Molecular Biology of the Cell
Vol. 11, 4359–4368, December 2000

Dual Requirement for Rho and Protein Kinase C in Direct Activation of Phospholipase D1 Through G Protein-coupled Receptor Signaling

Guangwei Du,*† Yelena M. Altshuller,*† Yong Kim,‡ Jung Min Han,‡ Sung Ho Ryu,‡ Andrew J. Morris,* and Michael A. Frohman*†§

*Department of Pharmacology and †the Center for Developmental Genetics, University Medical Center at Stony Brook, Stony Brook, New York 11794-5140; and the ‡Department of Life Science, Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, 790–784, Korea

Submitted July 26, 2000; Revised September 18, 2000; Accepted October 3, 2000
Monitoring Editor: Martin Raff

G protein-coupled and tyrosine kinase receptor activation of phospholipase D1 (PLD1) play key roles in agonist-stimulated cellular responses such as regulated exocytosis, actin stress fiber formation, and alterations in cell morphology and motility. Protein Kinase C, ADP-ribosylation factor (ARF), and Rho family members activate PLD1 in vitro; however, the actions of the stimulators on PLD1 in vivo have been proposed to take place through indirect pathways. We have used the yeast split-hybrid system to generate PLD1 alleles that fail to bind to or to be activated by RhoA but that retain wild-type responses to ARF and PKC. These alleles then were employed in combination with alleles unresponsive to PKC or to both stimulators to examine the activation of PLD1 by G protein-coupled receptors. Our results demonstrate that direct stimulation of PLD1 in vivo by RhoA (and by PKC) is critical for significant PLD1 activation but that PLD1 subcellular localization and regulated phosphorylation occur independently of these stimulatory pathways.

INTRODUCTION

The hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) generates the signaling lipid phosphatidic acid (PA) (reviewed in Frohman *et al.*, 1999; Jones *et al.*, 1999). PA has been hypothesized to act as a fusogenic lipid in vesicular transport such as exocytosis (Way *et al.*, 2000; Caumont *et al.*, 1998) or endocytosis (Schmidt *et al.*, 1999a). PA also may act as an anchor for signaling molecules that act in proliferation pathways, such as the Raf-1 kinase (Rizzo *et al.*, 2000), as regulators of cell shape through the stimulation of PI4P5-kinase (Honda *et al.*, 1999) or as the substrate to generate diacylglycerol or lysophosphatidic acid (LPA) through further metabolism (reviewed in Frohman *et al.*, 1999; Jones *et al.*, 1999). Many cell types express the known PLD1 (Hammond *et al.*, 1995) and/or PLD2 (Colley *et al.*, 1997) isoenzymes, which become activated in response to a wide variety of agonists that signal through heterotrimeric G protein-coupled or tyrosine kinase receptors (reviewed in Liscovitch *et al.*, 2000). It has been proposed that the link between receptor stimulation and PLD activation is mediated by protein kinase C (PKC), ADP-ribosylation factor (ARF), and Rho family members (Singer *et al.*, 1995; Siddiqi *et al.*, 1995; Liscovitch *et al.*, 2000), which have been shown to stimulate PLD1 directly using in vitro reconstitution assays (Hammond *et al.*, 1997). Since ARF, Rho, and PKC stimulate multiple downstream effector pathways that ultimately regulate cellular morphology, proliferation, and secretion, there has been intense interest in determining the relationship of PLD stimulation through each activator to these cell biological events.

Such studies generally have been approached through the manipulation of PKC, Rho, or ARF activity using the overexpression of activated or inactive alleles, toxins, or pharmacologic agents to activate or inhibit them (Liscovitch *et al.*, 2000). A key issue raised by many of these studies has been whether PLD1 is actually activated in vivo through direct stimulation by its effectors, or whether indirect pathways are more critical. Specifically, inhibitors of the enzymatic activity of PKC have been shown to block PLD activation, even though the direct stimulation of PLD1 by PKC in vitro involves an interaction with the regulatory domain of PKC and is ATP-independent (Liscovitch *et al.*, 2000). This suggests that a PKC target other than PLD is critical for the

§ Corresponding author. E-mail: michael@pharm.sunysb.edu.

Abbreviations used: AT1a, angiotensin II; ARF, ADP-ribosylation factor; LPA, lysophosphatidic acid; mAChR, muscarinic acetylcholine receptor; PA, phosphatidic acid; PC, phosphatidylcholine; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate.

activation of PLD by PKC in vivo. Similarly, it has been proposed that the primary mechanism through which Rho activates PLD1 is an indirect one in which Rho activates Rho-kinase, which then activates PLD via an unknown pathway (Schmidt *et al.*, 1999b).

However, these approaches involve complicated interpretations, since manipulation of PKC, Rho, and ARF levels of activity affect many things aside from PLD regulation. These include cross-regulation of the other stimulators and alterations in availability of $PI4,5P_2$ (a required cofactor for PLD). In addition, the pharmacologic agents are inevitably found to exhibit less than complete specificity (Zhang *et al.*, 1999). Moreover, reagents such as Rho-dominant negative alleles cause undesired widespread effects by sequestering the guanine–nucleotide exchange factors required to activate other small G-proteins.

In recent years, we have described an alternative approach based on generating alleles of PLD that exhibit altered regulation. These include mutants that are inactive (Sung *et al.*, 1997), selectively nonresponsive to PKC (Zhang *et al.*, 1999), or insensitive to $PI4,5P_2$ (Sciorra *et al.*, 1999). The rationale for this scheme was given in earlier studies that demonstrated that the stimulators of PLD1 physically interact with it at distinct sites in the molecule (Hammond *et al.*, 1997; Sung *et al.*, 1997; Sung *et al.*, 1999b; Yamazaki *et al.*, 1999). Our previous study using an allele selectively nonresponsive to PKC revealed that direct stimulation of PLD1 by PKC is required for much of the response of PLD1 to G protein-coupled agonists.

In this report, we demonstrate that direct stimulation of PLD1 by Rho is also critical using novel, selectively nonresponsive alleles. Taken together, our results demonstrate that signal integration through both PKC and Rho via direct contact is required for significant physiologic in vivo activation of PLD1 during agonist signaling.

MATERIALS AND METHODS

General Reagents

All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). $PI4,5P_2$ was isolated as described (Frohman *et al.*, 2000). L-dipalmitoyl phosphatidylcholine [choline-methyl-3H] ([3H]phosphatidylcholine) [3H]palmitic acid were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). 3F10 antibody (rat monoclonal anti-HA tag antibody) was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Antirat IgG from goat conjugated with horseradish peroxidase or Cy3 were from Jackson ImmunoResearch Laboratories (West Grove, PA). All cell culture media (DMEM and Opti-MEM-I) and LipofectAmine Plus were from Life Technologies (Gaithersburg, MD). Thin-layer chromatography plates were obtained from Fisher (Pittsburgh, PA). The yeast split-hybrid system was obtained from Bio101, Inc (Carlsbad, CA). SuperSignal West Pico Trial Kit for detection of HRP was from Pierce Chemical (Rockford, IL). Phorbol 12-myristate 13-Acetate (PMA) was from Sigma Chemical (St. Louis, MO). All other reagents were of analytical grade, unless otherwise specified.

Construction of Plasmids

The C-terminus of wild-type PLD1 (D4 fragment, amino acids 711-1074, but not the stop codon) was inserted into the *Bam*HI site of pSHM-lacZ vector using appended *Bcl*I and *Bgl*II restriction enzyme sites in frame with the VP16 DNA binding domain at the N-terminus and LacZ at the C-terminus of the PLD1 D4 fragment. To facilitate subsequent subcloning steps, *Sac*II and *Kpn*I restriction sites were generated at nucleotides 2342 and 3284 (amino acids 748 and 1062) by altering wobble codons and an *Xba*I site was appended to the end of the D4 coding sequence. Mutated D4 fragments were subcloned into pSHM-D4-lacZ using the *Sac*II and *Xba*I sites to replace the corresponding wild-type fragment and were recovered using the *Sac*II and *Kpn*I sites for subsequent subcloning/replacement into the full-length PLD1 cDNA (a version lacking the amino terminal *Kpn*I site at nt 388 was used, Zhang *et al.*, 1999) in the mammalian expression vector pCGN that contained the same engineered *Sac*II and *Kpn*I sites. Finally, the full-length PLD1 wild-type and mutant cDNAs were subcloned into pFASTBAC modified to contain the sequence MEYMPMEG (Glu-Glu tag) at the amino terminus to express the proteins in sf9 cells using baculovirus. PEA-15 (amino acids 32–129) was cloned into the *Eco*RI and *Pst*I sites of pBTM116 in frame with the LexA DNA-binding domain.

Site-directed Mutagenesis

Site-directed mutagenesis of expression plasmids was carried out using the Quik-Change kit (Stratagene, La Jolla, CA). Plasmids were sequenced to confirm the intended mutation and the integrity of the surrounding sequences for at least 500 bp using a DNA automated sequencer (model 373A, Applied Biosystems, Foster City, CA).

Random Mutagenesis and PLD1 Mutant Library Construction

Random mutations were introduced using mutagenic polymerase chain reaction (PCR) (Cadwell and Joyce, 1994). The PCR conditions were selected to obtain the desired low level of mutagenesis (1–2 amino acid substitution). Two synthetic oligonucleotides, which flanked the engineered *Sac*II and *Xba*I restriction sites, were used as primers for mutagenic PCR. PCR was carried out using 10 ng of the pSHM-D4-lacZ plasmid as the template, 0.5 μM each primer, 50 mM KCl, 10 mM Tris (pH 8.3), 5 mM MgCl2, 0.7 mM dCTP and 0.7 mM dTTP, 0.2 mM dATP and 0.2 mM dGTP, and 2.5 U *Taq* polymerase (Roche Molecular Biochemicals) in a total volume of 50 μl. The reaction mixtures were subjected to the following PCR conditions: 94°C for 2 min; 25 cycles of 94°C for 40 s; 55°C for 40 s; 72°C for 2 min; followed by one cycle of incubation at 72°C for 10 min. The amplification products were used to replace the corresponding wild-type fragments in pSHM-D4-lacZ using the engineered *Sac*II and *Xbal* I sites. DH5α cells were electroporated with the resulting plasmids and were plated to generate a primary library with a complexity of 2.3×10^6 individual clones. The resulting colonies were washed from the agar plates and grown overnight before purifying the pooled plasmid library.

Screening for PLD1 D4 Mutants That Lost Interaction with RhoA in Yeast

The yeast strain YI584 was cotransformed with the pSHM-D4-lacZ mutant library and pBTM116-$RhoA^{V14}$. Two milliliters of lithium-treated cells were transformed with 40 μg of library DNA, 10 μg of pBTM116-RhoAV14, and 2 mg of salmon sperm DNA and were plated on SDA-His-Leu-Lys-Trp-Ura media containing 30 mM 3AT. Approximately 90,000 clones were plated. After 5 days, the 550 large colonies recovered were streaked to new SDA-Leu-Lys-Trp-Ura plates and were tested for the ability to turn blue using 5-bromo-4-chloro-3-indoyl β-galactoside. White and pale blue colonies indicated mutants characterized by stop codons, frame shifts, or protein instability. The pSHM-D4-lacZ plasmids were isolated from dark blue colonies (147 total) using transformation into *Escherichia coli* strain DH5α and colony hybridization with a D4 cDNA fragment probe. Plasmids from 121 pSHM-D4-lacZ mutants were recovered, and the (loss of) interaction of 50 of them with $RhoA^{V14}$ was confirmed by retransforming them into YI584 cells as well as into L40 cells (standard two-hybrid system). In the latter system, interactions

with PEA-15 (Zhang *et al.*, 2000) also were scored using L40 cells and the standard two-hybrid system. Positive controls were pSHM-D4-lacZ: pBTM116-RhoA^{V14} and pSHM-D4-lacZ: pBTM116-PEA-15ΔN; the negative control was pSHM-D4-lacZ: pBTM116-RhoA-wild-type. Candidate alleles were sequenced using BigDye terminator chemistry (Perkin Elmer-Cetus, Norwalk, CT) on a DNA automated sequencer.

Baculovirus Expression of PLD1 Proteins

Recombinant bacmids were prepared by transformation of DH10Bac cells with the pFASTBAC wild-type and mutant PLD1 cDNAs. Recombinant baculoviruses were amplified and propagated using standard procedures. Monolayer cultures of exponentially growing Sf9 cells were infected with baculoviruses at a multiplicity of 10 and were cultured for 48 h at 27°C. These proteins were purified by affinity chromatography using an immobilized anti-Glu–Glu monoclonal antibody and were eluted using Glu–Glu peptide. The concentrations of the proteins were measured by Coomassie Plus-200 protein assay reagent (Pierce Chemical).

Cell Culture and Transfection

HEK 293 and COS-7 cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. HEK 293 cells stably expressing the m2 and m3 mAChR were maintained in DMEM medium containing 0.5 mg/ml G418.

For transfections, the cells were grown in 35-mm dishes (3–4 × 10^5 cells/dish) and then were switched into Opti-MEM I media before transfection. The cells were transfected with 1 μg of DNA/dish using Lipofectamine Plus. Four hours post-transfection, the medium was replaced with complete DMEM and the cells were incubated for a further 24 h. For in vivo PLD assays, the transfection mixtures were replaced with complete DMEM containing 2 μCi of [3H]palmitic acid.

Western Analysis and Immunofluorescent Staining

Both were performed as described as before (Colley *et al.*, 1997; Sung *et al.*, 1997; Sung *et al.*, 1999b) using rat anti-HA 3F10 antibody and Cy3- or HRP-conjugated secondary antibodies/chemiluminescence (Pierce Chemical) to detect the HA-tag fused in frame to the PLD1 proteins.

Phosphorylation Analysis of PIM87

Immunoprecipitation and immunoblot analyses were performed as described previously (Kim *et al.*, 2000). Phosphorylation analysis of PLD1 in COS-7 cells and two-dimensional phosphopeptide mapping were performed as described previously (Kim *et al.*, 1999).

PLD Activity Assays

PLD activity assays were carried out using the in vitro head-group release assay for a 30-min time period and the in vivo transphosphatidylation assay as previously described (Morris *et al.*, 1997; Zhang *et al.*, 1999). Recombinant ARF1 and RhoA were purified and were activated using 50 μM GTPS as previously described (Hammond *et al.*, 1997; Liang *et al.*, 1997). Error bars in the figures (Figures 2, 3, 6, 7, 8) depict one SD, based on analysis of the replicate samples in the representative experiments shown.

RESULTS

Generation of Rho-noninteracting PLD1 Alleles Using the Yeast Split-hybrid System

We previously reported that the C-terminus of hPLD1 (amino acids 712-1074) interacts in a GTP-dependent manner with RhoA in the two-hybrid system (Sung *et al.*, 1997; Yamazaki *et al.*, 1999). Further activity and interaction analyses using a large series of mutant alleles characterized by random 5-amino acid insertions narrowed the probable site of interaction (Zhang *et al.*, 1999, 2000). However, all of the mutant alleles that lost responsiveness to Rho also lost responsiveness in parallel to ARF1 and PKC, presumably reflecting disruptions of the basic enzymatic process. As the first goal of this study, we sought to generate more subtle changes in PLD1, such as point mutants, to identify selectively Rho-insensitive alleles.

The yeast split-hybrid system is a positive selection method designed to identify factors or mutations that disrupt protein–protein interactions (Shih *et al.*, 1996). In brief, it is a reversed two-hybrid system in which a successful interaction activates a tet transcription factor, which in turn suppresses a histidine synthase gene (*HIS3*) under the control of the tet operon. Accordingly, yeast lacking other means of generating histidine fail to grow in histidine-deficient media unless the interaction is lost. Loss of interaction is achieved through PCR mutagenesis of the cDNA corresponding to one of the protein partners. Mutated alleles containing stop codons or frame shifts are eliminated by fusing LacZ to the C-terminus of the mutated protein with concomitant monitoring of the retention of blue color in the yeast colonies recovered after selection.

RhoA^{V14}, a dominant active form of RhoA, was cloned into the pBTM116 vector as a fusion protein with the LexA DNA-binding domain (Yamazaki *et al.*, 1999). The C-terminal D4 fragment (amino acids 711-1074) of PLD1 was cloned into the pSHM-lacZ vector as a fusion protein linked to the transcriptional activator VP16 at the N-terminus of D4 and to LacZ at the C-terminus of D4 (pSHM-D4-LacZ). Rho and PLD1 successfully interacted in this setting despite the C-terminal addition of LacZ to the PLD1 fragment (our unpublished results). A subset of the D4 region (amino acids 749-1061) then was amplified using mutagenic PCR conditions that generated an average of one mutation per DNA fragment and was used to replace the corresponding sequence in pSHM-D4-LacZ. A library of such plasmids was cotransformed with pBTM116-RhoA^{V14} into yeast and was screened, as described in the MATERIALS AND METHODS section, to obtain RhoA^{V14}-noninteracting PLD1 alleles. Sequence analysis revealed that 15 of the alleles were single-point mutants and that 17 had two mutations (Figure 1A).

The single- and double-point mutants then were scored in the yeast two-hybrid system for interaction with PEA-15, the only other protein known to interact with the PLD1 C-terminus (Zhang *et al.*, 2000). Some of the alleles failed to interact, suggesting that both interaction sites had been disrupted or that the D4 fragment was not folding well. However, 12 of the mutants still interacted with PEA-15 as strongly as the wild-type D4 fragment, suggesting that, in general, these mutated D4 fragments were folded correctly (Figure 1A).

Identification of PLD1 Alleles Selectively Nonresponsive to Rho Stimulation

The 11 best candidates (alleles encoding mutations not located at residues known to be critical for functioning of the catalytic site and preferentially still interacting with PEA-15) were subcloned into the mammalian pCGN-PLD1 expres-

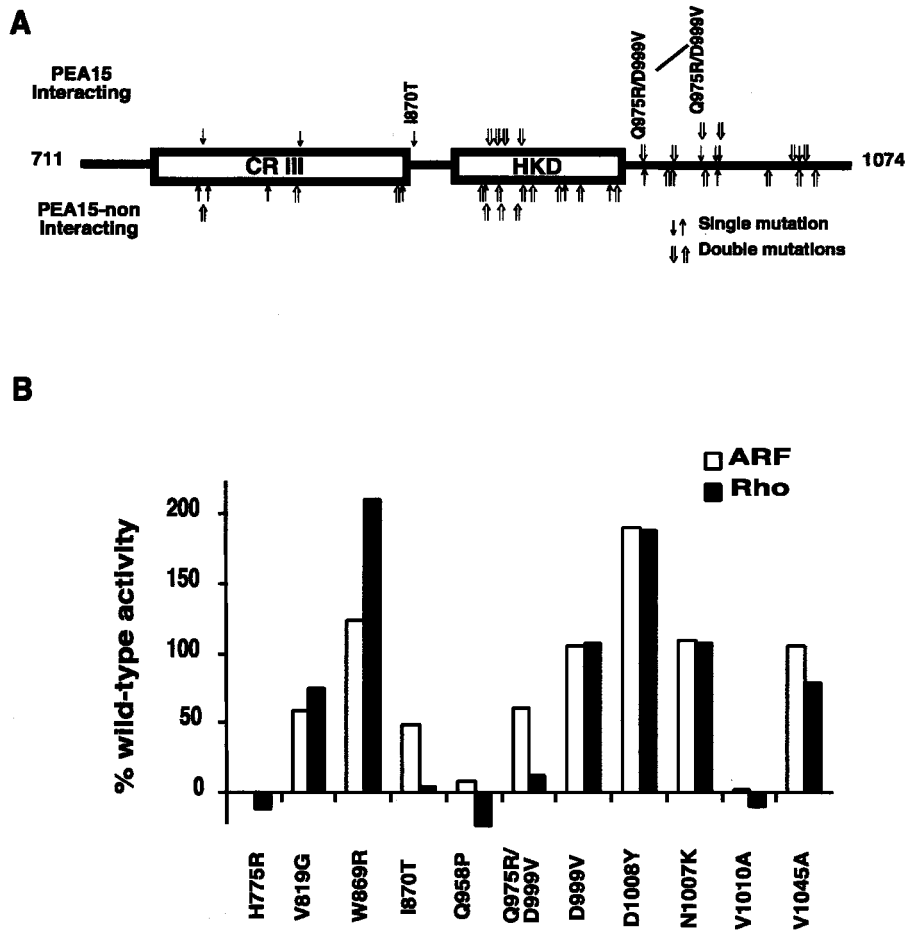


Figure 1. Identification of two candidate RhoA-nonresponsive PLD1 mutants. (A) Distribution of single- and double-point mutations in the RhoA-noninteracting D4 fragments recovered from the split-hybrid screen and scoring of their interaction with PEA15. Arrows above the schematic diagram indicate alleles that continued to successfully interact with PEA-15 (H775R, V819G, I870T, N905Y/K981N, T906S/Q1046H, N913/D1008N, G921E/K1039E, Q975R/D999V, D999V, N1007K, V1010A, and V1045A); arrows below the schematic diagram indicate alleles that did not interact with PEA-15 (I770 M, H775L/E1054G, I777N, I805T, V817E/S979R, N867K/D980V, W869R, D903V/R1032P, D904N, D904G/A912T, A912G/L964R, K922 M/S939L, S925R/V1001N, M941T, A948V/V1045 M, Q958P, D976V, and D1008Y). Only the C-terminal D4 fragment of PLD1 (amino acids 711-1074) is shown. Depicted within it is conserved region III (amino acids 743–864) and the HKD domain (amino acids 888–965), both of which are critical for catalytic functioning independent of RhoA stimulation. The mutants I870T and Q975R/D999V are discussed further in the text. (B) Wild-type PLD1, PLD1-K898R (a catalytically inactive allele) (Sung *et al.*, 1997), and candidate nonresponsive alleles recovered from the split-hybrid screen were overexpressed in COS-7 cells. The transfected cells were lysed and assayed for PLD activity in the presence of GTPγS-activated ARF1 and RhoA using the in vitro head-group release assay. Activities are shown in comparison to those exhibited by wild-type PLD1 and by PLD1-K898R, which define the 100% and 0% activities, respectively. Each mutant was assayed in duplicate a minimum of twice. Note that the PLD1-K898R behaves as a null allele, not as a dominant negative one, in both the in vitro and in vivo PLD assays (Zhang *et al.*, 1999).

sion vector using engineered *Sac*II and *Kpn*I restriction enzyme sites. COS-7 cells then were transiently transfected with these PLD1 mutants, harvested, lysed, and assayed in vitro for PLD activity in the presence of activated RhoA and ARF (Figure 1B). Three of the mutants appeared to be inactive, and six of the mutants responded normally to both ARF and Rho. However, two mutants, I870T and Q975R/D999V, selectively lost most of their response to Rho (5–10% residual activity, part or all of which could have resulted from GTPγS activation of the endogenous ARFs in the lysates). Neither the D999V mutation alone (Figure 1B) nor the Q975R point mutation alone (our unpublished results) exhibited more than minimally decreased responsiveness to RhoA; thus, the loss of responsiveness exhibited by the Q975R/D999V allele requires both mutations. The allele I870R also was generated since PLD2 does not coimmunoprecipitate with RhoA, interact with it in the two-hybrid assay, or become activated by it (Sung *et al.*, 1999a and unpublished data), and PLD2 encodes an arginine at that residue (Colley *et al.*, 1997). I870R interacted with PEA-15 but not RhoA in the two-hybrid assay and exhibited a loss of response similar to that of I870T in the in vitro PLD assay (our unpublished results).

Characterization of the candidate Rho-nonresponsive alleles and an allele that combined all three mutations was continued using a transphosphatidylation PLD assay to assess in vivo responses to $RhoA^{V14}$ and PKC stimulation (using PMA to activate PKC). Q975R/D999V exhibited a very small response to $RhoA^{V14}$, which is consistent with its behavior in the in vitro assay; the other alleles did not exhibit responses above background (Figure 2A). In contrast, all four mutant alleles exhibited wild-type responses to PMA (Figure 2B), demonstrating that the mutant proteins are fully active in vivo when stimulated through a non-Rho pathway.

Finally, we expressed and purified I870R and also a mutant (PIM87/I870R) that combined the I870R point mutation and the previously described 5-amino acid insertion at amino acid 87 that results in a loss of PKC responsiveness (PIM87). Both mutant alleles exhibited wild-type responses to ARF and a complete loss of responsiveness to RhoA (Figure 3). Taken together, these findings established the

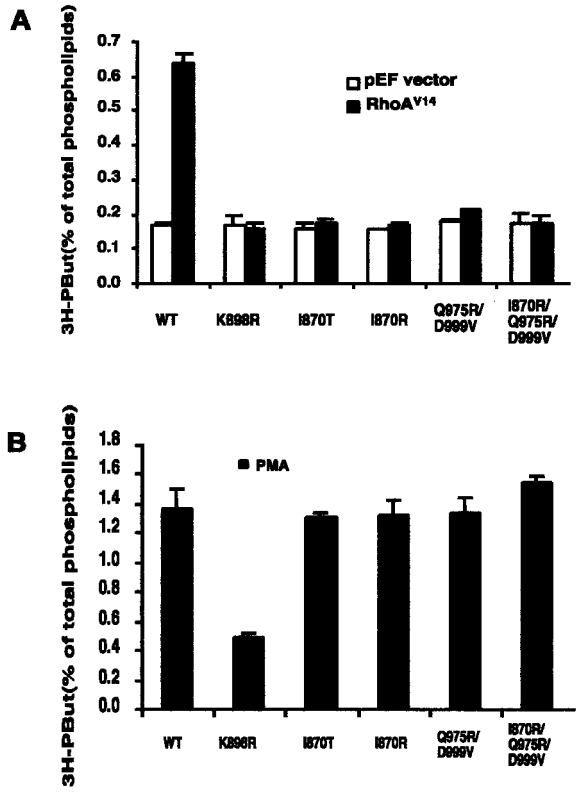


Figure 2. The mutant alleles I870T, I870R, and Q975R/D999V respond in vivo to the activation of PKC but not to coexpressed $RhoA^{V14}$. (A) HEK293 cells cotransfected with wild-type or mutant PLD1 alleles and pEF-Bos-HA-$RhoA^{V14}$ or the empty pEF-Bos-HA vector were assayed in vivo for PLD activity. (B) HEK293 cells overexpressing wild-type and mutant PLD1 alleles were stimulated with 100 nM PMA and were assayed in vivo for PLD activity. Assays were performed over a 30-min time period, and the data shown are representative of three experiments.

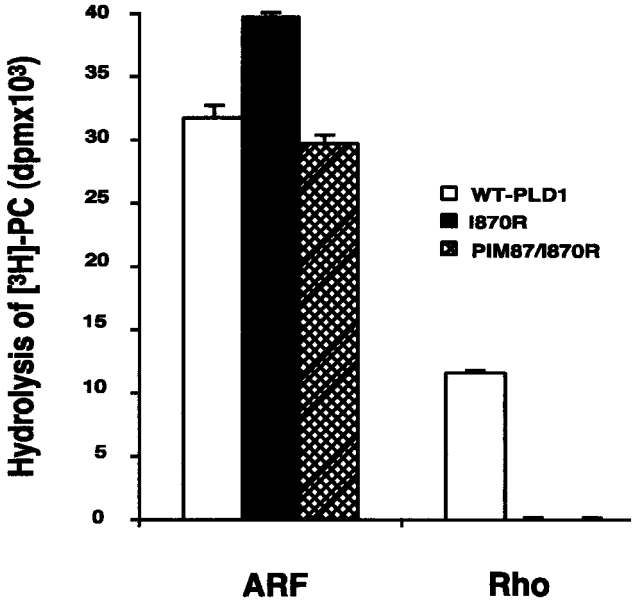


Figure 3. I870R is selectively nonresponsive to RhoA in vitro but otherwise is similar to wild-type PLD1. Glu–Glu tagged wild-type, I870R and PIM87/I870R PLD1 proteins were expressed in sf9 cells using the baculoviral system and were immunopurified. PLD activation was determined for 30 ng of each PLD protein using the head-group release assay in the presence of maximally effective concentrations of ARF1 and RhoA. Note that the maximal response of wild-type PLD1 to ARF1 is severalfold stronger than to Rho, as reported previously (Hammond *et al.*, 1997). The experiment was conducted in triplicate. The values shown reflect activity after the subtraction of basal activity and assay background, and represent one of three experiments performed.

new mutants as potentially useful alleles for dissection of PLD1 regulation and function. The results using the in vivo assay also suggest that the stimulation of PLD1 by $RhoA^{V14}$ occurs in cells through direct interaction rather than through the activation of other Rho-responsive pathways such as Rho-Kinase (Schmidt *et al.*, 1999b) or alterations in levels of $PI4,5P_2$ (Carpenter and Cantley, 1996).

PLD1 Subcellular Localization and Regulated Phosphorylation Occurs Independently of Direct PKC and Rho Stimulatory Pathways

The activation of RhoA and PKC leads to their translocation to specific membrane sites, raising the issue of whether they regulate PLD1 in part through changes in its subcellular localization. However, the mutant PLD1 alleles were expressed at wild-type levels in HEK293 cells (Figure 4) and exhibited the wild-type electrophoresis doublet pattern that signifies phosphorylation (Kim *et al.*, 1999, 2000), which occurs only when the proteins are membrane associated (Zhang *et al.*, 1999). Furthermore, the mutant alleles appear to localize similarly to wild-type PLD1 at a gross level in COS-7 cells, as visualized using immunofluorescence (our unpublished results).

It was recently reported that PLD1 undergoes regulated phosphorylation upon PMA stimulation of PKC (Kim *et al.*, 1999, 2000). One site in particular, amino acid 147, is very dramatically phosphorylated in this setting, and this has been proposed to affect the activation of PLD1 in vivo. The PIM87 mutation lies near this site, raising the possibility that interference with PKC-mediated phosphorylation of PLD1 amino acid 147 or other important sites might underlie its loss of PKC responsiveness. To address this, we examined the phosphopeptide map of PIM87 and its specific phosphorylation at amino acid 147. PIM87 was efficiently phosphorylated at Thr147, as visualized using a phospho-Thr147 specific monoclonal antibody (Figure 5A), eliminating this possibility. The phosphopeptide maps of PLD1b wt and PIM87 were generally similar (Figure 5, B–E) and were indistinguishable for the peptides previously reported to be specifically phosphorylated by PKC (one is on the middle left of the gel, and several are in the center) (Kim *et al.*, 1999). Several spots that differ compared with those in Figure 5, C and E, can be observed at the upper left of the gels. These spots do not represent PKC-specific phosphorylation and are not consistently observed (Kim *et al.*, 1999). Taken together, the PIM87 mutation does not seem to alter PKC-

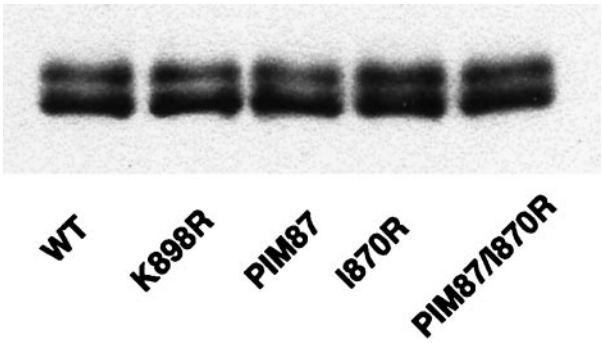


Figure 4. The mutant PLD1 proteins are expressed at wild-type levels and exhibit wild-type membrane association. HEK293 cells overexpressing wild-type and mutant PLD1 alleles were lysed and assayed using Western blot analysis. The PLD1 proteins were detected using a rat anti-HA tag monoclonal antibody. Wild-type PLD1 migrates as a doublet; the more slowly migrating band results from phosphorylation, which occurs only when PLD is membrane associated (Kim *et al.*, 1999, 2000; Zhang *et al.*, 1999).

mediated phosphorylation to a significant extent. Combined with the fact that purified PLD1 can be activated by PKC in the absence of ATP in vitro, this indicates that PKC-mediated phosphorylation proceeds in the absence of direct activation of PLD1 by PKC, and that the mechanism underlying the direct activation is distinguishable from the phosphorylation.

Direct Stimulation by PKC and RhoA Is Critical for PLD1 Activation Through G Protein-coupled Receptors

We next examined the stimulation of alleles of PLD1 by carbachol in HEK293 cells stably overexpressing the m3 muscarinic acetylcholine receptor (mAChR). We previously reported that 90% of the stimulatory response was lost for an allele (PIM87) that was nonresponsive to PKC, using cotransfection of the mutant allele and the receptor (Zhang *et al.*, 1999). Using the stably transfected cell line, we observed in this setting an 80% loss of activity for PIM87 (Figure 6A). A similar loss was observed for the Rho-nonresponsive I870R allele (see also Figure 7), and the combined mutant PIM87/I870R exhibited a virtually complete loss of response. Similar analyses were carried out using the M2 mAChR receptor, the Edg2 and Edg4 LPA receptors, and the angiotensin II (AT1a) receptor. We were unable to generate substantial agonist-induced PLD responses for the M2 mAChR and Edg2 receptors, which couple through Gi proteins (Offermanns *et al.*, 1994; An *et al.*, 1998). In contrast, good stimulation was observed for Edg4, which couples through Gq and Gi (An *et al.*, 1998), and for AT1a, which couples to PLC through Gq/11, G12, or G13 (Macrez-Lepretre *et al.*, 1997; Ushio-Fukai *et al.*, 1998). For the latter receptors, results similar to that described above for the M3 mAChR, which preferentially couples through Gq (Offermanns *et al.*, 1994), were obtained (Figure 6B), except that in some cases direct stimulation by RhoA was less critical than simulation by PKC. Nonetheless, the combined mutants were essentially unresponsive in all cases, demonstrating that PLD1 activation through G protein-coupled receptors requires direct stimulation by a combination of Rho and PKC family members.

Our finding above (Figure 2A) that PLD1 activation by the constitutively activated Rho^{V14} in vivo required direct binding between RhoA and PLD1 implies that other Rho-activated pathways do not suffice to stimulate PLD1 independently. It has been proposed, though, that some of these pathways function only in the context of agonist signaling (e.g., Rho-kinase) (Schmidt *et al.*, 1999b). To address this, we increased the levels of wild-type Rho before agonist stimulation, since we had previously reported that this manipulation resulted in increased responses for both wild-type PLD1 and PIM87, the PKC-nonresponsive allele (Zhang *et al.*, 1999). We examined the response of I870R in this setting; it exhibited no increase in PLD stimulation in the presence of increased levels of RhoA (Figure 7), again suggesting that increased stimulation of the many other Rho effector pathways does not play a role in regulating PLD1 during G protein-coupled signaling.

Finally, we examined the role of ARF proteins in the stimulation of PLD in vivo in HEK293 cells. The overexpression of dominant active ARF1 and ARF6 did not increase PLD1 activity (Figure 8), although a response was observed for PLD2. Synergism between the overexpression of wild-type ARFs and agonist signaling through the M3 mAChR also was not observed for PLD1 (our unpublished results). Taken together, in the context of the cell type and receptors that we have examined, we cannot demonstrate the involvement of ARF proteins in the agonist activation of PLD1.

DISCUSSION

Generation of RhoA-nonresponsive PLD1 Alleles

We report here the development of alleles of PLD1 that do not respond to Rho stimulation. These alleles now permit the regulation and cellular roles of PLD1 to be studied in the context of Rho input without manipulating Rho itself or the other pathways that it mediates.

Our reverse-two hybrid (split-hybrid) screen uncovered mutations located throughout the C-terminus that eliminated the RhoA:PLD1 D4 fragment interaction (Figure 1A). The dispersed nature of the mutations suggests that the RhoA binding site in PLD1 is not a relatively linear sequence, in contrast to what has been proposed for the Rho-binding motifs (REM-1 and REM-2), which are found in the many other Rho downstream effectors (Fujisawa *et al.* 1996; Yamazaki *et al.*, 1999). These results are consistent with our earlier studies on site-directed mutagenesis of the CRIII and HKD regions (Sung *et al.*, 1997) as well as transposon-induced mutagenesis of the C-terminus (Zhang *et al.*, 1999, 2000), which had generated dispersed mutants that no longer interacted with RhoA. Some of these mutations may have resulted in misfolded proteins; but even the mutants generated in the present study that should have been folded, which was reasonably based on their continued interaction with PEA-15, were targeted at sites dispersed throughout the C-terminus (Figure 1A, arrows above the line).

Surprisingly, six of the eight active candidate RhoA-nonresponsive mutant PLD1 proteins appeared to react normally to RhoA stimulation in the in vitro PLD assay (Figure 1B). It is possible that there were very low levels of interac-

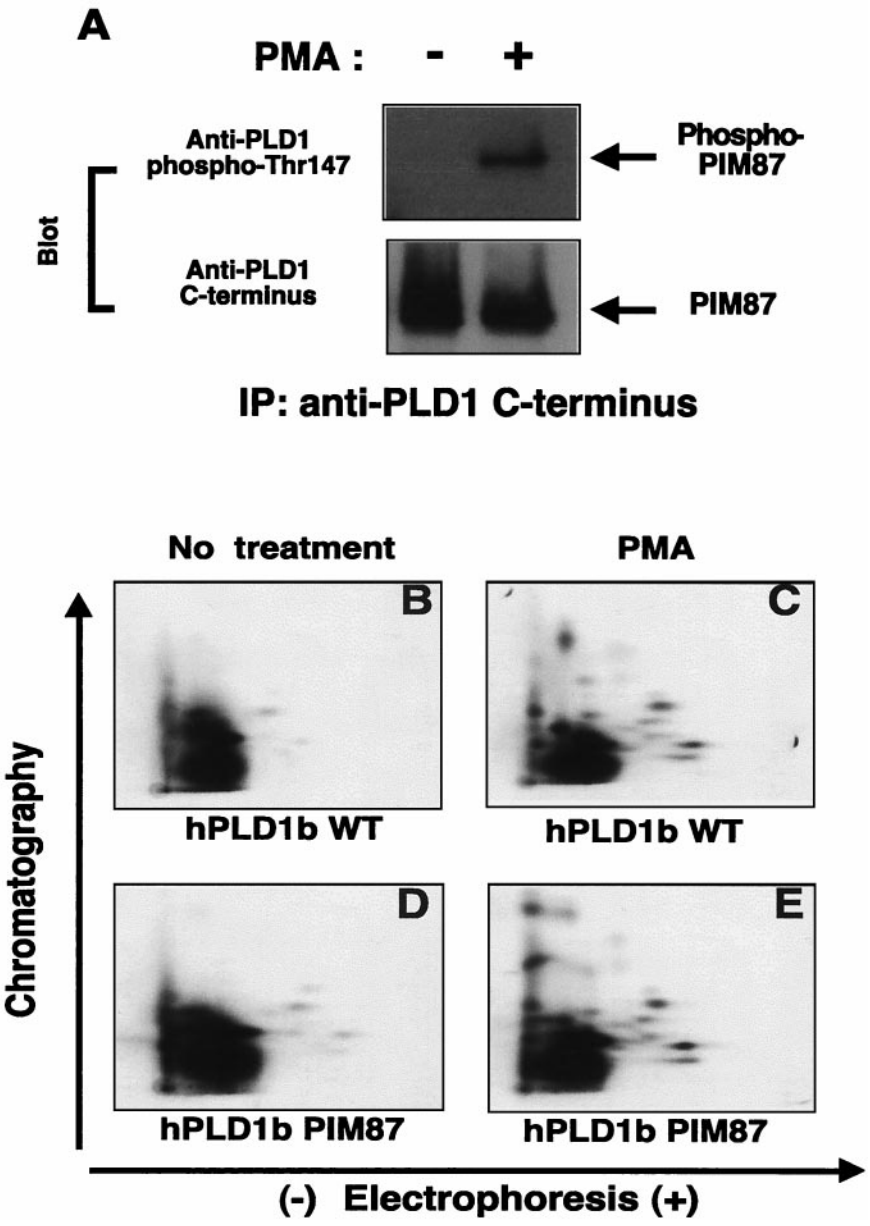
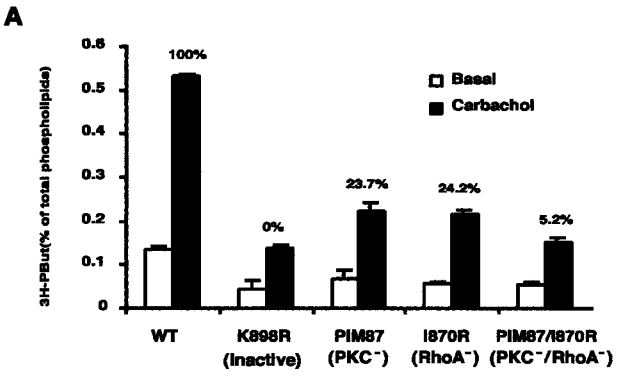


Figure 5. The PIM87 mutation does not alter PKC-induced phosphorylation of PLD1. (A) After the transfection of PIM87 followed by serum starvation, COS-7 cells were stimulated with 100 nM PMA for 15 min. PIM87 was immunoprecipitated from 2 mg of lysate using an anti-C-terminal PLD1 antibody. The proteins were separated by 8% SDS-PAGE and were subjected to immunoblot analysis using a monoclonal phosphorylation-specific anti-PLD1 antibody or an anti-C-terminal PLD1 antibody. The data are representative of three independent experiments. IP, immunoprecipitating antibody. COS-7 cells were transfected with hPLD1 WT (B, C) or PIM87 (D, E) cDNAs. After the COS-7 cells were loaded with [^{32}P]orthophosphate (0.4 mCi/ml) for 3 h, the cells were stimulated with 100 nM PMA for 15 min. Protein extracts (1.5 mg) then were immunoprecipitated with anti-PLD1 antibody. Immunoprecipitated proteins were separated by 8% SDS-PAGE. The bands of phosphorylated hPLD1 WT and PIM87 were excised and digested with TPCK-trypsin. The tryptic peptides were subjected to two-dimensional phosphopeptide mapping. These data are representative of two independent experiments.

tion in the two-hybrid system for these mutants or that they exhibit decreased affinity to RhoA but that this did not affect activation in the presence of the high concentration of RhoA used in our assay. Alternatively, there may be some differences in the way the D4 fragment and full-length PLD1 interact with RhoA. The interaction with D4 is stronger (Yamazaki *et al.*, 1999; our unpublished results) but also may be more sensitive to mutation-induced structural changes, since in the full-length protein the PLD1 C-terminus is associated with the N-terminus and is presumably stabilized by it (Frohman *et al.*, 1999; Liscovitch *et al.*, 2000). In a separate report, we have described an insertional mutant at amino acid 751 (and to a lesser extent, others at 759 and 1027) that interacted with RhoA but not PEA-15. In this report, we identified 17 mutants that interacted with PEA-15 but not RhoA, confirming that those interaction sites overlap but are not identical. Although the significance of the interaction of PEA-15 with PLD1 is not yet well defined, we chose for further study Rho-noninteracting mutants that did not eliminate this interaction.

The two useful mutants identified, I870T/R and Q975R/D999V, are located outside of the CRIII and HKD catalytic regions conserved in mammalian, yeast, and plant PLDs (Frohman *et al.*, 1999; Liscovitch *et al.*, 2000). The amino acids at these three sites are perfectly conserved in human, mouse, rat, and Chinese hamster PLD1 and are distinct from the conserved amino acids at the corresponding sites in the mammalian PLD2s, which is consistent with the lack of



B

	PIM87	I870R	PIM87/I870R
Edg4 receptor	20.7 ± 2.2%	38.1 ± 6.8%	10.0 ± 1.3%
AT1a receptor	24.4 ± 1.4%	57.9 ± 0.2%	5.4 ± 0.2%

Figure 6. Direct stimulation by PKC and RhoA are critical for PLD1 activation by G protein-coupled receptors. (A) M3 mAChR-expressing HEK293 cells were transfected with PLD1 alleles as indicated and in vivo assays were performed in the absence (basal) or presence of 1 mM carbachol. The data shown are representative of three similar experiments carried out in duplicate or triplicate. The percentage activation of each mutant was calculated by subtracting its basal activity and the agonist-dependent increase of K898R (which represents the activation of endogenous PLD) from agonist-activated activities of the mutant, and comparing this value with the agonist-activated wild-type PLD1 sample treated similarly. (B) Agonist-stimulated activation of PLD mutants using other G protein-coupled receptors. HEK293 cells were cotransfected with PLD1 mutants and the Edg4 or AT1a receptors. In vivo assays were performed in the absence or presence of 100 nM angiotensin II for the AT1a receptor or 100 μM LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; 18:1 Lyso PA) for the Edg-4 receptor. The values shown represent the percentage of activation of each mutant compared with that of the wild-type mutant (100%) and were calculated as described above. The experiments were conducted in duplicate or triplicate, and the values shown are representative of two experiments.

response of PLD2 to RhoA (Sung *et al.*, 1999a). The mutant sites flank the HKD domain, suggesting that they may juxtapose each other in the three-dimensional structure.

Direct Activation of PLD1 by Rho Occurs In Vivo and Is Important for G Protein-coupled Signaling

Studies on the activation of PLD1 by RhoA using the in vitro assay have demonstrated that RhoA, ARF, and PKC can activate PLD1 independently. Our current study confirms that these in vitro findings on Rho regulation of PLD activation are physiologically relevant: the Rho-noninteracting alleles are not responsive to RhoA or RhoA^{V14} in vivo (Figures 2 and 7), demonstrating that it is the direct interaction of Rho and PLD1 in vivo that leads to Rho-mediated activation of PLD1; however, the mutant alleles can be activated by PKC normally (after PKC is activated by PMA). As reported earlier (Zhang *et al.*, 1999), the opposite holds true for PIM87, the Rho-responsive, PKC-nonresponsive allele. This raises the possibility that Rho and PKC could act independently in vivo to regulate PLD1. However, as demonstrated in Figures 6 and 7, substantially reduced PLD1 stimulation is observed if either a Rho or PKC functional interaction is eliminated, suggesting that PLD1 is most strongly activated when Rho and PKC synergize. Whether either activator alone activates PLD1 sufficiently well to permit PLD1 to carry out its downstream cell biological role(s) will need to be established on a case-by-case basis. The alleles described

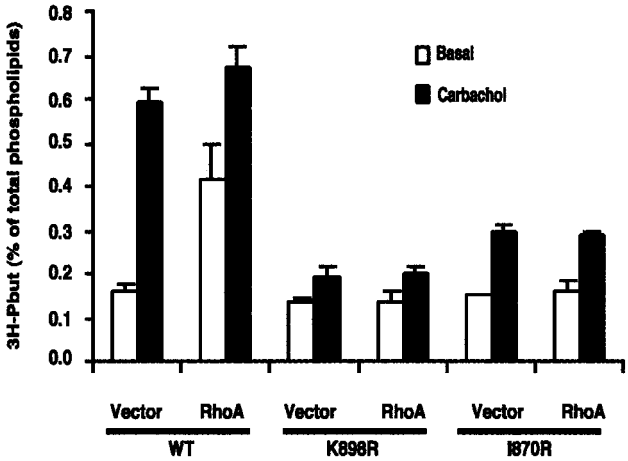


Figure 7. Insensitivity of I870R to increased levels of wild-type RhoA during agonist stimulation. M3 mAChR-expressing HEK293 cells were transfected with pCGN-wild-type PLD1, PLD-K898R, or I870R and pEF-Bos-HA-wild-type RhoA or the empty pEF-Bos-HA vector. After 24 h, a PLD in vivo assay was performed in triplicate in the absence (basal) or presence of 1 mM carbachol. The data are representative of two experiments.

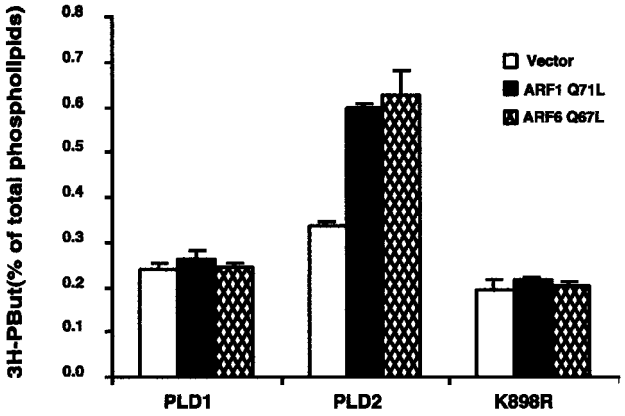


Figure 8. Constitutively active ARF1 and ARF6 increase PLD2 activity in HEK 293 cells but not that of PLD1. HEK 293 cells cotransfected with pCGN-hPLD1, mPLD2, or hPLD1-K8989R and the empty pcDNA vector, pcDNA-ARF1 Q71L or pcDNA-ARF6 Q67L (the dominant active alleles of ARF1 and ARF6) were assayed in vivo for PLD activity. The experiments were conducted in triplicate. The values shown are representative of four experiments.

here represent tools that can now be used to delineate Rho and PKC stimulation of PLD1 to specific downstream cellular roles. Preliminary studies suggest that the elimination of the ability to be activated by PKC and/or RhoA decreases the ability of PLD1 to mediate regulated exocytosis (unpublished data).

The elimination of direct signaling by both Rho and PKC renders PLD1 essentially inactive in the model system that we used in this report, suggesting that ARF does not suffice to activate PLD1 by itself. This is further supported by the observation that dominant active ARF1 and ARF6 activate PLD2 when coexpressed with it but do not similarly activate PLD1 (Figure 8). Physiologic roles for the activation of PLD2 (Honda *et al.*, 1999; Rizzo *et al.*, 1999) and PLD1 (Caumont *et al.*, 1998; Emoto *et al.*, 2000; Way *et al.*, 2000) by ARFs have been suggested. Based on our results, and on those of others who failed to demonstrate a role for ARF in agonist activation of PLD1 (Meacci *et al.*, 1999), we propose that ARF may activate PLD1 independently of signaling events (Emoto *et al.*, 2000), or that it may do so solely in a synergistic manner with Rho and/or PKC, or that it may activate PLD1 in a signaling context only in other settings, such as regulated exocytosis (Caumont *et al.*, 1998; Way *et al.*, 2000).

Many other factors that potentially influence PLD activity have been described, including PLD inhibitors, regulation of Ca^{2+} and $PI4,5P_2$ levels, and PKC- and Rho-kinase-mediated phosphorylation of PLD1 or of unknown targets (Frohman *et al.*, 1999; Liscovitch *et al.*, 2000). We would propose that these factors act, perhaps incidentally, to establish a permissive environment in which PLD can be activated, but that the actual activation of PLD occurs through direct stimulation by RhoA and PKC.

One role that the RhoA and PKC interactions do not seem to undertake is the influencing of the subcellular localization of PLD1; all of the mutant alleles examined localized similarly to the wild-type protein. In contrast, the altered localization for PLD is observed when mutations are present in regions specifically involved in membrane association, such as the PH domain, the $PI4,5P_2$ interacting site, and the catalytic domains (Sugars *et al.*, 1999; Zhang *et al.*, 1999; and our unpublished results).

Finally, it is noteworthy that we were able to examine PLD signaling in combination with G protein-coupled receptors that signal through Gq/G11 and G12, but not in ones that signal through Gi only, for which only very small agonist responses were observed. It has been proposed that receptors that signal through Gi only activate PKC but do not activate Rho effectively (Sah *et al.*, 2000). This further suggests that Rho is critical for the activation of PLD1 in signaling through G protein-coupled receptors.

ACKNOWLEDGMENTS

The authors thank M. Schmidt for providing HEK293 cells stably expressing the m2 and m3 muscarinic acetylcholine receptors. pcDNA3-ARF1 and pcDNA3-ARF6, pcDNA-rAT1a, and pCR3.1-hEdg-4 were kindly provided by Y. Kanaho, J. Haendeler, and K. Lynch, respectively. The pFastBacGlu vector and protein G sepharose beads conjugated with a mouse anti-GluGlu monoclonal antibody were a kind gift from N. Pryer. We would also like to thank J. Engebrecht, S. Tsirka, Y. Zhang, and members of the laboratory for critical reading of the manuscript and discussion. This work was supported by grants from the National Institutes of Health to MAF (GM54813) and AJM (GM50388). GD is a fellow of the American Heart Association.

REFERENCES

An, S., Bleu, T., Zheng, Y., and Goetzl, E. (1998). Recombinant human G. protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. Mol. Pharmacol. *54*, 881–888.

Cadwell, R.C., and Joyce, G. (1994). Mutagenic PCR. PCR Methods Appl. *3*, S136–S140.

Carpenter, C.L., and Cantley, L.C. (1996). Phosphoinositide kinases. Curr. Opin. Cell Biol. *8*, 153–158.

Caumont, A.S., Galas, M.C., Vitale, N., Aunis, D., and Bader, M.F. (1998). Regulated exocytosis in chromaffin cells: translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. J. Biol. Chem. *273*, 1373–1379.

Colley, W., Sung, T.C., Roll, R., Hammond, S.M., Altshuller, Y.M., Bar-Sagi, D., Morris, A.J., and Frohman, M.A. (1997). Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Curr. Biol. *7*, 191–201.

Emoto, M., Klarlund, J., Waters, S., Hu, V., Buxton, J., Chawla, A., and Czech, M. (2000). A role for phospholipase D in GLUT4 glucose transporter translocation. J. Biol. Chem. *275*, 7144–7151.

Frohman, M.A., Engebrecht, J., and Morris, A.J. (1999). Molecular and cell biological roles for the Phospholipase D genes. Chem. Phys. Lipids *98*, 127–140.

Frohman, M.A., Kanaho, Y., Zhang, Y., and Morris, A.J. (2000). In: Regulation of Phospholipase D1 activity by Rho GTPases, vol. 325, ed. A. Hall, 177–189.

Fujisawa, K., Fujita, A., Ishizaki, T., Saito, Y., and Narumiya, S. (1996). Identification of the Rho-binding domain of p160ROCK, a Rho-associated coiled-coil containing protein kinase. J. Biol. Chem. *271*, 23022–23028.

Hammond, S.M., Altshuller, Y.M., Sung, T.C., Rudge, S.A., Rose, K., Engebrecht, J., Morris, A.J., and Frohman, M.A. (1995). Human ADP-rybosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new, and highly conserved gene family. J. Biol. Chem. *270*, 29640–29643.

Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Cook, S., Nozawa, Y., Frohman, M.A., and Morris, A.J. (1997). Characterization of two alternately spliced forms of phospholipase D1: activation of the purified enzymes by PIP2, ARF, and Rho family monomeric G-proteins and protein kinase C-α. J. Biol. Chem. *272*, 3860–3868.

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A.J., Frohman, M.A., and Kanaho, Y. (1999). Phosphatidylinositol 4-phosphate 5-kinaseα is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell *99*, 521–532.

Jones, D., Morgan, C., and Cockcroft, S. (1999). Phospholipase D and membrane traffic. Biochem. Biophys. Acta *1439*, 229–244.

Kim, Y., Han, J.M., Han, B.R., Lee, K.A., Kim, J.H., Lee, B.D., Jang, I.H., Suh, P.G., and Ryu, S.H. (2000). Phospholipase D1 is phosphorylated and activated by protein kinase C in caveolin-enriched microdomains within the plasma membrane. J. Biol. Chem. *275*, 13621–13627.

Kim, Y., *et al.* (1999). Phosphorylation and activation of phospholipase D1 by protein kinase C in vivo: determination of multiple phosphorylation sites. Biochemistry *38*, 10344–10351.

Liang, J.O., Sung, T.-C., Morris, A.J., Frohman, M.A., and Kornfeld, S. (1997). Different domains of mammalian ADP-ribosylation factor

1 mediate interaction with selected target proteins. J. Biol. Chem. *272*, 33001–33008.

Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000). Phospholipase D: molecular and cell biology of a novel gene family. Biochem. J. *345*, 401–415.

Macrez-Lepretre, N., Kalkbrenner, F., Morel, J.L., Schultz, G., and Mironneau, J. (1997). G protein heterotrimer $G\alpha_{13}\beta_1\gamma_3$ couples the angiotensin AT_{1A} receptor to increases in cytoplasmic Ca^{2+} in rat portal vein myocytes. J. Biol. Chem. *272*, 10095–10102.

Meacci, E., Vasta, V., Moorman, J.P., Bobak, D.A., Bruni, P., Moss, J., and Vaughan, M. (1999). Effect of Rho, and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A459 cells. J. Biol. Chem. *274*, 18605–18612.

Morris, A.J., Frohman, M.A., and Engebrecht, J. (1997). Measurement of phospholipase D activity. Anal. Biochem. *252*, 1–9.

Offermanns, S., Wieland, T., Homann, D., Sandmann, J., Bombien, E., Spicher, K., Schultz, G., and Jakobs, K. (1994). Transfected muscarinic acetylcholine receptors selectively couple to Gi-type G proteins and Gq/11. Mol. Pharmacol. *45*, 890–898.

Rizzo, M.A., Shome, K., Vasudevan, C., Stolz, D.B., Sung, T.-C., Frohman, M.A., Watkins, S.C., and Romero, G. (1999). Phospholipase D. and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the MAP kinase pathway. J. Biol. Chem. *274*, 1131–1139.

Rizzo, M.A., Shome, K., Watkins, S.C., and Romero, G. (2000). The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. J. Biol. Chem.

Sah, V., Seasholtz, T., Sagi, S., and Brown, J. (2000). The role of Rho in G protein-coupled receptor signal transduction. Annu. Rev. Pharmacol. Toxicol. *40*, 459–489.

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B., and Soling, H.D. (1999a). Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature *401*, 133–141.

Schmidt, M., Voss, M., Oude Weernink, P.A., Wetzel, J., Amano, M., Kaibuchi, K., and Jakobs, K.H. (1999b). A role for Rho-kinase in Rho-controlled phospholipase D stimulation by the m3 muscarinic acetylcholine receptor. J. Biol. Chem. *274*, 14648–14654.

Sciorra, V.A., Rudge, S.A., Prestwich, G.D., Frohman, M.A., Engebrecht, J., and Morris, A.J. (1999). Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. EMBO J. *18*, 5911–5921.

Shih, H.M., Goldman, P.S., DeMaggio, A.J., Hollenberg, S.M., Goodman, R.H., and Hoekstra, M.F. (1996). A positive genetic selection for disrupting protein-protein interactions: identification of CREB mutations that prevent association with the coactivator CBP. Proc. Natl. Acad. Sci. USA *93*, 13896–13901.

Siddiqi, A.R., Smith, J.L., Ross, A.H., Qiu, R.G., Symons, M., and Exton, J.H. (1995). Regulation of phospholipase D in HL60 cells: evidence for a cytosolic phospholipase D. J. Biol. Chem. *270*, 8466–8473.

Singer, W.D., Brown, H.A., Bokoch, G.M., and Sternweis, P.C. (1995). Resolved phospholipase D activity is modulated by cytosolic factors other than ARF. J. Biol. Chem. *270*, 14944–14950.

Sugars, J., Cellek, S., Manifava, M., Coadwell, J., and Ktistakis, N. (1999). Fatty acylation of phospholipase D1 on cysteine residues 240 and 241 determines localization on intracellular membranes. J. Biol. Chem. *274*, 30023–30027.

Sung, T.-C., Altshuller, Y.M., Morris, A.J., and Frohman, M.A. (1999a). Molecular analysis of mammalian phospholipase D2. J. Biol. Chem. *274*, 494–502.

Sung, T.-C., Zhang, Y., Morris, A.J., and Frohman, M.A. (1999b). Structural analysis of human Phospholipase D1. J. Biol. Chem. *274*, 3659–3666.

Sung, T.C., Roper, R., Zhang, Y., Rudge, S.A., Temel, R., Hammond, S.M., Morris, A.J., Moss, B., Engebrecht, J., and Frohman, M.A. (1997). Mutational analysis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. EMBO J. *16*, 4519–4530.

Ushio-Fukai, M., Griendling, K.K., Akers, M., Lyons, P.R., and Alexander, R.W. (1998). Temporal dispersion of activation of phospholipase C-beta1 and -gamma isoforms by angiotensin II in vascular smooth muscle cells: role of αq/11, α12, and $\beta\gamma$G protein subunits. J. Biol. Chem. *273*, 19772–19777.

Way, G., O'Luanaigh, N., and Cockcroft, S. (2000). Activation of exocytosis by cross-linking of the IgE receptor is dependent on ADP-ribosylation factor 1-regulated phospholipase D in RBL-2H3 mast cells: evidence that the mechanism of activation is via regulation of phosphatidylinositol 4,5-bisphosphate synthesis. Biochem. J. *346*, 63–70.

Yamazaki, M., Zhang, Y., Watanabe, H., Yokozeki, T., Ohno, S., Kaibuchi, K., Shibata, H., Mukai, H., Ono, Y., Frohman, M.A., and Kanaho, Y. (1999). Interaction of the small G protein RhoA with the C-terminus of human phospholipase D1. J. Biol. Chem. *274*, 6035–6038.

Zhang, Y., Altshuller, Y.A., Hammond, S.A., Hayes, F., Morris, A.J., and Frohman, M.A. (1999). Loss of receptor regulation by a phospholipase D1 mutant unresponsive to protein kinase C. EMBO J. *18*, 6339–6348.

Zhang, Y., Redina, O., Altshuller, Y.M., Yamazaki, M., Ramos, J., Chneiweis, H., Kanaho, Y., and Frohman, M.A. (2000). Regulation of expression of phospholipase D1 and D2 by PEA-15, a novel protein that interacts with them. J. Biol. Chem. *275*, 35224–35232.