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Purification and partial characterization of phospholipase A_2 isoforms from human placenta

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ABSTRACT

Five isoforms of the human placental phospholipase A_2 were identified and purified to near homogeneity. The purification of these enzymes involved gel permeation, ion-exchange and affinity chromatography. The apparent relative molecular mass of these proteins is 70 000 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. These enzymes have pH optima of 7 and 8. Two-dimensional gel electrophoresis of these enzymes revealed distinct pH optima for each of the isoforms with values ranging from 4.0 to 6.5. Three of the isoforms require calcium for activity whereas the other two forms exhibit 50% of their maximum activity without the presence of calcium.

INTRODUCTION

Several types of phospholipase A_2 (PLA_2) have been purified from various sources. These enzymes may be found in the cytosol [1–3] or the membrane of the cell [4–7]. PLA_2s play key roles in phospholipid turnover, the regulation of arachidonic acid release and prostaglandin synthesis [5–7]. The intracellular PLA_2s are hydrophobic in nature and usually require calcium for activity. Several intracellular PLA_2s have been isolated from various cells [4,8–12]. These phospholipases have varied calcium dependences, pH optima and relative molecular masses. The relative molecular masses of recently isolated PLA_2s range from 56 to 110, and are large when compared with the low-molecular-mass PLA_2s that were previously isolated [1–3].

Calcium-independent PLA_2s have been found in human platelets [13] and the canine myocardium [14]. These enzymes hydrolyze phospholipids at the *sn*-2-acyl position in the absence of calcium. The enzymatic activities of these calcium-independent enzymes are comparable to those of the calcium-dependent enzymes.

The purification of different isoenzymes of PLA_2 have been described for various species. Snake venom [1–3,15–17], sheep platelet [18] and porcine pancreas [19,20] PLA_2s all possess isoenzymes.

PLA_2 plays a significant role in prostaglandin biosynthesis [21]. The human placenta and the fetal membrane are of particular interest because of their involvement in the initiation of labor [22].

Human placental PLA_2s have been isolated from blood vessels [23] and placental membranes [24]. Both enzymes have characteristics that are distinct from the isoenzymes that we have purified. The cDNA clone for a low-molecular-mass human placental PLA_2 has been isolated and characterized. A comparative analysis of the nucleotide sequence of this enzyme shows that this human placental PLA_2 has a sequence which is 69% homologous to the rat platelet PLA_2 [25].

This paper describes the chromatographic resolution of five isoforms of human placental PLA_2.

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Calcium-dependent and calcium-independent enzymes were purified to near homogeinity.

EXPERIMENTAL

Materials

L-3-Phosphatidylcholine-1-stearoyl-2-[^{14}C]-arachidonyl (58.3 mCi/mmol), L-3-phosphatidylcholine-2-lyso-1-[^{14}C]palmitoyl (58.5 mCi/mmol) and L-3-phosphatidylcholine-1,2-[^{14}C]dipalmitoyl (117 mCi/mmol), organic counting scintillant (OCS) and streptavidin biotinylated horseradish peroxidase anti-rabbit immunoglobulin G (IgG) complex were purchased from Amersham (Paris, France). Sepharose 6B and PD-10 desalting columns were purchased from Pharmacia (Uppsala, Sweden). DEAE-Cellulose DE-52 was purchased from Whatman (Maidstone, UK). DL-α-Phosphatidylcholine-β-oleoyl-γ-O-hexadecyl, ethylenedioxydiethylenedinitrilotetraaceticacid (EGTA), sodium deoxycholate, β-mercaptoethanol, dithiothreitol (DTT), sucrose, phenylmethanesulfonyl fluoride (PMSF), aprotinin and other chemicals were purchased from Sigma (St. Louis, MO, USA). Purified polyclonal antibodies to the *Naja naja* PLA_2 was a generous gift from Dr. G. Bezeiat, Hospital St. Antoine (Paris, France). Organic solvents and silica gel plates were purchased from Merck (Darmstadt, Germany).

Protein assays

Protein concentrations were determined by the modified method of Lowry [26]. Bovine serum albumin (BSA) was used as a protein standard.

Enzymatic assays

PLA_2 activities were routinely assayed in a 0.5-ml reaction volume containing 50 m*M* Tris–HCl (pH 7.5), 10 m*M* $CaCl_2$ and phosphatidylcholine-1-stearoyl-2-[^{14}C]arachidonyl in the presence of 0.1% sodium deoxycholate. The substrate was prepared as previously described [27]. Aliquots of 50 μl of the column eluates were assayed. The reaction was initiated by the addition of 50 μl (50 μ*M*) of radiolabelled substrate. Hydrolysis was allowed to occur for 30 min at 37°C. The reaction was terminated by the addition of 50 μl of 2 *M* HCl. The product was extracted with 2 ml of hexane and vortex mixed for 10 s. The two phases were allowed to separate at room temperature for 10 min and 1 ml of the hexane layer was added to 5 ml of OCS scintillation fluid. The samples were analyzed by scintillation spectrometry.

pH optima

The pH optima for the different protein samples were determined at a substrate concentration of 0.25 μ*M* using 50 m*M* 2-(N-morpholino)ethanesulfonic acid (MES) buffer for the pH range 3–5. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 m*M*) was used for the pH range 6–7 and 50 m*M* Tris–HCl for the pH range 8–10. All assays were conducted in a 1.0-ml volume.

Calcium dependence

The calcium dependences of the isoenzymes were examined in the calcium concentration range 0–10 m*M*. The buffer solution (50 m*M* Tris–HCl–200 μ*M* EGTA) was prepared in doubly distilled, deionized water which contained a minimal amount of metal ions. EGTA (200 μ*M*) was added to the assay mixture in order to chelate the residual calcium in the water. The assay mixture contained 0.25 μ*M* of substrate and the indicated amounts of calcium. The total assay volume was 1.0 ml. The assays were conducted at pH 7.5.

Fluorescent assay

Fractions were assayed in the presence of BSA and fluorescent phospholipids as described elsewhere [28]. The sample was introduced into an assay mixture containing 2 μ*M* of negatively charged substrate [1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycerol monomethylphosphatidic acid] in 50 μ*M* Tris–HCl–100 m*M* NaCl–1 m*M* EDTA (pH 7.5).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was performed according to the method of Cambell [29]. The antibodies against the *Naja naja* enzyme [30] were used as the primary antibody in a 1:500 dilution. Streptavidin biotinylated horseradish peroxidase anti-rabbit IgG was used to monitor antigen–antibody interaction.

Preparation of placenta

Fresh placenta was skinned and cut into cubes. The tissue (*ca.* 400 g) was washed with ten 300-ml volumes of ice-cold buffer containing 50 m*M* Na_2HPO_4, 150 m*M* NaCl and 5% sucrose (pH 7.2).

The cleaned placenta was rapidly immersed in liquid nitrogen and stored at −80°C until use.

Protein purification

Frozen placental material (145 g) was thawed and suspended in 500 ml of buffer containing 50 m*M* Tris–HCl, 0.1% sodium deoxycholate, 10 U/ml of aprotinin and 0.25 m*M* of PMSF. The scissor-minced tissue was homogenized for 3 × 60 s at 42 000 rpm using a Virtis homogenizer. The placental homogenate was then centrifuged at 10 000 rpm for 25 min and the supernatant was descanted. The pellet which contained cellular debris was discarded. All steps were done at 4°C unless noted otherwise. The supernatant was frozen in a dry-ice–acetone bath and lyophilized.

The lyophilized protein was suspended in a minimum volume of buffer A [50 m*M* Tris–HCl–2 m*M* EDTA–10 m*M* β-mercaptoethanol–0.2% sodium azide–10% glycerol (pH 7.5)]. The protein sample was equilibrated and concentrated in the same buffer, using the Amicon system, to a minimum volume of 24 ml (1070 mg).

Gel permeation chromatography

The concentrated protein sample (24 ml, 1070 mg) was loaded on to two 90 cm × 2.5 cm I.D. columns in series. The Sepharose CL-6B resin column was pre-equilibrated with buffer A. Fractions of 3 ml were collected at a flow-rate of 10 ml/h. The enzymatic activity was measured using both radiolabeled and fluorimetric assays.

DEAE-cellulose chromatography

The most active post-Sepharose CL-6B fractions were pooled, dialyzed and concentrated by vacuum dialysis against a buffer containing 20 m*M* Tris–HCl, 0.25 m*M* PMSF and 10 U/ml of aprotinin. The concentrated protein material (65 mg) was loaded on to a 20 cm × 2.5 cm I.D. DEAE-cellulose column. The column was washed with three column volumes of the same buffer followed by the initiation of a linear gradient of KCl. Fractions of 3 ml were collected at a flow-rate of 15 ml/h.

DL-α-Phosphatidylcholine-β-oleoyl-γ-O-hexadecyl-AH Sepharose chromatography

A dialkylphosphatidylcholine column was prepared and utilized with slight modifications as described by Rock and Snyder [31]. Post-DEAE-cellulose fractions were separately loaded on to a 30 cm × 0.5 cm I.D. column which was pre-equilibrated with buffer containing 50 m*M* Tris–HCl, 1 m*M* EDTA, 200 m*M* NaCl and 25 m*M* $CaCl_2$. The samples were allowed to bind to the phospholipid for 48 h at 4°C. After the incubation period, the bound protein was washed with five column volumes of the same buffer and eluted with a buffer containing 50 m*M* Tris–HCl and 50 m*M* EDTA (pH 7.5). Fractions of 1 ml were collected at a flow-rate of 10 ml/h.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was run with 10% gels either by the method of Laemmli [32] or by the use of the Pharmacia PHAST system. In the latter instance, gradient gels with a concentration of acrylamide ranging from 10 to 15% were utilized. The gels were stained using silver nitrate [33] or Coomassie Brilliant Blue.

Two-dimensional gel electrophoresis

Two-dimensional PAGE was run for each of the isoenzymes according to the method of Rickwood and Hames [34]. In the first dimension, the samples were electrofocused in the pH range 3.5–10. In the second dimension, the samples were electrophoresed based on their size on a 12.5% SDS–polyacrylamide gel.

Renaturation of the PLA_2 isoenzymes

The phospholipase isoforms were renatured according to the method of Hager and Burgess [35] with slight modifications. The protein bands were revealed by soaking the gels in 0.25 m*M* KCl–2 m*M* DTT solution for 10 min at 4°C. The appropriate region of the gel was excised and the gel slices were soaked in 2 m*M* DTT solution for an additional 10 min. Elution buffer (0.8 ml of 200 m*M* Tris–HCl–5 m*M* EDTA–5 m*M* DTT–0.1% SDS–10% glycerol) was added to the gel slice and the gel was subsequently crushed with a glass stirring rod.

The suspension was incubated for 2 h at 25°C with occasional mixing and centrifuged for 3 min at 4°C in an Eppendorf centrifuge. The supernatant was collected. The SDS was removed from the protein extract by selective precipitation with acetone. The

precipitate was collected by centrifugation at 10 000 rpm for 10 min at 4°C. The pellet was resuspended in a solution containing 80% acetone, 40 m*M* Tris–HCl and 1 m*M* DTT (pH 7.5). The suspension was centrifuged at 10 000 rpm for 10 min and the resulting pellet was collected and dried under vacuum. The dried pellets were dissolved and incubated for 20 min in a solution containing 6 *M* guanidine–HCl, 200 m*M* Tris–HCl, 1.6 m*M* EDTA and 20% glycerol. The protein was then dialyzed overnight against a buffer containing 200 m*M* Tris–HCl and 20% glycerol at 4°C. The enzymatic activity of the sample was monitored using the radiolabeled assay as described above.

RESULTS

Gel permeation chromatography

Fig. 1A–D display the column profiles for the Sepharose 6B column. Fractions numbered 30–60 were pooled and dialyzed.

DEAE-cellulose chromatography

The column profile for the anion-exchange resin is shown in Fig. 2. Three major peaks were eluted at different KCl concentrations (peak I 350 m*M*, peak II 750 m*M* and peak III 900 m*M*). Each peak was separately pooled and dialyzed. The radiolabeled and fluorimetric assays were both utilized to measure the enzymatic assay.

DL-α-*Phosphatidylcholine-β-oleoyl-γ-O-hexadecyl-AH Sepharose chromatography*

The three major protein samples which were pooled from the DEAE-cellulose column were separately chromatographed on a dialkyl-AH Sepharose column. The column profiles for these three samples are shown in Figs. 3, 4 and 5, respectively.

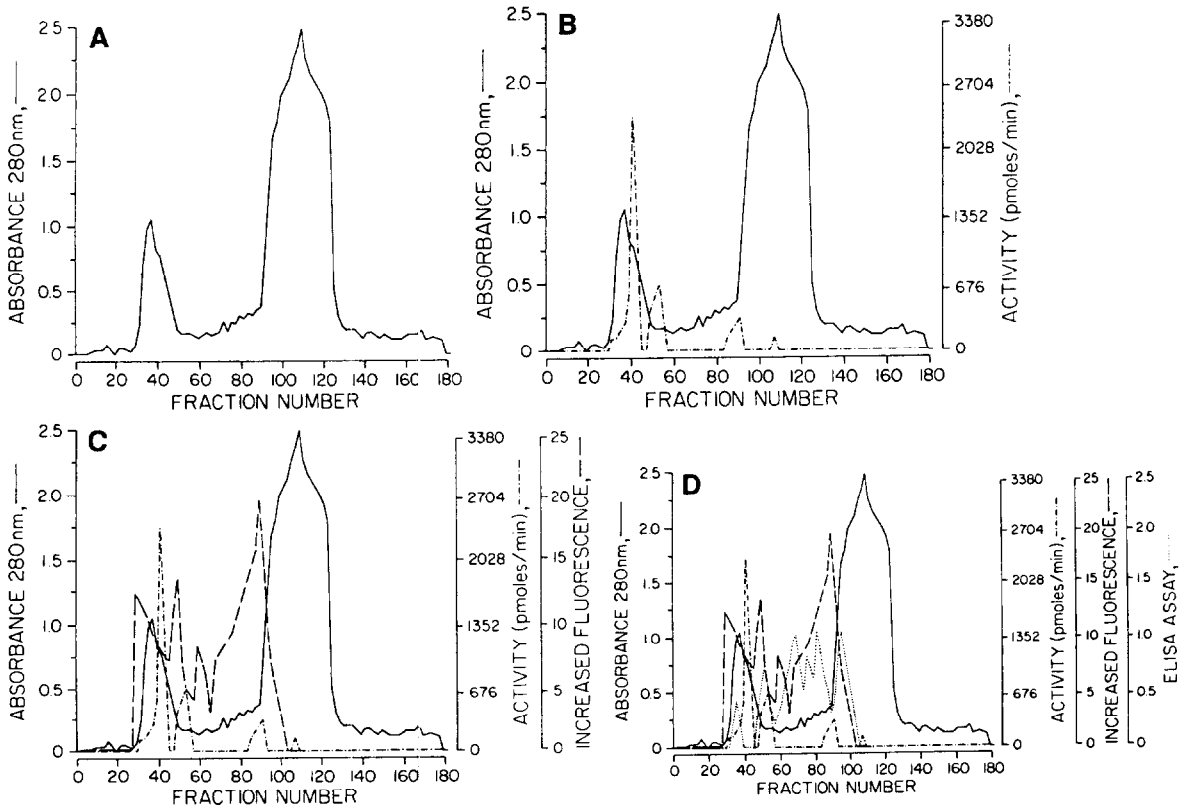

Fig. 1. Sepharose 6B chromatography. Column profiles of the placental homogenate chromatographed on Sepharose 6B resin; 3-ml fractions were collected. Fractions 30–50 were pooled. (A) Protein profile, absorbance at 280 nm. (B) Activity profile, radiolabeled assay. (C) Activity profile, fluorescence assay. (D) ELISA, assay profile.

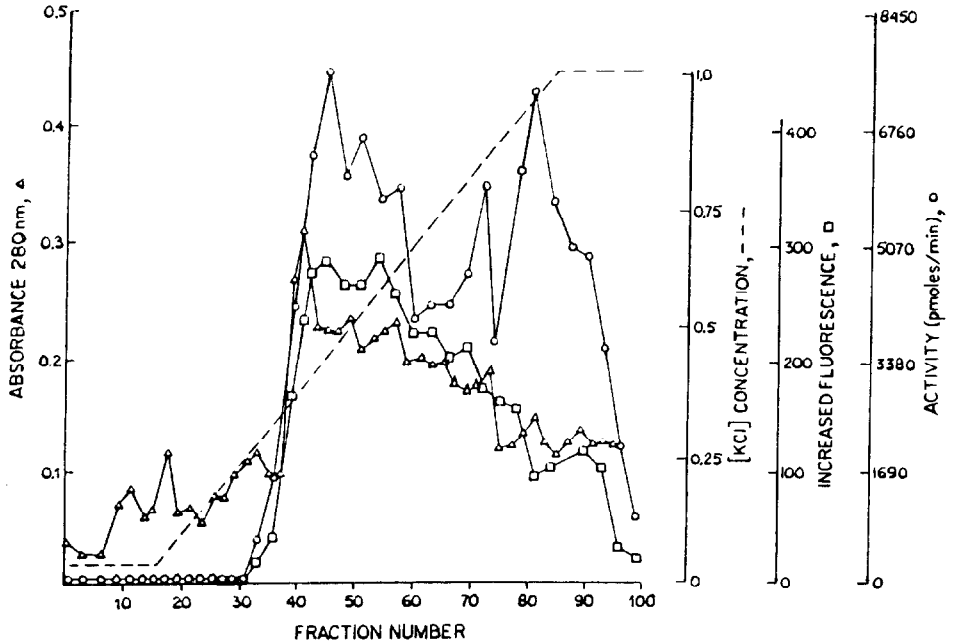

Fig. 2. DEAE-Cellulose chromatography. Elution profile of the post-Sepharose 6B fraction. Three different peaks of enzyme activity were eluted at 350, 750 and 900 m*M* concentration of KCl; 3-ml fractions were collected and screened by (○) the radiolabeled assay, (□) the fluorimetric assay and (△) the absorbance at 280 nm. Three fractions were separately pooled (I, II and III).

Peak I was chromatographed into two distinct PLA_2 species and corresponds to fractions 30–38 (Ia) and 42–48 (Ib) (Fig. 3). Peak II chromatographed into one major peak corresponding to fractions 32–38 (Fig. 4). Chromatography of peak III on this column separated two active protein samples corresponding to fractions 13–16 (IIIa) and 25–30 (IIIb) (Fig. 5).

The results of the purification scheme described are given in Table I.

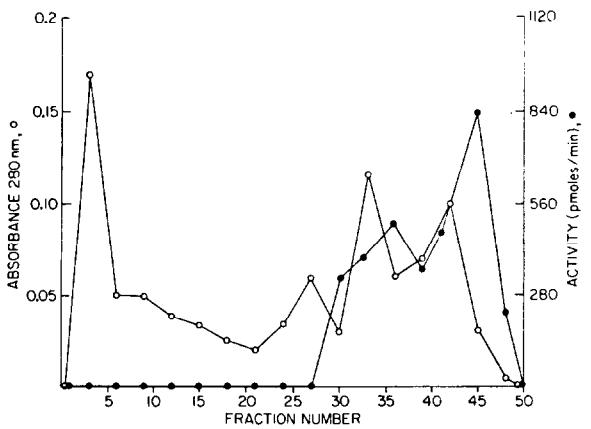

Fig. 3. Dialkylphosphatidylcholine chromatography. Elution profile for fraction I (post-DEAE-cellulose column); 1-ml fractions were collected. The column profile was constructed based on (○) the absorbance at 280 nm and (●) hydrolysis of the radiolabeled substrate. Two active protein samples, fractions 30–38 (Ia) and fractions 42–48 (Ib), were isolated and pooled.

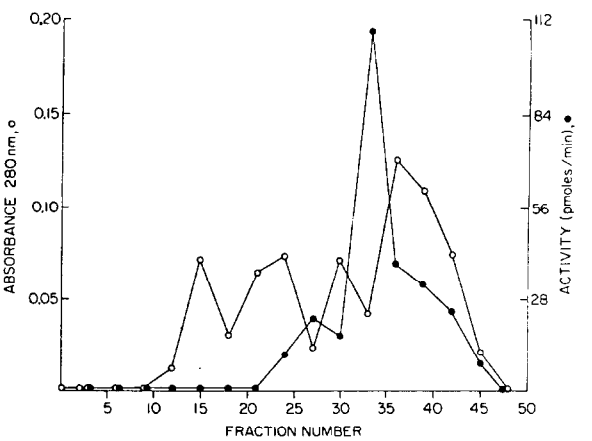

Fig. 4. Dialkylphosphatidylcholine chromatography. Elution profile from sample II from the DEAE-cellulose chromatographic step. The activity of the fractions was determined by the radiolabeled assay; 1-ml fractions were collected.

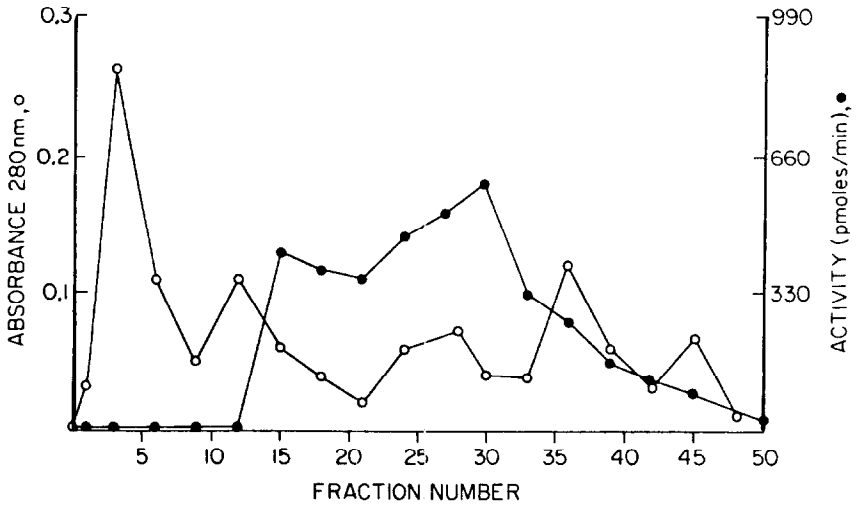

Fig. 5. Dialkylphosphatidylcholine chromatography. Elution profile for the sample III fractions (post-DEAE-cellulose); 1-ml fractions were collected. Two active protein samples were pooled based on the activity profile using the radiolabeled assay, fractions 13–18 (IIIa) and fractions 25–30 (IIIb).

Polyacrylamide gel electrophoresis

SDS-PAGE of the post-dialkyl-AH Sepharose PLA_2 active species displayed one major band with a relative molecular mass of 70 000 (Figs. 6 and 7).

Two-dimensional gel electrophoresis

Each of the isoforms migrated as a single band with a relative molecular mass of 70 000. The isoelectric points for the samples were determined as follows: Ia, p*I* 5.0; Ib, p*I* 6.0; II, p*I* 4.2; IIIa, p*I* 5.5; IIIb, p*I* 6.5 (Fig. 8).

Renaturation of PLA_2

The protein bands which were eluted from the SDS gel exhibited 70% of the initial activity (data not shown).

TABLE I

PURIFICATION PROFILE OF THE DIFFERENT ISOFORMS OF PLA_2

Step	Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)	Specific activity (pmol/min · mg)	Purification (-fold)	Recovery (%)
Starting material	44.6	24	1070	0.012	–	–
Post-Sepharose 6B	1.0	65	65	37	3083	6
Post-DEAE-cellulose:						
Peak I	0.37	60	22	142	11 833	2
Peak II	0.15	24	3.6	226	18 833	0.3
Peak III	0.14	60	8.4	207	17 250	0.65
Post-dialkylphosphatidylcholine chromatography:						
Peak Ia	0.27	5	1.35	239	19 916	0.13
Peak Ib	0.11	5	0.55	242	20 166	0.05
Peak IIIa	0.23	6	1.38	356	29 666	0.13
Peak IIIb	0.23	6	1.38	289	24 083	0.13

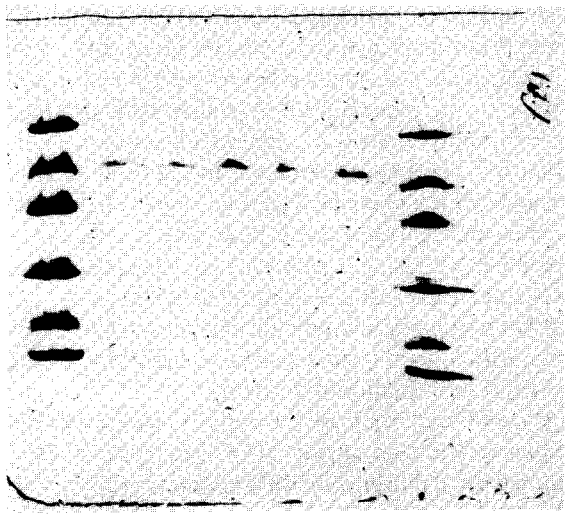

Fig. 6. SDS-PAGE. A 20-μg amount of each of the indicated protein fractions was electrophoresed on a 10% SDS reducing gel. One major band protein with a relative molecular mass of 70 000 was observed. Molecular mass standards with the following relative molecular masses: 94 000, 68 000, 30 000, 20 000 and 15 000.

Calcium profiles

Calcium profiles for the three types of PLA_2 are shown in Fig. 9. The calcium-independent PLA_2 has a maximum activity at 50 μM calcium (IIIa, IIIb). The calcium-dependent isoform has a maximum activity at 10 mM calcium (II). The optimum

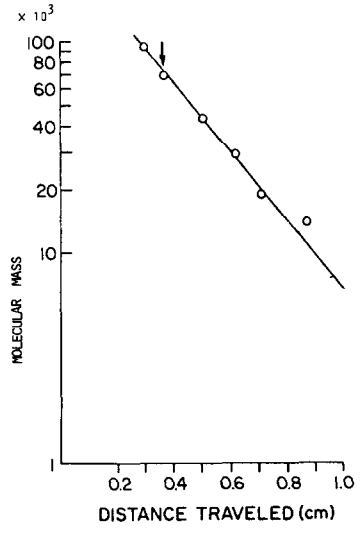

Fig. 7. Molecular mass profile. The arrow indicates the calculated molecular mass of the PLA_2 isoenzymes.

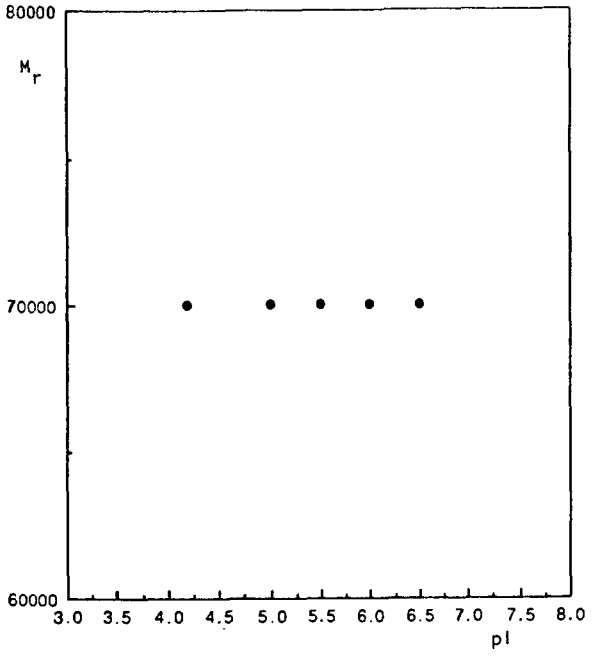

Fig. 8. Schematic representation of the two-dimensional gel electrophoresis pattern of the PLA_2 isoenzymes. M_r = Relative molecular mass.

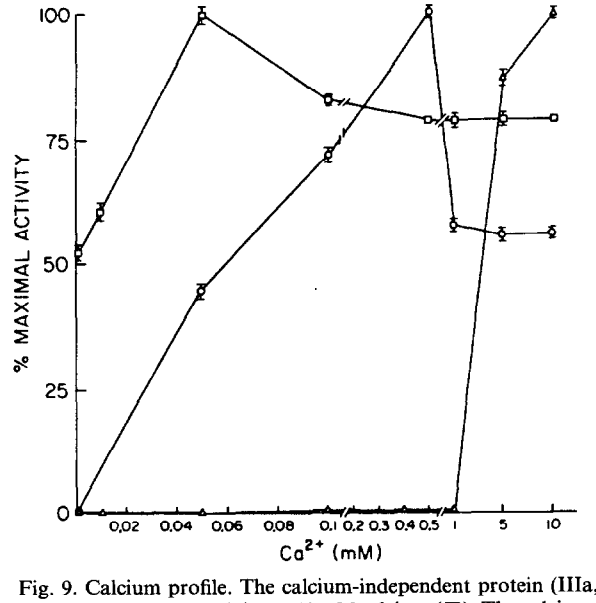

Fig. 9. Calcium profile. The calcium-independent protein (IIIa, IIIb) has maximum activity at 50 μM calcium (□). The calcium-dependent proteins have maximum activity at calcium concentrations of 500 μM (○, Ia, Ib) and 10 mM (△, II).

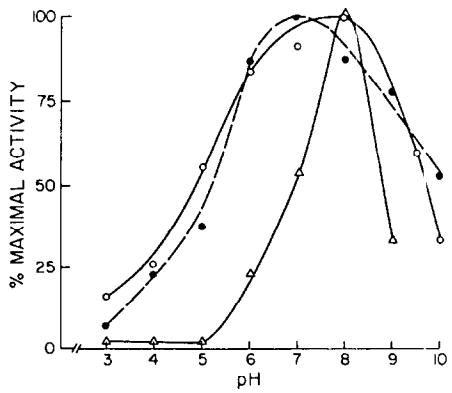

Fig. 10. pH profiles. The optimum pH values for the three different types of isoforms were determined to be 7 for calcium-independent (●) and 8 for the calcium dependent protein (△, fraction II; ○, fraction Ia).

calcium concentration for a second type of calcium-dependent PLA_2 is 500 μM (I). It should be noted that each subgroup of the protein samples identified as Ia, Ib and IIIa, IIIb gave similar calcium and pH profiles. Therefore, the figures represent the profiles for the five isoenzymes of type I, II and III, respectively.

pH profiles

The pH optima for the isoenzymes were determined as 7 and 8 for the calcium-independent and the calcium-dependent PLA_2s, respectively (Fig. 10).

DISCUSSION

We have been able to purify five isoforms of the human placental phospholipase A_2. Each of these isoforms has the ability to hydrolyze phospholipids at the *sn*-2-acyl position. These isoforms have the same relative molecular mass and distinctive p*I* values. Each subgroup of the isolated isoforms (Ia, Ib and IIIa, IIIb) have similar calcium and pH profiles. This cumulative evidence strongly suggests that these proteins are isoenzymes.

There are at least six different types of cells in the human placenta which may account for the amount of phospholipase that is found in the preparation. The question of the cell type from which these proteins have been isolated is currently under investigation.

These enzymes were purified 20 000–30 000-fold as calculated from the specific activities. It should be noted that this preparation was initiated with 145 g of whole placenta, which corresponded to *ca.* 1 g of protein in the placental homogenate as determined by the modified Lowry method of protein determination. There was a decrease in the total activity for some of the chromatographic steps. The reason for this apparent decrease is unknown, yet the same phenomenon has been observed for the membrane-associated PLA_2 isolated from the rat spleen [9].

Preliminary studies indicate that these isoenzymes are purified from the membrane fraction of the placental cells (data not shown).

A significant increase in activity was observed following the Sepharose 6B column stage. The removal of an inhibitor in this chromatographic step is not an unreasonable explanation as the isolation of several PLA_2 inhibitors from human placenta has been reported [33–36].

The problem of the high lysophospholipase and phospholipase A activities [20] has been circumvented by lyophilization of the placental homogenate prior to further purification. Lyophilization seems to retard the lysophospholipase activity. No effect is seen on the PLA_2 activity.

The hydrolysis of the phospholipid by these isoforms is specific for the *sn*-2-acyl position. PLA_2 was detected by both the ELISA and the fluorescence assays. Picomole amounts of hydrolyzed phospholipids per minute can be measured in the presence of serum albumin with the fluorescence assay [37].

The immunological recognition of the placental PLA_2 isoforms by the *Naja naja* enzyme proved to be beneficial for the localization of the phospholipases in the placental homogenate. There have been several other observations of interspecies cross-reactivity of this antibody with PLA_2s from various mammalian sources.

As shown in Fig. 1A–D, the fluorimetric and the ELISA assays gave positive results for other fractions (60–80). It is evident that there are other PLA_2s that are present in the placenta [23,24]. It may be that these PLA_2s have selective specificities for the phosphatidylcholine head group. Isoforms of the PLA_2 isolated from human platelets exhibited distinct substrate specificities [10,14] toward the phosphatidylcholine and phosphatidylethanolamine head groups.

Fig. 2 demonstrates there are three major PLA_2s that are eluted at different KCl concentrations. These fractions, I, II and III, were individually chromatographed on the dialkylphosphatidylcholine column.

SDS-PAGE (Fig. 6) exhibited one major band for each of these protein samples. The relative molecular mass of 70 000 was determined for all five isoforms as shown from the calibration graph (Fig. 7).

To answer the question of whether or not these enzymes were indeed PLA_2, renaturation experiments were conducted. The protein band was eluted from the gel and a 70% recovery of enzymatic activity was observed.

The five samples (Ia, Ib, II, IIIa, IIIb) all had different specific activities (Table I) and different p*I* values (Fig. 8). Samples Ia and Ib are both calcium dependent and have the same pH and calcium profiles. Samples IIIa and IIIb are both calcium independent and have the same pH and calcium profiles. Sample II has a requirement for calcium distinct from what is observed for samples Ia and Ib. Figs. 9 and 10 are the calcium and pH profiles which represent the three different types of PLA_2s isolated from human placenta.

The calcium dependences of these protein species show that the calcium-independent proteins (IIIa and IIIb) do not require calcium to hydrolyze the *sn*-2-acyl position of L-3-phosphatidylcholine-1-stearoyl-2-[^{14}C]arachidonyl. Fifty percent of the maximum activity is seen without calcium present, yet their maximum activity is observed in the presence of 50 μM of calcium. These proteins behave similarly to other mammalian PLA_2s recently purified [10,11] which also hydrolyze the *sn*-2-acyl position of phospholipids in the absence of calcium.

Sample Ia and Ib require calcium for activity. The maximum activity for these enzymes is observed at 500 μM calcium. Sample II behaves similarly to the porcine pancreas PLA_2 and no hydrolysis occurs until 5 mM calcium is present whereas maximum activity is seen in the presence of 10 mM calcium.

Interestingly, the purification of two distinct isoforms of PLA_2, each having different specific activities, one calcium dependent and the other calcium independent, from human platelets has been reported [10]. It is suggested that these proteins are purified in an aggregated form and have different specificities for phosphatidylcholine and phosphatidylethanolamine head groups [10].

The human placental calcium-independent isoforms have a pH optimum of 7 with a typical bell-shaped curve. The two types of calcium-dependent isoforms have a pH optimum of 8. However, the shapes of these curves are different. Sample I has residual activity at pH 3 which steadily increases at increasing pH until it reaches its optimum at pH 8. Sample II has no activity until pH 6. The differences in the pH optima for the different isoforms are consistent with the differences in calcium dependences.

The canine myocardial calcium-independent PLA_2 has a pH optimum of *ca.* 6 when plasminogencholine and phosphatidylcholine are used as substrates [11]. The human placental blood vessel PLA_2 has a pH optimum of 10 when phosphatidylcholine is used as substrate. The maximum activity for this enzyme is observed at 6 mM calcium and the enzymatic activity is inhibited by 50% at higher calcium concentrations [24]. The same phenomenon was also observed for the isoforms that we isolated. Samples IIIa and IIIb exhibited a 20% inhibition above a 50 μM calcium concentration, and samples Ia and Ib exhibited a 50% inhibition above 500 μM calcium. The enzymes that we have isolated have different calcium and pH requirements to other isolated placental PLA_2s. It would be interesting to compare the kinetic properties and head-group specificities of these various placental phospholipases.

At least five PLA_2 isoforms from the human placenta were found in this study. However, it is not known from what cell type(s) these proteins have been isolated. It would be interesting to determine the physiological role of the calcium-independent PLA_2.

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