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Characterization of Heparin Low-Affinity Phospholipase A_1 Present in Brain and Testicular Tissue[1]

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We identified a unique phospholipase A (PLA) with relatively low heparin affinity, which was distinguishable from the heparin-binding secretory PLA_2s, in rat, mouse, and bovine brains and testes. The partially purified enzyme was Ca^{2+}-independent at neutral pH but Ca^{2+}-dependent at alkaline pH. It predominantly hydrolyzed phosphatidic acid (PA) in the presence of Triton X-100 and phosphatidylethanolamine (PE) in its absence. When rat brain-derived endogenous phospholipids were used as a substrate, the enzyme released saturated fatty acids in marked preference to unsaturated ones. Consistent with this observation, the enzyme hydrolyzed *sn*-1 ester bonds in the substrates about 2,000 times more efficiently than *sn*-2 ones, thereby acting like PLA_1. The enzyme also exhibited weak but significant *sn*-1 lysophospholipase activity. On the basis of its limited tissue distribution, substrate head group specificity and immunochemical properties, this enzyme appears to be identical to the recently cloned PA-preferring PLA_1.

Key words: brain, heparin, phosphatidic acid, phospholipase, testis.

Cellular membranes have highly dynamic structures and play a crucial role in cellular homeostasis. A number of phospholipid-hydrolyzing enzymes [phospholipase (PL) A_1, PLA_2, PLB, PLC, PLD, lysophospholipases, sphingomyelinases, and so on] have been implicated in membrane dynamics, remodeling, and lipid mediator generation in response to extracellular and intracellular signals. The diversity of these enzymes is further complicated by the presence of various isozymes that regulate the same reaction: for instance, there are three classes of PLC (β, γ, and δ); at least three classes of PLD (PLD1, D2, and an as yet uncloned oleate-stimulated form); and more than ten members of the PLA_2 family, including cytosolic ($cPLA_2$), secretory ($sPLA_2$), and Ca^{2+}-independent ($iPLA_2$) PLA_2s as well as the platelet-activating factor acetylhydrolases (*1-3*).

We are currently studying the diversity and functions of mammalian PLA_2s and have demonstrated that Ca^{2+}-sensitive $cPLA_2\alpha$ and particular $sPLA_2$ isozymes (types IIA and V) are "signaling" PLA_2s involved in lipid mediator generation, whereas $iPLA_2$ and probably $sPLA_2$-X play a major role in membrane "remodeling" (*4, 5*). It is known that the $sPLA_2$ isozymes implicated in signaling (*4*) and some other phospholipid-hydrolyzing enzymes (*6-9*) display significant affinity for heparin. The latter group includes several PLC isozymes (*6*), lipoprotein lipase (*7*), and phosphatidylserine (PS)-specific PLA_1 (*8*). We therefore used heparin-Sepharose column chromatography to search for unique PLA activities in mammalian tissues. We identified a PLA with such activity, which exhibited weaker affinity for heparin than the heparin-binding $sPLA_2$s, in the brains and testes of various animals. Subsequent studies revealed that this heparin low-affinity PLA (HLA-PLA) displayed considerable PLA_1, weak lysophospholipase, and minimal PLA_2, activities with restricted substrate specificity, and that it is likely to be identical to the recently cloned phosphatidic acid (PA)-PLA_1 (*9, 10*).

MATERIALS AND METHODS

Materials—9-Anthryldiazomethane (ADAM) was purchased from Funakoshi. Dithiothreitol (DTT) was obtained from Wako. *p*-Bromophenacyl bromide (*p*-BPB), *n*-heptadecanoic acid, dioleoyl phosphatidylethanolamine (PE), and 1-oleoyl-lysophosphatidylethanolamine (lysoPE) were purchased from Sigma. Methyl arachidonylfluorophosphonate (MAFP) (*11*) was purchased from Cayman Chemical. Dioleoyl phosphatidylcholine (PC), dioleoyl phosphatidylserine (PS), dioleoyl phosphatidic acid (PA), and 1-oleoyl-lysophosphatidic acid (lysoPA) were purchased from Avanti. 1-Palmitoyl-2-[^{14}C]linoleoyl-PE was obtained from Amersham Pharmacia Biotech. Recombinant $sPLA_2$s-IIA, -IIC, and -V were prepared as described pre-

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Abbreviations: HLA-PLA, heparin low-affinity phospholipase A; PLA_1, phospholipase A_1; $sPLA_2$, secretory PLA_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; PLB, phospholipase B; PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; MAFP, methyl arachidonylfluorophosphonate; *p*-BPB, *p*-bromophenacyl bromide; DTT, dithiothreitol; ADAM, 9-anthryldiazomethane.

viously (*5*).

PLA Assay—In most experiments, PLA activity was measured using ADAM as described previously (*12*). Briefly, aliquots of samples were mixed with 80 μM substrate in 250 μl of 100 mM Tris-HCl buffer (pH 6–9) in the presence or absence of 4 mM $CaCl_2$ and incubated for appropriate periods at 37°C. In some assays, 1 mM DTT and/or 330 μM Triton X-100 were also added to the reaction mixtures as required for the particular experiment. The samples were then dried and mixed with 10 nmol *n*-heptadecanoic acid as an internal standard plus 50 μg ADAM in 150 μl methanol/ethyl acetate (9/1, v:v). After incubation for 1 h at room temperature in the dark, fatty acid ADAM derivatives were extracted by *n*-hexane and analyzed on a Capcell Pak C18 column (AG120, 4.6× 250 mm) (Shiseido) using a solvent system comprising acetonitrile/2-propanol/water (90:11:5, v/v/v) at a flow rate of 1.5 ml/min, with LC-6A HPLC system (Shimadzu). The ADAM derivatives were detected with the aid of a fluorescence detector (RF-535, Shimadzu) with excitation at 365 nm and emission at 412 nm.

To assess PLA_2 activity, the samples were incubated with 1-palmitoyl-2-[^{14}C]linoleoyl-PE. The [^{14}C]linoleic acid released was extracted by Dole's method (*13*), and its radioactivity was counted. To assess lysophospholipase activity, the samples were incubated with 1-oleoyl-lysoPE or 1-oleoyl-lysoPA, and the release of oleate was quantified by the ADAM method described above. To determine the positional specificity of HLA-PLA, samples were incubated with 1-palmitoyl-2-[^{14}C]linoleoyl-PE, and the resulting products were extracted as described previously (*14*), spotted onto silica gel 60 plates (Merck), and developed with a solvent system comprising chloroform/methanol/ammonia (120:65:15, v/v/v). The zones on the silica gel corresponding to [^{14}C]linoleic acid and [^{14}C]linoleoyl-lysoPE were identified by comparison with the mobilities of authentic standards and visualized using iodine vapor. The radioactivity associated with each zone was analyzed by exposure to BAS 2000 film (Fuji Film).

Purification Procedures—The brains or testes of rats (Nippon Bio-Supply Center), two strains of mouse, A/J (Japan SLC) and BALB/cJ (Nippon Bio-Supply Center), and cattle (Shibaura slaughterhouse) were homogenized using 10 strokes of a Potter homogenizer (B. Braun) at 1,000 rpm in SET buffer, comprising 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4). The homogenates were centrifuged at 100,000×*g* for 1 h at 4°C, and the resulting supernatants were used as enzyme sources.

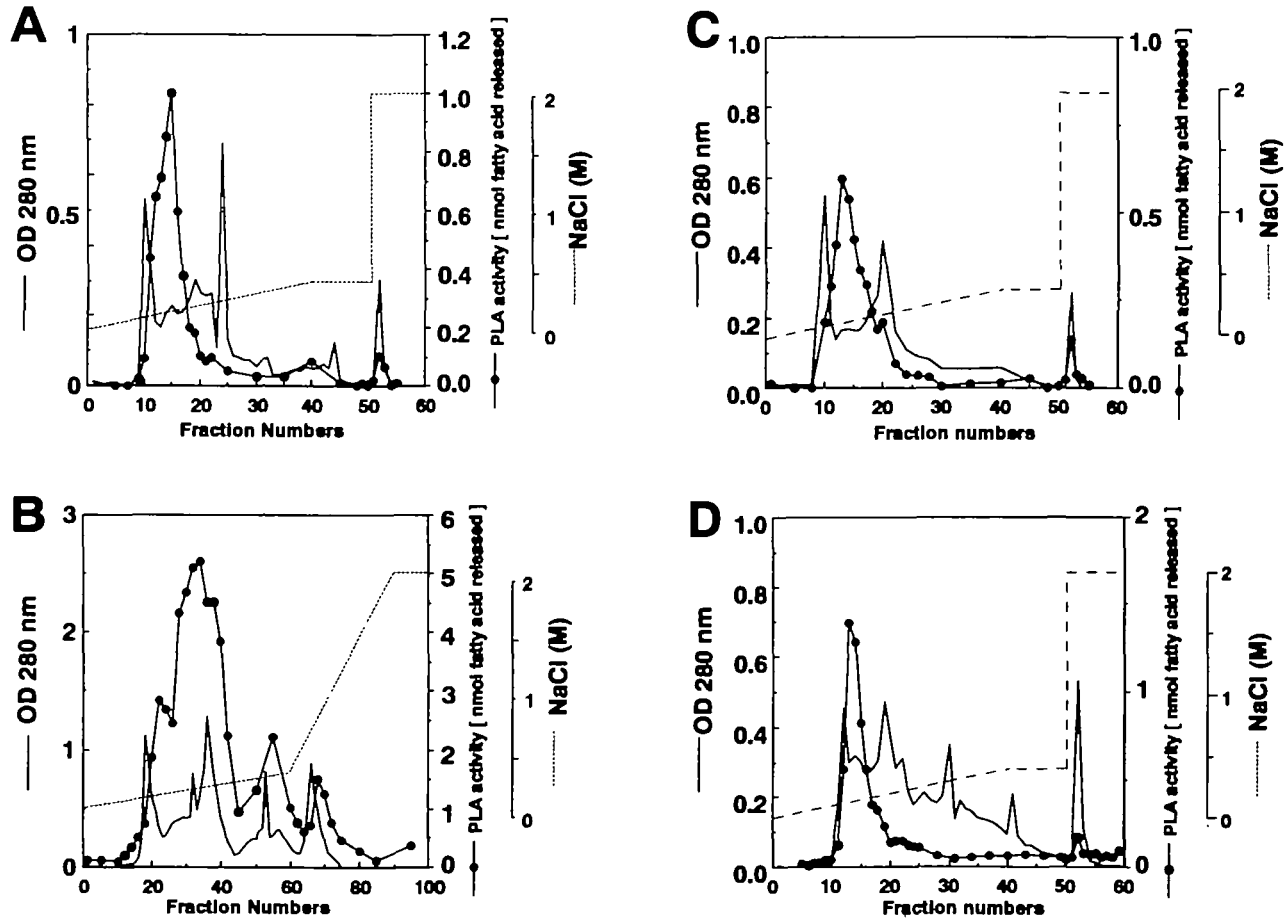

Fig. 1. **Detection of HLA-PLA activity in brain homogenates from various animal species by heparin-Sepharose column chromatography.** The 100,000×*g* supernatants of brain homogenates from rats (A), cattle (B), A/J mice (C), and BALB/cJ mice (D) were applied to Hitrap heparin FPLC, and the bound proteins were eluted with increasing NaCl gradients (dotted lines), starting from fraction 1. Aliquots of each fraction were taken for PLA assay by the ADAM method, using dioleoyl-PE as a substrate (solid circles). PLA activity is expressed as nmol oleic acid released.

The samples (10 rat, 25 mouse, or 1 bovine tissue equivalents) were applied to a heparin-Sepharose CL-6B column (Pharmacia) (15 × 300 mm), pre-equilibrated with SET buffer, and run at a flow rate of 25 ml/h. After washing with SET buffer, the bound proteins were eluted with SET buffer containing a gentle 0–0.4 M NaCl gradient, followed

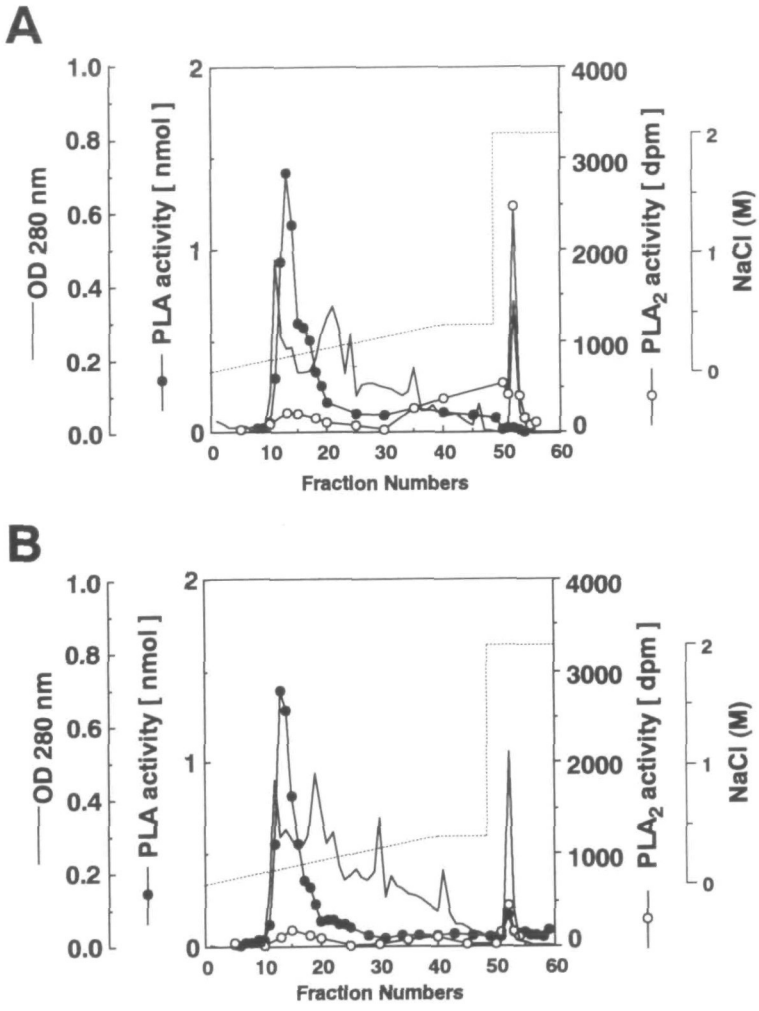

Fig. 2. **Different elution profile of HLA-PLA from that of recombinant sPLA$_2$-IIA on heparin-Sepharose column chromatography.** Recombinant mouse sPLA$_2$-IIA was added to the 100,000 × *g* supernatant of BALB/cJ mouse brain homogenate and the mixture was applied to a heparin-Sepharose column. The bound proteins were eluted with increasing NaCl gradients (dotted lines), starting from fraction 1. HLA-PLA (solid circles) and PLA$_2$ (open circles) activities in each fraction were assayed by the ADAM method, using dioleoyl-PE as a substrate, and by Dole's method, using 1-palmitoyl-2-[^{14}C]linoleoyl-PE as a substrate, respectively. (A) with additional sPLA$_2$-IIA; (B) without sPLA$_2$-IIA.

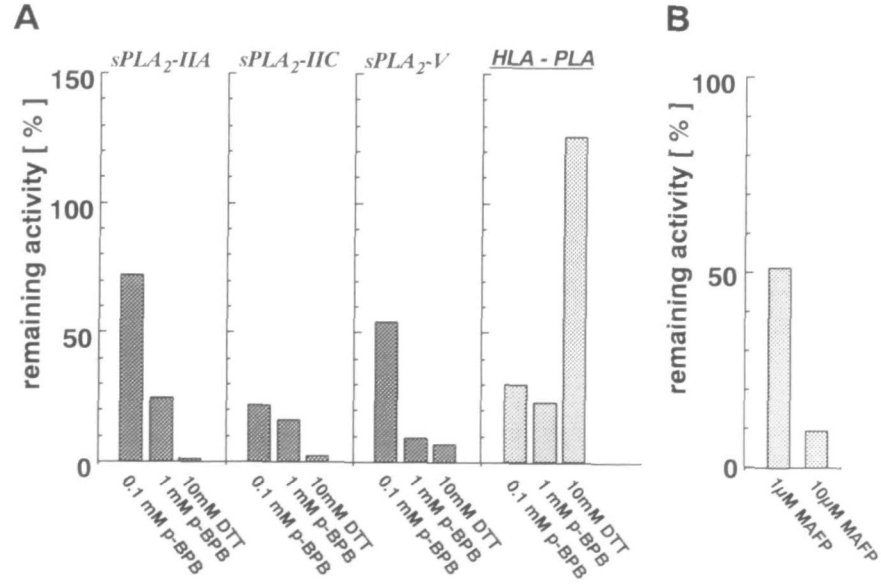

Fig. 3. **Effects of various chemical reagents on HLA-PLA activity.** (A) The effects of *p*-BPB and DTT on recombinant sPLA$_2$s-IIA, -IIC, and -V, which were expressed in human embryonic kidney 293 cells (*5*), and rat brain-derived HLA-PLA. Each sample was preincubated with the indicated concentrations of *p*-BPB for 30 min at 37 C or DTT overnight at 4 C. (B) The effect of MAFP on HLA-PLA activity. MAFP was added directly to the enzyme assay mixtures without preincubation. HLA-PLA and sPLA$_2$ activities were measured by the ADAM method, using dioleoyl-PE as a substrate, and by Dole's method, using 1-palmitoyl-2-[^{14}C]linoleoyl-PE as a substrate, respectively.

by a steep gradient up to 2 M. HLA-PLA was eluted in the fractions containing 0.2–0.3 M NaCl (see "RESULTS"). The fractions containing HLA-PLA were pooled and applied to a Phenyl-Sepharose 6FF column (Pharmacia) (10×300 mm) pre-equilibrated with 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 1 mM EDTA. After extensive washing, the bound proteins were eluted step-wise with 10 mM Tris-HCl (pH 7.4) buffers containing 0.5 M and then 0 M NaCl. Next, the fractions containing HLA-PLA activity were pooled and applied to a Mono Q FPLC column (Pharmacia) pre-equilibrated with 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, and run at a flow rate of 0.5 ml/min. After extensive washing, the bound proteins were eluted with a linear NaCl gradient up to 1 M. Fractions containing HLA-PLA activity were then subjected to Superose 12 gel filtration FPLC in a buffer comprising 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5 M NaCl. Throughout these purification steps, HLA-PLA activity

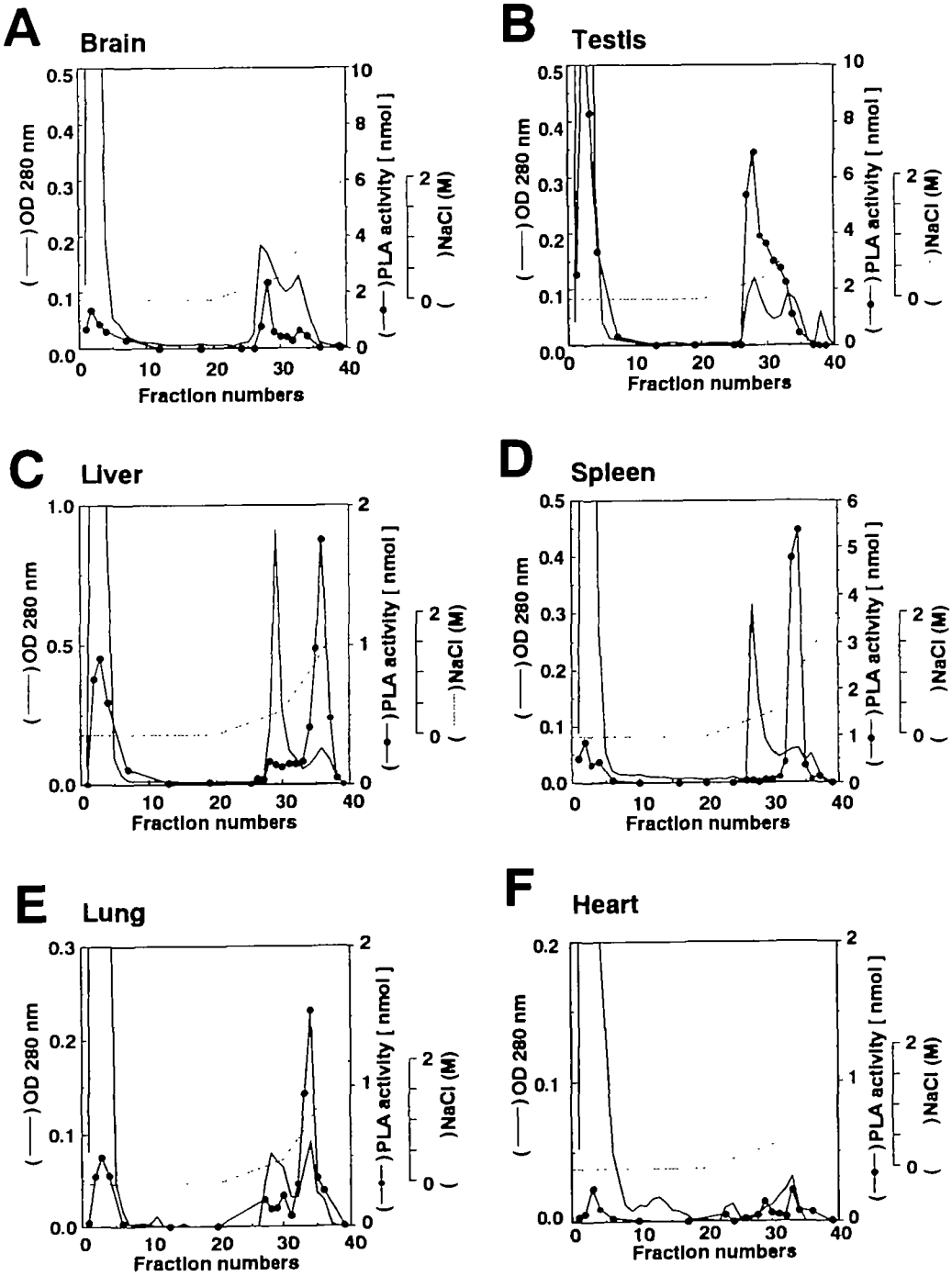

Fig. 4. **Tissue distribution of HLA-PLA.** The 100,000×*g* supernatants of homogenates from rat brain (A), testes (B), liver (C), spleen (D), lung (E), and heart (F) were applied to a heparin-Sepharose column, and the bound proteins were eluted with a two-step NaCl gradient (dotted lines). PLA activities (circles) were monitored by the ADAM method using dioleoyl-PE as a substrate.

was monitored by the ADAM method using dioleoyl-PE as a substrate, as described above. Protein concentrations were monitored by measuring the optical densities of the fractions at 280 nm and subsequently checked with a BCA protein assay kit (Pierce) as required for the particular experiment.

SDS-PAGE/Immunoblotting—Samples were subjected to SDS-PAGE (6–7.5% gel), and the separated proteins were visualized using a silver staining kit (Wako). For immunoblot analysis, the separated proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell) using a semi-dry blotter (MilliBlot-SDE system; Millipore), according to the manufacturer's instructions. The membranes were probed with a rabbit polyclonal anti-PA-PLA$_1$ antibody (a generous gift from Dr. J.A. Glomset, University of Washington, Seattle) and visualized using the ECL Western blot analysis system (Amersham) as described previously (*4*).

Extraction of Rat Brain Phospholipids—The total lipids extracted from the rat brain homogenates were reconstituted in chloroform and applied to a Wako gel C-200 column pre-equilibrated with chloroform. The column was then washed sequentially with chloroform to remove neutral lipids, with acetone to remove glycolipids, and finally with methanol to extract phospholipids. The phospholipids thus obtained were reconstituted in chloroform/methanol (2:1, v/v) and used as a HLA-PLA substrate.

RESULTS

Detection of HLA-PLA in the Brains and Testes of Various Animal Species—We prepared 100,000 × *g* supernatants of brain homogenates from various animals and subjected them to heparin-Sepharose column chromatography. The bound proteins were eluted with a 0 to 0.4 M NaCl gradient, followed by a step-wise increase in the elution gradient to 2 M NaCl. Each fraction was subjected to PLA assay using dioleoyl-PE as a substrate, as described in "MATERIALS AND METHODS." A single PLA activity peak, which was eluted from the heparin-Sepharose column with 0.2–0.3 M NaCl, was identified in brain homogenates from rats (Fig. 1A), cattle (Fig. 1B), and two strains of mouse (Fig. 1, C and D). Similar HLA-PLA activity was detected in the 100,000 × *g* supernatant of a lysate of Neuro 2a, a mouse neuronal cell line (data not shown). When recombinant sPLA$_2$-IIA was added to the BALB/cJ mouse brain homogenates, two PLA activity peaks appeared: one was eluted with 0.2 M NaCl, and the other was recovered after stepwise elution with 2 M NaCl (Fig. 2A). The latter peak was considered to represent sPLA$_2$-IIA, because it was barely detectable if sPLA$_2$-IIA was not added to the homogenates (Fig. 2B). Moreover, brain homogenates from A/J mice, which are intrinsically deficient in sPLA$_2$-IIA (*15*), contained HLA-PLA activity comparable to that in the BALB/cJ mouse brain homogenates (Fig. 1C). As shown in Fig. 3A, HLA-PLA was sensitive to *p*-BPB, whereas DTT, which inactivates all known sPLA$_2$ isozymes (*16*), produced no inhibitory effect whatsoever. These

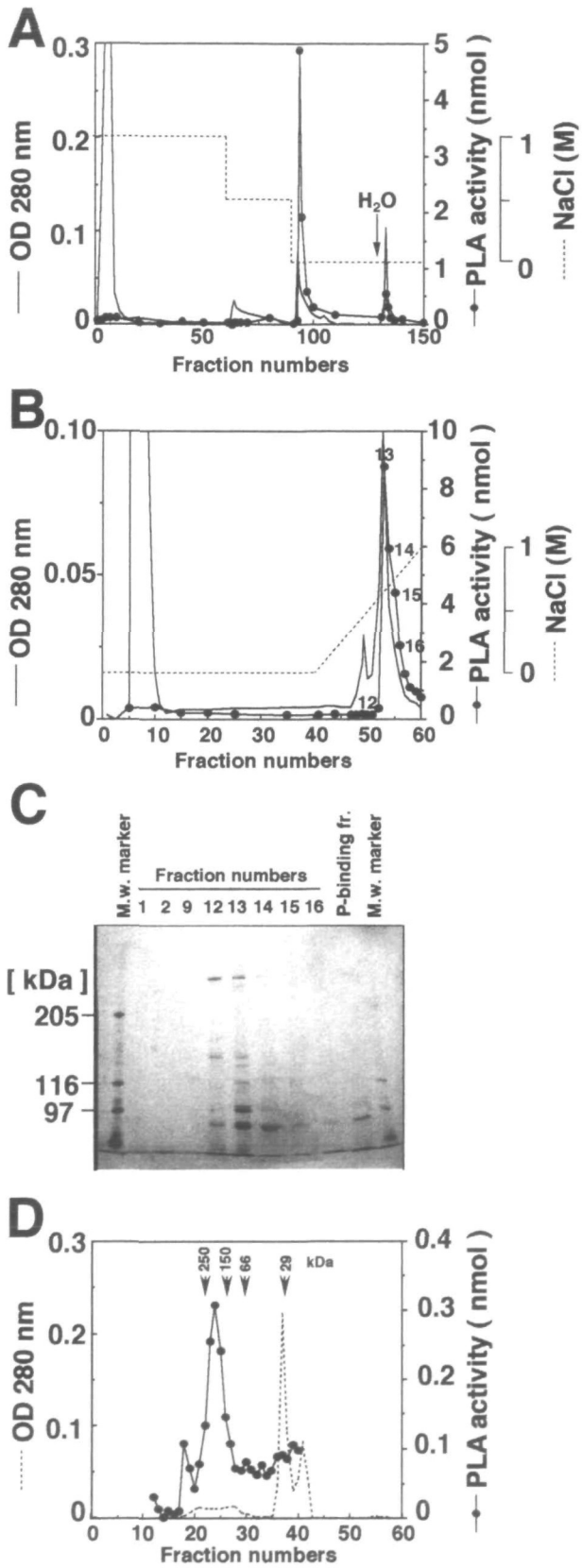

Fig. 5. **Purification of HLA-PLA.** Elution profiles of HLA-PLA activity (circles) on Phenyl-Sepharose 6FF (A) and Mono Q FPLC (B). (C) Fractions containing HLA-PLA activity [fractions 1, 2, 9, and 12 16 from Mono Q FPLC (B) and fraction 95 from the Phenyl-Sepharose column (P-binding fr.) (A)] were subjected to SDS-PAGE, followed by silver staining. (D) Elution profile of HLA-PLA activity on Superose 12 gel filtration FPLC.

results imply that HLA-PLA, which is present in the brains of various animal species, differs from the heparin-binding $sPLA_2$-IIA and all other known $sPLA_2$ isozymes. As reported previously (*17*), $sPLA_2$-IIA is present in $100,000 \times g$ pellets of rat brain homogenates, and is particularly abundant in synaptosomes. HLA-PLA was inhibited by the $cPLA_2$ inhibitor MAFP (Fig. 3B). However, HLA-PLA also differed from $cPLA_2\alpha$, which was recovered exclusively from heparin-nonbinding fractions (data not shown).

The fact that HLA-PLA was present in the brains of various animal species led us to examine its distribution in other tissues. We found that $100,000 \times g$ supernatants of rat testes contained HLA-PLA, which bound to heparin-Sepharose and was eluted with 0.3 M NaCl (Fig. 4B), a similar concentration to that at which brain HLA-PLA was eluted from the same column (Fig. 4A). In contrast, $100,000 \times g$ supernatants of rat liver, spleen and lung contained a PLA activity that was eluted from the heparin-Sepharose column with 1 M, but only minimally with 0.3 M, NaCl (Fig. 4, C–E). The main peak appeared to correspond to $sPLA_2$-IIA, showing the high affinity for heparin and tissue distribution previously reported (*18*). In rat heart, minimal HLA-PLA and $sPLA_2$-IIA-like activities were detected (Fig. 4F). Similar results were obtained using mouse and bovine tissues (data not shown). Thus, we concluded that the expression of HLA-PLA is largely restricted to the brain and testes, and as estimated by the total enzymatic activities, the testes contains approximately 5 times more HLA-PLA than the brain.

Purification of HLA-PLA—Purification of HLA-PLA from $100,000 \times g$ supernatants of rat and bovine brain and testicular tissue was carried out by sequential heparin-Sepharose, Phenyl-Sepharose 6FF, and Mono Q FPLC procedures. The elution profiles of rat brain HLA-PLA on Phenyl-Sepharose 6FF chromatography and Mono Q FPLC are shown in Fig. 5, A and B, respectively. Similar chromatographic profiles were obtained when rat testes or bovine brain were used as the enzyme source (data not shown). Post-Mono Q FPLC fractions, when analyzed by SDS-PAGE followed by silver staining, still contained several protein bands, among which two major bands with molecular masses of approximately 90 and 100 kDa, as well as two minor bands of 110 and 130 kDa, roughly correlated with the elution profile of the enzyme activity (Fig. 5C). At this stage, the specific activity of the partially purified enzyme had increased approximately 3,000-fold compared with that in the starting materials. When a pooled post-Mono Q fraction containing HLA-PLA activity was further subjected to Superose 12 gel filtration FPLC, the activity was eluted as a single major peak with an estimated molecular mass of about 200 kDa (Fig. 5D). Assuming that the 90–100 kDa molecular species were proteins responsible for HLA-PLA activity (see below), this enzyme might form a homo- or hetero-oligomer.

Enzymatic Characteristics of Partially Purified HLA-PLA—HLA-PLA activity was independent of Ca^{2+} at pHs below 7.4 (Fig. 6A). Its activity declined at alkaline pHs in the absence of Ca^{2+}, thereby peaking in the neutral pH range. However, it showed striking Ca^{2+} dependence at alkaline pHs. Under our standard assay conditions (see "MATERIALS AND METHODS"), HLA-PLA hydrolyzed PE efficiently yet exhibited no appreciable activity toward PA, PC, or PS (Fig. 6B, left). However, in the presence of Triton X-100, HLA-PLA hydrolyzed PA, but not PE, PC, or PS (Fig. 6B, right). Thus, the Ca^{2+} dependence and substrate specificity of this enzyme varied according to the assay conditions employed.

We measured the activity of the enzyme using vesicles composed of membrane phospholipids extracted from rat brain homogenates. Remarkably, HLA-PLA efficiently released saturated fatty acids, such as palmitic and stearic

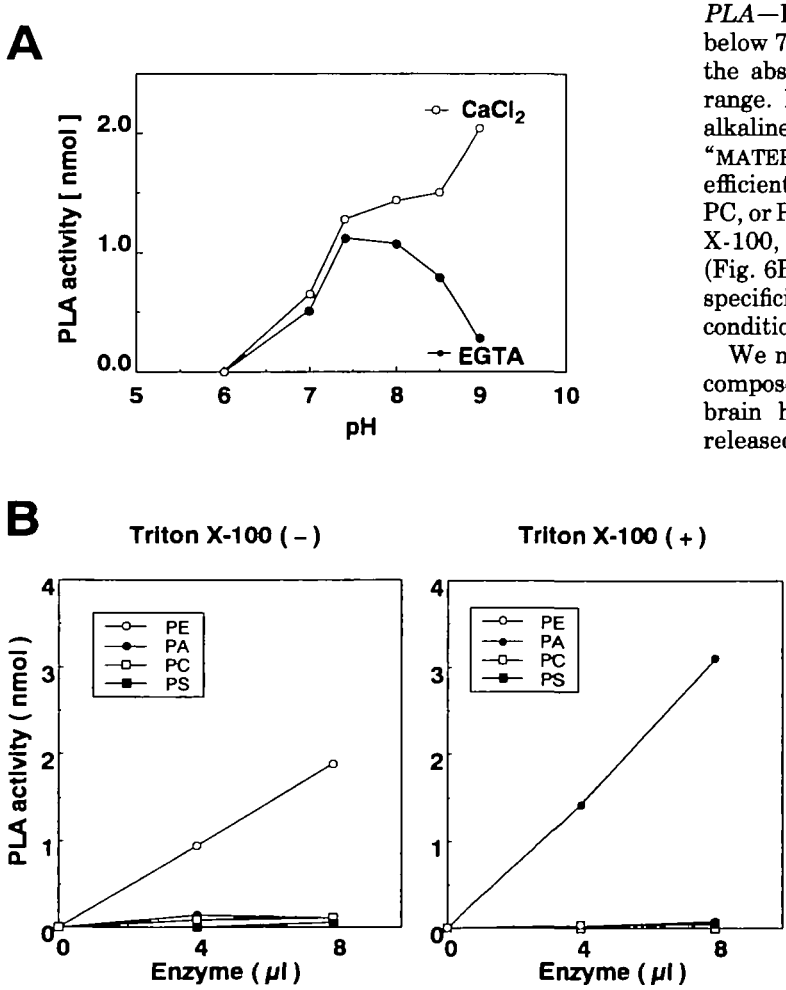

Fig. 6. **Enzymatic properties of partially purified HLA-PLA.** (A) Ca^{2+} requirements and pH dependence of HLA-PLA. The activity of partially purified HLA-PLA (post-Mono Q) was measured in the presence of either 4 mM $CaCl_2$ (open circles) or 2 mM EGTA (solid circles) at various pHs. (B) Substrate specificity in the absence (left) or presence (right) of 330 μM Triton X-100.

acids, and monounsaturated oleic acid, which are abundant at the *sn*-1 position of glycerophospholipids. In contrast, polyunsaturated fatty acids, which are usually esterified onto the *sn*-2 position, were poorly released (Fig. 7A). This was particularly notable with arachidonic acid. These results strongly suggest that HLA-PLA acts like PLA_1. However, when 2-[^{14}C]linoleoyl-PE was used as a substrate, significant liberation of free [^{14}C]linoleic acid, which was co-eluted with HLA-PLA activity even during the final purification step (Mono Q FPLC), was observed (Fig. 7B). This raises the possiblility that HLA-PLA might also possess PLA_2 activity. To confirm its positional specificity, HLA-PLA was incubated with 2-[^{14}C]linoleoyl-PE, and the accumulation of free [^{14}C]linoleic acid (indicative of PLA_2-like reactivity) and [^{14}C]linoleoyl-lysoPE (indicative of PLA_1-like reactivity) was quantified. The resulting specific PLA_1 and PLA_2 activities were 517 and 0.22 nmol/min/mg, respectively. This approximately 2,000-fold more efficient hydrolysis of *sn*-1 than of *sn*-2 ester bonds implies that this enzyme predominantly acts like PLA_1. Whether the release of traces of free [^{14}C]linoleic acid by HLA-PLA is due to its intrinsic weak PLA_2 activity, or reflects the *sn*-1 hydrolysis of linoleic acid that has migrated from the *sn*-2 to the *sn*-1 position during the enzyme reaction, remains uncertain.

We also found that HLA-PLA exhibited lysophospholipase activity, releasing oleic acid from 1-oleoyl-lysoPA or 1-oleoyl-lysoPE in the presence or absence of Triton X-100, respectively (Fig. 8). The specific lysophospholipase activity of HLA-PLA was about one-tenth that of PLA_1 for both substrates.

Relationship with PA-PLA$_1$—While our study was underway, Glomset and colleagues (*9, 10*) reported the molecular cloning of a 98-kDa PLA_1 from bovine testes. This enzyme is expressed in the testes and brain but not other tissues, predominantly hydrolyzes PA in the presence of Triton X-100, and is Ca^{2+}-independent at neutral pHs. These close similarities between PA-PLA_1 and HLA-PLA prompted us to ask whether they were identical.

To explore this possibility, we carried out an immunoblot analysis using an antibody raised against bovine testicular PA-PLA_1 (a generous gift from Dr. Glomset). When heparin-Sepharose FPLC fractions of bovine brain homogenates showing HLA-PLA activity were subjected to immunoblotting, two bands with estimated molecular masses of 90 and 100 kDa appeared. Both of these paralleled the elution profile of the enzyme activity (Fig. 9A). As noted in Fig. 5, these two proteins comigrated until the final purification step. When similar experiments were carried out with rat testicular HLA-PLA after Mono Q FPLC, a single 100-kDa band was visualized by the anti-PA-PLA_1 antibody only in fractions containing HLA-PLA activity (Fig. 9B).

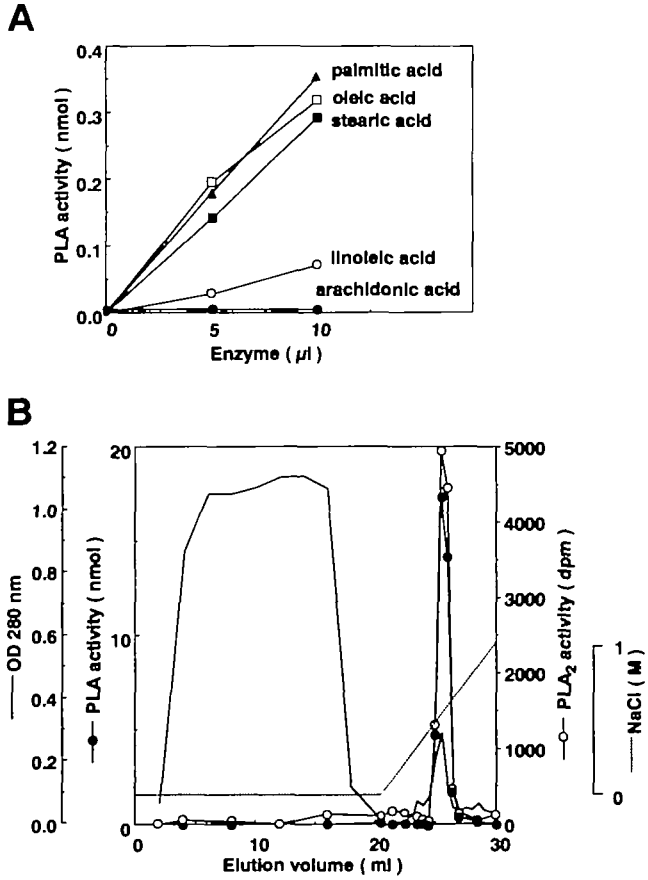

Fig. 7. **HLA-PLA has PLA_1 activity and very slight PLA_2 activity.** (A) Release of fatty acids from vesicles composed of rat brain-derived phospholipids by partially purified HLA-PLA (post-Mono Q). (B) Co-elution of HLA-PLA (solid circles) and PLA_2 (open circles) activities, as assessed by the ADAM method, using dioleoyl-PE as a substrate, and by Dole's method, using 1-palmitoyl-2-[^{14}C]-linoleoyl-PE as a substrate, respectively, on Mono Q FPLC.

DISCUSSION

Several types of phospholipases have been reported to show significant affinity for heparanoids, and this property is crucial for the functions of at least some of them. For instance, types IIA and V $sPLA_2$ associate with cell surface heparan sulfate proteoglycans and contribute to the augmentation of cellular prostanoid biosynthesis by autocrine, paracrine, and juxtacrine mechanisms (*4, 5, 19*). Lipoprotein lipase, which also possesses PLA_1 activity, anchors onto endothelial cell surface heparan sulfate proteoglycans and plays a crucial role in the metabolism of plasma lipoproteins (*7*). In the present study, by means of heparin-Sepharose chromatography, we identified a unique HLA-PLA activity in 100,000×*g* supernatants of brain and testicular homogenates from various animal species. Detailed analysis of the partially purified enzyme revealed that the HLA-PLA we have examined in this study is very similar, and probably identical, to PA-PLA_1, which was initially detected in bovine brain by Exton and coworkers (*20, 21*) and recently cloned from bovine testes by Glomset and coworkers (*9, 10*).

Several lines of evidence support the idea that HLA-PLA is identical to PA-PLA_1. First, both were detected mainly in the brain and testes (*9*), the latter containing several times more enzyme activity than the former. Second, HLA-PLA is an "A_1"-type phospholipase. Third, when HLA-PLA activity was measured in the presence of Triton X-100, an assay condition based on that employed by Glomset and colleagues (*9, 10*), it displayed remarkable specificity for PA. Conversely, cloned PA-PLA_1 has been shown to hydrolyze PE in preference to PA under certain assay

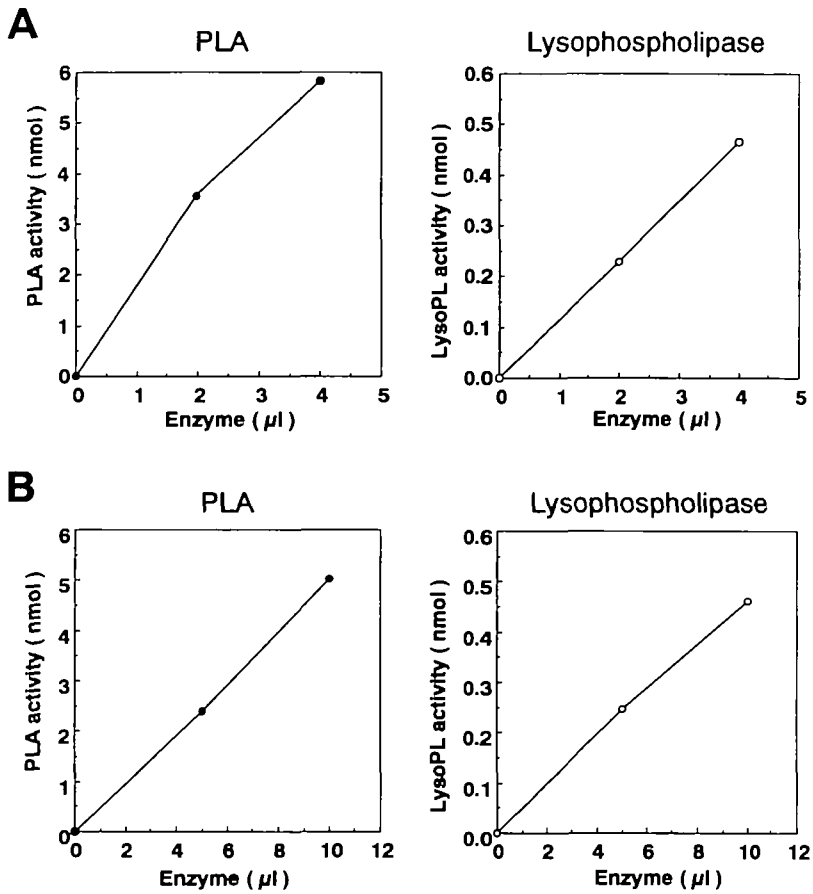

Fig. 8. **HLA-PLA has significant lysophospholipase activity.** The PLA (left) and lysophospholipase (LysoPL) (right) activities of partially purified HLA-PLA (post-Mono Q) were assessed using PA (A), lysoPA (B), PE (C), and lysoPE (D) as substrates. The assays in (A) and (B) were carried out in the presence of Triton X-100.

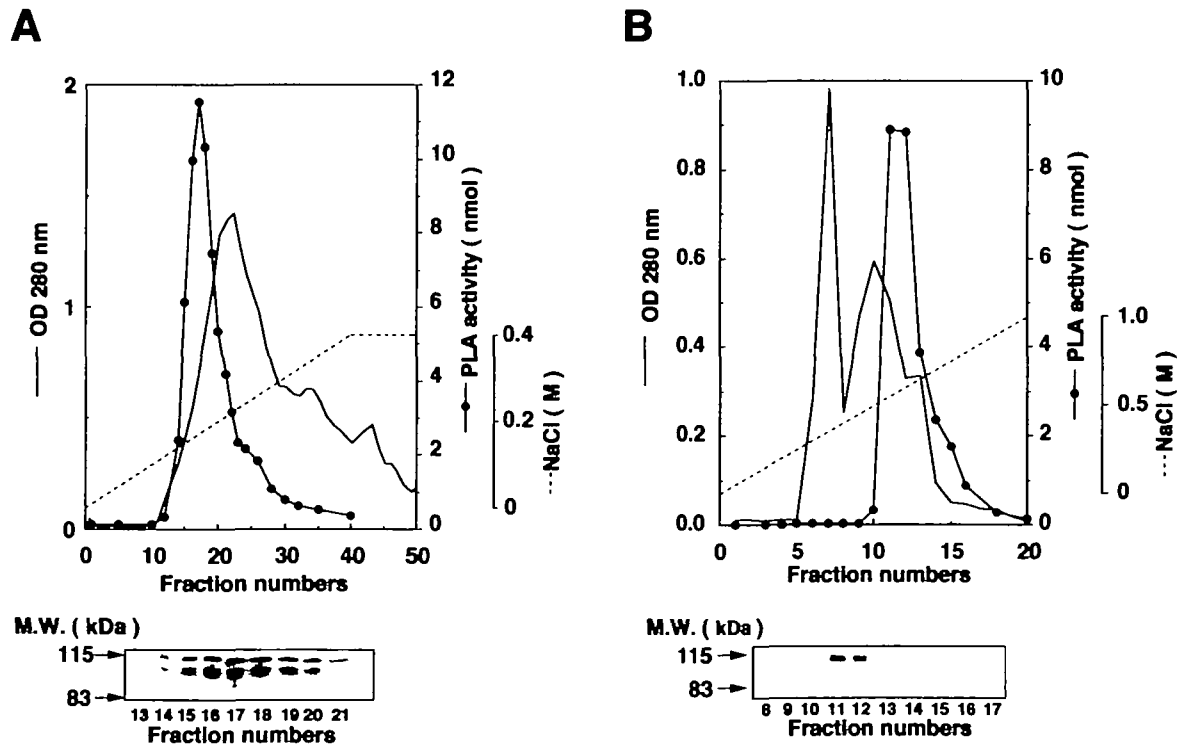

Fig. 9. **HLA-PLA is immunochemically identical to PA-PLA$_1$.** The elution profiles of HLA-PLA activity (solid circles) on heparin-Sepharose chromatography (A) and Mono Q FPLC (B) (top panels). HLA-PLA-containing fractions from each type of chromatography were subjected to SDS-PAGE, followed by immunoblotting with an anti-PA-PLA$_1$ antibody (bottom panels).

conditions (*9, 20, 21*). Fourth, both enzymes were Ca^{2+}-independent at neutral pHs and were inhibited by MAFP (*9*). The fact that HLA-PLA was activated by Ca^{2+} at alkaline pHs is reminiscent of the stimulatory effect of Mg^{2+} on PA-PLA_1 (*21*); both metal ion dependencies were assessed using PE as a substrate. Fifth, both enzymes showed similar chromatographic behaviors on several types of columns (*9, 20*). Sixth, the final preparation stage of partially purified HLA-PLA yielded protein bands of approximately 90–100 kDa that correlated with the presence of enzyme activity, and the predicted size of the PA-PLA_1 protein, estimated from its cDNA sequence, is 98 kDa (*10*). Seventh, based on gel filtration analyses, HLA-PLA, like cloned PA-PLA_1 (*9*), appeared to form an oligomer. Finally, an antibody raised against cloned PA-PLA_1 crossreacted with HLA-PLA purified from both bovine brain and rat testes, revealing 90- and 100-kDa bands. The lower 90-kDa band detected in a sample purified from bovine brain might be a proteolytic fragment of the mature 100-kDa protein, as has been suggested to occur with PA-PLA_1 (*9*).

We noted that *sn*-2 linoleic acid was consistently liberated by HLA-PLA/PA-PLA_1. It is unlikely that this was due to contamination by a heparin-binding PLA_2, since this activity was always coeluted with HLA-PLA activity throughout all the purification steps and displayed similar inhibitor sensitivity to HLA-PLA activity. Indeed, we initially regarded HLA-PLA as a novel type of PLA_2, as we had used 2-[^{14}C]linoleoyl-PE as a substrate in an attempt to purify it during the preliminary stages of this study. However, the subsequent observation that the PLA_2 activity of this enzyme was no more than 0.5% of its PLA_1 activity, together with the finding that saturated and monounsaturated fatty acids were liberated in marked preference to polyunsaturated fatty acids when the assay was performed using a brain-derived phospholipid mixture as a substrate, led us to conclude that the significance of the PLA_2 activity of this enzyme would be, at best, only minimal. In contrast to its weak PLA_2 activity, HLA-PLA/PA-PLA_1 was found to have substantial *sn*-1 lysophospholipase activity, corresponding to nearly one-tenth of its PLA_1 activity. These results indicate that the HLA-PLA/PA-PLA_1 molecule has a pocket near the catalytic triad that results in fairly strict recognition of the ester bonds at the *sn*-1 position of a substrate. Although the cellular functions of HLA-PLA/PA-PLA_1 remain uncertain, it is probable that one role is to inactivate lysoPA, a potent lipid mediator.

Comparison of the enzymatic characteristics of phospholipid-metabolizing enzymes has been hampered by the fact that different investigators have used different assay systems. Because of subtle differences in the structures formed by a single phospholipid, due to varying headgroups and acyl chain compositions, the results obtained with *in vitro* assay systems are difficult to interpret and may not always be applicable to physiologic circumstances. For instance, the preferential hydrolysis of PE by HLA-PLA/PA-PLA_1 under certain assay conditions might reflect the special physiologic properties of PE, which readily forms a non-bilayer, inverted micellar conformation. Because of the complexities of currently available artificial assay systems, "PA"-PLA_1 is a convenient name at this stage, but may not be the proper designation. The next important step will be to determine the physiological substrates of this enzyme.

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REFERENCES

1. Rhee, S.G. and Bae, Y.S. (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* **272**, 15045-15048
2. Exton, J.H. (1997) New developments in phospholipase D. *J. Biol. Chem.* **272**, 15579-15582
3. Dennis, E.A. (1997) The growing phospholipase A_2 superfamily of signal transduction enzymes. *Trends Biochem. Sci.* **22**, 1-2
4. Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M.V., Tischfield, J.A., and Kudo, I. (1998) The functions of five distinct mammalian phospholipase A_2s in regulating arachidonic acid release: type IIA and type V secretory phospholipase A_2s are functionally redundant and act in concert with cytosolic phospholipase A_2. *J. Biol. Chem.* **273**, 14411-14423
5. Murakami, M., Kambe, T., Shimbara, S., and Kudo, I. (1999) Functional coupling between various phospholipase A_2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *J. Biol. Chem.* **274**, 3103-3115
6. Suh, P.G., Ryu, S.H., Moon, K.H., Suh, H.W., and Rhee, S.G. (1988) Cloning and sequence of multiple forms of phospholipase C. *Cell* **54**, 161-169
7. Sendak, R.A. and Bensadoun, A. (1998) Identification of a heparin-binding domain in the distal carboxyl-terminal region of lipoprotein lipase by site-directed mutagenesis. *J. Lipid Res.* **39**, 1310-1315
8. Sato, T., Aoki, J., Nagai, Y., Dohmae, N., Takio, K., Doi, T., Arai, H., and Inoue, K. (1997) Serine phospholipid-specific phospholipase A that is secreted from activated platelets: a new member of the lipase family. *J. Biol. Chem.* **272**, 2192-2198
9. Higgs, H.N. and Glomset, J.A. (1996) Purification and properties of a phosphatidic acid-prererring phospholipase A_1 from bovine testis: examination of the molecular basis of its activation. *J. Biol. Chem.* **271**, 10874-10883
10. Higgs, H.N., Han, M.H., Johnson, G.E., and Glomset, J.A. (1998) Cloning of a phosphatidic acid-prererring phospholipase A_1 from bovine testis. *J. Biol. Chem.* **273**, 5468-5477
11. Balsinde, J., Balboa, M.A., and Dennis, E.A. (1998) Functional coupling between secretory phospholipase A_2 and cyclooxygenase-2 and its regulation by cytosolic group IV phospholipase A_2. *Proc. Natl. Acad. Sci. USA* **95**, 7951-7956
12. Fujita, K., Murakami, M., Yamashita, F., Amemiya, K., and Kudo, I. (1996) Phospholipase D is involved in cytosolic phospholipase A_2-dependent selective release of arachidonic acid by fMLP-stimulated rat neutrophils. *FEBS Lett.* **395**, 293-298
13. Dole, V.P. and Menertz, H. (1960) Microdetermination of long-chain fatty acid in plasma and tissues. *J. Biol. Chem.* **235**, 2595-2599
14. Atsumi, G., Tajima, M., Hadano, A., Nakatani, Y., Murakami, M., and Kudo, I. (1998) Fas-induced arachidonic acid release is mediated by Ca^{2+}-independent phospholipase A_2 but not cytosolic phospholipase A_2 which undergoes proteolytic inactivation. *J. Biol. Chem.* **273**, 13870-13877
15. MacPhee, M., Chepenik, K.P., Liddell, R.A., Nelson, K.K., Siracusa, L.D., and Buchberg, A.M. (1995) The secretory phospholipase A_2 gene is a candidate for the *Mom1* locus, a major modifier of Apc^{Min}-induced intestinal neoplasia. *Cell* **81**, 957-966
16. Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., and Kudo, I. (1997) Regulatory functions of phospholipase A_2. *Crit. Rev. Immunol.* **17**, 225-283
17. Matsuzawa, A., Murakami, M., Atsumi, G., Imai, K., Prados, P., Inoue, K., and Kudo, I. (1996) Release of secretory phospholipase A_2 from rat neuronal cells and its possible function in the regulation of catecholamine scretion. *Biochem. J.* **318**, 701-710
18. Kramer, R.M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E.P., Tizard, R., and Pepinsky, R.B. (1989) Structure and properties of a human non-pancreatic phospholipase A_2. *J. Biol.*

Chem. **264**, 5768-5775
19. Tada, K., Murakami, M., Kambe, T., and Kudo, I. (1998) Induction of cyclooxygenase-2 by secretory phospholipase A_2s in nerve growth factor-stimulated rat serosal mast cells is facilitated by interaction with fibroblasts and mediated by a mechanism independent of their enzymatic functions. *J. Immunol.* **161**, 5008-5015
20. Pete, M.J., Ross, A.H., and Exton, J.H. (1994) Purification and properties of phospholipase A_1 from bovine brain. *J. Biol. Chem.* **269**, 19494-19500
21. Pete, M.J. and Exton, J.H. (1995) Phospholipid interactions affect substrate hydrolysis by bovine brain phospholipase A_1. *Biochim. Biophys. Acta* **1256**, 367-373