Purification of a 100 kDa phospholipase A₂ from spleen, lung and kidney: antiserum raised to pig spleen phospholipase A₂ recognizes a similar form in bovine lung, kidney and platelets, and immunoprecipitates phospholipase A₂ activity

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Phospholipase A₂ (PLA₂) plays a key role in the production of intracellular and extracellular chemical mediators such as arachidonic acid, eicosanoids and platelet-activating factor, which modulate membrane channel activity, signal transduction, are vasoactive and chemotactic, and are implicated in many pathophysiological mechanisms of inflammation and tissue injury. We previously identified, purified and characterized an arachidonic acid-selective cytosolic 100–110 kDa PLA₂ from bovine platelets and rat kidney that is activated during cell stimulation. The purification schemes previously published resulted in low yields of enzyme, insufficient for extensive biochemical characterization. We report the purification of a large-molecular-mass (100 kDa) PLA₂ from pig spleen, bovine kidney and bovine lung, using a novel large-scale purification scheme. The enzyme was purified to near homogeneity from an acidified extract obtained from 4.8 kg of pig spleen by sequential use of DEAE-cellulose anionic exchange, Butyl-Toyopearl hydrophobic chromatography and DEAE-5PW h.p.l.c., and further purified by non-denaturing

PAGE. This purification scheme will permit the preparation of quantities of purified native enzyme sufficient to study its properties and regulation. To generate antiserum against the PLA₂ enzyme, the 100 kDa protein was excised and electroeluted from SDS/PAGE gels of the active fractions after DEAE-5PW h.p.l.c., and this was used as antigen. This polyclonal antibody against pig spleen 100 kDa PLA₂ protein reacted with 100 kDa bands in preparations partially purified from bovine platelets, kidney and lung as well as pig spleen, and immunoprecipitated PLA₂ activity from these sources. The antibody also immunoprecipitated a 100 kDa protein from cytosolic fractions of cultured renal mesangial cells, human erythroleukaemia cells and human monocytic U937 cells. Considerable PLA₂ activity was present in the immunoprecipitates. To our knowledge this antibody is unique in its ability to permit measurement of PLA₂ activity in the immunoprecipitate itself, and will be a useful tool for the study of the regulation and the activation mechanisms of the native PLA₂ enzyme.

INTRODUCTION

Phospholipase A₂ (PLA₂, EC 3.1.1.4) specifically hydrolyses the fatty acyl ester bond at the *sn*-2 position of glycerophospholipids [1,2]. The arachidonic acid (AA) released from membrane phospholipids by intracellular PLA₂ is subsequently metabolized to eicosanoids, such as prostaglandins, thromboxanes and leukotrienes. When the substrate for PLA₂ is 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine, the product is the precursor for platelet-activating factor. These lipid mediators play important roles in membrane channel activation, signal transduction, haemodynamics and pathophysiological mechanisms involved in inflammation and tissue injury [2,3]. An intracellular large-molecular-mass cytosolic PLA₂ has been implicated in signal-transduction processes and receptor-mediated liberation of AA [4–6]. A cDNA encoding a 85.2 kDa PLA₂ protein from the human monocyte U937 cell line has been cloned [7,8]. Unlike mammalian secretory Group I and Group II 14–18 kDa PLA₂s, this high-molecular-mass cytosolic PLA₂ enzyme has been reported to exhibit a preference for AA esterified at the *sn*-2 position of phospholipid substrates and had considerable activity when Ca²⁺ concentrations were lower than 1 μM [4,9–13]. By

analogy with the 14–18 kDa forms of PLA₂, the large-molecular-mass form may constitute a family of enzymes. Potential relationships among the large-molecular-mass enzymes in various organs are not defined. No such form has previously been identified in the lung. Furthermore, because of the low abundance of the enzyme in cells, previously published purification schemes result in insufficient amounts of the native enzyme for study of its regulation and enzymology *in vitro* or its susceptibility to putative inhibitory agents. Although the protein has been expressed in Chinese Hamster Ovary (CHO) cells, this does not obviate the need for obtaining native protein, since post-translational modifications are often cell-, tissue-, organ- and species-specific [14–16], and therefore a recombinant protein produced by a cultured cell may demonstrate properties very different from those of the native protein. We report the purification of a large-molecular-mass (100 kDa) PLA₂ from pig spleen, bovine lung and bovine kidney, using a novel large-scale purification scheme. Ca²⁺ induces translocation of the purified enzyme from the soluble to the membrane fraction. A polyclonal antibody generated against the 100 kDa splenic PLA₂ protein immunoprecipitated PLA₂ activity from bovine platelets, lung and kidney and pig spleen as well as cultured renal mesangial

Abbreviations used: PLA₂, phospholipase A₂; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; AA, arachidonic acid; LA, linoleic acid; HEL, human erythroleukaemia cells; MDCK, Madin–Darby canine kidney cells; DEDA, 7,7-dimethyleicosadienoic acid; NDGA, nordihydroguaiaretic acid; BPB, *p*-bromophenacyl bromide.

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cells, Madin–Darby canine kidney (MDCK) cells, human erythroleukaemia (HEL) cells and human monocytic U937 cells and reacted with 100 kDa proteins from these sources on Western blots. This antibody is unique in its ability to retain considerable activity in the immunoprecipitates.

EXPERIMENTAL

Materials

1-Stearoyl-2-[1-¹⁴C]arachidonoyl-GPC (where GPC is *sn*-glycero-3-phosphocholine) (2-[1-¹⁴C]AA-GPC; 56.7 mCi/mmol), 1-palmitoyl-2-[1-¹⁴C]linoleoyl-GPC (2-[1-¹⁴C]LA-GPC; 53.6 mCi/mmol), 1-acyl-2-[1-¹⁴C]arachidonoyl-GPE (where GPE is *sn*-glycero-3-phosphoethanolamine) (55.1 mCi/mmol), 1-palmitoyl-2-[1-¹⁴C]linoleoyl-GPE (54 mCi/mmol) and 1-stearoyl-2-[5,6,8,9,11,12,14,15-³H]arachidonoyl-GPC (2-[³H]AA-GPC; 132 Ci/mmol) were purchased from Amersham (U.K.) or New England Nuclear. 1-Stearoyl-2-arachidonoyl-GPC and 1-palmitoyl-2-linoleoyl-GPC were obtained from Sigma, St. Louis, MO, U.S.A. 7,7-Dimethyleicosadienoic acid (DEDA) was obtained from Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A. Nordihydroguaiaretic acid (NDGA), *p*-bromophenacyl bromide (BPB) and quinacrine were obtained from Sigma. Indomethacin was obtained from Calbiochem, La Jolla, CA, U.S.A. DEAE-cellulose (DE 52) was obtained from Whatman, Maidstone, Kent, U.K. Butyl-Toyopearl 650 M and DEAE-5PW columns were purchased from Tosoh, Tokyo, Japan. Protein A–Sepharose CL-4B was obtained from Pharmacia LKB Biotechnology. All other chemicals were of the highest purity available from commercial sources. Protein concentration was determined using the BCA protein assay (Pierce Chemical Co.) with BSA as the standard.

Assay of PLA₂ activity

PLA₂ activity was assayed by using sonicated liposomes prepared as described previously [11], containing 2-[1-¹⁴C]AA-GPC (56.7 mCi/mmol, from Amersham) unless otherwise specified. The standard PLA₂ assay buffer (200 µl) contained 75 mM Tris/HCl, 5 mM CaCl₂, 1 mg/ml BSA and 5.0 nmol of the phospholipid (approx. 65000 d.p.m.) at pH 9.0. The reaction was carried out at 37 °C for 3 min and was stopped by adding 1.25 ml of Dole's reagent (78 % propan-2-ol, 20 % *n*-heptane, 2 % of 2 M H₂SO₄ in water [17]). Non-esterified fatty acid was extracted as follows. First 0.55 ml of water was added and the sample was vortex-mixed and centrifuged at 5600 *g* for 5 min. Then 0.75 ml of the upper phase was transferred to a new tube, to which 100 mg of silica gel and 0.75 ml of heptane were added. The samples were vortex-mixed and centrifuged again for 5 min each, after which 1.4 ml of supernatant was counted for radioactivity in a liquid-scintillation counter. In some experiments designed to examine site-specificity of the enzyme, the reaction was stopped by adding 1 ml of chloroform/methanol (1:2, v/v) and lipids were extracted by the method of Bligh and Dyer [18]. Solvents were removed by a stream of nitrogen gas and the lipids were resuspended in chloroform/methanol (1:1, v/v). Phospholipids and neutral lipids were separated by double migration in a single direction on layers of silica gel G, first in chloroform/methanol/water (12:6:1, by vol.) and, after drying, in hexane/diethyl ether/acetic acid (160:40:3, by vol.). After drying, the plates were subjected to iodine vapour and lipids identified by their co-migration with authentic non-esterified fatty acids, diacylglycerol and lyso-phospholipids. Products were quantified

by scraping their corresponding spots into counting vials containing 10 ml of Aquasol II. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrophotometer.

Purification of PLA₂

Step 1: preparation of spleen extract

Pig spleen was freshly obtained from a local slaughterhouse and frozen at –70 °C. A 4.8 kg portion of spleen was thawed in 16 litres of homogenizing buffer A (50 mM Tris/HCl, 1 mM EDTA and 0.12 M NaCl, pH 9.0) and homogenized with a Waring blender (3 × 20 s). The homogenate was centrifuged at 13000 *g* for 60 min in a Sorvall centrifuge. The resulting supernatant was adjusted to pH 6.0 with acetic acid and stirred for 30 min at 4 °C, and centrifuged again as above. The resulting precipitates were collected and dissolved in 4 litres of buffer A. This redissolved solution was designated as spleen extract.

Step 2: ion-exchange chromatography on DEAE-cellulose

The spleen extract was chromatographed in a batch procedure. DEAE-cellulose (bed volume 2 litres) previously equilibrated with buffer A was added to 4 litres of the spleen extract. After equilibration for 2 h with constant stirring, the DEAE-cellulose gel was poured into a sintered-glass (coarse) filter funnel, and was extensively washed with buffer A until it was free of turbid lipid materials and unbound protein. The washed gel was stepwisely eluted at a flow rate of 18 ml/min with elution buffer consisting of 50 mM Tris/HCl, 1 mM EDTA and 0.3 M NaCl, pH 9.0. Approx. 4 litres of eluate was collected. The active fractions were adjusted to approx. 0.5 M NaCl by adding 5.0 M NaCl solution for the next purification step.

Step 3: hydrophobic chromatography on Butyl-Toyopearl 650 M

The total active pool from step 2 was applied to a Butyl-Toyopearl column (5 cm × 20 cm) equilibrated with 50 mM Tris/HCl/1 mM EDTA/0.5 M NaCl, pH 9.0, and washed with the same buffer until no protein was eluted. The column was stepwisely eluted at a flow rate of 6 ml/min with elution buffer containing 50 mM Tris/HCl and 1 mM EDTA, pH 9.0. The fractions containing PLA₂ activity were pooled for the next column-chromatography step.

Step 4: ion-exchange chromatography on DEAE-5PW h.p.l.c.

The pooled sample (140 ml) was filtered with MILEX-HA (0.45 µm pore size; Millipore, Bedford, MA, U.S.A.) and applied to a DEAE-5PW column (21.5 mm × 15 cm; Tosoh, Tokyo, Japan) pre-equilibrated with 50 mM Tris/HCl (pH 9.0)/1 mM EDTA/0.12 M NaCl. The column was eluted at a flow rate of 5 ml/min with a 200 ml linear gradient of 0.12–0.5 M NaCl, and 5 ml fractions were collected.

Step 5: PAGE

Native PAGE was performed to purify the PLA₂ enzyme further. Samples of the active fractions obtained from the DEAE-5PW h.p.l.c. were mixed with 0.1 vol. of 50 % sucrose containing 0.1 % Bromophenol Blue and loaded on a 4 % polyacrylamide minigel (1.5 mm thick) prepared in 50 mM Tris/HCl, pH 8.5. Electrophoresis was performed at 4 °C with 37.5 mM Tris/HCl, pH 8.8, as buffer. One of the two lanes was sliced into 50 pieces (1 mm × 5 mm). Individual slices were transferred into 250 µl of 25 mM Tris/HCl (pH 9.0)/0.1 M NaCl/1 mM EDTA, and then

homogenized. The homogenized gels were incubated overnight at 4 °C to elute the PLA₂ enzyme. Other lanes of the gel were made visible by staining with Coomassie Brilliant Blue.

Substrate specificity of the purified PLA₂ in mixed vesicles with 2-[³H]AA-GPC and 2-[1-¹⁴C]LA-GPC

2-[³H]AA-GPC and 2-[1-¹⁴C]LA-GPC in various amounts were mixed with non-radioactive lipids in chloroform and dried under nitrogen gas. To prepare phospholipid vesicles, 300 µl of 50 mM Tris/HCl (pH 7.5) containing 0.98 mM CaCl₂ and 1 mM EGTA (free [Ca²⁺] ≈ 1 µM) was added to each sample and sonicated at 4 °C for 5 min in an ultrasonic cleaner (Branson Ultrasonic cleaner, model no. 8200). PLA₂ activity was determined by incubation of 20 ng of purified PLA₂ with vesicles for 3 min at 37 °C. Released free [³H]AA and [¹⁴C]linoleic acid ([¹⁴C]LA) were quantified by double-channel β-scintillation counting.

Translocation of the purified spleen PLA₂ to washed spleen membrane in a Ca²⁺-dependent manner

To prepare pig spleen membrane vesicles, 13000 g supernatants of spleen homogenates, prepared as described previously, were centrifuged twice at 200000 g for 60 min, and the resulting washed pellets were resuspended in 50 mM Tris/HCl (pH 7.4) containing 2 mM EGTA and 0.12 M NaCl by brief sonication at 4 °C with a Branson Sonifier model 450. Purified PLA₂ enzyme (100 ng) from native PAGE was incubated with membrane vesicles (250 µg of protein) for 30 min at 37 °C in buffer containing 50 mM Tris/HCl (pH 7.4) and 2 mM EGTA, and the appropriate amount of Ca²⁺ to generate the free [Ca²⁺] indicated. The reaction mixture was then centrifuged at 200000 g for 60 min at 4 °C. A sample of the resulting supernatant was assayed for PLA₂ activity and the pellet was resuspended in buffer containing 50 mM Tris/HCl, 0.12 M NaCl and 1 mM EDTA, pH 9.0, without or with 10 mg/ml BSA, and ultracentrifuged as described above. The BSA concentration of each sample was adjusted to 10 mg/ml. A 20 µl portion of the resulting supernatant was assayed for PLA₂ activity translocated to membranes.

Preparation of polyclonal antibody

Active fractions obtained from DEAE-5PW h.p.l.c. were further purified. The fractions were pooled and run on SDS/PAGE (10% gel). The 100 kDa bands were excised and electroeluted with an electroelutor/concentrator (Isco Little Blue Tank; Isco, Lincoln, NE, U.S.A.), using procedures suggested by the manufacturer. Antisera against the purified 100 kDa PLA₂ were raised in female rabbits (2.5–3.0 kg). A portion (80 µg) of the protein was mixed with complete Freund's adjuvant and subcutaneously injected at six different sites of the rabbits' back. Three injections were given at 3-week intervals. At 2 weeks after each injection, the antiserum titre was tested by immunoblotting; 1 week before collecting blood, the rabbit was further boosted by an intravenous injection of 80 µg of 100 kDa PLA₂ in PBS (10 mM phosphate, pH 7.2, 150 mM NaCl).

Immunoprecipitation of PLA₂

Rabbit antiserum made against 100 kDa PLA₂ or rabbit pre-immune serum was mixed with packed Protein A-Sepharose beads pre-equilibrated with 20 mM Tris/HCl (pH 7.5) buffer containing 5 mg/ml BSA (2:1, v/v) and incubated for 24 h at 4 °C with constant shaking. The beads were washed six times

with 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 5 mg/ml BSA. Protein A-Sepharose beads of various amounts were incubated with 20 µg of protein of the active pool of PLA₂ activity, collected from DEAE-5PW h.p.l.c., for 4 h at 4 °C with constant shaking. The beads were then pelleted and washed with 3 × 1 ml of 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 5 mg/ml BSA. The supernatants and the washed pellets were assayed for PLA₂ activity.

Immunoblotting

Proteins were separated by SDS/PAGE (10% gels) [19] and then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) in 25 mM Tris/HCl (pH 8.3)/190 mM glycine/20% methanol. Non-specific binding of antisera to nitrocellulose was prevented by preincubation of the nitrocellulose in 3% BSA in PBS containing 0.1% NaN₃ for 1 h at room temperature. The blocked nitrocellulose membrane was incubated with diluted antiserum overnight at room temperature with constant shaking. Unbound antibodies were removed with three washes of PBS, and the sites of antibody binding were detected by using peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and the chromogenic substrate 4-chloro-1-naphthol.

Cell culture and immunochemical analysis of the 100 kDa PLA₂ from the cultured cells

Glomerular mesangial cells were isolated from both Sprague-Dawley and Wistar rats by using previously described methods [20] and maintained in RPMI 1640 culture medium supplemented with 20% fetal-calf serum. Strain II MDCK cells were grown in minimal essential media, supplemented with 2 mM glutamate and 10% fetal-calf serum. HEL cells and human monocytic line U937 cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal-calf serum and in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1, v/v), supplemented with 2 mM glutamine and 10% fetal-calf serum, respectively. All cells types were maintained at 37 °C under 5% CO₂ in air at a density of (1–3) × 10⁶/ml. Cultures were passaged 1–2 times a week to maintain exponential-phase growth. For immunoprecipitation experiments and Western-blot analysis, mesangial, MDCK, HEL and U937 cells were centrifuged at 400 g for 10 min and washed once in 50 mM Tris/HCl (pH 7.5) containing 0.15 M NaCl and 1 mM EDTA (buffer B). Mesangial and MDCK cells were put into suspension by scraping them off the dishes with a rubber policeman. The cells were resuspended in 1 ml of buffer B and sonicated for 20 s in an ice bath at 40 W output and 40% duty cycle (Sonicator, Ultrasonic Processor; Heat Systems-Ultrasonics Inc.). The lysates were centrifuged for 1 h at 100000 g. The resulting supernatants were incubated with 40 µl of packed Protein A-Sepharose beads saturated with antiserum raised against pig spleen 100 kDa PLA₂ enzyme at 4 °C for 4 h. The non-adsorbed supernatants were assayed for PLA₂ activity with 2-[1-¹⁴C]AA-GPC as substrate. The immunobeads were washed with 4 × 1 ml of buffer B and proteins were solubilized by adding 100 µl of 2 × Laemmli sample buffer [19], followed by heating at 100 °C for 5 min. The samples were subjected to SDS/PAGE (10% gels) and Western-blot analysis.

RESULTS

Detection of crude pig spleen cytosolic PLA₂ activity

To characterize spleen PLA₂ activity, 100000 g supernatants and pellets were prepared from pig spleen as described in the

Table 1 Distribution of PLA₂ activity in soluble fractions and membrane fractions from pig spleen

PLA₂ activity was determined in the supernatant of the first 100 000 *g* centrifugation. PLA₂ activity was measured in the 1st pellet after resuspension in buffer A (see the text). This suspension of the 1st pellet was then centrifuged at 100 000 *g*, and PLA₂ activity was determined in the pellet (2nd) after resuspension. Total activity is that calculated to be present in 4.8 kg of pig spleen. ND, not detected after incubation for 30 min.

Substrate	Total activity (mmol/min)		
	100 000 <i>g</i> supernatant	100 000 <i>g</i> pellet	
		1st	2nd
1-Stearoyl-2-[1- ¹⁴ C]arachidonoyl-GPC	7.1	1.4	0.1
1-Palmitoyl-2-[1- ¹⁴ C]linoleoyl-GPC	1.1	0.3	ND
1-Palmitoyl-2-[1- ¹⁴ C]palmitoyl-GPC	0.2	ND	ND
1-Acyl-2-[1- ¹⁴ C]arachidonoyl-GPE	11.0	1.7	0.3
1-Palmitoyl-2-[1- ¹⁴ C]linoleoyl-GPE	1.8	0.5	ND

Experimental section. Portions of supernatants and pellets were incubated with 10 μ M phospholipids containing [¹⁴C]AA in the *sn*-2 position (approx. 130 000 d.p.m./nmol). Reaction products were separated by t.l.c. and quantified by liquid-scintillation spectrometry (Table 1). There was no detectable radiolabelled diacylglycerols, suggesting that there was little phospholipase C activity present. These results indicate that the spleen PLA₂ preferentially hydrolyses phospholipids containing AA at the *sn*-2 position, whether the head group is choline or ethanolamine, with no marked preference for one over the other. Repeated washing of the pellets with buffer A (50 mM Tris/HCl/1 mM EDTA/0.12 M NaCl, pH 9.0) resulted in a considerable decrease in the PLA₂ activity in the 100 000 *g* pellets. Negligible pellet PLA₂ activity was detected after two washes of the 100 000 *g* pellets, suggesting that most of the PLA₂ activity in pig spleen was present in the soluble fraction. When the 100 000 *g* supernatant of pig spleen was applied to a Heparin-5PW column pre-equilibrated with 50 mM Tris/HCl (pH 7.4) containing 0.1 M NaCl and 1 mM EDTA and assayed for enzymic activity with 2-[1-¹⁴C]AA-GPC as substrate, approx. 70 % of the total PLA₂ activity was detected in the flow-through fractions (results not shown). On the basis of our previous results that the high-molecular-mass cytosolic PLA₂ did not bind to the heparin, whereas the secretory 14 kDa Group II PLA₂ in rabbit platelets was bound to the heparin column [11], this result indicated that the major form of PLA₂ in heparin-non-binding fractions is likely to be a cytosolic form of high molecular mass.

Purification of pig spleen cytosolic PLA₂

To examine the biochemical properties of the protein responsible for the observed AA-selective PLA₂ activity, a cytosolic PLA₂ was purified to homogeneity by sequential pH 6.0 extraction, anion-exchange, hydrophobic and h.p.l.c.-anion-exchange chromatographies, and native PAGE. Since preliminary studies identified an increase in hydrophobicity of the PLA₂ enzyme under more acidic pH conditions and considerable loss of activity when exposed to conditions of lower than pH 5.5, PLA₂ activity in pH 9.0 tissue homogenates was precipitated by adjusting the pH to 6.0, followed by centrifugation at 13 000 *g*. As shown in

Table 2 Summary of purification of PLA₂ from pig spleen

Sequential steps used in purification of the pig spleen 100 kDa PLA₂, resulting in a 15 920-fold purification with 44 % recovery.

Step	Protein (mg)	Total activity (mmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
100 000 <i>g</i> supernatant	198 400	7.14	0.036	1	100
pH 6 extract	14 200	9.94	0.70	19	139
DEAE-cellulose	1 400	7.90	5.62	156	111
Butyl-Toyopearl	54	5.20	95.5	2650	73
DEAE-5PW	5.5	3.15	573	15 920	44

Table 2, this initial step typically resulted in a 19-fold purification of spleen cytosolic PLA₂. Nearly 93 % of protein in the homogenate could be excluded by this step.

Most of the activity was solubilized from the particulate fraction by resuspending the pH 6.0 extracts in the pH 9.0 buffer. PLA₂ activity was further purified by translocating selectively the enzyme to the membrane fractions by addition of 2 mM CaCl₂. After ultracentrifugation of the sample at 100 000 *g* for 60 min at 4 °C, the enzyme activity was solubilized from the membrane fractions by resuspending the resulting pellets in buffer containing 2 mM EDTA. This procedure yielded 80 % recovery and 5-fold purification (results not shown). PLA₂ activity in the pH 9.0 buffer was stable for several days at 4 °C. Since the spleen extract resuspended in the pH 9.0 buffer was very turbid and it was difficult to clarify the large volume of sample by ultracentrifugation, further purification was carried out by a batch procedure.

The spleen extract was fractionated by DEAE-cellulose anion-exchange chromatography with a sintered funnel, and PLA₂ activity was quantitatively eluted by application of a 0.3 M NaCl stepwise gradient. The active fractions were pooled, and loaded on to a previously equilibrated hydrophobic column as described in the Experimental section. PLA₂ activity was eluted by a salt-free stepwise gradient. This hydrophobic-interaction step resulted in a 17-fold purification of spleen cytosolic PLA₂ in quantitative yield, accompanied by a 30-fold decrease in volume. The activity from the Butyl-Toyopearl column was stable for several months at -80 °C.

The Butyl-Toyopearl-eluate PLA₂ activity was further purified by DEAE-5PW anion-exchange h.p.l.c. and elution with a shallow linear gradient of NaCl (Figure 1a). The yield of total activity relative to the initial homogenate fraction was 44 %. To assess the purity of spleen PLA₂, each active fraction from the DEAE-5PW column was separated on SDS/PAGE, followed by Coomassie Brilliant Blue staining, as shown in Figure 1(b). Although a number of bands were observed, the relative intensity of the 100 kDa band paralleled the elution profile of PLA₂ activity during DEAE-5PW chromatography (compare Figures 1a and 1b). We estimate that these sequential steps yielded 1.5–3.0 mg of an 100 kDa protein comprising approx. 25–50 % of total protein in a 16 000-fold purification from 4.8 kg of pig spleen. Furthermore, when a part of the active fraction was subjected to TSK G 3000 SW gel-filtration h.p.l.c., the highest peak of the PLA₂ activity was eluted at 100–105 kDa as calibrated with molecular-mass standards (results not shown), further indicating that the 100 kDa protein was likely to be the PLA₂ enzyme.

In order to examine whether the 100 kDa protein was spleen PLA₂, and to obtain purified enzyme, the partially purified

Figure 1 Ion-exchange chromatography by DEAE-5PW h.p.l.c. and SDS/PAGE of the active fractions

(a) The sample enriched by pH 6 extraction, DEAE-cellulose and Butyl-Toyopearl chromatography was filtered with MILEX-HA (0.45 μ m pore size) and applied to a preparative DEAE-5PW column (21.5 mm $\times$ 15 cm) pre-equilibrated with 50 mM Tris/HCl (pH 9.0) containing 0.12 M NaCl and 1 mM EDTA. The column was eluted at a flow rate of 5 ml/min, with a 200 ml linear gradient of 0.12–0.5 M NaCl, and 5 ml fractions were collected. After each fraction was diluted by 1:100, 5 μ l was assayed at 37 °C for 3 min for PLA₂ activity using 2-[¹⁴C]AA-GPC. (b) 10 μ l of fractions, marked 1–8, were heated at 100 °C for 5 min in the presence of 100 mM 2-mercaptoethanol and 10% SDS, loaded on a 10%-polyacrylamide gel and detected by Coomassie Brilliant Blue stain.

enzyme preparation (pooled from fractions 2, 3 and 4 of the DEAE-5PW column) was subjected to electrophoresis under non-denaturing conditions as described in the Experimental section (50 μ g of protein was applied in duplicate lanes). After overnight elution, a sample of each fraction was assayed for PLA₂ activity. Over 40% of the applied PLA₂ activity was recovered with a broad band (Figure 2). To assess the purity, a portion of each fraction was analysed by SDS/PAGE, followed by silver staining. The relative PLA₂ activity shown in native PAGE precisely paralleled the intensity of the 100 kDa band, and only a single band was observed (Figure 2b), indicating that this 100 kDa band represents spleen PLA₂ protein.

Ca²⁺ requirements and substrate specificity

The activity of the purified PLA₂ activity was enhanced by Ca²⁺. The Ca²⁺-sensitivity was examined in 25 mM Tris/HCl/1 mM EGTA/CaCl₂ buffers [21] at pH 7.4 with 2-[1-¹⁴C]LA-GPC used as substrate. As shown in Figure 3, although there was a detectable PLA₂ activity at 0.1 μ M Ca²⁺, the activity was elevated nearly 10-fold when Ca²⁺ concentration was increased to 0.32 μ M. A further increase in PLA₂ activity was seen, with almost full activity achieved at 1 mM Ca²⁺. The dependency of the activity on [Ca²⁺] was essentially similar to that observed with 2-[1-¹⁴C]AA-GPC, with preference for AA in the *sn*-2 position of the substrate observed in all Ca²⁺ concentrations tested.

Figure 2 Further purification by native PAGE and SDS/PAGE of active fractions

The active fractions (2–5, Figure 1) obtained from DEAE-5PW h.p.l.c. were mixed with 0.1 vol. of 50% sucrose containing 0.1% Bromophenol Blue, loaded on a 4%-polyacrylamide minigel (1.5 mm thick) prepared in 50 mM Tris/HCl (pH 8.5), and electrophoresed at 25 mA for 16 h at 4 °C in 50 mM Tris/HCl (pH 8.8) as electrophoresis buffer. (a) One of the two lanes was sliced into 20 pieces (1 mm $\times$ 5 mm) and each piece was transferred into 250 μ l of 50 mM Tris/HCl (pH 9.0) containing 0.12 M NaCl and 1 mM EDTA. Each fraction was assayed for PLA₂ activity. After homogenization the gels were incubated overnight at 4 °C to elute the PLA₂ enzyme. The other lane of the gel was stained with Coomassie Brilliant Blue. (b) Equal amounts of fractions 1–9 were heated at 100 °C for 5 min in the presence of 10 mM 2-mercaptoethanol and 10% SDS, loaded on a 10%-polyacrylamide gel and revealed by silver staining. P, purified bovine platelet 100 kDa PLA₂.

Substrate specificity in mixed vesicles

Experiments were performed to investigate the specificity of the purified PLA₂ enzyme for AA-containing phospholipid. PC containing [³H]AA in the *sn*-2 position was mixed in varying proportions with PC containing [1-¹⁴C]linoleic acid in the *sn*-2 position. When increasing amounts of 2-[³H]AA-GPC were mixed with a fixed concentration of 2-[1-¹⁴C]LA-GPC (final concn. 10 μ M), the hydrolysis of 2-[1-¹⁴C]LA-GPC by the purified PLA₂ enzyme was increased by 2–3-fold (Figure 4a). On the other hand, when increasing amounts of 2-[1-¹⁴C]LA-GPC were mixed with a fixed concentration of 2-[³H]AA-GPC (final concn. 10 μ M), the amount of free AA released was significantly decreased (Figure 4b). Thus the selectivity of the purified spleen 100 kDa PLA₂ for AA that was observed in pure preparations of 2-LA- or 2-AA-GPC, as shown in Figure 3, was no longer as great in phosphatidylcholine vesicles containing both LA and

Figure 3 $[Ca^{2+}]$ requirement of the purified PLA_2 with 2-[1- ^{14}C]AA-GPC and 2-[1- ^{14}C]LA-GPC as substrates

The purified pig spleen PLA_2 (20 ng) was incubated with 25 μM 2-[1- ^{14}C]AA-GPC and 2-[1- ^{14}C]LA-GPC [65 000 d.p.m. (5 nmol)/tube] in 50 mM Tris/HCl (pH 7.4) containing 2 mM EGTA and various concentrations of $CaCl_2$ in a total volume of 200 μl for 10 min at 37 $^{\circ}C$. Results are shown as means of three separate experiments. Free $[Ca^{2+}]$ is indicated on the abscissa.

Figure 4 Substrate-specificity of the purified PLA_2 when the substrate consists of vesicles with various mixtures of 2-[3H]AA-GPC and 2-[1- ^{14}C]LA-GPC

2-[3H]AA-GPC and 2-[1- ^{14}C]LA-GPC in varying amounts were mixed in chloroform and dried under nitrogen. Substrate vesicles were made in buffer containing 1 μM Ca^{2+} , as described in the Experimental section. Substrate vesicles were then incubated with purified PLA_2 for 3 min at 37 $^{\circ}C$, and released free [3H]AA (□) and [1- ^{14}C]LA (▲) were quantified. (a) Indicated amounts of 2-[3H]AA-GPC were mixed with 10 μM 2-[1- ^{14}C]LA-GPC. (b) Indicated amounts of 2-[1- ^{14}C]LA-GPC were mixed with 10 μM 2-[3H]AA-GPC.

AA in the *sn*-2 positions of the phospholipids. As shown in Figures 4(a) and 4(b), when purified PLA_2 was exposed to vesicles produced with a ratio of 2-[3H]AA-GPC/2-[1- ^{14}C]LA-GPC of approx. 0.6, equal amounts of AA and LA were released.

Figure 5 Inhibition of pig spleen 100 kDa PLA_2 activity by various Group I and II PLA_2 inhibitors

Quinacrine, BPB, indomethacin, DEDA and NDGA, at various concentrations, were tested for their ability to inhibit the PLA_2 activity of the DEAE-5PW h.p.l.c. active fractions from the spleen. A 100 ng sample was incubated in a reaction mixture of 100 μl containing 75 mM Tris/HCl, pH 9.0, 5 mM $CaCl_2$ and 0.5 nmol of 1-stearoyl-2-[1- ^{14}C]AA-GPC dissolved in 10 μl of dimethyl sulphoxide. In the absence of added inhibitor there was approx. 20% hydrolysis of the substrate in 10 min. Dimethyl sulphoxide alone had no effect on the activity. The results represent the means of three experiments.

Inhibition of pig spleen 100 kDa PLA_2 activity by various group I and II PLA_2 inhibitors

We examined the effects of various putative Group I and Group II PLA_2 inhibitors on the partially purified 100 kDa cytosolic PLA_2 from pig spleen (Figure 5). DEDA and NDGA inhibit the hydrolysis of 2-[3H]AA-GPC, with 50% inhibition at approx. 10 μM for the former and between 10 and 100 μM for the latter. Quinacrine and BPB had no effect on the PLA_2 enzymic activity, even at concentrations of 1 mM. Indomethacin inhibited activity only at high concentrations (50% inhibition at 1 mM).

Translocation of the purified PLA_2 activity to washed spleen membranes

When subcellular fractions of spleen homogenates are examined for PLA_2 activity, over 95% of the enzyme is found in the cytosolic fraction (100 000 *g* supernatant). However, since the substrate phospholipids are derived from membranes, association of the enzyme with the membrane may be one of the important activation mechanisms for PLA_2 [4,23]. To examine Ca^{2+} -dependent association of the cytosolic PLA_2 with the membrane, purified enzyme and washed membranes were prepared as described in the Experimental section. Incubation of the enzyme and the membranes at 30 $^{\circ}C$ for 30 min in a buffer with the $[Ca^{2+}]$ expected in resting cells resulted in no considerable loss of the activity in soluble fractions. By contrast, when incubation was carried out at the $[Ca^{2+}]$ expected in stimulated cells there was a considerable loss of activity in soluble fractions. To determine the fate of PLA_2 activity under the indicated conditions, the 100 000 *g* pellets were resuspended by sonication in buffer containing 1 mM EDTA with or without 10 mg/ml BSA and ultracentrifuged as above. The resulting 100 000 *g* supernatants were assayed for PLA_2 activity. In the absence of BSA, the enzymic activity lost from the soluble fractions when incubated with membranes at free $[Ca^{2+}]$ greater than 0.32 μM was not recovered from the membranes. By contrast, when the membrane

Figure 6 Translocation of the purified PLA₂ activity to washed pig spleen membrane vesicles in a Ca²⁺-dependent manner

Pig spleen membrane vesicles were prepared by sonicating 100 000 *g* pellets of tissue homogenates which were washed twice in 50 mM Tris/HCl (pH 9.0) containing 0.12 M NaCl. Purified spleen PLA₂ was mixed with membrane vesicles and incubated for 30 min at 30 °C in 50 mM Tris/HCl (pH 7.4) containing 2 mM EGTA and the appropriate amount of Ca²⁺ to generate the free Ca²⁺ concentration indicated. The reaction mixture was then centrifuged for 60 min at 100 000 *g* at 4 °C, and a sample of the resulting supernatant was assayed for PLA₂ activity. To dissociate the PLA₂ enzyme bound to membrane vesicles, the resulting pellet was resuspended in the same buffer containing 2 mM EGTA with or without BSA and re-centrifuged under the same conditions. A sample of the second supernatant was then assayed for PLA₂ activity.

Figure 7 Immunoprecipitation of pig spleen PLA₂ activity by anti-(pig spleen 100 kDa PLA₂) polyclonal antibody

Rabbit antiserum against 100 kDa PLA₂ was prepared as described in the Experimental section. Packed Protein A-Sepharose beads pre-equilibrated with 20 mM Tris/HCl (pH 7.5) buffer, containing 5 mg/ml BSA, were mixed with rabbit antiserum or pre-immune serum (2:1, v/v) for 24 h at 4 °C with constant shaking. The beads were washed with 6 × 1.0 ml of 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 5 mg/ml BSA. Indicated volumes of washed beads were incubated with 20 μg of the active fractions from DEAE-5PW h.p.l.c. for 4 h at 4 °C with constant shaking. To estimate rabbit-antiserum-specific binding of 100 kDa PLA₂, the samples were centrifuged and the beads washed with 3 × 1 ml of 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 5 mg/ml BSA. The supernatants and washed immunobeads were assayed for PLA₂ activity.

pellets were sonicated in the presence of 10 mg/ml BSA, 62–80 % of the activity was recovered (Figure 6).

Immunoprecipitation of the partially purified spleen PLA₂ activity

The effect of anti-PLA₂ antiserum on PLA₂ enzymic activity was evaluated by measuring 2-AA-GPC-hydrolysing activity in the

Table 3 Immunoprecipitation of PLA₂ activity from various tissues

Partially purified PLA₂ from each tissue was diluted with 50 mM Tris/HCl (pH 9.0) containing 0.12 M NaCl, 1 mM EDTA and 10 mg/ml BSA to result in nearly equivalent activities. Each sample (800 μl) was mixed with 50 μl of packed antiserum-saturated Protein A-Sepharose beads and incubated for 4 h at 4 °C. The beads were pelleted by centrifugation for 10 min at 10 000 *g*. Samples of the supernatants were assayed for PLA₂ activity with 25 μM 2-[³H]AA-GPC as substrate.

Tissue	Total activity (nmol/min)	Residual supernatant PLA ₂ activity (% of total)
Bovine lung	0.78	36
Bovine platelets	0.50	25
Bovine kidney	0.72	53
Pig spleen	0.95	22

presence of antibody. Whereas incubation of the DEAE-5PW h.p.l.c. active fractions with increasing amounts of pre-immune serum did not result in any loss of PLA₂ activity, antiserum against the 100 kDa protein immunoprecipitated the PLA₂ activity in a dose-dependent fashion. As shown in Figure 7, 40 μl of packed Protein A-Sepharose beads, saturated with the antiserum, precipitated 87 % of the PLA₂ activity. Some 15 % of total PLA₂ activity could be recovered in the immunoprecipitates after resuspension in homogenization buffer A. Experiments were also designed to immunoprecipitate PLA₂ activities from bovine lung, platelet and kidney. The 100 kDa PLA₂ enzyme was partially purified from bovine lung and kidney by sequential steps of pH 6.0 extraction, DEAE-cellulose chromatography, Butyl-Toyopearl and DEAE-5PW h.p.l.c. as described for the pig spleen PLA₂, resulting in 1500-fold and 2400-fold purifications respectively. PLA₂ from bovine platelets was partially purified by 2000-fold as described previously [12]. A 50 μl portion of packed Protein A-Sepharose beads saturated with the antiserum precipitated 64, 75, 47 and 78 % of the PLA₂ activity from bovine lung, platelets and kidney and pig spleen respectively (Table 3).

Western-blot analysis of PLA₂ derived from various tissues by using anti-(pig spleen 100 kDa PLA₂) antiserum

Western-blot analysis was performed by using the partially purified PLA₂ enzymes from bovine lung, kidney and platelets. As shown in Figure 8, the antiserum reacted strongly with the 100 kDa bands from each of these sources, as well as pig spleen.

Immunochemical analysis of 100 kDa protein from rat glomerular mesangial cells, MDCK cells, HEL cells and human U937 cells

By Western-blot analysis, 100 kDa bands were detected in the immunoprecipitates of (6–8) × 10⁶ glomerular mesangial cells, 5 × 10⁶ MDCK cells, (1–2) × 10⁸ HEL cells and (2–3) × 10⁸ U937 cells (Figure 9). The antiserum immunoprecipitated the PLA₂ activity in the soluble fractions of these culture cells, and a considerable amount of PLA₂ activity (10–15 %) was detected in the immunoprecipitates formed from the soluble fractions of these cells and Protein A-Sepharose beads saturated with the antiserum (results not shown). To establish further the specificity of the antibody, we performed Western-blot analysis of the 100 000 *g* supernatant of LLC-PK₁ kidney epithelial cells and LLC-PK₁ cells stably transfected with the cytosolic PLA₂ (cPLA₂) cDNA [7] obtained from Genetics Institute. The parental cell line

Figure 8 Western-blot analysis of PLA₂ enzymes from various tissues by anti-(pig spleen 100 kDa PLA₂) polyclonal antibody

The PLA₂ enzymes from bovine lung and kidney and pig spleen were partially purified by sequential steps of pH 6.0 extraction, DEAE-cellulose, Butyl-Toyopearl chromatography and DEAE-5PW h.p.l.c. as described in the Experimental section. Bovine platelet PLA₂ was partially purified 2000-fold. The PLA₂s were separated by SDS/PAGE (10% gels) and blotted with 1/4000-diluted antiserum as described in the Experimental section. Samples (5–10 µg) of lung, kidney and spleen PLA₂ and 2.4 µg of platelet PLA₂ preparations were loaded on the gel. Positions of protein molecular-mass standards are marked (kDa).

Figure 9 Western-blot analysis of 100 kDa protein from cultured cells

Cultured glomerular mesangial cells (MES; 6×10^6 cells), MDCK cells (6×10^6 cells), HEL cells (2×10^6 cells) and human monocytic U937 cells (2×10^6 cells) were harvested 2 days after passage. The 100 000 g supernatants of these cells were incubated with 50 µl of packed Protein A–Sepharose beads saturated with antiserum. Immunoprecipitates were then submitted to SDS/PAGE and blotted with 1/2000-diluted antiserum as described in the Experimental section. Arrow indicates 100 kDa PLA₂ protein. Abbreviations: N, normal serum; A, antiserum.

expresses very low levels of cPLA₂, and no band was seen when the extract was loaded on a lane and SDS/PAGE was carried out and the transferred protein exposed to the antibody raised to the spleen enzyme. By contrast, a single band at 100 kDa was observed in extracts from the transfected cell line (results not shown). Thus the antibody reacted with the high-molecular-mass PLA₂, and there was no evidence for reaction of the antibody with other proteins.

DISCUSSION

We have purified a 100 kDa PLA₂ from the soluble fraction of pig spleen, bovine lung and bovine kidney, using a novel large-scale purification scheme. This represents the first purification of a large-molecular-mass PLA₂ from spleen or lung and the first

large-scale purification from the kidney. A polyclonal antibody against spleen 100 kDa PLA₂ has been raised in rabbits. This antibody recognizes the lung, kidney, platelet, mesangial, MDCK-, HEL- and U937-cell 100 kDa PLA₂.

Tojo and co-workers have purified and sequenced Group I and II PLA₂s from soluble fractions and membrane fractions of rat spleen respectively [24,25]. Since their assay buffer for PLA₂ activity included 6 mM deoxycholate, they did not detect a deoxycholate-inhibited large-molecular-mass form of PLA₂. Very recently, a high-molecular-mass form of PLA₂ was partially purified from cytosolic fractions of mouse spleen [26]. The activity migrated as a 70 kDa protein on gel-filtration chromatography. Under our assay conditions, the 100 kDa cytosolic Ca²⁺-dependent PLA₂ is the major measurable phospholipase activity in pig spleen. Little activity was detected in membrane fractions (Table 1).

A highly efficient purification of a 100 kDa PLA₂ was achieved by a unique purification scheme employing, as the first step, adsorption of spleen cytosolic PLA₂ to the particulate fraction by adjustment to pH 6.0. This step obviates tedious ultracentrifugation of large amounts of sample for a large-scale purification. The purity of the final preparation was demonstrated by the presence of a single 100 kDa protein band identified by native PAGE. The specific activity of the purified PLA₂ was over 2000 nmol/min per mg of protein. The 100 kDa PLA₂ activity was stable at –80 °C for several months and activity was not changed by freeze-drying.

We purified a PLA₂ enzyme from pH 6.0 extracts of bovine lung homogenates, using the same procedure as that for pig spleen PLA₂. The column profiles and biochemical properties of the partially purified enzyme were very similar to those of pig spleen PLA₂, and the active fractions from DEAE-5PW h.p.l.c. yielded a 100 kDa band, comprising 30–50% of total protein, which correlated with PLA₂ activity (results not shown). Furthermore, the lung 100 kDa protein was confirmed to be immunochemically similar to the 100 kDa spleen enzyme by Western-blot analysis (Figure 7). Pierik et al. [27], relying on gel-filtration column profiles, postulated that two Ca²⁺-independent and two Ca²⁺-dependent PLA₂ enzymes were present in delipidated rat lung cytosol. Recently, several groups have purified small-molecular-mass forms of PLA₂ from soluble fractions of guinea-pig lung [28] and rat lung [29] and from 1 M KCl extracts of microsomal fractions of rabbit lung [30]. A larger-molecular-mass PLA₂ was not detected, however, perhaps because previous investigators have used organic solvents or adjusted the sample to pH 5.1 for delipidation [27] and determined PLA₂ activity in assay buffers containing 2.4 mM deoxycholate [28], 4 mM taurodeoxycholate and 1-palmitoyl-2-[1-¹⁴C]oleoyl-GPC [30], or used [1-¹⁴C]oleate-labelled *Escherichia coli* [29] 1-acyl-2-[1-¹⁴C]linoleoyl-GPC [27] as substrates. The large-molecular-mass form of PLA₂ is irreversibly labile to most organic solvents or exposure to pH lower than 5.5, very sensitive to most detergents, including deoxycholate (D. K. Kim and J. V. Bonventre, unpublished work), and not very efficient at cleaving oleic acid at the *sn*-2 position of phospholipids. PLA₂ administered by bronchial lavage can produce a pathological state in animals that mimics acute respiratory distress syndrome [31]. Enhanced lung PLA₂ activity and eicosanoids have been identified in association with a number of clinical and experimental pulmonary inflammatory disorders [32].

Our purification scheme was also suitable for a 100 kDa PLA₂ from the soluble fraction of bovine kidney. The purification column profiles and biochemical properties were similar to those of the large-molecular-mass PLA₂ from pig spleen and bovine lung (results not shown). Thus a 100 kDa PLA₂ is present in

various tissues. Since this purification scheme is simple and convenient compared with procedures previously reported for the enzyme [9–11,33], this scheme will provide a useful way to obtain a large amount of purified native PLA₂ enzyme.

The purified spleen 100 kDa PLA₂ catalyses the release of an arachidonoyl residue more efficiently than a linoleoyl residue from the *sn*-2 position of phosphatidylcholine and phosphatidylethanolamine in vesicles containing a single phospholipid at any Ca²⁺ concentration examined (Figure 3). As shown in Figure 4, however, the selectivity for hydrolysis of arachidonoyl-GPC over linoleoyl-GPC was decreased when these lipids were mixed. These results are consistent with those found using human platelet cytosolic fractions and substrates in doubly labelled mixtures [34]. It has been reported, however, that stimulation of human platelets with thrombin or collagen results in selective hydrolysis of the molecular species of phosphatidylcholine and phosphatidylethanolamine bearing AA [35]. Clark et al. [7] have reported that recombinant cytosolic PLA₂ preferentially hydrolysed arachidonoyl phospholipids almost 20-fold more than phospholipids containing other fatty acids in the *sn*-2 position when using natural membrane vesicles as substrates. The discrepancy between our results and the observations of Clark et al. [7] may be due to the differences in physical state of the substrate between natural membranes and artificial vesicles, an AA-rich domain preferentially susceptible to PLA₂ in the natural membranes, an integral membrane factor responsible for the AA-selectivity shown in natural membranes or intact cells, or differences between the native and recombinant PLA₂ enzyme.

Previous studies have revealed that cytosolic PLA₂ activity associates with the membrane fractions when cells are homogenized in Ca²⁺-containing buffers [4,23]. Although purified recombinant PLA₂ protein translocated to natural membranes in response to physiological concentrations of Ca²⁺ as determined by Western-blot analysis [7], translocated enzymic activity could not be quantitatively recovered from the particulate fractions from macrophage cell line RAW 264.7 [23]. In the present study, the fate of the enzyme activity during translocation was examined by using the purified spleen 100 kDa PLA₂ and washed natural membranes. The soluble-fraction PLA₂ activity was decreased and membrane-associated activity was increased as the Ca²⁺ concentration was increased. Most of the enzymic activity in the particulate fraction could be recovered by resuspension of the pellets with a buffer containing 1 mM EDTA and 10 mg/ml BSA. This indicated that the loss of the activity in previous studies may have arisen from incomplete dissociation of the enzyme from the particulate fractions in Ca²⁺-free buffer, and not from inactivation of PLA₂ by interaction of the enzyme with its substrate or degradation by membrane associated-proteases.

The large-molecular-mass PLA₂ enzymes from bovine platelets, bovine lung and bovine kidney are biochemically and immunochemically indistinguishable from that of pig spleen by a number of criteria: (1) each of the enzymes showed a substrate selectivity for AA at the *sn*-2 position when single phospholipid substrates were used, and increased activity at sub-micromolar Ca²⁺ concentrations; (2) during purification, the column-chromatography profiles of all the enzyme sources were very similar; (3) only a 100 kDa band was detected in all the tissues by Western-blot analysis using our antibody raised against pig spleen PLA₂. The 100 kDa PLA₂ was also detected when the immunoprecipitates formed by 100 000 g supernatants of various cultured cells and Protein A-Sepharose beads saturated with anti-(100 kDa spleen PLA₂ antiserum) were subjected to SDS/PAGE followed by Western-blot analysis.

In order to delineate the role played by PLA₂ in signal transduction, it is necessary to understand how the enzymic

activity is regulated. To study activation mechanisms of the enzyme during cell stimulation, the detection of the activity in the immunoprecipitates formed by specific interactions of the antigen-specific antibody with the PLA₂ enzyme will provide a useful tool for studying how the PLA₂ activity increases during cell stimulation. We found that Protein A-Sepharose saturated with the antiserum immunoprecipitated the PLA₂ activity from all enzyme sources examined. More importantly, we detected considerable enzyme activity in the immunoprecipitates from partially purified spleen PLA₂ preparation (Figure 7) and cultured cells examined (results not shown).

In summary, we report a simple and convenient large-scale purification scheme for a 100 kDa cytosolic PLA₂ isolated from pig spleen, bovine lung and bovine kidney. This is the first demonstration of a 100 kDa PLA₂ in spleen and lung. This purification scheme provides a large amount of native PLA₂ enzyme and may be useful for studies of the kinetics of the enzyme, its mechanisms of regulation and physical structure, and screening of potential inhibitors. Based on Western-blot analysis, bovine platelets, mesangial, MDCK, HEL and U937 cells contained a 100 kDa PLA₂ immunochemically related to spleen 100 kDa PLA₂. The purified enzyme translocates from the cytosolic to the membrane fraction in a Ca²⁺-dependent manner. Antiserum raised to the native enzyme is unique among antibodies previously raised against a high-molecular-mass PLA₂ in that it can immunoprecipitate the active enzyme in a form where activity is maintained in the immunoprecipitates.

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