Phospholipase A₂ from plasma of patients with septic shock is associated with high-density lipoproteins and C3 anaphylatoxin: some implications for its functional role

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Phospholipase A₂ (PLA₂) activity was purified 12 544-fold with a 13 % yield from the plasma of patients diagnosed of septic shock by the sequential use of heparin-agarose affinity chromatography, gel filtration, and reverse-phase f.p.l.c. Gel-filtration chromatography of plasma omitting high-ionic-strength buffer revealed a molecular mass different from that of purified PLA₂ and co-elution with apolipoprotein A-I peaks, which suggests its association with high-density lipoproteins (HDL). N-terminal analysis of the enzyme activity protein band, electroblotted from a SDS-acrylamide gel and with an assessed molecular mass of 19 kDa, showed an identical sequence to that of α -chain of human C3 complement component, suggesting the presence in this band of a complex formed by a complement C3-derived anaphylatoxin (C3a)-related fragment and the PLA₂ linked side-by-side. Because the preparation of plasma enzyme showed lower activity than the enzyme obtained from fibroblasts transfected with the coding sequence of human group-II PLA₂, and because the addition of C3-derived anaphylatoxins from human serum inhibited the activity of this recombinant PLA₂, it was

considered that C3a-related peptides behave as inhibitors of group-II PLA₂. The enzyme showed optimal activity on [¹⁴C]oleate-labelled autoclaved *E. coli*, on synthetic phosphatidylethanolamine, and on [³H]arachidonate-labelled membranes of the monoblast cell line U937, but it did not show any activity on the release of [³H]arachidonate from pre-labelled human polymorphonuclear leukocytes (PMNs). In short, PLA₂ from plasma of sepsis patients shows unique associations with other plasma proteins which may influence its functional properties. The association with C3-related peptides shows an inhibitory effect on the enzyme activity, whereas the association with HDL might influence its environment and/or its interaction with cells. The study of the catalytic properties shows a prominent effect on bacterial phospholipids, synthetic phosphatidylethanolamine, and membranes from U937 monoblasts, but not on synthetic phosphatidylcholine or on PMNs, even when these cells were maintained in culture to allow spontaneous apoptosis and became a good substrate for pancreatic type PLA₂.

INTRODUCTION

Phospholipases A₂ (phosphatide *sn*-2-acylhydrolases, PLA₂, EC 3.1.1.4) from mammalian tissues play a role in physiological functions such as defence mechanisms and the production of bioactive lipids [1,2]. Although these functions of PLA₂ seem relevant to the pathogenesis of various clinical conditions including rheumatoid arthritis and septicaemia [3–6], the pathobiology related to abnormal production of extracellular PLA₂ is not clearly defined. Within the past two years recombinant human group-II PLA₂ of 14 kDa molecular mass, identical to that found in platelets [7,8], placenta [9], synovial fluid [8], spleen [10] and plasma [11], has become available and has been employed to evaluate its extracellular function in different *in vitro* models. Unlike snake venom PLA₂, pure recombinant human group-II PLA₂ does not express inflammatory activity in normal rat tissues [12,13], although a role for this enzyme in the propagation of cell-mediated inflammatory processes has been proposed because of its ability to produce activation of T-lymphocytes [14] and degranulation of mast cells [15]. Current views stress the role of the 14 kDa group-II PLA₂ as an acute-

phase protein secreted from liver cells in response to inflammatory stimuli [16], and the recent cloning of a 1 kbp cDNA encoding a novel human low-molecular-mass PLA₂ [17] lends support to previous biochemical studies pointing to the existence of distinct PLA₂ genes in humans. On the other hand, the characterization of phospholipases from mononuclear phagocytes [18–21], kidney tissues [22], guinea-pig intestinal brush-border membranes [23] and, most recently, rabbit [24] and human [25] platelet cytosol has disclosed both functional and structural properties different from those of the above reported enzymes, being of remarkable importance higher molecular sizes and coupling to the release of arachidonic acid induced by hormones and growth factors.

The data presented in this paper indicate that the group-II PLA₂ from septic patients' plasma shows a unique pattern of interaction with plasma proteins: an association with high-density lipoproteins (HDL) that can be reversed by high concentrations of salt, and a more stable interaction with peptide chains with complete identity to the N-terminal portion of the α -chain of human C3. The association of PLA₂ with complement component C3-derived anaphylatoxin (C3a) seems to explain the inhibition of activity observed in the samples of enzyme purified

Abbreviations used: apo, apolipoprotein; C3a, complement component C3-derived anaphylatoxin; C3a des Arg, C3a lacking C-terminal arginine; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; HDL, high-density lipoproteins; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA₂, phospholipase A₂; PMN, polymorphonuclear leukocyte.

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from plasma compared with that purified from the culture media of fibroblasts stably transfected with the coding sequence of group-II PLA₂. Plasma PLA₂ shows activity on bacteria, synthetic phosphatidylethanolamine, and isolated plasma membrane fraction from [³H]arachidonate-labelled U937 monoblasts, but not on synthetic phosphatidylcholine or the phospholipids of [³H]arachidonate-labelled human polymorphonuclear leukocytes (PMNs). The different effects of PLA₂ on membranes from U937 monoblasts and PMNs points to cell-specific mechanism(s) for the interaction of the enzyme and its substrate in cell membranes.

EXPERIMENTAL

Patients and materials

Plasma from patients with sepsis was obtained from venous blood anticoagulated with heparin. Sepsis syndrome was defined according to the criteria of Bone et al. [26], and APACHE II score was used for disease severity classification [27]. [1-¹⁴C]Oleate (53.9 mCi/mmol), [9,10(*n*)-³H]palmitate (53.4 Ci/mmol), 1-stearoyl-2[5,6,8,9,11,12,14,15-³H]arachidonoyl-GPC (GPC, *sn*-glycero-3-phosphocholine) (132 Ci/mmol) and 1-acyl-2[1-¹⁴C]-arachidonoyl-GPE (GPE, *sn*-glycero-3-phosphoethanolamine) (54 mCi/mmol) were from Amersham International (Bucks, U.K.). 1-Palmitoyl-2[1-¹⁴C]oleoyl-GPC (52.6 mCi/mmol) and 1-hexadecyl-2[5,6,8,9,11,12,14,15-³H]arachidonoyl-GPC (74 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). Essentially fatty-acid-free BSA was from Miles Laboratories. Reagent for the measurement of proteins according to the method of Bradford [28] was purchased from Bio-Rad Labs (Richmond, CA, U.S.A.). Antibodies for the assay of plasma apolipoprotein (apo) B and A-I were from Behring Diagnostics (La Jolla, CA, U.S.A.), and were used for radial immunodiffusion in agarose plates at a concentration of 1 µl of antiserum/mm² of plate. Heparin-agarose type I, protein A insolubilized on Sepharose CL-4B, and porcine pancreatic PLA₂ were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

A C127 mouse fibroblast line stably transfected with the coding sequence of group-II PLA₂ from human placenta [29] was used as a source of human recombinant group-II PLA₂. Plasma membranes from U937 cells were a kind gift from Dr. E. Diez, of SmithKline Beecham Pharmaceuticals. These cells were labelled with [³H]arachidonate for times long enough to allow a substantial incorporation of the label into the phosphatidylethanolamine (PE) pool [30]. The membrane fraction was obtained by ultracentrifugation of cells broken by nitrogen cavitation. Before use the fraction was heated for 20 min at 56 °C to inactivate membrane-associated acyltransferases.

Assays of PLA₂ activity

The assay was routinely carried out with either 0.5 µl of patients' plasma, 20 µl of the chromatography fractions, or the appropriate volume of concentrated pooled fractions in a total volume of 0.1 ml, according to the procedure of Elsbach et al. [31]. Samples were incubated with ~ 5000 d.p.m. of [¹⁴C]oleate-labelled autoclaved *Escherichia coli* of a K12 strain, containing 10–20 nmol of phospholipid, as assessed by the measurement of phospholipid-associated phosphate. The assay medium contained 0.1 M Tris/HCl, 1 mg/ml fatty-acid-free BSA and 0.5 mM CaCl₂, pH 7.4. The reaction proceeded for 30 min and was stopped by addition of 0.04 ml of ice-cold 2 M HCl and 0.02 ml of 10% (w/v) BSA, followed by centrifugation for 5 min at 10000 g in an Eppendorf microcentrifuge. The radioactivity released into the supernatant was assayed by liquid scintillation counting. Studies directed to assess positional specificity of the

phospholipase activity were carried out with [¹⁴C]palmitate-labelled autoclaved *E. coli*, as well as with synthetic phospholipid. Mixed lamellar substrate was made with unlabelled 1-stearoyl-2-arachidonoyl-GPC and 1,2-dioleoyl-GPE at a molar ratio of 1.0, and 50000 d.p.m. of the corresponding labeled substrate. The mixture was sonicated three times for 15 s with a tip-sonifier, and distributed in 0.1 M Tris/HCl, pH 7.4, containing 0.5 mM CaCl₂, and 1 mg/ml BSA. The reaction was initiated by the addition of enzyme and allowed to proceed for 30 min at 37 °C, until it was stopped with 0.36 ml of chloroform/methanol (1:2, v/v). The reaction products were extracted by the procedure of Bligh and Dyer [32] with 3 µg of unlabelled fatty acid as a carrier, and then separated by t.l.c. on silica G plates in the system hexane/diethyl ether/acetic acid (80:20:1, by vol.). Studies with [³H]arachidonate-labelled U937 cell membranes were carried out with 11 nmol of phospholipid substrate (~ 500 d.p.m./nmol), under analogous conditions to those described for synthetic substrate, but the t.l.c. separation of lipids was carried out by developing first in the system chloroform/methanol/acetic acid/water (50:25:8:6, by vol.) until the solvent front reached two thirds up the plate, drying, and then developing in a second system, hexane/diethylether/acetic acid (80:20:1, by vol.), to assess the extent of hydrolysis on each class of labelled phospholipid [33].

Purification of PLA₂ from human plasma

Heparin-agarose, which possesses affinity for group-II PLA₂ [24,25], was used to bind PLA₂ from patients' plasma. Fractions showing PLA₂ activity in the [³H]oleate-labelled *E. coli* assay were concentrated in an Amicon ultrafiltration cell fitted with a YM 5 membrane and loaded into a HiLoad Superdex 75 column (Pharmacia-LKB, Uppsala, Sweden) equilibrated in a medium containing 50 mM Tris/HCl, 0.4 M NaCl, pH 7.4, and eluted at a flow rate of 1 ml/min. Fractions containing PLA₂ activity in the Superdex 75 column were made in 0.1% (v/v) trifluoroacetic acid, and applied into a C₁₈/C₈ reverse-phase f.p.l.c. column (ProRPC HR 5/2, Pharmacia-LKB). The column was eluted at 1 ml/min with a 40 min gradient [0–50% (v/v) acetonitrile in 0.1% trifluoroacetic acid]. Fractions showing PLA₂ activity were pooled and evaporated to dryness in a Speed-Vac concentrator. A batch of plasma was exposed to sulphuric acid extraction [7,8] before the aforementioned purification procedure to assess the effect of this treatment on the removal of possible inhibitors of PLA₂.

Purification of human recombinant group-II PLA₂ and preparation of a rabbit polyclonal antibody

Supernatants from cultures at superconfluence of line C127 mouse fibroblasts stably transfected with the coding sequence of group-II PLA₂ from human placenta [29] were used as a source of human recombinant group-II PLA₂. To facilitate the purification of the secreted enzyme, the concentration of heat-inactivated fetal calf serum was reduced to 5% and supernatants were collected every 48 h. The purification protocol used to obtain a preparation suitable for the assay of enzyme activity was similar to that applied for the plasma PLA₂.

When the purpose of the experiment was to obtain a homogeneous preparation that could serve for both unambiguous characterization of its molecular mass and to produce a rabbit polyclonal antibody, an additional chromatography was performed using a C₁₈ reverse-phase h.p.l.c. column (Nova-Pak 15 cm × 3.9 mm, Waters), which was operated at room temperature at a flow rate of 0.5 ml/min with an 80 min gradient using the acetonitrile-based mobile phase described above. This

yielded a single protein band on SDS/PAGE with the N-terminal amino acid sequence of group-II human PLA₂. The portion of gel containing the Coomassie-stained protein band was removed, crushed, mixed with 80 µl of complete Freund's adjuvant, and used for injection into the popliteal lymph node. The rabbit was boosted 4 weeks later by intradermal injection and bled 8 days later to obtain anti-(group-II PLA₂) antibody. The immunoglobulin fraction of the serum was purified by sequential precipitation with octanoic acid and ammonium sulphate.

SDS/PAGE and determination of N-terminal sequences

SDS/PAGE was carried out in a Phast Gel system (Pharmacia-LKB) using 20% (w/v) homogeneous polyacrylamide gels [34]. Standards for molecular-mass estimation were obtained from Bio-Rad, and proteins were visualized by silver staining. Addition of 5% (v/v) 2-mercaptoethanol and boiling were omitted when the purpose of the experiment was the identification of the PLA₂ activity in the protein bands, which was carried out by enzyme assay of the eluted protein. Electrophoretic transfer to poly(vinylidene difluoride) protein-transfer membranes (Immobilon-P, Millipore, Bedford, MA, U.S.A.) was carried out by using a commercially available method (Pharmacia-LKB). Coomassie-Blue-stained bands were excised and used for N-terminal sequencing by automated Edman degradation.

Amino acid sequence analysis was performed using a Knauer modular liquid-phase protein sequencer model 810 equipped with a Knauer phenylthiohydantoin amino acid derivative analyser. Samples were analysed in the presence of polybrene employing the wet-filter technique in a flow-through reactor. The phenylthiohydantoin amino acid derivatives were identified and quantified using a reverse-phase h.p.l.c. system based upon a Lichrospher RP 18 column (0.2 cm × 25 cm) eluted with 6.5 mM sodium acetate, pH 4.77, and a 15% (v/v) acetonitrile gradient.

Preparation of C3a des Arg from human serum

C3-derived anaphylatoxins were obtained by inducing the generation of C3a in the absence of carboxypeptidase B inhibitors, which allows its conversion into C3a lacking C-terminal arginine (C3a des Arg) [35]. Fresh human serum was incubated with zymosan particles at the concentration of 10 mg/ml at 37 °C for 30 min. Zymosan particles were removed by centrifugation and the activated serum was loaded into a HiLoad Superdex 75 column equilibrated in a medium containing 50 mM Tris/HCl and 0.1 M NaCl, pH 7.4, and eluted at a flow rate of 1 ml/min. Fractions were checked for both PLA₂ activity and ability to inhibit recombinant group-II PLA₂.

Labelling of human PMNs with [³H]arachidonate

PMNs were obtained from peripheral blood of normal volunteers anticoagulated with EDTA. Red cells were removed by Dextran T-500 (Pharmacia-LKB) sedimentation, and PMNs were separated from mononuclear cells by centrifugation on Ficoll-Hypaque cushions. Isolated PMNs [(1–2) × 10⁶ cells/ml] were labelled for 60 min at 37 °C with 1 µCi/ml [³H]arachidonate in a Hepes-buffered medium, pH 7.4, containing 0.25% essentially fatty-acid-free BSA, in the absence of Ca²⁺ ions, according to the procedure described in [36]. At the end of this period, cells were washed twice, resuspended at the concentration of 5 × 10⁶ cells/ml and stimulated with secretagogues or purified PLA₂. Fifteen minutes after addition of the stimuli, or 30 min after addition of the enzyme, the reaction was stopped by Bligh and Dyer extraction [32]. Separation of non-esterified [³H]arachidonate

from 1-radyl-2-[³H]arachidonoyl-GPC was carried out by t.l.c. in the system hexane/diethylether/acetic acid (80:20:1, by vol.). [³H]Arachidonate-labelled plasma membranes from PMNs were obtained following a protocol analogous to that used in the case of U937 cells, except that the period of labelling with [³H]arachidonate was 60 min. Under these conditions, labelling predominantly occurs in choline-containing glycerophospholipids [30].

RESULTS

PLA₂ from patients' plasma is associated with HDL and fragments from complement C3 component

Initial strategies of purification were based on the utilization of gel-filtration chromatography in view of the low molecular mass of most PLA₂s, which allows a good separation of these polypeptides from plasma proteins [37]. Other criteria were the use of samples from the same patient until the purification procedure was found to give reliable results, and omission of drastic treatments such as sulphuric-acid extraction which might influence the environment of the enzyme, and has been shown to inactivate high-molecular-mass PLA₂ [38]. Table 1 summarizes clinical data of the group of patients used to carry out the study. Initial attempts to use gel-permeation chromatography techniques were carried out with an analytical Superose 12 HR 10/30 column. We found a changing pattern of elution of the enzyme activity depending on the molarity of the buffer. In fact, when the column was eluted with buffer containing 0.4 M NaCl, PLA₂ eluted as a single peak, with a molecular mass smaller than that of serum albumin (Fig. 1a). In contrast, when the chromatography was carried out in a medium containing 0.10–0.25 M NaCl, enzyme activity eluted with the peak of protein being excluded. This finding prompted us to use supplementary gel-permeation chromatographic conditions before drawing conclusions on the apparent molecular mass of PLA₂. As shown in Fig. 1(b), PLA₂ from patients' plasma eluted with a molecular mass > 160 kDa when chromatographed in a Superose 6 HR 10/30 column in a medium containing 0.1 M NaCl. PLA₂ co-eluted with two apo A-I peaks, but no activity was found associated with either of the apo B peaks. This suggests an association with some particles of HDL of plasma PLA₂ [39] but not with very-low-density lipoprotein or low-density lipoprotein.

The final schedule of purification (Table 2) took advantage of the affinity of group-II PLA₂s for heparin-agarose. Thus, all of the activity was found to bind strongly to heparin-agarose, and was rapidly recovered upon elution of the column with 1 M

Table 1 Summary of clinical data of septic patients and control individuals

Platelet count is expressed as platelets/µl. PLA₂ activity is expressed as nmol of oleate/30 min per mg of protein. The group of septic patients with fungal micro-organisms includes two patients with a previous diagnosis of aplastic anaemia. Results are given as means ± S.E.M. (*n* = 3–16).

Microorganism	APACHE II score	Platelet count	PLA ₂ specific activity	<i>n</i>
None	—	246 890 ± 14 345	0.4 ± 0	16
Fungi	23 ± 2.96	36 333 ± 15 387	20 ± 2	3
Combination*	29 ± 1.98	94 625 ± 34 386	16 ± 3	8
Gram-positive	25 ± 2.80	127 000 ± 31 391	14 ± 2	5
Gram-negative	24 ± 2.28	122 600 ± 30 135	15 ± 2	5

* Both Gram-positive and Gram-negative bacteria.

Figure 1 Elution profile of PLA₂ from plasma on Superose 12 HR 10/30 (a) and on Superose 6 HR 10/30 (b) f.p.l.c. gel-filtration columns

(a) Plasma (~200 µl) was applied to the Superose 12 column and eluted with 50 mM Tris/HCl, pH 7.4, containing 0.4 M NaCl. (b) For Superose 6 column chromatography, 400 µl of plasma was loaded on top of the column and eluted in 50 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl. Apo B (hatched bars) and apo A-I (open bars) were estimated in the different fractions by radial immunodiffusion, and are expressed as the distance in millimetres that separates the centre of the wells from the precipitation line. PLA₂ activity (○) was measured using the *E. coli* assay in 20 µl aliquots from all of the fractions. The solid line indicates on-line A₂₈₀. Numbers at the top of the Figures indicate the molecular masses of molecular-mass markers.

NaCl. The pooled fractions containing PLA₂ activity were concentrated and loaded into a HiLoad Superdex 75 column. The enzyme activity eluted as a wide peak over a range of apparent molecular masses between 40 and 15 kDa, well separated from protein u.v.-absorbing peaks. This observation, together with the aforementioned patterns of elution in gel-filtration chromatography, were considered to be evidence of an interaction of plasma PLA₂ with a well-defined fraction of plasma lipoproteins that could be reversed by high ionic strength. Fractions with PLA₂ activity in the Superdex 75 column were made in 0.1% trifluoroacetic acid, and applied to a C₁/C₈ reverse-phase f.p.l.c. column. All of the enzyme activity eluted at 25–30% acetonitrile in a single peak (Figure 2). Direct sequencing of the fraction containing PLA₂ activity allowed us to determine unambiguously the following first 30 amino acid residues: SVQLTEKRMDKVGKYPKELRKCCEDGMREN. This N-

Figure 2 Reverse-phase f.p.l.c. chromatogram of plasma PLA₂ after Superdex 75 column

The ProRPC HR 5/2 column was eluted at 1 ml/min with a 40 min gradient (0–50% acetonitrile in 0.1% trifluoroacetic acid), as indicated by the dashed line. See the legend to Figure 1 for further details.

terminal sequence was identical to the α-chain of human C3 α-chain [40]. On the other hand, analysis by SDS/PAGE of the corresponding PLA₂ activity fraction showed two prominent protein bands of 12 and 19 kDa (results not shown). Attempts to assign the enzyme activity to any of these bands were carried out by assaying the activity in eluates of fractions excised from gel slabs, in the absence of 2-mercaptoethanol and boiling. The pattern of protein bands under these conditions was almost identical to that observed with boiled and reduced samples. PLA₂ activity was only associated with the 19 kDa band (Figure 3). The N-terminal amino acid sequence of the active protein band was SVQLTEKRMD, which is identical to the reported α-chain of human C3 complement component and also to the other 12 kDa protein band. The relative yields in each degradation cycle of both protein bands were nearly the same.

These findings could be consistent with several hypotheses, but taking into account the sequence of the PLA₂ from septic shock patients [11] and its molecular mass, the most likely interpretations suggest either N-terminal blockade of the plasma PLA₂ or small amounts of protein strongly associated with C3a-related peptides. Because attempts to further separate PLA₂ activity from an associated C3a-related contamination using ion-exchange, hydroxylapatite-based chromatography and analytical gel filtration were unsuccessful, the existence in the 19 kDa activity band of a complex formed by a C3a-related fragment and the PLA₂ linked side-by-side, which could explain the unique N-terminal sequence obtained in such a protein gel band, can not be discarded (see below).

Table 2 Purification of patients' plasma PLA₂

PLA₂ activity was assayed using [¹⁴C]oleate-labelled *E. coli*.

Step	Protein (mg)	PLA ₂ total activity (nmol/30 min)	PLA ₂ specific activity (nmol/30 min per mg of protein)	Recovery (%)	Purification (-fold)
Plasma	7620	163 388	21	100	1
Heparin-agarose	155	158 630	1023	97	49
Superdex 75 HiLoad	1.3	63 420	48 784	39	2323
ProRPC HR 5/2	0.08	21 074	263 428	13	12 544

Figure 3 SDS/PAGE electrophoresis of both plasma and recombinant PLA₂ at the end of the purification procedure

A 4 μ l sample of the pooled active fractions of plasma PLA₂ (lane A) was made in 2.5% (w/v) SDS in the absence of both 2-mercaptoethanol and boiling, and run in a 20% (w/v) homogeneous Phast system gel. Protein bands were visualized by silver staining. Numbers on the abscissa indicate the molecular mass (kDa) of the markers. A lane with the same content as lane A was used for the assay of PLA₂ activity (○). For this purpose, gel fractions (1 mm long) were excised, mechanically disrupted and eluted overnight in 100 μ l distilled water, before PLA₂ assay on [¹⁴C]oleate-labelled *E. coli*. To avoid a possible interference of SDS on PLA₂ assay as a result of solubilization of bacterial phospholipids, the release of [¹⁴C]oleate was monitored after solvent extraction and t.l.c. of the supernatants obtained in the [¹⁴C]oleate-labelled *E. coli* assay. A gel analogous to that shown was electroblotted and used for the determination of the N-terminal sequences. Lane B shows the recombinant secreted PLA₂ after Nova-Pak C₁₈ chromatography. The sample was treated with 5% (v/v) 2-mercaptoethanol and boiling and processed as in lane A (except that the gel was stained with Coomassie Blue), and the protein band was used for immunization after confirmation that the N-terminal amino sequence was homologous to that of human group-II PLA₂. Lane C shows Bio-Rad high- and low-molecular-mass markers stained with Coomassie Blue.

Figure 4 Hydrolysis of [¹⁴C]oleate-labelled *E. coli* by various PLA₂ samples

The samples used were: patients' plasma PLA₂ (○), patients' plasma PLA₂ obtained after sulphuric-acid extraction (●), recombinant human PLA₂ (■), and porcine pancreatic PLA₂ (△). The assay was carried out in a 100 μ l volume as described in the Experimental section in the presence of different amounts of enzymes. The extent of hydrolysis is expressed as percentage of total label. Data represent means of a typical experiment carried out with duplicate samples.

The enzyme activity obtained from cultures of C127 mouse fibroblasts transfected with the coding sequence of group-II PLA₂ [29] showed a behaviour similar to that of the plasma enzyme in these chromatographic steps, except that ~20% of the enzyme activity eluted with the protein bulk at a higher

Figure 5 Inhibition of PLA₂ activity by the fraction containing C3 des Arg from zymosan-activated human serum

Fractions eluting with a molecular mass of ~9 kDa in HiLoad Superdex 75 column chromatography were concentrated and incubated with 20 ng of recombinant PLA₂ for 1 h before the addition of [¹⁴C]oleate-labelled *E. coli* substrate. Controls with pooled fractions eluting with a molecular mass of >60 or <5 kDa showed no inhibitory activity. Data represent means $\pm$ S.E.M. of three independent experiments in duplicate.

molecular mass in the gel-filtration chromatography step, even though the elution medium contained a high-salt concentration. SDS/PAGE analysis of the recombinant secreted PLA₂ after Nova-Pak C₁₈ chromatography (Figure 3, lane B) showed a unique protein band with a molecular mass analogous to that assigned to the enzyme activity obtained from human plasma, and an N-terminal amino acid sequence identical to that of human group-II PLA₂.

Biochemical characterization of plasma PLA₂ and inhibition by C3a

Hydrolysis of [³H]oleate-labelled *E. coli* by plasma-purified PLA₂ showed a linear pattern up to 40 ng of protein (Figure 4). Recombinant purified PLA₂ and pancreatic PLA₂ showed higher activities under these conditions. Unlike plasma PLA₂, they hydrolysed up to 90% of total phospholipid (Figure 4). As this finding suggested the presence of an inhibitor in the samples of plasma PLA₂, and this preparation contained fragments of C3 α -chain, the inhibitory effect was attributed to this peptide(s). This possibility was tested by looking for PLA₂ inhibitory activity in serum activated by zymosan. The fraction corresponding to ~9 kDa obtained by gel-filtration chromatography of zymosan-activated human serum induced a dose-dependent inhibition of the recombinant enzyme (Figure 5), whereas no inhibitory effect could be detected in any other fraction. As the size of this fraction corresponds with that of C3a des Arg, this result is in keeping with the inhibitory effect of C3 α -chain related products on group-II PLA₂. The possibility that sulphuric-acid extraction could produce the removal of inhibitory peptides for PLA₂ activity in plasma was tested in a sample exposed to this treatment before the standard purification protocol. As shown in Figure 4, ~80% of the substrate could then be hydrolysed.

PLA₂ from plasma showed a preference for substrate presented as [¹⁴C]oleate-labelled autoclaved *E. coli* compared with [¹⁴C]palmitate-labelled *E. coli* (Table 3). This can be explained by the preferential incorporation of oleate at the *sn*-2 position of phospholipid, as the percentage release of palmitate seems similar to the percentage incorporation of this fatty acid at the *sn*-2 position [5]. A similar preference could be demonstrated for pure PE substrate compared with phosphatidylcholine (PC) (Table 3). This is in keeping with the results obtained in the *E. coli* assay, as bacteria contain abundant amounts of PE and PC is not a

Table 3 Specificity of patients' plasma PLA₂

The reaction was performed as described in the Experimental section using 40 ng of purified plasma PLA₂ and 12 nmol of phospholipid in the form of either autoclaved *E. coli*, pure PE substrate*, or mixed PE/PC substrate§. Results are expressed as both percentage of substrate and amount of phospholipid hydrolysed, and represent means of two experiments with duplicate samples.

Substrate	Hydrolysis of phospholipid	
	(%)	(nmol/30 min per mg of protein)
[¹⁴ C]palmitate-labelled <i>E. coli</i>	11.65	34938
[¹⁴ C]oleate-labelled <i>E. coli</i>	50.60	151750
1-Acyl-2-[¹⁴ C]arachidonoyl-GPE*	38.10	114262
1-Palmitoyl-2-[¹⁴ C]oleoyl-GPC§	2.75	8247
1-Stearoyl-2-[³ H]arachidonoyl-GPC§	2.75	8247
1-Hexadecyl-2-[³ H]arachidonoyl-GPC§	2.70	8097
1-Acyl-2-[¹⁴ C]arachidonoyl-GPE§	4.20	12595

Table 4 Effect of anti-(group II PLA₂) antibody on PLA₂ activity

PLA₂ (~10 ng) was incubated in a 200 µl volume with the amounts of anti-(group II PLA₂) antibody indicated for 1 h at 37 °C. At the end of this period 10 µg of Protein A insolubilized on Sepharose CL-4B was added and the incubation maintained for an additional 30 min period. PLA₂ activity was measured in the supernatants using [¹⁴C]oleate-labelled *E. coli*. A rabbit anti-ovalbumin antiserum containing 15 µg of protein used as control for non-specific trapping produced <10% variation of the enzyme activity detected in the absence of antibody.

PLA ₂ source	Amount of anti-(group II PLA ₂ antibody) (µg)	[¹⁴ C]Oleate released (% of total)
Patients' plasma	0	34
	1	30
	5	17
	10	11
Group II recombinant	0	63
	5	29
	10	8

component of bacterial membranes. Additional experiments directed to assess PLA₂ selectivity towards the fatty acid at the *sn*-2 position were carried out on mixed substrate at a PC/PE ratio of 1.0, which is consistent with lamellar structure [41], to avoid a possible bias on the appraisal of specificity caused by the effect of the polar head on the physical conformation of vesicles [42,43]. As shown in Table 3, a preference for arachidonoyl-containing molecular species or for alkyl-ether-linked GPC was not observed. Plasma PLA₂ showed optimal activity at micromolar Ca²⁺ concentration as well as at physiological pH (results not shown).

Effect of anti-(group II PLA₂) antibody on PLA₂ activity from patients' plasma

Both patients' plasma PLA₂ and recombinant PLA₂ were incubated for 1 h at 37 °C with different amounts of the antiserum raised against recombinant PLA₂. At the end of this period, protein A insolubilized on Sepharose CL-4B was added to the medium. The mixture was centrifuged at 10000 *g* in an Eppendorf microcentrifuge and the supernatants used for the assay of the

Figure 6 Hydrolysis of [³H]arachidonate-labelled membranes from U937 monoblasts: dose-dependence (a) and time-course (b)

The experimental conditions were analogous to those used in Figure 4, except that t.l.c. was carried out at the end of the incubation period to separate free fatty acid from phospholipids. The samples used were: patients' plasma PLA₂ (○), recombinant human PLA₂ (■), and porcine pancreatic PLA₂ (△).

remaining PLA₂ activity. As shown in Table 4, PLA₂ activity was significantly removed by the polyclonal rabbit antibody.

Effect of purified PLA₂ on [³H]arachidonate-labelled plasma membranes from U937 cells

In keeping with the results obtained in the *E. coli* assay, saturation occurred at different extents of hydrolysis for each enzyme (Figure 6a). It was considered again that the association of C3 α -chain fragments with the plasma enzyme could account for the differences in activity. Figure 6(b) shows the time course of hydrolysis of [³H]arachidonate from membranes of U937 cells. Analysis of the extent of hydrolysis of the different phospholipid classes showed hydrolysis of 19 ± 4% (mean ± S.E.M., *n* = 3) of the [³H]arachidonate associated with PE by the plasma enzyme, and a slightly smaller activity on PC (12 ± 2%), whereas the rate of hydrolysis of either phospholipid class with the recombinant enzyme was 45 ± 7% on PC and 37 ± 6% on PE (*n* = 3). This result in combination with the aforementioned data on synthetic substrates indicates that the preferential action of group-II PLA₂ on different phospholipid classes could be explained by structural features influencing enzyme-substrate interaction, rather than by selectivity of the enzyme for either the polar head or the fatty acid substituting at the *sn*-2 position.

Effect of purified PLA₂ on [³H]arachidonate-labelled PMNs

Purified PLA₂ from either plasma or transfected fibroblasts did not induce the release of [³H]arachidonate from labelled PMNs

Table 5 Effect of patients' plasma PLA₂ on [³H]arachidonate release from prelabelled PMNs

PMNs were labelled with [³H]arachidonate as described and incubated for 15 min in the presence of fMLP + cytochalasin B and ionophore, and 30 min in the presence of PLA₂. The reaction was stopped by Bligh and Dyer extraction, and non-esterified arachidonate was separated from phospholipids by t.l.c. on the system hexane/diethyl ether/acetic acid (60:40:1, v/v). Results represent a typical experiment out of three with identical results.

Addition	Concentration	[³ H]Arachidonate (c.p.m.)
None	—	310
fMLP + cytochalasin B	1 μ M + 5 μ g/ml	1329
Ionophore A23187	5 μ M	1456
Patients' purified PLA ₂	1 μ g/ml	283
Group II recombinant PLA ₂	1 μ g/ml	301

Figure 7 Time in culture of [³H]arachidonate-labelled PMNs and hydrolysis by PLA₂

Isolated PMNs were maintained in culture for the times indicated, and then incubated with 1 μ g of recombinant PLA₂ (■), pancreatic group I PLA₂ (△) or medium (◇) for 30 min. At the end of this period lipids were extracted and separated by t.l.c. Closed circles (●) indicate the percentage of PMNs taking up Trypan Blue. Data represent means $\pm$ S.E.M. of four experiments in duplicate.

under conditions where both ionophore A23187 and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) did so (Table 5). Similar experiments performed on [¹⁴C]oleate-labelled PMNs also showed the lack of fatty acid release. Because it has been reported that the normal location of phosphatidylserine on the inner leaflet of the plasma membrane is lost during apoptotic death of PMNs [44–46], and because this may lead to an enhanced expression of anionic phospholipid to the outer leaflet, with possible consequences for the interaction of the enzyme with the lipid membranes, PMNs were maintained at 37 °C for different periods of time, in order to allow for the appearance of spontaneous apoptosis and expression of phosphatidylserine on the outer layer. Under these conditions only group-I PLA₂ provoked release of free fatty acid above the control level (Figure 7). When the experiment was carried out using isolated plasma membranes from PMNs, again only the pancreatic PLA₂ displayed hydrolysis of the phospholipid ($44 \pm 4\%$ on PC and $40 \pm 3\%$ on PE, $n = 3$). Taken together, these data indicate a poor capacity for group II PLA₂ to hydrolyse phospholipids on PMN membranes, whereas group-I PLA₂ showed this ability

both in populations of PMNs which contained a high number of apoptotic PMNs and on PMN isolated membranes.

DISCUSSION

This paper provides new results on PLA₂ activity in plasma of patients with sepsis syndrome regarding association with proteins which could influence its catalytic properties and its functional role. Our data indicate that the plasma enzyme exists in a high-molecular-mass complex which elutes under gel-permeation chromatography within a volume that is characteristic of the molecular mass of some particles of HDL [39]. This association with HDL particles is also suggested by the co-elution with both apo A-I apolipoprotein peaks, although preferentially with that showing a higher molecular mass. The chance of a reversible association with PLA₂ of some HDL particles is supported by the effect of high-ionic-strength treatment, which yields a PLA₂ showing a lower molecular mass.

The retarded elution of PLA₂ compared with apo B strongly suggests that this enzyme is not the form of PLA₂ associated with apo B, which acts on oxidized phospholipids, associates with low-density lipoprotein and shows a molecular mass > 66 kDa [47]. This finding is reminiscent of reports on some enzyme activities secreted by liver cells and mononuclear phagocytes that are incorporated into a lipoprotein particle, e.g. lecithin-cholesterol acyltransferase [48] and PAF-acetylhydrolase [49,50]. This is also consistent with the report of the induction of group-II PLA₂ gene expression in human hepatoma cells by mediators of the acute-phase response [16], and with the stimulation of PLA₂ synthesis and secretion by rat liver after injection of bacterial endotoxin [51].

Another association with PLA₂ herein disclosed is that of a polypeptide from the complement system. This is sustained on our inability to isolate the plasma enzyme from peptides showing the N-terminal sequence of the C3 α -chain [38] after a number of treatments. The formation of cross-linking between C3a and PLA₂ via intermolecular disulphide bridges seems a likely mechanism that could explain a side-by-side interaction of PLA₂ with a fragment of C3a. The reported ability of group-II PLA₂ to produce multimeric forms even in the presence of SDS and dithiothreitol [52], and the existence of six cysteine residues in C3a support this interpretation, but further analysis of this possibility was thwarted by the sensitivity of PLA₂ activity to sulphhydryl reducing agents [53]. This association seems of functional significance, as the production of group-II PLA₂ and C3 appears to take place simultaneously. Thus, expression of the human C3 gene is tissue-specific, with hepatocytes being the main site of synthesis [54].

Production of C3 has also been found in the human hepatoma-derived cell line HepG2 [55], and C3 is an acute-phase reactant whose production is increased during acute inflammation in response to interleukin-1 [56] and tumour necrosis factor- α [57] in a similar manner to the cytokine-mediated induction of the PLA₂ gene in HepG2 cells [15]. In addition, bacterial endotoxin is a strong initiator of complement activation that produces the generation of C3as. Because the association with C3as influences the catalytic properties of group-II PLA₂, our data indicate a new functional role for C3a-related peptide(s) as modulator(s) of group-II PLA₂. In fact, comparison of the catalytic activity of the plasma enzyme with that of recombinant group-II PLA₂ showed a lower activity of the plasma PLA₂, which could not be enhanced by increasing the amount of protein in the assay. Furthermore, addition of the Superdex 75 fraction from zymosan-activated serum corresponding to the molecular mass

of C3a des Arg to the recombinant enzyme produced inhibition of the activity.

We have not addressed as yet an analysis of the mechanism of inhibition of PLA₂ activity by the C3a-related moiety, but the close association with the enzyme, and the finding in preliminary experiments that optimal effect is obtained when the inhibitor is incubated with recombinant PLA₂ before the addition of substrate, would suggest the need for interaction of both peptides in the aqueous phase, rather than association at the interface or depletion of substrate [58]. As far as we know, there is only one report indicating that degraded products of C3 show inhibitory effects on rat peritoneal group-II PLA₂, but not on phospholipases from bee venom, *Naja naja* venom, and porcine pancreas [59]. However, that study was directed to investigate inhibitory proteins on the PLA₂ assay, rather than to search for inhibitory proteins associated with the enzyme. In addition, the portions of the C3 molecule isolated start from either residue 289 or 326 of the α -chain of human C3, and belong to a region of C3 complement component with a functional role different from that of the C3a fragment.

Another association of complement and PLA₂ has emerged from the recent description of the requirement for membrane-associated PLA₂ in platelet cytotoxicity induced by receptors for IgG and complement [60]; however, neither the type of PLA₂ involved in this reaction nor the precise nature of the bound C3 fragment has been characterized, although it has been shown that the complete cytotoxic system may exist as a preformed complex in platelet membranes.

As regards functional properties, the group-II PLA₂ used in this study showed a preferential activity on the *E. coli* assay, on synthetic PE and on U937 monoblast cell membrane fractions. Because U937 cell-line membranes should resemble the structure found in host cells, this prompts a search for an explanation for the lack of effect of group-II PLA₂ on PMNs maintained in culture to undergo spontaneous apoptosis and expression of phosphatidylserine on the outer leaflet of the plasma membrane [44–46], and even on isolated membranes from [³H]arachidonate-labelled PMNs. This puzzling finding contrasts with the concomitant evidence of hydrolysis of phospholipids from both ageing PMNs and PMN isolated membranes by group-I PLA₂.

Two conclusions can be drawn from these findings: (a) ageing of PMNs is sufficient to convert these cells into a good substrate for group-I PLA₂, but not for group-II PLA₂; and (b) some structural features in U937 monoblast cell membrane allow its recognition by group-II PLA₂. This observation could be related to recent reports on macrophage-like P388D₁ cells [61], rat connective tissue mast cells [14], and endothelial cells [62], that indicate regulation of group-II PLA₂ by interaction between cell-surface heparan sulphate and the heparin-binding domain of the enzyme. Alternatively, we can not rule out the existence of cell-surface binding sites similar to that demonstrated for group-I PLA₂ in several cell types [63]. Although our data do not show a role for group-II PLA₂ in the initiation of the lipid mediator cascade in PMN, or in the generation of prostaglandin D₂ from rat mast cells [14], our results would agree with the possible involvement of group-II PLA₂ in the production of eicosanoids from macrophage-type cells [61,64], and with a role for this enzyme in the modulation of arachidonate metabolism in chronic inflammation rather than in the acute setting of bacterial invasion where PMNs operate. Moreover, from studies in animal species other than human, it is now apparent that both normal rabbits [5] and rats [65] have plasma concentrations of PLA₂ similar to those detected in humans with sepsis, whereas they do not show the pathophysiological consequences of an enhanced production of lipid autacoids. Finally, taking into account the association

with lipoproteins of PLA₂, and the newly described pathway selectively related to the low-density lipoprotein receptor [66] that allows the delivery of arachidonate for eicosanoid production, we can not rule out a similar mechanism linked to HDL.

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