Biochemistry

 Volume 38, Number 6 February 9, 1999

Articles

A Novel Phosphatidylglycerol-Selective Phospholipase A_2 from Macrophages[†]

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Received September 2, 1998; Revised Manuscript Received December 14, 1998

ABSTRACT: In our recent studies on the synthesis of bis(monoacylglycero)phosphate (BMP), we postulated that the first step involved a PLA_2 that cleaved the 2-acyl group from phosphatidylglycerol (PG). In the present study, a novel lysosomal PLA_2 was partially purified and characterized from RAW 264.7, macrophage like cells. Cells were homogenized and delipidated, and the PLA_2 activity in the soluble fraction was purified by Sephacryl S100 and DEAE Sephacel. Further purification was performed using Con-A Sepharose, Phenyl Sepharose, DEAE Sephacel, and Superdex 75 FPLC. The enzyme at this stage of purification showed a dominant band around 45 kDa plus several minor bands on SDS−PAGE. The molecular mass determined by Superdex 75 column FPLC was about 45 kDa. The highly purified fraction hydrolyzed at the *sn*-1 position, implying that this PLA_2 also has some intrinsic PLA_1 activity. This enzyme preferentially hydrolyzed PG, has an acidic pH optima, and does not require divalent metal ions. Comparison using PG with various acyl chains on the *sn*-2 position showed that oleate and linoleate were preferred relative to arachidonate. MAFP, a known cytosolic PLA_2 inhibitor, strongly inhibited this PLA_2 activity. MJ33, $AACOCF_3$, DENP, and Amiodarone also gave moderate inhibition. The characteristics of this enzyme showed this to be a new type of PLA, and the overwhelming preference for PG as substrate suggests its physiological role is in the biosynthesis of BMP.

We postulated that the initial step in the synthesis of BMP[1] involves a PLA_2 that cleaved the 2-acyl group from PG (*1*). Our original studies were done using rabbit alveolar macrophages (*2*), and recently we have used the transformed murine macrophage cell line RAW 264.7 as a model. We postulated that the alveolar macrophage, in contact with lung surfactant, took up PG from the surfactant and specifically converted it to BMP. Current evidence indicates that the reactions responsible for the conversion of PG to BMP are in the endosomal/lysosomal compartment of the macrophage (*3*). The enzymes isolated thus far, the PLA_2 and a LPG-specific transacylase, have optimal activity in the acidic pH range, in support of their lysosomal origin. These two enzymes were separated from each other using a gel filtration and some of their properties described (*4*). It has been known since 1973 that alveolar macrophages contain Ca^{2+}-independent lysosomal PLA_1 and PLA_2. Although the lysosomal PLA_1 has been purified and characterized by us (*5*) and others (*6*), little work on the PLA_2 had been done until the groups of first Heath (*7*) and then Fisher (*8*) undertook its pursuit. The lysosomal PLA_2 is unique when compared with the other PLA_2's described thus far (*9*, *10*). Wang et al. (*11*) reported

[†] This work was supported by National Institutes of Health Grants HL 50395 and CA 12197.

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10.1021/bi982123q CCC: $18.00
Published on Web 01/22/1999

the purification of a Ca^{2+}-independent PLA_2 from lung homogenates that was optimally active at pH 4−4.5 and inhibited by the serine-targeted lipid analogue MJ33. The PLA_2 had an apparent mass of 15 kDa and an N-terminal sequence distinct from other known PLA_2's. Recently, another paper appeared from the group of Fisher on the human c-DNA clone for a lysosomal PLA_2 whose mass is 26 kDa (*12*). Although its properties more closely agree with our results presented here than their earlier report (*11*), the enzyme in our present study has far higher specific activity and different substrate specificity, inhibition susceptibility, and molecular weight. Our work shows that this PLA_2 has a strong preference for PG, supporting its role in the synthetic pathway for BMP from PG.

EXPERIMENTAL PROCEDURES

Materials. RAW 264.7 (murine monocytic, macrophage-like, Abelson leukemia virus transformed BALB/c) cells were obtained from American Type Culture Collection. Tissue culture medium was from GIBCO (Gaithersburg, MD), and fetal bovine serum was from Flow Laboratories (McLean, VA). [9,10,12,13-^{3}H]Linoleic acid and [1-^{14}C]linoleic acid were purchased from American Radiolabeled Chemicals (St. Louis, MO). Sephacryl S100, DEAE Sephacel, Con-A Sephacel, Phenyl Sepharose Fast 6, and Superdex S75 were from Pharmacia (Picataway, NJ). All nonradiolabeled lipid substrates were purchased from Avanti (Alabaster, AL). Silica G and H plates were from Analtech (Newark, DE). Centriprep 10 protein concentrators were purchased from Amicon (Denver, MA). $AACOCF_3$, MAFP, and BEL were from Biomol (Plymouth Meeting, PA). Amiodarone, DTT, and DENP were purchased from Sigma (St. Louis, MO). MJ33 was the generous gift of Prof. Mahendra Jain, University of Delaware.

Preparation of Radiolabeled Substrates. 1-Acyl-2-[9,10,12,13-^{3}H]linoleoyl-PG (2-[^{3}H]PG) and 1-alkyl-2-[14C]linoleoyl-PG (alkyl-2-[14C]PG) were synthesized from corresponding LPC and [^{3}H] or [14C]linoleic acid using rat liver microsomes followed by PLD-catalyzed transphosphatidylation of the labeled PC's with glycerol as previously described (*4*). The synthesis of PLPG and PAPG was also accomplished using a PLD-catalyzed transphosphatidylation reaction from PLPC and PAPC, respectively.

1-Acyl-2-[^{3}H]linoleoyl-PC (2-[^{3}H]PC), 1-acyl-2-[^{3}H]linoleoyl-PE (2-[^{3}H]PE), and 1-acyl-2-[^{3}H]linoleoyl-PI (2-[^{3}H]-PI) were prepared by the following method. [^{3}H]Linoleic acid (50 μCi) was incubated with RAW 264.7 cells in a 150 cm^2 flask at 37 °C for 30 min. After the medium was removed and the cells were washed with saline, the lipids in the cells were extracted and separated by acidic TLC (chloroform/methanol/acetic acid 63:35:8, v/v/v) (*4*). The identification of each phospholipid was based on the R_f value of each relative to standard phospholipids. 2-[^{3}H]PI was purified again by basic TLC (chloroform/methanol/ammonium hydroxide 65:35:5, v/v/v). 1-Acyl-2-[^{3}H]linoleoyl-PA (2-[^{3}H]-PA) was prepared from 2-[^{3}H]PC using PLD (*13*) and isolated using the basic TLC system. The purity of each phospholipid was determined by TLC, and the lipid phosphorus was quantitated by the method of Chalvardjian and Rudnicki (*14*). Each *sn*-3-labeled phospholipid was treated by snake venom, and more than 94% of the radioactivity was found with free fatty acid, showing that the [^{3}H]linoleic acid was primarily at the *sn*-2 position of each phospholipid.

Samples of *sn*-1:*sn*-1′ BMP and *sn*-1:*sn*-3′ BMP were synthesized from [1,2,3-^{3}H]PG using RAW cells and RAW cells homogenate, respectively, as described before (*1*, *4*).

Enzyme Purification. RAW 264.7 cells were harvested from suspension cultures, homogenized, and delipidated with 1-butanol as previously described (*4*). Between 5×10^8 and 10×10^8 cells were used for experiments for determination of the catalytic properties, and $(4-8) \times 10^9$ cells were used for enzyme purification. The delipidated homogenate was loaded onto a Sephacryl S-100 column (100 cm × 2.5 cm) that was equilibrated and eluted with 10 mM Tris buffer, pH 7.4, containing 50 mM NaCl. The fractions from the column were assayed for PLA_2 activity, and the active fractions were pooled and dialyzed against 10 mM Tris buffer, pH 7.4, to remove NaCl. The preparation was then loaded onto a DEAE Sephacel column (30 cm × 1.6 cm) equilibrated with 10 mM Tris buffer. The column was washed with 10 mL of Tris buffer and eluted with a linear gradient of NaCl (0.1−0.225 M) at a flow rate of 30 mL/h. Fractions were assayed for PLA_2 activity, and active fractions were pooled.

For further purification, the pooled active fractions from the Sephacryl S100 column were loaded onto a Con-A Sepharose column which was equilibrated with 10 mM Tris, pH 7.4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (running buffer). After washing with 10 mL of 0.5 M NaCl in the running buffer, the enzyme was eluted with 0.5 M methyl α-D-mannopyranoside in the running buffer. After dialyzing against 10 mM Tris, pH 7.4, the active fractions were loaded on a Phenyl Sepharose 6 Fast Flow column equilibrated with 1.0 M ammonium sulfate in 10 mM Tris, pH 7.4. A linear gradient elution from 1.0 to 0 M ammonium sulfate was used to elute PLA_2. The active fractions were pooled and dialyzed against 10 mM Tris, pH 7.4. This fraction was used as the enzyme source to compare acyl chain preference. For further purification, this preparation was then loaded onto a DEAE Sephacel column (0.5 cm × 1.0 cm) for concentration. After the column was washed with 10 mM Tris, pH 7.4, the PLA_2 was eluted with a small volume (2.0−3.0 mL) of 10 mM Tris, pH 7.4, containing 0.225 M NaCl. This fraction was further concentrated to about 500 μL using a Centricon 10 centrifuge concentrator. The final step of the purification was performed using Superdex S 75 FPLC. The active fraction (250 mL) was injected onto the column equilibrated with 50 mM Tris, pH 7.4, 150 mM NaCl, and 5 mM EDTA and eluted with the same buffer at a flow rate of 0.5 mL/min.

SDS−PAGE. After each purification step, an aliquot of the preparation was analyzed by SDS−PAGE (12%) and

[1] Abbreviations: BMP, bis(monoacylglycero)phosphate; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; LPG, lysophosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac* -glycerol; PLPG, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phospho-*rac*-glycerol; PAPG, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phospho-*rac*-glycerol; DPPG, 1-palmitoyl-2-palmitoyl-*sn*-glycero-3-phospho-*rac*-glycerol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CTMB, cetyltrimethylammonium bromide; $AACOCF_3$, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; DENP, diethyl *p*-nitrophenyl phosphate; MAFP, methyl arachidonoyl fluorophosphonate; DTT, dithiothreitol; pBPB, *p*-bromophenacyl bromide.

Table 1: Typical Purification of the PLA_2 from RAW 264.7 Cells[a]

step	total protein (mg)	total activity (nmol)	yield (%)	specific activity [nmol min^{-1} (mg of protein)$^{-1}$]	purification (*n*-fold)
homogenate	252	4536	100	0.4	1
delipidated homogenate	66	6534	144	2.2	6
Sephacryl S100	5.3	5008	110	21	53
Con-A Sepharose	0.18	1441	32	178	445
Phenyl Sepharose	0.036	513	11	317	792
DEAE Sephacel	0.008	208	5	580	1450

[a] The data were obtained from a single, representative enzyme preparation starting with 5×10^9 cells. Enzyme activity was assayed using 2-[^{3}H]linoleoyl-PG as a substrate. Total activity is nanomoles hydrolyzed under standard assay conditions.

visualized with silver staining.

PLA_2 Assay. Radiolabeled substrate (200 μM) was sonicated in 250 μL of 100 mM sodium acetate, pH 4.5, 5.0 mM mercaptoethanol, and the enzyme fraction (250 μL) was added. The incubation was carried out at 37.0 °C for 45 min. Previous work had shown that hydrolysis was linear over this time if hydrolysis did not exceed about 35% of total substrate. Products were extracted and separated by acidic TLC, and the radioactivity was determined by liquid scintillation counting. PLA_2 activity was assayed by the amount of [^{3}H]LPG produced and is expressed as percent of substrate or nanomoles substrate hydrolyzed.

PLA_2 Assay Using Mass Spectrometry. In the experiment comparing fatty acid hydrolysis at the *sn*-1 and *sn*-2 positions, the quantitation of products was performed using mass spectrometry. Before the extraction of total lipids from the incubation mixture, margaric acid (17:0) (10 nmol) was added to each sample as an internal standard. The extracted lipids were analyzed by mass spectrometer (MS/MS system, Quattro II, Micomass, Beverly, MA). The amount of each fatty acid was determined by the ratio of the response to that of a 17:0 internal standard. The relative response of each fatty acid was established in advance for quantitation.

RESULTS

The first step in the purification scheme using Sephacryl S-100 filtration is a modification of the previously used procedure to separate the PLA_2 from the transacylase (*4*). The PLA_2 activity eluted from the Sephacryl S-100 column in the sharp peak with a small percentage of protein (Figure 1A). The majority of PLA_1 activity, measured by the formation of [^{3}H]LPG, eluted with the late fractions. The fractions with high PLA_2 activity were pooled, dialyzed, and chromatographed on a Con-A column. Most of the proteins passed though the column without binding, or were eluted from the column with 0.5 M NaCl. The activity of PLA_2 was found on the trailing edge of the small protein peak eluted with methyl α-D-mannopyranoside (Figure 1B). In the next step, the PLA_2 was adsorbed to a Phenyl Sepharose column and eluted after the ammonium sulfate concentration of the gradient elution buffer reached zero, (Figure 1C). These elution profiles from Con-A and Phenyl Sepharose columns indicated that this PLA_2 is a hydrophobic and glycosylated enzyme. After concentration on the DEAE column, the specific enzymatic activity was 580 nmol min^{-1} (mg of protein)$^{-1}$ and was about 1450-fold purified relative to the 1-butanol extracted homogenate (Table 1). To estimate size, the enzyme was chromatographed by FPLC (Figure 2A). Because of extremely low protein concentration, the total and specific activity of the fraction from the Superdex

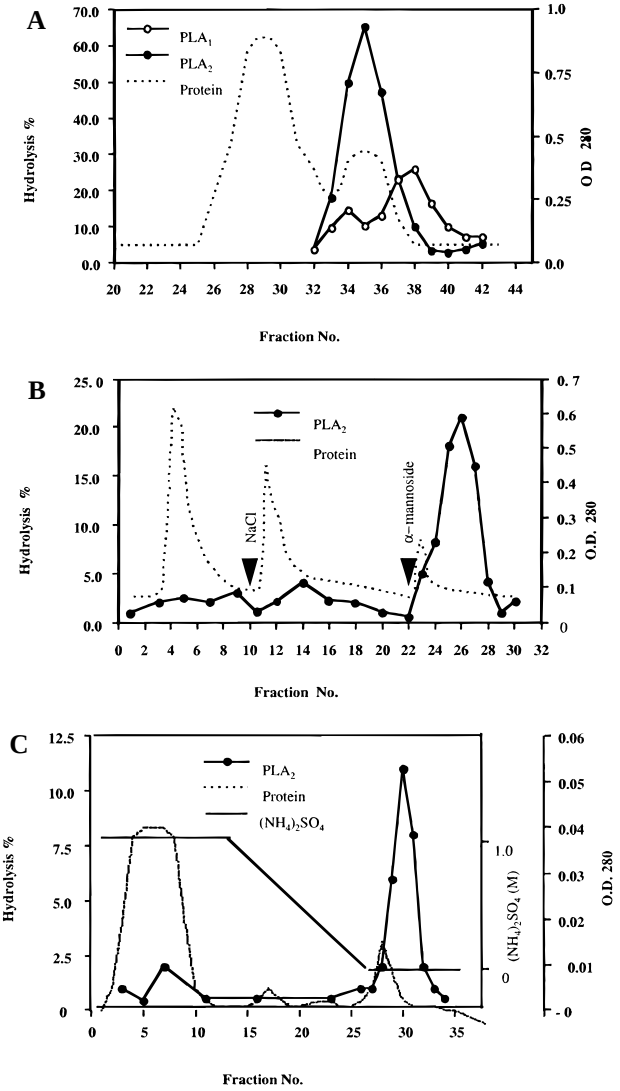

FIGURE 1: Column chromatography profiles of PLA_2 from RAW cells. (A) Sephacryl S100; (B) Con-A Sepharose; (C) Phenyl Sepharose Fast 6. PLA_2 activity obtained with 2-[^{3}H]linolate-labeled PG as a substrate is shown by closed circles and the absorbance of the protein at 280 nm is shown by a dotted line. Details of the purification procedure are under Experimental Procedures.

S75 column could not be determined. The molecular mass calculated for the PLA_2 based on a calibration curve of Superdex 75 with three known protein standards was 45 kDa (Figure 3). This corresponds to a Stokes' radius of 5.31 nm based on the average radii published for the protein standards (*15*). SDS−PAGE analysis of active fraction from Superdex S75 displayed a dominant band around 45 kDa, and a few weak bands between 30 and 45 kDa were seen when silver staining was utilized (Figure 2B). Until final purification of

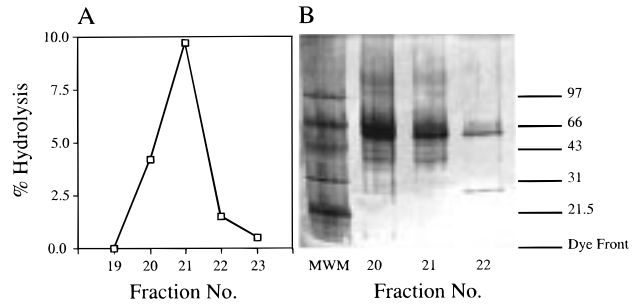

FIGURE 2: SDS−PAGE and PLA_2 activity of fractions from Superdex S75 chromatography. (Panel A) An aliquot of individual fractions was assayed under standard assay conditions (only active fractions shown). (Panel B) Another aliquot of the active fractions was analyzed by 12% SDS−PAGE. MWM, molecular weight markers; 20, 21, and 22, fractions 20, 21, and 22 from the Superdex S75 column, respectively.

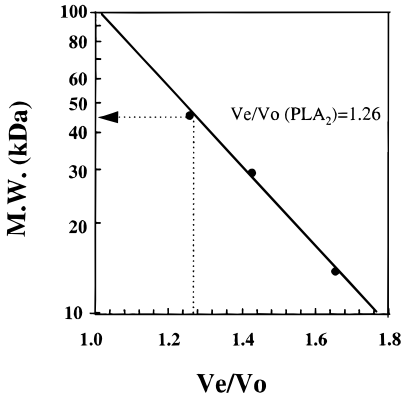

FIGURE 3: Calibration curve from Superdex S75. Standard proteins (ovalbumin 45 kDa; carbonic anhydrase 29 kDa; ribonuclease A 13.7 kDa), were applied to the column and eluted with the running buffer at a flow rate of 0.5 mL/min. The *x* axis shows the ratio of the elution volume of the proteins to that of the void volume (8.3 mL). The elution volume (10.5 mL) of the PLA_2 yields a mass of about 45 kDa.

the PLA_2 is achieved, the molecular size of the protein remains open.

For most experiments used to characterize the PLA_2, we used enzyme purified by Sephacryl S-100 and DEAE Sephacel chromatography. The PLA_2 was eluted with 0.2 M NaCl, based on extrapolation of the NaCl gradient (Figure 4). A lower level of PLA_1 activity was seen to coelute with the PLA_2. The hydrolysis of the 1-acyl moiety was seen at all steps of the enzyme purification including the preparation obtained after the Superdex 75 chromatography. The PG concentration dependency was examined using 20−400 μM PG and 45 min incubation time (Figure 5). A double-reciprocal plot of the substrate dependency yielded a linear plot from which apparent "K_m" and "V_{max}" values could be calculated: "K_m" = 147 μM and "V_{max}"= 500 nmol min^{-1} (mg of protein)$^{-1}$. It is recognized that these are conventional calculations and do not take into account the complexities of kinetic analysis of lipid interfacial enzymes (*16*).

The effect of pH was examined using sodium acetate buffer (pH 3.0−6.0) or Tris buffer (pH 6.0−10.0). The PLA_2 activity was highest at pH 4.5, and there was no activity seen with pH 6.0 or higher (data not shown). The enzyme was fully active in the presence of 5 mM EDTA, and most metal ions had little, if any, effect on hydrolysis (data not shown). Calcium ions had a slight inhibitory effect, similar

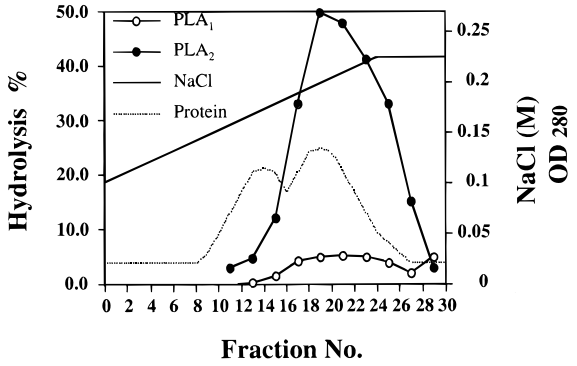

FIGURE 4: Typical chromatography profile of lysosomal PLA's on the DEAE column. Pooled and dialyzed active fractions from the Sephacryl S100 column were loaded on the DEAE Sephacel column preequilibrated with 10 mM Tris, pH 7.4. The proteins were eluted by gradient elution of NaCl (0.1−0.225 M, indicated by the solid line). The activity of PLA_1 (open circles) and PLA_2 (closed circles) in each fraction was determined using 2-[^{3}H] PG as described under Experimental Procedures.

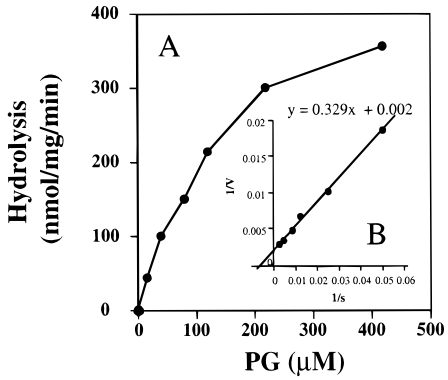

FIGURE 5: Effect of substrate concentration on PLA_2 activity. 20−400 μM PG was incubated with semi-purified PLA_2 (2 μg) for 45 min at 37.0 °C. (B) Lineweaver plot shows the linearity from which apparent K_m and V_{max} values could be calculated: "K_m" = 147 μM and "V_{max}" = 500 nmol min^{-1} (mg of protein)$^{-1}$.

to our findings with the lysosomal PLA_1 (*5*, *17*). In that case, and probably with the PLA_2, the inhibition is the result of Ca^{2+} interaction with the anionic substrate.

The effect of detergents on hydrolysis was tested prior to carrying out substrate specificity studies. Since different phospholipids assume various physical states (*16*), especially in the acidic pH range, we believe it to be most informative to use mixed micelles for substrate specificity analysis. We found that most detergents used with PG as substrate were inhibitory under the conditions chosen. These include Triton X-100, Triton N-101, deoxycholate, and CTMB (Figure 6). On the other hand, both CHAPS and Tween 20 were stimulatory. A concentration study of Tween 20 showed that optimal activation was seen at about 60−200 μM and amounted to a 1.5-fold stimulation (cmc of Tween 20 = 60 μM) (*18*). Substrate specificity studies were then carried out using an equal mixture of lipid and detergent (200 μM). As seen in Table 2, the enzyme has an overwhelming preference for PG as substrate. Under these conditions, 51.2 nmol of PG was hydrolyzed while no more than 1.0 nmol of PE and PC, or a few nanomoles of PA and PI, was attacked. Thirty-four nanomoles of alkyl-PG was hydrolyzed, showing that the replacement of the 1-acyl with a 1-alkyl group had only a slight effect on hydrolysis. The PLA_2 showed little hydrolysis on *sn*-1:*sn*-1′ BMP and *sn*-1:*sn*-3′ BMP. Using

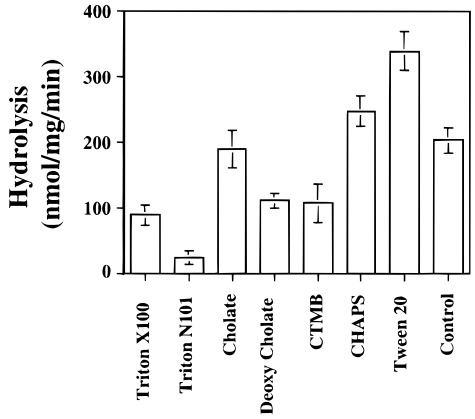

FIGURE 6: Effect of detergent on PLA_2 activity. The effect of various detergents (200 μM) on PLA_2 activity was examined. Each detergent was dissolved with PG in 100 mM sodium acetate, 50 mM mercaptoethanol, pH 4.5. Then enzyme fraction was added, and the mixture was incubated for 45 min. Mean values and standard errors of triplicated sample are shown.

Table 2: Substrate Preference of PLA_2[a]

substrate	nmol hydrolysis
PG	42.3 ± 2.7
1-alkyl-PG	34.9 ± 1.7
PC	0.8 ± 0.17
PE	0.2 ± 0.1
PA	4.9 ± 0.8
PI	2.2 ± 0.4
sn-1:*sn*-1′ BMP	1.1
sn-1:*sn*-3′ BMP	3.2

[a] 2-[^{3}H]Linolate-labeled substrates were prepared as described under Experimental Procedures. Each substrate (200 μM) was mixed with Tween 20 (200 μM) and incubated with PLA_2 for 45 min at 37.0 °C. Enzyme activity was calculated by the release of [^{3}H]linoleic acid from the *sn*-2 position, and the mean values and standard errors of results from three different enzyme preparations are shown except for the values of BMP's. Hydrolysis of BMP's are the mean values of two different enzyme preparations.

Table 3: Acyl Chain Preference of PLA_2[a]

	hydrolysis %		
substrate	*sn*-1	*sn*-2	total
POPG	palmitate	oleate	17.4 ± 8.8
	4.1 ± 2.2	12.7 ± 6.6	
PLPG	palmitate	linolate	20.7 ± 8.6
	6.9 ± 2.8	13.8 ± 5.8	
PAPG	palmitate	arachidonate	17.3 ± 11.8
	11.8 ± 10.6	1.9 ± 1.3	
DPPG	palmitate		11.6 ± 2.1
	11.6 ± 2.1		

[a] Each species of PG (100 nmol) was incubated with Phenyl Sepharose purified PLA_2 for 45 min. The products were analyzed utilizing mass spectrometry, and hydrolysis was determined based on the ratio to the internal standard. Values shown here are means ± SDs from triplicated samples from one enzyme preparation, and similar results were obtained using different enzyme preparations.

enzyme purified by Phenyl Sepharose chromatography, the acyl chain preference was examined. The PLA_2 hydrolyzed oleate and linolate at the *sn*-2 position at equal rates whereas arachidonate was hardly cleaved (Table 3). Significant hydrolysis was also seen at the *sn*-1 position. Interestingly, the hydrolysis at the *sn*-1 position was highest when arachidonate was present at the *sn*-2 position. The combined hydrolysis at the *sn*-1 and *sn*-2 positions was similar among all the PG's except the dipalmitoyl species. Hydrolysis at the *sn*-1 position was observed with the enzyme purified by Superdex S-75 FPLC. It is likely, therefore, that the enzyme has intrinsic PLA_2 activity.

Table 4: Effect of Various Inhibitors on PLA_2 Activity[a]

inhibitor	concn (μM)	% of control
$AACOCF_3$	10	95 ± 2.5
	100	49 ± 6.3
DENP	10	61 ± 6.3
	100	18 ± 2.5
Amiodarone	10	95 ± 2.0
	100	54 ± 2.5
DTT	2000	78 ± 5.1
BEL	10	102 ± 3.4
	100	88 ± 8.3
MAFP	10	2.6 ± 0.5
	100	1.4 ± 0.8
pBPB	20	82 ± 10.3
	2000	52 ± 12.0
control		100

[a] $AACOCF_3$, DENP, Amiodarone, and DTT were added to 2-[^{3}H]linoleoyl-PG (200 μM) and then added to PLA and incubated for 45 min at 37.0 °C. BEL and MAFP were preincubated with PLA_2 in Tris buffer, pH 7.4, for 5 min and then added to 2-[^{3}H]linoleoyl-PG (200 μM). pBPB was preincubated with enzyme for 10 min. The percentage was calculated as the specific activity of the positive control [minus inhibitor, 374−666 nmol min^{-1} (mg of protein)$^{-1}$] to that obtained with the inhibitors. The mean values of two or three different enzyme preparations are shown.

To better understand the mechanism of this PG-specific PLA_2, several known PLA_2 inhibitors were tested. $AACOCF_3$, DENP, or Amiodarone was added to PG in buffer prior to the addition of the PLA_2 whereas BEL and MAFP were preincubated with enzyme for 5 min at 37.0 °C before the incubation with substrate. The pBPB was preincubated with enzyme for 10 min. All the inhibitors were dissolved in DMSO, and the activity with inhibitor was compared to a control with DMSO. As seen in Table 4, MAFP was the most potent inhibitor while DENP was not quite as effective. $AACOCF_3$, Amiodarone, and pBPB inhibited at high concentrations only, and BEL and DTT did not inhibit significantly. Some of the characteristics of the PLA_2 resembled those of the acidic Ca^{2+}-independent PLA_2 reported by Kim et al. (*12*). Therefore, we also examined the effect of MJ33, a phospholipid transition-state analogue, which is reported to inhibit their PLA_2 preparation by 95% with 3 mol % of inhibitor. MJ33 showed only moderate inhibition on this lysosomal PLA_2 with an IC_{50} of 15 mol % (Figure 7).

DISCUSSION

In this study, we report the purification and characterization of an acid-active Ca^{2+}-independent PLA_2 from macrophages. Its substrate specificity gives strong support that at least one of its functions is to catalyze the first step in the conversion of PG to BMP. Previous work by us and others demonstrated that BMP is synthesized from exogenous phosphatidylglycerol by macrophages (*1*) and LPG has been shown to be an intermediate in the synthesis of BMP (*19*). In unpublished preliminary studies, we found that the PG in lung surfactant was preferentially taken up and metabolized by macrophages to BMP. These results then would account for the high content of BMP in alveolar macrophages and

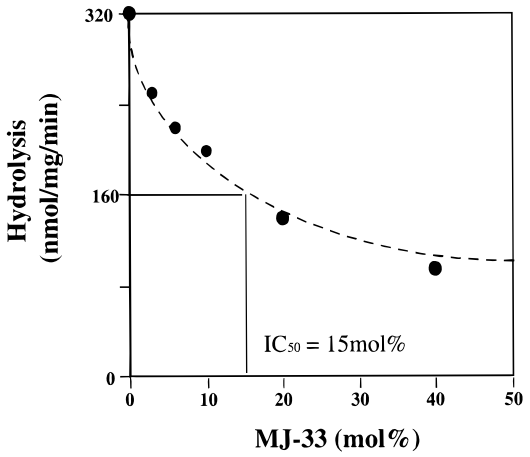

FIGURE 7: Effect of MJ33 on PLA_2. MJ33 (3−40 mol %) was incubated with 2-[^{3}H]linoleoyl-PG and partially purified PLA_2. Values were expressed as percentage of the control without the inhibitor (32% hydrolysis). Mol % is defined as ([MJ33]/([MJ33] + [PG]) × 100.

suggest that as surfactant PG is taken into the macrophage the lysosomal PLA_2 acts to hydrolyze it to LPG. The most likely PLA to degrade DPPC is active in the alkaline pH range and is stimulated by bile salts (*20*).

Some of the properties of the PLA_2 in this study are similar to the PLA_2 recently purified by Fisher's group (*12*). It is important to note significant differences, however. They used lung homogenate for their purification and found multiple forms of acidic active, Ca^{2+}-independent PLA_2. Their use of MJ33 inhibition allowed them to select a single form of the PLA_2's for purification. We, on the other had, began with a single cell type and have no evidence for additional PLA_2's active at pH 4.5. The two enzymes showed different susceptibility to PLA_2 inhibitors, especially to MJ33. Some of the purification schemes used by us were similar with one noticeable difference. Their preparation eluted from the DEAE column at about 20 mM NaCl while our enzyme required 10-fold higher NaCl for elution. A recent study indicates that the primary catalytic activity of the protein isolated by them may be a peroxidase that is localized to the cytosol (*21*).

Inhibitor studies showed that the sensitivity of our enzyme preparation is unique among known PLA_2's. MAFP, thought to phosphorylate the active site serine-residue (*20*), showed the most potent inhibition. DENP, another serine-directed inhibitor, also inhibited this enzyme at 10−100 mM. These results suggests that serine is involved in the active site of this enzyme. Inhibition by DENP was also reported by Kim et al. (*12*) with the PLA_2 activity of the expressed protein from lung although higher concentrations (500 μM) were used. $AACOCF_3$, which presumably binds the active site of c-PLA_2, inhibited our enzyme but did not significantly inhibit the PLA_2 by Kim et al. at the same concentration (100 μM). It is of interest that Amiodarone, reported to inhibit lysosomal PLA in vivo (*21*), inhibited our PLA_2 activity in dose dependent manner.

It is clear from the work of Kim et al. and our lab that a new class of lysosomal PLA_2's has emerged even though its existence has been known for over 2 decades (*2*, *7*, *23*). Kim et al. (*12*) have shown by Northern blot analysis that the mRNA is present in granular pneumocytes. It will be of considerable interest to establish the range of distribution of the enzyme described here. The function of this enzyme in lysosomes, like that of the PLA_1 (*5*), may relate to the processing of internalized membranes and phagocytized particles in addition to its postulated role in the alveolar macrophage to degrade surfactant. It has been shown that cardiolipin, derived from mitochondria, can serve as a precursor of BMP, presumably with PG and/or LPG as an intermediate (*24*, *25*). If our hypothesis is correct that this PLA_2 is obligatory in the synthesis of BMP from PG, we predict that, at a minimum, it will be found in phagocytic cells that degrade bacterial lipids, a component of which is PG. Since PG with arachidonate was a poor substrate, it is not likely this PLA_2 is involved in eicosanoid production.

In this study, the lysosomal PLA_1 and PLA_2 were separated on the first gel filtration column. Since the PLA_2 preparation showed hydrolysis at the *sn*-1 position through all purification steps, we believe this PLA_2 has intrinsic PLA_1 activity, similar to the properties of the cytosolic PLA_2 (c-PLA_2). The finding that the acyl composition can influence activity at the *sn*-1 and *sn*-2 positions could complicate substrate class studies if the phospholipid substrate differed significantly. We do not believe that to be the case here since the PG used here was derived from rat liver PC, known to contain oleic and linoleic acid residues at the *sn*-2 position. PA was derived from PG and therefore should have an identical acyl composition as PG. Since PE and PI are expected to have a somewhat higher content of arachidonic acids and other polyunsaturates, their hydrolysis, shown in Table 2, could be reduced. However, in situ, both PI and PE would be poor substrates based on both the acyl composition and headgroup characteristics. In this regard, both enzymes are similar to the known PLB (*26*). Until more is known about the catalytic mechanism of this lysosomal PLA_2, the final nomenclature should remain open.

The lysosomal phospholipases A_1 and A_2 have a number of features in common. Both are active at pH 4.0−4.5 and are inhibited by Ca^{2+}. The PLA_1 has multiple sizes most likely due to variable states of glycosylation (*27*). Both PLA_1 and PLA_2 under the appropriate conditions, prefer PG as substrate (*17*). However, as we showed with PLA_1, when assayed on neat substrate, PLA_1 is most active on PE with little activity on PG. In the presence of Triton X-100, the activity on PE is reduced while that on PG is increased manyfold. We found that this was due in part to the surface charge of the substrate. These factors that may regulate PLA_2 are under study in our laboratory.

ACKNOWLEDGMENT

Use of the Tissue Culture, Technical Chemistry, and Protein Analysis Core Laboratories of the Wake Forest University Comprehensive Cancer Center is gratefully acknowledged. We especially thank Dr. Mahendra Jain from the University of Delaware for generously providing us MJ33 and Dr. Mark O. Lively, Mark Morris, Dr. Mike Thomas, Dr. Roy Hantgan, and Mike Samuel from Wake Forest University for their advice and technical support.

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BI982123Q