Purification of a phosphatidic-acid-hydrolysing phospholipase A_2 from rat brain

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A phosphatidic-acid-hydrolysing phospholipase A_2 was purified from rat brain and characterized. This phospholipase A_2 was purified by sequential cation, hydrophobic, heparin and gel-filtration chromatography. The purified protein had a mass of ~ 58 kDa as assayed by SDS/PAGE, had a pH optimum of 6.0, and was Ca^{2+}-independent. This enzyme was apparently phosphatidic-acid-selective and had little measurable catalytic activity when phosphatidylcholine, phosphatidylethanolamine or diacylglycerol was used as substrate. On the basis of its physical and catalytic properties, we conclude that this phospholipase A_2 is unique from those previously purified, and we speculate that it may be important for the production of the bioactive lipid lysophosphatidic acid.

INTRODUCTION

Lysophosphatidic acid (1-acyl-*sn*-glycero 3-phosphate) (LPA) is a potent biologically active lipid with pleiotropic effects. These actions include induction of platelet aggregation [1,2], smooth-muscle contraction [3,4], DNA synthesis and cell division [5,6], alterations in neuronal cell morphology [7] and promotion of chemotaxis [8]. At least some of these effects are mediated by an LPA-specific G-protein-coupled receptor that we have recently characterized [9]. A consequence of LPA-receptor activation is the production of a variety of second messengers [5,6,10–14] and transcription of genes controlled by serum response elements [15]. Given the multiplicity of LPA's effects, it is essential that its levels be tightly regulated.

We have identified and purified a LPA-specific lysophospholipase that may attenuate the LPA-driven response [16]. Although less well understood, it is crucial that the synthesis of LPA also be tightly regulated. Phospholipase A_2 (PLA_2) catalysis of phosphatidic acid is one pathway of LPA synthesis. Indeed, a phosphatidic-acid-hydrolysing PLA_2 activity has been previously identified in platelets, but has not yet been purified [17]. Several different PLA_2 enzymes have been purified, however none has been shown to hydrolyse phosphatidic acid. This study reports the purification and characterization of a novel phosphatidic-acid-selective PLA_2 from rat brain.

MATERIALS AND METHODS

Materials

1-Stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol ([^{14}C]diacylglycerol) (specific radioactivity 55 mCi/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.). Non-radioactive 1-stearoyl-2-arachidonyl-*sn*-glycerol-3-phosphate (Avanti Polar Lipids, Alabaster, AL) was used to lower the specific radioactivity of [2-^{14}C]phosphatidic acid to 27.5 mCi/mmol. 1-Stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol-3-phosphatidylcholine ([^{14}C]phosphatidylcholine) (specific radioactivity 52 mCi/mmol) and 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol-3-phosphatidylethanolamine ([^{14}C]phosphatidylethanolamine) (specific radioactivity 52 mCi/mmol) were purchased from DuPont New England Nuclear (Boston, MA, U.S.A.). 1-Stearoyl-2-arachidonyl-*sn*-glycerol was purchased from Sigma (St. Louis, MO, U.S.A.). All other non-radioactive lipids were purchased from Avanti Polar Lipids and were reported to be > 98% pure by the manufacturer. N-octyl glucoside and ATP were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Diacylglycerol kinase (*Escherichia coli*) was purchased from Calbiochem (La Jolla, CA, U.S.A.). All buffers were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). All other reagents were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Synthesis of [2-^{14}C]phosphatidic acid

Two separate methods for synthesizing radiolabelled phosphatidic acid were employed in these studies. The substrate that was used in all studies except those designed to examine the *sn*-2 specificity of the PLA_2 was [2-^{14}C]phosphatidic acid. This substrate was synthesized by diacylglycerol-kinase-catalysed phosphorylation of [^{14}C]diacylglycerol as described previously [18]. The [^{14}C]diacylglycerol was dried under a stream of N_2 and redissolved in 0.05 ml of a mixture comprising 500 mM n-octyl glucoside and 50 mM Pipes/NaOH, pH 6.8. The phosphorylation assay contained 100 mM NaCl, 50 mM $MgCl_2$, 5 mM ATP, 0.5 mM cardiolipin, 50 mM n-octyl glucoside, 2 mM 2-mercaptoethanol, 400 μg/ml DAG kinase and 1 mM [^{14}C]-diacylglycerol in 50 mM Pipes/NaOH, pH 6.8 (0.5 ml final volume). The reaction mixture was incubated for 14 h at 37 °C in a shaking water bath. The lipids were extracted using chloroform and methanol as previously described [19]. The purity of the phosphatidic acid product was quantified by t.l.c. (Silica gel G plates, Analtech, Newark, DE, U.S.A.) using a mobile phase comprising light petroleum (boiling range 35–60 °C)/diethyl ether/acetic acid (70:30:1; by vol.). The radiolabelled phosphatidic acid product was > 95% pure and was stored in chloroform at −20 °C.

Abbreviations used: LPA, lysophosphatidic acid; PLA_2, phospholipase A_2.

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The second method for synthesizing radiolabelled phosphatidic acid was to utilize DAG kinase, [γ-^{32}P]ATP and the reaction conditions indicated above. This method produced phosphatidic acid that was radiolabelled in the polar head group and had various fatty acids in the *sn*-2 position.

PLA_2 purification

A number (40) of frozen rat brains (Pel-Freeze, Rogers, AR, U.S.A.) were thawed at room temperature in 20 mM Hepes (pH 7.5)/10 mM EDTA (1 brain/4 ml) containing 100 μg/ml bacitracin, 10 μg/ml soybean trypsin inhibitor and 1 mM phenylmethanesulphonyl fluoride. The tissue was disrupted using a Polytron homogenizer, and clarified by centrifugation (100000 *g* for 45 min at 4 °C) to yield a supernatant.

Cation-exchange chromatography

The supernatant was applied on to a 21.5 mm × 15 cm CM-5PW carboxymethyl (CM)-silica h.p.l.c. column (Toso Haas, Philadelphia, PA, U.S.A.) which had been pre-equilibrated in buffer A (20 mM Hepes, pH 7.5, 10 mM EDTA) at a flow rate of 10 ml/min. Protein was eluted from the column by linearly increasing the NaCl concentration in buffer A from 0 to 1 M over a time period of 25 min. A flow rate of 10 ml/min was used throughout and fractions were collected at 1 min intervals and assayed for phospholipase A2 activity.

Hydrophobic chromatography of a phosphatidic-acid-specific PLA_2

Fractions 19–23 from the cation column were pooled and further purified by hydrophobic-interactive chromatography. Ammonium sulphate was added to the sample to achieve a final concentration of 0.5 M and the precipitate was removed by filtration through a 0.22 μm pore size membrane (Gilson Scientific, Ann Arbor, MI, U.S.A.). The sample was then applied to a 7.5 mm × 7.5 cm phenyl–5PW silica h.p.l.c. column (Toso Haas) which had been pre-equilibrated in buffer A containing 0.5 M ammonium sulphate. The protein was eluted by linearly decreasing the ammonium sulphate concentration from 0.5 to 0 M over a period of 25 min, using a flow rate of 1 ml/min throughout. Fractions were collected at 1 min intervals and assayed for PLA_2 activity.

Heparin-affinity chromatography

Fractions 25–30 from the hydrophobic interactive column were pooled. The salt concentration was decreased by dilution in 20 mM Hepes (pH 7.5)/10 mM EDTA and the sample was then concentrated by ultrafiltration using a 3000 Da molecular mass cut-off filter (Amicon, Danvers, MA, U.S.A.). The concentrated sample was then applied to a 7.5 mm × 7.5 cm heparin–5PW silica h.p.l.c. column (Toso Haas). PLA_2 activity was eluted from the column by linearly increasing the NaCl concentration in buffer A from 0 to 1 M over a time period of 25 min. A flow rate of 1 ml/min was used throughout. Fractions were collected at 1 min intervals and assayed for PLA_2 activity.

Gel-filtration chromatography

The PLA_2 activity obtained from the heparin column (fractions 25–31) was concentrated by ultra filtration as described above before being applied to a GF-250 h.p.l.c. column (Zorbax 250, DuPont, Wilmington, DE, U.S.A.). PLA_2 activity was eluted using buffer A containing 0.5 M NaCl at a flow rate of 1 ml/min.

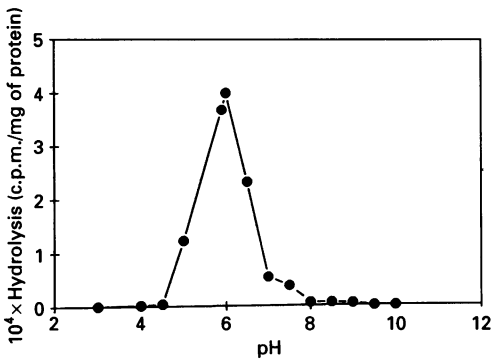

Figure 1 Effect of pH on phosphatidic acid hydrolysis by rat-brain phospholipase A2

The hydrolysis of [^{14}C]phosphatidic acid to free fatty acid by rat-brain homogenates was determined over a pH range of 3.5–10. Data are shown from one experiment representative of three experiments performed.

Fractions were collected at 1 min intervals and assayed for PLA_2 activity.

PLA_2 assay

All PLA_2 assays except those designed to examine the *sn*-2 specificity of the enzyme were performed in 100 mM Mes (pH 6.0)/10 mM EDTA/10 μM [^{14}C]phosphatidic acid (final volume 0.05 ml). Reactions were initiated by addition of substrate, incubated for 30 min at 37 °C, then terminated by organic extraction of the lipids [19]. The reaction product, free fatty acid, was separated from unreacted substrate by t.l.c. using silica-G plates and a mobile phase comprising light petroleum/diethyl ether/acetic acid (70:30:1, by vol.).

PLA_2 assays to examine the *sn*-2 specificity were performed using the reaction conditions described above, except that the substrates used were radiolabelled with ^{32}P in the polar head group. The reaction products were separated using Silica-G plates and a mobil phase comprising chloroform/methanol/acetic acid/water (100:60:16:8 by vol.). The reaction product, LPA, was quantified. The specific activity of the enzyme was the same regardless of the assay method used (results not shown).

Under the conditions used, all reactions were linear with respect to protein concentration and time, and $< 10\%$ of the substrate was hydrolysed.

To determine the effect of pH on this enzyme activity, assays were performed as described above, using the following buffers: pH 3.5–5.0, sodium acetate; pH 5.5–6.5, Mes; pH 7.0–7.5, Hepes; pH 7.5–9.0, Tris/HCl; pH 9.5–11.0, 3-(cyclohexylamino)-1-propanesulphonic acid. All buffers were used at a concentration of 100 mM.

Protein measurements

Protein concentrations were determined by the method of Bradford [20] using reagents obtained from BioRad (Richmond, CA, U.S.A.). Samples having a low concentration of protein were quantified using Quanti-Gold (Diversified Biotech, Newton, Center, MA, U.S.A.) as directed by the manufacturer. BSA was used as a standard.

RESULTS

PLA_2 purification

[^{14}C]Phosphatidic acid was readily hydrolysed by crude homogenates of rat brain, as assessed by measuring [1-^{14}C]arachidonate release. This enzyme activity was unaffected by EDTA or the addition of $\leqslant 1$ mM $CaCl_2$ (results not shown). This reaction had a pH optimum of 6.0 (Figure 1). Of this enzyme activity $\sim 70\%$ was associated in the particulate fraction and only partially solubilized by a variety of detergents tested. We have attempted to increase the amount of this enzyme solubilized using anionic, cationic and non-ionic detergents. Of all detergents tested only the non-ionic detergents (n-octyl glucoside) were partially successful, solubilizing $\sim 10\%$ of the membrane-bound activity. The remaining soluble PLA_2 activity bound to a cation-exchange column and eluted as a single peak (Figure 2). This activity was further enriched by sequential hydrophobic interactive (Figure 3), heparin-affinity (Figure 4) and gel-filtration (Figure 5) chromatography. The PLA_2 activity chromatographed with an apparent molecular mass of ~ 60 kDa. This purification procedure resulted in an enrichment of ~ 40000-fold with a final yield of $\sim 0.4\%$ (Table 1). The enzyme was unstable throughout the purification procedure and it was essential that each step was carried out as rapidly as possible.

The purity of the isolated phospholipase was assessed by SDS/PAGE (Figure 6). A protein with a molecular weight of ~ 58 kDa was observed.

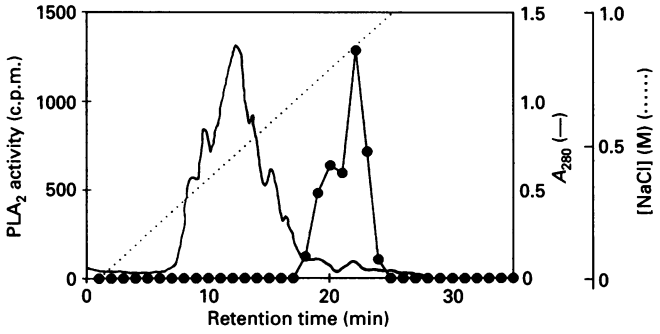

Figure 2 Cation-exchange chromatography of rat brain PLA_2

Soluble PLA_2 activity from rat brain was applied to a CM-5PW h.p.l.c. column. The material was eluted by increasing the NaCl concentration. A_{280} (——) was monitored and fractions were collected and assayed for PLA_2 activity using [^{14}C]phosphatidic acid as substrate (●).

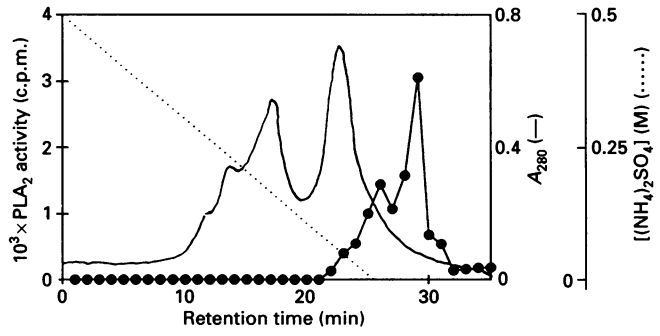

Figure 3 Hydrophobic chromatography of rat brain PLA_2

Fractions 19–23 from the CM-5PW h.p.l.c. column were pooled and $(NH_4)_2SO_4$ was added to a final concentration of 0.5 M before being applied to a phenyl-5PW h.p.l.c. hydrophobic-interaction column. The material was eluted by decreasing the $(NH_4)_2SO_4$ concentration. A_{280} was monitored (——) and fractions were collected and assayed for PLA_2 activity using [^{14}C]-phosphatidic acid as a substrate (●).

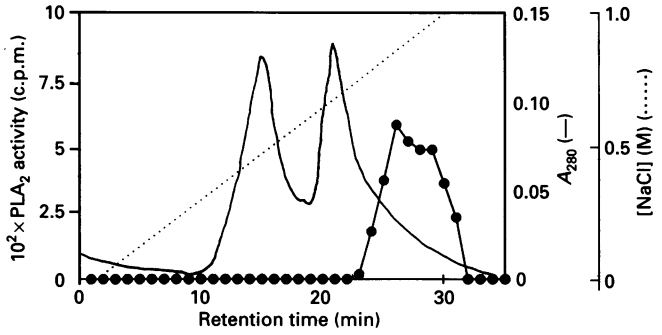

Figure 4 Heparin-affinity chromatography of rat brain PLA_2

Fractions 25–30 from the phenyl column were pooled, and the salt concentration was lowered by adding 22 vol. of 20 mM Hepes/10 mM EDTA (pH 7). The sample was concentrated by ultrafiltration before being applied to a heparin–5PW h.p.l.c. column. The material was eluted by increasing the NaCl concentration. A_{280} (——) was monitored and fractions were collected and assayed for PLA_2 activity using [^{14}C]phosphatidic acid as substrate (●).

PLA_2 substrate specificity

The ability of the purified PLA_2 to hydrolyse a variety of phospholipids and diacylglycerol was determined using a variety of radiolabelled substrates (Table 2). Although phosphatidic acid was readily hydrolysed, this enzyme had little measurable activity when either phosphatidylcholine, phosphatidylethanolamine or diacylglycerol were used as substrates. The *sn*-2 specificity of this enzyme was tested using phosphatidic acid that had either myristic, palmitic, oleic or arachidonic acid in the *sn*-2 position; however, no selectivity was found with any of the substrates used (results not shown).

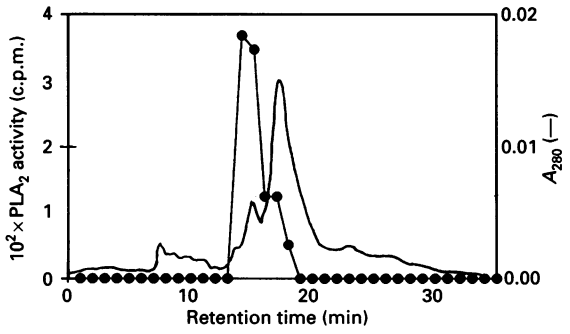

Figure 5 Gel filtration of rat brain PLA_2

Fractions 25–31 from the heparin column were pooled, concentrated to a final volume of 1 ml by ultrafiltration, applied to a gel-filtration column and the material was eluted. A_{280} (——) was monitored and fractions were collected and assayed for PLA_2 activity (●). The material that eluted in fractions 14 and 15 was pooled and used in all subsequent experiments.

Table 1 Summary of PLA_2 purification

The data shown are representative of three independent experiments.

	Total protein (mg)	Specific activity (nmol/h per mg)	Total activity (nmol/h)	Enrichment	Total enrichment	Yield (%)
Starting material	7791	2.3	18000	–	–	100
Supernatant	2111	2.69	5680	1.2	1.2	31.6
CM column	91	118	1074	43.8	51	6.0
Phenyl column	0.1	1100	110	9.3	478	0.6
Heparin column	0.004	24000	97.6	22.2	10434	0.54
Gel filtration	0.0008	96062	76.8	4.0	41766	0.43

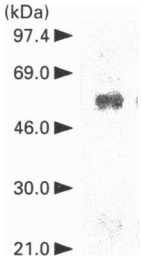

Figure 6 SDS/PAGE of the purified rat-brain PLA_2

The material in fractions 15 and 16 from the gel-filtration column was analysed by SDS/PAGE. The resulting gel was stained with silver. The molecular masses (kDa) of a set of protein standards are given on the left.

Table 2 Substrate specificity of purified PLA_2

Purified PLA_2 (10 ng/ml) was incubated with the indicated substrates and the rates of hydrolysis were quantified. The data shown above represent means ± S.D. from three separate experiments using two different preparations of enzyme.

Substrate	Specific activity (nmol/h per mg)
Phosphatidic acid	94000 ± 2000
Phosphatidylcholine	61 ± 24
Phosphatidylethanolamine	36 ± 16
Diacylglycerol	10 ± 8

DISCUSSION

Hydrolysis of phospholipids by PLA_2 produces a variety of biologically active and physiologically important metabolites. Much of the research has focused on the phospholipases A_2 that are responsible for the liberation of arachidonic acid and subsequent eicosanoid production (for review, see [21]). However, there is increasing evidence that the lysophospholipids, such as lysophosphatidylcholine, lysophosphatidylserine and LPA, have both physiological and pathophysiological roles [22–24].

One of the most interesting lysophospholipids, LPA, has recently received a great deal of attention largely because of its wide spectrum of biological actions (for review, see [25]). As a consequence, both LPA synthesis and degradation must be tightly regulated. In this study, we have described the purification and characterization of a PLA_2 which selectively hydrolyses phosphatidic acid and thus is involved in LPA synthesis.

Multiple phospholipases A_2 have been purified which differ from each other in terms of their physical properties, substrate specificities and cofactor requirements. Both low- (14 kDa) and high-molecular-mass (86 kDa) proteins have been well documented (for review, see [21]). The enzyme described here is intermediate in size (58 kDa) and Ca^{2+}-independent and in these respects is similar to the PLA_2 purified from canine heart [26]. However, unlike canine heart PLA_2, this enzyme is cationic and has an acidic pH optimum. Although few phospholipases A_2 have been examined for the ability to hydrolyse phosphatidic acid, all enzymes purified so far hydrolyse either phosphatidylcholine or phosphatidylethanolamine. In contrast, the PLA_2 described here fails to effectively utilize any lipid tested except phosphatidic acid. Other phosphatidic-acid-hydrolysing PLA_2 activities probably exist, including a membrane-bound, Ca^{2+}-dependent form identified in platelets [17]. Rat brain also has insoluble phosphatidic-acid-hydrolysing activity that may be related to the platelet enzyme. It is possible that the enzyme described here may undergo covalent modification and become membrane-associated, as has been reported for the 86 kDa PLA_2 [27]. Alternatively, it is possible that we have isolated a proteolytic fragment of the membrane-bound form of the enzyme.

Cellular concentrations of phosphatidic acid are low in comparison with phosphatidylcholine and phosphatidylethanolamine. This may indicate that a phosphatidic-acid-selective PLA_2 would be substrate-limited. However, agonist-induced phospholipase D activation would elevate intracellular levels of phosphatidic acid which, in turn, could be metabolized by the PLA_2 described here to produce LPA. This water-soluble reaction product is released into the extracellular milieu [28–30] and interacts with specific membrane receptors on target cells [9]. Following LPA-receptor activation a variety of second messengers are produced [5,6,10–14] and induction of specific genes occurs [15]. The LPA signal may be attenuated by a lysophospholipase which specifically degrades this lipid [16]. We conclude that the enzyme described here is a unique and novel member of the PLA_2 family which may regulate the synthesis of LPA, a potent and biologically important lipid.

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