Available online at www.sciencedirect.com
SCIENCE DIRECT®
Chemistry and Physics of Lipids 133 (2005) 1–15
CPL
CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

Modulation of the in vitro activity of lysosomal phospholipase A1 by membrane lipids

Jocelyne Piret[a,1], André Schanck[b], Sylvie Delfosse[a], Françoise Van Bambeke[a,2], Bellamkonda K. Kishore[a,3], Paul M. Tulkens[a], Marie-Paule Mingeot-Leclercq[a,*]

[a] *Unité de Pharmacologie Cellulaire et Moléculaire, Université catholique de Louvain 73.70, Avenue E. Mounier 73, B-1200 Brussels, Belgium*
[b] *Unité de chimie structurale et des mécanismes réactionnels, Université catholique de Louvain, Place Louis Pasteur 1, 1348 Louvain-la-Neuve, Belgium*

Received 20 February 2004; received in revised form 6 August 2004; accepted 19 August 2004
Available online 27 October 2004

Abstract

Lysosomal phospholipases play a critical role for degradation of cellular membranes after their lysosomal segregation. We investigated the regulation of lysosomal phospholipase A1 by cholesterol, phosphatidylethanolamine, and negatively-charged lipids in correlation with changes of biophysical properties of the membranes induced by these lipids.

Lysosomal phospholipase A1 activity was determined towards phosphatidylcholine included in liposomes of variable composition using a whole-soluble lysosomal fraction of rat liver as enzymatic source. Phospholipase A1 activity was then related to membrane fluidity, lipid phase organization and membrane potential as determined by fluorescence depolarization of DPH, ^{31}P NMR and capillary electrophoresis.

Phospholipase A1 activity was markedly enhanced when the amount of negatively-charged lipids included in the vesicles was increased from 10 to around 30% of total phospholipids and the intensity of this effect depended on the nature of the acidic lipids used (ganglioside GM1 < phosphatidylinositol ∼ phosphatidylserine ∼ phosphatidylglycerol ∼

Abbreviations: Chol, cholesterol; DPH, diphenylhexatriene; EDTA, ethylene-diamine-tetra-acetic acid; GM1, ganglioside GM1; LBPA, lysobisphosphatidic acid; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PP, phosphatidylpropanol; PS, phosphatidylserine; SM, sphingomyelin; sPLA2, secreted phospholipase A2; SUV, small unilamellar vesicles

* Corresponding author. Tel.: +32 2 764 73 74; fax: +32 2 764 73 73.
E-mail addresses: jocelyne.piret@crchul.ulaval.ca (J. Piret), Bellamkonda.Kishore@hsc.utah.edu (B.K. Kishore), mingeot@facm.ucl.ac.be (M.-P. Mingeot-Leclercq).
[1] Present address: Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, 2705 Boulevard Laurier RC-709, Sainte-Foy, Qué., Canada G1V 4G2. Tel.: +1 418 654 2705; fax: +1 418 654 2715.
[2] F.V.B. is Chercheur qualifié of the Belgian Fonds National de la Recherche Scientifique.
[3] Present address: Division of Nephrology and Hypertension, University of Utah Health Science and VA Medical Centers, 50 North Medical Drive, Room 4R312, Salt Lake City, UT 84132, USA. Tel.: +1 801 581 6709; fax: +1 801 581 4343.

0009-3084/$ – see front matter
doi:10.1016/j.chemphyslip.2004.08.002

phosphatidylpropanol < phosphatidic acid). For liposomes containing phosphatidylinositol, this increase of activity was not modified by the presence of phosphatidylethanolamine and enhanced by cholesterol only when the phosphatidylinositol content was lower than 18%.
Our results, therefore show that both the surface-negative charge and the nature of the acidic lipid included in bilayers modulate the activity of phospholipase A1 towards phosphatidylcholine, while the change in lipid hydration or in fluidity of membrane are less critical. These observations may have physiological implications with respect to the rate of degradation of cellular membranes after their lysosomal segregation.

Keywords: Lysosomal phospholipase A1; Cholesterol; Phosphatidylethanolamine; Negatively-charged lipid; Fluidity; Lamellar phase; Surface potential

Enzymes that hydrolyse lipids usually access their substrate from the membrane phase and must therefore undergo a process of interfacial activation in which the enzyme attaches to the membrane before acting on the substrate. This process, which has been extensively studied for phospholipase A2 (PLA2), critically depends on the physico-chemical nature as well as the organization and dynamics of the interface (Roberts, 1996; Berg et al., 2001; Berg and Jain, 2002). Kinetic and X-ray structural studies of sPLA2s have established that these enzymes contain a recognition site that allows their attachment to the interface, which is distinct from the catalytic site where the esterolysis of a phospholipid molecule occurs. As a consequence, the substrate specificity and the level of activity of sPLA2s is dictated by the type of membrane interface to which the enzyme preferentially binds (interfacial specificity) as well as by the type of phospholipid that is accommodated in the catalytic site (catalytic site specificity) (Singer et al., 2002; Tatulian, 2003).

In contrast to the large body of data available for sPLA2, little is known concerning intracellular phospholipases and especially the lysosomal phospholipase A1. This enzyme however plays a crucial role for the degradation of intracellular phospholipids (Mellors and Tappel, 1967; Stoffel and Greten, 1967; Stoffel and Trabert, 1969; Franson et al., 1971; Shinozaki and Waite, 1999). Impairment of its activity by polycationic antibiotics (Laurent et al., 1982; Montenez et al., 1999) or amphiphilic cationic drugs (Lullmann-Rauch, 1979; Kodavanti and Mehendale, 1990; Halliwell, 1997; Schneider et al., 1997; Reasor and Kacew, 2001) is considered to be responsible for the development of lysosomal phospholipidosis observed in cells and tissues upon exposure to those drugs.

The mode of access of the phospholipid molecule to the catalytic site of phospholipase A1 implies its removal from the bilayer, the physico-chemical structure of which is therefore susceptible to strongly affect the overall activity of the enzyme. For instance, the rate of hydrolysis of DL-α-dipalmitoylphosphatidylcholine and L-α-dimyristoylphosphatidylcholine incorporated into liposomes, by a soluble fraction of liver lysosomes is maximal near the transition temperature (Vandenbranden et al., 1985). We also know that the activity of lysosomal phospholipase A1 is reduced when the surface pressure exceeds 32 dynes/cm^2 (Robinson and Waite, 1983) and increases markedly when the amount of negatively-charged phospholipid present in the vesicles is raised from 10 to 30% of the total phospholipid content (Mingeot-Leclercq et al., 1988, 1990; Piret et al., 1992), which is within the range found in most natural membranes (Bode et al., 1976).

Unfortunately, no systematic data on the role played by the organization and dynamics of the interface (Zhou et al., 1997) on lysosomal phospholipase A1 activity, are currently available. Using models mimicking biological membranes, we investigated the effect of two major lipids present in these membranes (cholesterol and phosphatidylethanolamine) as well as the effect of an increase in the content of negatively-charged lipids (phospholipid versus glycosphingolipid) on the hydrolysis of phosphatidylcholine by phospholipase A1 present in lysosomal extracts. In parallel, we also examined whether the variations observed in terms of activity are related to potential modifications induced by these lipids on critical membrane properties such as fluidity, lipid organization and surface potential of the bilayer.

Table 1
Composition of lipid vesicles

Percentage of negatively-charged lipid (in mol% of total phospholipids)	Standard vesicles (in concentration)	Vesicles minus cholesterol (in concentration)	Vesicles plus phosphatidylethanolamine (in concentration)
0	Chol/PC/SM/PX: 5/3.64/6.36/0	PC/SM/PI: 3.64/6.36/0	Chol/PC/SM/PI/PE: 5/3.64/3.64/0/2.72
2.3	Chol/PC/SM/PX: 5/3.64/6.13/0.23	PC/SM/PI: 3.64/6.13/0.23	Chol/PC/SM/PI/PE: 5/3.64/3.41/0.23/2.72
4.5	Chol/PC/SM/PX: 5/3.64/5.91/0.45	PC/SM/PI: 3.64/5.91/0.45	Chol/PC/SM/PI/PE: 5/3.64/3.19/0.45/2.72
6.8	Chol/PC/SM/PX: 5/3.64/5.68/0.68	PC/SM/PI: 3.64/5.68/0.68	Chol/PC/SM/PI/PE: 5/3.64/2.96/0.68/2.72
9.1	Chol/PC/SM/PX: 5/3.64/5.45/0.91	PC/SM/PI: 3.64/5.45/0.91	Chol/PC/SM/PI/PE: 5/3.64/2.73/0.91/2.72
13.6	Chol/PC/SM/PX: 5/3.64/5.00/1.36	PC/SM/PI: 3.64/5.00/1.36	Chol/PC/SM/PI/PE: 5/3.64/2.28/1.36/2.72
18.2	Chol/PC/SM/PX: 5/3.64/4.54/1.82	PC/SM/PI: 3.64/4.54/1.82	Chol/PC/SM/PI/PE: 5/3.64/1.82/1.82/2.72
27.3	Chol/PC/SM/PX: 5/3.64/3.63/2.73	PC/SM/PI: 3.64/3.63/2.73	Chol/PC/SM/PI/PE: 5/3.64/0.91/2.73/2.72
36.4	Chol/PC/SM/PX: 5/3.64/2.72/3.64	PC/SM/PI: 3.64/2.72/3.64	Chol/PC/SM/PI/PE: 5/3.64/0/3.64/2.72

PX = PI or PS or PA or GM1 or PG or PP. The net concentration of lipids is 15 mM including cholesterol and 10 mM without cholesterol.

1. Materials and methods

1.1. Lipid vesicles

All vesicles used in this work contained a constant amount (3.64 mM) of phosphatidylcholine (PC). The molar proportions of other lipids (cholesterol [Chol], sphingomyelin [SM], phosphatidylethanolamine [PE] and one acidic lipid [phosphatidylinositol [PI], phosphatidylserine [PS], phosphatidylglycerol [PG], phosphatidylpropanol [PP], phosphatidic acid [PA] or ganglioside GM1 [GM1]) varied as indicated in Table 1. The amount of phosphatidylethanolamine or the variations of negatively-charged lipids were compensated by a commensurate and inverse variation of the sphingomyelin content in order to maintain the phospholipid:cholesterol molar ratio constant (2:1). The concentration of total phospholipids was set at 10 mM throughout. For kinetic studies, only vesicles containing a constant amount of negatively-charged lipid (27 mol% [of total phospholipids] in phosphatidylinositol) were used.

Lipid vesicles were prepared as described previously (Laurent et al., 1982). Briefly, the required quantities of lipids were dissolved in chloroform:methanol (2:1, v:v) in a round bottomed flask. The solvent was evaporated under vacuum (Rotavapor® Buchi RE-111, Buchi, Flawil, Switzerland) to obtain a thin film of lipids which was dried overnight in a vacuum dessicator. Lipids were then resuspended in the required volume of buffer (40 mM Na acetate pH 5.4 for enzymatic and NMR studies or in 40 mM citrate/phosphate buffer pH 5.4 for electrophoretic studies), flushed with nitrogen and kept in a water bath at 37 °C (or 45 °C for liposomes containing gangliosides) for 1 h. This procedure yields multilamellar vesicles (MLV) used for ^{31}P NMR studies of lipid phase organization. Except for control experiments of lysosomal phospholipase activity performed on large unilamellar vesicles (LUV) prepared by extrusion, all other studies were made with small unilamellar vesicles (SUV) obtained by sonication of the MLV under a nitrogen flow (Branson Sonifier™, Branson Sonic Power Company, Danburg, CT) at 50 W for 5×2 min with 1 min intervals or until the opaque suspension became translucent. The preparation was then centrifuged ($800 \times g$ for 15 min) to remove particulate matter, stored at 4 °C under nitrogen, and used within 24 h. The average diameter of SUV liposomes

was evaluated by quasielastic light spectroscopy using a Nano-Sizer N4MD particle analyzer (Coulter Electronics Ltd., Luton, UK) and an unimodal analysis of data.

1.2. Soluble native and delipidated fractions of rat liver lysosomes

Purified lysosomes were isolated from livers of rats injected with Triton WR-1339 (Trouet, 1974) and a soluble fraction obtained by hypotonic shock followed by centrifugation as previously described (Schneider et al., 1979). Compared to the unfractionated liver homogenate, this extract was enriched approx. 45-fold in *N*-acetyl-β-hexosaminidase (used as a marker of lysosomes) (Sellinger et al., 1960) with a yield of 18%. The influence of endogenous lipids present in the soluble fraction on the activity of phospholipase A1 was tested using delipidated fractions of lysosomes as described by Matsuzawa et al. (1978). Briefly, the preparation was diluted with 40 mM acetate buffer pH 5.4 (incubation buffer) to obtain a protein concentration of 2.2 mg/ml, and then mixed thoroughly with an equal volume of ice-cold:water-saturated *n*-butanol. The mixture was centrifuged at 100,000 × *g* in a Beckman rotor Ti50 at 4 °C for 1 h. The pellet was resuspended in 40 mM Na acetate buffer pH 5.4. The butanol phase was dried at 37 °C under a stream of nitrogen and resuspended in 40 mM Na acetate buffer pH 5.4. The aqueous phase was separated from the butanol phase and the pellet, and thereafter subjected to gel filtration through Sephadex® G-50 to remove the contaminating butanol. This preparation is henceforth referred to as delipidated extract. The delipidation was freshly done each time just before assaying the phospholipase A1 activity. All fractions were assayed for protein (Lowry et al., 1951), total lipid phosphorus (Bligh and Dyer, 1959; Bartlett, 1959) and individual phospholipid (Ibrahim et al., 1989) contents as well as for phospholipase A1 activity as described below.

1.3. Assay of phospholipase A1 activity

The activity of phospholipase A1 present in soluble fractions of purified lysosomes was measured towards phosphatidylcholine as described previously (Laurent et al., 1982; Carlier et al., 1983) by following the release of labeled β-lysophosphatidylcholine from L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C] oleoyl (140 μCi of labeled phosphatidylcholine per mmol of cold phosphatidylcholine). In experiments aimed at determining the importance of the nature of the fatty acid chains or the polar head group on phospholipase A1 activity, we used L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]linoleoyl or L-3-phosphatidylethanolamine-1-palmitoyl-2-[1-^{14}C]oleoyl, respectively.

Labeled vesicles prepared in 40 mM Na acetate buffer pH 5.4 were mixed with an equal volume of soluble lysosomal fraction (15 μg proteins) and incubated at 37 °C for 30 min. The final concentration of the substrate during the assay was 1.82 mM. Substrate hydrolysis was kept below 25% of the total amount available. Appropriate blanks without enzyme were run in parallel. After incubation, the reaction was stopped by the addition of 50 μl methanol at 4 °C. The mixture was dried at 37 °C under a gentle nitrogen stream, the residue dissolved in 15 μl of chloroform–methanol (1:1; v/v) and spotted on a precoated thin-layer silica gel plate (E. Merck, AG, Darmstadt, Germany). Ascending chromatography was performed with chloroform–methanol–acetic acid–water (25:25:18:4, v/v) in parallel to internal standards. Plates were cut in strips after the phospholipids had been visualized by spraying bromophenol blue, and radioactivity was measured by scintillation counting. In preliminary experiments, we checked that the recovery of unlabeled phospholipid was reproducible between 95 and 110%. Results are expressed in nmol of β-lysophosphospholipids released per mg total proteins and per minute. In control experiments, we checked that the use of 40 mM citrate/phosphate buffer pH 5.4 (i.e. the buffer used for the electrophoretic mobility studies) in place of 40 mM acetate buffer did not significantly modify the enzyme activity.

For kinetic studies with soluble, native and delipidated fractions of lysosomes, concentrations of phosphatidylcholine spanning between 0.05 and 4 mM in the incubation mixtures were used.

1.4. Fluorescence polarization studies

Fluorescence polarization studies were performed with lipid vesicles diluted to a final concentration of 3.14 mM of total lipids. Incorporation of diphenylhexatriene (DPH; a totally hydrophobic fluorescent probe)

was achieved by a vigorous mixing followed by preincubation at 37 °C for 1 h in the dark (at a molar ratio to total lipids of 1:250). The fluorescence values emitted in the planes parallel (I_{par}) and perpendicular (I_{per}) to that of the polarized excitation light were measured at 37 °C. Results are expressed as polarization values ($P = [I_{par} - I_{per}]/[I_{par} + I_{per}]$). Fluorescence was measured on a LS-50 Perkin-Elmer fluorimeter (Perkin-Elmer, Beaconsfield, UK), equipped with a special adaptor for polarization measurements, and operating at an excitation wavelength of 365 nm and an emission wavelength of 427 nm. The samples were kept under gentle stirring throughout the experiment and the temperature was continuously monitored by a sensor placed into the measuring unit coupled with a programmable circulator bath DC5 (Haake, Karlsruhe, Germany).

1.5. ^{31}P NMR studies

^{31}P nuclear magnetic resonance (NMR) spectroscopy was used to examine the effect of the nature of the negatively-charged lipid and phosphatidylethanolamine on the size and organization of the membrane. A Bruker AC 250 spectrometer operating at 101.3 MHz for ^{31}P observation was used with an internal ^{2}H lock (15% D_2O) for field frequency stabilization; 10 mm tubes containing 2 cm^3 of the dispersion were employed. The Fourier-transform conditions were: spectral width, 25 KHz; 45° (12 μs) flip angle; 8 K data points; and 1.2 s repetition time. Accumulated free induction decays were obtained from 5000 transients. We used the power gated ^{1}H decoupling mode, and a 25 or 50 Hz line-broadening was applied to the free induction decays of SUV and MLV, respectively. The sample temperature was regulated at 25 ± 1 °C for SUV. MLV spectra were recorded upon warming of the sample from 25 to 62 °C with an equilibration time of 15 min before acquiring data at a new temperature.

1.6. Capillary electrophoresis studies

Free solution capillary electrophoresis was used to determine the surface potential of liposomes containing an acidic phospholipid using a HPETM 100 Capillary Electrophoresis System from Bio-Rad Laboratories (Regotta, CA). Liposomes introduced by electrophoretic loading migrated as electrical force drives them through a coated silica capillary tube (50 cm × 50 μm; No. 1483013 cartridge), filled with electrolyte (citrate-phosphate buffer 40 mM pH 5.4). Liposomes were monitored by an UV detector as they migrated through a segment of the capillary, and the detector signal was displayed as peaks on an electropherogram. Capillary electrophoresis was performed with negative polarity (i.e., liposomes have a net negative charge and migrated towards the positively-charged anode) and in constant voltage mode. The loading and running conditions were 5 s and 30 min, and 8 and 12 kV, respectively. A standard calibration curve using substance P and fragments thereof was performed before the injection of the liposomes according to the manufacturer's instructions. The temperature was maintained at 30.0 ± 0.2 °C for all experiments. For gangliosides-containing liposomes, the electrophoretic analysis was performed on a Biofocus 3000 from Bio-Rad Laboratories (Regotta, CA). All experimental conditions were the same as above except that the injection time, length of the capillary and temperature were fixed at 20 s, 36 cm and 20 °C respectively. The surface potential (ψ_o), defined as the electrical potential at the membrane surface with reference to the potential in the bulk aqueous phase (ζ), was estimated from electrophoretic mobility measurement (μ) (Glaser, 2001).

1.7. Statistical analysis

Differences between levels of enzymatic activity were assessed using unpaired *t*-tests. All statistical analyses were performed with the Statview + SE Software (Abacus Concepts, Berkeley, CA) with $P < 0.05$ considered as significant. The kinetic parameters of phospholipase A1 activity (V_{max}, K_m) were estimated by fitting the corresponding data to an hyperbolic function using a non-linear regression analysis based on the damping Gauss–Newton's iterations method (Yamaoka et al., 1981).

1.8. Source of major products

Phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, phosphatidylpropanol, phosphatidic acid, egg yolk phosphatidylcholine and phosphatidylethanolamine) were purchased from Lipid Product (Nr. Redhill, UK) as grade 1 products. Bovine brain sphingomyelin, monosialo-

ganglioside GM1, and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). Labeled phospholipids (L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]oleoyl, L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]linoleoyl, L-3-phosphatidylethanolamine-1-palmitoyl-2-[1-^{14}C]linoleoyl; specific radioactivity 53 mCi/mmol) were purchased from Amersham International plc (Buckingamshire, UK). Diphenylhexatriene (DPH) was obtained from Molecular Probes (Eugene, OR). Other reagents were of analytical grade.

2. Results

2.1. Nature of endogenous phospholipids present in the soluble fraction of lysosomes

The soluble lysosomal fraction was found to contain 244 nmol of phospholipids per mg protein. Thin-layer chromatography showed the following typical relative composition: phosphatidylethanolamine plus phosphatidylglycerol, 40.0%; total acidic phospholipids (phosphatidylinositol plus phosphatidylserine), 24.2%; lysophospholipids, 15.7%; sphingomyelin, 13.8%; and phosphatidylcholine, 6.5%. This composition, which is quite different from that of the lysosomal or pericellular membrane (Bode et al., 1976) was considered to reflect the content of the lysosomal matrix. Delipidation allowed to remove 99.1% of these phospholipids, while about 34% of proteins were precipitated in this process. Phospholipase A1 activity (measured towards phosphatidylcholine) was recovered in the aqueous phase with a yield of approx. 73% and an enrichment of approx. 1.6 on a protein basis compared to the non-delipidated extract.

2.2. Influence of phosphatidylinositol and kinetic characterization of phospholipase A1 activity in native and in delipidated soluble lysosomal fraction

Since acidic phospholipids represent a major component of the phospholipids present in the lysosomal matrix and biological membranes, we examined the influence of variation of the phosphatidylinositol content of the vesicles on the activity of phospholipase A1 using phosphatidylcholine as substrate and comparing the native and the delipidated, soluble lysosomal fraction as enzyme source. These experiments were made with vesicles for which the increase in phosphatidylinositol was compensated by a commensurate decrease in sphingomyelin content, and the results are shown in Fig. 1. The most striking observation was that the activities were quite low in the absence of phosphatidylinositol in the vesicles for both the native and the delipidated soluble fractions of lysosomes, but markedly increased when this content was brought to 9 mol% or more. A plateau was observed for the native extracts when the content in phosphatidylinositol reached about 18 mol% of total phospholipids, which is close to the value observed for acidic phospholipids in the lysosomal matrix (see above) and in biological membranes. For the delipidated fraction, activity rose continuously when the phosphatidylinositol content of the vesicles was brought from 10 to 27 mol%, but a plateau was thereafter reached at a value about twice that of the native extract. The percentage of phosphatidylinositol needed for half-maximal activation for native and delipidated fractions was 8.2 and 15.0%, respectively. The kinetic parameters of these activities were studied thereafter. The data describing the velocity of the enzyme reactions for both the native and the delipidated fractions of lysosomes could be fitted to an hyperbolic equation

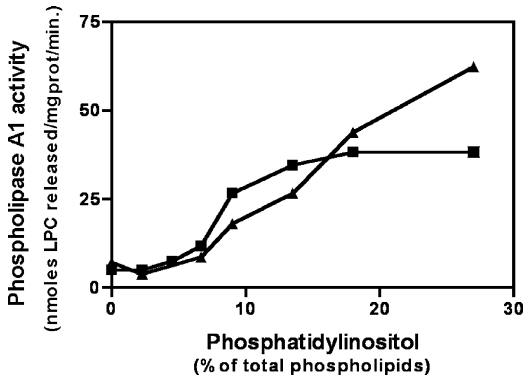

Fig. 1. Effect of endogenous lipids on the activity of phospholipase A1 towards L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]oleoyl (140 μCi/mmol) included in liposomes composed of Chol:PC:SM:PI, as a function of their content in phosphatidylinositol. The final phosphatidylcholine concentration in the incubation mixture was set constant at 1.82 mM. The ordinate indicates the amount of labeled lysophosphatidylcholine (LPC) released (nmol mg protein^{-1} min^{-1}) at 37 °C in presence of soluble, native (■) or delipidated (▲) fractions of lysosomes. Each data point shown is the mean of triplicate incubations of three independent experiments (n = 9), with less than 4% variation.

Table 2
Kinetic parameters (K_m and V_{max}) of phospholipase A1 in native and delipidated fractions of lysosomes towards phosphatidylcholine

Parameter	Enzyme state	
	Native	Delipidated
K_m (mM PC)	1.5 ± 0.2	4.0 ± 0.3
V_{max} (nmol of LPC released × mg protein^{-1} min^{-1})	80.2 ± 3.9	155.0 ± 6.0

Lipid vesicles contained 27 mol% phosphatidylinositol with respect to total phospholipids. Kinetic parameters were estimated by non-linear regression analysis using the damping Gauss–Newton's iterations method. The matrix of normal equations gives the values of the parameters and the inverse matrix their standard deviations.

of the Michaelis-Menten type, and the corresponding kinetic parameters (V_{max}, K_m) are given in Table 2. Delipidation caused approximately a two-fold increase of both kinetic parameters as compared to that observed with the native enzyme. Thus, the delipidated enzyme had a lower affinity, but a higher capacity to hydrolyse phosphatidylcholine included in these vesicles.

2.3. Modulating effect of membrane lipids on native lysosomal phospholipase A1 activity

Because lysosomal phospholipase A1 is able to recognize and hydrolyze both phosphatidylcholine and phosphatidylethanolamine, we addressed, using native enzymes, the question as to whether the dependency of an acidic phospholipid for optimal activity was observed to the same extent for both phospholipids. As shown in Fig. 2, following the hydrolysis of L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]oleoyl or L-3-phosphatidylethanolamine-1-palmitoyl-2-[1-^{14}C]oleoyl, an increased activity of phospholipase A1 upon an increase of the vesicles content in phosphatidylinositol was observed independently of the nature of the substrate. The absolute level of activity, however, was lower towards phosphatidylethanolamine as compared to phosphatidylcholine. In subsequent experiments, it was checked that phosphatidylcholine hydrolysis was independent of the nature of the fatty acid chain in position 2 (oleoyl instead of linoleoyl) and of the type of liposomes used (SUV or LUV) (data not shown).

Focusing on phosphatidylcholine as substrate and on native soluble fractions of lysosomes as enzyme source, we then explored the influence of biophysical membrane properties on the effect of negative charge on the activity of lysosomal phospholipase A1 towards phosphatidylcholine by examining the influence of cholesterol, phosphatidylethanolamine, and the nature of the acidic lipids.

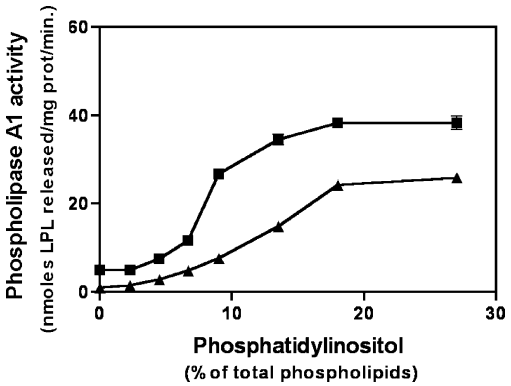

Fig. 2. Effect of the nature of the substrate (phosphatidylcholine vs. phosphatidylethanolamine) on the activity of phospholipase A1 present in soluble native fractions of lysosomes as a function of the content in phosphatidylinositol included in liposomes composed of Chol:PC:SM:PI or composed of Chol:PE:SM:PI. The ordinate indicates the amount of labeled lysophospholipids (LPL; lysophosphatidylcholine) (■) or lysophosphatidylethanolamine (▲) released (nmol mg protein^{-1} min^{-1}) at 37 °C from L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]linoleoyl or L-3-phosphatidylethanolamine-1-palmitoyl-2-[1-^{14}C]linoleoyl included in liposomes (140 μCi/mmol). Each data point shown is the mean of triplicate incubations of three independent experiments (n = 9), with less than 6% variation.

2.3.1. Effect of cholesterol

Fig. 3 (left panel) shows that the presence of cholesterol modified the activity of phospholipase A1 when tested in vesicles containing less than 18 mol% of phosphatidylinositol. In the absence of cholesterol, the activity remained higher at lower phosphatidylinositol contents than in the presence of cholesterol. The dependency of the activity upon a variation of phosphatidylinositol was however maintained with a marked rise in activity for phosphatidylinositol concentrations ranging between 4.5 and 9.0 mol% (of total phospholipids). Since the presence of cholesterol is known to affect the fluidity of the membrane (Yeagle et al., 1990; Mitchell and Litman, 1998), we determined this parameter in both types of vesicles. The fluorescence polarization value of DPH as a function of the phosphatidylinositol content in-

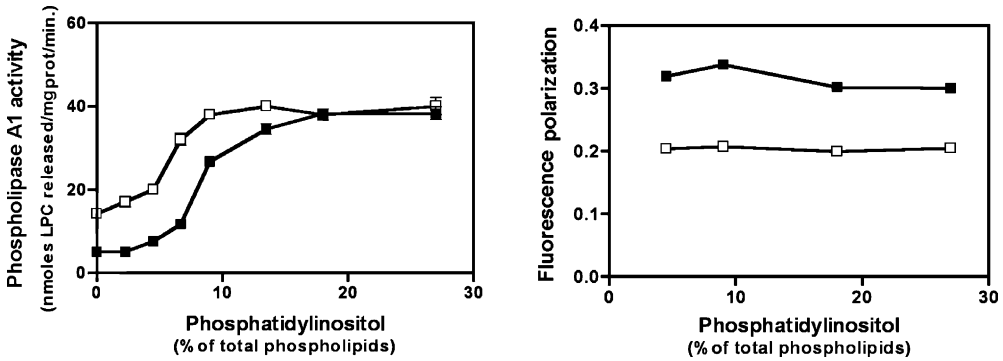

Fig. 3. Left panel: effect of cholesterol on the activity of phospholipase A1 present in soluble fractions of lysosomes towards L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]oleoyl (140 μCi/mmol) included in liposomes composed of Chol:PC:SM:PI or PC:SM:PI, as a function of their content in phosphatidylinositol. The final phosphatidylcholine concentration in the incubation mixture was set constant at 1.82 mM. Vesicles containing a fixed amount of cholesterol (■) (molar ratio phospholipid to cholesterol of 2:1); vesicles containing no cholesterol (□). The ordinate indicated the amount of labeled lysophosphatidylcholine (LPC) released (nmol mg protein^{-1} min^{-1}) at 37 °C. Each data point shown is the mean of triplicate incubations of three independent experiments ($n = 9$), with less than 10% variation. Right panel: fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) probe incorporated in the bilayer of lipid vesicles containing increasing amounts of phosphatidylinositol and a fixed amount of cholesterol (■) or no cholesterol (□). Each data point shown is the mean of triplicate, with less than 4% variation (S.D. values have not been shown for the sake of clarity).

cluded in cholesterol-containing and cholesterol-free vesicles is shown in Fig. 3 (right panel). As anticipated, cholesterol markedly increased the rigidity of the bilayers, since the corresponding fluorescence polarization value was considerably increased as compared to cholesterol-free vesicles. A variation in phosphatidylinositol content, however, did not affect the fluidity of the bilayers, since the polarization value remained essentially constant for both cholesterol-containing and cholesterol-free vesicles during the variations in phosphatidylinositol content explored (4.5–27 mol%).

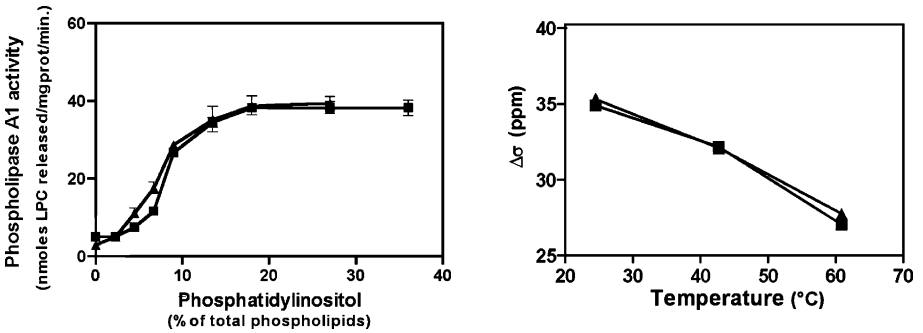

Fig. 4. Left panel: effect of phosphatidylethanolamine on the activity of phospholipase A1 present in soluble fractions of lysosomes towards L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]oleoyl included in liposomes composed of Chol:PC:SM:PI:PE or Chol:PC:SM:PI as a function of their content in phosphatidylinositol. The final phosphatidylcholine concentration in the incubation mixture was set constant at 1.82 mM. The amount of phosphatidylethanolamine was fixed to 0% (■) or 27% (▲). The ordinate indicates the amount of labeled lysophosphatidylcholine (LPC) released (nmol mg protein^{-1} min^{-1}) at 37 °C. Results are mean ± S.D. of triplicate incubations of three independent experiments ($n = 9$). Right panel: variation of the effective chemical shift anisotropy ($\Delta\sigma$) in ^{31}P NMR of large multi-lamellar vesicles as a function of temperature. The different curves correspond to liposomes containing no (■) or 27% (▲) of phosphatidylethanolamine. Each sample was successively examined at increasing temperatures and recordings were made over ≈1 h at each temperature investigated. ($\Delta\sigma$) values were calculated as indicated in Section 1. Each data point shown is the mean of three experiments, with less than 10% variation (S.D. values are not shown for the sake of clarity).

2.3.2. Effect of phosphatidylethanolamine

Fig. 4 (left panel) shows that a partial replacement of sphingomyelin by phosphatidylethanolamine did not change the phospholipase A1 activity (measured towards phosphatidylcholine) whatever the phosphatidylinositol content over the whole range investigated (2.3–27 mol% of total phospholipids). Since phosphatidylethanolamine is known to induce changes in membrane organization (Silvius, 1986), we examined by ^{31}P NMR the organization of the lipids in MLV containing a fixed amount of phosphatidylethanolamine (27 mol% of total phospholipids) in comparison with phosphatidylethanolamine-free vesicles. The ^{31}P NMR spectra showed the characteristic line shape of multilamellar phospholipids with a maximum at high field and a shoulder at low field (Seelig, 1978). Since the shoulder at low field was not well-defined, the effective chemical shift anisotropy $\Delta\sigma$ was deduced by measuring the difference between the high field maximum chemical shift and the isotropic shift which corresponds to one-third of the $\Delta\sigma$ value (Seelig, 1978). Fig. 4 (right panel) summarizes the results of the $\Delta\sigma$ variation as a function of temperature for the two types of vesicles (0 or 27 mol% phosphatidylethanolamine). No effect was seen. In subsequent experiments, we also observed that phosphatidylethanolamine had no significant effect on membrane fluidity (data not shown).

2.3.3. Importance of the nature of the negatively-charged lipids

Fig. 5 (left panel) shows that not only the proportion but also the nature of the acidic lipids included in the bilayer had a significant influence on phospholipase A1. Phosphatidic acid caused a much steeper rise, which maintained itself up to a content of approx. 36 mol% of the total phospholipids, than what was observed with phosphatidylinositol and phosphatidylserine. A plateau was then reached at a value about twice that observed with phosphatidylinositol or phosphatidylserine. To verify the importance of the negative charge for lysosomal phospholipase activity, we run parallel experiments with phosphatidylglycerol or phosphatidylpropanol. The activity was strictly similar to that found with phosphatidylinositol or phosphatidylserine (data not shown). In sharp contrast, inclusion of ganglioside GM1 only increased sluggishly the activity of phospholipase A1, which reached values higher than that of neutral liposomes only when its amount exceeded 27 mol% of total phospholipids. The activity of phospholipase A1 in vesicles containing 36 mol% GM1 was still lower than that seen for vesicles containing the same content of phosphatidylinositol or phosphatidylserine, and was

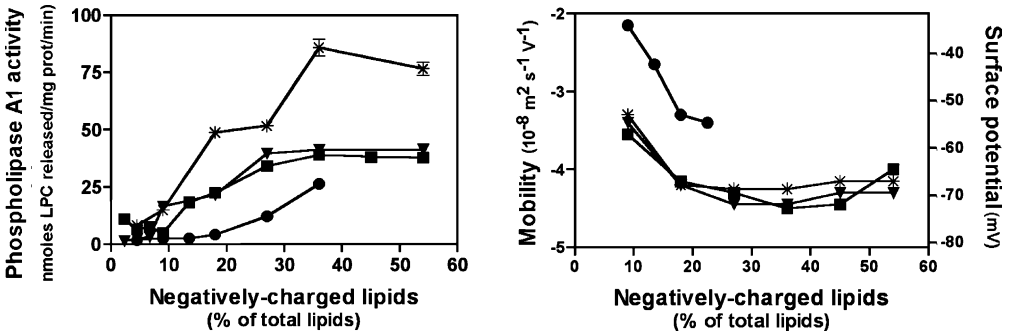

Fig. 5. Left panel: effect of negatively-charged lipids on the activity of phospholipase A1 present in soluble fractions of lysosomes towards L-3-phosphatidylcholine-1-palmitoyl-2-[1-^{14}C]oleoyl included in liposomes composed of Chol:PC:SM:PI, Chol:PC:SM:PS, Chol:PC:SM:PA or Chol:PC:SM:GM1 as a function of their content in negatively-charged lipid; phosphatidylinositol (■), phosphatidylserine (▼), phosphatidic acid (✳), ganglioside GM1 (●). The final phosphatidylcholine concentration in the incubation mixture was set constant at 1.82 mM. The ordinate indicates the amount of labeled lysophosphatidylcholine (LPC) released (nmol mg protein^{-1} min^{-1}) at 37 °C. Each data point shown is the mean of triplicate incubations of three independent experiments ($n = 9$), with less than 10% variation. Right panel: electrophoretic mobility of liposomes containing increasing amounts of negatively-charged lipid; phosphatidylinositol (■), phosphatidylserine (▼), phosphatidic acid (✳), ganglioside GM1 (●) measured by capillary electrophoresis (12 kV; 30 min). The abscissa shows the percentage of negatively-charged lipid present in liposomes. The left ordinate shows the actual mobility, and the right ordinate the corresponding surface potential of each liposome preparation. Each data point shown is the mean of triplicate, with less than 6% variation (S.D. values have not been shown for the sake of clarity).

actually similar to that observed for vesicles containing only approx. 20 mol% of these acidic phospholipids. Ganglioside GM1 contents higher than 36 mol% were not investigated since biological membranes never contain such a large proportion of gangliosides (Curatolo, 1987). In parallel, we measured the electrophoretic mobility of the vesicles used to assay for phospholipase A1 activity with the aim to determine their surface potential. The surface potential of all vesicles containing an acidic phospholipid (phosphatidic acid, phosphatidylinositol, and phosphatidylserine) was very similar and decreased when the percentage of acidic phospholipid increased from 9 to 18 mol% but remained almost constant thereafter (with a slight rise, however, for vesicles containing 55 mol% phosphatidylinositol). In GM1-containing vesicles, the mobility was also negatively related to the amount of ganglioside present, but the surface potential was always higher (i.e., less negative) than that of vesicles containing a similar molar proportion of acidic phospholipids. The calculations allowing to derive the surface potential from electrophoretic mobility values depend on both the viscosity of the medium and the size of the particles. These two parameters were determined for all the liposome preparations studied using the Oswald capillary approach and direct determination of vesicles size by quasi-elastic scattering spectroscopy using unimodal analysis and ^{31}P NMR spectroscopy. No difference in viscosity was seen, and no significant effect of the nature and content of acidic lipid on the average sizes of the different vesicles was noted (data not shown).

3. Discussion

Lysosomes play an important role in the metabolism of both endogenous and exogenous phospholipids that gain access to them by the processes of autophagy and phagocytosis, respectively. Phospholipase A1-catalyzed deacylation is the preferred reaction initiating diacylphosphoglyceride catabolism in rat liver lysosome, since neither phospholipase C nor phospholipase D seem to act appreciably on L-3-phosphatidylcholine-1-palmitoyl-2-oleoyl (Laurent et al., 1982; Kunze et al., 1982). Because phospholipids are most often organized in the form of bilayers or micelles, phospholipase A1 has to act at or close to an hydrophobic/hydrophilic interface (Dennis, 1983) and must probably bind to the membrane to be active. Interface recognition region is, therefore, likely to be an essential determinant in its activity and kinetic properties. In the present study, we have investigated in detail the respective roles of three types of lipids (cholesterol, phosphatidylethanolamine, and acidic lipids) known to modify the physico-chemical properties of bilayers.

Phospholipase A1 activity has been examined mainly with respect to phosphatidylcholine hydrolysis since this phospholipid is the major constituent of cellular membranes (Bode et al., 1976). Our experiments have been designed to mimic the composition and physico-chemical properties of biological membranes (Robinson and Waite, 1983) on one hand and the enzymatic environment present in lysosomes on the other. For this purpose, we used substrate-vesicles containing the major types of lipids found in biological membranes (cholesterol, phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and one negatively-charged phospholipid), and as enzyme source a total soluble fraction of lysosomes to remain as closely as possible to the mixture of the various enzymes and co-factors naturally present in lysosomes (Kunze et al., 1988). One advantage of this approach is to avoid artefacts due to phospholipases purification like selection of one isoenzyme, modification of enzyme structure by lysosomal proteases or glycosidases, alteration of enzymatic activity by ethylene glycol required to improve the stability of the purified enzyme (Hostetler et al., 1982; Robinson and Waite, 1983; Loffler and Kunze, 1989). The use of such soluble fractions of lysosomes has successfully allowed to study the mechanism of various forms of drug-induced phospholipidosis, since a close correlation has been demonstrated between the capacity of the drugs to inhibit phospholipase A1 activity in vitro and their capacity to induce a lysosomal phospholipidosis in cell culture models as well as in vivo (Lullmann-Rauch, 1979; Laurent et al., 1982; Kacew, 1987; Schneider et al., 1997; Mingeot-Leclercq and Tulkens, 1999; Montenez et al., 1999). The drawback in our approach, however, is that it does not allow to study specific interactions between substrate and enzyme. We nevertheless have been able to characterize many pertinent aspects of the enzymatic model used. Thus, we (i) established which endogenous lipids are present in the native soluble fractions of lysosomes, (ii) examined on a comparative fashion the influence exerted by

one specific acidic phospholipid, phosphatidylinositol, on the activity of both the native and the delipidated fractions of lysosomes, and (iii) compared the activity of phospholipase A1 towards two major zwitterionic phospholipids, namely phosphatidylcholine and phosphatidylethanolamine, which only differ by their polar head group. These studies not only confirmed that delipidation did not grossly affect the regulation of phospholipase A1 activity by negatively-charged phospholipids (Kunze et al., 1988) also unambiguously demonstrated that phosphatidylcholine is a preferred substrate compared to phosphatidylethanolamine, as observed with a purified enzyme (Hostetler et al., 1982; Robinson and Waite, 1983). This preferential hydrolysis of phosphatidylcholine could result from the differences in its orientation at the interface, compared to phosphatidylethanolamine, in the size of their respective polar groups, and/or from an enhanced diffusion of products from the enzyme (Waite, 1985; Kucera et al., 1988).

A first major observation made in this study is that the increase of phosphatidylcholine hydrolysis by phospholipase A1 activity due to negatively-charged lipids included in the vesicles is not influenced by phosphatidylethanolamine and is largely independent of the presence of cholesterol. This may result from two properties of membranes which we analyzed here and may be critical with respect to the access of enzyme–lipid substrates, namely the organization of the lipids in the bilayer and its fluidity. With respect to phosphatidylethanolamine, when this lipid is included in the liposomes at 27 mol%, a value which is within the physiological range, the lamellar phospholipid organization is not altered (for hexagonal phase to appear, the proportion of phosphatidylethanolamine needs, indeed, to be larger (Silvius, 1986)). Concerning the cholesterol, we noted that its effect consists essentially in making the enzyme activity more susceptible to the stimulating effect exerted by an increase in the phosphatidylinositol content of the vesicles. Our fluorescence polarization studies show that cholesterol decreases the membrane fluidity. We may, therefore, suggest that fluid bilayers provide a more appropriate interface at low phosphatidylinositol content than rigid ones, perhaps by allowing the active site of the enzyme to more easily access individual molecules of substrate. This effect may, however, be important only at low phosphatidylinositol contents. At higher contents, the large density of negative charges will allow a more efficient anchoring of the enzyme on the bilayer surface, making the role of membrane fluidity less noticeable.

A second and perhaps the most interesting feature of the present work is to give insights about the role of the membrane potential and/or the nature of the negatively-charged lipids in lysosomal phospholipase A1 activity. The influence of the surface-negative charge on the activity of phospholipases has already been described not only for phospholipase A1 activity (acting on phosphatidylcholine in mixed micelles containing dicetylphosphate and stearylamine (Robinson and Waite, 1983)) but also for several other enzymes acting at a lipid–water interface, such as the pancreatic phospholipase A2 (Volwerk et al., 1986), the human non-pancreatic secretory phospholipase A2 (Kinkaid et al., 1997), the glucocerebrosidase (Vaccaro et al., 1990), and the glucosylceramidase (Vaccaro et al., 1997; Wilkening et al., 1998; Ciaffoni et al., 2001). We show here that this effect of the negative charge is not simply the consequence of a decrease in the sphingomyelin content of the vesicles when the acidic phospholipid is increased (since these two phospholipids are most often exchanged from one another when the charge of the membrane has to be varied in order to maintain constant cholesterol:phospholipid ratio). This is in clear contrast with what is observed for sPLA2 and lipoprotein lipase (Lobo and Wilton, 1997; Koumanov et al., 1998), and identifies the mode of membrane recognition by lysosomal phospholipases A1 as clearly distinct from that of sPLA2. Because the membrane-surface potential is sensitive to differences in the average conformation of phospholipid head groups relative to the membrane-normal, we would expect this potential to be modified according to the type of phospholipid included in the vesicles (Seelig et al., 1987). The variations in acidic lipids composition that we made did not change the vesicle size or the lipid organization. We may, therefore, reasonably assume that the changes in activity observed are related to the presence of the acidic lipids themselves and to changes in the surface potential. The membrane potential values we observed for vesicles containing acidic phospholipids are in accordance with those obtained by the determination of the distribution of methylene blue between negatively-charged phospholipid membrane and the bulk aqueous phase (Nakagaki et al., 1981; Kuate and Mingeot-Leclercq, unpublished data) as well

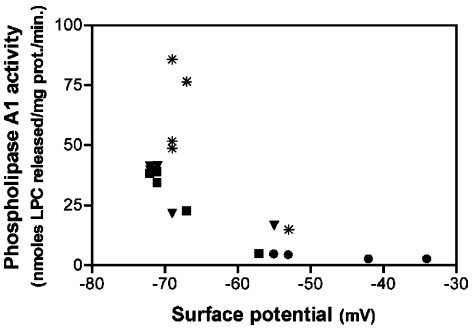

Fig. 6. Correlation between the activity of phospholipase A1 and the surface charge of the various liposome preparations used: phosphatidylinositol (■), phosphatidylserine (▼), phosphatidic acid (✳), and ganglioside GM1 (●).

as from the prediction of the Gouy Chapman theory of the diffuse double layer (McLaughlin, 1977). The lower surface charge associated with the inclusion of the GM1 ganglioside, in comparison with the acid phospholipids and at equimolar concentrations, probably results from the fact that the head group of the ganglioside, when extended maximally, protrudes up to 2 nm above the bilayer surface with its negative charge moving unrestricted at approx. 1 nm of this surface (Lee et al., 1980; Delmelle et al., 1980; McDaniel et al., 1984), which is much farther away than for acidic phospholipids. Actually, within the range of concentrations investigated, GM1 ganglioside exerts a somewhat inhibitory effect on the activity of lysosomal phospholipase A1, compared to acidic phospholipids. This phenomenon had been observed previously for pancreatic phospholipase A2 (Bianco et al., 1989). An alternative explanation, however, could be the formation of domains (Maggio et al., 1988; Bianco et al., 1989) which could exclude phosphatidylcholine (Tillack et al., 1982; Masserini and Freire, 1986) and, therefore, separate the substrate from the negative charges necessary for enzyme activity (Romero et al., 1987). Finally, gangliosides may simply have a steric effect by virtue of their large oligosaccharide head group, decreasing thereby the access of lysosomal phospholipase to the substrate in the membrane interfacial region.

Whatever the precise molecular or structural mechanism, our results show unambiguously that the surface-negative charge is one critical determinant in the modulation of the activity of phospholipase A1 (see Fig. 6). This was ascertained by the observation that at least two other negatively-charged phospholipids (phosphatidylglycerol and phosphatidylpropanol) increased the lysosomal phospholipase activity towards phosphatidylcholine exactly as is observed for phosphatidylinositol. The fact that phosphatidic acid is a much more effective activator than the other acidic phospholipids can also be interpreted as an evidence for the role of the negative charges. Phosphatidic acid, indeed, has two negative charges, and could, therefore, more easily anchor the enzyme close to its substrate, explaining its higher potency to activate phospholipase activity (Tocanne and Teissie, 1990). Moreover, phosphatidic acid is probably more accessible from the aqueous phase and more susceptible to pack itself around phosphatidylcholine. This may not be the case for phosphatidylinositol or phosphatidylserine, the polar heads of which are more bulky.

In conclusion, the present study shows that the phospholipase A1 activity observed in soluble extracts from purified lysosomes shares with other phospholipases, the intriguing property of changing its activity as a result of the presence of non-substrate lipids (Feng et al., 2003). This effect, which depends on the properties of the membrane-surface and on the specific characteristics of the individual lipid components, may play a crucial role in the activities of the corresponding enzymes in vivo. Taken together, the results suggest that the bilayer surface charge, and probably also the phospholipids packing are important modulators of lysosomal phospholipase A1. Purification and site-mediated mutagenesis studies could be useful to identify the aminoacid residues that are responsible for membrane binding and interfacial activation of phospholipase A1. All these studies could also be helpful to understand the lipidic alterations of membranes observed in various pathological situations.

Acknowledgments

This work was supported by the Belgian Fonds National de la Recherche Scientifique Médicale (grant no. 1.5.213.87F), Region wallone (grant no. 115020), and Fonds de la Recherche Scientifique Médicale (grant no. 3.4553.88, 3.4589.96, 3.4546.02) and Service de la Politique et de la Programmation Scientifique (grants no. 88-93/122 Actions concertées and 7bis PAI). We

thank Dr. P. Gilbert and Dr. J.M. Dethy (Continental Pharma/Searle, s.a., Louvain-la-Neuve, Belgium) as well as Dr. J. Caspers (Laboratoire de Chimie Physique des Macromolécules aux Interfaces, Université Libre de Bruxelles, Brussels, Belgium) for teaching us the capillary electrophoresis technique. We gratefully acknowledge the help of A. Loomis for correcting the English.

References

Bartlett, G.R., 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234, 466–468.

Berg, O.G., Gelb, M.H., Tsai, M.D., Jain, M.K., 2001. Interfacial enzymology: the secreted phospholipase A(2)-paradigm. Chem. Rev. 101, 2613–2654.

Berg, O.G., Jain, M.K., 2002. Interfacial Enzymes Kinetics. Wiley, Chichester, UK.

Bianco, I.D., Fidelio, G.D., Maggio, B., 1989. Modulation of phospholipase A2 activity by neutral and anionic glycosphingolipids in monolayers. Biochem. J. 258, 95–99.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Bode, F., Baumann, K., Kinne, R., 1976. Analysis of the pinocytic process in rat kidney. II. Biochemical composition of pinocytic vesicles compared to brush border microvilli, lysosomes and basolateral plasma membranes. Biochim. Biophys. Acta 433, 294–310.

Carlier, M.B., Laurent, G., Claes, P.J., Vanderhaeghe, H.J., Tulkens, P.M., 1983. Inhibition of lysosomal phospholipases by aminoglycoside antibiotics: in vitro comparative studies. Antimicrob. Agents Chemother. 23, 440–449.

Ciaffoni, F., Salvioli, R., Tatti, M., Arancia, G., Crateri, P., Vaccaro, A.M., 2001. Saposin D solubilizes anionic phospholipid-containing membranes. J. Biol. Chem. 276, 31583–31589.

Curatolo, W., 1987. Glycolipid function. Biochim. Biophys. Acta 906, 137–160.

Delmelle, M., Dufrane, S.P., Brasseur, R., Ruysschaert, J.M., 1980. Clustering of gangliosides in phospholipid bilayers. FEBS Lett. 121, 11–14.

Dennis, E.A., 1983. Phospholipases. In: Boyer, P.D. (Ed.), The Enzymes — liid enzymology. Academic Press, New York, NY, pp. 307–353.

Feng, J., Bradley, W.D., Roberts, M.F., 2003. Optimizing the interfacial binding and activity of a bacterial phosphatidylinositol-specific phospholipase C. J. Biol. Chem. 278, 24651–24657.

Franson, R., Waite, M., LaVia, M., 1971. Identification of phospholipase A 1 and A 2 in the soluble fraction of rat liver lysosomes. Biochemistry 10, 1942–1946.

Glaser, R., 2001. Molecular structure of biological systems. In: Glaser, R. (Ed.), Biophysics. Springer-Verlag, Berlin, Germany, pp. 5–104.

Halliwell, W.H., 1997. Cationic amphiphilic drug-induced phospholipidosis. Toxicol. Pathol. 25, 53–60.

Hostetler, K.Y., Yazaki, P.J., van den, B.H., 1982. Purification of lysosomal phospholipase A. Evidence for multiple isoenzymes in rat liver. J. Biol. Chem. 257, 13367–13373.

Ibrahim, S., Van der, A.P., Meunier, F., Tulkens, P.M., 1989. Effect of netilmicin and amikacin on urinary phospholipids excretion in humans. Arch. Toxicol. (Suppl.) 13, 413–416.

Kacew, S., 1987. Cationic amphiphilic drug-induced renal cortical lysosomal phospholipidosis: an in vivo comparative study with gentamicin and chlorphentermine. Toxicol. Appl. Pharmacol. 91, 469–476.

Kinkaid, A.R., Voysey, J.E., Wilton, D.C., 1997. Cholesterol-3-sulfate enhances phospholipase A2 activity by promoting interfacial binding. Biochem. Soc. Trans. 25, 497S.

Kodavanti, U.P., Mehendale, H.M., 1990. Cationic amphiphilic drugs and phospholipid storage disorder. Pharmacol. Rev. 42, 327–354.

Koumanov, K.S., Quinn, P.J., Bereziat, G., Wolf, C., 1998. Cholesterol relieves the inhibitory effect of sphingomyelin on type II secretory phospholipase A2. Biochem. J. 336 (Pt 3), 625–630.

Kucera, G.L., Sisson, P.J., Thomas, M.J., Waite, M., 1988. On the substrate specificity of rat liver phospholipase A1. J. Biol. Chem. 263, 1920–1928.

Kunze, H., Bohn, E., Loffler, B.M., 1988. Inhibitors of liver lysosomal acid phospholipase A1. Eur. J. Biochem. 177, 591–595.

Kunze, H., Hesse, B., Bohn, E., 1982. Hydrolytic degradation of phosphatidylethanolamine and phosphatidylcholine by isolated rat-liver lysosomes. Biochim. Biophys. Acta 711, 10–18.

Laurent, G., Carlier, M.B., Rollman, B., Van Hoof, F., Tulkens, P., 1982. Mechanism of aminoglycoside-induced lysosomal phospholipidosis: in vitro and in vivo studies with gentamicin and amikacin. Biochem. Pharmacol. 31, 3861–3870.

Lee, P.M., Ketis, N.V., Barber, K.R., Grant, C.W., 1980. Ganglioside headgroup dynamics. Biochim. Biophys. Acta 601, 302–314.

Lobo, L.I., Wilton, D.C., 1997. Combined effects of sphingomyelin and cholesterol on the hydrolysis of emulsion particle triolein by lipoprotein lipase. Biochim. Biophys. Acta 1349, 122–130.

Loffler, B.M., Kunze, H., 1989. An improved purification procedure for rat liver lysosomal phospholipase A1. Biochem. Int. 18, 129–139.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lullmann-Rauch, R., 1979. Drug-induced Lysosomal Storage Disorders. North-Holland, Amsterdam, The Netherlands.

Maggio, B., Montich, G.G., Cumar, F.A., 1988. Surface topography of sulfatide and gangliosides in unilamellar vesicles of dipalmitoylphosphatidylcholine. Chem. Phys. Lipids 46, 137–146.

Masserini, M., Freire, E., 1986. Thermotropic characterization of phosphatidylcholine vesicles containing ganglioside GM1 with homogeneous ceramide chain length. Biochemistry 25, 1043–1049.

Matsuzawa, Y., Poorthuis, B.J., Hostetler, K.Y., 1978. Mechanism of phosphatidylinostiol stimulation of lysosomal bis (monoacylglyceryl) phosphate synthesis. J. Biol. Chem. 253, 6650–6653.

McDaniel, R.V., McLaughlin, A., Winiski, A.P., Eisenberg, M., McLaughlin, S., 1984. Bilayer membranes containing the ganglioside GM1: models for electrostatic potentials adjacent to biological membranes. Biochemistry 23, 4618–4624.

McLaughlin, S., 1977. Electrostatic Potentials at Membrane–Solution Interfaces. Academic Press, London, UK.

Mellors, A., Tappel, A.L., 1967. Hydrolysis of phospholipids by a lysosomal enzyme. J. Lipid Res. 8, 479–485.

Mingeot-Leclercq, M.P., Laurent, G., Tulkens, P.M., 1988. Biochemical mechanism of aminoglycoside-induced inhibition of phosphatidylcholine hydrolysis by lysosomal phospholipases. Biochem. Pharmacol. 37, 591–599.

Mingeot-Leclercq, M.P., Piret, J., Brasseur, R., Tulkens, P.M., 1990. Effect of acidic phospholipids on the activity of lysosomal phospholipases and on their inhibition by aminoglycoside antibiotics—I. Biochemical Analysis. Biochem. Pharmacol. 40, 489–497.

Mingeot-Leclercq, M.P., Tulkens, P.M., 1999. Aminoglycosides: nephrotoxicity. Antimicrob. Agents Chemother. 43, 1003–1012.

Mitchell, D.C., Litman, B.J., 1998. Effect of cholesterol on molecular order and dynamics in highly polyunsaturated phospholipid bilayers. Biophys. J. 75, 896–908.

Montenez, J.P., Van Bambeke, F., Piret, J., Brasseur, R., Tulkens, P.M., Mingeot-Leclercq, M.P., 1999. Interactions of macrolide antibiotics (Erythromycin A, roxithromycin, erythromycylamine [Dirithromycin], and azithromycin) with phospholipids: computer-aided conformational analysis and studies on acellular and cell culture models. Toxicol. Appl. Pharmacol. 156, 129–140.

Nakagaki, M., Katoh, I., Handa, T., 1981. Surface potential of lipid membrane estimated from the partitioning of methylene blue into liposomes. Biochemistry 20, 2208–2212.

Piret, J., Kishore, B.K., Tulkens, P.M., 1992. Effect of substrate organization on the activity and on the mechanism of gentamicin-induced inhibition of rat liver lysosomal phospholipase A1. Biochem. Pharmacol. 43, 895–898.

Reasor, M.J., Kacew, S., 2001. Drug-induced phospholipidosis: are there functional consequences? Exp. Biol. Med. (Maywood) 226, 825–830.

Roberts, M.F., 1996. Phospholipases: structural and functional motifs for working at an interface. FASEB J. 10, 1159–1172.

Robinson, M., Waite, M., 1983. Physical-chemical requirements for the catalysis of substrates by lysosomal phospholipase A1. J. Biol. Chem. 258, 14371–14378.

Romero, G., Thompson, K., Biltonen, R.L., 1987. The activation of porcine pancreatic phospholipase A2 by dipalmitoylphosphatidylcholine large unilamellar vesicles. Analysis of the state of aggregation of the activated enzyme. J. Biol. Chem. 262, 13476–13482.

Schneider, P., Korolenko, T.A., Busch, U., 1997. A review of drug-induced lysosomal disorders of the liver in man and laboratory animals. Microsc. Res. Tech. 36, 253–275.

Schneider, Y.J., Tulkens, P., de Duve, C., Trouet, A., 1979. Fate of plasma membrane during endocytosis. II. Evidence for recycling (shuttle) of plasma membrane constituents. J. Cell Biol. 82, 466–474.

Seelig, J., 1978. 31P nuclear magnetic resonance and the head group structure of phospholipids in membranes. Biochim. Biophys. Acta 515, 105–140.

Seelig, J., Macdonald, P.M., Scherer, P.G., 1987. Phospholipid head groups as sensors of electric charge in membranes. Biochemistry 26, 7535–7541.

Sellinger, O.Z., Beaufay, H., Jacques, P., Doyen, A., de Duve, C., 1960. Tissue fractionation studies. 15. Intracellular distribution and properties of beta-*N*-acetylglucosaminidase and beta-galactosidase in rat liver. Biochem. J. 207, 450–456.

Shinozaki, K., Waite, M., 1999. A novel phosphatidylglycerol-selective phospholipase A2 from macrophages. Biochemistry 38, 1669–1675.

Silvius, J.R., 1986. Solid- and liquid-phase equilibria in phosphatidylcholine/phosphatidylethanolamine mixtures — a calorimetric study. Biochim. Biophys. Acta 857, 217–228.

Singer, A.G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G., Gelb, M.H., 2002. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. J. Biol. Chem. 277, 48535–48549.

Stoffel, W., Greten, H., 1967. Studies on lipolytic activities of rat liver lysosomes. Hoppe Seylers. Z. Physiol. Chem. 348, 1145–1150.

Stoffel, W., Trabert, U., 1969. Studies on the occurrence and properties of lysosomal phospholipases A1 and A2 and the degradation of phosphatidic acid in rat liver lysosomes. Hoppe Seylers. Z. Physiol Chem. 350, 836–844.

Tatulian, S.A., 2003. Structural effects of covalent inhibition of phospholipase A2 suggest allosteric coupling between membrane binding and catalytic sites. Biophys. J. 84, 1773–1783.

Tillack, T.W., Wong, M., Allietta, M., Thompson, T.E., 1982. Organization of the glycosphingolipid asialo-GM1 in phosphatidylcholine bilayers. Biochim. Biophys. Acta 691, 261–273.

Tocanne, J.F., Teissie, J., 1990. Ionization of phospholipids and phospholipid-supported interfacial lateral diffusion of protons in membrane model systems. Biochim. Biophys. Acta 1031, 111–142.

Trouet, A., 1974. Isolation of modified liver lysosomes. Methods Enzymol. 31, 323–329.

Vaccaro, A.M., Tatti, M., Ciaffoni, F., Salvioli, R., Barca, A., Scerch, C., 1997. Effect of saposins A and C on the enzymatic hydrolysis of liposomal glucosylceramide. J. Biol. Chem. 272, 16862–16867.

Vaccaro, A.M., Tatti, M., Salvioli, R., Ciaffoni, F., Gallozzi, E., 1990. Correlation between the activity of glucosylceramidase and its binding to glucosylceramide-containing liposomes. Role of acidic phospholipids and fatty acids. Biochim. Biophys. Acta 1033, 73–79.

Vandenbranden, M., De Gand, G., Brasseur, R., Defrise-Quertain, F., Ruysschaert, J.M., 1985. Hydrolysis of phosphatidylcholine liposomes by lysosomal phospholipase A is maximal at the phase transition temperature of the lipid substrate. Biosci. Rep. 5, 477–482.

Volwerk, J.J., Jost, P.C., de Haas, G.H., Griffith, O.H., 1986. Activation of porcine pancreatic phospholipase A2 by the presence of negative charges at the lipid–water interface. Biochemistry 25, 1726–1733.

Waite, M., 1985. Phospholipases. In: Vance, D.E., Vance, J.E. (Eds.), Biochemistry of Lipids and Membranes. The Benjamin/Cummings Pub. Co., Menlo Park, CA, pp. 299–324.

Wilkening, G., Linke, T., Sandhoff, K., 1998. Lysosomal degradation on vesicular membrane surfaces. Enhanced glucosylceramide degradation by lysosomal anionic lipids and activators. J. Biol. Chem. 273, 30271–30278.

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T., 1981. A pharmacokinetic analysis program (multi) for microcomputer. J. Pharmacobiodyn. 4, 879–885.

Yeagle, P.L., Albert, A.D., Boesze-Battaglia, K., Young, J., Frye, J., 1990. Cholesterol dynamics in membranes. Biophys. J. 57, 413–424.

Zhou, C., Qian, X., Roberts, M.F., 1997. Allosteric activation of phosphatidylinositol-specific phospholipase C: specific phospholipid binding anchors the enzyme to the interface. Biochemistry 36, 10089–10097.