Purification and characterization of a calcium-independent acidic phospholipase A₂ from rat lung

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Several phospholipase A₂ (PLA₂) activities have been identified in rat lung homogenate and shown to be important in metabolism of lung phospholipids. One PLA₂ activity is Ca²⁺-independent, active *in vitro* at pH 4, and inhibited by a substrate analogue, 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol (MJ33). Purification of this rat lung PLA₂ by approx. 550-fold was carried out by sequential column chromatographies using DE-52, Sephacryl-100, heparin–Sephacryl, and phenyl–Sepha-

rose columns. The purified activity had an acidic pH optimum, was Ca²⁺-independent, was inhibited by MJ33 in a dose-dependent manner (50 % inhibition at 3 mol %), was unaffected by treatment with *p*-bromophenacyl bromide or mercaptoethanol, and had a unique N-terminal amino acid sequence. The apparent molecular mass was 15 kDa on gel electrophoresis and activity was recovered in part by renaturation of protein from the gel. The properties of this enzyme suggest a new class of PLA₂.

INTRODUCTION

Phospholipase A₂ (PLA₂) (EC 3.1.1.4) is a diverse and ubiquitous family of enzymes that hydrolyse the acyl ester bond at the *sn*-2-position of phospholipids to produce a free fatty acid and lysophospholipid. In recent years at least two major classes of PLA₂ (secretory and cytoplasmic) have been well characterized. The secretory 14 kDa PLA₂s are of three evolutionary types [1]: type I is present in pancreatic juices and Old-World snakes; type II is present in macrophages and New-World snakes; type III is found in bee venom. These enzymes have little selectivity for different classes of phospholipids and the apparent dissociation constant for the essential cofactor, calcium, is about 0.1 to 1 mM [2]. The cytoplasmic 85 kDa PLA₂ is relatively specific for arachidonate-containing phospholipids and requires calcium (*K*_{Ca} approx. 1 μM) for binding to the substrate interface [3]. Besides these well-characterized enzymes, PLA₂ activity with various other properties such as Ca²⁺ independence and acid-pH optimum have been reported from a variety of tissues [4–6].

Several different members of the PLA₂ family have been identified in lung tissue including secretory (type I) and cytosolic PLA₂ enzymes [7,8]. Type-II secretory enzyme is also present [9], although a possible contribution from trapped blood cells has not been excluded. In addition to these 'classical' PLA₂ enzymes, a calcium-independent PLA₂ activity has been detected in the lung by several laboratories [10–16]. This activity shows an acidic pH optimum and has been localized to the secretory organellar (lamellar body) fraction of lung homogenate [11,16]. A specific function of this PLA₂ in lung is metabolism of endocytosed alveolar dipalmitoylphosphatidylcholine (DPPC), the predominant component of pulmonary surfactant [16]. In a previous publication, this acidic phospholipase A₂ activity in rat lung was shown to be competitively inhibited by a phospholipid transition-state analogue, MJ33 (1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol), while activity of the Ca²⁺-dependent alkaline enzymes was unaffected [16]. Availability of the inhibitor enabled

us to selectively study the enzyme in lungs despite the heterogeneity of PLA₂s. In this paper, we present a method for purification and an initial characterization of this Ca²⁺-independent PLA₂. The purified enzyme showed novel properties which distinguish it from the other major classes of PLA₂.

EXPERIMENTAL

Materials

DPPC, phosphatidylglycerol (PG) and egg phosphatidylcholine (PC) were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Cholesterol, Sephacryl-100, phenyl–Sepharose CL-4B, *p*-bromophenacyl bromide (pBPB), dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF) and mercaptoethanol were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Aquacide III was obtained from Calbiochem (La Jolla, CA, U.S.A.). DEAE-cellulose (DE-52) was purchased from Whatman (Maidstone, U.K.). Heparin–Sepharose CL-6B was from Pharmacia (Piscataway, NJ, U.S.A.). 1-Palmitoyl-2-[9,10-³H(N)]palmitoyl-*sn*-glycero-3-phosphocholine ([³H]DPPC) was obtained from New England Nuclear (Boston, MA, U.S.A.) and reported as > 99 % purity. Antiserum to pig pancreatic PLA₂ was from UBI (Lake Placid, NY, U.S.A.). MJ33 was synthesized as described previously [17]. Arachidonyl trifluoromethyl ketone (AACOCF₃) was kindly provided by Dr. Michael Gelb (University of Washington, Seattle, WA, U.S.A.). Surfactant protein A (SP-A) was isolated from bovine lungs as described previously [18]. Sprague–Dawley rats weighing approx. 200 g were obtained from Charles River Breeding Laboratories (Kingston, NY, U.S.A.).

Assay of PLA₂ activity

PLA₂ assay was performed using DPPC in liposomal form as substrate [16]. Unilamellar liposomes were prepared from a mixture of DPPC:PC:PG:cholesterol in molar ratio 10:5:2:3

Abbreviations used: PLA₂, phospholipase A₂; MJ33, 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; AACOCF₃, arachidonyl trifluoromethyl ketone; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; pBPB, *p*-bromophenacyl bromide; PC, phosphatidylcholine; PG, phosphatidylglycerol; SP-A, surfactant protein A; PVDF, poly(vinylidene difluoride).

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with radioactive label (9,10-³H-palmitoyl) in the *sn*-2 position of DPPC. The lipid mixture was dried under N₂, resuspended in water, and sonicated at 55 °C using a cup-horn sonicator (Heat System Ultrasonics, Plainview, NY, U.S.A.) at 70 % of maximum power with six 3 min bursts at 1 min intervals. The liposomes were stored at 4 °C overnight and centrifuged before use to remove large aggregates.

PLA₂ activity was measured according to the methods previously described [12,16]. The standard assay cocktail for acidic PLA₂ contained 40 mM sodium acetate, pH 4, and 5 mM EDTA. The alkaline assay cocktail contained 50 mM Tris, pH 8.5, 10 mM CaCl₂ and 1 mM EGTA. The reaction was initiated by adding substrate (final concentration 1 mM DPPC) to 1 ml of assay cocktail and incubating at 37 °C for 60 min. The reaction was stopped with chloroform/methanol (1:2, v/v). Lipids were extracted and phospholipids and free fatty acids were separated by t.l.c. [16]. PLA₂ activity was calculated from the decrease in radioactivity (d.p.m.) of DPPC, expressed as μ mol/mg of protein per min. Alternatively, the reaction was terminated by adding 5 ml hexane/diethyl ether (1:1) and vortexed for 10 s [18]. The two phases were allowed to separate under unit gravity at room temperature for 5 min. The organic phase, which contained released fatty acid, was collected and evaporated under N₂. The amount of radiolabelled free fatty acid hydrolysed from substrate was determined by scintillation counting with quench correction based on internal standards. The two methods for isolating PLA₂ metabolic products gave similar results and values for PLA₂ activity were combined. Protein was determined by Coomassie Blue assay (Bio-Rad kit) using bovine IgG as standard.

The effect of potential PLA₂ inhibitors was determined following pre-incubation of the enzyme and inhibitor for 10 min at 25 °C. To determine the effect of pBPB, purified protein was pre-incubated with 2 mM pBPB in 25 mM Tris/HCl, containing 0.5 mM EDTA at pH 8.5 for 10 min. PLA₂ activity was then measured at pH 4 using the standard assay. The same pre-incubation procedure was used to determine the effect of 5 mM 2-mercaptoethanol. The pH profile of the isolated enzyme was determined using the following buffers: pH 3, 50 mM glycine; pH 4–5, 50 mM acetate; pH 6–7, 50 mM phosphate; and pH 8–10, 50 mM Tris.

Purification of PLA₂

Preparation of lung homogenates

Rat lungs were perfused *in situ* before removal from the chest in order to minimize possible contamination of the preparation with PLA₂ from blood. Rats were anaesthetized with pentobarbital (50 mg/kg body wt., intraperitoneally) and continuously ventilated. The chest was opened rapidly and the lungs were cleared of blood by perfusion for 4 min through the pulmonary artery with 20 ml of ice-cold saline. The lungs were frozen immediately after the perfusion and stored at –70 °C.

Isolation of PLA₂

Frozen rat lungs (approx. 100 g of starting material) were thawed to 4 °C and homogenized, first with a commercial blender three times for 1 min at 1 min intervals in 50 mM Tris buffer, pH 7.4, containing 1 mM EDTA, 1 mM PMSF, 5 % (w/v) glycerol, and then with a Polytron PT-10 homogenizer (Brinkmann, Westbury, NY, U.S.A.) three times for 1 min at 1 min intervals. Homogenization and subsequent isolation steps were all performed at 0–4 °C. The homogenate was centrifuged at 10000 *g* for 10 min, the pellet was discarded and the supernatant was recentrifuged at 100000 *g* for 1 h. The supernatant was collected and concentrated

either by 55 % (w/v) ammonium sulphate precipitation for 1 h followed by centrifugation at 16000 *g*, or by dialysis against Aquacide III to a protein concentration of approx. 100 mg/ml.

The concentrated protein sample was then loaded on to a DEAE-cellulose column (DE-52, 38 cm × 2.2 cm) equilibrated with 50 mM Tris buffer, pH 7.4, containing 1 mM EDTA and 5 % glycerol. The buffer described above was used throughout the purification unless otherwise noted. The column was eluted by a linear gradient of KCl (50 mM to 450 mM). Fractions were collected at a flow rate of 30 ml/h. Every third fraction was assayed for protein and PLA₂ activity. The fractions containing PLA₂ activity inhibited by MJ33 were pooled and concentrated by an Amicon concentrator with PM-10 membrane (Amicon, Beverly, MA, U.S.A.). The concentrated protein was then applied to a Sephacryl-100 column (120 cm × 2.2 cm) which was equilibrated with Tris buffer. Protein was eluted using the same buffer at a flow rate of 20 ml/h. Active fractions (PLA₂ activity inhibited by MJ33) were pooled and concentrated by the Amicon concentrator. Pooled fractions from the Sephacryl-100 column were loaded on to a heparin-Sepharose CL-6B column (30 cm × 1.0 cm) equilibrated with Tris buffer. The column was washed with a linear gradient of 0 to 1.5 M KCl at a flow rate of 10 ml/h. The active fractions were collected and dialysed. The dialysate was concentrated and subjected to a phenyl-Sepharose CL-4B column (30 cm × 1.0 cm). The column was eluted with a gradient of 1.5 M KCl and 0 % ethylene glycol to 0 M KCl and 10 % ethylene glycol at a flow rate of 10 ml/min. Active fractions were collected, dialysed against Tris buffer overnight and stored at –20 °C for biochemical characterization.

Characterization of PLA₂

PAGE

SDS/PAGE was run under reducing conditions with a 15 % acrylamide gel using the Laemmli system [19]. Protein samples were heated at 100 °C for 10 min in the presence of 5 % (v/v) 2-mercaptoethanol before electrophoresis. Protein bands were stained with Coomassie Blue or silver nitrate.

Renaturation of PLA₂

PLA₂ was renatured by the method of Hager and Burgess [20] with slight modification. The enzyme sample, without prior heating, was electrophoresed by SDS/PAGE and the protein bands were visualized by soaking the gel in a solution containing 0.25 M KCl and 2 mM DTT for 15 min at 4 °C. After destaining with 2 mM DTT and water sequentially, the appropriate band was excised and the protein was eluted with elution buffer (150 mM NaCl, 0.1 % SDS, 50 mM Tris/HCl, 0.1 mM EDTA, 5 mM DTT, 0.1 mg/ml BSA, pH 7.9). Protein was precipitated with acetone, and renatured in 6 M guanidine hydrochloride followed by 50-fold dilution with modified elution buffer (20 % glycerol substituted for SDS). The eluted protein was assayed for PLA₂ activity after 12 h at room temperature. The enzymic activity and protein content were determined as described above.

Western blot

The proteins from SDS/PAGE were transferred by electroblotting at 40 mA for 12 h to nitrocellulose paper (Bio-Rad). The blotted proteins were incubated with antiserum to pig pancreatic PLA₂ as primary antibody followed by goat anti-rabbit IgG antibody labelled with horseradish peroxidase as secondary antibody. Colour development was with 4-chloro-1-naphthol following the manufacturer's instructions (Bio-Rad kit).

N-terminal amino acid sequencing

Sequencing was done according to the method of Matsudaira [21]. After SDS/PAGE (without prior heating) of the purified enzyme, the protein was blotted to Immobilon P transfer membrane [poly(vinylidene difluoride); PVDF] following the manufacturer's instructions (Millipore, Bradford, MA, U.S.A.). The PVDF membrane was stained with Ponceau S. The band at approx. 15 kDa was excised and subjected to N-terminal amino acid sequencing with an Applied Biosystems (Foster City, CA, U.S.A.) 470A protein sequencer by standard procedures recommended by the manufacturer. An amino acid sequence on-line search was carried out on the National Center for Biotechnology Information (NCBI) comprehensive database.

RESULTS

Identification of Ca²⁺-independent PLA₂ in rat lung

Assay of homogenate of perfused rat lung showed a similar PLA₂ activity at pH 4 (minus Ca²⁺) and pH 8.5 (plus Ca²⁺) (Table 1). The activity at pH 4 was significantly inhibited by MJ33 whereas the alkaline PLA₂ activity was not, confirming our previous observations [12,16]. PLA₂ activity detected at pH 8.5 required calcium in the assay medium, while activity at pH 4 was Ca²⁺-independent. PLA₂ activity of the acidic enzyme in the homogenate was enhanced about 3-fold after storage for 1 week at -20 °C (*n* = 3, results not shown), as also reported by Nijssen et al. [13]. In contrast, no significant change was seen with the alkaline PLA₂ activity. The mechanism for activation during storage remains unknown. Subsequent isolation steps focused on the acidic, MJ33-sensitive, Ca²⁺-independent PLA₂.

Purification of Ca²⁺-independent PLA₂

About 85% of total acidic, Ca²⁺-independent PLA₂ activity remained in the supernatant after centrifuging the homogenate at 100000 *g* and that activity was significantly inhibited by MJ33. Storage of the supernatant at -20 °C enhanced activity as described above for the frozen homogenate. The 100000 *g* supernatant was concentrated as described in the Experimental section and chromatographed on an ion-exchange column (DE-52) (Figure 1). PLA₂ activity present in the void volume was not inhibited by MJ33 and it was discarded. The major fraction of PLA₂ activity was eluted in a broad peak that was slightly retarded with respect to the main protein peak. The initial fractions of this peak of PLA₂ activity responded poorly to the inhibitor (MJ33), possibly due to aggregation of protein with residual membrane lipid which could have protected the enzyme. Subsequent fractions in this peak of PLA₂ activity were quite sensitive to the inhibitor (Figure 1). Fractions 12–18 were collected and pooled. This column resulted in a 15-fold purification of activity.

The pooled fractions from the DE-52 column were loaded on to a Sephacryl-100 column and eluted by Tris buffer (Figure 2). PLA₂ was eluted as a major peak of enzymic activity (fractions 18–22) that was sensitive to MJ33 (results not shown). This chromatographic step resulted in an approx. 70-fold increase in the specific activity.

Concentrated PLA₂ fractions from the Sephacryl-100 column were fractionated on a heparin-Sepharose column (Figure 3). The major activity was eluted at low-salt concentration (fractions 10–23). This activity was inhibited by MJ33 (results not shown).

Table 1 Assay of acidic (pH 4.0) and alkaline (pH 8.5) PLA₂ activity of lung homogenate

Values are mean ± S.D. for *n* = 3.

Assay conditions	Acidic PLA ₂ activity (nmol/mg per min)	Alkaline PLA ₂ activity (nmol/mg per min)
Plus 10 mM Ca ²⁺	0.9 ± 0.20	1.2 ± 0.26
Minus Ca ²⁺ + 5 mM EDTA	1.0 ± 0.23	0.1 ± 0.05
MJ33 (3 mol %)*	0.5 ± 0.13	1.1 ± 0.20

*Added under minus Ca²⁺ conditions for acidic PLA₂ and plus Ca²⁺ for alkaline PLA₂.

Figure 1 Elution profile of Ca²⁺-independent PLA₂ on a DE-52 column

The column was equilibrated with 50 mM Tris, 0.5 mM EDTA (pH 7.0). The protein was eluted by a linear KCl gradient from 50 to 450 mM at a flow rate of 30 ml/h (indicated by the dotted line). Fractions were collected and measured for PLA₂ activity in the absence (□) and presence (■) of MJ33 and for protein content (○).

Figure 2 Elution profile of Ca²⁺-independent PLA₂ on Sephacryl-100

Pooled fractions from the DE-52 columns that showed inhibition of PLA₂ by MJ33 were applied to a Sephacryl-100 column. The column was eluted at a flow rate of 20 ml/h with 50 mM Tris buffer (pH 7.0) containing 0.5 mM EDTA. Fractions were assayed for protein (●) and PLA₂ (□) activity.

Figure 3 Elution profile of Ca^{2+} -independent PLA_2 on heparin-Sepharose CL-4B

Pooled fractions from the Sephacryl-100 column were applied to a heparin-Sepharose CL-4B column. The column was eluted by a linear KCl gradient (not shown) from zero to 1.5 M at a flow rate of 10 ml/h. Fractions were assayed for protein (●) and PLA_2 (□) activity.

Figure 4 Elution profile of Ca^{2+} -independent PLA_2 on phenyl-Sepharose

Pooled fractions from the heparin-Sepharose CL-4B column were applied to a phenyl-Sepharose column. The column was eluted with a gradient (not shown) of 1.5 M KCl and 0% ethylene glycol to 0 M KCl and 10% ethylene glycol at a flow rate of 10 ml/h. Fractions were assayed for protein (●) and PLA_2 (□) activity.

Another peak of activity was eluted by a high-salt wash, but it was not significantly inhibited by MJ33 and accounted for only a small percentage of total PLA_2 activity. This second peak of activity, possibly representing the type-II secretory enzyme, was not further investigated.

The main active fractions from the heparin-Sepharose column were purified with a phenyl-Sepharose CL-4B column. The most active fractions (fractions 12–16) were eluted as a major peak on the chromatogram (Figure 4). This peak displayed a high sensitivity to MJ33 and a major band on SDS/PAGE (see below).

The enzyme purification procedure described above gave essentially identical results for three different preparations; values obtained at each stage during one such preparation are shown in Table 2. The specific activity increased 560-fold and the yield of

the purified enzyme was 0.6% of initial activity of the MJ33-sensitive enzyme. The low yield of activity suggests instability of the enzyme during isolation and/or losses due to the absorption on surfaces because of the small quantities of material.

Characterization of Ca^{2+} -independent PLA_2

The purity of the PLA_2 preparation was evaluated by SDS/PAGE. Electrophoresis of pooled fractions post-phenyl-Sepharose CL-4B column displayed a major band with an apparent molecular mass of approx. 15 kDa (Figure 5). In order to determine whether the 15 kDa band had PLA_2 activity, it was excised from the SDS/PAGE gel and subjected to the renaturation protocol. The renatured protein showed activity of 40 nmol/mg of protein per min; this activity was inhibited more than 90% by MJ33. Although the specific activity of the renatured protein was only 10% compared with the native enzyme, the relatively low recovery of activity can be explained by lability of the enzyme.

The kinetic properties of the isolated PLA_2 were determined by measuring PLA_2 activity as a function of substrate concentrations (Figure 6). Double-reciprocal plot analysis of the data revealed an apparent K_a for the interface of 140 μM for DPPC and an apparent initial rate (V_0) of 250 nmol/mg per min. These apparent kinetic parameters must be interpreted cautiously because of the complexity of PLA_2 interfacial catalysis [22]. The K_a value obtained from the bulk-concentration-dependence of the aggregates relates to the affinity of the enzyme for the interface, i.e. it is the concentration at which 50% of the enzyme is at the interface [22]. Thus the maximum rate of hydrolysis is the rate of hydrolysis when all enzyme is at the interface. Since the maximum available substrate is mol fraction 1, the calculated value (V_0) relates to such a limiting rate.

Various compounds were tested for effects on the activity of purified PLA_2 . There was no change in activity in the presence of 1 mM Ca^{2+} (Table 3). Inhibition was observed at higher Ca^{2+} concentrations (30% decrease at 5–10 mM Ca^{2+}). Inhibition with high calcium may have been due to an effect on the anionic liposome substrate which showed significant aggregation at pH 4, as measured by absorbance at 540 nm, and hence a reduction in the effective available substrate concentration. High concentrations (10 mM) of other bivalent metal ions (BaCl_2 , MgCl_2) also reduced the apparent PLA_2 activity at pH 4 by 10–30%. ATP has been shown to activate cardiac Ca^{2+} -independent PLA_2 [6] but had no effect on the lung enzyme (Table 3). pBPPB, an inhibitor of secretory PLA_2 [23], was ineffective with the lung enzyme (Table 3). The enzyme was also resistant to inactivation by reduction with 2-mercaptoethanol (Table 3). MJ33 demonstrated a strong inhibitory effect on the enzyme (Table 3); the IC_{50} for MJ33 is approx. 20 μM , which can be expressed as 0.3 mol% with reference to the substrate [17]. In contrast, no significant effect on activity was observed with the cytosolic PLA_2 inhibitor, AACOCF₃ [24] (Table 3). SP-A has been shown to inhibit lung Ca^{2+} -independent acidic PLA_2 activity in lung homogenate and lung lamellar bodies [25] and it also inhibited activity of the purified enzyme (Table 3).

The pH-activity profile of the isolated Ca^{2+} -independent enzyme was measured. The enzyme had maximal activity at pH 4, which is in agreement with the previous observations [26], and had only 20% activity at pH 6.

Immunoblot analysis (results not shown) using antiserum raised against pig pancreatic PLA_2 , gave a positive reaction with the lung homogenate, indicating the presence of secretory PLA_2 ; the antibody did not react with the purified Ca^{2+} -independent enzyme.

Table 2 Enzyme activity during purification of Ca²⁺-independent PLA₂ from rat lung

Total activity was measured using DPPC as substrate at pH 4 in Ca²⁺-free medium by the standard assay.

Purification step	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol of protein per min)	Enzyme recovery (%)	Purification (fold)
Homogenate	2000	2000	1.0	100	1
Supernatant	450	1800	4.0	90	4
DE-52	40	600	15	20	15
Sephacryl-100	0.4	28	70	1.4	70
Heparin-Sepharose	0.05	20	400	1.0	400
Phenyl-Sepharose	0.02	11	560	0.6	560

Figure 5 SDS/15% polyacrylamide gel electrophoresis of rat lung Ca²⁺-independent PLA₂

Lane 1 was loaded with 5 μ g of protein representing the PLA₂ active fraction from the phenyl-Sepharose CL-4B column. Lane 2 shows molecular-mass standards. The gel was visualized by staining with Coomassie Blue.

Figure 6 Substrate concentration dependence of rat lung Ca²⁺-independent PLA₂

Enzyme activity was assayed at pH 4 using DPPC as substrate. Insert shows a double-reciprocal plot of PLA₂ activity versus DPPC concentration.

N-terminal amino acid sequence

Amino acid sequence was obtained for 14 N-terminal amino acids from the lung enzyme (Table 4) and compared with the published sequences for rat pancreatic (type-I secretory) PLA₂ [27], rat platelet (type-II secretory) PLA₂ [28], and mouse cytosolic PLA₂ from a RAW 264.7 macrophage cell line [3]. The Ca²⁺-independent enzyme N-terminal sequence is rich in leucine (43% of the amino acid residues) and has two cysteines in relatively close proximity, possibly forming an internal bridge. N-terminal sequences from other rat PLA₂s reveal little or no similarity to the rat lung Ca²⁺-independent PLA₂ sequence. Likewise, this sequence was not identified in any other protein by a search of the NCBI database.

DISCUSSION

This report describes the isolation to near homogeneity of a Ca²⁺-independent PLA₂ from rat lung that is optimally active at

Table 3 Effect of various agents on Ca²⁺-independent PLA₂ from rat lung

The purified enzyme was preincubated at 25 °C for 10 min in the presence of the indicated reagents. The activity of PLA₂ was measured at pH 4 by the standard assay. Values are mean $\pm$ S.D. for three experiments or mean $\pm$ range for $n = 2$ (*).

Reagent	Remaining activity (% of control)
None (control)	100
CaCl ₂ (1 mM)	105 $\pm$ 5
ATP (5 mM)	98 $\pm$ 6
pBPB (2 mM)	95 $\pm$ 3
2-Mercaptoethanol (5 mM)	95 $\pm$ 5*
MJ33 (3 mol %)	5 $\pm$ 2
AACOCF ₃ (70 μ M)	90 $\pm$ 1
SP-A (15 nM)†	50 $\pm$ 9

† Calculated from an assumed molecular mass of 650 kDa [18].

Table 4 N-terminal amino acid sequences for PLA₂ enzymes from rat

Source	Reference	Sequence
Rat lung (Ca ²⁺ -independent)	Present study	NH ₂ V L L I C L L K C F K L T L
Rat pancreas (type-I secretory)	[27]	NH ₂ A V W Q F R N M I K C T I P
Rat platelet (type-II secretory)	[28]	NH ₂ S L L E F G Q M I L F K T G
Mouse macrophage (cytosolic)	[3]	NH ₂ M S F I D P Y Q H I I V E H

acidic pH values. Ca²⁺-independent PLA₂ activity has been known in lung for some time [10,11], but isolation and purification have been hampered by heterogeneity of PLA₂s, low abundance of the enzyme, and the unusual profile of activity versus pH. Our studies with rat lung homogenates and isolated organelles have indicated that the transition-state substrate analogue, MJ33, inhibits only Ca²⁺-independent acidic PLA₂ activity [16,29] and this inhibitor was used to define enzyme activity of fractions during the purification steps. By choosing carefully the assay conditions and with the use of the inhibitor MJ33, we have developed an isolation scheme and obtained a relatively pure protein.

The choice of columns used for isolation and purification was based on general strategies for protein purification and PLA₂ properties. DE-52 was followed by Sephacryl-100 as a method to handle a large amount of crude material with good recovery of activity. Heparin-Sepharose has been used previously for isolation of other types of PLA₂ [30]. The hydrophobic phenyl-Sepharose column was chosen based on the probable hydrophobicity of PLA₂. This scheme was successful in purifying the PLA₂ activity by a factor of 560.

Biochemical characterization of the enzyme showed an apparent molecular mass of 15 kDa, on the basis of SDS/PAGE; renaturation from the gel confirmed the identity of the activity and the isolated protein. Activity was insensitive to 2-mercaptoethanol, suggesting that the enzymic function of the protein does not require disulphide linkages. Activity was independent of Ca²⁺; decreased reaction at high Ca²⁺ concentrations (5–10 mM) is more likely due to an effect on the substrate rather than the enzyme, since aggregation of liposomes by Ca²⁺ occurred at acidic pH values. A similar decrease in activity of PLA₂ (acidic enzyme) with high Ca²⁺ was reported by other investigators [5,26]. This effect of Ca²⁺ at high concentration would be dependent on the nature of the substrate.

Previous reports have identified secretory PLA₂ and cytosolic PLA₂ in lung homogenates [7,8]. The PLA₂ activity isolated at the present study has characteristics that distinguish it from these and other previously identified PLA₂ types. Differentiation from type-I secretory PLA₂ is based on the pH optimum of 4, the absence of calcium requirement, the stable activity with chemical reduction, and insensitivity to the inhibitor pBBP [31]. In addition, low affinity for heparin-Sepharose resin (Figure 3) and for the lectin concanavalin A (results not shown) indicate a difference from type-II secretory PLA₂ [30]. Cytosolic PLA₂ is characterized by a high molecular mass (approx. 85 kDa), alkaline pH optimum, and a micromolar Ca²⁺ requirement [3,8,9]; it is also, unlike the Ca²⁺-independent lung enzyme, inhibited by AACOCF₃ [24]. Myocardial Ca²⁺-independent PLA₂ has a molecular mass of approx. 40 kDa, has a pH optimum of 6.4, is stimulated by ATP, and has little activity against PC (plasmalogens are the preferred substrate) [6,32]. A PLA₂ with acidic pH optimum has been described in mast cells. Unlike the enzyme isolated from lung, the mast-cell enzyme has a micro-

molar Ca²⁺ requirement, does not hydrolyse PC (phosphatidylserine is the preferred substrate), has a pH optimum of 6.5 and is sensitive to pBBP [33]. Therefore, the isolated Ca²⁺-independent lung PLA₂ with molecular mass of 15 kDa, a pH optimum of 4.0, activity towards PC, and insensitivity to pBBP and to reducing agents appears to be a new class of PLA₂. This conclusion is supported by N-terminal amino acid sequence analysis, which shows little similarity to other known PLA₂s.

The subcellular origin of this enzyme has not yet been precisely identified. We have demonstrated MJ33-inhibitable Ca²⁺-independent PLA₂ activity in a lamellar body fraction [16] and in a lysosomal fraction (C. R. Dodia and A. B. Fisher, unpublished work) of lung, both acidic organelles. Although PLA₂ activity in these organelles has been known for some time [26,34], the present study represents the first known isolation of this activity to essential homogeneity.

We thank Dr. Yu-Chu Huang (University of Delaware) for amino acid sequence analysis, Ms. Kay Speicher (Wistar Institute Protein Microchemistry Laboratory, Philadelphia, PA, U.S.A.) for on-line amino acid search, Dr. Michael Gelb (University of Washington) for supplying the AACOCF₃ inhibitor, Dr. Lin Liu for suggesting the experiments on liposome aggregation and Elaine Primerano for expert secretarial assistance. Grant support was provided by HL19737 from the National Institutes of Health. This work was presented in preliminary form at the 1993 Experimental Biology (FASEB) meeting in New Orleans, LA, U.S.A.

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Received 14 March 1994/2 June 1994; accepted 16 June 1994