Phospholipase D Regulation by a Physical Interaction with the Actin-Binding Protein Gelsolin

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ABSTRACT: Increases in intracellular phosphatidic acid levels caused by receptor-mediated activation of phospholipase D (PLD) have been implicated in many signal transduction pathways leading to cellular activation. PLD is known to be regulated by several means, including tyrosine kinase activity, increases in Ca^{2+}, receptor-coupled G proteins, small GTP binding proteins, ceramide metabolism, and protein kinase C. We have investigated an additional regulatory effect on PLD activity involving nucleoside triphosphates (NTPs). A NTP binding protein copurifies with PLD activity from rabbit brains using a GTP−agarose affinity column, and this protein stimulates PLD activity only in the absence of NTPs. The NTP effect is reversible and labile, and the binding protein is separable from the PLD activity by heparin−agarose chromatography. We identified this protein as the actin-binding protein gelsolin by amino acid sequencing following peptide mapping. This finding was verified by the co-immunoprecipitation of gelsolin and PLD activity as well as by the reconstitution of gelsolin-dependent nucleotide sensitive PLD activity by the addition of purified gelsolin to gelsolin-free PLD. Our data indicate that actin rearrangements and PLD signaling are coordinately regulated through the physical association between PLD and gelsolin and that this interaction may also serve to amplify both PLD signaling and actin reorganization.

The hydrolysis of phosphatidylcholine (PC)[1] by phospholipase D (PLD) to yield choline and phosphatidic acid (PA) has been implicated in a variety of signal transduction pathways (Billah, 1993; Exton, 1994). Increased PA is associated with several important cellular activities, including stimulation of c-*fos* and c-*myc* transcription (Kanuss et al., 1990), activation of protein kinases (Ha & Exton, 1993; Limatola et al., 1994), activation of phospholipase C-γ (Jones & Carpenter, 1993), influx of Ca^{2+} (Putney et al., 1980), and increased DNA synthesis (Boarder, 1994) as well as other effects (Moritz et al., 1992). Increased intracellular PA concentrations are manifested in a diversity of cellular changes, including cellular activation and cell cycle control (Fukami & Takenawa, 1992; McPhail et al., 1993; Stutchfield & Cockroft, 1993; Yasui et al., 1994). In addition to producing the lipid messenger PA, PLD activity leads to the formation of diacylglycerol (DAG) through dephosphorylation of PA by the action of PA phosphohydrolase (Kanoh et al., 1992). This DAG response derived from PC is often of longer duration relative to the DAG formed by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C and is the major source of DAG in some cell types (Billah et al., 1989). Thus, the stimulation of PLD activity is a component of multiple signaling cascades.

The control of PLD activity is known to be complex. Receptor-mediated activation of PLD occurs in cells treated with cytokines, growth factors, hormones, and neurotransmitters (Natarajan & Iwamoto, 1994; Zhou et al., 1993). Many of these responses are dependent on trimeric guanyl nucleotide regulatory proteins (G proteins) (Cockroft, 1992). PLD can be activated by protein kinase C (PKC), tyrosine phosphorylation (Dubyak et al., 1993; Gomez-Cambronero, 1995), ceramides (Gomez-Munoz et al., 1994), and *ras* superfamily GTP binding proteins (Cockroft et al., 1994; Kuribara et al., 1995; Lambeth et al., 1995; Massenburg et al., 1994). There are also unidentified regulators of PLD activity (Geny et al., 1995; Singer et al., 1995). Despite the intensive study dedicated to PLD and its regulatory importance, the purification of a mammalian PLD to homogeneity and the cloning of human PLD have only been reported recently (Hammond et al., 1995; Okamura & Yamashita, 1994).

In this paper, we show that partially purified PLD activity extracted from rabbit brain membranes is regulated by the nucleoside triphosphates (NTPs) ATP, GTP, CTP, and UTP. Further, we identify the regulatory NTP binding protein responsible for this phenomenon as cytosolic gelsolin. Gelsolin and its homologs are important actin-binding proteins that function in actin filament reorganization [for a review, see Cunningham (1992), Rozycki et al. (1994), and Wegner et al. (1994)]. Actin rearrangements involve severing of actin filaments, formation of nucleation sites, and subsequent repolymerization. Such events are important for cellular activities such as mobility, proliferation, and secretion, events in which PLD activation has been shown to play a regulatory role (Boarder, 1994; Stutchfield & Cockroft,

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[1] Abbreviations: ARF, ADP-ribosylation factor; PC, phosphatidylcholine; PLD, phospholipase D; PA, phosphatidic acid; DAG, diacylglycerol; G proteins, guanyl nucleotide regulatory proteins; PKC, protein kinase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP kinase, phosphatidylinositol 4-phosphate kinase; NTP, nucleoside triphosphate; ATPγS, adenosine 5′-*o*-(3-thiotriphosphate); GTPγS, guanosine 5′-*o*-(3-thiotriphosphate).

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Table 1: Partial Purification of Phospholipase D from Rabbit Brain

purification step	total protein (mg)	units of PLD activity[a] (nmol/h)	PLD specific activity [nmol h^{-1} (mg protein)$^{-1}$]
rabbit brain homogenate	1.51×10^4	4983	0.33
Triton X-100 extract	1.90×10^3	4389	2.31
Q-Sepharose FF	266	3510	13.2
GTP−agarose/UTP elution	12	2109	175.8
heparin−agarose post-GTP−agarose purification	3.2	1992	623.4
heparin−agarose/no GTP−agarose purification	53.1	2949	55.5

[a] PLD assays were performed in the presence of 1.0 mM ATP. The substrate concentration used (3.0 μM) was below the K_m in order to avoid problems created by phase separation of the lipid substrate.

1993; Yasui et al., 1994). Additionally, PLD signaling has recently been found to have a stimulatory effect on actin filamentation (Ha & Exton, 1994).

In this paper, we further demonstrate that PLD activity is stimulated by the addition of phosphatidylinositol 4,5-bisphosphate (PIP_2) only in the presence of gelsolin, indicating that the regulatory relationship between actin reorganization and PLD is reciprocal. These findings are discussed in terms of the central role of PLD in signal transduction.

MATERIALS AND METHODS

Phospholipase D Assay. The PLD assay measured the release of [^{3}H]choline from [*methyl*-^{3}H]choline L-α-PC dipalmitoylphosphatidylcholine essentially as described (Kanoh et al., 1991). Choline was separated from PC substrate by partitioning into water from a chloroform−methanol extraction. [^{3}H]PC substrate was prepared by drying stock (in methanol) with argon and redissolving into minimal volumes of methanol (2−10 μL). After dilution with a buffer of 20 mM Tris-HCl (pH 7.0), 1.0 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% Triton X-100 (PLDT buffer) to a 5X concentration, the substrate was sonicated for 30 s and used immediately. The assay was performed with 1.0 μM [^{3}H]choline (Dupont-NEN, Boston MA NET654; 0.1 μCi/assay) and 1−10 units of enzyme with PLDT buffer added to a final volume of 50 μL using polypropylene tubes. Following a 2 h incubation at 37 °C, the reaction was stopped by the addition of 250 μL of water−methanol (2:3), followed by the addition of 250 μL of chloroform followed by vigorous vortexing and centrifugation. The [^{3}H]choline in the aqueous phase (200 μL) was counted in either a Beckman LS 3801 (Beckman Instruments, Columbia, MD) or a Wallac 1450 Microbeta Plus (Wallac Inc., Gaithersburg, MD) liquid scintillation counter after the addition of scintillant. Preliminary experiments established that the assay was linear with time and enzyme concentration. The estimated K_m for PLD with this assay is 50 μM, but solubility constraints for PC preclude its use above approximately 30 μM. At 1 μM PC, therefore, the substrate is a limiting reagent. We verified that the activity being measured is PLD using a transphosphatidylation assay with methanol as a substrate and a TLC mobile phase of chloroform−methanol−ammonia hydroxide−water (200:140:12:8, v:v) with a silica gel thin layer chromatography (TLC) system. This allows for separation of DAG from PA, and no PLC activity was evident using the partially purified rabbit brain PLD because product migrated as phosphatidylmethanol. This is consistent with previously reported results using rat brain PLD purified in the same manner (Kanoh et al., 1991).

Partial Purification of PLD Activity from Rabbit Membranes. All procedures were performed at 4 °C or on ice. Tris-HCl (50 mM) (pH 7.2) and 1.0 mM EDTA (D buffer) were added to 50 frozen, stripped, mature rabbit brains (Pel-Freez Biological, Rogers, AK; approximately 500 g) to a final volume of 1.0 L. This material was homogenized in a Waring blender for 2 min and further homogenized with a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY) on ice using three periods of 5 min. The homogenized tissue was centrifuged at 1000*g* for 15 min and the supernatant collected. D buffer was added (400 mL) to the cellular debris, and the homogenization and centrifugation were repeated and the supernatants pooled and centrifuged at 30000*g* for 2 h. The membrane pellet was suspended in buffer supplemented with 2.0 M NaCl, the suspension recentrifuged, and the resulting pellet resuspended in 800 mL of buffer. Protein concentrations were determined using a Coomassie blue dye-binding kit (Bio-Rad). Triton X-100 was added to a final concentration of 1.0% and the suspension stirred for 2 h and then centrifuged at 30000*g* for 2 h. The Triton X-100 extract had a specific activity of 3.34×10^{-4} nmol h^{-1} (mg protein)$^{-1}$. This material (400 mL, 15.1 g of total protein) was filtered through a 0.45 μm filter and applied to a 50 mm × 300 mm Waters AP glass column (Millipore Corp., Milford, MA) packed with Q-Sepharose Fast Flow (approximately 600 mL; Pharmacia Biotech, Piscataway, NJ). PLD activity was eluted with a linear gradient over 60 min from 0 to 0.6 M NaCl in PLDT buffer at a flow rate of 15 mL/min. Fractions (15 mL) were collected and assayed for PLD activity. Table 1 shows the level of purification for each chromatography step for one purification of eight total. The level of purification and specific activity obtained with our protocol for rabbit brain PLD agrees closely with a recent report of the partial purification of porcine brain PLD (Brown et al., 1995), though we do not resolve which of the potential PLD isoforms is found in rabbit brain.

Purification of PLD Activity on GTP−Agarose. Pooled Q-Sepharose fractions (240 mL, 1.9 g of total protein) were dialyzed against 15 volumes of 20 mM MES (pH 6.5), with 1.0 mM EDTA and 0.05% Triton X-100 (PLDM buffer) for 4 h. The PLD activity was loaded onto a 20 mm × 300 mm Waters AP glass column (bed volume of 90 mL) packed with GTP−agarose (Sigma Chemical Co., St. Louis, MO; catalog no. G 1771). The column was washed with 2 column volumes of PLDM buffer and 1 column volume of PLDM buffer supplemented with 0.1 M NaCl. PLD activity was eluted with PLDM buffer with 1.0 mM UTP. The column was run at a flow rate of 1.0 mL/min, and 8 min fractions were collected. Due to the limited capacity of the GTP−agarose column, no more than 10% (10−30 mg of total protein per run) of the pooled PLD-containing fractions from the Q-Sepharose step was loaded at a time on the GTP−

agarose column. Samples were run on a Bio-Rad 10% mini-PROTEAN gel (Bio-Rad Laboratories, Hercules, CA) and visualized using silver staining.

Purification of PLD Activity on Heparin−Agarose. Heparin−agarose chromatography was used following Q-Sepharose or GTP−agarose as indicated. PLD activity (5−20 mg of total protein per run) was loaded onto a 5 mm × 100 mm Waters AP column packed with heparin−agarose (bed volume of 2.0 mL; Sigma catalog no. H-6508) and equilibrated with PLDT buffer. After the column was washed with 2 volumes of PLDT buffer, PLD activity was eluted with a linear gradient from 0 to 1.0 M NaCl over 100 min at a flow rate of 0.25 mL/min. Fractions of 4 min were collected and the active fractions pooled. Alternatively, 200−300 mg of total protein (an entire run of pooled Q-Sepharose fractions) was purified on a 5 mm × 100 mm Waters AP column (bed volume of 7.0 mL) using the same gradient run at 2.0 mL/min.

Purification of PLD Activity on Sephacryl S300 HR. A 20 mm × 300 mm Waters AP glass column was packed with Sephacryl S300 HR (Pharmacia) and equilibrated with PLDT buffer. The column was calibrated using standards obtained from Pharmacia: blue dextran 2000 (void volume), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa). The sample was concentrated to approximately 200 μL (0.5−5.0 mg of total protein) with an Amicon stirred cell concentrator using a YM100 membrane (Amicon, W. R. Grace & Co., Danvers, MA) and injected into the column run at 0.25 mL/min. Fractions of 4 min were collected.

Protein Sequence Analysis. Protein sequences were determined as previously described (Aebersold et al., 1987). Samples of approximately 5 μg were separated on 7.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels and transferred to nitrocellulose using a Bio-Rad mini-transfer apparatus. Amino acid sequences were determined at the Harvard Microsequencing facility from fragments generated by *in situ* digestion with trypsin.

Photolabeling of the Nucleotide Binding Protein. Approximately 2 mg of total protein of the GTP−agarose-purified PLD activity was used for photolabeling experiments. Buffer was exchanged to PLDT buffer by passage of the sample through PD-10 size exclusion columns (Pharmacia Biotech). In a 12-well microtiter plate, 1.65 μM [γ-^{32}P]-8-azidoadenosine 5′-triphosphate (6.10 Ci/mmol) (ICN Biomedicals, Irvine, CA) was added to the enzyme in a total volume of 1.0 mL. ATP, GTP, or UTP (1.0 mM) was added in separate wells as a nonradioactive, competitive displacer of the label. All mixtures were incubated for 20 min on ice. The plate was then illuminated while on ice with 3 × 3.0 min pulses with a mineral lamp (Ultra-Violet Products, San Gabriel, CA). Samples were concentrated using Centricon 30 spin concentrators (Amicon), run on 10% SDS−PAGE gels (Daiichi Pure Chemicals, Tokyo, Japan), silver stained (Bio-Rad), and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY).

Purification and Immunoprecipitation of Gelsolin. Gelsolin was purified from rabbit plasma (East Acres Biologicals, Southbridge, MA) as previously described (Yamamoto et al., 1989). For immunoprecipitation experiments, aliquots of rabbit anti-pig smooth muscle gelsolin polyclonal antibody or nonimmunized rabbit serum (East Acres Biologicals) were incubated in PLDT buffer at room temperature for 60 min with approximately 5 units (1 unit = 1.0 nmol of [^{3}H]choline produced/min) of PLD activity in a total volume of 100 μL. The antibody was then precipitated with 30 μL of *Streptomyces aureus* cells (10% solution, Pansorbin cells; Calbiochem, La Jolla, CA). The cells were added, and the mixture was vortexed and the incubation continued at room temperature for 20 min. After the cells were pelleted by centrifugation for 5 min at 15 000 rpm in a microcentrifuge, the supernatant was assayed for PLD activity.

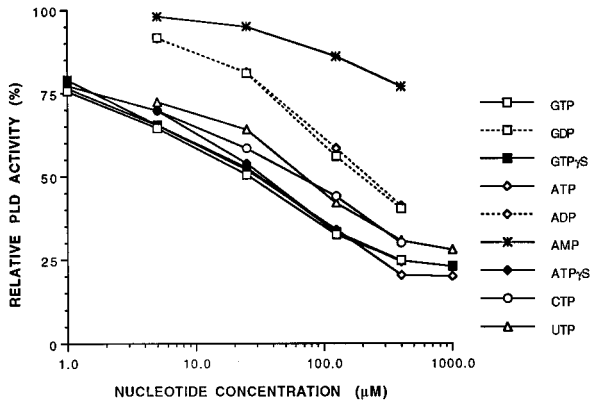

FIGURE 1: Effect of nucleoside phosphates on the activity of partially purified PLD. Nucleoside phosphates and their analogs were added at the indicated final concentrations in the PLD assay using Q Sepharose FF-purified PLD in PLDT buffer. PLD activity is expressed as a percent of activity in the absence of nucleoside phosphates. Data represent an average of three separate experiments. The error for all data points is less than 5%. The nucleoside mono-, di-, and triphosphates tested are listed.

Addition of PIP_2 to the PLD Assay. PIP_2 stock was maintained in chloroform at a concentration of 1.0 mg/mL. The solution was dried under nitrogen and resuspended in methanol by vortexing and sonication (30 s) to a final concentration of 1.0 mg/mL. This stock was diluted into PLDT buffer for use in PLD assays.

RESULTS

Effect of Nucleotides on Partially Purified PLD Activity. The enzyme used in initial experiments was extracted from rabbit brain membranes with Triton X-100 and purified by a Q-Sepharose column, representing an enrichment of approximately 40-fold for PLD activity. The specific activities of the rabbit brain homogenate, Triton X-100 extract, and pooled Q-Sepharose fractions were 0.33, 2.31, and 13.2 nmol h^{-1} (mg protein)$^{-1}$, respectively. PLD activity was measured in the presence of ATP because, as described below, a labile stimulatory factor copurifies with PLD through several steps and the effect of this stimulatory factor on PLD activity is not present when ATP is added to 1.0 mM. Thus, ATP must be present so that the PLD activity at different levels of purification can be compared.

Micromolar amounts of GTP and a nonhydrolyzable analog inhibited partially purified PLD activity (Figure 1). The inhibition of this PLD activity by GTP did not require Mg^{2+} and was also seen with micromolar amounts of ATP, CTP, and UTP. The presence of 0.1 mM Mg^{2+} had no effect on the inhibition by ATP and GTP. The addition of 1.0 mM or greater Mg^{2+} (or Ca^{2+}) inhibited PLD activity regardless of the presence of nucleoside triphosphates (data not shown). While purine nucleoside triphosphates were 1.5-fold more potent inhibitors than pyrimidine nucleoside triphosphates, ADP and GDP were 10-fold less effective at inhibiting PLD activity and AMP only showed a marginal effect at 300 μM.

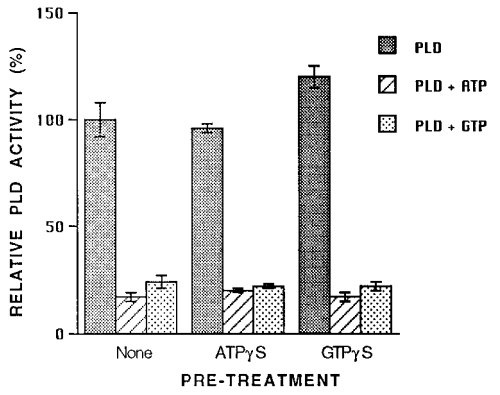

FIGURE 2: Nucleoside triphosphate effect on partially purified PLD activity is reversible. After purified PLD was incubated for 20 min in the presence of 1.0 mM ATPγS or GTPγS in PLDT buffer (total volume of 0.5 mL), the nucleoside triphosphates were removed by passage of the enzyme through a Sephacryl S300 column run with PLDT buffer (as described in Materials and Methods). The resulting PLD activities were tested for nucleoside triphosphate sensitivity by incubation in the presence of 1.0 mM ATP and 1.0 mM GTP. PLD activity is expressed as a percent of activity in the absence of nucleoside triphosphates. Data represent the mean ± the standard error of the mean (SEM) of triplicate assays for a representative of three separate experiments.

Interestingly, the inhibitory effects of NTPs on PLD activity plateaued when millimolar amounts of NTPs were used. The maximum amount of NTP inhibition for a fresh preparation of partially purified PLD was 80−85%. However, nucleotide sensitivity was lost over time with a half-life of approximately 3 days at 4 °C and 2 weeks at −80 °C (data not shown).

To determine whether the inhibitory effects of NTPs were reversible, preparations of partially purified PLD were pretreated with ATP, GTP, ATPγS, or GTPγS for 20 min and passed through a Sephacryl S300HR size exclusion column to remove the NTPs. When PLD assays were terminated after 20 min, the inhibitory effect of NTPs is present, indicating that a 20 min pretreatment is sufficient to cause PLD inhibition (data not shown). The resulting PLD activity increased to near uninhibited levels and was subsequently affected by ATP and GTP to the same degree as the untreated enzyme (Figure 2). Thus, the nucleotide effect on PLD activity is reversible and was not separated from the enzyme activity on a gel filtration column under these conditions, implying that NTPs do not cause dissociation of a regulatory polypeptide.

Changes in PLD Activity following Further Purification. PLD activity was purified by a heparin−agarose column. Following this step, all inhibition of PLD activity by ATP, UTP, and CTP was lost. Whereas overall recovery of PLD activity was 15−50% (when assayed in the absence of NTPs), an effect of GTPγS was retained (Figure 3A). This effect is specific to GTP and its analogs (data not shown) and may represent the influence of a GTP binding regulatory protein. While these data were obtained using PLD activity eluted from Q-Sepharose, similar results were seen with GTP−agarose-purified enzyme. The cause for the loss of enzyme activity on heparin−agarose was not obvious since less than 5% of the PLD activity passed through the column. Significantly, there was an inverse relationship between yield and the level of ATP/GTP sensitivity of the starting material. The inhibition of PLD activity by NTPs was variable because, as described above, the level of ATP/GTP inhibition

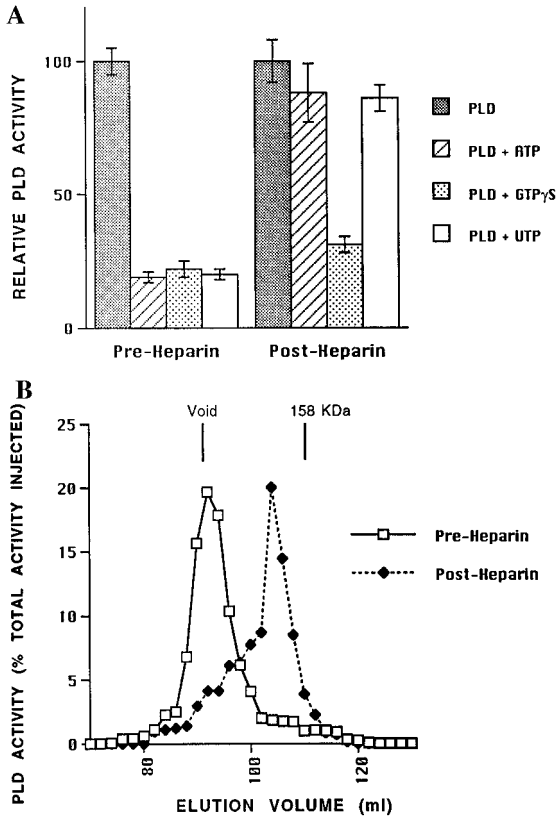

FIGURE 3: Changes in the characteristics of PLD following purification on heparin−agarose. (A) Nucleoside triphosphate sensitivity of PLD following purification on heparin−agarose. Partially purified PLD activity was eluted from heparin−agarose using a 0.0 to 0.5 M NaCl gradient in PLDT buffer as described in Materials and Methods. The nucleotide sensitivity of PLD activity was tested by addition of ATP, GTPγS, or UTP to a final concentration of 1.0 mM in the PLD assay using PLDT buffer. PLD activity is expressed as a percent of activity in the absence of nucleoside triphosphates. Absolute activity of post-heparin PLD was 3-fold lower than that of the pre-heparin PLD (see text). Data represent the mean ± SEM of triplicate assays for two separate experiments. (B) Shift in apparent molecular mass of PLD activity following purification on heparin−agarose. PLD activity (0.5−1.0 mg of total protein) was passed through a Sepharose S300HR column with 20 mM Tris-HCl and 1.0 mM EDTA prior to and following purification on heparin−agarose. The fractions were assayed in the PLD assay with PLDT buffer.

decreases over time. PLD activity that was maximally inhibited 50% by ATP or GTP produced a yield of 40% on heparin−agarose, while the highly ATP/GTP sensitive PLD activity of a fresh preparation (maximal inhibition of 80%) gave a yield of only 20%. These data indicate that NTPs were not directly inhibiting PLD; rather, there was a stimulatory effect on partially purified PLD activity that was lost upon further purification. This stimulatory effect was blocked by the addition of millimolar amounts of NTPs. The stimulatory factor was also labile under the conditions used here. Further verification of this conclusion was the observation that unstimulated PLD activity (in the presence of NTPs or heparin−agarose-purified) remained stable for months at 4 °C while stimulated (without NTPs prior to heparin−agarose purification) PLD activity decreased rapidly (data not shown).

The loss of a stimulatory factor and responsiveness to NTPs, other than GTP, was accompanied by a shift in the apparent molecular mass of PLD activity. PLD activity prior to purification on heparin−agarose elutes in the excluded

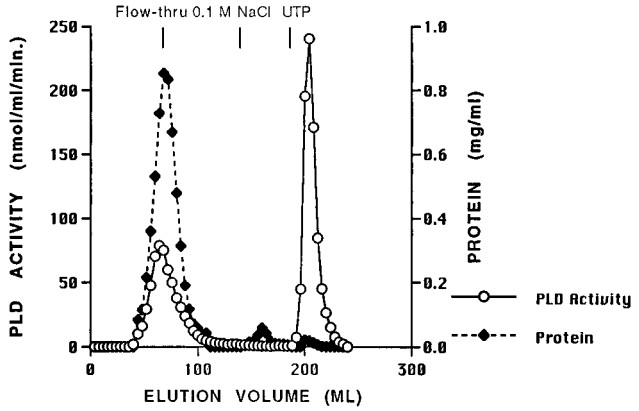

FIGURE 4: GTP−agarose chromatography of PLD activity. PLD activity was loaded onto GTP−agarose, washed with 0.1 M NaCl, and eluted with 1.0 mM UTP as described in Materials and Methods. All fractions were assayed for PLD activity in PLDT buffer with 1.0 mM UTP. The eluent corresponding to the flow-through, the 0.1 M NaCl wash, and the UTP elution are indicated.

volume (greater than 300 kDa) of a Sephacryl S300HR column, while heparin−agarose-purified PLD activity elutes at approximately 250 kDa (Figure 3B). Thus, a NTP-binding protein that stimulates activity is complexed with PLD when extracted from rabbit brain membranes. This protein(s) was labile under the conditions optimized for PLD activity and can be removed by purification of PLD activity on heparin−agarose.

Affinity Purification of the Stimulated PLD Complex. Since the interaction of PLD with this stimulatory protein(s) survived purification on Q-Sepharose and Sephacryl S300HR, we attempted to purify the complex on a GTP−agarose column prior to the application of heparin−agarose chromatography. The column was washed with 0.1 M NaCl without elution of PLD activity (Figure 4), and UTP was found to be the most selective displacer of the complex when compared to ATP and GTP (data not shown). The use of GTP−agarose resulted in an approximately 13-fold purification of PLD activity. The Q-Sepharose fractions had a specific activity of 13.2 nmol h^{-1} (mg protein)$^{-1}$ which increased to 175.8 nmol h^{-1} (mg protein)$^{-1}$ following purification on GTP−agarose. This PLD activity was still responsive to NTPs and remained a large macromolecule which eluted in the excluded volume of a Sephacryl S300HR column; however, the NTP sensitivity of this PLD activity had a half-life of only a few hours (data not shown).

The use of GTP−agarose chromatography further verified that the NTP binding activity was independent of PLD activity. Heparin−agarose-purified PLD activity, lacking the NTP binding domain, was not retained by GTP−agarose (data not shown). While PLD activity eluted from GTP−agarose with UTP was retained by GTP−agarose when reapplied to the column, PLD activity eluted from GTP−agarose with a gradient of NaCl (0 to 1.0 M, with activity eluting at approximately 0.4 M) rather than UTP was not retained when reapplied to a GTP−agarose column (data not shown). This NaCl-eluted activity was like heparin−agarose-purified PLD in that it was unresponsive to ATP and UTP (data not shown), implying that the NaCl separated PLD from the NTP binding site.

Identification of a Nucleotide Binding Protein Associated with PLD. The NTP binding protein associated with PLD was identified by cross-linking [γ-^{32}P]-8-azidoadenosine 5′-triphosphate to purified PLD activity. A protein of approximately 90 kDa was identified by autoradiography (Figure 5). This cross-linking was displaced by ATP, GTP, and UTP, as would be expected for the PLD-associated NTP binding protein. As further verification, the 90 kDa protein was not retained by heparin−agarose, thereby separating it from PLD activity (Figure 6). The heavy arrow denotes the protein that was photolabeled using the protein that flowed thru the heparin−agarose column (data not shown). There are slight differences between the GTP−agarose-purified material in Figures 5 and 6. This is due to the higher protein concentration used in Figure 5, the treatment of the material with UV radiation in Figure 5, or differences between the batches of material used for these experiments.

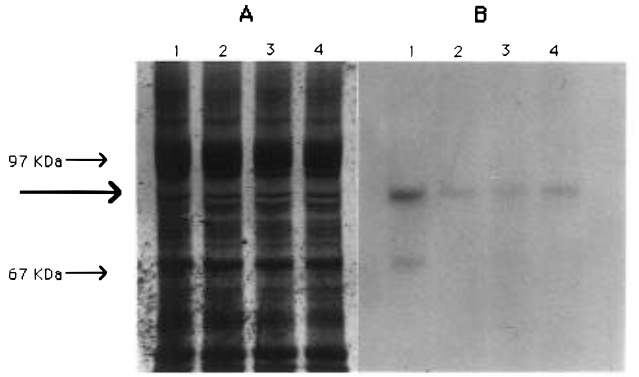

FIGURE 5: Photolabeling of the PLD-associated nucleotide triphosphate binding protein. The procedure for photolabeling PLD activity eluted from GTP−agarose with azido-ATP is described in Materials and Methods. The silver stain (A) and the autoradiogram (B) of the photolabeling experiment are indicated. The locations of molecular mass standards are shown. Lane 1 contains no displacer, and lanes 2−4 contain 1.0 mM ATP, GTP, or UTP as displacer, respectively. The large arrow indicates the major protein labeled by azido-ATP.

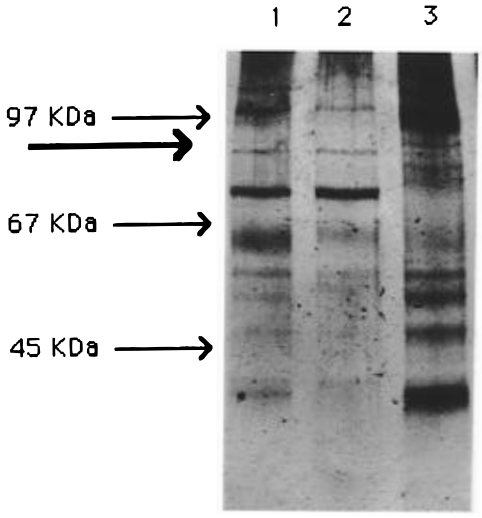

FIGURE 6: The photolabeled 90 kDa protein is not retained by heparin−agarose. PLD activity eluted from GTP agarose (lane 1) was loaded onto heparin−agarose in PLDT buffer with 1.0 mM UTP. The protein not retained by the column was collected (lane 2), and PLD activity was eluted by a gradient of NaCl (0.0 to 0.5 M) in PLDT buffer with a peak of activity concentrated and presented in lane 3. The respective fractions were run on a 12.5% Diaiichi precast SDS−PAGE gel and stained with silver as described in Materials and Methods. The photolabeled 90 kDa protein is indicated by the heavy arrow. The locations of molecular mass standards are shown.

Human Plasma Gelsolin	194	GRRVVRATEV PVSWESFNNG DCFILDLGNN IHQ
PLD-Associated Protein	1	GRRVVRATEV PVSWDSFNNG DNFILDLGND IYQ
Human Plasma Gelsolin	616	TGAQELLRVL RAQPVQVAEG SEPDGFWEAL GGK
PLD-Associated Protein	1	TGAQELLRVL RAQPVQVAEG SEPDGFWEAL GGK

FIGURE 7: Identification of the PLD-associated nucleotide binding protein. Amino acid sequence analysis indicates that the nucleotide binding protein is gelsolin. Two proteolytic fragments of the PLD-associated nucleotide binding protein yielded a sequence of 33 amino acids for each. Sixty-two of 66 amino acids for this protein matched the sequence for human plasma gelsolin. Sequence differences are boxed, and the amino acid numbers for gelsolin are shown.

The heparin−agarose flow-through (Figure 6, lane 3) was concentrated and purified with a preparative SDS−PAGE gel, yielding approximately 100 pmol of the 90 kDa protein. This material was blotted onto nitrocellulose and digested with trypsin, and two of the resulting proteolytic fragments were sequenced. A sequence of 33 amino acids from each fragment was obtained, and 62 of the 66 (33 of 33 and 29 of 33) amino acids matched exactly with the protein sequence deduced from the human gene encoding the actin-binding protein gelsolin (Figure 7). The tryptic digest of this protein matched the predicted digest of gelsolin (data not shown).

Gelsolin Is Verified as the Protein Responsible for Stimulating Partially Purified PLD Activity. Gelsolin exists in two forms, both encoded by the same gene [for a review, see Pollard and Cooper (1986)]. Cytosolic gelsolin is very abundant in many cell types and is responsible for severing actin filaments during cytoskeletal rearrangements. The secreted form of gelsolin has a 21-amino acid leader sequence and is a component of blood plasma. This form of the protein is responsible for clearing actin filaments from the vascular system. In order to directly test the effect of gelsolin on PLD activity, gelsolin was purified from rabbit plasma as described in Materials and Methods. The data show that heparin-purified PLD activity, which is unaffected by 1.0 mM ATP, is stimulated by the addition of purified gelsolin. This stimulation is blocked by the addition of 1.0 mM ATP (Figure 8A). These results indicate that purified gelsolin restores the stimulation of PLD activity that is lost upon purification on heparin−agarose.

To verify further that gelsolin is responsible for the nucleotide triphosphate sensitive stimulation of PLD activity extracted from rabbit brain membranes, anti-gelsolin antibody (antiserum) was used to immunoprecipitate PLD activity. Rabbit anti-pig smooth muscle gelsolin polyclonal antibody was added to nucleotide triphosphate sensitive PLD activity in increasing amounts. In this experiment, the nonimmunized rabbit control serum stimulated PLD activity (Figure 8B). This effect was expected because serum is a rich source of gelsolin (Yamamoto et al., 1989) and gelsolin directly stimulates PLD activity (Figure 8A). Despite the activation of PLD by the plasma gelsolin, the anti-gelsolin antisera precipitated more than 90% of the nucleotide sensitive PLD activity (Figure 8B). These data confirm the interaction between PLD and gelsolin and support the hypothesis that nucleoside triphosphate sensitive PLD activity represents the amount of gelsolin-coupled complex present.

The Gelsolin−PLD Complex Is Regulated by PIP_2. It has been reported that gelsolin binds PIP_2 and that this phospholipid also causes increased actin filamentation in certain cell types (Janmey et al., 1987; Pike et al., 1991). PIP_2 is also involved in the regulation of PLD because optimal PLD

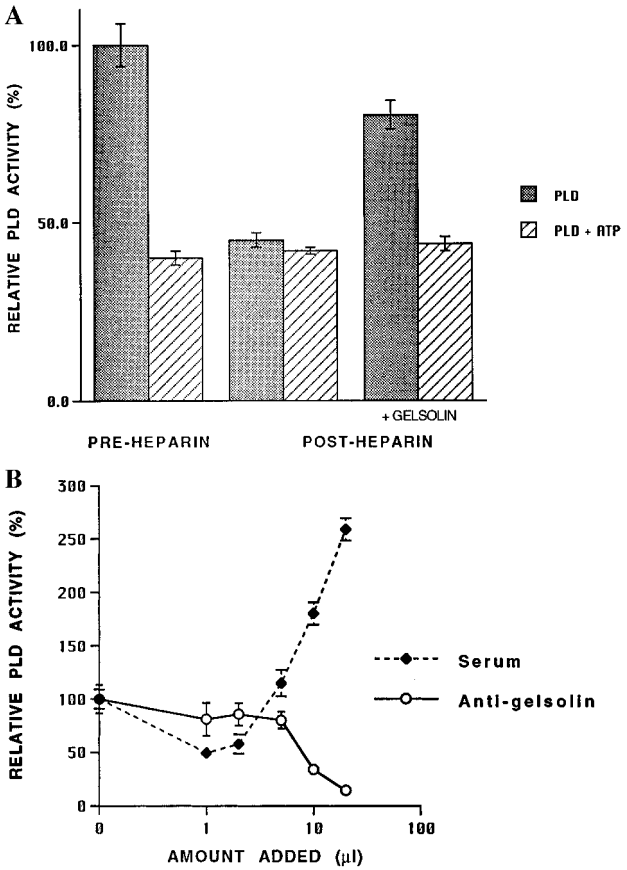

FIGURE 8: Purified gelsolin and anti-gelsolin antibody affect partially purified PLD activity. (A) The addition of purified gelsolin to partially purified PLD. Gelsolin, approximately 1.0 μg purified from rabbit plasma as described in Materials and Methods, was added to approximately 250 units (1 unit = 1.0 nmol of PC hydrolyzed per milliliter per minute; approximately 4.5 mg of total protein) of heparin-purified PLD activity in the PLD assay. Pre-heparin PLD was used as a control. PLD activity is expressed as a percent of untreated control PLD activity (denoted as pre-heparin PLD on the figure). All assays are standard PLD assays in PLDT buffer with the nucleoside triphosphate sensitivity evaluated by the addition of ATP to a final concentration of 1.0 mM. Data represent the mean and SEM of three experiments performed with triplicate assays. Gelsolin alone, without PLD, had no PLD activity (not shown). (B) Immunoprecipitation of PLD activity by anti-gelsolin antibody. Serum containing rabbit anti-pig smooth muscle gelsolin (East Acres Biologicals) as well as control rabbit serum was added in increasing amounts to partially purified PLD activity that was nucleotide triphosphate sensitive (prior to heparin−agarose purification). PLD activity is given as a percentage of the nucleotide sensitive portion of the PLD activity, defined in the presence of 1.0 mM ATP. Assays were performed in PLDT buffer, and the data represent the mean and SEM of triplicate assays for two experiments.

activity in HL-60 polymorpholeukemic cell membranes requires the presence of PIP_2 (Brown et al., 1993), and PIP_2 is a potent stimulator of rat brain membrane PLD activity (Liscovitch et al., 1994). Since PIP_2 levels affect both PLD and gelsolin, we tested the effect of PIP_2 on PLD activity both in the presence and in the absence of gelsolin. PIP_2 increased gelsolin-associated PLD activity 3−5-fold regardless of whether ATP was present in the assay (Figure 9). In contrast, there was no significant effect of PIP_2 on PLD in the absence of gelsolin. Our data suggest that the effect of PIP_2 on PLD activity *in vitro* and in isolated membranes is due to the presence of gelsolin.

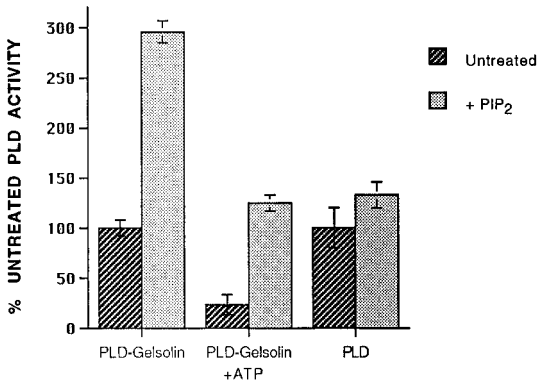

FIGURE 9: Effect of PIP_2 on gelsolin-associated PLD activity. PIP_2 was prepared as described in Materials and Methods and used in the standard PLD assay at a final concentration of 100 μg/mL. PLD activity prior to heparin−agarose purification was assayed either in the absence (left) or in the presence (center) of ATP (1.0 mM). Heparin−agarose-purified PLD activity was also measured in the presence of PIP_2 (right). Data are presented as percent of the untreated (no PIP_2 or ATP) activity of pre- and post-heparin−agarose-purified PLD, respectively. The mean and SEM of quadruplicate assays is shown.

DISCUSSION

We have shown that PLD activity extracted from rabbit brain membranes by detergent and partially purified through two columns was reversibly inhibited by millimolar amounts of NTPs. Our data indicate that this NTP effect is most likely due to the presence of a labile NTP-binding protein that copurifies with PLD activity up to the heparin−agarose chromatography step. The NTP binding protein interaction is removed by heparin−agarose chromatography. In this model, the NTP binding protein stimulates PLD activity only in the absence of millimolar amounts of NTPs. There are several lines of evidence presented here that support this hypothesis. (1) PLD activity is separable from NTP binding, as shown by the ability of PLD to bind to GTP−agarose only when the NTP effect is present. (2) The NTP inhibition and PLD activity are both labile until the NTP effect is lost, whereupon PLD activity remains stable. PLD activity determined in the presence of NTPs is stable. (3) PLD activity cannot be completely inhibited by NTPs, and the noninhibitable activity correlates with the yields on heparin−agarose. (4) Since the NTP effect is labile, PLD activity with varying amounts of NTP sensitivity was used for heparin−agarose chromatography. The yields from this column correlate highly with the NTP sensitivity of the starting material. However, when PLD activity is assayed in the presence of ATP, the results were very reproducible and virtually all of the PLD activity could be accounted for (Table 1). (5) The loss of NTP sensitivity coincides with a loss of apparent size on a size fractionation column.

A recent report by Kanfer and McCartney mentions an inhibitory effect of NTPs on the PLD activity of isolated rat brain plasma membranes (Kanfer & McCartney, 1994), a phenomenon similar to the one we have characterized in detail for the partially purified enzyme. Similarly, Xie and Dubyak described a GTP and ATP effect on PLD in electropermeabilized HL-60 cells (Xie & Dubyak, 1991). Therefore, our data further showed that this regulatory interaction survives the extraction of PLD from membranes using Triton X-100.

Photo-cross-linking experiments indicated that PLD activity extracted from rabbit brain membranes with Triton X-100 is in a complex with a NTP binding protein of approximately 90 kDa. Amino acid sequence analyses of proteolytic fragments of this protein indicate that it is the actin-binding protein gelsolin; 62 of 66 amino acids matched those of human gelsolin. Since the rabbit gene encoding gelsolin is not present in GenBank version 86, the four differing amino acids likely represent species differences. Gelsolin catalyzes the severing of actin filaments and is thought to be intimately involved in the control of actin reorganizations through the regulation of filament severing as well as repolymerization (Cunningham, 1992; Deaton et al., 1992; Ditsch & Wegner, 1994; Janmey et al., 1987; McLaughlin et al., 1993; Rozycki et al., 1994; Wegner et al., 1994; Yu et al., 1992).

Our data show that the interaction between gelsolin and PLD caused stimulation of PLD activity which can be prevented by the addition of nucleotides or by the separation of PLD from gelsolin by heparin−agarose chromatography. Consistent with these data, gelsolin has been shown to bind nucleoside triphosphates in the presence of EDTA (Pollard & Cooper, 1986; Yamamoto et al., 1990) and has been demonstrated to associate with other phospholipases (Akiba et al., 1993; Banno et al., 1992; Vaziri & Downes, 1992; Yang et al., 1994). The PLD−gelsolin complex is stable enough to be purified on a GTP column and to be precipitated by anti-gelsolin antibodies. Since a similar NTP effect on PLD activity was seen with rat brain plasma membranes (Kanfer & McCartney, 1994) and electropermeabilized HL-60 granulocytes (Xie & Dubyak, 1991), the association with gelsolin is presumably a general mechanism of PLD regulation. Our results show that the stimulatory effect of gelsolin is augmented by the addition of PIP_2. Activation of PLD by PIP_2 has been previously reported (Liscovitch et al., 1994), but our data indicate that this requires gelsolin and is not a direct effect on PLD. Further, since there is a GTP-dependent effect on PLD activity that is retained following purification on heparin−agarose, a member of either the Rho or ARF family may be interacting with PLD. Thus, the regulatory interactions involving PLD and gelsolin are complex.

The stimulation of PLD by the actin-binding protein gelsolin is consistent with several reports implicating a relationship between PLD activity and regulation of the cytoskeleton. First, PLD activity is dependent upon a small molecular mass GTP binding protein of the Rho family (Bowman et al., 1993). In addition to their involvement in PLD stimulation, these proteins have been shown to play a role in actin reorganization (Leffers et al., 1993). Second, PLD activity in myeloid cells requires pretreatment of the cells with the cytoskeletal disrupter cytochalasin B. These cells must be "primed" by the addition of cytochalasin B for maximal receptor-mediated stimulation of PLD. Since PLD plays a pivotal regulatory role in these cells, priming is also required for maximal cellular response, such as degranulation and oxidative burst, upon activation (Bonser et al., 1989; Pai et al., 1988; Reinhold et al., 1990). Cytochalasin B is a fungal metabolite that binds actin at the barbed end, and because this activity mimics that of actin-binding proteins like gelsolin, cytochalasin B has been used to study the mechanisms of actin filamentation [for a review, see Cooper (1987)]. Thus, the priming effect of cytochalasin B is indicative of a dependence of PLD signaling on the

level of actin polymerization and may be a result of altering the interactions between PLD and gelsolin.

While PLD activity is dependent upon the state of the cytoskeleton, there is also evidence for a reciprocating regulatory effect of PLD activity on microfilament assembly. By using exogenously added PA and PLD, Ha and Exton (1994) showed that PC-derived PA stimulates actin polymerization in IIC9 fibroblasts. Clearly, PLD is intimately involved in the cytoskeletal rearrangements required for many responses to extracellular stimuli. Further, PA has been shown to indirectly affect gelsolin function. PIP_2 has been shown to cause gelsolin to dissociate from actin, thereby promoting actin polymerization, and this event likely causes the prolonged stimulation of actin assembly seen during the activation of phagocytes (Pike et al., 1991). During cellular activation, PIP_2 is produced by the action of phosphatidylinositol-4-phosphate (PIP) kinase (Pike et al., 1991, 1992; Yu et al., 1992) and PA directly stimulates PIP kinase (Jenkins et al., 1994; Moritz et al., 1992). Since we found that PIP_2 also stimulates PLD in the presence of gelsolin, this positive feedback involving these signaling interactions may represent an amplification step in the activation of the PLD pathway and the control of cytoskeletal rearrangements.

Our data suggest the presence of a regulatory element in addition to the PLD/gelsolin interaction. The GTP-specific regulation of PLD activity following loss of the NTP-binding protein may indicate the presence of a GTP binding regulatory protein. There have been several reports of PLD regulation by members of the *ras* superfamily of small molecular mass GTP binding proteins (Bowman et al., 1993; Brown et al., 1993; Cockroft et al., 1994; Lambeth et al., 1995). Similarly, there have been a number of studies indicating that PLD activity is regulated by receptor-coupled trimeric GTP binding proteins in various tissues (Cockroft, 1992; Qian et al., 1990). Heparin−agarose may disrupt some of the protein/protein interactions present in the partially purified PLD preparation because it has been found to disrupt GTP binding protein-mediated interactions (Dasso & Taylor, 1991), and heparin−agarose is commonly used to purify these proteins (Brown et al., 1993; Lambeth et al., 1995). The GTP effect on our partially purified enzyme will be studied in greater detail to determine whether a GTP binding regulatory protein copurifies with PLD. With a greater understanding of the components of the macromolecular complex that includes PLD, it should be possible to elucidate all of the functions of this complex in intracellular signaling.

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