Purification of Lysosomal Phospholipase A and Demonstration of Proteins That Inhibit Phospholipase A in a Lysosomal Fraction from Rat Kidney Cortex†

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Received January 2, 1986; Revised Manuscript Received April 14, 1986

ABSTRACT: Phospholipase A has been isolated from a crude lysosomal fraction from rat kidney cortex and purified 7600-fold with a recovery of 9.8% of the starting activity. The purified enzyme is a glycoprotein having an isoelectric point of pH 5.4 and an apparent molecular weight of 30 000 by high-pressure liquid chromatography gel permeation. Naturally occurring inhibitors of lysosomal phospholipase A are present in two of the lysosomal-soluble protein fractions obtained in the purification. They inhibit hydrolysis of 1,2-di[1-^{14}C]oleoylphosphatidylcholine by purified phospholipase A_1 with IC_{50} values of 7–11 μg. The inhibition is abolished by preincubation with trypsin at 37 °C, but preincubation with trypsin at 4 °C has no effect, providing evidence that the inhibitors are proteins. The results suggest that the activity of lysosomal phospholipase A may be regulated in part by inhibitory proteins. Lysosomal phospholipase A from rat kidney hydrolyzes the *sn*-1 acyl group of phosphatidylcholine, does not require divalent cations for full activity, and is not inhibited by ethylenediaminetetraacetic acid. It has an acid pH optimum of 3.6–3.8. Neither *p*-bromophenacyl bromide, diisopropyl fluorophosphate, nor mercuric ion inhibits phospholipase A_1. In contrast to rat liver, which has two major isoenzymes of acid phospholipase A_1, kidney cortex has only one isoenzyme of lysosomal phospholipase A_1.

Lysosomes are involved in the intracellular processing of complex lipids that originate outside the cell, such as plasma lipoproteins (Goldstein & Brown, 1977) and pulmonary surfactant (Hallman et al., 1981; Jacobs et al., 1983). In addition, lipids from intracellular membranes may reach the lysosome by autophagy or by the action of phospholipid transfer proteins (Matsuzawa & Hostetler, 1980). Certain cationic amphiphilic drugs interfere with intralysosomal processing of complex lipids and cause the accumulation of phospholipid in lysosomes (Lüllmann-Rauch, 1979). In the kidney, aminoglycoside antibiotics concentrate in proximal tubule cell lysosomes (Morris et al., 1980), where they appear to interfere with polar lipid catabolism (Feldman et al., 1982), resulting in the formation of multilamellar bodies which accumulate in these cells in the early phase of aminoglycoside nephrotoxicity (Kosek et al., 1974).

We have previously shown that acid phospholipase A_1 is the principal enzyme of intralysosomal phospholipid catabolism in the kidney cortex (Hostetler & Hall, 1982). In order to further characterize the enzymes of phospholipid catabolism in kidney, we isolated and purified acid phospholipiase A_1 from a rat kidney lysosomal preparation. This paper describes the purification and properties of kidney lysosomal phospholipase A_1 and demonstrates the presence of inhibitory proteins in two lysosomal soluble protein fractions obtained during purification of phospholipase.

Materials and Methods

Chemicals. 1,2-Di[1-^{14}C]oleoylphosphatidylcholine, 1,2-di[1-^{14}C]palmitoylphosphatidylcholine, 2-[1-^{14}C]oleoylphosphatidylcholine, and [1-^{14}C]palmitoyllysophosphatidylcholine were obtained from Amersham, Arlington Heights, IL. The following were purchased from Sigma Chemical Co, St. Louis, MO: rabbit gamma globulin, Sephadex G-150, diisopropyl flurophosphate, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, tris(hydroxymethylamino)-methane, methyl α-mannoside, EDTA, *p*-bromophenacyl bromide, and 1-palmitoyllysophosphatidylcholine. Bio-Gel HTP (hydroxyapatite) and the Coomassie brilliant blue protein assay kit, were purchased from BioRad, Richmond, CA. Superose 12 HPLC column, PBE 94 exchanger, and Polybuffer 74 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ.

Isolation and Solubilization of Lysosomal Phospholipase A. Eighteen to thirty rats of the Fischer 344 strain were fasted overnight and killed by cervical fracture, and the kidneys were removed and placed in iced buffer A consisting of 0.25 M sucrose, 5 mM Tris (pH 7.4), and 2 mM EDTA. The tissue was excised, weighted, and rinsed in ice-cold buffer. Kidney cortex weighing 18–40 g was cut into small pieces, and a 5% homogenate in buffer A was prepared as previously described (Hostetler & Hall, 1982). The homogenate was passed through four layers of cheesecloth and centrifuged at 160*g* for 6 min. The pellet was resuspended in buffer A and recentrifuged at 160*g* for 6 min; this washing was repeated three times, and the respective supernatants were combined with the original postnuclear supernatant. The nuclear pellet was discarded, and the combined supernatants were centrifuged 20000*g* for 20 min. The pellet was taken up 70–80 mL of buffer containing 10 mM sodium phosphate buffer, pH 7.2, and 50 mM NaCl and subjected to five cycles of freezing followed by thawing. The resulting suspension was centrifuged at 20000*g* × 60 min to sediment membranous material, which was discarded. The supernatant containing the soluble proteins was diluted with 0.25 volumes of cold glycerol, and this material was purified further as noted. Protein was measured

† This work was supported in part by NIH Grants AM 32159 and GM 24979 and by the Research Service of the Veterans Administration Medical Center, La Jolla, CA.

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by the method of Bradford (1979) using rabbit gamma globulin as standard.

Purification of Phospholipase A. Hydroxyapatite/Concanavalin A–Sepharose. All operations were carried out at 4 °C unless otherwise noted. The soluble protein in 8 mM sodium phosphate (pH 7.2)/50 mM NaCl containing 20% glycerol was applied to a column of hydroxyapatite (1.6 × 20 cm) connected in series to a column (1 × 4 cm) of concanavalin A–Sepharose. The sample (approximately 60 mL) was applied to the hydroxyapatite at 20 mL/h, and the columns were washed with an equal volume of the starting buffer solution. The columns were then disconnected, and the concanavalin A-Sepharose column was eluted with 90 mL of 0.5 M methyl α-mannoside at a flow rate of 20 mL/h. The column was warmed to 20 °C and eluted with an additional 35 mL of methyl α-mannoside containing 5 mM EDTa. The methyl α-mannoside eluates were combined and taken to a small volume by using a 100-mL Amicon ultrafiltration cell with a YM-10 membrane (Amicon Corporation, Lexington, MA).

Chromatofocusing. The sample was diluted to 50 mL with 25 mM Tris (pH 7.0) containing 20% glycerol and concentrated to a small volume as noted above. This process was repeated once, and the resulting sample (approximately 4 mL) was applied to a 1 × 4 cm column of PB94 exchanger which had been equilibrated with 25 mM Tris (pH 7.0)/20% glycerol. The column eluted at 14 mL/h with 60 mL of Polybuffer 74 pH (4.0) which was diluted 1:8 with distilled water and adjusted to a glycerol concentration of 20% (w/v). Fractions of 4 mL were collected and assayed for phospholipase A activity.

Sephadex G-150. The active fractions from the chromatofocusing step were combined, concentrated rapidly to a small volume as noted above, and diluted with 0.15 M NaCl/5 mM Tris (pH 7.4)/20% glycerol. The process was repeated twice, and the sample (1.0 mL) was applied to a 1 × 30 cm column of Sephadex G-150 previously equilibrated with this buffer. The column was eluted, the active fractions were combined. The combined fractions were adjusted to 50% glycerol and stored at −60 °C until use. These preparations were stable at −60 °C for at least 8–12 months with negligible loss of activity. The time required to purify phospholipase A from the soluble protein fraction was approximately 60–72 h.

Enzyme Assays. Phospholipase A activity of the respective column fractions was measured as follows: assay mixtures containing 50 mM sodium acetate (pH 4.4), 0.4 mM di[1-^{14}C]oleoylphosphatidylcholine, and protein were incubated for 20 min at 37 °C. The release of [1-^{14}C]oleic acid was measured by using the Dole extraction (Dole, 1956) as modified by van den Bosch and Aarsman (1979), and aliquots of the heptane layer were counted by liquid scintillation spectrometry as described earlier (Hostetler et al., 1982). The sonicated small unilamellar vesicles of 1,2-di[1-^{14}C]oleoylphosphatidylcholine were prepared as previously described (Pappu & Hostetler, 1984). In some experiments 1,2-di[1-^{14}C]palmitoylphosphatidylcholine and 0.5 mg of Triton X-100/mL was used as substrate (mixed micelles). Further details are given in the respective figure legends. Lysophospholipase was assayed by measuring the release of [1-^{14}C]palmitic acid from [1-^{14}C]palmitoyllysophosphatidylcholine as previously described (Hostetler et al., 1982).

Molecular Weight Determination by HPLC Gel Permeation Chromatography. Purified phospholipase A (5 mL) from the Sephadex G-150 step (0.1 mg/mL) was diluted 1:1 with cold distilled H_2O, concentrated to a small volume in a 10-mL Amicon ultrafiltration cell with a YM10 membrane, and then further concentrated in a Centricon tube (Amicon) by centrifugation at 5000*g* for 1 h at 4 °C. The final volume was 120 μL. This material (100 μL) was injected into a Superose 12 HPLC column and eluted at room temperature by using a Beckmann HPLC (Beckman Instruments, Fullerton, CA). The elution buffer consisted of 0.15 M NaCl containing 5 mM Tris (pH 7.4) and 20% ethylene glycol (v/v), at a flow rate of 0.4 mL/min. Fractions were collected (0.52 mL) and kept at 4 °C until they were assayed for phospholipase A activity as noted above.

Trypsin Treatment of Fractions Containing the Phospholipase A Inhibitor. To study the effect of trypsin on the fractions containing putative phospholipase A inhibitors, 0.5 mg of the respective proteins was incubated with 100 μL of trypsin (0.5 mg/mL in 1 mM HCl) at 37 °C for 4 h after which 100 μL of phenylmethanesulfonyl fluoride (PMSF) (saturated solution in 20 mM NaCl, 2 mM Tris, pH 7.4) was added to block trypsin action. Other aliquots were incubated with trypsin as above at 4 °C for 4 h followed by addition of PMSF. Trypsin-hydrolyzed samples and the 4 °C trypsin controls were incubated at 37 °C for 20 min in the presence of 0.46 μg of purified phospholipase A and 0.4 mM di[1-^{14}C]oleoylphosphatidylcholine. Controls containing only trypsin and PMSF as noted above were also incubated with purified phospholipase A and substrate to determine the basal rates obtained in the absence of any inhibitors. All samples were preincubated for 15 min at 4 °C before adding the enzyme and ^{14}C-labeled substrate. The final incubation mixture contained aliquots representing 68 μg of HAPB protein or 40 μg of ConA protein, respectively.

RESULTS

The purification results representing the average of three separate preparations are shown in Table I. The sequence of steps was designed to allow rapid processing of the protein fractions. Dialysis steps were avoided by the use of ultrafiltration when changing buffers or concentrating to a small volume because lysosomal phospholipase A is moderately labile at 4 °C. The enzyme was solubilized from a crude lysosomal pellet by repeated cycles of freezing and thawing. It passed through hydroxyapatite when applied in 8 mM phosphate buffer and was subsequently bound by concanavalin A–Sepharose attached in series. This step reduced the protein from 680 to about 5 mg because substantial amounts of protein either bound to hydroxyapatite or passed through concanavalin A–Sepharose. Phospholipase A was eluted from concanavalin A–Sepharose with methyl α-mannoside. This combined step results in a 254-fold purification relative to the soluble protein fraction. Interestingly, the total activity increased 2.3-fold at this step, suggesting that naturally occurring inhibitors may have been removed at this combined step. Chromatofocusing of phospholipase A using columns of PBE exchanger gave a very reproducible, single peak at pH 5.4 as shown in Figure 1. In contrast to liver, there was almost no activity eluted from the column by 0.1 M KCl after pH 4.0 had been reached (data not shown). The combined material from chromatofocusing was treated further by gel permeation chromatography with Sephadex G-150. A large protein peak was noted at the void volume which did not have any phospholipase A activity. Phospholipase A activity was eluted from the Sephadex G-150 column as a broad peak between M_r 70 000 and 25 000. The purification resulted in a yield of 0.134 ± 0.051 mg of protein in the combined phospholipase A fractions with a specific activity of 15 ± 3.5 μmol mg^{-1} h^{-1}. The recovery was 9.8 ± 4.2%, and the purification factor was 7600

Table I: Purification of Kidney Lysosomal Phospholipase A[a]

purification step	protein (mg)	PLA activity (nmol mg^{-1} h^{-1})	total activity (nmol h^{-1})	% recovery	purification factor
homogenate	10000	1.96	19100	100	1.0
lysosome fraction	2510	6.90	17200	88	3.5
soluble	680	4.58	2640	14	2.4
hydroxyapatite/ConA	4.93	1200	6200	32	610
chromatofocusing	1.63	2600	4270	22	1300
Sephadex G-150	0.154	15000	1850	9.8	7600

[a] The data are the average of three separate preparations where the amount of homogenate protein has been set equal to 10000 mg. The actual amount of protein in the three homogenates ranged from 10300 to 27800 mg. PLA activity was measured as described under Materials and Methods by using sonicated 0.2 mM (1-^{14}C]dioleoylphosphatidylcholine present as small unilamellar vesicles. Four different protein concentrations were used, and only the data from the linear portion of the protein curve were used to calculate the rate. When the protein used was in the linear range, PLA activity was linear with time to at least 20 min. The results for protein, phospholipase A (PLA), and total activity have been rounded to three significant figures while the % recovery and purification factors represent two significant figures. The data in each column represents the mean of three experiments. For example, since the results represent the average of the three separate experiments the data for total activity obtained by multiplying protein × PLA activity given in the table dose not agree exactly with the values given for total activity. The mean ±SD at the final purification step are as follows: protein, 0.154 ± 0.051; PLA activity, 15000 ± 3,500; total activity, 2200 ± 462; % recovery 9.8 ± 4.2; purification factor, 7600 ± 1800.

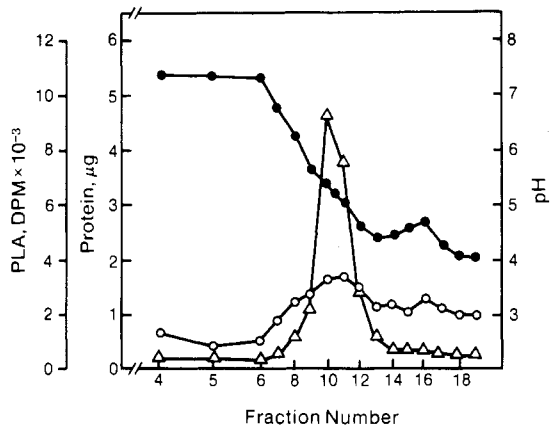

FIGURE 1: Chromatofocusing of kidney phospholipase A. Purification of the soluble protein fractions obtained from the hydroxyapatite/concanavalin–Sepharose step was carried out as described under Materials and Methods. Results are expressed as follows: (○) μg of protein per 50 μL of sample; (△) fatty acid release from 1,2-di-[^{14}C]palmitoylphosphatidylcholine as DPM × 10^{-3} per 50 μL; (●) pH. Experimental details of the phospholipase A assay are given under Materials and Methods.

± 1800 fold relative to the homogenate (n = 3).

As noted above, two- to threefold increases in total activity were noted at the combined hydroxyapatite/concavalin A–Sepharose step of the purification. We studied the protein fractions that were removed at this stage by incubating increasing amounts of protein with purified lysosomal phospholipase A, and the results are shown in Figure 2. Both the material that was bound to hydroxyapatite (HAPB) and the material that was not bound by either hydroxyapatite or concanavalin A–Sepharose (ConA) inhibited the release of [^{14}C]oleic acid from sonicated di[1-^{14}C] oleoylphosphatidylcholine in vitro with IC_{50} values of 7 and 11 μg, respectively. Several other "control" proteins added to the medium in concentrations of 1–20 μg either stimulated the phospholipase A activity slightly (bovine serum albumin, fatty acid free bovine serum albumin, and poly-L-glutamic acid) or had no effect (rabbit gamma globulin) on the activity of purified lysosomal phospholipase A_1 (data not shown). The inhibitory fractions contained <0.5 pmol of lipid phosphorus/μg of protein, and no neutral lipids were detected by iodine staining after thin-layer chromatography of the lipid extract representing 5 mg of protein, suggesting strongly that the inhibitory factor(s) in these two protein fractions cannot be lipids. The two inhibitor fractions (10–40 μg) were also tested with another lysosomal enzyme, acid phosphatase from human spleen

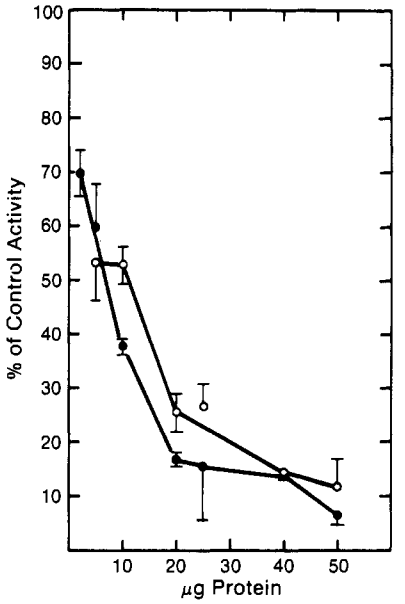

FIGURE 2: Effect of protein fractions on purified kidney phospholipase A. The incubation medium contained 50 mM sodium acetate, pH 4.4, 10 μg of bovine serum albumin, HAPB (●) or ConA (○) protein as noted, 1.32 μg of purified phospholipase A, and sonicated 0.2 mM 1,2-di[1-^{14}C]oleoylphosphatidylcholine. The incubations were started by addition of substrate; after incubation at 37 °C × 20 min the release of [1-^{14}C]oleic acid was determined as described under Materials and Methods.

(Calbiochem., La Jolla, CA), and found to have a slight stimulatory effect (data not shown).

To further verify that protein components of the HAPB and ConA fractions are responsible for inhibition of purified lysosomal phospholipase A_1 we preincubated the HAPB and ConA fractions with trypsin at 37 or 4 °C for 4 h; subsequently PMSF, sonicated di[1-^{14}C]oleoylphosphatidylcholine, and purified phospholipase A_1 were added, and the release of [1-^{14}C]oleic acid was measured. The results are shown in Table II. Untreated HAPB and ConA reduced the activity to 27% or 28% of control, respectively. Treatment of HAPB and ConA with trypsin at 37 °C completely abolished the inhibition and restored the phospholipase A activity to control levels. However, if the preincubation with trypsin was done at 4 °C for 4 h, the inhibitory activity remained. These results show clearly that the inhibitors in these fractions are proteins since their activity is destroyed by trypsin.

The molecular weight of the purified phospholipase A was estimated by HPLC gel permeation chromatography at room

Table II: Effect of Trypsin Treatment of Crude Inhibitor Fractions from Rat Kidney and Their Effect on Purified Phospholipase A_1[a]

addition	preincubation	PLA activity (μmol mg^{-1} h^{-1})	% of control
control + trypsin	none	7.8 ± 1.5	100
HAPB	none	2.1 ± 0.2	27
HAPB + trypsin	37 °C × 4 h	8.2 ± 0.6	105
HAPB + trypsin	4 °C × 4 h	2.1 ● 0.2	27
ConA	none	2.2 ● 0.3	28
ConA + trypsin	37 °C × 4 h	7.7 ● 0.5	99
ConA + trypsin	4 °C × 4 h	2.4 ± 0.5	31

[a] Preincubations with trypsin were carried out at pH 7.4 as indicated in Materials and Methods. Subsequently, purified 0.46 μg of kidney phospholipase A, PMSF, and 0.4 mM 1,2-di[1-^{14}C]oleoylphosphatidylcholine in 50 mM sodium acetate buffer (pH 4.4) were added as noted under Materials and Methods. The mixture was incubated at 37 °C for 20 min, and the phospholipase A activity was determined. The data are expressed as μmol of fatty acid mg^{-1} h^{-1} and represent the mean and standard deviation of three replicates. Abbreviations: HAPB, The protein eluted from the hydroxyapatite column; ConA, the protein not retained by either hydroxyapatite or concanavalin A-Sepharose (see Table I); PLA, phospholipase A.

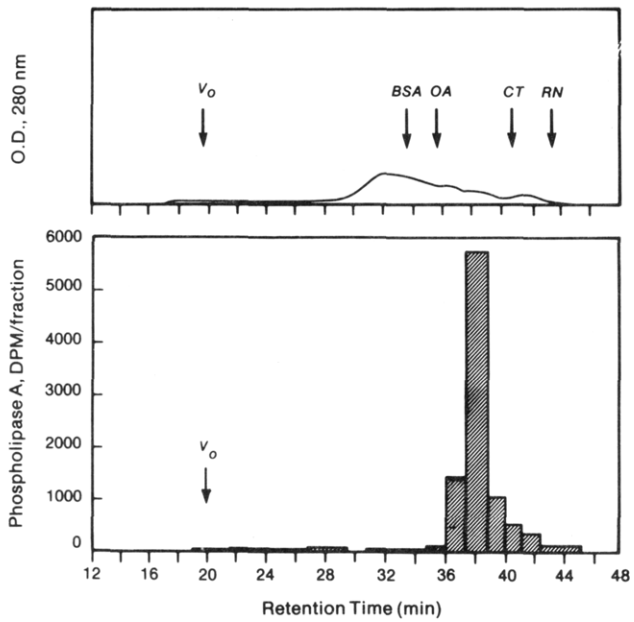

FIGURE 3: Analysis of the purified kidney lysosomal phospholipase A by HPLC gel-permeation chromatography. The upper panel shows the OD 280 (arbitrary units) and the position of pure reference proteins: BSA, bovine serum albumin; OA, ovalbumin; CT, chymotrypsinogen A; RN, RNase. The lower panel shows the total activity of phospholipase A per fraction. The void volume is indicated by the arrow.

temperature, and the results are shown in Figure 3. The phospholipase A activity corresponds with protein eluting at a retention time of 38 ± 2 min. Three separate preparations of kidney phospholipase A were analyzed by HPLC and had nearly identical V_e/V_0 values of 1.96, 1.96, and 2.05, respectively, corresponding to an average molecular weight of 30000. HPLC methodology could not used in the purification sequence shown above (Table I) because the enzyme lost activity rapidly when the gel-permeation chromatography was done at room temperature. Analysis of the HPLC-purified preparation by SDS-polyacrylamide gel electrophoresis (not shown) showed a band at 34000 roughly corresponding to the HPLC phospholipase peak with an apparent molecular weight of 30000. A contaminating protein was present at 66000 and a minor band was noted at 18000.

Table III shows the effects of divalent cations and inhibitors on the purified enzyme. Kidney acid phospholipase A did not require Ca^{2+} for full activity and was not inhibited by 5 mM EDTA. Mg^{2+} and Mn^{2+} both at 5 mM were without effect while Hg^{2+} at 2.0 mM stimulated phospholipase A activity. Preincubation of the enzyme with 2 mM *p*-bromophenacyl bromide or 1 mM diisopropyl fluorophosphate did not affect the activity of phospholipase A significantly. The pH dependence was determined (data not shown). Phospholipase A exhibited maximal activity at pH 3.6–3.8. Fifty percent of the maximal activity is present at pH 4.5, and 10% remains at pH 5.2. Although the activity of the enzyme declines sharply as the pH increases above 4.0, substantial activity remains into the acid range below 3.5. For example, at pH 2.6, 83% of maximal activity was present (data not shown).

Table III: Effect of Divalent Cations and Inhibitors on Kidney Acid Phospholipase A_1[a]

addition	control
5 mM Ca^{2+}	89 ± 7
5 mM EDTA	101 ± 1
5 mM Mg^{2+}	95 ± 2
5 mM Mn^{2+}	103 ± 5
2 mM Hg^{2+}	137 ● 2
2 mM *p*-bromophenacyl bromide[b]	85 ± 11
1 mM diisopropyl fluorophosphate	90 ± 12

[a] Sonicated dispersions of 0.2 mM 1,2-di[1-^{14}C]oleoylphosphatidylcholine were incubated with 1 g of purified phospholipase A in triplicate as described under Materials and Methods with the addition noted above. The results are expressed as a percentage of control values obtained with no additions. [b] These samples were preincubated with enzyme for 5 min at 20 °C before starting the reaction by adding substrate. The results are expressed as % of control which was also preincubated at 20 °C for 5 min without additions.

Table IV: Hydrolysis of ^{14}C-Labeled Phosphatidylcholines by Kidney Lysosomal Phospholipase A[a]

	substrate			
	1,2-di[1-^{14}C]-oleoyl-phosphatidyl-choline		2-[1-^{14}C]-oleoyl-phosphatidyl-choline	
product	DPM-B	%	DPM-B	%
fatty acid	16300	50.8	34	4.3
lysophosphatidylcholine	15800	49.2	757	95.7
% fatty acid release due to lysophospholipase		1.5		

[a] Results given as DPM product minus the blank incubated without protein and as % of recovered product (fatty acid and lysophosphatidylcholine. Sonicated phosphatidylcholine, as indicated above (0.2 mM), were incubated at 37 °C for 20 min with 1 g of purified phospholipase A as noted in Materials and Methods. Total lipid extracts were prepared by the method of Folch et al. (1957). The extracts were applied to 20 × 20 plates (0.25 mm) of silica gel H prepared with magnesium acetate and developed as previously described (Matsuzawa & Hostetler, 1980b). The areas corresponding to fatty acid and lysophosphatidylcholine references were scraped into liquid scintillation vials and counted (Hostetler et al., 1982). The % fatty acid released due to lysophospholipase was calculated as previously described (Nalbone & Hostetler, 1985) by using the equation FA/LPC = (P + L)/(P − L), where P and L represent the fraction of fatty acid release due to phospholipase A (P) and lysophospholipase (L), respectively.

Table IV shows the results of incubations of the purified acid phospholipase A with sonicated dispersion of 1,2-di[1-^{14}C]oleoylphosphatidylcholine or 2-[1-^{14}C]oleoylphosphatidylcholine. The reaction products after a 20-min incubation were analyzed by thin-layer chromatography to determine the positional specificity of the enzyme and to determine the contribution of lysophospholipase activity to fatty acid release. 1,2-Di[1-^{14}C]oleoylphosphatidylcholine gave nearly equal amounts of fatty acid (50.8%) and lysophosphatidylcholine (49.2%) which is equivalent to a negligible

contribution for lysophospholipase of 1.5% of the fatty acids released on the basis of the previously formulated relationship: FA/LPC = (P + L)/(P − L) (Nalbone & Hostetler, 1985). When incubated with sonicated phosphatidylcholine specifically ^{14}C-labeled in the *sn*-2-acyl group, 96% of the radioactivity was recovered in lysophosphatidylcholine, indicating that the kidney phospholipase A hydrolyzes the acyl ester at the *sn*-1 position.

The substrate dependence of purified kidney phospholipase A_1 was studied with sonicated vesicles of 1,2-di[1-^{14}C]-oleoylphosphatidylcholine (DOPC) and with mixed micelles of 1,2-di[1-^{14}C]palmitoylphosphatidylcholine(DPPC)/Triton X-100. Double reciprocal plots of phospholipase A_1 activity and substrate gave straight line plots in each instance. The apparent K_m for DPPC/Triton X-100 mixed micelles was 0.57 mM vs. 1.1 mM for DOPC vesicles, and the observed values for V_{max} were 53 and 48 μmol h^{-1} mg^{-1}, respectively. The purified enzyme also exhibited weak lysophospholipase activity with 1-[1-^{14}C]palmitoyllysophosphatidylcholine as substrate. The observed V_{max} for lysophospholipase was only 3.3 μmol mg^{-1} h^{-1}, and the apparent K_m for lysophosphatidylcholine was 42 μM.

Discussion

The phospholipase A of kidney cortex lysosomes is a glycoprotein on the basis of its binding to concanavalin A–Sepharose. Its molecular weight is 30000 by HPLC gel permeation, and the isoelectric point is pH 5.4. Phospholipase A has been purified 7600-fold over the homogenate with a recovery of 9.5%. This is less than the degree of purification reported earlier for one of the liver phospholipase A_1 isoenzymes (17600-fold), but the former data represent a single fraction from a gel filtration step (Hostetler et al., 1982), while the data in the present study (7600-fold) represent combined phospholipase A_1 obtained from several fractions. Phospholipase A from kidney lysosomes has an acid pH optimum in the 3.6–3.8 range.

Our studies indicate that two soluble fractions isolated from a crude lysosomal fraction obtained from rat kidney contain proteins which inhibit purified lysosomal phospholipase A_1 as shown in Figure 2. Trypsin destroys the inhibitor activity, confirming the protein nature of the substances (Table II). Several other groups have demonstrated the synthesis and secretion of phospholipase A_2 inhibitory from cultured neutrophils or renomedullary intestitial cells which are referred to as macrocortin (Blackwell et al., 1980), lipomodulin (Hirata et al., 1980) or renocortin (Cloix et al., 1983), respectively. These proteins are secreted in response to cortisone treatment and have been implicated in the antiinflammatory effects of glucocorticosteroids.

Intracellular inhibitors of phospholipase A have not previously been described. In principle, these naturally occurring proteins could stabilize lysosomes by inhibiting phospholipase A_1 activity directed against the lysosomal phospholipid bilayer. The crude inhibitor fractions do not reduce acid phosphatase activity in vitro, suggesting that they are not aspecific inhibitors of lysosomal enzymes. The two inhibitor fractions each contain more than 10 distinct bands by SDS–gel electrophoresis (data not shown), and preliminary purifications indicate that many of the protein components are not strong inhibitors of phospholipase A. Demonstration that the inhibitor proteins are lysosomal has not yet been accomplished, and it should be pointed out that the soluble proteins in the HAPB and ConA fractions could have been released from either mitochondria or lysosomes which are present in the 20000*g* pellet. We are presently purifying the protein inhibitors of lysosomal phospholipase A and determining their subcellular localization.

The lysosomal phospholipase A_1 purified from kidney is similar in many ways to the enzyme previously purified from liver lysosomes. Both are glycoproteins that hydrolyze the *sn*-1 ester of phospholipids and exhibit a similar acid pH optimum; neither requires Ca^{2+} or other divalent cations for full activity (Hostetler et al., 1982; Robinson & Waite, 1983). Neither kidney nor liver lysosomal phospholipase A_1 is inhibited significantly by *p*-bromophenacyl bromide (Hostetler et al., 1982), indicating the absence of a histidine at the active site equivalent to that found in venom and pancreatic phospholipases A_2 (Slotboom et al., 1982). Both lysosomal phospholipases exhibit a weak lysophopholipase activity. The only apparent difference between the purified enzymes is that the molecular weight of the kidney phospholipase A_1, 30000, is somewhat smaller than that of the two major isoenzymes of phospholipase A_1 which we purified previously from liver, 44000 and 34000, respectively (Hostetler et al., 1982). The phospholipase A_1 purified from liver lysosomes by Robinson and Waite (1983) had an apparent molecular weight of 56000.

The properties of kidney lysosomal phospholipase A differ greatly from those of snake venom and pancreas phospholipases A. As shown in Tables III and IV, the kidney enzyme is a phospholipase A_1, and it does not require Ca^{2+} for activity. In contrast, the venom and pancreas phospholipases attack the *sn*-2-acyl ester of phospholipids and have a absolute requirement for Ca^{2+} (Slotboom et al., 1982). Purified kidney phospholipase A_1 also has a weak lysophospholipase activity. However, the apparent V_{max} of phospholipase A activity is 14- to 16-fold greater than that of lysophospholipase. Thus, it is appropriate to classify this enzyme as a phospholipase A rather than a phospholipase B (an enzyme having roughly equivalent phospholipase A and lysophospholipase activity) (Dennis, 1983).

Acknowledgments

We thank Dr. Edward A. Dennis for helpful discussions of the manuscript.

Registry No. PLA, 9043-29-2.

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Importance of the Unsaturated Fatty Acyl Group of Phospholipids in Their Stimulatory Role on Rat Adrenal Mitochondrial Steroidogenesis†

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Received March 19, 1986; Revised Manuscript Received June 16, 1986

ABSTRACT: We have investigated the relationship between chemical properties of various phospholipids and their steroidogenic activity for adrenal mitochondria prepared from dexamethasone/cycloheximide-treated quiescent rats. Phospholipids studied include those purified from bovine and rat adrenal mitochondria, obtained from commercial sources, and reduced by catalytic hydrogenation. All phospholipids were subjected to analysis of their fatty acyl groups and examined for their steroidogenic activities. From these experiments, we came to the following conclusions: (i) The degree of unsaturation in the fatty acyl moiety correlates with their steroidogenic activities regardless of head groups. Namely, polyunsaturation appears to be more important than monounsaturation with a relative insensitivity toward their head groups. (ii) Saturated phospholipids exhibit an inhibition for steroidogenic activity. (iii) Cardiolipins, which are steroidogenic, appear exceptional. Their head groups may partially participate in the activity in addition to their high content of unsaturated fatty acids. (iv) The importance of the adrenoyl (C22:4) group in phospholipids is suggested.

The rate-limiting step in adrenocorticotropic hormone (ACTH)[1]-dependent steroidogenesis is the conversion of cholesterol to pregnenolone (Stone & Hechter, 1954). An important conclusion is that ACTH stimulates this step by producing cAMP-dependent, cycloheximide-sensitive factor(s) (Garren et al., 1965). This factor seems to enhance the movement of cytosolic cholesterol to P-450$_{scc}$ in the mitochondrial inner membrane (Simpson, 1979; Kimura, 1981; Simpson & Waterman, 1983; Cheng et al., 1985). In our previous study (Igarashi & Kimura, 1984), we demonstrated that the quantities of mitochondrial PC, PE, and PI increase by ACTH administration and this increase was sensitive to cycloheximide inhibition but not to aminoglutethimide. On the other hand, CL and polyphosphoinositides were reported as activators of both mitochondrial and reconstituted steroidogenesis (Lambeth, 1981; Pember et al., 1983; Greenfield et al., 1981; Farese & Sabir, 1980; Tanaka & Strauss, 1982; Kowluru et al., 1983; Hsu et al., 1985). We have shown recently that by model experiments using cholesterol-containing liposomes and purified steroid-free P-450$_{scc}$ the unsaturated fatty acid moiety and its carbon chain length are important in the enhancement of the binding rate of cholesterol to the cytochrome (Hsu et al., 1984, 1985; Kido & Kimura, 1981; Kido et al., 1981). Although the effects of phospholipids on reconstituted liposomes may not be the same as those on mitochondria, we had a question on the role of head groups in phospholipids for their activating function.

In order to gain further insight into the action of these phospholipids, we have studied here the relationship between fatty acid compositions and steroidogenic activities of various phospholipids. We have revealed that a high content of polyunsaturated fatty acids is required for steroidogenic activities. This relationship stands for many phospholipids, except

† This study was supported by a research grant from the National Institutes of Health (AM-12713).

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[1] Abbreviations: ACTH, adrenocorticotropic hormone; P-450$_{scc}$, cytochrome P-450 for cholesterol side-chain cleavage reaction; CL, cardiolipin; DPI, diphosphoinositide; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; lysoPG, lysophosphatidylglycerol; lysoPS, lysophosphatidylserine; PAF, platelet activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TPI, triphosphoinositide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-pressure liquid chromatography.

0006-2960/86/0425-6461$01.50/0