Purification of a Lysophospholipase from Bovine Brain That Selectively Deacylates Arachidonoyl-substituted Lysophosphatidylcholine*

(Received for publication, January 26, 1996, and in revised form, April 24, 1996)

Matthew J. Pete‡ and John H. Exton§
From the Howard Hughes Medical Institute and the Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0295

A high activity lysophospholipase A (lysoPLA) was purified from the soluble fraction of bovine brain. The separation included sequential DEAE-Sepharose, phenyl-Sepharose FF, heparin-Sepharose CL-6B, and Q-Sepharose FF column chromatography. Mono Q, Sephacryl S300HR, and hydroxyapatite column chromatography in the presence of the detergent CHAPS (3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate) and glycerol further purified the activity to 17,000-fold. The enzyme was purified to homogeneity by polyacrylamide gel electrophoresis using non-denaturing conditions. The pure enzyme migrated as a single polypeptide of 95 kDa mass by SDS-polyacrylamide gel electrophoresis and deacylated arachidonoyl-lysophosphatidylcholine (ara-lysoPC) at a rate of 70 μmol/(min mg). The enzyme showed selectivity for arachidonoyl-substituted lysoPC, since palmitoyl-lysoPC was deacylated at a much lower rate (7 μmol/(min mg)). LysoPLA activity was maximal at pH 7.4–8.0 and was increased 1.3-fold by MgCl₂ (5 mM). By including MgCl₂, however, the range of optimal activity was expanded to pH values up to 9.0. The 95-kDa protein also deacylated arachidonoyl groups from 1-O-hexadecyl-2-arachidonoyl-PC (PLA₂ activity) at a rate of 15 μmol/(min mg). Moreover, the deacylation of arachidonoyl groups from diacylPC was greatly increased by including purified bovine brain PLA₁ in the reaction mixture. Thus, the same 95-kDa polypeptide catalyzed both lysoPLA and PLA₂ activities, but the rate of arachidonoyl group deacylation was increased by prior sn-1 deacylation. Finally, the 95-kDa polypeptide cross-reacted with antibodies raised against a human recombinant cPLA₂, implying that the 95-kDa protein is structurally similar to cPLA₂. Additionally, these data suggest that the combined actions of PLA₁ and the 95-kDa protein generate significant amounts of free arachidonic acid in the brain.

In brain and other tissues, arachidonic acid may be metabolized to potent, bioactive substances that may diffuse from the cell to evoke a response in adjacent cells (1, 2). For example, in addition to yielding various eicosanoids, brain preparations enzymatically condense arachidonic acid and ethanolamine to yield anandamide (3, 4), which inhibits conductance through astrocyte gap junctions (5) and, perhaps, glutamate-mediated neurotrans-astrocyte signaling (6).

The production of arachidonic acid metabolites is limited by the availability of arachidonic acid, which is stored primarily in the sn-2 position of phospholipids (1, 2). Deacylation of this position, catalyzed by phospholipase A₂ (PLA₂), mostly frees arachidonic acid. Many PLA₂ forms have been purified and cloned (7), but it is uncertain which of these contribute to brain PLA₂ activity. In addition to PLA₂, many tissues, including brain (8–10), liver (11), heart (12), and pulmonary artery endothelial cells (13), show phospholipase A₁ (PLA₁) activity. One form of PLA₁, which catalyzes deacylation of the sn-1 position of phospholipids, has been purified from the soluble fraction of bovine brain (14). In brain (8–10) and endothelial cells (13), the activity of PLA₁ may be much greater than that of PLA₂, but PLA₁ activity is often masked by further deacylation of 2-acyllysophospholipids by lysophospholipase (lysoPLA) activity (10). This is significant, since sequential sn-1 and sn-2 deacylation may yield free arachidonic acid and contribute to signaling in the brain. In this report, the purification of an enzyme that catalyzes the deacylation of lysophosphatidylcholine is detailed. This enzyme shows high activity and selectivity toward arachidonoyl-substituted lysoPC and may act with brain PLA₁ to yield arachidonic acid from diacylPC.

EXPERIMENTAL PROCEDURES

Enzyme Purification—All purification procedures were performed at 4 °C. Five bovine brains (300–400 g each) were obtained fresh from a local slaughterhouse, stripped of meninges, and washed free of blood. Bovine brain (stripped of meninges) was also purchased from PelFreez Biologicals (Rogers, AR). We observed no difference in the enzyme preparation using either frozen or fresh brain. The cerebra were homogenized in 5 liters of a buffer containing HEPES (20 mM, pH 7.6), sucrose (320 mM), EDTA (2 mM), EGTA (2 mM), and DTT (0.5 mM) using three high speed bursts (5 s duration) in a Waring blender (Waring Laboratory Products, New Hartford, CT). The homogenate was centrifuged (40 min, 12,000 × g), the supernatant collected and adjusted to pH 5.0 by the dropwise addition of 1 N acetic acid. This mixture was stirred for 1 h and centrifuged (20 min, 12,000 × g). The precipitate was collected and suspended in 2 liters of a buffer (buffer A) consisting of Tris (20 mM, pH 7.8), NaCl (20 mM), EDTA (1 mM), and DTT (0.5 mM). This preparation was stirred (2 h) and then mixed with DEAE-Sepharose (1100 ml) that had been pre-equilibrated in buffer A. After a period of 2–3 h, the resin was washed with 4 liters of buffer A using a sintered glass funnel and poured into a column (5 × 55 cm). The bound lysophospholipase activity was washed free of the resin using buffer A (2 liters) that increased linearly from 20 to 600 mM NaCl. Flow rate was 2 ml/min, and fraction size was 20 ml.

The abbreviations used are: PLA₁, phospholipase A₁; PLA₂, phospholipase A₂; lysoPLA, lysophospholipase A₁; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate; PMSF, phenylmethylsulfonyl fluoride; ara-lysoPC, [H]arachidonoyl-sn-glycero-3-phosphocholine; hex-ara-PC, 1-O-hexadecyl-2-[H]arachidonoyl-sn-glycero-3-phosphocholine; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
The active fractions collected from the DEAE-Sephacel column were pooled, slowly made 1.2 M NaCl using solid NaCl, and adjusted to pH 7.0 by the addition of solid HEPES. The pool was stirred (2 h) and then applied (flow rate, 2 ml/min) to a column (5.0 x 16 cm) of phenyl-Sepharose FF (flow substitution) that was pre-equilibrated in buffer B (20 mM HEPES, pH 7.0, 1 mM EDTA, 0.5 mM DTT) containing NaCl (1.2 M). The column was washed (flow rate, 2 ml/min) with buffer C containing 1.2 M NaCl until absorbance (A = 280 nm) of the column effluent neared base-line values. The column was then washed (flow rate, 2 ml/min) with buffer B (1200 ml) in which the NaCl concentration decreased linearly from 1200 to 0 mM. Finally the column was washed with buffer B (800 ml) in the absence of NaCl. The column effluent was collected when the column void volume was reached.

Fractions that showed lysPLA and PLA2 activity from phenyl-Sepharose FF were pooled and then directly applied (flow rate, 1.6 ml/min) to a column (2.6 x 34 cm) of heparin-Sepharose CL-6B that was pre-equilibrated in buffer B containing NaCl (20 mM). Following sample application, the column was washed (flow rate, 1.6 ml/min) with equilibration buffer until the effluent absorbance neared base-line values, then buffer C (720 ml) in which the NaCl concentration increased linearly from 20 to 360 mM. The column effluent was collected in 16 ml fractions.

The fractions collected from heparin-Sepharose CL-6B chromatography were pooled according to lysPLA activity. The mixed mixture was diluted with two volumes of a buffer consisting of Tris (20 mM, pH 7.8), EDTA (1 mM), and DTT (0.5 mM, buffer C) and applied (flow rate, 1.2 ml/min) to a column (16 x 1.1 cm) of Q-Sepharose FF that was pre-equilibrated in buffer C containing 100 mM NaCl. The column was washed (flow rate, 1.2 ml/min) with this buffer until the effluent absorbance neared base-line values, then buffer C (720 ml) in which the NaCl concentration increased linearly from 100 to 400 mM. The column effluent was collected in 4.8 ml fractions.

The active fractions from the Q-Sepharose FF column were pooled, diluted with 2 volumes of buffer C that contained CHAPS (15 mM) and glycerol (15%, v/v) and applied (flow rate, 0.8 ml/min) to a Mono Q HR 5/5 column that had been pre-equilibrated in buffer C that also contained NaCl (150 mM), CHAPS (10 mM), and glycerol (10%, v/v; buffer D). The column was washed (flow rate, 0.8 ml/min) with buffer D (80 ml) that contained the same concentration of NaCl from 100 to 400 mM NaCl. Fraction size of the effluent was 0.8 ml.

LysPLA activity from Mono Q chromatography was pooled and applied (flow rate, 0.5 ml/min) to a column (2.6 x 110 cm) of Sephacryl S300HR which had been pre-equilibrated in buffer D. The column was washed (flow rate, 0.5 ml/min) with buffer D (600 ml), and the column effluent was collected in 4 ml fractions. LysPLA activity from the gel filtration column was pooled and applied (flow rate, 0.5 ml/min) to a Mono Q HR 5/5 column that was pre-equilibrated in buffer D. The column was washed (50 ml) with buffer D that increased linearly from 150 to 500 mM NaCl.

Following the second Mono Q step, the activity was pooled and the pH of this mixture was adjusted to 7.0 by the addition of solid HEPES. The mixture was applied (flow rate, 0.5 ml/min) to a column (1.5 x 16 cm) of hydroxylapatite (HPLC grade, Calbiochem) that had been pre-equilibrated in buffer E (20 mM, pH 7.2, NaCl (150 mM), CHAPS (10 mM), DTT (0.5 mM), and glycerol (10%, v/v)). The column was washed with buffer E (24 ml) that increased linearly from 0 to 180 mM sodium phosphate. The column effluent was collected in 0.8 ml fractions. The active fractions were pooled, diluted with two volumes of buffer C that contained CHAPS (10 mM) and glycerol (10%, v/v), and then applied and washed from a Mono Q column as described for the second Mono Q step.

Polyacrylamide Gel Electrophoresis—Active material from the third Mono Q column was separated by polyacrylamide gel electrophoresis (PAGE) according to the manufacturer's instructions. Following electrophoresis, the lane containing the separated material was excised from the gel and sectioned (5 x 5 mm), and each section was suspended in a buffer (100 μl) consisting of Tris (20 mM, pH 7.8), NaCl (500 mM), CHAPS (10 mM), EDTA (1 mM), DTT (0.5 mM), and glycerol. Following an overnight incubation (4°C, 10–12 ml of 0.5 ml containing 3% sodium deoxycholate and 25 mg/ml of serum albumin (4.5 mg/ml), sodium deoxycholate (3 mg/ml, Utest grade, Calbiochem), and R. arrhizus lipase (40 μg, Type X, Sigma), the resulting reaction was monitored by spotting an aliquot (1 μl) on a silica gel LK6D thin layer chromatography plate (Whatman, Millisboro, OR). The plate was developed using a chloroform:methanol:H2O solvent (19:81:1, v/v/v). The plate was dried under a current of warm air (37°C) in chloroform:methanol (85:15, v/v) for less than 1 week.

For lysPLA determinations ([1H]arachidonoyl-lysPC was prepared as a stock solution (100 μl), total volume) in which the substrate concentration was 10-fold greater than that used in the assay. To this, the appropriate amount of [1H]arachidonoyl-lysPC was transferred to a test tube (12 x 75 mm). Additionally, 25 μl of chloroform:methanol:water solution containing Coomassie Brilliant Blue R-250 (0.03%, w/v), NaCl (100 mM), and methanol (30%, v/v) was added. The reaction was terminated by the addition of chloroform (1 ml), and the lysPC substrate was purified by column chromatography as described previously (17). Purity and yield of the resulting lysospholipid was judged by thin layer chromatography using 1-palmitoyl-lysPC (Avanti Polar Lipids, Birmingham, AL) as a standard. The pure (always greater than 95%) product was stored at -70°C in chloroform:methanol (85:15, v/v) for less than 1 week.

Polyacrylamide gel electrophoresis (PAGE) using nondenaturing conditions and a 6% (w/v) polyacrylamide gel was used to separate the separated proteins. Following electrophoresis, the lane containing the separated proteins was stained with Coomassie Brilliant Blue (0.03%, w/v) in 25% glacial acetic acid (1 ml/liter) and an aliquot of a column fraction (1–10 μl), and hydrolysis was initiated by adding an aliquot (5 μl) of the substrate stock solution (see above). Following an incubation period (37°C, 5–10 min), the reaction was stopped by the addition (0.1 ml) of 2-propanol:hexane:1 mM H2SO4 (20:20:1, v/v/v). Solid silicic acid (Bio-Sil A, 100–200 mesh, Bio-Rad) was added (20–25 mg) to the reaction vial and the suspension was mixed using a vortex mixer. Finally, H2O (150 μl) was added and the suspension mixed as before. Following centrifugation, the top layer was transferred into a scintillation vial, 7.5 ml of an aqueous-based scintillant was added, and the amount of decay quantified.

The protocol described above was a fast and convenient measure of lysPLA activity. For a more precise determination of activity (19), the reaction was terminated by the addition (500 μl) of chloroform:methanol:chloroform:methanol:2-propanol:hexane:1 mM H2SO4 (20:20:1:0.1:0.1:0.1) v/v/v, and the solvent was evaporated using a stream of N2. The dried lipids were redissolved in chloroform: methanol (85:15, v/v) and spotted onto a silica gel LK6D thin-layer chromatography plate (Whatman), and the plate was developed in a chloroform:methanol:water:ammonia (90:85:3:0.8, v/v/v/v) mixture to the top of the plate. The plate was developed until the solvent reached about halfway to the top of the plate. After drying, the lipids were visualized using Coomassie Brilliant Blue R-250 (0.03%, v/v) and the radioactive lipid was visualized by phosphor imaging (Bio-imaging System, Bio-Rad). The plate was developed until the solvent front reached about halfway to the top of the plate. The plate was removed from the solvent, dried, and chromatographed in the
hexane:diethyl ether:formic acid solvent system described above. The first solvent system separated lysoPC (RF = 0.3) from PC (RF = 0.6), and PE (RF = 0.77), whereas free fatty acid was carried along the solvent front. The second solvent separated the free fatty acid as before without affecting the migration of lysoPC, PC, or PE. The chromatographic zone corresponding to each lipid was scraped from the plate and the radioactivity was determined as before. It is important to note that the radioactivity in the scintillation vials containing lysoPC or PC fractions increased with time. However, the counts had stabilized by the next day.

Materials—All radiolabeled substrates were purchased from Dupont/NEN. All unlabeled lipids were from Avanti Polar Lipids (Birmingham, AL). Phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate, and CHAPS were from Sigma. All chromatographic materials were from Pharmacia.

RESULTS

Enzyme Purification—Low speed centrifugation (12,000 × g, 40 min) of a homogenate of bovine brain yielded a supernatant (protein concentration = 6.3 mg/ml) that deacylated [3H]arachidonoyl-lysoPC yielding [3H]arachidonic acid (lysoPLA activity) at a rate of 1.07 nmol/(min mg) (step 1, Table I). The rate was lowered to a value of 0.24 nmol/(min mg), however, by pretreating the supernatant with the serine esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF, 2 mM, Table I). Similar results were obtained using diisopropyl fluorophosphate (2 mM, data not shown). Hence, the supernatant fraction showed a PMSF-sensitive and insensitive lysoPLA activity. In contrast, the deacylation of the sn-2 position of 1-O-hexadecyl-2-[3H]arachidonoyl-sn-glycero-3-phosphocholine (ara-lysoPC) by the supernatant fraction was not detected (step 1, Table I). The pH of the supernatant fraction was lowered to a value of 5.0 using 1 N acetic acid. This precipitated both PMSF-sensitive and insensitive lysoPLA activities, which resulted in a 2-fold purification of both lysoPLA activities (step 2, Table I), and facilitated the exchange of buffers. Moreover, following acid precipitation, PLA2 activity (0.28 nmol/(min mg)), which also showed sensitivity to PMSF, was detected (step 2, Table I).

The acid-precipitated material was resuspended in a low conductance buffer and mixed with DEAE-Sephacel chromatography resin. Using these conditions, the PMSF-insensitive, but not PMSF-sensitive, lysoPLA and PLA2 activities were adsorbed to the resin. The DEAE-Sephacel with its bound material was poured into a chromatography column, and activity was washed from the column using a linear NaCl gradient. The column profile is shown in Fig. 1A. Fractions collected

**Table 1**

| Step | LysoPLA activity | PLA2 activity |
|------|------------------|---------------|
|      | Total protein    | Specific activity | Total activity | Yield |
|      | mg               | nmol/(min mg)  | nmol/min | %   | nmol/(min mg) | nmol/min | %   |
| 1. Supernatant | 23,100 | 1.07 | 24,700 | <0.01 |
| + PMSF | 0.24 | 5540 | 0.01 |
| 2. Acid ppt. | 8640 | 2.57 | 22,200 | 0.28 |
| + PMSF | 0.43 | 3720 | 0.06 |
| 3. DEAE-Sephacel | 2130 | 1.69 | 3600 | 0.81 |
| + PMSF | 1.57 | 3340 | 0.81 |
| 4. Phenyl-Sepharose FF | | | | |
| I | 330 | 4.76 | 1570 | <0.01 |
| II | 195 | 73.8 | 14,400 | 100 |
| 5. Heparin-Sepharose CL-6B | 60 | 182 | 10,900 | 76 |
| 6. Q-Sepharose FF/Mono Q | 0.27 | 4030 | 1090 | 8 |
| 7. Sepharil S300/Mono Q | 0.26 | 4460 | 1160 | 8 |
| 8. Hydroxylapatite/Mono Q | 0.016 | 17,000 | 272 | 2 |

**Fig. 1**. Elution profiles of the separation of lysoPLA activity. A, DEAE-Sephacel; B, phenyl-Sepharose FF (flow substitution). Fractions were collected, and aliquots (10 μl) were assayed for lysoPLA (●), PLA2 (○), or PLA2 (×) activity as described under "Experimental Procedures." The solid line represents absorbance of the column effluent at 280 nm. The bars designate the portions pooled for lysoPLA purification and correspond to NaCl concentrations of 140–220 mM (A), 750–350 mM (B, I), and 150–0 mM (B, II).
from the DEAE-Sephacel column showed lysoPLA activity, PLA2 activity, and a third activity that hydrolyzed 1-palmitoyl-2-[3H]palmitoyl-PC to yield 2-[3H]palmitoyl-lysoPC (PLA1 activity). Column chromatography using phenyl-Sepharose FF matrix separated the pooled lysoPLA activity from DEAE-Sephacel into two portions (Fig. 1B). Portion I (I, Fig. 1B) showed a lysoPLA activity of 4.8 nmol/(min mg), but no PLA2 activity (step 4, Table I). The PLA1 activity of this pool, however, was 51.3 nmol/(min mg), yielding a total PLA1 activity of 16900 nmol/min. The second portion (II, Fig. 1B) comprised 93% of the lysoPLA activity, deacylated ara-lysoPC at a rate of 73.8 nmol/(min mg) (pool II, step 4, Table I) and hexara-PC at a rate of 20.5 nmol/(min mg). A large increase in total lysoPLA and PLA2 activities was observed in pool II (step 4, Table I). This gain was probably due to the separation of activity from factors (e.g., phospholipids) that interfered with the enzyme assays of previous pools (steps 1-3, Table I), however, this was not determined. It is noteworthy that the total PLA1 activity was comparable to the total PMSF-insensitive lysoPLA activity (step 4, pools I, II, Table I) but much greater than the PLA2 activity (step 4, Table I). Additionally, assays of phenyl-Sepharose FF fractions (Fig. 1B) suggested that the two pools (I and II, Fig. 1B) contain similar portions of lysoPLA activity. This is deceptive since large sample sizes and prolonged incubation periods were employed in assays of column fractions (Fig. 1) to maximize hydrolysis. This probably resulted in the nonlinear accumulation of reaction products in some of the column fractions assayed. More accurate determinations of the pool activities were achieved using small sample sizes (≤1 μg of protein) and short (3 min) incubations (Table I). Nevertheless, all of the PMSF-insensitive lysoPLA and PLA2 activities of the bovine brain-soluble fraction were accounted for by the PLA1-associated and PLA2-associated activities. The PLA1-associated (pool I, step 4, Table I) lysoPLA activity remained tightly associated with PLA1 activity through multiple purification steps (data not shown). Since it comprised only a small fraction of total lysoPLA activity (step 4, pool I, Table I), however, the PLA2-associated lysoPLA activity was not characterized further at this time.

Successive column chromatography purifications of pool II from phenyl-Sepharose FF each yielded a single lysoPLA activity peak (Fig. 2) but did not separate PLA2 from lysoPLA activity (steps 5-8, Table I) despite including the detergent, CHAPS (10 mM), and glycerol (10%, v/v) in buffers of steps 7 and 8. These two components maintained high enzymatic activity despite the very low protein concentrations (μg of protein/ml) yielded by the later purification steps. It is also important to note that the lysoPLA activity in fractions from the hydroxylapatite column was low and difficult to measure (Fig. 2D). This may be due to the presence of phosphate, since the activity was recovered following a third Mono Q column chromatography step (Fig. 3A). SDS-PAGE (Fig. 3B) of some of the Mono Q fractions showed that the active fractions (fractions 18-20, Fig. 3A) each contained more than one polypeptide. Nevertheless, the relative intensity of a 95-kDa band in fractions 18-20 (Fig. 3B) corresponded to the activity (Fig. 3A)
each of these fractions. In contrast, the other bands are either not present in all active fractions or are abundant in inactive fractions (Fig. 3, A and B). This implied that the lysoPLA activity was associated with this 95-kDa protein.

To optimize purity, only fractions 18 and 19 (Fig. 3) from the Mono Q column were pooled. As observed following each previous purification step, the pooled material (step 8, Table I) showed lysoPLA and PLA₂ activity. Since each activity copurified with the 95-kDa polypeptide, structural similarity between the pooled material (fractions 18 and 19, Fig. 3) and an 85-kDa PLA₂ (cPLA₂) cloned from human monocytic U937 cells (20) was examined using antibodies generated against the recombinant cPLA₂.

Western blot analysis showed identity between the brain 95-kDa protein (Fig. 3C, lanes 2 and 3), the recombinant cPLA₂ (Fig. 3C, lane 1) and protein(s) found in cell lysates of mouse peritoneal macrophages (Fig. 3C, lane 4). This provided additional evidence that the 95-kDa polypeptide catalyzed both lysoPLA and PLA₂ reactions. To confirm this, the pooled material from the Mono Q columns (fractions 18 and 19, Fig. 3; step 8, Table I) was separated by PAGE using nondeaturing conditions. Following the separation (represented in Fig. 4A), the gel was fractioned and each fraction was assayed for lysoPLA and PLA₂ activities (Fig. 4B) or separated by SDS-PAGE (Fig. 4C). The fractions that showed lysoPLA activity (fractions 6–9, Fig. 4B) contained the 95-kDa protein (Fig. 4C), whereas other bands were absent or more prominent.
or hex-ara-PC at rates of 70 and 15. Reaction mixtures, pH 7.5, included MgCl2 (5 mM) and PE (90 mM). Reactions were terminated at the indicated time and assayed for arachidonic acid by TLC. Each data point represents the mean value of triplicate determinations.

In fractions not showing lysoPLA activity. Additionally, the relative intensity of this 95-kDa band corresponded to the amount of lysoPLA activity in these fractions. These data are further evidence that lysoPLA activity was associated with the 95-kDa protein. It was also demonstrated that fractions showing lysoPLA activity also showed PLA2 activity (Fig. 4B). Furthermore, the ratio of lysoPLA to PLA2 activity in each fraction (Fig. 4B) was equal to that of the pooled sample (step 8, Table I). Hence, both lysoPLA and PLA2 activities are probably associated with this 95-kDa protein.

Laser densitometric scanning of the pooled material (step 8, Table I) following separation by SDS-PAGE (Fig. 4C, lane Q) was used to estimate that the 95-kDa protein comprised about 23% of the total protein in this pool. From these data and those of Table I, it can be calculated that the 95-kDa protein catalyzed the deacylation of arachidonoyl groups from ara-lysoPC or hex-ara-PC at rates of 70 and 15 mmol (min mg), respectively. This purification protocol, including native PAGE, has been performed three times and always yielded the 95-kDa protein (data not shown). Some minor contaminating proteins were variably present, but these separated from the 95-kDa protein on native PAGE. Additionally, in every separation PLA2 activity was also observed.

In the same reaction mixture, however, 2-arachidonoyl-lysoPC was not deacylated, this has not yet been determined. PLA2 activity of the enzyme 5-fold (data not shown). This PE effect has been previously observed using purified PLA1 (19) or PLA2 (21). At a substrate concentration of 10 μM, the enzyme catalyzed 1-[3H]palmitoyl-lysoPC (Fig. 7, column 5) or 2-[3H]palmitoyl-lysoPC (Fig. 7, column 6) deacylation slowly regardless of the presence or absence of Mg2+ (data not shown) of PE.

The ara-lysoPC substrate used in the above studies was prepared by sn-1 deacylation of 1-2-[3H]arachidonoyl-sn-glycero-3-phosphocholine and purified prior to the assay. Thus, the positional specificity of the purified lysoPLA cannot be strictly defined since some of the arachidonoyl groups might have migrated to the unoccupied sn-1 position. It was, thus, important to determine if the purified lysoPLA deacylated the sn-2 arachidonoyl groups from PC faster if a purified sample of brain PLA1 (shown in Fig. 3B, lane P) was included in the same reaction mixture. Brain PLA1 incubated with 1-palmitoyl-2-[14C]arachidonoyl-PC yielded a small amount of arachidonic acid that increased linearly with time (Fig. 8A). This gain in arachidonic acid may be due to the contamination of the PLA1 sample by lysoPLA, however, this has not yet been determined.

In the same reaction mixture, however, 2-arachidonoyl-lysoPC accumulated rapidly (Fig. 8B) until about 30–40% of the substrate was hydrolyzed. This experiment was repeated, but after 15 min, an aliquot of the purified lysoPLA was added. The added lysoPLA produced a rapid gain in arachidonic acid (Fig. 8A). This rapid gain was accompanied by a similar loss in radiolabel from the 2-arachidonoyl-lysoPC fraction (Fig. 8B). The coincident gain in arachidonic acid and loss in 2-arachidonoyl-lysoPC implied that the added lysoPLA deacylated a portion (about 20%) of the 2-arachidonoyl-lysoPC. Mixing the pu
purified lysoPLA and PLA₂ prior to adding the diacylPC, produced a modest 1.3-fold gain in arachidonic acid, compared with the sum of the two yields, over the same time period (data not shown). As before, this gain in arachidonic acid was accompanied by a like decrease in the amount of 2-arachidonoyl-lysoPC produced (data not shown).

**DISCUSSION**

This report outlines the purification of an enzyme that catalyzes the deacylation of arachidonoyl-substituted lysoPC with high velocity (Fig. 5) and specificity (Fig. 7). The activity was analyzesthe deacylation of arachidonoyl-substituted lysoPC with lysoPLA activity obtained in fractions from native PAGE (Fig. 4) or Mono Q column chromatography (Fig. 3A) and native PAGE (Fig. 4B) that showed high lysoPLA activity. Other proteins were either not present in every active fraction or were present at high concentration in inactive fractions (Figs. 3 and 4). The native PAGE fractions that showed high lysoPLA activity (Fig. 4B) also showed PLA₂ activity, thus implying that PLA₂ activity was associated with the 95-kDa protein. The strong immunological identity of this 95-kDa polypeptide (Fig. 3C, lanes 2 and 3) with a recombinant cPLA₂ (Fig. 3C, lane 1), the ratio of lysoPLA:PLA₂ activity obtained in fractions from native PAGE (Fig. 4) or Mono Q chromatography (step 8, Table I) and the copurification of the two activities through multiple purification steps (Table I) support this conclusion.

To our knowledge, this is the first purification of an enzyme that selectively catalyzes the hydrolysis of arachidonoyl-substituted lysophospholipids. A variety of smaller lysoPLAs (20–28 kDa by SDS-PAGE) have been previously purified from bovine liver (22), rabbit heart (23), and macrophage-like P388D₁ (18) or WEHI 265.1 (24) cells. The activities of these enzymes are generally low (1 μmol/(min mg)); however, it is interesting that the lysoPLAs from macrophage-like cells (18, 24) are inhibited by PMSF (1–5 mM). This may be important since high PMSF-sensitive lysoPLA activity was also measured in preparations of bovine brain (steps 1 and 2, Table I). This implies that bovine brain contains a second, PMSF-sensitive lysoPLA analogous to one of those described above.

In addition to the high lysoPLA activity (70 μmol/(min mg)), the 95-kDa protein catalyzed arachidonoyl-group deacylation of an alkyl-arachidonoyl PC at a slower (15 μmol/(min mg)) but significant rate. This dual lysoPLA/PLA₂ activity has been observed previously for an enzyme (88 kDa by SDS-PAGE) purified from the cytosolic fraction of rabbit platelets (25, 26). Interestingly, this platelet enzyme (26) hydrolyzed 1-palmitoyl-lysoPC in PC vesicles at a rate (8 μmol/(min mg)) that was close to that observed using the brain enzyme and the same substrate in PE vesicles (7 μmol/(min mg); Fig. 7). Moreover, the platelet enzyme hydrolyzed oleoyl groups from lysoPC (26) or arachidonoyl groups from 1-stearyl-2-[³H]arachidonoyl-PC (25) at rates of 23 or 11 μmol/(min mg), respectively. These data suggest that the 95-kDa brain lysoPLA may be an isoform of the platelet enzyme. An enzyme that shows both lysoPLA (27) and PLA₂ activities has also been purified (28) and cloned (20) from human monocytic U937 cells. This U937-derived enzyme...
(cPLA₂) is thought to account for most agonist-induced arachidonic acid formation in these cells. The strong immunological identity between the 95-kDa bovine brain protein and recombinant cPLA₂ (Fig. 3C) implies that the two enzymes are structurally related. It is important to determine this as well as the 95-kDa enzyme contribution to brain agonist-stimulated arachidonic acid release.

It is clear that prior sn-1 deacylation greatly increased the rate of arachidonoyl group hydrolysis by brain lysoPLA (Table I, Fig. 5). Yet, in incubations that contained lysoPLA and PLA₁ purified from bovine brain and a diacyl arachidonoyl-PC-only a 1.3-fold gain in arachidonic acid was observed when the two enzymes were mixed prior to reaction initiation. Presumably this was due to the small amounts of each enzyme (≤5 ng each) used and, thus, the low probability that the two enzymes were acting on the substrate in close proximity. The arachidonic acid yield from diacylPC was increased 3-fold if the substrate was preincubated with PLA₁ prior to lysoPLA (Fig. 8A). However, only about 20% of the 2-arachidonoyl-lysoPC was hydrolyzed before the lysoPLA activity slowed (Fig. 8B). This rapid initial velocity followed by a slower rate of hydrolysis by the lysoPLA was also observed with ara-lysoPC in PE vesicles (Fig. 5) and has been previously described for both lysoPLA (27, 29) and PLA₁ (28, 30).

Ghomashchi et al. (30) have proposed that, as the enzyme depletes local substrate, it becomes tightly adsorbed to product-containing vesicles. As a result, substrate is obstructed from the active site of the enzyme and the reaction slowed. Thus, the progressive slowing of the lysoPLA reaction and the physical separation between the two enzymes probably combined to attenuate the arachidonic acid yield from the diacylPC (Fig. 8A). Nevertheless, it can be concluded that the lysoPLA will hydrolyze the 2-arachidonoyl-lysoPC generated by PLA₁ with high velocity (Fig. 8). Further work is required to determine if conditions such as enzyme modification or association with other proteins provide a closer relationship between the two enzymes, PLA₁ and lysoPLA, in brain. Such a relationship would be expected to result in a much larger yield in arachidonic acid than if the 95-kDa lysoPLA acted as a PLA₂ alone.

Acknowledgments—We express our appreciation to Dr. Christina C. Leslie (National Jewish Center, Denver, CO) for Western blot analysis of the purified material. We also thank Dr. Joseph P. Provost for generating Fig. 3 and Judy Childs for expert preparation of this manuscript.

REFERENCES
1. Piomelli, D., and Greengard, P. (1990) Trends Pharmacol. Sci. 11, 367–373
2. Shimizu, T., and Wolfe, L. S. (1990) J. Neurochem. 55, 1–15
3. Devane, W. A., Husan, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gilson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) Science 258, 1946–1949
4. Devane, W. A., and Axelrod, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6698–6701
5. Venance, L., Piomelli, D., Giowinski, J., and Glauze, C. (1995) Nature 376, 590–594
6. Parpura, V., Basarsky, T. A., Liu, F., et al. (1995) J. Neurochem. 67, 613–620
7. Hiratsawa, K., Irvine, R. F., and Dawson, R. M. C. (1995) Eur. J. Biochem. 229, 53–58
8. Pete, M. J., Wu, D. W., and Exton, J. H. (1996) Biochim. Biophys. Acta 1299, 325–332
9. Dawson, R. M. C., Irvine, R. F., Hemington, N. L., and Hiratsawa, K. (1983) Biochim. Biophys. Acta 709, 865–872
10. Nalbone, G., and Hostetler, K. Y. (1985) J. Lipid Res. 26, 104–114
11. Martin, T. W., and Wysolmerski, R. B. (1987) J. Biol. Chem. 262, 13086–13092
12. Pete, M. J., Ross, A. H., and Exton, J. H. (1994) J. Biol. Chem. 269, 19494–19500
13. Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) Anal. Biochem. 118, 197–203
14. de Carvalho, M. S., McCormack, F. X., and Leslie, C. C. (1993) Arch. Biochem. Biophys. 306, 534–540
15. Itabe, H., King, W. C., Reynolds, C. N., and Glomset, J. A. (1992) J. Biol. Chem. 267, 15319–15326
16. Zhang, Y., and Dennis, E. A. (1988) J. Biol. Chem. 263, 9965–9972
17. Pete, M. J., and Exton, J. H. (1995) Biochim. Biophys. Acta 1256, 367–373
18. Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gambba, C. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, B. L., and Pepper, W. R. (1981) J. Biol. Chem. 256, 19494–19500
19. Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) Anal. Biochem. 118, 197–203
20. de Carvalho, M. S., McCormack, F. X., and Leslie, C. C. (1993) Arch. Biochem. Biophys. 306, 534–540
21. Leslie, C. C., and Channon, J. Y. (1990) Biochim. Biophys. Acta 1045, 261–270
22. Dej, O., J. G. N., Van den Bosch, H., Rijken, D., and van Deenen, L. L. M. (1974) Biochim. Biophys. Acta 369, 50–63
23. Gross, R. W., and Sobel, B. E. (1983) J. Biol. Chem. 258, 5221–5226
24. Garsetti, D. E., Ozgür, L. E., Steiner, M. R., Egan, R. W., and Clark, M. A. (1992) Biochim. Biophys. Acta 1165, 229–238
25. Kim, D. K., Kudo, I., and Inoue, K. (1991) Biochim. Biophys. Acta 1083, 80–88
26. Fujimori, Y., Kudo, I., Fujita, K., and Inoue, K. (1993) Eur. J. Biochem. 218, 629–635
27. Reynolds, L. J., Hughes, L. L., Louis, A. I., Kramer, R. M., and Dennis, E. A. (1993) Biochim. Biophys. Acta 1299, 13057–13060
28. Kramer, R. M., Roberts, E. F., and Exton, J. H. (1991) J. Biol. Chem. 266, 14850–14853
29. Piomelli, D., and Greengard, P. (1990) Trends Pharmacol. Sci. 11, 367–373
30. Ghomashchi, F., Schüttel, S., and Gelb, M. H. (1992) Biochemistry 31, 3814–3824