Purification and characterization of a lysophosphatidic acid-specific phosphatase

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Lysophosphatidic acid (LPA)-specific phosphatase was purified 3300-fold from bovine brain cytosol. The purification was achieved by $(NH_4)_2SO_4$ fractionation and several chromatography steps, such as Q-Sepharose, DEAE-5PW, Superdex 200 and heparin–Sepharose. The final enzyme preparation showed a single band of molecular mass 44 kDa on SDS/PAGE under reducing conditions. The enzyme activity was completely dependent on the presence of detergents such as Triton X-100, CHAPS, cholate and octyl-β-glucoside. The activity was independent of Mg^{2+}; other cations were inhibitory. The enzyme hydrolysed LPA specifically but not cardiolipin, tetraoleoyl-bisphosphatidic acid, ceramide 1-phosphate or sphingosine 1-phosphate, although phosphatidic acid was hydrolysed slightly. The purified enzyme hydrolysed 1-oleoyl LPA at a rate of 1.1 μmol/min per mg of protein when assayed with LPA as Triton X-100 mixed micelles. The K_m value for LPA was 38 μM. NaF and *N*-ethylmaleimide markedly inhibited the activity, but propranolol had a less potent inhibitory effect. The LPA-specific phosphatase might have an important role in LPA elimination.

INTRODUCTION

Lysophosphatidic acid (LPA) is the simplest phospholipid in Nature that elicits growth-factor-like activation [1–3]. LPA is rapidly produced and released from activated platelets as a product of the blood-clotting process. Therefore it has been suggested that LPA has a role in wound healing and regeneration. In addition it has become clear that LPA induces the rapid retraction of neurites and transient rounding of the cell body in neuronal cells. These biological activities are believed to be caused by a G-protein-coupled LPA receptor [3]. In fact, the isolation of cDNA species encoding two putative LPA receptors has been achieved [4,5], although these two clones are not homologous.

With regard to LPA production, LPA is synthesized by two pathways: production from monoacyl glycerol by monoacyl glycerol kinase and from phosphatidic acid (PA) by phospholipase A1 or A2 [6]. However, LPA produced in response to stimuli is probably derived predominantly from the latter pathway, because LPA formation occurs slightly later than that of PA [6].

Thompson and Clark [7] purified 58 kDa phospholipase A2 specific for PA from rat brain that acts independently of Ca^{2+}. Furthermore, cDNA encoding a Ca^{2+}-independent phospholipase A2, which hydrolyses PA 20-fold more effectively than PC, has been cloned [8–10]. Although it is not clear whether these enzymes are responsible for LPA production, their molecular characterization will provide clues to the LPA productive pathway.

In contrast, there are three possible LPA-hydrolysing pathways: LPA phospholipase A, LPA phosphatase and LPA acyltransferase [6,11]. Because LPA is a biologically active lipid, its elimination by these enzymes is important, to terminate the signal. LPA phospholipase A was purified from rat brain [12]. The enzyme has a molecular mass of 80 kDa; it is membrane-bound and hydrolyses LPA but not other lysophospholipids. Concerning LPA phosphatase, the existence of an ecto-type LPA phosphatase that also hydrolysed PA was reported in PAM212 cells [13]. So far, membrane-bound PA phosphatases have been purified from pig thymus, which are relatively PA-specific and with weak activity towards LPA [14,15], and from rat liver, which also hydrolyse LPA, ceramide 1-phosphate and sphingosine 1-phosphate [16,17]; an LPA-specific phosphatase has not yet been purified.

In the present study we purified an LPA-specific phosphatase from bovine brain. The enzyme hydrolysed LPA, and PA only slightly.

MATERIALS AND METHODS

Materials

Mono-oleoyl[9,10-^{3}H]LPA, [γ-^{32}P]ATP and En^{3}Hance spray were purchased from NEN Research Products. LPA, ceramide 1-phosphate, 1-mono-oleoylglycerol, *N*-ethylmaleimide (NEM), D,L-α-glycerophosphate and *p*-nitrophenyl phosphate were purchased from Sigma. Dioleoyl PA, 1,2-dioleolyglycerol and cardiolipin (beef heart) were obtained from Doosan Serdary Research Laboratories. Sphingosine 1-phosphate was obtained from Bio-Mol Research Laboratories. NaF, $HClO_4$, ammonium molybdate, L-ascorbic acid and propranolol hydrochloride were obtained from Wako Pure Chemical Industries. *Escherichia coli* DG kinase was obtained from Calbiochem. X-ray film was purchased from Kodak. Q-Sepharose Fast Flow was obtained from Amersham Biotech. TSK-Gel DEAE-5PW GL was from Tosoh Corp. (Tokyo, Japan); Hi Trap Heparin and Hi Load 26/60 Superdex 200 were from Pharmacia Biotech. Silica gel 60 H was obtained from Merck.

Preparation of labelled PA and LPA

Radiolabelled PA and LPA were prepared by incubating dioleoylglycerol and 1-oleoylgylcerol respectively with [γ-^{32}P]ATP and *E. coli* DG kinase as described previously [17]. The

Abbreviations used: LPA, lysophosphatidic acid; NEM, *N*-ethylmaleimide; PA, phosphatidic acid.

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Table 1 Purification of LPA phosphatase from bovine brain

LPA phosphatase activity was determined as described in the Materials and methods section. The reaction product was measured by using TLC. The results are averages of two independent experiments. A unit of enzymic activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of product/min.

Purification step	Protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
1. Cytosol	9600	3.23	100	0.00034	1
2. $(NH_4)_2SO_4$ (45%-satd.)	800	1.16	36.0	0.00145	4.3
3. Q-FF	65.57	2.30	71.3	0.0351	104.4
4. DEAE	9.25	1.60	49.7	0.1735	516.0
5. Gel filtration	1.46	0.374	11.6	0.2558	761.0
6. Heparin	0.02	0.022	0.69	1.1118	3306.9

labelled PA and LPA were purified by TLC and mixed with unlabelled PA and LPA to specific radioactivities ranging from 1000 to 5000 c.p.m./nmol.

LPA phosphatase assay

LPA phosphatase activity was measured in a 50 μl reaction mixture [containing 50 mM Tris/maleate, pH 7.5, and 50 μM [^{3}H]LPA (5×10^4 d.p.m.)] together with the indicated concentration of Triton X-100 and enzyme. The mixture was incubated at 37 °C for 15 min; the enzyme activity was determined as the amount of [^{3}H]mono-oleoylglycerol produced. For measurement of enzyme activity during the purification steps, a simple method was used. In this system, recovery of the monoacylglycerol produced was approx. 80 %. The reaction was terminated by the addition of 250 μl of Dole reagent [isopropanol/heptane/1 M H_2SO_4 (39:14:1, by vol.)], followed by the addition of 125 μl of water and 150 μl of heptane. After mixing, the two phases were created by centrifugation at 1200 ***g*** for 5 min. The upper phase (150 μl) was taken into another tube, to which 150 μl of heptane and a microspatula amount of silica gel 60H were then added. After mixing and centrifugation at 1200 ***g*** for 5 min, 200 μl of the supernatant was mixed with 2 ml of Scintisol EX-H and the radioactivity was determined. Specific activity and kinetic experiments were performed by using the following separation method. After the reaction, the mixture was freeze-dried and analysed by TLC with the solvent system n-butanol/acetic acid/water (30.4:4.8:4.8, by vol.) after the addition of mono-oleoylglycerol as carrier. The TLC plates were exposed to iodine vapour, after which the mono-oleoylglycerol spots were scraped off and the radioactivity was determined. The optimal pH of LPA phosphatase was measured by P_i quantification. The reaction was done at various pH values with Tris/maleate or Tris/HCl buffer as described, after which 25 μl of $HClO_4$, 75 μl of water, 25μl of 10 % (w/v) ammonium molybdate and 50 μl of 10 % (w/v) ascorbic acid were added. The mixture was boiled at 95 °C for 5 min; the absorbance was then measured at 795 nm. The enzyme activity of the assay with ^{32}P-labelled PA and LPA was determined as the release of water-soluble [^{32}P]P_i. The reaction was terminated by the addition of 100 μl of 0.1 M HCl in methanol, followed by the addition of 200 μl each of $CHCl_3$ and 1 M $MgCl_2$. After being mixed vigorously, the two phases were created by centrifugation at 1000 ***g*** for 5 min. The aqueous phase (175 μl) was removed and the radioactivity was determined in toluene/Triton X-100 scintillation mixture.

All assays were performed in the linear range with regard to the protein concentration in each assay system and the incubation time. A unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of product/min. Specific activity was defined as units/mg of protein. Protein was quantified by the method of Bradford with BSA as standard.

Purification of LPA phosphatase

All procedures were performed at 0–4 °C. The tissues were homogenized with 1.5 ml/g of tissues in buffer A [20 mM Tris/HCl (pH 7.5)/1 mM EDTA/ 0.1 mM PMSF/1 mg/ml aprotinin/1 mg/ml leupeptin]. The homogenates (four brains per lot) were centrifuged at 12000 ***g*** for 30 min. The resulting supernatant was further centrifuged at 100000 ***g*** for 1 h.

$(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ was used. The step 1 extract was first brought to 35 % satn. with $(NH_4)_2SO_4$. The mixture was stirred for 1 h at 4 °C and then centrifuged at 12000 ***g*** for 30 min. The supernatant was then brought to 45 % satn. with $(NH_4)_2SO_4$. The precipitate was suspended in 70 ml of 20 mM Tris/HCl, pH 7.5, and was dialysed against 20 mM Tris/HCl (pH 7.5).

Q-Sepharose Fast Flow column chromatography

The fraction was centrifuged at 100000 ***g*** for 1 h and the supernatant was applied to a Q-Sepharose Fast Flow column (600 ml) equilibrated with 20 mM Tris/HCl, pH 7.5. The column was washed once with the same buffer. LPA phosphatase was eluted with 2 litres of a linear NaCl gradient (0–1 M) in 20 mM Tris/HCl, pH 7.5, and 120 fractions of 15 ml were collected. The active fractions (fractions 63–69) were combined and dialysed against 20 mM Tris/HCl, pH 7.5.

TSK-Gel DEAE-5PW GL column chromatography

Active fractions from the Q Fast Flow column were applied to a DEAE-5PW GL column equilibrated with 20 mM Tris/HCl, pH 7.5. The column was washed with the same buffer, and the enzyme eluted with a 240 ml linear NaCl gradient (0–0.5 M) in 20 mM Tris/HCl, pH 7.5. The active fractions were combined and precipitated with 60 %-satd. $(NH_4)_2SO_4$.

Hi Load 26/60 Superdex 200 gel filtration

The precipitate was suspended in 3 ml of buffer B [20 mM Tris/HCl (pH 7.5)/200 mM NaCl/0.1 % sodium cholate] and was subjected to gel filtration on a Superdex 200 column equilibrated with buffer B. The column was eluted at a flow rate of 2 ml/min with 300 ml of buffer B and fractions of 2 ml were

collected. The active fractions (fractions 108–113) were combined and dialysed against 20 mM Mes/NaOH, pH 5.8.

Hi Trap heparin column chromatography

The fractions eluted from the Superdex 200 column were applied to a small column (1 ml) of heparin equilibrated with 20 mM Mes/NaOH, pH 5.8. The column was washed with the same buffer and the enzyme was eluted with a 30 ml linear gradient of NaCl (0–1 M) in 20 mM Mes/NaOH, pH 5.8.

RESULTS

LPA phosphatase purification

We found that the LPA phosphatase activity was present in the cytosolic fraction (100000 ***g*** supernatant). It converted 1-oleoyl LPA into 1-mono-oleoylglycerol at a rate of 0.34 m-unit/mg (Table 1). The supernatant was precipitated in the ranges 0–35%-satd., 35–45%-satd., 45–55%-satd. and 55–80%-satd. $(NH_4)_2SO_4$. The 45–55%-satd. $(NH_4)_2SO_4$ precipitate had a higher specific activity than other fractions (results not shown).

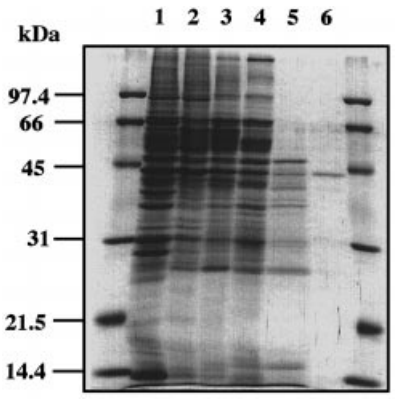

Figure 2 SDS/PAGE of separated material from bovine brain

Samples from each purification step were separated by SDS/PAGE [12.5% (w/v) gel]. Lanes 1–6 correspond to pooled materials (14, 10, 5.5, 4.6, 2.1 and 0.27 μg respectively) from steps 1–6 (Table 1). The standard proteins were rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66 kDa), hen's-egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen's-egg white lysozyme (14.4 kDa).

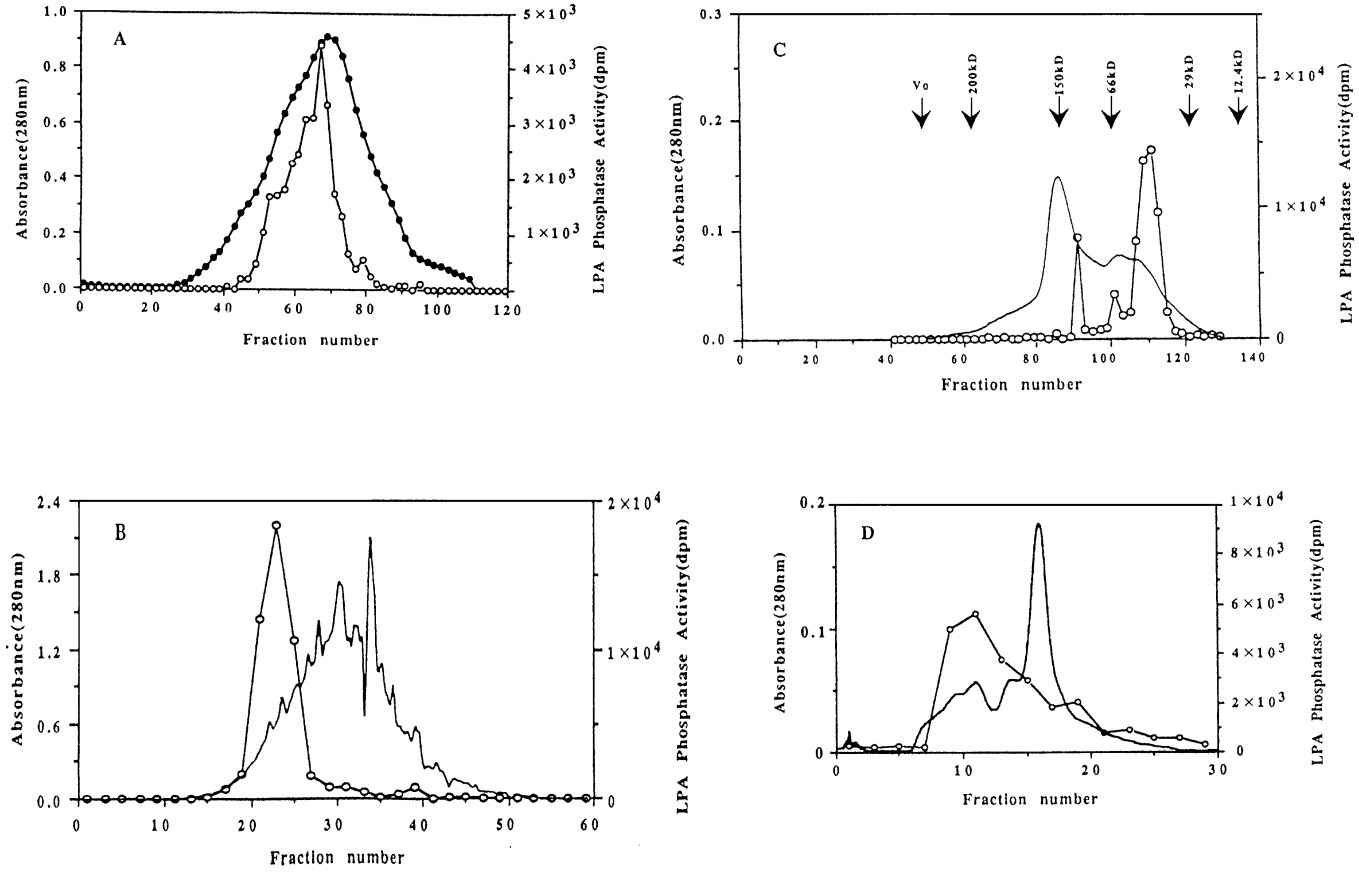

Figure 1 Fractionation of bovine brain by column chromatography

(**A**) Q Fast Flow column; (**B**) DEAE column; (**C**) Superdex 200 gel filtration; (**D**) heparin column. Aliquots (2–3.5 μl) of the respective fractions were assayed for phosphatase activity (○) as described in the text. The column effluent absorbance at 280 nm was monitored (● or continuous line without symbols). The arrows in (**C**) indicate the volumes at which proteins of known molecular mass were eluted from the column.

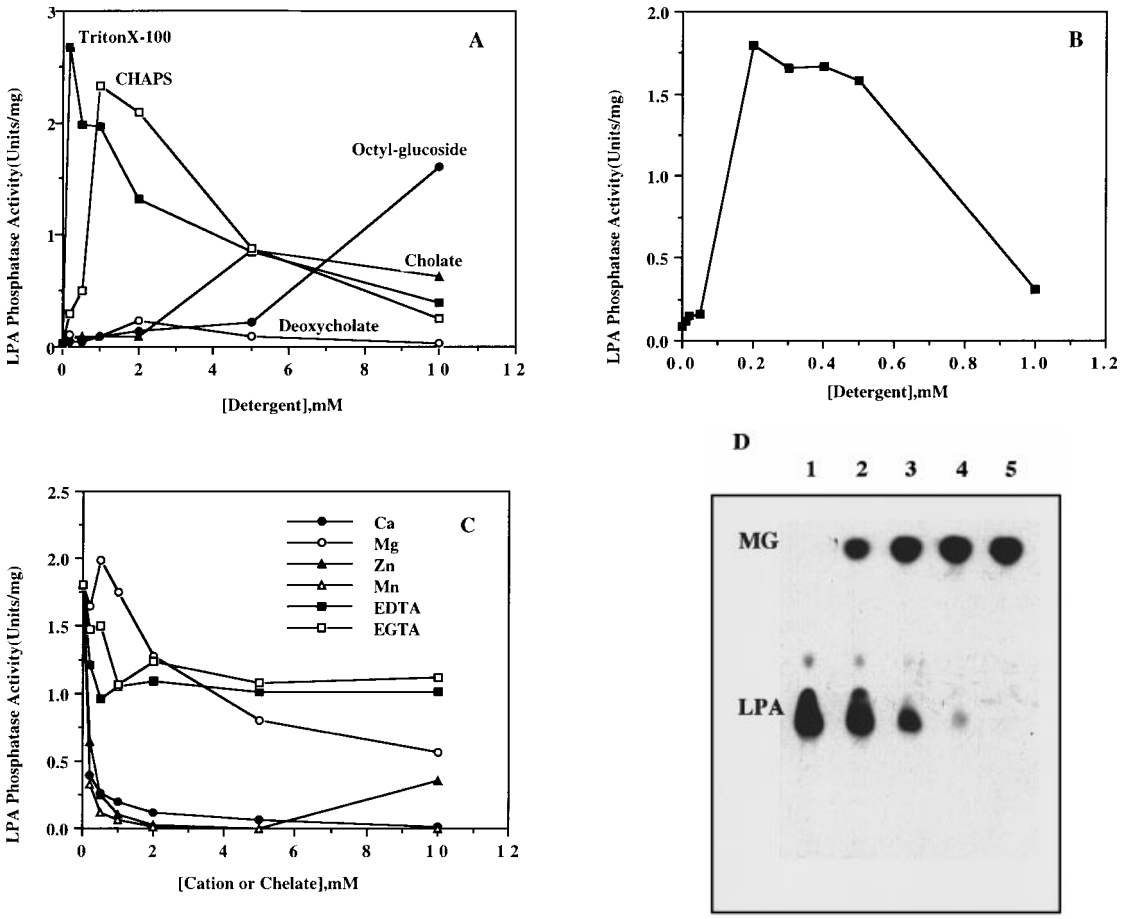

Figure 3 Catalytic properties of the LPA phosphatase

(**A**) The purified enzyme was diluted 1:10 with Triton X-100-free buffer. The diluted enzyme (17.5 ng) was assayed in the presence of various detergent concentrations. Other assay conditions were as described. (**B**) The enzyme was assayed in the presence of various concentrations of Triton X-100. (**C**) The enzyme was diluted 1:10 with cation-free buffer. The diluted enzyme (21 ng) was assayed in the presence of various concentrations of cations and chelates. The results are averages of two independent experiments. Symbols: ●, $CaCl_2$; ○, $MgCl_2$; ▲, $ZnCl_2$; △, $MnCl_2$; ■, EDTA; □, EGTA. (**D**) The activity of the purified enzyme was determined at various amounts (0, 15, 37.5, 75 and 150 ng; corresponding to lanes 1–5) of the enzyme by TLC. In this assay system, the substrate (5 nmol, 10^5 d.p.m.) was used in the presence of Triton X-100 at a four times greater molar concentration than that of the lipid substrate. Similar results were obtained in a separate experiment. Abbreviation: MG, 1-mono-oleoylglycerol.

Typical column profiles during separation of the LPA phosphatase are shown in Figure 1.

LPA phosphatase was eluted from Q-Sepharose FF at pH 7.5 with a linear NaCl gradient (Figure 1A). A broad peak of activity was eluted between approx. 0.3 and 0.6 M NaCl (Figure 1A); the fractions eluted between approx. 0.52 and 0.57 M NaCl were used for the following step. This step gave 104-fold purification over the first step (Table 1, steps 1 and 3). Next the active fractions were applied to a DEAE column. LPA phosphatase activity eluted as a single symmetrical peak between 0.16 and 0.22 M NaCl (Figure 1B). The recovery of LPA phosphatase activity from the DEAE column was nearly 70 % of that applied (Table 1). The pooled active fractions were applied to a Superdex 200 column.

Phosphatase activity was eluted mainly as two peaks, centred at fractions 91 and 111 (Figure 1C). We collected the major active fractions around fraction 111, and after cholate had been removed by dialysis against 20 mM Mes/NaOH, pH 5.8, the fractions were applied to a heparin column as the final step (Figure 2). In this way the enzyme (20 μg) was purified approx. 3300-fold from the bovine brain cytosol fraction and hydrolysed 1-oleoyl LPA to 1-mono-oleoylglycerol at a rate of 1.1 μmol/min per mg of protein (Table 1). The purified enzyme showed a single band with a molecular mass of 44 kDa on SDS/PAGE [12.5 % (w/v) gel] under reducing conditions (Figure 2).

Catalytic properties of the LPA phosphatase

The enzyme had no activity in the absence of detergent. Triton X-100 and CHAPS activated phosphatase activity maximally at 0.2 mM (substrate-to-Triton X-100 ratio of 1:4) and 0.5 mM respectively (Figures 3A and 3B). Cholate (between 5 and 10 mM) activated the phosphatase to approx. one-third that of 0.2 mM Triton X-100, whereas deoxycholate had no activating effect. Activation by octyl-β-glucoside was dose-dependent up to 10 mM (Figure 3A).

The effects of cations on enzyme activity were determined in the presence of 0.2 mM Triton X-100. All cations examined inhibited phosphatase activity, especially Ca^{2+}, Mn^{2+} and Zn^{2+} (Figure 3C). Mg^{2+} had no effect on the enzyme activity up to 1 mM, but inhibited the activity at higher concentrations in a dose-dependent manner, suggesting that this enzyme activity is

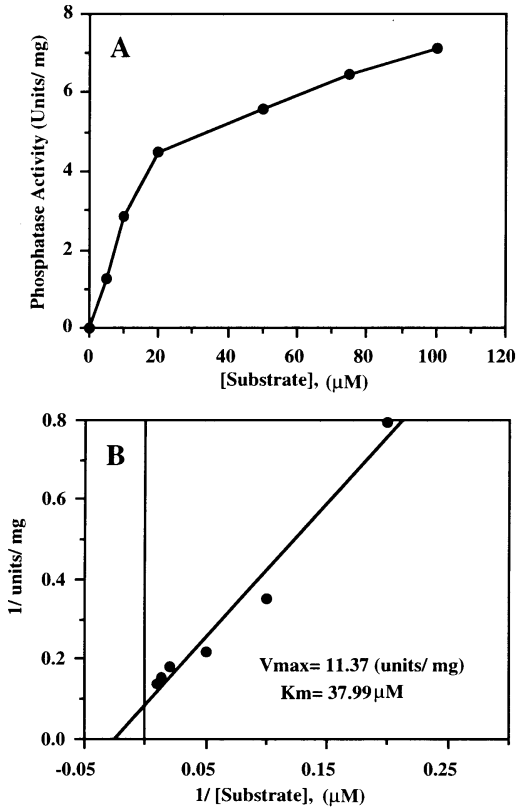

Figure 4 Kinetics of LPA phosphatase activity

(**A**) The ability of the purified enzyme (3.5 ng) to hydrolyse LPA was determined at the substrate concentrations indicated. Triton X-100 was present at a 4-fold higher molar concentration than that of the lipid substrates. Reaction velocities were determined in duplicate.The reaction product was measured by using TLC. (**B**) A Lineweaver–Burk plot of the data.

Mg^{2+}-independent. The addition of EDTA or EGTA also slightly inhibited the phosphatase activity (Figure 3C).

The purified enzyme hydrolysed [^{3}H]LPA to [^{3}H]mono-oleoylglycerol in a dose-dependent manner as determined by TLC. It was shown that this enzyme was a phosphatase, but not phosphatase A (Figure 3D).

Kinetics of LPA phosphatase activity

The kinetics of LPA phosphatase activity was examined with Triton X-100/LPA mixed micelles at a constant 4:1 molar ratio. LPA phosphatase was shown to hydrolyse LPA with typical Michaelis–Menten kinetics. Analysis of the results with a Lineweaver–Burk plot yielded a V_{max} of 11.37 units/mg and a K_m for LPA phosphatase of 38 μM (Figure 4).

Specificity of the hydrolysing activity of the LPA phosphatase

To test the specificity of the enzyme we examined its hydrolysing activity for LPA analogues such as dioleolyl PA, tetraoleoyl-bisphosphatidic acid, cardiolipin, ceramide 1-phosphate and sphingosine 1-phosphate. We examined the activity with non-radiolabelled lipids for all lipids as substrates. After the reaction, products were subjected to TLC (silica gel G 60 plates); diacylglycerol and monoacylglycerol were detected as described above.

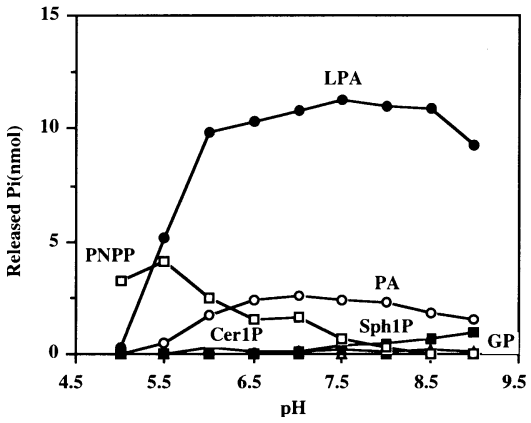

Figure 5 Specificity analysis of LPA phosphatase and effect of pH on LPA phosphatase activity

The specificity and pH dependence of the phosphatase activity were examined at various pH values in Tris/maleate or Tris/HCl buffer in the presence of a variety of non-radiolabelled lipids as substrates. In this assay system the enzyme (105 ng) and substrates (30 nmol) were used in the presence of Triton X-100 at a 4-fold higher molar concentration than that of the lipid substrates. The results are averages of two independent experiments. Abbreviations: PNPP, *p*-nitrophenyl phosphate; PA, dioleoyl PA; GP, glycerol phosphate; Cer1P, ceramide 1-phosphate; Sph1P, sphingosine 1-phosphate.

The purified enzyme hydrolysed mono-oleoyl LPA to mono-oleoylglycerol in a dose-dependent manner, but the enzyme had no hydrolysing activity towards tetraoleoylbisphosphatidic acid, cardiolipin, ceramide 1-phosphate or sphingosine 1-phosphate. The enzyme seemed to hydrolyse dioleoyl PA to diacylglycerol slightly (results not shown). Next we examined the pH-dependence and the specificity of the phosphatase with a variety of lipids as substrates. In this assay system we used 30-fold enzyme and 12-fold substrate concentrations compared with the usual assay conditions to detect a weak phosphatase activity towards various lipids. The optimal pH on LPA degradation was 7.5 and the activity was maintained over a wide pH range from 6.0 to 8.5. The enzyme showed a slight activity towards PA (dioleolyl) and *p*-nitrophenyl phosphate in the neutral pH range around 7.5 and in the acidic pH range around 5.5 respectively. However, it did not hydrolyse glycerol phosphate, ceramide 1-phosphate or sphingosine 1-phosphate in all pH ranges examined (Figure 5).

Next we further examined how specifically this enzyme hydrolysed LPA by using a highly sensitive method with [^{32}P]PA and [^{32}P]LPA. This phosphatase was found to hydrolyse LPA very specifically (Figure 6). In this experiment [^{32}P]LPA and [^{32}P]PA were purified by TLC before use. This might be the reason why the enzyme hydrolysed PA slightly, suggesting that the non-radiolabelled PA used in Figure 5 was contaminated with LPA.

Effects of enzyme inhibitors

A variety of drugs have been shown to inhibit PA phosphatase activity by using subcellular fractions and intact cells [14,18]. We tested the effects of several substances that have previously been described as phosphatase inhibitors of the purified enzyme. As shown in Figure 7, NaF exhibited the most marked inhibitory effect of the substances tested: at 2.5 mM it inhibited 85% of the phosphatase activity. NEM exhibited a 50% inhibitory effect at

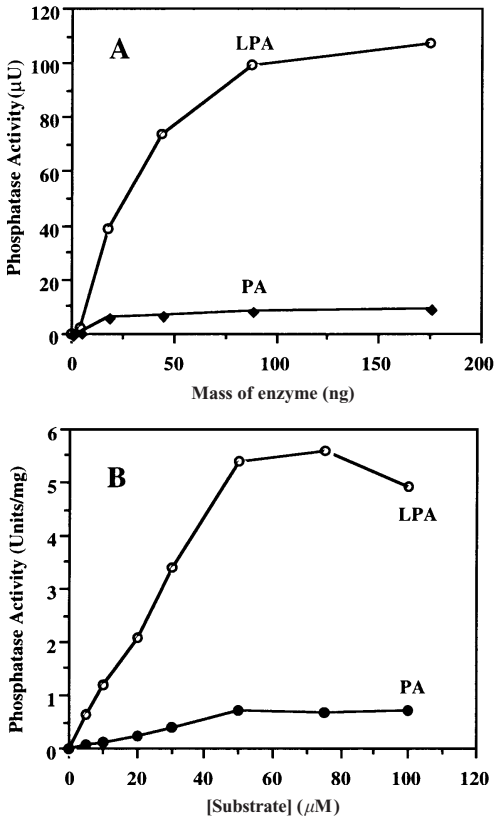

Figure 6 Comparison of phosphatase activity on PA and LPA

The phosphatase activity was measured with [^{32}P]PA and [^{32}P]LPA. **(A)** The activity of the purified enzyme was determined at various amounts (0, 4.3, 17.5, 43.8, 87.5 and 175 ng) of the enzyme using 50 μM LPA. **(B)** The ability of the purified enzyme (35 ng) to hydrolyse LPA was determined at the concentrations of substrate indicated (0, 5, 10, 20, 30, 50, 75, 100 μM). Triton X-100 was added at a 4-fold higher molar concentration than that of the lipid substrates. Each point was determined in duplicate.

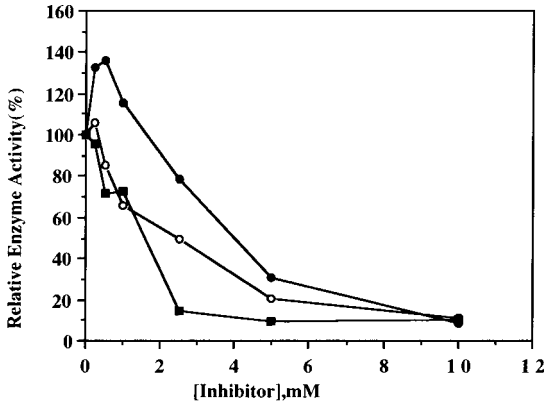

Figure 7 Effects of inhibitors on enzyme activity

LPA phosphatase activity was measured under standard assay conditions with the purified enzyme (21 ng) and 50 μM LPA in the presence of various concentrations of inhibitor. Triton X-100 concentration was constant at 0.2 mM. Results are averages of two independent experiments. Symbols: ●, propranolol; ○, NEM; ■, NaF.

2.5 mM. Propranolol activated the enzyme activity by up to 136%, with a peak at 0.5 mM, and then inhibited it at higher concentrations in a dose-dependent manner (Figure 7).

DISCUSSION

The biological activities of LPA are diverse, ranging from the induction of cell growth, the stimulation of neurite retraction, fibroblast chemotaxis and membrane depolarization in quiescent fibroblasts [1–3]. This variety of activities seems to be induced through novel G-protein-mediated pathways. It is clear that LPA receptors couple to at least three distinct G-proteins, G_q, G_i and $G_{12/15}$, which link the receptor to signalling mediated by phospholipase C, Ras and Rho.

As LPA induces a variety of biological activities in each tissue, attenuation of the LPA signal is crucial for homoeostasis. LPA is metabolized by phosphatase or phospholipase A. We first purified from bovine brain an LPA-specific phosphatase that hydrolyses LPA and also, weakly, PA. This enzyme has LPA-specific phosphatase activity and seems different from that found by Thompson and Clark [12], which has lysophospholipase A activity including LPA phospholipase A activity. Therefore the LPA phosphatase might more closely resemble a PA phosphatase. So far two PA phosphatase isoforms have been reported in rat liver [18,19]. One isoform, designated PAP1, is predominantly present in cytosol and microsomes; the other, PAP2, is localized in the plasma membrane. PAP1 requires Mg^{2+} and is completely inhibited by NEM. In contrast, PAP2 does not require Mg^{2+} and its activity is not inhibited by NEM. These characters seem to be different from the LPA phosphatase described here, because the LPA phosphatase is a soluble enzyme and does not require Mg^{2+}. Thus these results show that there must be an LPA phosphatase in addition to the PA phosphatase.

LPA and PA are key intermediates in the biosynthesis of glycerolipids [20,21]. These two simple phospholipids are formed in both the microsomes and mitochondria by the sequential acylation of glycerol 3-phosphate catalysed by glycerophosphate acyltransferase (EC 2.3.1.15) and monoacylglycerophosphate acyltransferase (EC 2.3.1.51) [22–26]. In addition, LPA, but not PA, can be synthesized in peroxisomes by the acylation of dihydroxyacetone phosphate followed by its reduction [27]. However, the principal site for the synthesis of complex phospholipids is the microsomes [20,21]; mitochondria and peroxisome-derived LPA must be transported to the microsomes for lipid biosynthesis [28]. Thus an LPA-specific phosphatase might have an important role in phospholipid metabolism.

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