An activator of rat brain phospholipase D (PLD) that is distinct from the already identified PLD activator, ADP-ribosylation factor (ARF), was partially purified from bovine brain cytosol by a series of chromatographic steps. The partially purified preparation contained a 22-kDa substrate for Clostridium botulinum C3 exoenzyme ADP-ribosyltransferase, which strongly reacted with anti-rho p21 antibody, but not with anti-rac1 p21 or anti-cdc42Hs p21 antibody. Treatment of the partially purified PLD-activating factor with both C3 exoenzyme and NAD significantly inhibited the PLD-stimulating activity. These results suggest that rho p21 is, at least in part, responsible for the PLD-stimulating activity in the preparation. Recombinant isoprenylated rhoA p21 expressed in and purified from Sf9 cells activated rat brain PLD in a concentration- and GTPγS (guanosine 5′-O-(3-thiotriphosphate))-dependent manner. In contrast, recombinant non-isoprenylated rhoA p21 (fused to glutathione S-transferase) expressed in Escherichia coli failed to activate the PLD. This difference cannot be explained by a lower affinity of non-isoprenylated rhoA p21 for GTPγS, as the rates of [35S]GTPγS binding were very similar for both recombinant preparations and the GTPγS-bound form of non-isoprenylated rhoA p21 did not induce PLD activation. Interestingly, recombinant isoprenylated rhoA p21 and ARF synergistically activated rat brain PLD; a similar pattern was seen with the partially purified PLD-activating factor. The synergistic activation was inhibited by C3 exoenzyme-catalyzed ADP-ribosylation of recombinant isoprenylated rhoA p21 in a NAD-dependent manner. Inhibition correlated with the extent of ADP-ribosylation. These findings suggest that rhoA p21 regulates rat brain PLD in concert with ARF, and that isoprenylation of rhoA p21 is essential for PLD regulation in vitro.

**Phospholipase D (PLD)** catalyzes the hydrolysis primarily of phosphatidylcholine (PC) to produce choline and phosphatic acid; PET, phosphatidylethanol; small G proteins, low molecular weight GTP-binding proteins; ARF, ADP-ribosylation factor; C3 exoenzyme, Clostridium botulinum C3 exoenzyme; C3 ADP-ribosyltransferase; GTPγS, guanosine 5′-O-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.
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EXPERIMENTAL PROCEDURES

Materials—1,2-Dipalmitoyl phosphatidylcholine (DPPC), phosphatidyl-
diniositol (PI), and phosphatidylethanolamine (PE) from egg were obtained from Avanti, and streptolysin O was from Eiken Chemical Co. (Tokyo, Japan). Guanosine 5'-O-(3-thiotriphosphate) (GTP S) was purchased from Boehringer Mannheim, phosphatidylinositol 4,5-bisphosphate (PIP2), and NAD were from Sigma, and choline methyl-h-125DPPC (labeled-h-125DPPC), [3H]-palmitoyl-9,10-125DPPC, [35S]GTP, [3H]NAD, and [3H]ADP-ribosylation of recombinant ARF (2) were obtained from Du Pont NEN. Affinity-purified rabbit polyclonal anti-rhoA p21, anti-rac p21, and anti-cdc42Hs p21 antibodies were obtained from Santa Cruz Biotechnology. Anti-rhoA p21 antibody was raised against a synthetic peptide corresponding to amino acids 119-132 of rhoA p21, and specifically recognizes rhoA p21, but not rhoB p21, rhoC p21, or other members of the ras p21 supergene family. Anti-rac p21 antibody was raised against a peptide mapping within the carboxy-terminal domain of the predicted human rac gene product, and anti-cdc42Hs p21 antibody against a peptide corresponding to amino acid residues 546-562 of the predicted human cdc42h gene product; these antibodies specifically react with rac p21 and cdc42Hs p21, respectively. Recombinant C3 exoenzyme was prepared as described (12). PEt, as an internal standard, was prepared as reported (3). Isoprenylated rhoA p21 homopolymers and non-isoprenylated rhoA p21 fused to glutathione S-transferase were expressed in and purified from Sf9 cells and Escherichia coli, respectively, as already published (13, 14).

Partial Purification of PLD and ARF—ARF-sensitive PLD was partially purified from rat brain membranes by a method based on that of Brown et al. (8). Rat brain was homogenized in TEDP (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.3% phenylmethylsulfonat fluoride (PMSF)), filtered through two layers of cheesecloth, and centrifuged at 20,000 x g for 30 min. To extract the PLD, the crude membranes (5 mg of protein/ml) were incubated for 1 h at 4°C with 0.6 mM NaCl in 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 10 mM EDTA, 5 mM DTT, and 0.1 mM PMSF. After centrifugation at 100,000 x g for 30 min, the clear supernatant was diluted 3-fold with 20 mM Na-Hepes, pH 7.5, 10 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (HEDP), and subjected to heparin-Sepharose CL-6B column chromatography. The column was washed, and then PLD was eluted with a linear gradient of 0.3-2 mM NaCl in HEDP. The ARF-sensitive PLD activity was eluted as a single peak at 1.1 mM NaCl. This partially purified PLD preparation was used as rat brain PLD in this study.

ARF was partially purified from bovine brain cytosol. Briefly, bovine brain was homogenized, filtered, and centrifuged as described above with rat brain. Proteins from the cytosol fraction were precipitated with ammonium sulfate at 25-70% saturation and dissolved in TEDP. After dialysis against TEDP, the proteins were subjected to DEAE-Sephacel chromatography on Mono Q resulted in elution of both the PLD-stimulating activity and the C3 exoenzyme substrate as peaks (Fig. 1). The major, second peak of PLD-stimulating activity from the Mono Q column contained a 22-kDa protein assessed by SDS-PAGE (Fig. 2A). This protein strongly reacted with anti-rhoA p21 antibody, but not with anti-rac p21 or anti-cdc42Hs p21 antibody (Fig. 2B). The PLD-stimulating activity of the partially purified factor was suppressed upon incubation with both NAD and C3 exoenzyme, which specifically
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**FIG. 1.** Mono Q column chromatography of the PLD-activating factor of bovine brain cytosol. The PLD-activating factor that is distinct from ARF was partially purified by DEAE-Toyopearl 650S, phenyl-Sepharose CL-4B, and TSK-GEL 3000 SW XL column chromatography (as described under “Experimental Procedures”). Fractions containing the PLD-stimulating activity eluted from TSK-GEL 3000 SW XL column were subjected to Mono Q column chromatography. Each fraction was reconstituted with the partially purified rat brain PLD in the presence of 40 μM GTPγS. After incubation at 37 °C for 1 h, the PLD activity (●) was determined by measuring [3H]choline formation (as described under “Experimental Procedures”). C3 exoenzyme (○) or with 40 μM GTPγS (●) in the presence of 1% ethanol. [3H]PEt formation over 1 h was analyzed as described under “Experimental Procedures.”

**FIG. 2.** Protein staining and immunoblotting of the partially purified preparation of PLD-activating factor probed with anti-rhoA p21, anti-rac1 p21, and anti-cdc42Hs p21 antibodies. Proteins in fraction 34 of the Mono Q column eluate containing the PLD-stimulating activity were analyzed by SDS-PAGE on 15% gels and Coomassie Brilliant Blue (CBB) staining (A). Immunoblots of the partially purified preparation of PLD-activating factor were probed with anti-rhoA p21, anti-rac1 p21, and anti-cdc42Hs p21 antibodies (B), as described under “Experimental Procedures.”

ADP-ribosylates and inhibits rho proteins (Fig. 3); no suppression occurred with either agent alone. These results, taken together, imply that rhoA p21 is an activator of rat brain PLD, in agreement with the finding of Malcolm et al. (11).

To confirm this hypothesis, isoprenylated rhoA p21 was expressed in and purified from Sf9 cells (13), and reconstituted with partially purified rat brain PLD. As shown in Fig. 4A, recombinant isoprenylated rhoA p21 activated in a concentration-dependent manner the rat brain PLD in the presence of GTPγS, but not in its absence. In contrast, non-isoprenylated rhoA p21, expressed in and purified from E. coli, failed to activate PLD (Fig. 4B). Recombinant isoprenylated and non-isoprenylated rhoA p21s both bound [35S]GTPγS with similar kinetics (Fig. 5A). Furthermore, recombinant non-isoprenylated rhoA that had been preloaded with GTPγS was also unable to effect PLD activation (Fig. 5B). These findings rule out a difference in binding of GTPγS as the explanation for the difference in PLD activation between the two recombinant forms of rhoA p21. The results further indicate that rhoA p21 directly or indirectly activates rat brain PLD, and that post-translational modification of rhoA p21, like ARF (8, 9), is essential for it to function as a PLD activator.

Whereas the PLD in rat liver membranes is activated by rhoA p21 but insensitive to ARF (11), rat brain PLD was activated by ARF (Fig. 6) as well as by rhoA p21 (Figs. 4 and 6). When the partially purified PLD-activating factor or recombinant isoprenylated rhoA p21 was reconstituted together with ARF, synergistic activation of rat brain PLD was observed (Fig. 6). Consistent with the result illustrated in Fig. 3, incubation of recombinant isoprenylated rhoA p21 with C3 exoenzyme plus a series of concentrations of NAD attenuated the synergistic activation of PLD in a NAD concentration-dependent manner (Fig. 7). The degree of inhibition correlated well with the extent of ADP-ribosylation of recombinant isoprenylated rhoA p21 by the C3 exoenzyme (Fig. 7).

**DISCUSSION**

Malcolm et al. (11) have recently reported that rhoA p21 activates rat liver PLD. We have shown in the present study that rat brain PLD is also activated by rhoA p21 (Fig. 4A). The mechanism for activation of rat brain PLD by rhoA p21, however, seems to be different from that of rat liver PLD. In
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Fig. 5. [35S]GTP•S binding to recombinant rhoA p21s and effects of the GTP•S-bound form of recombinant rhoA p21s on rat brain PLD activity. A, recombinant isoprenylated (C) or non-isoprenylated rhoA p21 (D) (10 nm) was incubated for the indicated time at 37°C with 1 μM [35S]GTP•S. [35S]GTP•S bound to rhoA p21s was determined as described under “Experimental Procedures.” B, a series of concentrations of recombinant isoprenylated (C) or non-isoprenylated rhoA p21 (D) were incubated for 5 min at 37°C with 80 μM GTP•S to effect preloading, and reconstituted with rat brain PLD. [3H]IPet production during a 1-h incubation at 37°C was determined. Bars represent differences between duplicate determinations in a typical study.

Fig. 6. Synergistic activation of rat brain PLD by ARF and the partially purified PLD-activating factor or isoprenylated rhoA p21. Partially purified rat brain PLD was reconstituted without (C) or with (●) 1 μM of the PLD-activating fraction obtained from the DEAE-Toyopearl 650S column (A) or 10 nm recombinant isoprenylated rhoA p21 (B) in the presence of the indicated concentrations of ARF. After incubation for 1 h at 37°C in the presence of 40 μM GTP•S and 1% ethanol, [3H]IPet formation was assessed (as described under “Experimental Procedures”). Bars represent differences between duplicate determinations in a typical study.

In particular, ADP-ribosylation of rhoA p21 by C3 exoenzyme perturbs the ability of rhoA p21 to activate rat brain PLD (Figs. 3 and 7), whereas rat liver PLD is still activated by ADP-ribosylated rhoA p21 (11).

Another difference between rat brain PLD (our observations) and rat liver PLD (data from Malcolm et al.) is the sensitivity of the PLD(s) to ARF; we found rat brain PLD to be activated by ARF (Fig. 6), whereas rat liver PLD is insensitive to ARF (11). This supports the concept that the isoforms of PLD in rat liver and brain are distinct. A possible alternative is that rat brain contains two isoforms of PLD, one of which is ARF-sensitive and another rhoA p21-sensitive, whereas rat liver contains only the rhoA p21-sensitive PLD. However, this is unlikely. If there were two isoforms of PLD in rat brain, the activation of rat brain PLDs by ARF plus rhoA p21 would be expected to be additive. On the other hand, if rat brain PLD (considered a distinct isoform from that in rat liver) were regulated by both ARF and rhoA p21 by different mechanisms, activation by these agents might be synergistic. The results of the present study provide strong support for the latter possibility, as recombinant isoprenylated rhoA p21 and ARF activated rat brain PLD in a synergistic manner (Fig. 6). Thus, it is likely that rat brain PLD differs from rat liver PLD. Very recently, Siddiqi et al. (18) and Singer et al. (19) have reported that PLDs in membranes of HL-60 cells and porcine brain, respectively, are activated synergistically by ARF and rhoA p21. Their data suggest that the PLDs in membranes of HL-60 cells and porcine brain are analogous to that in rat brain membranes.

The PLD-stimulating activity partially purified from bovine brain cytosol was inhibited only incompletely (by about 50%) on ADP-ribosylation of rhoA p21 by C3 exoenzyme and NAD (Fig. 3). Although rac1 p21 and cdC247s p21 were not detected in this preparation (Fig. 2B), it is still possible that another C3 exoenzyme-insensitive PLD activator(s) contaminated the preparation. It has not proven practicable to assess whether exhaustive ADP-ribosylation of the preparation results in complete inhibition, because prolonged incubation (more than 20 min at 30°C) of the partially purified PLD-activating factor drastically diminishes its activity even in the absence of C3 exoenzyme and NAD. Nevertheless, the results obtained (Fig. 3) imply that rhoA p21 is responsible, at least in part, for the PLD-stimulating activity of the preparation. Perturbation by C3 exoenzyme-catalyzed ADP-ribosylation of the ability of rhoA p21 to modulate rat brain PLD activity was clearly demonstrated in the studies employing recombinant rhoA p21 (Fig. 7).

Although the rate of ADP-ribosylation of partially purified bovine brain rhoA was very slow (reaching a plateau after 60–120 min of incubation), ADP-ribosylation of the recombinant isoprenylated rhoA reached a maximum within 5 min under the conditions employed in Fig. 7 (data not shown). Taking advantage of this characteristic of the recombinant rhoA, we were able to demonstrate that the extent of ADP-ribosylation of rhoA correlated well with the degree of inhibition of the synergistic activation of PLD by rhoA in concert with ARF (Fig. 7).

Synergistic activation of PLD by rhoA p21 and ARF has also been found with permeabilized, cytosol-depleted rabbit neutro-
phils, suggesting that rabbit neutrophil PLD is regulated by the same (or similar) mechanism as rat brain PLD. Lambeth et al. and Bourgoin et al. have recently reported that ARF functions in concert with a 50-kDa cytosolic factor in the activation of PLDs in human neutrophils and HL-60 cells, respectively (20, 21). In their studies, the molecular weight of the cytosolic factor was estimated by gel filtration chromatography. In the present study, the PLD-stimulating activity and C3 exoenzyme substrate in the bovine brain cytosol co-chromatographed on gel filtration at a position corresponding to ~45 kDa (data not shown). It has been established that rhoA p21 associates with a Rho GTP/GDP exchange inhibitor, Rho GDI, with a molecular mass of 27 kDa (22). Thus the complex of rhoA p21 with Rho GDI should elute from the chromatograph with an apparent molecular mass of ~50 kDa. Bourgoin et al. (21) found that rhoA p21 eluted with an apparent molecular mass of ~45 kDa, but concluded that it is not the 50-kDa PLD-activating factor of HL-60 cells as they failed to detect rhoA p21 in several PLD-activating fractions. Although the ~50-kDa PLD-activating factor(s) in HL-60 cells and human neutrophils remains to be identified, PLD in these cells may be regulated differently from that in rat brain. Thus, the mechanism of PLD activation may vary between one isoform of PLD and another.

Another finding in this study was that, like ARF, rhoA p21 required post-translational modification to effect its regulation of PLD: non-isoprenylated rhoA p21 failed to activate rat brain PLD, even in its GTPγS-bound state (Figs. 4 and 5B). It has been reported that GTP-bound isoprenylated rhoA p21, but not non-isoprenylated rhoA p21, translocates to plasma membranes (23). Consistent with the latter report, rhoA p21 (iso- prenylated) in the partially purified preparation of PLD-activating factor bound to phospholipids vesicles containing the PLD substrate, PC, only in the presence of GTPγS, whereas non-isoprenylated rhoA p21 did not bind under any circumstances (data not shown). This result leads us to speculate that the GTPγS-bound isoprenylated rhoA p21 also translocates to the phospholipid vesicles, and thereby attracts PLD to the vesicles, making the enzyme more accessible to its substrate and increasing hydrolysis. Alternatively, PLD might interact specifically with PIP2 in the vesicles and the GTPγS-bound isoprenylated rhoA p21 translocate to the vesicles to interact with PLD. The latter notion is based on the finding that translocation of isoprenylated rhoA p21 to the vesicles does not require the presence of PIP2, whereas PIP2 is essential for PLD activity (8).2 We are currently investigating whether PLD interacts specifically with PIP2.

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