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Hydrolysis of phosphatidylcholine liposomes by lysosomal phospholipase A is maximal at the phase transition temperature of the lipid substrate

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We have measured the rate of hydrolysis of liposomes made of DL-α-dipalmitoylphosphatidylcholine (DPPC) and L-α-dimyristoylphosphatidylcholine by a soluble fraction of highly purified lysosomes isolated from rat liver. Phospholipids are hydrolyzed into lysophospholipids and fatty acids at a rate which is maximal near the temperature characteristic of the gel to liquid crystalline phase transition of the lipid bilayer. This strong influence of the physical properties of the substrate on the enzyme activity suggests a structural analogy between the lysosomal phospholipases of the A type (EC 3.1.1.32 and EC 3.1.1.4) and the pancreatic phospholipase A_2.

The mechanism of action of non-lysosomal phospholipases, mainly of the A_2 type, has been extensively investigated (Verger, 1980). The maximum hydrolysis rate has been observed at the transition temperature of the lipid substrate (Op Den Kamp et al., 1974) and was attributed to the preferential binding of the enzyme to the border regions between the solid and the fluid lipid zones during the phase transition process (Goormaghtigh et al., 1981).

The lipid binding site of the protein (IRS: 'interface recognition site') has been identified in pancreatic phospholipase A_2 and is distinct from the catalytic site involved in lipid hydrolysis (Pieterson et al., 1974). In contrast with extracellular phospholipases, little is known about the structure and mode of action of intracellular phospholipases like lysosomal phospholipases, although they play an important role in the degradative and recycling pathways of lipids inside the cell. It is the purpose of this work to analyze how the lipid organization mediates the lysosomal phospholipase A activity.

Materials and Methods

DL-α-dipalmitoylphosphatidylcholine (DPPC) and L-α-dimyristoylphosphatidylcholine were purchased from Sigma Chemical Co. L-α-1-palmitoyl-2-[1-^{14}C]palmitoylphosphatidylcholine (59 mCi/nmol) and L-α-1,2-di[1-^{14}C]myristoylphosphatidylcholine (63 mCi/mmol) were

from Amersham. The soluble fraction of rat liver lysosomes, generously supplied by Dr. Tulkens (Lab. Chimie Physiologique, Université Catholique de Louvain) was isolated according to the method of Trouet (1974). Unilamellar vesicles were formed by ethanolic injection (Batzri & Korn, 1973). 14 µl of phospholipid in ethanol was injected in 1 ml of acetate buffer (4 mM, pH 4.4) at 50°C. The liposome suspension was vortexed for 10 s. Final concentrations were $3.22\ 10^{-2}$ µmol DPPC/ml and $4.91\ 10^{-1}$ DMPC/ml. [^{14}C]-labelled phospholipid was $3.5\ 10^{-4}$ µmol/ml in each case. In enzyme assays, 250 µl of ethanol injected liposomes were incubated with 25 µl of a 40-fold diluted sample of the soluble fraction of liver lysosomes for DPPC and with 1.25 µl of the soluble fraction for DMPC, giving respectively a $1.04\ 10^{-2}$ mg and $2.23\ 10^{-2}$ mg of total lysosomal protein/ml of final solution. Kinetics were stopped at 20 min for DPPC and at 45 min for DMPC by freezing the samples in liquid nitrogen. After overnight lyophilization, lipids were extracted three times with 20 µl of a 1:1 v/v methanol/chloroform mixture. Lipids were chromatographed on silica F plates (Merck) with chloroform/methanol/water (65:25:4 v/v) as developing system. Lipids and corresponding standards were visualized with iodine staining. The spots corresponding to lysolecithin, fatty acid and unhydrolyzed phospholipid were cut out of the plate and incubated overnight in scintillation liquid (Dynagel) before counting. Percentage of hydrolysis was given by lyso/(lyso + PC) for DPPC, and lyso/{lyso + (PC/2)} for DMPC, where lyso is the radioactivity associated with the lysophosphatidylcholine spot and PC is the radioactivity associated with the DPPC or DMPC spot.

The fluorescence polarization associated with the hydrophobic probe 1,6-diphenylhexatriene was used to detect the gel-liquid crystalline phase transition. Liposomes were labelled by addition of diphenylhexatriene dissolved in tetrahydrofuran (diphenylhexatriene/lipid molar ratio is 1/500). Fluorescence polarization measurements were carried out with an Elscint Microviscosimeter MV-1a (Elscint Ltd., Haifa, Israel) designed to give directly the degree of fluorescence polarization P. $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation beam. The heating rate was 1°C/min.

Results and Discussion

The two synthetic phospholipids used as substrate, DMPC and DPPC, tend to form lipid vesicles (liposomes) when hydrated in an excess of water at a temperature above their gel (rigid phase) to liquid crystalline phase (fluid phase) transition temperature. DMPC and DPPC vesicles were formed above their transition temperature (DMPC, 23.4°C; DPPC, 41.2°C) by the ethanol injection technique (Batzri & Korn, 1973) and were incubated with the phospholipases contained in a soluble extract of lysosomes purified by the Triton WR 1339 method (Trouet, 1974). A phospholipase A activity (release of fatty acids and lysophospholipids) was easily detected for a phospholipid-lysosomal soluble protein ratio of 2.5 (w/w) for DPPC and 14.4 (w/w) for DMPC. The hydrolysis rate was measured with L-α-1,2-di[1-^{14}C]myristoylphosphatidylcholine and L-α-1-palmitoyl-2-[1-^{14}C]palmitoylphosphatidylcholine. The availability of this last

compound gave us the opportunity to discriminate between phospholipase A_1 (EC 3.1.1.32; release of acyl chain in position 1 of the glycerol backbone) and A_2 (EC 3.1.1.4; release of acyl chain in position 2 of the glycerol backbone) activities. Release of acyl chain in position 1 was predominant (95%), revealing a strong phospholipase A_1 activity. As shown in Fig. 1, release of labelled lysophospholipids by lysosomal phospholipases was maximal around the phase transition temperature of DMPC (Fig. 1A) and DPPC (Fig. 1B). Rates were linear within time of incubation and protein concentration employed.

In order to demonstrate that in our experimental conditions TWR 1339 (which could form PC/detergent micelles) or any other compound (protein) contained in the lysosomal soluble fraction had not drastically modified the bilayer structure, we monitored the phase transition of our vesicles in the presence of the lysosomal soluble fraction using the fluorescence polarization technique (see Materials and Methods). This technique measures the mobility of a hydrophobic fluorescent probe (1,6-diphenylhexatriene, DPH) inserted into the phospholipid bilayers of the liposomes (Shinitzky & Inbar, 1974). The mobility of the probe is restricted by the fluidity of the surrounding hydrocarbon acyl chains of the phospholipids. Upon heating, the fluorescence polarization decreases strongly as the phospholipids undergo the phase transition and become more mobile. Fig. 2 shows the fluorescence

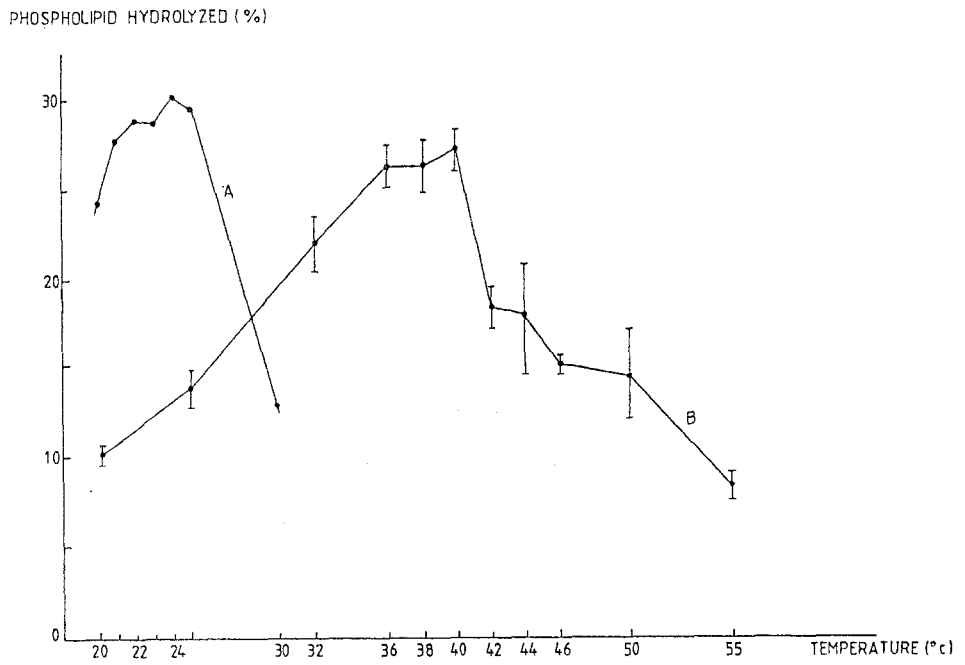

Fig. 1. Hydrolysis by lysosomal phospholipase A_1 of DMPC (A) and DPPC (B) vesicles formed by ethanol injection. For experimental conditions and evaluation of percentage of hydrolysis, see Materials and Methods.

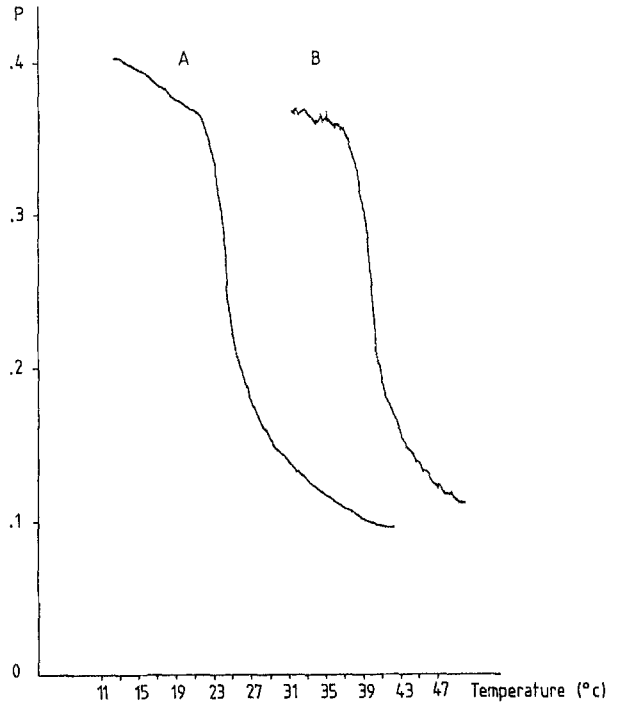

Fig. 2. Fluorescence polarization curves of:
(A) DMPC vesicles (4.91 10^{-1} µmol/ml buffer) formed by ethanol injection; lysosomal total protein extract was added at a final concn. of 2.23 10^{-2} mg/ml buffer. Buffer: 4mM sodium acetate, pH 4.4.
(B) DPPC vesicles (3.22 10^{-2} µmol/ml buffer) formed by ethanol injection; lysosomal total protein extract was added at a final concn. of 1.04 10^{-2} mg/ml buffer. Buffer: 4 mM sodium acetate, pH 4.4.

polarization curve observed for DMPC and DPPC liposomes in the presence of the lysosomal soluble extract. The transition temperatures are those expected for DMPC and DPPC liposomes, demonstrating that the bilayer structural organization is maintained after addition of the lysosomal extract.

From all the results illustrated above, we can conclude that the soluble fraction of the rat liver lysosomes contains a phospholipase A, mainly of the A_1 type (Waite et al., 1976; Kunze et al., 1982; Hostetler et al., 1982; Robinson & Waite, 1983), which is sensitive to the phase transition of the phosphatidylcholine substrate. The maxima were not as sharp as those previously reported for pancreatic phospholipase A_2 (Op den Kamp et al., 1979; Goormaghtigh et al., 1981). The phospholipase activity observed outside of the phospholipid phase transition zone could result from the presence of residual TWR 1339 in the crude lysosomal soluble fraction. Indeed, Hostetler et al. (1982) and Robinson and Waite (1983) have shown that detergents of the

Triton family stimulated the hydrolysis of phosphatidylcholine by purified lysosomal phospholipase A_1. The fact that for two different phospholipids with their own phase transition temperature, the maximum of hydrolysis rate was in each case centered on the phase transition temperature of the substrate indicates that the hydrolysis process is directly mediated by the mode of organization of the phospholipid bilayers at the phase transition temperature. To the best of our knowledge, this is the first time that such a phenomenon has been described for lysosomal phospholipases.

The effect of phase transition on lipid barrier permeability (Blok et al., 1975; Marsh et al., 1976) and protein binding has been observed in numerous studies (Goormaghtigh et al., 1981; Klausner et al., 1981; Okimasu et al., 1982). Although these phenomena are not fully understood, they are generally related to the presence of defects in the packing of the phospholipid molecules at the border region existing between fluid zones and rigid zones when the transition occurs. Phospholipase A_2 from porcine pancreas has been shown to hydrolyze phospholipids preferentially in their phase transition domain (Op den Kamp et al., 1979). This fact has been related to the presence of an interface recognition site (IRS) (distinct from the catalytic site of the enzyme molecule) which binds the protein to the interface (Pieterson et al., 1974). This binding is favored when phospholipids undergo the phase transition (Goormaghtigh et al., 1981), probably because packing defects created during the transition are favorable sites for the anchorage of the IRS. Our results suggest that some lysosomal phospholipases could act similarly to phospholipase A_2.

Our approach does not make it possible to attribute the observed properties to a single species of phospholipase (Hostetler et al., 1982; Robinson & Waite, 1983), since we used the whole lysosomal soluble fraction. Robinson and Waite (1983) demonstrated the importance of the 'quality of interface' in regulating the lysosomal phospholipase A_1 activity. Indeed, sonicated PE suspensions were the preferred substrate, PC was hydrolyzed at 20% the rate of PE while PS, PI and PE were poor substrates. These authors suggested that these modifications of enzyme activity could reflect the physical structure adopted by each phospholipid in aqueous solution. Our data demonstrate also a direct relationship between this lipid physical state (lipid packing) and the enzyme activity.

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