Ca^{2+}-independent cytosolic phospholipase A in HL-60 cells differentiating to granulocytes

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Abstract The release of various fatty acids (FAs) from permeabilized HL-60 cells, predominantly oleic acid (OA) rather than arachidonic acid, was greatly enhanced by GTP-γ-S and vanadate [Tsujishita, Y., Asaoka, Y. and Nishizuka, Y., Proc. Natl. Acad. Sci. USA 91 (1994) 6274–6278]. The present study shows that phospholipase A (A_2/A_1) activity which cleaves the acyl group from both *sn*-2 and *sn*-1 positions of phosphatidylethanolamine (PtdEtn) is increased in HL-60 cells during differentiation to granulocyte-like cells. This enzyme does not require Ca^{2+} and releases various FAs, preferentially OA from PtdEtn and, to lesser extent, from lysoPtdEtn. Other phospholipids including phosphatidylcholine and phosphatidic acid serve as very poor substrates. Although further studies are necessary to show the direct link of this enzyme activation to receptor stimulation, the results described here imply that this enzyme is responsible for the release of various FAs, particularly OA, from permeabilized HL-60 cells.

Key words: Phospholipase A_2; Phospholipase A_1; Oleic acid; Arachidonic acid

1. Introduction

Several metabolites derived from signal-induced hydrolysis of membrane lipid components serve as messengers or mediators to regulate intracellular events (for reviews, see [1–3]). Although unsaturated fatty acids (FAs) have been shown to exert various biological actions, such as modulation of ion channel properties, membrane fusion, long-term potentiation, and activation of protein kinases [3,4], little attention has been paid to FAs other than arachidonic acid (AA). After cell stimulation, FAs may be produced from membrane phospholipids through several pathways initiated by activation of phospholipases C (PLC), D (PLD), and A_2/A_1 (PLA_2/A_1).

PLA_2 is a large family of enzymes. The best characterized cytosolic PLA_2 is group IV 85 kDa ($cPLA_2$), which is linked to receptor stimulation through protein phosphorylation (for reviews, see [5,6]). This enzyme preferentially releases AA and requires Ca^{2+} for its association with membranes. Group VII 45 kDa (secreted) and group VIII 29 kDa (cytosolic) enzymes are platelet activating factor acetylhydrolases (for reviews, see [5,7]). More recently, group VI 80–85 kDa enzymes ($iPLA_2$) were cloned and sequenced from CHO cells and mouse $P388D_1$ macrophages, which cleave various FAs non-selectively from phosphatidic acid (PtdOH) and phosphatidylcholine (PtdCho), and, to lesser extents, from phosphatidylethanolamine (PtdEtn) and other phospholipids [8,9]. These enzymes are postulated to play a role in remodeling of membrane phospholipids (for reviews, see [5,10]).

In our early studies with permeabilized HL-60 and U-937 cells, various FAs, predominantly oleic acid (OA) rather than AA, were released spontaneously. This release reaction was enhanced greatly by GTP-γ-S and vanadate, and this enhancement was attenuated by tyrosine kinase inhibitor [11]. However, no evidence is available to date indicating that the AA-non-selective type of cytosolic PLA_2 (or PLA_1) is directly linked to receptor stimulation. To explore the mechanism responsible for the release reaction of FAs, especially OA, from permeabilized cell preparations, the present studies were undertaken.

2. Materials and methods

2.1. Chemicals

Leupeptin was obtained from Peptide Institute (Osaka). Dioleoyl-PtdEtn, dioleoyl-PtdCho and (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF) were from Wako (Osaka). 1,2-Diolein was a product of Doosan Serdary (Englewood Cliffs, NJ). Other phospholipids and lysophospholipids, FAs, FA-free bovine serum albumin were products of Sigma. 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi (Tokyo), dissolved in acetone at a concentration of 2.5% (w/v), and stored at −20°C in the dark. 1,2-Dioleoyl-phosphatidyl[2-^{14}C]ethanolamine (54 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]linoleoyl-PtdEtn (57 mCi/mmol) were products of Amersham. Arachidonyl trifluoromethyl ketone ($AACOF_3$) and bromoenol lactone (BEL; (*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one) were from Calbiochem. Rabbit polyclonal antibody against the N-terminal domain (N-216) of human $cPLA_2$ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Culture and differentiation of HL-60

HL-60 cells were maintained as described [11]. When required, the cells were plated at 4.0×10^5 cells/ml, and treated with 1.25% dimethyl sulfoxide (DMSO) for various periods to differentiate to granulocyte-like cells. The degree of differentiation was examined by the expression of a cell surface marker, CD11b [12]. The granulocytic differentiation was also assayed by the release of β-glucuronidase [13]. When cultured with 1.25% DMSO, HL-60 cells displayed a progressive decrease of their cytosolic protein as well as their total phospholipid content as reported [14,15]. Both expression of CD11b and release of β-glucuronidase showed maximal values at 5 days after the addition of DMSO. Electro-permeabilized cells were prepared as described [11].

2.3. Preparation of cytosol

HL-60 cells were collected and washed in phosphate-buffered saline. All subsequent steps were performed at 4°C. The cells were suspended at a density of 0.5 or 1.0×10^8 cells/ml for undifferentiated or 3-day differentiated HL-60 cells, respectively, in a buffer containing 25 mM Tris-HCl at pH 8.0, 2 mM EDTA, 250 mM sucrose, 50 μM *p*-APMSF, and 20 μg/ml leupeptin. The cells were then disrupted by sonication. The cytosol was obtained by centrifugation for 30 min at $100\,000\times g$.

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2.4. MonoQ column chromatography

The salt concentration of cytosol was adjusted to 250 mM NaCl before filtration through a Millipore filter (pore size 0.45 μm). Then, it was loaded onto a MonoQ HR5/5 column (0.5 cm×5 cm) pre-equilibrated with a buffer containing 25 mM Tris-HCl at pH 8.0, 2 mM EDTA, 2 mM 2-mercaptoethanol and 250 mM NaCl (buffer A). After extensive washing with buffer A, elution was made with a 20-ml linear concentration gradient of NaCl (250–600 mM) in buffer A at a flow rate of 0.5 ml/min. Fractions (0.5 ml each) were collected.

2.5. Endogenous lipid substrate

Undifferentiated HL-60 cells were washed, suspended in phosphate-buffered saline, sonicated for 1 min, and centrifuged for 10 min at 1000×*g*. Then, the supernatant was centrifuged for an additional 30 min at 100 000×*g*. The pellet was extracted with chloroform-methanol (2:1, v/v) by the modified procedure of Bligh and Dyer [16] including acidification with HCl. The lipid mixture was dried, weighed, and suspended at 50 mg/ml (w/v) in chloroform-methanol. This endogenous lipid mixture (ELM) was used as a substrate for lipase assay.

2.6. PLA assay

For non-radioactive assay, the reaction mixture (250 μl) contained 20 μM chemically defined phospholipid or 300 μg/ml ELM, 20 mM Tris-HCl at pH 8.0, 2 mM EDTA, 8 mM $MgCl_2$, 0.4 mg/ml of bovine serum albumin, 20% glycerol, and enzyme. The lipid vesicles were prepared by sonication on ice for 2 min in the assay buffer. After incubation for 30 min at 37°C, the reaction was terminated by adding 1.25 ml of Dole reagent [17].

For radioactive assay, the phospholipid substrate was evaporated under N_2, sonicated, and added to the reaction mixture at a final concentration of 20 μM. After incubation for 30 min at 37°C, lipids were extracted by the method of Bligh and Dyer [16].

2.7. FA and phospholipid determinations

Non-radioactive FAs were determined with an HPLC system using ADAM as described [11] except that FAs were separated on an Octyl 80-Ts column (4.6 mm×250 mm, Tosoh, Tokyo).

Radioactive lipids were separated by TLC on a Merck silica gel 60 plate with chloroform/methanol/water, 65/25/4 (v/v/v) as a solvent [18]. Radioactivity of each lipid was determined with a BAS-2000 Bioimage analyzer (Fuji, Tokyo).

2.8. Other procedures

Protein was determined by the method of Bradford [19] using bovine serum albumin as a standard. Immunoblot analysis of $cPLA_2$ was made as described [20].

3. Results

3.1. Release of fatty acids

Electro-permeabilized HL-60 cells released various FAs spontaneously, and this reaction was significantly enhanced by the addition of GTP-γ-S, ATP and vanadate at pH 8.4 as described [11] (Table 1). The release of OA far exceeded that of AA, and some saturated FAs were also released. When the cells were disrupted, the FA release from endogenous lipids became more predominant, but GTP-γ-S, ATP and vanadate showed no effect. The cytosol per se was inert, but could release FAs from ELM, suggesting the existence of enzyme(s) responsible for this FA release reaction in the cytosol.

Table 1
Release of various fatty acids from endogenous lipids

	Fatty acids released (pmol/min/10^7 cells)			
	Permeabilized cells		Cell-free system	
Activator	−	+	−	+
Palmitic acid	11.4	63.1	103.8	78.3
Stearic acid	8.9	36.1	74.3	66.8
Oleic acid	37.3	143.9	137.4	118.6
Linoleic acid	5.2	20.7	24.7	18.7
Linolenic acid	4.7	15.7	22.0	15.5
Arachidonic acid	8.5	38.3	58.7	48.2

HL-60 cells were electro-permeabilized as described [11] in the presence of 1 mM $CaCl_2$ and 1 mM EGTA at pH 8.4 with or without activator (250 μM ATP, 25 μM GTP-γ-S, and 100 μM vanadate), and incubated at 37°C for 60 min. For cell-free system, cells were suspended in the same buffer, sonicated for 1 min, and incubated with or without activator at 37°C for 20 min. The FAs released were extracted and determined as described.

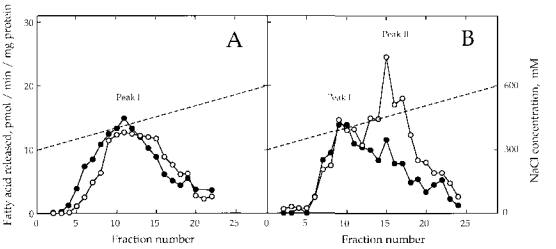

Fig. 1. Two major fractions for fatty acid release from endogenous lipid mixture (ELM). Cytosolic supernatant was prepared from (A) undifferentiated or (B) 3-day differentiated HL-60 cells, fractionated by MonoQ HR5/5 chromatography (pH 8.0 at 4°C). Fractions (0.5 ml each) obtained were assayed with ELM as substrate for the release of OA (open symbols) and AA (closed symbols).

3.2. Two major fractions for fatty acid release

The cytosol from differentiated cells released more OA from ELM than the cytosol from undifferentiated cells. Both cytosols were separately subjected to MonoQ column chromatography, and assayed for the ability to release FAs from ELM (Fig. 1) and from structurally defined phospholipids as substrates (Fig. 2). The results indicate that the cytosol contained apparently two major fractions, peak I and peak II, which were able to release FAs from phospholipids. Peak I released AA as well as OA, and did not appear to show much difference in its specific activity between the differentiated and non-differentiated cells. On the other hand, peak II released predominantly OA rather than AA, and increased its specific activity during cell differentiation.

3.3. Peak I contains $cPLA_2$

Peak I contained the well-defined enzyme, $cPLA_2$, since this fraction preferentially cleaved AA from 1-stearoyl-2-arachidonoyl-PtdCho (Fig. 2), and its reaction required a millimolar order of Ca^{2+} concentrations to reveal its maximal activity.

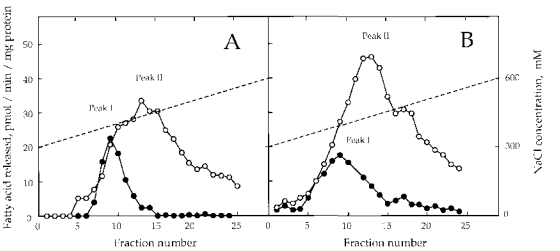

Fig. 2. Two major fractions for fatty acid release from chemically defined phospholipid. Cytosolic supernatant was prepared from (A) undifferentiated or (B) 3-day differentiated HL-60 cells, fractionated by MonoQ HR5/5 chromatography (pH 8.0 at 4°C). Fractions (0.5 ml each) obtained were assayed for the release of OA (open symbols) and AA (closed symbols) with 20 μM dioleoyl-PtdEtn or 20 μM 1-stearoyl-2-arachidonoyl-PtdCho as substrate, respectively. 2 mM $CaCl_2$ was added when assayed with the latter substrate.

Table 2
Characterization of the phospholipase A activity of peak II with radiolabeled substrates

Substrate	1,2-Dioleoyl-Ptd[^{14}C] PtdEtn	1-Palmitoyl-2-[^{14}C]linoleoyl-PtdEtn	
	LysoPtdEtn	LysoPtdEtn	Linoleic acid
Control	0.33	0.12	0.02
Peak II	2.32	0.76	1.49

The PLA activity of partially purified peak II was determined in the absence of Ca^{2+} (with 2 mM EDTA). The reaction was carried out as described in Section 2. The control incubation was made without enzyme fraction. After incubation for 30 min, lipids were extracted and separated by TLC. The radioactivity corresponding to lysophospholipids and free FA was determined. Results are expressed as percentage of the total radioactivity in each assay.

Also, peak I fraction reacted with anti-cPLA$_2$ antibody in immunoblot analysis. This peak I enzyme was inhibited by AACOF$_3$, a cPLA$_2$ inhibitor, but not by BEL, an iPLA$_2$ inhibitor [10].

3.4. Peak II contains iPLA$_2$

To characterize the FA release reaction in peak II, an enzyme responsible for the production of OA was purified partially with dioleoyl-PtdEtn as substrate over 1200-fold from the cytosol of differentiated cells by sequential column chromatographies on TSK SuperQ-5PW, TSK Phenyl-5PW, and TSK heparin-5PW. The overall yield was approximately 5% starting from the crude cytosol.

With chemically defined radioactive PtdEtn as substrate, the enzyme preparation was shown to cleave FA at position 2 as well as FA at position 1 (Table 2). In addition to this PLA$_2$/A$_1$ activity, the enzyme preparation also showed a weak lysophospholipase activity (Table 3). The activity to release FAs in this peak II fraction was insensitive to Ca^{2+}, and was not affected by the addition of EGTA.

3.5. Lipid specificity

The enzyme preparation described above cleaved PtdEtn most rapidly, and lyso-PtdEtn slowly (Table 3). PtdOH having OA did not serve as a substrate. PtdCho and other phospholipids including PtdInt and PtdSer were practically inert. Among various FA moieties, OA was cleaved most rapidly, whereas AA and linoleic acid were released very slowly. The catalytic activity described above was slightly sensitive to BEL but not to AACOF$_3$.

4. Discussion

The results presented above seem to indicate that HL-60 cells contain a soluble Ca^{2+}-insensitive PLA$_2$ which releases unsaturated FAs, preferentially OA rather than AA, most rapidly from PtdEtn. Although it is premature to discuss the specificity of this enzyme, it also shows PLA$_1$ as well as lysophospholipase activity. PtdOH and PtdCho are very poor substrates. The iPLA$_2$ recently cloned and identified from CHO cells and mouse macrophages by Tang et al. [8] and Balboa et al. [9] cleaves PtdOH and PtdCho rapidly in a mixed micelle assay. The CHO cell-derived PLA$_2$ purified by Wolf and Gross [21] shows PLA$_2$ as well as PLA$_1$ and lysophospholipase activities in a vesicle assay. This enzyme cleaves AA from PtdCho, and interacts with ATP and calmodulin as was described for the 40 kDa iPLA$_2$ from myocardium [22].

The enzyme described in this report shows properties that resemble those of PLA$_1$ purified partially from bovine brain cytosol by Pete et al. [23] which cleaves the acyl groups from the *sn*-1 position of PtdEtn most rapidly and PtdCho and lysoPtdCho to some extent. PLA$_1$ purified from bovine testis by Higgs and Glomset [24] appears to react with PtdOH selectively, but its recently described relation to human PtdOH-specific secretory PLA$_2$ [25] remains unknown, although both enzymes are postulated to play a role to generate a potent lipid mediator, lysoPtdOH. The apparent iPLA (A$_2$/A$_1$) activity described in this report increases significantly during the differentiation of HL-60 cells to granulocytes. During HL-60 cell differentiation by butyric acid a single lysophospholipase appears to be expressed [26]. The identity of this enzyme with that described herein is unclear. In short, numerous reports have recently appeared in the literature describing the existence of PLA$_1$/PLA$_2$ activities in the cytosol of mammalian tissues. The fatty acid preference and specificity of phospholipid polar head groups appear to depend markedly on the assay conditions, particularly lipid compositions of the substrate presented. Perhaps, sequence analysis is needed to discuss the identity or difference of various enzymes so far described.

Receptor-mediated activation of PLA$_2$ was proposed first by Axelrod et al. [27]. cPLA$_2$ has been well established to reveal its activity after phosphorylation by mitogen-activated protein kinase [5,6]. The C-terminal portion of cPLA$_2$ is multiply phosphorylated by PKC [28]. The enzyme reported here appears to be responsible for the observation described earlier [11] that, with permeabilized HL-60 and U-937 cells, various FAs, particularly OA, are released in response to GTP-γ-S, and that the release reaction is affected by ATP as well as the inhibitors of tyrosine kinase and PKC. It has been proposed that in guinea pig heart microsomes PtdEtn-hydrolyzing PLA$_1$ is directly activated by isoprenaline and guanine nucleotides [29]. Nevertheless, no definitive evidence is yet available that any iPLA$_2$ is coupled to receptor stimulation. Further exploration may provide the answer to the question whether the GTP-γ-S-induced release of OA from the permeabilized

Table 3
Substrate specificity of PLA activity of peak II

Substrate	Fatty acid release (pmol)
None	N.D.
Dioleoyl PtdEtn	985
Dioleoyl PtdCho	71
Dioleoyl POH	10
Dioleoylglycerol	N.D.
Oleoyl-lysoPtdEtn	248
Oleoyl-lysoPtdCho	61
Oleoyl-lysoPOH	N.D.
Dilinoleoyl PtdEtn	142

The enzymatic activity of partially purified peak II obtained from 3-day differentiated HL-60 cell was determined in the presence of 0.5 mM Mg^{2+} with various lipids (20 μM) as substrate. The reaction was carried out as described in Section 2. After incubation for 30 min, FA released was extracted and determined.

cells results from its direct coupling to receptors or from an indirect secondary effect from some perturbation of the cell membrane structure by other lipid messengers or mediators.

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