Multiple Forms of Phospholipase D Inhibitor from Rat Brain Cytosol

PURIFICATION AND CHARACTERIZATION OF HEAT-LABILE FORM*

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Rat brain cytosol contains proteins that markedly inhibit the activity of partially purified brain membrane phospholipase D (PLD) stimulated by ADP-ribosylation factor (Arf) and phosphatidylinositol 4,5-bisphosphate (PIP2). Sequential chromatography of the brain cytosol yielded four inhibitor fractions, which exhibited different kinetics to heat treatment at 70 °C. Purification of the most heat-labile inhibitor to homogeneity yielded two preparations, which displayed apparent molecular masses of 150 kDa and 135 kDa, respectively, on SDS-polyacrylamide gels. Tryptic digests of the 150- and 135-kDa proteins yielded similar elution profiles on a C18 reverse-phase column, suggesting that the 135-kDa form is a truncated form of the 150-kDa form. Sequences of two tryptic peptides were determined. A data base search revealed no proteins with these sequences.

The purified 150-kDa inhibitor negated the PLD activity stimulated by Arf, RhoA, or Cdc42. The concentration required for half-maximal inhibition was 0.4 nM. Concentration dependence on the 150-kDa inhibitor was not affected by changes in the concentrations of Arf, PIP2, or phosphatidylcholine used in the assays, suggesting that the inhibition is not due to competition with the activators or substrate for PLD. The purified inhibitor did not affect the PIP2-hydrolyzing activity of a phospholipase C isozyme that was measured with substrate vesicles of lipid composition identical with that used for the PLD assay. Thus, the mechanism of inhibition appears to be a specific allosteric modification of PLD rather than disruption of substrate vesicles.

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The abbreviations used are: PLD, phospholipase D; DPLC, phospholipase C; PC, phosphatidylcholine; (pam)PC, dipalmityloxyphosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PIP2, phosphatidylinositol 4,5-bisphosphate; Arf, ADP-ribosylation factor; G protein, guanine nucleotide-binding protein; Smg, small guanine nucleotide-binding protein; Rho-GDI, Rho GDP dissociation inhibitor; GTPyS, guanosine 5’-O-(thiotriphosphate); HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.
**PLD Inhibitor**

**EXPERIMENTAL PROCEDURES**

**Materials**

Rat brains were purchased from Pel-Freez Biologicals (Rogers, AR). Bovine brain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Alabaster, AL), and GTPyS and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) from Boehringer Mannheim. [chol:mehdilipid:PIP$_2$] (50 Ci/mmol) and [3H]PIP$_2$ from DuPont NEN. Rabbit antiserum to Rho-GDI was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and [3H]GTP$_s$ from NEN. Bovine brain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Rat brains (400 g) were thawed in 4 liters of Buffer A (20 mM Hepes (pH 7.0), 1 mM EGTA, 1 mM EDTA, 0.7% n-octyl-$\beta$-D-glucopyranoside containing 1.5 mM MgCl$_2$ and 2 mM CaCl$_2$). The final concentration of CaCl$_2$ was 3.4 mM, and the free Ca$^{2+}$ concentration is estimated to be 300 nM. Assays were incubated at 37 °C for 30 min before addition of 1 ml of stop solution containing CHCl$_3$, CH$_3$OH, and concentrated HCl (50:50:0.3, v/v) and 0.35 ml of 1 M HCl in 5 mM EGTA. After separation of the organic and aqueous phases by centrifugation, the released [3H]choline in 0.5 ml of the aqueous phase was quantitated by liquid scintillation spectrometry. Unless otherwise indicated, PLD inhibitor was assayed by measuring its capacity to inhibit the Arf-dependent activity measured under the conditions described above. Partially purified PLD (100 ng) from the TS-K gel Sepharose column (see below) was used as the PLD source, resulting in the production of 20,000 to 30,000 cpm of choline in the absence of inhibitor. Carrier of buffer used in preparation of this PLD source introduced 0.16 mM n-octyl-$\beta$-D-glucopyranoside into the assay.

**Preparation of Brain Membranes and Ammonium Sulfate Fractionation of Cytosol**

All procedures were carried out at 4 °C unless otherwise indicated. Rat brains (400 g) were thawed in 4 liters of Buffer A (20 mM Hepes (pH 7.0), 1 mM EGTA, 1 mM EDTA, 0.7% n-octyl-$\beta$-D-glucopyranoside containing 1.5 mM MgCl$_2$ and 2 mM CaCl$_2$) and were kept at 4 °C for at least 30 min. The brain was homogenized by 2 to 3 passes of 30 s each through a glass/glass French press pasting chamber at 0°C. The homogenate was centrifuged at 100,000 g at 4°C for 20 min. The resulting membrane pellet was saved for the purification of PLD. The supernatant was further fractionated by adding solid ammonium sulfate to yield a final saturation of 35%. The solution was stirred gently for 1 h and then centrifuged at 100,000 g for 20 min. The new pellet (0–35% ammonium sulfate fraction of cytosol) was saved for the purification of PLD inhibitor. The resulting supernatant was brought to 70% saturation with ammonium sulfate, stirred for 1 h, and centrifuged at 100,000 g for 20 min. The 35–70% ammonium sulfate fraction of cytosol was stored at −80 °C for the purification of Arf.

**Assays of PLD and PLD Inhibitor**

Arf-dependent PLD activity was measured using an assay described previously (17) with a minor modification. Briefly, 25 μl of mixed lipid vesicles containing PE, PIP$_2$, and PC in molar ratio of 16:1:4 with [chol:mehdilipid:PIP$_2$](50 Ci/mmol) to yield ~200,000 cpm per assay were added to 2 to 5 μl of PLD source, 100 μl Arf, and 5 μl GTPyS in a total volume of 125 μl containing 50 mM Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl$_2$, and 2 mM CaCl$_2$. The final concentration of PC was 3.4 μM, and the free Ca$^{2+}$ concentration is estimated to be 300 nM.

**Separation of Heat-labile and Heat-stable PLD Inhibitors and Purification of the Heat-labile Form**

Anion-exchange chromatography on DEAE-Sephadex column—The 0–35% ammonium sulfate fraction of rat brain cytosol was suspended in −400 ml of buffer E (10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM DTT, and leupeptin and aprotinin (each at 1 μg/ml)) and diluted 4-fold with buffer E and centrifuged to remove insoluble particles. The supernatant (10 g of protein in 400 ml) was loaded onto a DEAE-Sephadex column (5 × 20 cm) that had been equilibrated with Buffer E. Fractions (20 ml) were assayed for PLD-activating activity in the presence of GTPyS and Arf protein by immunoblot analysis with antibodies to Arf. Peak fractions of Arf (800 mg of protein in 100 ml) were pooled and were brought to 1.5 M ammonium sulfate by adding solid salt, and the mixture was centrifuged to remove particles. The supernatant was applied to a TSK-gel Phenyl-SPW (21.5 × 150 mm) that had been equilibrated with Buffer F. Fractions (20 ml) were assayed for PLD-activating activity in the presence of GTPyS and Arf protein by immunoblot analysis with antibodies to Arf. Peak fractions of Arf (800 mg of protein in 100 ml) were pooled and were brought to 1.5 M ammonium sulfate by adding solid salt, and the mixture was centrifuged to remove particles. The supernatant was applied to a Mono S HR 5/5 cation exchange column (200 ml) equilibrated with Buffer F. Proteins were eluted at a linear gradient of 0 to 0.5 M NaCl in Buffer F. Peak fractions (fractions 32–36) were pooled, concentrated to 5 ml in an ultracentrifugal filter apparatus with a YM 10 membrane (Amicon) and applied to a Aca54 gel filtration column (2.5 × 110 cm) that had been equilibrated with Buffer G (20 mM Hepes (pH 8.0), 1 mM EDTA, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM sodium azide, 2 mM DTT, and 250 mM sucrose). Proteins were eluted at a flow rate of 0.5 ml/min. Fractions of 6 ml were collected and assayed for Arf protein and PLD-activating activity. Peak fractions (fractions 61–65) were pooled and stored in aliquots at −70 °C. This preparation (12.6 mg) was greater than 90% pure based on SDS-PAGE.
portions, which were then chromatographed independently. Each portion was applied to a TSK-gel DEAE-5PW column (21.5 × 150 mm) that had been equilibrated with Buffer H. The column was eluted at 5 ml/min with a 400-ml linear gradient from 0 to 0.3 M NaCl in Buffer H followed by a 200-ml linear gradient from 0.3 to 1.0 M NaCl in the same Buffer. Fractions of 5 ml were collected and assayed for PLD-inhibiting activity. The peak fractions (fractions 39–47) from each of these chromatography runs were pooled (Fig. 1B).

Hydrophobic Chromatography on Phenyl-5PW Column—Saturated ammonium sulfate solution was added to the combined peak fractions from the DEAE-5PW column to give a concentration of 0.8 M ammonium sulfate, and the mixture was then centrifuged to remove insoluble material. The supernatant containing 200 mg of protein was applied to a TSK-gel Phenyl-5PW column (21.5 × 150 mm) that had been equilibrated with Buffer H containing 0.8 M ammonium sulfate. Proteins were eluted at a flow rate of 5.0 ml/min with a decreasing ammonium sulfate linear gradient from 0.8 to 0 M in Buffer H for 60 min. Two activity peaks, centered at fractions 20 and 49, were observed. Peak fractions 18–23 and 46–52, designated inhibitors IA and IB, were pooled separately (Fig. 1C).

Cation-exchange Chromatography on Heparin-5PW Column—Peak fractions of inhibitor IA (7.4 mg of protein) and inhibitor IB (38.4 mg of protein) from the Phenyl-5PW HPLC column were dialyzed against Buffer H and loaded separately onto a TSK-gel Heparin-5PW column (7.5 × 75 mm) that had been equilibrated with the same buffer. Both activities were eluted at 1.0 ml/min with a linear NaCl gradient from 0 to 0.6 M in Buffer H for 60 min. Fractions of 1.0 ml were collected. The inhibitor IA was eluted in a peak centered at fraction 30 (Fig. 1D), whereas the inhibitor IB was eluted in a peak centered at fraction 26 (Fig. 1E).

Gel Filtration Chromatography Inhibitor IA on Superose 12 PC 3.2/30 Column—Each of the inhibitor IA fractions (29 (170 μg of protein) and 32 (230 μg of protein) from the TSK-gel Heparin-5PW HPLC column was concentrated to 30 μl on a Microcon-30 (Amicon), and applied to a Superose 12 PC 3.2/30 column (3.2 × 300 mm, Pharmacia). This sizing chromatography was performed on a Pharmacia-LKB SMART System equipped with a μ separator unit and a μ precision pump. The column was eluted at a flow rate of 40 μl/min with 20 mM Hepes (pH 7.5) containing 150 mM NaCl. Fractions of 40 μl were collected and assayed for PLD-inhibiting activity and analyzed on SDS-polyacrylamide gel (Fig. 3).

The above purification procedure starting with the 0–35% ammonium sulfate precipitate was repeated three times to accumulate enough purified inhibitor IA for characterization.

RESULTS

Multiple Forms of PLD Inhibitor—We found that most PLD-inhibiting activity in rat brain cytosol could be precipitated by adding ammonium sulfate to 35% saturation (data not shown). Fractionation of the precipitated proteins on a DEAE-Sephacel column yielded two main peaks containing PLD-inhibiting activity peaks I, II, and III, which eluted sequentially as shown in Fig. 1A. Rho-GDI had previously been identified as an inhibitor of Rho-stimulated PLD but appeared not to inhibit Arf-stimulated PLD activity (27). Furthermore, immunoblotting with antibodies to Rho-GDI did not detect any reactive bands in these three peak fractions (data not shown).

In an effort to identify any physical property that distinguishes one inhibitory activity from the others, the kinetics of heat inactivation of the three fractions was evaluated. Upon incubation at 70 °C, peak I lost ~50% of its inhibiting activity in 3 min but retained ~40% of its activity after 30 min. However, the activities of peaks II and III increased slightly and remained at that level during the incubation period (Fig. 2A).

At 85 °C, the three inhibitor fractions lost their activities completely within 30 min but with different kinetics. Whereas peaks II and III showed slow, monophasic decreases in activity, peak I displayed a biphasic time course with a rapid inactivation phase followed by a slow one (data not shown). These results suggested that the inhibitor in peak I is most likely distinct from those in peaks II and III.

Sequential fractionation of peak I proteins on a preparative DEAE-5PW HPLC column (Fig. 1B) and a Phenyl-5PW column (Fig. 1C) yielded two inhibitory peaks, IA and IB. Both peak IA and IB proteins were chromatographed separately under identical conditions on a Heparin-5PW column. IA activity eluted as a single peak centered at fraction 30 (Fig. 1D), whereas IB activity eluted in a main peak centered at fraction 26 and two minor peaks (Fig. 1E). The kinetics of heat inactivation was followed at 70 °C using peak fraction 30 (IA) and peak fraction 26 (IB) from the Heparin-5PW column steps (Fig. 2B). More than 95% of IA activity was abolished within 1 min. Under the same conditions, IB lost rapidly only ~30% of its activity and retained ~65% of its activity after 30 min. The peak fraction from the DEAE-5PW step (mixture of IA and IB) lost ~50% of its activity in the rapid phase and retained ~35% after 30 min in a fashion similar to the result shown in Fig. 2A with the post-DEAE-Sephacel fraction I. These results suggest that the rapid phase in the inactivation of the post-DEAE Sephaloc fraction I was due largely to the inactivation of inhibitor IA. Whether the biphasic time course of IB is an intrinsic property of a single inhibitor or reflects yet another mixture of inhibitors with different heat stabilities awaits further purification of IB.

Further purification of the heat-stable peak II and III inhibitors from the DEAE-Sephacel column is underway but has not progressed enough to be reported here. Nevertheless, the two peak activities remained heat-stable through several chromatography steps (data not shown).

Purification of Heat-labile Inhibitor IA—Peak fraction 26 (IB) from the Heparin-5PW column showed a number of protein bands on SDS-polyacrylamide gels (figure not shown) and was not further purified. In contrast, SDS-PAGE analysis of 8 fractions across the peak from the Heparin-5PW column containing IA showed three broad bands with apparent molecular masses of 150, 135, and 120 kDa. The 120-kDa band and probably the 135-kDa band also appeared to comprise multiple proteins with different mobility (Fig. 1F). No other protein bands were apparent in the silver-stained gel. Furthermore, while repeating the purification procedure, we noticed that the relative abundance of the three proteins bands changed and that the inhibitory activity correlated with none of the three bands but rather with the sum of the three. This suggests that inhibitor IA activity is attributable to multiple proteins and that smaller proteins might have been derived from the 150-kDa protein by proteolysis. Indeed, when protease inhibitors were not added to buffers during the first trial of purification or when the purification time was longer, the intensity of the lower molecular mass bands became stronger (figure not shown).

Two post-Heparin-5PW column fractions, fractions 29 and 32 in Fig. 1D, were subjected to gel filtration chromatography (Fig. 3A). Fraction 29 yielded two protein peaks, one small and one large, centered at fractions with an apparent molecular mass of 300 kDa and 140 kDa, respectively. In contrast, fraction 32 yielded a large peak at the fraction corresponding to 300 kDa followed by a shoulder. For both chromatographies, the PLD-inhibiting activity profiles were parallel to protein elution profiles. SDS-PAGE analysis of the fractions across the gel filtration peaks revealed that the early peak is due to a 150-kDa protein and the late peak (or shoulder) to a mixture of 135- and 120-kDa proteins (Fig. 3B). These results clearly indicated that the inhibitory activity is associated with both the 150-kDa protein and the mixture of 135- and 120-kDa proteins. It was also apparent that the 150-kDa protein exists in a dimeric form, whereas the 135- and 120-kDa proteins exist in monomeric forms. Thus, the segment cleaved by proteolysis might be involved in dimerization.

In another preparation that proceeded swiftly, we resolved an inhibitor IA preparation into a fraction containing nearly homogeneous 150-kDa protein (>95%) and a fraction contain-
FIG. 1. Purification of PLD-inhibiting proteins from rat brain cytosol. The 0–35% ammonium sulfate precipitate from the cytosol of 400 g of rat brains was subjected to sequential chromatography on a DEAE-Sephacel column (A), a preparative HPLC DEAE-5PW column (B), a preparative HPLC Phenyl-5PW column (C), and HPLC Heparin-5PW columns (D and E). Detailed procedures are described under “Experimental Procedures.” Bars above the elution profiles indicate those inhibitor fractions that were pooled for subsequent purification or characterization. Fractions thus pooled are hereafter collectively referred to as a “peak fraction.” Peak fractions I, II, and III refer respectively to fractions 73–77, 82–88, and 94–98 of A. Peak fraction IA (fractions 27–34) from the HPLC Heparin-5PW column was subjected to SDS-PAGE on an 8% gel, and proteins were visualized by silver staining (F).
ing largely 135-kDa protein (90%) with a small amount of 120-kDa protein (10%) (Fig. 4A). These two preparations were digested separately with trypsin and subjected to a C18 column. The elution profiles were similar as shown in Fig. 4B, in agreement with the notion that the 135-kDa protein is a truncated fragment of the 150-kDa protein. At the present time, we do not know whether the 135-kDa form exists in cells or is an artifact of the purification procedure. However, the 120 kDa proteins(s) appeared to be generated during purification.

The sequences of two peptides through the first eight residues were determined to be EANAPAFD and GSVPLFWE. A search of the data base did not reveal any proteins with these sequences. Upon incubation at 70 °C, both the 150- and 135-kDa proteins rapidly lost their inhibitory activity as did the mixture of both proteins as shown in Fig. 2B.

Characterization of Inhibitor IA—In addition to Arf proteins, other small G proteins, including RhoA and Cdc42, are known to activate PLD (12, 16, 17, 25–27, 30). Our brain PLD preparation could be activated by Cdc42 and RhoA, and, in both cases, the resulting activation was diminished by inhibitor IA, as in the case of Arf-stimulated activity (Fig. 5). The Arf- and Cdc42-stimulated PLD activities were inhibited to a similar extent (~75%) by 0.5 nM inhibitor IA, whereas the same concentration of inhibitor reduced the RhoA-stimulated PLD activity by 50%.

The concentration-dependent inhibition of Arf-stimulated PLD activity by the 150-kDa inhibitor IA was studied at 3 different concentrations of Arf (Fig. 6A). Half-maximal inhibition was observed at 0.4 nM inhibitor IA (calculated based on monomeric molecular mass) in the presence of 100 nM Arf, and the inhibition profile was nearly unchanged when the Arf concentration was reduced by 20-fold. The inhibitor concentration dependence was also measured with respect to PIP2 and PC (Fig. 6, B and C). Neither PIP2 nor PC concentration variance affected the profiles of inhibitor IA concentration dependence.

**DISCUSSION**

Frequently, inhibitors identified for the enzymes acting on lipid substrates are hydrophobic molecules that interact nonspecifically with lipid vesicles. However, the inhibitory action of inhibitor IA is unlikely to be due to a hydrophobic interaction...
because the inhibitor is a cytosolic protein that elutes in very early fractions from a hydrophobic column (Fig. 1C). Another possible interaction target for the inhibitor is PIP2, which is a cofactor for the PLD reaction. A number of cytosolic proteins are known to bind PIP2 tightly and inhibit the hydrolysis of PIP2 by phospholipase C (PLC) (33). However, the concentrations (up to 5 nM) of inhibitor IA used in this study are too low to cause inhibition directly by binding PIP2 and do not interfere with the presumed binding of PIP2 to PLD (Fig. 6B). Furthermore, when the effect of inhibitor IA was studied with a phospholipase C (PLC) isoform, PLC-γ1, using substrate vesicles of lipid composition identical to that used for the PLD assay, PLC activity was not affected by inhibitor IA (data not shown).

A growing body of evidence suggests the existence of multiple PLD isoforms, and the PLD preparation used in this study is likely to contain more than one isoform of PLD (25, 26, 29). The effect of Arf and RhoA on rat brain PLD was shown to be additive. However, it is not clear whether the additivity is due to the existence of separate PLD enzymes or separate interaction sites for Arf and RhoA on the same PLD. In the former case, inhibitor IA inhibits both Arf- and RhoA-dependent enzymes. In the latter case, inhibitor IA reduces the activity of a specific PLD independently of the activator. Neither mechanism eliminates the possibility that inhibitor IA inhibits basal activity in the absence of activators like PIP2 and Smg proteins. However, we could not evaluate the effect of inhibitor IA on the basal activity, because the basal activity of our partially purified PLD preparation is too low to be measured quantitatively.

The concentration of inhibitor IA required for half-maximal inhibition is 0.4 nM, while the cellular concentration of inhibitor IA appears to be much higher than 0.4 nM, as homogeneous preparation of inhibitor IA from cytosol required only several thousandfold purification according to rough estimates. Furthermore, the interaction of inhibitor IA with PLD is not affected by the presence of activated (GTPγS-bound) Arf. Therefore, an increase in the concentration of a GTP-bound Smg protein in response to extracellular signals is not likely to be sufficient to relieve the inhibition. A specific mechanism by which receptor signaling can modulate the interaction between the inhibitor and PLD might be involved. Modulation by phosphorylation is one possibility. Considering that inhibitor IA is extremely sensitive to proteolysis, degradation of the inhibitor in response to a PLD-activating signal is also a possibility.

While our work was in progress, Geny et al. (34) reported the identification of a bovine brain cytosolic protein that inhibits Arf-dependent PLD activity associated with HL-60 cell membrane. This inhibitory protein was eluted from a Superose 12 column in the void volume which corresponds to an apparent molecular mass larger than 300 kDa. The partially purified bovine brain inhibitor was heat-stable. Thus, inhibitor IA is unlikely to be a rat homolog of the heat-stable bovine brain inhibitor. Preliminary results from gel filtration chromatography of the peak II inhibitor from the DEAE-Sepharose column (Fig. 1A) indicate that the apparent size of inhibitor II is much smaller than the peak I inhibitor.
larger than that (300 kDa) of untruncated IA. The peak II inhibitor, which was also heat-stable, is a better candidate for a rat homolog of the bovine brain inhibitor.

REFERENCES
1. Billah, M. M., and Anthes, J. C. (1990) Biochem. J. 269, 281–291
2. Cockcroft, S. (1992) Biochim. Biophys. Acta 1113, 135–160
3. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42
4. Gustavsson, L., Moehren, G., Torres-Marquez, M. E., Benistant, C., Rubin, R., and Hoek, J. B. (1994) J. Biol. Chem. 269, 849–859
5. Ahmed, A., Plevin, R., Shoaibi, M. A., Fountain, S. A., Ferriani, R. A., and Smith, S. K. (1994) J. Biol. Chem. 266, C206–C212
6. Lee, Y. H., Kim, H. S., Pai, J.-K., Ryu, S. H., and Suh, P.-G. (1994) J. Biol. Chem. 269, 26842–26847
7. Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. E., and Siegel, M. I. (1989) J. Biol. Chem. 264, 17069–17077
8. Conricode, K. M., Brewer, K. A., and Exton, J. H. (1992) J. Biol. Chem. 267, 7199–7202
9. Conricode, K. M., Smith, J. L., Burns, D. J., and Exton, J. H. (1994) FEBS Lett. 342, 149–153
10. Moehren, G., Gustavsson, L., and Hoek, J. B. (1994) J. Biol. Chem. 269, 838–848
11. Martin, T. W., and Michaelis, K. (1989) J. Biol. Chem. 264, 8847–8856
12. Brown, H. A., Gutowski, S., Mooham, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144
13. Siddiqui, R. A., and Exton, J. H. (1992) J. Biol. Chem. 267, 5755–5761
14. Huang, C., Wykle, R. L., Daniel, L. W., and Cabot, M. C. (1992) J. Biol. Chem. 267, 16859–16865
15. Kanfer, J. N., and McCartney, D. (1994) FEBS Lett. 337, 251–254
16. Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Trioung, O., and Hsuan, J. J. (1994) Science 263, 523–526
17. Masseburg, D., Han, J. S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J., and Vaughan, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11718–11722
18. Chalifa, V., Möhn, H., and Liscovitch, M. (1990) J. Biol. Chem. 265, 17512–17519
19. Möhn, H., Chalifa, V., and Liscovitch, M. (1992) J. Biol. Chem. 267, 11131–11136
20. Kiss, Z., and Anderson, W. H. (1994) Biochem. J. 300, 751–756
21. Ullings, I. J., Thompson, N. T., Randall, R. W., Spacey, G. D., Bonser, R. W., Hudson, A. T., and Garland, L. G. (1992) Biochem. J. 281, 597–600
22. Inoue, H., Shimooku, K., Akiu, E., and Nakamura, S. (1994) Biochem. Biophys. Res. Commun. 210, 542–548
23. Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) J. Biol. Chem. 270, 5130–5135
24. Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-S., and Cantley, L. (1994) J. Biol. Chem. 269, 24103–24106
25. Brown, H. A., Gutowski, S., Kahn, R. A., and Sternweis, P. C. (1995) J. Biol. Chem. 270, 14935–14943
26. Siddiqui, R. A., Smith, J. L., Ross, A. H., Qiu, R.-G., Symons, M., and Exton, J. H. (1995) J. Biol. Chem. 270, 8466–8473
27. Singer, W. D., Brown, H. A., Bokoch, G. M., and Sternweis, P. C. (1995) J. Biol. Chem. 270, 14944–14950
28. Malcolm, K. C., Ross, A. H., Qiu, R.-G., Symons, M., and Exton, J. H. (1994) J. Biol. Chem. 269, 25951–25954
29. Hammond, S. M., Altshuller, Y. M., Sung, T.-C., Rudge, S. A., Rose, K., Engelbrecht, J., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29643
30. Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1993) J. Biol. Chem. 268, 21509–21512
31. Bourgoin, S., Harbour, D., Desmarais, Y., Takai, Y., and Beaulieu, A. (1995) J. Biol. Chem. 270, 3172–3178
32. Hong, J.-X., Haun, R. S., Tsai, S.-C., Moss, J., and Vaughan, M. (1994) J. Biol. Chem. 269, 9743–9745
33. Lee, S. B., and Rhee, S. G. (1995) Curr. Opin. Cell Biol. 7, 183–189
34. Geny, B., Paris, S., Dubois, T., Franco, M., Lukowski, S., Chardin, P., and Russo-Marie, F. (1995) Eur. J. Biochem. 231, 31–39

Fig. 6. Effect of changes in the concentration of Arf, PIP2, or PC on PLD inhibition by the 150-kDa inhibitor IA. Concentration dependence of PLD inhibition by the 150-kDa inhibitor IA was measured in the presence of three different concentrations of Arf (A) and four different concentrations of PIP2 (B) or PC (C). PLD activity profiles were determined as described under “Experimental Procedures” for the case of fixed concentrations of Arf, PIP2, or PC.
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