Activation of Phospholipase Cϵ by Free Fatty Acids and Cross Talk with Phospholipase D and Phospholipase A_2[†]

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Received April 3, 2006; Revised Manuscript Received July 18, 2006

ABSTRACT: This paper uses phospholipase Cϵ as a model to demonstrate that lipids can act as ligands to bind to specific motifs and regulate protein activity via allosteric effects. Phospholipids such as phosphatidic acid and free fatty acids such as arachidonate are potent activators of PLCϵ, increasing the rate of PI hydrolysis by 8-fold and 50-fold, respectively. The mechanism appears to be a reduction of K_m, as the substrate dependence curve is shifted to the left and K_m is reduced 10-fold. The regulation of PLCϵ by lipids appears to be physiologic, as reconstitution or cotransfection of either cPLA$_2$ or PLD with PLCϵ leads to activation of phosphodiesterase activity. Additionally, TSA-201 cells transfected with PLCϵ and fed arachidonic acid complexed with BSA had increased (4−5-fold) hydrolysis of polyphosphoinositides. This study demonstrates the ability of lipids to act as potent and direct mediators of protein function and identifies cross talk between different classes of phospholipase (PLD and PLA$_2$ with PLC) mediated via lipid products.

Many hormones, neurotransmitters, and growth factors elicit intracellular responses by activating a family of inositol phospholipid-specific phospholipase C (PLC)[1] isozymes. The mammalian PLC family consists of six subfamilies that differ in structure, mode of regulation, and function: β, γ, δ, ϵ, ζ, and η (*1*−*5*). Commonly these isoforms all cleave the polar headgroup from inositol phospholipids to generate the second messengers inositol 1,4,5-trisphophate (IP_3) and diacylglycerol (DAG). IP_3 is a potent mobilizer of intracellular calcium from the endoplasmic reticulum, and DAG is an activator of protein kinase C (PKC).

PLCϵ has emerged as a fascinating member of the PLC family. There are two isozymes that differ predominantly in the length of the N-terminus. One human isoform is 1994 amino acids in length, while the other is 2303 (Table 1). It is not known how the isoforms might differ in function, though some differences have been described. For instance, the short isoform appears to be highly membrane associated in contrast to the long isoform which is cytosolic (*6*, *7*). The short isoform may also have a different specificity for small G proteins at the N-terminal CDC25 domain. The short isoform can activate Ras while the long isoform appears to have specificity for Rap1 (*6*, *8*). To date, there have been a number of excellent reports describing how PLCϵ interacts with a myriad of both large and small G proteins. As an effector for G proteins, PLCϵ is regulated by Gα_{12}, Gα_{13}, and Gαs (indirectly via the cAMP-dependent guanine nucleotide regulatory protein EPAC), $\beta\gamma$ subunits of large heterotrimeric G proteins, and members of the small G protein family such as Ras, Rap1, Rap2b, Rho, Rac, and Ral (*6*−*16*). In addition to generating the second messengers IP_3 and DAG, PLCϵ also acts as a guanine nucleotide exchange factor for members of the Ras superfamily of small G proteins (*6*, *8*). Therefore, upstream signals from both small and large G proteins converge on PLCϵ. Downstream signals diverge into traditional PLC second messengers, such as DAG and IP_3, and small G protein pathways.

Table 1: Phospholipase Cϵ

species	size (AA)	% identity[a]	ref
human	1994		*6*
human	2303	84	*7*
rat	2281	76	*10*
mouse	2282	76	*52*
C. elegans	1898	29	*51*

[a] Compared with human PLCϵ [Lopez et al. (*6*)].

During our own in vitro studies investigating the mechanisms by which PLCϵ is regulated, we noticed that the enzyme, when purified to homogeneity, had basically no basal phosphodiesterase activity and was very unresponsive in vitro to G proteins known to activate the enzyme in cellular systems. Hence, we came to the conclusion that we had eliminated a necessary cofactor during the purification. Since some of our previous work had demonstrated the ability of

[†] This work was supported by NIH Grants HL55591 and HL63407 provided to J.W.L.

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; AA, arachidonic acid; BSA, bovine serum albumin; cPLA$_2$, cytosolic phospholipase A$_2$; DAG, diacylglycerol; DDM, *n*-dodecyl maltoside; FFA, free fatty acid; IP_3, inositol 1,4,5-trisphophate; LPA, lysophosphatidic acid; TAME, N^{α}-*p*-tosyl-L-arginine methyl ester; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; PLC, phospholipase C; PS, phosphatidylserine; PH, pleckstrin homology; PKC, protein kinase C; SA, stearic acid; STI, soybean trypsin inhibitor.

10.1021/bi060648+ CCC: $33.50
Published on Web 08/18/2006

lipids to activate another family member, PLCδ1 (*17*, *18*), we investigated the effect of lipids on PLCϵ. The results of these studies identify a number of lipid products, such as phosphatidic acid (PA) and arachadionic acid (AA), that result from the activation of other classes of phospholipases, such as phospholipase D (PLD) and phospholipase A_2 (PLA_2), that can markedly activate PLCϵ. The results also suggest another mechanism by which small G proteins such as Rho can activate PLCϵ via activation of PLD and generation of PA.

EXPERIMENTAL PROCEDURES

Materials

Baculovirus transfer vector, pAcHLT-A, and the transfection kit were purchased from BD Biosciences Pharmingen. Phosphatidylinositol (PI, bovine liver), phosphatidylinositol 4,5-bisphosphate (PIP_2, porcine brain), phosphatidylcholine (PC, bovine liver), phosphatidylserine (PS, porcine brain), phosphatidic acid (PA, chicken egg), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (lysophosphatidic acid, LPA, synthetic), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were from Avanti Polar Lipids. Arachidonic acid (AA) and *n*-dodecyl D-maltoside (DDM) were from Calbiochem. ^{3}H-PI (7.2 Ci/mmol), ^{3}H-PIP_2 (5.5 Ci/mmol), [^{3}H]-*myo*-inositol (18.5 Ci/mmol) were from Perkin-Elmer Life Sciences. The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. The lipofectamine, polymerase chain reaction (PCR) reagents, sf9 insect cells, and SF II-900 SFM medium were from Invitrogen. T4 DNA ligase, peroxidase-conjugated anti-mouse IgG, and mouse monoclonal IgG to the histidine tag were from Amersham Pharmacia Biotech. Restriction enzymes were from New England Biolabs. Stearic acid, phenylmethanesulfonyl fluoride (PMSF), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N^{α}-*p*-tosyl-L-arginine methyl ester (TAME), leupeptin, pepstatin A, soybean trypsin inhibitor (STI), benzamidine, peroxidase-conjugated anti-rabbit IgG, and essentially fatty acid-free bovine albumin were from Sigma. Trasylol was from Bayer. Ni-NTA agarose and purification kits were from Qiagen. Acetylated bovine serum albumin (AcBSA) was from Promega. Nitrocellulose (BA83, 0.2 μm pore size) was from Schleicher and Schuell. Competent DH5α cells were from Strategene. Purified $cPLA_2$ was obtained from Dr. Wonhwa Cho (University of Illinois, Chicago, IL) and PLD from Dr. Mary Roberts (Boston College, Chestnut Hill, MA). $cPLA_2$ plasmid was obtained from Dr. Christina Leslie (National Jewish Medical and Research Center, Denver, CO), and PLD plasmid was obtained from Dr. John Exton (Vanderbilt University, Nashville, TN). Purified truncated rPLCϵ-(EF-RA2) was obtained from Dr. Ken Harden (University of North Carolina, Chapel Hill, NC). All other reagents were of analytical grade or better.

Methods

Subcloning of PLCϵ into Baculovirus Transfer Vector pAcHLT-A. PLCϵ was subcloned in-frame into unique sites of *Nde*I and *Not*I in baculovirus transfer vector pAcHLT-A. To construct the *Nde*I site, a set of oligo DNA primers were used to perform PCR using PLCϵ in pcDNA3 as template. The forward primer (5′-GGAATTCCATATGCATGGTTT-CAGAAGGAAGTGCAGCAGG-3′) contained the *Nde*I site (CATATG) followed by the PLCϵ sequence starting from the first nucleotide (*1*−*26*), and the reverse primer (5′-GCAGCTCCGGATGACCTG-3′) spanned from nucleotides 1407 to 1390. The PCR mixture contained 1 μL (12 ng) of template (PLCϵ in pcDNA3), 1 μL of each of 20 μM forward and reverse primers, and 45 μL of PCR Supermix High Fidelity. The PCR was carried out in the GeneAmp PCR system (Perkin-Elmer) for 30 cycles. The PCR product was purified using a PCR purification kit. The PCR product was amplified by subcloning and transformation into TOPO vector. This 1.4 kbp of DNA contained *Nde*I and *Eco*RI sites.

PLCϵ in pcDNA3 (*6*) was digested with *Eco*RI and *Not*I, the generated 5 kbp fragment PLCϵ DNA was separated by electrophoresis on 0.9% low melting agarose, and DNA was purified by gel extraction. The baculovirus transfer vector, pAcHLT-A, was also digested with *Eco*RI and *Not*I, and the product was purified by gel extraction following electrophoresis on agarose. The ligation of PLCϵ (5 kbp) and baculovirus transfer vector pAcHLT-A was carried out for 18 h at 16 °C using T4 DNA ligase. The reaction mixture contained 1 μL of *Not*I and *Eco*RI digested pAcHLT-A, 8 μL of PLCϵ 5 kbp DNA, 0.5 μL of 100 mM ATP, 2 μL of one Phor all plus Pharmacia buffer, 1 μL of T4 DNA ligase, and 7.5 μL of water. The ligation product was transformed into DH5α competent cells, and plasmid was purified. The PCR product of 1.4 kbp of PLCϵ was then subcloned into this plasmid at *Nde*I and *Eco*RI sites.

Incorporation of the Poly-His Tag at the C-Terminus of PLCϵ. For the purposes of incorporating the poly-His tag at the carboxy-terminal end of PLCϵ, two PCR products were amplified and then subcloned into baculovirus transfer vector. For the first PCR, the forward primer (5′-CGGGTCCT-TCTGGATCAG-3′) spanning nucleotides 5704−5721 and the reverse primer (5′-TCAGTGATGATGATGATGAT-GTCCCTGTCGGTAATCCATTGTGTC-3′) contained a stop codon, codons for six histidines, one glycine, and PLCϵ nucleotides 5972−5962. For the second PCR, the forward primer (5′-CGACATCATCATCATCATCACTGACTAA-GGGCAGCATGTTTAACC-3′) contained codons for glycine, six histidines, a stop codon, and PLCϵ nucleotides 5986−6006 and the reverse primer (5′-GAGGCCCGAT-CAGCGAGC-3′) spanning nucleotides 1040−1023 in pcDNA3 downstream from the *Not*I site. The two PCR products were purified, annealed at 100 °C for 5 min, and cooled at room temperature. The annealed product was used as template for PCR using the forward primer (5′-CGGGTC-CTTCTGGATCAG-3′) spanning nucleotides 5704−5721 and the reverse primer (5′-GAGGCCCGATCAGCGAGC-3′) spanning nucleotides 1040−1023 in pcDNA3. This 900 bp PCR product was digested with *Not*I and *Xho*I, and the purified product (677 bp) was subcloned into baculovirus transfer vector pAcHLT-A at *Xho*I and *Not*I sites. The plasmid was purified from bacterial cell culture following transformation into DH5α competent cells. The sequence of inserted DNA was verified. Recombinant baculovirruses were produced by cotransfection into sf9 cells according to manufacturer's instructions.

Expression and Purification of PLCϵ. Insect cells, sf9 cells, were infected with PLCϵ virus for 48 h at 27.7 °C in SF II-900 SFM medium. The cells (∼500−800 mL) were

harvested by centrifugation at 6000 rpm for 15 min in Beckman JA-10 rotor at 4 °C. The cell pellets were washed once by resuspending in 40 mL of PBS containing 0.1 mM EGTA, 0.1 mM EDTA, 130 μM PMSF, 60 μM TLCK, and 60 μM TPCK, sedimented by centrifugation, and stored at −80 °C until further use. The cell pellets obtained from 2.25 L of sf9 cell culture were resuspended in lysis buffer (30 mL/L) containing 50 mM HEPES, pH 8.0, 0.8 M KCl, 0.25 M sucrose, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM β-mercaptoethanol, and protease inhibitors [130 μM PMSF, 60 μM TPCK, 60 μM TLCK, 55 μM TAME, 2 μg/mL leupeptin, 1 μg/mL pepstatin A, 10 μg/mL (STI), 1 mM benzamidine, 10 units/mL Trasylol], sonicated briefly on ice, and centrifuged at 17000 rpm in a Beckman JA-20 rotor for 60 min. The supernatant was recentrifuged at 40000 rpm in a Beckman 70Ti rotor for 60 min. The supernatant, lysate, was used for purification of PLCε by affinity chromatography on Ni-NTA agarose. Approximately 2 mL of the Ni-NTA agarose suspension was washed once with 50 mL of PBS by brief centrifugation at 900 rpm and once with lysis buffer containing 10 mM imidazole. Concentrated imidazole solution was added to lysate to 10 mM and allowed to bind Ni-NTA agarose overnight at 4 °C. The resin was packed into a column and washed with 50 mL of lysis buffer containing protease inhibitors and 10 mM imidazole, with 50 mL of wash buffer 1 (50 mM HEPES, pH 8.0, 1 M NaCl, 10% glycerol, 2 mM β-mercaptoethanol, 10 mM imidazole plus protease inhibitors), and with 50 mL of wash buffer 2 (50 mM HEPES, pH 8.0, 400 mM NaCl, 10% glycerol, 20 mM imidazole, 2 mM β-mercaptoethanol plus protease inhibitors except STI and Trasylol). The bound proteins were eluted with 100 mM imidazole in 50 mM HEPES, pH 8.0, 10% glycerol, 400 mM NaCl, 2 mM β-mercaptoethanol, and protease inhibitors PMSF, TPCK, and TLCK. Approximately 1 mL fractions were collected, and the elution was monitored by absorbance at 280 nm and PLC activity.

PLCε Activity Assay. PLCε activity assays were carried out in mixed micelles containing either sodium deoxycholate or *n*-dodecyl maltoside (DDM) as detergent and either ^{3}H-PI or ^{3}H-PIP$_2$ as substrate as described previously (*19*). Indicated amounts of PI containing ^{3}H-PI or PIP$_2$ containing ^{3}H-PIP$_2$ in chloroform−methanol (2:1) were dried under argon. The dried samples were resuspended in 50 mM HEPES, pH 7.3, 100 mM NaCl, and 0.1% deoxycholate or DDM, vortexed, and sonicated briefly on ice for 20 s. Acetylated BSA (50 μg/mL) and EGTA (1 mM) were added to the samples. In experiments involving DDM, the concentration of DDM was always kept above 200 μM (CMC of DDM is 150 μM), and the total concentration of substrate, lipids, and DDM was kept constant at 300 μM except in those experiments involving high concentrations of substrate. In the experiments involving lipids, appropriate concentrations of lipids were included with the substrates while drying under argon. The activity assays were carried out at 30 or 37 °C as indicated in the figure legends. The reaction was initiated by the addition of an appropriate concentration of $CaCl_2$. The reaction was inhibited by the addition of 50 μL of reaction mixture to the tubes containing 100 μL of chloroform−methanol−HCl (100:100:0.6) plus 50 μL of 1 N HCl and 5 mM EDTA. The aqueous and organic phases were separated by centrifugation, and the aqueous phases were used for counting in a liquid scintillation counter.

SDS−PAGE and Immunoblotting. Electrophoresis in the presence of SDS was carried out using the discontinuous buffer system of Laemmli (*20*) on 6% acrylamide in the separating gel. The proteins were visualized by staining with Coomassie blue. Alternatively, following SDS−PAGE, the proteins were electroblotted onto nitrocellulose using the method of Towbin et al. (*21*) in 25 mM Tris-HCl, 192 mM glycine, and 20% methanol for 2 h at 4 °C. The remaining sites on the blots were blocked with 3% non-fat dry milk powder and 0.1% Tween-20 in 100 mM Tris-HCl, pH 7.6, and 150 mM NaCl. For the purposes of immunostaining, the nitrocellulose blots were treated for 2 h at room temperature with either a rabbit antiserum to PLCε (no. R6698, 3/21, developed in the laboratory) diluted 1:10000 in the blocking buffer or a mouse monoclonal to His tag diluted 1:5000 in the blocking buffer. Following extensive washing with blocking buffer, the blots were treated with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as secondary antibody for 1 h at room temperature with 1:10000 dilution in the blocking buffer. The blots were developed using the SuperSignal West Dura kit (Pierce) according to manufacturer's instructions.

Protein concentrations were estimated spectrophotometrically using the bicinchoninic acid method with bovine serum albumin as a standard (Pierce document no. 1296.3) according to manufacturer's protocol.

Protein−Lipid Vesicle Binding Assay. The vesicle binding assay was conducted as described previously (*18*, *22*). The appropriate amount of DMPC and arachidonic acid dissolved in chloroform−methanol (2:1) were mixed and dried under argon gas. The dried materials were resuspended in 1 mL of 20 mM HEPES, pH 7.3, and 170 mM sucrose. After vigorous vortexing, the suspension was kept overnight at −80 °C. After three cycles of freezing (at −80 °C) and thawing (at 37 °C), the suspension was passed through 100 nm membranes using Mini Extruder (Avanti Polar Lipids). The resulting large unilamellar vesicles (800 μL) were diluted with 0.2 M KCl and 20 mM HEPES, pH 7.3, buffer (800 μL) and collected by centrifugation at 20000*g* for 1 h. Vesicles were resuspended in 100 mM KCl and 20 mM HEPES, pH 7.3. Fixed amounts of PLCε were added to 50 μL of vesicle suspension in 200 μL of 100 mM KCl and 20 mM HEPES, pH 7.3, and allowed to bind at room temperature for 1 h. The vesicles were collected by centrifugation at 100000*g* at 4 °C for 30 min. The pelleted vesicles were dissolved in SDS−PAGE buffer and analyzed by electrophoresis followed by immunostaining with an antiserum to PLCε.

Construction of cPLA$_2$ Plasmid DNA. cPLA$_2$ plasmid DNA was received from Dr. Christina Leslie in the vector pEGFP-C3. It was suspected that the GFP tag of pEGFP-C3 may cause aggregation and decrease activity of the protein in cellular studies. Therefore, we cloned cPLA$_2$ from pEGFP-C3 using the forward primer (5′-GGATCACTCTCGGCATG-GAC-3′) and the reverse primer (5′-ACTGCTCGAGATTTT-TATATTGATGTATTT-3′) and purified the product by gel extraction. The insert was ligated into pcDNA3 at the sites of *Hin*dIII and *Xho*I with T4 DNA ligase and transformed into DH5α competent cells. Colonies were screened for proper ligation, grown, and purified.

PLC Activity in TSA-201 Cells. PLC activity in TSA-201 cells was measured as described in Lopez et al. (*6*). TSA-201 cells were transiently transfected using lipofectamine

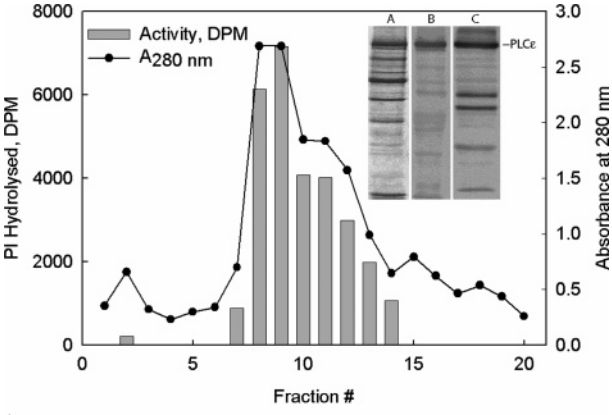

FIGURE 1: Affinity purification of PLCϵ on an Ni-NTA agarose column. PLCϵ virus-infected sf9 cell lysates were allowed to bind Ni-NTA agarose overnight at 4 °C. Following washing (see Experimental Procedures for details), the proteins were eluted with 100 mM imidazole, and 1 mL fractions were collected. The elution was monitored by absorbance at 280 nm. The PLC activity assays were performed in ^{3}H-PI and deoxycholate mixed micelles using a 3 μL aliquot of eluted fractions. The main figure shows the elution profile. Fractions 1−4 represent elution with 20 mM imidazole, and fractions 5−20 represent elution with 100 mM imidazole. The inset (A) shows the Coomassie blue stained profile of purified PLCϵ following SDS−PAGE. Immunostaining with rabbit anti-serum to PLC (B) and antibody to His tag (C) shows that ~220−230 kDa protein is recognized by both of the antibodies.

in six-well plates with pcDNA3 vector (control), PLCϵ, cPLA$_2$, or PLD, alone or in combination, keeping the total plasmid uniform (8 μg/well). Cells were labeled with [^{3}H]-*myo*-inositol (4 μCi/well) in serum-free media 36 h posttransfection. Cells were harvested for PLC activity 48 h posttransfection. Inositol phosphates (inositol 1-phosphate, inositol 1,4-bisphosphate, and IP$_3$) were determined using anion-exchange chromatography (*23*). The percentage of PI hydrolyzed is expressed as the total inositol phosphates formed relative to the amount of [^{3}H]-*myo*-inositol incorporated into the phospholipid pool.

Free Fatty Acid Loading of TSA-201 Cells. TSA-201 cells were transfected with either 4 μg of pcDNA3 vector (control) or PLCϵ in the same manner described above. Cells were labeled with [^{3}H]-*myo*-inositol (4 μCi/well) and fed 500 μM AA and SA complexed to 60 μM fatty acid-free serum albumin 36 h posttransfection. Cells were harvested for PLC activity 48 h posttransfection and assayed for PLC activity in the same manner described above. Free fatty acids were stored in ethanol and added to essentially fatty acid-free bovine albumim dissolved in serum-free media (*24*, *25*). Ethanol was added to wells that were not fed free fatty acids to keep conditions uniform.

RESULTS

Expression and Purification of PLCϵ. PLCϵ was subcloned into the baculovirus transfer vector pAcHLT-A which contained a polyhistidine tag at the C-terminus. The sf9 cells were cotransfected with this PLCϵ plasmid and baculogold DNA, and expression of PLCϵ was monitored in virus-infected sf9 cells by measuring the hydrolysis of polyphosphoinositides. Activity reached a maximum after 48 h of infection and then declined (data not shown). Most of the PLC activity was found to be in the membrane fraction of the PLCϵ-infected insect cells. We were unable to solubilize

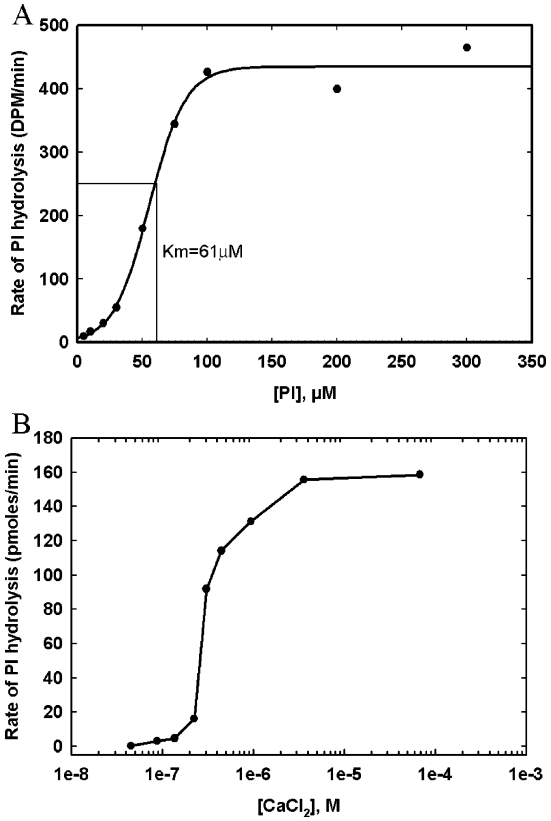

FIGURE 2: PLCϵ is dependent on the concentration of substrate and Ca^{2+}. PLCϵ activity assay was carried out in ^{3}H-PI and deoxycholate mixed micelles in duplicate at 37 °C for 0−8 min. The reaction mixture contained 180 μL of 300 μM PI, 3 μL of PLCϵ, and 12 μL of various concentrations of $CaCl_2$. At intervals of time, 50 μL of the reaction mixture was aliquoted, and the reaction was terminated as described before. The rate of hydrolysis was calculated on the basis of slopes from 0 to 8 min where it is linear. (A) The K_m for hydrolysis of PI by PLCϵ is 61 μM. (B) The free Ca^{2+} concentration was calculated using computer software. The mean of the rate of hydrolysis from duplicate experiments was plotted against free Ca^{2+} concentration. At approximately 20 nM Ca^{2+} PLCϵ exhibits 50% activity.

PLCϵ despite using a variety of detergents such as Tween-20, NP-40, NP-10, Triton X-100, *n*-octyl glucopyranoside, DDM, digitonin, and polyoxyethelene 10 lauryl ether. However, high salt concentrations, 0.5−0.8 M NaCl or KCl, were able to solubilize the protein in its active form. A typical chromatographic profile of PLCϵ purification on the Ni column is shown in Figure 1. Most of the PLC activity is eluted with 100 mM imidazole. The Coomassie blue stained SDS−PAGE profile (Figure 1A) shows that the expressed protein has an appropriate molecular mass, ~220−230 kDa, and is recognized by rabbit antiserum to PLCϵ (Figure 1B) as well as by the monoclonal His-tag antibody (Figure 1C). We can express ~500 μg of PLCϵ/L of infected sf9 cells. Unfortunately, we can solubilize only ~20% of the activity in the particulate fraction and elute only 30% of the activity that was loaded onto the Ni resin, so our overall yield is low, ~5%. Further purification of this protein by extensive washing of an affinity resin with detergents resulted in the loss of activity. Hence, the studies shown below utilized a partially purified preparation of PLCϵ (~10% of total protein is PLCϵ). As will be discussed below, we now believe that the extensive washing with detergents leads to a stripping of essential lipid cofactors necessary for activity.

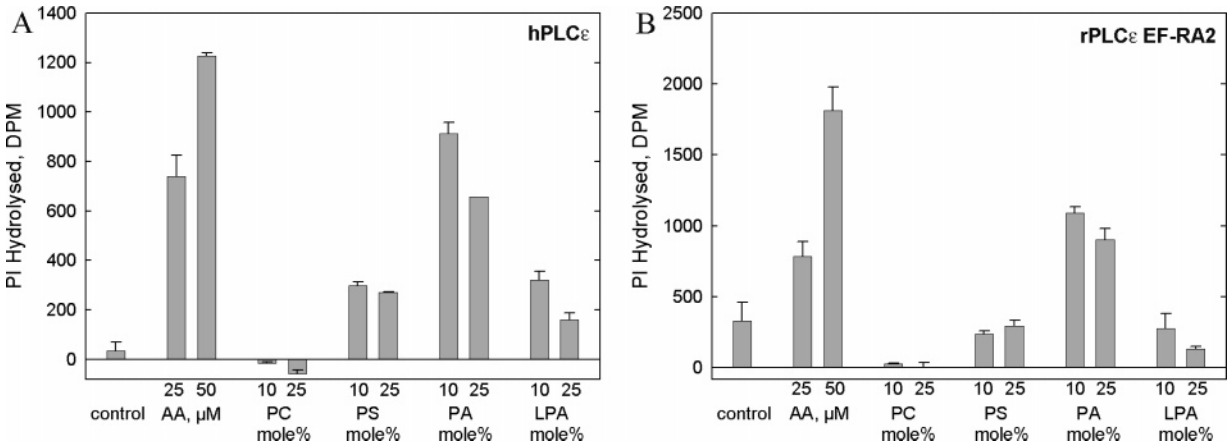

FIGURE 3: The phospholipid activation profile of PI hydrolysis by PLCϵ is different. Assays were carried out in ^{3}H-PI and appropriate amounts of lipids and DDM for 10 min at 30 °C using 20 μM PI and keeping the total concentration of PI, lipids, and DDM constant at 300 μM and either human PLCϵ (A) or rat PLCϵ-(EF-RA2) (B). The experiment was carried out in duplicate, and the values were corrected for hydrolysis in the absence of enzyme.

PLCϵ Activity Is Dependent on the Concentration of Substrate and Ca^{2+}. Phosphodiesterase activity was assessed using detergent mixed micelles. We have found that this is the best system to describe the complex behavior of interfacial enzymes such as PLCϵ. While we have also performed activity studies using vesicles, a system that more closely resembles the plasma membrane, we have not been able to find a neutral diluent lipid. Therefore, it is difficult to identify regulators using vesicles because enzyme activity is the result of alterations in the concentration of substrate as well as diluent. Since the detergent DDM appears to be a true neutral diluent (PLC activity is independent of DDM concentration), we feel that this system is best to assess function. The mixed micelle assay is a well-established assay for phospholipases. As can be seen in Figure 2A, the K_m for PI is approximately 61 μM, very similar to the K_m determined for other PI−PLCs. All mammalian PI−PLCs require free Ca^{2+} for activity, and in fact some isoforms, notably the δ isoforms, are most likely primarily activated by increases in free Ca^{2+}. As shown in Figure 2B, PLCϵ reaches maximal activity at nanomolar concentrations of free Ca^{2+} (a physiological concentration) similar to the Ca^{2+} requirement seen with other PI-PLCs. At approximately 20 nM free Ca^{2+}, PLCϵ exhibits 50% activity.

Select Phospholipids and Free Fatty Acids Directly Activate PLCϵ. Lipids and FFAs are increasingly being recognized as ligands that bind and activate proteins. Since the activity of PLCϵ is almost nonexistent when pure, we wondered if lipids might serve at least as a cofactor if not an activator for this enzyme. We have previously shown the importance of lipids such as PIP_2, PS, and FFAs for the activity of the δ1 isoform (*17*, *18*, *26*, *27*). Another isoform of PLC, namely PLCγ, is also activated by arachidonic acid (*28*). The lipids were incorporated into micelles with PI as substrate and the neutral detergent DDM. The PI concentration was kept constant at 20 μM (well below the K_m), while the lipid concentration was varied between 10 and 25 mol %. The concentration of DDM was varied such that the total concentration of PI, lipids, and DDM was constant at 300 μM. The DDM concentration was always above 200 μM, well above the CMC for the detergent. As shown in Figure 3, the effect of various lipids on PI hydrolysis was very dissimilar, suggesting specificity. While PC inhibited PLCϵ activity, PS and PA activated PI hydrolysis (Figure 3) about 4-fold and 8-fold, respectively. LPA as well as the fatty acids AA and SA also profoundly activate PLCϵ (Figures 3 and 4). FFAs at physiological concentrations can activate PLCϵ up to 60-fold.

PLCϵ contains several protein motifs that have been shown to interact with lipids. These include the pleckstrin homology (PH) domain, C2 domain, and the catalytic site made up of the conserved X and Y domains. On the basis of our very recent work with PLCδ1, we would add the EF-hand domains as another site of lipid−protein interaction (*18*). An N-terminal truncated enzyme was tested for its ability to be regulated by lipids such as AA. This rPLCϵ-(EF-RA2) enzyme was a kind gift of Dr. Kendall Harden (University of North Carolina) and does not contain the CDC25 or PH domains (*13*). It does however contain motifs 3 and 4 of the EF-hand and the C2 domains. Motifs 1 and 2 are not present in PLCϵ. This abbreviated protein retains the ability to hydrolyze substrate. The ability of lipids to regulate this low molecular weight PLCϵ has been assessed **(**Figure 3B). It appears to be activated by lipids like the full-length wild-type protein. It can therefore be deduced that the CDC25 and PH domains are not necessary for lipid regulation.

Since we have used a partially purified preparation, we had to make sure that FFAs were not acting on a PLC other than PLCϵ from sf9 cells. FFAs did not have any effect on any endogenous PLCs in sf9 cells, since FFAs had no effect on noninfected sf9 cell lysates (Figure 4A) or cell homogenates (data not shown). The effect of these FFAs on PLCϵ is dose dependent, with maximal activation at 50 μM (Figure 4B). Unsaturated FFAs such as AA were more potent since AA activated PLCϵ by about 50−60-fold, and the saturated FFA, SA, activated PLCϵ only about 8-fold.

The Mechanisms of Arachidonic and Phosphatidic Acid Activation Appear To Be a Lowering of K_m. In order to investigate the mechanism by which the FFAs activate PLCϵ, we studied the rate of PI hydrolysis at various concentrations of PI and in the absence or presence of 50 μM AA and 50 μM PA. The concentration of DDM was varied so that the concentration fell between 200 and 300 μM. Arachidonate shifted the substrate dependence curve to the far left (Figure 5A). The K_m for PI, in the presence of either AA or PA

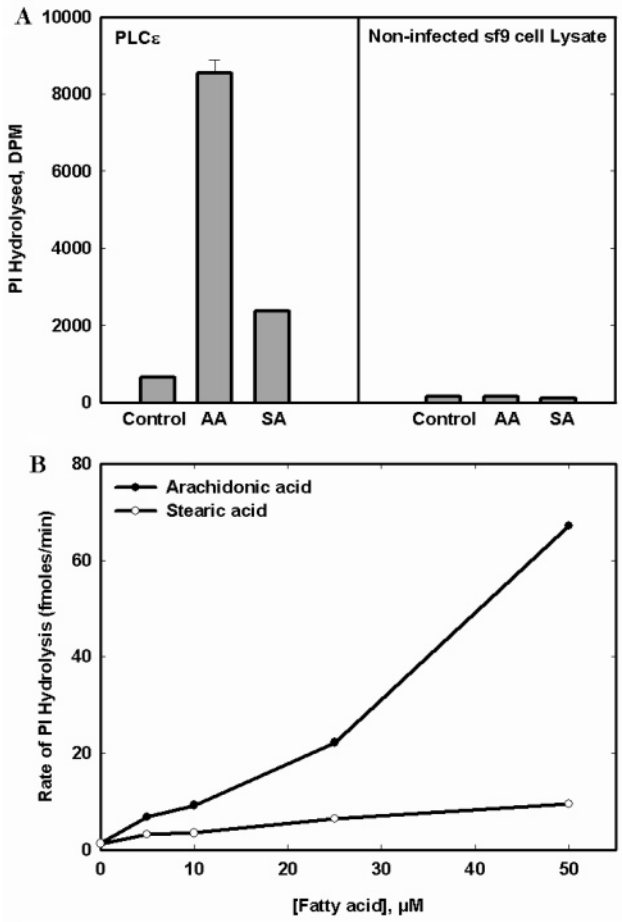

FIGURE 4: Free fatty acids are potent activators of PLCϵ. Activity assays were carried out in duplicate in ^{3}H-PI and DDM micelles in the presence or absence of either AA or SA for 10 min at 30 °C. The total concentration of PI, fatty acids, and DDM was kept constant at 300 μM. (A) The reaction mixture contained 60 μL of 20 μM PI, 50 μM AA or SA, 1 μL of PLCϵ, and 0.9 mM free Ca^{2+}. Noninfected cell lysate (right panel) was used as a control, which shows no activation of any endogenous PI hydrolyzing enzymes. Activation of purified PLCϵ by FFAs is shown in the left panel. The values were corrected for hydrolysis in the absence of enzyme. (B) The activation is dose dependent. The reaction was carried out as described above except the reaction mixture contained 160 μL of PI and various concentrations of either AA or SA. At intervals of time 50 μL was used to estimate the amount of hydrolysis. The rate of reaction was calculated on the basis of the slope of the reaction between 0 and 8 min.

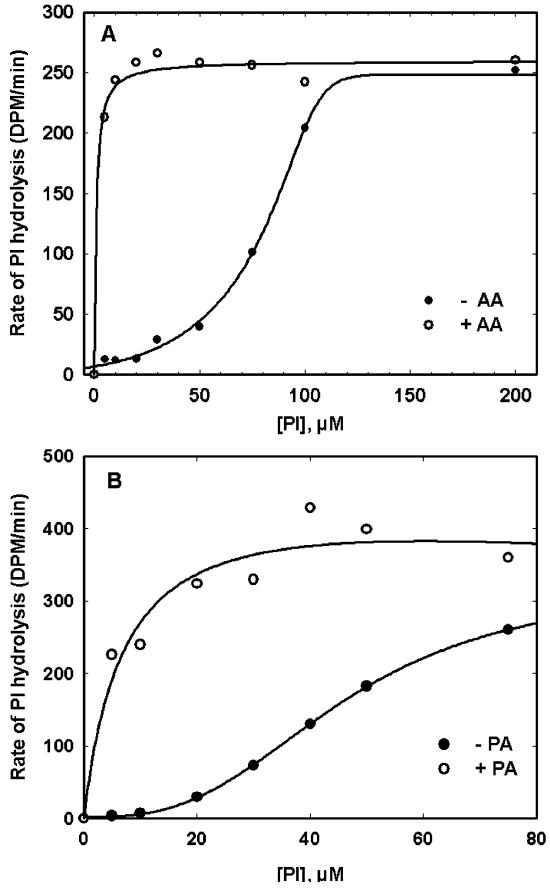

FIGURE 5: The effect of arachidonic and phosphatidic acids is on K_m. The experimental conditions were the same as described in the legend to Figure 3B except that reaction was carried out in the absence or presence of 50 μM AA (A) or 50 μM PA (B) and various concentrations of ^{3}H-PI. The rate of the reaction was calculated from the slopes between 0 and 8 min and was plotted as a mean of duplicate experiments.

(Figure 5B), shifted by almost an order of magnitude without any change in the V_{max}.

Lipids and FFAs Also Increase Hydrolysis of PIP$_2$ by PLCϵ. Since PIP$_2$ seems to be the preferred substrate for PLCs (it has a higher affinity or lower K_m for the catalytic site), we investigated the effect of lipids on the hydrolysis of PIP$_2$ mediated by PLCϵ. The phospholipids were incorporated into micelles with PIP$_2$ and DDM. The PIP$_2$ concentration was kept constant at 1.4 μM, and the phospholipid concentration was varied between 10 and 20 mol %, keeping the total concentration of PIP$_2$, lipids, and DDM at 300 μM by varying the DDM concentration. The DDM concentration was always above 200 μM. The FFAs were added to the substrate just before the activity experiments. As was found for the hydrolysis of PI, AA activates hydrolysis of PIP$_2$ by up to 10-fold (Figure 6**)**. The rank order of phospholipids for activation of PIP$_2$ hydrolysis

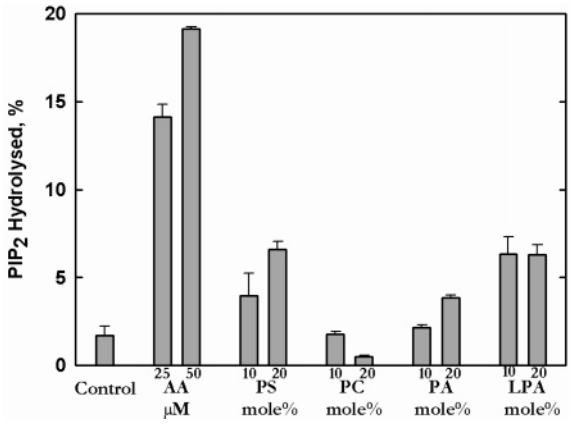

FIGURE 6: The effect of lipids on PIP$_2$ hydrolysis by PLCϵ is varied. Assay conditions were similar to those described in Figure 5, except 1.4 μM ^{3}H-PIP$_2$ was used instead of PI.

(LPA > PS > PA) was slightly different and distinct for the rank order for PI (PA > PS = LPA). This may reflect differences in which the two substrates bind to the catalytic site and may represent a mechanism for modulation of substrate preferences in vivo.

PLCϵ Binds to Arachidonic Acid Containing Vesicles. To assess if enzyme binding to AA correlated with activation, we performed a vesicle binding ultracentrifugation assay.

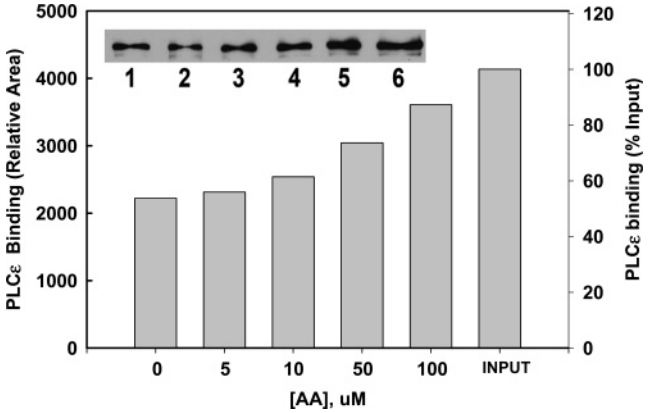

FIGURE 7: PLCε binds to unilamellar vesicles containing arachidonic acid. PLCε-bound vesicle pellets were analyzed by SDS−PAGE followed by immunostaining with an antiserum to PLCε as described in Experimental Procedures. The inset represents the immunostaining of PLCε. Lanes 1−5 contained 0, 5, 10, 50, and 100 μM AA with 2 mM DMPC, respectively. Lane 6 represents the amount of PLCε applied to vesicles. The binding percentage was determined by densiometric scanning of the immunoblots, and for computational purposes, the input (lane 6) was taken as 100% and without AA (lane 1) as nonspecific binding.

This assay is considered by many to be the gold standard for detecting protein−lipid interactions. Sucrose-loaded unilamellar vesicles were prepared with AA. PLCε bound to AA containing vesicles in a concentration-dependent manner (Figure 7). PLCε binding was specific for AA since the enzyme bound much better to vesicles containing AA than to those without it.

Reconstitution of either cPLA$_2$ or PLD with PLCε Leads to Activation of Phosphodiesterase Activity. Since the products of the phospholipases cPLA$_2$ and PLD, AA and PA, respectively, have profound effects on the phosphodiesterase activity of PLCε, we wondered whether these two enzymes may be able to be upstream regulators of PLCε. The effect of these two purified enzymes on the activity of PLCε was investigated in vitro. PC, the substrate for both cPLA$_2$ and PLD, was incorporated into micelles with PI. The concentrations of PI (20 μM), PC (75 μM), and DDM (220 μM) were kept constant. The concentration of cPLA$_2$ was 90 nM, that of PLD was 75 nM, and that of PLCε was ∼5 nM. Co-incubation of cPLA$_2$ or PLD led to the robust activation PLCε (Figure 8**)**. The hydrolysis of PI by cPLA$_2$ was very minimal under these conditions (1.5-fold greater than the basal hydrolysis of PLCε despite the concentration of cPLA$_2$ being 20-fold greater than that of PLCε). When cPLA$_2$ was co-incubated with PLCε, PI hydrolysis was 9.6-fold greater than PLCε alone (Figure 8A). This results in an 8-fold net gain when one corrects for the amount of PI hydrolyzed by cPLA$_2$. PLD can also hydrolyze PI to some extent as can be seen by the 6.5-fold increase over PLCε alone, though its preferred substrate is PC. PLD appears to be better at hydrolyzing PI than PLCε only because it was in excess (15-fold). The hydrolysis of PI by PLCε was increased 23-fold (corrected for PI hydrolyzed by PLD) by co-incubation with PLD (Figure 8B). Hydrolysis of PI was time dependent and linear for 20 min under our experimental conditions (Figure 8C).

Activation of PLCε by cPLA$_2$, PLD, and Free Fatty Acids in TSA-201 Cells. Cotransfection studies in TSA-201 cells support our in vitro results that illustrate PLCε activation by cPLA$_2$ and PLD. cPLA$_2$ (Figure 9A) and PLD (Figure 9B) are shown to be important activators of PLCε through their products AA and PA, increasing PLCε activity 2-fold and 3-fold, respectively.

In order to determine the effect of FFAs in cells, PLCε-transfected TSA-201 cells were fed 500 μM AA and 500 μM SA complexed to 60 μM essentially fatty acid-free bovine albumin for 12 h before harvesting (Figure 10**)**. The physiological concentration of FFAs in the human body varies from 90 to 1200 μM with basal values of 300−500 μM. The basal value of serum albumin in the human body is 600 μM (*25*). AA significantly increases the activity of PLCε, as well as endogenous PLCs in the pcDNA3 control. These cellular data support the in vitro results for PLCε activation by AA. This FFA−BSA assay also shows specificity through differing results between AA and SA.

DISCUSSION

In the present study we demonstrate that lipids can act as ligands to bind to specific motifs and regulate protein activity via allosteric effects by using PLCε as a model. Lipids and FFAs, such as a PA, PC, PS, LPA, AA, and PS, were shown to regulate PLCε activity in vitro and in cellular experiments. Phospholipids such as PA and FFAs such as AA are potent activators of PLCε, increasing the rate of PI hydrolysis by 8-fold and 50-fold, respectively. Because the basal activity of PLCε is virtually nonexistent in the absence of lipids, one could almost think of FFAs such as AA as a cofactor for the enzyme, suggesting that the lipid environment of the plasma membrane is not an inert one for this class of membrane-associated enzyme. While the mechanisms by which Ras activates PLCε are still debated (*29*), one study proposes activation via translocation to the membrane (*7*). Perhaps Ras-induced translocation causes activation by promoting PLCε binding to its necessary lipid cofactors or activators that reside and/or are generated in the plasma membrane.

FFAs are increasingly being recognized as bioactive molecules. While most of the effects of AA are attributed to its conversion by oxygenases to prostaglandins, and leukotrienes, direct effects have been described (*30*). For example, AA is a ligand for a novel family of G protein-coupled receptors termed GPR40 (*31*). AA will also activate PPAR receptors and NADPH oxidase (*30*). FFAs play an important role in diabetes by inhibiting insulin-stimulated glucose uptake and glycogen synthesis (*32*). Most obese individuals have elevated circulating plasma levels of FFAs. Many of these individuals develop insulin resistance and type 2 diabetes. The mechanism involves several aspects of insulin signaling including FFA inhibition of insulin-induced protein kinase B (Akt) activation and activation of PKC. Unsaturated or cis-fatty acids (oleic acid and AA) have been demonstrated to directly activate PKC (*33*, *34*). The mechanism appears to be due to an allosteric effect that increases V_{max}. Another mechanism for the increase in PKC activity seen in diabetic patients could be explained by our findings of PLC activation by FFA, since a product of PLC is DAG, a potent activator of many isoforms of PKC.

Interrelationships between different classes of phospholipase have been shown to significantly affect signal transduction systems in activated cells. PLC-mediated production of inositol triphosphate triggers intracellular Ca^{2+} mobiliza-

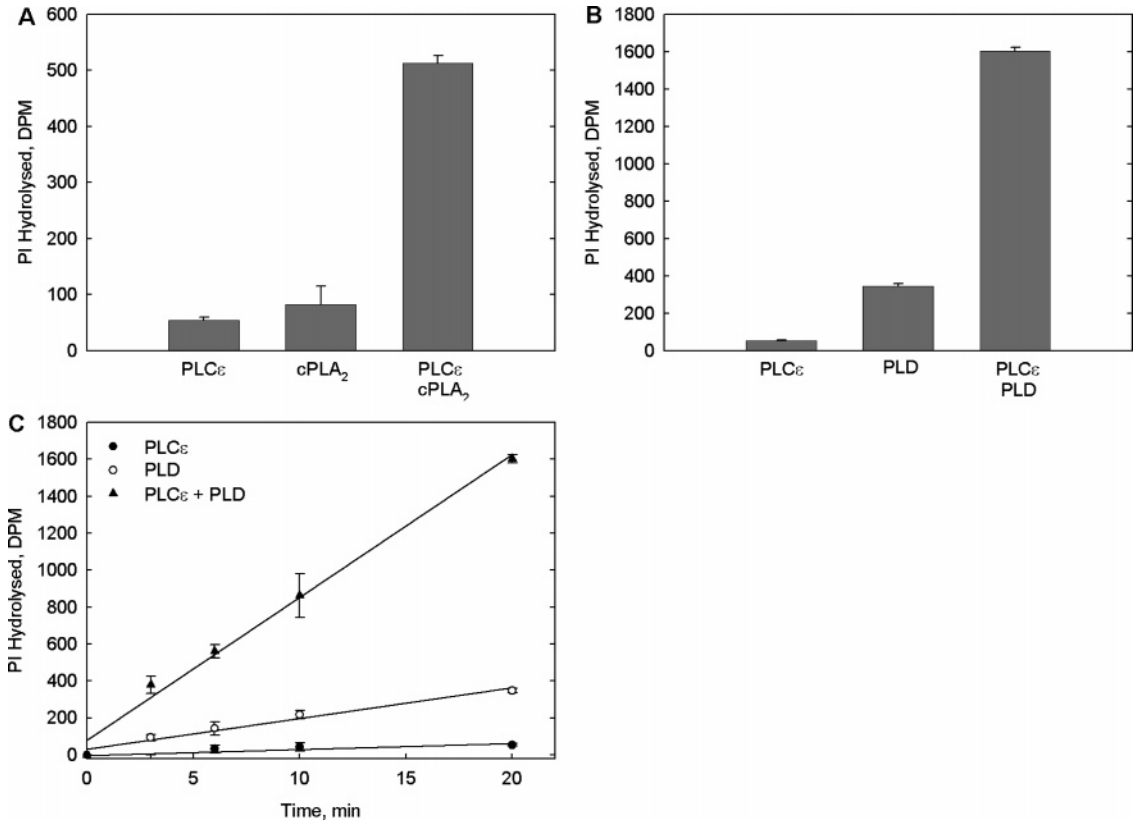

FIGURE 8: In an in vitro study both $cPLA_2$ and PLD activate PLC. Assays were carried out in duplicate at 30 °C for 20 min in ^{3}H-PI (20 μM), PC (75 μM), and DDM (220 μM) mixed micelles in the absence or presence of $cPLA_2$ (A) or PLD (B). The reaction contained 20 μM PI, 90 nM $cPLA_2$ or 75 nM PLD, PLCϵ, and 0.5 mM free $CaCl_2$. (C) Time course of PI hydrolysis by PLC, PLD, or PLCϵ with PLD in 20 μM PI, 75 μM PC, and 220 μM DDM mixed micelles.

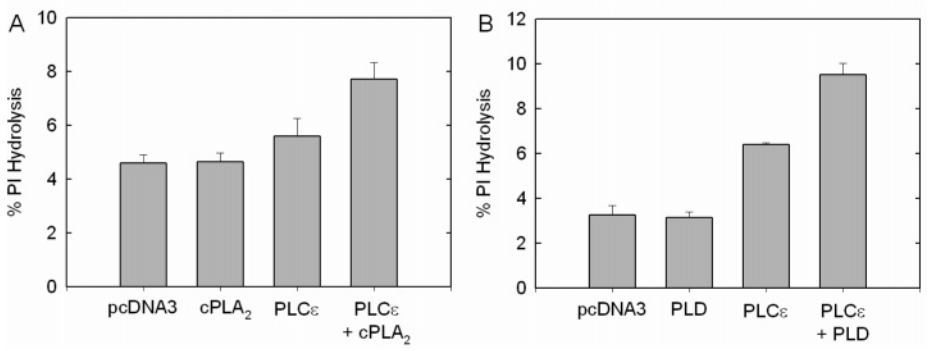

FIGURE 9: In an in vivo study both $cPLA_2$ and PLD activate PLC in TSA-201 cells. TSA-201 cells were cotransfected with PLCϵ and either $cPLA_2$ (A) or PLD (B) in duplicate and assayed for PLC activity by measuring percent PI hydrolysis. Data shown are the average of three separate experiments.

tion, which is a prerequisite for $cPLA_2$ activation (*35*). Conversely, the tyrosine kinase-coupled PLC isozymes are stimulated by AA, which is supplied by $cPLA_2$, in the presence of the microtubule-associated protein τ or τ-like protein AHNAK (*36*−*38*). In this study, the ability of both PLD and PLA_2 to activate PLCϵ is described. PLA_2 and PLD are important effector molecules which mediate a variety of important physiological effects. PLA_2 catalyzes the hydrolysis of membrane phospholipids, liberating free AA and lysophospholipids, which are precursors for bioactive lipid mediators. Of a number of PLA_2 enzymes identified to date, $cPLA_2$ and several secretory PLA_2 enzymes have been implicated in stimulus-induced AA release, which is coupled with cyclooxygenase-catalyzed prostaglandin formation (*35*, *39*). Activation of $cPLA_2$ is tightly regulated by postreceptor signal transduction events, including Ca^{2+} signaling promoted by phosphoinositide-specific PLC enzymes and phosphorylation directed by the mitogen-activated protein kinase members (*35*).

PLD catalyzes the hydrolysis of PC to produce PA, which by itself acts as a second messenger or is metabolized to other signaling molecules, such as LPA and DAG. PLD activation has been implicated in a wide range of cellular responses, including vesicular transport, mitogenesis, and respiratory burst (*40*). There are at least two PLD isoforms, PLD1 and PLD2, in mammals. PLD1, which exists as two splicing variants (PLD1a and PLD1b), has low basal activity and is activated by small G proteins (ARF, Rho, and Ral), PIP_2, and PKC in vitro. Additional splice variants of PLD1a2 and PLD1b2 lacking the C-terminal 113 amino acids of

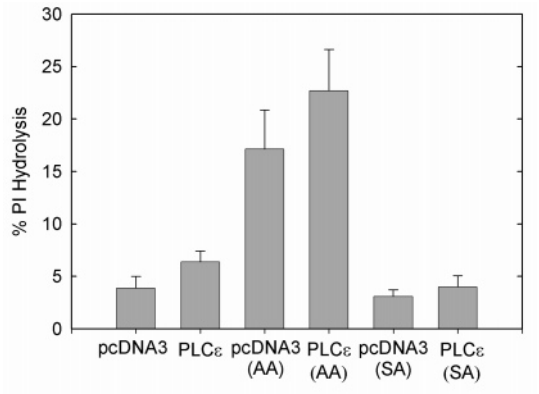

FIGURE 10: Free fatty acids activate PLCϵ in TSA-201 cells. TSA-201 cells transfected with PLCϵ were fed free fatty acids (arachidonic or stearic) complexed to essentially fatty acid-free bovine albumin in serum-free media. Transfections were conducted in duplicate and assayed for PLC activity, measuring percent PI hydrolysis. Data shown are the average of three separate experiments.

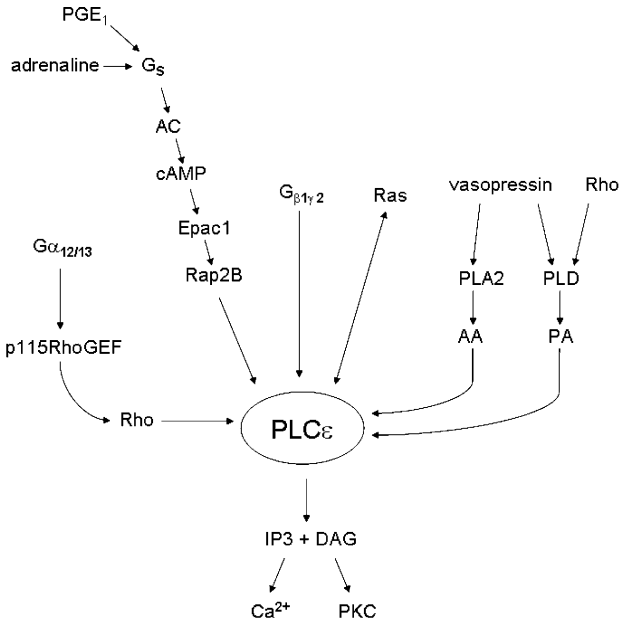

FIGURE 11: Graphical representation of PLCϵ signaling pathways.

PLD1a and PLD1b, respectively, have been identified and shown to be expressed in vivo (*41*). These shorter variants also have much less activity than their larger counterparts. PLD1 is distributed primarily on intracellular membranes, where it may regulate the formation of transport intermediates. Our splice variant of human PLCϵ is also membrane-associated, predicting possible spatial specificity for interaction with PLD1. PLD2 is constitutively active and is further stimulated by PIP_2 in vitro and is localized in caveolae (*42*−*44*). Human PLD2 exists in two alternatively spliced forms, PLD2b and PLD2c, with a deletion of 11 amino acid residues at the C-terminus and an insertion of 56 bp, respectively (*45*).

The identification of significant cross talk between PLCϵ, PLD, and PLA_2 may help to explain some hitherto unsolved mysteries in signal transduction occurring in the heart. We have shown that the products of PLD and PLA_2, PA and AA, respectively, can activate PLCϵ (Figure 11**)**, identifying that PLD and PLA_2 are upstream activators of PLC. Though angiotensin has been recognized to activate PLD and calcium signaling in cardiomyocytes, the link between the two signaling pathways (PLD and calcium release) was unknown (*46*). We suggest that this angiotensin pathway consists of the following: angiotensin activates PLD, and the resulting release of PA activates PLCϵ which in turn generates IP_3, a potent activator of sarcoplasmic reticulum calcium release. PLCϵ is highly expressed in cardiac tissues and plays an important physiological role. Recent studies show that PLCϵ plays a crucial role downstream of the epidermal growth factor receptor in controlling semilunar valvulogenesis through inhibition of bone morphogenic signaling (*47*). PLCϵ has also been implicated in cardiogenesis due to its interaction with small G proteins such as Ras and its variation in expression levels between normal and cancerous colonic tissues (*48*, *49*).

The demonstration of cross talk between PLCϵ, PLD, and PLA_2 via lipid mediators may also shed light on the mechanisms by which small G proteins such as Rho and Ral can activate PLC signaling. Many of the studies demonstrating activation of PLCϵ have been done via cotransfection in cells, so some uncertainty exists as to whether small G proteins activate directly or via an indirect mechanism. In vitro studies strongly implicate a direct mechanism for Rho activation of PLCϵ (*2*, *14*), though it is not known if this is the only mechanism. The fact that the deletion of 70 amino acids in the catalytic region of PLCϵ leads to a loss of functional regulation by Rho, but not to Rho binding, leaves the door open to an additional mode of regulation of this enzyme by Rho (*2*, *14*). Since both Rho and Ral are known activators of PLD (*50*), it is plausible that these small G proteins may activate PLCϵ in part via lipid mediators such as PA generated by activation of PLD.

The physiological importance of the recently discovered PLCϵ enzyme is just beginning to take shape. In this report the importance of lipids as cofactors or activators has been described. In addition, the paradigm that significant cross talk exists between phospholipases has been expanded and the complexity of signaling pathways influencing the activity of PLCϵ increased. The roles of FFAs and other lipids in cell signaling and protein−lipid interactions may be crucial to understanding the molecular basis of disease and in our efforts to develop new therapeutics against disease.

ACKNOWLEDGMENT

We thank Dr. Wonhwan Cho for providing $cPLA_2$ protein, Dr. Mary Roberts for providing PLD protein, Dr. Christina Leslie for providing $cPLA_2$ plasmid, Dr. John Exton for providing PLD plasmid, and Dr. Ken Harden for providing truncated rPLCϵ-(EF-RA2) protein.

REFERENCES

1. Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C, *Annu. Rev. Biochem. 70*, 281−312.
2. Wing, M. R., Bourdon, D. M., and Harden, T. K. (2003) PLC-epsilon: a shared effector protein in Ras-, Rho-, and G alpha beta gamma-mediated signaling, *Mol. Interv. 3*, 273−280.
3. Hwang, J. I., Oh, Y. S., Shin, K. J., Kim, H., Ryu, S. H., and Suh, P. G. (2005) Molecular cloning and characterization of a novel phospholipase C, PLC-eta, *Biochem. J. 389*, 181−186.
4. Swann, K., Larman, M. G., Saunders, C. M., and Lai, F. A. (2004) The cytosolic sperm factor that triggers Ca^{2+} oscillations and egg activation in mammals is a novel phospholipase C: PLCzeta, *Reproduction 127*, 431−439.

5. Rebecchi, M. J., and Pentyala, S. N. (2000) Structure, function, and control of phosphoinositide-specific phospholipase C, *Physiol. Rev. 80*, 1291−1335.
6. Lopez, I., Mak, E. C., Ding, J., Hamm, H. E., and Lomasney, J. W. (2001) A novel bifunctional phospholipase c that is regulated by Galpha 12 and stimulates the Ras/mitogen-activated protein kinase pathway, *J. Biol. Chem. 276*, 2758−2765.
7. Song, C., Hu, C. D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohge, M., Wu, D., Satoh, T., and Kataoka, T. (2001) Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras, *J. Biol. Chem. 276*, 2752−2757.
8. Jin, T. G., Satoh, T., Liao, Y., Song, C., Gao, X., Kariya, K., Hu, C. D., and Kataoka, T. (2001) Role of the CDC25 homology domain of phospholipase Cepsilon in amplification of Rap1-dependent signaling, *J. Biol. Chem. 276*, 30301−30307.
9. Evellin, S., Nolte, J., Tysack, K., vom Dorp, F., Thiel, M., Weernink, P. A., Jakobs, K. H., Webb, E. J., Lomasney, J. W., and Schmidt, M. (2002) Stimulation of phospholipase C-epsilon by the M3 muscarinic acetylcholine receptor mediated by cyclic AMP and the GTPase Rap2B, *J. Biol. Chem. 277*, 16805−16813.
10. Kelley, G. G., Reks, S. E., Ondrako, J. M., and Smrcka, A. V. (2001) Phospholipase Cepsilon: a novel Ras effector, *EMBO J. 20*, 743−754.
11. Kelley, G. G., Reks, S. E., and Smrcka, A. V. (2004) Hormonal regulation of phospholipase Cepsilon through distinct and overlapping pathways involving G12 and Ras family G-proteins, *Biochem. J. 378*, 129−139.
12. Wing, M. R., Houston, D., Kelley, G. G., Der, C. J., Siderovski, D. P., and Harden, T. K. (2001) Activation of phospholipase C-epsilon by heterotrimeric G protein betagamma-subunits, *J. Biol. Chem. 276*, 48257−48261.
13. Wing, M. R., Snyder, J. T., Sondek, J., and Harden, T. K. (2003) Direct activation of phospholipase C-epsilon by Rho, *J. Biol. Chem. 278*, 41253−41258.
14. Seifert, J. P., Wing, M. R., Snyder, J. T., Gershburg, S., Sondek, J., and Harden, T. K. (2004) RhoA activates purified phospholipase C-epsilon by a guanine nucleotide-dependent mechanism, *J. Biol. Chem. 279*, 47992−47997.
15. Schmidt, M., Evellin, S., Weernink, P. A., von Dorp, F., Rehmann, H., Lomasney, J. W., and Jakobs, K. H. (2001) A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase, *Nat. Cell Biol. 3*, 1020−1024.
16. Song, C., Satoh, T., Edamatsu, H., Wu, D., Tadano, M., Gao, X., and Kataoka, T. (2002) Differential roles of Ras and Rap1 in growth factor-dependent activation of phospholipase C epsilon, *Oncogene 21*, 8105−8113.
17. Kobayashi, M., Gryczynski, Z., Lukomska, J., Feng, J., Roberts, M. F., Lakowicz, J. R., and Lomasney, J. W. (2005) Spectroscopic characterization of the EF-hand domain of phospholipase C delta1: identification of a lipid interacting domain, *Arch. Biochem. Biophys. 440*, 191−203.
18. Kobayashi, M., Mutharasan, R. K., Feng, J., Roberts, M. F., and Lomasney, J. W. (2004) Identification of hydrophobic interactions between proteins and lipids: free fatty acids activate phospholipase C delta1 via allosterism, *Biochemistry 43*, 7522−7533.
19. Cifuentes, M. E., Honkanen, L., and Rebecchi, M. J. (1993) Proteolytic fragments of phosphoinositide-specific phospholipase C-delta 1. Catalytic and membrane binding properties, *J. Biol. Chem. 268*, 11586−11593.
20. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
21. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
22. Buser, C. A., and McLaughlin, S. (1998) Ultracentrifugation technique for measuring the binding of peptides and proteins to sucrose-loaded phospholipid vesicles, *Methods Mol. Biol. 84*, 267−281.
23. Martin, T. F. (1983) Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH3 pituitary cells. Evidence for the role of a polyphosphoinositide-specific phospholipase C in hormone action, *J. Biol. Chem. 258*, 14816−14822.
24. Cupp, D., Kampf, J. P., and Kleinfeld, A. M. (2004) Fatty acid-albumin complexes and the determination of the transport of long chain free fatty acids across membranes, *Biochemistry 43*, 4473−4481.
25. Potter, B. J., Sorrentino, D., and Berk, P. D. (1989) Mechanisms of cellular uptake of free fatty acids, *Annu. Rev. Nutr. 9*, 253−270.
26. Lomasney, J. W., Cheng, H. F., Wang, L. P., Kuan, Y. S., Liu, S. M., Fesik, S. W., and King, K. (1996) Phosphatidylinositol 4, 5-bisphosphate binding to the pleckstrin homology domain of phospholipase C-delta 1 enhances enzyme activity, *J. Biol. Chem. 271*, 25316−25326.
27. Bromann, P. A., Boetticher, E. E., and Lomasney, J. W. (1997) A single amino acid substitution in the pleckstrin homology domain of phospholipase C delta1 enhances the rate of substrate hydrolysis, *J. Biol. Chem. 272*, 16240−16246.
28. Hwang, S. C., Jhon, D. Y., Bae, Y. S., Kim, J. H., and Rhee, S. G. (1996) Activation of phospholipase C-gamma by the concerted action of tau proteins and arachidonic acid, *J. Biol. Chem. 271*, 18342−18349.
29. Harden, T. K., and Sondek, J. (2006) Regulation of phospholipase C isozymes by ras superfamily GTPases, *Annu. Rev. Pharmacol. Toxicol. 46*, 355−379.
30. Brash, A. R. (2001) Arachidonic acid as a bioactive molecule, *J. Clin. Invest. 107*, 1339−1345.
31. Itoh, Y., Kawamata, Y., Harada, M., Kobayashi, M., Fujii, R., Fukusumi, S., Ogi, K., Hosoya, M., Tanaka, Y., Uejima, H., Tanaka, H., Maruyama, M., Satoh, R., Okubo, S., Kizawa, H., Komatsu, H., Matsumura, F., Noguchi, Y., Shinohara, T., Hinuma, S., Fujisawa, Y., and Fujino, M. (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40, *Nature 422*, 173−176.
32. Boden, G. (2003) Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes, *Exp. Clin. Endocrinol. Diabetes 111*, 121−124.
33. McPhail, L. C., Clayton, C. C., and Snyderman, R. (1984) A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase, *Science 224*, 622−625.
34. Khan, W. A., Blobe, G. C., and Hannun, Y. A. (1995) Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C, *Cell. Signalling 7*, 171−184.
35. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP, *Cell 65*, 1043−1051.
36. Rhee, S. G., and Bae, Y. S. (1997) Regulation of phosphoinositide-specific phospholipase C isozymes, *J. Biol. Chem. 272*, 15045−15048.
37. Sekiya, F., Bae, Y. S., Jhon, D. Y., Hwang, S. C., and Rhee, S. G. (1999) AHNAK, a protein that binds and activates phospholipase C-gamma1 in the presence of arachidonic acid, *J. Biol. Chem. 274*, 13900−13907.
38. Lee, I. H., You, J. O., Ha, K. S., Bae, D. S., Suh, P. G., Rhee, S. G., and Bae, Y. S. (2004) AHNAK-mediated activation of phospholipase C-gamma1 through protein kinase C, *J. Biol. Chem. 279*, 26645−26653.
39. Shinohara, H., Balboa, M. A., Johnson, C. A., Balsinde, J., and Dennis, E. A. (1999) Regulation of delayed prostaglandin production in activated P388D1 macrophages by group IV cytosolic and group V secretory phospholipase A2s, *J. Biol. Chem. 274*, 12263−12268.
40. Exton, J. H. (1997) New developments in phospholipase D, *J. Biol. Chem. 272*, 15579−15582.
41. Mcdermott, A. M., and Haslam, R. J. (1996) Chemical cross-linking of pleckstrin in human platelets: Evidence for oligomerization of the protein and its dissociation by protein kinase C, *Biochem. J. 317*, 119−124.
42. Czarny, M., Lavie, Y., Fiucci, G., and Liscovitch, M. (1999) Localization of phospholipase D in detergent-insoluble, caveolin-rich membrane domains. Modulation by caveolin-1 expression and caveolin-182-101, *J. Biol. Chem. 274*, 2717−2724.
43. Lopez, I., Arnold, R. S., and Lambeth, J. D. (1998) Cloning and initial characterization of a human phospholipase D2 (hPLD2). ADP-ribosylation factor regulates hPLD2, *J. Biol. Chem. 273*, 12846−12852.

44. Sung, T. C., Altshuller, Y. M., Morris, A. J., and Frohman, M. A. (1999) Molecular analysis of mammalian phospholipase D2, *J. Biol. Chem. 274*, 494−502.
45. Steed, P. M., Clark, K. L., Boyar, W. C., and Lasala, D. J. (1998) Characterization of human PLD2 and the analysis of PLD isoform splice variants, *FASEB J. 12*, 1309−1317.
46. Li, Y., Shiels, A. J., Maszak, G., and Byron, K. L. (2001) Vasopressin-stimulated Ca^{2+} spiking in vascular smooth muscle cells involves phospholipase D, *Am. J. Physiol. Heart Circ. Physiol. 280*, H2658−H2664.
47. Tadano, M., Edamatsu, H., Minamisawa, S., Yokoyama, U., Ishikawa, Y., Suzuki, N., Saito, H., Wu, D., Masago-Toda, M., Yamawaki-Kataoka, Y., Setsu, T., Terashima, T., Maeda, S., Satoh, T., and Kataoka, T. (2005) Congenital semilunar valvulogenesis defect in mice deficient in phospholipase C epsilon, *Mol. Cell. Biol. 25*, 2191−2199.
48. Bai, Y., Edamatsu, H., Maeda, S., Saito, H., Suzuki, N., Satoh, T., and Kataoka, T. (2004) Crucial role of phospholipase Cepsilon in chemical carcinogen-induced skin tumor development, *Cancer Res. 64*, 8808−8810.
49. Sorli, S. C., Bunney, T. D., Sugden, P. H., Paterson, H. F., and Katan, M. (2005) Signaling properties and expression in normal and tumor tissues of two phospholipase C epsilon splice variants, *Oncogene 24*, 90−100.
50. Ping, P., Anzai, T., Gao, M., and Hammond, H. K. (1997) Adenylyl cyclase and G protein receptor kinase expression during development of heart failure, *Am. J. Physiol. 273*, H707−H717.
51. Shibatohge, M., Kariya, K., Liao, Y., Hu, C. D., Watari, Y., Goshima, M., Shima, F., and Kataoka, T. (1998) Identification of PLC210, a Caenorhabditis elegans phospholipase C, as a putative effector of Ras, *J. Biol. Chem. 273*, 6218−6222.
52. Wu, D., Tadano, M., Edamatsu, H., Masago-Toda, M., Yamawaki-Kataoka, Y., Terashima, T., Mizoguchi, A., Minami, Y., Satoh, T., and Kataoka, T. (2003) Neuronal lineage-specific induction of phospholipase Cepsilon expression in the developing mouse brain, *Eur. J. Neurosci. 17*, 1571−1580.

BI060648+