2, or LPA_3 receptor. COS-7 cells were transfected with an expression vector for the LPA_1, LPA_2, or LPA_3 receptor in the absence or presence of an expression vector for PLC-ε. [^{3}H]Inositol phosphate accumulation was quantified in the absence or presence of 10 μM LPA as described under *Materials and Methods*.

nonspecific effect on inositol lipid signaling because the capacity of GTPase-deficient $G\alpha_q$ ($G\alpha_q$Q209L) to promote phosphoinositide hydrolysis was largely retained. To determine whether the reduction of PLC-ε activity was due to a decrease in the expression level of PLC-ε, we immunoblotted cell lysates expressing PLC-ε alone or PLC-ε in the presence

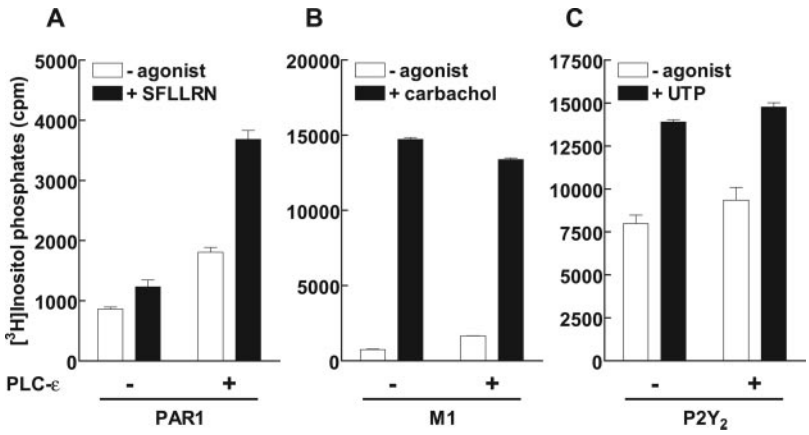

Fig. 3. Differential effects of PLC-ε on agonist-stimulated [^{3}H]inositol phosphate accumulation in cells expressing the PAR1 receptor versus the M1 muscarinic cholinergic or $P2Y_2$ receptor. COS-7 cells were transfected with an expression vector for the PAR1 (A), M1 muscarinic cholinergic (B), or $P2Y_2$ (C) receptor in the absence or presence of an expression vector for PLC-ε. [^{3}H]Inositol phosphate accumulation was quantified in the PAR1, M1, or $P2Y_2$ receptor-expressing cells in the absence or presence of 50 μM SFLLRN, 100 μM carbachol, or 100 μM UTP, respectively.

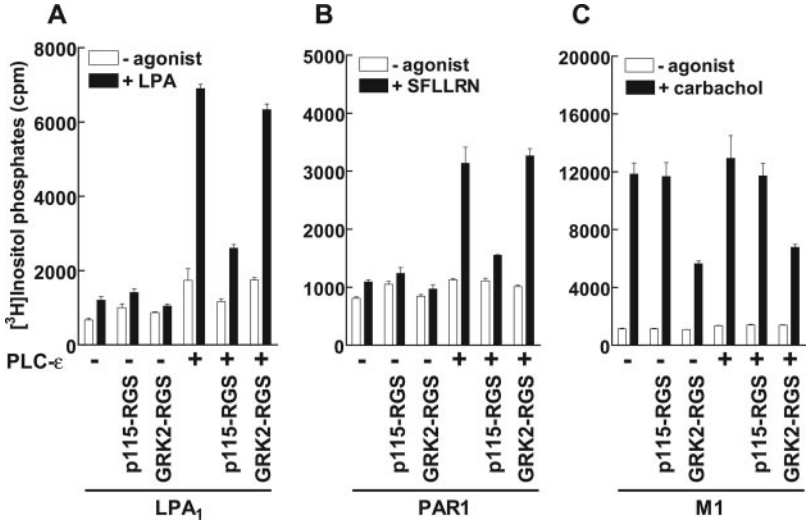

Fig. 4. Differential effects of RGS proteins on LPA_1 receptor- and PAR1 receptor- versus M1 muscarinic receptor-promoted [^{3}H]inositol phosphate accumulation in PLC-ε-expressing cells. COS-7 cells were transfected with the LPA_1 receptor, the PAR1 receptor, or the M1 muscarinic cholinergic receptor in the absence or presence of PLC-ε and with either p115-RGS or GRK2-RGS. [^{3}H]Inositol phosphate accumulation was quantified in the LPA_1, PAR1, or M1 receptor-expressing cells in the absence or presence of 10 μM LPA, 50 μM SFLLRN, or 100 μM carbachol, respectively, as described under *Materials and Methods*.

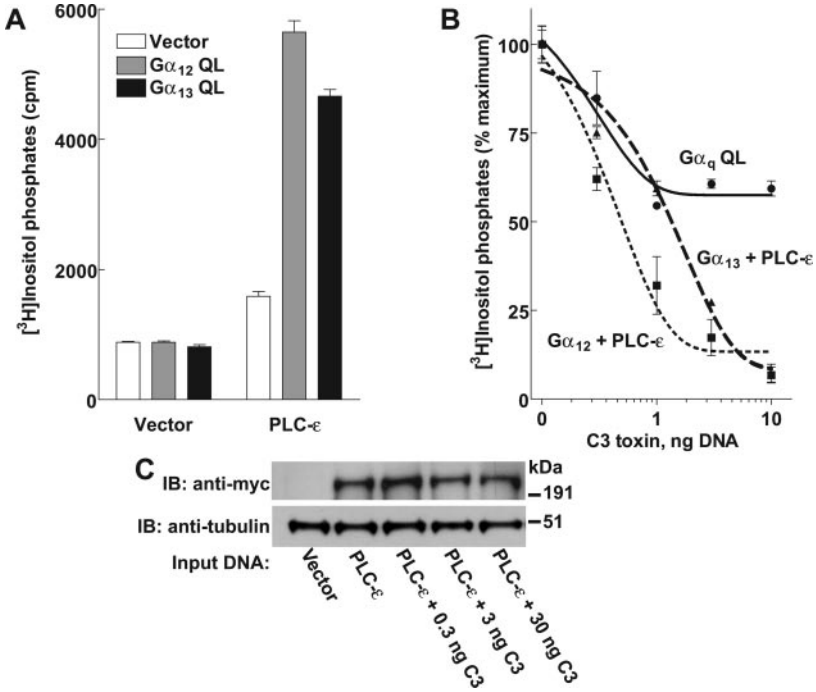

Fig. 5. C3 toxin-mediated inhibition of the activation of PLC-ε by $G\alpha_{12}$ and $G\alpha_{13}$. A, COS-7 cells were transfected with empty vector, GTPase-deficient $G\alpha_{12}$ ($G\alpha_{12}$ Q226L), or GTPase-deficient $G\alpha_{13}$ ($G\alpha_{13}$ Q229L) in the absence or presence of transfection of an expression vector for PLC-ε. B, COS-7 cells were transfected with PLC-ε + $G\alpha_{12}$, PLC-ε + $G\alpha_{13}$, or $G\alpha_q$(Q209L) and with the indicated amounts of an expression vector for C3 toxin. [^{3}H]Inositol phosphate accumulation was measured as described under *Materials and Methods*. Data are expressed as the percentage of maximum, with the average maximum for each as follows: PLC-ε + $G\alpha_{12}$, 6693 cpm; PLC-ε + $G\alpha_{13}$, 12,447 cpm; $G\alpha_q$(Q209L), 7947 cpm. C, COS-7 cells were transfected with empty vector, myc-PLC-ε, or myc-PLC-ε in the presence of 0.3 ng of C3 toxin, 3 ng of C3 toxin, or 30 ng of C3 toxin. Forty-eight hours after transfection, the cells were lysed and mixed 1:1 with 5× Laemmli sample buffer. The lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted for the presence of myc-PLC-ε and α-tubulin as indicated.

of increasing amounts of C3 toxin. The expression of PLC-ε in the presence of 0.3, 3, or 30 ng of C3 toxin was not significantly altered (Fig. 5C), suggesting that the lack of PLC-ε-dependent activity in the presence of C3 toxin is not due to the inhibition of PLC-ε expression. Thus, Rho is downstream of $G\alpha_{12}$ and $G\alpha_{13}$ in the PLC-ε-dependent signaling response measured under these conditions.

The potential contribution of Rho to the PLC-ε-dependent stimulation of inositol lipid signaling by the endogenous LPA and thrombin receptors of COS-7 cells also was examined by measuring inositol phosphate accumulation after expression of C3 toxin. Whereas the capacity of LPA (Fig. 6A) or SFLLRN (Fig. 6B) to stimulate phosphoinositide hydrolysis in the absence of PLC-ε was not affected by transient expression of C3 toxin, PLC-ε-dependent elevation of inositol phosphates in response to both agonists was entirely lost in C3 toxin-expressing cells in a concentration-dependent manner (Fig. 6, A and B). These results suggest that endogenous LPA and thrombin receptors of COS-7 cells activate a Rho GTPase(s), which in turn activates PLC-ε.

C3 Toxin-Mediated Inhibition of PLC-ε-Dependent Inositol Lipid Signaling by Molecularly Defined LPA and Thrombin Receptors. To determine whether Rho also is involved in PLC-ε-dependent inositol lipid signaling by molecularly defined LPA receptors and the PAR1 receptor, C3 toxin was coexpressed with each of these receptors in the absence or presence of PLC-ε. The increased response to LPA conferred by LPA_1 receptor expression was not affected by C3 toxin (data not shown). In contrast, the large PLC-ε-dependent response to LPA observed in LPA_1 receptor-expressing cells was completely inhibited in a concentration-dependent manner by coexpression of C3 toxin (Fig. 7A). C3 toxin also had no effect on the LPA-promoted inositol phosphate response in COS-7 cells expressing either the LPA_2 or LPA_3 receptor alone (data not shown) but blocked completely and in a concentration-dependent manner the PLC-ε-dependent effects of LPA mediated through these two receptors (Fig. 7, B and C). Expression of C3 toxin also resulted in the loss of PLC-ε-dependent but not -independent inositol lipid signaling of the PAR1 receptor (Fig. 8A). In contrast, agonist-stimulated inositol phosphate accumulation promoted by the M1 muscarinic cholinergic receptor (Fig. 8B) or the $P2Y_2$ purinergic receptor (Fig. 8C) was neither enhanced by expression of PLC-ε nor inhibited by coexpression of C3 toxin.

Although expression of C3 toxin did not affect the expression of PLC-ε (Fig. 5C and data not shown), the elevated levels of basal [^{3}H]inositol phosphate accumulation after PLC-ε expression were suppressed by coexpression of C3 toxin (Figs. 7 and 8). PLC-ε-dependent elevation of [^{3}H]inositol phosphate accumulation also was inhibited by p115-RGS (Fig. 4A and data not shown), suggesting that a $G\alpha_{12/13}$/Rho-dependent pathway activates expressed PLC-ε in the absence of added receptor agonists. An analogous effect is observed with overexpression of G_q-coupled P2Y receptors (Fig. 8C) in the absence of application of exogenous agonist as a result of autocrine release of cognate adenine and uridine nucleotide agonists (Filtz et al., 1994; Lazarowski et al., 2000; Alvarado-Castillo et al., 2005). Nonetheless, these results do not rule out the possibility that C3 toxin nonspecifically inhibits the activation of PLC-ε by all activators. Thus, we also examined EGF receptor-mediated activation of PLC-ε, which occurs via a $G\alpha_{12/13}$/Rho-independent mechanism involving the binding of Ras subfamily GTPases to the carboxyl-terminal RA domains of the enzyme (Kelley et al., 2001, 2004; Song et al., 2001; Stope et al., 2004). As illustrated in Fig. 9B, PLC-ε-dependent effects of EGF on [^{3}H]inositol phosphate accumulation were observed in COS-7 cells coexpressing the EGF receptor. The effect of C3 toxin on EGF-promoted activation of PLC-ε was examined in a series of experiments in which PLC-ε-dependent accumulation of [^{3}H]inositol phosphates was quantified in the presence of EGF versus LPA in EGF receptor-expressing cells versus LPA_1 receptor-expressing cells, respectively. Whereas the expression of increasing amounts of C3 toxin inhibited LPA_1 receptor-promoted [^{3}H]inositol phosphate accumulation (Figs. 7A and 9A), little if any effect of C3 toxin on PLC-ε-dependent stimulatory effects of the EGF receptor was observed (Fig. 9B).

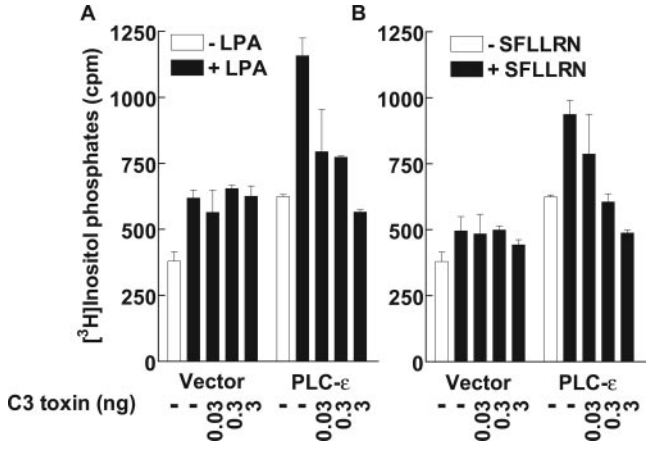

Fig. 6. Effect of C3 toxin on PLC-ε-dependent signaling of endogenous LPA and thrombin receptors. COS-7 cells were cotransfected with the indicated amounts of an expression vector for C3 toxin and either empty vector or an expression vector for PLC-ε. [^{3}H]Inositol phosphate accumulation was measured in the absence or presence of 10 μM LPA or 50 μM SFLLRN as described under *Materials and Methods*.

Discussion

PLC isozymes contain a highly conserved catalytic core and additional domains that render these isozymes susceptible to different modes of regulation (Rhee, 2001). PLC-ε is activated by Ras and Rho GTPases and by subunits of heterotrimeric G proteins, including $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_s$, and $G\beta\gamma$ (Kelley et al., 2001; Lopez et al., 2001; Schmidt et al., 2001; Song et al., 2001; Wing et al., 2001, 2003). Activation by Ras subfamily GTPases occurs as a consequence of direct interaction of these G proteins with RA domains in the carboxyl terminus of PLC-ε (Kelley et al., 2001; Song et al., 2001). We illustrated recently that Rho binds to an undefined sequence in the catalytic core of PLC-ε, activating the isozyme through a mechanism that does not require the RA domains (Wing et al., 2003; Seifert et al., 2004). In contrast, the mechanism(s) whereby PLC-ε is activated by subunits of heterotrimeric G proteins remains largely unclear and may not be direct. Data reported in the current study indicate that activation of PLC-ε by $G\alpha_{12}$ and $G\alpha_{13}$ after expression in COS-7 cells is dependent on functional Rho. Moreover, these results are consistent with the conclusion that activation of PLC-ε by receptors for LPA and thrombin is dependent on the activation of both $G\alpha_{12/13}$ and Rho.

The downstream signaling responses promoted by GPCRs through $G\alpha_{12/13}$-dependent mechanisms have been less clearly

defined than those dependent on activation of Gα subunits of the G_s, G_i, and G_q families. Nonetheless, marked morphological and cell proliferative changes are consistently observed with the introduction of GTPase-deficient mutants of $G\alpha_{12}$ or $G\alpha_{13}$ in various cell types (Sah et al., 2000; Kurose, 2003), and a variety of effectors are stimulated downstream of activation of $G\alpha_{12/13}$ (Kurose, 2003; Riobo and Manning, 2005). Some of the cellular responses promoted by $G\alpha_{12/13}$ are mimicked by activated Rho, and activation of Rho occurs in many if not all cells in which $G\alpha_{12}$ or $G\alpha_{13}$ is activated (Sah et al., 2000). A large family of RhoGEFs (Hart et al., 1998; Fukuhara et al., 1999; Booden et al., 2002; Suzuki et al., 2003; Dutt et al., 2004) (e.g., p115RhoGEF, leukemia-associated RhoGEF, PDZ-RhoGEF, and Lbc-RhoGEF) are among the best studied of the effector proteins directly regulated by $G\alpha_{12/13}$. Moreover, the observation that certain $G\alpha_{12/13}$-coupled GPCRs produce cellular effects that involve Rho (Sah et al., 2000) or RhoGEFs (Wang et al., 2004) suggests that the putative Rho-activated PLC-ε signaling pathway implied from our previous studies is logically extended to GPCRs that activate Rho GTPases through activation of $G\alpha_{12/13}$ (Buhl et al., 1995; Sah et al., 2000; Sagi et al., 2001; Kurose, 2003).

The recent observation by Kelley and coworkers (2004) of activation of PLC-ε by natively expressed LPA and PAR receptors of COS-7 cells was confirmed by the results reported here. Whereas the inositol lipid signaling response of these GPCRs was not affected by C3 toxin in the absence of PLC-ε expression, the complete inhibition of PLC-ε-dependent signaling from these receptors by C3 toxin is consistent with the conclusion that LPA and thrombin receptor-promoted activation of PLC-ε occurs through activation of Rho. Previous studies of the three subtypes of LPA receptors have suggested that these GPCRs couple to multiple G proteins (Anliker and Chun, 2004; Riobo and Manning, 2005). Expression of the LPA_1, LPA_2, or LPA_3 receptors all resulted in an enhanced inositol phosphate response to LPA in COS-7 cells. The large increase in LPA-stimulated response observed when the LPA_1 receptor was coexpressed with PLC-ε, and the inhibition of this augmented response by p115-RGS or C3 toxin is consistent with the known coupling of this GPCR to $G\alpha_{12/13}$ (Anliker and Chun, 2004; Riobo and Manning, 2005). Moreover, these results are consistent with the conclusion that the LPA_1 receptor activates PLC-ε through $G\alpha_{12/13}$-promoted activation of Rho. Observation of a large PLC-ε-dependent signaling response with expression of the PAR1 receptor also was consistent with the known coupling of this receptor to $G\alpha_{12/13}$ and Rho signaling pathways (Trejo, 2003; Riobo and Manning, 2005).

More than 50 RhoGEFs exist (Rossman et al., 2005), and therefore, Rho is activated by many different signaling pathways in addition to those involving $G\alpha_{12/13}$. Indeed, $G\alpha_q$ also promotes activation of Rho through mechanisms that appar-

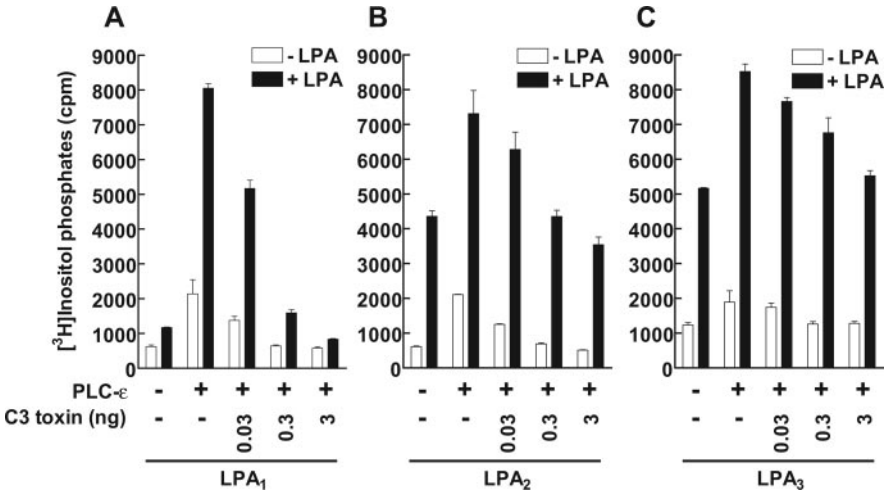

Fig. 7. C3 toxin-mediated inhibition of LPA-stimulated [^{3}H]inositol phosphate accumulation in COS-7 cells expressing PLC-ε and the LPA_1, LPA_2, or LPA_3 receptor. COS-7 cells were transfected with the indicated amounts of C3 toxin DNA and expression vectors for PLC-ε and the LPA_1, LPA_2, or LPA_3 receptor. [^{3}H]Inositol phosphate accumulation was quantified in the absence or presence of 10 μM LPA as described under *Materials and Methods*.

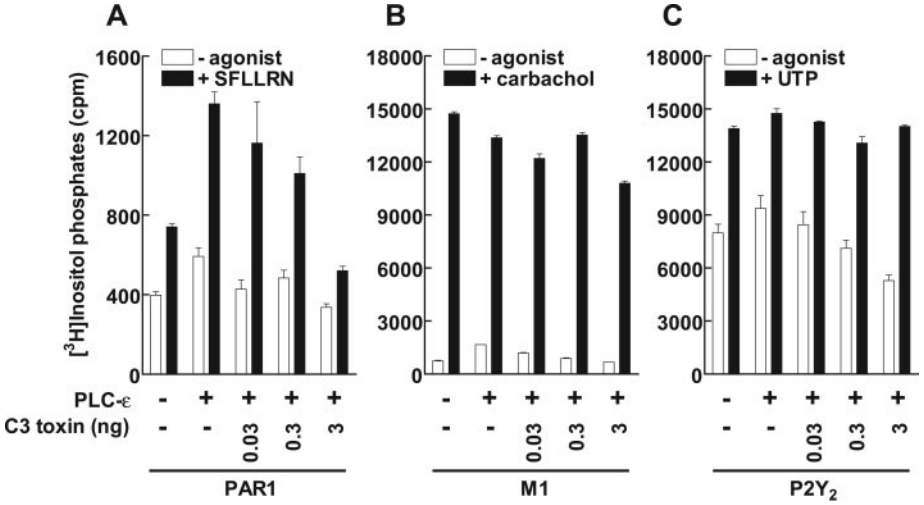

Fig. 8. Differential effect of C3 toxin on PAR1 receptor- versus M1 muscarinic cholinergic and $P2Y_2$ receptor-promoted [^{3}H]inositol phosphate accumulation in PLC-ε-expressing cells. COS-7 cells were transfected with the indicated amounts of C3 toxin DNA and expression vectors for PLC-ε and the PAR1 (A), M1 muscarinic cholinergic (B), or $P2Y_2$ purinergic receptor (C). [^{3}H]Inositol phosphate accumulation was quantified in the PAR1, M1, or $P2Y_2$ receptor-expressing cells in the absence or presence of 50 μM SFLLRN, 100 μM carbachol, or 100 μM UTP, respectively.

ently are independent of inositol lipid hydrolysis (Sah et al., 2000; Lutz et al., 2005), and $G\alpha_q$-activated RhoGEFs have been proposed to exist (Booden et al., 2002; Vogt et al., 2003; Lutz et al., 2005). Thus, GPCRs potentially regulate PLC-ε through $G\alpha_q$-dependent signaling pathways, although this apparently does not occur in COS-7 cells with the M1 muscarinic or $P2Y_2$ purinergic receptors. That is, whereas large increases in agonist-stimulated inositol lipid hydrolysis were observed after the expression of the G_q-coupled M1 muscarinic or $P2Y_2$ receptors, no activation of PLC-ε by these two receptors was observed. Perhaps G_q-regulated RhoGEFs are not expressed in COS-7 cells or lack cellular localization with the LPA and thrombin receptors and PLC-ε.

The RhoGEF responsible for LPA and thrombin receptor-promoted activation of PLC-ε in COS-7 cells remains to be identified. Indeed the work of Wang et al. (2004) in PC-3 prostate cancer cells indicates that the LPA and thrombin receptors may activate Rho through distinct RhoGEFs. Whereas the PAR1 receptor used the $G\alpha_{12/13}$-activated RhoGEF leukemia-associated RhoGEF, LPA receptor-promoted signaling involved another $G\alpha_{12/13}$-activated RhoGEF, PDZ-RhoGEF. It is noteworthy that Yamada et al. (2005) recently reported that the carboxyl termini of the LPA_1 and LPA_2 receptors, but not the LPA_3 receptor, interact with the PDZ domain of PDZ-RhoGEF and that mutation of the carboxyl terminus of the LPA_1 and LPA_2 receptors results in loss of capacity of LPA to promote the activation of Rho.

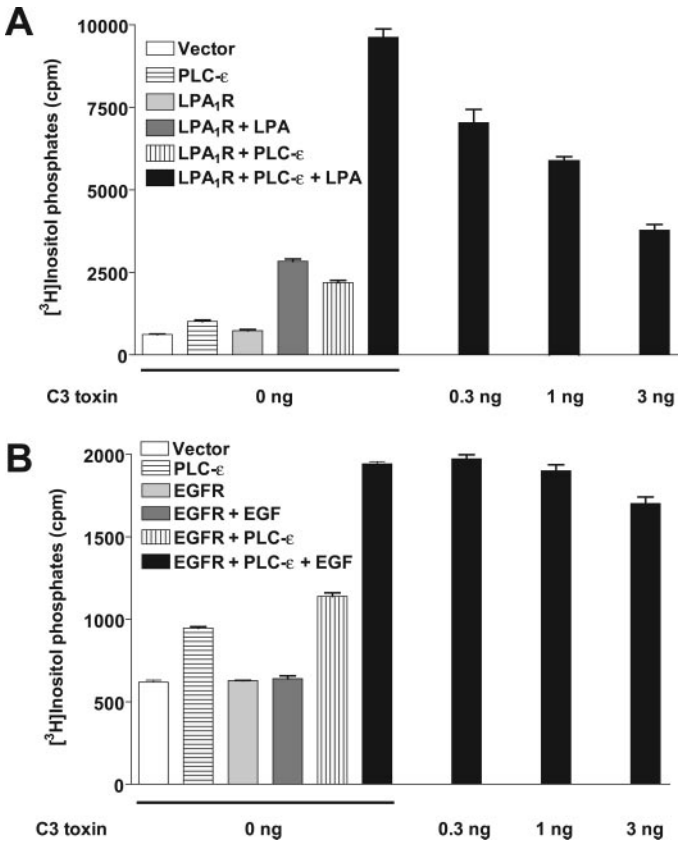

Fig. 9. Lack of effect of C3 toxin on EGF receptor-promoted activation of PLC-ε. COS-7 cells were transfected with the indicated amounts of C3 toxin DNA and expression vectors for PLC-ε and the LPA_1 (A) or EGF receptor (B). [^{3}H]Inositol phosphate accumulation was quantified in the LPA_1 or EGF receptor-expressing cells in the absence or presence of 10 μM LPA or 100 ng/ml EGF, respectively.

The most parsimonious interpretation of the current data is that LPA and thrombin receptors natively expressed in COS-7 cells and recombinant LPA_1 and PAR1 receptors overexpressed in these cells all potently activate PLC-ε as a downstream consequence of the activation of $G\alpha_{12/13}$ and Rho. LPA and thrombin receptors also are known to activate G_q and G_i and therefore also regulate inositol lipid signaling through the activation of PLC-β isozymes. The relative contribution of these different inositol lipid signaling pathways almost certainly will vary widely across cell types, and it will be important to establish the relative contribution of Rho-dependent activation of PLC-ε in the physiological responses to LPA and thrombin.

Independent binding of activated Rho and Ras subfamily GTPases to PLC-ε implies complex physiological regulation of this inositol lipid-hydrolyzing isozyme from multiple cell surface receptors. Activation of PLC-ε by EGF receptors was shown to be dependent on intact RA domains and apparently involves GEF-promoted activation of Ras or Rap (Kelley et al., 2004). Activation of Rap1A and consequential binding of GTP-bound Rap1A to the carboxyl-terminal RA domains also account for activation of PLC-ε by $G\alpha_s$-coupled GPCRs, which activate adenylyl cyclase, elevate cAMP levels, and therefore activate a cAMP-regulated GEF for Rap1A (Schmidt et al., 2001; Evellin et al., 2002; Keiper et al., 2004). $G\alpha_{12}$, $G\alpha_{13}$, and/or other $G\alpha$ subunits may yet prove to be direct activators of PLC-ε. However, the data presented here indicate that Rho accounts for much if not all of the activation of this isozyme by $G\alpha_{12}$ and $G\alpha_{13}$, and the direct regulation of PLC-ε illustrated to date involves binding of Rho family GTPases in the catalytic core of the isozyme and binding of Ras family GTPases in the RA domains of the carboxyl terminus.

The physiological roles played by PLC-ε have yet to be defined. However, mice lacking functional PLC-ε exhibit defects in heart semilunar valve development (Tadano et al., 2005), increased susceptibility to hypertrophy under chronic stress (Wang et al., 2005), and marked reduction of the incidence of skin tumors in a chemical carcinogen-induced model (Bai et al., 2004). Furthermore, PLC-ε is specifically induced in the developing mouse brain (Wu et al., 2003), suggesting a role for this phospholipase in neuronal differentiation. Rho-mediated pathways downstream of receptors for LPA, thrombin, and other extracellular signaling molecules subserve important roles in neuronal growth and differentiation (Govek et al., 2005). The current study suggests that it will be important to establish the contribution of Rho-regulated PLC-ε in various aspects of neuronal development and function.

In summary, the current work together with previous studies illustrating that Rho directly activates PLC-ε are consistent with the idea that PLC-ε is a downstream effector of GPCRs that activate $G\alpha_{12/13}$ and consequentially activate Rho. LPA, thrombin, and other receptors that activate $G\alpha_{12/13}$ also predictably activate G_q. The relative contribution of PLC-ε versus PLC-β isozymes in the physiological action of these $G\alpha_{12/13}$-activating GPCRs will be important to establish. Likewise, RhoA, RhoB, and RhoC robustly activate PLC-ε, and it will be important to understand the extent to which this PLC isozyme is involved in the wide range of cellular processes known to be regulated by these GTPases.

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