Purification of a high-molecular-mass form of phospholipase A₂ from rat kidney activated at physiological calcium concentrations

Joseph H. GRONICH,[†] Joseph V. BONVENTRE[†] and Raphael A. NEMENOFF^{*†}

Medical Services, *Diabetes Unit and †Renal Unit, Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston, MA 02114, U.S.A.

Rat kidney contains a soluble phospholipase A₂ (PLA₂), which is chromatographically identical with a previously identified hormonally regulated form of the enzyme in rat renal mesangial cells. This kidney enzyme has been purified by sequential column fractionation. The purified enzyme is a 110 kDa polypeptide which can hydrolyse arachidonoyl phosphatidylcholine and arachidonoyl phosphatidylethanolamine, but has low activity towards arachidonoyl phosphatidylinositol. The enzyme is considerably larger than most previously isolated forms of secretory or intracellular PLA₂, and is stimulated by physiological concentrations of Ca²⁺, with half-maximal activation occurring at 500 nM-Ca²⁺. The hormonal regulation and Ca²⁺-dependency of this enzyme strongly suggest that it plays a role in hormonally regulated arachidonic acid release and prostaglandin production in the kidney.

INTRODUCTION

The release of arachidonic acid from membrane phospholipids is the rate-limiting step in the production of a variety of vasoactive inflammatory mediators with cell-signalling properties, e.g. prostaglandins, thromboxanes and leukotrienes [1]. This process has been shown to be under stringent hormonal control in a wide variety of cell types, and represents an important cell-signalling pathway, with diverse physiological effects. A number of enzymic pathways have been described which result in the production of free arachidonic acid: (1) direct hydrolysis of phospholipids by phospholipase A₂ (PLA₂) to produce non-esterified fatty acid and lysophospholipid [2]; (2) hydrolysis by phospholipase C to produce diacylglycerol, which in turn is hydrolysed by diacylglycerol lipase to produce non-esterified fatty acid [3,4]; and (3) hydrolysis by phospholipase D to produce phosphatidic acid [5], which is then hydrolysed by a phosphatidic acid-specific PLA₂. The relative contribution of each pathway varies with cell type; however, it appears that in most cells PLA₂ is the dominant enzyme regulating this process [6].

PLA₂ is a ubiquitous class of enzymes; both secretory and intracellular forms have been identified. The secretory forms of the enzyme have been extensively studied, and appear to be small proteins of approx. 15–18 kDa. Much less is known about intracellular forms of the enzyme. Several recent studies suggest that a number of isoenzymes of intracellular PLA₂ exist [7–12], and at least some of these are immunologically distinct from secretory forms of the enzyme [13]. It has not been established which of these intracellular forms are subject to modulation by hormones, growth factors, and other important regulatory influences.

We have recently reported the identification and initial characterization of a hormonally regulated form of PLA₂ in rat renal mesangial cells [14,15]. These cells are important producers of prostaglandins, which play a critical role in glomerular haemodynamics and inflammation in the kidney. In the mesangial cell, PLA₂ appears to be the dominant enzyme regulating arachidonic acid release [16]. This Ca²⁺-dependent form of PLA₂ was stimulated by [arginine]vasopressin, phorbol myristate acetate and

epidermal growth factor. The stimulated activity survived cell disruption and fractionation, suggesting a stable modification of the enzyme [14]. The mesangial-cell enzyme appeared identical with the major phosphatidylcholine-hydrolysing PLA₂ in soluble extracts prepared from whole rat kidney. Using rat kidney as a source, we report here the purification and characterization of this novel form of PLA₂, which is considerably larger than previously purified PLA₂ enzymes.

EXPERIMENTAL

Preparation of extracts

Rats (male Sprague–Dawley, 150–200 g) were killed by cervical dislocation. Kidneys were removed, rinsed in ice-cold phosphate-buffered saline (150 mM-NaCl, 10 mM-sodium phosphate, pH 7.5), and homogenized with a Potter–Elvehjem motor-drive pestle in 50 mM-Hepes, pH 7.5, containing 0.25 M-sucrose, 1 mM-EDTA, 1 mM-EGTA, 20 μM-pepstatin, 20 μM-leupeptin, 100 kallikrein-inhibitory units of trasylol/ml and 0.1 mM-phenylmethanesulphonyl fluoride. All subsequent steps were performed at 4 °C. The homogenate was spun at 1000 g for 5 min to remove debris and fat. The low-speed supernatant was spun at 200 000 g for 1 h in a Ti45 ultracentrifuge rotor. The resulting high-speed supernatant was filtered through glass wool, and is termed the original extract.

Assay of PLA₂ activity

PLA₂ activity was assayed with 1-stearoyl 2-[1-¹⁴C]arachidonoyl phosphatidylcholine as substrate as previously described [14]. Radiolabelled phospholipid was dried under N₂, and resuspended in dimethyl sulphoxide. Substrate (2 μl) was added to an Eppendorf tube, followed by 4 μl of 30 mM-Ca²⁺. Substrate resuspended in 50 mM-Hepes, pH 7.5, containing 0.1% (w/v) octyl glucoside, followed by sonication, gave results identical with those obtained with dimethyl sulphoxide. Reactions were initiated by addition of 34 μl of enzyme extract. Our standard assay had a final concentration of 15 μM phospholipid, and 1 mM free Ca²⁺ (in excess of EGTA and EDTA). Incubations were

Abbreviation used: PLA₂, phospholipase A₂.

† To whom reprint requests should be addressed.

carried out for 30 min at 37 °C, and the reaction was terminated by addition of 40 μ l of ethanol/acetic acid (49:1, v/v) containing 100 μ g of unlabelled arachidonic acid/ml. Samples were spotted on silica-gel t.l.c. plates (LK5D, Whatman), and developed in the organic phase of ethyl acetate/iso-octane/water/acetic acid (55:75:100:8, by vol.). Lipids were detected by I_2 staining. Authentic standards were co-chromatographed with the samples. Phospholipid, free arachidonic acid and diacylglycerol bands were scraped from the plate and counted for radioactivity in 0.25 ml of water and 3 ml of Dimiscint (National Diagnostics). Blank samples contained no extract protein. Extracts were diluted to ensure that not more than 20% of the label was hydrolysed during the incubation. Results were expressed as units of enzyme activity, where 1 unit represents 1 nmol of arachidonic acid released from [14 C]arachidonoyl phosphatidylcholine/minute at 37 °C, with a substrate concentration of 15 μ M. In experiments requiring higher concentrations of substrate, unlabelled 1-stearoyl 2-arachidonoyl phosphatidylcholine was mixed with [14 C]arachidonoyl phosphatidylcholine before being dried under N_2 . For experiments examining substrate specificity, [14 C]arachidonoyl phosphatidylethanolamine and [14 C]arachidonoyl phosphatidylinositol were prepared in an identical manner.

Purification of PLA₂

DEAE-Sephacel anion-exchange chromatography. High-speed supernatants from 30–50 rats were incubated with approx. 100 ml of settled DEAE-Sephacel, which had been equilibrated with 50 mM-Hepes (pH 7.5)/1 mM-EDTA/1 mM-EGTA (buffer A) for 1 h at 4 °C. Proteins not binding to the resin were removed by filtering the DEAE-Sephacel with a funnel with a glass frit. The resin was washed with 2–3 column vol. of equilibration buffer, and poured into a 2.6 cm $\times$ 20 cm column. The column was washed further with equilibration buffer until the A_{280} was less than 0.1. Proteins were eluted with an 800 ml linear salt gradient from 0–0.5 M-NaCl. Fractions were assayed for PLA₂ activity and protein.

Phenyl-Sepharose chromatography. The peak of PLA₂ activity eluted from the DEAE-Sephacel column was made 1.0 M in NaCl by addition of 50 mM-Hepes (pH 7.5)/1 mM-EDTA/1 mM-EGTA/4.8 M-NaCl. The material was loaded on a 30 ml phenyl-Sepharose column (1.6 cm $\times$ 15 cm) which had been equilibrated in 50 mM-Hepes (pH 7.5)/1 mM-EDTA/1 mM-EGTA/1.0 M-NaCl. The column was washed with equilibration buffer (high-salt wash), followed by washing in the same buffer containing no NaCl (low-salt wash). PLA₂ activity was then eluted with a linear gradient of octyl glucoside from 0 to 3% (w/v). High concentrations of octyl glucoside reversibly inhibit PLA₂ activity. Therefore, in assaying fractions from the phenyl-Sepharose column, samples of all fractions were diluted 1:10 with buffer A before assay.

Sephacryl S-300 gel filtration. The peak of PLA₂ activity from the phenyl-Sepharose column was concentrated to a final volume of 15–20 ml, by using an Amicon ultrafiltration apparatus with Pharmacia low-protein-binding filters, having a 10 kDa cut-off. The concentrated material was applied to a Sephacryl S-300 column (2.6 cm $\times$ 100 cm) which had been equilibrated in 50 mM-Hepes (pH 7.5)/1 mM-EDTA/1 mM-EGTA/150 mM-NaCl; 8 ml fractions were collected. The column was calibrated with a mixture of Blue Dextran, bovine serum albumin, ovalbumin, catalase and ribonuclease.

MONO-Q anion-exchange chromatography. The peak from the Sephacryl S-300 column was applied to a prepacked 2 ml MONO-Q (HR 5/5) column (Pharmacia), which had been

equilibrated in buffer A. After washing the column with equilibration buffer, PLA₂ activity was eluted with a 35 ml linear gradient from 0 to 0.6 M-NaCl; 1 ml fractions were collected and assayed for PLA₂ activity.

Hydroxyapatite chromatography. The peak of enzyme activity recovered from the MONO-Q column was diluted 1:1 with 50 mM-Hepes (pH 7.5)/1 mM-EDTA/1 mM-EGTA/4 mM-CaCl₂, and applied to a 2 ml hydroxyapatite column which had been equilibrated with 3 mM-potassium phosphate (pH 7.5)/0.2 M-KCl/10% (v/v) glycerol. The column was washed with equilibration buffer, and PLA₂ activity was eluted with a linear gradient from 0 to 300 μ M-potassium phosphate. To inhibit proteolysis by Ca²⁺-dependent proteases, all fractions were made 1 mM in EGTA and EDTA.

Gel electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [17]. After electrophoresis, the gel was silver-stained with a Bio-Rad kit.

Ca²⁺-dependency

Partially purified PLA₂ (extracts fractionated by DEAE-Sephacel) were diluted with Ca²⁺/EGTA buffers. Buffer free Ca²⁺ concentration was measured with fura-2 when the Ca²⁺ concentration was less than 1 μ M. For Ca²⁺ concentrations above 1 μ M, a Ca²⁺-sensitive electrode was used. Fura-2 free acid was added to the buffer at a final concentration of 167 nM. Fluorescence of the solution was determined at excitation wavelengths of 340 and 380 nm, and emission wavelength of 510 nm, by using a Photon Technology Incorporated Deltascan Spectrofluorimeter. The K_d for the dye was taken to be 224 nM, and the Ca²⁺ concentration was calibrated by previously described methods [18]. For Ca²⁺ concentrations above 1 μ M, Ca²⁺ electrodes were constructed and calibrated as previously described [19]. PLA₂ activity was determined with [14 C]arachidonoyl phosphatidylcholine and [14 C]arachidonoyl phosphatidylethanolamine, both at 15 μ M.

N-terminal sequencing

Preparations of highly purified PLA₂ were run on 15% acrylamide gels, and transferred to Immobilon membranes (Millipore, Bedford, MA, U.S.A.) with a Hoefer transfer apparatus. The Immobilon was stained with Coomassie Blue for 15 min, and destained with 10% (v/v) acetic acid. Bands were excised from the Immobilon and subjected to N-terminal amino acid sequencing, by using the Diabetes Research Laboratory Applied Biosystems Protein Sequencer 470A, equipped with PTL Analyzer 120A.

Materials

Radiolabelled phospholipids were from Amersham Corp. or Dupont-New England Nuclear. Unlabelled phospholipids, arachidonic acid and standards for t.l.c. were from Sigma. DEAE-Sephacel, phenyl-Sepharose, Sephacryl S-300 and a prepacked MONO-Q (HR 5/5) column were from Pharmacia LKB Biotechnology Inc. Hydroxyapatite was from Calbiochem. Protein was measured with a Bio-Rad protein kit. Molecular-mass markers for polyacrylamide gels and silver-staining reagents were from Bio-Rad. Chemicals for buffers were from Sigma and other standard suppliers.

RESULTS

Purification of PLA₂

High-speed supernatants from rat kidneys were fractionated by DEAE-Sephacel anion-exchange chromatography. Over 80%

Fig. 1. Phenyl-Sepharose chromatography

The peak of PLA₂ from the DEAE-Sephacel column was made 1.0 M in NaCl and applied to a 30 ml phenyl-Sepharose column, which had been equilibrated with 50 mM-Hepes (pH 7.5)/1 mM-EGTA/1 mM-EDTA/1.0 M-NaCl. The column was washed, and PLA₂ activity was eluted with a linear gradient (0–3 %, w/v) of octyl glucoside (—); 6 ml fractions were collected. ●, PLA₂ activity; —, A₂₈₀ (arbitrary units). The indicated fractions were pooled.

Fig. 2. Sephacryl S-300 gel filtration

The peak of PLA₂ activity recovered from the phenyl-Sepharose column was concentrated to 15 ml with an Amicon ultrafiltration apparatus, with a Pharmacia low-protein-binding filter. The concentrated material was fractionated by Sephacryl S-300 gel filtration; 8 ml fractions were collected. ●, PLA₂ activity; —, A₂₈₀ (arbitrary units). Arrows indicate the elution position of marker proteins (BSA, bovine serum albumin; OA, ovalbumin). The indicated fractions were pooled.

Fig. 3. Hydroxyapatite chromatography

The peak of PLA₂ activity recovered from the MONO-Q column was applied to a 2 ml hydroxyapatite column which had been equilibrated with 3 mM-potassium phosphate (pH 7.4)/0.2 M-KCl/10 % (v/v) glycerol. PLA₂ activity was eluted with a linear gradient of 3–300 mM-potassium phosphate (with the same other components) (—); 1 ml fractions were collected. ●, PLA₂ activity; —, A₂₈₀ (arbitrary units).

of the enzyme activity bound to the column. A major peak of enzyme activity was eluted from the column at 0.22 M-NaCl. A minor peak of activity, occasionally observed to be eluted earlier from the column, was not characterized further. Overall recovery was over 80 %, with the major peak representing over 60 % of the activity applied to the column.

The peak from the DEAE-Sephacel column was further fractionated by phenyl-Sepharose hydrophobic chromatography. Over 90 % of applied activity bound to the column. Small amounts of activity were eluted during the low-salt wash. The major peak of activity was eluted from the column in a broad peak of approx. 2 % octyl glucoside, and preceded a major peak of protein (Fig. 1). Overall recovery of enzyme activity on this column was over 75 %.

The major peak from the phenyl-Sepharose column was concentrated with no loss of enzyme activity, and applied to a Sephacryl S-300 gel-filtration column. A single peak of enzyme activity was recovered, migrating at 55 kDa (Fig. 2). Recovery was over 60 %. The S-300 peak was applied to a Pharmacia f.p.l.c. MONO-Q column. Again a single peak of activity was eluted from this column, at approx. 0.3 M-NaCl. Recovery was over 90 %.

Table 1. Purification of PLA₂ from rat kidney

Extracts were prepared from the kidneys of 30 rats by homogenization and centrifugation, as described in the Experimental section. PLA₂ activity was assayed with phosphatidylcholine as substrate (15 μM) in the presence of 1 mM-Ca²⁺ in excess of the combined concentrations of EGTA and EDTA. A unit of enzyme activity corresponds to the production of 1 pmol of free arachidonic acid/min at 37 °C, under standard assay conditions. Protein was determined with a Bio-Rad protein kit. Final protein recovered from the hydroxyapatite column was determined by amino acid analysis.

	Protein (mg)	Activity (units)	Sp. activity (units/mg)	Purification (fold)	Recovery (%)
Original	6885	35196	5.1	1	100
DEAE-Sephacel	606.3	21658	35.7	7.0	61.5
Phenyl-Sepharose	46.4	11140	240.1	47	31.7
S-300	5.6	9334	1672	327	26.5
MONO-Q	0.40	5428	13704	2682	15.4
Hydroxyapatite	0.04	1460	36514	7146	4.1

Fig. 4. SDS/PAGE of purified PLA₂.

(a) Duplicate fractions from the peak of PLA₂ activity recovered from the hydroxyapatite column were run on a 15% acrylamide/0.2% *NN*-methylenebisacrylamide gel, by the method of Laemmli [17]. The gel was silver-stained, with a Bio-Rad kit. Molecular-mass markers (Bio-Rad) are shown on the right, their values in kDa. (b) Partially purified preparation of PLA₂ (fractionated through MONO-Q) was run on an identical gel. The gel was sliced into 0.5 cm slices, and proteins were eluted from the gel by the method of Hager & Burgess [20]. Proteins were precipitated with acetone, and renatured by using 6 M-guanidine/HCl, followed by dilution. Eluted proteins were assayed for PLA₂ activity. Arrows indicate molecular masses (kDa) of known markers on a parallel lane of the same gel. Overall recovery of PLA₂ activity was 9%.

The MONO-Q peak was diluted with buffer containing Ca²⁺ to bind the free EGTA and EDTA, and applied to a 2 ml hydroxyapatite column. PLA₂ was eluted with a phosphate gradient. The peak of PLA₂ activity was separated from the major peak of protein, and was eluted at 0.17 M-sodium phosphate (Fig. 3). Table 1 summarizes the results of a typical purification. The enzyme was purified approx. 7200-fold, with an overall recovery of 4%.

The purified material was analysed by SDS/PAGE, followed by silver staining, as shown in Fig. 4(a). Two bands, of 110 kDa and 55 kDa, were observed in fractions containing PLA₂ activity. The intensity of the 110 kDa band correlated best with PLA₂ activity across the hydroxyapatite column. A number of much less intense bands were occasionally observed at approx. 48 and 35 kDa. These bands were not detected in all preparations, and

Fig. 5. Lineweaver-Burk analysis of PLA₂ activity

Highly purified PLA₂ was assayed for activity at several concentrations of 1-stearoyl 2-arachidonoyl phosphatidylcholine. Data represent means of triplicate determinations.

Table 2. Substrate specificity of kidney PLA₂

Purified PLA₂ was assayed for its ability to release [¹⁴C]arachidonic acid from [¹⁴C]arachidonoyl phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylinositol (PI). All substrates were present at 15 μM, and were prepared as described in the Experimental section. Samples were analysed by t.l.c., by using authentic standards. Extracts were assayed in the absence of exogenous Ca²⁺ (Ca²⁺ < 100 nM) (–Ca²⁺), or in the presence of 1 mM-Ca²⁺ in excess of EDTA and EGTA (+Ca²⁺). Data are means ± S.D. of triplicate determinations.

	PLA ₂ activity (nmol/min per mg)		
	PC	PE	PI
–Ca ²⁺	21.9 ± 13.3	126.0 ± 10.4	4.8 ± 6.0
+Ca ²⁺	126.1 ± 21.0	401.4 ± 23.4	3.1 ± 4.3

their intensity did not correlate with PLA₂ activity. To establish convincingly which band corresponded to PLA₂ activity, we eluted the protein from the SDS gel, and renatured PLA₂ activity by the procedure of Hager & Burgess [20]. This procedure has recently been successful with a Ca²⁺-independent form of PLA₂ [21]. As shown in Fig. 4(b), enzyme activity was recovered as a single peak, precisely coinciding with the migration position of the 110 kDa band. No significant activity was reproducibly recovered anywhere else on the gel, including the position of the 55 kDa band.

We attempted to determine the *N*-terminal sequence of both of these proteins. After electrophoresis, proteins were transferred to Immobilon membranes, and subjected to *N*-terminal amino acid sequencing. The sequence obtained from the 55 kDa protein indicated that it was the rat form of protein disulphide-isomerase [22] (results not shown). This is a 55 kDa protein involved in the formation of disulphide bonds (see [23] for a review). Two separate attempts to obtain the *N*-terminal sequence of the 110 kDa protein were unsuccessful, suggesting that the *N*-terminal of this protein may be blocked.

Characterization of purified PLA₂

The Lineweaver-Burk analysis for the purified enzyme, with phosphatidylcholine as substrate, is shown in Fig. 5. The plot is

Fig. 6. pH-dependency of PLA₂ activity

Partially purified preparations of PLA₂ were diluted with 2 M-Tris/HCl or 1% acetic acid to the indicated pH. PLA₂ activity was measured with the standard assay. Results are means ± S.D. of triplicate determinations.

Fig. 7. Ca²⁺-dependency of PLA₂ activity

Partially purified preparation of PLA₂ (through the DEAE-Sephacel column) was diluted in Ca²⁺/EGTA buffers, and assayed for PLA₂ activity, with either [¹⁴C]arachidonoyl phosphatidylcholine (●) or [¹⁴C]arachidonoyl phosphatidylethanolamine (○), both at 15 μM, as substrate. Actual free Ca²⁺ was measured as described in the Experimental section. Results represent means ± S.D. of triplicate determinations.

linear, indicating orthodox Michaelis–Menten kinetics under the conditions used. The K_m for phosphatidylcholine is approx. 57 μM, with a V_{max} of 500 nmol/min per mg. This latter value is within the range reported for other intracellular forms of PLA₂ [10]. The substrate specificity of the enzyme is shown in Table 2. At equal concentrations of phospholipid substrate (15 μM), the enzyme can hydrolyse both [¹⁴C]arachidonoyl phosphatidylcholine and [¹⁴C]arachidonoyl phosphatidylethanolamine, but has much lower activity towards [¹⁴C]arachidonoyl phosphatidylinositol. The pH-dependency of the enzyme is shown in Fig. 6. The highest activity is observed at alkaline pH values, with little activity at pH values below 6.0. This is similar to the pH-dependency of the mesangial-cell enzyme [14].

This soluble high-molecular-mass form of PLA₂ has an absolute requirement for Ca²⁺. Mn²⁺ can substitute for Ca²⁺, but Mg²⁺, Cu²⁺, or Zn²⁺ do not activate the enzyme (results not shown). Using the dye fura-2 and a Ca²⁺-sensitive electrode, we have investigated the Ca²⁺-dependency in greater detail, with both phosphatidylcholine and phosphatidylethanolamine as substrates. Fig. 7 shows that half-maximal activation occurred at approx. 500 nM-Ca²⁺ with both substrates, and maximal activity was achieved at micromolar Ca²⁺ concentrations. Interestingly, PLA₂ activity in the absence of Ca²⁺ (concentrations < 50 nM) is much higher against phosphatidylethanolamine than against phosphatidylcholine.

DISCUSSION

Both secretory and intracellular forms of PLA₂ have been identified [6]. A large number of secretory forms of the enzyme have been purified, principally from snake and insect venoms, pancreatic extracts and synovial fluid [24,25]. These are generally small proteins, with molecular masses of 12–18 kDa, and very high specific activities. Although most demonstrate stimulated activity in the presence of Ca²⁺, maximal activation requires millimolar Ca²⁺ concentrations. Progress in characterizing intracellular forms of the enzyme has been much slower; these enzymes are less abundant, and in general have been found to have lower specific activities. The relationship between the intracellular forms of PLA₂ and the secretory forms has not been completely delineated. For example, Tojo and co-workers [9]

have purified and sequenced a soluble form of PLA₂ from rat spleen, which is identical with the secretory pancreatic form of the enzyme. On the other hand, de Jong *et al.* [13] found that monoclonal antibodies raised against a rat liver mitochondrial PLA₂ failed to cross-react with several secretory forms of the enzyme, but did recognize several intracellular forms, such as rat liver cytosolic and solubilized rat platelet PLA₂. Taken together, these data suggest that, although some intracellular forms of the enzyme may be related to the secretory enzymes, there do exist distinct intracellular forms of PLA₂.

The complex nature of intracellular PLA₂ regulation has become better appreciated over the past few years. It was generally perceived that PLA₂ is a Ca²⁺-dependent enzyme, and would be regulated by hormones which elevate intracellular Ca²⁺ levels. A growing body of data suggests that the regulation is more complex, involving a variety of interdependent pathways. In several cell types PLA₂ may be directly coupled to a G-protein [26–29]. In addition, both inhibitory [30–32] and stimulatory [33,34] PLA₂-modulatory proteins have been identified. Relatively little is known about the form(s) of PLA₂ involved in these mechanisms. Both membrane-associated and soluble forms of intracellular PLA₂ have been described.

We have recently identified a soluble form of the enzyme in rat renal mesangial cells, whose activity is stably modified by [arginine]vasopressin and phorbol esters, presumably through the action of protein kinase C [14]. In addition, the same form of the enzyme is stimulated by epidermal growth factor, through a mechanism that is independent of protein kinase C [15]. This mesangial-cell enzyme appears to be identical with the major soluble form of PLA₂ in whole rat kidney, with phosphatidylcholine as a substrate. In the present study we have purified this enzyme over 7000-fold. At this stage of purity, two proteins were detected by gel electrophoresis and silver staining, of 110 kDa and 55 kDa. The 55 kDa protein is, in all likelihood, the rat homologue of protein disulphide-isomerase.

Recent reports have suggested several potential enzymic functions for protein disulphide-isomerase [23]. Moreover, the enzyme has been shown to have a region of sequence identity with an isoenzyme of phospholipase C [35]. This raises the possibility that protein disulphide-isomerase in fact may have PLA₂ activity. In our view, this is unlikely. No PLA₂ activity corresponding to the 55 kDa band was recovered during the gel renaturation experiment (see Fig. 4b). In addition, protein disulphide-isomerase does not have a Ca²⁺-dependency for enzyme activity; from examination of the full sequence of the isomerase [22], no consensus region coding for known Ca²⁺-binding domains can be identified.

The 110 kDa band is the only other detectable band observed with silver staining (see Fig. 4a), and the intensity of the band correlates with the amount of PLA₂ activity recovered. Elution of the protein from the SDS gel, followed by renaturation, results in recovery of PLA₂ enzyme activity precisely coinciding with the migrational position of the 110 kDa protein. This is considerably larger than most other forms of PLA₂ described [8–10,24,25,36]. In addition, this protein appears to be a fairly scarce enzyme. By examination of the intensity of silver staining, it is likely that the 110 kDa protein represents no more than 10% of the total protein analysed on the gel. This implies that the specific activity of the homogeneous enzyme is greater than 0.4 μmol/min per mg, under standard assay conditions, and has a V_{max} > 3 μmol/min per mg. It has generally been assumed that intracellular forms of PLA₂ have a lower activity than secretory forms of the enzyme. There may exist, however, relatively scarce intracellular forms with high specific activities, comparable with those reported for secretory forms of PLA₂. The 110 kDa protein appears to migrate anomalously on gel-filtration chromato-

graphy. This may be due to the hydrophobic nature of the protein, which causes it to bind to and be retarded by the Sephacryl resin. This explanation is consistent with the observation that at high ionic strength (2 M-NaCl) this protein eluted later from the column (results not shown).

This high-molecular-mass enzyme is extremely sensitive to Ca^{2+} , with half-maximal activation occurring at approx. 500 nM- Ca^{2+} . Several other forms of intracellular PLA_2 are only activated at millimolar concentrations of Ca^{2+} [10], or at greater than 10 μM [9]. Since these levels of Ca^{2+} are not attained in cells under normal physiological conditions, the role of these enzymes in arachidonic acid release and prostaglandin synthesis is not well understood. The Ca^{2+} -dependency of the purified kidney enzyme closely parallels the Ca^{2+} -dependency of arachidonic acid release and prostaglandin production in the mesangial cell [19], further suggesting that this enzyme is responsible for the regulation of these events. We have previously shown that the soluble mesangial-cell form of PLA_2 associates with microsomal membranes in a Ca^{2+} -dependent fashion [14]. Thus, in addition to activating the enzyme, hormonally induced elevations of intracellular Ca^{2+} may cause a translocation of this enzyme to the membrane.

A soluble form of PLA_2 has been purified from sheep platelets [7]. This enzyme has a similar Ca^{2+} -dependency to the kidney enzyme; however, its apparent molecular mass by SDS/PAGE appeared to be 30 kDa. The relationship between this enzyme and the kidney enzyme remains to be determined. A high-molecular-mass form of PLA_2 has recently been isolated from guinea-pig intestinal brush-border membranes [21]. However, this enzyme appears to be inhibited by Ca^{2+} . Leslie *et al.* [12] have reported the purification of a soluble form of PLA_2 from RAW 764.7 macrophages. Their enzyme appears similar to our kidney enzyme; it is a soluble protein and has similar elution properties on a variety of fractionation techniques. SDS/PAGE of their purified preparation shows a major band at approx. 55 kDa, which they suggest is the enzyme. However, a less prominent band at 110 kDa is also observed on their gels. In the absence of definitive renaturation data it appears likely to us that their enzyme is in fact also of 110 kDa. Wijkander & Sundler [11] have also partially characterized a soluble form of PLA_2 in mouse peritoneal macrophages. This is a soluble enzyme, which appears to be closely related to the kidney form which we have purified; it has a similar Ca^{2+} -dependency, and migrates at the same molecular mass on gel filtration columns. Thus this high-molecular-mass form of PLA_2 is likely to be widely distributed in tissues, and to play a critical role in regulation of physiological responses.

PLA_2 may have other roles in cell signalling. In platelets, Sweatt *et al.* [37] have shown that a component of arachidonic acid release precedes adrenaline-induced elevations of diacylglycerol. This implies that direct activation of PLA_2 may regulate the activity of phospholipase C. Recent reports have also implicated PLA_2 in a G-protein-mediated regulation of K^+ channels in rat atrial membranes [38,39]. The activity of this membrane form of PLA_2 is inhibited by antibodies raised against bovine pancreatic PLA_2 [39,40]. The kidney form of the enzyme, which we have purified, is not recognized by these antibodies (J. H. Gronich & R. A. Nemenoff, unpublished work). These data raise the possibility that distinct forms of intracellular PLA_2 may have separate physiological functions, which are regulated by different signal-transduction pathways. To determine the function of a particular form of PLA_2 it will be necessary to develop specific reagents for that enzyme.

In summary, we have purified a novel form of PLA_2 . The enzyme is a soluble high-molecular-mass form, which is activated at physiological intracellular Ca^{2+} concentrations, and is chromatographically identical with the regulated mesangial-cell form of

the enzyme. Purification of this enzyme will enable us to develop reagents to study its regulation in detail, and to determine the role of this enzyme in cell function.

This work was supported by National Institutes of Health Grants DK 39902 (R. A. N.), DK 39773, DK 38452, and DK 38165 and NS 10828 (J. V. B.). J. H. G. is supported by National Research Service Award F32 DK 07903. J. V. B. is an Established Investigator of the American Heart Association. We thank Dr. Joseph Avruch for use of his protein sequencer, and for many helpful discussions.

REFERENCES

1. Flower, R. G. & Blackwell, G. J. (1976) *Biochem. Pharmacol.* **25**, 285–291
2. Billah, M. M., Lapetina, E. G. & Cuatrecasas, P. (1980) *J. Biol. Chem.* **255**, 10223–10231
3. Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. S. & Wilson, D. B. (1986) *Science* **234**, 1519–1526
4. Prescott, S. M. & Majerus, P. W. (1983) *J. Biol. Chem.* **258**, 764–769
5. Bocchino, S. B., Blackmore, P. F., Wilson, P. B. & Exton, J. H. (1987) *J. Biol. Chem.* **262**, 15309–15315
6. Waite, M. (1985) *J. Lipid Res.* **26**, 1379–1388
7. Loeb, L. A. & Gross, R. W. (1986) *J. Biol. Chem.* **261**, 10467–10470
8. Ono, T., Tojo, H., Kuramitsu, S., Kagamiyama, H. & Okamoto, M. (1988) *J. Biol. Chem.* **263**, 5732–5738
9. Tojo, H., Ono, T., Kuramitsu, S., Kagamiyama, H. & Okamoto, M. (1988) *J. Biol. Chem.* **263**, 5724–5731
10. Ulevitch, R. J., Watanabe, Y., Sano, M., Lister, M. D., Deems, R. A. & Dennis, E. A. (1988) *J. Biol. Chem.* **263**, 3079–3085
11. Wijkander, J. & Sundler, R. (1989) *FEBS Lett.* **244**, 51–56
12. Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M. & Zelarney, P. T. (1988) *Biochim. Biophys. Acta* **963**, 476–492
13. de Jong, J. G. N., Amesz, H., Aarsman, A. J., Lenting, H. B. M. & Van den Bosch, H. (1987) *Eur. J. Biochem.* **164**, 129–135
14. Gronich, J. H., Bonventre, J. V. & Nemenoff, R. A. (1988) *J. Biol. Chem.* **263**, 16645–16651
15. Bonventre, J. V., Gronich, J. H. & Nemenoff, R. A. (1990) *J. Biol. Chem.* **265**, 4934–4938
16. Schlondorff, D., DeCandido, S. & Satriano, J. A. (1987) *Am. J. Physiol.* **253**, C113–C120
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
18. Bonventre, J. V., Skorecki, K. L., Kreisberg, J. I. & Cheung, J. Y. (1986) *Am. J. Physiol.* **251**, F94–F102
19. Bonventre, J. V. & Swidler, M. (1988) *J. Clin. Invest.* **82**, 168–176
20. Hager, D. A. & Burgess, R. R. (1980) *Anal. Biochem.* **109**, 76–86
21. Gassama-Diagne, A., Fauvel, J. & Chap, H. (1989) *J. Biol. Chem.* **264**, 9470–9475
22. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A. & Rutter, W. J. (1985) *Nature (London)* **317**, 267–270
23. Freedman, R. B. (1989) *Cell* **57**, 1069–1072
24. Seilhamer, J. J., Pruzanski, W., Vadas, P., Plant, S., Miller, J. A., Kloss, J. & Johnson, L. K. (1989) *J. Biol. Chem.* **264**, 5335–5338
25. Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizard, R. & Pepinsky, R. B. (1989) *J. Biol. Chem.* **264**, 5768–5775
26. Burch, R. M. & Axelrod, L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6374–6378
27. Burch, R. M., Luini, A. & Axelrod, L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7201–7205
28. Jelsema, C. L. (1987) *J. Biol. Chem.* **262**, 163–168
29. Jelsema, C. L. & Axelrod, J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3623–3627
30. Pepinsky, R. B. & Sinclair, L. K. (1986) *Nature (London)* **321**, 81–84
31. Rothbut, B., Russo-Marie, F., Wood, J., DiRosa, M. & Flower, R. J. (1983) *Biochem. Biophys. Res. Commun.* **117**, 878–884
32. Hirata, F. (1981) *J. Biol. Chem.* **256**, 7730–7733
33. Clark, M. A., Conway, T. M., Shorr, R. G. L. & Crooke, S. T. (1987) *J. Biol. Chem.* **262**, 4402–4406
34. Clark, M. A., Conway, T. M., Mong, S., Steiner, S. & Crooke, S. T. (1986) *J. Biol. Chem.* **261**, 10713–10718

35. Bennett, C. F., Balcerek, J. M., Varrichio, A. & Crooke, S. T. (1988) *Nature (London)* **334**, 268–270
 36. DeWinter, J. M., Vianen, G. M. & Van den Bosch, H. (1982) *Biochim. Biophys. Acta* **712**, 332–341
 37. Sweatt, J. D., Blair, I. A., Cragoe, E. J. & Limbird, L. E. (1986) *J. Biol. Chem.* **261**, 8660–8666
 38. Kurachi, Y., Ito, H., Sugimoto, T., Shimizu, T., Miki, I. & Ui, M. (1989) *Nature (London)* **337**, 555–557
 39. Kim, D., Lewis, D. L., Graziadei, L., Neer, E. J., Bar-Sagi, D. & Clapham, D. E. (1989) *Nature (London)* **337**, 557–560
 40. Bar-Sagi, D., Suhan, J. P., McCormick, F. & Feramisco, J. R. (1988) *J. Cell Biol.* **106**, 1649–1658
-

Received 9 April 1990/8 June 1990; accepted 21 June 1990