

# Activation of rat brain phospholipase D by ADP-ribosylation factors 1, 5, and 6: Separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes

(dipalmitoylphosphatidylcholine/transphosphatidylolation/guanine nucleotide-binding protein/cholera toxin)

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Contributed by Martha Vaughan, August 8, 1994

**ABSTRACT** Two major forms of phospholipase D (PLD) activity, solubilized from rat brain membranes with Triton X-100, were separated by HPLC on a heparin-5PW column with buffer containing octyl glucoside. One form was completely dependent on sodium oleate for activity. The other, which was dramatically activated by the addition of ADP-ribosylation factor (ARF) 1 and guanine 5' [ $\gamma$ -thio]triphosphate, required the presence of phosphatidylinositol 4,5-bisphosphate in the phosphatidylcholine substrate for demonstration of activity, as described by others. Oleate-dependent activity was unaffected by guanine 5' [ $\gamma$ -thio]triphosphate, or phosphatidylinositol 4,5-bisphosphate. Both sodium oleate- and ARF-dependent activities catalyzed transphosphatidylolation, thus identifying them as PLDs. ARF-dependent PLD was activated by recombinant ARF5 (class II) and ARF6 (class III), as well as ARF1 (class I). Myristoylated recombinant ARFs were more effective than their nonmyristoylated counterparts. ARFs were originally identified as activators of cholera toxin ADP-ribosyltransferase activity. The effects of recombinant ARF proteins from the three classes on cholera toxin activity (assayed under conditions identical to those used to assay PLD activity) did not, however, correlate with those on PLD, consistent with the notion that different aspects of ARF structure are involved in the two functions.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PtdCho), producing phosphatidic acid (PtdOH) and choline (for review, see ref. 1). PtdOH is a central metabolite in both phospholipid and triglyceride metabolism and an effector in several physiological processes including secretion, DNA synthesis, and cell proliferation. PtdOH is metabolized by PtdOH phosphohydrolase to diacylglycerol, which can activate protein kinase C. Thus, PLD action generates not only PtdOH, a molecule with potential second-messenger functions, but also the well known second-messenger diacylglycerol, and is a major source of both. PLD activity was first identified in plants (2) and then in bacteria and fungi (3). Its presence in mammalian tissues was reported in 1973 (4). PtdCho-preferring PLD has been found in numerous tissues and cells—including brain, lung, liver, adipose tissue, endothelial cells, HL-60 cells, and spermatozoa; lung and brain are the richest sources (5–15).

Membrane-associated PLD activities appear specific for PtdCho (16–18), whereas cytosolic forms hydrolyze phosphatidylethanolamine (PtdEtn) and phosphatidylinositol (PtdIns), as well as PtdCho (17, 18). PLD activities partially purified from rat brain (19) and HL-60 cells (20) were associated primarily with particulate fractions. PLD has not been purified to homogeneity, and its subcellular localization

remains to be definitively identified. It is possible that the enzyme can be present in membranes as well as in the cytosol, as shown for phospholipase C isozymes (21). Lis-covitch and coworkers (12, 22) described membrane-bound PLD activity that was absolutely dependent on sodium oleate and was markedly activated by Mg<sup>2+</sup> and Ca<sup>2+</sup>. Under those assay conditions with exogenous PtdCho as a substrate, activity of the membrane-bound PLD was not affected by the addition of GTP analogues (12, 18), which activate PLD in other systems.

Activation of PLD by guanine nucleotide-binding cytosolic protein(s) was suggested in studies that showed stimulation by guanosine 5' [ $\gamma$ -thio]triphosphate (GTP[ $\gamma$ S]) of PLD activity in liver plasma membranes (6, 23) and HL-60 cells (14, 15). The cytosolic factors were purified and identified as ADP-ribosylation factor 1 (ARF1) and/or ARF3, which stimulated PLD activity from HL-60 cell membranes in the presence of GTP[ $\gamma$ S] (20, 24). These ARFs are  $\approx$ 20-kDa proteins initially identified as activators of cholera toxin ADP-ribosyltransferase activity (25) and are now known to play a critical role in vesicular membrane trafficking in all eukaryotic cells (26). We report here the separation, after solubilization from rat brain membranes, of two forms of PLD designated as oleate-dependent and ARF-dependent and describe some of their characteristics, including activation by three different mammalian ARF proteins.

## EXPERIMENTAL PROCEDURES

**Materials.** Rat brains were purchased from Bioproducts, Inc., or Pel-Freez Biologicals and stored at  $-70^{\circ}\text{C}$  until use. L- $\alpha$ -Dipalmitoylphosphatidylcholine [(Pam)<sub>2</sub>PtdCho] was purchased from Calbiochem; phosphatidylethanolamine (PtdEtn) and phosphatidylserine were from Avanti Polar Lipids; GTP[ $\gamma$ S] and L- $\alpha$ -phosphatidyl-D-*myo*-inositol 4,5-bisphosphate (PtdInsP<sub>2</sub>) were from Boehringer Mannheim; [choline-methyl-<sup>3</sup>H](Pam)<sub>2</sub>PtdCho (50 Ci/mmol; 1 Ci = 37 GBq) and [2-palmitoyl-9,10-<sup>3</sup>H](Pam)<sub>2</sub>PtdCho (42 Ci/mmol) were from DuPont/NEN; L- $\alpha$ -dioleoylphosphatidyl[2-<sup>14</sup>C]ethanolamine (54 mCi/mmol), L- $\alpha$ -dioleoylphosphatidyl-L-[3-<sup>14</sup>C]serine (53 mCi/mmol), and [adenine-<sup>14</sup>C]NAD (280 mCi/mmol) were from Amersham. Sources of other materials have been published (27).

Abbreviations: ARF, ADP-ribosylation factor; rARF, recombinant ARF; mARF, myristoylated rARF; PLD, phospholipase D; PtdCho, phosphatidylcholine; (Pam)<sub>2</sub>PtdCho, L- $\alpha$ -dipalmitoylphosphatidylcholine; PtdOH, phosphatidic acid; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdInsP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PtdEtnOH, phosphatidylethanol; GTP[ $\gamma$ S], guanosine 5' [ $\gamma$ -thio]triphosphate; CTA, cholera toxin A subunit.

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**Chromatography of Rat Brain PLD.** All procedures were done at 4°C. Rat brains were homogenized in 10 vol (wt/vol) of homogenization buffer (20 mM Hepes, pH 7.0/1 mM EGTA/1 mM EDTA/0.1 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride) two or three times for 1 min at maximum speed with a Polytron homogenizer (Brinkmann). The homogenate was centrifuged (1000 × *g*, 20 min), and the supernatant was centrifuged (100,000 × *g*, 1 hr). Pelleted membranes from this centrifugation were extracted for 1 hr with homogenization buffer containing 0.5% Triton X-100 (volume equal to twice the initial weight of tissue, ml/g). Insoluble material was discarded after centrifugation (100,000 × *g*, 1 hr) leaving solubilized membrane proteins (8–10 mg/ml).

Solubilized proteins (4 g) from 200 g of rat brain were applied (flow rate, 20 ml/min) to a column (50 × 150 mm) of heparin-Sepharose CL-6B (Pharmacia) equilibrated in 20 mM Hepes, pH 7.0/1 mM EGTA/1 mM EDTA/0.1 mM dithiothreitol/0.7% octyl-β-D-glucopyranoside followed by 400 ml of equilibration buffer. Proteins were eluted with a linear gradient of 0–1.5 M NaCl in a total volume of 2 liters of equilibration buffer (20 ml/min). Fractions (20 ml) were collected, and samples (5 μl) were assayed for ARF-dependent and oleate-dependent hydrolysis of PtdCho (Fig. 1A).

Complete separation of ARF-dependent and oleate-dependent activities by HPLC was achieved by applying 30 mg of solubilized membrane proteins to a Tosohaas heparin-5PW column (7.5 × 75 mm) equilibrated in 20 mM Hepes, pH 7.0/1 mM EGTA/1 mM EDTA/0.1 mM dithiothreitol/0.7% octyl-β-D-glucopyranoside. Proteins were eluted (flow rate, 1 ml/min) with equilibration buffer for 10 min, followed by a linear gradient from 0 to 0.5 M NaCl in 15 min, 0.5 M NaCl for a further 20 min, a second linear gradient from 0.5 to 1 M NaCl in 5 min, and equilibration buffer containing 1 M NaCl for 30 min. Fractions (1 ml) were collected and assayed for ARF-dependent and oleate-dependent activities (Fig. 1B).

**PLD Assays.** For experiments shown in Figs. 1 and 2, ARF-dependent PtdCho hydrolysis was determined by using an assay recently described by Brown *et al.* (20) with slight modifications. Briefly, 25 μl of mixed lipid vesicles (PtdEtn/PtdInsP<sub>2</sub>/(Pam)<sub>2</sub>PtdCho, molar ratio 16:1.4:1) with [*choline-methyl*-<sup>3</sup>H](Pam)<sub>2</sub>PtdCho to yield ≈200,000 cpm per assay was added to 5 μl of enzyme extract in a total of 125 μl containing 50 mM Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl<sub>2</sub>, and 300 nM of free calcium. Assays were done without or with 10 μM GTP[γS] and 1.67 μM myristoylated ARF1 (mARF1, 5 μg per assay). The total amount of (Pam)<sub>2</sub>PtdCho was 0.51 nmol per assay. Assays were incubated at 37°C for 1 hr before addition of 1 ml of CHCl<sub>3</sub>/CH<sub>3</sub>OH/concentrated HCl, 50:50:0.3 (vol/vol), and 0.35 ml of 1 M HCl/5 mM EGTA. After centrifugation (5 min, 3000 rpm, 4°C, Sorvall RC3B, H2000 B swinging bucket rotor), <sup>3</sup>H in 0.5 ml of the aqueous phase was quantified by liquid scintillation spectroscopy.

Oleate-dependent activity was assayed by a slight modification of a published procedure (12). (Pam)<sub>2</sub>PtdCho vesicles (25 μl) containing 10 nmol of (Pam)<sub>2</sub>PtdCho and ≈200,000 cpm of [*choline-methyl*-<sup>3</sup>H](Pam)<sub>2</sub>PtdCho were added to 5 μl of enzyme in a total volume of 175 μl containing 50 mM Hepes (pH 7.0), 2 mM EGTA, 4 mM sodium oleate, 500 mM KCl, 4 mM MgCl<sub>2</sub>, and 0.1 mM free calcium. Reaction was done as described for ARF-dependent PLD, except the incubation temperature was 30°C, and only 0.30 ml of 1 M HCl/5 mM EGTA was added at termination.

Transphosphatidylase assays of ARF- or oleate-dependent PLD were done under the same conditions used for PtdCho hydrolysis, except that the radiolabeled substrate was replaced with 0.5% ethanol (vol/vol) and 1 or 2 μCi, respectively, of [*2-palmitoyl*-9,10-<sup>3</sup>H](Pam)<sub>2</sub>PtdCho. After

phase separation, 400 μl of organic phase containing [<sup>3</sup>H]-PtdEtOH was dried down (vacuum speed centrifugation), and the lipids, suspended in 20 μl of CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH, 65:15:5 (vol/vol), were applied to thin-layer silica gel plates (silica gel 60 F-254; Merck). The plates were developed in the same solvent, dried completely, and sprayed twice with En<sup>3</sup>Hance (NEN) before autoradiography.

For experiments of Figs. 3 and 4, PLD assays contained the components described by Brown *et al.* (20) plus those for assay of cholera toxin NAD:agmatine ADP-ribosyltransferase activity. Substrate was prepared by drying down a mixture of PtdEtn, PtdInsP<sub>2</sub>, and (Pam)<sub>2</sub>PtdCho, 16:1.4:1 with 0.2 μCi of [*choline-methyl*-<sup>3</sup>H](Pam)<sub>2</sub>PtdCho per 0.51 nmol of (Pam)<sub>2</sub>PtdCho under N<sub>2</sub>. The lipid was then suspended in 50 mM Hepes, pH 7.5/80 mM KCl/3 mM EGTA/1 mM dithiothreitol by sonification (two or three times for 1 min at a power setting of 5–6 on a W-385 sonicator using a cup-type probe; Heat Systems, Ultrasonics, Farmingdale, NY). To initiate assays, 25 μl of 250 mM Hepes, pH 7.5/400 mM KCl/15 mM EGTA/15 mM MgCl<sub>2</sub>/10 mM CaCl<sub>2</sub> was added to 12 × 75-mm glass tubes kept in an ice bath, followed by cholera toxin A subunit (CTA) (1 μg in 10 μl) and ARF in 60 μl of buffer B (20 mM Tris-HCl, pH 8.0/0.25 M sucrose/100 mM NaCl/5 mM MgCl<sub>2</sub>/2 mM dithiothreitol/1 mM EDTA/1 mM NaN<sub>3</sub>). Five microliters of stock PLD, which was diluted with 5 μl of 50 mM Hepes, pH 7.5/80 mM KCl/3 mM EGTA/3 mM MgCl<sub>2</sub>/2 mM CaCl<sub>2</sub>, was added; then 25 μl of PLD substrate was added, and finally, 20 μl of a solution containing 1.5 mM NAD, 75 mM agmatine, 7.5 mM dithiothreitol, and 750 μM GTP[γS] was added. Typically, assays contained 0.51 nmol of (Pam)<sub>2</sub>PtdCho, 53 mM Hepes (pH 7.5), 85 mM KCl, 3.2 mM EGTA, 4.7 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 8 mM Tris, 0.1 M sucrose, 40 mM NaCl, 1.0 mM dithiothreitol, 0.4 mM EDTA, 0.4 mM NaN<sub>3</sub>, 100 μM GTP[γS], 10 mM agmatine, 200 μM NAD, and 0.023% 1-octyl-β-D-glucopyranoside in a total volume of 150 μl (final pH 7.5). After incubation at 37°C as indicated, tubes were transferred to an ice bath, and 1 ml of chloroform/methanol/concentrated HCl, 50:50:0.3 (vol/vol), was added followed by 0.3 ml of 1 M HCl/5 mM EGTA. Tubes were centrifuged as described above and 500-μl samples of supernatant were collected for radioassay.

**Preparation of Recombinant ARF (rARF) Proteins.** rARF1 (28) and rARF5 and mARF5 (29) were prepared and purified by published procedures, except that lysozyme (10 mg/ml) and sonification were used to disrupt the *Escherichia coli*. rARF6 was prepared as described by Price *et al.* (30), except that the ARF6 cDNA was subcloned into the pT7/Nde expression vector, as described by Hong *et al.* (28). Coexpression of plasmids containing human ARF-coding sequences with a vector containing *N*-myristoyltransferase (pAcYC177/ET3d/yNMT) (29) produced myristoylated forms of rARF1, rARF5, and rARF6, designated mARF1, mARF5, and mARF6, respectively. The cytosol was applied to a column of Ultrogel Aca 54 (Sepracore, Marlborough, MA), which was eluted with buffer B. Fractions containing ARF activity were pooled and stored at –20°C. Protein concentrations were determined by the Bio-Rad assay. ARF preparations were usually >90% pure, as judged by Coomassie staining of samples (≈5–10 μg) after SDS/PAGE.

## RESULTS AND DISCUSSION

**Separation of Rat Brain Oleate- and ARF-Dependent PLD Activities.** When the Triton X-100 extract of rat brain membranes was fractionated on a heparin-Sepharose column, activity that was eluted at 0.4 M NaCl (peak I) was identified in assays with 4 mM sodium oleate. Additional PLD activity (peak II) that could not be detected in the presence of oleate

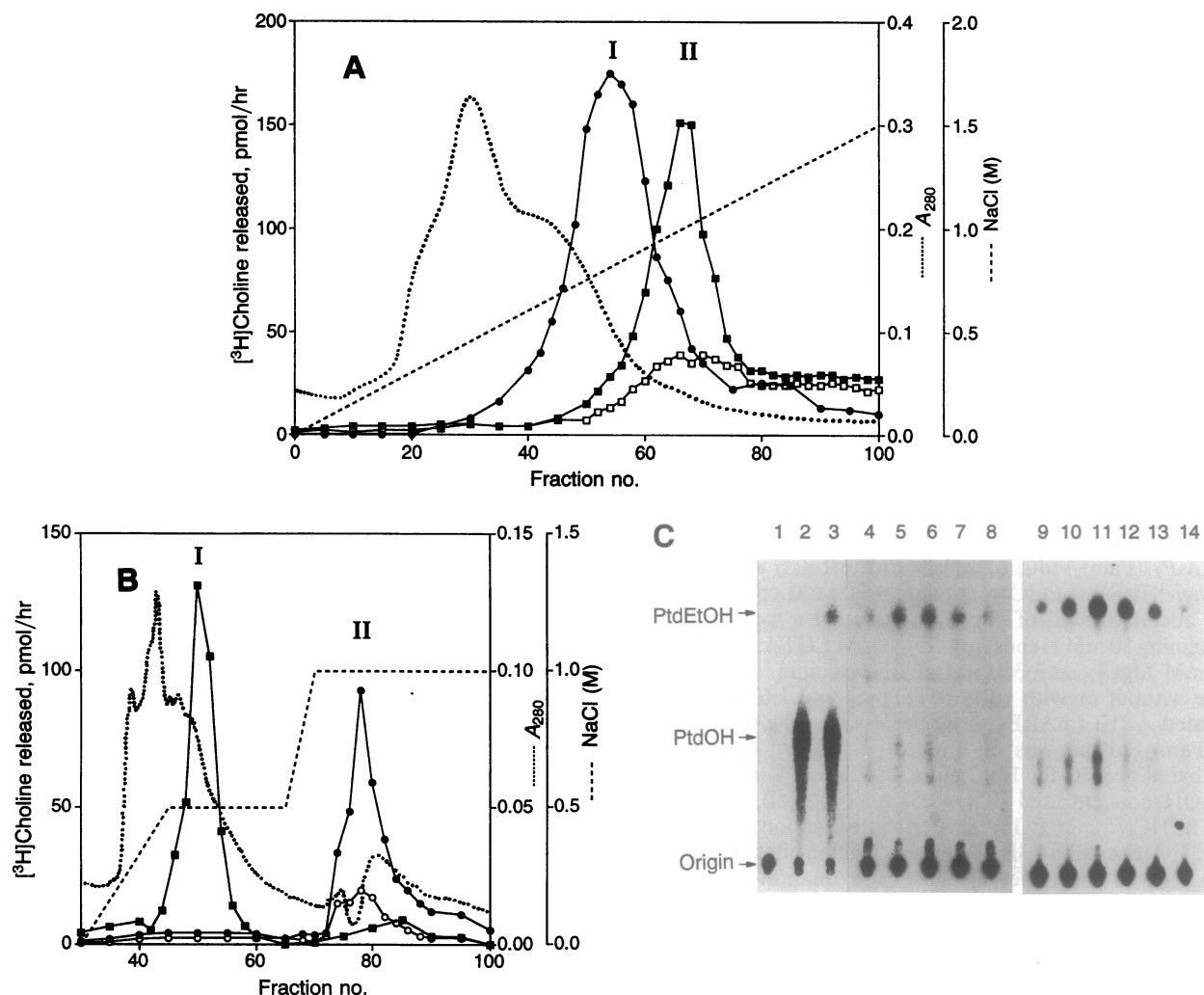
was found with assays containing mARF1 and GTP[ $\gamma$ S] (Fig. 1A). These two activities, which are referred to as "oleate-dependent PLD" and "ARF-dependent PLD," were separated completely using the heparin-5PW HPLC with combined linear and stepwise gradients of NaCl (Fig. 1B).

To confirm that both oleate- and ARF-dependent activities were due to PLD, phosphatidylethanol (PtdEtOH) formation by the two forms of PLD under their optimal assay conditions was monitored. In fractions from heparin-5PW HPLC, PtdEtOH generation corresponded with both peaks of PLD activity identified in the [ $^3$ H]choline release assays (Fig. 1C). PtdEtOH and PtdOH are, respectively, the products of the transphosphatidylation and hydrolytic reactions catalyzed by PLD. Because PtdEtOH is exclusively a product of PLD (31–33) and is relatively stable metabolically (32, 33), it is a reliable indicator of PLD activity, whereas release of  $^3$ H from [methyl- $^3$ H]phosphatidylcholine in a water-soluble form could result also from phospholipase C activity.

**Properties of Rat Brain Oleate- and ARF-Dependent PLD Activities.** Oleate-dependent PLD activity was maximal with 4 mM sodium oleate but was  $\approx$ 50% less with 8 mM; 0.3 mM sodium oleate completely inhibited ARF-dependent PLD (Fig. 2A).

mARF1 did not affect the activity of oleate-dependent PLD in the presence of 10  $\mu$ M GTP[ $\gamma$ S], whereas activation of ARF-dependent PLD was proportional to mARF1 concentration with maximal activity at 1.4–2  $\mu$ M mARF1 in the presence of 10  $\mu$ M GTP[ $\gamma$ S] (Fig. 2B). In the presence of 1.67  $\mu$ M mARF1, the activity of oleate-dependent PLD was not affected by GTP[ $\gamma$ S], whereas GTP[ $\gamma$ S] stimulated ARF-dependent PLD activity, with maximal activation at 10–20  $\mu$ M and half-maximal at 4  $\mu$ M GTP[ $\gamma$ S] (Fig. 2C).

As reported by Brown *et al.* (20), PtdInsP<sub>2</sub> was required for ARF stimulation of PLD activity, which was maximal with  $\approx$ 8  $\mu$ M. Higher concentrations were inhibitory; with 16  $\mu$ M PtdInsP<sub>2</sub>, ARF-dependent PLD activity was negligible (Fig.



**FIG. 1.** Triton X-100 extract of rat brain chromatography on heparin-Sepharose CL-6B and heparin-5PW. (A) Triton X-100 extract of rat brain (4 g of proteins in 400 ml) was applied to a heparin-Sepharose CL-6B column, which was eluted with a linear gradient of NaCl (0–1.5 M) in 20 mM Hepes buffer, pH 7.0/0.7% octylglucoside (flow rate 20 ml/min); 20-ml fractions were collected. Peak I (oleate-dependent, ●) and peak II (ARF-dependent) activities assayed without (□) or with 5  $\mu$ g of mARF1 and 10  $\mu$ M GTP[ $\gamma$ S] (■) were only partially separated. (B) Triton X-100 extract of rat brain (30 mg of protein in 3 ml) was applied to a heparin-5PW HPLC column, which was eluted with gradients of NaCl as shown, in 20 mM Hepes buffer, pH 7.0/0.7% octylglucoside (flow rate 1.0 ml/min); 1.0-ml fractions were collected. Samples (5  $\mu$ l) of fractions from both columns were assayed for oleate-activated (■) or ARF-activated PLD without (○) or with (●) 5  $\mu$ g of mARF1 ( $\approx$ 1.7  $\mu$ M) and 10  $\mu$ M GTP[ $\gamma$ S], as described. (C) Samples (5  $\mu$ l) of fractions from heparin-5PW HPLC were assayed for formation of PtdEtOH. Lane 1, substrate alone. PLD purified from *Streptomyces chromofuscus* was used as standard in assays without (lane 2) and with ethanol (lane 3). Lanes 4–8, samples from fractions 46, 48, 50, 52, and 54, respectively, in oleate-dependent PLD assay with ethanol. Lanes 9–14, samples from fractions 74, 76, 78, 80, 82, and 84, respectively, in ARF-dependent PLD assay with ethanol. Arrows indicate positions of phospholipid standards: (Pam)<sub>2</sub>PtdCho (at origin), PtdOH, and PtdEtOH.

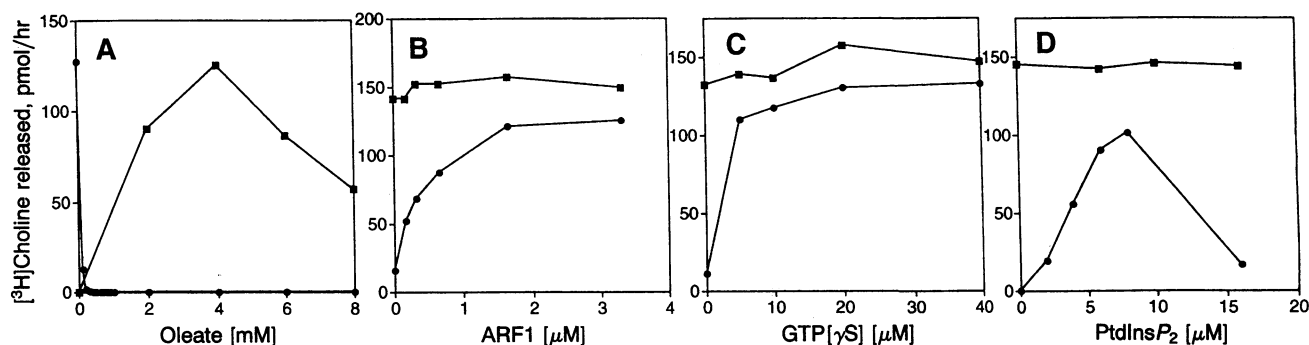


FIG. 2. Effects of sodium oleate, ARF, GTP[ $\gamma$ S], and PtdInsP<sub>2</sub> on oleate-dependent and ARF-dependent PLD. Samples (5  $\mu$ l) of peak fractions of peak I or II from heparin-5PW HPLC (Fig. 1B) were assayed for oleate-dependent (■) or ARF-dependent PLD (●) except that the indicated concentrations of sodium oleate (A), mARF1 (with 10  $\mu$ M GTP[ $\gamma$ S]) (B), GTP[ $\gamma$ S] (with 1.3  $\mu$ M mARF1) (C), or PtdInsP<sub>2</sub> (D) were present. Data are means of values from duplicate assays; variance was <5%.

2D). Oleate-dependent PLD activity was not altered by PtdInsP<sub>2</sub>. Both oleate- and ARF-dependent PLD failed to hydrolyze PtdEtn or phosphatidylserine under conditions used for PtdCho hydrolysis (data not shown).

With ARF-dependent PLD fractions from heparin-Sepharose CL-6B chromatography, addition of mARF1 after 30 or 60 min of incubation produced essentially the same marked stimulation as it did when added at zero time (Fig. 3). Thus, the PLD seemed to be stable for at least 90 min under assay conditions. With a 60-min incubation period, PtdCho hydrolysis was proportional to the amount of PLD added over only a relatively narrow range (data not shown), probably due to the crude nature of the enzyme preparation.

ARF1 (and ARF3), which have been described as activators of PLD in HL-60 cells (20, 24) are class I ARFs that are ubiquitous in eukaryotic cells and have been implicated in vesicular membrane trafficking in the secretory pathway (26). The function(s) of class II and III ARFs, identified by cDNA cloning, is less well understood (34), and it seemed important to determine whether they also are capable of activating PLD. ARF5 was used as a representative of class II, and

ARF6 was used as a representative of class III. mARF5 and mARF6 were similarly effective in stimulating PLD activity, whereas somewhat larger amounts of mARF1 were apparently required to achieve comparable activation (Fig. 4). The significance of these differences cannot, however, be evaluated precisely, as it is not known just what fractions of the ARF proteins were, in fact, myristoylated. In addition, some ARFs may be isolated with bound GTP (35), and in the preparations used here, the amounts of GTP-liganded ARF were not known. The maximal effect of mARF6 was not defined because the concentration of the ARF preparation made it impossible to achieve >0.3  $\mu$ M. For the same reason, maximal levels of activation by nonmyristoylated rARFs were not established. Each was less active than the corre-

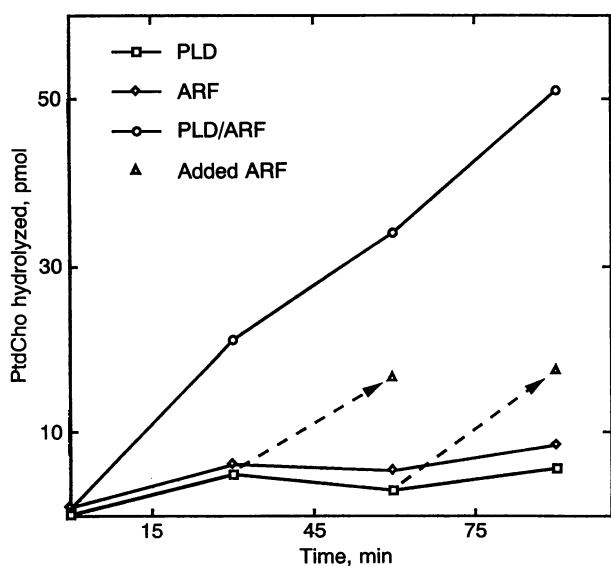


FIG. 3. Time course of PLD activity. Assays containing 5  $\mu$ l of ARF-activated PLD from heparin-Sepharose CL-6B chromatography without ARF (□), 0.67  $\mu$ M of mARF1 without PLD (◇), or PLD plus mARF1 (○) were incubated for the indicated time in the combined CTA-PLD assay. Some samples of PLD were incubated for 30 or 60 min before addition of mARF1 followed by incubation for 30 min (Δ). Data are means of values from duplicate assays. This experiment was repeated twice.

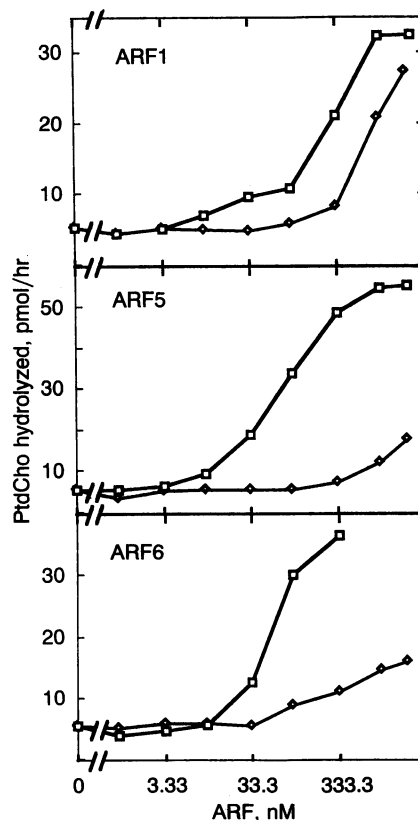


FIG. 4. Effects of rARF proteins on PLD activity. Samples of ARF-activated PLD (5  $\mu$ l) from heparin-Sepharose CL-6B chromatography were assayed in duplicate with the indicated concentrations of mARF (□) or rARF (◇) in the combined CTA-PLD assay with [*choline-methyl-<sup>3</sup>H](Pam)<sub>2</sub>PtdCho.*

sponding myristoylated form, although in the case of rARF1, the difference was only <10-fold.

**ARF Stimulation of CTA Activity.** Comparing ARF proteins at a concentration of 0.33  $\mu\text{M}$  under the same conditions used to assay PLD in Figs. 3 and 4, mARF6 caused greatest activation of CTA and was considerably more effective than rARF6 or mARF1, which had only a small effect; rARF1, mARF5, and rARF5 had no effect (Table 1). Thus, there was no correlation between ARF activation of PLD and CTA under identical assay conditions. Under standard cholera toxin assay conditions, the activities of ARF proteins were more similar to each other; the myristoylated protein in each case was somewhat more effective than the unmyristoylated counterpart (29).

Considerable differences in the effects of phospholipids and detergents on the activation of cholera toxin by individual ARF proteins have been noted, and it was hoped that these might reflect characteristics of the interactions of individual ARFs with their physiological targets, which were at that time unidentified. If PLD is, indeed, that target, it appears that this hypothesis may not be true. Activation of ARF (i.e., GTP-binding) was not, however, separated from activation of the target by ARF-GTP in most assays. Thus, either or both processes could be responsible for any observed effects. Quantification of amounts of activated ARF is complicated by the fact that high-affinity binding of GTP (which can be demonstrated under certain conditions) is not required for ARF activation of cholera toxin (36). It will be important to use preparations of ARF already bound to GTP (or other guanine nucleotide) to evaluate quantitatively factors that influence activation of PLD and/or cholera toxin. It will be necessary also to determine whether PLD is directly activated by interaction with ARF-GTP or whether ARF-GTP acts indirectly to enhance substrate-PLD interaction. In any case, under identical assay conditions, effects of individual ARF proteins on toxin and PLD activities are clearly different, consistent with the notion that different aspects of ARF structure are involved in the two functions.

The complete separation of oleate- and ARF-dependent PLD activities after their solubilization from rat brain membranes establishes their existence as specific enzymes with different responses to cofactors. Whether the ARF-dependent PLD preparation is a single enzyme that responds similarly (but perhaps not identically) to mARF1, mARF5, and mARF6 remains to be established, as does the role of PLD activity in ARF function in vesicular membrane trafficking (37). For example, is PLD-catalyzed PtdCho hydrolysis in some way involved in the events that follow association of ARF1-GTP or ARF3-GTP with membranes resulting

in coatomer binding and bud formation? Or does PLD activity participate in the last step of this kind of process, when the transport vesicle fuses with the target membrane?

We thank Carol Kosh for expert secretarial assistance and Eleanor Cavanaugh for purification of the mARF5 used in these experiments.

Table 1. Effect of rARF proteins on CTA activity under conditions used to assay phospholipase D activity

ARF (0.33 $\mu\text{M}$ )	CTA activity, nmol/hr
None	0.10
rARF1	0.07
mARF1	0.71
rARF5	0.17
mARF5	0.12
rARF6	0.93
mARF6	2.12

Assays were done under conditions like those used for the PLD assays in Figs. 3 and 4, except that the radiolabeled substrate was [adenine- $^{14}\text{C}$ ]NAD instead of [ $^3\text{H}$ ](Pam) $_2$ PtdCho. After the reaction had been stopped in an ice bath, one-half of the total volume was applied to a column of AG1-X2 (200–400 mesh, chloride form, from Bio-Rad) followed by 5 ml of water; total eluate was collected for radioassay. Data are means of values from duplicate assays.

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