Cytosolic phospholipase A₂ from human monocytic cells: characterization of substrate specificity and Ca²⁺-dependent membrane association

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A cytosolic phospholipase A₂ (PLA₂) has been purified to homogeneity from the human monocytic tumour cell line THP-1 by a combination of ion-exchange, heparin-agarose and hydrophobic-interaction chromatography and non-denaturing PAGE. The purified PLA₂ exhibited a molecular mass of 100 kDa on SDS/PAGE, whereas non-denaturing PAGE showed a molecular mass of about 80 kDa. Investigation of the substrate specificity of PLA₂ revealed no preference for arachidonic acid- over linoleic acid-containing phospholipids when labelled THP-1 membranes were used as substrate. Treatment of THP-1 cells for 1–72 h with the phorbol ester phorbol 12-myristate 13-acetate (PMA) led to a stable enzyme activation, which was also found after partial purification of PLA₂ from PMA-stimulated THP-1 cells. The properties of the partially purified PLA₂ from

PMA-stimulated cells were compared with those of the PLA₂ from unstimulated cells. Both enzymes showed an identical Ca²⁺-dependent enzyme activation ($EC_{50} = 300 \text{ nM Ca}^{2+}$). A significant difference was found in the Ca²⁺-dependent membrane association of the enzymes obtained from the two sources. PLA₂ from PMA-stimulated cells showed membrane association of PLA₂ activity at significantly lower Ca²⁺ concentrations (100–300 nM) compared with the PLA₂ from unstimulated THP-1 cells (300–500 nM). The altered Ca²⁺-dependency was due to a stable modification of the enzyme. Changes in the ability of PLA₂ to associate with cellular membranes may thus be regarded as an important molecular mechanism underlying the increase in fatty acid metabolism observed in activated cells.

INTRODUCTION

Phospholipases A₂ (PLA₂s) are widely distributed intracellular and extracellular enzymes which selectively hydrolyse the *sn*-2 fatty acyl ester bond of membrane phospholipids [1,2]. These enzymes play an important role in generating polyunsaturated fatty acids such as arachidonic acid, the precursor of the biologically active eicosanoids, e.g. prostaglandins and leukotrienes.

Mammalian PLA₂s are divided into two groups. Group I (pancreatic type) enzymes have been characterized in lung, spleen and pancreas, whereas group II PLA₂s are found in platelets, spleen and liver. Group II PLA₂s are also found in inflammatory exudates, and their expression and extracellular release is enhanced upon stimulation of cells participating in inflammatory reactions [3–5].

Recent studies have demonstrated the existence of a new class of intracellular PLA₂s having no sequence similarity to either group I or II enzymes. Such intracellular PLA₂s have been characterized in and purified from the cytosol of various cell types [6–15]. Biochemical characterization has revealed some common properties among this group of enzymes. Specifically, they show a molecular mass of about 100 kDa (by SDS/PAGE) and substrate specificity for arachidonic acid. In addition, they appear to be regulated by Ca²⁺. This subgroup of PLA₂ requires both physiological concentrations of Ca²⁺ and translocation to the cellular membrane for its activation [16,17].

cDNA cloning and sequencing of PLA₂ from U937 cells revealed that the N-terminal of the 85 kDa protein contains a 140-amino-acid fragment that binds to cellular membranes in a Ca²⁺-dependent manner [18]. The functional aspects of the Ca²⁺-dependent PLA₂ translocation are not yet clear, and little is

known about the regulatory mechanisms leading to changes in PLA₂ activity.

Protein kinase C (PKC) is involved in the regulation of intracellular PLA₂ in several cell types (monocytes/macrophages [19–22], as well as mesangial cells [23], platelets [24], keratinocytes [25] and endothelial cells [26]). In a recent study, Lin et al. [27] demonstrated that activation of PLA₂-transfected CHO cells with several agonists [ATP, phorbol 12-myristate 13-acetate (PMA), Ca²⁺-ionophore] stimulates the phosphorylation of PLA₂ on serine residues. The phosphorylation was accompanied by an increased PLA₂ activity. We have previously identified an intracellular PLA₂ in the human monocytic tumour cell line THP-1 [12]. Activation of PKC by the phorbol ester PMA leads to an increased activity of this PLA₂, which is accompanied by a translocation of the enzyme to cellular membranes.

In the present study, we report the purification and biochemical characterization of an 80 kDa PLA₂ from the human monocytic tumour cell line THP-1 which cleaves arachidonic acid and linoleic acid containing phospholipids equally well. Furthermore, these studies provide the first evidence that PKC is able to modulate the capability of PLA₂ to associate in a Ca²⁺-dependent manner with cellular membranes, supporting the biological relevance of PLA₂ translocation.

EXPERIMENTAL

Cell culture

THP-1 cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were grown in RPMI 1640 (Gibco) containing 5% fetal-calf serum and cultured at 37 °C in a humidified atmosphere with 5% CO₂ in a 3.0-litre spinner flask. When the cell density was approx.

Abbreviations used: PKC, protein kinase C; PLA₂, phospholipase A₂; PMA, phorbol 12-myristate 13-acetate.

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$(0.5-1) \times 10^6$ cells/ml, the cells were washed once with buffer A [10 mM Tris/HCl (pH 7.6), 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, 1 mM EGTA, 0.1 mM phenylmethanesulphonyl fluoride and 1 μ g/ml leupeptin] and collected by centrifugation. In activation experiments THP-1 cells were incubated with PMA (5 nM) up to 72 h.

Cell fractionation

The cell pellet of 1×10^{10} cells was resuspended in 200 ml of buffer A and sonicated at 4 °C (50 W; 3×15 s) with a probe sonicator (Labsonic Sonifier; Braun, Melsungen, Germany). The homogenate was centrifuged at 6500 *g* for 20 min to remove cellular organelles. Membranes and cytosol were separated by centrifugation at 100 000 *g* for 45 min. The membrane fraction was resuspended in buffer A. Protein was determined by a microtitre Bradford assay with BSA as standard [28].

Assay for PLA₂ activity

PLA₂ activity was determined by using α -1-palmitoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine (NEN, Boston, MA, U.S.A.) as substrate as described previously [29]. If not indicated otherwise, the assay mixture contained 100 mM Tris/HCl (pH 9.5), 5 mM CaCl₂, 5 mg/ml BSA (essentially fatty-acid-free) and 1 μ M phospholipid (approx. 30 000 c.p.m.). The reaction was carried out at 37 °C for 30 min. The liberated fatty acid was extracted by heptane extraction and the radioactivity was determined by liquid-scintillation counting.

DEAE-52 anion-exchange chromatography

The cytosol of THP-1 cells was applied to a 2.2 cm $\times$ 15 cm DEAE-cellulose 52 (Whatman) column equilibrated with buffer A. The column was washed with the same buffer, and the PLA₂ activity was eluted by a 500 ml linear gradient of NaCl (0–0.5 M) in buffer A. PLA₂ activity was eluted as a single peak at about 0.25 M NaCl.

Phenyl-Sepharose chromatography

The fractions with PLA₂ activity were pooled and adjusted to 1 M (NH₄)₂SO₄. The fractions were loaded on a phenyl-Sepharose column (2.5 cm $\times$ 5 cm) (Pharmacia) equilibrated with buffer A containing 1 M (NH₄)₂SO₄. Elution was carried out with a decreasing linear gradient (400 ml) of (NH₄)₂SO₄ from 1 to 0 M, followed by washing (150 ml) at 0 M salt. The enzyme activity was only weakly bound to the phenyl-Sepharose column and was eluted during the low-salt wash with 0 M (NH₄)₂SO₄.

Heparin-agarose chromatography

The active fractions from the phenyl-Sepharose column were applied to a 1 cm $\times$ 5 cm heparin-agarose column (Sigma) equilibrated with buffer A. After 70 ml of the solution had passed through the column, the elution was continued with 20 ml of buffer A containing 1 M NaCl. Most of the PLA₂ activity was found in the flow-through, whereas contaminating proteins and a small fraction of PLA₂ (left uncharacterized) were bound to the column.

Mono P chromatography

The pooled active fractions obtained from heparin-agarose chromatography were loaded on to a Mono P column (Pharmacia LKB) equilibrated with buffer A. Elution was performed at a flow rate of 0.5 ml/min with a 24 ml discontinuous gradient of 0–1.0 M NaCl in buffer A (Figure 1).

Native PAGE

The active fractions from Mono P chromatography were concentrated to a final volume of 100 μ l with a Centricon 10 micro-concentrator (Amicon). Electrophoresis of PLA₂ under non-denaturing conditions, i.e. in the absence of SDS and 2-mercaptoethanol, was carried out at 4 °C in a 4%-acrylamide stacking gel and a 8% separating gel by using the 2001 vertical electrophoresis system (Pharmacia LKB) with 25 mM Tris/190 mM glycine, pH 8.9, as cathode buffer and 100 mM Tris/HCl, pH 8.9, as anode buffer. The concentrated PLA₂ fraction was applied and the gel was run at 25 mA for 30 min and then at 30 mA for 3.5 h.

After electrophoresis, the gel was cut into 2 mm slices. All slices were crushed and then incubated at 4 °C for 15 h in 0.5 ml of buffer A. After centrifugation (10 000 *g*, 5 min), the supernatant was assayed for PLA₂ activity.

SDS/PAGE

Samples for gel electrophoresis were dried in a Speed Vac Concentrator (SVC 100; Savant). Protein pellets were redissolved in Laemmli buffer and then boiled for 5 min. PAGE was carried out on 5–20% linear gradient gels as described by Laemmli [30]. The gels were stained for protein by the silver-staining method [31]. The molecular mass was estimated by comparison with SDS/PAGE standards (Bio-Rad).

Molecular mass estimation by non-denaturing gel electrophoresis

Samples (20 μ g) of partially purified PLA₂ (via DE-52 column separation) and several standard proteins were electrophoresed on a set of gels of various polyacrylamide concentrations (6–10%) as described above. To determine the relative mobilities (*R_f*) of the proteins in each gel, the gel slabs were cut into two pairs. Gel protein standards were stained for protein-band localization with Coomassie Blue. For elution of PLA₂ activity, the gel stripe was cut into slices (2 mm) and treated as described above.

The molecular mass of the native PLA₂ was calculated by the method of Hedrick and Smith [32]. The logarithm of the relative mobility was plotted against the gel concentration for each protein. The logarithm of the negative slopes for the standards was then plotted as a function of the corresponding molecular masses. The molecular mass of the native PLA₂ was then derived from the measured slope. The molecular-mass markers used were catalase (232 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and α -lactalbumin (14 kDa).

Preparation of radiolabelled THP-1-cell membrane lipids

THP-1 cells were incubated overnight with either 0.1 μ Ci of [¹⁴C]arachidonic acid (Amersham Buchler) (sp. radioactivity 50 mCi/mmol) or 0.1 μ Ci [¹⁴C]linoleic acid (NEN) (sp. radioactivity 55.6 mCi/mmol)/ 3×10^5 cells per ml in medium without fetal-calf serum or BSA. After 1 h BSA (essentially fatty-acid-free; Sigma) was added for overnight incubation (final concn. 1 mg/ml). After labelling, the cells were washed once with PBS and collected by centrifugation.

The radiolabelled phospholipids were extracted by methods described in detail elsewhere [33].

Substrate specificity of PLA₂

Partially purified PLA₂ (via DE-52 column separation) was incubated with the lipid extracts from radiolabelled THP-1

membranes (40000 c.p.m. of either [¹⁴C]arachidonic acid- or [¹⁴C]linoleic acid-labelled lipids) under standard assay conditions. After the indicated intervals, the phospholipids were extracted and separated by t.l.c. on silica-gel plates (F 1500; Schleicher & Schuell) in the solvent system chloroform/methanol/acetic acid/0.9% NaCl (100:50:16:5, by vol.). The radioactivity corresponding to the different phospholipid species was detected by radioscanning. Phospholipid standards were made visible with iodine vapour.

Ca²⁺-dependent association of PLA₂ with cellular membranes

The membranes from THP-1 control cells were prepared as described above with buffer supplemented with 1 mM EGTA to remove adhering PLA₂. Samples of the partially purified PLA₂ from PMA-treated as well as from control THP-1 cells were incubated with THP-1 membranes (100 µg) at different Ca²⁺ concentrations (0–1000 nM). The free Ca²⁺ concentrations were calculated by a computer program, by using EGTA/CaCl₂ mixtures in 20 mM Tris/HCl (pH 7.6). The incubation was carried out for 45 min at 4 °C to enable binding but to preclude cleavage. After centrifugation (100000 g, 45 min) PLA₂ activity in the supernatant and in the membrane pellet was determined under standard assay conditions (5 mM CaCl₂, 100 mM Tris/HCl, pH 9.5).

RESULTS AND DISCUSSION

Purification of PLA₂ from THP-1 cells

The monocytic THP-1 cells contain an intracellular PLA₂ activity comparable with that obtained from the closely related U937 cell line [12]. About 90% of the total PLA₂ activity was recovered in the soluble fraction when the cells were disrupted in the presence of Ca²⁺-chelating agents such as EDTA/EGTA. The buffer contained 2-mercaptoethanol to inhibit the low-molecular-mass PLA₂s, which might also exist in this cell line. The enzyme activity was determined with α-1-palmitoyl-2-arachidonoyl phosphatidylcholine as substrate.

The intracellular PLA₂ activity was purified to homogeneity by column chromatography and preparative gel electrophoresis (for details see the Experimental section). The results of a typical purification procedure are summarized in Table 1. Interestingly, the Mono P column, designed as a chromatofocusing column, was most effectively used as an anion-exchange column. The PLA₂ activity was eluted as a single peak at 0.8 M NaCl, yielding

Table 1 Purification of a cytosolic PLA₂ from the human monocytic cell line THP-1

PLA₂ activity was determined with 1-palmitoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine as substrate as described in the Experimental section. N.D., not detectable.

	Protein (mg)	Sp. activity (nmol/min per mg)	Total activity (nmol/min)	Yield (%)	Purification (fold)
Cytosol	485	0.0505	24.5	100	1
DEAE-52	52	0.24	12.5	51	5
Phenyl-Sepharose	3.3	0.86	2.83	11.6	17
Heparin-agarose	0.4	3.41	1.4	5.6	68
Mono P	0.003	48.2	0.14	0.6	954
Centricon 10	< 0.003	60.9	0.18	0.7	1206
Native gel electrophoresis	N.D.	—	—	—	> 1206

Figure 1 Mono P chromatography

Elution of the Mono P column was performed as described in the Experimental section. ---, NaCl gradient; ■, PLA₂ activity (nmol/min per mg of protein); ▲, A₂₈₀.

Figure 2 SDS/PAGE analysis of PLA₂ purification

Samples were subjected to SDS/PAGE (5–20% linear gradient). Proteins were detected by silver staining. Lanes: a, cytosol (10 µg of protein); b, DEAE-cellulose fraction (10 µg); c, phenyl-Sepharose fraction (5 µg); d, heparin-agarose fraction (5 µg); e, Mono-P fraction (1 µg); f, eluate from native PAGE (2 mm slice).

a purification of more than 10-fold (Figure 1). After concentration of the active fractions with a Centricon 10 micro-concentrator, the concentrate was submitted to electrophoresis on an 8% polyacrylamide gel under non-reducing conditions in the absence of SDS. As described in the Experimental section, PLA₂ activity was recovered from the polyacrylamide gel and was further analysed by SDS/PAGE (Figure 2). The eluted PLA₂ showed essentially a single protein band corresponding to a molecular mass of 100 kDa. Only a very slight contamination by a minor protein band (~65 kDa) was observed. This band represents a typical contaminating protein of PLA₂ purifications which has been identified in rat kidney as protein disulphide-isomerase [6].

High-molecular-mass PLA₂ enzymes were purified from a number of cell lines and tissues, including human [11], bovine [9] and rabbit [8] platelets, the macrophage cell lines RAW 264.7 [13] and J774 [14], rat kidney [6] and the human monocytic cell line U937 [7,10].

The molecular masses were estimated by SDS/PAGE to be about 90–110 kDa, whereas a value of 85 kDa was determined by molecular cloning of PLA₂ cDNA from U937 cells [18,34]. Recently, it has been supposed that the abnormal migration of PLA₂ on SDS/PAGE is due to a proline-rich amino acid sequence, as deduced from its cDNA [34]. Moreover, the conformation of the denatured PLA₂ protein may retard its mobility in SDS/PAGE, possibly due to an increased affinity of the enzyme for the gel matrix.

The enzyme became considerably labile during the last two purification steps. Loss of enzyme activity was only partially avoided by concentrating the enzyme preparation and storing it at -70°C in the presence of BSA. Due to enzyme lability and the relatively small amount of highly purified PLA_2 obtained after purification, partially purified enzyme from the DEAE-52 column was used for further characterization.

Molecular mass of the native PLA_2

In order to define further the molecular mass of the native PLA_2 from THP-1 cells, the methods described by Hedrick and Smith [32] were adapted. The partially purified protein was electrophoresed under non-denaturing conditions on gels with different concentrations of polyacrylamide (see the Experimental section). The R_f values of PLA_2 activity and several standard proteins were plotted versus the corresponding gel concentrations. The slopes of the resulting linear curves were further plotted against the molecular masses of the standard proteins. From this plot (Figure 3), the molecular mass of the native PLA_2 was estimated to be 78 kDa. The same value was determined for the native PLA_2 from U937 cells by radiation inactivation [35]. This value, along with the results obtained by molecular cloning of PLA_2 (85 kDa) [18,34], indicates that the native enzyme exists as a monomer.

Substrate specificity of PLA_2

It has been reported that the new class of intracellular high-molecular-mass PLA_2 s preferentially hydrolyses cellular phospholipids with arachidonic acid in the *sn*-2 position. However, most of these specificity studies were carried out with pure radiolabelled phospholipids, in the presence of BSA, or presented to the enzyme in the form of sonicated (mixed) phospholipid vesicles solubilized with or without detergents [7–9,11,13,14,17]. The results of these studies need to be interpreted with some caution, since the apparent selectivity of the enzyme may be related to the physical form of the phospholipid aggregate rather than to the real substrate specificity [36].

Figure 3 Determination of the molecular mass of PLA_2 by non-denaturing PAGE

Partially purified PLA_2 (x) and several standard proteins (each $20\text{ }\mu\text{g}$: a, α -lactalbumin; b, carbonic anhydrase; c, ovalbumin; d, BSA monomer; e, BSA dimer; f, catalase) were electrophoresed on a set of gels of various polyacrylamide concentrations (6–10%) under non-reducing conditions in the absence of SDS. The standard proteins were detected by staining with Coomassie Blue, and PLA_2 activity was detected by determination of the activity in the sliced gel. The R_f value of each protein was calculated and plotted versus the concentration of the corresponding gel. Insert: the slopes of the resulting linear curves were further plotted against the molecular masses of the standard proteins.

Table 2 Incorporation of [^{14}C]arachidonic acid and [^{14}C]linoleic acid into cellular phospholipids of THP-1 cells

THP-1 cells were incubated with [^{14}C]arachidonic acid or [^{14}C]linoleic acid overnight and the phospholipids were extracted as described (see the Experimental section). The radioactive products were resolved by t.l.c. Bands corresponding to the different phospholipid species were detected by radioscanning. Radioactivity in each phospholipid class was quantified by computer-aided analysis of the radioactivity scans.

	Incorporation (%)	
	Arachidonic acid ($n = 3$, $\pm$ S.D.)	Linoleic acid ($n = 2$, $\pm$ range)
Phosphatidylcholine	33.6 ± 2.9	45.6 ± 1.4
Phosphatidyl-inositol/-serine	19.0 ± 5.1	19.7 ± 1.3
Phosphatidylethanolamine	47.4 ± 8.5	34.7 ± 2.6

Table 3 Decrease in fatty acid content of radiolabelled phospholipids

Partially purified PLA_2 from THP-1 cells was incubated with lipid extracts from radiolabelled THP-1 cells under standard assay conditions as described in the Experimental section. The decrease in fatty acid content of the linoleic acid- or arachidonic acid-containing phospholipids is expressed relative to the fatty acid content of the phospholipids from the control experiment (assay in the presence of 5 mM EGTA). Data are means $\pm$ S.D. of three experiments.

	Decrease (%)	
	Arachidonic acid	Linoleic acid
Phosphatidylcholine	26.8 ± 8.5	29.4 ± 3.5
Phosphatidyl-inositol/-serine	34.1 ± 13.3	32.6 ± 5.6
Phosphatidylethanolamine	38.6 ± 13.0	43.0 ± 6.5

To circumvent these problems, we used lipids of THP-1 membranes labelled with arachidonic acid or linoleic acid for the determination of PLA_2 substrate specificity. THP-1 cells were incubated with [^{14}C]arachidonic acid or [^{14}C]linoleic acid overnight, and the lipids were extracted as described [33]. The distribution of radioactivity in each phospholipid class is shown in Table 2. These results concur with findings in the human monocytic cell line U937 and mouse macrophages which indicate that arachidonic acid is primarily incorporated into phosphatidylethanolamine [16,37,38], whereas linoleic acid is incorporated preferentially into phosphatidylcholine [37]. After incubation of partially purified PLA_2 with a mixture of phospholipid extract and BSA, the radioactive products were resolved by t.l.c. Bands corresponding to phosphatidylcholine, phosphatidyl-inositol/-serine, phosphatidylethanolamine and non-esterified fatty acid were detected by radioscanning. The result of the quantification of three independent experiments is shown in Table 3. The enzyme preferentially hydrolysed phosphatidylethanolamine, followed by phosphatidylinositol and phosphatidylcholine. A PLA_2 with a similar substrate specificity has been identified in J774 mouse macrophages: when mixed vesicles were used as substrate, the enzyme cleaved arachidonic acid from phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine, with a slight preference for phosphatidylethanolamine [14]. Furthermore, our results demonstrate that the enzyme hydrolysed arachidonic acid- and linoleic acid-containing phospholipids equally well, whereas lipids labelled with palmitic acid were not hydrolysed (results not shown). These data differ from a number of studies using PLA_2 from

other cellular sources, which suggest that PLA₂ is highly specific for arachidonic acid-containing phospholipids [8,9,11,14,18,39].

The conflicting results may in part be explained by the use of different substrate preparations. Most of the studies were made with single types of aggregated or vesicular phospholipids. It has been concluded from monolayer studies that phospholipid vesicles containing arachidonic acid at the *sn*-2 position have a more loose structure compared with oleic acid- and linoleic acid-containing phospholipid vesicles [40]. Thus the apparent substrate specificity may be due to an increased availability of the arachidonic acid-containing phospholipid for the PLA₂.

The finding *in vitro* that the high-molecular-mass PLA₂ from THP-1 cells has a specificity for polyunsaturated fatty acids, and not exclusively for arachidonic acid, may have important implications for the biological function of the intracellular PLA₂. Until now, the proposed function of intracellular PLA₂ has been limited to the generation of arachidonic acid for further metabolism to eicosanoids. Linoleic acid, on the other hand, has been shown to be a potent activator of membrane-bound enzymes, e.g. PKC [41]. Activation of the intracellular PLA₂ might thus have more implications besides providing arachidonic acid as a precursor molecule.

PMA-induced change of PLA₂ activity

The mechanisms by which the new class of intracellular high-molecular-mass PLA₂s is regulated has been the subject of intensive research during recent years. It has been demonstrated that PMA is able to stimulate the arachidonic acid release in various monocyte/macrophage cell lines [12,20,21]. We have shown in a previous study that short-term incubation of THP-1 cells with the phorbol ester PMA induced PLA₂ activity, as indirectly determined by arachidonic acid release and also with exogenous substrate [12].

To investigate further the effect of PMA on the modulation of PLA₂ activity, the enzyme was partially purified from stimulated and control THP-1 cells. The THP-1 cells were incubated with 5 nM PMA for 1, 4 and 72 h. In addition, the cells were further stimulated with PMA for 4 h in the presence of cycloheximide (1 µg/ml). The cytosol of the cells was prepared as described above, in the presence of 1 mM EGTA, to shift the Ca²⁺-dependent membrane-adhering PLA₂ into the cytosol. PLA₂ from control and stimulated THP-1 cells was then partially purified by DEAE-cellulose anion-exchange chromatography. The chromatographic profiles were identical (results not shown), and PLA₂ activity was eluted as a single peak at 250 mM NaCl in both instances.

Incubation of the cells with PMA caused a time-dependent increase in cytosolic PLA₂ activity (Table 4). The activity was enhanced after 1 h of incubation with PMA. Incubation of the cells with PMA for 4 h in the presence of cycloheximide did not affect the increase in PLA₂ activity, suggesting that the short-term induction of PLA₂ activity was not due to protein synthesis. After partial purification of PLA₂ from PMA-stimulated and control cells, the enhanced enzyme activity was still measurable (Table 4), indicating a stable modification.

These data are in good agreement with those obtained by Lin et al. [27], who showed that PLA₂ was phosphorylated in response to external stimuli such as thrombin, ATP, PMA and the Ca²⁺-ionophore A23187. The phosphorylation was associated with an increased PLA₂ activity, confirming the important role for protein kinase(s) in regulation of PLA₂. PMA-induced enhancement of PLA₂ activity and the subsequent purification of the activated enzyme have also been shown in renal mesangial cells [42].

The action of the tumour promoter PMA is primarily mediated

Table 4 Stimulation of PLA₂ activity by PMA

THP-1 cells were incubated with PMA (5 mM) or cycloheximide (CHX, 1 µg/ml) for the times indicated. The cells were fractionated in the presence of 1 mM EGTA, and PLA₂ activity was determined in the cytosolic fraction. Additionally, the enzyme was partially purified by DEAE-cellulose chromatography and the activity determined in the peak fractions. Data are means ± range of two preparations with triplicate determinations.

Incubation condition	PLA ₂ activity (pmol/min per mg of protein)	
	Cytosol	Partially purified enzyme
Control cells	175.9 ± 3.3	307.6 ± 9.0
PMA 1 h	227.3 ± 8.4	494.5 ± 9.2
PMA 4 h	263.7 ± 13.5	538.4 ± 2.5
PMA + CHX 4 h	280.0 ± 15.2	484.5 ± 54.5
PMA 72 h	335.4 ± 21.3	1494.9 ± 453.0

by PKC [43]. In the study by Wijkander and Sundler [14], PKC-dependent phosphorylation of PLA₂ from a macrophage cell line was demonstrated *in vitro*. However, a corresponding increase in enzyme activity could not be detected. In addition, two previous studies showed that PLA₂ was not activated in response to short-term treatment with PMA, although PMA promoted the translocation of PKC to the membrane within 5–10 min [12,44]. From these data it is reasonable to conclude that, besides the possibility of direct PKC-dependent enzyme phosphorylation, there may exist additional steps to activate PLA₂ in monocytes/macrophages. Thus it is more likely that activation of PKC will further activate other kinases, which finally might phosphorylate PLA₂.

Prolonged incubation of monocytic cell lines with the tumour promoter PMA leads to differentiation to macrophage-like cells. These differentiated cells are morphologically and functionally altered compared with undifferentiated cells. They show an enhanced ability to release arachidonic acid and prostanoids [45]. PLA₂ activity measured in the cytosol was enhanced about 2-fold compared with the cytosols of untreated cells. Partial purification enhanced the stimulation to about 4-fold. This might be the result of the removal of an enzyme inhibitor, which was induced during differentiation. Long-term incubation of the cells was not possible in the presence of an inhibitor of protein synthesis. The enhanced activity in differentiated cells may thus well be due to protein modification as well as to enhanced gene expression and protein synthesis.

Ca²⁺-dependent activation of PLA₂

In contrast with group I and II PLA₂s, which need millimolar Ca²⁺ concentrations for their activity, enzyme activation at physiological Ca²⁺ concentrations is a typical feature of the intracellular high-molecular-mass PLA₂s [6–11,14,15,39]. Therefore, we compared the effect of various Ca²⁺ concentrations on PLA₂ activity of the partially purified enzyme from control cells and PMA-differentiated (72 h) cells. Free Ca²⁺ concentrations were stabilized by using Ca²⁺/EGTA buffers. As shown in Figure 4, there was no difference in Ca²⁺-dependence of PLA₂ activity from control cells compared with the enzyme from PMA-treated cells. Both enzymes showed the same biphasic dependence of the activity on Ca²⁺. The enzyme was activated in the presence of sub-micromolar concentrations of Ca²⁺, and the activity was further enhanced when the free Ca²⁺ concentration was increased from 10 µM to 10 mM.

These data indicate that enhanced PLA₂ activity observed in

Figure 4 Ca^{2+} -dependency of PLA₂ from PMA-differentiated and control cells

The activity of partially purified PLA₂ from control cells (a) and PMA-differentiated (72 h) cells (b) was assayed at pH 7.6 (20 mM Tris) either in the presence of 5 mM EGTA (zero Ca^{2+}) or in the presence of 1 mM EGTA and 0.45–11 mM $CaCl_2$, giving the free Ca^{2+} concentrations indicated. Data are means $\pm$ S.D. of three preparations.

PMA-differentiated cells is not accompanied by a measurable change of the Ca^{2+} -dependence of PLA₂ activity. Moreover, in two recent studies [46,47] it has been suggested that the role of Ca^{2+} is to promote the binding of the enzyme to the membrane, rather than to be involved in the catalytic mechanism of PLA₂ action, as described for group I and II PLA₂s [48].

Effect of PMA on Ca^{2+} -dependent translocation of PLA₂ to cellular membranes

Ca^{2+} -dependent membrane translocation of PLA₂ has been studied previously by using homogenized cells [12,15–17,49] as well as cell-free systems [50], and has been confirmed recently by immunological and molecular-biological methods [18]. This type of translocation, which permits the interaction of the enzyme with the membrane (representing its natural substrate), plays an important role in regulation of the intracellular high-molecular-mass PLA₂.

Most of the PLA₂ activity is found in the cytosol at basal Ca^{2+} concentrations (100 nM) [16]. Changes in the intracellular Ca^{2+} concentration may be necessary to promote the translocation process.

In the present study we have examined the effect of PMA on

Figure 5 Effect of PMA on Ca^{2+} -dependent translocation of PLA₂ to cellular membranes

THP-1 cells were stimulated with PMA (5 nM) for 1 h (a) or 72 h (b). The cytosolic fractions were prepared from control and stimulated cells in the presence of 1 mM EGTA and fractionated by DEAE-cellulose chromatography. Samples of partially purified PLA₂ from PMA-treated ($\square$) and control cells ($\triangle$) were incubated with THP-1 membranes (100 μ g of protein) in the presence of 1 mM EGTA (control) and various concentrations of free Ca^{2+} , in 20 mM Tris (pH 7.6). After centrifugation, PLA₂ activity was determined in the supernatant and membrane fractions. Data are means $\pm$ S.E.M. of 3–7 experiments.

the Ca^{2+} -dependent distribution of the intracellular PLA₂. Partially purified PLA₂ from PMA-treated THP-1 cells (1, 4 and 72 h) or from control cells were mixed with THP-1 membranes from control cells, which were isolated in the presence of EGTA, and thus showed no PLA₂ activity. They were incubated with various concentrations of Ca^{2+} at 4 $^{\circ}$ C to prevent hydrolysis. After centrifugation, PLA₂ activity was determined in the membrane fraction and the supernatant. Figure 5 demonstrates that the enzyme distribution between supernatant and membrane fraction was dependent on the Ca^{2+} concentration in the reconstitution buffer.

In the presence of increasing Ca²⁺ concentrations, the decrease in PLA₂ activity in the supernatant fractions was paralleled by an increase of PLA₂ activity in the membrane fractions. A significant membrane translocation of PLA₂ from control cells was observed at Ca²⁺ concentrations of 300–500 nM. The enzyme from PMA-treated cells required lower Ca²⁺ concentrations for translocation to the membrane fraction (100–300 nM). Analysis of the time course for this PMA-mediated modulation of Ca²⁺-dependent enzyme translocation revealed that the activation was rapid and long-lasting. We observed the shift to lower Ca²⁺ concentrations after 1 h of exposure of the cells to PMA (Figure 5a). The same effect was also detected after 4 h (results not shown) and remained constant for up to 72 h (Figure 5b).

The rapid modulation of the PLA₂'s ability to translocate Ca²⁺-dependently to membranes suggests that this regulatory event may also involve a stable modification of PLA₂ rather than synthesis *de novo* of a modified enzyme. A recent study has shown that a structure involved in the Ca²⁺-dependent membrane association is located on a 140-amino-acid fragment in the N-terminal region of PLA₂ [18]. It is therefore conceivable that the PMA-induced activation process modifies the physicochemical properties of the Ca²⁺-dependent membrane-binding domain and thereby facilitates the enzyme translocation in the presence of lower Ca²⁺ concentrations.

These data represent the first demonstration that incubation of whole cells may lead to a change in the Ca²⁺-dependent ability of the intracellular PLA₂ to associate with membranes, supporting the biological relevance of PLA₂ translocation for the regulation of fatty acid release.

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REFERENCES

- Van den Bosch, H. (1980) *Biochim. Biophys. Acta* **604**, 191–246
- Waite, M. (1985) *J. Lipid Res.* **26**, 1379–1388
- Kaiser, E., Chiba, P. and Zaky, K. (1990) *Clin. Biochem.* **23**, 349–370
- Dennis, E. A., Rhee, S. G., Billah, M. M. and Hannun, Y. A. (1991) *FASEB J.* **5**, 2068–2077
- Pruzanski, W. and Vadas, P. (1991) *Immunol. Today* **12**, 143–146
- Gronich, J. H., Bonventre, J. V. and Nemenoff, R. A. (1990) *Biochem. J.* **271**, 37–43
- Clark, J. D., Milona, N. and Knopf, J. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7708–7712
- Kim, D. K., Kudo, I. and Inoue, K. (1991) *Biochim. Biophys. Acta* **1083**, 80–88
- Kim, D. K., Suh, P. G. and Ryu, S. H. (1991) *Biochem. Biophys. Res. Commun.* **174**, 189–196
- Kramer, R. M., Roberts, E. F., Manetta, J. and Putnam, J. E. (1991) *J. Biol. Chem.* **266**, 5268–5272
- Takayama, K., Kudo, I., Kim, D. K., Nagata, K., Nozawa, Y. and Inoue, K. (1991) *FEBS Lett.* **282**, 326–330
- Rehfeldt, W., Hass, R. and Goppelt-Strube, M. (1991) *Biochem. J.* **276**, 631–636
- Leslie, C. C. (1991) *J. Biol. Chem.* **266**, 11366–11371
- Wijkander, J. and Sundler, R. (1991) *Eur. J. Biochem.* **202**, 873–880
- Goppelt-Strube, M. and Rehfeldt, W. (1992) *Biochim. Biophys. Acta* **1127**, 163–167
- Channon, J. Y. and Leslie, C. C. (1990) *J. Biol. Chem.* **265**, 5409–5413
- Diez, E. and Mong, S. (1990) *J. Biol. Chem.* **265**, 14654–14661
- Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) *Cell* **65**, 1043–1051
- Wrightman, P. D., Dahlgren, M. E. and Bonney, R. J. (1982) *J. Biol. Chem.* **257**, 6650–6652
- Emilsson, A. and Sundler, R. (1986) *Biochim. Biophys. Acta* **876**, 533–542
- Goppelt-Strube, M. and Moeller, A. (1990) *Biol. Chem. Hoppe-Seyler* **371**, 369–374
- Balsinde, J., Fernandez, B. and Diez, E. (1990) *J. Immunol.* **144**, 4298–4304
- Bonventre, J. V. and Swidler, M. (1988) *J. Clin. Invest.* **82**, 168–176
- Halenda, S. P., Banga, H. S., Zavoico, G. B., Lau, L.-F. and Feinstein, M. B. (1989) *Biochemistry* **28**, 7356–7363
- Kast, R., Fürstenberger, G. and Marks, F. (1991) *Eur. J. Biochem.* **202**, 941–950
- Chakraborti, S., Michael, J. R. and Sanyal, T. (1992) *Eur. J. Biochem.* **206**, 965–972
- Lin, L.-L., Lin, A. Y. and Knopf, J. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6147–6151
- Redinbaugh, M. G. and Campbell, W. H. (1985) *Anal. Biochem.* **147**, 144–147
- Goppelt-Strube, M., Wolter, D. and Resch, K. (1989) *Br. J. Pharmacol.* **98**, 1287–1295
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
- Hedrick, J. L. and Smith, A. J. (1968) *Arch. Biochem. Biophys.* **126**, 155–164
- Goppelt, M. and Resch, K. (1984) *Anal. Biochem.* **140**, 152–156
- Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., Roberts, E. F. and Kramer, R. M. (1991) *J. Biol. Chem.* **266**, 14850–14853
- Tremblay, N. M., Nicholson, D., Potier, M. and Weech, P. K. (1992) *Biochem. Biophys. Res. Commun.* **183**, 121–127
- Wijkander, J. and Sundler, R. (1989) *FEBS Lett.* **244**, 51–56
- Bomlaski, J. S., Freundlich, B., Steiner, S. and Clark, M. A. (1988) *J. Leukocyte Biol.* **44**, 51–57
- Flesch, I. and Ferber, E. (1986) *Biochim. Biophys. Acta* **889**, 6–14
- Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M. and Zelarney, P. T. (1988) *Biochim. Biophys. Acta* **963**, 476–492
- Demel, R. A., Geurts van Kessel, W. S. M. and van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* **266**, 26–40
- Murakami, K., Chan, S. Y. and Routtenberg, A. (1986) *J. Biol. Chem.* **261**, 15424–15429
- Gronich, J. H., Bonventre, J. V. and Nemenoff, R. A. (1988) *J. Biol. Chem.* **263**, 16645–16651
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Shibata, Y., McCaffrey, P. G., Minowada, J., Volkman, A. and Oghiso, Y. (1992) *J. Leukocyte Biol.* **51**, 32–38
- Koehler, L., Hass, R., Wessel, K., DeWitt, D. L., Kaever, V., Resch, K. and Goppelt-Strube, M. (1990) *Biochim. Biophys. Acta* **1042**, 395–403
- Wijkander, J. and Sundler, R. (1992) *Biochem. Biophys. Res. Commun.* **184**, 118–124
- Ghomashchi, F., Schüttel, S., Jain, M. K. and Gelb, M. H. (1992) *Biochemistry* **31**, 3814–3824
- Verheij, H. M., Volwerk, J. J., Jansen, E. H. J. M., Puyk, W. C., Dijkstra, B. W., Drenth, J. and de Haas, G. H. (1980) *Biochemistry* **19**, 743–750
- Krause, H., Dieter, P., Schulze-Specking, A., Ballhorn, A. and Decker, K. (1991) *Eur. J. Biochem.* **199**, 355–359
- Yoshihara, Y. and Watanabe, Y. (1990) *Biochem. Biophys. Res. Commun.* **170**, 484–490