A specific protein inhibitor of partially purified bovine brain phospholipase D (PLD) was identified from bovine brain cytosol. The PLD inhibitor has been enriched through several chromatographic steps and characterized with respect to size and mechanism of inhibition. The inhibitor showed an apparent molecular mass of 30 kDa by Superose 12 gel exclusion chromatography and inhibited PLD activity with an IC50 of 7 nM. The inhibitor had neither proteolytic activity nor phospholipase D activity nor phospholipase C (PLC)-phosphatidic acid; PIP2, phosphatidylinositol 4,5-bisphosphate; PE-domain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, lipase C; PKC, protein kinase C; PH domain, pleckstrin homology phospholipid-hydrolyzing activity. Because phosphatidyl-

The inhibitor had neither proteolytic activity nor phosphorylation and inhibited PLD activity with an IC50 of 7 nM. The inhibitor showed an apparent molecular mass of 30 kDa by Superose 12 gel exclusion chromatography.

Inhibition of both PLC-phosphatidic acid; PIP2. In contrast, a PH domain derived from PLC-

The inhibition of both PLC-

The inhibitor showed an nearly identical sequestration of PIP2. PIP2 hydrolysis by phospholipase C (PLC)-β1 was not affected by the inhibitor and the inhibitor did not bind to substrate vesicles containing PIP2. In contrast, a PH domain derived from PLC-β1, which could bind to PIP2, showed a nearly identical inhibition of both PLC-β1 and PLD activities. Thus, the PLD inhibition by the inhibitor is due to the specific interaction with not PIP2 but PLD. The suppression of PLD activity by the inhibitor was largely eliminated by the addition of ADP-ribosylation factor (ARF) and GTPγS. We propose that the inhibitor plays a negative role in regulation of PLD activity by PIP2 and ARF.

Phospholipase D (PLD) plays an important role in signal transduction of a variety of cells (1, 2). The enzyme hydrolyzes phosphatidylcholine (PC) to produce phosphatic acid (PA) and choline. PA has been implicated as a biologically active molecule and can also be metabolized via PA phosphohydrolase to form diacylglycerol (3–5). The diacylglycerol from PC has been known as a sustained activator of protein kinase C (PKC) (5).

Activation of PLD occurs through multiple mechanisms involving PKC, Ca2+ ions, tyrosine kinases, and/or GTP-binding proteins (6–8). Two recent reports have implicated ADP-ribosylation factor (ARF), a low molecular weight GTP-binding protein (small G protein), in the activation of PLD (9, 10). Members of the Rho family of small G-proteins, PKC, and calmodulin were also identified as PLD-activating factors (11–19). Many of these activating factors stimulate PLD activity synergistically with ARF (15–19). In addition to these PLD-activating factors, phosphoinositide 4,5-bisphosphate (PIP2) functions as a cofactor for PLD in assays using exogenous lipid substrate (20, 21).

While PLD-activating mechanisms have been described, the negative regulation of PLD is poorly understood. The existence of PLD-inhibitory factors has been suggested by several research groups (22–26). It was recently suggested that an inhibitor of ARF-dependent PLD activity exists in bovine brain-derived membranes (25). In addition, an inhibitor of ARF-dependent PLD activity in permeabilized HL-60 cells was detected in bovine brain cytosol (26).

The present work was performed to characterize the inhibitor and its mechanism of inhibition of PLD. Herein, we demonstrate that a 30-kDa inhibitor from bovine brain cytosol specifically inhibits PLD activity.

EXPERIMENTAL PROCEDURES

Materials

All reagents were analytical grade unless otherwise indicated. Dioleoylphosphatidylethanolamine (dioleoyl-PE), dipalmitoylphosphatidylethanolamine (dipalmitoyl-PC), phosphatidylinositol 4,5-bisphosphate (PIP2), and sodium cholate were purchased from Sigma. GTPγS was obtained from Boehringer Mannheim. Dipalmitoyl[2-3H]phosphatidylcholine, dipalmitoylphosphatidyl[3H]choline and phosphatidyl[3H]inositol 4,5-bisphosphate were from Amersham. Heparin-Sepharose, butyl-Sepharose, and Superose 12 columns were purchased from Pharmacia Biotech Inc. TSK gel DEAE-5PW, TSK gel blue-5PW, TSK gel hydroxyapatite-5PW, and TSK gel SP-5PW were from Toso (Japan).

Measurement of PLD Activity

PLD activity was measured essentially as described previously by Brown et al. (9) with slight modifications. Partially purified bovine brain PLD was reconstituted in mixed phospholipid vesicles with the column fractions containing PLD-inhibitory activity. The phospholipid vesicles (65.5 μm phospholipids) comprised PE, PIP2, and dipalmitoyl-[methyl-3H]choline in a molar ratio of 16:1:4:1 (total volume of 150 μl), and the 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 0.3 ml of 1 N HCl. The mixture was shaken and centrifuged at 2000 × g for 5 min. An
 aliquot of the supernatant (0.5 ml containing released \[^{3}H\]choline) was removed, and the released \[^{3}H\]choline was counted by liquid scintillation spectroscopy. To determine whether the PLD inhibitor decreases the transphosphatidyl dation activity of PLD, PLD activity was measured under the same condition except that the radiolabeled substrate was replaced with dipalmityl[2-palmitoyl-9–10-\[^{3}H\]HPC (0.5 \(\mu\)Ci) and 1% ethanol (v/v). The produced \[^{3}H\]phosphatidylethanol (PEtOH) was separated by thin layer chromatography as described (27). The PEtOH-producing activity as well as the choline-releasing activity of PLD was decreased by addition of the inhibitory fraction (data not shown).

**Cell-free Assay of PLD Using Human Neutrophils**

PLD activities of plasma membrane from human neutrophil were measured as described (14). Cells were labeled with \[^{3}H\]alkyllyosphosphatidylcholine (1.5 \(\mu\)Ci/2 \times 10\(^7\) cells/ml) for 90 min at 37°C. Plasma membranes and cytosol were isolated as described (28). Incubations containing plasma membrane plus cytosol were carried out in the presence of 10 \(\mu\)M GTP-\(\alpha\)-S, 1 \(\mu\)M Ca\(^{2+}\), and 1.8% ethanol, and the reaction was terminated by transfer to chloroform:methanol (1:2). The produced \[^{3}H\]inositol 1,4,5-trisphosphate was measured by liquid scintillation spectroscopy. To determine whether the PLD inhibitor decreases the transphosphatidyl dation activity of PLD, PLD activity was measured under the same condition except that the radiolabeled substrate was replaced with dipalmityl[2-palmitoyl-9–10-\[^{3}H\]HPC (0.5 \(\mu\)Ci) and 1% ethanol (v/v). The produced \[^{3}H\]phosphatidylethanol (PEtOH) was separated by thin layer chromatography as described (27).

**Purification of an Inhibitor of PLD Activity**

To determine whether the PLD inhibitor decreases the transphosphatidyl dation activity of PLD, PLD activity was measured under the same condition except that the radiolabeled substrate was replaced with dipalmityl[2-palmitoyl-9–10-\[^{3}H\]HPC (0.5 \(\mu\)Ci) and 1% ethanol (v/v). The produced \[^{3}H\]phosphatidylethanol (PEtOH) was separated by thin layer chromatography as described (27).

**Preparation of Bovine Brain Membranes and Cytosol**

Bovine brains were obtained from a local slaughter house. All steps were performed at 4°C. Six brains (about 2 kg) were homogenized with a polytron homogenizer in 4 volumes (8000 ml) of buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) containing 1% (w/v) sodium cholate, and the mixture was incubated for 1 h at 4°C with stirring. Insoluble material was removed by centrifugation at 100,000 \(\times\) g for 60 min. Membranes were washed once by suspension in buffer B and the repeated centrifugation.

**Partial Purification of PLD from Bovine Brain Membrane**

PLD was prepared from bovine brain membranes essentially as described previously (25). Membranes were solubilized by buffer B (20 mM Heps, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.4 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride, and 0.2 mM \(\beta\)-mercaptoethanol). The homogenate was filtered through two layers of cheesecloth, and the resultant pellet (membranes) and supernatant (cytosol) were obtained by centrifugation at 100,000 \(\times\) g for 60 min. Membranes were washed once by suspension in buffer B and the repeated centrifugation.

**TABLE I**

| Fractons | Total protein | Specific activity | Total activity (units) | Purification yield |
|----------|---------------|-------------------|------------------------|-------------------|
| Cytosol  | 31000         | 50                | 1,500,000              | 1                 |
| Heparin-agarose | 1800         | 430               | 774,000                | 8.6               |
| Butyl-Toyopearl | 380          | 820               | 320,000                | 16.4              |
| DEAE-5PW | 62            | 910               | 56,400                 | 18.2              |
| Blue-5PW | 9.3           | 2,680             | 25,000                 | 53.6              |
| HA-5PW  | 2.1           | 4,760             | 10,000                 | 95.2              |
| SP-5PW  | 0.5           | 8,000             | 4,000                  | 160               |
| Superoxide 12 | 0.025       | 48,000            | 1,200                  | 960               |

One unit of the inhibitory activity was defined as the amount of the inhibitory fraction which decreased the hydrolysis of \[^{3}H\]phosphatidylcholine by PLD to 50% of control.
PLD-inhibitory activity was eluted around 0.3 M KCl.

Step 5: Chromatography with TSK Gel Hydroxyapatite-5PW—The PLD-inhibitory fractions from Step 4 were pooled and dialyzed against buffer D (20 mM potassium phosphate, pH 7.5) and loaded onto a TSK gel hydroxyapatite-5PW column (7.5 × 75 mm) equilibrated with buffer D. Bound proteins were eluted with 40 ml of a linear gradient from 0.02 to 0.3 M potassium phosphate in 40 min. The PLD-inhibitory activity was eluted around 0.2 M potassium phosphate.

Step 6: Chromatography with TSK Gel SP-5PW—The PLD-inhibitory fractions from Step 5 were pooled and dialyzed in buffer E (20 mM Mes, pH 6.5, 1 mM EGTA, 1 mM EDTA). The dialyzed fraction was loaded onto TSK gel SP-5PW column (7.5 × 75 mm) equilibrated with buffer E. Proteins were eluted with 40 ml of a linear gradient from 0.02 to 0.3 M NaCl in 40 min. PLD-inhibitory activity was eluted around 0.2 M NaCl. 

Step 7: Gel Filtration with Superose 12—Fractions containing PLD-inhibitory activity from Step 6 were pooled and concentrated to 0.4 ml with an Centricon-10. The preparation was applied to a Superose 12 column (7.5 × 75 mm) equilibrated with buffer F (20 mM Mes, pH 6.5, 1 mM EGTA, 1 mM EDTA, 3 mM MgCl₂, 1 mM dithiothreitol, 80 mM KCl, and 15 μM of bovine serum albumin plus the indicated concentration of phospholipid vesicles and PLD-inhibitory fraction). After incubation at room temperature for 10 min, the mixture was centrifuged at 100,000 × g for 60 min. An aliquot of the supernatant was removed for measurement of the PLD-inhibitory activity.

Measurement of Binding of the Inhibitor to Phospholipid Vesicles

The binding ability of the PLD-inhibitory factor to substrate vesicles was estimated by centrifugation assay as described previously (30). The final assay volume was 0.15 ml containing 50 mM HEPES (pH 7.5), 3 mM EGTA, 2 mM CaCl₂, 3 mM MgCl₂, 1 mM dithiothreitol, 80 mM KCl, and 15 μM of bovine serum albumin plus the indicated concentration of phospholipid vesicles and PLD-inhibitory fraction. After incubation at room temperature for 10 min, the mixture was centrifuged at 100,000 × g for 60 min. An aliquot of the supernatant was removed for measurement of the PLD-inhibitory activity.

Preparation of ARF

Recombinant ARF was overexpressed in E. coli as described (31). For the N-myristoylation of ARF, pBB131 vector containing the N-myristoyltransferase gene-1 (NMT) was cotransformed into the cells. The overexpressed ARF was purified to near homogeneity through sequential chromatography on DEAE-cellulose and Superose 12 gel filtration column.

Miscellaneous Methods

Bovine plasma gelsolin was purified from bovine serum as described (32). The final preparations of plasma gelsolin were about 70% pure, as judged by Coomassie staining of sample after SDS-PAGE. PLC-β1 was purified from bovine brain as described previously (33). Protein concentrations were estimated by the assay of Bradford and Rubin (34) with bovine serum albumin as the standard. SDS-PAGE was according to Laemmli (35).

RESULTS

Bovine Brain Cytosol Contains an Inhibitor of PLD Activity—Previous studies have demonstrated several PLD-activating factors, such as ARF, RhoA, and Cde42 in cytosolic fractions (9–19). However, we found that bovine brain cytosol inhibited the PIP₂-dependent PLD activity both in the absence and in the presence of GTPγS (Fig. 1). This result suggests that strong inhibitory constraints of PLD activity may exist.

Partial Purification of an Inhibitor of PLD Activity—A PLD-inhibitory factor was purified from bovine brain cytosol about 1000-fold with a yield of 0.08% (Table I) through seven sequential column chromatographic steps as described under “Experimental Procedures.” The PLD inhibitor was resolved from several PLD-regulating factors, such as ARF, RhoA, and...
RhoGDI (GDP dissociation inhibitor) by heparin-agarose chromatography (data not shown). The inhibitor was eluted at a volume corresponding to a molecular mass of approximately 30 kDa using Superose 12 column chromatography (Fig. 2A). When the column fractions from the Superose 12 were loaded onto SDS-PAGE, a protein band with a molecular mass of about 50 kDa correlated with the inhibitory activity (Fig. 2B). However, the protein band did not coincide with the inhibitory activity in the TSK gel SP-5PW column chromatographic step (data not shown). Although we tried five times to purify the inhibitor with many different combinations of column chromatographic steps, any candidate protein band was not observed in SDS-PAGE with either Coomassie or silver staining (data not shown). The inhibitory fraction from the Superose 12 column decreased PLD activity in a dose-dependent manner (Fig. 3). When the inhibitory fractions were boiled at 100 °C for 5 min, the inhibitory activity disappeared (Fig. 4A). Tryptic digestion also resulted in the loss of inhibitory activity (Fig. 4B). From these results, the inhibitory factor appears to be a protein.

Specific Inhibition of PLD Activity by the Inhibitor—Since PLD requires PIP2 in the substrate vesicles as a cofactor, a PIP2-binding protein is a candidate for the PLD-inhibitory factor. In addition to its role as a cofactor, PIP2 is used as substrate for PLC. Therefore, we investigated the effect of the inhibitory factor and PIP2-binding protein on PLC and PLD activities. As shown in Fig. 5A, PLD activity decreased by increasing concentrations of the inhibitor, while PIP2-hydrolyzing activity by PLC-β1 (0.1 μg) were determined as described under “Experimental Procedures.” Aliquots of PLC-β1 enzyme or PLD activities were incubated for 15 min at 37 °C in the presence of the indicated amounts of the inhibitory fraction (TSK gel blue-5PW) (A) or GST-PHPLCδ1 (B). The data shown are the mean ± S.E. of three independent experiments.

The PLD Inhibitor Does Not Bind to Substrate Vesicles—We investigated the ability of the PLD inhibitor to bind to sub-
The PLD inhibitor does not bind to lipid vesicles containing PIP$_2$. 2 μg of the PLD-inhibitory fraction (TSK gel blue-5PW, open bar) or 3 μg of GST-PHPLC61 (closed bar) were mixed with the indicated amounts of phospholipid vesicles. The concentration of PIP$_2$ (μM) was a fraction of phospholipid vesicle containing PE/PIP$_2$/PC with a molar ratio of 16:1:4.1. All of the centrifugation assays were carried out in a 0.15-ml total volume using a Beckman TL-100 table top ultracentrifuge and TLA-100 rotor (see “Experimental Procedures”). The PLD-inhibitory activity of the unbound fractions (supernatant) were quantified by reconstitution with an aliquot (1 μg) of PIP$_2$-hydrolyzing PLD enzyme (A). The data shown are the mean ± S.E. of three independent experiments. The bound fraction (pellets) of GST-PHPLC61 was precipitated by addition of phospholipid vesicles containing PIP$_2$ in a dose-dependent manner, whereas the PLD-inhibitory factor did not bind to substrate vesicles under PLD assay conditions (Fig. 6).

The Inhibitor Fraction Contains Neither Protease nor Lipase—In addition to PIP$_2$ binding, an alternative mechanism is that the inhibitor may be a PIP$_2$-hydrolyzing PLC. However, this is unlikely because we could not detect PIP$_2$-hydrolyzing activity by the inhibitor, and PIP$_2$-hydrolyzing activity did not coincide with the PLD-inhibitory activity during chromatographic steps (data not shown). Hydrolysis of the phospholipids in the substrate vesicle by lipases might inhibit the PLD activity by changing the physical environments of the substrate vesicles. To test whether the PLD inhibitor might have lipase activity, we incubated the inhibitor with substrate vesicles. The phospholipids were then analyzed by thin layer chromatography. The PLD inhibitor did not hydrolyze PE, PC, and PIP$_2$ (data not shown). A protease might also inhibit the PLD by hydrolyzing the enzyme. To exclude the possibility of a contaminating protease in the PLD-inhibitory fraction, the PLD was preincubated with the inhibitor for 30 min at 37 °C before adding substrate vesicles. Preincubation failed to affect the potency of the inhibitor (data not shown), indicating that a time-dependent hydrolysis of the PLD enzyme was not taking place. Also, the PLD-inhibitory activity was not affected by the addition of several protease inhibitors, including phenylmethylsulfonyl fluoride, leupeptin, and aprotinin (data not shown).

The Inhibitor Has Little Effect on PLD Activity in the Presence of ARF—PIP$_2$-dependent PLD activities of partially purified bovine brain PLD (1 μg, SP-Sepharose fraction) were measured in the presence of the indicated amounts of ARF and 10 μM GTPγS (A). The PLD activities were measured in the absence (open circle) or in the presence (closed circle) of ARF (1.0 μM), and different amounts of inhibitory fraction (TSK gel blue-5PW) were added in the reaction mixtures (B). The data shown are the mean ± S.E. of three independent experiments.

**DISCUSSION**

The present study has shown that an inhibitor from bovine brain cytosol potently inhibits PIP$_2$-dependent PLD activity. The inhibitor specifically regulated PLD activity and did not bind to substrate vesicles containing PIP$_2$. In addition, the suppression of PLD activity by the inhibitory protein was re-
PIP2 are involved in the activation of PLD (9–21). In this report, we showed that the PIP2-dependent PLD activity was inhibited by the inhibitor. The inhibitor did not bind to the substrate vesicles containing PIP2. Moreover, the inhibitor exhibits a far greater potency. Moreover, the inhibitor seems to decrease PLD activity at a concentration considerably less than 7 nM, indicating that the inhibitor is very potent. In contrast, the inhibitor had no effect on PLC-mediated PIP2 hydrolysis.

To examine the specificity of inhibition, the effects of PIP2-binding protein versus the inhibitor on PLD activity were tested. Because PIP2 is essential for PLD activity as a cofactor, masking of PIP2 by PIP2-binding proteins should cause the inhibition of PLD activity. The PH domain of PLC-ζ1 decreased PLD activity at a concentration 100-fold higher than inhibitor (Fig. 5B). We also found that gelsolin, which has PIP2-binding properties, inhibited PLD activity at a concentration above 1 μM (data not shown). Compared to the PIP2-binding proteins, the inhibitor exhibits a far greater potency. Moreover, the inhibitor did not bind to the substrate vesicles containing PIP2. These results suggest that the inhibitor does not mask the PIP2, but suppresses PLD activity by specific binding to PLD. In addition to PIP2 binding, an alternative mechanism for the PLD-inhibitory effects is that the inhibitor may be a PIP2-hydrolyzing PLC or other lipase. This is unlikely because we could not detect the hydrolysis of PE, PC, and PIP2 by the inhibitor.

It has been reported that several GTP-binding proteins and PIP2 are involved in the activation of PLD (9–21). In this report, we showed that the PIP2-dependent PLD activity was potently decreased by the inhibitor and that the suppression of PLD activity was overcome by ARF. Recent reports demonstrate that ARF-mediated PLD activation is synergistically modulated by RhoA, PKC, calmodulin, or unidentified proteins (15–19, 31, 38). In concert with ARF, these activating factors may play a role in reversing the suppression of PLD activity by the inhibitor. However, nothing is known about the cellular role of the PLD inhibitor and how the PLD-activating factors relate to the negative regulation by the inhibitor. Because the substrate of the PLD enzyme, PC, is abundant in cell membranes and PIP2 stimulates the PLD activity, we speculate that this inhibitor suppresses the PIP2-dependent PLD activity in unstimulated cells. If PLD enzyme were activated by the PLD-stimulating factors, the inhibitory constraint might be eliminated.

Recently, it was reported that an inhibitor from bovine brain cytosol eluted with a high molecular mass of 300 kDa from a Superose 12 gel filtration column (26). These data were obtained using streptolysin-O-permeabilized HL-60 cells to measure PLD-inhibitory activity. However, we could not detect an inhibitory fraction with such a high molecular mass when exogenous substrate vesicles were used for measurement of PLD activity. Thus, different forms of PLD inhibitor proteins, which can be detected under different assay conditions, may exist in bovine brain cytosol. In this report, we showed that the low molecular weight PLD inhibitor did not decrease PLD activity of neutrophil membranes. The clear indication for the different PLD isoforms between neutrophil and brain has not been reported yet. However, previous studies have shown that neutrophil PLD might be activated by distinct regulation mode, such as the existence of 50-kDa protein as a major activating factor, and little activation of PLD by ARF (14, 31). Thus, it is tempting to speculate that PLD inhibitors may have a specificity toward different PLD isoforms.

In summary, our observations have elucidated specific inhibition of PLD activity by an inhibitory protein with a low molecular mass, and have demonstrated the relationship between the inhibitor and ARF-dependent activation on the regulation of PLD activity. To elucidate the cellular role and the biochemical mechanism of the inhibitor, it will be important to know the molecular identity.

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FIG. 8. The PLD inhibitor does not decrease PLD activity of neutrophil plasma membranes. Plasma membranes (25 μg) were incubated with 1 μM CaCl2, 10 μM GTPγS, and 1.6% ethanol in the absence (open bar) or in the presence (closed bar) of neutrophil cytosol (50 μg) for 20 min at 37°C. The indicated amounts of PLD-inhibitory fraction (TSK gel blue-5PW) were added in the reaction mixtures. PLD-catalyzed transphosphatidylation was monitored by PEtOH formation, expressed as a percentage of the total radiolabeled lipids. The data shown are the mean ± S.E. of three independent experiments.
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