Fluorimetric Assay of Phospholipase A Acting on Biomembrane Phospholipids

Nils-Erik L. Saris and Pentti Somerharju

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10, SF-00170 Helsinki, Finland

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A sensitive phospholipase A assay suitable for organelle activities is described. Activation of the enzyme produces an increase in membrane lysophospholipids. The amino groups of extracted lipids were derivatized with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole and the amounts of phosphatidylethanolamine and its lysoform were quantitated using HPLC equipped with a SiO_2 column and a fluorescence detector. The procedure was tested with porcine enzyme acting on liposomes and mitochondria, and with endogenous mitochondrial enzyme.

Intracellular PLA_1 and PLA_2* activities (EC 3.1.1.4) are much lower than pancreatic and other secreted activities. The enzymes are localized in contact with their substrates in various organelles and membranes. Their activation is often of great physiological interest. Most assays are based on the liberation of fatty acids and may therefore be unspecific when assaying low intracellular activities. Fatty acids may be produced by other hydrolases such as triacylglycerol lipase (EC 3.1.1.3), cholesterol esterase (EC 3.1.1.13), acylcarnitine hydrolase (EC 3.1.1.28) and acylethanolamine hydrolase[1] or they may be consumed by acylCoA synthetases (EC 6.2.1.3). Thus, in liver mitochondria, the formation of fatty acids may exceed the formation of lysophospholipids[2] even though mitochondria lack lysophospholipase activity[3]. More specific assays are based on the formation of lysophospholipids. For the assay of hydrolysis of endogenous substrates, which is of considerable interest, four main approaches have been used: (1) *in vivo* labeling of endogenous phospholipids with radioisotopes,[4] (2) introduction of radioactive phospholipids with the aid of a phospholipid transfer protein,[5] (3) introduction of fluorescent phospholipids by passive diffusion from liposomes,[6] or (4) by chemical quantitation of the lysophospholipids formed during the reaction. In the last case highest sensitivity is obtained by labeling of the phospholipids with radioisotopes such as [^{14}C]acetic anhydride[7] or reagents giving fluorescent products. Since acetic anhydride reacts both with amino and alcohol groups, the label is introduced to many compounds and therefore one of the products of the PLA_2 reaction, lysoPC, had to be isolated before being labeled[7] which makes this procedure rather laborious. In the method used here, NBD-F was used to convert PE and lysoPE into the crude lipid extract into fluorescent derivatives under conditions where decomposition of PE and lysoPE was negligible. NBD-F was used for derivatization instead of the corresponding Cl-derivative as labeling proceeds faster and produces fewer side products.[8]

Experimental

Phosphatidylethanolamine was prepared from egg yolk phosphatidylcholine by transphosphatidylation[9] and was purified by SiO_2 column chromatography using chloroform–methanol (9:2, v/v) as the eluent. NBD-F was obtained from Molecular Probes Inc. (Eugene, OR) and porcine pancreatic PLA_2 from Boehringer (Mannheim). Rat liver mitochondria were prepared conventionally in mannitol (210 mmol l^{-1}), sucrose (70 mmol l^{-1}), EGTA (1 mmol l^{-1}) and Hepes-chloride (10 mmol l^{-1}), pH 7.3, omitting EGTA in the media used for resuspension of the mitochondrial pellets after centrifugation. Mitochondrial protein was estimated with a biuret procedure using bovine serum albumin as the standard. Phospholipid phosphate was determined with a colorimetric procedure[10] using pure phosphatidylcholine (Sigma Chemical Co, St Louis, MO) as the standard.

The PLA_2 assays were carried out at 37 °C in the media specified in the figure legends. The reaction was stopped by addition of 3 ml of chloroform and 1.5 ml of methanol to 1.0 ml of the incubation medium with simultaneous Vortex mixing. 300 µl of 2.5 % (w/v) NaCl were then added and after mixing, the lipid fraction in the chloroform layer was removed and the chloroform was evaporated under a stream of nitrogen. Derivatization was carried out by adding 5 µl of NBD-F in dichloromethane, 300 µl of methanol, and 150 µl of methylene chloride. After mixing, 45 µl of water and 5 µl of 5 % (w/v) aqueous $NaHCO_3$ were added and incubation was carried out for specified time periods.

* Abbreviations: EGTA, ethyleneglycol-*bis*-(2-aminoethyl)-tetraacetate; Hepes, 4-(2-hydroxyethyl)piperazinoethanesulfonic acid; HPLC, high-pressure liquid chromatography; NBD-F, 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole; PE, phosphatidyl ethanolamine; PLA_2, phospholipase A_2.

The reaction was stopped by addition of 50 µl of 1 M acetic acid and the reaction mixture was filtered through 0.45 µm pore-size filters obtained from Nihon Millipore Kogyo K. K., Yonezawa, Japan. A sample of 10 µl was analysed with a Zorbax SIL 4.6 mm × 25 cm column (DuPont Biotechnology Systems, Boston, MA) connected to a LKB (Stockholm, Sweden) 2150 pump and a Merck-Hitachi F-1000 fluorescence detector with excitation and emission wavelengths set to 470 and 530 nm, respectively. Hexane–isopropyl alcohol–H_2O–H_2SO_4 (730:260:9:1, v/v) was used as the eluent at a rate of 1.5 ml min^{-1}. The extent of hydrolysis was calculated from the peak area of NBD-lysoPE relative to that of NBD-PE.

Results and discussion

Conditions for labeling. PE and lysoPE could be rapidly derivatized at room temperature. The height of the NBD-lysoPE peak increased with reaction time but so did the background which impaired resolution between NBD-PE and NBD-lysoPE (not shown). Heterogeneity was observed in both peaks and is most probably due to the presence of several PE and lysoPE molecular species of different acyl-chain structure, which, owing to their slightly different polarity, have unequal retention times. Within the PE peak area there was a shift in favor of the slower eluting components observable with reaction times longer than 6 min. This may be due to the appearance of side-products. However, the ratio of NBD-lysoPE to NBD-PE did not change noticeably over the first 10 min. A reaction time of 2 min was selected since it gave a narrow NBD-lysoPE peak of sufficient magnitude for quantitation, low background and little evidence of decomposition.

Fig. 1 shows the increase in NBD-lysoPE brought about by varying amounts of PLA_2. In order to test the method further, lysoPE was produced by the action of porcine PLA_2 on PE and, after extraction into chloroform, mixed with known amounts of PE, treated with NBD-F and analysed by HPLC as described in the Experimental section.

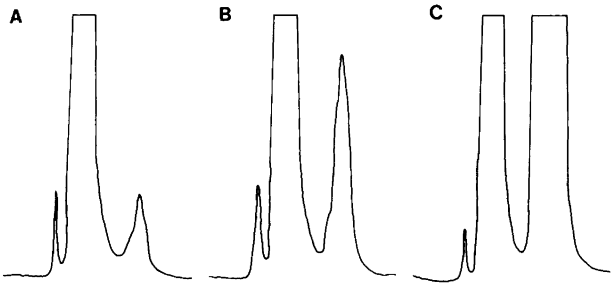

Fig. 1. Separation of NBD-PE and NBD-lysoPE by HPLC. PE, 7.9 nmol of a PE preparation containing 97 % PE and 1.73 % lysoPE, was treated with A, no PLA_2; B, 25 ng PLA_2; and C, 250 ng PLA_2, in (100 mmol l^{-1}), Tris pH 8.0, Ca^{2+} (1 mmol l^{-1}) at 37 °C for 15 min. The lipid extracts were subjected to derivatization and HPLC as described in the Experimental section.

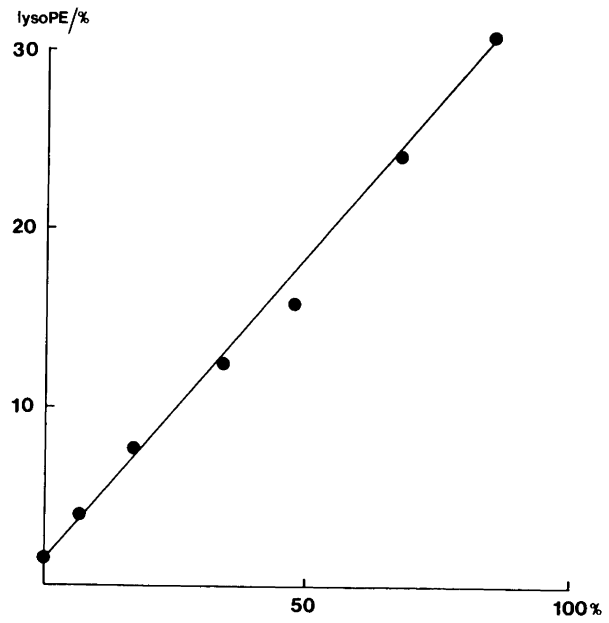

Fig. 2. Calibration of lysoPE content. Porcine PLA_2 was allowed to act on PE and the lipid extract was mixed in various proportions with substrate PE before derivatization. The abscissa gives the percentage of PLA_2-treated PE in the mixture.

As shown in Fig. 2, the percentage NBD-lysoPE of the total NBD-lipids is linearly related to the amount of PLA_2-treated PE present in the reaction mixture. This indicates

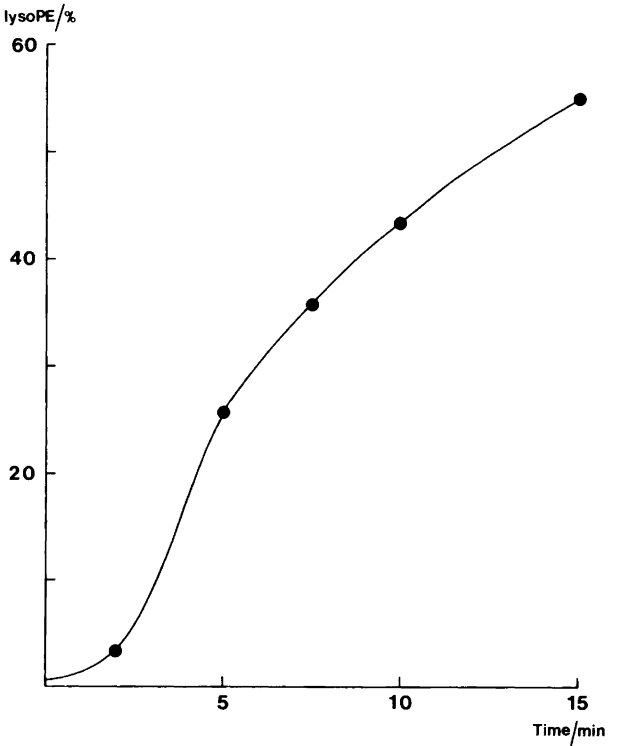

Fig. 3. Time course of lysoPE formation catalysed by 250 ng porcine PLA_2. The amount of the PE substrate was 7.9 nmol. The medium contained Tris-Cl, (100 mmol l^{-1}) pH 7.4 and $CaCl_2$ (1 mmol l^{-1}).

that derivatization of both lysoPE and PE are independent of the ratio of these lipids.

Application of the method to the assay of PLA_2. Porcine PLA_2 acting on PE was employed to test the assay. Fig. 3 shows the increase in the NBD-lysoPE peak as a function of time. The curve is sigmoidal, i.e. the rate of production of lysoPE increases initially and then decreases when 50 % hydrolysis is approached. Also, when a fixed incubation time of 5 min and variable amounts of the enzyme is used a non-linear relationship is observed between the amount of enzyme used and the amount of lysoPE (Fig. 4). These non-linear responses may be rationalized in terms of changes in the physical properties of the substrate bilayer which is known to affect profoundly the activity of phospholipases.[11] Thus the initial acceleration can be ascribed to activation of the enzyme by the negatively-charged fatty acid residues liberated from the substrate[12] but remaining bound to the bilayer,[13–15] while the decrease of the reaction at the later stages of hydrolysis is probably due to the decrease in substrate concentration and/or product inhibition.[12] Incomplete hydrolysis of PE even at high PLA_2 concentrations (Fig. 4) may also result from the restriction of the enzymatic degradation of the outer monolayer of the substrate vesicles.[13,14]

The procedure was also tested on mitochondria in the presence of rotenone (5 μmol l^{-1}) in order to inhibit endogenous respiration both with mitochondrial PLA_2 acting on endogenous phospholipids and with added PLA_2. (Fig. 5). With Ca^{2+} (1 mmol l^{-1}) and a 10 min incubation time only 2.0 % of the mitochondrial PE was hydrolysed by the endogenous activity. Fig. 5 also shows that PE in a membrane is a poorer substrate for added PLA_2 than PE liposomes. The

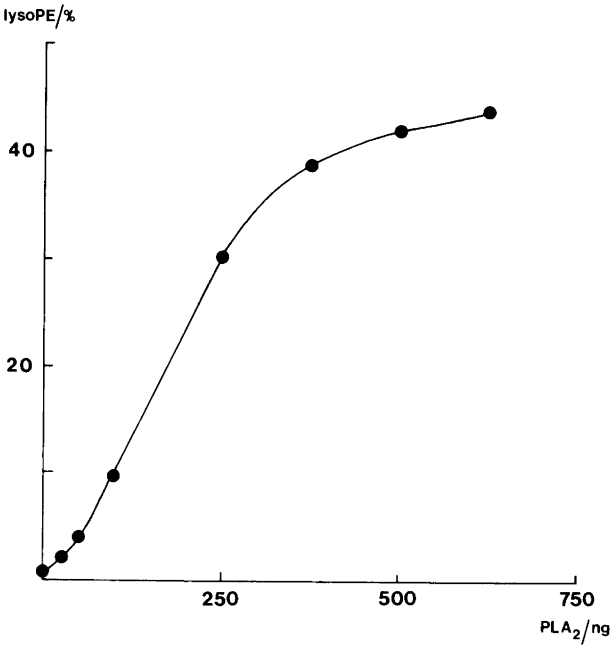

Fig. 4. LysePE formation with varying amounts of porcine PLA_2. An incubation time of 5 min was used. Experimental details as in Fig. 3.

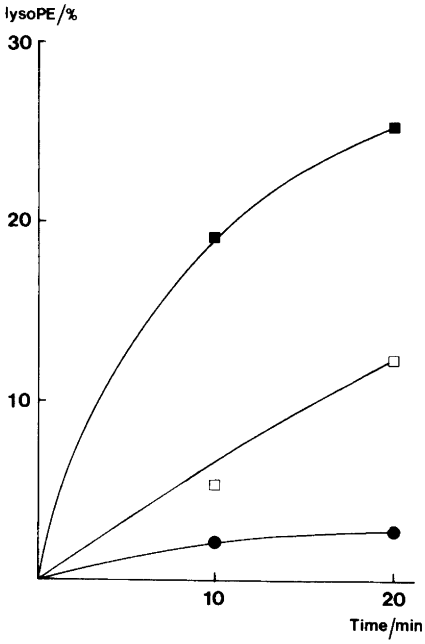

Fig. 5. LysoPE formation with pancreatic and endogenous mitochondrial PLA_2s. The medium contained sucrose (200 mmol l^{-1}) Hepes, (20 mmol l^{-1}), pH 7.4, $CaCl_2$ (1 mmol l^{-1}) and rotenone (5 μol l^{-1}). ■, PE (7.9 nmol l^{-1}) as the substrate, 100 ng porcine PLA_2; ○, 2 mg mitochondrial protein ml^{-1} instead of PE as substrate; ○, mitochondria without added PLA_2.

amount of PE hydrolysed by the endogenous mitochondrial PLA_2 is quite low compared with the extent of hydrolysis found in Ca^{2+}-induced large-amplitude swelling.[15–18] No additional peaks were forund, indicating that PE and lysoPE are the only phospholipids present in sufficient quantities to be detectable with this assay, but the possibility that phosphatidylserine and lysophosphatidylserine also form NBD-derivatives which run together with the corresponding PE derivatives cannot be excluded. It should be noted, however, that PE is the main substrate of mitrochondrial PLA_2.[3,4,12]

The HPLC procedure gave day-to-day imprecision of 0.29 % lysoPE (standard deviation) in duplicate runs of samples containing between 1.4 and 4.3 % lysoPE, e.g. the low range of interest in Ca^{2+}-induced swelling in mitochondria. This gives a detection limit of production of 0.6 % lysoPE.

A method similar to the present one, but employing fluorescamine as the labeling reagent and TLC to separate the PE and the lyso derivatives, has been described previously.[19] The major advantage of the present method over the reported one is that it is less tedious owing to fewer steps being involved. Both methods are unspecific in that the are not able to differentiate between PLA_2 and PLA_1 activities.

Exogenous NBD-labeled phospholipids have been used previously to assay phospholipases in intact cells.[6] A disadvantage of such a method that the exogenous substrate does not have access to all cellular membranes.[20]

Conclusion

Derivatization of PE and lysoPE with NBD-F and the quantitation of these phospholipids by HPLC may be used as a sensitive assay for studying intracellular PLA's acting on biomembranes.

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