We have recently cloned a cDNA encoding a phospholipase D (PLD) from rat brain and named it rPLD1. It shows 90% amino acid identity with the human PLD isoform hPLD1b. We have expressed rPLD1 as a histidine-tagged fusion protein in insect (Sf9) cells using the expression vector pBlueBacHis and purified the recombinant protein to homogeneity by Ni²⁺-agarose affinity chromatography. Phosphatidylinositol 4,5-P₂ and phosphatidylinositol 3,4,5-P₃ activated the PLD equally, but other acidic phospholipids were ineffective. The activity of rPLD1 was dependent on both Mg²⁺ and Ca²⁺. It was specific for phosphatidylincholine and showed a broad dependence on pH with optimum activity at pH 6.5–7.5. The enzyme was inhibited by oleate and activated by the small G proteins ARF3 and RhoA in the presence of guanosine 5′-3-O-[(thio)triphosphate. Protein kinase C (PKC)-α and -βIII, but not PKC-γ, -δ, -ε, or -ζ, activated rPLD1 in a manner that was stimulated by phorbol ester but did not require ATP. Neither synergistic interactions between ARF3 and RhoA nor between these G proteins and PKC-α or -βII were observed. Recombinant PKC-α and -βII phosphorylated purified rPLD1 to high stoichiometry in vitro, and the phosphorylated PLD exhibited a mobility shift upon electrophoresis. Phosphorylation of the PLD by PKC was correlated with inhibition of its catalytic activity. rPLD1 bound to concanavalin A-Sepharose beads, and its electrophoretic mobility was altered by treatment with endoglycosidase F. The amount of PLD bound to the beads was decreased in a concentration-dependent manner when tunicamycin was added to the Sf9 expression system. Tunicamycin also decreased membrane localization of rPLD1. These results suggest that rPLD1 is a glycosylated protein and that it is negatively regulated by phosphorylation by PKC in vitro.

Phospholipase D type I (PLD1) plays an important role in signal transduction in a variety of cells. PLD catalyzes the hydrolysis of phosphatidyicholine (PC), the major phospholipid of membranes, to phosphatidic acid and choline in response to a variety of hormones, neurotransmitters, growth factors, and cytokines (1). Phosphatidic acid (PA), the direct product of PLD action, has been implicated in increased DNA synthesis, activation of protein kinases and a protein-tyrosine phosphatase, stimulation of the respiratory burst in neutrophils, stimulation of c-fos and c-myc transcription, activation of certain enzymes of inositol phospholipid metabolism, and effects on actin polymerization and the GTPase-activating proteins of small G proteins (1–4). PA can be further metabolized by PA phosphohydrolase to yield diacylglycerol and by phospholipase A₂ to form the intracellular messenger lysophosphatidic acid. Diacylglycerol derived from PC via PA can result in prolonged activation of certain PKC isoforms (1, 5). Thus, agonist-induced stimulation of PLD through its activation of PKC could play a role in long term cellular responses such as proliferation and differentiation.

Regulation of PLD activity is not fully understood. PLD is known to be activated via multiple pathways involving PKC, heterotrimeric and small G proteins, protein-tyrosine kinases, Ca²⁺, and unsaturated fatty acids (1, 5). However, there is little information on the biochemical and molecular properties of mammalian PLDs because homogenous preparations of the enzyme have not been available until very recently.

Much attention has focused on the role of PKC in the regulation of PLD. Studies using down-regulation of PKC and PKC inhibitors suggest the involvement of PKC-dependent mechanisms in the activation of PLD by many agonists (1, 5). Surprisingly, the stimulation of PLD by PKC in membranes from Chinese hamster lung (CCL39) fibroblasts (6) and in a partially purified preparation of PLD from brain (7) has been shown to occur in an ATP-independent manner, suggesting that PKC may interact directly with PLD and activate it by a nonphosphorylation mechanism. PKC-α and PKC-β isoforms have also been found to activate HL-60 cell membrane-bound PLD in an ATP-independent manner (8). In contrast, Lopez et al. (9) reported that conventional isoform(s) of PKC activated neutrophil PLD by phosphorylating a target protein located in the plasma membrane. Although there are many reports indicating PKC involvement in PLD activation, there is evidence that PKC is not required by some agonists, suggesting an alternate pathway(s) (1, 5).

Early reports showed that GTPγS-activated PLD in membranes or permeabilized cells, suggesting the involvement of G proteins (10–12). Members of the ADP-ribosylation factor (ARF) family of small G proteins were first recognized as activators of PLD (13, 14), and the presence of phosphatidylinositol 4,5-bisphosphate (PIP₂) in substrate vesicles was shown to be essential for the ARF-regulated PLD activity (13, 15). ARF is also required for coatomer assembly and vesicle trafficking in Golgi (16). ARF-activated PLD is enriched in Golgi membranes, and PLD may play a role in ARF-dependent coatomer assembly (17). Two PLD activities have been separated from rat brain. One is activated by ARF and PIP₂ but inhibited by oleate, whereas the other is completely dependent on oleate for activation.
ity but insensitive to ARF and PIP2 (18).

Members of Rho family of small G proteins, including RhoA, Cdc42, and Rac have also been found to activate PLD in isolated membranes (8, 19–21). It has also been reported that treatment of Rat 1 fibroblasts with Clostridium botulinum C3 transference, which ADP-ribosylates and inactivates Rho, results in decreased activation of PLD in response to stimuli (22). Rho proteins are involved in structural rearrangements of the actin cytoskeleton (23, 24) and in the regulation of phosphatidylinositol 3-kinase (25) and phosphatidylinositol-4-phosphate 5-kinase (26). Membrane-associated PLDs from HL-60 cells and porcine brain were shown to be synergistically activated by ARF and RhoA (20, 27), whereas rat liver membrane PLD was activated by RhoA but minimally by ARF (19).

Evidence for the existence of multiple isoforms of agonist-activated PLDs in mammalian cells is substantial (1, 18). It has been obtained in a variety of biochemical studies, which indicate differences in the subcellular localization of PLD activity and in its responses to Ca2+, small G proteins, fatty acids, detergents, and pH, and differences in substrate specificities and chromatographic properties of partially purified PLD enzymes.

Recently, Hammond et al. (29, 30) cloned alternatively spliced forms of human PLD (hPLD1a and hPLD1b) and expressed them in insect cells using baculovirus vectors. The enzymes were purified to homogeneity using immunoaffinity chromatography and shown to be activated by ARF, Rho family proteins, PIP2, phosphatidylinositol 3,4,5-P3 (PIP3), and PKC-

MATERIALS AND METHODS

All reagents were analytical grade unless otherwise indicated. Dioleoylphosphatidylethanolamine, dipalmitoyl-PC, PIP2, and other unlabeled phospholipids were obtained from Sigma. Dipalmitoyl-Cl3-PC, phosphatidylinositol 3,4-biphosphate were kind gifts from Dr. Ching-Shih Chen (University of Kentucky). β-Octylglucopyranoside, GTPyS, and tunicamycin were purchased from Boehringer Mannheim, and concanavalin A-Sepharose beads were from Amersham Pharmacia Biotech. All radioactive reagents were from NEN Life Science Products except for 1-palmitoyl-2-14C(1)linoleoyl PC and 1-palmitoyl-2-[14C]linoleoyl PE, which were from Amersharm Pharmacia Biotech. LK60 silica gel thin layer plates were purchased from Whatman. Nickel-agarose resin was from Qiagen, and anti-X press antibody was from Invitrogen. Affinity-purified anti-C-terminal dodecapeptide of hPLD antibody was a kind gift of Dr. S. H. Ryu (POSTECH, Korea). Purified recombinant PKC isoforms expressed in Sf9 cells were from Panvera Corp. The cDNAs for RhoA, ARF3, and myristoyltransferase were kind gifts of Drs. R. A. Cerione (Cornell University), J. Moss (National Institutes of Health), and J. I. Gordon (Washington University), respectively.

Constitution of Recombinant Baculovirus—The rPLD1 cDNA was inserted into the baculovirus transfer vector pBlueBacHis2B (Invitrogen) such that the PLD coding sequence was in frame with the sequence encoding the hexahistidine tag. This transfer plasmid and linearized AcMNPV viral DNA were cotransfected into Spodoptera frugiperda (Sf9) cells by lipofection (Invitrogen) according to the manufacturer’s instructions.

Putative recombinant viral plaques were identified by color screening. Virus from several putative recombinant plaques was tested for the ability to induce expression of (His6)-rPLD1 in infected Sf9 cells using Western blots of cell lysates with affinity-purified antibodies to the C terminus of hPLD1 (data not shown). The positive plaques were purified, and the pure viruses were amplified by infection of spinner cultures of Sf9 cells, which were stirred at 70 rpm and incubated at 27 °C. Insect Cell Culture and Buculovirus Expression—Growth of Sf9 (High Five) Cells (Invitrogen) was maintained at 27 °C in SF 900 II SFM (Life Technologies, Inc.) supplemented with 5% FBS, 5% fetal bovine serum containing antibiotic and antifungal agents. Production of high titer viral stocks was performed in spinner flasks with 2 × 106 Sf9 cells/ml. The high titer virus stocks were stored at 4 °C and protected from light to ensure maintenance of titer. Monolayers of Sf9 cells (5 × 106 cells in a 100-mm dish) were infected with recombinant baculovirus expressing (His6)-rPLD and aMultiplicity of 10 and cultured at 27 °C for various times. The cells were washed in ice-cold phosphate-buffered saline, scraped into lysis buffer (200 mM HEPEs, pH 8.0, 50 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl2, and 10% glycerol), and lysed by five passages through a 25-gauge needle. Trypan blue staining of the lysate indicated >95% disruption of the cells. The lysate was centrifuged at 10,000 × g for 1 h to prepare cytosolic and particulate (membrane) fractions. Membranes were washed once by suspension in buffer and repeated centrifugation. PLD activity was higher in the soluble fraction than in the particulate fraction, and activity was detectable at 12 h, reached a maximum after 40 h of infection, and then declined slightly (data not shown). Western blotting using affinity-purified antibodies to the C-terminal dodecapeptide of hPLD also showed higher expression of rPLD1 in the soluble fraction, and the time course of enzyme expression measured by immunoblotting was similar to that measured by enzyme activity (data not shown).

Purification of (His6)-rPLD1 by Ni2+-NTA-Agarose Affinity Chromatography—Monolayers of Sf9 cells (3 × 105 cells/150-mm dish; 10 dishes of cells) were used for each purification. They were infected at a multiplicity of 10 for 48 h with recombinant baculovirus encoding (His6)-rPLD1. The medium was removed, and the cells were lysed by incubation with 10 ml of buffer containing 20 mM HEPEs, pH 8.0, 1% β-octylglucopyranoside, 0.6 mM NaCl, 5 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 15% glycerol for 30 min at 4 °C.

The suspension was centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant (10 ml) was incubated with 0.5 ml of Ni2+-NTA-agarose (Qiagen) at 4 °C overnight with constant agitation. The incubation mixture was placed in a Bio-Rad chromatography column, and the resin was washed with 50 column volumes of buffer containing 20 mM HEPEs, pH 8.0, 1% β-octylglucopyranoside, 1 mM NaCl, 50 mM imidazole, 5 mM MgCl2, 10 mM β-mercaptoethanol, and 20% glycerol. Bound protein was eluted in four fractions with an elution buffer containing 0.6 mM NaCl, 50 mM imidazole in 0.6 mM NaCl, 20 mM HEPEs, pH 8.0, 5 mM MgCl2, and 20% glycerol. The fractions were pooled and analyzed by SDS-PAGE and Western blotting. Protein was assayed by the Bio-Rad microassay.

Purification of Small G Proteins—RhoA cDNA was subcloned into the baculovirus transfer vector, pBlueBacHis (Invitrogen) encoding a hexahistidine tag. The protein was expressed in Sf9 cells by cotransfection of the transfer plasmid and linearized AcMNPV viral DNA, and purified by affinity chromatography using Ni2+-NTA-agarose as described. Human ARF3 was coexpressed with myristoyltransferase in Escherichia coli and purified using DEAE-Sephacel and Superdex 75 column chromatography as described by Weiss et al. (32). Both preparations of small G proteins were shown by SDS-PAGE and silver staining to be near homogeneous and were stored in aliquots at −70 °C.

Measurement of PLD Activity—PLD activity was measured by the formation of phosphatidylinositolbutanol in the presence of 1% butanol using phospholipid vesicles comprised PE, PIP2, and PC in a molar ratio of 16:1:4:1, as described by Brown et al. (13). In some assays, the lipid composition of the vesicles was altered as indicated. The lipid reconstitution buffer was 50 mM HEPEs (pH 7.5), 3 mM EGTA, 2 mM CaCl2, 3 mM MgCl2, 1 mM dithiothreitol, and 80 mM KCl. The reaction mixture was incubated at 37 °C and terminated by the addition of 1 ml of chloroform/methanol/HCl (50:50:0.3) and 0.35 ml of 1 N HCl in 5 mM EGTA. The incubation time was 30 min unless otherwise noted. The mixture was centrifuged at 2000 × g for 5 min, and the lower phase was dried under N2, resuspended in 300 μl of chloroform/methanol (2:1), and spotted onto silica gel 60 A thin layer chromatography plates (Whatman). Spots were visualized as described. The lipid standard system of ethyl acetate/isooctane/H2O/acetic acid (55:25:50:10) and then stained with iodine. A phosphatidylinositol standard (Avanti) was used to locate the bands, which were scraped into scintillation vials containing 500 μl of methanol and 7.5 ml of Ready Organic scintillation mixture (Beckman).

In Vitro Phosphorylation Studies—Phosphorylation reactions were
carried out in a total volume of 25 μl containing kinase buffer (25 mM Tris-HCl, pH 7.5, 1.32 mM CaCl2, 5 mM MgCl2, 1 mM EDTA, 1.25 mM EGTA, 1 mM dithiothreitol) supplemented with 4β-phorbol 12-myristate 13-acetate (PMA) (100 nM), phosphatidylinerine (100 μg/ml), 50 μM ATP, 10 μCi of [γ-32P]ATP (NEF Life Science Products), 50 ng of purified PKC-α or β, in the presence or absence of purified PLD for 20 min at 30 °C. Incubations were stopped in Laemmli sample buffer and analyzed by SDS-PAGE on 4–12% gradient gels followed by autoradiography. For the time course studies, the incubations were performed with or without 10 μM Ro-31-8220 for the indicated times. An assay of PLD activity was performed as described above. For the phosphorylation reaction, incubations were stopped in Laemmli sample buffer. PLD was separated from PKC-α by SDS-PAGE on 4–12% gradient gels, and the gel was dried. To quantitate the amount of 32P incorporated into PLD, the gel was exposed to film, and the autoradiogram was used as a template for excising radioactive PLD bands, which were added into scintillation vials containing 10 ml of Ready Organic scintillation mixture. The stoichiometry of phosphate incorporation was determined from the specific radioactivity of the [γ-32P]ATP (counted under the same conditions) and the amount of PLD protein applied to the gels (determined by Bio-Rad protein microassay).

Binding of PLD to Concanavalin A-Sepharose Beads—Sf9 cells were incubated at a multiplicity of 10 for 60 h with recombinant baculovirus encoding rPLD1 in the presence of various concentrations of tunicamycin (Boehringer Mannheim Corp.) and lysed by incubation with a lysis buffer containing 20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 15,000 Ci of [γ-32P]ATP (counted), 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 15,000 × g for 10 min, and the supernatant was incubated for 2 h at 4 °C with Sepharose beads coupled to Concanavalin A (ConA) (Amersham Pharmacia Biotech). The beads were washed five times with a washing buffer, followed by the addition of SDS-sample buffer, and boiling. The recovered protein was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were probed with anti-PLD antibody. Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and ECL reagent (Amersham Pharmacia Biotech).

Endoglycosidase F Digestion—Purified rPLD1 (200 ng) was denatured in 0.5% SDS, 1% β-mercaptoethanol for 10 min at 100 °C and then incubated for 5 h at 37 °C with 1000 units of endoglycosidase F (New England Biolabs) in 50 mM sodium phosphate (pH 7.5), 1% Nonidet P-40. The reaction was stopped by adding SDS sample buffer and heating for 4 min at 100 °C, followed by Western blotting with anti-PLD antibody. Immunoreactive bands were visualized by transferring to polyvinylidene difluoride membranes, which were probed with a washing buffer, followed by the addition of SDS-sample buffer, and boiling. The recovered protein was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were probed with anti-PLD antibody. Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and ECL reagent (Amersham Pharmacia Biotech).

Expression and Affinity Purification of (His6)-rPLD1—To produce amounts of enzymatically active rPLD1 sufficient for in vitro characterization, we expressed the enzyme using the baculovirus expression system and the transfer vector pBlueBacHis, which contains an epitope for the Anti-Xpress antibody to allow affinity purification of the recombinant enzyme. We compared the (His6)-rPLD1 expression level in two different insect cell lines, the S. frugiperda cell line Sf9 and the Trichoplas mytani cell line High Five, respectively. Although it is reported that High Five cells offer up to 25-fold higher expression of secreted proteins than Sf9 cells, the Sf9 line was used, since infection of these cells yielded approximately 10-fold more fusion protein than High Five cells (data not shown). Four different detergents (β-octyl glucoside, Triton X-100, Nonidet P-40, sodium cholate) were tested for solubilization of the expressed PLD, and octyl glucoside was found to be most suitable (data not shown). Membrane-bound PLD could also be extracted with 0.6 M NaCl. The 10 × 150-mm dishes of Sf9 cells each containing 2 × 107 cells were infected at a multiplicity of infection of 10 with recombinant baculovirus for expression of the PLD. At 48 h postinfection, the cells were washed with phosphate-buffered saline and extracted with buffer containing 1% β-octyl glucoside and 0.6 M NaCl. After centrifugation, supernatants were adsorbed to 0.4 ml of Ni2+-NTA resin overnight at 4 °C. The resin was then washed with buffer containing 50 mM imidazole and 800 mM NaCl. (His6)-rPLD1 was eluted in 80 ml imidazole-containing fractions and analyzed by SDS-PAGE and Western blotting. The molecular mass of rPLD1 was estimated to be 120 kDa by SDS-PAGE (Fig. 1). The enzyme was purified to apparent homogeneity as shown by staining with Coomassie Brilliant Blue (Fig. 1A), and approximately 50 μg of pure rPLD1 protein was obtained. The purified protein was recognized by an antibody to the C-terminal dedcapeptide of hPLD1 (Fig. 1B) as well as the Anti-Xpress antibody (Fig. 1C).

Regulation by Polyphosphoinositides—We studied the effects of acidic phospholipids on the activity of purified rPLD1 using substrate vesicles containing 5 μM test phospholipid in the presence of PE and PC. The assays also contained 50 μM GTPγS and the small G protein ARF, since this greatly enhances the activity of PLD (13, 14). PIP2 and PIP3 stimulated the activity of rPLD1 significantly, and phosphatidylinositol 4-phosphate and phosphatidylinositol 3,4-bisphosphate were largely ineffective (Fig. 2A). The activation of PLD by PIP2 and PIP3 was dependent on the molar fraction of polyphosphoinositide in the vesicles (Fig. 2B). Significant activation was observed with 1 μM and maximal activation occurred at 5 μM.

Effect of pH and Oleate on PLD Activity—The effect of pH on rPLD1 activity was investigated using pH values ranging from 4.0 to 9.0 in the presence of 1 mM free Mg2+. The activity was assayed in the presence of GTPγS and ARF. PLD activity showed a broad dependence on pH, with optimum activity at pH 6.5–7.5 (data not shown).

Since previous reports have shown divergent effects of oleate on PLD (1), we tested the effects of this fatty acid on rPLD1 in the presence of GTPγS and ARF. Oleate had a strong inhibitory effect on the enzyme, whereas other fatty acids (palmitate, stearate, arachidonate) were ineffective (data not shown). The effect of oleate was concentration-dependent, with about 70% inhibition occurring at 0.01 μM.

Substrate Specificity—We examined the substrate selectivity of purified rPLD1 toward different phospholipids. The enzyme was incubated with 1-palmitoyl-2-[1-14C]linoleoyl PE and 1-stearoyl-2-[1-14C]arachidonoyl phosphatidylinositol under standard assay conditions. [14C]Phosphatidylbutanol was produced only from PC (data not shown), thus demonstrating that rPLD1 is PC-specific.

Dependence on Ca2+ and Mg2+—Ca2+ and Mg2+ have been implicated in the activation of PLD (28). To determine the influence of these divalent cations on PLD activity assayed in the presence of GTPγS and ARF, free Ca2+ and Mg2+ in the assay medium were controlled using Ca2+/Mg2+-EGTA buffers at pH 7.5. ARF-dependent rPLD1 activity was very low in the absence of Ca2+ and Mg2+, and stimulation by both cations was observed in the micromolar range and reached a peak at 1 mM (Fig. 3). The PLD activity showed two phases of stimulation by the cations. The first phase occurred at 1–100 μM Ca2+ and...
Mg$^{2+}$ concentrations, and the second one was a sharp increase at 1 mM concentration of both cations. At higher concentrations of Ca$^{2+}$, there was a sharp decrease in activity, but this was not observed with Mg$^{2+}$.

Activation by ARF and Rho—The activation of rPLD1 by purified ARF3 and RhoA in the presence of GTP$\gamma$S is shown in Fig. 4A. The activation of the enzyme by these G proteins was dependent on GTP$\gamma$S, since in the absence of the nucleotide, no stimulation was seen (data not shown). The PLD activity was increased by ARF in a dose-dependent manner. Stimulation was detectable at 50 nM and was maximal at 2 mM ARF plus 50 µM GTP$\gamma$S. The concentration of the indicated phospholipids in the lipid vesicles was varied as shown.

Activation of rPLD1 by the $\alpha$- and $\beta$II-Isozymes of PKC—It has been reported that classical isozymes of PKC stimulate PLD in several in vitro systems (6–9). To investigate the effects of PKC, rPLD1 was incubated with increasing concentrations of purified recombinant PKC isozymes. Among the isozymes tested, PKC-$\alpha$ and -$\beta$II (Fig. 4B), but not PKC-$\gamma$, -$\delta$, -$\epsilon$, or -$\zeta$ (data not shown), stimulated the enzyme in a concentration-dependent manner with half-maximal effects at approximately 10 nM and with similar maximal effects. The addition of 100 nM PMA increased the stimulation by PKC-$\alpha$ and PKC-$\beta$II 1.5- and 1.3-fold, respectively. The effects of the two isozymes were observed in the absence of ATP, and, in fact, the addition of 100 µM ATP inhibited the effects of the isozymes by 53 and 77% in the presence and absence of PMA, respectively. This suggested, contrary to expectation, that phosphorylation of the enzyme might be inhibitory.

Lack of Synergism among Arf, Rho, and PKC on rPLD1 Activity—It has been reported that ARF and RhoA synergistically activate PLD in some systems (27, 33) and that PKC-$\alpha$...
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Phosphorylation of rPLD1 by PKC Inhibits Its Enzymatic Activity—It was observed that when ATP was included in activity assays, stimulation of PLD by PKC-α or -βII was reduced. This observation was also reported by Hammond et al. (30). To investigate the effect of PKC phosphorylation on rPLD1 activity, in vitro phosphorylation and PLD activity assays were performed in the presence or absence of the PKC inhibitor, Ro-31-8220 (Fig. 7). Phosphorylation of rPLD1 by PKC-α was markedly inhibited by the addition of Ro-31-8220, as expected. In the presence of ATP and PKC-α, there was an initial increase in PLD activity, but thereafter the activity declined. The addition of the PKC inhibitor increased PLD activity at all times (Fig. 7). These data indicate that phosphorylation of rPLD1 by PKC negatively regulates its activity.

rPLD1 Is a Glycosylated Protein—We investigated whether rPLD1 is glycosylated in cells, since the rPLD1 sequence revealed 5 putative PKC phosphorylation sites. Purified recombinant PKC-α and -βII were added to an in vitro phosphorylation assay using [γ-32P]ATP and purified rPLD1. Interestingly, PKC-α and PKC-βII phosphorylated the PLD (Fig. 6A) in a time-dependent manner (Fig. 7). The phosphorylation occurred at high stoichiometry (approximately 4 mol of phosphate/mol of enzyme at 60 min, determined as described under “Materials and Methods”) and was accompanied by a mobility shift on SDS-PAGE (Fig. 6B). Under the assay conditions containing PMA, Ca2+, and phosphatidylserine, PKC-α and PKC-βII were autophosphorylated (Fig. 6A).

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has a synergistic effect with the two G proteins (7). This has also been shown for the 1a and 1b isoforms of hPLD1 (30). To investigate possible synergism among ARF, Rho, and PKC on PLD activity, rPLD1 was incubated with GTPγS and with ARF3, RhoA, PKC-α, and PKC-βII and their combinations. However, the combination of maximally effective concentrations of ARF3 and RhoA produced no greater stimulation than seen with ARF3 alone (Fig. 5). Likewise, the combination of either ARF3 or RhoA with PKC-α or PKC-βII resulted in no increase in activity above that observed with either G protein alone. Finally, the combination of ARF3 plus RhoA with either PKC isozyme produced no increase above that seen with ARF3 alone (Fig. 5). These results with pure rPLD1 provide no indication of synergism among ARF, Rho, and PKC in the regulation of this enzyme, in agreement with results obtained using intact COS-7 cells expressing rPLD1 and using membranes from these cells (31).

PKC-α and -βII Phosphorylate rPLD1 in Vitro—Although the results of Fig. 4B indicated that the stimulatory effects of PKC-α and -βII did not require ATP and previous work has shown that the regulatory domain alone of PKC may be sufficient to provide stimulation of PLD by PKC (7), we investigated whether PKC isozymes could phosphorylate purified rPLD1 in vitro, particularly since a search of the rPLD1 sequence revealed 14 putative PKC phosphorylation sites. Purified recombinant PKC-α and -βII were added to an in vitro phosphorylation assay using [γ-32P]ATP and purified rPLD1. Interestingly, PKC-α and PKC-βII phosphorylated the PLD (Fig. 6A) in a time-dependent manner (Fig. 7). The phosphorylation occurred at high stoichiometry (approximately 4 mol of phosphate/mol of enzyme at 60 min, determined as described under “Materials and Methods”) and was accompanied by a mobility shift on SDS-PAGE (Fig. 6B). Under the assay conditions containing PMA, Ca2+, and phosphatidylserine, PKC-α and PKC-βII were autophosphorylated (Fig. 6A).

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of the enzyme with endoglycosidase F caused a shift in its mobility on electrophoresis (Fig. 8B).

We next examined the subcellular localization and activity of rPLD1 after treatment of Sf9 cells with tunicamycin (Fig. 9). As the concentration of tunicamycin increased, the amount and activity of the PLD decreased in the membrane fraction and increased in the cytosolic fraction, suggesting that tunicamycin affects membrane localization of PLD. These results suggest that rPLD1 is a glycosylated protein and that glycosylation is a factor in membrane localization.

**DISCUSSION**

PLD activity has been found in many mammalian tissues and cells (1), but its study has been hindered by difficulties in purifying the enzyme. A major breakthrough came when the gene for PLD in the plant *Ricinus communis* was cloned (34). With information from the plant and yeast PLD sequences, the cDNA for a mammalian PLD was cloned (29). Recently, Hammond et al. (30) have cloned and expressed alternatively spliced forms of human hPLD1 and also a second PLD isozyme (PLD2) (35), which is regulated very differently from PLD1. PLD2 has also been cloned from rat brain (36).

Many biochemical studies have indicated the existence of multiple forms of PLD (1). To identify other PLD isozymes, we have cloned several PLD cDNAs from mammalian tissues. The most fully characterized is rPLD1, which was cloned from a rat brain cDNA library and is regulated by ARF, Rho, and PKC (31). This enzyme is present in both soluble and membrane fractions when expressed in Sf9 cells (present study) and COS-7 cells (31). The present results provide an explanation for this finding, namely that the enzyme undergoes N-glycosylation, presumably in the Golgi, and that the glycosylated form is targeted to the membranes (Fig. 9).

Affinity purification of (His<sub>6</sub>)-rPLD1 from baculovirus-infected Sf9 cells with β-octyl glucoside using Ni<sup>2+</sup>-NTA agarose resin provided a homogeneous active enzyme. Although rPLD1 was extracted with the detergent, the elution of enzyme from the resin was performed in a buffer without detergent. The purified enzyme was stable for 5 months when maintained in detergent-free condition in the presence of 20% glycerol. The molecular mass of purified enzyme is 120 kDa, and it is specific for PC.

The original detection of ARF-stimulated PLD activity, assayed with exogenous substrate, required the presence of PIP<sub>2</sub> in the substrate vesicles (13). PIP<sub>2</sub> and PIP<sub>3</sub> stimulated rPLD1 activity almost equitably, but a variety of other inositol phospholipids were ineffective (Fig. 2A). Surprisingly, phosphatidylinositol 3,4-bisphosphate was ineffective, suggesting that the stereochemical position of the inositol phosphate is critical for the regulation of PLD. The regulation of PLD activity by PIP<sub>2</sub> and PIP<sub>3</sub> may be of physiological significance, since PIP<sub>3</sub> is a recognized signaling molecule (37), and PIP<sub>2</sub> and PIP<sub>3</sub> bind to the pleckstrin homology (PH) domain of various proteins (38) and modify their localization and function. The mechanism by which PIP<sub>2</sub> and PIP<sub>3</sub> activate PLD is unclear, since there is no evident PH domain or other binding site for phosphoinositides in its sequence. It has been reported that PIP<sub>2</sub> stimulates the rate of GDP dissociation from ARF1 and the rate of GTPγS binding (39). It has also been shown that ARF1 directly binds PIP<sub>2</sub> (40), and it has been suggested that this binding is required for ARF1 to interact with target proteins such as its GTPase-activating protein or PLD (13, 40–43). However, it is clear that the action of PIP<sub>2</sub> on PLD is not just related to ARF, since the lipid is required for the stimulation of PLD by RhoA and PKC in vitro (Fig. 4 and Refs. 7, 8, 27, and 30).

rPLD1 has a calculated PI value of 9.0, but the optimal pH for ARF-activated PLD in vitro was between 6.5 and 7.5. The enzyme was similarly dependent on Ca<sup>2+</sup> and Mg<sup>2+</sup> and was most active at a 1 mM concentration of these divalent cations. ARF-stimulated rPLD1 was similarly sensitive to changes in Mg<sup>2+</sup> (30), but Ca<sup>2+</sup> was not tested alone. The cations appeared to act on the same site, since the addition of Ca<sup>2+</sup> produced no stimulation above that seen with 1 mM Mg<sup>2+</sup> alone (30). Since
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the Mg$^{2+}$ concentration of cytosol is in the millimolar range, at which hPLD1 and rPLD1 would be fully stimulated by this cation, these data indicate that these isozymes are unlikely to be regulated by changes in cytosolic Ca$^{2+}$, which occur in the micromolar and submicromolar range.

As found for hPLD1, olate strongly inhibited rPLD1 (Fig. 5). This is in contrast to some other forms of the enzyme that are stimulated by this fatty acid (28). The finding that PLD isozymes are regulated by unsaturated fatty acids raises the possibility that alterations in phospholipase $A_2$ activity in vivo could secondarily alter PLD activity. However, to date, there is no experimental support for this possibility.

In agreement with Hammond et al. (30), who studied two alternatively spliced forms of hPLD1, rPLD1 was stimulated by ARF, RhoA, and PKC in vitro. The role of ARF in intracellular (Golgi) protein trafficking is well established (16), and it has been suggested that PLD plays a part in this process, perhaps by modulating membrane lipids to facilitate formation and fusion of transport vesicles (17, 44). Although ARF-responsive PLD activity has been found in other subcellular fractions besides Golgi (plasma membrane, nucleus, and cytosol) (1), its role at these sites is unknown. Some studies have shown agonist-induced membrane translocation of ARF (45, 46), but the nature of the membrane target is unclear. Rho regulates the formation of actin stress fibers and focal adhesions (23, 24) and has also been implicated in the regulation of phosphatidylinositol 3-kinase (25) and phosphatidylinositol 4-phosphate 5'-kinase (26), which synthesize PIP$_2$ andPIP$_2$ respectively. Although the present and previous (30) findings indicate that Rho can directly activate PLD, the possibility that the G protein exerts part of its stimulatory effects on PLD in vivo through alterations in PIP$_2$ is suggested by the findings of Jakobs and co-workers (47). Irrespective of the mechanisms by which Rho regulates PLD, there is growing evidence that this protein plays a role in coupling cell surface receptors to PLD activation and other cellular responses (22, 48), and has also been implicated in the regulation of phosphatidylinositol 3-kinase (25) and phosphatidylinositol 4-phosphate 5'-kinase (26), which synthesize PIP$_2$ and PIP$_2$ respectively.

A surprising finding of the present study was the lack of synergism in the effects of ARF, Rho, and PKC on rPLD1 (Fig. 5). This is in contrast to the findings with the two forms of hPLD1 (30) but in agreement with our observations on rPLD1 expressed in COS-7 cells (31). These data suggest that the domains at which these regulatory proteins bind to rPLD1 are noninteracting. Preliminary data$^2$ indicate that PKC interacts with an N-terminal sequence in the enzyme, whereas ARF and RhoA interact at other sites. It is possible that ARF and PKC activate rPLD1 by a shared mechanism, since their maximum effects were not additive (Fig. 5). The failure of these proteins to interact with Rho in the regulation of rPLD1 versus hPLD1 could be due to the substantial differences in the sequences of the two isozymes between residues 507 and 574, i.e. in the middle of the enzymes (31). However, these explanations are highly speculative and obviously need experimental support.

An interesting finding is that rPLD1 is a glycoprotein, as assessed by its binding to ConA and the effects of endoglycosidase F and tunicamycin (Figs. 8 and 9). The findings suggest that N-linked glycosylation is a factor in the membrane localization of the enzyme. Except when the enzyme is transiently overexpressed in cells, it is almost exclusively membrane-bounded (1, 4). This suggests that membrane translocation of PLD is probably not a factor in its regulation. However, translocation of its regulatory proteins may be very important (1).

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REFERENCES

1. Exton, J. H. (1997) J. Biol. Chem. 272, 15579–15582
2. Boarder, M. R. (1994) Trends Pharmacol. Sci. 15, 57–62
3. Olson, S. C., and Lambeth, J. D. (1996) Chem. Phys. Lipids 80, 3–19
4. Kinass, T. C., Jaffer, F. E., and Abboud, H. E. (1990) J. Biol. Chem. 265, 14457–14463
5. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42
6. Conricode, K. M., Brewer, K. A., and Exton, J. H. (1992) J. Biol. Chem. 267, 7999–7992
7. Singer, W. D., Brown, H. A., Jiang, X., and Sternweis, P. C. (1996) J. Biol. Chem. 271, 4504–4510
8. Ohguchi, K., Banno, Y., Nakashima, S., and Nozawa, Y. (1996) J. Biol. Chem. 271, 4366–4372
9. Lopez, I., Burns, D. J., and Lambeth, J. D. (1995) J. Biol. Chem. 270, 19465–19472
10. Beckeino, S. B., Blackmore, P. F., Wilson, P. B., and Exton, J. H. (1987) J. Biol. Chem. 262, 15309–15315
11. Olson, S. C., Bowman, E. P., and Lambeth, J. D. (1991) J. Biol. Chem. 266, 17296–17292
12. Kusner, D. J., Schomisch, S. J., and Duhayg, G. R. (1993) J. Biol. Chem. 268, 19973–19982
13. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–11144
14. Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, R., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) Science 263, 523–526
15. Brown, H. A., Gutowski, S., Kahn, R. A., and Sternweis, P. C. (1995) J. Biol. Chem. 270, 14935–14943
16. Knauss, T. C., Jaffer, F. E., and Abboud, H. E. (1990) J. Biol. Chem. 265, 17117–17122
17. Knauss, T. C., Jaffer, F. E., and Abboud, H. E. (1990) J. Biol. Chem. 265, 17117–17122
18. Malcolm, K. C., Ross, A. H., Qiu, R.-G., Symons, M., and Exton, J. H. (1994) J. Biol. Chem. 269, 19591–19594
19. Malcolm, K. C., Ross, A. H., Qiu, R.-G., Symons, M., and Exton, J. H. (1995) J. Biol. Chem. 270, 8466–8473
20. Kwak, J.-Y., Lopez, I., Uhlinger, D. J., Stry, Y. S., and Lambeth, J. D. (1995) J. Biol. Chem. 270, 27052–27058
21. Cabell, M., Kek, K., and Exton, J. H. (1996) J. Biol. Chem. 271, 13105–13139
22. Ridley, A. R., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
23. Nobes, C. D., and Hall, A. (1995) Cell 81, 53–62
24. Zheng, Y., Bagrodia, S., and Cerione, R. A. (1994) J. Biol. Chem. 269, 17127–17130
25. Cheng, L. D., Tryon-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994) Cell 79, 507–513
26. Singer, W. D., Brown, H. A., and Bokoch, G. M., and Sternweis, P. C. (1995) J. Biol. Chem. 270, 14944–14950
27. Leskovitch, M., and Chalifa-Caspi, V. (1996) Chem. Phys. Lipids 80, 37–44
28. Hammond, S. M., Alshuller, Y. M., Sung, T.-C., Rudge, S. A., Rose, K., Engberscht, J., Morris, A. J., and Fruhman, M. A. (1995) J. Biol. Chem. 270, 29640–29643
29. Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S.-K. Park, D. S. Min, and J. H. Exton, unpublished observations.
