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Direct Modulation of Phospholipase D Activity by Gβγ

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ABSTRACT

Phospholipase D-mediated hydrolysis of phosphatidylcholine is stimulated by protein kinase C and the monomeric G proteins Arf, RhoA, Cdc42, and Rac1, resulting in complex regulation of this enzyme. Using purified proteins, we have identified a novel inhibitor of phospholipase D activity, Gβγ subunits of heterotrimeric G proteins. G protein-coupled receptor activation alters affinity between Gα and Gβγ subunits, allowing subsequent interaction with distinct effectors. $G\beta_1\gamma_1$ inhibited phospholipase D1 and phospholipase D2 activity, and both $G\beta_1\gamma_1$ and $G\beta_1\gamma_2$ inhibited stimulated phospholipase D1 activity in a dose-dependent manner in reconstitution assays. Reconstitution assays suggest this interaction occurs through the amino terminus of phospholipase D, because $G\beta_1\gamma_1$ is unable to inhibit an amino-terminally truncated phospholipase D construct, PLD1.d311, which like full-length phospholipase D isoforms, requires phosphatidylinositol-4,5-bisphosphate for activity. Furthermore, a truncated protein consisting of the amino-terminal region of phospholipase D containing the phox/pleckstrin homology domains was found to interact with $G\beta_1\gamma_1$, unlike the PLD1.d311 recombinant protein, which lacks this domain. In vivo, expressed recombinant $G\beta_1\gamma_2$ was also found to inhibit phospholipase D activity under basal and stimulated conditions in MDA-MB-231 cells, which natively express both phospholipase D1 and phospholipase D2. These data demonstrate that Gβγ directly regulates phospholipase D activity in vitro and suggest a novel mechanism to negatively regulate phospholipase D signaling in vivo.

Phospholipase D (PLD) mediates the regulated hydrolysis of phosphatidylcholine (PC), producing the second messenger phosphatidic acid (PA). PA can be further metabolized to two other signaling lipids, lysophosphatidic acid (LPA) and diacylglycerol, through actions of a phospholipase A and lipid phosphate phosphohydrolase, respectively. There are two mammalian PLD isoforms, PLD1 and PLD2, both with splice variants. PLD1 is found at plasma membranes and intracellular membranes, including Golgi and nuclear membranes (Liscovitch et al., 1999). PLD2 is predominantly localized to the plasma membrane and is found in membrane fractions containing caveolin (Xu et al., 2000). PLD1 is known to be regulated by PKC, Arf, and Rho family proteins. Although PLD2 was initially described as constitutively active, stimulation by Arf and PKC have been reported (Lopez et al., 1998; Chen and Exton, 2004) as well as modulation by inhibitory factors (Jenco et al., 1998; Lee et al., 2001). Both PLD1 and PLD2 share a common domain structure, consisting of PX/PH and catalytic domains. The PX/PH domains in the amino terminus contribute to membrane and lipid binding, and this region is also known to participate in PKC-mediated activation of PLD, although carboxyl-terminal regions have been implicated as well. The amino-terminal region is also thought to be autoinhibitory, because removal of this domain increases the basal activity of the protein (Sung et al., 1999a,b). The catalytic domains contain characteristic His-Lys-Asp motifs and encompass regions implicated in lipid binding. The lipid binding regions are important to both PLD isoforms, because both PLD1 and PLD2 are membrane-associated, use PC as substrate, and depend on PIP_2 for their activity. The carboxyl terminus of PLD is implicated in interactions with RhoA, and this well conserved region contains residues critical for the enzymatic activity of the protein (Sung et al., 1999b).

A relatively small number of negative regulators of PLD have been identified, including synucleins (Jenco et al.,

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ABBREVIATIONS: PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; LPA, lysophosphatidic acid; PKC, protein kinase C; PX, phox; PH, pleckstrin homology; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; GPCR, G protein-coupled receptor; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; h, human; Sf, *Spodoptera frugiperda*; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MBP, maltose binding protein; N-PLD, amino-terminal phospholipase D; MIANS, M8, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; PMA, phorbol 12-myristate 13-acetate; r, recombinant; mPLD, membranes from Sf21 cells expressing phospholipase D; GRK, G protein-coupled receptor kinase; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein.

1998), amphiphysins, clathrin assembly protein (Lee et al., 2000), and munc-18-1 (Lee et al., 2004). In this work, we identify Gβγ as a novel inhibitor of PLD activity, using purified proteins to demonstrate this direct effect on PLD. Gβγ is known to regulate a number of effector proteins, including adenylyl cyclase, PLC-β, phosphatidylinositol 3-kinase, and potassium and calcium channels, among others. The reduced affinity of Gβγ for activated Gα subunits upon receptor activation reveals new and distinct sites for protein-protein interaction on Gα and Gβγ subunits, which regulate GPCR signaling cascades. PLD is the downstream target of a number of GPCRs, including LPA receptors (Kim et al., 2004), endothelial differentiation gene/sphingosine 1-phosphate receptors (Meacci et al., 2003), and M_3 muscarinic receptors (Nieto et al., 1994). In recent years, the number of Gβγ effectors has grown to include those involved in neurotransmitter release (SNAREs; Blackmer et al., 2005), guanine nucleotide exchange factor activity (P-Rex1, a guanine nucleotide exchange factor for Rac1; Hill et al., 2005), and clathrin-mediated endocytosis (tubulin; Popova and Rasenick, 2004). We now extend that list to include PLD, which has been shown to play roles in vesicle transport (Roth et al., 1999), trafficking and exocytosis (for review, see Jones et al., 1999), cell migration, proliferation, and tumor formation (for review, see Foster and Xu, 2003; Buchanan et al., 2005).

Materials and Methods

Protein Expression. Expression and purification of recombinant hPLD1, Cdc42, PKCα, RhoA, and Arf were carried out as described previously (Walker et al., 2000). Recombinant PLD isoforms from membranes were obtained from monolayers of *Spodoptera frugiperda* (Sf) 21 cells infected with baculovirus encoding human PLD1 and PLD2, respectively, as described previously (Gidwani et al., 2003).

$G\beta_1\gamma_1$ subunits were prepared from holotransducin as described previously (Mazzoni et al., 1991). Purified proteins were stored at −80°C in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, and 10% glycerol. $G\beta_1\gamma_2$ subunits were obtained from recombinant baculovirus expression in Sf9 cells as described previously (Ford et al., 1998) and stored in 50 mM Tris, 100 mM NaCl, 1 mM EDTA, 3 mM DTT, and 0.03% CHAPS. Before use, detergent was removed, and Gβγ subunits were concentrated in buffer containing 50 mM Tris, 50 mM NaCl, and 1 mM DTT, pH 7.5, by ultrafiltration (Vivascience, Stonehouse, UK).

PLD1.d311 consisting of amino acids 312 to 1036 of rat PLD1b (with N-terminal MBP affinity tag) was expressed in baculovirus infected Sf21 cells. Recombinant PLD1.d311 protein was extracted in detergent and purified by fast-performance liquid chromatography as described previously (Henage et al., 2006). In brief, sequential metal-chelating chromatography, size exclusion chromatography, and ultrafiltration steps were used to purify PLD1.d311 to homogeneity.

Purified PLD1.d311, amino-terminal PLD (N-PLD) domains, Gβγ subunits, and Arf, Cdc42, and RhoA proteins were shown to be >85% pure by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining, as shown in Henage et al. (2006) for various proteins used in this study, and Fig. 1C for Gβγ subunits, PLD.d311, and N-PLD. The protein concentrations of recombinant proteins were determined with Bradford reagent (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard. PLD1 and PKCα enzymes were prepared as described previously (Brown et al., 1995; Henage et al., 2006). Given the low concentrations of these enzymes in purified form, estimated from immunoblot analysis, their suitability for use in exogenous assays was further evaluated by their ability

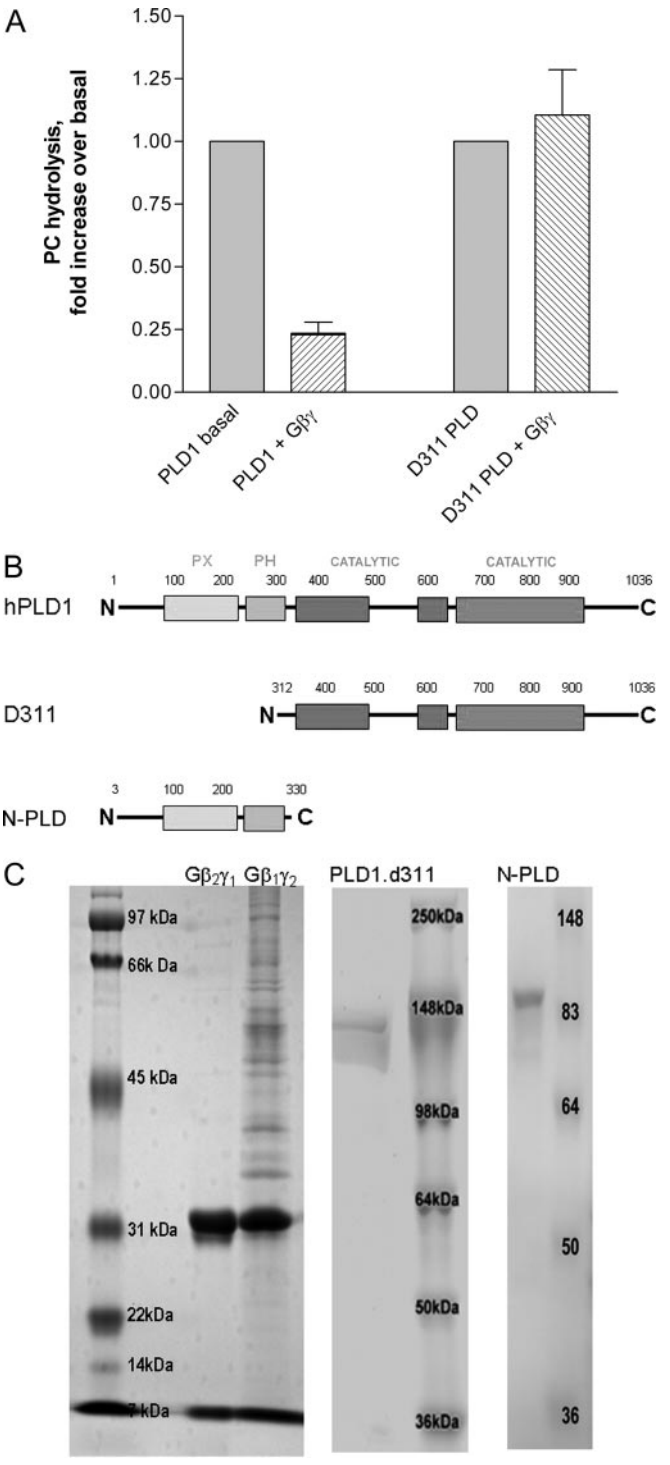

Fig. 1. Gβγ inhibits full-length PLD1 basal activity. A, $G\beta_1\gamma_1$ effect on basal PLD activity in an exogenous substrate assay (Brown et al., 1995). Left columns, 1 μM Gβγ and 1.6 nM purified PLD1, mean basal PLD1 activity was 1.9 pmol of PC hydrolyzed/30 min. Right columns, 2 nM PLD1.d311 (D311 PLD), 5 μM Gβγ; mean basal PLD1.d311 activity was 10.5 pmol of PC hydrolyzed/30 min. Data are mean ± S.E.M. from three independent experiments. B, domain structure of full-length PLD, PLD1.d311 (D311 PLD), and N-PLD. C, Gβγ, PLD1.d311, and N-PLD proteins used in this study were analyzed by SDS-polyacrylamide gel electrophoresis and stained by Coomassie Blue. Left, Gβγ proteins (5 μg of total protein) were analyzed using a 10% Tris-glycine gel. Middle, PLD1.d311 and N-PLD with N-terminal MBP affinity tags (see *Materials and Methods*) results in molecular masses of ~130 and 90 kDa, respectively. PLD1.d311 (2 μg of total protein) was analyzed using a 4 to 20% Tris-glycine gel. Right, N-PLD, 2 μg, was analyzed by a 10% Tris-glycine gel.

to support PLD hydrolysis in the exogenous PLD assay described below; in the case of PLD1, this value ranges from 1 to 10 pmol of PC hydrolyzed per 30 min without addition of any activators (basal activity). For activator PKCα, the ability to stimulate PLD-mediated PC hydrolysis was 30 to 40 times greater than basal PLD measured without activator stimulation.

The amino-terminal 331-amino acid region of PLD1 (N-PLD) containing the PX/PH domain of PLD1 was expressed as a hexahistidine-tagged MBP fusion protein in *Escherichia coli*, purified by nickel-chelating chromatography, and stored in buffer containing 50 mM Tris, 150 mM NaCl, and 2 mM β-mercaptoethanol. The N-terminal sequence from rat PLD1 (residues 3–330) was amplified by polymerase chain reaction using the following primers: 5′-GACTTGCGCACTAAGAAGTGAGGC-3′ and 5′-GGTCTTTGAGGAAGGTAC-CTCAGTGCTTCTGGATGAAC-3′. The amplified PCR product was digested with FspI and HindIII and cloned in frame into the psV282 expression vector (courtesy of Laura Mizoue, Vanderbilt Center for Structural Biology, Nashville, TN) to generate a PLD1 fragment corresponding to amino acids 3 to 330 with N-terminal hexahistidine and maltose-binding protein purification tags. The N-terminal PLD1 protein was expressed in BL21 (DE3) Rosetta *E. coli* (Novagen, Madison, WI) and purified by nickel-chelating chromatography. One-liter cultures were grown in Luria broth (30 μg/ml kanamycin and 34 μg/ml chloramphenicol). Expression was induced at optical density$_{600}$ = 0.9 with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside. Cultures were grown overnight at 25°C and harvested by centrifugation. Cells were resuspended in 10 ml of lysis buffer [50 mM phosphate buffer, pH 7.5, 250 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 1% (w/v) *n*-octyl-β-D-glucopyranoside, and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)] and lysed by incubation on ice with 10 mg/ml lysozyme and sonication (6 × 20-s pulses at 6W RMS). Insoluble material was removed by centrifugation (40,000*g* for 30 min at 4°C), and lysate was further clarified by centrifugation (100,000*g* for 1 h at 4°C). Clarified lysate was applied to a 1-ml HiTrap Chelating HP column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in 25 mM phosphate buffer, pH 7.5, 150 mM NaCl, 0.2 mM DTT, and 0.2% (w/v) *n*-octyl-β-D-glucopyranoside. Purified (>90%) N-PLD eluted at 220 mM imidazole in a linear imidazole gradient. Detergent and DTT were removed from pooled fractions, and protein was concentrated to 1.5 mg/ml by ultrafiltration (Vivascience). Protein aliquots were frozen in liquid nitrogen and stored at −80°C.

Exogenous PLD Activity Assay. PLD activity was measured in an exogenous substrate assay as described previously (Brown et al., 1995). The hPLD1 source for the exogenous assays was generated with an SP-Sepharose-purified fraction of Sf21 cytosol expressing hPLD1 and is described in detail in Walker et al. (2000). Reactions were incubated for 30 min at 37°C with 10 μM guanosine 5′-*O*-(3-thio)triphosphate in the presence of lipid vesicles containing [^{3}H]dipalmitoylphosphatidylcholine as well as phosphatidyletholamine, unlabeled phosphatidylcholine, cholesterol, and PIP_2 as described previously (Brown et al., 1995). Activity was measured by scintillation counting of the soluble [^{3}H]choline released as a result of PLD-mediated PC hydrolysis.

Fluorescent Labeling of Proteins and Spectrofluorometric Assays. $G\beta_1\gamma_1$ subunits were exchanged into labeling buffer (50 mM Tris and 150 mM NaCl, pH 7.5) before modification with fluorescent probe. MIANS (M8) [2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid] was used to label $G\beta_1\gamma_1$ subunits for fluorescent assays. In brief, Gβγ was incubated with a 5-fold molar excess of M8 at 4°C for 4 h before quenching with 5 mM β-mercaptoethanol, followed by ultrafiltration to remove unreacted probe from labeled protein. Because of the presence of multiple Cys residues, stoichiometry of labeling for Gβγ was between 1.5 and 3.2 mol M8/mol Gβγ (M8 extinction coefficient 27,000 cm^{-1} M^{-1}, excitation 320 nm, and Bradford assay, respectively). Fluorescence assays were conducted in 50 mM Tris, 50 mM NaCl, and 5 mM β-mercaptoethanol, pH 7.5, at excitation/emission wavelengths 320/420 nm, respectively. Emission increases upon binding of M8-$G\beta_1\gamma_1$ to MBP-tagged N-PLD was compared with basal emission of M8-$G\beta_1\gamma_1$ in the absence of interacting proteins, less contributions from MBP-tagged N-PLD alone in assay buffer.

Cell Culture and Transfection. MDA-MB-231 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were seeded in six-well tissue culture plates (2×10^5 cells/well) the day before transfection with either empty vector pcDNA3.1 (control) or the combination of pcDNA 3.1 encoding $G\beta_1$ and the vector encoding $G\gamma_2$ (Chen et al., 2004). Transfections were performed with a total of 2 μg of DNA/well and 3 μl of LipofectAMINE (Invitrogen, Carlsbad, CA) per microgram of DNA in 0.5% fetal bovine serum. Cells were labeled for in vivo PLD analysis 30 h after transfection.

PLD Activity Measurements in Intact Cells. PLD activity in cells was measured as described previously (Walker and Brown, 2004). In brief, cells were incubated for 16 h in serum-free media containing [^{3}H]oleic acid, washed, and then incubated with 0.4% 1-butanol in serum-free Dulbecco's modified Eagle's medium for 1 h at 37°C in 5% CO_2 to allow formation of phosphatidylbutanol. For stimulated PLD activity, this incubation was reduced to 15 min under stimulated conditions. Products were separated by thin layer chromatography, and bands comigrating with nonradioactive phosphatidylbutanol standards were scraped, counted by scintillation, and counts (less any background measured in the absence of butanol) were compared with total radioactivity in the extract. For PMA- and LPA-stimulated PLD activity, the percentage of phosphatidylbutanol formed was compared with basal levels in control (vector-transformed) cells.

Immunoblot Analysis. Transient expression of $G\beta_1\gamma_2$ was determined by protein immunoblot analysis. Whole-cell radioimmunoprecipitation assay buffer lysates were denatured by boiling cells in Laemmli sample buffer and then resolved on a 10% Tris-glycine gel (5 μg of total protein lysate/lane) and transferred to polyvinylidene difluoride membranes. Membranes were probed with rabbit anti-Gβ antibody and mouse anti-actin antibody to control for protein loading, followed by incubation with appropriate secondary antibody conjugated to horseradish peroxidase. Detection was performed with chemiluminescence (GE Healthcare) and imaging with Fluor-S (Bio-Rad, Hercules, CA).

Results

To determine the effect of $G\beta_1\gamma_1$ (Gβγ) on basal, unstimulated PLD1 activity, Gβγ was incubated with soluble, purified hPLD1 in complex lipid vesicles containing PIP_2, and PLD activity was measured in this reconstituted system. [^{3}H]PC was cleaved over 30 min by PLD, and soluble [^{3}H]choline released as a result of PLD activation was recovered and measured by scintillation counting. As seen in Fig. 1A, Gβγ inhibited basal activity of purified full-length PLD1. To determine the role of the amino-terminal domain of PLD in this interaction, which contains the PX/PH domains of PLD, we used a truncated rPLD1 isoform lacking residues 1 to 311 of full-length PLD (PLD1.d311) (Fig. 1B). PLD1.d311, like full-length PLD1, requires PIP_2 for activity and is regulated by Arf and Rho family proteins Cdc42, RhoA, and Rac1 (Henage et al., 2006). This truncated rPLD1 isoform, expressed as a MBP fusion protein, demonstrates high expression (>100-fold compared with wild-type hPLD) and robust enzymatic activity in vitro and in vivo. PLD1.d311 retains sensitivity to monomeric G proteins, with activation kinetics similar to that of wild type, but it demonstrates a reduced sensitivity to PKCα, because of elimination of one of two putative PKC interaction sites located within the amino terminus. We

tested the ability of Gβγ to inhibit both full-length and the N-terminally truncated PLD isoform PLD1.d311, which are both PIP_2-dependent (Henage et al., 2006). Although full-length PLD1 was inhibited by a relatively modest $G\beta_1\gamma_1$ concentration (1 μM), PLD1.d311 was not inhibited by even higher levels of $G\beta_1\gamma_1$, compared with their respective basal activities in the absence of Gβγ (Fig. 1A), suggesting $G\beta_1\gamma_1$ inhibits full-length PLD1.

PLD1 activity is stimulated by PKCα, Arf, and RhoA family proteins, and PKCα acts synergistically to activate PLD in the presence of monomeric G proteins. To determine the effect of Gβγ on stimulated PLD activity, PLD1 was incubated with indicated activators (Fig. 2) in the presence and absence of $G\beta_1\gamma_1$, and PC hydrolysis was measured as indicated above. We found that Gβγ inhibited PLD-mediated PC hydrolysis in the presence of each activator tested (Fig. 2), whereas using boiled Gβγ, Gβγ storage buffer, or bacterial PLD had no effect on PC hydrolysis, nor did Gβγ or activators alone in the absence of PLD (data not shown). In addition, synergistic PLD1 activation was also strongly inhibited by $G\beta_1\gamma_1$ (Fig. 2, far right).

Because $G\beta_1\gamma_1$ was found to inhibit both basal and stimulated PLD1 activity, we next examined the isoform specificity of the interaction. Because PLD1 is more sensitive to regulation by monomeric G proteins and PKC activators, PLD2 stimulation by selected activators has also been reported, albeit to a lesser extent than PLD1 (Lopez et al., 1998; Chen and Exton, 2004). Membranes from Sf21 cells expressing PLD1 or PLD2 (mPLD1and mPLD2) were incubated with a combination of Gβγ and activators PKCα and Arf. PLD-mediated PC hydrolysis was measured, relative to basal PLD activity, for each isoform in the absence of Gβγ and activators. We found $G\beta_1\gamma_1$ inhibited both PLD1 and PLD2 activity (Fig. 3) stimulated by Arf and PKCα. Despite higher basal activity of PLD2 (results normalized to basal), only modest increases were seen upon activation of PLD2 by PKC and Arf, consistent with the observation that this isoform is less sensitive to activators (Lopez et al., 1998). Although $G\beta_1\gamma_1$ inhibited both stimulated PLD1 and PLD2 activity, $G\beta_1\gamma_1$ reduced stimulated PLD2 activity to a level below that of basal, an effect not seen with mPLD1, nor with soluble PLD1 stimulated by activators (Fig. 2), suggesting a strong inhibition of PLD2 by Gβγ subunits. The Gβγ-mediated inhibition of PLD1 in membrane preparations is less complete than that seen using purified PLD1 (compare Fig. 2 with Fig. 3), which may reflect the accessibility of Gβγ to the PLD enzyme in a membrane environment.

To determine the potency of inhibition of PLD1 by Gβγ, increasing concentrations of Gβγ were used to inhibit PKC-stimulated PLD1 activity. Because Gβγ inhibited both basal and stimulated PLD activity, Gβγ-mediated inhibition of PLD was measured after stimulation with PKCα (to increase signal amplitude in these assays). We found that increasing amounts of $G\beta_1\gamma_1$ inhibited stimulated PLD1 activity in a dose-dependent manner, compared with the maximal activation in the absence of Gβγ (Fig. 4). For comparison, $G\beta_1\gamma_2$ was also examined for its ability to inhibit PLD. $G\beta_1\gamma_2$ was a more potent inhibitor of PLD activation than $G\beta_1\gamma_1$ (Fig. 4);

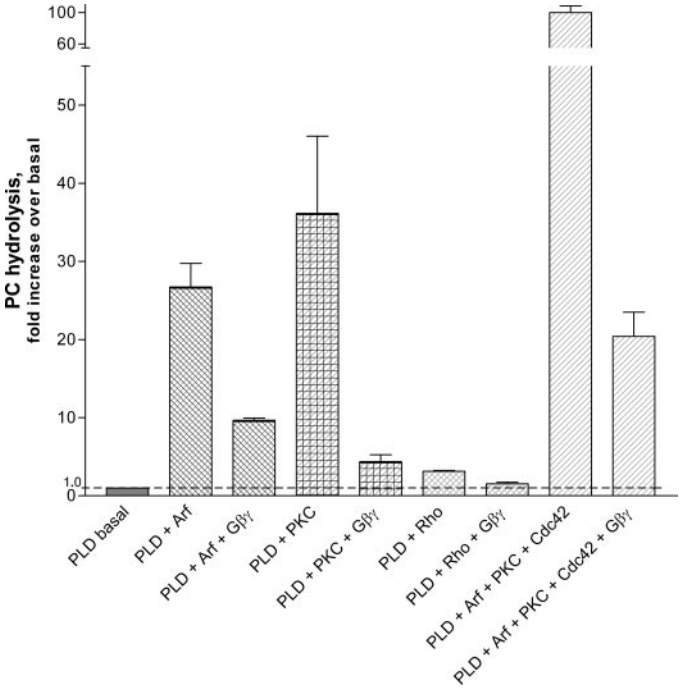

Fig. 2. Gβγ inhibits stimulated PLD1 activities. Comparison of effect of 5 μM $G\beta_1\gamma_1$ on 1.6 nM PLD1-mediated PC hydrolysis (mean basal PLD activity, 3.4 pmol of PC hydrolyzed/30 min) in the presence and absence of PLD activators 166 nM Arf1, 650 nM PKCα, 65 nM RhoA, and 50 nM Cdc42, measured by release of soluble [^{3}H]choline from [^{3}H]PC after a 30-min incubation of the indicated proteins at 37°C. Data are mean ± S.E.M. from three independent experiments. Basal activity is defined as the amount of PC hydrolysis in the presence of PLD alone.

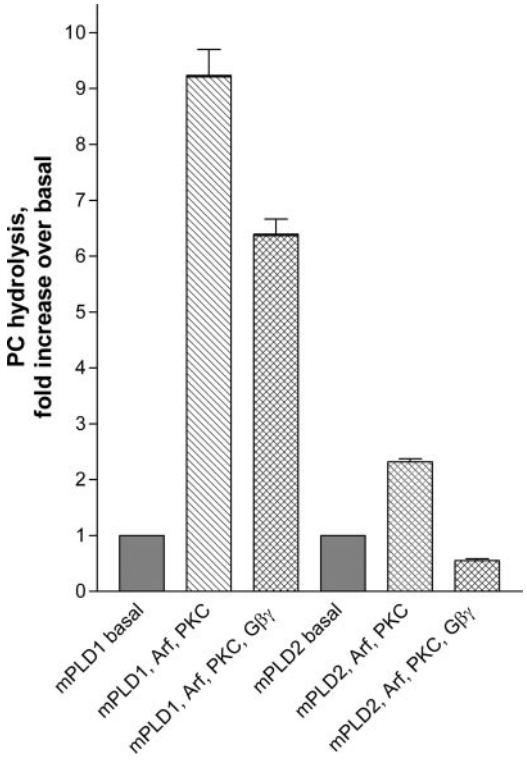

Fig. 3. Gβγ inhibits PLD1 and PLD2 activities in membranes. PC hydrolysis was measured in the presence and absence of purified $G\beta_1\gamma_1$ added to membranes expressing either PLD1 or PLD2 (26 and 10 ng of total membrane protein, respectively), amounts chosen to normalize basal activity (set to 1.0) measured in the absence of activators or Gβγ. For membrane-derived PLD1, mean basal activity was 9.5 pmol of PC hydrolyzed/30 min, and for membrane-derived PLD2, mean basal activity was 21.1 pmol of PC hydrolyzed/30 min. Activities compared with basal upon addition of activators 18 nM Arf and 1 μM PKCα and after addition of 4.4 μM $G\beta_1\gamma_1$. Data are the mean ± S.E.M. (n = 3).

$G\beta_1\gamma_2$ (1.2 μM) was sufficient to inhibit PLD1 activation by 50%, compared with 2.4 μM $G\beta_1\gamma_1$ required to mediate the same level of PLD inhibition.

The higher potency of $G\beta_1\gamma_2$ is not surprising; in other signaling systems, $G\beta_1\gamma_2$ has been shown to interact with higher affinity to effectors than $G\beta_1\gamma_1$. For example, $G\beta_1\gamma_2$ more potently stimulated PLCβ (Ueda et al., 1994) and inhibited SNARE fusion machinery (Blackmer et al., 2005) than $G\beta_1\gamma_1$. This may be due largely to post-translation modifications of these G$\beta\gamma$ isoforms. $G\beta_1\gamma_1$ is farnesylated, whereas $G\beta_1\gamma_2$ is geranylgeranylated, and these modifications influence membrane association. Geranylgeranylation of $G\beta_1\gamma_2$ may allow this isoform to more effectively bind phospholipids, thus conferring greater potency toward membrane associated effectors such as PLD.

The ability of G$\beta\gamma$ ability to inhibit full-length PLD is in contrast to its lack of effect on PLD1.d311, in which the first 331 residues of full-length PLD1 have been ablated (Fig. 1). To further investigate this result, residues 3 to 311 encompassing the PX/PH of PLD1 domain were expressed and purified as MBP-fusion proteins (N-PLD), as was PLD1.d311. Because the PX/PH domain itself is relatively insoluble, creation of a MBP fusion protein allows for a significant improvement in solubility and moderate levels of protein expression. Binding of purified N-PLD to G$\beta\gamma$ was measured using fluorescently labeled G$\beta\gamma$. $G\beta_1\gamma_1$ was labeled with M8, a thiol-reactive, environmentally sensitive fluorescent probe that increases its emission upon a binding event as a result of an increase in the hydrophobicity of the environment of the probe. Binding is detected as an increased emission from the labeled protein compared with emission in the absence of the interacting protein. M8-$G\beta_1\gamma_1$ bound N-PLD in a dose-dependent manner (Fig. 5A), in contrast to quenched label alone (data not shown). Both the amino-terminally truncated PLD1.d311 and the N-PLD proteins contain an MBP tag; however, the PLD1.d311 protein did not interact with labeled G$\beta\gamma$ (Fig. 5B). Only the N-PLD protein demonstrated increases in fluorescence upon incubation with M8-G$\beta\gamma$. These results indicate the amino-terminal region of PLD is necessary and sufficient for interaction with $G\beta_1\gamma_1$ subunits. PLD1 constructs lacking this domain do not interact with $G\beta_1\gamma_1$, consistent with the inability of G$\beta\gamma$ to regulate PLD1.d311 activity (Fig. 1).

The ability of G$\beta\gamma$ to inhibit full-length PLD activity in vitro, both basal and stimulated (in contrast to the N-terminally truncated PLD1.d311), suggests G$\beta\gamma$ directly inhibits PLD. This interaction is likely to be mediated through the amino-terminal 311-amino acid stretch of PLD1, which binds to G$\beta\gamma$ with a dose-response relationship consistent with its effect on PC hydrolysis as measured in reconstitution assays.

To determine the effect of G$\beta\gamma$ on PLD activity in cells, we overexpressed $G\beta_1\gamma_2$ in MDA-MB-231 cells (Fig. 6), which express both PLD1 and PLD2 (Meier et al., 1999). G$\beta\gamma$ reduced basal PLD activity in these cells significantly compared with control (Fig. 6B). Furthermore, G$\beta\gamma$ modestly reduced PLD activation in PMA and LPA stimulated MDA-MB-231 cells (Fig. 6C), indicating G$\beta\gamma$ may play a modulatory role in PLD activation in vivo.

Discussion

PLD activity is highly regulated by a number of factors, which together combine to determine signaling output to

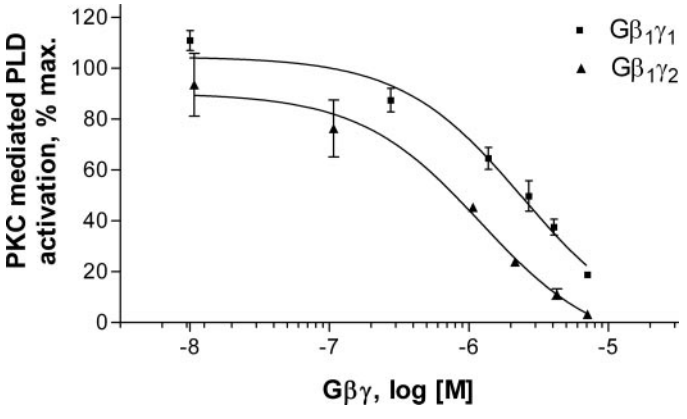

Fig. 4. $G\beta_1\gamma_2$ inhibits PLD1 with greater potency than $G\beta_1\gamma_1$. PKCα (654 nM) and increasing concentrations of indicated G$\beta\gamma$ subtypes were incubated with purified PLD1 (1.6 nM) in PC vesicles containing PIP_2 for 30 min at 37°C in an exogenous substrate assay. Mean basal PLD1 activity, 2.26 pmol of PC hydrolyzed/30 min; maximal PKCα-stimulated PLD1 activity, 95.01 pmol of PC hydrolyzed/30 min. Maximum PKCα-stimulated PLD activation measured in the absence of G$\beta\gamma$. Data are expressed as the mean ± S.E.M. (n = 3).

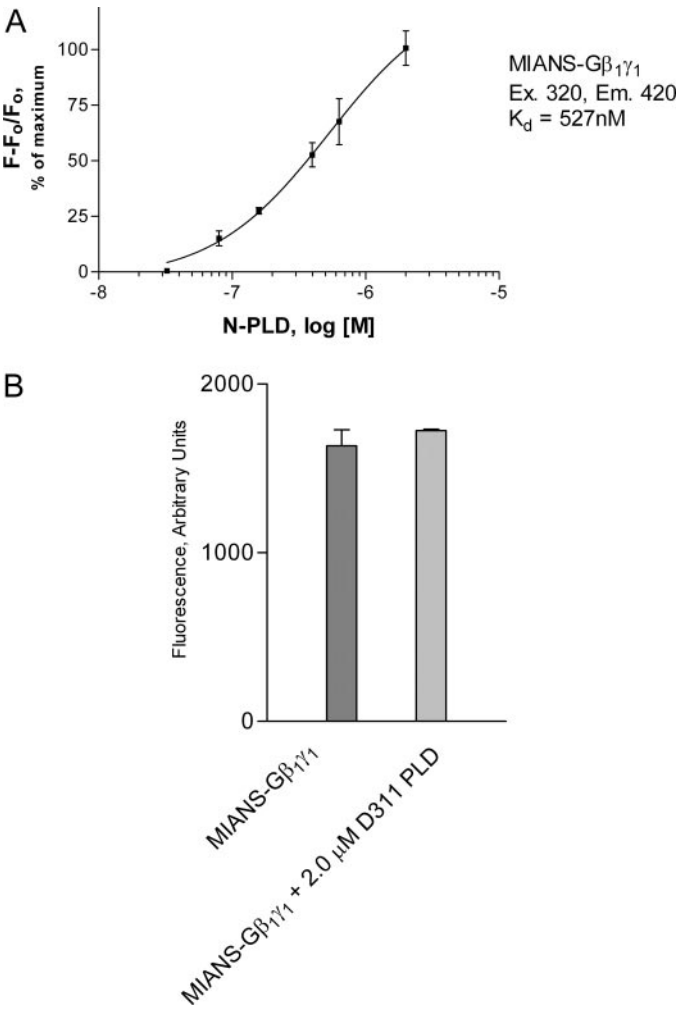

Fig. 5. G$\beta\gamma$ binds to the amino-terminal domain of PLD1. Fluorescence of MIANS-labeled $G\beta_1\gamma_1$ (100 nM), excitation/emission 320/420 nm, upon interaction with increasing concentrations of N-PLD, encompassing the PX/PH domain of PLD (A) or 2.0 μM PLD1.d311 (D311) (B), which lacks the first 311 residues of PLD. Data are expressed as the mean ± S.E.M. (n = 3).

PLD. Although PKC, monomeric G proteins, and PIP_2 are well known activators, less is known about the negative regulation of PLD activity. Because PLD is known to be involved in a myriad of processes from tumor formation to exocytosis to membrane remodeling, activation of PLD is likely to be tightly regulated. Increases in PLD1 expression and activity were demonstrated in colorectal tumors, compared with normal adjacent tissues, and the same study identified PLD1 as a downstream effector of oncogenic Ras transformation (Buchanan et al., 2005).

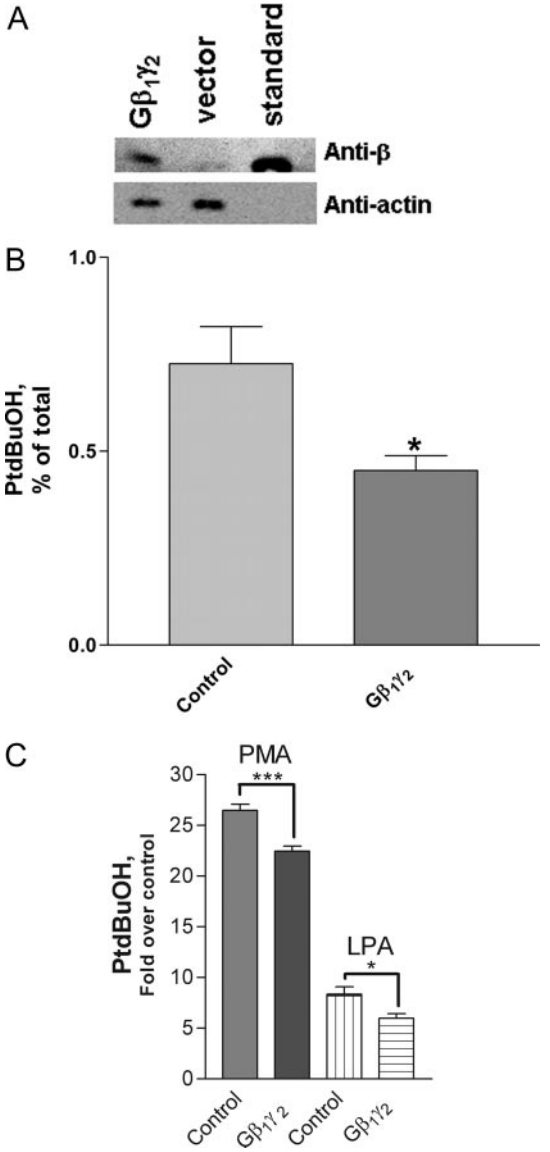

Fig. 6. Inhibition of PLD activity in vivo. MDA-MB-231 cells were transiently transfected with plasmid DNA encoding either empty vector (control) or cotransfected with plasmid DNA encoding $G\beta_1$ and $G\gamma_2$. A, transfected MDA-MB-231 cells were analyzed by Western blotting as described under *Materials and Methods*. $G\beta_1\gamma_1$ protein, 1 μg, is shown as standard in lane 3. B and C, PLD activity in transfected cells was measured by the transphosphatidylation of radiolabeled endogenous substrate to form phosphatidylbutanol, measured by scintillation counting. Results are the mean (± S.E.M.) for two separate experiments performed in triplicate. Asterisk denotes values significantly lower than control. Student's *t* test was used to determine significant differences (two-tailed: *, $p < 0.02$; ***, $p < 0.001$). B, transfected cells were assayed for basal PLD activity over a 1-h period in the presence of primary butanol. C, PLD activity in transfected cells measured after a 15-min stimulation by either 1 μM PMA or 5 μM LPA in the presence of primary butanol, compared with vector control (average 0.15% of total).

We found that $G\beta\gamma$ inhibits PLD activation, and reconstitution assays suggest the inhibition is mediated by the N-terminal region of PLD. The N terminus of PLD contains PX/PH domains, which contain palmitoylation sites that, along with these domains, are important for membrane association (Ktistakis et al., 2003). This region is also critical for interaction with $G\beta\gamma$, as shown by our direct binding data. A number of $G\beta\gamma$ effectors contain PH domains, such as protein kinase D (Jamora et al., 1999), PLCβ (Wang et al., 2000), and GRK proteins (Carman et al., 2000), to name a few. PH domains may serve to localize $G\beta\gamma$ to effector proteins and position distinct sites on $G\beta\gamma$ for interactions with other regions on effector molecules to regulate their activity. A recent study demonstrates that the amino terminal coiled-coil region of $G\beta\gamma$ mediates inhibitory contacts with PLCβ catalytic domains, whereas the switch II binding regions on $G\beta\gamma$ mediate stimulatory contacts with distinct regions on PLCβ (Chen et al., 2004; Bonacci et al., 2005). Likewise, the N terminus of PLD may bind $G\beta\gamma$ and position it for inhibitory interaction with residues important for the catalytic activity of PLD on a distinct surface of $G\beta\gamma$. Mutational studies of residues on $G\beta\gamma$ mediating the interaction with PLD will shed light on the molecular determinants of this interaction. Such studies have revealed that the residues on $G\beta\gamma$ mediating effector interactions vary, with some overlap between effectors (Ford et al., 1998). It will therefore be of interest to determine which effector proteins can compete with PLD for binding to $G\beta\gamma$, or whether simultaneous binding can occur, resulting in a scaffold of interacting proteins. The structure of GRK2 in complex with $G\beta_1\gamma_2$ (Lodowski et al., 2003) suggests a signaling scaffold of receptor, GRK2, and $G\beta\gamma$ (and $G\alpha$ proteins, which may interact with RGS domains of GRK2). These interactions lead to fast, efficient signal termination by localizing players involved in signal down-regulation into one complex. In this case, $G\beta_1\gamma_2$ binds the PH domain of GRK2, which enhances receptor phosphorylation and assists in orienting the complex toward the membrane and membrane bound effectors.

Because almost all agonists that activate PLD also stimulate phosphatidylinositol hydrolysis, and because $G\beta\gamma$ has been shown to mediate activation of PLCβ isoforms leading to PKC stimulation, a potent activator of PLD, we used purified proteins to demonstrate the direct effect of $G\beta\gamma$ on PLD. Findings from these in vitro studies are consistent with our results in MDA-MB-231 cells, which express both PLD1 and PLD2. Overexpression of $G\beta_1\gamma_2$ reduces PLD activity in these cells, suggesting $G\beta\gamma$ modulates PLD signaling in vivo. Although the effect seems modest in this cell line, even small changes in the enzymatic activity of PLD may result in large downstream effects, as a result of signal amplification, requiring tight regulation of the enzymatic activity of PLD. The role of $G\beta\gamma$ may be to fine-tune PLD signaling and maintain membrane homeostasis under a variety of conditions. $G\beta\gamma$ may also play a greater modulatory role in other cell types and under conditions that remain to be tested.

Previous studies by other groups have also suggested some interaction between PLD and $G\beta\gamma$. In myometrial homogenates containing PLD, Arf, and heterotrimeric G proteins, reduction in PLD activity was measured upon treatment with the heterotrimeric G protein activator AlF_4^- (Le Stunff

et al., 2000), which liberates Gβγ upon activation of heterotrimeric G proteins. Dissociation of Gα and Gβγ subunits may reveal activation-dependent interaction sites on Gβγ for PLD. Although these results do not definitively rule out Gβγ binding to Arf, preventing Arf-mediated activation of PLD, we demonstrate Gβγ inhibits both basal PLD and PLD activity stimulated by Arf (and other activators). This may suggest a broader role for Gβγ in modulating PLD signaling that does not require indirect regulation through Arf or PLCβ.

Subcellular localization of PLD may regulate its activity in vivo, modulating interactions with regulators generated at signaling nodes within the cell. Gβγ subunits are highly localized to cell membranes and may well serve as physiologically relevant modulators of PLD activation. Redistribution between intracellular membranes may be mediated by specific lipid binding or protein-protein interactions, which together regulate PLD activity. PLD1 activity has been detected in multiple cellular membrane fractions, including Golgi, endoplasmic reticulum, secretory vesicles, and plasma membrane (for review, see Liscovitch et al., 1999). PLD1 activity is regulated through access to various activators, and in the absence of such activators, it has a relatively lower basal activity than PLD2. In PC12 cells, which express predominantly PLD2, overexpression of the α_{2A}-adrenergic receptor impaired PMA stimulation of PLD, which was restored by treatment with either α_2-adrenergic antagonist or pertussis toxin (Ella et al., 1997). Consistent with these results, we found that $G\beta_1\gamma_1$ inhibited PLD2 (and PLD1) in vitro. Although PLD2 activity was reduced to a level below that of basal upon addition of Gβγ in our reconstitution assay, this may be due in part to the high affinity of both PLD2 and Gβγ for membrane components, whereas PLD1 is found in both membrane and cytosolic fractions. In vivo, Gβγ may dissociate from PLD to mediate activation, similar to other identified PLD inhibitors, such as the cytoskeletal protein β-actin (Lee et al., 2001), or neuronal proteins such as synucleins (Pronin et al., 2000) and munc-18-1 (Lee et al., 2004), initially identified through the use of reconstitution assays to characterize their effects on PLD activity.

In a cellular context, Gβγ and PLD may work in concert to regulate exocytosis. In *Saccharomyces cerevisiae*, a chimeric SNAP-25 ortholog deficient in vesiculation was rescued by up-regulation of a gene encoding a phosphatidylinositol 4′-kinase ortholog responsible for the production of phosphatidylinositol-4,5-biphosphate, an essential cofactor for PLD activity. Furthermore, the amino terminus of the SNAP ortholog was observed to bind PA, the direct product of PLD activation (Coluccio et al., 2004), which has been shown to stimulate vesicle formation (Kaldi et al., 2002). Exocytotic processes may require PLD activation and PA production to lower the energy barrier to membrane vesiculation. Modulation of PLD activity may restrict membrane remodeling and maintain membrane homeostasis, because sustained hydrolysis of cationic phosphatidylcholine to the anionic phospholipid PA would be expected to have significant effects on membrane electrostatics, architecture, and phospholipid content. Gβγ may play a role in fine tuning such activity. $G\beta_1\gamma_2$, highly enriched in brain tissue (Betty et al., 1998), has been shown to interact with SNAP-25 and syntaxin in SNARE complexes, inhibiting exocytosis (Blackmer et al., 2005). Gβγ may regulate both SNAREs and PLD to efficiently inhibit exocytosis, or it may function to maintain a low level of basal PLD activity until exocytosis is triggered. Relief of this inhibition by liberation of Gβγ, or in the case of SNARE binding, by competitive binding of synaptotagmin, could allow fast, efficient exocytosis to occur.

The data suggest Gβγ inhibits both basal and activator-mediated PLD activity, consistent with a direct interaction between PLD and Gβγ, which our data suggest is mediated by the N-terminal domain of PLD. This negative regulation of PLD may play a role in vivo to fine-tune the outcome from various signaling pathways that converge on PLD to activate it, or Gβγ may function to modulate effects of multiple stimuli that would be detrimental to membrane homeostasis. It may further define precisely which subcellular membranes tolerate sustained activation of PLD. The effects of Gβγ on PLD function is likely to be context-dependent and need to be deconstructed in terms of regulatory molecules (identified in biochemical assays) that have been shown to directly modulate PLD activity. The spatial and temporal distribution of these regulatory molecules is likely to influence the eventual output in terms of PLD signaling. Small changes in the enzymatic activity of PLD may have large effects on signaling downstream from PLD. Together, these data suggest a previously unappreciated role for direct Gβγ modulation of PLD and present new opportunities for the intersection of these key components in fine-tuning network signaling processes.

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