Gα_{12/13}- and Rho-Dependent Activation of Phospholipase C-ε by Lysophosphatidic Acid and Thrombin Receptors

Melinda D. Hains, Michele R. Wing, Savitri Maddileti, David P. Siderovski, and T. Kendall Harden

Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina

ABSTRACT

Because phospholipase C ε (PLC-ε) is activated by Gα_{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 LPA₁ 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 LPA₁ 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 the RGS domain of Gα_{12/13} but not by expression of the RGS domain of GRK2. Expression of the Rho inhibitor C3 botulinum toxin did not affect LPA₁- 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 LPA₁, LPA₂, LPA₃, or PAR1 receptor but had no effect on the agonist-promoted inositol phosphate response of the M1 or P2Y₂ 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 Gα_{12/13} and Rho.

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.

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)P₂ into the Ca^{2+}-mobilizing second-messenger inositol (1,4,5)P₃ and the protein kinase C-activating second-messenger diacylglycerol (Irving 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 Gα family of G proteins (Smrcka et al., 1991; Taylor et al., 1991; Waldo et al., 1991) or by the release of Gβγ dimers from activated Gi (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, Gα₂-coupled GPCRs promote PLC-ε-dependent inositol lipid signaling through activation of the
cAMP-activated GEF, EPAC, which in turn activates Rap1A (Schmidt et al., 2001). Initial studies of mammalian PLC-e revealed activation by G<sub>12</sub> and G<sub>13</sub> but not by G<sub>q</sub> (Lopez et al., 2001; Wing et al., 2001), and G<sub>q</sub>γ also has been shown to activate this PLC isozyme (Wing et al., 2001).

Coexpression of Rho family GTPases with PLC-e results in marked stimulation of inositol lipid hydrolysis (Wing et al., 2003). PLC-e 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-e 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<sub>12/13</sub> (Hart et al., 1998; Fukuhiara et al., 1999; Boonden et al., 2002; Suzuki et al., 2003; Dutt et al., 2004). Thus, the observation of Rho-dependent activation of PLC-e suggests that GPCRs that activate G<sub>12/13</sub> 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<sub>12/13</sub>, Rho, and PLC-e, we studied the regulation of PLC-e-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<sub>12/13</sub>- and Rho-dependent activation of PLC-e occurs downstream of both LPA- and thrombin-activated receptors and that the regulation of PLC-e by G<sub>12/13</sub> 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<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> receptors were purchased from the University of Missouri–Rolla DNA Resource Center (Rolla, MO). An expression vector encoding the human P2Y<sub>12</sub> 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-e 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 p115RhoEGF 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 178 to 478 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-rac-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 N-terminal tandem haemagglutinin-epitope tag into a modified pcDNA3.1 vector provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). 1-Oleoyl-rac-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 N-terminal tandem hemagglutinin-epitope tag into a modified pcDNA3.1 vector provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). 1-Oleoyl-rac-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 N-terminal tandem hemagglutinin-epitope tag into a modified pcDNA3.1 vector provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA).

Results

PLC-e-Dependent Promotion of Inositol Lipid Signaling by Endogenous LPA and Thrombin Receptors. We reported recently that Rho GTPases directly activate PLC-e (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-e-dependent activation of inositol lipid signaling. Incubation of cells with histamine, prostaglandin E<sub>2</sub>, 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-e-dependent manner (data not shown). In contrast, as was reported recently by Kelley et al. (2004), incubation of PLC-e-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-e-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<sub>1</sub>, LPA<sub>2</sub>, or LPA<sub>3</sub> receptors in COS-7 cells with the goal of determining the extent to which these signaling proteins exhibit PLC-e-dependence in their action. LPA-stimulated inositol phosphate accumulation was reproducibly increased in LPA<sub>1</sub> receptor-expressing cells compared with empty vector-transfected cells and was enhanced by ~10-fold in LPA<sub>2</sub> or LPA<sub>3</sub> receptor-expressing cells (Fig. 2). Coexpression of the LPA<sub>1</sub> receptor with PLC-e markedly enhanced the
independent stimulation of 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 P2Y2 receptor (Lazarowski et al., 1995, 2000; Alvarado-Castillo et al., 2005).

RGS Protein-Selective Inhibition of PLC-ε-Dependent Signaling by the LPA1 and PAR1 Receptors. Activation of both LPA (Anliker and Chunt, 2004; Riobo and Manning, 2005) and thrombin (Trejo, 2003; Riobo and Manning, 2005) receptors has been reported previously to result in activation of Ga_{12/13} and Rho-promoted signaling pathways. To address the potential roles of Ga_{12/13} and Ga_{13} in the responses to these GPCRs, we applied the RGS domain of p115RhoGEF (p115-RGS), which acts as a GTPase-activating protein for Ga_{12} and Ga_{13} (Kozasa et al., 1998; Hains et al., 2004), and GRK2-RGS, which is known to bind selectively to Ga_{13} and inhibit Ga_{13} signaling (Carman et al., 1999; Hains et al., 2004). Coexpression of p115-RGS with the LPA1 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 P2Y2 receptor (data not shown) in the absence or presence of PLC-ε. Conversely, expression of Ga_{13}-binding GRK2-RGS significantly inhibited M1 (Fig. 4C) and P2Y2 receptor (data not shown) receptor-promoted signaling but had no effect on the inositol lipid signaling response to the LPA1 (Fig. 4A) or PAR1 (Fig. 4B) receptor, either in the absence or presence of the expression of PLC-ε.

C3 Toxin Inhibits Ga_{12/13}-, LPA-, and Thrombin-Promoted Activation of PLC-ε. The data presented thus far implicate Ga_{12/13} in the mechanism of activation of PLC-ε by LPA and thrombin receptors. In contrast, the M1 muscarinic and P2Y2 receptors apparently regulate inositol lipid signaling by mechanisms that involve neither Ga_{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 Ga_{12} (Ga_{12(Q229L)} or Ga_{13} (Ga_{13(Q226L)}) had no effect on the accumulation of inositol phosphates in control COS-7 cells (Fig. 5A). However, coexpression of these Ga_{12} or Ga_{13} mutants with PLC-ε resulted in a marked increase in [H]inositol phosphate accumulation compared with [H]inositol phosphate levels in cells transfected with PLC-ε alone. The capacity of Ga_{12} and Ga_{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
nonspecific effect on inositol lipid signaling because the capacity of GTPase-deficient Gaq (GaQ209L) 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 [3H]inositol phosphate accumulation in cells expressing the PAR1 receptor versus the M1 muscarinic cholinergic or P2Y2 receptor. COS-7 cells were transfected with an expression vector for the PAR1 (A), M1 muscarinic cholinergic (B), or P2Y2 (C) receptor in the absence or presence of an expression vector for PLC-ε. [3H]Inositol phosphate accumulation was quantified in the PAR1, M1, or P2Y2 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 LPA1 receptor- and PAR1 receptor- versus M1 muscarinic receptor-promoted [3H]inositol phosphate accumulation in PLC-ε-expressing cells. COS-7 cells were transfected with the LPA1 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. [3H]Inositol phosphate accumulation was quantified in the LPA1, 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 Ga12 and Ga13. A, COS-7 cells were transfected with empty vector, GTPase-deficient Ga12 (Ga12 Q226L), or GTPase-deficient Ga13 (Ga13 Q226L) in the absence or presence of transfection of an expression vector for PLC-ε. B, COS-7 cells were transfected with PLC-ε + Ga12, PLC-ε + Ga13, or GaQ (Q209L) and with the indicated amounts of an expression vector for C3 toxin. [3H]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 + Ga12, 6693 cpm; PLC-ε + Ga13, 12,447 cpm; GaQ (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× Laemli 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 Gtq12 and Gtq13 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 was also examined by measuring inositol phosphate accumulation after expression of C3 toxin. Whereas the capacity of LPA (Fig. 6A) or SPLLRRN (Fig. 6B) to stimulate phosphoinositide hydrolysis in the absence of PLC-ε was not affected by transient expression of C3 toxin, the 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 LPA1 receptor expression was not affected by C3 toxin (data not shown). In contrast, the large PLC-ε-dependent response to LPA observed in LPA1 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 LPA2 or LPA3 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 P2Y2 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 [3H]inositol phosphate accumulation after PLC-ε expression were suppressed by coexpression of C3 toxin (Figs. 7 and 8). PLC-ε-dependent elevation of [3H]inositol phosphate accumulation also was inhibited by p115-RGS (Fig. 4A and data not shown), suggesting that a Gtq12/13/Rho-dependent pathway activates expressed PLC-ε in the absence of added receptor agonists. An analogous effect is observed with overexpression of Gtq-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; Lazdunski 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 Gtq12/13/Rho-independent mechanism involving the binding of Ras subfamily GTPases to the carboxy-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 [3H]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 [3H]inositol phosphates was quantified in the presence of EGF versus LPA in EGF receptor-expressing cells versus LPA1 receptor-expressing cells, respectively. Whereas the expression of increasing amounts of C3 toxin inhibited LPA1 receptor-promoted [3H]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).

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 Gtq12, Gtq13, Gαq, and Gβγ (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 Gαq12 and Gαq13 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 Gtq12/13 and Rho.

The downstream signaling responses promoted by GPCRs through Gtq12/13-dependent mechanisms have been less clearly...
defined than those dependent on activation of Gα subunits of the Gs, Gi, and Gq families. Nonetheless, marked morphological and cell proliferative changes are consistently observed with the introduction of GTPase-deficient mutants of Gα12 or Gα13 in various cell types (Sah et al., 2000; Kurose, 2003), and a variety of effectors are stimulated downstream of activation of Gα12/13 (Kurose, 2003; Riobo and Manning, 2005). Some of the cellular responses promoted by Gα12/13 are mimicked by activated Rho, and activation of Rho occurs in many if not all cells in which Gα12 or Gα13 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 Gα12/13. Moreover, the observation that certain Gα12/13-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 Gα12/13 (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 LPA1, LPA2, or LPA3 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 LPA1 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 Gα12/13 (Anliker and Chun, 2004; Riobo and Manning, 2005). Moreover, these results are consistent with the conclusion that the LPA1 receptor activates PLC-ε through Gα12/13-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 Gα12/13 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 Gα12/13. Indeed, Gαq also promotes activation of Rho through mechanisms that appar-

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**Fig. 7.** C3 toxin-mediated inhibition of LPA-stimulated [3H]inositol phosphate accumulation in COS-7 cells expressing PLC-ε and the LPA1, LPA2, or LPA3 receptor. COS-7 cells were transfected with the indicated amounts of C3 toxin DNA and expression vectors for PLC-ε and the LPA1, LPA2, or LPA3 receptor. [3H]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 P2Y2 receptor-promoted [3H]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 P2Y2 purinergic receptor (C). [3H]Inositol phosphate accumulation was quantified in the absence or presence of 10 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 Goαi-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 Goαi-dependent signaling pathways, although this apparently does not occur in COS-7 cells with the M1 muscarinic or P2Y2 purinergic receptors. That is, whereas large increases in agonist-stimulated inositol lipid hydrolysis were observed after the expression of the Goαi-coupled M1 muscarinic or P2Y2 receptors, no activation of PLC-ε by these two receptors was observed. Perhaps Goαi-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 Go12/13-activated RhoGEF leukemia-associated RhoGEF, LPA receptor-promoted signaling involved another Go12/13-activated RhoGEF, PDZ-RhoGEF. It is noteworthy that Yamada et al. (2005) recently reported that the carboxyl termini of the LPA1 and LPA2 receptors, but not the LPA3 receptor, interact with the PDZ domain of PDZ-RhoGEF and that mutation of the carboxyl terminus of the LPA1 and LPA2 receptors results in loss of capacity of LPA to promote the activation of Rho.

The most parsimonious interpretation of the current data is that LPA and thrombin receptors natively expressed in COS-7 cells and recombinant LPA1 and PAR1 receptors overexpressed in these cells all potently activate PLC-ε as a downstream consequence of the activation of Go12/13 and Rho. LPA and thrombin receptors also are known to activate Goε and Goq 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 carboxy-terminal RA domains also account for activation of PLC-ε by Goαi-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). Go12, Go13, and/or other Goα 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 Go12 and Go13, and the direct regulation of PLC-ε illustrated to date involves binding of the 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 seminal 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 Go12/13 and consequently activate Rho. LPA, thrombin, and other receptors that activate Go12/13 also predictably activate Goε. The relative contribution of PLC-ε versus PLC-β isozymes in the physiological action of these Go12/13-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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At the University of North Carolina School of Medicine, Chapel Hill, NC 27599-7965. E-mail: tkhard@med.unc.edu

Address correspondence to: Dr. T. K. Harden, Department of Pharmacology,